Chemoprofiling and Antioxidant Potential of Selected *Piper* Species

Thesis submitted to the
University of Calicut

For the award of degree of
Doctor of Philosophy
(Biochemistry)

By

SRUTHI D.

Under the guidance of
Dr. T. John Zachariah

ICAR-Indian Institute of Spices Research
Kozhikode, Kerala, India
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CERTIFICATE

This is to certify that the thesis entitled **"Chemoprofiling and Antioxidant Potential of Selected *Piper* Species"** submitted to University of Calicut by **Ms. Sruthi D** for the award of the degree of **Doctor of Philosophy** in **Biochemistry** is a bonafide record of research work carried out by her at **ICAR-Indian Institute of Spices Research, Kozhikode, Kerala,** under my supervision and guidance. No part of the work has formed the basis for the award of any other degree or diploma previously. The plagiarism has been checked at CHMK library, University of Calicut and the values are well within the acceptable limit.

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DECLARATION

I hereby declare that the thesis entitled **"Chemoprofiling and Antioxidant Potential of Selected *Piper* Species"** submitted to University of Calicut by me for the award of the degree of **Doctor of Philosophy** in **Biochemistry** is a record of bonafide research work done by me at ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, under the supervision and guidance of **Dr. T. John Zachariah,** Acting Director, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala. This thesis or part of it has not been previously formed the basis for the award of any other degree or diploma previously and no plagiarism is made in the thesis. The plagiarism has been checked at CHMK library, University of Calicut and the values are well within the acceptable limit. All sources of help received by me during the period of this study have been duly acknowledged.

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Sruthi D.

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In the Name of God....

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ABBREVIATIONS

g	:	Gram
Kg	:	Kilogram
mg	:	Milligram
µg	:	Microgram
µL	:	Microliter
m	:	Meter
mm	:	Millimeter
µm	:	Micrometer
cm	:	Centimeter
nm	:	Nanometer
cm^3	:	Centimeter cube
%	:	Percentage
g%	:	Gram percentage
mg%	:	Milligram percentage
wt%	:	Weight percentage
M	:	Molar
mM	:	Millimolar
µM	:	Micromolar
^{o}	:	Degree
t	:	Tonne
KV	:	Kilovolt
V	:	Volt
hrs	:	Hours
min	:	Minute
Kcal	:	Kilocalorie
α	:	Alpha
γ	:	Gamma
β	:	Beta

Δ	:	Delta
p	:	Para
o	:	Ortho
rpm	:	Revolutions per minute
ppm	:	Parts per million
m/z	:	Mass-to-charge ratio
m above MSL	:	Meters above mean sea level
Nos.	:	Numbers
Fig.	:	Figure
UV-VIS	:	Ultraviolet-Visible
g/kg	:	Gram per kilogram
mg/kg	:	Milligram per kilogram
mg/gm	:	Milligram per gram
g/mol	:	Gram per mole
g/cm^3	:	Gram per cubic centimeter
ha^{-1}	:	Per Hectare
mL/min	:	Milliliter per minute
g/L	:	Gram per liter
mmol/L	:	Millimole per liter
µg/mL	:	Microgram per milliliter
mg/mL	:	Milligram per milliliter
HPLC	:	High performance liquid chromatography
GC-MS	:	Gas chromatography-mass spectrometry
AAS	:	Atomic absorption spectrophotometry
NMR	:	Nuclear magnetic resonance
IR	:	Infrared
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CO_2	:	Carbon dioxide
N	:	Nitrogen
P	:	Phosphorus

K	:	Potassium
Ca	:	Calcium
Mg	:	Magnesium
Zn	:	Zinc
C	:	Carbon
Na	:	Sodium
Al	:	Aluminium
Ba	:	Barium
Fe	:	Iron
Sr	:	Strontium
B	:	Boron
Cu	:	Copper
Mn	:	Manganese
Mo	:	Molybdenum
HCl	:	Hydrochloric acid
NaOH	:	Sodium hydroxide
H_2SO_4	:	Sulphuric acid
$FeCl_2$	:	Ferrous chloride
K_2O	:	Potassium oxide
K_2SO_4	:	Potassium sulphate
$CuSO_4$	:	Copper sulphate
KCl	:	Potassium chloride
$KMnO_4$	:	Potassium permanganate
HNO_3	:	Nitric acid
ATP	:	Adenosine triphosphate
PBS	:	Phosphate Buffered Saline
BHT	:	Butylated hydroxytoluene
ABTS	:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
MTT	:	(3-(4,5 Dimethylthiazol-2yl)-2,5-Diphenyl Tetrazolium Bromide)

MTS	:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
EC_{50}	:	Half maximal effective concentration
IC_{50}	:	Half maximal inhibitory concentration
LD_{50}	:	Lethal Dose, 50%
TEAC	:	Trolox equivalent antioxidant capacity

"All that man needs for health and healing has been provided by God in nature, the challenge of science is to find it"

- Paracelsus

Chapter 1

Introduction

Piper is the largest and the most representative genus among Piperaceae family and has great economical, commercial and medicinal importance. *Piper* species occupies an important place in all systems of medicine for thousands of years to enhance flavour, taste, aroma and medicinal properties. The genus *Piper* was established by Linnaeus (1753) in his *Species Plantarum,* where he included 17 *Piper* species. It consists of about 3000 species of which 115 are of Indian origin. The members of the genus *Piper* are mainly herbs, shrubs, creepers, climbers and trees. *Piper,* in general, is characterized by very small, highly reduced flowers which closely packed to form spikes. The male flower is represented by 2-3 anthers subtended by a bract and the female flower is represented by naked ovary. The distribution of genus *Piper* is pantropical in nature but the members also occur in the neotropics. Majority of the members distributed in the neotropics are bisexual and are small trees or shrubs whereas those from the Asian origin are unisexual and are woody climbers. Many of the *Piper* species like *P. nigrum* L. (Black pepper), *P. longum* L. (Long pepper), *P. chaba* Hunter (Java long pepper), *P. betle* L. (Betel vine) and *P. cubeba* L. (Cubeb) have gained a lot of attention because of their economic and medicinal values (Yunker, 1958; Saji, 2006; Saji *et al*., 2007; Anupama *et al.,* 2015).

Apart from their value as a spice to impart flavour and taste to food, many of the *Piper* species have excited the pharmaceutical world due to their diverse therapeutic potential and chemical profile. Even though the members of this genus are the hoard of high value compounds with tremendous health benefits, many of them are underexplored or not identified. Therefore, *Piper* species has assumed great significance in

the field of biological research and hence, four medicinally valued *Piper* species viz., *Piper nigrum* L., *Piper longum* L., *Piper chaba* Hunter and *Piper colubrinum* Link. were selected for the proposed study.

***Piper nigrum* L. (Black Pepper),** dubbed the 'King of Spices' and 'black gold', is the most important and widely consumed spice in the world (Ravindran, 2000a). The tropical evergreen forests of Western Ghats are considered as the centre of origin for black pepper (Hooker, 1886) and Malabar Coast of India is the traditional home of this most popular spice. Currently, black pepper is cultivated in about 26 countries including India, Sri Lanka, Indonesia, Malaysia, Vietnam, Brazil, Thailand, China, Mexico and Guatemala. Major black pepper growing states in India include Kerala, Karnataka and Tamil Nadu and to some extent in Andhra Pradesh, Maharashtra, Andaman and Nicobar Islands and North Eastern states *viz*. Meghalaya, Assam, Manipur and Arunachal Pradesh (IPC, 2015; Saji *et al*., 2007). Black pepper is a woody climber that usually grows between 20^{o} North and 20^{o} South of Equator and from almost sea level to an elevation of about 1500 m above mean sea level. It requires an annual rainfall of 2000-3000 mm with a relative humidity of 60-95%. The texture of soil to which black pepper plantations are established, varies from sandy loam to clayey loam (Thankamani & Kandiannan, 2012).

Apart from wild *P. nigrum,* more than 100 black pepper cultivars are also known to India. Black pepper cultivars are bisexual and are evolved directly from wild *P. nigrum.* Diversity in cultivars is created through natural selection and conscious selection for various traits. Besides, high yielding, good quality black pepper varieties with tolerance to diseases and pests have also been developed by different

institutions. About 19 improved varieties are developed or recommended for release (Saji, 2006; Saji *et al*., 2007; Saji & Krishnamoorthy, 2012; http://www.aicrps.res.in/pdf/Spices%20varieties%2017%20May.pdf).

Black pepper of commerce is the matured and dried berries (fruits) of *P. nigrum.* Malabar black pepper is fabled for its quality and thus, it has been the paramount item for trade between India and Europe for centuries (Parry, 1969). Peppercorn has earned high local, national and international markets because of its intrinsic quality, wide use in pharmaceuticals, household consumption and flavouring industries.

Intrinsic quality of black pepper is attributed by pungency and aroma. Its spiciness is due to the presence of piperamides, which are the pungent bioactive alkaloids concentrated in the skin and seeds of the berries. Among them, piperine is the major compound responsible for the bitter taste of black pepper. Besides, black pepper has characteristic aroma due to their essential oil constituents. The black pepper contains 2-4% essential oil and 2-6% piperine. Essential oil constituents are mainly terpenoids and also contain phenyl propanoids and aliphatic compounds. Monoterpenes and sesquiterpenes are the major terpenoid constituents of black pepper oil. Pinene, sabinene, camphene, limonene, etc. are the major monoterpenes whereas β-caryophyllene is the major sesquiterpene in black pepper essential oil. Other high value constituents (phenolic acids, flavonoids, alkaloids, fatty acids, etc.) ascribed to medicinal and therapeutic values were also identified from black pepper. Thus, black pepper is renowned for profuse therapeutic and pharmacological potentials like analgesic, antiseptic, antispasmodic, digestive, diuretic, laxative, antitoxic, antioxidant, antimicrobial, anticancer and antiinflammatory

activities. This ‘spice gold’ has thus, great commercial, medicinal and industrial potential (Narayanan, 2000; Zachariah & Parthasarathy, 2008; Rani *et al*., 2013). However, many of its bioactive constituents are still unidentified and further pharmacological studies are needed to assess their biological effects.

Piper longum **L. (Long Pepper)** is a flowering aromatic climber cultivated mainly for its fruit. This dioecious plant is native to North East India and occurs mainly in the hotter parts of India, from central Himalayas to Assam, the lower hills of West Bengal, Khasi and Mikir hills and evergreen forests of Western Ghats from Konkan to Kanyakumari. It also occurs in the Car Nicobar Islands. Considering the global allocation, Indo-Malaysian regions and Sri Lanka are suggested as the native of *P. longum*. Good rainfall, high relative humidity, well drained sandy soil with pH 5.5-8.5 are the suitable conditions for *P. longum* to grow well. *P. longum* presumably came to Europe even before the present dominant black pepper. *P. longum* was highly priced during the Roman Empire, since it was highly attracted for Roman cookery due to its pungent and sweet taste. *P. longum* is the source for ‘Pippali’ and ‘Pippalimulam’. ‘Pippali’ is its dried and matured fruit and used as spice and seasoning whereas ‘Pippalimulam’ is its root (Sivarajan & Balachandran, 1994; Oommen *et al*., 2000; Manoj *et al*., 2004).

The pungency of *P. longum* is due to the alkaloid piperine (0.3-1.3%). It also contains essential oil (0.6-1.5%), which mainly includes aliphatic compounds like n-pentadecane and also monoterpenes and sesquiterpenes. *P. longum* also contains diverse chemical groups like phenolics, alkaloids, flavonoids, resins, etc. Because of this diverse and highly active chemical profile, *P. longum* has great heed to medical

field and hence became a major component of Ayurvedic medicines. It is a good remedy for treating tuberculosis, respiratory tract infections, menstrual pain, sleeping problems, leprosy, anaemia, cardiac and spleen disorders, chronic fever, colic indigestion, gout and arthritic conditions. Other reported beneficial effects of *P. longum* include antibacterial, antiallergic, antitumor and antioxidant activities (Manoj *et al*., 2004; Rameshkumar *et al*., 2011).

Ascribable to this commercial, economic and medical importance, *P. longum* has been studied pharmacognostically, pharmacologically and chemically. Thus, valuable phytochemicals with significant upshot in our life has been identified. Potentials of some of these constituents were also realised. However, many of the high value compounds and therapeutic potential of this medicinally valued spice is still unknown to the scientific world.

Piper chaba **Hunter (Java long pepper)** is a climbing glabrous creeper originated from South East Asia. The plant is cultivated in various parts of India and Malaya Islands. The plant grows well in Bangladesh particularly in the Satkhira–Bagerhat area. Like other *Piper* species, the plant shows extensive folklore uses, as traditional medicine. Various parts of this plant (fruit, leaf, root and stem) have vast ethno medical uses. Traditionally, the leaves and stem of this plant are used as spices in foods and also as an alternative for *P. longum.* The root is a good remedy for asthma, bronchitis and tuberculosis. The fruit is pungent in nature and is thermogenic, expectorant, carminative and improves appetite and taste. It is also useful in asthma, bronchitis, fever, inflammation, piles and pain in the abdomen. The stem is used to diminish the post-delivery and rheumatic pains. The spice also attained pharmacological attention due to its activities like

antimicrobial, antioxidant, anticancer and antiinflammatory effects. Studies have been conducted to identify many of its phytochemicals and these include alkaloids, alkamides, lignans, terpenoids, etc. These bioactive compounds might be the reason for this plant to be a medicinally valued crop. Piperine (0.9-1.3%), chabamide, piplartine, piperlonguminine, piperidide, kusunokinin, pellitorine, etc. are some of the alkaloids identified from *P. chaba.* Sesquiterpenes (β-caryophyllene, germacrene D, etc.), monoterpenes (pinene, limonene, etc.) and aliphatic hydrocarbons (n-pentadecane, 1-heptadecene, etc.) are the major classes of constituents from essential oil (0.8-1.2%) of *P. chaba* fruit (Kirtikar & Basu, 1980; Yusuf *et al*., 1994; Rahman *et al*., 2005; Vaghasiya *et al*., 2007; Rahman *et al*., 2011; Rameshkumar *et al*., 2011; Plengsuriyakarn *et al*., 2012).

However, this crop has not received a deserved pharmacological and medicinal importance due to dearth in the information regarding their detailed chemical profile and medicinal values. Thus, the therapeutic and chemical world of research demands much exploration of this plant with more attention to their hidden medicinal importance and bioactive compounds.

Piper colubrinum **Link.** is a distant relative of black pepper. This exotic *Piper* species is a woody shrub, native to Northern part of South America. *P. colubrinum* is known for its multiple-disease resistance. It has resistance to plant pathogens like *Phytophthora capsici*, *Radopholus similis* and *Meloidogyne incognita*. *P. colubrinum* has thus gained great attention in crop breeding since it can be biotechnologically exploited for transferring their resistance genes to black pepper varieties against the specific pathogens. However in the phytochemical and medicinal point of view, the studies on

P. colubrinum are scanty (Nambiar & Sarma, 1977; Ramana & Mohandas, 1987; Ravindran & Remashree, 1998; Devasahayam, 2000; Varma *et al*., 2009).

SIGNIFICANCE OF THE STUDY

Chemical examination spanning over a century contributed exceedingly to the understanding of black pepper, one of the most wonderful spices. However, an area relatively disregarded by the black pepper researchers is the chemical and pharmacological diversity among black pepper varieties. Detailed study on their chemical composition and medicinal potential will help to locate black pepper varieties with high flavour and quality and also with high medicinal values. Such lines can then be considered in breeding programmes in future to develop black pepper varieties with promising characteristics like high quality and yield. Such an approach calls for a close collaboration between the breeders and the chemists. Likewise, black pepper showed variability in their essential oil profile and pungent principles with regard to maturity at harvest, extraction techniques, etc. However, a systematic study for variability in intrinsic quality of black pepper varieties in relation to different locations has not been observed. Furthermore, information is scanty regarding the correlation among the chemical constituents of black pepper berries.

Human body produces free radicals, mainly, Reactive Oxygen Species (ROS) as a part of normal metabolic processes. Many acute and chronic diseases like cancer, diabetes, inflammation, arthritis, aging, atherosclerosis and various neurodegenerative disorders mainly arise from oxidative stress initiated by these highly reactive and unstable free radicals. So, the oxidation and anti-oxidation balance should be

maintained for a healthy biological system and thus it is imperative to find a permanent solution for this oxidative stress and related disorders. This can be achieved by exploring compounds with antioxidant activity. Plants are the tremendous source for bioactive compounds with antioxidant, anticancer and other medicinal values. Hence, in the modern research, there is a great inducement to discover biologically active natural compounds from plants due to their medicinal potential and fewer side effects compared to synthetic chemicals. They are safer from health and environmental point of view. However, many of the plants remain underexplored with regard to their chemical profile, antioxidant, anticancer and other therapeutic values. *P. longum, P. chaba* and *P. colubrinum* are a few among such plant species which require much more attention in these aspects. Likewise, information is scanty on comparative chemoprofiling, antioxidant and anticancer potential of *P. nigrum, P. longum, P. chaba* and *P. colubrinum*.

OBJECTIVES

Based on the above mentioned background information, the following objectives have been formulated in the present study.

- Variability in physico-chemical and biochemical profile among selected *Piper* species and also among selected black pepper varieties.
- Variability in physico-chemical and biochemical profile of black pepper variety Panniyur-1 in relation to different locations.

- Antioxidant potential of selected *Piper* species and selected black pepper varieties.
- *In vitro* cytotoxicity of selected *Piper* species and selected black pepper varieties on cancer cell line CaSki.

Plate 1. *Piper* species selected. A) *P. nigrum* L., B) *P. longum* L., C) *P. chaba* Hunter, D) *P. colubrinum* Link.

Plate 2. Matured and dried fruits/berries of *Piper* species selected. A) *P. nigrum* L. (wild), B) *P. longum* L., C) *P. chaba* Hunter, D) *P. colubrinum* Link.

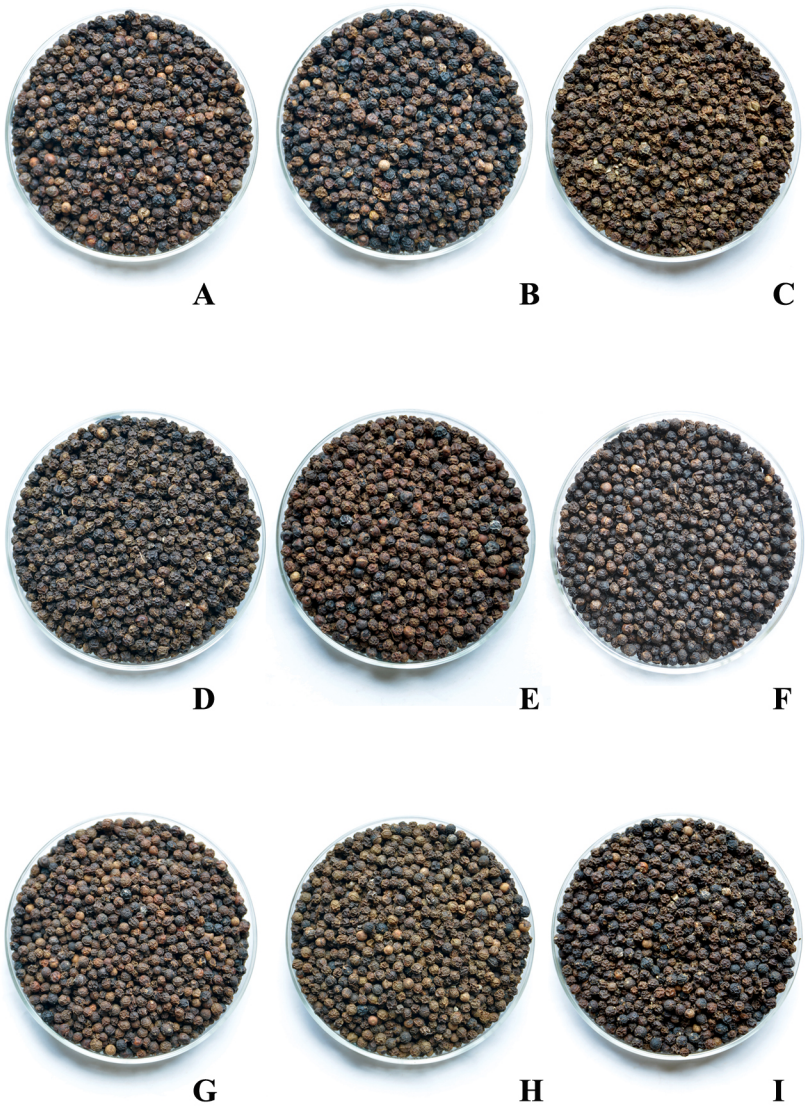

Plate 3. Matured and dried berries of black pepper (*P. nigrum* L.) varieties selected. A) IISR Girimunda, B) IISR Malabar Excel, C) Panchami, D) Panniyur-1 E) Panniyur-5, F) IISR Sakthi, G) Sreekara, H) Subhakara, I) IISR Thevam

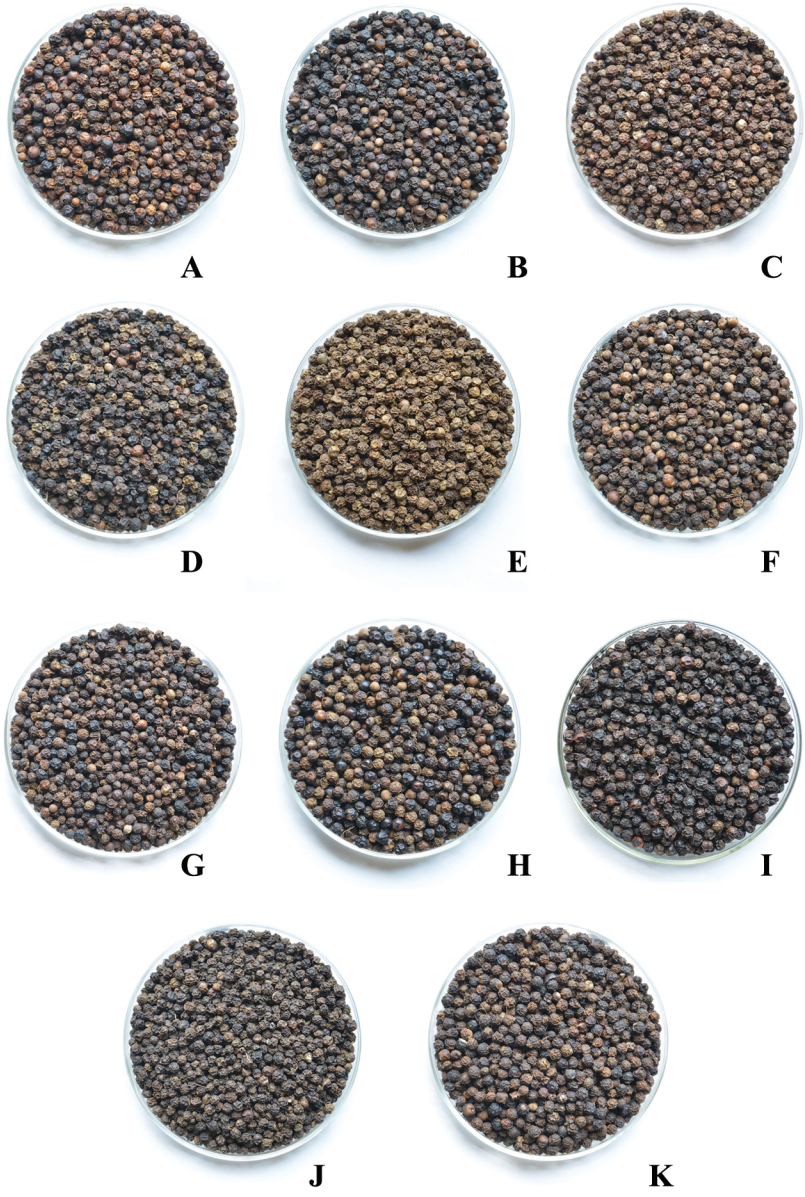

Plate 4. Matured and dried berries of black pepper (*P. nigrum* L.) variety Panniyur-1 from different locations. A) Ambalavayal, B) Appangala, C) Chelavoor, D) Dapoli, E) Kasaragod, F) Mudigere, G) Pampadumpara, H) Panniyur, I) Pechiparai, J) Peruvannamuzhi, K) Thadiankudisai

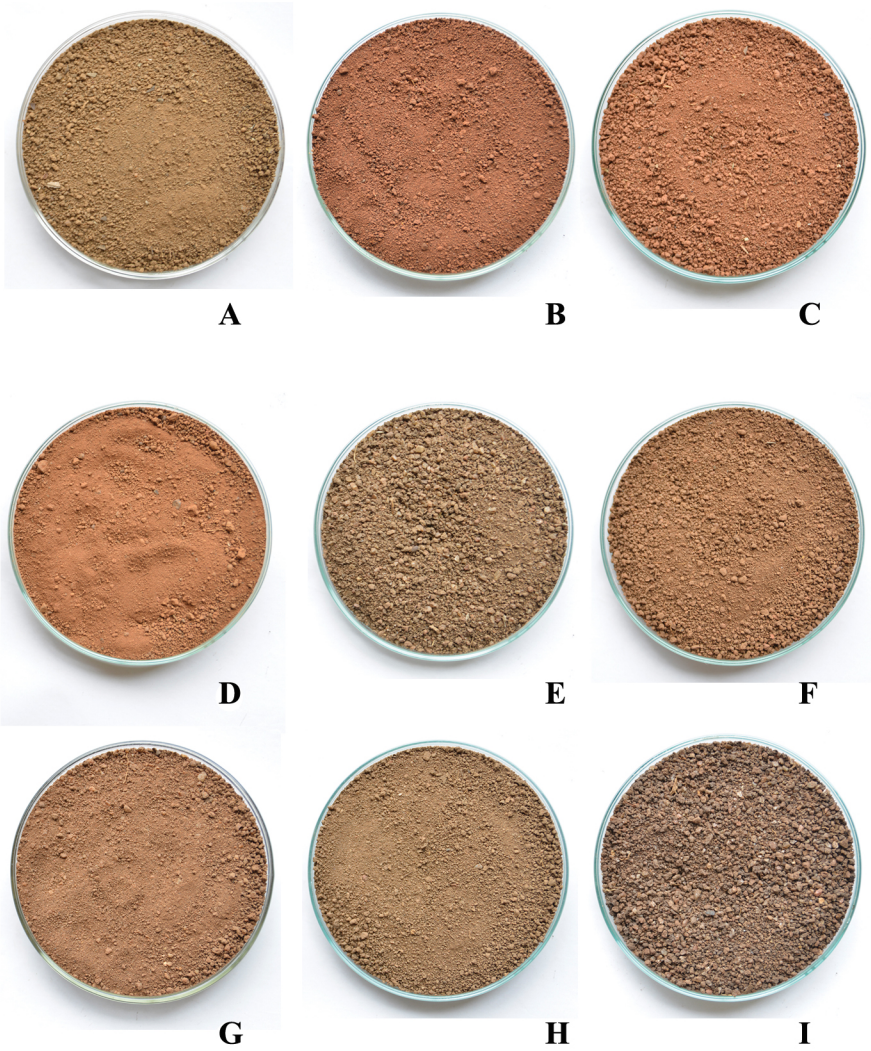

Plate 5. Soil samples collected from Panniyur-1 black pepper (*P. nigrum* L.) growing locations. A) Ambalavayal, B) Appangala, C) Chelavoor, D) Dapoli, E) Pampadumpara, F) Panniyur, G) Pechiparai, H) Peruvannamuzhi, I) Thadiankudisai

Chapter 2

Review of Literature

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2.1 THE GENUS *PIPER*

The genus *Piper*, the largest among the Piperaceae family, comprises economically, medicinally and commercially important species. The name *Piper* was derived probably from the Greek word, *Peperi* (Ravindran *et al*., 2000). The genus *Piper,* one of the major components of tropical forest ecosystem, was first described by Linnaeus in 1753 in his *Species Plantarum. Piper* species are distributed in both hemispheres, throughout the tropical and sub tropical regions of the world. Central and Northern South America, India, Indonesia, Malaysia, China and Sri Lanka are the major *Piper* species growing countries (Hartemink, 2001; Saji *et al*., 2007; Saji & Krishnamoorthy, 2012).

The genus *Piper* consist about 3000 species of which 115 are of Indian origin. Neotropical species which are mostly shrubs and rarely climbers and paleotropic species which are climbers, creepers or bushy forms are the two major groups of *Piper* species. Central America is the main centre of distribution for neotropical species whereas paleotropic species are mainly seen in the moist evergreen forests and to some extent, in the semi evergreen forests. Trans-Gangetic region and the South Indian Deccan are the two independent centres recognized for the distribution of the genus *Piper* in India (Hooker, 1886; Jaramillo & Manos, 2001; de Figueiredo & Sazima, 2004; Saji, 2006; Anupama *et al.,* 2015).

Piper species are mainly herbs, shrubs, creepers, climbers and trees and are characterized by very small, highly reduced flowers, closely

packed to form spikes. The male flower is represented by 2-3 anthers subtended by a bract and the female flower is represented by naked ovary. *Piper* species that occur in India are unisexual whereas that from Central and South America are bisexual in nature (Saji, 2006).

The genus *Piper* has great economic value since many of its members, especially *P. nigrum* L. (Black pepper), *P. longum* L. (Long pepper), *P. chaba* Hunter (Java long pepper), *P. betle* L. (Betel vine) and *P. cubeba* L. (Cubeb or tailed pepper) have medicinal, therapeutic and culinary importance. Hence, it is believed that *Piper* species have great significance and scope in the field of research and thus, four medicinally valued *Piper* species *viz*., *Piper nigrum* L*., Piper longum* L*., Piper chaba* Hunter and *Piper colubrinum* Link. were selected for the proposed study.

2.2 GENERAL INTRODUCTION FOR *PIPER* SPECIES SELECTED FOR THE STUDY

2.2.1 *Piper nigrum* L.

Piper nigrum L., commonly known as 'black pepper', is the most important and widely consumed spice in the world. Western Ghats of India is regarded as the centre of origin of this humid tropical crop. Black pepper of commerce is the matured and dried berries of *P. nigrum* (Ravindran, 2000a). Indian pepper, mainly familiar as 'Malabar pepper', is considered to be the best in the world for its excellent aroma and pungency. Because of its intrinsic quality, wide use in pharmaceuticals, household consumption and flavouring industries, peppercorn has gained attention in global markets and thus

has become an important item for the trade between India and Europe for centuries (Parry, 1969).

India is one of the major producers, exporters and consumers of black pepper. India contributes more than 40% of black pepper cultivation area of world with about 23% of contribution for the world pepper production. An average area for black pepper cultivation in India for the period of 2003-2012 was estimated as 2, 09,625 hectares (ha) with a production of 54,315 metric tonne (mt). During this period, average export of black pepper was 21,620 mt and average import was 15,392 mt for India. The area for black pepper cultivation in India was estimated as 117760 ha with a production of 37000 t for the period of 2013-14. As per the reports of 2014-2015, the total production of black pepper in India was 50,870 mt from an area of 23, 810 ha (IPC, 2015; Spices board, 2015; NHB, 2015). Kerala, Karnataka and Tamil Nadu are the major black pepper cultivation areas in India. Other states like Andhra Pradesh, Orissa, West Bengal, Tripura, Maharashtra, Manipur, Arunachal Pradesh, Andaman and Nicobar Islands are the other black pepper cultivating areas in India (Saji *et al*., 2007; Saji & krishnamoorthy, 2012).

Black pepper is a woody climber characterized with swollen internodes, simple, thick, glabrous and petiolate leaves (10-24 cm), pendant, filiform, long and glabrous spikes (green, greenish white or purple colour), spherical and pungent seeds and fruit, which is green when young and red after ripening (Saji *et al*., 2007).

Black pepper grows between 20^{o}North and 20^{o}South of Equator and from almost sea level to an elevation of about 1500 m above MSL. An annual rainfall pattern of 2000-3000 mm with a relative humidity of

60-95% is required for proper growth of black pepper (Thankamani & Kandiannan, 2012). The ideal temperature for black pepper growth is 23-32^{o}C with a tolerable limit of 10-40^{o}C. Though exposure to direct solar radiation causes physiological disorders, black pepper demands adequate sunlight for its essential growth and development (de Waard, 1969; Vijayakumar *et al*., 1985; Wahid & Sitepu, 1987; Srinivasan *et al*., 2012).

A wide variety of soils with their texture varying from sandy loam to clayey loam is suitable to establish black pepper plantations. Well drained, deep loamy soil, rich in organic matter, good water holding capacity and pH ranging from 5-6 are good for black pepper growth (Mathew *et al*., 1995; Thankamani & Kandiannan, 2012).

The seed and vegetative cuttings are the two sources for black pepper propagation, of which, stem cutting is adopted as the traditional propagation method. Micro propagation is also under practice (Nair & Gupta, 2006; Thankamani & Kandiannan, 2012). To reach full maturity, black pepper takes 7-8 months after flowering. It is harvested during December to January in plains and January to April in high altitudes of Western Ghats. It should be harvested at proper maturity to achieve a dried black pepper with good colour, appearance and quality (Thankamani & Kandiannan, 2012).

Apart from their multidimensional medicinal values, black pepper attained great demand in the international market because of its various culinary uses. It is mainly used in curry powders. Pepper cookies, pepper tofu, etc. are the other products prepared from black pepper. Pepper extract is a valuable part in the flavouring of sausages, canned meat, table sauces, soups, certain beverages and liquor. Black pepper

also has considerable industrial uses. It is valued as essential preservative for meat and other perishable foods and thus, used in meat packing, canning, pickling and also in the preparations of beverages. Black pepper oil is used in perfumery and also used in carnation compounds of soaps (Jayashree & Zachariah, 2012).

Cultivar diversity is one of the major components of black pepper diversity and the cultivars are evolved directly from wild *P. nigrum*. Natural selection and conscious selection by human endeavor for various traits have created cultivar diversity. Today more than 100 cultivars are known, of which, 60-65 are prevalent in cultivation. Cultivar diversity is maximum in Kerala, followed by Karnataka (Saji *et al*., 2007). Besides, developing high yielding, good quality black pepper varieties with tolerance to disease and pests are one of the major aims of different research institutions. High yield per vine, adaptation to high altitude, high quality along with high yield, resistance to different diseases and resistance to drought are the important objectives for developing improved black pepper varieties. Clonal selection, hybridization, open pollinated progeny selection and polyploidy breeding are the important strategies used for developing improved black pepper varieties. About 19 improved varieties are developed or recommended for release. Among these, Panniyur-1 to 8 black pepper varieties were developed by Pepper Research Station, Panniyur, Kerala whereas Sreekara, Subhakara, Panchami, Pournami, IISR Girimunda, IISR Malabar Excel, IISR Sakthi, IISR Thevam and PLD-2 were developed by ICAR-Indian Institute of Spices Research, Kozhikode, Kerala. The other two black pepper varieties *viz.,* Vijaya and Arka Coorg Excel were developed by Kerala Agricultural University (Thrissur, Kerala) and Central Horticultural Experiment

Station (Chettalli, Karnataka) respectively (Ravindran *et al*., 2000; Saji *et al*., 2007; Krishnamoorthy & Parthasarathy, 2010; http://www.aicrps.res.in/pdf/Spices%20varieties%2017%20May.pdf).

2.2.2 *Piper longum* L.

Piper longum L., commonly known as 'Indian long Pepper', is a slender creeping plant distributing throughout India. This flowering, aromatic climber with perennial woody root also occurs in Sri Lanka, Burma, Malaysia and other South Asian countries. In India, it is grown in Assam, West Bengal, Nepal, Bihar, Uttar Pradesh, Kerala, Tamil Nadu, Andhra Pradesh, etc. (Ravindran, 2000b).

P. longum fruit (Pippali) and root (Pippalimulam) are among the most important in the Indian systems of medicines- Ayurveda, Sidha and Unani. Pippali is also used as spice and seasoning (Sivarajan & Balachandran, 1994; Jayashree & Zachariah, 2012). It is believed that long pepper possibly reached Europe even before the now dominant black pepper. Because of its pungent and sweet taste, long pepper was highly attracted to Roman cookery and hence it was highly priced during the time of Roman Empire (Manoj *et al*., 2004).

This perennial creeping plant is dioecious in nature with creeping vegetative branches, erect fruiting branches, dimorphic leaves (around 7 x 5 cm), long, cylindric and erect spikes (2-4 cm), laterally fused flowers and very small, laterally fused, spicy and pungent fruits (Ravindran *et al*., 2000).

P. longum is mainly found in tropical, humid climate, preferably in shady, moist conditions. The area with good rainfall and high relative humidity is suitable for the successful growth of this plant. They grow

well in laterite soil with sufficient organic matter and water holding capacity. The plant demands 50% shade for better fruiting. An altitudinal range of 900-1500 m above MSL is recommended for the optimum growth of *P. longum*. However, it can also grow in the plains as an intercrop in the coconut plantations. *P. longum* can be propagated by vegetative means like mature branches or by suckers planted during the beginning of rainy season and also by tissue culture technique (Oommen *et al*., 2000; Soniya & Das, 2002; Manoj *et al*., 2004).

Efforts to develop *P. longum* varieties with improved characters are in progress by researchers and 'Viswam' is one of such improved variety of *P. longum* (Saji, 2012).

2.2.3 *Piper chaba* Hunter

P. chaba Hunter (Java long pepper) is a perennial, glabrous and fleshy climber, native to Moluccas. It is also cultivated in Malaysia and in India, especially North Eastern regions (Saji, 2012).

This dioecious vine shows pronounced dimorphic branching with short, petioled, oblong, oblong-ovate or elliptic lanceolate leaves (6-7.5 x 3.2-6.5 cm), adhesive roots, erect spikes (3.0-6.5 cm long) and more or less united fruits, which are partly or fully embedded in the rachis. The mature spike is pungent and the spikes lose their pungency and quality on ripening. Therefore, harvesting should be done when the spikes are matured but still green (Ravindran, 2000b; Saji, 2012).

P. chaba can also grow as an intercrop in arecanut or coconut plantations. It is found in hot and moist climate. There is no evidence on organized cultivation of *P. chaba*. Tissue culture method for

propagation is reported (Rema *et al*., 1995; Kandiannan *et al*., 2005; Saji, 2012).

P. chaba exhibits many culinary uses especially in Thailand and Bangladesh. Their stem and roots are usually used to cook with meat and fish to get its pungent flavour. It is an ingredient in many of the Thai sauces and soups. The fruits of *P. chaba* are used as spice and also in pickles and preserves. It is also important in the traditional systems of medicine and also as an alternative to fruits of *P. longum* (Saji, 2012; https://en.wikipedia.org/wiki/Piper_chaba).

2.2.4 *Piper colubrinum* Link.

Piper colubrinum Link. is an exotic *Piper* species and is native to Northern part of South America. It is a woody shrub with dimorphic branching pattern. The orthotropic shoots have monopodial growth whereas plagiotropic branches have sympodial growth. During the stages of development, vegetative apical buds of the latter modify as spikes. The mature stem follows anomalous secondary growth. The aerial root is externally bound by the epidermis with a thick cuticle and for the underground roots, there is a thick cuticle with root hairs. *P. colubrinum* is considered as a distant relative of black pepper and is resistant to *Phytophthora capsici* and *Radopholus similis,* the causative agents of foot rot and slow decline diseases in black pepper respectively. Hence, this plant has attained importance in crop breeding (Ravindran & Remashree, 1998).

2.3 PHYTOCHEMISTRY

Primary and secondary metabolites are the two main classes of chemical constituents in plants. Primary metabolites are mainly

responsible for growth, development and reproduction whereas secondary metabolites are for defense mechanisms in plants.

2.3.1 Primary metabolites

Primary metabolites are mainly involved in respiration, transport, assimilation and differentiation of plants. They are also involved in constitutive or structural defense mechanisms in plants and also provide strength and rigidity to them. They are ubiquitous in the plant kingdom and are produced by all plant cells. Carbohydrates, proteins and lipids are the major primary metabolites (Shamina & Sarma, 2001; Freeman & Beattie, 2008).

2.3.1.1 Carbohydrates

Carbohydrates, one of the most abundant organic compounds in the plants, contribute to the greatest portion of its dry weight. They form important energy reserves, which are utilized during growth and development of the plant. The main carbohydrates in plants can be categorized as monosaccharides, disaccharides, oligosaccharides and polysaccharides (Avigad & Dey, 1997; Miller, 2005). Carbohydrates in plants can be quantified by spectrophotometric and chromatographic techniques.

Glucose, the simplest form of carbohydrate, is the first evident product formed by green plants and forms the foundation of most of the other organic compounds in plants. Fructose, mannose and galactose are the other monosaccharides present in plants. Sucrose is the most abundant, most widely distributed disaccharide in plants and is found in all living plant cells in varying amount. This major photosynthetic product is considered as the transport form of plant world. Maltose is another

disaccharide present in plants. The oligosaccharides which include trisaccharides (raffinose, umbelliferose, planteose, etc.) and tetrasaccharides (stachyose, lychnose, isolychnose, sesamose, etc.) are important as storage carbohydrates and transport sugars in plants (Stumpf & Conn, 1980; Avigad & Dey, 1997; Miller, 2005). The polysaccharides in plants include two classes, *viz*., structural and storage polysaccharides. Structural polysaccharides are responsible for the cell wall formation and provide strength and rigidity to them and thus are involved in the first line of defense mechanism of plants. Storage polysaccharides, on the other hand, form temporary or permanent stores of fixed carbon and energy. Cellulose is the most important structural polysaccharide whereas starch is the most important storage polysaccharide in plants (Goodwin & Mercer, 2003).

2.3.1.2 Proteins

Protein, the polymer of amino acids, is one of the essential components for life. The plant proteins can be classified as seed protein, leaf protein and isoenzymes. Seed proteins are confined to and characteristics of the seeds whereas leaf proteins are located in the chloroplast and mainly concerned with the CO_2 fixation. On the other hand, isoenzymes are mainly assigned for metabolic regulation (Goodwin & Mercer, 2003).

Different extraction procedures for plant proteins were described by different researchers using different reagents or chemicals like trichloroacetic acid, acetone, phenol-based method, dodecyl sulphate mediated protein extraction, etc. (Saravanan & Rose, 2004; Mounicou *et al.,* 2004). However, a number of factors like liberation of highly acidic vacuolar sap, highly active oxidative enzymes and liberation of

proteolitic enzymes on maceration, usually, make the plant proteins much difficult to handle experimentally (Goodwin & Mercer, 2003). SDS-PAGE is the most popular technique for quantification, purification and molecular weight determination of proteins (Weber & Osborn, 1969; Figeys *et al*., 1998). Protein can also be quantified spectrophotometrically.

Amino acids are the building blocks of proteins. In addition to their involvement in protein building, amino acids are also responsible for other important functions in plants and these includes stress resistance, role in photosynthesis, chelating action, in pollination and fruit formation, for maintaining equilibrium of soil flora and for phytohormone synthesis. Thus, amino acids are essential for plants to increase its quality and yield (http://www.servpro.com.my/ Folio_intro.pdf).

Apart from being bound as proteins, amino acids can also exist in the free form in many tissues and are called as free amino acids. Free amino acids are mainly water soluble in nature. In plants, usually there is a change in free amino acid composition during diseased conditions and thus, the estimation of total free amino acids helps to know the physiological and health status of the plants. Chromatographic techniques like paper chromatography, HPLC, etc. can be useful for separation, quantification and identification of amino acids. Spectrophotometric method is widely used for the quantification of total free amino acids (Sadasivam & Manickam, 2008).

2.3.1.3 Lipids

Lipids are heterogeneous group of biomolecules, mainly function as storage form of energy. They are the structural component of biomembranes and also act as metabolic regulators. They contain fatty acids, alcohols and isoprenoids. Simple lipids (neutral fats and waxes), compound lipids (phospholipids, glycolipids, etc.), and derived lipids (fatty acids, steroids, etc.) are the major classes of lipids. Neutral fats are fatty acid esters of glycerol. Fat in the liquid form is called oil. Neutral fats are accumulated as food reserves mainly in seeds and fleshy parts of fruits whereas waxes, phospholipids and glycolipids have structural functions (Goodwin & Mercer, 2003; Chatterjea & Shinde, 2005; Sadasivam & Manickam, 2008).

Fatty acid is a long hydrocarbon chain capped by a carboxyl group. More than 200 different fatty acids are identified in the plant kingdom and these include 'major' (palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, lauric acid and myristic acid), 'minor' (palmitoleic acid, myristoleic acid, erucic acid, 11-eicosenoic acid, γ-linolenic acid, etc.) and 'unusual' (labellenic acid, crepenynic acid, stearolic acid, vernolic acid, etc.) fatty acids (Goodwin & Mercer, 2003). Fatty acids in plants can be identified by several techniques like GC-FID and GC-MS.

The fixed oils and fats are important industrially, pharmaceutically and as food. They are used in pharmaceuticals for their emollient properties. They can also serve as vehicle for other medicaments. A few oils have cathartic and antileprosy activities and certain fatty acids have antifungal activities. In the industries, they are used for the

manufacturing of soaps, as lubricant and emulsifying agents, as drying oils in the manufacture of varnishes and paints, etc. (Bhat *et al*., 2009).

2.3.1.4 Primary metabolites in Piper species selected for the study

The monosaccharide composition of hot water extract from black pepper seeds was studied by Chun *et al*., (2002) and revealed the presence of glucose, galactose, galacturonic acid, arabinose and rhamnose.

Pradeep *et al*. (1993) have given amino acid composition (g/100 g N) of black pepper as follows: alanine (5.02), aspartic acid (6.03), arginine (5.00), glutamic acid (6.61), cystine (0.94), glycine (3.78), histidine (1.83), isoleucine (2.02), leucine (5.43), lysine (1.15), methionine (0.80), phenylalanine (2.19), proline (4.73), serine (3.59), threonine (1.74), tryptophan (0.59), tyrosine (2.46) and valine (3.10).

The fat content and fatty acid composition of black pepper was also studied. Black pepper contains fat content ranging from 1.9-9.0%. The acid constituents of this fat consist of 19-38% saturated fatty acid and 51-83% unsaturated fatty acids. The major fatty acids identified in black pepper includes palmitic (16-30%), oleic (18-29%), linoleic (25-35%) and linolenic acids (8-19%) (Bedi *et al*., 1971; Salzer, 1975a; Narayanan, 2000). Parmar *et al*. (1997) also listed stearic, sterculic, lauric, melvalic, myristic, capric and vernolic acids from black pepper. Al-Jasass & Al-Jasser (2012) reported that, black pepper from Saudi Arabia contained 81.34% of total unsaturated fatty acid and 18.66% of total saturated fatty acids. They have identified linoleic acid as major fatty acid (33.03%) along with palmitic, palmitoleic, oleic, linolenic and arachidic acids.

Biochemical variability studies were conducted in the leaves and berries of seven black pepper varieties (Karimunda, OP Karimunda, Panniyur-1, P 24, HP 813, Coll. 1041 and HP 105) by Sumeshkumar (2004). The starch content ranged from 0.415-4.964% in leaves and 14.14-57.81% in berries. The reducing sugar was in the range of 0.144-1.526 mg/100 mg in leaves and 0.90-2.31 mg/100 mg in berries. The total free amino acid was in the range of 0.02-0.136% in leaves whereas 0.02-0.201% in berries. However, total protein ranged from 2.907-7.22% in leaves and 3.54-6.61% in berries.

Primary metabolites, *viz*., total carbohydrate, starch, reducing sugar and protein were analyzed in leaf and stem of juvenile vines of five high yielding and five low yielding black peppers. The mean starch and carbohydrate content were more in leaves than stem while reducing sugar and protein content were more in stem than leaves (Shujari, 2005).

Jayashree *et al*. (2009) evaluated five black pepper varieties (Panniyur-1, Panniyur-2, Panniyur-5, Sreekara, and Subhakara) for their starch, crude fibre and protein content. The starch content was in the range of 34.7-52.3% whereas protein and crude fibre were in the range of 9.6-13.9% and 6.8-14.6% respectively.

Zachariah *et al*. (2010) evaluated leaf and berries of 26 black pepper cultivars for variability in biochemical parameters. The results showed that total carbohydrate of black pepper berries was in the range of 38.6-51.2 mg%. On the other hand, starch, total free amino acid and total protein of black pepper berries ranged from 32.1-43.2 mg%, 0.3-0.8 mg% and 2.1-6.0 mg% respectively. However, the leaf total carbohydrate, starch, total free amino acid and total protein were in the

range of 1.6-4.8 mg%, 0.6-2.1 mg%, 0.2-0.9 mg% and 1.0-2.8 mg% respectively.

Shankaracharya *et al*. (1997) reported 40% starch in *P. longum.* Jiang *et al*. (2013) reported longumosides A (5-formyl-(+)-angelicoidenol 2-O-b-D-glucopyranoside) and longumosides B (*Trans*-cinnamyl-(6-(3-O-3-methyl-pentanedioic acid))-b-D-glucopyranoside) in *P. longum.* The amino acid L-aspartic acid, DL-serine, L-cysteine hydrochloride and L-tyrosine have also been reported in *P. longum* fruits (Khushbu *et al*., 2011). Palmitic, stearic, linoleic, hexadecenoic, oleic, linolenic, arachidic and behenic acids were the fatty acids reported from *P. longum* (Bedi *et al*., 1971; Khushbu *et al*., 2011). In addition, Zaveri *et al*. (2010) listed Z-12-octandecenoic–glycerol-monoester from *P. longum* fruit.

Primary metabolites (mg/g fresh weight) of *P. longum* leaf, fruit, stem and root have been reported by Sindhu *et al*. (2013). Total protein, total carbohydrate and total free amino acid of leaf were 11.47±0.87, 2.5±0.21 and 8.7±0.81 respectively whereas that of stem were 11.56±0.56, 2.79±0.11 and 7.01±0.27 respectively. The root contained 14.72±0.22, 7.3±0.21 and 2.6±0.27 and the fruit contained 13.47±0.77, 8.2±0.55 and 2.75±0.72 total protein, total free amino acid and total carbohydrate respectively.

Leela (2002) has reported a fatty acid *viz*., n-triacontanoic acid from hexane extract of *P. colubrinum* leaves. However, very few reports are available on primary metabolites of *P. chaba* and *P. colubrinum*.

2.3.2 Secondary metabolites

The secondary metabolites are mainly responsible for plant defense and have no direct involvement in their growth and development. Terpenes, alkaloids and phenolics are the three main classes of secondary metabolites in plants (Shamina & Sarma, 2001).

2.3.2.1 Terpenes (Terpenoids)

Terpenes are ubiquitous in plants and are considered as the largest class of secondary metabolite with more than 22,000 compounds. The high concentration of compounds in turpentine oil has given the alternate name 'terpenoid' to these compounds. All terpenes are considered to be derived from basic, branched, 5C unit, isoprene (C_5H_8). Isoprene is a volatile gas emitted at the time of photosynthesis by leaves and that may protect the plant cell membranes from damage by light or high temperature. Terpenes are essential for plant life, photosynthesis and also to regulate plant metabolisms. Though majority of the terpenoids are included in the secondary metabolite category, very few of them like steroids are considered as primary metabolites (Bramley, 1997; Goodwin & Mercer, 2003; Freeman & Beattie, 2008).

The terpenoids are synthesized from acetyl-CoA by mevalonate or isoprenoid pathway. Two acetyl-CoA molecules combined to form mevalonic acid and from which, Δ^3 - isopentenyl pyrophosphate (IPP) is formed. IPP is then converted into all the different terpenoids found in nature. The cytosol and the plastids are the site of biosynthesis of terpenoids (Waterman, 1993; Bramley, 1997; Goodwin & Mercer, 2003; Aharoni *et al*., 2006).

Based on the number of isoprene units, terpenes are classified as monoterpenes (two isoprene units), sesquiterpenes (three isoprene units), diterpenes (four isoprene units), sesterterpenes (five isoprene units), triterpenes (six isoprene units), tetraterpenes (eight isoprene units) and polyterpenes (more than eight isoprene units) (Goodwin & Mercer, 2003).

Monoterpenes are the simplest class of terpenes with a C_{10} skeleton. They are distributed widely in higher plants and have a strong characteristic smell which gives them great importance in perfumery industry. They have also applications in food flavourings and pharmacology. Monoterpenes are usually seen in secretory glands, as major component of plant essential oil. They are mainly non-volatile in nature and can further be classified as acyclic, cyclohexanoid, cyclopentanoid and irregular monoterpenes. Thujene, pinene, camphene, myrcene, limonene, careen, etc. are some of the monoterpenes (Goodwin & Mercer, 2003).

Sesquiterpenes are the largest group of terpenes with a C_{15} skeleton. They are less volatile and less organoleptic than monoterpenes. However, they also co-exist with monoterpenes and form the essential part of essential oils. Besides, they have a wide range of biological properties such as insect antifeedant, insect juvenile hormone, pheromones, phytoalexines, mycotoxins, antibiotics and plant growth regulators. The sesquiterpenes are further classified as acyclic, monocyclic, bicyclic and tricyclic sesquiterpenes. Sesquiterpenes include caryophyllene, humulene, nerolidol, elemol, γ-bisabolene, α-cadinene, etc. (Bramley, 1997; Goodwin & Mercer, 2003).

Diterpenes with C_{20} skeleton include acyclic, monocyclic, bicyclic, tricyclic or tetracyclic diterpenes. More than 500 diterpenes have been reported. Phytol, α-camphorene, cassaic acid, etc. are some of the diterpenes. The sesterterpenes with C_{25} skeleton are found along with diterpenoids in fungi, lichens, seaweeds and higher flowering plants. Ophiobolin A is an example of sesterterpene (Bramley, 1997; Goodwin & Mercer, 2003).

Triterpenes with C_{30} skeleton represents another vast group of isoprenoid compounds. It consists of tetracyclic derivatives and pentacyclic compounds. Lanosterol, cycloartenol, euphol, ursolic acid, lupeol, etc. are the examples for cyclic triterpenoids. Phytosterols with cyclopentanoperhydrophenanthrene ring system is the derivatives of tetracyclic triterpenes whereas saponins are the water soluble glycosidic triterpenes. The tetraterpenes with C_{40} skeleton consists of only one group, the carotenoid pigments (α and β carotene, xanthophylls, etc.). They are localized mainly in chloroplasts and protect it against photodynamic sensitization. They also contribute to the light harvesting of photosynthesis. Polyterpenes (e.g. natural rubber) on the other hand, is the terpenoids with higher molecular weight and are widely seen in plants (Bramley, 1997; Goodwin & Mercer, 2003).

Terpenoids play significant roles in plant-plant communication, plant-environment, plant-insect and plant-animal interactions. They have commercial value due to their wide applications in a large number of industrial products like flavouring agents, pharmaceuticals, perfumes, insecticides and antimicrobial agents (Pichersky & Gershenzon, 2002).

2.3.2.1.1 Essential oil

The aromatic plants have their own characteristic fragrance due to certain compounds which collectively form an oily product called essential oil. The essential oil, the 'living essence' of aromatic plants, is the volatile liquid extracted from their various parts. The strong aroma and volatile nature distinguishes essential oils from fatty oils. Each plant species has their own characteristic blend of volatiles which contain species specific compounds. Piperaceae, Lamiaceae, Myrtaceae, Zingiberaceae, etc. are some plant families that have their own essential oil constituents (Janardhanan & Thoppil, 2004).

Essential oil is synthesized in special secretory structures and accumulated outside the cell, between cuticle and the rest of the cell wall. The distinctive quality of essential oils is due to their chemical compositions. It is a heterogeneous, complex mixture composed of hydrocarbons of all kinds, mono and sesquiterpenoids, aromatics (benzenoids and phenylpropanoids), oxygen containing compounds, aliphatic compounds, aromatic aldehydes, ketones, alcohols, esters, ethers, lactones, complex oxides and peroxides. However, the more volatile fractions of essential oils are contributed by plant terpenes, especially, mono and sesquiterpenes (Janardhanan & Thoppil, 2004).

Considering the physical characteristics of essential oil, most mono and sesquiterpenes have optical activity whereas phenylpropenes usually do not. However, the oil as a whole has optical activity. Refractive index and specific gravity are the other physical parameters of essential oil, which are useful as indicators of oil quality (Waterman, 1993). The volatile oils are colourless or may have wide range of pale colours. The odour, another important physical parameter

of essential oil, can be classified into six major notes *viz*., woody (odour of wood like sandal wood), herbaceous (smell of green herbs like dill), fruity (odour of fruits), spicy (smell of spices), green (smell of cut grass, leaves, etc.) and flowery (odour of flowers) notes (Janardhanan & Thoppil, 2004).

Hydrodistillation using Clevenger trap apparatus is the traditional method for essential oil extraction. Upon boiling, essential oil escapes along with steam and form a separate layer in the Clevenger trap, which can be collected. This method is cheap, easy to conduct, suitable for field operation and the extracted oil will be free from oil glands/ducts/cells in the plant tissues. Steam distillation, hydrosteam distillation, solvent extraction, maceration, enfleurage and expression are the other essential oil extraction methods. Supercritical fluid extraction with supercritical CO_2 is an advanced technique for volatile oil extraction. The selection of extraction method depends on the type of plant material used and the desired end products (Janardhanan & Thoppil, 2004; Kumoro *et al*., 2010).

The essential oils are analyzed by various chromatographic techniques like Thin Layer Chromatography (TLC), High Pressure Liquid Chromatography (HPLC), Column chromatography and Gas Chromatography (GC). NMR spectroscopy is used to elucidate the structure of essential oil components and Mass spectroscopy (MS) is used for direct analysis of mixtures. Recently, GC coupled with MS (GC-MS) is used for separation, identification and quantification of essential oil constituents (Janardhanan & Thoppil, 2004).

Gas chromatography-mass spectrometry (GC-MS) is mainly used for identification of essential oil constituents. GC-MS is

also useful to determine the molecular mass of compounds, its fragmentation pattern and concentration of components in the mixture. GC involves the partition of volatile solutes between an inert gas. Once the essential oil sample is injected into the injection port of GC, the carrier gas, which act as mobile phase, carries the sample to the column, where the individual components get separated. Helium is the widely used carrier gas and capillary column is the widely used column in GC. The separated compounds are then taken into the Mass spectrometer (MS) where compounds are bombarded with sub atomic particles and mass of the charged particles produced, is determined. The bombardment causes a fragmentation pattern that is characteristic for a given compound. GC-MS system has a large library of fragmentation patterns, which facilitate easy identification of the component (Waterman, 1993; Sharma, 1995; Settle, 1997).

Apart from their role as aroma contributor, essential oil has many other functions in plants. By attracting certain insects, essential oils help in pollination. Certain volatile oils can protect plants from the attack of animals, microbes and plant parasites and also protect the plants from becoming too warm. Some essential oils help to heal the wounds and some of their constituents have allelopathic potential. Thus, they are involved in the ecology and physiology of plants (Janardhanan & Thoppil, 2004).

Essential oil is known for its application as medicaments, perfumery and flavouring agents and thus, has great importance in food, perfumery and pharmaceutical industries. They are used for perfuming soaps, deodorants, oral care products, for flavouring food, beverage products, etc. They are also used as laboratory reagents, as solvent in paint industry, as insecticides, as a component of polishes, pastes, ink,

etc. They have also use in pharmaceutical industries due to their antiseptic, carminative, stimulative, expectorant, diuretic, rubifacient, counter irritant and flavouring properties. They are also known for their disinfectant, antispasmodic, antihelmintic, bactericidal, stomachic, fungicidal and antiinflammatory activities. They are used for the manufacture of ointments, lotions and various syrups. Essential oils are also widely used in aromatherapy (Janardhanan & Thoppil, 2004).

2.3.2.1.1.1 Essential oil and its constituents in Piper species selected for the study

Researchers identified about 135 compounds from black pepper essential oil, which can be mainly categorized as monoterpenes, sesquiterpenes and miscellaneous compounds (Table 2.1).

Table 2.1. Different classes of essential oil constituents identified from black pepper

Essential oil constituents
Monoterpene hydrocarbons: Alpha-thujene, α- and β-pinene, limonene, camphene, δ-3-carene, *p*-cymene, myrcene, α-and β-phellandrene, sabinene, α- and γ-terpinene, terpinolene, etc.
Monoterpene oxygenated compounds: Linalool, α-terpeneol, *cis*- and *trans*-carveol, nerol, 1,8-cineole, α-terpenyl acetate, etc.
Sesquiterpene hydrocarbons: Beta-caryophyllene, *cis* and *trans*-bergamotene, β-bisabolene, δ- and γ-cadinene, β-and δ-elemene, γ-muurolene, α-copaene, α- and β-cubebene, α-and γ-humulene, α-guaiene, α- and β-selinene, etc.
Sesquiterpene oxygenated compounds: Caryophyllene oxide, cubenol, elemol, cubebol, γ-eudesmol, etc.
Miscellaneous compounds: eugenol, methyl eugenol, myristicin, safrole, phenylacetic acid, 2-undecanone, n-nonane, n-tridecane, n-nonadecane, etc.

(*Sources*: Hasselstrom *et al*., 1957; Ikeda *et al*., 1962; Nigam & Handa, 1964; Wrolstad & Jennings, 1965; Muller & Jennings, 1967; Muller *et al*., 1968; Richard & Jennings, 1971; Russel & Else, 1973; Debrauwere & Verzele, 1975a,b; Gopalakrishnan *et al*., 1993; Menon *et al*., 2000, 2002, 2003; Menon & Padmakumari, 2005; Zachariah *et al*., 2010).

Variability in the chemical composition of black pepper oil was reported by different researchers. It may be due to the use of oils from different cultivars/varieties, differences in the oil extraction methods, different plant parts, variation in maturity of raw material, etc. (Narayanan, 2000).

Variability in black pepper oil composition in relation to the raw material (cultivars/varieties) was demonstrated by different researchers by adopting techniques like GC and GC-MS. Lewis *et al.* (1969) extracted essential oil from 17 black pepper cultivars of Kerala by steam distillation and variability in oil constituents were studied. The yield of the essential oil ranged from 2.4 to 3.8% and it contained monoterpene hydrocarbons (69.4-85%), sesquiterpene hydrocarbons (15-27.6%) and the rest was oxygenated constituents. The major monoterpene hydrocarbons were α-pinene (5.9-12.8%), β-pinene (10.6-35.5%) and limonene (22-31.1%). The major sesquiterpene hydrocarbon was β-caryophyllene (10.3-22.4%). They have also analyzed essential oil from Sri Lankan variety and identified α-pinene (22.1%), β-pinene (11.1%), sabinene (21.3%), limonene (11.1%) and β-caryophyllene (16.6%).

Richard *et al.* (1971) extracted volatile oil components from 17 black pepper cultivars from Kerala with pentane-ether and found that the oil contained monoterpene hydrocarbons (49.5-73.3%), sesquiterpene hydrocarbons (19.8-45.1%) and oxygenated constituents (2.0-15.7%). Twelve samples of Lampong and 16 samples from Sarawak were

studied by Russel & Else (1973) for their essential oil constituents and significant differences have been established among the varieties.

Gopalakrishnan *et al.* (1993) examined four genotypes of black pepper (Panniyur-1, 2, 3 and 4) by GC-MS. The oils from the first three Panniyur genotypes contained α-pinene (5.07-6.18%), β-pinene (9.16-11.08%), sabinene (8.50-17.16%), limonene (21.06-22.71%) and β-caryophyllene (21.57-27.7%). On the other hand, oil from Panniyur-4 contained α-pinene (5.32%), β-pinene (6.40%), sabinene (1.94%), myrcene (8.40%), limonene (16.74%), *p*-cymene (9.70%) and caryophyllene (21.19%).

Zachariah (1995) evaluated 42 accessions of black pepper germplasm for essential oil constituents. Good variability was observed among the accessions, especially for pinene (3.8-16.6%), limonene (3.6-21.2%), sabinene (2.2-33%) and β-caryophyllene (11.8-41.8%). Arya *et al*. (2003) reported volatile oil content in black pepper varieties, Panniyur-6 and 7 as 1.33% and 1.50% respectively.

Seven black pepper cultivars, namely Panniyur-2, 3 and 4, Sreekara, Subhakara, KS-88 and Neelamundi were evaluated for essential oil contents. Sreekara and Subhakara recorded the highest essential oil content (7.0 and 6.0%, respectively) whereas Panniyur-4 recorded the lowest (2.1%) (Radhakrishnan *et al*., 2004).

Zachariah *et al*. (2005) reported variability in essential oil recovery (2.4-4.4%) among major black pepper cultivars and it was found to be highest in Panniyur-2 and lowest in Panniyur-5 and Balankotta. They have also reported essential oil constituents in non-grafted and grafted (on *P. colubrinum)* black pepper cultivars. The major essential oil

constituents in grafts and non-grafts of black pepper cultivars include pinene, sabinene and β-caryophyllene. Beta caryophyllene varied from 12-27% in graft and 7-29% in non-graft.

The chemical composition of pepper oil also depends on the method of oil extraction. The black pepper oil extracted by steam distillation usually contains about 70-80% monoterpene hydrocarbons, 20-30% sesquiterpene hydrocarbons and less than 4% oxygenated compounds (Lewis *et al*., 1969). On the other hand, essential oil obtained by vacuum distillation of pepper oleoresin extracts contains less monoterpene hydrocarbons and relatively more sesquiterpene hydrocarbons and oxygenated compounds (Eiserle & Rogers, 1972; Richard, 1972; Salzer, 1975b). The constituents from essential oil of *P. nigrum* and *P. longum* extracted by MD-HS-SPME (microwave distillation and headspace solid-phase micro extraction) and conventional HS-SPME were identified and compared. The results showed that microwave distillation had a high extract efficiency and good precision (Liu *et al*., 2007). The black pepper oil obtained by supercritical CO_2 extraction and hydrodistillation was subjected to oil profiling by GC-FID and GC-MS. The main constituents in oil obtained by both extraction methods were β-caryophyllene, sabinene, limonene, 3-carene, β-and α-pinene. Some of these constituents showed variability with regard to the extraction methods (Bagheri *et al*., 2014).

Variability in oil constituents of *P. nigrum* in relation to different plant parts was also investigated. The essential oils from *P. nigrum* berries (green and black) and pepper leaves were identified and compared by GC and GC-MS. The major compound in both pepper berry oils was β-caryophyllene followed by β-pinene, limonene, α-pinene and

humulene. The green pepper oil has more oxygenated compounds compared to black pepper oil. The main compounds in pepper leaf oil include α-bisabolol, α-cubebene, elemol, bisabolene and α-guaiene (Sasidharan & Menon, 2010).

The volatile oils of leaves and berries of different black pepper cultivars collected from Panniyur and Peruvannamuzhi, Kerala, was evaluated by Zachariah *et al*. (2010). The oil recovery was in the range of 0.1-0.3 mL/100 g for leaves and 1.6-6.0 mL/100 g for berries. The oil constituents were identified by gas chromatography (GC). Germacrene-D and elemol were the major constituents in leaf essential oil whereas β-caryophyllene was high in berries. Berry oil constituents like sabinene, pinene, limonene and myrcene were not detected in the leaf volatile oil.

By capillary GC, GC-MS and HPLC methods, previously unreported, nitrogen-containing compounds (pyridine, piperidine, methylpyrazine, 2,6-dimethylpyridine, 2-isopropylpyridine, 2-heptylpyridine, etc.) were also identified from black pepper oil (Clery *et al*., 2006).

The essential oil extracted from long pepper (0.95%) was subjected to oil profiling by Shankaracharya *et al*. (1997). Forty eight compounds were identified and it includes 8-10% oxygenated compounds and 35-40% straight chain hydrocarbons. Beta-caryophyllene (17%), pentadecane (17.8%) and β-bisabolene (11.16%) were the major compounds. Heptadecane, spathulenol, globulol, pentadecene, germacrene B and D, α-zingeberene, α-humulene, β-farnesene, α-copaene, tridecane, etc. were the other compounds detected in considerable quantities. Myrcene, α- and β-pinene, *p*-cymene,

α-phellandrene, linalool, camphor, β-selenene, γ-muurolene, etc. were also identified in minute quantities.

Upon steam distillation, 0.7-1.5% essential oil was extracted from *P. longum* and oil constituents were identified. The constituents include: n-hexadecane (0.7%), n-heptadecane (6.0%), n-octadecane (5.8%), n-eicosane (4.7%), n-heneicosane (2.5%), α-thujene (1.7%), terpinolene (1.3%), zingeberene (7.0%), dihydrocarveol (4.3%), phenethyl alcohol (2.1%) and *p*-methoxyacetophenone (trace) (Jayasinha, 1999).

P. chaba essential oil was also subjected to its profiling by researchers. By GC-MS analysis, 93 compounds were identified from leaf essential oil of *P. chaba.* Terpinolene (33.03%), β-pinene (8.23%), δ-cadinol (6.86%), cedren-13-ol (4.77%), γ-elemene (4.90%), cyclosativene (3.43%) and nerolidyl acetate were the main oil constituents identified (Bhuiyan *et al*., 2010).

Essential oil was extracted from the air dried leaves of *P. chaba* by hydrodistillation and the oil constituents were identified by GC-MS. The oil yield was 0.31% and 54 constituents were identified. Alpha humulene (16.4%), caryophyllene oxide (12.2%), globulol (7.4%), veridiflorol (8.1%), spathulenol (6.2%), β-selinene (7.1%), *trans*-nerolidol (5.1%), 3-pentanol (3.5%), linalool (4.5%), tricyclene (2.2%) and *p*-cymene (1.6%) were the major compounds. Farnesol (1.1%), citronellyl acetate (1.0%), α-selinene (1.1%), 1, 8-cineole (0.8%), β-pinene (0.9%), α-pinene (0.5%) and camphene (0.3%) were also identified as minor compounds (Rahman *et al*., 2011).

A monoterpene ester, bornyl piperate, has been reported for the first time from the root of *P. chaba* by Naz *et al*. (2012) whereas β-sitosterol has been identified from *P. chaba* stem by Mishra & Tewari (1964).

Comparative essential oil profiling of these *Piper* species is also reported. The oil profiling of *P. nigrum* from Malaysia, *P. chaba* from Thailand and *P. longum* from Indonesia were performed with GC and GC-MS. Unlike black pepper oil, *P. longum* and *P. chaba* contained few monoterpene hydrocarbons, moderate sesquiterpenes and high content of aliphatic hydrocarbons (Tewtrakul *et al*., 2000).

Gas chromatography-mass spectrometry following microwave distillation and headspace solid phase micro extraction was developed for analysis of essential oils from *P. nigrum* and *P. longum*. Thirty compounds were identified from black pepper, of which, β-caryophyllene (23.49%), D-limonene (18.68%), 3-carene (22.20%), β-pinene (8.92%) and α-pinene (4.03%) were predominated. Among 45 compounds identified from *P. longum,* β-caryophyllene (33.44%), Δ^3-carene (7.58%), eugenol (7.39%), D-limonene (6.70%), zingiberene (6.68%) germacrene-D (3.41%) and cubenol (3.64%) were the major compounds (Liu *et al*., 2007).

Leaf and fruit essential oils from *P. longum* and *P. chaba* were identified and compared by Rameshkumar *et al*. (2011). *P. longum* leaf oil was rich in phenylpropanoids with apiole (50%) and myristicin (26.9%) as the major compounds whereas sesquiterpene hydrocarbons, especially β-caryophyllene (28.60%), α-humulene (22.8%) and germacrene D (14.6%), were predominant in *P. chaba* leaf oil. Aliphatic hydrocarbons, mainly n-pentadecane (15.8%), predominated

in the fruit oil of *P. longum* whereas sesquiterpenes hydrocarbons like β-caryophyllene (18.5%), germacrene D (21.5%) and α-humulene (11.4%) predominated in the fruit oil of *P. chaba*.

The essential oil of some *P. colubrinum* samples showed that its chemical composition is entirely formed by terpenoids (Maia & Andrade, 2009). Beta-sitosterol and sitosterol-3-O-β-D glucopyranoside have been reported in hexane extract of *P. colubrinum* leaves (Leela, 2002). Ursolic acid, a pentacyclic triterpenoid has been identified by Chandra *et al.* (2015), from fruit of *P. longum* and also from leaf and fruit of *P. nigrum* and *P. colubrinum*.

2.3.2.2 *Alkaloids*

Alkaloids represent another large group of secondary metabolites. More than 2000 alkaloids have been isolated from plants. All alkaloids contain nitrogen and most of them are basic in nature. The biosynthetic precursors of alkaloids are almost always amino acids. Other multi carbon units like acetate are also incorporated into the final structure of some alkaloids. The alkaloids with heterocyclic rings are commonly known as true alkaloids and those without such rings are called protoalkaloids. The alkaloids (both with and without heterocyclic rings) that are not derived from amino acids are called pseudoalkaloids, in which, carbon skeleton is derived from isoprenoids. Besides, steroidal alkaloids are also present and which include, cholestane derivatives, pregnane derivatives and C-nor-D-homosteroidal alkaloids (Goodwin & Mercer, 2003).

Alkaloids have many biochemical and physiological roles in plants. They act as poisonous agents to protect the plants from insects and

herbivores, as end products of detoxification reactions, as nitrogen reserve and growth regulators. They also help to maintain ionic balance by virtue of their chelating power (Goodwin & Mercer, 2003).

The alkaloids have great importance in pharmaceutical industries due to their various pharmacological activities like antiamoebic, anticholinergic, antimalarial, antihypertensive, cardiac depressant, central stimulant and antitumour properties (Bhat *et al*., 2009).

2.3.2.2.1 *Piperine*

Piperine, a piperidine alkaloid, is the major compound responsible for pungency of black pepper. It also contributes pungency to *P. longum* and *P. chaba.* It is a needle shaped or short rod shaped, yellow or white, crystalline substance, with a molecular formula of $C_{17}H_{19}O_3N$ and molecular weight of 285.34 g/mol. The melting point and density of piperine was found to be 128-130^{o}C and 1.193 g/cm^3 respectively (Zachariah & Parthasarathy, 2008; Wadhwa *et al*., 2014).

Piperine is synthesized from the amino acid L-lysine. The piperidine, formed from L-lysine, reacts with piperoyl-CoA, in the presence of the enzyme piperidine piperoyltransferase to give piperine (Okwute & Egharevba, 2013). Piperine (*trans, trans*-5-(3,4-methylenedi-oxyphenyl)-2, 4-pentadienoic acid piperidide) was first isolated by Oersted (1820). Piperine is shown to be a weak base (Zachariah & Parthasarathy, 2008). Pure piperine degraded quickly under UV lamp exposure (half-life of about 40 min) and it indicated that photolysis is mainly responsible for piperine degradation (Scott *et al*., 2004).

Piperine exists in four different isomeric forms, which include *trans-trans* piperine (piperine), *cis-trans* piperine (isopiperine), *cis-cis*

piperine (chavicine) and *trans-cis* piperine (isochavicine). The *trans-trans* piperine is believed to be the main constituent for the pungency of black pepper. The other three isomers are formed by the light induced and/or enzyme catalyzed isomerization of the parent piperine compound (Kozukue *et al*., 2007). Some researchers reported that chavicine is more pungent than piperine whereas others proved that piperine in a solution is highly pungent (Buchheim, 1876; Verzele *et al*., 1979). This leads to a controversy regarding the pungency of the four isomers. However, later research demonstrated that piperine is the major pungent principle of black pepper and chavicine is the mixture of piperine and several other alkaloids (Purseglove *et al*., 1981).

In addition to its contribution to pungency, piperine is reported to possess several beneficial biological and pharmacological properties which include, antimicrobial (Hiwale *et al*., 2002; Khan *et al*., 2006), anticarcinogenic (Selvendiran *et al*., 2004; Sunila & Kuttan, 2004; Bezerra *et al*., 2006), antidiarrhoeal (Bajad *et al*., 2001a), antidepressant (Lee *et al*., 2005; Li *et al*., 2007), insecticidal (Scott *et al*., 2005), antioxidant (Vijayakumar *et al*., 2004; Vijayakumar & Nalini, 2006), antiinflammatory (Hu *et al.,* 2015) and antiulcer (Bajad *et al*., 2001b) properties. Piperine is reported to enhance the bioavailability of various nutrients and drugs and their efficacies *in vivo* (Bano *et al*., 1991; Shoba *et al*., 1998; Pattanaik *et al*., 2006) and also to modulate the permeability characteristics of intestine, by altering the membrane dynamics (Khajuria *et al*., 2002). The effectiveness of piperine to treat vitiligo disease was investigated by Vinod *et al*. (2011) and formulated a piperine based cream for that disease. The efficacy of piperine, isolated from black pepper, to inhibit

the corrosion of C38 steel in 1M HCl solution was reported by Dahmani *et al*. (2010).

Piperine comprises about 95% of the total pungent alkaloids present in black pepper. Thus, the estimation of piperine content is a good measure for the pungency of this spice (Purseglove *et al*., 1981). Different methods are adopted for piperine analysis and these include Kjeldahl method (Rogers, 1966), spectrophotometric and colorimetric methods (Lupina & Cripps, 1987), reverse phase HPLC method (Wood *et al*., 1988), NMR spectroscopy (Catchpole *et al*., 2003), vibrational spectroscopy (Schulz *et al*., 2005), TLC, LC-MS (Scott, *et al*., 2005) and GC-MS (Marutoiu *et al*., 2006). HPLC is the widely accepted method for identification and quantification of piperine content, along with other pungent alkaloids (Purseglove *et al*., 1981).

High performance liquid chromatography (HPLC) is a technique for the separation, identification and quantification of non-volatile compounds. The pump, column, detector and computer are the major components of HPLC. It involves the injection of a small volume of liquid sample into a column, which is packed with tiny particles of about 3-5 μm in diameter (stationary phase). The individual components of the sample move down the column with a liquid (mobile phase), that is forced through the column by high pressure delivered by a pump. The components in the sample are separated by the column through various chemical and/or physical interactions between these molecules and the packing particles. The separated components are then detected at the exit of column by a 'flowthrough' device (detector). The main detection principles used to detect the compounds eluting from an HPLC column include spectroscopic detection, refractive index detection and fluorescence detection. The

liquid chromatogram (output from the detector) is thus formed and that can be obtained in output devices like computer (http://polymer.ustc.edu.cn/xwxx_20/xw/201109/P020110906263097048536.pdf).

2.3.2.2.2 Piperine content in Piper species selected for the study

Seven black pepper cultivars, namely, Panniyur-2, 3 and 4, Sreekara, Subhakara, KS-88 and Neelamundi, were evaluated for piperine content and the highest value (6.6%) was recorded for Panniyur-2 (Radhakrishnan *et al*., 2004). Sasikumar *et al*. (2004) reported high piperine content in HP-813 (IISR Malabar Excel) in comparison with the other recently released black pepper cultivars, IISR Thevam and IISR Girimunda. Varietal variation of piperine content has also been reported by Kurian *et al*. (2002) in black pepper from Idukki District, Kerala. Arya *et al*. (2003) reported piperine content in black pepper varieties (Panniyur-6 and Panniyur-7) as 4.94% and 5.57% respectively. Piperine content in different black pepper cultivars grafted on *P. colubrinum* and also in non-grafts has been reported by Zachariah *et al*. (2005). Among the different non-grafted (2.79-3.87%) and grafted (2.7-4.44%) cultivars, piperine content was found to be highest in Sreekara. The piperine content of leaves and berries of black pepper cultivars from Panniyur and Peruvannamuzhi was also evaluated by Zachariah *et al*. (2010) and revealed that piperine content was in the range of 0.00006-0.008 g% for leaves and 1.6-4.2 g% for berries. Dutta & Bhattacharjee (2015) reported that α-amylase-assisted, supercritical carbon dioxide extraction of black pepper significantly increased the yield and phytochemical properties of piperine-rich extracts.

Shankaracharya *et al.* (1997) reported 1.25% piperine from *P. longum.* Jayasinha (1999) reported that piperine content of *P. longum* fruit increases with maturity from 14-16 days (0.53%) to 40-45 days (0.9%). Piperine content of fresh and old *P. longum* was also compared as 0.76 and 3.26% respectively by Vinay *et al*. (2012).

Rameshkumar *et al*. (2011) reported that piperine content in *P. longum* fruit was lower (0.03%), compared to that of *P. chaba* (1.32%). By RP-HPLC method, Santosh *et al*. (2005) quantified the piperine from fruit and root of *P. longum* and fruit of *P. nigrum* as 0.879%, 0.31% and 4.5% respectively whereas it was quantified in black pepper and *P. longum* as 1.2% and 1.58% respectively by Trivedi *et al*. (2011). Hamrapurkar *et al*. (2011) also extracted piperine from black pepper and *P. longum* by supercritical fluid extraction and soxhlet extraction methods. The extracted piperine was quantified by HPLC. The yield of piperine obtained by supercritical fluid extraction was 8.76% and 4.96% in *P. nigrum* and *P. longum* respectively whereas in soxhlet extract, the piperine yield for black pepper was 8.13% and that for *P. longum* was 4.32%. Piperine content from *P. nigrum, P. longum* and *P. chaba* has also been reported by Rao *et al*. (2011) as 3.5661%, 0.0011% and 1.1219% respectively.

Chandra *et al.* (2015) reported piperine content in the fruit of *P. nigrum, P. chaba* and *P. longum* as 7220±0.62 µg/g, 5530±3.12 µg/g and 2150±1.23 µg/g respectively. They have also quantified 4.67±0.07 µg/g of piperine content in the leaf of *P. colubrinum* and also reported that the piperine in the fruit of *P. colubrinum* was below detectable limit.

2.3.2.3 *Phenolics*

Phenolics are ubiquitous secondary metabolites in plants and they occur as free phenols or their glycosides. This class of compounds consists of more than 8000 biologically active compounds which range from simple phenolic molecules to polymeric structures with molecular weight of above 30000 Da (Marinova *et al*., 2005). They include simple phenols, phenolic acids, phenylacetic acids, hydroxycinnamic acids, phenylpropenes, coumarins, quinones, stilbenes, xanthones, lignans, neolignans, melanins, tannins and flavonoids. All the phenolics, except flavonoids, are synthesized from phenylalanine, tyrosine or its close precursor, shikimic acid. The conversion of phenylalanine to cinnamic acid by the elimination of an ammonia molecule, catalyzed by phenylalanine ammonia lyase (PAL), is the key step for the biosynthesis of phenolic compounds (Goodwin & Mercer, 2003).

Simple phenols are not widely distributed and the most common simple phenol is hydroquinone. The phenolic acids are widely distributed throughout the plant kingdom. Protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid and syringic acid are some of the phenolic acids. Aldehyde or alcohols corresponding to the parent carboxylic acids (e.g. vanillin) are also known. Very little is known about the distribution of phenylacetic acids in plants, but, 2- and 4-hydroxyphenylacetic acids were reported. The hydroxycinnamic acids like *p*-coumaric acid and caffeic acid, and their methylated derivatives like ferulic acid and synapic acid, are universally present in higher plants. Phenylpropenes are not widely distributed, but they occur sporadically in essential oils (e.g.myristicin). Coumarins are lactones, derived from *o*-hydroxycinnamic acid and occur abundantly

in plants. The quinones mainly include benzoquinones, naphthoquinones and anthraquinones, whereas, lunalaric acid is the most common plant stilbene. Over 70 xanthones were reported in plants and their distribution is restricted to Guttiferae and Gentianaceae families (e.g. mangiferin) (Goodwin & Mercer, 2003).

The lignans are group of naturally occurring phenylpropanoid dimers in which, the two C_6-C_3 units are joined tail-to-tail by carbon-carbon bond between the middle carbons of their side chains (e.g.conidendrin). If the C_6-C_3 units are joined head-to-tail, the resultant phenolics are called neolignans (e.g.eusiderin). Melanins are brown or black, naturally occurring pigments. Melanin, characteristics of plants are called catechol melanins, since they yield catechol on alkali fusion. The tannins are group of high molecular weight chemical compounds with tanning properties and mainly include hydrolysable, condensed and pseudo tannins (Goodwin & Mercer, 2003; Bhat *et al*., 2009).

The flavonoids are the largest group of naturally occurring phenolic compounds, and occur in different plant parts both in free form (aglycones) and as glycosides. These coloured compounds are also known as co-pigments and are responsible for various colours exhibited by different plant parts. The flavonoid glycosides are found to be occurring as O-glycosides and C-glycosides, where the latter is less common. The structure of flavonoid consists of two parts, *viz*., ring A with six carbon atoms and phenylpropanoid moiety (C_6-C_3). The flavonoids can be classified into the following categories. Flavones (contains benzo-γ-pyrone ring; e.g. apigenin), flavanone (2, 3 dihydroderivative of flavones; e.g. naringenin), flavonol (3-hydroxy derivative of flavones; e.g. kaempferol), dihydroflavonol (3-hydroxy

derivative of flavanone; e.g. taxifolin), isoflavones (contain benzo-γ-pyrone ring with phenyl substitution at position 3 of the pyrone ring; e.g. genistein), chalcones (open chain flavonoids; e.g. butein) and dihydrochalcones (dihydro derivatives of chalcones; e.g. phloretin). They also include the aurones (possess the 2-benzylidene-coumaranone skeleton; e.g. sulphuretin), anthocyanins (glycosides of anthocyanidins; e.g. pelargonidin), leucoanthocyanidins (have flavan-3,4-diol skeleton) and biflavonoids (dimers of various flavonoids) (Goodwin & Mercer, 2003).

The phenolic compounds have wide range of medicinal potentials like antioxidant (Chen & Ho, 1997), antidiabetic (Adisakwattana *et al*., 2008), antimicrobial (Narasimhan *et al*., 2004) and antiinflammatory (Sun *et al*., 2007) activities.

Various techniques including HPLC, GC-MS and LC-MS are widely used for the identification and quantification of phenolic compounds (Justesen *et al*., 1997; Proestos *et al*., 2006). Liquid chromatography-mass spectrometry (LC-MS) is the technique used for identification, quantitation and mass analysis of compounds. It permits the structural elucidation of unknown compounds through fragmentation. As in HPLC, LC-MS utilizes a compound's intrinsic affinity for both mobile phase and stationary phase. The mobile phase is liquid, usually a buffered solvent and the stationary phase is porous solid support with specialized coating. The pump is used to provide a continuous flow of a solvent, into which, a dissolved sample is introduced. When the sample is in the solvent flow, it travels through an analytical column and the compounds present in the sample mixture are then separated based on their affinity towards the coated particles in the column. After the separation of each components of the sample, they will pass

through a mass detector. The mass detector response and retention time of the compound of interest are then compared to a reference standard and thus, the unknown compounds can be identified (http://www.chemir.com/liquid-chromatography-mass-spectrometry.html). Mass spectrometer (MS), the detector of LC-MS, operates by ionizing molecules and then identifying the ions as per their mass-to-charge (*m/z*) ratios. The ion source, which generates the ions and the mass analyzer, which sorts the ions, are the two key components in this process. The MS can thus generate mass spectral data, which can provide information about the molecular weight, quantity, structure, identity and purity of a sample. Electrospray ionization (ESI) is one of the commonly used ion sources, whereas, Time-of-flight, Quadrupole, Ion trap and Fourier transform-ion cyclotron resonance are the most often used mass analyzers (Pitt, 2009; http://ccc.chem.pitt.edu/wipf/Agilent%20LC-MS%20primer.pdf).

2.3.2.3.1 Phenolics in Piper species selected for the study

Nine phenolic acids including hydroxybenzoic acids and hydroxycinnamic acids, along with considerable quantities of quercetin and kaempferol, were obtained from black pepper (Bandyopadhyay *et al*., 1990). Gallic acid, trans-*p*-feruloyl-a-D-glucopyranoside, trans-*p*-sinapyl-a-D-glucopyranoside, quercetin 3-*O*-R-L-rhamnopyranoside-7-*O*-a-D-glucopyranosyl, quercetin 3-*O*-R-L-rhamnopyranoside, luteolin 6-C-a-D-glucopyranoside-8-C-R-L-arabinopyranoside, luteolin 7-*O*-[2-(a-D-apiofuranosyl)-a-D-glucopyranoside-8-C-R-L-arabinopyranoside, luteolin 7-*O*-[2-(a-D-apiofuranosyl)-4-(a-D-glucopyranosyl and coumarins were also reported in black pepper (Al-Shahwany, 2014). Salicylic acid, protocatechuic acid, ferulic acid, vanillic acid, syringic acid, gentisic acid, guaiacol, 4-hydroxybenzoic

acid and synapic acid were the other phenolic compounds reported from *P. nigrum* (Swain *et al*., 1985; Variyar & Bandyopadhyay, 1994; Jagella & Grosch, 1999a; Chatterjee *et al*., 2007). Parmar *et al*. (1997) listed the flavones (isorhamnetin-3-*O*-*β*-D-rutinoside, isoquercitrin, kaempferol 3-*O*-arabinoside-7-rhamnoside, 3-*O*-*β*-D-rutinoside, kaempferol-3-*O*-*β*-glucoside, quercetin 3-*O*-*β*-D-rutinoside, quercetin 3-*O*-*β*-D-galactoside quercetin 3-*O*-*β*-D-rhamnoside and rhamnetin-*O*-triglucoside) and other phenolic compounds (caffeic acid, *p*-coumaric acid, *p*-and *m*-cresol) from black pepper.

Chandra *et al.* (2015) identified quercetin in the leaf and protocatechuic acid, ferulic acid, vanillic acid, rosamarinic acid, apigenin and kaempferol in leaf and fruit of *P. nigrum.* The lignans, *viz.,* cubebin, 3,4-dimethoxy-3,4-dimethylenedioxycubebin and 3',4'-dimethoxy-3',4'-dimethylenedioxycubebin and the neolignan piperenone were also reported in *P. nigrum* (Grewe *et al*., 1970; Parmar *et al*., 1997).

Total phenolic content of leaves and berries of seven black pepper varieties *viz.,* Karimunda, OP Karimunda, Panniyur-1, P 24, HP 813, Coll. 1041 and HP 105 has been reported by Sumeshkumar (2004) as 1.245-2.35% (lowest in P 24 and highest in Karimunda) and 1.24-1.73% (lowest in P 24 and highest in Coll. 1041) respectively. The total phenolic and flavonoid contents in the methanolic extract of black pepper were reported to be 1.728 mg/g and 1.087 µg/g, respectively (Ahmad *et al.,* 2015).

Dutt *et al*. (1975) isolated three lignans, *viz*., sylvatin, (+)-diaeudesmin and sesamin from the seeds of *P. longum.* Sesamin, pluviatilol, fargesin and (+)-asarinine were the lignans reported from

P. longum fruits (Parmar *et al*., 1997; Kumar *et al*., 2011). Das *et al*. (1998) isolated two long chain esters (tridecyl-dihydro-*p*-coumarate and eicosanyl-(E)-*p*-coumarate) and Kumar *et al*. (2005) isolated 3',4',5'-trimethoxycinnamate from *P. longum* fruit.

Gentisic acid, syringic acid, melilotic acid and apigenin were the phenolics reported in *P. longum* by Mammen & Daniel (2014). Bisdemethoxycurcumin and demethoxycurcumin were identified from the chloroform fraction of ethanolic extract of *P. longum* by Liu *et al*. (2009). Vanillic acid and quercetin (from the leaf), protocatechuic acid, ferulic, rosamarinic acid and apigenin (from fruit and leaf) were also identified from *P. longum* (Chandra *et al.,* 2015).

Total phenolic content in methanolic extract of leaf, root and fruit of *P. longum* was reported as 24. 27± 0.008, 1.26±0.013 and 0.04±0.010 mg/gm respectively and total flavonoid content of them were reported as 0.39±0.014, 3.27 ±0.020 and 0.45±0.011 mg/gm respectively (Rami *et al*., 2013).

The lignan sylvatin was reported in the root of *P. chaba* (Patra & Ghosh, 1974). Through NMR and Mass spectral evidences, Bhandari *et al*. (1998) identified a new lignan (epimers of [8*R*,8′*R*]-9-hydroxy,3,4-dimethoxy,3′,4′-methylene dioxy-9,9′ epoxy lignin) from chloroform soluble fraction of alcohol extract of *P. chaba.* The lignin kusunokinin was also reported in *P. chaba* (Parmar *et al.,* 1997). Haribabu *et al*. (2014) identified two epimeric furofuran lignans *viz*., asaranin and sesamin from *P. chaba* fruit by HPTLC-MS method. Chandra *et al.* (2015) identified protocatechuic acid, caffeic acid, luteolin and quercetin from the fruit, ferulic and vanillic acids from the

leaf, rosamarinic acid and apigenin from both fruit and leaf of *P. chaba*.

Leela & Pillai (2008) had reported two flavones *viz*., 5, 4'-dihydroxy-7-methoxy-flavone and 5, 3'- 4'-trihydroxy-7-methoxy-flavone from chloroform extract of *P. colubrinum* leaves. Phenolic acids like protocatechuic acid, ferulic acid, rosamarinic acid, vanillic acid and flavonoids like luteolin, kaempferol and apigenin were identified in detectable quantities from the fruit and leaf of *P. colubrinum* by Chandra *et al.* (2015). They have also identified caffeic acid and quercetin in its leaf and fruit respectively.

2.3.3 Oleoresin

The extraction of plant material with suitable solvent and the subsequent evaporation of solvent yields a residue called oleoresin. It functions as a flavouring agent in the food processing industry and is important for the colour, taste, texture and antioxidant properties of the product. Various solvents can be used for the extraction of oleoresin, which may be polar or non- polar. The oleoresin consists of all the volatile and non volatile components soluble in the extracting solvent. The volatile components include the essential oil constituents, that are responsible for the flavouring and aromatic properties of the spice. The non-volatile components of the oleoresin include carotenoids, steroids, anthocyanins, alkaloids, glycosides, fixed oils, natural resins, etc. The spice oleoresins are capable of reproducing their respective characteristics and have given a close approximation to the total flavour of spices. They are heat stable and extremely powerful in their flavouring effect. They are cleaner, more economical and easy to control the quality than the equivalent ground spices (Boelens &

Boelens, 2000; Shamina & Sarma, 2001). The solvent extracted oleoresins are used as such and in encapsulated form and both are substitutes for the whole or ground spice (Prajapati *et al*., 2003).

The black pepper oleoresin is a viscous, dark green, heavy liquid with strong aroma and on standing, piperine crystals appear. Acetone, alcohol and ethylene dichloride have been used as solvents to optimize the extraction of active constituents from black pepper (Purseglove *et al*., 1981). It is reported that 1 kg of pepper oleoresin equals 20-25 kg of ground pepper. Black pepper yields 10-12% oleoresin, of which 20-30% is volatile oils and 40-45% is piperine (Shankaracharya & Natarajan, 1975; Shamina & Sarma, 2001).

Nambudri *et al*. (1970) reported oleoresin yield as 10-13% for Indian Malabar pepper. Zachariah *et al*. (2005) reported oleoresin content in different black pepper cultivars grafted on *P. colubrinum* and also in non-grafts. In non-grafted (6.9-12.77%) and grafted (7.39-10.57%) cultivars, oleoresin content was found to be highest in Panniyur-2.

The oleoresin content of seven black pepper cultivars, namely Panniyur-2, 3 and 4, Sreekara, Subhakara, KS-88 and Neelamundi were evaluated by Radhakrishnan *et al*. (2004) and found that, Neelamundi, KS-88 and Sreekara showed the highest (13.9, 13.1 and 13.0% respectively) and Panniyur-4 showed the lowest (9.2%) oleoresin content. Sasikumar *et al*. (2004) reported high oleoresin content in HP-813 (IISR Malabar Excel) in comparison with other recently released black pepper cultivars, IISR Thevam and IISR Girimunda. Varietal variation in oleoresin content has also been reported by Kurian *et al*. (2002) in black pepper grown at Idukki District, Kerala. Arya *et al*. (2003) reported that, black pepper variety

Panniyur-6 contained 8.27% and Panniyur-7 contained 10.61% oleoresin. The oleoresin content of berries of black pepper cultivars from Panniyur and Peruvannamuzhi was evaluated by Zachariah *et al*. (2010) and was in the range of 5.9-13.9 g%.

2.4 PHYSICO-CHEMICAL CHARACTERIZATION

The physical quality parameters of black pepper, like the size of the berries, colour, bulk density (426-850 g/L), moisture content (9.73%) and chemical parameters like volatile ether extract (3.1%), non volatile ether extract (4.4%), alcohol extract (10.8%), total ash (4.55%) and acid insoluble ash (0.08%) were reported by Pruthi (1980, 1993).

Ash and acid insoluble ash of black pepper were also reported by Trivedi *et al*. (2011) as 4.5% and 0.45% respectively and by Ahmad *et al.* (2015) as 4.31±0.32% and 0.48±0.44% respectively. Physical properties like true density (kg m^{-3}), bulk density (kg m^{-3}), porosity (^{o}) and spherecity (%) of black pepper were reported as 987.71-1012.24, 542.71-556.85, 43.62-46.38 and 94.22-95.08 respectively (Meghwal & Goswami, 2012).

Black pepper varieties (Panniyur-1, Panniyur-2, Panniyur-5, Sreekara and Subhakara) were graded with rotary sieve having perforations of sizes 3.5, 3.8 and 4.8 mm diameter. The result showed that black pepper variety Subhakara belongs to TGSEB (Tellicherry Garbled Special Extra Bold) grade, whereas, Panniyur-1, Panniyur-2 and Panniyur-5 were under the grade TG (Tellicherry Garbled) and variety Sreekara in MG (Malabar Garbled) grade. The bulk density of these varieties was also checked and it varied from 450-571 g/L. Bulk density increased with increase in size. However, it decreased when the

berry size was > 4.8 mm (Jayashree *et al*., 2009). By studying the moisture isotherm and thermodynamic properties of whole black peppercorn, Yogendrarajah *et al.*(2015) revealed that it can be stored at 22, 30 and 37^{o}C, by reducing their moisture content to 10, 8 and 7 g/100 g respectively, with water activity below 0.60 a_w.

Ash and acid insoluble ash of *P. longum* fruit were reported as 4.31 and 0.41% respectively (Trivedi *et al*., 2011). The ash value of fresh and old *P. longum* was also reported by Vinay *et al*. (2012) as 2.5 and 5.5% respectively. Total ash content in stem and root of *P. longum* (10.5% and 9.2% respectively) and its acid insoluble ash (0.8% and 0.56% respectively) were reported by Nitin *et al*. (2012). However, reports are scanty on physico-chemical properties of *P. chaba* and *P. colubrinum* fruits.

2.5 MINERAL/NUTRIENT ANALYSIS OF SELECTED *PIPER* SPECIES AND BLACK PEPPER GROWING SOILS

Minerals (nutrients) are necessary for growth, development and yield of the plants and their influence depends on their ratios in the soil as well as in the plant system. Most of the plants obtain them through their roots, mainly from the soil. So, soil must be maintained with adequate nutriens, which are necessary for proper growth, chemical composition and health of the plants. Fertilizers especially rich in N, P, K and Ca are nowadays widely under practice for soil nutrient management (Srinivasan *et al*., 2012).

Atomic absorption or emission spectrophotometry is the most convenient technique for quantifying such elements, both in the plant

tissues and soil (Sutcliffe & Baker, 1974). Atomic absorption spectrophotometry (AAS) is an analytical technique that measures the concentrations of chemical elements, by measuring the radiation absorbed by the element of interest. This is performed by reading the spectra produced while the sample is excited by radiation. The technique makes use of the wavelengths of light specifically absorbed by an element. The atoms absorb ultraviolet light or visible light and make transitions to higher energy levels. Thus, atomic absorption method measures the amount of energy, in the form of photons of light that are absorbed by the sample. The light source, a sample cell to produce gaseous atoms and detector are the three major components of AAS. A hollow cathode lamp, with a tungsten anode and a cylindrical hollow cathode, made of the element to be determined, is the common light source in AAS. Aspiration and electrothermal atomization are the two commonly used systems for atomization of samples. The specific wavelength of light is selected by a monochromator and directed to the detector, which is usually a photomultiplier tube. The detector produces an electrical signal proportional to the light intensity. The detector measures the wavelengths of light transmitted by the sample and compares them to the wavelengths, which originally passed through the sample. A signal processor then integrates the changes in wavelength absorbed, which appear in the readout as peaks of energy absorption at discrete wavelengths. The concentration of element is calculated based on the Beer-Lambert law. Absorbance is directly proportional to the concentration of the analyte absorbed for the existing set of conditions. The concentration is usually determined from a calibration curve, obtained using standards of known concentration

(http://www.kau.edu.sa/Files/130002/Files/6785_AAs.pdf; http://cdn.intechopen.com/pdfs-wm/26275.pdf).

A moist, well drained soil with water holding capacity, rich in organic matter and essential plant nutrients is required for the optimum growth and yield of black pepper. It can grow in forest loam soils, red loam and laterite soils and coastal sand soils. Poor soil fertility, low level of inputs like fertilizers and manures, will result in various diseases of black pepper and their low productivity (Srinivasan *et al*., 2012). The studies at ICAR-Indian Institute of Spices Research (IISR) revealed that, proper nutrient management of black pepper nursery is essential for healthy plants and thereby giving high establishment rate and growth in the field (Sadanandan, 2000).

The soil and leaf nutrient data obtained from the surveys of major black pepper growing tracts in Kerala and Karnataka revealed that, the order of limiting nutrients was organic carbon>Zn>P>Ca>K>Mg for soil and Mg>Cu>P=K=Zn>Mn for leaf samples (Hamza *et al*., 2007). Hamza *et al*. (2004) collected pepper growing soils from low and high altitudes and based on the yield, these samples were categorized as high, moderate and low yielding gardens. They found that, nutrients from the collected soil samples like organic carbon (1.24-1.32%), P (2.1-8.5 ppm), K (126-254 ppm), Ca (226-1850 ppm), Mg (49-516 ppm), Fe (23-48 ppm), Mn (3.8-40 ppm), Zn 0.51-5.1 ppm) and Cu (1.7-3.4 ppm) showed variability among locations. The investigation also revealed that, soils of high yielding gardens showed high pH, organic carbon, P, K, Ca, Mg and Zn and thus, these factors favoured good black pepper growth with high productivity.

Srinivasan *et al*. (2012) reported that, among the major black pepper nutrients studied, N uptake was highest followed by K and Ca and among micronutrients, uptake of Fe was the highest. It is reported that, higher levels of N adversely affected the yield of Panniyur-1 hybrid and it is not necessary to increase N level beyond 50 g $vine^{-1}$ $year^{-1}$ (Pillai *et al*., 1979). Pillai *et al*. (1987) worked out an optimum K dose of 200g of K_2O $vine^{-1}$ for Panniyur-1 variety whereas Sadanandan (1993) optimized a dose of 270 Kg K_2O ha^{-1} for high yield. Different black pepper cultivars showed significant differences in utilization of nutrients. The cultivar Ampirian utilizes more K and N than the other black pepper cultivars (Sadanandan, 2000).

The major black pepper growing area of Kerala and Karnataka were surveyed by Sadanandan & Hamza (1992) and revealed that, the black pepper yield was correlated with Fe (r=0.55*) and Cu (r=0.41*) in healthy gardens. The yield was also correlated with leaf Fe in both healthy and diseased gardens and also with leaf Mn and leaf Cu in diseased gardens. Sadanandan (2000) listed the desired level of nutrients in the soil and these include soil pH (6-7), organic matter (2.5%), P (18), K (150), Ca (1500), Mg (300), S (20), Fe (10), Mn (10), Zn (1.6), Cu (0.6), Mo (0.3) and B (0.8). All the nutrient values are given in mg/kg.

The N concentration in various parts of black pepper plant was reported by Azmil & Yau (1993) which includes leaves (2.3%), branches (2.07%), stem (1.96%), fruit spikes (2.21%) and flower (2.11%). Black pepper requires large quantities of K for growth and fruiting. Potassium requirement depends on the content of other nutrients, mainly N in the plant, whereas P availability by plants depends on fixation and release in the soil, soil pH and organic matter

(Srinivasan *et al*., 2012). Zinc, Molybdenum and Boron are the most important micronutrients for black pepper nutrition (de Waard, 1969). Kato (1978) reported that, black pepper adult plant requires 90 g N, 10 g P, 120 g K, 80 g Ca and 11 g Mg to grow and produce fruits.

Christensen *et al*. (1968) revealed variability in different black pepper samples for macro and micronutrients by emission spectroscopy and these include Ca (0.34-0.43%), K (0.85-1.5%), Na (0.005-0.019%), P (0.16-0.17%), Mg (0.095-0.20%), Ba (2.0-35 ppm), Al (48-237 ppm), Fe (39-270 ppm), Sr (1.8-36 ppm), Cu (8.8-14 ppm), B (4-13 ppm), Mn (21-75 ppm), Zn (5.2-14 ppm) and Cr (<3-<5 ppm). Veloso & Carvalho (1999) have quantified the macronutrients in different black pepper parts. The leaves contained 23.5, 2.10, 14.2, 21.7 and 4.3 g/kg, fruit contained 22.8, 2.18, 12.5, 7.8 and 2.4 g/kg, root contained 22.4, 1.34, 5.1, 20.9 and 7.6 g/kg, plagiotropic branches contained 15.2, 1.62, 11.3, 8.9 and 1.9 g/kg and orthotropic branches contained 18.3, 1.63, 16.9, 11.5 and 2.9 g/kg of N, P, K, Ca and Mg respectively.

Neelam & Kamala (2001) reported minerals (mg/100g) like Ca-1230, P-190 and Fe-62.1 from *P. longum* fruit. However mineral data of *P. chaba* and *P. colubrinum* fruits are scanty.

2.6 PROXIMATE COMPOSITION OF *PIPER* SPECIES SELECTED FOR THE STUDY

The proximate composition of black pepper is listed in Table 2.2

Table 2.2. Proximate composition of black pepper

Component	Quantity/100g
Energy	255 Kcal
Moisture content	8.7-14%
Total ash	3.4-6.0 g
Acid insoluble ash	0.03-0.55%.
Non-volatile ether extract	3.9-11.5%
Volatile ether extract	0.3-4.2%
Alcohol extract	4.4-12.0%
Carbohydrates	65.75 g
Starch	28-49%
Glucose	0.35% Dry Matter (DM)
Sucrose	0.40% DM
Fructose	0.07% DM
Protein	10.9-12.7 g
Crude fibre	8.7-18%
Fat	10.2 g
Piperine	4.9-7.7%
Essential oil	1.0-1.8%
Niacin	1.142 mg
Riboflavin	0.109 mg
Thiamin	0.109 mg
Vitamin A	299 IU
Vitamin C	21 mg
Vitamin E	4.56 mg

Component	Quantity/100g
Vitamin K	163.7 µg
Sodium	44 mg
Potassium	1259 mg
Calcium	437 mg
Copper	1.127 mg
Total nitrogen	1.55-2.6%
Magnesium	194 mg
Manganese	5.626 mg
Phosphorus	173 mg
Zinc	1.42 mg
β-Carotene	156 µg

(*Sources*: Pruthi, 1993; Tainter & Grenis, 1993; Pradeep *et al.,* 1993; Nelson & Cannon-Eger, 2011**;** de Waard & Anunciado, 1999; Gutierrez *et al*., 2013; http://spices.indianetzone.com/1/black_ pepper.htm)

Proximate composition of *P. longum* has been reported by Shankaracharya *et al*. (1997) on moisture free basis (%) and which includes: moisture content-7.52, volatile oil by Clevenger method-0.95, volatile oil by steam distillation-0.40, total ash-5.2, acid insoluble ash-0.85, crude fibre-6.8, crude starch-40.5, crude protein-11.94, reducing sugars-5.07, mucilage-2.08, piperine-1.25, acetone extract-9.95, non-volatile ether extract-6.53 and oleoresin yield-7.84. Proximate composition of *P. longum* was also listed by Jayasinha (1999) on moisture free basis (%) and which include: moisture content-9.5, protein-12.2, starch-39.5, fibre-5.8, total ash-5.9, insoluble ash-4.2, volatile oil-0.7-1.5, fixed oil-6.6 and piperine-4.5. The proximate composition of *P. longum* was also given by Srivastava (2012) as moisture-11.0±0.04%, ash-6.4±0.03%, fat-17.6±0.02%, crude fibre-

4.5±0.03%, protein-1.30±0.02%, carbohydrate-59.2±0.03% and total phenol-22.8 mg/g.

2.7 VARIABILITY IN BLACK PEPPER CONSTITUENTS IN RELATION TO LOCATION

Twenty three types of black pepper from Kerala, North and South Kanara, Coorg and Assam were analyzed and variability was found for moisture content (8.7-14.1%), total nitrogen (1.55-2.60%), non volatile ether extract (3.9-11.5%), volatile ether extract (0.3-4.2%), alcohol extract (4.4-12.0%), total ash (3.6-5.7%), acid insoluble ash (0.03-0.55%), starch (28-49%), crude fibre (8.7-18%) and piperine (1.7-7.4%). The piperine content was found to be high in Kerala types and low in North Kanara types of black pepper.The Assam types had low moisture content and, high alcohol and ether extracts (Pruthi, 1993).

Salzer (1975b) proposed the variability in Sarawak, Tellicherry, Malabar, Brazilian and Lampong black pepper oils on the basis of the relative abundance of β-pinene, sabinene, δ-3-carene, limonene and caryophyllene. Politeo *et al*. (2006) identified essential oil constituents from black pepper berries purchased from local market of Croatia by GC-MS. Beta-caryophyllene was the major oil constituent (57.6%). Sabinene, α-pinene, limonene, α-terpinolene, α-terpinene, α-phellandrene, germacrene B and D, α-humulene, γ-elemene, etc. were the other compounds identified.

Variability in physical and biochemical characters of black pepper samples collected from India, Indonesia, Vietnam and Malaysia was studied by Thomas (2009). The Indian black pepper showed

superiority for the physical properties like bulk density and weight of 100 berries. All the black pepper from Indonesia and one each from Vietnam and Malaysia showed more oleoresin and essential oil content than the Indian black pepper. Vietnam and Malaysian black pepper showed slight variation in β-caryophyllene (10.5-12.78%) whereas the percentage of α-phellandrene was comparatively higher in Malaysian black pepper. Nerolidol was identified only in Indian black peppers. Piperine content showed higher values for Vietnam/Indonesian produces (4.2-4.75%).

Essential oils extracted by hydrodistillation of green and black pepper berries (cultivar Thevanmundi) of Indian origin were subjected to GC and GC-MS analysis and the constituents obtained were compared with the reported constituents from Sri Lankan green and black pepper essential oils. The monoterpene hydrocarbons from Indian oils were similar to those of corresponding Sri Lankan oils, but the oils differed in relation to their sesquiterpene and oxygenated compounds (McCarron *et al*., 1995). The essential oils from dried fruits of black pepper collected from Cameroon were extracted by solid-phase micro extraction (SPME) and the aroma compounds were identified by GC-FID and GC-MS. The main compounds included: limonene (10.26%), germacrene D (11.01%), β-pinene (10.02%), α-pinene (6.40%), α-phellandrene (8.56%), β-caryophyllene (7.29%) and *cis*-β-ocimene (3.19%) (Jirovetz *et al*. 2002).

Kumoro *et al*. (2010) analyzed black pepper sample from Sarawak (Malaysia) for its physical and chemical constituents and it includes: oleoresin (with hexane)-10.6 wt%, piperine-5.8 wt%, essential oil-1.7 (v/w) %, water content-11 wt% and bulk density-793 kg/m^3.

The black pepper from Saudi Arabia was subjected to proximate analysis by Al-Jasass & Al-Jasser (2012). The result indicated that, black pepper has the following chemical composition (%): moisture content-4.68±0.3, crude fat-5.34±0.6, crude protein-25.45±0.4, crude fibre-23.6±0.3, ash-3.57±0.1 and total carbohydrate-37.36. Its mineral content (mg/100g) includes: K-663±25.0, Mg-52.0±8.0, Ca-195.0± 15.0, Zn-0.9±0.1, Mn-3.5±0.2, Cu-1.3±0.1 and Fe-20.5±0.5.

The leaves of 180 black pepper accessions were collected from different regions of Kerala and Karnataka and subjected to total phenol, chlorophyll and carotene content analysis to study the spatial influence on these biochemical components using GIS tool. The results indicated a clear latitudinal variation in phenol concentration. The studies also revealed a wide variability in leaf chlorophyll and carotene contents in a narrow geographical range (Parthasarathy *et al*., 2008a).Thirty five black pepper accessions were collected from 35 locations of the Western Ghats of Karnataka and Kerala and the oil profiling of their leaves were performed with GC. The results were plotted in a map using Arc-GIS software to understand the influence of location. Among the 7-15 compounds detected from volatile oils in different accessions, maximum variability was obtained for β-caryophyllene and nerolidol and the influence of location on these components was significant (Parthasarathy *et al*., 2008b).

Three black pepper varieties *viz*., Hainan variety (Sample A), Batangas variety (Sample B) and unidentified variety (Sample C), grown at Galay's Farm, Kidapawan, Philippines were subjected to variability studies. Sample C showed highest pungency, oleoresin (15.18%) and volatile ether extract (2.71%) whereas sample B showed lowest. However, sample B had the highest amount of total nitrogen (2.49%),

acid insoluble ash (1.32%), total ash (4.77%), extraneous matter (0.95%), crude fibre (16.51%) and light berries (33.64%). Sample A had the highest non volatile ether extract (7.14%) (Buenaflor *et al*., 2008).

One black pepper variety from Bangladesh and one from India were subjected to essential oil extraction and the extracted oils were compared for their physico-chemical properties and oil constituents. There was not much variation in their organoleptic properties and solubility characters. But they showed variation in refractive index, specific gravity, acid value, optical rotation and ester value. They have reported great variation in the chemical composition of these two black pepper oils. Eighteen chemical constituents, with a total monoterpene of 65.421% and sesquiterpene of 34.579%, were obtained for Bangladesh oil. On the other hand, 14 chemical constituents were obtained from the Indian black pepper oil, with a total monoterpene of 77.673% and sesquiterpene of 14.195% (Aziz *et al*., 2012).

2.8 MEDICINAL AND PHARMACOLOGICAL POTENTIAL

2.8.1 Antioxidant potential

Free radicals produced during metabolism are a health concern. In the present day living style, free radicals and resultant oxidative stress are one of the major issues which can cause a great number of problems. A highly reactive and unstable molecule with one or more unpaired electron in its outer shell is termed as free radicals. Free radicals at moderate or low levels exhibit beneficial effects on cellular responses and immune functions. However, at high levels, free radicals produce a state of oxidative stress that could damage cellular structures and thus,

leads to the development of chronic and degenerative diseases like cancer, aging, arthritis, cardiovascular disorders, autoimmune and neurodegenerative diseases (Pham-Huy *et al*., 2008). Free radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and lipid peroxyl (LOO•) radicals and among them, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important (Pham-Huy *et al*., 2008; Halliwell & Gutteridge, 2007). Though the oxygen that we breathe is a non reactive chemical, its exposure to high energy or electron transferring chemical reaction converts it to various, highly reactive, chemical forms collectively termed as ROS (Rodriguez & Redman, 2005). Oxidative stress is the term used when oxidants outnumber the antioxidants due to excessive generation of ROS, and, when antioxidants cannot scavenge these free radicals (Sharma *et al*., 1999).

The human body has several mechanisms to prevent this oxidative stress by producing antioxidants which are either endogenous (naturally produced *in situ*) or exogenous (externally supplied through foods or supplements) (Pham-Huy *et al*., 2008). Antioxidants have the unique ability to neutralize the free radicals and create stable molecules, and thus, protect body from various diseases. Antioxidants can remove radical intermediates and thereby terminate the chain reactions triggered by free radicals. They can also inhibit other oxidation reactions by being oxidized themselves (Sies, 1997).

The endogenous antioxidants include enzymatic and metabolic antioxidants. The superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRx) and glutathione peroxidase (GPx) are the major antioxidant enzymes. Superoxide dismutase, the first line of defense against free radicals, is responsible for the dismutation of

superoxide anion radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) by reduction. The H_2O_2 is converted into water and oxygen by catalase or glutathione peroxidase (GPx). The GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase regenerates GSH from GSSG with NADPH, a source of reducing power. Besides H_2O_2, GPx can also reduce lipid or non lipid hydroperoxides, while oxidizing glutathione (Pham-Huy *et al*., 2008). Glucose-6-phosphate dehydrogenase and glutathione-S-transferase are the other endogenous antioxidant enzymes (Noori, 2012; Pham-Huy *et al*., 2008). The metabolic antioxidants produced as a part of metabolic reactions include glutathione, melatonin, L-ariginine, coenzyme Q10, uric acid, bilirubin, metal-chelating proteins, etc. (Pham-Huy *et al*., 2008).

The exogenous antioxidants like nutrient antioxidants (vitamin E, vitamin C and carotenoids), natural antioxidants (phenolic acids and flavonoids) and trace metals (selenium, manganese, zinc, etc.) must be supplied through diet to neutralize the free radical load in our body (Pham-Huy *et al*., 2008). Beta-carotene is an excellent antioxidant for scavenging singlet O_2. Vitamin C can directly interact with hydroxyl and superoxide radicals whereas vitamin E can prevent the oxidative damage of poly unsaturated fatty acids in the membrane (Rao, 2003). The phenolic acids, isoflavones, flavonoids, flavones, coumarins, anthocyanins, lignans, isocatechin, catechin, gallic acid, esculatin, etc. are the natural antioxidants present in several herbs, spices, vegetables and fruits (Noori, 2012). They can act as effective free radical scavengers by donating hydrogen to unstable and highly reactive free radicals. The studies thus revealed that vegetables, spices, fruits, etc. have potent antioxidant activity since they have high content of

antioxidant polyphenols and other phytochemicals and thus, the increased consumption of them can lower the risk of oxidative stress disorders (Bunea *et al*., 2012; Palozza *et al*., 2012; Singh *et al*., 2008). The trace metals like manganese, zinc and selenium are responsible for the enhancement of the endogenous antioxidant enzyme activities (Noori, 2012). Synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and propyl gallate are commonly used to control lipid oxidation in foods but are suspected to cause liver damage, cancer and other diseases (Ito *et al*., 1986; Safer & al-Nughamish, 1999). So, nowadays the interest for finding antioxidants from natural source has been increased tremendously to replace the synthetic antioxidants.

2.8.1.1 Antioxidant activity studies on Piper species selected for the study

Several investigations revealed that black pepper and its compounds, especially phenolic amides, have good antioxidant property. The antioxidant effectiveness of this spice was reported in 1955 by Chipault *et al*. Later, Revankar & Sen (1974) stated that, the antioxidant property of black pepper is due to its polyphenolic content whereas Saito & Asari (1976) reported that, antioxidant activity of black pepper is due to its tocopherol content. Nakatani *et al*. (1986) reported very good antioxidant property for five phenolic amides of *P. nigrum* and also reported that, their activity was superior to that of synthetic antioxidants, BHA and BHT.

The antioxidant efficacy of black pepper and the alkaloid piperine in rats with high-fat-diet-induced oxidative stress has been studied by Vijayakumar *et al*. (2004). The result indicated that, supplementation

with black pepper or piperine can reduce high-fat-diet-induced oxidative stress. The antioxidant potential of essential oil and oleoresin of black pepper extracted by supercritical carbon dioxide extraction has been reported by Tipsrisukond *et al*. (1998) whereas antioxidant activity of black pepper essential oils has also been reported by Dorman *et al*. (2000). Ethanol and water extracts of black pepper were prepared and their antioxidant activity was checked and compared with synthetic antioxidants. Results showed that both the extracts have good antioxidant activity and radical scavenging ability against various *in vitro* antioxidant systems. Water extract showed higher activity in all assays except ferric reducing ability (Gulcin, 2005).

Methanolic extracts of green and black pepper collected from USA and white pepper from France were reported to have antioxidant activity of 11.15±0.007, 4.56±0.013 and 8.97±0.007 mmol of trolox equivalent/100 g of dry weight, respectively. They have also reported that volatile oils and phenolic amides were the major contributors to its antioxidant activity (Shan *et al*., 2005).

The antioxidant activity of essential oil extracted from black pepper has been reported by Politeo *et al*. (2006) and compared its activity with essential oils from other eleven species. They have reported good DPPH scavenging ability for black pepper oil (14-61% inhibition for 5-50 g/L). Its antioxidant activity was also checked by Ferric reducing antioxidant power (FRAP) method (1-11 mmol/L for 5-50 g/L), Thiobarbituric acid reactive species (TBARS) assay (16-36 Antioxidant Index% for 5-50 g/L) and Rancimat test (0.9 Antioxidant Activity Index for 50 g/L).They have also found that antioxidant activity of pepper oil was lesser than that of clove, basil, laurel, coriander and nutmeg but higher than that of mint, marjoram,

cinnamon, sage and fennel. The antioxidant activity of hydrolyzed and non hydrolyzed extracts from black and white peppers were compared by Agbor *et al*. (2006) and the result revealed that, black pepper extracts have more polyphenols and thus, more effectively scavenges the free radicals than white pepper extracts. Khalaf *et al*. (2008) reported that methanolic extract of black pepper showed DPPH radical scavenging activity with an IC_{50} value of 144.1±2.2 µg/mL.

The free radical scavenging activity of ethanolic extract of black pepper from Brazil was investigated by DPPH radical and ABTS radical cation decolorization assay. They have reported an EC_{50} value of 110±0.2 g spice/kg DPPH (DPPH assay) and a TEAC value of 5.7±0.2 mM/g spice (ABTS radical assay) for ethanolic extract of black pepper (Mariutti *et al*., 2008).

The essential oil, ethanol and ethyl acetate extracts of black pepper showed strong antioxidant activity by Ferric thiocynate method, DPPH radical scavenging activity and FRP method (Kapoor *et al*., 2009). By analyzing the antioxidant activity of water extract from thermally treated black pepper, Nikousaleh & Prakash (2009) reported that thermal treatment increased its antioxidant efficacy.

The black pepper purchased from local market of Mysore was powdered and extracted with methanol and methylene chloride. The extracts were subjected to antiradical and antioxidant analysis and the results showed that black pepper extracts exhibited significant radical scavenging and antioxidant potential in all the systems tested (Krishna *et al*., 2010). The antioxidant activities of essential oil from black (BPEO) and white pepper (WPEO) were evaluated by superoxide anion, hydroxyl radical scavenging activities, phosphomolybdenum

method, reducing power method and inhibition of lipid peroxidation in linoleic acid. The results clearly indicated that both possess a marked antioxidant activity, however, BPEO was better than WPEO (Ying *et al*., 2009). Ahmad *et al*. (2010) reported the antioxidant activity of regenerated tissues of black pepper and concluded that the activity may probably due to flavonoids and other phenolic contents.

The dried black pepper berries were powdered and extracted successively with water, ethanol and methanol. Each extract was subjected to DPPH radical scavenging activity and ethanol extract showed highest scavenging activity (74.61±0.02%) followed by methanolic extract (63.84±0.05%) and aqueous extract (39.92±0.02%) (Nahak & Sahu, 2011). Shanmugapriya *et al*. (2012) proved the antioxidant efficacy of ethyl acetate, acetone and water extracts of dried black pepper leaves by adopting different antioxidant assays.

Antioxidant capacity of crude water and ethyl acetate extracts of black pepper was tested by DPPH and FRAP methods and found that ethyl acetate extract showed more activity than water extract and both showed concentration dependent increase in activity (Asimi *et al*., 2013). The antioxidant activity of black pepper extracts obtained by super critical fluid extraction (SFE) with different temperature and pressure was compared with that obtained by soxhlet extraction and ultrasound solvent extraction with ethanol. The results showed that the extracts obtained through ethanol as the solvent, has given the best results (Andrade & Ferreira, 2013). The antioxidant efficacy of piperine, purified from ethanolic extract of black pepper, and piperic acid, synthesized from alkaline hydrolysis of purified piperine, were compared with that of chloroform, ethyl acetate, methanol, ethanol and water extracts of black pepper by DPPH assay, reducing power

method, β-carotene-linoleic acid assay and phosphomolybdenum assay. Results indicated that piperic acid was superior for antioxidant efficacy by all the systems tested (Zarai *et al*., 2013).

Antioxidant activity of methanol and water extracts of black pepper alone and in combination with tea, ginger and tulsi were checked by DPPH radical scavenging method. Black pepper extracts alone showed 35.2% and 45.66% DPPH radical activity for water and methanol extract respectively, and in combination, their activity was found to be increased (Gupta *et al*., 2014). Radical scavenging activity of the black pepper essential oil obtained by supercritical CO_2 extraction and hydrodistillation was compared by Bagheri *et al*. (2014) and results showed that, essential oil obtained by supercritical CO_2 extraction had more DPPH radical scavenging activity (EC_{50}-103.28 µg/mL) than those obtained by hydrodistillation (EC_{50}-316.27 µg/mL).

The antioxidant activity of black pepper was also reported by other researchers (Singh *et al*., 2004; Suhaj *et al*., 2006; Su *et al*., 2007; Singh *et al*., 2008; Gacche *et al*., 2010; Jain *et al*., 2011; Ranjan *et al*., 2011; Bai *et al*., 2011; Madhu *et al*., 2012; Pingili *et al*., 2012; Hritcu *et al*., 2014; Aliakbarlu *et al*., 2014).

P. longum also showed promising antioxidant potential against free radical-induced oxidative damage. The antioxidant activity of ethanol and water extracts of leaf samples of *P. longum* plantlets developed through micropropagation technique were carried out by DPPH assay. The result revealed more radical scavenging activity for its ethanolic extract (Parida & Dhal, 2011). The antioxidant efficacy of methanolic extract of *P. longum* was reported on Wistar rats with Adriamycin-induced cardiotoxicity and isoproterenol-induced myocardial

infarction. They have helped to retain the activity of endogenous antioxidant enzymes in rats, which is lowered due to Adriamycin-induced cardiotoxicity and isoproterenol-induced myocardial infarction (Wakade *et al*., 2008; Wakade *et al*., 2009).

The effect of storage conditions on free radical scavenging activities of *P. longum* was studied and the result indicated that the samples of long term studies had better antioxidant activity and higher total phenolic content than the samples of accelerated and real time studies (Srivastava *et al*., 2011). Powdered *P. longum* sample was extracted in succession with ethyl acetate, methanol and water by soxhlet apparatus and the extracts were subjected to antioxidant activity by FRAP method. Result revealed that water extract was more potent for its antioxidant activity followed by methanol and ethyl acetate extracts (Sawhney *et al*., 2011).

In vitro antioxidant activity of ethyl acetate, hexane, chloroform, ethanol, hydro-ethanol and aqueous extracts of *P. longum* seeds were evaluated by DPPH radical, hydroxy radical and nitric oxide scavenging activity, reductive ability assay and ABTS assay. The result indicated highest antioxidant activity for its chloroform extract (Barua *et al*., 2014). Reddy *et al*. (2014) reported powerful antioxidant activity for aqueous extract of *P. longum* fruit and also for silver nanoparticles synthesized using aqueous extract of *P. longum* fruit.

The *in vitro* antioxidant activity of ethanolic extract from *P. longum* has been reported by Samudram *et al*. (2009) and Ramesh *et al*. (2011) and that of hexane, ethyl acetate and water extracts by Joy *et al*. (2010), petroleum ether extract by Jagdale *et al*. (2009), hydro-

alcoholic extract and its fractions by Chaudhary *et al*. (2013) and methanolic extract by Veeru *et al*. (2009) and Srivastava (2012).

The antioxidant activity of methanol and water extracts from the fruits of *P. chaba, P. longum* and *P. nigrum* has been investigated by DPPH radical scavenging assay. Results showed that the methanolic extracts of these *Piper* species exhibited significant antioxidant activity with LD_{50} values of 47.8, 45.1 and 48.7 µg/mL respectively, followed by their aqueous extracts (LD_{50}: 57.6, 69.4 and 56.9 µg/mL respectively). They have also reported that volatile oils of these species were almost inactive for their antioxidant efficacy (Tewtrakul, 1998).

A combination of spices (*P. longum, P. nigrum* and *Z. officinale*), herbs (*P. zeylanica* and *C. rotundus*) and salts, used to make up *Amrita Bindu,* was tested for antioxidant activity. The antioxidant potential of the ingredients was in the following order: *P. nigrum* > *P. longum* > *C. rotundus* > *P. zeylanca* > *Z. officinale* (Natarajan *et al*., 2006).

A comparative antioxidant activity study was conducted on methanolic extract of *P. longum* and *P. chaba* by Rameshkumar *et al*. (2011) and found that *P. longum* showed high activity. Likewise, a comparative antioxidant and radical scavenging activity of methanolic extracts of *P. nigrum* and *P. longum* along with other *Piper* and *Clerodendrum* species were also carried out by reducing power, total antioxidant capacity and DPPH assays. Black pepper showed highest antioxidant activity (reducing power assay) and DPPH radical scavenging ability followed by *P. longum,* whereas, in total antioxidant capacity assay, *P. longum* showed highest activity followed by *P. nigrum* (Prasad & Sushant, 2014).

P. chaba extracts were not explored much for their antioxidant activity studies. Likewise, information is scanty regarding the antioxidant potential of *P. colubrinum*.

Ascorbic-acid, camphene, eugenol, carvacrol, γ-terpinene, β-carotene, lauric acid, linalyl acetate, myrcene, methyl eugenol, myristicin, myristic acid, palmitic acid, terpinen-4-ol, piperine and ubiquinone were some of the antioxidant compounds isolated from black pepper fruit (Suhaj, 2006).

A hot, pungent compound chabbarin was isolated from acetone extract of *P. chaba* stem and its antioxidant activity was checked by DPPH assay. The result revealed that the compound is a potent DPPH radical scavenger with very low IC_{50} value (3.0 µg/mL) (Biswas *et al.*, 2012). Mishra (2010) reported significant DPPH radical scavenging ability for piperaldehyde, isolated from long pepper.

The free radical scavenging ability and antioxidant activity of phenolic compounds (salicylic acid, 4-hydroxybenzoic acid, gallic acid, gentisic acid, vanillic acid, protocatechuic acid, syringic acid, caffeic acid, synapic acid, apigenin, coumaric acid, luteolin, ferulic acid, quercetin, kaempferol, etc.) indentified from these *Piper* species were also reported by researchers (Romanova *et al*., 2001; Karamac *et al*., 2005; Wang *et al*., 2006; Chatterjee *et al*., 2007; Jiang *et al*., 2013).

2.8.2 *In vitro* cytotoxicity and anticancer activity

Cytotoxicity is the cell-killing property of a chemical compound or a mediator cell, independent from the mechanisms of death (Rode, 2008). Compounds that can thus inhibit the proliferation of cancer cells are used to treat cancer.

Cancer is characterized by uncontrolled division of cells and has the ability to invade other tissues, either by direct growth into adjacent tissues through invasion or by implantation into distant sites by metastasis. Occasionally, dividing and differentiating cells deviate from their normal genetic programme and give rise to tissue tumours or neoplasm. This process is known as transformation. If the transformed cells stay together in a single mass, the tumour is said to be benign and if the tumour cells can invade and disrupt surrounding tissues, it is called malignant and is identified as cancer (Chatterjea & Shinde, 2005; Karp, 2007). The critical events that lead to cancer include the loss of proliferative control, the failure to undergo apoptosis, the onset of neoangiogenesis, tissue remodeling, invasion of tumour cells into surrounding tissues and finally metastatic distribution of tumour cells to distant organs (Herzig & Christofori, 2002).

The causative agents of cancer are termed as carcinogens and these mainly include genetic (DNA damage or gene mutation), chemical (tobacco smoking, alcohol intake, improper diet, long term use of certain drugs, etc.), physical (exposure to UV rays, X rays, polluted air, etc.) and biological (DNA or RNA virus) factors. The oncogenes, tumour-suppressor genes and stability genes are the three types of genes whose changes cause cancer (Halliwell & Gutteridge, 2007; Sakarkar & Deshmukh, 2011).

Cancers due to chemical and environmental carcinogens can be effectively prevented through education and social policies. The cancers due to infections by pathogens can be prevented by known interventions like vaccines, antibiotics, etc. Through regular screening and examination, many cancers can be diagnosed and cured easily at the early stages of their development. Chemotherapy, radiotherapy,

surgery, immunotherapy, hormonal therapy, etc. are the main methods for cancer treatment (Anand, 2013). Recently, complementary and alternative medicine (CAM) is becoming a popular treatment for various cancers. CAM is a group of diverse medical and healthcare systems, practices and products that are not presently considered to be a part of conventional medicine. Herbal medicine is one of the methods among CAMs to treat cancer (Cassileth, 1999: Yildirim, 2010).

For many years, cancer chemotherapy was dominated by potent drugs. But their toxicity is not limited to cancer cells and normal cells are also harmed (Baxevanis *et al*., 2009). So, efforts to develop less toxic drugs that can affect only cancer cells and a mechanism based approach is necessary in cancer therapy. To attain this goal, numerous phytochemicals have been investigated for their chemopreventive potential and numerous scientific studies support herbal medicine as a potent anticancer drug (Buchanan *et al*., 2005; Kwon *et al*., 2009).

The measurement of cell viability and growth is a common method used in assessing the cytotoxicity of drugs on cancer cells. A variety of assays have been developed to measure the cellular sensitivity towards the drug. It involves the isolation of cancer cells and their incubation with drugs. It is followed by the assessment of surviving cells (*in vitro* cytotoxicity). Cells exposed to a cytotoxic compound can respond in a number of ways. The cells may undergo necrosis (cell lose membrane integrity and die rapidly as a result of cell lysis), they can stop growing and dividing or they can activate apoptosis. There are many ways to measure cytotoxicity, most involve assessment of cell membrane integrity. Membrane integrity can be evaluated by using vital dyes like trypan blue, by protease biomarkers, by MTT or MTS redox potential assays, or by measuring ATP content. Many of these assays involve

colorimetric, fluorescence or luminescence detection (Rode, 2008; http://www.moleculardevices.com/applications/areas-research/cytotoxicity). The cell distribution by cytotoxic drugs follows the first order kinetics. Thus, it reduces a constant percentage and not a constant number of cancer cells (Vasudevan & Sreekumari, 2005). *In vitro* cytotoxicity is considered as the preliminary analysis for the anticancer activity of various drugs, phytochemicals, plant extracts, etc.

Cervical cancer, the third most common cancer among women worldwide**,** starts in the cell lining the cervix (especially in the cells in the transformation zone of the cervix). The cervix is the lower part of the uterus and is the connection between the body of the uterus and vagina. The squamous cells and the glandular cells are the two main types of cells that cover the cervix. Adenocarcinoma and squamous cell carcinoma are the two main types of cervical cancer, of which, about 80-90% of cervical cancers are squamous cell carcinomas. Rarely, cervical cancers have features of both squamous cell carcinomas and adenocarcinomas and are called adenosquamous carcinomas or mixed carcinomas (American Cancer Society, 2014).

The most important risk factor for cervical cancer is the Human Papilloma Virus (HPV) infection. HPV is a group of more than 150 related viruses and among this, two-third of all cervical cancers are caused by HPV 16 and 18 (American Cancer Society, 2014). Infection caused by Human Papilloma Virus (HPV) of any type accounts for 82% of cervical cancers in developed countries and 91% in developing countries (Sakarkar & Deshmukh, 2011). Smoking, Human Immunodeficiency Virus (HIV), Chlamydia infection, overweight, diet low in fruits and vegetables, use of intrauterine device, long-term use

of oral contraceptives (birth control pills), having multiple full-term pregnancies, being younger than 17 at the first full-term pregnancy, hereditary, poverty etc. are the other risk factors of cervical cancer (American Cancer Society, 2014).

Abnormal vaginal bleeding, an unusual discharge from the vagina, pain during sex, etc. are the major signs and symptoms of cervical cancer. Since the most common form of cervical cancer starts with pre-cancerous changes, there are two ways to prevent this from developing. One way is to find and treat pre-cancers before they become true cancers and the other is, to prevent the pre-cancers in the first place. Surgery, radiation therapy, chemotherapy and targeted-therapy are the main treatment methods for cervical cancer and the selection of the treatment method depends on the stage of the disease (American Cancer Society, 2014). HeLa, CaSki, HtTA-1, HR5, C-4I, Bu25-TK, etc. are the important cervical cancer cell lines. CaSki cell lines, epithelial by morphology, are the epidermoid cervical cancer cells. It can secrete the β-subunit of human chorionic gonadotropin (β-hCG), express tumour-associated antigen and Glucose-6-Phosphate Dehydragenase type B. CaSki cells have been reported to contain an integrated Human Papilloma Virus 16 genome and HPV-18 related sequences (Pattillo *et al*., 1997; http://www.sigmaaldrich.com/catalog/product/sigma/87020501?lang=en&region=IN).

2.8.2.1 Anticancer studies on Piper species selected for the study

Black pepper essential oil is reported for antiproliferative effect on murine leukemia and human mouth epidermal carcinoma cell lines (Manosroi *et al*., 2006) and its extracts were reported to inhibit tumour formations in experimental models (Lin *et al*., 2007). The cytotoxicity

of different *P. nigrum* extracts was analyzed against HL60 (Human promyelocytic leukemia cells) by Lim *et al*. (2009) and found that petroleum ether (IC_{50}-11.2 µg/mL) and chloroform extracts (IC_{50}-9.8 µg/mL) were bioactive against HL-60 cell line but the ethyl acetate extract had no activity.

The alcoholic and water extracts of black pepper were tested against esophageal squamous cell carcinoma (TE-13) and found that aqueous extract was more potent than ethanolic extract **(**Dwivedi *et al*., 2011). Yen *et al*. (2012) reported that essential oil from black pepper was not cytotoxic at the concentration of 200 µg/mL for different cancer cell lines including human liver (Hep 3B and Hep G2), lung (A549) and breast (MCF-7 and MDA-MB-231) cancer cell lines. *In vitro* cytotoxicity of black pepper oil in combination with methanolic extract of *Terminalia arjuna* and volatile oil of cumin were also reported against lung cancer cell line A-549 (Pingili *et al*., 2012).

The methanolic and dichloromethane extracts from black pepper were checked for cytotoxicity against different breast cancer cell lines (MCF-7, MDA-MB-468, MDA-MB-231 and MCF-12A) by MTT assay for 72 hrs. Result indicated that methanolic extract showed highest cytotoxicity for MCF-7 (IC_{50}-20.25±0.01 µg/mL) and MDA-MB-231 (IC_{50}-22.37±2.31 01 µg/mL) whereas dichloromethane extract showed highest cytotoxicity for MDA-MB-468 (IC_{50}-7.94±4.52 µg/mL) and MCF-12A (IC_{50}-35.65±0.27 µg/mL) (Sriwiriyajan *et al*., 2014).

The anticancer activity of black pepper was also reported by other researchers (Liu *et al*., 2010; Majdalawieh & Carr, 2010).

P. longum also showed efficacy against various cancers. Sunila & Kuttan (2004) reported anticancer effect of alcohol extract of *P. longum* fruit on Dalton's lymphoma ascites and Ehrlich ascites carcinoma cells. The chemopreventive effect of ethanolic extract of *P. longum* fruit on 7,12-dimethyl benz(a)anthracene (DMBA) induced oral carcinogenesis was reported by measuring lipid peroxidation and antioxidants status (Senthil *et al*., 2007). The hot ethyl acetate and cold hexane: water (1:1) extracts of *P. longum* were tested for *in vitro* cytotoxicity against leukemic cell line K562. The extracts showed dose- dependent cytotoxicity and the antitumour activity was found to be higher for hot extract than that of cold extract (Joy *et al*., 2010).

The anticancer activity of ethyl acetate, methanol and water extracts of *P. longum* fruits were checked against lung epithelial adenocarcinoma cell line HCC-827. All the extracts showed a dose-dependent antitumour activity and methanol extract was found to be superior (Sawhney *et al*., 2011). Kumar *et al*. (2011) reviewed that the alcohol extract of *P. longum* (10 mg/dose/animal) and piperine (1.14 mg/dose/animal) inhibits solid tumour development in mice, induced with Dalton's lymphoma ascites cells.

The silver nanoparticle loaded with *P. longum* has been studied against HEp-2 cell line by MTT test and a significant cytotoxicity (94.02%) was observed at 500 µg/mL concentration of silver nanoparticles. In room temperature, *P. longum* extract is capable of producing silver nanoparticles (Jacob *et al*., 2012). Reddy *et al*. (2014) revealed that, the silver nanoparticles synthesized using aqueous extract of *P. longum* fruit had potent cytotoxic effect on breast cancer cell line MCF-7, with an IC_{50} value of 67µg/mL for 24 hrs. The *in vitro* and *in vivo* studies of ethanolic extract of *P. longum* fruit revealed that it is a potential, safe

and non-toxic alternative for cancer therapy especially against colon cancer (Ovadje *et al*., 2014).

The ethanol and water extracts of *P. chaba* fruit collected from Thailand were subjected to cytotoxicity studies against human breast adenocarcinoma cell line (MCF7) at an exposure time of 72 hrs. The result showed that ethanolic extract was more cytotoxic (IC_{50}-35.17±1.91 µg/mL) than water extract (IC_{50}- >100 µg/mL) (Sakpakdeejaroen & Itharat, 2009). *In vitro* cytotoxicity of crude ethanol extract of *P. chaba* fruit was tested against human cholangiocarcinoma (CL-6), human laryngeal (Hep-2) and human hepatocarcinoma (HepG2) cell lines. The extract showed potent activity against CL-6 (IC_{50}-40.74 µg/mL), HepG2 (IC_{50}-68.09±22.58 µg/mL) and Hep-2 (IC_{50}-18.93±5.03 µg/mL) cell lines (Mahavorasirikul *et al*., 2010).

In vitro cytotoxicity of ethanolic extract of *P. chaba* fruits on large lung carcinoma cell line (COR-L23), cervical cancer cell line (Hela) and liver cancer cell line (HepG2) has been investigated by Ruangnoo *et al*. (2012) and results revealed that it was more cytotoxic to COR-L23 (IC_{50}-15.82 µg/mL) than other three cell lines. The *in vitro* cytotoxicity of crude ethanolic extract of *P. chaba* fruit against cholangiocarcinoma cell line CL-6 is also reported (Plengsuriyakarn *et al*., 2012).

The anticancer efficacy of individual phytochemicals from these *Piper* species was also investigated. Pradeep & Kuttan (2002) reported the inhibitory effect of the alkaloid piperine on lung metastasis-induced B16F/10 melanoma cells in mice. The cytotoxic efficacy of the alkaloid piplartine on A-549 (lung carcinoma), KB (nasopharyngeal

carcinoma), HT-29 (colon carcinoma) and P-388 (lymphocytic leukaemia) cell lines has been reported by Bezerra *et al*. (2005). Piperine exhibited a chemopreventive effect on benzo(a)pyrene induced lung cancer in Swiss albino mice, through modulating the protein bound carbohydrate levels (Selvendiran *et al*., 2006). Bezerra *et al*. (2006) studied the effect of piperine and piplartine on Sarcoma 180 tumours in mice and observed a significant reduction in tumour weight in piplartine- and piperine-treated animals. The *in vivo* antitumour activity of piplartine and piperine were also reported by Bezerra *et al*. (2008). The anticancer activity of piperine against colon cancer (Duessel *et al*., 2008), oral cancer (Manoharan *et al*., 2009) and fibrosarcoma metastasis (Hwang *et al*., 2011) has also been reported. The ability of piperine to potentiate the cytotoxicity of anticancer drugs in resistant sublines like MCF-7/DOX and A-549/DDP, which were derived from MCF-7 and A-549 cell lines, has been reported by Li *et al*. (2011). The effect of piperine on the inhibition of leukemic cell lines has been reported by Chuchawankul *et al*. (2012) and that of triple-negative breast cancer cells by Greenshields *et al.* (2015). Kumar *et al*. (2011) and Gutierrez *et al*. (2013) reviewed that piperine is also cytotoxic towards Dalton's lymphoma ascites and Ehrlich ascites carcinoma cells. The anticancer activity of piperine has also been reported by other researchers against different cancers (Sakpakdeejaroen & Itharat, 2009; Makhov *et al*., 2012; Doucette *et al*., 2013; Samykutty *et al*., 2013).

In vitro antitumour activity of the alkaloid piperidine, isolated from *P. nigrum,* has been reported by Reshmi *et al*. (2010) against HEp2 cell lines (Human epithiloma cells of laryax), with an inhibition of 51.38% at a concentration of 5 µg/mL. The cytotoxicity of pellitorine

against HL60 (Human promyelocytic leukemia cells) and MCT-7 (breast cancer) cell lines were evaluated and compared with *P. nigrum* extract. Results showed that pellitorine exhibited less ctotoxicity (IC_{50}-13.0 μg/mL) compared to petroleum ether extract of *P. nigrum* (IC_{50}-9.8 μg/mL) for HL-60 cell lines, whereas high activity against MCF-7 cell lines (Ee *et al*., 2010).

The terpenoid compound D-limonene present in the black pepper oil was found to reduce the carcinogenic activity of the potent carcinogen, methylcholanthrene (Wrba *et al*., 1992).

Piperlongimin A and B isolated from *P. longum* fruit inhibited cell proliferation of human leukemia cell line (HL-60) and displayed apoptosis-inducing effects (Mishra *et al*., 2011). The antiproliferative effect of pipernonaline from *P. longum* has been reported by Lee *et al*. (2013) on human prostate cancer PC-3 cells. The anticancer effect of piperlongumine has been reported against prostate cancer (Golovine *et al*., 2013), lymphoma (Han *et al*., 2013), ovarian cancer (Gong *et al*., 2014), breast cancer (Bharadwaj *et al*., 2015), leukemia (Han *et al*., 2014) and hepatocellular carcinoma (Chen *et al*., 2015).

The efficacy of *trans*-piplartine isolated from the roots of *P. chaba* has been reported against rat histiocytoma (BC-8), mouse macrophages (P388D1 and J774), mouse embryonal carcinoma (PCC4) and human neuroblastoma (IMR32) tumour cells by Jyothi *et al*. (2009). They have found that *trans*-piplartine induced a dose-dependent cytotoxicity (2-24 μM) in these tumour cells. They have also reported that combinatorial treatment of piplartine with curcumin significantly enhanced the piplartine-induced cytotoxicity in tumour cells. The dimeric alkamides *viz*., chabamide H, I, J, K, F and G isolated from

methanolic extracts of *P. chaba* root were subjected to cytotoxic activities on cervical (HELA), breast (MCF-7), liver (HEPG2) and colon (HT-29 and COLO-205) cancer cell lines and found that certain isolates exhibited potent cytotoxic activity against the COLO-205 cell line (Rao *et al*., 2011).

The anticancer activity of phenolic compounds like 4-hydroxybenzoic acid, protocatechuic acid, gallic acid, coumaric acid, vanillic acid, ferulic acid, apigenin, syringic acid, quercetin, caffeic acid and kaempferol, indentified from these *Piper* species, were also reported by researchers (Hudson *et al*., 2000; Bestwick *et al*., 2005; Indap *et al*., 2006; Patel *et al*., 2007; Joshi *et al*., 2011; Tanaka *et al*., 2011; Intisar *et al*., 2013; Karthik *et al*., 2014).

Beta-sitosterol, compound reported from *P. colubrinum* leaves, shown anticancer activity against prostate and colon cancers (Awad *et al*., 1996; Wilt *et al*., 1999). However, the anticancer studies on *P. colubrinum* fruits are scanty.

2.8.3 Other medicinal values of *Piper* species selected for the study

Apart from antioxidant and anticancer activities, the selected *Piper* species are also reported for other medicinal properties. *P. nigrum* is reported for antimicrobial (Rani *et al*., 2013), antimutagenic (EI-Hamss *et al*., 2003), antithyroid (Panda & Kar, 2003), antidiarrhoeal (Shamkuwar & Shahi, 2012), antispermatogenic and infertility effect on mice (Mishra & Singh, 2009), antihypertensive (Taqvi *et al*., 2008), antiasthmatic (Parganiha *et al*., 2011), antiinflammatory and antiarthritic (Bang *et al*., 2009), hepatoprotective (Nirwane & Bapat, 2012), digestive (Platel & Srinivasan, 2000), anticonvulsant and

analgesic (Bukhari *et al*., 2013) and antidepressant (Li *et al*., 2007) activities. The efficacy of *P. nigrum* to prevent Alzheimer's disease was also reported (Subedee *et al.,* 2015).

P. longum exhibits other therapeutic potentials like antiinflammatory (Choudhary, 2006), immunomodulatory (Tripathi *et al*., 1999), antimicribial (Ali *et al*., 2007) antiplatelet (Iwashita *et al*., 2007), antihyperlipidemic (Jin *et al*., 2009), analgesic (Vedhanayaki *et al*., 2003), adulticidal (Choochote *et al*., 2006), antiamoebic (Ghoshal & Lakshmi, 2002), antifertility (Lakshmi *et al*., 2006), larvicidal (Chaithong *et al*., 2006), radioprotective (Sunila & Kuttan, 2005) cardioprotective (Wakade *et al*., 2008), antifungal (Lee *et al*., 2001) and antihepatitis (Jiang *et al*., 2013) activities.

P. chaba is reported for antifungal, antileishmanial, antibacterial, antiamoebic, antigiardial, CNS depressant, sedative and anxiolytic activities (Sawangjaroen *et al*., 2005; Sawangjaroen *et al*., 2006; Naz *et al*., 2012; Sarfaraz *et al*., 2014). The hepatoprotective, gastroprotective, antimalarial, antituberculosis, analgesic, antiinflammatory, antidiarrhoeal, anthelmintic and antilisterial activities of *P.chaba* is also reported (Rukachaisirikul *et al*., 2002; Morikawa *et al*., 2004; Taufiq-Ur-Rahman *et al*., 2005; Matsuda *et al*., 2008; Atjanasuppat *et al*., 2009; Rahman *et al*., 2011).

Even though *P. colubrinum* is well established for its plant pathogenic resistance, it is not explored much with regard to its medicinal and pharmaceutical potentials.

Chapter 3

Materials and Methods

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3.1 MATERIALS

3.1.1 Sample materials

The present study was conducted at ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala during the period of 2011-2015. The sample materials used in the study are given below.

- Matured berries of wild *P. nigrum* L., matured fruits of *P. longum* L., *P. chaba* Hunter and *P. colubrinum* Link. were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala. The samples were dried to desired moisture level (~10%) and subjected to various analysis.

- Matured berries of nine black pepper varieties (Panniyur-1, Panniyur-5, Sreekara, Subhakara, Panchami, IISR Malabar Excel, IISR Sakthi, IISR Girimunda and IISR Thevam) were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala. All the samples were sun dried and subjected to various analysis after maintaining desired moisture level (~10%).

- Matured berries of high yielding black pepper variety Panniyur-1 were collected from eleven locations of India. The locations include Kasaragod (ICAR-CPCRI), Chelavoor (ICAR-IISR headquarters), Peruvannamuzhi (ICAR-IISR Experimental Farm), Panniyur (Pepper Research Station, KAU), Ambalavayal (Regional Agricultural Research Station, KAU), Pampadumpara (Cardamom Research Station, KAU), Appangala (ICAR-IISR Regional Station), Mudigere (University of Horticultural Sciences, Bagalkot),

Pechiparai (Horticultural Research Station, TNAU), Thadiankudisai (Horticultural Research Station, TNAU) and Dapoli (Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth). All the samples were dried and subjected to variability studies after attaining desired moisture level (~10%). The features of selected locations are given in Table 3.1.

Table 3.1. Locations and its features

Location	State	Latitude	Longitude	Altitude (m above MSL)
Kasaragod	Kerala	12°12'5.97"N	75°9'48.51"E	10.7
Chelavoor	Kerala	11°17'35.16"N	75°49'10.2"E	45
Peruvannamuzhi	Kerala	11°36' 45"N	75°49'27"E	60
Panniyur	Kerala	12°4'35.18"N	75°24' 19.21"E	95
Ambalavayal	Kerala	9°37'32.46"N	76°43'50.52"E	974
Pampadumpara	Kerala	9°47' 48.12"N	77°9'30.89"E	1100
Appangala	Karnataka	12°26'0"N	75°45'0"E	800-1000
Mudigere	Karnataka	13°7'54.02"N	75°38' 27.29"E	1175
Pechiparai	Tamil Nadu	8°14' 23.10"N	77°20'4.02"E	58.3
Thadiankudisai	Tamil Nadu	10°0'0"N	77°0'0"E	1098
Dapoli	Maharashtra	17°46' 0.12"N	73°10'59.88"E	170-240

- *P. colubrinum* fresh leaves were collected from ICAR-IISR headquarters, Chelavoor, Kozhikode, Kerala, for essential oil extraction. The extracted oil was subjected to antioxidant and *in vitro* cytotoxicity analysis.
- Soil samples were collected from Panniyur-1 black pepper plot of different locations (Chelavoor, Peruvannamuzhi, Panniyur,

Ambalavayal, Pampadumpara, Appangala, Pechiparai, Thadiankudisai and Dapoli) at the same period of berry collection. The soil samples were air dried, sieved and subjected to various analysis.

3.1.2 Sources of chemicals and solvents used for the study

The chemicals, organic solvents and other reagents used for the study were purchased from the following companies and stored according to the recommendation of manufacturers.

- HiMedia, India
- Merck specialities private limited, India.
- Nice Chemicals, India
- Life Technologies, USA.
- Sisco Research Laboratories (SRL), India.
- Sigma-Aldrich, USA

3.1.3 Instruments/apparatus used for the study

- UV-VIS Spectrophotometer (UV-1800) : Shimadzu, Japan.
- HPLC (LC-10AT VP) : Shimadzu, Japan
- GC-MS (GC-2010 with MS QP-2010) : Shimadzu, Japan
- LC-MS (UHPLC 1290 with 6530 Q-TOF MS) : Agilent Technologies, USA
- AAS (AA 240 FS) : Varian, Inc., USA
- Fibra Plus (FES 6) : Pelican equipments, India.
- Moisture balance (MOC-120H) : Shimadzu, Japan.
- Weighing balance (BS 223 S) : Sartorius, Germany

- Sonicator (Power Sonic 405) : Hwashin Technology, Korea
- pH meter (Cyber Scan pH Totor) : Eutech, Singapore
- Electrical conductivity meter (Cyber Scan CON11) : Eutech, Singapore
- Kjeldahl apparatus (Kjeltec ^{TM} 2100) : Foss India Pvt. Ltd., India
- Refrigerated centrifuge (5417 R) : Eppendorf, Germany
- High speed centrifuge (Avanti J 301) : Beckman Coulter, USA
- Mechanical Shaker (Orbit) : Labline, India
- Rotary evaporator (R-205) : BUCHI, Switzerland
- Micro plate spectrophotometer : BioTek, USA
- CO_2 incubator : NuAire, USA
- Inverted microscope : Olympus, USA
- Chemical digestion system : Foss India Pvt. Ltd., India
- Muffle furnace : Labline, India
- Soxhlet apparatus : Vensil, India
- Clevenger trap apparatus : Vensil, India
- Deep freezer (-20^{o}C) : Arctiko, Denmark
- Biological safety cabinet : NuAire, USA

3.2 METHODS

3.2.1 Physico-chemical constituents

3.2.1.1 Moisture content

Moisture content of dried, powdered sample was analyzed using fully automated moisture balance with a heat source of Medium Frequency

Infrared Quartz heater. Moisture balance determined the moisture content by heating the sample using heat source and change in mass due to evaporation was measured (drying loss method).

3.2.1.2 Bulk density

Bulk density of sample was obtained by exactly filling the sample in a one litre volume measuring cylinder and weight of the sample occupied in it was recorded (Gupta & Das, 1997).

3.2.1.3 Total ash

The total ash content of the sample was analyzed using the method of ASTA (1968). The crucible was ignited to dull red colour, cooled to room temperature and weighed. Dried and finely powdered sample was weighed, taken in the crucible and subjected to ashing at $600^{o}C \pm 20^{o}C$ for 2 hrs using muffle furnace. The ash was filtered with hot water and after filtration, the filter paper and the contents were transferred to the original crucible and heated in the muffle furnace at $600^{o}C$. When the carbon free ash was obtained, crucible was allowed to cool in a desiccator and the final weight was noted. The ash content of the sample was calculated by taking into account the difference of empty weight of crucible and that of crucible with ash.

3.2.1.4 Acid insoluble ash

The acid insoluble ash of the sample was analyzed using ASTA method (1968). The crucible was ignited to a dull redness, cooled to room temperature and weighed. Dried and finely powdered sample was weighed, taken in the crucible and subjected to ashing at $600^{o}C \pm 20^{o}C$ for 2 hrs using muffle furnace. The crucible was taken out, transferred

the contents into boiling tube, added 25 mL HCl (1.0 mL conc. HCl: 2.5 mL distilled water) and boiled for 5 minutes. The contents were filtered and the filter paper along with its contents was transferred to the original crucible. The crucible was then ignited in muffle furnace at 600^{o}C. When the carbon free ash was obtained, crucible was allowed to cool and the final weight was noted. The differences in weight denote the acid insoluble ash content of the sample.

3.2.2 Primary metabolites

3.2.2.1 Carbohydrates

3.2.2.1.1 Total carbohydrate (Phenol sulphuric acid method)

The carbohydrates present in the sample were first hydrolyzed to simple sugars by 2.5N HCl. The sample was then neutralized with solid sodium carbonate until the effervescence ceases. It was then centrifuged, supernatant was collected and final volume was made up to 100 mL with distilled water (Sadasivam & Manickam, 2008). The total carbohydrate present in the extract was then estimated by phenol sulphuric acid method. Here, glucose is dehydrated to hydroxymethyl furfural in hot acidic medium. The orange-yellow coloured product developed with phenol was read spectrophotometrically at 490 nm. Glucose standard was used for the preparation of calibration curve (Dubois *et al.,* 1956).

3.2.2.1.2 Starch (Anthrone method)

The sample was treated with 80% alcohol to remove sugars and then starch was extracted with 52% perchloric acid. It was then centrifuged, saved the supernatant and made up the final volume to 100 mL with

distilled water (Sadasivam & Manickam, 2008). Starch was then estimated from this extract by Anthrone method. In this method, starch is hydrolyzed to glucose and dehydrated to hydroxymethyl furfural in hot acidic medium. This compound formed a green coloured product with anthrone, which was measured at 630 nm. Glucose standard was used to prepare the calibration curve (Hodge & Hofreiter, 1962).

3.2.2.1.3 Reducing sugars (Nelson-Somogyi method)

The reducing sugars present in the sample were extracted with hot 80% ethanol. The supernatant was collected and the final volume was made up to 10 mL with 80% ethanol (Sadasivam & Manickam, 2008). The estimation of reducing sugars was then carried out from this alcoholic extract by Nelson-Somogyi method. In this method, while heating with alkaline copper tartrate, reducing sugars reduce copper, resulting in the formation of cuprous oxide. The cuprous oxide is then converted to molybdenum blue after the addition of arsenomolybdic acid and the resultant blue colour was measured at 620 nm with spectrophotometer. The calibration curve was prepared using glucose standard (Somogyi, 1952).

3.2.2.2 Protein

3.2.2.2.1 Total protein (Lowry's method)

The powdered sample was extracted with hot 80% ethanol. The residue obtained after the complete extraction with ethanol was treated with 10% trichloroacetic acid. After centrifugation at 10,000 rpm for 30 minutes at 4^{0}C, the residue was again treated with absolute alcohol followed by alcohol-diethyl ether mixture (3:1) for twice. The dried

precipitate obtained after centrifugation was dissolved in 1N NaOH, boiled for 4-5 minutes and transferred to a 50 mL volumetric flask and made up the volume with distilled water. The protein content was determined from this extract by Lowry's method. The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components of Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by biuret reaction of protein with alkaline cupric tartrate were measured at 660 nm. Bovine Serum Albumin standard was used for the preparation of calibration curve (Lowry *et al.,* 1951).

3.2.2.2.2 Total free amino acid (Ninhydrin method)

The total free amino acid present in the sample was estimated by Ninhydrin method using its alcoholic (80%) extract (Sadasivam & Manickam, 2008). In this method, the α-amino acids are decarboxylated to an intensely coloured bluish purple product by the powerful oxidising agent ninhydrin and the resultant colour was measured at 570 nm using spectrophotometer. Calibration curve was prepared using Glycine standard (Yapinlee & Takahashi, 1996).

3.2.2.3 Lipids

3.2.2.3.1 Total fat

Dried and powdered sample was weighed and extracted with petroleum ether for 4-5 hrs using Soxhlet apparatus. After concentrating the extract, the contents were transferred to a pre-weighed beaker. The solvent was then completely evaporated and the increased weight of the beaker was noted. The difference in the

weights gives the total fat content of the sample (Sadasivam & Manickam, 2008).

3.2.2.3.2 Fatty acid profiling (GC-MS)

Extraction of free fatty acids and its conversion to fatty acid methyl esters (FAME)

The fatty acids present in the sample were extracted and converted into fatty acid methyl esters by modified method of Odham & Stenhagen (1972). The powdered sample (0.5 g) was refluxed in 3N NaOH-methanol (1:9) at 70^{o}C for 2 hrs. After refluxing, methanol was evaporated and the residue was dissolved in distilled water. It was then transferred to a separating funnel and extracted with petroleum ether (boiling range: 60-80^{o}C). The petroleum ether layer was removed to get rid of the unsaponifiable matters. The lower layer was collected and again transferred to separating funnel and 2-3 drops of conc. HCl were added to hydrolyze the saponified material. The acidified fraction was further extracted with petroleum ether to release the free fatty acids. The upper petroleum ether fraction was collected and evaporated to dryness and then methanol-HCl mixture (5:1) was added to it. It was then refluxed for 5 hrs at 70^{o}C to convert free fatty acids to fatty acid methyl esters. After incubation, the excess methanol was removed and petroleum ether was added. It was then transferred to a separating funnel containing 0.5N sodium carbonate, shaken well and the aqueous layer was removed. The petroleum ether layer containing fatty acid methyl esters was collected, de-moisturized using anhydrous sodium sulphate, filtered and stored in airtight vials at 0-4^{o}C until analysis. Fatty acids are non-volatile and cannot be analyzed in Gas-liquid

chromatography and thus they were converted into volatile fatty acid methyl esters.

Identification of FAME by Gas Chromatography-Mass Spectrometry (GC-MS)

The fatty acid methyl esters thus formed were identified using Shimadzu gas chromatograph (GC-2010) coupled with mass spectrometer (MS QP-2010). The separation was done using the capillary column RtX-5 (diphenyl dimethyl polysiloxane) with a dimension of 30 m X 0.25 mm X 0.25 µm and the carrier gas used was helium at 1.0 mL/min. The injection port and detector temperatures were 250^{o}C and 240^{o}C respectively. The column oven was programmed as follows: 60^{o}C for 5 min followed by 60-110^{o}C @ 5^{o}C/min, 110-200^{o}C @ 3^{o}C/min and 200-220^{o}C @ 5^{o}C/min and held for 15 min. The ion source temperature and interface temperature of the detector (MS) was 220^{o}C and 240^{o}C respectively with a detector voltage of 1.5 KV. The mass analysis was performed in the range of 40-650 m/z. The data were analyzed by Lab solutions-GCMS solution software (V 2.40). The fatty acid methyl esters were identified by comparison of their mass spectra with those in NIST and WILEY libraries and also by literature survey. Only those compounds that showed considerable similarity with NIST and WILEY libraries were taken and the compounds were quantified by area normalization.

3.2.2.4 Crude fibre

The crude fibre content was estimated as per ASTA (1968) method using Fibra-Plus apparatus. Dried and powdered sample was weighed into an oven dried crucible and the crucible was placed in Fibra-Plus

hot extraction unit. The sample in the crucible was allowed to digest with dilute H_2SO_4 (1.25%) followed by dilute NaOH (1.25%). After digestion, the crucible was taken out from the apparatus and allowed to dry and weighed. It was then placed in muffle furnace at 500^{o}C for ashing. After ashing, cooled down the hot crucible and weighed again. The difference in the two weights gives the weight of crude fibre present in the sample.

3.2.3 Secondary metabolites

3.2.3.1 *Essential oil*

3.2.3.1.1 Extraction of essential oil (Hydrodistillation using Clevenger trap)

The powdered sample was distilled in water for 3 hrs using Clevenger trap. The distilled oil is condensed and collected over water. As it is immiscible with water and lighter than water, essential oil forms a separate layer and can be quantified (AOAC, 1975).

3.2.3.1.2 Essential oil profiling (GC-MS)

The characteristic constituents of the extracted essential oil were identified and quantified by GC-MS.

Gas Chromatography-Mass Spectrometry (GC-MS):

Identification of essential oil constituents was carried out using Shimadzu gas chromatograph (GC-2010) coupled with mass spectrometer (QP-2010). The instrument parameters were same as mentioned for identification of FAME by GC-MS (section 3.2.2.3.2) and the column oven was programmed as follows: 60^{o}C for 5 min

followed by 60-110°C @ 5°C/min, 110-200°C @ 3°C/min and 200-220°C @ 5°C/min and held for 5 min. The essential oil constituents were identified by comparing their mass spectra with those in NIST and WILEY libraries, by comparing their retention indices with authentic standards and also by literature survey. Only those compounds that showed considerable similarity with NIST and WILEY libraries were taken and the compounds were quantified by area normalization.

Retention Indices (RI) of the compounds were calculated relative to C_8-C_{20} n-alkanes standards using the formula proposed by Dool & Kratz (1963) and compared with those of authentic standards listed in Adams (2007).

$$\mathbf{RI_t = C_n + \left(\frac{RT_t - RT_x}{RT_y - RT_x}\right) \times 100}$$

Where, RI_t = Retention indices of test

C_n = Number of carbon atoms of preceding n-alkane standard.

RT_t = Retention time of test

RT_x = Retention time of preceding alkane standard

RT_y = Retention time of following alkane standard

3.2.3.2 Estimation of piperine (HPLC)

Sample preparation

One gram of powdered sample was taken in a round bottom flask. After adding 50 mL of ethanol (95%), it was refluxed for 3 hrs. After cooling, the extract was filtered into a 100 mL standard flask and made

up the volume with ethanol. Ten mL of sample from this stock solution was then diluted to 25 mL with mobile phase. This aliquot is used for HPLC analysis.

Standard preparation

Stock solution (0.2 mg/mL) was prepared by dissolving 10 mg of authentic piperine standard (99% purity) in 50 mL ethanol. Working solution with a concentration of 0.016 mg/mL was prepared by diluting 2.0 mL of sample from stock solution to 25 mL with mobile phase.

High Performance Liquid Chromatography (HPLC)

Identification and quantification of piperine was carried out in Shimadzu liquid chromatograph (LC-10AT VP) equipped with a pump, UV-VIS detector (SPD-10A VP), system controller (SCL-10A VP) and the software Lab solutions-LC solution (V 1.22). The stationary phase was C-18 reversed phase column (250 mm X 4.6 mm; particle size of 5 µm). The filtered sample (test sample and standard piperine) was injected (20 µL) into the injection port and the separation was achieved with a mobile phase of 1% acetic acid and acetonitrile (52:48), in isocratic elution. The flow rate was adjusted to 1.5 mL/min and measurement was taken at 342 nm (Wood *et al.,* 1988).

Quantification of piperine using HPLC

Percentage of piperine in the sample was calculated by comparing with the authentic standard, using the following formula:

$$\textbf{Piperine}\ (\%) = \frac{\mathbf{A} \times \mathbf{K} \times \mathbf{2\ 5} \times \mathbf{100}}{\mathbf{10} \times \mathbf{m}} \times \mathbf{100}$$

Where, A = Area of piperine sample

m = Mass of the sample in milligrams

K = Response factor

$$\mathbf{K} = \frac{\mathbf{Conc.\ of\ piperine\ std.\ \times\ std.\ purity}}{\mathbf{Area\ of\ piperine\ std.\ \times\ 100}}$$

3.2.3.3 Phenolics

3.2.3.3.1 Total phenol (Folin-Ciocalteau method)

The total phenol present in the sample was extracted with 80% alcohol (Sadasivam & Manickam, 2008) and estimated by Folin-Ciocalteau method (Malick & Singh, 1980). In this method, the alcoholic extract was allowed to react with Folin-Ciocalteau reagent and sodium carbonate. In alkaline medium, the phenols present in the extract react with phosphomolybdic acid component of Folin-Ciocalteau reagent and the resultant blue coloured complex (molybdenum blue) was measured at 650 nm using spectrophotometer. Gallic acid was used for the preparation of calibration curve.

3.2.3.3.2 Total flavonoid (Aluminium chloride colorimetric assay)

The total flavonoid present in the samples was extracted with 80% alcohol and estimated with Aluminium chloride colorimetric assay. In this method, the extract was allowed to react with aluminium chloride and sodium nitrite in aqueous and alkaline medium. The aluminium can attract the atomic nuclei of the aromatic rings in flavonoids due to its high electropositive nature and thus a charge transfer resonance hybrid which is highly stable in the aqueous medium is created. This hybrid is then interacts with sodium nitrite in an alkaline medium and a pink coloured complex was formed, which was measured spectrophotometrically at 510 nm. The calibration curve was prepared using quercetin standard (Kamtekar *et al.,* 2014).

3.2.3.3.3 Phenolic profiling (LC-MS & HPLC)

Extraction of phenolic compounds

The powdered sample (1.0 g) was subjected to acid digestion with 2N HCl for 20 minutes in boiling water bath. After cooling, the contents were filtered and the filtrate was extracted thrice with diethyl ether. The pooled ether layer was then extracted with 5% anhydrous sodium carbonate. The collected sodium carbonate layer was adjusted to pH 3.0 with 5% H_2SO_4. The acidified fraction was re-extracted with diethyl ether. After complete evaporation of diethyl ether, the residue was dissolved in methanol and stored in amber coloured bottle at 4^oC until analysis (Bate-Smith, 1954).

Identification of phenolic compounds by Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS analysis was performed using Agilent 1290 Infinity UHPLC system coupled with an Agilent 6530 Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (MS/MS) with JetStream ESI ion source. The chromatographic separation of phenolic compounds was performed by injecting 1.0 µL of filtered phenolic extract in UHPLC system equipped with Agilent Zorbax Eclipse Plus C18 column (3.0 mm X 150 mm; particle size of 1.8 µm) with temperature 40^oC. The mobile phase used for separation was 10 mM ammonium acetate in water (solvent A) and methanol (solvent B) with a flow rate of 0.5 mL/min (gradient programme: 10% B - 0 to 5 min; 95% B -12 to 18 min; 10% B - 20 to 25 min) and the chromatogram was developed at 254 nm using UV-VIS detector. The separated compounds were then ionized using JetStream Electron Spray Ionization (ESI) ion source in a

negative mode with a capillary voltage of 3.5 KV and fragment voltage of 160 V. The drying gas used was high purity nitrogen with a flow rate of 7.0 L/min and temperature of 350^{o}C. The nebulizer pressure was set at 20 psig (pounds per square inch gage). The sheath gas (nitrogen) flow rate was maintained at 11 L/min at a temperature of 400^{o}C. The mass analysis was performed in the range of 90-1700 m/z. The Q-TOF data obtained by MS and MS/MS was analyzed using MassHunter software (V.B.05.01). Molecular Feature Extraction (MFE) algorithm and Metlin database were used to identify the compounds and its empirical formula. The structure of individual compound was predicted based on the empirical formula generated, MS/MS fragment spectrum through Molecular Structure Correlator (MSC) programme and also, based on literature survey. The mass accuracy of LC-MS data was <2 ppm in most of the cases.

Confirmation of phenolic compounds by High Performance Liquid Chromatography (HPLC)

The confirmation of phenolic compounds was performed in Shimadzu liquid chromatograph (LC-10AT VP) equipped with a pump, UV-VIS detector (SPD-10A VP), system controller (SCL-10A VP) and the software Lab solutions-LC solution (V 1.22). The stationary phase was C-18 reversed phase column (250 mm X 4.6 mm; particle size of 5 µm). Mobile phase was acetic acid-water, 5:95 (solvent A) and acetonitrile-water, 40:60 (solvent B) with a flow rate of 1.0 mL/min. The gradient programme was as follows: 100% A - 0 to 50 min; 100% B - 50 to 55 min; 100% A - 55 to 60 min. The measurement was taken at 280 nm after injecting 20 µL samples. HPLC analysis was carried out to confirm the phenolic compounds identified in LC-MS, with

available standards (10 mg/mL methanol). The confirmation was done by comparing their retention time with those of standards and also by peak enrichment.

3.2.4 Oleoresin (Cold percolation technique)

Oleoresin content of the sample was extracted by cold percolation technique as per the method of ASTA (1968). The powdered sample was weighed and transferred to a glass column, acetone was added and kept overnight. The extract was drained into a pre-weighed beaker and the solvent was evaporated to dryness and the increased weight of the beaker was noted. The difference between these two weights gives the amount of oleoresin present in the sample.

3.2.5 Plant mineral analysis

3.2.5.1 Nitrogen (Kjeldahl method)

While digesting the sample with conc. H_2SO_4 in the presence of catalytic mixture of K_2SO_4 and $CuSO_4$, nitrogen will be converted to ammonium sulphate, that on neutralization with NaOH solution and steam distillation, liberates ammonia. This was performed using Kjeldahl distillation unit. The liberated ammonia can be absorbed in boric acid solution containing mixed indicator. The ammonia absorbed in boric acid can be found out by back titration of boric acid using 0.1N H_2SO_4. The nitrogen in sample was calculated from the titre value (Jackson, 1973).

3.2.5.2 Processing of plant sample (Wet oxidation method)

Powdered sample was weighed (1.0 g) and added into a 100 mL volumetric flask. Added 10 mL of diacid mixture and kept for 2 hrs for

cold digestion. It was then heated in hot plate slowly for 1 hr and then strongly, until a clear white solution or emulsion was obtained at the bottom of the flask. The flask was cooled, the volume was made up to 100 mL, and used for estimation of P, K, Ca, Mg, Fe, Cu, Zn and Mn (Dinesh *et al.,* 2006).

3.2.5.3 Phosphorous (Vanado molybdate method)

Phosphorus content in the digested sample obtained in section 3.2.5.2 was estimated by Vanado molybdate method. In this method, the sample was allowed to react with HNO_3-Vanado molybdate reagent. Under acidic condition, phosphates react with molybdate and vanadate to form a yellow-coloured vanadomolybdophosphoric heteropoly complex, whose colour intensity is directly proportional to the concentration of phosphate present in the sample, which can be read on the spectrophotometer at 470 nm. The amount of P in the sample was calculated from the standard P curve (Jackson, 1973).

3.2.5.4 K, Ca, Mg, Fe, Cu, Zn and Mn (AAS)

Potassium, calcium, magnesium, iron, copper, zinc and manganese were measured directly from the diacid digest of plant sample obtained by wet oxidation method (section 3.2.5.2) by atomic absorption spectrophotometer (AAS) with appropriate standards (Dinesh *et al.,* 2006).

Atomic Absorption Spectrophotometer (AAS)

Analysis of the above mentioned nutrients in the selected plant sample was performed using Varian atomic absorption spectrometer (AA 240 FS). Measurements of Mg, Fe, Cu, Zn and Mn were made in

absorption mode using hollow cathode lamp at wavelengths of 285.2 nm, 248.3 nm, 324.7 nm, 213.9 nm and 279.5 nm respectively whereas K and Ca were measured in emission mode at 766.5 nm and 422.7 nm respectively. The fuel gas used was acetylene with air as support gas.

3.2.6 Soil analysis

The collected soil samples were air dried and passed through <2.0 mm sieve. These air dried, fractionated soil samples were used for the analysis.

3.2.6.1 pH

The pH of soil sample was measured potentiometrically from 1:2.5 soil-water suspension. The suspension was stirred well for about 5 minutes and kept for half an hour. Stirred well again and took the reading using pH meter, which was already calibrated with standard buffer solutions of known pH (Jackson, 1973).

3.2.6.2 Electrical conductivity (EC)

The clear supernatant of 1:2.5 soil-water suspension prepared for pH measurement was used for estimation of EC. The conductivity of the supernatant liquid was measured using electrical conductivity meter after calibrating it with 0.01N KCl (Sureshkumar *et al.,* 2010).

3.2.6.3 Organic carbon (Walkley and Black wet oxidation method)

Organic carbon of soil sample was analyzed by Walkley and Black wet oxidation method. In this method, soil organic matter is oxidized under standard conditions with potassium dichromate in H_2SO_4 solution. The remaining dichromate is back titrated with ferrous ammonium sulphate

hexahydrate solution (Mohr's salt) after adding ferroin indicator. The titre value is inversely related to the amount of carbon present in the soil sample (Walkley, 1947).

3.2.6.4 Available Nitrogen (Kjeldahl method)

A known weight of soil was mixed with $KMnO_4$ (0.32%), NaOH (2.5%) and liquid paraffin (2.0 drops) and distilled using Kjeldahl distillation unit. Ammonia gas formed was absorbed in 2% boric acid solution containing mixed indicator, which is then back titrated to original colour using standard acid (0.01N H_2SO_4). Available N in the soil sample was calculated using the titre value (Jackson, 1973).

3.2.6.5 Available Phosphorous (Ascorbic acid method)

Available P was extracted using Bray-1 reagent. Soil sample was weighed and mixed with Bray-1 reagent. The mixture was shaken for 30 minutes at 180 oscillations per minute after adding little P-free activated charcoal and then filtered (Bray & Kurtz, 1945). Phosphorus was estimated from this filtrate by ascorbic acid method. In this method, the orthophosphate ions get precipitated as phosphomolybdate complex in an acidic molybdate solution. This complex is then reduced by agents like ascorbic acid and the resultant blue coloured phosphomolybdate was measured spectrophotometrically at 660 nm. The concentration of P in the sample was computed from the standard P curve (Watanabe & Olsen, 1965).

3.2.6.6 Exchangeable K, Ca and Mg (AAS)

Exchangeable K, Ca and Mg in soil sample were extracted by neutral ammonium acetate extraction procedure. Here, neutral ammonium salt

solutions replace the cation present in the soil exchange complex, which can be measured using AAS. The concentrations determined by this method are referred to as 'Exchangeable plus soluble'. The soil sample was weighed and allowed to react with extracting reagent by shaking for 5 minutes using a mechanical shaker at 180 oscillations per minute. The mixture was filtered and the filtrate was used to determine the K, Ca and Mg by atomic absorption spectrophotometer (AAS) with appropriate standards. The instrumentation for AAS was same as that explained in section 3.2.5.4 (Jackson, 1973).

3.2.6.7 Available Fe, Cu, Zn and Mn (DTPA method)

Available Fe, Cu, Zn and Mn in soil sample were extracted by DTPA method. In this, diethylene triamine pentaacetic acid (DTPA) acts as a chelating agent for micronutrients like Fe, Cu, Zn, Mn, etc. The soil sample was weighed and allowed to react with DTPA extracting solution by shaking for 2 hrs using a mechanical shaker at 180 oscillations per minute. The mixture was filtered and the filtrate was used to determine the Fe, Cu, Zn and Mn by atomic absorption spectrophotometer (AAS) with appropriate standards. The instrumentation for AAS was same as that explained in section 3.2.5.4 (Page, 1982).

3.2.7 Sequential extraction of sample by Soxhlet apparatus

The sample was powdered and extracted sequentially with n-hexane, chloroform, methanol and water in the increasing order of polarity using Soxhlet apparatus. After the completion of extraction with one solvent, the sample left was dried at room temperature and extracted with next solvent. Each extract was filtered and evaporated to dryness

using rotary evaporator. The yield of concentrated extracts was noted and stored at 4^{o}C until analysis.

3.2.8 *In vitro* antioxidant activity

The *in vitro* antioxidant activity of sequential extracts and essential oils was carried out using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, phosphomolybdenum method, ferric reducing power method and ferrous chelating activity. Stock solution of all the sequential extracts and synthetic antioxidant butylated hydroxyanisole (BHA) was prepared in methanol with a concentration of 10 mg/mL. Stock solutions of essential oils were also prepared in methanol in different concentrations for different assays.

3.2.8.1 DPPH free radical scavenging activity

The antioxidant activity of the sample was determined in terms of its hydrogen donating or radical scavenging ability and thus to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl*)* free radical (Braca *et al*., 2001). The reduction of DPPH free radicals by hydrogen or electrons donated by free radical scavengers present in the sample, and the resultant change in colour from violet to yellow, was measured in this method. Working solutions with a concentration range of 10-500 µg/mL for methanol and chloroform extracts, and 100-2000 µg/mL for hexane and water extracts, were prepared from their respective stock solutions. In the case of essential oils, a stock solution of 0.5 g/mL was prepared in methanol for black pepper oil, 0.18 g/mL for *P. longum* and *P. chaba* oils, and 0.025 g/mL for *P. colubrinum* oil. The working solutions with a concentration range of 10-200 mg/mL for pepper oil,

10-75 mg/mL for *P. longum* and *P. chaba* oil and 2.5-125 µg/mL for *P. colubrinum* oil were prepared from the stock solutions.

An aliquot (1.0 mL) from each working solution was added into test tubes and final volume was made up to 4.0 mL with methanol. One mL of 0.004% DPPH was added to the samples. After proper mixing, samples were incubated at dark for 30 minutes and absorbance was taken at 517 nm with a UV-VIS spectrophotometer against methanol blank. Methanol and DPPH alone served as control. Radical scavenging activity was assessed as the inhibition percentage of free radical by the sample and was calculated using the formula:

$$\%\ DPPH\ radical\ scavenging\ activity = \frac{Absorbance\ of\ Control - \ Absorbance\ \ of\ Test}{Absorbance\ of\ Control} \times 100$$

Radical scavenging ability was expressed as IC_{50} value, which was obtained by plotting percentage of DPPH radical scavenging activity against the corresponding concentration of the extract. IC_{50} value represents the inhibitory concentration of extract/essential oil required to scavenge 50% DPPH free radicals and is inversely proportional to the antioxidant activity. IC_{50} value was expressed in µg/mL for sequential extracts and mg/mL for essential oils. The synthetic antioxidant BHA was taken as positive control to compare the efficacy of these samples to scavenge DPPH free radicals.

3.2.8.2 Total antioxidant activity by Phosphomolybdenum method

In Phosphomolybdenum method, the molybdenum present in the reagent gets reduced by the antioxidants present in the sample and the resultant green colour was measured. An aliquot of 50 µL for methanol and chloroform extracts, 100 µL for hexane and water extracts from the corresponding stock solutions, and 10 µL of essential oils from the

stock solution of 0.5 g/mL, were added to the test tubes. The final volume was made up to 3.0 mL with methanol. One mL of phosphomolybdenum reagent was added and incubated at 95^{o}C for 90 minutes. After incubation, the samples were read at 695 nm using UV-VIS spectrophotometer. Ascorbic acid (0.2-1.0 mM) was used as standard for the preparation of calibration curve. The results were expressed as molar ascorbic acid equivalents/g of extract (M AAE/ g of extract) for sequential extracts and molar ascorbic acid equivalents/g of oil (M AAE/ g of oil) for essential oils. Synthetic antioxidant BHA was used for comparison (Prieto *et al*., 1999).

3.2.8.3 Ferric reducing power (FRP) method

The reductive capacity of the sample was estimated by the method described by Oyaizu (1986). In this method, antioxidants present in the sample causes the reduction of Fe^{3+} to Fe^{2+} and the resultant coloured complex (Perl's Prussian blue) was measured. An aliquot of 50 µL of methanol, chloroform and hexane extracts, and 100 µL of water extract from the corresponding stock solutions, and 10 µL of essential oils from the stock solution of 0.5 g/mL, were added to the test tubes. The final volume was made up to 1.0 mL with distilled water. The samples were then mixed with 2.5 mL phosphate buffer (0.2M, pH 6.6). Potassium ferricyanide (2.5 mL; 1%) was added to the mixture and incubated at 50^{o}C for 30 min. The reaction was terminated by adding trichloroacetic acid (2.5 mL; 10%) and the mixture was centrifuged at 3000 rpm for 20 minutes. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL; 0.1%) solution and the absorbance was measured at 700 nm using UV-VIS spectrophotometer. Ascorbic acid (0.25-1.0 mM) was used as standard

for the preparation of calibration curve. Increased absorbance of the reaction mixture indicated greater reducing power and it was expressed in molar ascorbic acid equivalents/g of extract (M AAE/ g of extract) for sequential extracts and millimolar ascorbic acid equivalents/g of oil (mM AAE/ g of oil) for essential oils. Synthetic antioxidant BHA was kept as positive control.

3.2.8.4 Determination of ferrous chelating ability

The ability of samples to chelate ferrous ion was estimated by measuring the intensity of ferrous-ferrozine complex. The antioxidants present in the sample chelate the ferrous ions from the ferrous chloride and the remaining ferrous ions combine with ferrozine to form ferrous-ferrozine complex and the resultant red colour was measured (Carter, 1971). An aliquot of 25 µL for methanol and chloroform extracts, and 50 µL for hexane and water extracts from the corresponding stock solutions, and 10 µL of essential oil from the stock solution (0.5 g/mL), were taken into the test tubes. The final volume was made up to 3.0 mL with methanol. All the test solutions were then treated with $FeCl_2$ (0.1 mL; 2 mM). After incubation for 5 minutes, 0.4 mL of 5 mM ferrozine was added to the above mixture and incubated for 10 minutes. After incubation, the absorbance was recorded at 562 nm with UV-VIS spectrophotometer. EDTA (10-50 µg) was used as standard for the preparation of calibration curve. Ferrous chelating ability was expressed as milligram EDTA/gram of extract (mg EDTA/g of extracts) for sequential extracts and milligram EDTA/g of oil (mg EDTA/g of oil) for essential oils. The ferrous chelating ability of synthetic antioxidant BHA was also checked.

3.2.8.5 Total phenolic content of sequential extracts

The estimation of total phenolic content in the extracts used for antioxidant study is crucial since it is reported that the phenolics play major role in the antioxidant activity of plants (Cai *et al.,* 2004; Demiray *et al.,* 2009). The total phenolic content of these samples were estimated as per section 3.2.3.3.1 and expressed as milligram gallic acid equivalents/g of extract (mg GAE/g of extracts).

3.2.9 *In vitro* cytotoxicity

The sequential extracts prepared as per section 3.2.7, and the essential oils, were subjected to *in vitro* cytotoxicity study. The study was conducted in the division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram, Kerala on cervical cancer cell line CaSki by MTT assay. Cervical cancer cell line CaSki was cultured as adherent monolayer as per earlier method (Freshney, 2010) and maintained in 90% Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37^{0}C with 5% CO_2. Stock solution (250 mg/mL) of each extract was prepared in DMSO and stored at -20^{0}C until use.

MTT Assay

MTT (3-(4,5Dimethylthiazol-2yl)-2,5-Diphenyl Tetrazolium Bromide) assay (Mosmann, 1983) is based on the ability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of the pale yellow tetrazole MTT to a dark blue formazan crystals. Formazan crystals are impermeable to cell membranes which results in its accumulation within healthy cells. The crystals can be solubilized by adding lysis buffer and its colour can be measured

spectrophotometrically. The level of the coloured formazan product is directly proportional to the number of surviving cells.

The stock solutions of extracts were diluted in 10% DMEM to get lower concentration (0.5 mg/mL). Cells harvested in the log phase of growth were counted and seeded (5×10^3 cells/ well) in 96 well micro titer plates and incubated overnight at 37°C in a humidified 5% CO_2 incubator. The cells were then allowed to react with different amount of extracts (25, 50 and 100 µg) and essential oil (25%) for 24, 48 and 72 hrs in a humidified 5% CO_2 incubator at 37°C. Synthetic anticancer drug Doxorubicin served as positive control whereas 10% DMEM was taken as negative control. After incubation, the medium was discarded and wells were washed with PBS. 100 µL of the MTT (5% in 10% DMEM media) was added and incubated for 2 hrs. MTT lysis buffer (100 µL) was added to solubilize the coloured formazan crystals formed by the reduction of MTT. After incubation for 4 hrs, the absorbance was measured at 570 nm using a micro plate spectrophotometer and the percentage cytotoxicity was determined as follows:

$$\%\ \textbf{Cytotoxicity} = \frac{\textbf{Absorbance of Control} - \textbf{Absorbance of Test}}{\textbf{Absorbance of Control}} \times 100$$

IC_{50} (amount of extract required for 50% cytotoxicity) values for potential extracts were also calculated from the dose response curve for CaSki.

3.2.10 Preliminary phytochemical analysis of screened extracts

The extracts screened for high antioxidant activity and *in vitro* cytotoxicity were subjected to preliminary phytochemical screening. The extracts were tested for phenolics, alkaloids, flavonoids,

steroids/triterpenes, saponins, fixed oils and fats, carbohydrates and proteins by adopting standard protocols (Trease & Evans, 2002; Khandelwal, 2008; Kokate *et al.,* 2008).

3.2.10.1 Test for carbohydrates

a) Molisch's test: To 2.0 mL of the extract, added 1.0 mL of α-naphthol solution, followed by concentrated sulphuric acid through the side of the test tube. Purple or reddish violet colour at the junction of the two liquids revealed the presence of carbohydrates.

b) Fehling's test: To 1.0 mL of the extract, added equal quantities of Fehling's solution A and B and heated for 2 minutes. Formation of a brick red precipitate confirmed the presence of sugars.

c) Benedict's test: To 5.0 mL of Benedict's reagent added 1.0 mL of extract solution and boiled for 2 minutes and cooled. Formation of red precipitate again confirmed the presence of sugars.

3.2.10.2 Test for Protein

a) Biuret test: To 2.0 mL of the extract, added 2.0 mL of 10% sodium hydroxide solution followed by 2 drops of 0.1% copper sulphate solution. Formation of pinkish or purple violet colour indicated the presence of proteins.

b) Millon's test: 1.0 mL of the extract was acidified with sulphuric acid and then added Millon's reagent and boiled. Presence of protein was confirmed by the formation of yellow precipitate.

3.2.10.3 Test for fixed oils and fats

a) Spot test: A small quantity of extract was pressed between the filter paper. Oil stains on paper indicated the presence of fixed oils.

b) Saponification test**:** Few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of the extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hrs. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

3.2.10.4 Test for steroids/triterpenes

a) Salkowski test: The extract was dissolved in chloroform and equal volume of concentrated sulphuric acid was added to it. Formation of bluish red to cherry colour in chloroform layer and green fluorescence in the acid layer indicated the steroidal components in the extract.

b) Liebermann-Burchard (Acetic anhydride) test: The extract was dissolved in chloroform and 1.0 mL of acetic anhydride solution and two drops of concentrated sulphuric acid were added. Formation of dark green colour indicated the presence of steroids/triterpenes**.**

3.2.10.5 Test for alkaloids

a) Mayer's test: 1.0 mL of Mayer's reagent (potassium mercuric iodide solution) was added to 1.0 mL of the extract and formation of cream coloured precipitate indicated the presence of alkaloids.

b) Hager's test: To 1.0 mL of the extract, 3.0 mL of Hager's reagent (saturated aqueous solution of picric acid) was added. Presence of alkaloids was confirmed by the formation of yellow coloured precipitate.

c) Wagner's test: To 1.0 mL of the extract, 2.0 mL of Wagner's reagent (iodine in potassium iodide) was added. Presence of alkaloids was confirmed by the formation of reddish brown coloured precipitate.

3.2.10.6 Test for phenolic compounds

a) To 1.0 mL of the extract, ferric chloride solution was added. Formation of a dark blue or greenish black colour indicated the presence of phenolic compounds.

b) Strong potassium dichromate solution was added to the test extract, a yellow coloured precipitate confirmed the presence of phenolic compounds.

c) I.0 mL of the test solution was mixed with basic lead acetate solution and the formation of white precipitate also confirmed the presence of phenolic compounds

3.2.10.7 Test for flavonoids

a) Little quantity of extract was treated with amyl alcohol, sodium acetate and ferric chloride. A yellow colour was formed, which disappeared on addition of an acid. It indicates the presence of flavonoids.

b) Shinoda's test: The extract was treated with magnesium foil and concentrated hydrochloric acid. Formation of intense cherry red colour indicated the presence of flavonones or orange red colour indicated the presence of flavonols.

c) The extract was treated with sodium hydroxide and the formation of yellow colour indicated the presence of flavones.

d) The extract was treated with concentrated sulphuric acid and the formation of yellow or orange colour confirmed the presence of flavones.

3.2.10.8 Test for saponins

a) Foam test: To a small quantity of extract taken in a test tube, 20 mL of distilled water was added and shaken for 15 minutes. Formation of foam indicated the presence of saponins.

3.2.11 Identification of possible compounds from potential extract screened for both *in vitro* antioxidant activity and cytotoxicity

The possible high value compounds were identified from the potential extract screened for both *in vitro* antioxidant activity and cytotoxicity by LC-MS analysis. LC-MS analysis was performed using Agilent 1290 Infinity UHPLC system coupled with an Agilent 6530 Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (MS/MS) with JetStream ESI ion source. The stock solution of crude extract was prepared in methanol with a concentration of 23 mg/100 mL and 5.0 µL was taken from stock solution for LC-MS analysis. The chromatographic separation of its individual compounds was performed in UHPLC system equipped with Agilent Zorbax Eclipse Plus C18 column (4.6 mm X 100 mm; particle size of 3.5 µm) with a temperature of 40^{o}C. The mobile phase used for separation was 10 mM ammonium acetate (solvent A) and acetonitrile (solvent B) with a flow rate of 0.5 mL/min (gradient programme: 7.0% B - 0 min; 45% B - 7.0 min; 62% B - 15 min; 85% B - 20-25 min and 7.0% B - 30 min) and the chromatogram was developed at 254 nm using diode array detector. The separated compounds were then ionized using JetStream

Electron Spray Ionization (ESI) ion source in positive and negative modes with a capillary voltage of 3.5 KV and fragment voltage of 175 V. The drying gas used was high purity nitrogen with a flow rate of 7.0 L/min and temperature of 350^{o}C. The nebulizer pressure was set at 35 psig (pounds per square inch gage). The sheath gas (nitrogen) flow rate was maintained at 11 L/min at a temperature of 400^{o}C. The data were analyzed using MassHunter software (V.B.05.01). The possible compounds were identified by targeted screening approach using 'Find by Formula' available in MassHunter software. The compounds were confirmed using possible elemental composition generated by Molecular Formula Generator (MFG) feature of MassHunter software. MFG is working by consideration of accurate mass of compounds (TOF MS), accurate mass of the fragments of the compounds (TOF MS/MS), isotopic abundance and isotopic spacing.

3.2.12 Statistical analysis

Data were combined and analyzed by analysis of variance (ANOVA). The ANOVA was performed with the MSTATC software (version 1.41). Significant differences ($p \leq 0.05$) were estimated by Duncan's multiple range test (DMRT) using 'RANGE' procedure and the correlation studies were performed by determining correlation coefficient (r) using the 'CORR' procedure of MSTATC. Values were expressed as Mean of three replicates and superscripts was used to represent the significant difference as per DMRT ($p \leq 0.05$). IC_{50} values for *in vitro* antioxidant activity and cytotoxicity studies were calculated using the statistical software SAS (version 9.3) and Microsoft excel, 2007. The Average linkage cluster analysis of samples based on their biochemical composition was also performed using the statistical software SAS (version 9.3) and the resultant dendrogram was analyzed.

Chapter 4

Results and Discussion

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4.1 VARIABILITY IN PHYSICO-CHEMICAL AND BIOCHEMICAL PROFILE AMONG SELECTED *PIPER* SPECIES AND ALSO AMONG SELECTED BLACK PEPPER VARIETIES

4.1.1 Variability in physico-chemical and biochemical profile among selected *Piper* species

Matured berries of wild *P. nigrum* L. and matured fruits of *P. longum* L., *P. chaba* Hunter and *P. colubrinum* Link., were collected from ICAR-Indian Institute of Spices Research (ICAR-IISR) Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala. The samples were subjected to variability studies after proper drying.

4.1.1.1 Physico-chemical constituents

Moisture content, bulk density, total ash and acid insoluble ash were the physico-chemical constituents studied from selected *Piper* species as per section 3.2.1.

4.1.1.1.1 Moisture content

The moisture content of selected samples was in the range of 6.98 to 8.38% and it was highest in wild *P. nigrum* and and lowest in *P. colubrinum.* Wild *P. nigrum* and *P. longum* as well as *P. chaba* and *P. colubrinum* were statistically on par for their moisture content. The moisture content values are given in Fig. 4.1.

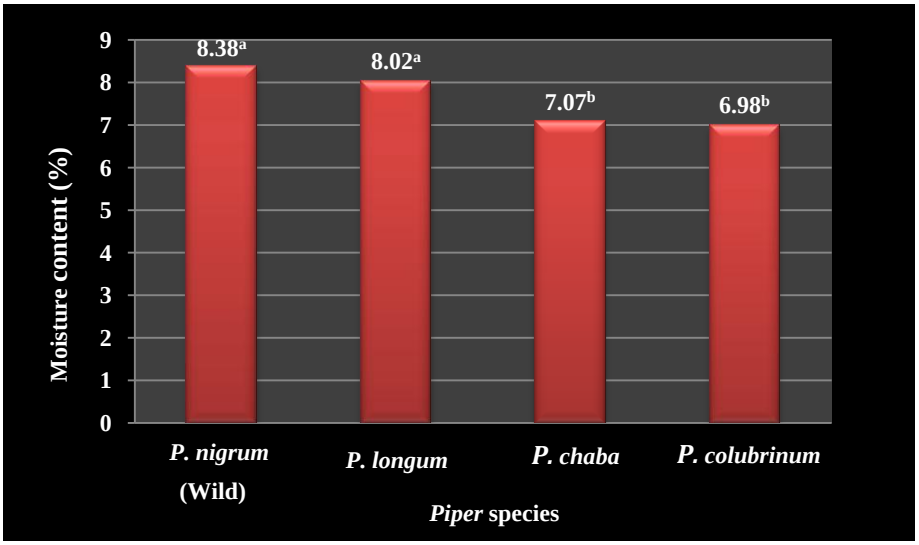

Fig. 4.1. Moisture content of selected *Piper* species

The moisture content is an important parameter for maintaining intrinsic quality of a product and also for their storage. So drying of harvested samples to normal/safe moisture level is essential to retain their quality attributes like essential oil, oleoresin and other important biochemical constituents like starch. Maintaining the normal moisture level is also critical to reduce the risk of fungal growth and consequent aflatoxin production in samples (Sindhu, 2011).

The moisture content of wild *P. nigrum* of present study is comparable to those of Pruthi (1993) and Nelson & Cannon-Eger (2011) who have given an average moisture content of 8.7 to 14% and 9.5 to 12.0 g/100 g respectively for black pepper berries. On the other hand, the moisture content of *P. longum* is given as 7.52% (Shankaracharya *et al*., 1997), 9.5% (Jayasinha, 1999) and 11.0±0.04% (Srivastava, 2012). Khan (2015) has compared the moisture content in *P. nigrum*, *P. longum*, *P. chaba*, *P. cubeba* and *P. betle* and found that *P. nigrum* showed

maximum moisture content whereas it is found to be least in *P. chaba.* This finding supports the present study.

4.1.1.1.2 Bulk density

Overall bulk density of selected *Piper* species was in the range of 502.8 to 530.4 g/L and was highest in wild *P. nigrum* (530.4 g/L) followed by *P. chaba* (521.4 g/L) and *P. colubrinum* (516.6 g/L). *P. longum* had comparatively low bulk density (502.8 g/L). However, on statistical interpretation, it was observed that there was no significant difference in the bulk density of selected *Piper* species. The results are illustrated in Fig. 4.2.

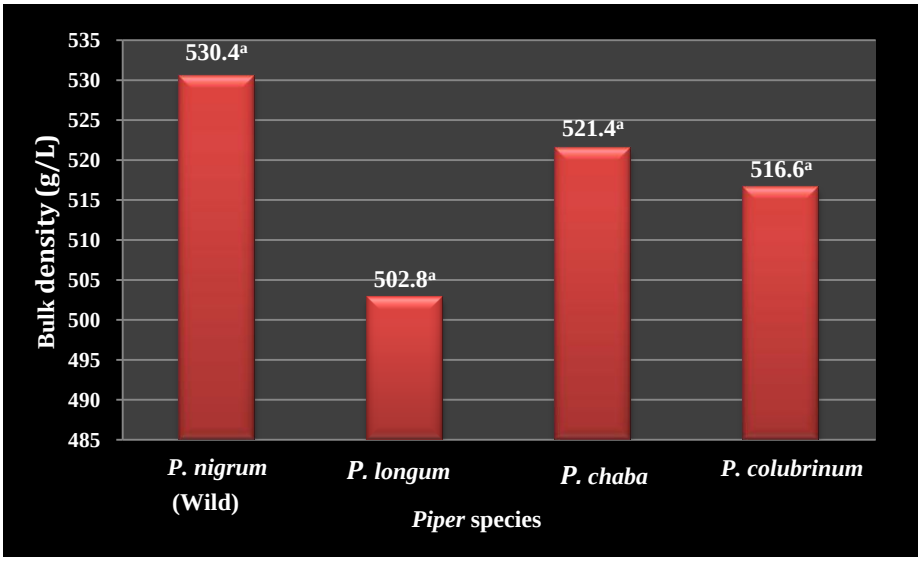

Fig. 4.2. Bulk density of selected *Piper* species

Bulk density is the major physical property of powder, bulk material, granules or other food material and it influences directly to the cost of the substances (Lam *et al.*, 2008; Meghwal & Goswami, 2011). It also impacts storage requirements, the sizing of the material handling

system and how the material behaves during subsequent thermo-chemical and biological processes (McKendry, 2002).

Bulk density of wild *P. nigrum* in the present study is in tune with Pruthi (1993), who has reported bulk density in the range of 426 to 850 g/L for black pepper samples.

4.1.1.1.3 Total ash

The total ash content had a range of 4.7 to 9.2% and it was found to be highest in *P. colubrinum* and lowest in *P. chaba*. Wild *P. nigrum* and *P. chaba* were statistically on par whereas remaining samples showed significant variability for their total ash content. The percentage of total ash content in the selected *Piper* species is summarized in Fig. 4.3.

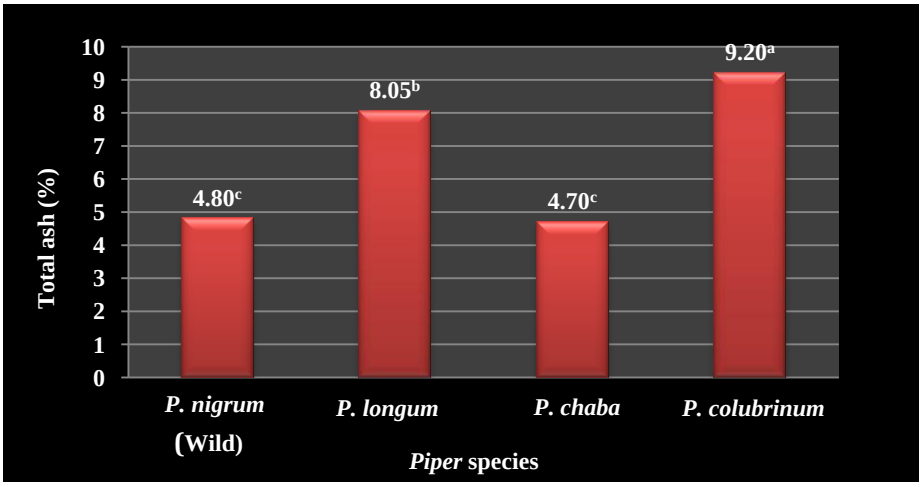

Fig. 4.3. Total ash content of selected *Piper* species

The ash content is the measure of mineral contents and other inorganic matters present in biomass and is used in conjunction with other procedures to determine their total composition (Sluiter *et al.*, 2005). The total ash content of wild *P. nigrum* obtained in the present study is

in agreement with those reported by Pruthi (1993) as 3.6 to 5.7% and de Waard & Anunciado (1999) as 3.4 to 6.0 g/100 g and also reasonably in line with the report of Trivedi *et al*. (2011) as 4.5% and Ahmad *et al.* (2015) as 4.31±0.32%. However, the total ash content obtained for *P. longum* in the present study is found to be higher than those compiled by Trivedi *et al*. (2011), Vinay *et al*. (2012), Shankaracharya *et al*. (1997), Jayasinha (1999) and Srivastava (2012) as 4.31%, 5.5%, 5.2%, 5.9% and 6.4±0.03% respectively. In addition, Khan (2015) has reported the total ash value of *P. nigrum*, *P. longum* and *P. chaba* fruits as 10.8±0.4, 5.7±0.2% and 6.85±0.8% respectively. Antia *et al*. (2006) stated in their publication that the high ash content is the reflection of mineral contents preserved in the sample.

4.1.1.1.4 Acid insoluble ash

The mean acid insoluble ash content of the selected *Piper* species (Fig. 4.4) ranged from 0.3 to 1.6%. *P. colubrinum* showed highest acid insoluble ash whereas wild *P. nigrum* and *P. chaba* had lowest acid insoluble ash content. The acid insoluble ash content showed the same statistical trend as total ash content of the samples.

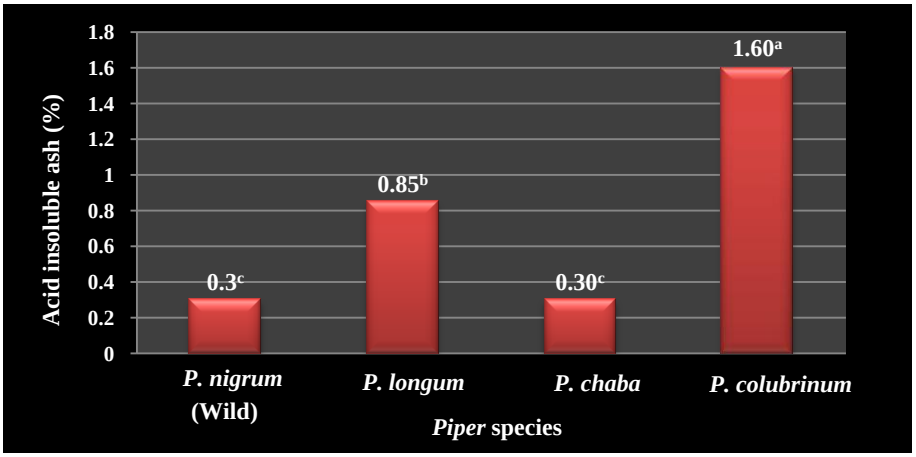

Fig. 4.4. Acid insoluble ash content of selected *Piper* species

The acid insoluble ash, an index of dirt or sand in a product is determined in selected *Piper* species by different research groups. The average acid insoluble ash content of wild *P. nigrum* determined in the present study is supported by the previous work done by Pruthi (1993) for different black pepper samples as 0.03 to 0.55%. Trivedi *et al*. (2011) and Ahmad *et al.* (2015) also reported acid insoluble ash content in black pepper sample as 0.45% and 0.48±0.44% respectively. Trivedi *et al*. (2011) also stated the acid insoluble ash content of *P. longum* as 0.41%. Likewise, Shankaracharya *et al*. (1997) reported acid insoluble ash content of *P. longum* as 0.85%, which support our finding. However, Khan (2015) has reported high acid insoluble ash value for *P. nigrum* (1.0±0.1%), *P. longum* (1.5±0.1%) and *P. chaba* (3.0±0.1%) fruits.

4.1.1.2 Primary metabolites

The primary metabolites are essential and ubiquitous to plant kingdom since they are directly involved in the growth and development of plants and they are the main source of energy. The primary metabolites investigated in the present study include total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat and crude fibre.

4.1.1.2.1 Carbohydrates

The total carbohydrate, reducing sugars and starch contents were estimated from the selected *Piper* species by UV-Visible spectrophotometric method as per section 3.2.2.1.

4.1.1.2.1.1 Total carbohydrate

The total carbohydrate content (61.53 to 70.74%) was found to be highest in *P. colubrinum* whereas lowest in *P. chaba.* Wild *P. nigrum* and *P. chaba* as well as *P. longum* and *P. colubrinum* were statistically on par for their total carbohydrate content. The percentage values for the total carbohydrate of the selected samples are given in Fig. 4.5.

Carbohydrates, the compounds produced during photosynthesis have two main functions in plants. First, they provide building blocks for plant structural components like cell wall. Secondly, carbohydrates are major contributor to deliver energy for plant growth. (http://passel.unl.edu/pages/informationmodule.php?idinformationmodule=1120069862&topicorder=4). In addition to these typical roles, carbohydrates are also recognized as signaling molecules in plants and thus contributing to innate immunity of plants (Moghaddam *et al.*, 2010; Moghaddam & Van den Ende, 2012).

Zachariah *et al*. (2010) have revealed the range for total carbohydrate of different black pepper cultivars as 38.6 to 51.2 mg%. The total carbohydrate content of wild *P. nigrum* in the present study is reasonably in agreement with Pradeep *et al.* (1993) and Gutierrez *et al*. (2013) who have reported total carbohydrate for black pepper sample as 65.75 g/100 g and 64.81 g/100 g respectively. Srivastava (2012) found out total carbohydrate content of *P. longum* as 59.2+0.03%.

The stages of plant growth, environmental factors (temperature, light intensity, etc.), availability of water and nutrients during plant growth, rate of photosynthesis, biotic and abiotic stress, etc. are the factors affecting carbohydrate contents of plants. They also vary in relation to species and varieties and also due to the interactions of the plants with their environment (http://www.safergrass.org/pdf/AAEP.pdf).

4.1.1.2.1.2 Starch

The starch content varied from 22.62 to 45.01% (Fig. 4.5) and was shown to be highest in *P. longum*. It was noted that starch content was found to be very low in *P. colubrinum* compared to other three species.

The reserve carbohydrate starch is an important constituent of human diet and has numerous industrial applications includes food and pharmaceuticals (Steinbuchel & Rhee, 2005a).

Starch content in the present study for wild *P. nigrum* is supported by previous reports of Pradeep *et al.* (1993), Pruthi (1993) and de Waard & Anunciado (1999) as 41.24%, 28.0 to 49.0% and 25.8 to 44.8 g/100 g respectively. However, starch content of *P. longum* in the present study is found to be higher than those reported by Shankaracharya *et al*. (1997) and Jayasinha (1999) as 40.5% and 39.5% respectively.

Starch content in plants varies based on environmental factors (light, wind, air, temperature, availability of water, location of plants, etc.) and plant factors like leaf surface (https://in.answers.yahoo. com/question/index?qid=20070407104556AAfAiXc).

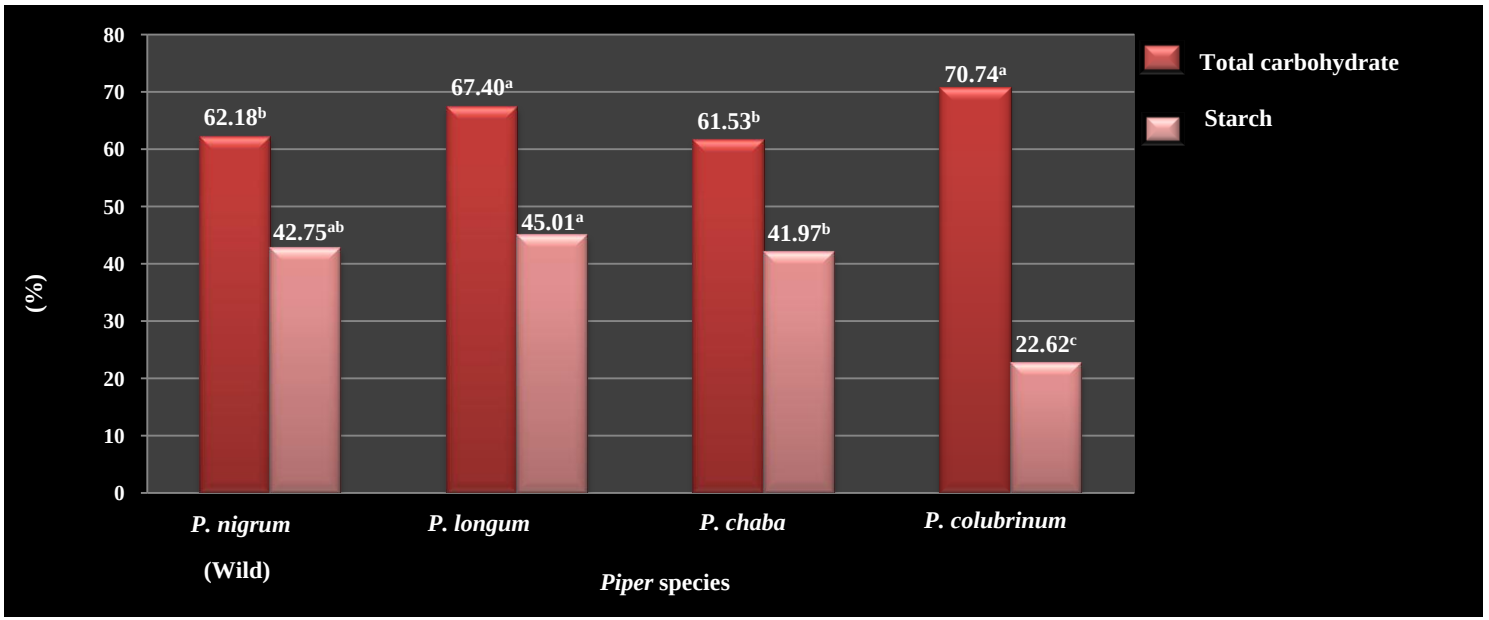

Fig. 4.5. Total carbohydrate and starch contents of selected *Piper* species

4.1.1.2.1.3 Reducing sugars

The reducing sugar content of matured berries/fruits of selected *Piper* species were in the range of 0.41 to 13.9%. *P. colubrinum* showed very high (13.9%) reducing sugar content and was found to be significantly different from other three *Piper* species (Fig. 4.6). Wild *P.nigrum* and *P.chaba* were statistically on par for their reducing sugar content.

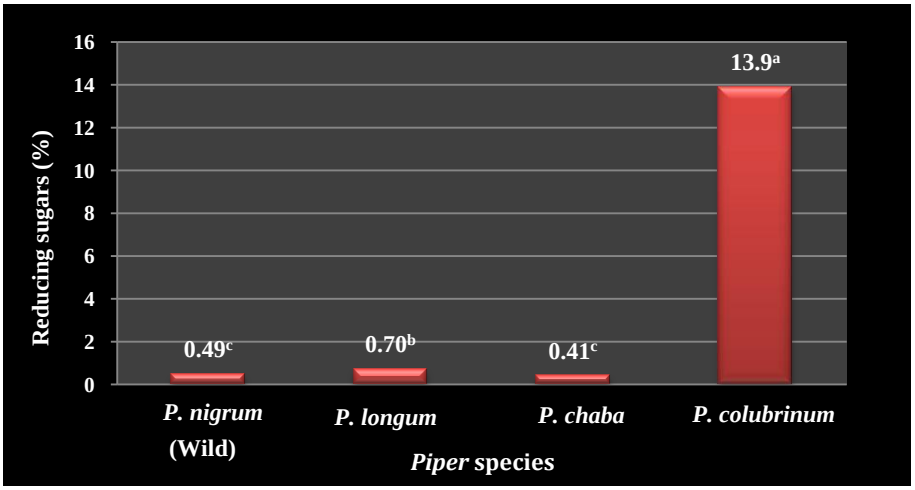

Fig. 4.6. Reducing sugar content of selected *Piper* species

Reducing sugar is able to act as reducing agent due to its free aldehyde or ketone group and thus can function as quality indicator of plant products like juice, wine, etc. (Pratt & Cornely, 2013; https://en.wikipedia.org/wiki/Reducing_sugar#Importance_in_medicine).

Sumeshkumar (2004) has stated 0.90 to 2.31 mg/100mg reducing sugars for different black pepper varieties. Shankaracharya *et al.* (1997) have reported reducing sugar for *P. longum* as 5.07%. Variation in the factors like light intensity, temperature, location and rate of photosynthesis, can be attributed as reasons for such variability.

4.1.1.2.2 Protein

4.1.1.2.2.1 Total protein

The total protein content, estimated by Lowry's method according to section 3.2.2.2.1 is given in Fig. 4.7. The total protein content (6.05 to 15.5%) was highest in *P. colubrinum* and lowest in wild *P. nigrum.* It was statistically on par for *P. longum* and *P. chaba* samples.

Protein, the catalysts of diverse capability, mediators of self assembly, agents of molecular recognition, transducers of energy and information, media of communication and librarians of the genetic program, is essential component of every biological system (Steinbuchel & Rhee, 2005b).

Sumeshkumar (2004) who has reported the total protein content of different black pepper varieties (3.54 to 6.61%), supports the reported protein value in this study for wild *P. nigrum*. In addition, Pradeep *et al*. (1993), Gutierrez *et al*. (2013) and Nelson & Cannon-Eger (2011) reported protein content of black pepper as 13.25, 10.95 and 10.9 to 12.7 g/100 g respectively. Report of protein in *P. longum* is in the range of 11.94 to 12.2% (Shankaracharya *et al*., 1997; Jayasinha, 1999). These reports endorse the report in the present study for *P. longum.* Sindhu *et al*. (2013) also reported protein content from *P. longum* as 13.47±0.77 mg/g fresh weight. The environmental factors like solar radiation, soil nutrients especially nitrogen, biochemical and nutritional factors of plants such as level of free amino acids, nitrogen content and genetic factors like level of RNA are the important factors affecting protein content of plants (Cruz *et al.*, 1970; Fabre & Planchon, 2000).

4.1.1.2.2.2 Total free amino acid

The total free amino acid was quantified from the selected *Piper* species by Ninhydrin method (section 3.2.2.2.2) and the result is shown in Fig. 4.7. It was in the range of 0.21 to 0.92%. The highest total free amino acid was recorded in *P. colubrinum* and lowest in *P. longum*. It was observed that the total free amino acid was comparatively higher in *P. colubrinum* fruit sample than in other three species.

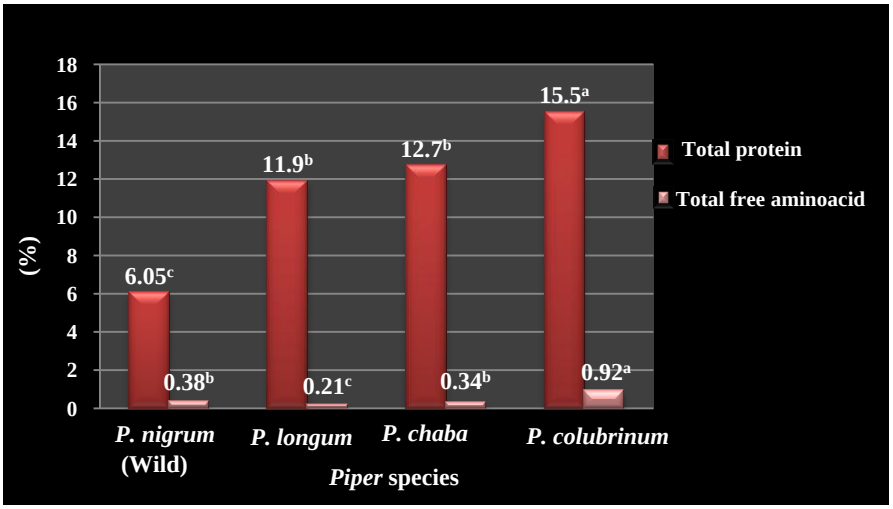

Fig. 4.7. Total protein and total free amino acid contents of selected *Piper* species

The amino acids which exist in free form in plant tissues will vary based on health condition of the plants (Sadasivam & Manickam, 2008). Sumeshkumar (2004) has studied total free amino acid content of different black pepper varieties and given the range as 0.02 to 0.201%. Sindhu *et al*. (2013) evaluated the total free amino acid content of *P. longum* fruit as 8.2±0.55 mg/g fresh weight.

4.1.1.2.3 Total fat

The total fat content estimated as per section 3.2.2.3.1, ranged from 5.82 to 8.04%. The highest total fat content was recorded in *P. colubrinum* (8.04%) and it was statistically on par with that of wild *P. nigrum* (7.56%). The lowest total fat content was recorded in *P. longum* sample. The result is illustrated in Fig. 4.8.

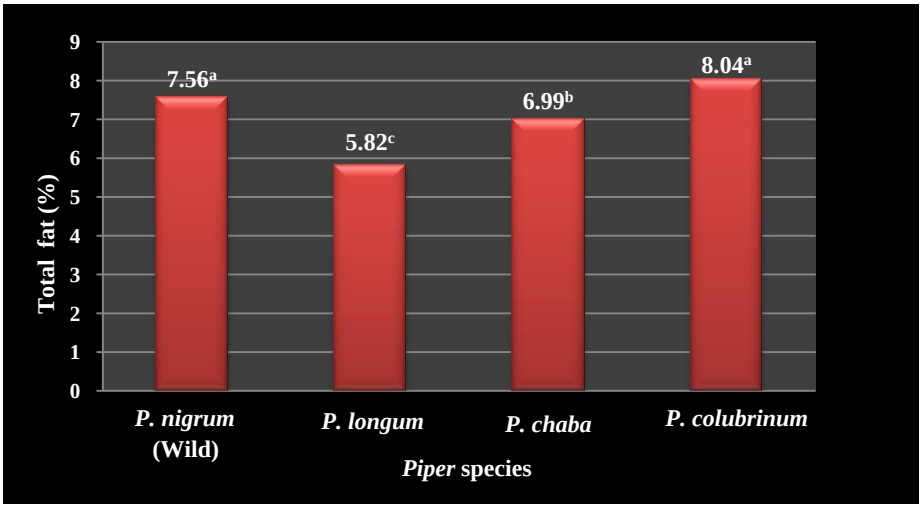

Fig. 4.8. Total fat content of selected *Piper* species

Fat is another primary metabolite in plants, which mainly involves in storage of metabolic energy, plant protection, carrying electrons and light absorption (http://lifeofplant.blogspot.in/2011/03/lipids.html). Pradeep *et al*. (1993) reported total fat content of black pepper as 6.77% whereas Srivastava (2012) has reported total fat from *P. longum* as 17.6±0.02%. Harwood (1994) reported that the plant lipid contents vary in response to environmental changes and the significance of this variation is not always clear. His report indicates that, this alteration may merely represent the acute responses and not necessarily true adaptation.

4.1.1.2.4 Crude fibre

The crude fibre content estimated as per section 3.2.2.4 varied from 6.65 to 12.6% (Fig. 4.9) and it was highest in wild *P. nigrum* followed by *P. longum* whereas it was comparatively low in *P. colubrinum* and *P. chaba*.

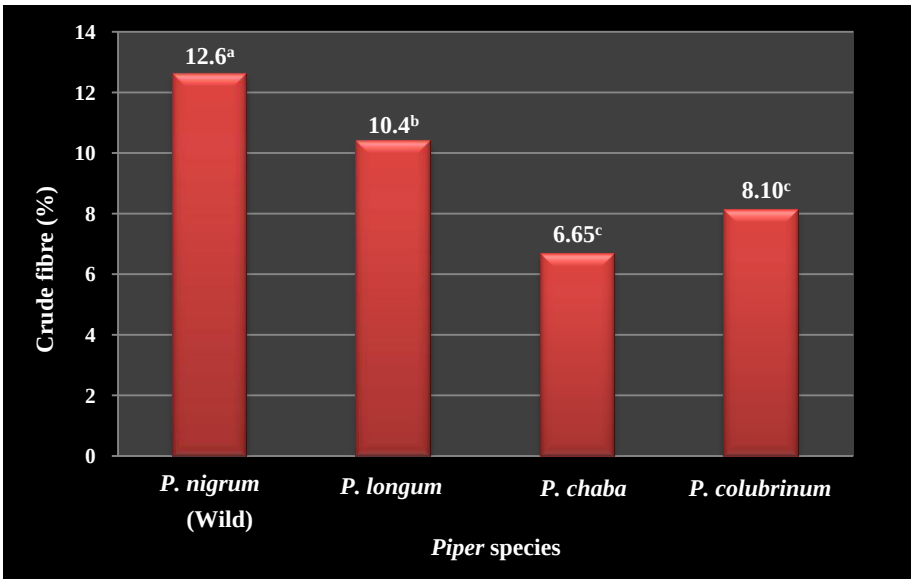

Fig. 4.9. Crude fibre content of selected *Piper* species

Crude fibre, the residue left after treatment with acid and alkali, primarily consists of cellulose and lignin and thus imparts structural rigidity to plant cell wall (http://www.livestrong.com/article/480986-differences-of-crude-and-dietary-fiber/). The range for fibre content is listed by Nelson & Cannon-Eger (2011**)** in their article as 9.7 to 17.2 g/100 g for black pepper which support the crude fibre value obtained for wild *P. nigrum* in the present study. The crude fibre content of *P. longum* in the present study is shown to be high in comparison with the earlier reports of Shankaracharya *et al*. (1997), Srivastava (2012)

and Jayasinha (1999) as 6.8%, 4.5±0.03% and 5.8% respectively. The interactions of environment and climate with plant physiology might impart variability in crude fibre content (http://www.fao.org/wairdocs /ilri/x5495e/x5495e06.htm).

4.1.1.3 Secondary metabolites

Secondary metabolites which play the major defensive and attractive role in the interactions between the plants and their environment mainly include terpenoids, alkaloids and phenolics (Figueiredo *et al.*, 2008).

4.1.1.3.1 Essential oil

4.1.1.3.1.1 Essential oil yield

The essential oil content, estimated by hydrodistillation method (section 3.2.3.1.1), varied between 0.2 to 2.4% (Fig. 4.10). The highest essential oil content was recorded in wild *P. nigrum* followed by *P. chaba* and *P. longum* whereas lowest in *P. colubrinum*. It was observed that the essential oil content in *P. colubrinum* was very low compared to other three *Piper* species.

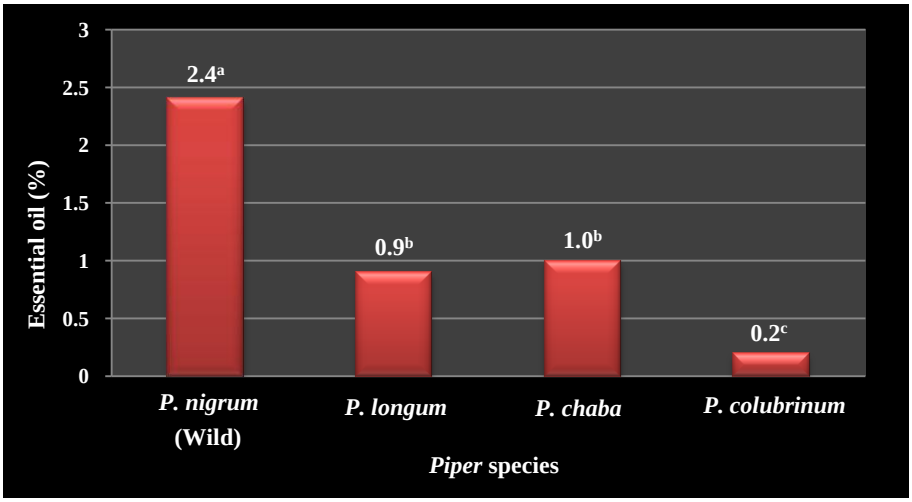

Fig. 4.10. Essential oil yield of selected *Piper* species

The essential oil is a heterogeneous complex mixture and is the contributor to aroma. The quantity of essential oil is affected by factors like age, growth and climatic conditions (Janardhanan & Thoppil, 2004). The essential oil content of black pepper is mentioned by de Waard & Anunciado (1999) as 1.0 to 1.8%. Radhakrishnan *et al*. (2004) and Zachariah *et al*. (2005) reported essential oil content for different black pepper cultivars as 2.1 to 7.0% and 2.4 to 4.4% respectively. The essential oil yield of wild *P. nigrum* in the present study is in accordance with these reports. Shankaracharya *et al*. (1997) reported essential oil content from *P. longum* as 0.95% whereas Jayasinha (1999) mentioned it as 0.7 to 1.5%. These reports support the essential oil yield for *P. longum* in the present study. Rameshkumar *et al*. (2011) reported essential oil yield of *P. chaba* and *P. longum* as 1.2% and 0.6% respectively. Khan (2015) has reported essential oil content of black pepper, *P. longum* and *P. chaba* collected from local market as 1.6, 0.2 and 0.8% respectively. The trend obtained in the present study is in tune with these two reports.

4.1.1.3.1.2 Essential oil profiling by GC-MS

Essential oil constituents have great importance because of their ability for being natural and biodegradable, having a low toxicity to mammals and being able to simultaneously accomplish the function of their synthetic equivalents. Furthermore, essential oils can also be employed for the protection of crops and also for other pharmacological and medicinal values (Figueiredo *et al*., 2008).

The essential oil extracted from the four *Piper* species were subjected to oil profiling by GC-MS and their characteristic constituents were identified (section 3.2.3.1.2). A range of 28-41 compounds were identified and good variability was observed among these *Piper* species for their essential oil constituents (Table 4.1).

Among 28 compounds (98.63%) identified from wild *P. nigrum* essential oil, major compounds were monoterpenes (α-thujene, α-and β-pinene, δ-3 carene, D-limonene, etc.) followed by sesquiterpenes (β-caryophyllene, α-humulene, caryophyllene oxide, etc). No aliphatic compounds were detected from this sample. Delta-3 carene (19.8%) and D-limonene (16.3%) were the major monoterpenes whereas β-caryophyllene (20.7%) was the major sesquiterpene identified in wild *P. nigrum* essential oil sample. Delta-3 carene, a bicyclic monoterpene is recommended for salty flavours whereas D-limonene, a cyclic monoterpene is known for hepatoprotective activity and chemopreventive efficacy against different cancers (Stratton *et al.*, 2000; Ozbek *et al.*, 2003; Parija & Das, 2003). On the other hand, β-caryophyllene, the bicyclic sesquiterpene is shown to have anticancer (Legault & Pichette, 2007), antiinflammatory (Gertsch *et al*., 2008), antinociceptive (Katsuyama *et al.*, 2013), anti-alcoholism

(Al Mansouri *et al*., 2014), anxiolytic and antidepressant (Bahi *et al*., 2014) activities. Alpha thujene, α- and β-pinene, β-myrcene, α-phellandrene, α-terpinolene, β-elemene, α-humulene, α-and β- selinene, caryophyllene oxide, etc. were the other major volatile constituents identified from this sample.

Forty one (65.68%) compounds were identified from essential oil of *P. longum* fruits and of which, aliphatic compounds predominated followed by monoterpenes. Sesquiterpenes were less compared to other three essential oils. n-Pentadecane (14.5%) followed by n-heptadecane (6.98%) were the major aliphatic compounds and β-pinene (14.6%) was the major monoterpene identified from *P. longum* essential oil. Beta-pinene is reported for therapeutic potential includes antimicrobial and anticancer activities (Wang *et al.*, 2012). However, the information is scanty regarding the medicinal properties of aliphatic compounds. A few miscellaneous constituents were also identified from this sample. Alpha pinene, D-limonene, 2-undecanone, methyl eugenol, β-caryophyllene, 1-pentadecene, *trans*-nerolidol, β-bisabolene, etc. were the other major essential oil constituents identified from this sample.

Thirty five compounds (93.17%) were identified from *P. chaba* fruit oil. Sesquiterpenes were the major class of compounds followed by aliphatic compounds. Only a trace amount of monoterpenes were identified from this sample. Beta-caryophyllene (9.73%) followed by zingiberene (7.44%) were the major sesquiterpenes identified in *P. chaba* oil. The importance of the monocyclic sesquiterpene zingiberene as insecticides, repellents, insect feeding deterrents, antioxidant, anticancer and antiproliferative agent has been reported (Antonious & Kochhar, 2003; Togar *et al*., 2015). On the other hand,

1-pentadecene (9.77%) and n-heptadecane (8.86%) were identified as the major aliphatic compounds. Beta-elemene, n-tridecane, *trans*-α-bergamotene, 1-heptadecene, n-pentadecane, 3-heptadecene, α-bisabolene, α-humulene, germacrene D, etc. were the other major compounds identified from this sample.

Among 36 (87.74%) compounds identified from essential oil of *P. colubrinum* fruits, sesquiterpenes have given the major contribution. Alpha-muurolol (12.5%), the underexplored compound for its therapeutic potential and β-caryophyllene (11.3%) were the major sesquiterpenes identified along with other sesquiterpenes like γ-muurolene, muurola-3,5 diene, δ-selinene, δ-cadinene, α-humulene, caryophyllene oxide, etc. Few aliphatic compounds like n-heptadecane and trace amount of monoterpenes were also identified from the sample. Alpha- and β-pinene and α-terpineol were the only monoterpenes identified from the *P. colubrinum* fruit oil.

Wild *P. nigrum* shared 17, 12 & 11 compounds common with *P. longum*, *P. chaba* and *P. colubrinum* respectively whereas 19 and 16 compounds of *P. longum* were common with *P. chaba* and *P. colubrinum* respectively. *P. chaba* and *P. colubrinum* showed 15 common essential oil constituents. Constituents such as β- pinene, β-caryophyllene, α-humulene, β-bisabolene and caryophyllene oxide were identified in all the four species. Beta pinene, known for its aroma and therapeutic properties was found to be highest in *P. longum* (14.6%) followed by wild *P. nigrum.* However, *P. chaba* and *P. colubrinum* contained trace amount of β-pinene content. Beta-caryophyllene that contributes to the spiciness was highest in wild *P. nigrum* (20.7%) and lowest in *P. longum* (3.83%). Alpha humulene, isomer of β-caryophyllene was highest in *P. chaba* (5.25%) and

comparatively very low in *P. longum* (0.14%). On the other hand, Beta-bisabolene, a natural sweetener and food additive was recorded highest in *P. colubrinum* (2.84%) and not much difference among other three *Piper* species. Caryophyllene oxide, a well known preservative in food, drugs and cosmetics was highest in *P. colubrinum* (5.16%) and comparatively low in *P. longum* (0.14%).

Alpha-phellandrene, *o*-cymene, *cis*-limonene oxide, α-phellandrene-8-ol and α-guaiene were identified only in wild *P. nigrum* whereas methyl eugenol, 2-heptanol, 2-nonanone, 4-terpineol, 2-acetoxytridecane, 2-undecanone, β-farnesene, β-eudesmol and geranyl linalool isomer were identified only in *P. longum* essential oil. Germacrene D, *trans*-α-bergamotene, zingeberene, α-bisabolene, selina-3,7(11)-diene, 1-heptadecene (Z), Z-5-nonadecene, 1-nonadecene and n-octadecanal were detected only in *P. chaba* essential oil whereas aromadendrene, β-bourbonene, 6,9 guaiadiene, muurola 3,5 diene, alloaromadendrene, γ-muurolene, δ-selinene, γ-cadinene, spathulenol, epi-α-cadinol, α-cadinol, α-muurolol, α-bisabolol, 3-farnesol and farnesyl acetone (5E 9E) were unique to *P. colubrinum* essential oil and not identified in other three samples.

Camphene, α-thujene, β-myrcene, α-terpinene, *p*-cymene, γ-terpinene and α-terpinolene were identified only in wild *P. nigrum* and *P. longum* whereas sabinene and δ-3-carene were identified only in wild *P. nigrum* and *P. chaba.* Z- and E-ocimene, 6- tridecene, n-tridecane, *cis*-α-bergamotene, 1-pentadecene and n-pentadecane were present only in *P. longum* and *P. chaba* whereas δ-cadinene, *trans*-nerolidol and phytol were the compounds identified only in *P. longum* and *P. colubrinum.* The volatile constituent cubenol was identified only in *P. chaba* and *P. colubrinum.*

Heneicosane, 3-Heptadecene (Z), 8-heptadecene (Z), n-heptadecane and n-nonadecane were identified in all samples except wild *P. nigrum.* Likewise, β-selinene, δ-and β-elemene were identified in all essential oils except *P. longum.* Alpha-pinene, α-terpineol and α-selinene were absent only in *P. chaba* oil. Furthermore, D-limonene and β-linalool were absent only in *P. colubrinum* oil.

GC-MS profiling of essential oils of four *Piper* species is given in Fig. 4.11. This is the first report regarding essential oil profile of *P. colubrinum* fruits cultivated in India and also comparison of oil profiles among these four species.

Table 4.1. Essential oil profiling of selected *Piper* species by GC-MS

Compound	**% Composition**				
	***P. nigrum* (wild)**	***P. longum***	***P. chaba***	***P. colubrinum***	**RI***
2-Heptanol	-	0.14	-	-	908
α-Thujene	0.11	0.12	-	-	932
α-Pinene	5.17	4.81	-	0.12	941
Camphene	0.12	0.37	-	-	954
Sabinene	0.10	-	0.02	-	979
β-Pinene	9.60	14.6	0.03	0.03	987
β -Myrcene	3.24	0.87	-	-	997
α-Phellandrene	4.83	-	-	-	1012
δ-3-Carene	19.8	-	0.01	-	1015
α-Terpinene	0.05	0.06	-	-	1022
p-Cymene	0.08	0.02	-	-	1031
o-Cymene	2.57	-	-	-	1032
D-Limonene	16.3	3.95	0.04	-	1037
Z-Ocimene	-	0.01	0.02	-	1044
E-Ocimene	-	0.15	0.04	-	1054
γ-Terpinene	0.13	0.12	-	-	1065
α-Terpinolene	1.53	0.25	-	-	1094
2-Nonanone	-	0.18	-	-	1099
β-Linalool	0.28	0.59	0.11	-	1108
cis-Limonene oxide	0.10	-	-	-	1143
α -Phellandrene-8-ol	0.10	-	-	-	1171

Compound	% Composition				
	***P. nigrum* (wild)**	***P. longum***	***P. chaba***	***P. colubrinum***	**RI***
4-Terpineol	-	0.26	-	-	1187
α-Terpineol	0.21	0.51	-	0.25	1202
2-Acetoxytridecane	-	0.15	-	-	1241
6- Tridecene	-	0.05	0.32	-	1291
2-Undecanone	-	1.03	-	-	1301
n-Tridecane	-	0.43	2.56	-	1303
δ-Elemene	0.89	-	0.15	0.31	1344
α-Copaene	-	-	0.39	1.70	1384
β-Bourbonene	-	-	-	0.13	1394
β- Elemene	1.67	-	2.06	0.52	1400
Methyl eugenol	-	1.65	-	-	1416
cis-α-Bergamotene	-	0.15	0.56	-	1423
β-Caryophyllene	20.7	3.83	9.73	11.3	1431
Aromadendrene	-	-	-	0.26	1437
trans-α-Bergamotene	-	-	4.21	-	1442
α-Guaiene	0.95		-	-	1446
6,9 Guaiadiene	-	-	-	0.91	1452
Muurola 3,5 diene	-	-	-	4.06	1461
β-Farnesene	-	0.64	-	-	1462
α-Humulene	2.25	0.14	5.25	2.22	1466
Allo-aromadendrene	-	-	-	1.87	1470
γ-Muurolene	-	-	-	8.49	1487
1-Pentadecene	-	2.63	9.77	-	1492
Germacrene D	-	-	4.43	-	1495
β- Selinene	3.22	-	1.93	1.22	1497
δ-Selinene	-	-	-	9.15	1502
Zingeberene	-	-	7.44	-	1505
α-Selinene	2.07	0.75	-	2.29	1506
n-Pentadecane	-	14.5	5.40	-	1508
β- Bisabolene	1.18	1.56	1.23	2.84	1519
α-Bisabolene	-	-	7.04	-	1520
γ-Cadinene	-	-	-	0.90	1525
7-epi α-selinene	0.11	-	-	-	1528
Selina-3,7 (11)-diene	-	-	3.31	-	1531
δ-Cadinene	-	0.72	-	3.36	1534
trans-Nerolidol	-	1.20	-	1.22	1584
Spathulenol	-	-	-	1.79	1593

Compound	% Composition				
	***P. nigrum* (wild)**	***P. longum***	***P. chaba***	***P. colubrinum***	**RI***
Caryophyllene oxide	1.27	0.14	1.10	5.16	1599
Cubenol	-	-	0.26	2.62	1600
Epi-α-cadinol	-	-	-	0.73	1634
α-Muurolol	-	-	-	12.5	1643
β -Eudesmol	-	0.07	-	-	1667
α-Cadinol	-	-	-	0.60	1670
3-Heptadecene (Z)	-	0.46	5.21	0.41	1683
1-Heptadecene (Z)	-	-	7.94	-	1692
8-Heptadecene (Z)	-	0.74	0.12	0.92	1697
α-Bisabolol	-	-	-	2.03	1698
n-Heptadecane	-	6.98	8.86	2.49	1706
3-Farnesol	-	-	-	0.51	1708
Z-5-Nonadecene	-	-	0.84	-	1879
1-Nonadecene	-	-	1.42	-	1889
n-Nonadecane	-	0.14	0.87	0.68	1899
Farnesyl acetone (5E 9E)	-	-	-	2.73	1926
Phytol	-	0.12	-	1.16	-
Geranyl linalool isomer	-	0.26	-	-	-
n-Octadecanal	-	-	0.40	-	-
Heneicosane	-	0.33	0.10	0.26	-
Total (Nos.)	28	41	35	36	
Total (%)	98.63	65.68	93.17	87.74	

*RI-Retention Indices

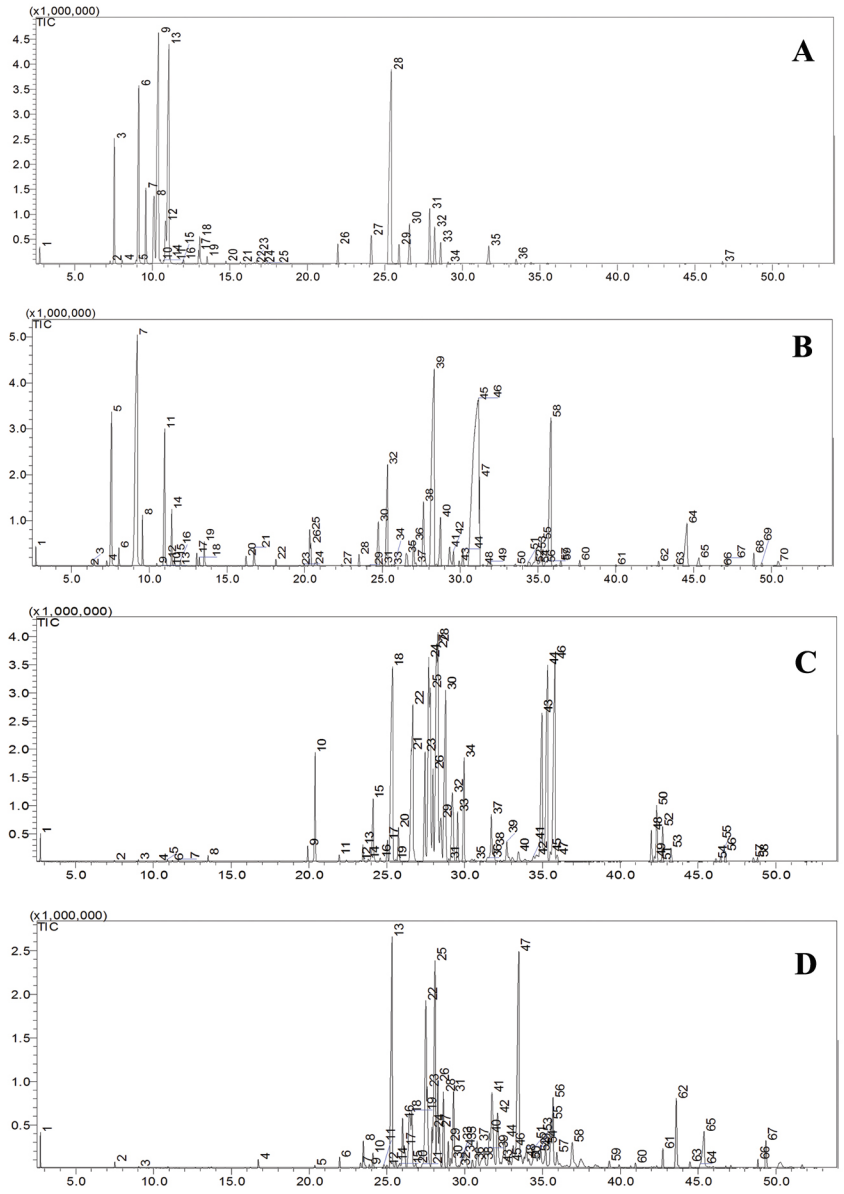

Fig. 4.11. GC-MS profiling of essential oil from selected *Piper* species. A) *P. nigrum* (Wild), B) *P. longum*, C) *P. chaba,* D) *P. colubrinum.* X-axis represents the retention time (Rt) and Y-axis represents the peak intensity (mV)

The essential oil constituents from the selected *Piper* species were reported by several researchers. Variations in the present study with these reports might be due to differences in the response to climatic, geographic or edaphic patterns and also due to variation in the maturity of raw material, extraction methods adopted, etc. (Rameshkumar *et al*., 2011). Barra (2009) mentioned that both exogenous and endogenous factors affect the essential oil composition. The endogenous factors mainly related to anatomical and physiological characteristics of the plants and also to the biosynthetic pathways of the volatiles, which in turn might be influenced by different tissues of the plants, different seasons and DNA adaptation. The exogenous factors might affect the genes responsible for the formation of volatiles.

Apart from the commercial importance of variability in yield and composition of essential oil, the possible changes are also important when the essential oil compounds are used as chemotaxonomic tools. The knowledge about the factors that determine the chemical variability and yield of oil for each species are thus very important. These factors include environmental conditions, physiological variations, genetic factors and evolution, geographic variations and also amount of plant material/space and manual labour needs (Figueiredo *et al.,* 2008).

Singh *et al*. (2004) identified 49 components from black pepper essential oil by GC and GC-MS analysis. The major components were β-caryophyllene (24.24%), limonene (16.88%), sabinene (13.01%), β-bisabolene (7.69%) and α-copaene (6.3%). On comparing with this report, wild *P. nigrum* essential oil of present study contained trace amount of sabinene (0.1%), less amount of β-bisabolene and no α-copaene, while it is in agreement with β-caryophyllene and limonene

contents. Sasidharan & Menon (2010) identified essential oil constituents from black pepper berries by GC and GC-MS. The major compound in pepper berry oil was β-caryophyllene followed by β-pinene, limonene, α-pinene and humulene.

Shankaracharya *et al*. (1997) reported 48 compounds from *P. longum,* which includes 8 to 10% oxygenated compounds and 35 to 40% straight chain hydrocarbons. Pentadecane (17.8%), β-caryophyllene (17%) and β-bisabolene (11.16%) were the major compounds. Heptadecane, spathulenol, globulol, pentadecene, germacrene B and D, α-zingeberene, α-humulene, β-farnesene, α-copaene, tridecane, etc. were the other compounds identified. Present study also showed pentadecane as a major component of *P. longum* fruit. However, β-caryophyllene and β-bisabolene reported as major constituents by these researchers were comparatively less in the present study.

Jayasinha (1999) reported compounds like zingeberene (7.0%), n-hexadecane (0.7%), n-heptadecane (6.0%), n-octadecane (5.8%), n-eicosane (4.7%), n-heneicosane (2.5%), α-thujene (1.7%), terpinolene (1.3%), dihydrocarveol (4.3%), *p*-methoxy acetophenone (trace) and phenethyl alcohol (2.1%) from *P. longum*. Among these compounds, zingeberene, n-hexadecane, n-octadecane, n-eicosane, dihydrocarveol, *p*-methoxy acetophenone and phenethyl alcohol were not identified in the present study.

The oil profiling of *P. nigrum* from Malaysia, *P. chaba* from Thailand and *P. longum* from Indonesia has been performed with GC and GC-MS by Tewtrakul *et al*. (2000). Unlike black pepper oil, *P. longum* and *P. chaba* contained few monoterpene hydrocarbons, moderate sesquiterpenes and high content of aliphatic hydrocarbons. *P. nigrum*

sample contained high β-caryophyllene content (39.7%) whereas 8-heptadecene (18.8%) has been reported as the major *P. longum* fruit oil constituent. They have also reported germacrene D (16.5%) and β-caryophyllene (10.2%) as major constituents in this sample. Heptadecane (15.1%), 8-heptadecene (15.0%) and pentadecane (10.8%) have been reported as the major constituents by these researchers from *P. chaba* fruit oil. By comparing this report with present study, it is observed that β-caryophyllene was low in wild *P. nigrum* (20.7%). Likewise, 8-heptadecene was obtained in minute quantity (0.74%) in *P. longum* fruit oil. Beta-caryophyllene was also comparatively low (3.83%) and germacrene D was not detected from *P. longum* fruit oil. On the other hand, pentadecane and 8-heptadecene were found to be less in *P. chaba* fruit oil of present study.

Liu *et al*. (2007) identified essential oil constituents of *P. nigrum* and *P. longum* by Gas chromatography-mass spectrometry following microwave distillation and headspace solid phase micro extraction. Thirty compounds have been identified from *P. nigrum*, of which, β-caryophyllene (23.49%), 3-carene (22.20%), D-limonene (18.68%), β-pinene (8.92%) and α-pinene (4.03%) were the major ones. This result supports our findings on wild *P. nigrum.* Among 45 compounds identified from *P. longum,* β-caryophyllene (33.44%), Δ^3-carene (7.58%), eugenol (7.39%), D-limonene (6.70%), zingiberene (6.68%), germacrene D (3.41%) and cubenol (3.64%) were the major compounds. Interestingly, β-caryophyllene was shown to be very less whereas Δ^3-carene, eugenol, zingiberene, germacrene D and cubenol were absent in *P. longum* fruit oil of the present study.

The essential oil constituents from *P. longum* and *P. chaba* fruits have been identified using GC-FID by Rameshkumar *et al*. (2011).

Aliphatic hydrocarbons mainly n-pentadecane (15.8%) predominated in the fruit oil of *P. longum* whereas *P. chaba* fruit oil mainly contained sesquiterpene hydrocarbons like germacrene D (21.5%), β-caryophyllene (18.5%) and α-humulene (11.4%). In the present study also, aliphatic hydrocarbons mainly n-pentadecane (14.5%) predominated in *P. longum* fruit oil whereas sesquiterpenes predominated in *P. chaba* fruit oil. In contrast to this report, zingeberene (7.44%) was comparatively high whereas β-caryophyllene (9.73) germacrene D (4.43) and α-humulene (5.25%) were comparatively low in *P. chaba* fruit oil of the present study.

It is reported that composition of essential oil of some *P. colubrinum* samples is entirely dominated by terpenoids (Maia & Andrade, 2009). However, reports on chemical profiling of *P. colubrinum* fruit oil are comparatively less.

4.1.1.3.2 Piperine

The pungent alkaloid piperine was quantified by HPLC method as per section 3.2.3.2 and the result is given in Fig. 4.12. It was in the range of 0.04 to 3.3%. Highest piperine content was recorded in *P. chaba* and it was statistically on par with wild *P. nigrum.* It was comparatively less in *P. longum* and found to be trace in *P. colubrinum*. HPLC chromatogram for piperine content of selected *Piper* species is shown in Fig. 4.13.

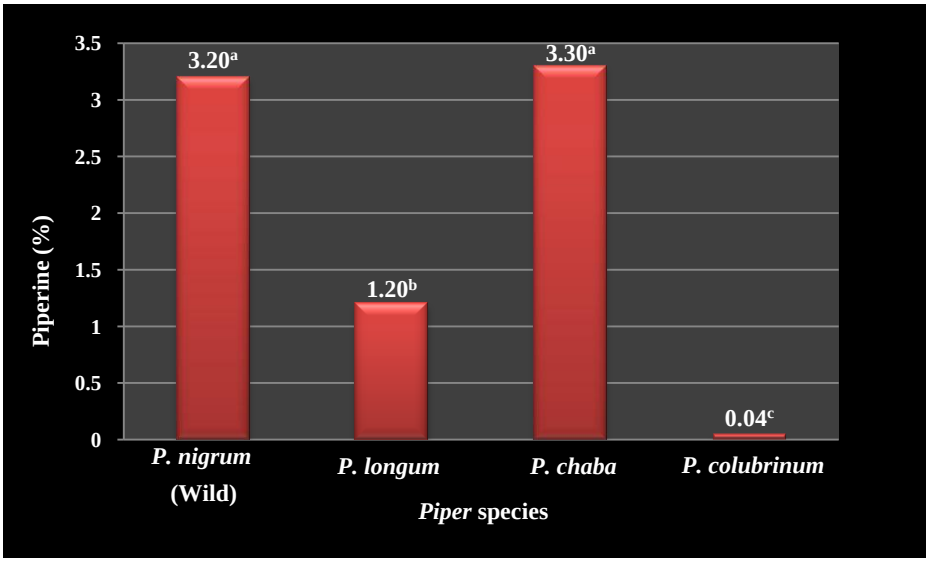

Fig. 4.12. Piperine content of selected *Piper* species

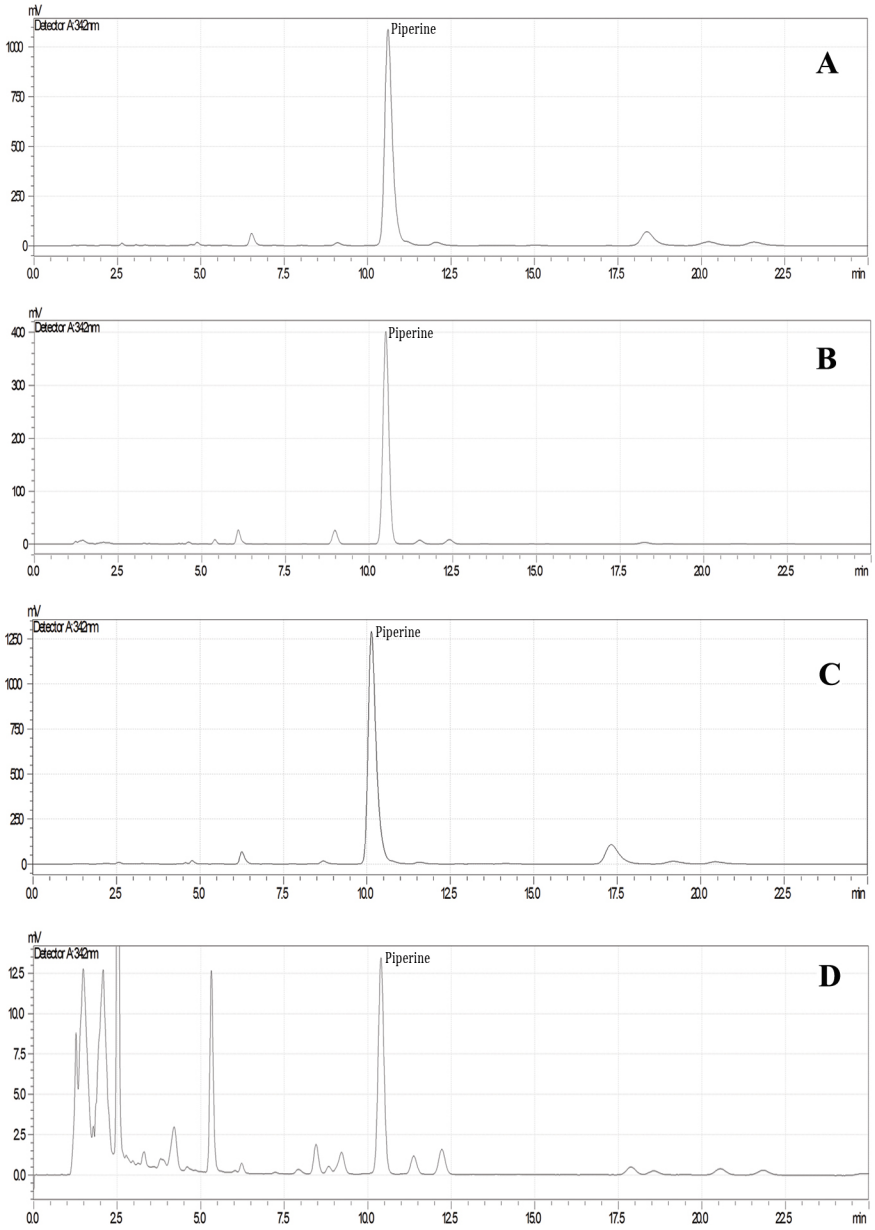

Fig. 4.13. HPLC chromatogram for piperine from selected *Piper* species. A) *P. nigrum* (Wild), B) *P. longum*, C) *P. chaba,* D) *P. colubrinum*

Piperine, the cyclobutane containing natural alkaloid imparts pungency and medicinal values to *Piper* species (Vasavirama & Upender, 2014) and is affected by maturation, planting site, harvesting time, climate, place of growth, etc. (Zachariah *et al.*, 2005; Honarvar *et al*., 2010; Haley & McDonald, 2016). Different research groups have studied piperine content from the *Piper* species selected for the study. Pruthi (1993) has recorded piperine content of different black pepper berries in the range of 1.7 to 7.4%. Piperine content of wild *P. nigrum* of present study is also within this range. The piperine content of *P. longum* on the other hand, has been reported by Shankaracharya *et al.* (1997) as 1.25%, which is closely in agreement with the present work. Rameshkumar *et al*. (2011) reported that piperine content in *P. longum* fruit is lower (0.03%) compared to *P. chaba* (1.32%). Even though the piperine content of *P. longum* and *P. chaba* of present study is high compared to this literature, the trend is same. The piperine from *P. longum* and *P. nigrum* fruits have also been illustrated by Santosh *et al*. (2005) as 0.879% and 4.5% respectively. Khan (2015) has studied piperine content of black pepper, *P. longum* and *P. chaba* collected from local market as 1.7, 0.3 and 0.95 g% respectively. Chandra *et al.* (2015) stated that piperine content in the fruit of *P. colubrinum* is below detectable limit. This finding is in agreement with present work which has given trace amount of piperine content for *P. colubrinum* fruit.

4.1.1.3.3 Phenolics

4.1.1.3.3.1 Total phenol

The total phenol content, estimated by Folin-Ciocalteau method as per section 3.2.3.3.1 ranged from 0.42 to 5.1% and it was extremely

high in *P. colubrinum* than other three species. There was no significant variability among wild *P. nigrum*, *P. longum* and *P. chaba* for their total phenolic content. The result is given in Fig. 4.14.

4.1.1.3.3.2 Total flavonoid

The total flavonoid content, estimated by Aluminium chloride colorimetric assay (section 3.2.3.3.2) was varied from 0.06 to 4.4%. It was found to be highest in *P. colubrinum* followed by wild *P. nigrum* whereas found to be trace in *P. chaba* and *P. longum*. It is noted that *P. colubrinum* showed comparatively very high total flavonoid content. The result is summarized in Fig. 4.14.

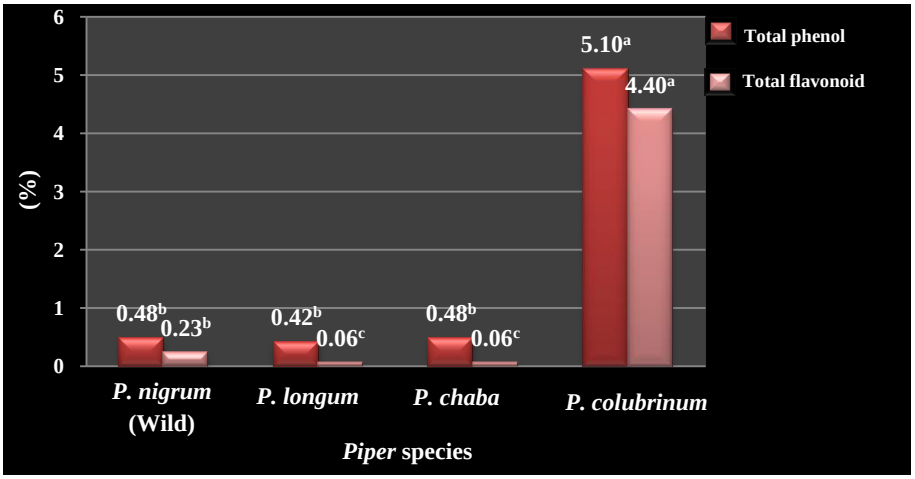

Fig. 4.14. Total phenol and total flavonoid contents of selected *Piper* species

Phenolic compounds are responsible for facilitating diverse functions in plants mainly defense against pathogens and pollination through colour and fragrance (Li *et al.*, 2010). Flavonoids, the largest and best studied natural phenols provide colour to the plant part and also act as chemical messengers, physiological regulators, cell cycle inhibitors

and inhibition/defense against pathogens (Galeotti *et al*., 2008; Cazarolli *et al*., 2008). The phenolic compounds are also reported to have very large range of biological activities.

The total phenolic and flavonoid contents in methanolic extract of black pepper were reported to be 1.728±0.049 mg/g and 1.087±0.002 µg/g respectively (Ahmad *et al.*, 2015). Chandra *et al.* (2015) revealed 59.31±2.69, 87.9±1.74, 42.7±0.21 and 222.72±3.13 µg/g of total phenol from the fruit of *P. nigrum, P. longum, P. chaba* and *P. colubrinum* respectively. They have also reported 13.4±0.32, 5.75±0.82, 28.98±1.81 and 943.25±0.83 µg/g of total flavonoids from the fruit of *P. nigrum, P. longum*, *P. chaba* and *P. colubrinum* respectively. This report endorses the present study for high phenolic and flavonoid contents in *P. colubrinum* than in other three *Piper* species.

Taxonomic position of plants and their specificity to biotic and abiotic environment are among the factors that affect the variability of polyphenols in plants (Gottlieb & Borin, 2000).

4.1.1.4 Oleoresin

The mean oleoresin content, estimated as per the section 3.2.4, varied from 7.88 to 22.3% (Fig. 4.15). Oleoresin content was found to be very high in *P. colubrinum*. There was no significant variability among wild *P. nigrum, P. longum* and *P. chaba* for their oleoresin content. Total phenol and oleoresin contents of selected *Piper* species showed same statistical trend.

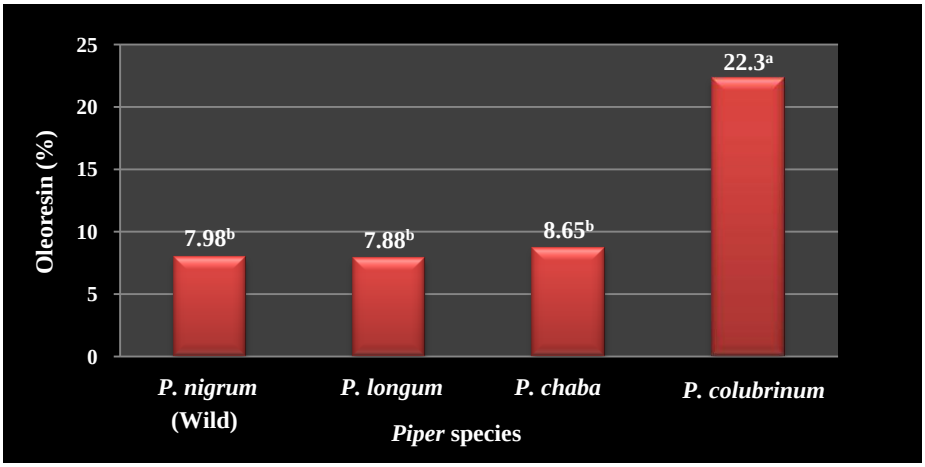

Fig. 4.15. Oleoresin content of selected *Piper* species

Oleoresins are natural isolates obtained by extracting the spice with suitable solvent and recovering the solvent by evaporation. It is a blend of volatile and non-volatile components that are soluble in the solvent. Oleoresins are commercially important because of the consistency in flavor, taste, antioxidant properties, increased shelf life and less storage space as it is a highly concentrated product (Zachariah, 2009). Zachariah *et al.* (2005) have reported oleoresin in the range of 6.9 to 12.77% for *P. nigrum* whereas Shankaracharya *et al*. (1997) have stated oleoresin yield for *P. longum* as 7.84%. Present study is in tune with these reports.

4.1.1.5 Minerals

Minerals are inorganic ions and though do not provide energy, plays a major role in the functioning of cell by being the co-factor of enzymes, cell signal and cell function mediators (Sathyanarayana, 2007). Mineral analysis of selected *Piper* species was conducted for N, P, K, Ca, Mg, Fe, Cu, Mn and Zn as per section 3.2.5 and significant

variability was observed. Wild *P. nigrum* showed highest Cu (45.5 mg/kg) whereas lowest N (2.1%), P (0.13%), K (0.54%), Ca (0.10%), Mg (0.08%) and Zn (12.2 mg/kg) contents. *P. longum* showed highest N (2.86%), K (1.54%) and Fe (145 mg/kg) contents and lowest Mn (13.0 mg/kg) content. *P. chaba* had highest Ca (0.43%) and lowest Fe (49.5 mg/kg) and Cu (10.0 mg/kg) contents. On the other hand, *P. colubrinum* showed highest P (0.4%), Mg (0.16%), Mn (36.0 mg/kg) and Zn (26.7 mg/kg) contents. The variability in mineral contents of selected *Piper* species is given in Table 4.2.

Table 4.2. Mineral contents of selected *Piper* species

Sample	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
P. nigrum (wild)	2.10^{b}	0.13^{c}	0.54^{d}	0.10^{d}	0.08^{c}	55.50^{c}	45.5^{a}	30.0^{b}	12.2^{d}
P. longum	2.86^{a}	0.31^{b}	1.54^{a}	0.18^{b}	0.13^{b}	145.0^{a}	12.0^{c}	13.0^{d}	20.8^{b}
P. chaba	2.27^{b}	0.14^{c}	0.72^{c}	0.43^{a}	0.13^{b}	49.50^{c}	10.0^{d}	16.5^{c}	14.1^{c}
P. colubrinum	2.69^{a}	0.40^{a}	1.44^{b}	0.12^{c}	0.16^{a}	121.0^{b}	20.0^{b}	36.0^{a}	26.7^{a}

Major nutrients (N, P and K), secondary nutrients (Ca and Mg) and micronutrients mainly Zn are the most important nutrients necessary for the growth, development and yield of plants (Srinivasan *et al*., 2012). Azmil & Yau (1993) have revealed nitrogen concentration in black pepper fruit spikes as 2.21% and is comparable with the present study. Zachariah & Parthasarathy (2008) have listed P, K, Ca and Fe contents of black pepper as 160 mg/100g, 1200 mg/100g, 0.4 g/100g and 17 mg/100g respectively whereas Gutierrez *et al.* (2013) have given Mg, Cu, Mn and Zn contents of black pepper as 194, 1.127, 5.626 and 1.42 mg/100g respectively. Neelam & Kamala (2001) reported that *P. longum* fruit contained minerals like Ca, P and Fe as 1230, 190 and 62.1 mg/100g respectively. The mineral constituents are

affected by several factors like geological locations, use of fertilizers, irrigation water and climatic conditions (Jabeen *et al*., 2015).

4.1.1.6 Average linkage cluster analysis based on biochemical constituents

Since the selected *Piper* species showed variability in biochemical constituents including both primary and secondary metabolites (sections 4.1.1.2, 4.1.1.3 and 4.1.1.4), they were subjected to average linkage cluster analysis based on these biochemical constituents as per section 3.2.12. Total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat, crude fibre, essential oil, piperine, oleoresin, total phenol and total flavonoid were the biochemical constituents used for the cluster analysis. The resultant cluster dendrogram (Fig. 4.16) consisted of two major clusters of which *P. colubrinum* formed a distinct species (cluster 1) and the remaining three were clustered together (cluster 2). Separate clustering of *P. colubrinum* is by noteworthy difference in biochemical compositions especially reducing sugars, starch, essential oil, oleoresin, piperine, total phenol and total flavonoid from other three species. Among the cluster 2, *P. longum* and *P. chaba* were closely related in terms of their biochemical composition (mainly total protein, essential oil and total flavonoid) and thus created a separate cluster. Thus it is clear that, by considering the biochemical composition, *P. colubrinum* is distinct whereas *P. longum* and *P. chaba* are closely related species.

The hierarchical clustering study based on biochemical features is one of the potential tools to study the proper and accurate phylogenetic status of species from the taxonomic point of view (Paul & Banerjee,

2015). The phylogenetic relationship among eight *Solanum* species (*S. nigrum, S. americanum, S. villosum, S. torvum, S. xanthocarpum, S. sisymbriifolium, S. macranthum* and *S. indicum*) has been studied by Paul & Banerjee (2015) by constructing dendrograms based on biochemical characters (total soluble seed protein content per gram of tissue as well as protein content per grain of seed) along with their morphological parameters and revealed that the genus *Solanum* can be divided into two sub genera.

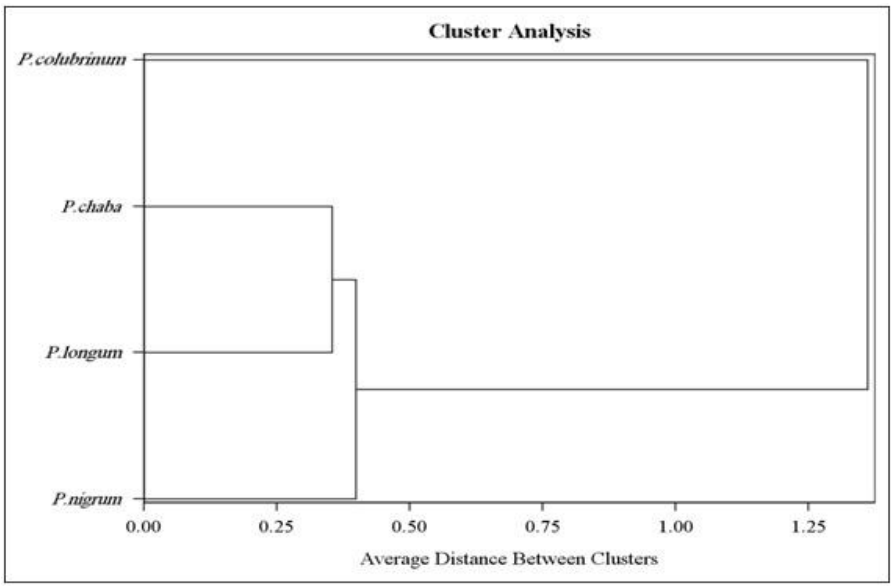

Fig. 4.16. Average linkage cluster dendrogram of selected *Piper* species based on biochemical composition

As far as the studies could ascertain, it is to be noted that the detailed physico-chemical and biochemical analysis of *P. chaba* and *P. colubrinum* fruits is the first report. Likewise, the study also contributes to the scarce knowledge regarding the cluster analysis of these *Piper* species based on their biochemical composition.

4.1.2 Variability in physico-chemical and biochemical profile among selected black pepper varieties

Matured berries of nine improved black pepper varieties (Panniyur-1, Panniyur-5, Sreekara, Subhakara, Panchami, IISR Malabar Excel, IISR Sakthi, IISR Girimunda and IISR Thevam) were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi (Kozhikode, Kerala) and subjected to variability studies after proper drying.

4.1.2.1 Physico-chemical constituents

The moisture content, bulk density, total ash and acid insoluble ash were the physico-chemical constituents analyzed for black pepper varieties as per section 3.2.1. The result indicated a significant variability for these constituents among selected black pepper varieties. Moisture content was in the range of 7.68 to 10.9% whereas bulk density ranged from 466.7 to 595.5 g/L. Highest bulk density was obtained for Sreekara sample and lowest for Panniyur-1 sample. Bulk density of IISR Malabar Excel and Panchami were also found to be low and were statistically on par with that of Panniyur-1 sample. The ash content and acid insoluble ash had a range of 3.5 to 5.5% and 0.15-0.5% respectively, with highest ash content in Panchami and lowest in IISR Sakthi. Black pepper varieties Subhakara and Panchami showed highest acid insoluble ash content. Lowest acid insoluble ash was recorded in IISR Sakthi and was statistically on par with that of Panniyur-1 sample. Variability in physico-chemical constituents of black pepper berries in relation to varieties is illustrated in Table 4.3.

Table 4.3. Variability in physico-chemical constituents among selected black pepper varieties

Sample	Moisture content (%)	Bulk density (g/L)	Total ash (%)	Acid insoluble ash (%)
IISR Girimunda	8.24^{d}	491.7^{cd}	5.00^{bc}	0.30^{d}
IISR Sakthi	10.1^{b}	539.5^{b}	3.50^{f}	0.15^{e}
IISR Malabar Excel	10.9^{a}	485.8^{d}	4.45^{d}	0.40^{b}
Panchami	9.66^{bc}	479.2^{d}	5.50^{a}	0.50^{a}
Panniyur-1	8.01^{d}	466.7^{d}	4.17^{de}	0.16^{e}
Panniyur-5	7.75^{d}	544.5^{b}	3.85^{e}	0.40^{b}
Sreekara	7.68^{d}	595.5^{a}	5.25^{ab}	0.38^{c}
Subhakara	7.79^{d}	524.2^{bc}	3.95^{e}	0.50^{a}
IISR Thevam	9.15^{c}	541.2^{b}	4.80^{c}	0.30^{d}

The moisture content is important since it influences the quality and storability of the sample. Maturity, optimum harvest time, mechanical damage, drying methods, storage conditions, pathogen infestation, etc. are the factors affecting the moisture content (http://hasanuzzaman.weebly.com/uploads/9/3/4/0/934025/moisture_test.pdf). The estimation of bulk density is essential since it is an important physical parameter used for black pepper grading and also to determine the market price of peppercorn. The size, shape, individual particle density, surface characteristics, etc. are few factors affecting bulk density (Lam *et al*., 2008). Total ash and acid insoluble ash contents are important indices to illustrate the nutritional value, quality as well as purity of herbal medicines, foods, feeds and other samples (Rao & Xiang, 2009; http://web.nchu.edu.tw/pweb/users/mushroom/lesson/6412.pdf). Total ash, which represents the inorganic part of the plant, varies according to the plant part, plant age, type of soil, type of

elements, time of collection, season, etc. (Tambe & Kadam, 2010; Kadam *et al*., 2013).

Different black pepper types were analyzed for moisture content (8.7 to 14.1%), total ash (3.6 to 5.7%), acid insoluble ash (0.03 to 0.55%) and bulk density (426 to 850 g/L) by Pruthi (1993). The bulk density and acid insoluble ash content obtained in the present study for black pepper varieties is within these limits. Bulk density values on black pepper varieties (Panniyur-1, Panniyur-2, Panniyur-5, Sreekara and Subhakara) reported by Jayashree *et al*. (2009), with a range of 450 to 571 g/L, also support the present study.

4.1.2.2 Primary metabolites

The total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat and crude fibre were the primary metabolites estimated from selected black pepper varieties. Table 4.4 illustrates a significant varietal-wise variability for these primary metabolites. Total carbohydrate ranged from 41.54 to 67.26% whereas starch content from 28.30 to 56.69% and reducing sugars from 0.53 to 2.4%. Panchami, Panniyur-5 and Sreekara samples were shown to be high and statistically on par for total carbohydrate content. Panniyur-5 and Sreekara samples showed high starch content and were statistically on par. However, lowest total carbohydrate and starch contents were obtained for Panniyur-1 black pepper sample. The mean value for reducing sugars was highest in IISR Thevam and lowest in IISR Sakthi. Total protein and total free amino acid were in the range of 3.99 to 8.3% and 0.2 to 0.59% respectively whereas crude fibre and total fat ranged from 11.9 to 17.7% and 8.14 to 10.07% respectively. IISR Girimunda and Panniyur-1 samples were found to be superior for

total protein whereas it was lowest in Panchami sample. Highest total free amino acid was recorded in Panniyur-1 sample whereas it was foumd to be low and statistically on par in IISR Sakthi, Panniyur-5 and Sreekara samples. In the case of crude fibre, IISR Malabar Excel, Panchami and Panniyur-1 samples were found to be superior and statistically on par whereas Sreekara showed lowest crude fibre content. Likewise, highest total fat was recorded in IISR Thevam and was statistically on par with that of Panniyur-1 sample whereas it was lowest in Sreekara sample.

Table 4.4. Variability in primary metabolites of selected black pepper varieties

Sample	Total carbohydrate (%)	Starch (%)	Reducing sugars (%)	Total protein (%)	Total free amino acid (%)	Crude fibre (%)	Total fat (%)
IISR Girimunda	54.15^{d}	42.46^{cd}	1.60^{d}	8.30^{a}	0.36^{d}	15.7^{b}	8.85^{b}
IISR Sakthi	58.99^{c}	44.03^{c}	0.53^{g}	6.15^{c}	0.20^{e}	14.2^{c}	8.52^{bc}
IISR Malabar Excel	63.27^{ab}	33.08^{e}	1.40^{e}	6.35^{bc}	0.36^{d}	17.4^{a}	9.19^{b}
Panchami	66.85^{a}	40.51^{d}	1.91^{c}	3.99^{e}	0.44^{c}	17.7^{a}	8.94^{b}
Panniyur-1	41.54^{f}	28.30^{f}	1.19^{f}	8.23^{a}	0.59^{a}	16.9^{a}	10.02^{a}
Panniyur-5	67.26^{a}	55.23^{a}	2.20^{b}	5.95^{c}	0.20^{e}	14.4^{c}	8.50^{bc}
Sreekara	65.21^{a}	56.69^{a}	1.54^{d}	6.10^{c}	0.21^{e}	11.9^{d}	8.14^{c}
Subhakara	49.88^{e}	42.82^{cd}	1.36^{e}	6.73^{b}	0.35^{d}	15.2^{bc}	8.87^{b}
IISR Thevam	60.93^{bc}	47.50^{b}	2.40^{a}	4.66^{d}	0.51^{b}	14.4^{c}	10.07^{a}

Carbohydrates, which make up three-fourths of the biomass of plants, is influenced by factors like stages of plant growth, temperature, light, rate of photosynthesis, biotic and abiotic stress, etc. They also vary in relation to species and varieties (http://www.safergrass.org/pdf/ AAEP.pdf). Starch, the predominant constituent of black pepper, varies

in relation to various plant and environmental factors (https://in.answers.yahoo.com/question/index?qid=20070407104556AAfAiXc). Various researchers also reported that, the important quality parameter starch in black pepper is affected by factors like cultivars, maturation of black pepper and temperature (Zachariah & Parthasarathy, 2008; Srinivasan, 2009). Purseglove *et al.* (1981) have reported variability in starch content of two black pepper cultivars (Karimunda and Panniyur-1) in relation to maturation and revealed that as maturity increases, starch content increases. Cruz *et al.* (1970) studied variability in protein content of different varieties of rice grains and revealed that the factors like the level of free amino nitrogen, level of RNA, capacity of amino acid incorporation are the certain factors affecting the level of this macromolecule. On the other hand, free amino acid content, which contributes to overall quality, is affected by several factors like geological locations, use of fertilizers, irrigation water, climatic conditions and health status of plants (Sadasivam & Manickam, 2008; Jabeen *et al*., 2015). Fat content, an agricultural commodity important to food, medical and manufacturing industries is influenced by various environmental factors like temperature, water stress, light, soil constituents, atmospheric constituents, xenobiotics and other factors like physical damage and pest attack (Harwood, 1994; http://lifeofplant.blogspot.in/2011/03/lipids.html). Crude fibre, the portion of total carbohydrate resistant to acid and alkali treatment, play an important role in reducing the risk of chronic diseases like diabetes, cardiovascular disease, obesity and diverticulitis (http://www.foodscience-avenue.com/2008/04/crude-fiber.html).

Jayashree *et al*. (2009) evaluated five black pepper varieties (Panniyur-1, Panniyur-2, Panniyur-5, Sreekara and Subhakara) for

starch, crude fibre and protein content. The starch content was in the range of 34.7 to 52.3% whereas protein and crude fibre was in the range of 9.6 to 13.9% and 6.8 to 14.6% respectively. Zachariah *et al*. (2010) collected 26 black pepper cultivars from Panniyur and Peruvannamuzhi, Kerala and evaluated for variability in biochemical parameters. The result showed that total carbohydrate of black pepper berries was in the range of 38.6 to 51.2 mg% whereas that of starch, total free amino acid and total protein ranged from 32.1 to 43.2 mg%, 0.3 to 0.8 mg% and 2.1 to 6.0 mg% respectively. Sumeshkumar (2004) has reported 0.90 to 2.31 mg/100 mg reducing sugars for different black pepper varieties. Black pepper is reported to have fat content in the range of 1.9 to 9.0% (Narayanan, 2000). The variability reported in the present study for certain primary metabolites with earlier reports might be due to the influence of above described factors.

4.1.2.3 Secondary metabolites

The essential oil and its constituents, piperine, total phenol and total flavonoid were the secondary metabolites studied from the selected black pepper varieties.

4.1.2.3.1 Essential oil

4.1.2.3.1.1 Essential oil yield

The average essential oil yield of black pepper varieties selected for the study varied from 2.0 to 4.0%. Significant variability was observed for essential oil content with highest yield for Panchami and lowest for Panniyur-5 samples (Fig. 4.17).

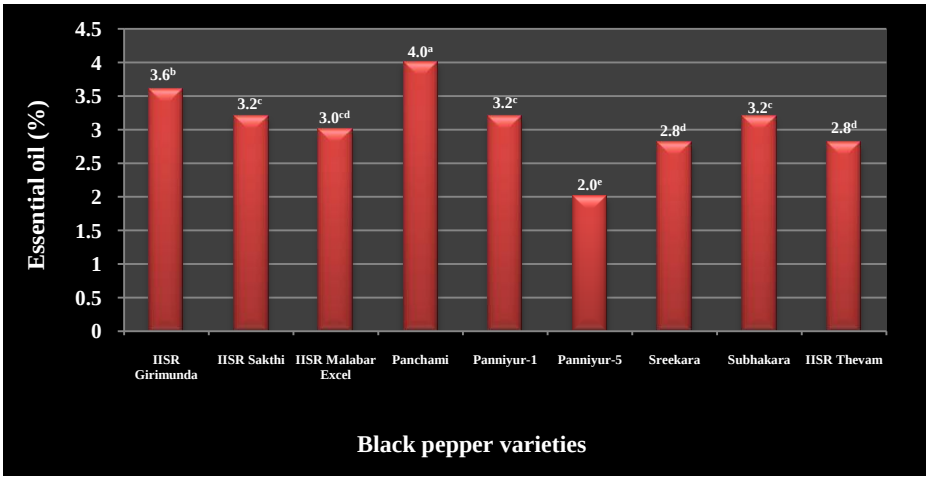

Fig. 4.17. Essential oil content of selected black pepper varieties

Essential oil, the mixture of odoriferous terpenes is responsible for the characteristic aroma of the plant. Moreover, it is well known for various medicinal, pharmacological and other industrial values (Figueiredo *et al*., 2008). It is reported that the percentage of plant essential oil is affected by factors like cultivars, maturation, planting site, harvesting time, etc. (Zachariah *et al.,* 2005; Honarvar *et al*., 2010). Purseglove *et al.* (1981) have reported variability in volatile oil content of two black pepper cultivars (Karimunda and Panniyur-1) in relation to maturation and revealed that as maturity increases, oil content decreases.

The essential oil yield of 17 black pepper cultivars of Kerala has been reported by Lewis *et al.* (1969) as 2.4 to 3.8% whereas Arya *et al*. (2003) revealed volatile oil content in black pepper varieties *viz.*, Panniyur-6 and 7 as 1.33% and 1.50% respectively. The essential oil recovery of seven black pepper cultivars (Panniyur-2, 3 and 4, Sreekara, Subhakara, KS-88 and Neelamundi) has been reported by Radhakrishnan *et al*. (2004) as 2.1 to 7.0% with highest yield for

Sreekara and Subhakara whereas lowest for Panniyur-4 variety. Zachariah *et al*. (2005) examined variability in essential oil content (2.4-4.4%) among major black pepper cultivars and it was found to be highest in Panniyur-2 and lowest in Panniyur-5 and Balankotta. Jayashree *et al*. (2009) studied essential oil content for Panniyur-1, Panniyur-2, Panniyur-5, Sreekara and Subhakara varieties and it ranges from 2.4 to 3.3%. Zachariah *et al*. (2010) have clearly mentioned the variability in essential oil yield from 26 black pepper cultivars with a range of 1.6 to 6.0 mL/100 g. Present study with selected black pepper varieties is also within this limit.

4.1.2.3.1.2 Essential oil profiling by GC-MS

Present study indicated about 26-37 compounds comprising 78.23 to 98.03% of the total essential oil from nine black pepper varieties (Table 4.5). Alpha thujene, α- and β-pinene, sabinene, myrcene, D-limonene and α-phellandrene were the major monoterpenes identified from the essential oils of black pepper varieties and of which, D-limonene predominated. Major sesquiterpene identified from these samples was β-caryophyllene. Linalool, δ-3-carene, *p*-cymene, ocimene (Z and E), germacrene-D, α-copaene, α-cubebene, α-humulene, α-bisabolene, δ-cadinene, α-guaiene, caryophyllene oxide, etc. were the other compounds identified from black pepper oils.

Good varietal variability was observed for essential oil constituents. Variability was profound in α-thujene (0.12 to 2.33%), α-pinene (3.05 to 6.11%), β-pinene (5.44 to 10.40%), sabinene (0.38 to 17.8%), myrcene (1.83 to 3.81%), δ-3-carene (0.04 to 14.8%), α-phellandrene (0.26 to 4.43%), D-limonene (10.6 to 18.1%), α-terpinolene (0.25 to 1.41%), β-linalool (0.47 to 1.83%), 4-terpineol (0.09 to 1.70%),

δ-elemene (0.32 to 2.75%), β-elemene (0.12 to 2.84%), β-caryophyllene (9.26 to 28.9%) and caryophyllene oxide (0.30 to 2.81%). High α-thujene content was obtained for Panchami and IISR Girimunda whereas it was very low in Sreekara and Subhakara samples. Alpha- pinene was found to be highest in Panchami followed by IISR Thevam whereas lowest in IISR Malabar Excel. Subhakara showed high and IISR Malabar Excel showed low β-pinene content. It was noted that varieties with high sabinene content showed low δ-3 carene and vice versa. Panchami, IISR Girimunda and Panniyur-1 showed high sabinene but low/absent δ-3 carene whereas Sreekara & Subhakara showed high δ-3 carene but low sabinene content. D-limonene was found to be highest in Panchami whereas lowest in IISR Malabar Excel samples. High β-myrcene content was noted in IISR Thevam whereas it was shown to be low in IISR Malabar excel. Alpha-phellandrene was very low in Panchami, IISR Girimunda and Panniyur-1 essential oils whereas α-terpinolene was comparatively high in Panniyur-5 and Subhakara oils. The percentage of β-linalool was high in IISR Thevam followed by IISR Sakthi and low in Panniyur-1. High 4-terpineol was observed in Panchami with low β-elemene content whereas low 4-terpineol was observed in Sreekara with high β-elemene content. IISR Thevam, Sreekara, Subhakara and Panchami samples showed comparatively less amount of δ-elemene content. Beta-caryophyllene was observed to be high in all samples except Panchami. Among the high values for Beta-caryophyllene, IISR Sakthi predominated followed by IISR Girimunda. Likewise, comparatively high caryophyllene oxide was observed in IISR Sakthi and IISR Girimunda.

The study showed the absence of δ-3-carene in Panchami sample and *p*-cymene in IISR Girimunda and Panniyur-1. Likewise α- terpineol was not detected in IISR Thevam. Alpha-cubebene was not detected in IISR Sakthi and Panchami whereas α-copaene was detected only in IISR Girimunda, IISR Malabar Excel, Panniyur-1, Panniyur-5 and IISR Thevam. Alpha-guaiene was obtained in all the samples except IISR Girimunda, Panchami, Panniyur-1 and IISR Thevam. Alpha-humulene was absent only in Panchami sample whereas germacrene D was absent in Panchami, Sreekara and Subhakara. Gamma-muurolene was detected only in IISR Malabar Excel, Panniyur-1 and Panniyur-5 whereas α-zingeberene in IISR Girimunda, Panchami and Panniyur-1. Beta-selinene was absent only in IISR Girimunda whereas α-selinene in IISR Girimunda and Panchami. Beta-bisabolene was not detected in IISR Sakthi, Sreekara and Subhakara whereas δ-cadinene was not detected in IISR Sakthi, Panchami, Sreekara and Subhakara samples. Elemol was absent in IISR Sakthi, Panniyur-1, Panniyur-5 and Subhakara whereas spathulenol in Panchami, Sreekara, Subhakara and IISR Thevam. Delta-cadinol was absent in IISR Sakthi, Panchami, Sreekara and Subhakara. Alpha-gurjunene, *trans*-nerolidol and amorphan-3en-9-ol were the constituents present only in Panniyur-1 sample whereas α-cadinol was identified only in IISR Malabar Excel and Panniyur-1. Representative GC-MS chromatograms for essential oil constituents from dried berries of black pepper varieties are given in Fig. 4.18.

Table 4.5. Essential oil profiling of selected black pepper varieties by GC-MS

Compound	% Composition									RI*
	IISR Girimunda	IISR Sakthi	IISR Malabar Excel	Panchami	Panniyur-1	Panniyur-5	Sreekara	Subhakara	IISR Thevam	
α-Thujene	2.25	0.94	0.83	2.33	1.24	0.91	0.12	0.22	1.15	932
α-Pinene	4.63	4.56	3.05	6.11	4.62	4.37	4.76	4.58	5.67	940
Camphene	0.12	0.08	0.06	0.13	0.12	0.10	0.08	0.08	0.16	953
Sabinene	14.7	7.44	6.73	17.8	10.0	7.18	0.38	1.38	7.51	983
β-Pinene	6.71	8.41	5.44	8.34	6.96	7.87	10.1	10.4	8.84	986
β –Myrcene	2.20	2.46	1.83	2.62	2.22	2.83	3.11	3.07	3.81	997
α-Phellandrene	0.66	2.36	2.40	0.52	0.26	3.81	3.87	4.43	3.40	1010
δ-3-Carene	0.88	7.90	7.64	-	0.04	10.8	14.2	14.8	8.33	1016
α-Terpinene	0.40	0.23	0.16	0.44	0.16	0.19	0.04	0.07	0.53	1022
p-Cymene	-	0.52	0.26	0.03	-	0.11	0.84	0.64	0.13	1032
D-Limonene	15.9	14.6	10.6	18.1	15.8	15.2	17.4	16.7	12.6	1040
Z-Ocimene	0.03	0.13	0.15	0.03	0.01	0.13	0.09	0.08	0.14	1044
E-Ocimene	0.33	0.02	0.03	0.32	0.16	0.05	0.05	0.06	0.03	1054
γ-Terpinene	0.69	0.43	0.33	0.77	0.28	0.39	0.15	0.21	0.48	1065
α-Terpinolene	0.39	0.67	0.93	0.39	0.25	1.41	0.95	1.12	0.72	1094
β-Linalool	0.69	1.15	0.92	1.02	0.47	0.65	0.90	0.96	1.83	1108
4-Terpineol	1.67	0.48	0.87	1.70	0.81	0.62	0.09	0.15	1.38	1187
α-Terpineol	0.25	0.15	0.24	0.11	0.17	0.15	0.25	0.22	-	1201
δ-Elemene	2.75	1.54	2.00	0.33	2.41	1.78	0.33	0.63	0.32	1344
α-Cubebene	0.32	-	0.78	-	0.45	0.21	0.27	0.34	0.23	1356
α-Copaene	5.29	-	6.37	-	5.19	4.43	-	-	3.86	1385
β- Elemene	0.53	1.58	1.38	0.12	0.91	0.86	2.84	2.69	0.38	1400
α-Gurjunene	-	-	-	-	0.18	-	-	-	-	1418
β-Caryophyllene	24.2	28.9	17.8	9.26	20.3	20.1	21.2	21.2	17.8	1437
α-Guaiene	-	0.17	0.09	-	-	0.15	0.58	0.56	-	1447
α-Humulene	1.86	2.97	1.90	-	2.02	1.75	2.87	2.71	1.62	1464

Compound	% Composition									RI*
	IISR Girimunda	IISR Sakthi	IISR Malabar Excel	Panchami	Panniyur-1	Panniyur-5	Sreekara	Subhakara	IISR Thevam	
γ-Muurolene	-	-	0.19	-	0.12	0.12	-	-	-	1485
Germacrene D	0.58	0.10	0.60	-	0.44	0.28	-	-	0.27	1491
β-Selinene	-	2.79	2.03	0.11	0.90	1.38	6.04	5.43	0.45	1497
α-Zingeberene	0.07	-	-	3.16	0.39	-	-	-	0.08	1502
α-Selinene	-	2.21	3.69	-	1.52	0.95	4.15	3.79	0.45	1506
β-Bisabolene	1.94	-	3.57	0.21	7.05	0.56	-	-	2.12	1516
δ-Cadinene	2.51	-	3.08	-	2.47	2.01	-	-	1.85	1533
Elemol	0.36	-	0.84	3.63	-	-	0.29	-	11.3	1561
trans-Nerolidol	-	-	-	-	0.06	-	-	-	-	1571
Spathulenol	0.59	0.68	0.16	-	0.14	0.08	-	-	-	1592
Caryophyllene oxide	2.09	2.81	0.56	0.52	0.51	0.30	0.49	0.34	0.34	1597
δ-Cadinol	0.21	-	0.15	-	0.16	0.16	-	-	0.18	1640
α-Cadinol	-	-	0.87	-	0.74	-	-	-	-	1656
Amorphan-3en-9-ol	-	-	-	-	3.89	-	-	-	-	1662
Pentadecanal	0.24	0.32	0.10	0.13	0.12	0.17	0.15	0.14	0.07	-
Total (Nos.)	32	29	37	26	34	35	29	28	33	
Total (%)	96.04	96.6	88.63	78.23	93.52	92.06	96.59	97.00	98.03	

*RI-Retention Indices

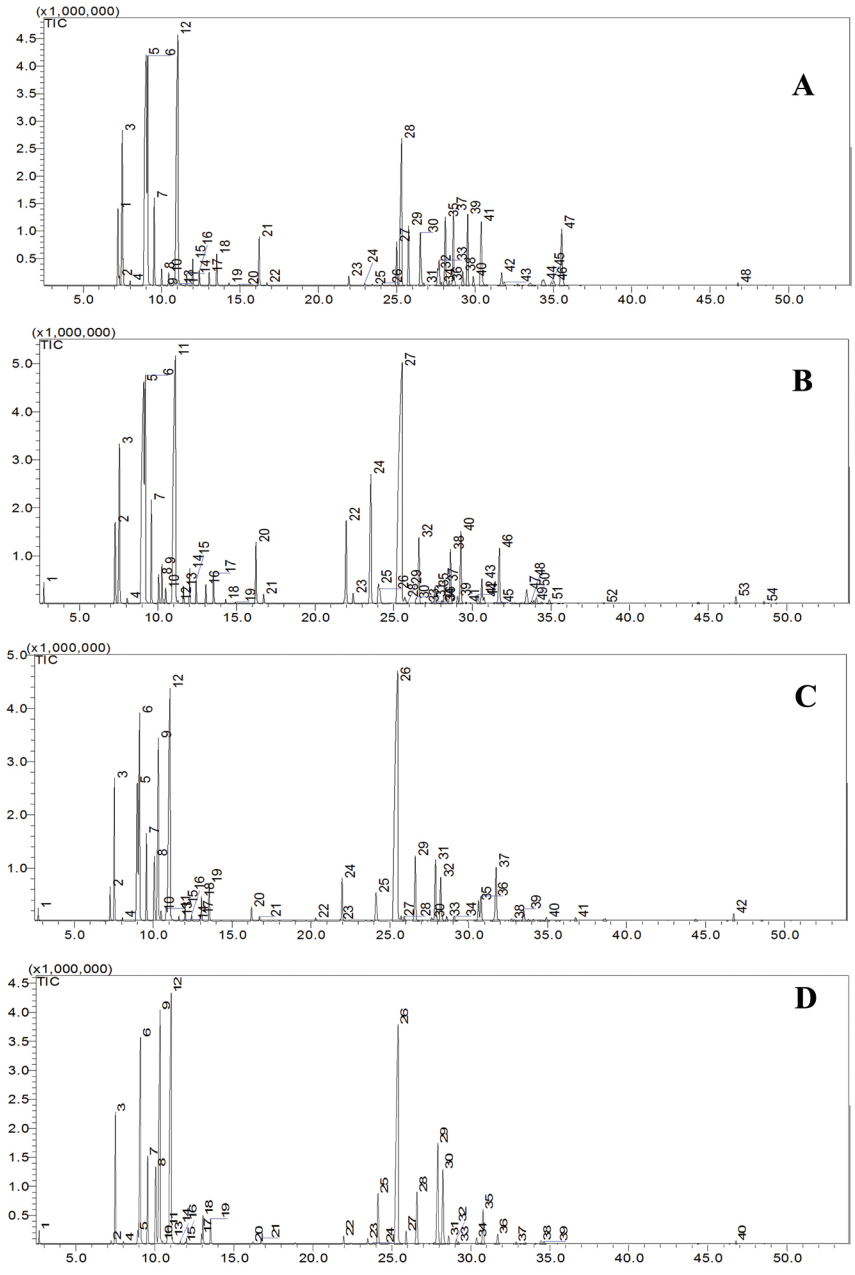

Fig. 4.18. Representative GC-MS chromatograms for essential oil from selected black pepper varieties. A) Panchami, B) IISR Girimunda, C) IISR Sakthi, D) Sreekara. X-axis represents the retention time (Rt) and Y-axis represents the peak intensity (mV)

The essential oil constituents identified from these black pepper varieties are reported to have diverse and sophisticated roles. The monoterpene pinene is an important contributor for aroma of pepper oil. Limonene and α-thujene are important flavor constituents of black pepper. Sabinene, the bicyclic monoterpene is the precursor of C-3 oxygenated thujene-type monoterpenes. Myrcene is a compound with pleasant odour and hence, wide application in perfumery industry. Likewise, α-phellandrene is used in fragrances because of its pleasing aroma (Zachariah *et al*., 2010; Nair, 2011; https://en.wikipedia. org/wiki/Phellandrene; https://en.wikipedia.org/wiki/Myrcene).

Beta-caryophyllene, the major sesquiterpene identified from black pepper oil is a bicyclic compound contributes to the spiciness of black pepper and also has commercial value as food additive and in cosmetics (Gertsch *et al.,* 2008). Ocimene and linalool are involved in the protection of plants against insect attacks (http://www.cyberlipid. org/simple/simp00041.htm). In addition, δ-3-carene, a bicyclic monoterpene with sweet and pungent odour and *p*-cymene, a monoterpene with terpenic odour are known flavor and fragrance agents (http://www.thegoodscentscompany.com/data/rw1032712.html; http://www.thegoodscentscompany.com/data/rw1014471.html).

Germacrene-D, a sesquiterpene hydrocarbon, exhibits the role in pollinator attraction and also as insect pheromone mimic (Nieuwenhuizen *et al*., 2010). Alpha-copaene, a tricyclic sesquiterpene attained strong economic significance since it has the ability to attract the Mediterranean fruit fly, the agricultural pest (Nishida *et al.,* 2000). Alpha-humulene, a monocyclic sesquiterpene has role as insecticide (da Silva *et al.,* 2015) whereas the sesquiterpene α-bisabolene is an intermediate in the biosynthesis of many other natural chemical

compounds (https://en.wikipedia.org/wiki/Bisabolene). Alpha guaiene and caryophyllene oxide, the sesquiterpenes with woody order, is used as flavor and fragrance agents (http://www.thegoodscentscompany.com/data/rw1054351.html; http://www.thegoodscentscompany.com/data/rw1023631.html).

The variability in essential oil composition of black pepper in relation to cultivars/varieties was demonstrated by different researchers. Lewis *et al.* (1969) showed variability in essential oil constituents of 17 black pepper cultivars, which mainly includes α-pinene (5.9 to 12.8%), β-pinene (10.6 to 35.5%), limonene (22 to 31.1%) and β-caryophyllene (10.3 to 22.4%). Gopalakrishnan *et al.* (1993) also showed variability in essential oil constituents of four black pepper genotypes (Panniyur-1, 2, 3 and 4). The oils contained α-pinene (5.07 to 6.18%), β-pinene (6.40 to 11.08%), sabinene (1.94 to 17.16%), limonene (16.74 to 22.71%) and β-caryophyllene (21.19 to 27.70%). Good variability is also observed by Zachariah (1995) among 42 black accessions for their essential oil constituents. Pinene content was varied from 3.8 to 16.6%, limonene from 3.6 to 21.2%, sabinene from 2.2 to 33% and β-caryophyllene from 11.8 to 41.8%. Zachariah *et al*. (2010) also established variability for pinene (3.9 to 16.2%), sabinene (12.7 to 29.7%), myrcene (2.2 to 23.3%), limonene (15.5 to 26.8%), β-caryophyllene (12.8 to 29.2%), germacrene-D (3.0 to 12.8%), nerolidol (0.5 to 4.5%) and elemol (0.9 to 9.6%) from 26 black pepper cultivars.

4.1.2.3.2 Piperine

The piperine content, estimated by HPLC method (section 3.2.3.2), also showed variability among black pepper varieties (2.12- 4.1%). It

was shown to be high and statistically on par for IISR Sakthi, IISR Malabar Excel, Panchami and Panniyur-1 samples whereas low and statistically on par for IISR Girimunda, Panniyur-5, Subhakara and IISR Thevam (Fig. 4.19). Representative HPLC chromatograms for piperine content estimated from dried berries of selected black pepper varieties are shown in Fig. 4.20.

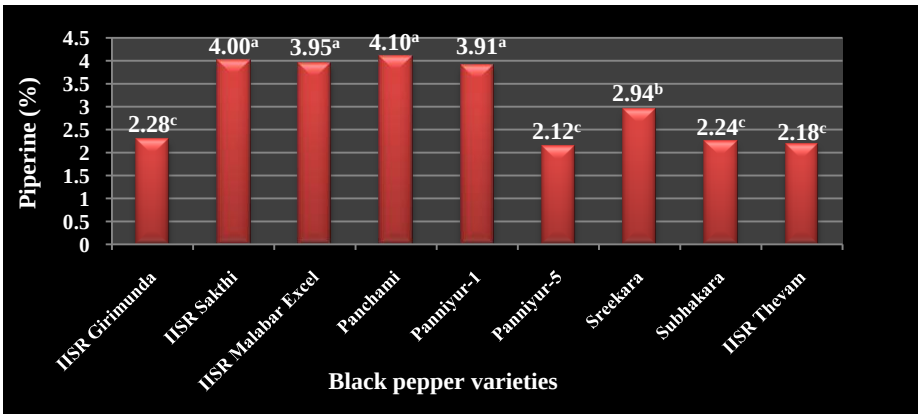

Fig. 4.19. Piperine content of selected black pepper varieties

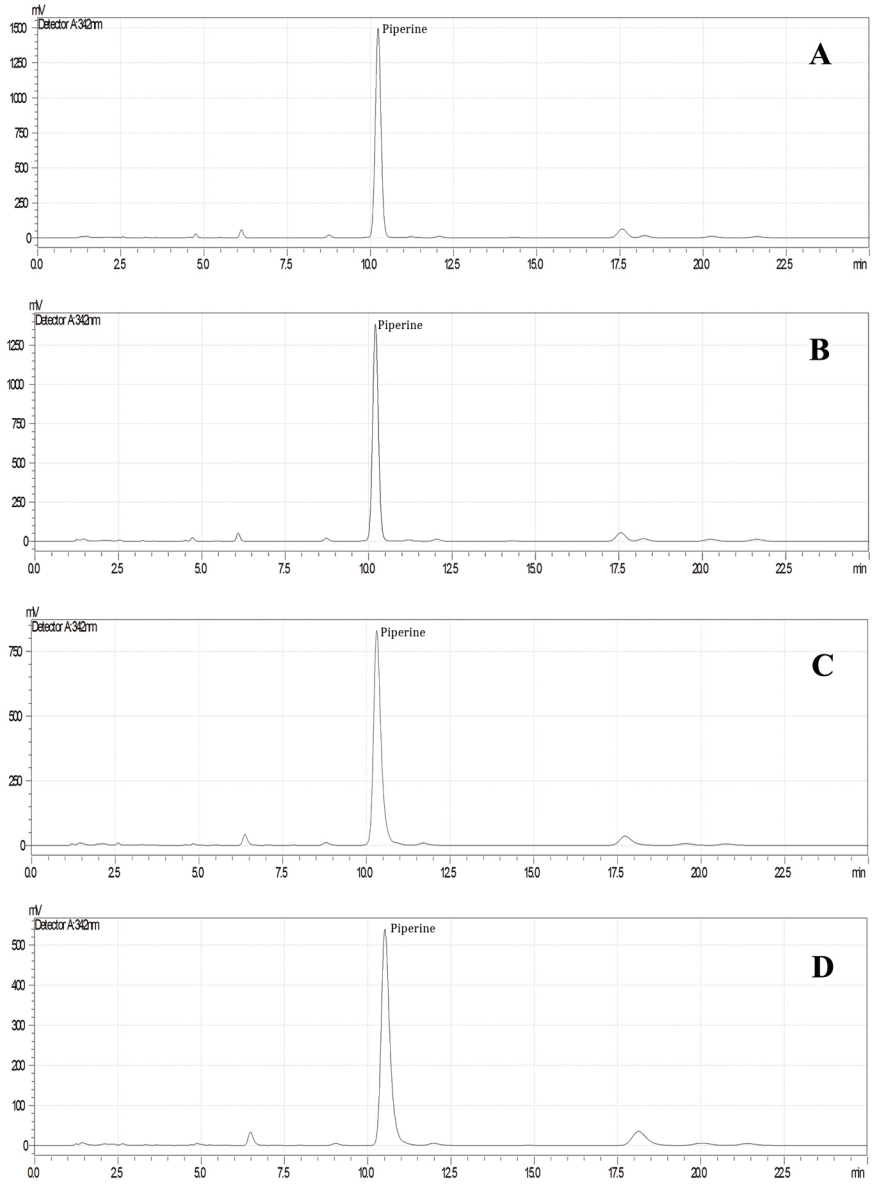

Fig. 4.20. Representative HPLC chromatograms for piperine from selected black pepper varieties. A) Panchami, B) IISR Malabar Excel, C) Sreekara, D) Panniyir-5

Piperine, the major compound responsible for pungency of black pepper is affected by various factors like cultivars, maturation, planting site, harvesting time, climate, location, etc. (Zachariah *et al.,* 2005; Honarvar *et al.,* 2010; Haley & McDonald, 2016). Purseglove *et al.* (1981) have reported variability in piperine content of two black pepper cultivars (Karimunda and Panniyur-1) in relation to maturation and revealed that as maturity increases, piperine content increases.

Varietal variation of piperine content was reported by various research groups. The piperine content obtained in the present study for different black pepper varieties is in accordance with that reported by Zachariah *et al*. (2010) for different black pepper cultivars (1.6 to 4.2 g%). Jayashree *et al*. (2009) studied piperine content for Panniyur-1, Panniyur-2, Panniyur-5, Sreekara and Subhakara varieties as 2.8 to 4.4%. Present study is in tune with the study of Sasikumar *et al*. (2004) who have reported high piperine content in HP-813 (IISR Malabar Excel) in comparison with other recently released black pepper cultivars, IISR Thevam and IISR Girimunda. Piperine content in two black pepper varieties *viz.,* Panniyur-6 and Panniyur-7 is also reported by Arya *et al*. (2003) as 4.94% and 5.57% respectively and that in different black pepper cultivars by Zachariah *et al*. (2005) as 2.79 to 3.87%.

4.1.2.3.3 Phenolics

4.1.2.3.3.1 Total phenol

The total phenolic content (0.55 to 0.91%) estimated by Folin-Ciocalteau method (section 3.2.3.3.1) was highest in IISR Malabar

Excel followed by Panchami and Subhakara samples whereas lowest in Panniyur-1 sample (Table 4.6).

4.1.2.3.3.2 Total flavonoid

The total flavonoid content was found to be highest in IISR Malabar Excel (0.51%) whereas lowest (0.30%) in Panniyur-1 (Table 4.6).

The black pepper varieties selected for the study showed significant variability for their total phenol and total flovonoid contents. It is noted that, same black pepper variety showed highest (IISR Malabar Excel) as well as lowest (Panniyur-1) total phenol and flavonoid contents.

Plant phenols, the electron rich molecules, are various derivatives of hydroxybenzene and are one of the major contributors to the medicinal utility of plants. Among the phenolic compounds identified, flavonoids are the largest group with diverse function (Gould & Lister, 1975; Parthsarathy & Nandakishore, 2014). The harvest time, maturation, cultivar, the year, environmental factors (temperature, light intensity, etc.), edaphoclimatic conditions, growing practices (farming system, irrigation, fertilization practices, pruning, etc.), incidence of pests and diseases, geographical areas where the plants are grown are the factors affecting variability in phenolic contents of the plant (Woodhead, 1981; Griffith & Yaish, 2004; Agostini-Costa *et al.*, 2015; Boskou, 2015).

Zachariah *et al*. (2010) studied total phenol content of leaves and berries of 26 black pepper cultivars as 0.3 to 1.5 and 0.3 to 0.6 mg% respectively whereas Sumeshkumar (2004) reported that of leaves and berries of seven black pepper varieties as 1.245 to 2.35% and 1.24 to

1.73% respectively. Ahmad *et al.* (2015) reported total flavonoid content in methanolic extract of black pepper as 1.087±0.002 μg/g.

4.1.2.4 Oleoresin

Oleoresin, the concentrated form of spice, was estimated by cold percolation technique (section 3.2.4) and varied between 7.29 and 11.7%. It was found to be high and statistically on par in IISR Malabar Excel and Panchami samples whereas lowest in Panniyur-5 sample (Table 4.6).

There are reports on variability in oleoresin content of black pepper varieties. The oleoresin content of different black pepper cultivars (5.9-13.9 g%) revealed by Zachariah *et al*. (2010) supports the present study. Likewise, Jayashree *et al*. (2009) also reported oleoresin content for five black pepper varieties (Panniyur-1, Panniyur-2, Panniyur-5, Sreekara and Subhakara) as 8.2 to 12.2%. On the other hand, Radhakrishnan *et al*. (2004) studied that, oleoresin content of seven black pepper cultivars (Panniyur-2, 3 and 4, Sreekara, Subhakara, KS-88 and Neelamundi) ranged from 9.2 to 13.9%. Arya *et al*. (2003) reported that, black pepper variety Panniyur-6 contained 8.27% and Panniyur-7 contained 10.61% oleoresin. In general, the oleoresin content in different black pepper cultivars varies with respective piperine content (Zachariah *et al*., 2010). It is also reported that climate is a factor affecting oleoresin content. The cooler climate with low temperature helps in high oleoresin yield (Srinivasan, 2009).

Table 4.6. Variability in total phenol, total flavonoid and oleoresin contents of selected black pepper varieties

Sample	Total phenol (%)	Total flavonoid (%)	Oleoresin (%)
IISR Girimunda	0.72^{c}	0.43^{d}	9.55^{c}
IISR Sakthi	0.63^{de}	0.38^{f}	10.7^{b}
IISR Malabar Excel	0.91^{a}	0.51^{a}	11.4^{a}
Panchami	0.81^{b}	0.49^{b}	11.7^{a}
Panniyur-1	0.55^{f}	0.30^{i}	9.74^{c}
Panniyur-5	0.60^{def}	0.36^{g}	7.29^{e}
Sreekara	0.58^{ef}	0.32^{h}	9.62^{c}
Subhakara	0.78^{b}	0.45^{c}	8.84^{d}
IISR Thevam	0.65^{d}	0.41^{e}	8.68^{d}

4.1.2.5 Minerals

Mineral contents of selected black pepper varieties were estimated as per the section 3.2.5. The results indicated significant variability in mineral contents in relation to black pepper varieties (Table 4.7). Nitrogen content (1.96 to 2.40%) was highest in IISR Malabar Excel and lowest in Panchami. Black pepper variety Panchami showed highest P (0.26%), Fe (181.5 mg/kg), Cu (43.5 mg/kg) and Zn (19.2 mg/kg) contents. Panniyur-1 sample recorded lowest P (0.16%) and Zn (12.5 mg/kg) contents whereas IISR Thevam recorded lowest Fe content (52.0 mg/kg). Black pepper variety IISR Thevam was also found to be lowest for Cu (14.5 mg/kg) and it was statistically on par with that of Panniyur-5 sample (15.0 mg/kg). Potassium content was found to be high and statistically on par for Subhakara (0.84%), Sreekara (0.79%) and Panchami (0.79%) samples whereas it was found to be low and statistically on par in IISR Sakthi (0.55%), IISR Malabar

Excel (0.58%) and Panniyur-1 (0.59%) samples. Black pepper varieties IISR Malabar Excel and IISR Thevam recorded highest Ca content (0.19%) and these samples were statistically on par with Panniyur-5 and Panchami for their Ca content (0.18%). Lowest Ca was recorded in Sreekara sample (0.10%). Magnesium content ranged from 0.08 to 0.15% and was found to be highest in panniyur-5 and lowest in IISR Sakthi samples. Likewise, Panniyur-1 sample had highest Mn content (48.3 mg/kg) and Subhakara had the lowest value (12.0 mg/kg).

Table 4.7. Variability in mineral contents of selected black pepper varieties

Sample	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
IISR Girimunda	2.32^{ab}	0.23^{c}	0.69^{b}	0.16^{b}	0.11^{cd}	69.0^{f}	18.0^{e}	33.1^{c}	17.8^{b}
IISR Sakthi	2.13^{c}	0.25^{ab}	0.55^{c}	0.14^{c}	0.08^{f}	71.5^{ef}	25.5^{c}	14.0^{g}	18.9^{ab}
IISR Malabar Excel	2.40^{a}	0.19^{e}	0.58^{c}	0.19^{a}	0.12^{bc}	77.0^{de}	18.5^{e}	31.0^{d}	16.4^{c}
Panchami	1.96^{d}	0.26^{a}	0.79^{a}	0.18^{a}	0.12^{bc}	181.5^{a}	43.5^{a}	26.5^{e}	19.2^{a}
Panniyur-1	2.32^{ab}	0.16^{f}	0.59^{c}	0.15^{bc}	0.11^{cd}	120.5^{b}	23.0^{d}	48.3^{a}	12.5^{f}
Panniyur-5	2.18^{bc}	0.24^{bc}	0.72^{b}	0.18^{a}	0.15^{a}	79.0^{d}	15.0^{f}	14.6^{fg}	15.5^{cd}
Sreekara	2.16^{c}	0.21^{d}	0.79^{a}	0.10^{d}	0.09^{ef}	95.5^{c}	24.5^{cd}	16.5^{f}	14.6^{de}
Subhakara	2.16^{c}	0.23^{c}	0.84^{a}	0.15^{bc}	0.10^{de}	76.5^{de}	32.0^{b}	12.0^{h}	16.0^{c}
IISR Thevam	2.32^{ab}	0.21^{d}	0.69^{b}	0.19^{a}	0.13^{b}	52.0^{g}	14.5^{f}	36.0^{b}	13.9^{e}

Minerals are necessary for growth, development and yield of the plants and their influence depend on their ratios in the soil as well as in the plant system. Most of the plants obtain minerals via their roots, mainly from the soil (Srinivasan *et al*., 2007; Srinivasan *et al*., 2012). The

primary macronutrients like N, P and K, the secondary macronutrients like Ca and Mg and micronutrients like Fe, Cu, Mn and Zn are the important nutrients/minerals for plants.

Nitrogen is an important element for many of the plant metabolites like amino acids. Phosphorus is necessary for photosynthesis whereas potassium is necessary for the formation of sugars, starch, carbohydrates, protein synthesis and cell division in all parts of the plant. Calcium forms the structural component of cell walls, influences water movement in cell and activates enzymes. Magnesium is the critical structural component of chlorophyll molecule and is necessary for the functioning of plant enzymes in the production of carbohydrates, sugars and fat. Iron acts as enzyme cofactor in plants and is essential for photosynthesis. Copper is also important for photosynthesis since it involves in many enzyme processes. Zinc is an essential factor for proper functioning of many enzymes and plays a key role in DNA transcription and manganese, an antioxidant micronutrient also involved in photosynthesis (Watts, 1997; Easwaran & Ramani, 2012; https://en.wikipedia.org/wiki/Plant_nutrition).

Variability in mineral constituents of black pepper is reported by researchers. Christensen *et al*. (1968) reported variability in different black pepper samples for P (0.16 to 0.17%), K (0.85 to 1.5%), Ca (0.34 to 0.43%), Mg (0.095 to 0.20%), Fe (39 to 270 ppm), Cu (8.8 to 14 ppm), Zn (5.2 to 14 ppm) and Mn (21 to 75 ppm). Veloso & Carvalho (1999) stated that black pepper fruit contained 22.8, 2.18, 12.5, 7.8 and 2.4 g/kg, N, P, K, Ca and Mg respectively. Gutierrez *et al*. (2013) have given P, K, Ca and Cu requirements for black pepper as 173, 1259, 437 and 1.127 mg/100 g respectively whereas Pradeep *et al.* (1993) have reported N, Fe, Mn and Zn for black pepper as 2.12 g/100 g, 17.8 mg/100 g, 6.33 mg/100 g and 0.90 mg/100 g respectively. The variability shown by mineral constituents of black pepper varieties

might be due to factors like use of fertilizers, irrigation water, climate and soil conditions, availability of minerals, etc.

4.1.2.6 Average linkage cluster analysis based on biochemical constituents

The results that revealed in sections 4.1.2.2**,** 4.1.2.3 and 4.1.2.4 clearly indicated variability in biochemical constituents (primary and secondary metabolites) of selected black pepper varieties. So based on this biochemical composition (total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat, crude fibre, essential oil, piperine, oleoresin, total phenol and total flavonoid), the black pepper varieties were clustered into different groups by adopting average linkage cluster analysis (section 3.2.12). The resultant dendrogram clearly revealed the clustering pattern of these black pepper varieties (Fig. 4.21). The cluster 1 is constituted by Panniyur-1 alone and the remaining black pepper varieties formed cluster 2. Thus, it is noted that Panniyur-1 is distinct from other varieties due to its unique biochemical pattern mainly for total carbohydrate, starch and total free amino acid contents. The cluster 2 comprised of two sub clusters and which include a distinct sub cluster of Sreekara and Panniyur-5 (cluster 3) and the sub cluster of remaining varieties (cluster 4). Sreekara and Panniyur-5 clustered together because their biochemical constituents like total carbohydrate, starch, total protein and total free amino acid were closely related. The cluster 4 consisted of a distinct sub cluster of Panchami and IISR Malabar Excel (cluster 5) and the sub cluster of remaining varieties (cluster 6). Clustering of Panchami and IISR Malabar Excel is due to similarity in their biochemical constituents specially crude fibre, total fat, oleoresin, piperine, total phenol and total flavonoid contents. Considering the closeness of biochemical parameters, cluster 6 consists of IISR Thevam, IISR Sakthi, Subhakara and IISR Girimunda samples. Black

pepper varieties in the same cluster indicated that they were more closely related in relation to their biochemical constituents than those present in separate clusters. Kottawa-Arachchi *et al.* (2011) have categorized 22 accessions of black tea (*Camellia sinensis*) into high, moderate and low quality categories using average linkage cluster analysis based on biochemical parameters including total polyphenol, total free amino acid and crude fibre contents.

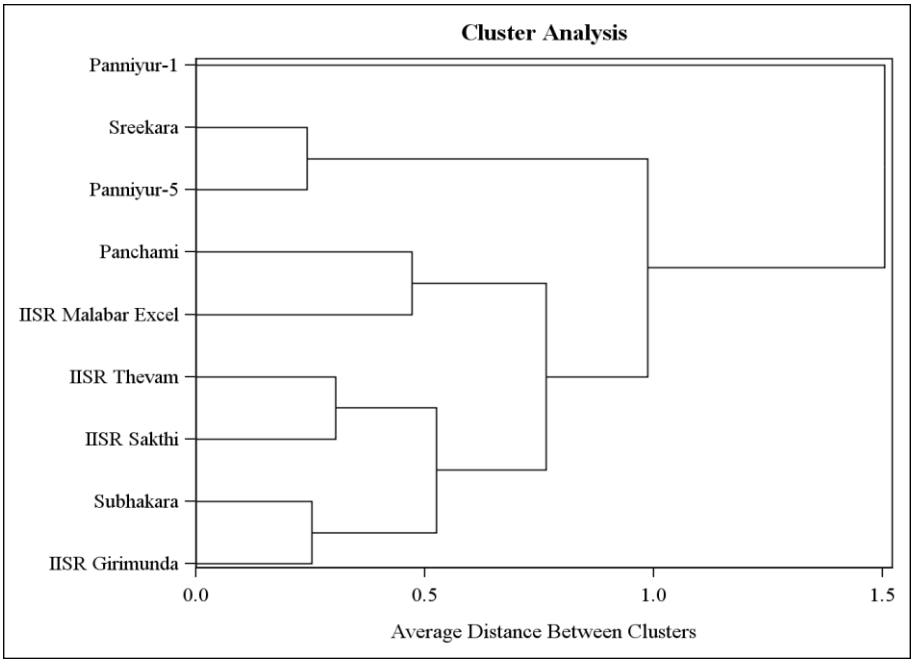

Fig. 4.21. Average linkage cluster dendrogram of selected black pepper varieties based on biochemical composition

4.1.3 Fatty acid profiling of selected *Piper* species

Fatty acids in spices also contribute to the human health along with its other medicinal properties. In this context, the fatty acid profiling of the selected *Piper* species requires special attention.

Matured fruits/berries of high yielding black pepper variety Panniyur-1, *P. longum, P. chaba* and *P. colubrinum* were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi (Kozhikode, Kerala) and subjected to fatty acid profiling after proper drying. Free fatty acids present in the samples were identified by GC-MS after converting to fatty acid methyl esters (FAME) as per the procedure described in section 3.2.2.3.2. The fatty acids identified from the samples and their percentage composition are given in Table 4.8.

Seven fatty acids, comprised 80.37% of the total fatty acids, were identified from black pepper variety Panniyur-1 and in which linoleic acid (27.7%) predominated followed by oleic and palmitic acids. Stearic and myristic acids in considerable amounts, whereas lauric and palmitoleic acids in trace amounts were also identified from black pepper sample. In the case of *P. longum,* palmitic acid (21.2%) was the dominant among five fatty acids (63.84%) identified, followed by linolenic and linoleic acids. Stearic and behenic acids were the other fatty acids identified from *P. longum* fruits. *P. chaba* also showed a predominated percentage of palmitic acid (18.9%) among six fatty acids (52.71%) identified. Linoleic and oleic acids were the other major fatty acids whereas lauric, myristic and stearic acids were the minor fatty acids identified from *P. chaba* fruits. In the case of *P. colubrinum*, seven fatty acids were detected which accounts for 89.54% of total fatty acids. Unlike other three samples, *P. colubrinum* fruit had oleic acid (41.3%) as the predominant fatty acid followed by palmitic and linoleic acids. Other fatty acids identified in this sample include lauric, myristic, stearic and arachidic acids.

By comparing the fatty acid profiling of four *Piper* species, it is noted that palmitic, linoleic and stearic acids were present in all the four samples whereas lauric, myristic and oleic acids were identified in all samples except *P. longum.* Palmitoleic acid was identified only in

black pepper. Likewise, behenic and linolenic acids were detected in *P. longum* alone whereas arachidic acid was unique to *P. colubrinum* sample. GC-MS chromatogram representing fatty acid profiling is given in Fig. 4.22.

Table 4.8. Fatty acid profiling of selected *Piper* species by GC-MS

	% Composition			
Fatty acid	***P. nigrum***	***P. longum***	***P. chaba***	***P. colubrinum***
Lauric acid	0.85	-	1.43	0.64
Myristic acid	1.80	-	1.22	0.86
Palmitoleic acid	0.32	-	-	-
Palmitic acid	20.1	21.2	18.9	25.2
Behenic	-	1.06	-	-
Linoleic acid	27.7	18.0	16.1	16.7
Linolenic acid	-	18.6	-	-
Oleic acid	26.1	-	12.4	41.3
Stearic acid	3.50	4.98	2.66	3.00
Arachidic acid	-	-	-	1.84
Total (Nos.)	7.0	5.0	6.0	7.0
Total (%)	80.37	63.84	52.71	89.54

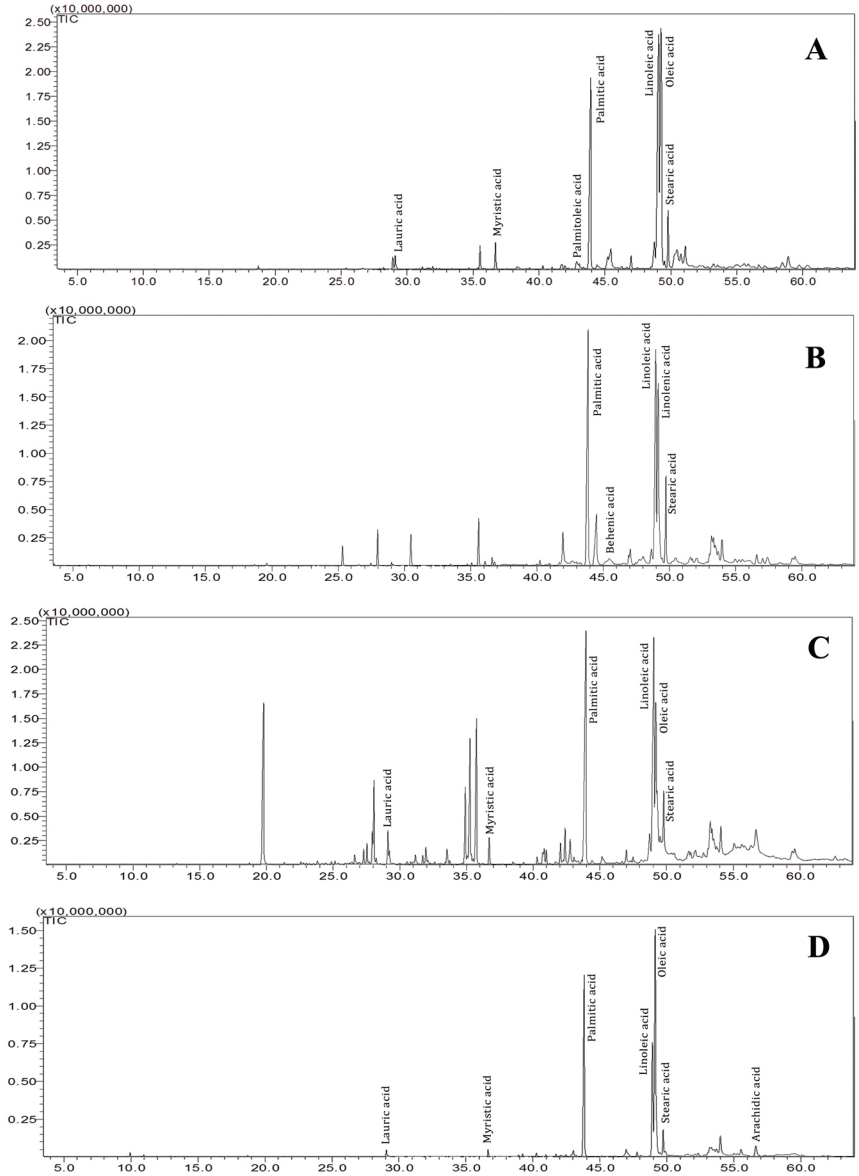

Fig. 4.22. Fatty acid profiling of selected *Piper* species by GC-MS. A) *P. nigrum*, B) *P. longum*, C) *P. chaba*, D) *P. colubrinum*. X-axis represents the retention time (Rt) and Y-axis represents the peak intensity (mV)

The major fatty acids already reported in black pepper include palmitic (16 to 30%), oleic (18 to 29%), linoleic (25 to 35%) and linolenic (8 to 19%) acids (Bedi *et al*., 1971; Salzer, 1975a). Parmar *et al*. (1997) also reported stearic, sterculic, lauric, malvalic, myristic, capric and vernolic acids from black pepper. Fatty acids identified in the present study for black pepper are comparable with these reports. Al-Jasass & Al-Jasser (2012) reported that black pepper from Saudi Arabia contains 81.34% of total unsaturated and 18.66% of total saturated fatty acids. They have identified linoleic acid as the major fatty acid (33.03%) as in the present study. They have also identified palmitic, palmitoleic, oleic, linolenic and arachidic acids. Present study is thus in tune with the earlier reports. Palmitic, stearic, linoleic, hexadecenoic, oleic, linolenic, arachidic and behenic acids were the fatty acids reported earlier from *P. longum* (Bedi *et al*., 1971; Khushbu *et al*., 2011). These reports endorse the present findings. A fatty acid *viz.,* n-triacontanoic acid has been reported in hexane extract of *P. colubrinum* leaves (Leela, 2002). However, information is scanty regarding fatty acid profiling of *P. chaba* and *P. colubrinum* fruits.

The major fatty acids identified in the present study are reported to have significance in various fields. Palmitic acid, the saturated long chain fatty acid is appeared to have many beneficial effects includes antioxidant and anti atherosclerotic effect (Cho *et al.,* 2010). It also impart many industrial uses as adhesives and sealant chemicals, agricultural chemicals, fillers, finishing agents, lubricants, processing aids and surface active agents (https://pubchem.ncbi.nlm.nih.gov/ compound/palmitic_acid#section=Top). Stearic acid, another saturated long chain fatty acid lowers serum cholesterol and has many industrial applications as anti-adhesive agent, finishing agent, fuels and fuel additive, lubricant additives, pigments and surface active agents (Mensink, 2005; https://pubchem.ncbi.nlm.nih.gov/compound/ stearic_

acid#section=Top). On the other hand, the unsaturated oleic acid is the most widely distributed and abundant fatty acid in nature. It helps to reduce blood pressure, increases fat burning and thus helps for weight loss, protects the cells from free radical damage, prevent type 2 diabetes and ulcerative colitis. It is used as plasticizers, solvents, finishing agents, intermediates, lubricants, paint and coating additives (http://mooscience.com/Oleic-Acid.html; https://pubchem. ncbi.nlm. nih.gov/compound/oleic_acid#section=Top). Linoleic acid, a polyunsaturated fatty acid is essential to human diet and is used in the biosynthesis of prostaglandins and cell membranes. It is also used to make quick drying oils and also as surfactant, lubricants and lubricant additives. Because of its antiinflammatory, acne reductive and moisture retention properties, it is also used in beauty product industries (Diezel *et al.*, 1993; Letawe *et al*., 1998; Darmstadt *et al*., 2002; https://pubchem.ncbi.nlm.nih.gov/compound/linoleic_acid# section=Top). Linolenic acid is another polyunsaturated and essential fatty acid involved in reduction of inflammation and prevention of certain chronic diseases and also helps to decrease cardiovascular diseases. It has industrial uses as adhesives and sealant chemicals, finishing agents, non-pesticidal agricultural chemicals, lubricants and lubricant additives and surface active agent (https://pubchem. ncbi.nlm.nih.gov/compound/linolenic_acid#section=Top).

Thus, the fatty acid profiling study indicated that the selected *Piper* species is a good source of fatty acids, which is shown to possess various applications and importance to human health.

4.1.4 Phenolic profiling of selected *Piper* species

Phenolics are the most abundant secondary metabolite in plant kingdom and are responsible for their defense mechanisms under different environmental stress conditions such as infection, UV

irradiation, wounding, etc. (Bennet & Wallsgrove, 1994; Dixon & Paiva, 1995). Plant phenolics ranges from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Flavonoids are the largest group among the natural phenolic compounds. The physical, chemical and biological properties of phenolics are determined by their structure (Kartsova & Alekseeva, 2008; Jain *et al.,* 2013). Recent studies revealed that phenolic compounds from plants exert several health promoting functions like reducing the risks of cancer, heart and neurodegenerative diseases (Indap *et al.,* 2006; Vita, 2005). The free-radical scavenging capability and consequent antioxidant properties of the phenolic compounds play an important role in protecting our body from oxidative stress and other biological effects which forms the root causes of these chronic diseases (Rimbach & De Pascual-Teresa, 2005). Because of these health-promoting effects, phenolics from various plant sources have been reported in recent years. So, present study focused on identification of phenolic compounds from *P. nigrum, P. longum, P. chaba* and *P. colubrinum.*

Matured fruits/berries of high yielding black pepper variety Panniyur-1, *P. longum*, *P. chaba* and *P. colubrinum* were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi (Kozhikode, Kerala). After proper drying, the samples were subjected to phenolic profiling by LC-MS and HPLC as per the section 3.2.3.3.3.

Identification of phenolic compounds by LC-MS

LC-MS analysis was adopted for phenolic extracts and the possible phenolic compounds were identified based on their MS, MS/MS spectra, monoisotopic mass, generated empirical formula, predicted structures, Molecular Feature Extraction (MFE) algorithm, metlin data base and also by literature survey. Overlaid chromatogram of UV at

254 nm and Total Ion chromatogram (TIC) for selected *Piper* species are illustrated in Fig. 4.23.

As illustrated in Table 4.9, thirteen compounds were identified from black pepper and these include hydroxybenzoic acid, hydroxycinnamic acid and flavonoid category. Apart from this, other phenolic compounds like guaiacol and 4-hydroxymandelic acid were also identified from this sample. The compound 4-hydroxymandelic acid was identified in *P. nigrum* for the first time. Even though luteolin-8-C glucoside was detected for the first time in Panniyur-1 black pepper, luteolin 6-C-a-D-glucopyranoside-8-C-R-L-arabinopyranoside, luteolin 7-O-[2-(a-D-apiofuranosyl)-a-D-glucopyranoside-8-C-R-L-arabinopyranoside and luteolin 7-O-[2-(a-D-apiofuranosyl)-4-(a-D-glucopyranosyl) were reported by Al-Shahwany (2014). Salicylic acid, caffeic acid, protocatechuic acid, coumaric acid, ferulic acid, vanillic acid, syringic acid, gentisic acid, guaiacol, apigenin and 4-hydroxybenzoic acid were already reported in black pepper (Swain *et al.,* 1985; Variyar & Bandyopadhyay, 1994; Parmar *et al.,* 1997; Jagella & Grosch, 1999a; Chatterjee *et al.*, 2007; Chandra *et al.,* 2015).

Out of six phenolic constituents identified from *P. longum,* gentisic acid has been reported earlier by Mammen & Daniel (2014). They have also reported syringic acid, melilotic acid and apigenin from *P. longum* and revealed that no other flavonoid or phenolic acids were identified from any part of this plant. Later, Chandra *et al.* (2015) identified protocatechuic acid, ferulic acid, rosamarinic acid and apigenin from *P. longum* fruits. They have also reported caffeic acid (below detection limit) from this sample. Thus, in the present study, salicylic acid, 4-coumaric acid and 2-pyrocatechuic acid have been reported for the first time in *P. longum* (Table 4.10).

Seven phenolic compounds were identified in *P. chaba* (Table 4.11) and except protocatechuic and caffeic acids, the remaining compounds were not reported earlier. Chandra *et al.* (2015) identified protocatechuic acid, caffeic acid, luteolin and quercetin from the fruit, ferulic and vanillic acid from the leaf, rosamarinic acid and apigenin from both fruit and leaf of *P. chaba.*

Total of ten phenolic compounds were identified from *P. colubrinum* fruit (Table 4.12). Phenolic acids like protocatechuic acid, ferulic acid, rosamarinic acid, vanillic acid and flavonoids like luteolin, kaempferol, quercetin and apigenin have been identified from *P. colubrinum* fruit by Chandra *et al.* (2015). They have also reported caffeic acid in below detection limit from this sample. Except these compounds identified by above researchers, all other phenolics identified from *P. colubrinum* fruit in the present study, is not reported earlier. Chandra *et al.* (2015) also identified protocatechuic acid, ferulic acid, caffeic acid, rosamarinic acid, vanillic acid and flavonoids like luteolin, kaempferol and apigenin from leaf of *P. colubrinum.* Leela & Pillai (2008) have reported two flavones viz., 5,4'-dihydroxy-7-methoxy-flavone & 5, 3'-4'-trihydroxy-7-methoxy-flavone from chloroform extract of *P. colubrinum* leaves.

The hydroxybenzoic acid, hydroxycinnamic acid, phenolic aldehydes and flovonoids were the main group of phenolic compounds identified from the selected *Piper* sepecies. The hydroxybenzoic acid category includes salicylic acid, 4-hydroxybenzoic acid, 2-pyrocatechuic acid, protocatechuic acid, gentisic acid, vanillic acid and syringic acid. Salicylic acid, a monohydroxy benzoic acid was detected in all the four *Piper* species selected. The protocatechuic acid was identified in *P. nigrum, P. chaba* and *P. colubrinum* whereas gentisic acid was detected in *P. nigrum* & *P. longum* and 2-pyrocatechuic acid in

P. longum and *P. colubrinum.* However 4-hydroxybenzoic acid, vanillic acid and syringic acid were identified only in *P. nigrum.*

Ferulic acid, caffeic acid and 4-coumaric acid were the hydroxycinnamic acids identified from the selected *Piper* species. Caffeic acid and ferulic acid were identified in black pepper, *P. longum* and *P. chaba* extracts whereas 4-coumaric acid was identified in black pepper and *P. longum*. However the hydroxycinnamic acids were not detected in *P. colubrinum.*

The flavonoid aglycones and flavonoid glycosides were included in the flavonoid group of compounds identified from the selected *Piper* species. The luteolin-8-C glucoside (flavone glycoside) and apigenin (flavone) were identified in black pepper whereas apigenin-7-galactoside, (flavone glycoside), kaempferol-5-glucoside and kaempferide-3-glucoside (flavonol glycosides), scutellarein-4'-methyl ether and 5,7,2',5'-tetrahydroxy flavanone were identified from *P. colubrinum*. However flavonoid compounds were not detected in *P. longum* and *P. chaba* samples. The identified phenolic aldehydes include caffeic aldehyde (*P. chaba*) and vanillin (*P. colubrinum*). The 4-hydroxymandelic acid (*P. nigrum*), guaiacol (*P. nigrum*), 5-methoxysalicylic acid (*P. chaba* & *P. colubrinum*) and salicylaldehyde (*P. chaba*) were the other phenolic compounds identified.

Even though LC-MS-Q-TOF showed the presence of these phenolic compounds, their molecular structure can be established further by additional techniques like NMR and IR.

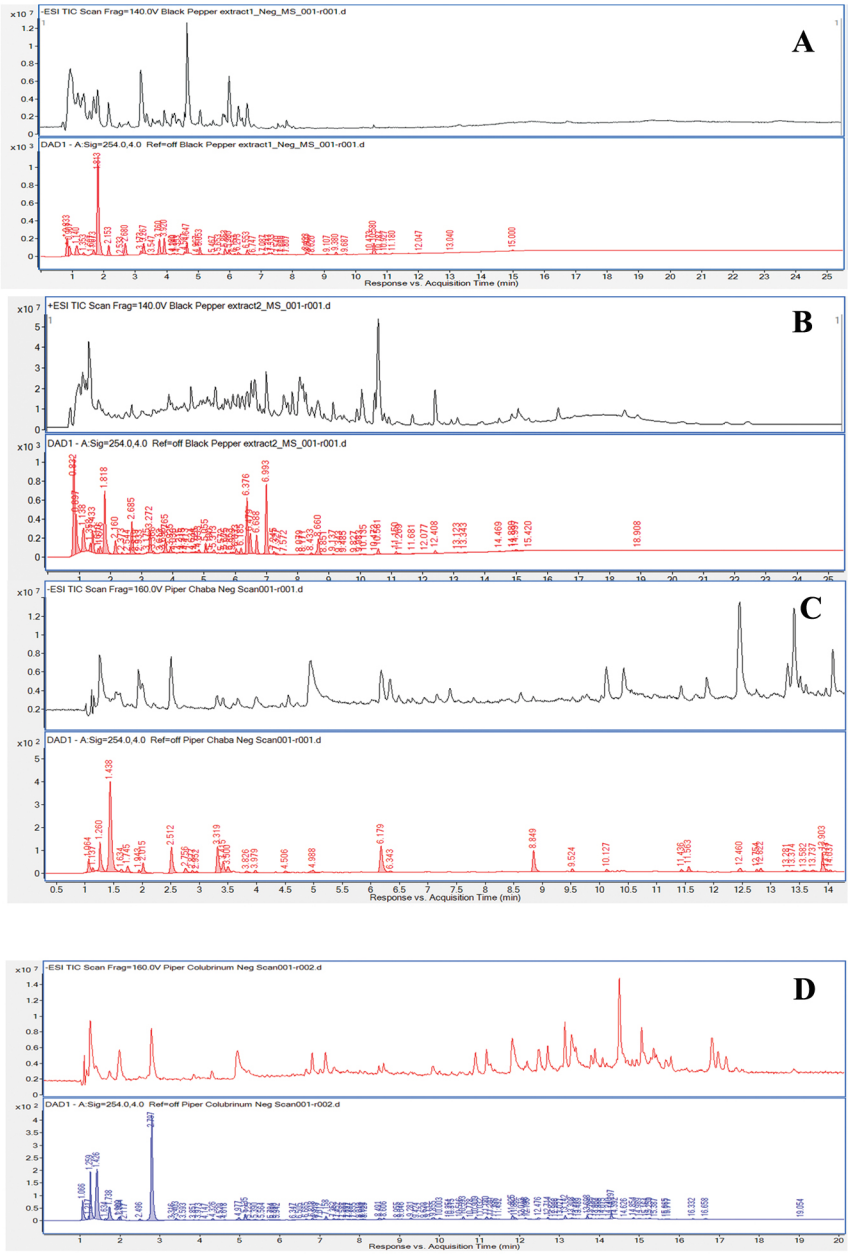

Fig. 4.23. LC-MS analysis of phenolic compounds: Overlaid chromatogram of UV at 254 nm and TIC. A) *P. nigrum*, B) *P. longum*, C) *P. chaba*, D) *P. colubrinum*

Table 4.9. Phenolic compounds identified from *P. nigrum* by LC-MS

Compound	$(M-H)^-$ (Da)	Monoisotopic mass (Da)	Ion formula	Molecular formula
Guaiacol	123.0453	124.0524	$C_7H_7O_2$	$C_7H_8O_2$
Salicylic acid	137.0243	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
4-Hydroxybenzoic acid	137.0245	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
Gentisic acid	153.0194	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Protocatechuic acid	153.0195	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
4-Coumaric acid	163.0401	164.0473	$C_9H_7O_3$	$C_9H_8O_3$
Vanillic acid	167.0349	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
4-Hydroxymandelic acid	167.0351	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
Caffeic acid	179.0352	180.0423	$C_9H_7O_4$	$C_9H_8O_4$
Ferulic acid	193.051	194.0579	$C_{10}H_9O_4$	$C_{10}H_{10}O_4$
Syringic acid	197.0456	198.0528	$C_9H_9O_5$	$C_9H_{10}O_5$
Apigenin	269.0459	270.0528	$C_{15}H_9O_5$	$C_{15}H_{10}O_5$
Luteolin-8-C-glucoside	447.0936	448.1006	$C_{21}H_{19}O_{11}$	$C_{21}H_{20}O_{11}$

Table 4.10. Phenolic compounds identified from *P. longum* by LC-MS

Compound	$(M-H)^-$ (Da)	Monoisotopic mass (Da)	Ion formula	Molecular formula
Salicylic acid	137.0243	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
2-Pyrocatechuic acid	153.0194	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Gentisic acid	153.0195	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
4-Coumaric acid	163.0402	164.0473	$C_9H_7O_3$	$C_9H_8O_3$
Caffeic acid	179.0353	180.0423	$C_9H_7O_4$	$C_9H_8O_4$
Ferulic acid	193.0509	194.0579	$C_{10}H_9O_4$	$C_{10}H_{10}O_4$

Table 4.11. Phenolic compounds identified from *P. chaba* by LC-MS

Compound	$(M-H)^-$ (Da)	Monoisotopic mass (Da)	Ion formula	Molecular formula
Salicylaldehyde	121.0297	122.0368	$C_7H_5O_2$	$C_7H_6O_2$
Salicylic acid	137.0247	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
Protocatechuic acid	153.0196	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Caffeic aldehyde	163.0404	164.0473	$C_9H_7O_3$	$C_9H_8O_3$
5-Methoxysalicylic acid	167.0353	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
Caffeic acid	179.0352	180.0423	$C_9H_7O_4$	$C_9H_8O_4$
Ferulic acid	193.0510	194.0579	$C_{10}H_9O_4$	$C_{10}H_{10}O_4$

Table 4.12. Phenolic compounds identified from *P. colubrinum* by LC-MS

Compounds	$(M-H)^-$ (Da)	Monoisotopic mass (Da)	Ion formula	Molecular formula
2-Pyrocatechuic acid	153.0199	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Salicylic acid	137.0247	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
Protocatechuic acid	153.0194	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Kaempferol-5-glucoside	447.0934	448.1006	$C_{21}H_{19}O_{11}$	$C_{21}H_{20}O_{11}$
5-Methoxysalicylic acid	167.0355	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
Apigenin-7-galactoside	431.0986	432.1056	$C_{21}H_{19}O_{10}$	$C_{21}H_{20}O_{10}$
5,7,2',5'-Tetrahydroxy flavanone	287.0564	288.0634	$C_{15}H_{11}O_6$	$C_{15}H_{12}O_6$
Kaempferide-3-glucoside	461.1090	462.1162	$C_{22}H_{21}O_{11}$	$C_{22}H_{22}O_{11}$
Vanillin	151.0404	152.0473	$C_8H_7O_3$	$C_8H_8O_3$
Scutellarein-4'-methyl ether	299.0565	300.0634	$C_{16}H_{11}O_6$	$C_{16}H_{12}O_6$

Confirmation of phenolic compounds by HPLC

HPLC analysis was performed to confirm the phenolic compounds identified by LC-MS, with authentic standards. The compounds were confirmed by comparing their retention time with those of standards and also by peak enrichment. Thus, HPLC clearly established the presence of 4-hydroxybenzoic acid, caffeic acid, syringic acid, 4-coumaric acid, ferulic acid and salicylic acid in black pepper, caffeic acid, 4-coumaric acid, ferulic acid and salicylic acid in *P. longum*, caffeic acid, ferulic acid and salicylic acid in *P. chaba* and salicylic acid in *P. colubrinum*. HPLC chromatogram for phenolic compounds from selected *Piper* species is given in Fig. 4.24.

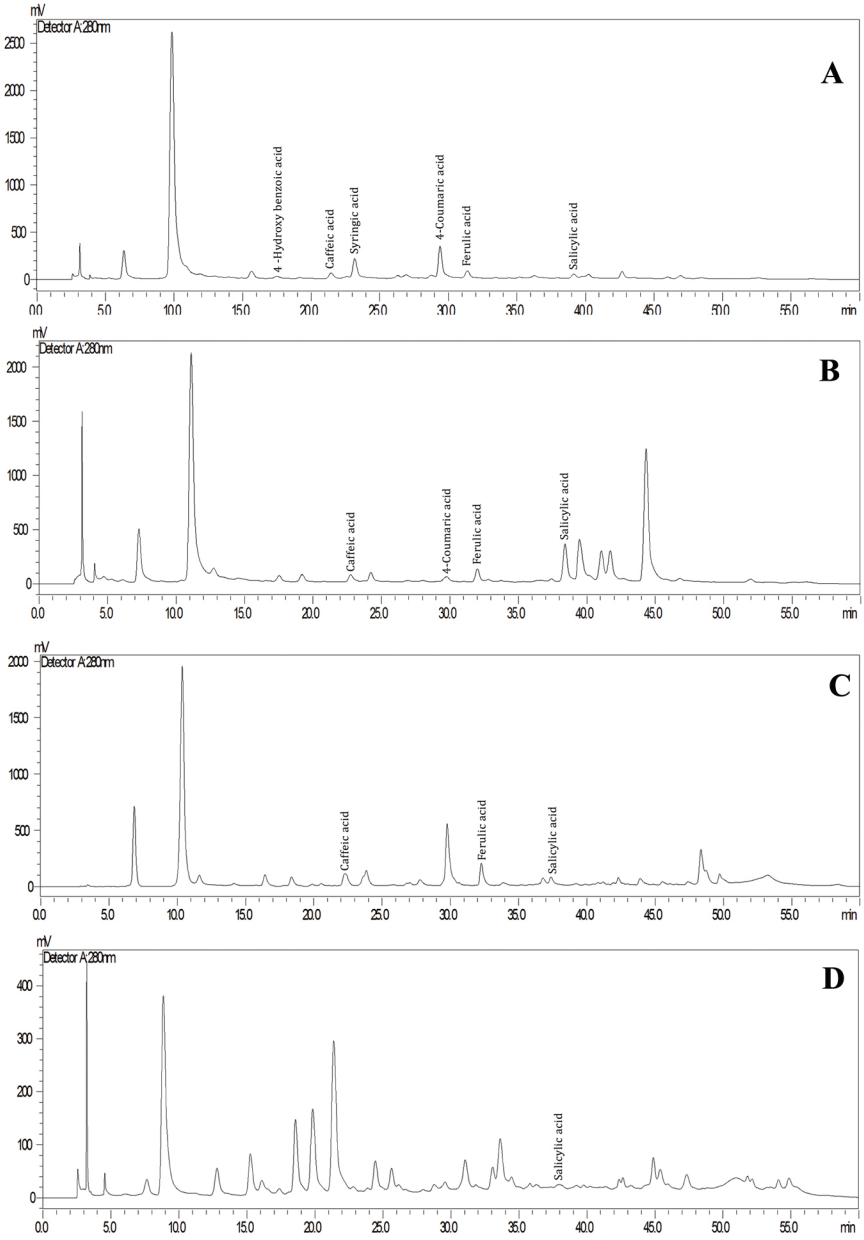

Fig. 4.24. HPLC profiling of phenolic compounds. A) *P. nigrum*, B) *P. longum*, C) *P. chaba*, D) *P. colubrinum*

The phenolic constituents identified from these *Piper* species are reported to have many medicinal values. Salicylic acid, caffeic acid, ferulic acid and coumaric acid are known for its antioxidant and anticancer properties (Hudson *et al*., 2000; Karamac *et al*., 2005; Indap *et al.,* 2006; Prasad *et al.,* 2011; Jiang *et al*., 2013; Djurendic *et al.,* 2014) whereas 2-pyrocatechuic acid is reported for its antimicrobial property (Benny *et al*., 2010; George *et al*., 2011; Shibumon *et al*., 2011). On the other hand, protocatechuic acid is known for its antioxidant, anticancer, antifungal, hepatoprotective and antiinflammatory properties (Herrmann & Nagel, 1989; Hudson *et al*., 2000; Liu *et al.,* 2002). Gentisic acid, vanillic acid and 4-hydroxybenzoic acid are reported for antioxidant and antimicrobial properties by Merkl *et al.* (2010) whereas syringic acid is reported for its antioxidant (Karamac *et al.,* 2005), hepatoprotective (Itoh *et al.,* 2009) and antidiabetic effects (Muthukumaran *et al.,* 2013). The flavonoids apigenin and luteolin are reported for antiinflammatory and antioxidant activities (Romanova *et al.,* 2001; Funakoshi-Tago *et al.,* 2011). Anticancer activity of apigenin is also studied (Patel *et al*., 2007). Kaempferol is reported for its anticancer (Cui *et al.,* 2008), antioxidant (Wang *et al.,* 2006), antiinflammatory (Park *et al.,* 2009), antidiabetic (Lee *et al.,* 2010) and neuroprotective (Lopez-Sanchez *et al.,* 2007) effects whereas kaempferide is known for its ability to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells (Matsuda *et al*., 2009). The effect of kaempferide on biomineralization of cultured osteoblasts is also studied by Song *et al.* (2012). Scutellarein 4'-methyl ether has been reported for its anticancer (Shan *et al*., 2006) and antiallergic (Kawasaki *et al*., 1994) properties. Antimicrobial activity of salicylaldehyde (both in substituted and unsubstituted form) is reported by Pelttari *et al.* (2007)

whereas caffeic aldehyde is studied for its antioxidant activity (Jiang *et al.,* 2013) and 5-methoxysalicylic acid is studied in tomato for their host resistance induction (Safari *et al.,* 2013). Antioxidant, antimicrobial and anticancer properties of vanillin are reported by researchers (Kamat *et al.*, 2000; Fitzgerald *et al.,* 2004; Liang *et al*., 2009). Guaiacol, on the other hand has medicinal applications as expectorant, local anesthetic and antiseptic (http://www. yourdictionary.com/guaiacol). Medicinal properties of 4-hydroxymandelic acid is not explored but mandelic acid is reported for improving skin conditions like abnormal pigmentation, skin texture, etc. (Taylor, 1999). However, medicinal properties of 5, 7, 2', 5'-tetrahydroxy flavanone is not explored much.

Thus, it is understood that some of these identified compounds are underexplored for their medicinal values or well studied compounds are still unknown for certain medicinal properties. Thus, these findings will help researchers to explore these natural phytochemicals for medicine and health.

4.2 VARIABILITY IN PHYSICO-CHEMICAL AND BIOCHEMICAL PROFILE OF BLACK PEPPER VARIETY PANNIYUR-1 IN RELATION TO DIFFERENT LOCATIONS

Panniyur-1 is a leading variety of black pepper at different pepper growing areas of India. Variability of this black pepper variety at different locations assumes great commercial significance. However, a systematic study for its intrinsic quality in relation to location has not been observed. Hence, variability in constituents of Panniyur-1 black pepper berries in relation to different locations has been conducted.

Matured berries of high yielding black pepper variety Panniyur-1 were collected from eleven locations of India (Kasaragod, Chelavoor, Peruvannamuzhi, Panniyur, Ambalavayal, Pampadumpara, Appangala, Mudigere, Pechiparai, Thadiankudisai and Dapoli). After proper drying, the samples were subjected to variability studies in relation to different locations.

4.2.1. Physico-chemical constituents

The moisture content, bulk density, total ash and acid insoluble ash were the physico-chemical constituents examined from Panniyur-1 black pepper berries of eleven locations using standard procedures (section 3.2.1) and the results are illustrated in Table 4.13. The moisture content was in the range of 8.01 to 10.6%. The bulk density showed clear cut variability in relation to location and it ranged from 460.6 to 608.7 g/L. Panniyur-1 sample from Pampadumpara showed highest and that from Kasaragod showed lowest bulk density. Considering the commercial importance of black pepper variety Panniyur-1, which is grown at all major pepper growing areas, bulk density assumes great significance.

The total ash and acid insoluble ash contents had a range of 3.43 to 5.09% and 0.07 to 0.31%, respectively. The highest ash content was observed at Kasaragod whereas it was found to be low at Pechiparai and Dapoli samples. The sample from Pechiparai showed highest whereas that from Appangala showed lowest acid insoluble ash content. Total ash and acid insoluble ash also showed significant variability among Panniyur-1 samples from different locations. Total ash determines the total amount of inorganic solutes present in the sample and thus a useful parameter to assess the nutritional value of

foods and feeds (Tambe & Kadam, 2010; Kadam *et al.*, 2013) whereas acid insoluble ash is an indication of the cleanliness or the amount of sand or dirt present in the sample (Pomeranz & Meloan, 1994).

Table 4.13. Variability in physico-chemical constituents of black pepper variety Panniyur-1 in relation to location

Location	Moisture content (%)	Bulk density (g/L)	Total ash (%)	Acid insoluble ash (%)
Kasaragod	10.6^{a}	460.60^{h}	5.09^{a}	0.13^{g}
Chelavoor	10.2^{ab}	505.80^{f}	3.89^{ef}	0.27^{c}
Peruvannamuzhi	8.01^{d}	466.70^{g}	4.17^{d}	0.16^{f}
Panniyur	10.2^{ab}	547.00^{d}	3.87^{f}	0.12^{g}
Ambalavayal	9.29^{c}	512.00^{e}	3.99^{ef}	0.19^{e}
Pampadumpara	8.15^{d}	608.70^{a}	4.41^{c}	0.12^{g}
Appangala	9.37^{c}	556.50^{c}	3.88^{f}	0.07^{i}
Mudigere	8.36^{d}	573.10^{b}	4.03^{e}	0.09^{h}
Pechiparai	9.91^{bc}	548.90^{d}	3.43^{g}	0.31^{a}
Thadiankudisai	8.63^{d}	570.70^{b}	4.93^{b}	0.28^{b}
Dapoli	10.6^{a}	510.60^{ef}	3.45^{g}	0.21^{d}

Twenty three types of black pepper from Kerala, North and South Kanara, Coorg and Assam have been analyzed for moisture content (8.7 to 14.1%), total ash (3.6 to 5.7%) and acid insoluble ash (0.03 to 0.55%) by Pruthi (1993). Variability in moisture content, bulk density, ash and acid insoluble ash content of black pepper samples collected from India, Indonesia, Vietnam and Malaysia has been studied by Thomas (2009) as 9.1±0.14 to 14.0±0.24%, 273.01±0.28 to 606.29±0.27 g/L, 4.09±0.02 to 5.24±0.01% and 0.49±0.34 to 0.85±0.08% respectively and revealed that Indian black pepper showed superiority for these physical properties. Al-Jasass & Al-Jasser (2012)

have reported the moisture and ash content of black pepper from Saudi Arabia as 4.68±0.3% and 3.57±0.1% respectively. The moisture content from Sarawak black pepper sample has been analyzed by Kumoro *et al*. (2010) as 11.0%. These studies indicate status of moisture content in different areas. International pepper community recommends upto 12 to 13% as desired moisture content for different grades of black pepper (Personal communication). Buenaflor *et al*. (2008) reported total ash and acid insoluble ash of black pepper from Galay's Farm, Kidapawan, Philippines as 4.77% and 1.32% respectively. Kolhe *et al*. (2011) reported total ash and acid insoluble ash of black pepper berries collected from local market as 1.0 and 0.55%, respectively. Zachariah & Parthasarathy (2008) have reported the limits for total ash and acid insoluble ash as 5.0 and 0.5%, respectively. The samples from all the locations except Kasaragod in the present study adhere to this limit. The high ash content is the reflection of mineral contents preserved in the sample (Antia *et al*., 2006).

4.2.2 Primary metabolites

The total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat and crude fibre were the major primary metabolites analyzed as per section 3.2.2, from Panniyur-1 black pepper berries collected from different locations. The results are tabulated and shown in Table 4.14. Panniyur-1 showed a significant location wise variability for primary metabolites. Total carbohydrate ranged from 41.54 to 57.34% whereas reducing sugars from 0.71 to 4.19%. The starch content ranged from 21.57 to 39.46%. Among locations, Panniyur, Kerala had the highest total carbohydrate and reducing sugars whereas high starch content was observed at

Thadiankudisai and Pampadumpara samples. The lowest total carbohydrate was observed at Peruvannamuzhi sample and it was statistically on par with that of Kasaragod sample. On the other hand, lowest reducing sugar and starch was recorded in Dapoli and Kasaragod respectively. Total protein and total free amino acid of Panniyur-1 from eleven locations ranged from 3.27 to 9.0% and 0.13 to 0.59%, respectively. The highest protein content was found at Kasaragod whereas lowest at Pechiparai sample. Total free amino acid content was shown to be highest at Peruvannamuzhi whereas samples from Mudigere and Panniyur showed low total free amino acid content. The total fat had a range of 6.16 to 10.34% and found to be highest in Kasaragod and lowest in Pampadumpara samples. The crude fibre was in the range of 10.79 to 18.6% and it was highest at Kasaragod and lowest at Pampadumpara samples.

Table 4.14. Variability in primary metabolites of black pepper variety Panniyur-1 in relation to location

Location	Total carbohydrate (%)	Reducing sugars (%)	Starch (%)	Total protein (%)	Total free amino acid (%)	Total fat (%)	Crude fibre (%)
Kasaragod	43.44^{e}	0.89^{gh}	21.57^{f}	9.00^{a}	0.47^{b}	10.34^{a}	18.60^{a}
Chelavoor	48.68^{d}	1.32^{e}	33.08^{d}	6.85^{d}	0.29^{d}	8.12^{e}	15.18^{c}
Peruvannamuzhi	41.54^{e}	1.19^{f}	28.30^{e}	8.23^{b}	0.59^{a}	10.02^{b}	16.86^{b}
Panniyur	57.34^{a}	4.19^{a}	35.87^{bc}	6.28^{e}	0.13^{g}	8.06^{f}	14.62^{d}
Ambalavayal	49.84^{cd}	2.08^{c}	36.15^{bc}	5.43^{f}	0.22^{f}	7.77^{g}	12.54^{f}
Pampadumpara	49.41^{cd}	3.40^{b}	38.91^{a}	4.04^{h}	0.29^{d}	6.16^{k}	10.79^{g}
Appangala	48.58^{d}	1.71^{d}	35.02^{bcd}	4.62^{g}	0.34^{c}	7.68^{h}	14.15^{e}
Mudigere	54.46^{ab}	1.14^{f}	36.28^{b}	7.39^{c}	0.15^{g}	7.30^{i}	14.17^{e}
Pechiparai	53.7^{b}	0.94^{g}	34.12^{cd}	3.27^{i}	0.20^{f}	8.88^{c}	14.61^{d}
Thadiankudisai	52.13^{bc}	0.82^{h}	39.46^{a}	6.85^{d}	0.29^{d}	6.28^{j}	12.45^{f}
Dapoli	52.36^{bc}	0.71^{i}	33.78^{d}	4.62^{g}	0.26^{e}	8.37^{d}	15.19^{c}

Primary metabolites are responsible for growth and development of the plant (Shamina & Sharma, 2001). Total carbohydrate, reducing sugar, starch and total protein influence the productivity of black pepper and also provide precursors for piperine biosynthesis. Factors like temperature, light, rate of photosynthesis, availability of water and nutrients, stages of plant growth, level of free amino nitrogen, level of RNA, etc., influence the carbohydrate and protein level of black pepper berries (Prejeena, 2003; Sumeshkumar, 2004; Shujari, 2005; Cruz *et al.*, 1970; http://www.safergrass.org/pdf/AAEP.pdf). Since total free amino acids tend to change during disease, their measurements will give an idea about physiological and health status of the plants (Sadasivam & Manickam, 2008). The total free amino acid level is also influenced by factors like geological locations, use of fertilizers, irrigation water and climatic conditions (Jabeen *et al*., 2015) whereas fat content, an agricultural commodity important to the food, medical and manufacturing industries is influenced by various environmental factors like temperature, water stress, light, soil constituents, atmospheric constituents, xenobiotics and other factors like physical damage and pest attack (Harwood, 1994; http://lifeofplant.blogspot.in/2011/03/lipids.html). Crude fibre is the portion of total carbohydrate remaining after acid and alkali digestion and thus may influence by the factors affecting the carbohydrate content (http://www.pelicanequipments.com/application.html). Thus, in the present study, variability in primary metabolites with respect to locations may be due to variation in the aforementioned factors.

Zachariah *et al.* (2010) reported that total carbohydrate of dried berries of 26 black pepper cultivars from Panniyur and Peruvannamuzhi locations of Kerala was in the range of 38.6 to 51.2% whereas starch,

total free amino acid and total protein ranged from 32.1 to 43.2%, 0.3 to 0.8% and 2.1 to 6.0% respectively. Al-Jasass & Al-Jasser (2012) have revealed total carbohydrate (37.36±1.4%), crude protein (25.45±0.4%), crude fat (5.34±0.6%) and crude fibre (23.6±0.3%) contents of black pepper from Saudi Arabia. Variability in total carbohydrate, starch, reducing sugar, total protein, total free amino acid and crude fibre contents of black pepper samples collected from India, Indonesia, Vietnam and Malaysia has been studied by Thomas (2009) as 42.74±0.14 to 57.58±0.12%, 38.59±0.35 to 45.53±0.42%, 1.27±0.02 to 1.79±0.04%, 1.74±0.05 to 3.32±0.03%, 0.24±0.04 to 1.18±0.02%, 8.2±0.01 to 9.6±0.07% respectively. Pruthi (1993) has reported starch (28 to 49%) and crude fibre (8.7 to 18%) contents from 23 types of black pepper collected from Kerala, North and South Kanara, Coorg and Assam. On the other hand, the black pepper berries collected from Galay's Farm, Kidapawan, Philippines has been reported to have crude fibre content of 16.51% (Buenaflor *et al*., 2008). These earlier reports are comparable with the respective primary metabolites obtained in the present study and discrepancies noticed might be due to location or other agronomic factors.

4.2.3 Secondary metabolites

4.2.3.1 Location wise variability in secondary metabolites and oleoresin

Table 4.15 illustrates the level of major secondary metabolites in Panniyur-1 from different locations and clear location wise variability was observed for secondary metabolites of Panniyur-1 sample. Essential oil, the contributor for characteristic odour of black pepper was extracted by hydrodistillation using Clevenger trap (section

3.2.3.1.1). Essential oil varied between 1.6 and 3.2%. The highest oil content was recorded at Peruvannamuzhi and the lowest at Pampadumpara. Piperine, the pungent principle of pepper was quantified by HPLC (section 3.2.3.2) and shown to be in the range of 2.13 to 4.49%. Piperine content was highest at Kasaragod and lowest at Pampadumpara samples. The representative HPLC chromatograms for piperine content of Panniyur-1 black pepper berries from different locations are given in Fig. 4.25. Total phenol content was estimated by Folin-Ciocalteau method (section 3.2.3.3.1) whereas total flavonoid by Aluminium chloride colorimetric method (section 3.2.3.3.2). The total phenol content had a range of 0.30 to 0.63% and was highest in Kasaragod and lowest in Pampadumpara samples. An important observation is that, the low altitude locations such as Kasaragod, Chelavoor, Pechiparai, Panniyur and Peruvannamuzhi showed relatively more total phenol as compared to the remaining high altitude locations. Thus, apart from location wise variation, phenolic content showed a significant altitudinal variation also. This is the first report regarding altitudinal variation of total phenol content in black pepper berries. Total flavonoid content (0.23 to 0.53%) was observed to be highest in Kasaragod sample whereas found to be low in Pampadumpara and Appangala samples. Oleoresin, the blend of volatile and non-volatile components, which are extractable with suitable organic solvents, was estimated by gravimetric method as explained in section 3.2.4**.** The oleoresin content ranged from 5.82 to 12.73%. Kasaragod sample showed the highest whereas Thadiankudisai sample showed the lowest oleoresin content. It is reported that the oleoresin content of different black pepper cultivars is very much correlated with their piperine content (Zachariah *et al*., 2010).

Table 4.15. Variability in secondary metabolites and oleoresin content of black pepper variety Panniyur-1 in relation to location

Location	Essential oil (%)	Piperine (%)	Total phenol (%)	Total flavonoid (%)	Oleoresin (%)
Kasaragod	2.8[b]	4.49[a]	0.63[a]	0.53[a]	12.73[a]
Chelavoor	2.5[d]	2.76[c]	0.53[c]	0.40[b]	8.17[cd]
Peruvannamuzhi	3.2[a]	3.91[b]	0.55[b]	0.30[e]	9.74[b]
Panniyur	2.0[f]	2.55[d]	0.51[d]	0.39[b]	7.42[e]
Ambalavayal	2.4[e]	2.22[g]	0.40[f]	0.29[e]	7.14[ef]
Pampadumpara	1.6[h]	2.13[h]	0.30[h]	0.23[g]	6.48[g]
Appangala	1.8[g]	2.46[e]	0.36[g]	0.24[g]	6.91[f]
Mudigere	2.0[f]	2.40[f]	0.37[g]	0.27[f]	7.17[ef]
Pechiparai	2.0[f]	2.56[d]	0.46[e]	0.39[b]	7.98[d]
Thadiankudisai	2.4[e]	2.40[f]	0.37[g]	0.34[d]	5.82[h]
Dapoli	2.7[c]	2.49[e]	0.46[e]	0.36[c]	8.43[c]

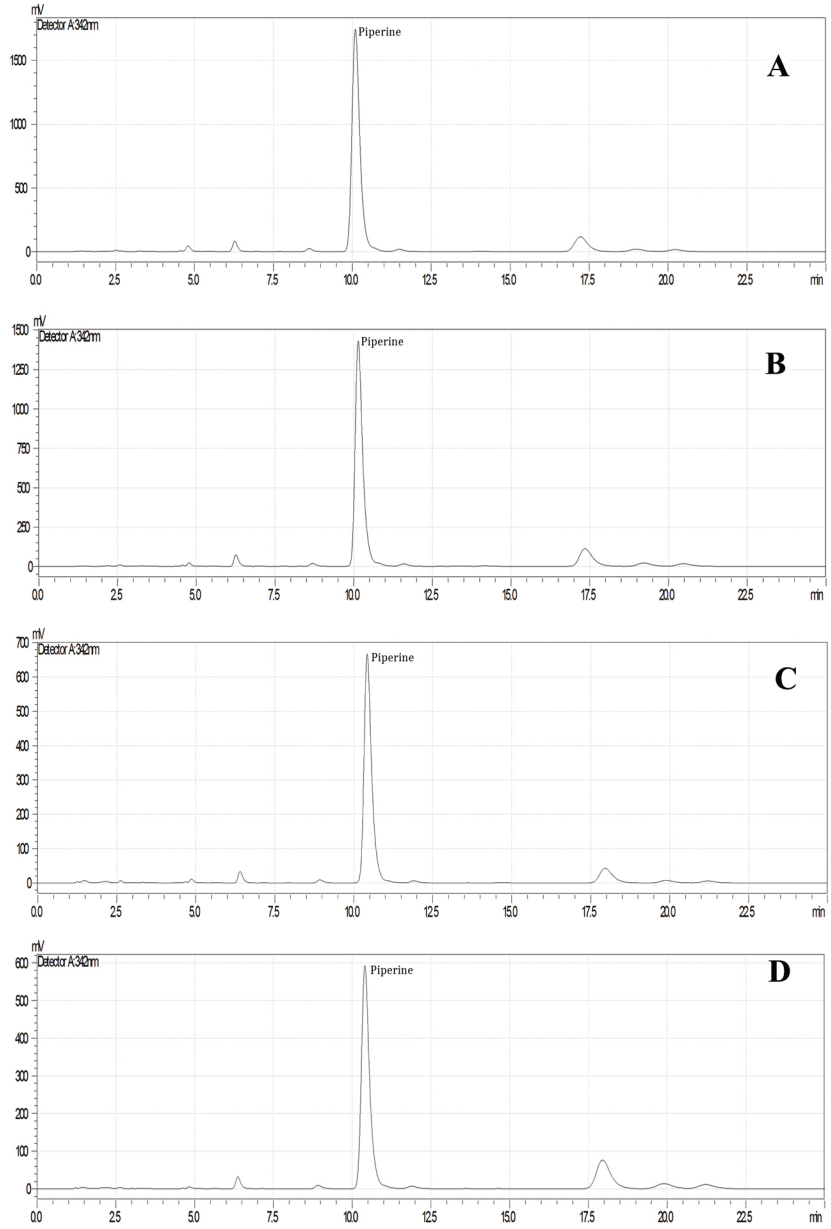

Fig. 4.25. Representative HPLC chromatograms for piperine content from Panniyur-1 berries of different locations. A) Kasaragod, B) Peruvannamuzhi, C) Appangala, D) Pampadumpara.

Secondary metabolites in black pepper are mainly responsible for defense mechanism, pungency and aroma (Shamina & Sharma, 2001). Physiological factors (organ development, pollinator activity cycle, type of plant material, type of secretory structure, seasonal variation, mechanical or chemical injuries, etc.), environmental conditions (climate, pollution, edaphic factors, diseases, pests, etc.), geographic variation, storage, amount of plant material/space, genetic factors and evolution are the factors influencing the secondary metabolite production and compositions (Figueiredo *et al*., 2008). In addition, precursors of secondary metabolic pathways are products of the primary metabolism. Therefore, a severe or long lasting stress factor could induce an excessive shift between primary and secondary metabolism and consequently a diversion of available resources from growth to defense (Iriti & Faoro, 2009). These factors can also be attributed for accounting the variability in secondary metabolites especially phenolic contents at different locations.

The range of piperine content obtained for present study is in agreement with those reported by Pruthi (1993) as 1.7-7.4% for piperine content of 23 types of black pepper collected from Kerala, North and South Kanara, Coorg and Assam. He had also reported that Kerala types of black pepper showed high whereas North Kanara types showed low piperine content. Present study also revealed highest piperine content for black pepper sample from Kerala.

Variability in essential oil, oleoresin, phenol and piperine contents of black pepper samples collected from India, Indonesia, Vietnam and Malaysia has been studied by Thomas (2009) as 2.3±0.14 to 4.5±0.25%, 8.35±0.42 to 15.82±0.68%, 0.90±0.02 to 3.32±0.06% and 1.81 to 4.75% respectively and revealed that all the black pepper from

Indonesia and one each from Vietnam and Malaysia showed more oleoresin and essential oil content than Indian black pepper. The study also revealed that piperine content was higher in Indonesian and Vietnam samples.

Kumoro *et al*. (2010) have reported oleoresin, piperine and essential oil contents as 10.6%, 5.8% and 1.7% respectively from black pepper sample of Sarawak (Malaysia). Parthasarathy *et al*. (2008a) studied spatial influence in biochemical constituents of black pepper leaves and found diversity in total phenol content in a narrow geographical range. Buenaflor *et al*., (2008) reported the oleoresin content of black pepper grown at Galay's Farm, Kidapawan, Philippines (15.18%). Variability in curcumin content and yield of same turmeric variety grown at different locations with varying climatic conditions, reported by Srinivasan (2009) as well as, the environmental impact on curcumin content of turmeric grown at different locations reported by Anandaraj *et al.* (2014), support the present study.

4.2.3.2 GC-MS analysis of black pepper essential oil from different locations

The essential oils extracted from Panniyur-1 black pepper samples of eleven locations were subjected to GC-MS analysis as per section 3.2.3.1.2 and their characteristic constituents were identified. A total of 49 compounds comprised 78.23 to 96.97% of the total essential oil were identified from these samples. Major monoterpenes identified in the pepper oil were α-thujene, α-and β-pinene, sabinene, β-myrcene, D-limonene and β-linalool and of which, D-limonene (15.13 to 20.78%), β-pinene (4.67 to 13.26%) and sabinene (4.31 to 19.23%) predominated. The constituent D-limonene was found to be highest in

Thadiankudisai and lowest in Kasaragod samples whereas Kasaragod and Pampadumpara samples showed highest and lowest β-pinene content respectively. Sabinene content was found to be highest in Thadiankudisai sample whereas lowest in Pechiparai sample. The major sesquiterpene, β-caryophyllene, ranged from 9.52 to 26.95% and it was highest at Pechiparai and lowest at Pampadumpara samples. Alpha-phellandrene, δ-3-carene, ocimene (Z and E), germacrene-D, linalool, α-copaene, δ-and β-elemene, α-cubebene, α-humulene, caryophyllene oxide, etc. were the other compounds identified from these pepper oil samples. Many of the compounds identified from Panniyur-1 pepper oil showed variability in relation to locations. Variability was profound in α-thujene, α- and β-pinene, sabinene, D-limonene, 4-terpineol, α-copaene, δ- and β-elemene, β-caryophyllene, α-humulene, β-bisabolene, caryophyllene oxide and α-cadinol.

It is noted that α-thujene, α-and β-pinene, camphene, β-myrcene, D-limonene, Z-ocimene, γ-terpinene, α-terpinolene, β-linalool, 4-terpineol, α- terpineol, δ-and β-elemene, α-copaene, β-caryophyllene, α-humulene, β-selinene, β-bisabolene, δ-cadinene, caryophyllene oxide and α-cadinol were identified from all the samples. However, sabinene, α-phellandrene and α-zingeberene were identified in all the samples except that from Kasaragod whereas α-terpinene and pentadecanal were identified from all the samples except that from Chelavoor. Likewise, Alpha-bergamotene and α-guaiene were identified only in Pampadumpara sample whereas *o*-cymene was present only in Mudigere sample.

It is observed that β-caryophyllene, the sesquiterpene hydrocarbon, was the major constituent of Panniyur-1 black pepper oil samples from

low elevations whereas monoterpene hydrocarbons such as D-limonene and sabinene were the major constituents of samples from high elevations. The significant finding is that, apart from location wise variability, clear altitudinal variability was also observed for β-caryophyllene. Beta-caryophyllene was low at high altitudes (>500 m MSL) and high at low altitudes. This kind of variability was not observed with other sesquiterpenes. Similarly, monoterpenes like thujene, α-pinene, sabinene, limonene, α-phellandrene and linalool were relatively high at higher altitudes as compared to plains. The differences in the oil constituents in relation to location may be due to certain factors mainly geographic divergence and ecological conditions (Aziz *et al*., 2012).

Jagella & Grosch (1999 a, b) demonstrated that, pinene, limonene, α- phellandrene, linalool, etc. are the potent odorants of pepper oil. In the present study, these constituents were relatively high at higher altitudes and it can be concluded that aroma profile is superior at higher altitudes. Variability in essential oil constituents of Panniyur-1 black pepper sample in relation to location and altitude is not reported earlier.

The detailed picture of percentage composition, location wise variability and altitudinal variability of the selected essential oil samples are given in Table 4.16. The representative GC-MS chromatograms for essential oil composition of Panniyur-1 samples from different locations are given in Fig. 4.26.

The different essential oil profile of a sample from different origins reflects the different environmental conditions of each location and culture conditions (different altitudes, different soil types, different

solar exposure, etc.). At the same time, all these factors are inter connected and moreover the differences in essential oil composition for different geographical origins may also be due to genetic differences, processing of the plant material after harvest, etc. (Figueiredo *et al*., 2008). Renjie *et al.* (2010) stated that chemical composition of essential oils is greatly influenced by different production area. The influence of planting site (location) on plant essential oil percentage has also been reported by Honarvar *et al*. (2010).

Variability in essential oil constituents of black pepper was reported by several researchers. This can be attributed to the effect of cultivar, agro climatic variation, variation in the maturity of raw material, oil extraction, etc. (Zachariah & Parthasarathy, 2008). However, information is scanty on variability in essential oil constituents of black pepper variety collected from different locations.

Krishnamurthy *et al.* (2012) reported that black pepper grown under low temperature (high elevation) had more α-pinene, limonene, sabinene+myrcene and less β-caryophyllene compared to that grown in high temperature (low elevation). Present study is in accordance with this finding.

Variability in essential oil constituents of different black pepper samples collected from India, Indonesia, Vietnam and Malaysia has been studied by Thomas (2009). Most abundant compounds observed in essential oil of traded Indian black peppers were α-phellandrene, limonene and β-caryophyllene. Vietnam and Malaysian black pepper showed slight variation in β-caryophyllene whereas the percentage of

α-phellandrene was comparatively high in Malaysian black pepper. Nerolidol was identified only in Indian black peppers.

Essential oil constituents from black pepper of Indian origin were compared with the reported constituents of Sri Lankan black pepper oils. The monoterpene hydrocarbons from Indian oils were similar to those of corresponding Sri Lankan oils but the oils differed with regard to their sesquiterpene and oxygenated compounds. Beta-pinene and β-caryophyllene occurred in all oils and sabinene in Sri Lankan oils only (McCarron *et al*., 1995). Jirovetz *et al*. (2002) reported essential oil constituents of black pepper collected from Cameroon and these include limonene (10.26%), germacrene D (11.01%), β-pinene (10.02%), α-pinene (6.40%), α-phellandrene (8.56%), β-caryophyllene (7.29%) and *cis*-β-ocimene (3.19%).

Parthasarathy *et al.* (2008b) reported maximum variability in β-caryophyllene and nerolidol in leaf oil of *P. nigrum* from Western Ghats of Kerala and Karnataka. Aziz *et al*. (2012) have revealed great variation in the chemical composition of black pepper samples collected from Bangladesh and India. Variability was profound for constituents like 3-carene, α-pinene, D-limonene, β-caryophyllene, etc. Indian pepper oil contained 65.421% of monoterpenes and 34.579% of sesquiterpenes whereas Bangladesh pepper oil contained 77.673% of monoterpenes and 14.195% of sesquiterpenes.

Table 4.16. Essential oil profiling of black pepper variety Panniyur-1 from different locations

Compound	% Composition											RI*
	K	C	Z	R	L	P	A	M	E	G	D	
α-Thujene	0.60	0.83	1.24	0.86	2.61	2.30	2.65	2.53	0.47	2.94	1.03	927
α-Pinene	3.88	4.18	4.62	3.96	5.77	4.52	5.19	5.73	4.00	6.48	4.86	937
Camphene	0.14	0.12	0.12	0.09	0.14	0.15	0.15	0.11	0.09	0.15	0.08	950
Sabinene	-	8.09	10.0	8.35	17.76	14.60	15.9	18.07	4.31	19.23	8.64	980
β-Pinene	13.26	6.82	6.96	8.93	7.76	4.67	5.16	9.90	10.21	8.98	10.65	985
β-Myrcene	1.76	2.27	2.22	2.34	2.62	2.28	2.28	2.06	1.95	2.74	2.20	995
α-Phellandrene	-	0.20	0.26	0.17	0.52	1.04	0.52	0.50	0.16	0.58	0.16	1011
δ-3-Carene	-	0.12	0.04	-	-	4.40	0.66	1.08	0.27	-	-	1017
α-Terpinene	0.07	-	0.16	0.06	0.23	0.28	0.45	0.11	0.06	0.38	0.07	1021
o-Cymene	-	-	-	-	-	-	-	0.81	-	-		1032
D-Limonene	15.13	15.99	15.8	16.60	19.53	17.70	18.4	19.33	16.6	20.78	17.4	1041
Z-Ocimene	0.03	0.14	0.01	0.14	0.02	0.21	0.01	0.09	0.06	0.02	0.09	1053
E-Ocimene	-	-	0.16	-	0.23	-	0.19	-	-	0.22	-	1054
γ-Terpinene	0.03	0.22	0.28	0.12	0.42	0.71	0.84	0.28	0.11	0.68	0.14	1064
α-Terpinolene	0.04	0.25	0.25	0.19	0.29	0.49	0.41	0.19	0.16	0.34	0.15	1093
β-Linalool	0.39	0.41	0.47	0.33	0.74	1.70	0.86	0.96	0.23	0.87	0.21	1107
p-Menth-2-en-1-ol	-	0.03	-	-	-	0.35	0.23	0.13	-	0.17	-	1131
4-Terpineol	0.71	0.57	0.81	0.57	1.19	3.62	2.91	2.09	0.32	1.95	0.47	1187
Cryptone	0.07	-	-	-	-	0.16	0.07	0.11	-	-	-	1197
α-Terpineol	0.18	0.22	0.17	0.01	0.19	0.40	0.24	0.22	0.13	0.18	0.10	1202
trans-Piperitol	-	-	-	-	-	0.02	0.04	-	-	-	-	1219
Nerol	0.04	0.03	-	-	-	0.02	-	-	-	-	-	1237
δ-Elemene	1.36	2.27	2.41	1.03	0.88	0.40	0.70	0.42	2.10	0.77	1.20	1344
α-Cubebene	0.49	0.12	0.45	0.39	0.41	0.37	0.47	-	0.38	0.32	0.26	1356
α-Copaene	4.75	0.20	5.19	5.31	4.79	3.06	4.65	3.92	5.51	3.91	5.06	1387
β- Cubebene	-	-	-	-	0.62	-	-	0.16	-	-	-	1398
β- Elemene	1.06	0.69	0.91	0.59	0.38	0.51	0.42	0.08	0.63	0.21	0.50	1400

Compound	% Composition											RI*
	K	C	Z	R	L	P	A	M	E	G	D	
α-Gurjunene	0.07	1.10	0.18	-	0.14	0.09	0.16	-	-	0.07	-	1418
β-Caryophyllene	21.41	21.9	20.30	22.99	13.49	9.52	12.8	12.48	26.95	10.71	26.3	1441
α-Bergamotene	-	-	-	-	-	0.07	-	-	-	-	-	1443
α-Guaiene	-	-	-	-	-	0.04	-	-	-	-	-	1446
β-Farnesene	0.47	0.36	-	-	-	-	-	-	-	-	-	1463
α-Humulene	2.43	0.83	2.02	2.15	1.16	1.53	1.24	1.26	3.03	1.11	2.44	1467
γ-Muurolene	0.17	0.07	0.12	0.11	0.06	0.04	0.06	-	0.12	0.02	-	1486
Germacrene-D	-	0.10	0.44	0.52	0.32	0.57	0.37	-	0.42	0.23	0.35	1491
β-Selinene	0.68	0.49	0.90	0.57	0.64	0.87	0.63	0.52	0.63	0.39	0.57	1498
α-Zingeberene	-	0.97	0.39	0.14	0.20	0.14	0.24	0.07	0.20	0.17	0.12	1503
α-Selinene	-	0.30	1.52	0.45	0.52	0.94	0.85	-	-	-	0.43	1506
β-Bisabolene	6.49	1.32	7.05	6.91	7.03	5.68	7.96	7.07	7.08	7.58	5.98	1521
γ-Cadinene	-	0.03	-	-	-	1.01	1.6	-	-	-	-	1530
δ-Cadinene	2.43	1.82	2.47	2.36	2.12	1.74	2.63	1.49	2.59	1.83	2.14	1536
trans-Nerolidol	0.16	0.18	0.06	0.07	0.08	0.97	0.07	-	0.09	-	-	1550
Spathulenol	2.31	-	0.14	0.15	0.10	0.71	0.48	0.26	0.14	-	0.10	1592
Caryophyllene oxide	4.91	0.06	0.51	0.83	0.54	1.56	1.00	1.32	0.86	0.16	0.73	1601
δ-Cadinol	0.35	0.16	0.16	0.12	0.12	0.19	0.24	-	0.14	0.23	-	1641
α-Cadinol	4.89	3.84	0.74	0.73	0.22	0.36	3.06	0.18	0.36	0.19	0.23	1654
Amorphan-3en-9-ol	-	0.64	3.89	4.29	2.16	2.40	-	2.15	3.59	1.97	2.29	1663
Pentadecanal	0.14	-	0.12	0.22	0.20	0.09	0.12	0.12	0.17	0.16	0.12	-
Nonadecanol	0.08	0.29	-	-	0.06	0.08	0.06	-	-	-	-	-
Total (Nos.)	34	39	38	35	39	45	42	34	35	35	32	
Total (%)	90.98	78.23	93.48	92.65	96.26	92.56	96.97	95.52	94.12	96.72	95.07	

*Retention Indices

K=Kasaragod, C=Chelavoor, Z=Peruvannamuzhi, R=Panniyur, L=Ambalavayal, P=Pampadumpara, A=Appangala, M=Mudigere, E=Pechiparai, G=Thadiankudisai, D=Dapoli

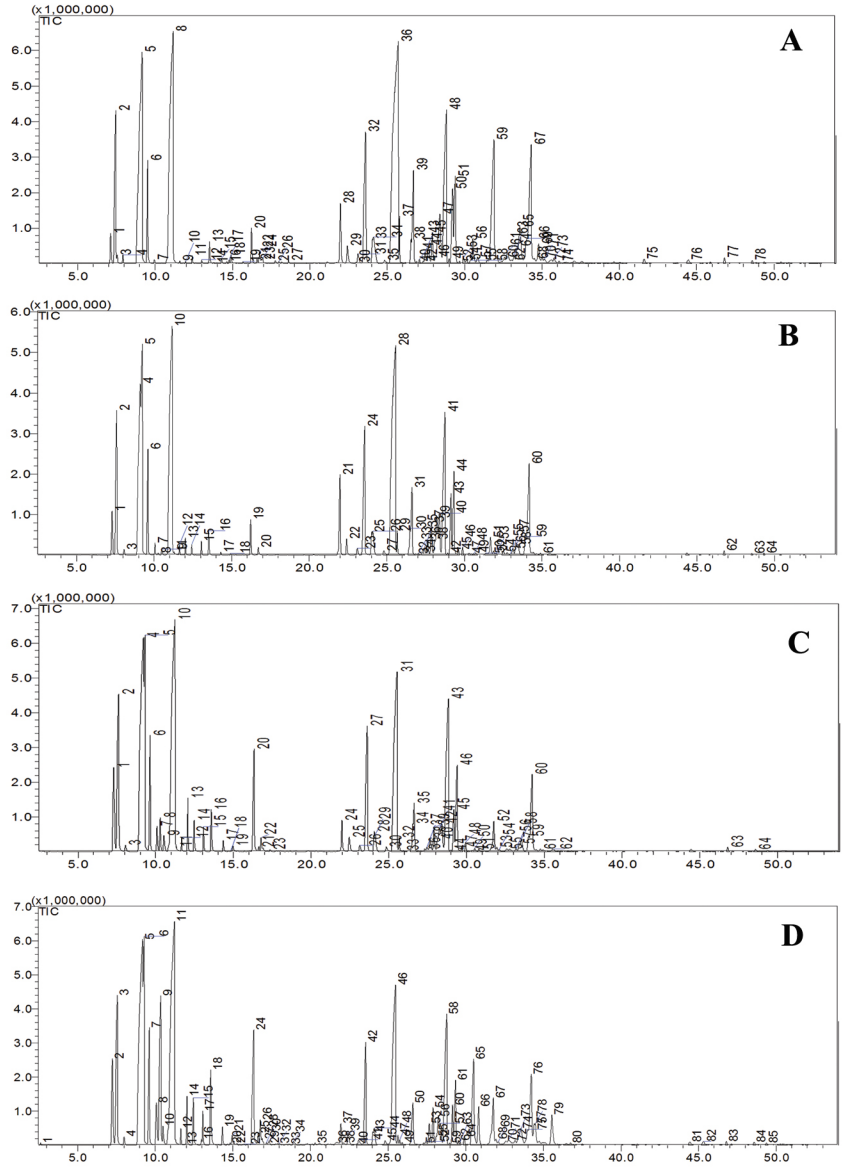

Fig.4.26. Representative GC-MS chromatograms for essential oil from Panniyur-1 berries of different locations. A) Kasaragod, B) Peruvannamuzhi, C) Appangala, D) Pampadumpara. X-axis represents the retention time (Rt) and Y-axis represents the peak intensity (mV)

4.2.4 Minerals

Nitrogen, phosphorus, magnesium, potassium and micronutrients are the most important minerals/nutrients for black pepper growth, development and yield and their influence depend on their ratios in the soil as well as in the plant (Srinivasan *et al.,* 2007). Mineral contents of Panniyur-1 berries collected from eleven locations were analyzed as per section 3.2.5 and the results indicated a significant variability in relation to location (Table 4.17). Kasaragod sample was found to be highest for P, K, Ca, Mg, Fe and Mn whereas Peruvannamuzhi sample for N, Chelavoor sample for Cu and Mudigere sample for Zn. Potassium and Fe contents of Thadiankudisai sample were also high and statistically on par with that of Kasaragod sample. Nitrogen was found to be lowest in sample collected from Panniyur whereas P was low and statistically on par for Pechiparai and Dapoli samples. Lowest K content was recorded in Pechiparai sample and it was statistically on par with that of Dapoli sample. Dapoli sample was recorded for lowest Mg, Appangala sample for lowest Cu and Peruvannamuzhi sample for lowest Mn content. On the other hand, Pechiparai sample showed lowest Ca and Fe contents whereas Zn content was found to be low at Pechiparai and Thadiankudisai samples.

Table 4.17. Variability in mineral contents of black pepper variety Panniyur-1 in relation to location

Sample	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)
Kasaragod	1.99^{cd}	0.24^{a}	1.20^{a}	0.27^{a}	0.15^{a}	163.0^{a}	12.4^{gh}	124.0^{a}	14.2^{d}
Chelavoor	2.05^{cd}	0.12^{d}	0.54^{c}	0.18^{de}	0.09^{ef}	81.75^{c}	71.0^{a}	101.5^{c}	15.9^{c}
Peruvannamuzhi	2.32^{a}	0.16^{bc}	0.59^{bc}	0.15^{f}	0.11^{cd}	120.5^{b}	23.0^{d}	48.25^{f}	12.5^{e}
Panniyur	1.91^{d}	0.12^{d}	0.54^{c}	0.18^{de}	0.10^{de}	80.50^{c}	37.0^{b}	88.00^{d}	15.7^{c}
Ambalavayal	2.02^{cd}	0.15^{c}	0.55^{c}	0.19^{cd}	0.08^{fg}	85.50^{c}	15.0^{f}	99.50^{c}	13.1^{de}
Pampadumpara	1.92^{cd}	0.17^{b}	0.63^{b}	0.22^{b}	0.12^{bc}	82.00^{c}	13.5^{fg}	103.5^{c}	26.0^{b}
Appangala	1.96^{cd}	0.13^{d}	0.56^{c}	0.19^{cd}	0.10^{de}	82.00^{c}	10.0^{i}	88.00^{d}	16.6^{c}
Mudigere	1.99^{cd}	0.16^{bc}	0.53^{c}	0.17^{e}	0.12^{bc}	78.00^{c}	18.5^{e}	103.3^{c}	32.6^{a}
Pechiparai	2.21^{ab}	0.09^{e}	0.42^{d}	0.14^{f}	0.09^{ef}	52.00^{d}	15.5^{f}	70.00^{e}	10.2^{f}
Thadiankudisai	2.08^{bc}	0.17^{b}	1.19^{a}	0.23^{b}	0.13^{b}	157.0^{a}	25.3^{c}	111.5^{b}	10.0^{f}
Dapoli	2.02^{cd}	0.10^{e}	0.46^{d}	0.20^{c}	0.07^{g}	52.50^{d}	11.0^{hi}	84.00^{d}	15.6^{c}

Plants are the rich source of minerals that are essential for human life. They have remarkable role against various degenerative diseases and also to prevent or reduce injury caused by environmental pollutant. They are also essential for many vital mechanisms of human body (Watts, 1997; Kadam *et al*., 2013).

The variability in mineral contents of black pepper berries from different locations has not been studied much. The black pepper leaf mineral analysis data of farmer's fields from major black pepper growing gardens of South India (Idukki, Kozhikode, Wayanad and Kannur districts of Kerala and Kodagu district of Karnataka) have been compiled by Hamza *et al.* (2007). The results showed variability in mineral contents and these include N (1.9 to 2.7%), P (0.06 to 0.50%), K (0.9 to 4.7%), Ca (1.1 to 4.9%), Mg (0.12 to 1.60%), Fe (110 to 537 mg/kg) and Mn (33 to 810 mg/kg). Al-Jasass & Al-Jasser (2012) reported mineral contents of black pepper from Saudi Arabia and these include K (663±25.0 mg/100 g), Ca (195.0±15.0 mg/100 g), Mg (52.0±8.0 mg/100 g), Zn (0.9±0.1 mg/100 g), Mn (3.5±0.2 mg/100 g), Cu (1.3±0.1 mg/100 g) and Fe (20.5±0.5 mg/100 g) contents. Mineral data of present study is comparable with this literature. The difference in geographical locations, use of fertilizers, irrigation water and climatic conditions may be the factors influencing variability in mineral contents of Panniyur-1 sample in relation to location.

4.2.5 Correlation analysis

Apart from physico-chemical and biochemical variability in Panniyur-1 berries from different locations, significant correlation was also established between many constituents by identifying their correlation coefficient (r). This correlation for different constituents of black

pepper berries observed in the present study in relation to different locations has not been reported so far.

4.2.5.1 Correlation between bulk density and metabolites

Bulk density, an important physical characteristic of biomass influences directly to its cost, determines the storage requirements, the sizing of the material handling system and how the material behaves during subsequent thermo-chemical and biological processes (McKendry, 2002; Lam *et al.*, 2008). The size, shape, individual particle density, surface characteristics, etc. are few factors affecting bulk density (Lam *et al*., 2008). In this study, bulk density was positively correlated with starch (r = +0.83) and had negative correlation with total phenol, piperine, oleoresin, crude fibre, essential oil, total fat, total protein and total free amino acid. Table 4.18 illustrates the correlation coefficient (r) for correlation between bulk density and each metabolite. A positive correlation between bulk density and starch content has been reported by Fife *et al.* (2008) in barley grains. All the correlations, except that between bulk density and total protein was found to be significant ($p \leq 0.05$) in relation to location. Jayashree *et al*. (2009) have related the bulk density and size of black pepper and found that bulk density increased with increase in size up to 4.8 mm and after that it was found to be decreased. Likewise, they have also correlated oleoresin and piperine contents of black pepper varieties with its size and revealed that these parameters were highest for the lower grade (<3.5 mm).

4.2.5.2 Correlation among metabolites

Starch, the important reserved carbohydrate, showed significant negative correlation with metabolites, *viz.*, total phenol, piperine,

oleoresin, essential oil, total fat, crude fibre, total protein and total free amino acid. Total phenol, piperine, oleoresin, essential oil, total fat, crude fibre, total protein and total free amino acid showed positive correlation with each other. Except the correlation of total protein with oleoresin and total fat as well as the correlation of total free amino acid with total phenol, crude fibre, total fat and total protein, all other correlations among constituents were found to be statistically significant ($p \leq 0.05$) in relation to location. The correlation matrix which represents the correlation coefficient (r) for each correlation among metabolites is given in Table 4.18

Zachariah *et al.* (2010) have revealed a strong positive correlation for piperine and phenols of black pepper leaf with its berry piperine. They have also reported that, caryophyllene, nerolidol, germacrene-D, elemol, piperine, essential oil, total phenol, total starch, total amino acids, total carbohydrate and protein of black pepper leaf had a residual effect on its berry piperine.

Table 4.18. Correlation matrix for different constituents of black pepper variety Panniyur-1 in relation to location

	P	**TP**	**O**	**BD**	**S**	**CF**	**EO**	**TF**	**PR**	**TFA**
P	1.00									
TP	+0.83*	1.00								
O	+0.92*	+0.86*	1.00							
BD	-0.80*	-0.87*	-0.82*	1.00						
S	-0.93*	-0.84*	-0.95*	+0.83*	1.00					
CF	+0.88*	+0.90*	+0.89*	-0.83*	-0.90*	1.00				
EO	+0.71*	+0.71*	+0.64*	-0.88*	-0.64*	+0.68*	1.00			
TF	+0.85*	+0.88*	+0.89*	-0.83*	-0.89*	+0.92*	+0.68*	1.00		
PR	+0.73*	+0.60*	+0.57	-0.57	-0.60*	+0.61*	+0.64*	+0.44	1.00	
TFA	+0.78*	+0.46	+0.62*	-0.63*	-0.68*	+0.54	+0.67*	+0.57	+0.50	1.00

P=Piperine; TP=Total phenol; O=Oleoresin; EO=Essential oil; BD=Bulk density; S=Starch; CF=Crude fibre; TF=Total fat; PR=Total protein; TFA=Total free amino acid

The superscript (*) is given for significant correlations ($p \leq 0.05$)

4.2.5.3 Correlation between total ash and mineral contents

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating. This residue mainly consists of oxides and salts containing anions (chlorides, phosphates, sulfates, other halides, etc.) and cations (potassium, sodium, calcium, iron, magnesium, manganese, etc.). Thus, total ash is a measure of total amount of minerals within the sample and high ash content is a reflection of high mineral contents of the samples (http://www.foodscience-avenue.com/2012/11/ash-content-in-food.html; Antia *et al*., 2006). So, correlation between total ash and the mineral contents of Panniyur-1 samples in relation to different locations will be helpful for better understanding of aforementioned statements. The correlation results obtained by calculating correlation coefficient (r) indicated a significant ($p \leq 0.05$) and strong positive correlation for total ash with P (r = +0.92), K (r = +0.93), Ca (r = +0.78), Mg (r = +0.89), Fe (r = +0.93) and Mn (r = +0.61). This correlation studies proved the relationship between total ash and mineral contents of the sample.

4.2.5.4 Correlation between biochemical and mineral contents

Mineral contents, essential for growth, development and yield of the plant, also influence the plant metabolites in different ways (Cruz *et al.*, 1970; Aminzadeh *et al.*, 2010; Srinivasan *et al.*, 2012). So, correlation between biochemical (total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat, crude fibre, essential oil, oleoresin, piperine, total phenol and total flavonoid) and mineral contents (N, P, K, Ca, Mg, Fe, Cu, Mn and Zn) of Panniyur-1 samples in relation to different locations was performed. Among the

correlation studied, the significant correlations ($p \leq 0.05$) were obtained for N and essential oil (r = +0.59), P and total protein (r = +0.68), P and piperine (r = +0.59), Mg and total protein (r = +0.60), Fe and total protein (r = +0.74), Fe and total free amino acid (r = +0.59) and Fe and piperine (r = +0.62). A moderate uphill correlation was also obtained for N and total free amino acid (r = +0.51), P and total free amino acid (r = +0.51), K and total protein (r = +0.57) and Mg and piperine (r = +0.51). Likewise, a moderate downhill correlation was also obtained for N and reducing sugars (r = -0.54), P and total carbohydrate (r = -0.56) and Fe and total carbohydrate (r = -0.53).

Sharafzadeh *et al.* (2011) have mentioned that nitrogen content affect the quantity and composition of volatile oil and also mentioned that a high nitrogen content cause secondary metabolite production. This statement support the positive correlation obtained in the present study for N with essential oil. Phosphorous influences the protein content significantly since it is an essential component of many of enzymes involved in protein synthesis (Shukla *et al.*, 2010). Potassium and Mg are important for protein synthesis (Watts, 1997; http://www.kali-gmbh.com/en/pdf-articles/article-201006-better-crops-magnesium.pdf) and also, iron is mainly a component of proteins and enzymes (http://www.drt.com.tr/doctoferro/Iron.aspx). These reasons might impart for positive correlation of P, K, Mg and Fe with total protein in the present study. Positive correlation between N and total free amino acid is obtained since N is the major component of amino acid (http://www.cropnutrition.com/efu-nitrogen#overview). Cruz *et al.* (1970) also reported that nitrogen content changes the level of free amino acid in the developing rice grain. The ability of phosphorus to activate the co-enzymes for amino acid production indicated the effect

of P on amino acid (http://www.privilifesciences.com/role-phosphorus.htm) and these assume to be the reason for positive correlation between P and amino acid in the present study.

Singh & Singh (2006) have revealed that P deficiency in mint leaf and root brought about an increase in all sugar fractions. This finding is a support to the negative correlation between P and total carbohydrate of present study. 'Phosphorous also function to decompose the carbohydrates produced in photosynthesis' (http://www .privilifesciences.com/role-phosphorus.htm) is another statement which support this correlation. The physiological effects of iron on the general plant metabolism like carbohydrate synthesis are intimately related to its chlorophyll status since the most important function of Fe is to take part in chlorophyll synthesis. However, the amount of Fe do not always correlate directly with those of chlorophyll (Sideris & Young, 1944) and thus with carbohydrate synthesis. This might be a possible reason for negative correlation between Fe and total carbohydrate.

4.2.6 Average linkage cluster analysis based on biochemical constituents

Panniyur-1 black pepper berries collected from different locations of India showed variability in biochemical constituents in relation to different locations (sections 4.2.2 and 4.2.3). For better understanding, these samples were clustered based on their biochemical data (total carbohydrate, reducing sugars, starch, total protein, total free amino acid, total fat, crude fibre, essential oil, piperine, oleoresin, total phenol and total flavonoid). The average cluster linkage analysis was adopted for clustering these samples. The respective dendrogram clearly

showed number of clusters and number of samples in each cluster. The cluster membership when viewed in the context of Panniyur-1 from different locations, indicate that samples with similar biochemical constituents were closely clustered. Looking at the dendrogram, sample from Kasaragod and Peruvannamuzhi were closely related and formed a single cluster (cluster 1) and found to be distinct from other samples (cluster 2). This is because Panniyur-1 from Kasaragod and Peruvannamuzhi samples had high secondary metabolites (essential oil, oleoresin, total phenol and piperine), total protein, total free amino acid, total fat, crude fibre and low total carbohydrate and starch. Among the cluster 2, samples from Thadiankudisai and Pampadumpara showed high starch content, similar pattern for total free amino acid and relatively low oleoresin and total phenol contents and thus formed a separate cluster (cluster 3). Like wise, samples from Mudigere and Panniyur clusterd together due to their similar essential oil and total free amino acid pattern (cluster 4) and the remaining samples (Dapoli, Pechiparai, Appangala, Ambalavayal and Chelavoor) clustered together (cluster 5). The dendrogram depicting different clusters of Panniyur-1 black pepper berries from different locations is shown in Fig. 4.27.

Based on biochemical parameters (fermentation rate, crude fibre content, total polyphenol content, total catechins, chlorophyll *a*, chlorophyll *b* and total carotenoids), hierarchical clustering (average linkage cluster analysis) of 35 tea germplasm accessions has been performed by Kottawa-Arachchi *et al.* (2013). Such attempts indicate successful clustering of samples based on their biochemical parameters.

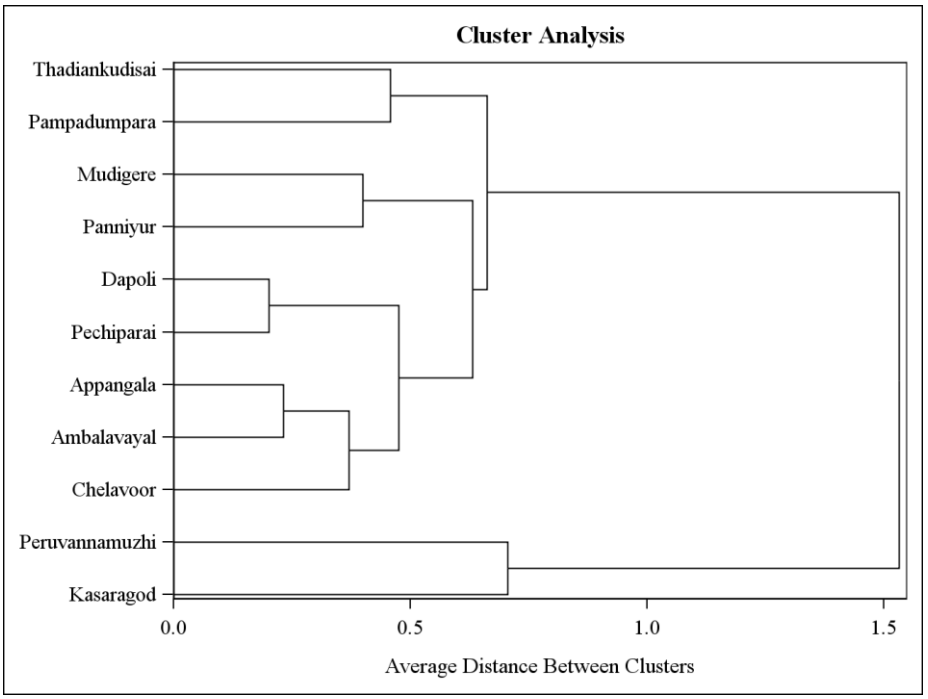

Fig. 4.27. Average linkage cluster dendrogram of Panniyur-1 black pepper berries of different locations based on biochemical composition

4.2.7 Soil analysis

4.2.7.1 Analysis of soil constituents

Soil is one of the most important environmental factors which play the major role in defining plant's growth, development and its distribution (Sahney *et al.*, 2010). It is also noted that soil parameters influences the metabolic pattern of plant and hence, influence its nutritional and pharmacological properties (Ramakrishna & Ravishankar, 2011). Thus, understanding of physico-chemical properties of black pepper growing soil is essential. So, physico-chemical parameters of black pepper growing soils and their effect on various black pepper constituents were studied in this section.

Soil samples were collected from panniyur-1 black pepper growing plot of locations *viz.,* Appangala, Thadiankudisai, Pampadumpara, Panniyur, Dapoli, Ambalavayal, Peruvannamuzhi, Chelavoor and Pechiparai, along with berry samples and soil parameters were analyzed (section 3.2.6). The pH, electrical conductivity, organic carbon, available N, available P, exchangeable K, Ca, Mg and available Fe, Cu, Zn and Mn were the physico-chemical parameters analyzed from the collected soil samples and results are summarized in Table 4.19.

Soil samples collected from all the locations were acidic in nature with a pH range of 4.3 to 6.9. Sample from Appangala showed nearly neutral pH compared to soil samples from other locations whereas soil from Pechiparai and Ambalavayal were more acidic than other soil samples. Mathew *et al.,* (1995) reported that soil with near neutral pH enhances the black pepper productivity whereas Wardani & Zaubin (1984) reported that black pepper differs in their growth with respect to soil pH and soil with pH above 7.5 inhibits the growth. Electrical conductivity (EC), method of determining the salt content was in the range of 20.9 to 372 microsiemens (µs). Low EC value was found in soil samples from Chelavoor, Appangala and Pechiparai whereas very high in Thadiankudisai sample. The greater the conductivity, the greater is its salt content and lesser is the yield (Ali, 2011; http://www.environment.nsw.gov.au/salinity/basics/production.htm).
The organic carbon (OC) varied from 1.46 to 3.7% and soil sample collected from Panniyur and Pechiparai showed highest and lowest OC respectively. The variability in other soil nutrients include available N (100 to 206.5 mg/kg), available P (1.05 to 270.6 mg/kg), exchangeable K (79.5 to 260.4 mg/kg), exchangeable Ca (197.6 to 1882 mg/kg),

exchangeable Mg (71.59 to 217.9 mg/kg), available Fe (21.91 to 49.1 mg/kg), available Cu (1.85 to 12.63 mg/kg), available Mn (22.99 to 34.6 mg/kg) and available Zn (1.77 to 4.98 mg/kg). The different nutrient management practices existing in the different Panniyur-1 black pepper growing locations might be the reason for this variability in soil nutrients.

Table 4.19. Physico-chemical parameters of Panniyur-1 black pepper growing soil samples collected from different locations

Sample	pH	EC (µs)	OC (%)	Available N (mg/kg)	Available P (mg/kg)	Exchangeable K (mg/kg)	Exchangeable Ca (mg/kg)	Exchangeable Mg (mg/kg)	Available Fe (mg/kg)	Available Cu (mg/kg)	Available Mn (mg/kg)	Available Zn (mg/kg)
Chelavoor	5.1^{c}	25.30^{e}	2.92^{b}	178.5^{b}	1.100^{e}	79.50^{f}	367.90^{g}	125.5^{d}	45.25^{b}	1.850^{f}	33.94^{a}	1.78^{e}
Peruvannamuzhi	5.7^{b}	63.70^{c}	2.23^{d}	159.0^{c}	16.27^{c}	133.9^{de}	626.40^{f}	106.7^{e}	33.86^{d}	12.31^{ab}	22.99^{b}	2.91^{c}
Panniyur	5.3^{c}	59.90^{c}	3.70^{a}	100.0^{e}	3.910^{de}	182.6^{b}	1217.0^{d}	200.8^{b}	27.63^{e}	12.56^{a}	33.51^{a}	2.46^{d}
Ambalavayal	4.4^{d}	38.20^{d}	2.29^{d}	144.2^{d}	10.12^{cd}	153.1^{c}	693.00^{f}	71.59^{f}	47.72^{ab}	7.100^{c}	33.61^{a}	2.72^{c}
Pampadumpara	5.3^{c}	46.70^{d}	2.35^{d}	139.3^{d}	1.100^{e}	128.6^{e}	1372.0^{c}	172.3^{c}	45.22^{b}	6.370^{d}	34.10^{a}	1.77^{e}
Appangala	6.9^{a}	23.90^{e}	2.17^{de}	147.0^{d}	1.050^{e}	141.5^{d}	1882.0^{a}	170.3^{c}	21.91^{f}	12.63^{a}	34.28^{a}	1.82^{e}
Pechiparai	4.3^{d}	20.90^{e}	1.46^{f}	206.5^{a}	36.88^{b}	141.3^{d}	197.60^{h}	108.8^{e}	34.36^{d}	3.290^{e}	32.89^{a}	3.94^{b}
Thadiankudisai	5.4^{bc}	372.0^{a}	2.69^{c}	171.5^{b}	270.6^{a}	260.4^{a}	1627.0^{b}	212.5^{a}	49.10^{a}	11.76^{b}	34.21^{a}	4.98^{a}
Dapoli	5.4^{bc}	104.9^{b}	2.01^{e}	150.0^{cd}	7.690^{d}	181.5^{b}	959.80^{e}	217.9^{a}	38.08^{c}	11.75^{b}	34.60^{a}	2.40^{d}

There are number of factors reported to have influence on soil parameters. Climate, organic matter, tillage, plants, roots and residues, animals that burrow in the soil, microorganisms, fertilizers, wetting and drying, exchangeable cations, inorganic cements, clay and water are some of such factors (http://www.agriinfo.in/?page=topic&superid=4&topicid=268). It is reported that high rainfall pattern of black pepper growing regions made the soil less productive, more acidic and low in P, Ca, K and Zn status due to leaching and erosion losses of these nutrients (Sadanandan, 2000). Another factor reported for proper growth and yield is proper / balanced manuring (Hamza *et al.*, 2007).

There are different factors affecting the availability of soil nutrients to plants and which include soil drainage, soil texture, slope steepness, soil aeration, salt content, soil pH, electrical conductivity, cation competition, alkaline-sodic soil, sub-soil, climate, soil parent material and interactions among nutrients (Murphy *et al*., 1981; Haldar & Mandal, 1981; Alloway, 2008; Srinivasan *et al.*, 2012; http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_053274.pdf; http://www.spectrumanalytic.com/support/library/ff/Ca_Basics.htm; http://www.spectrumanalytic.com/support/library/ff/Ca_Basics.htm).

The properties of rooting system, the successful use of fertilizers, light, temperature, air humidity, nature of spraying solutions, species, varieties, leaf surface, leaf age, nutritional status and plant development stage, etc. are the factors affecting the nutrient absorption (Clarkson, 1985; http://www.nutrico.co.za/factors-influencing-nutrient-absorption/).

Hamza *et al.* (2004) collected black pepper growing soils from low and high altitudes and reported variability in soil nutrients like OC (1.24 to 1.32%), P (2.1 to 8.5 ppm), K (126 to 254 ppm), Ca (226 to 1850 ppm), Mg (49 to 516 ppm), Fe (23 to 48 ppm), Mn (3.8 to 40 ppm), Zn (0.51 to 5.1 ppm) and Cu (1.7 to 3.4 ppm). The investigation also revealed that soils of high yielding gardens showed high pH, OC, P, K, Ca, Mg and Zn and thus found that these parameters favoured good black pepper growth with high productivity.

The soil nutrient analysis data of farmer's fields from major black pepper growing gardens of South India (Idukki, Kozhikode, Wayanad and Kannur districts of Kerala and Kodagu District of Karnataka) have been compiled by Hamza *et al.* (2007). The result revealed variability in soil nutrients from these locations as soil pH (4.4 to 6.7), OC (0.51 to 3.7%), P (1.6 to 154.0 mg/kg), exchangeable K (61 to 1194 mg/kg), exchangeable Ca (244 to 3310 mg/kg), exchangeable Mg (24 to 455 mg/kg), available Mn (5.8 to 31.0 mg/kg), available Zn (0.62 to 14.00 mg/kg) and available Cu (1.5 to 55.0 mg/kg). They have also revealed that the order of limiting nutrients for soil was organic carbon>Zn>P>Ca>K>Mg.

These reports are comparable with the respective nutrients obtained in the present study and certain discrepancies were noticed and that assume to be the effect of previously mentioned factors.

4.2.7.2 Correlation analysis

Physico-chemical properties of black pepper growing soil have great influence on productivity, quality and chemical constituents of black pepper (Srinivasan *et al.,* 2012). So, study on effect of soil parameters on various constituents of Panniyur-1 black pepper berries from

different locations is crucial and thus, it was performed using correlation analysis.

4.2.7.2.1 Correlation between soil and berry nutrients/minerals

The correlation between soil and berry nutrients/minerals was performed by identifying correlation coefficient (r) and certain significant relationships ($p \leq 0.05$) were obtained. Soil N showed significant positive correlation with berry N (r = +0.65) whereas soil P with berry K (r = +0.93) and berry Fe (r = +0.76). Soil N also showed a moderate negative correlation with berry Zn (r = -0.47). Likewise, soil P also showed moderate positive correlation with berry P (r = +0.40), berry Ca (r = +0.47), berry Mg (r = +0.59) and a moderate negative correlation with berry Zn (r = -0.47). Zn and P were already reported to have antagonistic nature (Murphy *et al.,* 1981). Soil K showed significant positive correlation with berry K (r = +0.71) and a moderate correlation with berry Ca (r = +0.49), berry Fe (r = +0.51) and berry Zn (r = -0.40). Likewise, soil Ca was in significant positive correlation with berry Ca (r = +0.71). Soil Ca also showed moderate uphill correlation with berry Mg (r = +0.51), berry P (r = +0.44) and berry K (r = +0.52) whereas moderate downhill correlation with berry N (r = -0.58). Soil Mg was in moderate uphill correlation with berry Ca (r = +0.61) and moderate downhill correlation with berry N (r = -0.49). Soil Zn showed significant negative correlation with berry Zn (r = -0.74). Soil Cu was in moderate negative correlation with berry Cu (r = -0.43). Soil Mn showed significant positive correlation with berry Mn (r = +0.81) and significant negative correlation with berry N (r = -0.78) and also moderate positive correlation with berry Ca (r = +0.56). Soil Fe showed moderate positive correlation with berry P (r = +0.43), K (r = +0.45), Ca (r = +0.43) and Mn (r = +0.55). Availability of these nutrients both in berries and soil, different interactions among these nutrients, etc. might be the factors which

contribute for such correlations. This correlation study has indicated the importance of soil nutrients in black pepper nutrition.

4.2.7.2.2 Correlation between soil nutrients and berry biochemical constituents

The correlation between soil nutrients and biochemical constituents of panniyur-1 black pepper berries collected from different locations were also performed since soil parameters have great influence on quality, growth and yield of black pepper. Certain significant ($p \leq 0.05$) relationships were recorded by calculating correlation coefficient (r) values. Soil N showed a significant negative correlation with reducing sugar content of black pepper berries (r = -0.79) whereas soil Ca with oleoresin content of berries (r = -0.66). Soil Mn was in significant correlation with total carbohydrate (r = +0.72), starch (r = +0.76), total free amino acid (r = -0.82), total fat (r = -0.70), essential oil (r = -0.66), piperine (r = -0.93) and oleoresin (r = -0.71) contents of Panniyur-1 black pepper berries in relation to location. Apart from this, soil P showed moderate positive correlation with starch (r = +0.47), total fat (r = -0.45) and oleoresin (r = -0.52) whereas soil K with starch (r = +0.50) and oleoresin (r = -0.48). Soil Ca was in moderate uphill correlation with berry starch (r = +0.56) and moderate downhill correlation with berry total fat (r = -0.64), crude fibre (r = -0.45), total phenol (r = -0.65) and total flavonoid (r = -0.56). Likewise, soil Mg was in moderate uphill correlation with berry total carbohydrate (r = +0.47) and moderate downhill correlation with berry total fat (r = -0.46). Soil Fe showed moderate negative correlation with total fat (r = -0.45) and crude fibre (r = -0.50) whereas soil Mn was found to be in considerable negative correlation with total protein (r = -0.59), crude fibre (r = -0.59) and total phenol (r = -0.53). This correlation study indicated the influence of soil nutrients on berry biochemical compositions. Hamza & Sadanandan (2005) reported that the

oleoresin and piperine contents increased significantly with zinc application. This report also revealed the influence of soil nutrients on berry quality parameters.

4.3 ANTIOXIDANT POTENTIAL OF SELECTED *PIPER* SPECIES AND SELECTED BLACK PEPPER VARIETIES

Human body produces free radicals mainly Reactive Oxygen Species (ROS) as a part of normal metabolic processes. Mitochondria, peroxisomes and immune cells like leukocytes and macrophages are the main endogenous sources for free radical production in cells. Many acute and chronic diseases like cancer, diabetes, inflammation, arthritis, aging, atherosclerosis and various neurodegenerative disorders mainly arise from oxidative stress initiated by highly reactive and unstable free radicals. So, the oxidation and antioxidation balance should be maintained for a healthy biological system. This can be achieved by exploring compounds with antioxidant activity. Antioxidants are compounds that inhibit or delay the oxidation of other molecules by terminating the initiation or propagation of oxidizing chain reactions. The restriction in the use of synthetic antioxidants due to their adverse effects has led to a growing interest in recent years on natural antioxidants (Kahl & Kappus, 1993; Agbor *et al.*, 2006; Krishnaswami *et al.*, 2013). Since the plants are tremendous source for such antioxidants, present study investigated the antioxidant activity of four medicinally valued *Piper* species (*P. nigrum*, *P. longum*, *P. chaba* and *P. colubrinum*).

Matured berries of six high yielding black pepper varieties (Sreekara, Subhakara, IISR Malabar Excel, IISR Thevam, Panchami and Panniyur-1) and fruits of four *Piper* species (wild *P. nigrum*, *P. longum*, *P. chaba* and *P. colubrinum*) were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi, (Kozhikode, Kerala). After

proper drying, the samples were subjected to sequential extraction using n-hexane, chloroform, methanol and water as per section 3.2.7 and the resultant extracts were used for *in vitro* antioxidant analysis.

4.3.1 Yield of sequential extracts of samples

The extracts obtained from the samples after sequential extraction, were evaporated to dryness using rotary evaporator and the yield of solvent free extracts was noted. The yield of water extracts was shown to be high compared to hexane, chloroform and methanol extracts. Among chloroform extracts, the yield of *P. colubrinum* extract was very high. The details of yield of sequential extracts are given in Table 4.20. Sequential extraction helps for effective and complete extraction of compounds with different polarity (Policegoudra *et al*., 2011).

Table 4.20. Yield of sequential extracts (%)

Sample	**Sequential extracts**			
	Hexane	**Chloroform**	**Methanol**	**Water**
Sreekara	6.34^{c}	5.45^{cd}	4.91^{e}	20.90^{g}
Subhakara	7.07^{b}	6.10^{c}	5.57^{d}	21.37^{fg}
IISR Malabar Excel	7.39^{b}	3.06^{e}	9.20^{a}	23.54^{e}
IISR Thevam	8.16^{a}	5.25^{d}	6.58^{bc}	20.82^{g}
Panniyur-1	8.22^{a}	7.26^{b}	6.53^{c}	29.24^{d}
Panchami	7.14^{b}	3.58^{e}	5.76^{d}	23.12^{ef}
P. nigrum (wild)	7.21^{b}	2.94^{e}	3.28^{g}	11.54^{h}
P. longum	5.15^{d}	3.37^{e}	6.77^{bc}	39.96^{b}
P. chaba	6.90^{b}	2.20^{f}	4.21^{f}	36.25^{c}
P. colubrinum	7.99^{a}	27.9^{a}	7.01^{b}	45.63^{a}

The recovery of crude water and ethanol extracts of black pepper has been given by Gulcin (2005) as 11.75 and 6.76% respectively whereas the yield of its methanolic extract has been reported by Khalaf *et al*. (2008) as 9.2 g%. The dried black pepper berries were powdered and extracted successively with water, ethanol and methanol and the extracts recovery have been recorded as 2.8, 3.35 and 2.45 g respectively from 50 g of sample (Nahak & Sahu, 2011). The recovery of ethanol and water extracts of *P. chaba* fruit collected from Thailand has been reported by Sakpakdeejaroen & Itharat (2009) as 12.3906 and 15.8965% respectively. Sawhney *et al*. (2011) reported the yield of ethyl acetate, methanol and water extracts of *P. longum* as 2.96, 40.68 and 15.16% respectively. Zarai *et al*. (2013) have reported the yield of chloroform, ethyl acetate, methanol, ethanol and water extracts of black pepper as 35.95±0.65, 16.14±0.78, 49.09±0.98, 55.91±1.42 and 8.72±1.05% respectively. Recovery of ethanol, chloroform, hexane, ethyl acetate, aqueous and hydro-ethanol extracts of *P. longum* has been reported as 9.45, 5.43, 1.65, 8.05, 4.36, and 4.1% respectively by Barua *et al*. (2014). The variation in yield may be due to the polarity of different solvents used and also due to varying extractability of solvents for different class of phytochemicals. On the other hand, this variation in the extract recovery can be attributed to the polarities of different compounds present in the samples (Zarai *et al*., 2013). The extract recovery also varies in relation to factors like method of extraction, solvent used for extraction, etc.

4.3.2 *In vitro* antioxidant activity of sequential extracts

The sequential extracts obtained in section 4.3.1 were subjected to *in vitro* antioxidant analysis using different assays include 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity,

phosphomolybdenum method, ferric reducing power method and ferrous chelating activity.

4.3.2.1 DPPH free radical scavenging activity

The antioxidant activity of the extracts was determined in terms of their hydrogen donating or radical scavenging ability and thus to scavenge DPPH free radicals (section 3.2.8.1). Table 4.21 showed the inhibitory concentration of each extract required to scavenge 50% DPPH free radicals (IC_{50} in µg/mL). Though all the extracts exhibited free radical scavenging ability, chloroform and methanol extracts predominated. Hexane extract of *P. colubrinum* also showed high DPPH radical scavenging ability. IC_{50} value for chloroform and methanol extracts was found to be very low (27.4 to 228.9 µg/mL for chloroform extract whereas 29.42 to 153.9 µg/mL for methanol extract) and this indicated their high ability to scavenge DPPH free radicals. Among methanol extracts, IISR Malabar Excel and among chloroform extracts, *P. colubrinum* showed highest DPPH radical scavenging activity. Methanol extract of Panchami and *P. colubrinum* also showed high ability to scavenge DPPH. Hexane extracts (except *P. colubrinum*) and water extracts showed comparatively low DPPH scavenging ability with high IC_{50} values. Significant variation was observed among varieties and also among *Piper* species in DPPH radical scavenging ability. DPPH radical scavenging activity of BHA was compared with that of extracts. It was found that BHA was found to be superior to extracts for scavenging DPPH free radicals (IC_{50}-5.2 µg/mL).

Table 4.21. DPPH radical scavenging activity of sequential extracts in terms of IC_{50} (µg/mL)

Sample	Sequential extracts			
	Hexane	**Chloroform**	**Methanol**	**Water**
Sreekara	1068.0^{d}	148.2^{e}	62.45^{c}	935.47^{e}
Subhakara	1240.0^{c}	228.9^{a}	54.09^{d}	800.00^{f}
IISR Malabar Excel	776.54^{f}	99.61^{f}	29.42^{f}	1942.0^{a}
IISR Thevam	1430.0^{b}	154.2^{de}	54.04^{d}	765.20^{f}
Panniyur-1	1019.0^{de}	170.1^{bc}	54.58^{d}	1784.0^{b}
Panchami	970.00^{e}	104.6^{f}	40.14^{e}	1620.0^{c}
P. nigrum (wild)	1830.0^{a}	164.9^{bcd}	153.9^{a}	1025.0^{e}
P. longum	1400.0^{b}	174.1^{b}	130.4^{b}	1660.0^{c}
P. chaba	1800.0^{a}	159.9^{cd}	152.1^{a}	1900.0^{a}
P. colubrinum	122.70^{g}	27.40^{g}	42.28^{e}	1207.0^{d}
BHA	5.200			

There are reports on DPPH free radical scavenging ability of these samples. DPPH free radical scavenging ability of sample is due to its hydrogen donating ability. The DPPH radical scavenging ability of crude ethanol and water extracts of black pepper has been investigated by Gulcin (2005) as 48 and 55% respectively at the concentration of 75 µg/mL and that of ethanol extract of black pepper from Brazil has been reported with EC_{50} value 110±0.2 g spice/kg DPPH by Mariutti *et al*. (2008). Khalaf *et al*. (2008) reported that methanolic extract of black pepper showed DPPH radical scavenging activity with IC_{50} value of 144.1±2.2 µg/mL. Petroleum ether extract of black pepper was also subjected to DPPH scavenging activity and found that there is a concentration dependent increase in their scavenging activity and

hence black pepper could be considered as a potent source of natural antioxidant (Singh *et al*., 2008).

Kapoor *et al*. (2009) have demonstrated a dose-dependent DPPH radical scavenging activity for ethanol and ethyl acetate oleoresins of black pepper and also revealed that ethyl acetate oleoresin is superior for their ability to scavenge DPPH. The DPPH radical scavenging activity of alcohol and methylene chloride extracts of black pepper from local market of Mysore has been studied by Krishna *et al*. (2010) with IC_{50} value as 900 and 750 µg respectively. Jain *et al*. (2011) reported DPPH radical scavenging ability of methanolic extract of *P. nigrum* berries as 57.22±1.05%. Nahak & Sahu (2011) extracted the powdered black pepper berries successively with water, ethanol and methanol and DPPH radical scavenging activity has been tested. The ethanol extract showed highest scavenging activity (74.61±0.02%) followed by methanolic extract (63.84±0.05%) and aqueous extract (39.92±0.02%). They have also recorded the IC_{50} value for ethanolic extract as 14.15±0.02 µg/mL. Asimi *et al*. (2013) reported DPPH radical scavenging activity of crude water and ethyl acetate extracts of black pepper. Ethyl acetate extract showed more activity (10.805±0.36 to 86.589±0.68% for 1.0 to 25 mg/mL extract) than water extract (0.82±0.46 to 83.695±0.22% for 1.0 to 25 mg/mL extract) and both showed concentration dependent increase in activity. Zarai *et al*. (2013) studied DPPH radical scavenging ability of chloroform, ethyl acetate, methanol, ethanol and water extracts of black pepper and revealed that the activity is concentration dependent and ethanolic extract showed high DPPH activity (65.59%) at a final concentration of 50 µg/mL. Gupta *et al.*, (2014) have reported DPPH inhibitory capacity of water and methanol extracts of black pepper as 35.2 and

45.66% respectively. Barua *et al*., (2014) reported the IC_{50} for DPPH radical scavenging activity of chloroform, ethyl acetate, hexane, ethanol, hydro-ethanol and aqueous extracts of *P. longum* as 6, 54, 70, 50, 26 and 19.5 µg/mL respectively.

The antioxidant activity of methanol and water extracts from the fruits of *P. chaba, P. longum* and *P. nigrum* has been investigated by DPPH radical scavenging assay (Tewtrakul, 1998). Results showed that, methanolic extracts of these *Piper* species exhibited potent antioxidant activity with low LD_{50} values of 47.8, 45.1 and 48.7 µg/mL respectively, followed by their aqueous extracts (LD_{50}-57.6, 69.4 and 56.9 µg/mL respectively). Rameshkumar *et al*. (2011) have conducted a comparative antioxidant activity study on methanolic extract of *P. longum* and *P. chaba* and revealed high DPPH radical scavenging activity for *P. longum* (IC_{50}-220.3±3.51 µg/mL) than *P. chaba* (IC_{50}-857.7±11.67 µg/mL). Likewise, the DPPH radical scavenging ability of methanolic extracts of *P. nigrum* and *P. longum* has been reported by Prasad & Sushant (2014) and revealed more ability for *P. nigrum* than *P. longum.*

4.3.2.2 Total antioxidant activity by Phosphomolybdenum method

Table 4.22 indicates total antioxidant capacity of sequential extracts by Phosphomolybdenum method (section 3.2.8.2), which measures the ability of sample to reduce Mo (VI) to Mo (V). Hexane extract was in the range of 0.41 to 0.75 M AAE/g of extract whereas chloroform, methanol and water extracts were in the range of 0.81 to 1.85, 0.65 to 1.87 and 0.09 to 0.49 M AAE/g of extract respectively. Methanol and chloroform extracts showed high total antioxidant capacity than hexane and water extracts. However, hexane extract of *P. colubrinum* also

showed comparatively high total antioxidant activity. Among the potential extracts, methanol extract of IISR Malabar Excel and chloroform extract of *P. colubrinum* were found to be superior for total antioxidant activity. Methanol extract of Panchami also showed high total antioxidant capacity. The results were compared with that of synthetic antioxidant BHA and found that BHA showed high ability to reduce molybdenum (4.6 M AAE/ g of extract).

Table 4.22. Total antioxidant activity of sequential extracts (Phosphomolybdenum method*)

Sample	**Sequential extracts**			
	Hexane	**Chloroform**	**Methanol**	**Water**
Sreekara	0.59^{bc}	1.19^{bc}	0.83^{d}	0.32^{bc}
Subhakara	0.53^{bcd}	1.08^{bc}	0.85^{d}	0.24^{cd}
IISR Malabar Excel	0.62^{b}	1.06^{bc}	1.87^{a}	0.09^{e}
IISR Thevam	0.51^{bcd}	0.81^{c}	1.13^{c}	0.49^{a}
Panniyur-1	0.55^{bcd}	0.83^{bc}	0.66^{d}	0.24^{cd}
Panchami	0.64^{ab}	1.24^{b}	1.52^{b}	0.15^{de}
P. nigrum (wild)	0.41^{d}	1.01^{bc}	0.73^{d}	0.45^{ab}
P. longum	0.43^{d}	1.16^{bc}	0.65^{d}	0.19^{de}
P. chaba	0.47^{cd}	0.85^{bc}	0.79^{d}	0.21^{cde}
P. colubrinum	0.75^{a}	1.85^{a}	1.15^{c}	0.33^{bc}
BHA	4.6			

*Molar Ascorbic acid equivalents/gram of extract (M AAE/ g of extract)

The ability of ethyl acetate, acetone and water extracts of dried black pepper leaves to reduce molybdenum was studied in terms of number of equivalent of ascorbic acid, using phosphomolybdenum method (Shanmugapriya *et al*., 2012). The result showed more activity for acetone extract (0.95±0.01) followed by ethyl acetate (0.76±0.02) and water (0.44±0.01) extracts.

The total antioxidant capacity (Phosphomolybdenum method) of chloroform, ethyl acetate, methanol, ethanol and water extracts of black pepper has been reported by Zarai *et al*. (2013) and revealed highest activity for ethanol extract (48.2 µmol/mL α-tocopherol equivalents) at a concentration of 25 µg/mL.

Prasad & Sushant (2014) also reported antioxidant activity of methanolic extracts of *P. nigrum* and *P. longum* by phosphomolybdenum method and revealed more ability for *P. longum* than *P. nigrum*.

4.3.2.3 Ferric reducing power (FRP) method

The antioxidant activity of sequential extracts was also checked on the basis of their ability to reduce ferric (III) ion to ferrous (II) ion using FRP method, as per section 3.2.8.3. The reducing power of the extracts could be due to the presence of reductones which have been shown to exert antioxidant efficacy by donating an electron (Barua *et al*., 2014). Table 4.23 shows ferric reducing capacity of each extract. Among hexane extracts, *P. colubrinum* showed highest activity followed by IISR Malabar Excel and Panchami. Chloroform and methanol extracts were in the range of 0.54 to 1.02 and 0.50 to 1.18 M AAE/ g of extract. For chloroform extract, *P. colubrinum* followed by

IISR Malabar Excel and for methanol extract, IISR Malabar Excel followed by Panchami showed highest ferric reducing power. Water extract showed comparatively very low reductive capacity. Among all extracts, methanol extract of IISR Malabar Excel and chloroform extract of *P. colubrinum* showed high ferric reducing power.

Table 4.23. Ferric reducing power of sequential extracts (FRP method*)

Sample	**Sequential extracts**			
	Hexane	**Chloroform**	**Methanol**	**Water**
Sreekara	0.54^{cde}	0.64^{cde}	0.61^{f}	0.27^{ab}
Subhakara	0.57^{bcd}	0.54^{e}	0.75^{de}	0.23^{cd}
IISR Malabar Excel	0.63^{ab}	0.91^{ab}	1.18^{a}	0.22^{de}
IISR Thevam	0.59^{abc}	0.78^{bc}	0.81^{cd}	0.29^{a}
Panniyur-1	0.59^{abc}	0.73^{cd}	0.71^{e}	0.25^{bc}
Panchami	0.63^{ab}	0.76^{bc}	0.93^{b}	0.21^{e}
P. nigrum (wild)	0.46^{e}	0.55^{e}	0.52^{g}	0.26^{b}
P. longum	0.49^{e}	0.59^{de}	0.50^{g}	0.22^{de}
P. chaba	0.52^{de}	0.57^{de}	0.56^{fg}	0.22^{de}
P. colubrinum	0.65^{a}	1.02^{a}	0.84^{c}	0.21^{e}
BHA	3.46			

*Molar Ascorbic acid equivalents/gram of extract (M AAE/g of extract)

Ferric reducing ability of different extracts from above selected *Piper* species were also reported by researchers. The ferric reducing power of crude ethanol and water extracts of black pepper has been investigated by Gulcin (2005) and revealed high reductive capacity for ethanolic extract than its water extract. Kapoor *et al*. (2009) have demonstrated that ethanol and ethyl acetate oleoresins of black pepper had ferric reducing power with superiority for ethyl acetate oleoresin.

Barua *et al*. (2014) reported high ferric reducing power for chloroform extract of *P. longum* compared to its ethanol, hexane, ethyl acetate, aqueous and hydro-ethanol extracts. Likewise, ferric reducing ability of methanolic extracts of *P. nigrum* and *P. longum* has been reported by Prasad & Sushant (2014) and revealed more ability for *P. nigrum* than *P. longum.*

4.3.2.4 Determination of ferrous chelating ability

The ability of extracts to chelate ferrous ion was also estimated by measuring the intensity of ferrous-ferrozine complex (section 3.2.8.4). The ability to chelate ferrous ion was in the range of 72.17 to 137, 228.5 to 320.1, 245.5 to 349.8 and 124.5 to 170.6 mg EDTA/g of extract for hexane, chloroform, methanol and water respectively (Table 4.24). Hexane and water extracts showed comparatively low ferrous chelating ability whereas it was found to be high for methanol and chloroform extracts. Chloroform extract of *P. colubrinum*, methanol extract of IISR Malabar Excel, Panchami and *P. colubrinum* were found to be superior for their ability to chelate ferrous ions.

Table 4.24. Ferrous chelating ability of sequential extracts*

Sample	Sequential extracts			
	Hexane	**Chloroform**	**Methanol**	**Water**
Sreekara	130.5^{b}	232.2^{cd}	294.5^{cde}	167.7^{ab}
Subhakara	108.6^{d}	228.5^{d}	314.7^{bc}	157.8^{bc}
IISR Malabar Excel	131.7^{ab}	251.6^{b}	349.6^{a}	126.6^{e}
IISR Thevam	131.1^{b}	234.3^{bcd}	297.2^{cd}	170.6^{a}
Panniyur-1	133.6^{ab}	230.3^{cd}	316.5^{bc}	153.4^{cd}
Panchami	132.8^{ab}	249.3^{bc}	320.8^{b}	143.4^{d}
P. nigrum (wild)	72.19^{e}	241.6^{bcd}	245.5^{f}	166.2^{ab}
P. longum	111.4^{d}	249.1^{bc}	273.4^{e}	147.9^{cd}
P. chaba	123.5^{c}	243.3^{bcd}	290.8^{de}	153.2^{cd}
P. colubrinum	137.0^{a}	320.1^{a}	319.8^{b}	124.5^{e}
BHA	--			

* Milligram EDTA equivalents/gram of extract (Mg EDTA/g of extract)

Iron, a prooxidant metal ion can stimulate lipid peroxidation through Fenton reaction and can also accelerate peroxidation through decomposition of lipid hydroperoxides into alkoxyl and peroxyl radicals, which can themselves attract hydrogen and thus perpetuate the chain reaction (Halliwell, 1991). In the present study, the extracts containing antioxidant compounds interfered with the formation of ferrous and ferrozine complex by capturing ferrous ion before ferrozine, suggesting that they have chelating ability. Secondary metabolites like phenolics and flavonoids can chelate such metal ions and often decrease the prooxidant activity of metal ions. Metal chelating potency of phenolic compounds is dependent upon their unique phenolic structure and the number and location of the hydroxyl

groups (van Acker *et al*., 1998; Santoso *et al*., 2004). Metal chelating ability of crude water and ethanolic extracts of black pepper has been reported as 84±2.20% and 83±4.36% respectively at the concentration of 75 μg /mL (Gulcin, 2005).

4.3.2.5 Total phenolic content of sequential extracts

It is reported that phenolics especially phenolic acids and flavonoids shows antioxidant activity and they are responsible for variation in antioxidant activity of plants (Cai *et al*., 2004; Demiray *et al*., 2009). Phenolics exhibit antioxidant activity by various mechanisms like radical scavenging activity, transition metal chelating activity, lipid peroxidation inhibition, etc. The antioxidant activity of phenolic compounds is considerably due to their high redox potential, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. The antioxidant activity of the phenolic compounds is essentially determined by their structure, in particular the electron delocalization over an aromatic nucleus. Due to this multiple mechanism of antioxidant activity, phenolics became an interesting class of compounds for researchers to find natural health beneficial phytochemicals (Rice-Evans *et al.*, 1997; Chen & Ahn, 1998; Yanishlieva & Marinova, 2001; Hotta *et al*., 2010). Thus, estimation of total phenol from the selected extracts is crucial to know their contribution in the antioxidant activities of these extracts. Total phenolic content of all the extracts was estimated by Folin-Ciocalteau method as per section 3.2.3.3.1 and the results are tabulated (Table 4.25). Chloroform and methanol extracts showed maximum total phenol content than hexane and water extracts. Hexane extract of *P. colubrinum* also showed high phenolic content. Total phenol content was in the range of 3.14 to 30.7, 14.02 to 100.6, 14.86 to 50.85 and

1.05 to 5.12 mg GAE/g of extract in hexane, chloroform, methanol and water extracts respectively. Chloroform extract of *P. colubrinum* followed by methanol extract of IISR Malabar Excel showed highest total phenol content among all extracts. Among black pepper varieties, methanol extract of IISR Malabar Excel followed by methanol extract of Panchami showed highest total phenol content. It was clear from the result that there is a significant variation in total phenol content among extracts of black pepper varieties and also among *Piper* species.

Methanol and ethanol has been reported as effective solvent to extract phenolic compounds (Nahak & Sahu, 2011). High total phenol content in methanol extracts of present study can be attributed to this fact. The chloroform extracts of the present study also showed high total phenol content. Flavonoids, the major class of phenolics are extracted with solvents based on their polarity. Less polar flavanones, isoflavones, dihydroflavonols, highly methylated flavones and flavonols can be extracted with chloroform, ether and ethyl acetate (Reenu *et al.*, 2015). The higher levels of total phenols in chloroform extract in the present study can be attributed to high flavonoid content. Zarai *et al*. (2013) have extracted black pepper with chloroform, ethyl acetate, ethanol, methanol and water and reported highest total phenolic content for ethanol followed by methanol extracts and highest flavonoid content for chloroform extract. This information endorses the present study.

Table 4.25. Total phenolic content of sequential extracts (Folin-Ciocalteau method*)

Sample	Sequential extracts			
	Hexane	**Chloroform**	**Methanol**	**Water**
Sreekara	5.98^{c}	22.32^{c}	17.44^{de}	3.41^{c}
Subhakara	4.90^{de}	14.02^{e}	21.28^{c}	1.73^{e}
IISR Malabar Excel	7.74^{b}	35.26^{b}	50.85^{a}	1.12^{f}
IISR Thevam	4.26^{e}	21.14^{c}	20.72^{c}	5.12^{a}
Panniyur-1	6.98^{b}	17.26^{d}	19.33^{cd}	1.62^{e}
Panchami	7.43^{b}	23.64^{c}	38.74^{b}	1.12^{f}
P. nigrum (wild)	3.14^{f}	15.06^{de}	14.86^{f}	3.84^{b}
P. longum	5.41^{cd}	15.66^{de}	17.38^{de}	2.16^{d}
P. chaba	5.37^{cd}	16.50^{de}	16.53^{ef}	2.22^{d}
P. colubrinum	30.7^{a}	100.6^{a}	37.26^{b}	1.05^{f}

* Milligram Gallic acid equivalents/gram of extract (Mg GAE/g of extract)

Since the phenolics are considered to have high antioxidant activity, different research groups studied the total phenolic contents of different black pepper extracts which was used to investigate their antioxidant activities. Gulcin (2005) has reported the total phenol content of water and ethanol extracts of black pepper as 54.3 and 42.8 µg gallic acid equivalent/mg of extract respectively whereas total phenolic content of methanolic extract of black pepper collected from USA has been reported by Shan *et al*. (2005) as 0.30 ± 0.002 g of gallic acid equivalents/100 g of dry weight.

Jain *et al*. (2011) have reported total phenolic content of methanolic extract of *P. nigrum* berries as 5.13 ± 0.61 mg GAE/g and it is very low compared to that obtained in present study. Nahak & Sahu (2011)

reported the total phenolic content of ethanolic extract of black pepper as 62.3±0.08 µg catechol equivalents/g.

The total phenol content of chloroform, ethyl acetate, methanol, ethanol and water extracts of black pepper has reported by Zarai *et al*. (2013) with highest total phenolic content (µg of gallic acid equivalent/g) in ethanol (45.08±2.21) followed by methanol (37.48±0.67), chloroform (25.03±0.38) and ethyl acetate (22.69±0.58) extracts whereas lowest in water extract (1.09±0.10). Barua *et al*., (2014) have reported total phenolic content of ethanol, chloroform, hexane, ethyl acetate, aqueous and hydro-ethanol extracts of *P. longum* as 22.39±0.32, 33.29±0.094, 8.95±0.098, 13.360±0.232, 23.875±0.151 and 16.61±0.25 µg GAE/mg of extract respectively with highest in chloroform and lowest in hexane extracts.

4.3.2.6 Correlation between total phenolic content and antioxidant activity

Several studies reported that phenolic constituents in spices and other plants have significant antioxidant properties and they are assumed to be the major contributor for the antioxidant activities of plants. (Romanova *et al.*, 2001; Shan *et al*., 2005; Gulcin, 2005; Wang *et al.*, 2006; Lu *et al.*, 2011; Maizura *et al*., 2011). In the present study, chloroform and methanol extracts were screened for high antioxidant activity by different assays. So correlation of total phenolic content of that two extracts from all the samples with their antioxidant activity by each assays was performed. Result showed significant ($p \leq 0.05$) negative correlation between total phenolic content of each extract and their IC_{50} to scavenge DPPH free radicals. Correlation coefficient (r) for total phenol of chloroform extracts and their IC_{50} for DPPH scavenging activity was -0.86 and that for total phenol of methanol

extract and their IC_{50} for DPPH was -0.72. This indicated that, extract with high total phenol shows less IC_{50} and thus more ability to scavenge DPPH. Correlation coefficient (r) for total phenolic content of chloroform extract with their antioxidant activity by phosphomolybdenum method, FRP and ferrous chelating activity was +0.86, +0.81 and +0.95 respectively ($p \leq 0.05$). Total phenolic content of methanol extracts of samples also showed significant ($p \leq 0.05$) positive correlation with their antioxidant activity by phosphomolybdenum method (r = +0.94), FRP (r = +0.92) and ferrous chelating activity (r = +0.79). High ability of *P. colubrinum* hexane extract to scavenge DPPH may also be an indication of radical scavenging ability of phenolics since that extract contains more total phenol than other hexane extracts. These correlation studies thus revealed that phenolic contents have direct correlation with the antioxidant activity of plant extracts and hence they have marked importance. Such linearity between total phenol of black pepper extracts and their antioxidant activity was reported by different researchers (Gulcin, 2005; Agbor *et al*., 2006; Nahak & Sahu, 2011). The linear relationship between total phenol content and antioxidant activity by different assays of 19 commonly consumed Chinese spices (including white pepper) has been clearly established by Lu *et al.* (2011) by performing correlation test. Jain *et al*. (2011) also established linear relationship between total phenol and antioxidant potential of different medicinal plants including *P. nigrum*. Barua *et al*. (2014) validated the correlation of total phenolic content of *P. longum* extracts and their antioxidant activity.

4.3.3 *In vitro* antioxidant activity of essential oils

Apart from plant extracts which containing mainly non-volatiles, essential oil can also consider for investigating antioxidant compounds. Essential oil, the major contributor for aroma in plants, is the mixture of mainly two important plant secondary metabolites *viz.,* terpenoids and phenyl propanoids. Monoterpenes and sesquiterpenes are the major terpenoid constituents of essential oil and they are volatile in nature. The essential oils are easily extractable and contain a large variety of compounds and their chemical profiling is easier with gas chromatographic techniques than other non-volatile compounds (Lesgards *et al.,* 2014). *Piper*, medicinally valued plant genera, is a major source for essential oil and hence, medicinally important *P. nigrum*, *P. longum* and *P. chaba* were subjected to essential oil extraction and its *in vitro* antioxidant analysis.

Matured berries of high yielding black pepper variety IISR Malabar Excel and matured fruits of *P. longum* and *P. chaba* were collected from ICAR-IISR Experimental Farm, Peruvannamuzhi (Kozhikode, Kerala), and after proper drying, they were subjected to essential oil extraction according to section 3.2.3.1.1. The extracted oils were then taken for *in vitro* antioxidant analysis using 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity, phosphomolybdenum method, ferric reducing power method and ferrous chelating activity. Among black pepper varieties, the essential oil from IISR Malabar Excel was selected for *in vitro* antioxidant analysis since its methanol extract was already screened for highest antioxidant activity (section 4.3.2).

4.3.3.1 DPPH free radical scavenging activity

DPPH free radical scavenging ability of selected essential oils were tested as per section 3.2.8.1 and compared with that of synthetic

antioxidant BHA (Table 4.26). The results were recorded in terms of IC_{50} value and oil with low IC_{50} showed high DPPH scavenging ability and vice versa. Significant variation was observed among selected oils for their DPPH scavenging ability. Black pepper essential oil showed lowest DPPH radical scavenging ability with high IC_{50} value (79.5 mg/mL) followed by *P. chaba* (62.0 mg/mL) whereas essential oil from *P. longum* predominated with low IC_{50} value (21.0 mg/mL). DPPH free radical scavenging ability of synthetic antioxidant BHA was found to be superior to the selected essential oils with an IC_{50} value of 0.0052 mg/mL). DPPH free radical scavenging ability of a sample is due to their hydrogen donating ability.

4.3.3.2 Total antioxidant activity by Phosphomolybdenum method

Total antioxidant capacity of each essential oil was estimated by phosphomolybdenum method (section 3.2.8.2) and was in the range of 0.35 to 0.61 M AAE/g of oil (Table 4.26). The selected essential oils showed a species wise, significant statistical variability for their ability to reduce Mo (VI) to Mo (V). Highest total antioxidant activity was shown by essential oil extracted from black pepper (IISR Malabar Excel) followed by *P. longum* whereas the activity was found to be lowest in *P. chaba.* Total antioxidant activity of synthetic antioxidant BHA was found to be higher than these essential oils.

4.3.3.3 Ferric reducing power (FRP) method

Antioxidant activity of essential oil samples was also checked for their ability to reduce ferric (III) ion to ferrous (II) ion (FRP method- section 3.2.8.3). Table 4.26 shows ferric reducing capacity of each essential oil. It was found to be highest in essential oil of *P. nigrum* (53.96 mM AAE/g of oil) whereas those from *P. longum* and *P. chaba* were statistically on par for their ferric reducing ability. Ferric reducing

power of BHA was recorded as 3460 mM AAE/g of oil and found to be higher than essential oil samples tested.

4.3.3.4 Determination of ferrous chelating ability

The ferrous chelating ability of essential oil samples were performed as per section 3.2.8.4. The ability to chelate ferrous ion was in the range of 5.6 to 9.2 mg EDTA/g of oil (Table 4.26). Significant variation was observed among essential oil for their ferrous chelating ability. Black pepper oil showed highest metal chelating ability whereas it was lowest for *P. chaba.*

Table 4.26. Antioxidant potential of essential oils from selected *Piper* species by adopted methods

Sample	**DPPH radical scavenging ability (IC_{50} - mg/mL)**	**Phosphomolybdenum method (M AAE/g of oil)***	**Ferric reducing power method (mM AAE/g of oil)****	**Ferrous chelating ability (mg EDTA/g of oil)*****
P. nigrum	79.5[a]	0.61[a]	53.96[a]	9.2[a]
P. longum	21.0[c]	0.43[b]	25.00[b]	7.7[b]
P. chaba	62.0[b]	0.35[c]	23.78[b]	5.6[c]
BHA	0.0052	4.6	3460	--

* Molar Ascorbic acid equivalents/gram of essential oil
** Milli molar Ascorbic acid equivalents/gram of essential oil
*** Milligram EDTA equivalents/gram of essential oil

Medicinal properties of essential oils from aromatic plants are mainly contributed by their active constituents. The chemical constituents of selected essential oils were already discussed in sections 4.1.1.3.1.2 and 4.1.2.3.1.2. Thus, the antioxidant property shown by these essential oils can be attributed to major and minor constituents or their cumulative action.

Researchers also evaluated the antioxidant activity of essential oil from these *Piper* species. The antioxidant potential of essential oil and oleoresin of black pepper extracted by supercritical carbon dioxide (SF-CO_2) and conventional methods has been compared by Tipsrisukond *et al*. (1998). The result revealed that essential oil extracted by SF-CO_2 and conventional method is less effective as antioxidants than oleoresin extracted by SF-CO_2 and conventional methods. Bagheri *et al*. (2014) compared radical scavenging activity of black pepper essential oil obtained by supercritical CO_2 extraction and hydro-distillation and result showed more DPPH radical scavenging activity for essential oil obtained by supercritical CO_2 extraction (EC_{50}-103.28 µg/mL) than those obtained by hydrodistillation (EC_{50}-316.27 µg/mL).

Tewtrakul (1998) reported that volatile oils of *P. chaba, P. longum* and *P. nigrum* fruits were almost inactive for their antioxidant efficacy by DPPH radical scavenging activity (LD_{50} -100 µg/mL) and also reported remarkable activity for their methanolic extracts. The antioxidant activity of black pepper essential oils has also been reported by Dorman *et al*. (2000). Volatile oil of black pepper along with its acetone extract has been reported as a good antioxidant for linseed oil, in comparison with BHA and BHT (Singh *et al*., 2004).

The antioxidant activity of essential oil extracted from black pepper has been reported by Politeo *et al*. (2006) and compared its activity with essential oils from other eleven species. They have reported good DPPH scavenging ability for black pepper oil (14 to 61% inhibition for 5 to 50 g/L). They have also found that antioxidant activity of pepper

oil is lesser than that of clove, basil, laurel, coriander and nutmeg but higher than that of mint, marjoram, cinnamon, sage and fennel.

The antioxidant activity of black pepper oil has been compared with its ethanol and ethyl acetate oleoresins and also with synthetic antioxidants like BHA, BHT and propyl gallate by Kapoor *et al*. (2009). The result indicated that, pepper oil and its ethyl acetate extract showed better ferric reducing power than its ethanol extract and their ability to reduce ferric ion is higher than that of BHA and BHT but lower than that of propyl gallate. The result also indicated that pepper oil is superior for DPPH radical scavenging power than the pepper oleoresins, BHA and BHT but lower than propyl gallate.

Thus, the antioxidant evaluation of sequential extracts and essential oils from the samples revealed that the variability in concentration of phytoconstituent which contribute to antioxidant activity might be the reason for variability in the antioxidant efficacy of these extracts and essential oils. These reasons, along with other factors like method of extraction, type of solvent used and extractability of solvents may also reflect for their similarities and discrepancies with previous reports.

Reports on comparative *in vitro* antioxidant activity of the sequential extracts of the black pepper varieties selected for the present study is almost nil. Likewise, antioxidant activity of sequential extracts (n-hexane, chloroform, methanol and water) of *P. chaba* and *P. colubrinum* fruits are also reported for the first time. Likewise, studies on the antioxidant activity of essential oil from these *Piper* species especially *P. longum* and *P. chaba* were comparatively less than their extracts.

Thus, present study on antioxidant activity could clearly establish hydrogen donating ability, the effectiveness to scavenge DPPH free radicals, reductive capacity and metal chelating ability of the *Piper* species used in the study. These species can therefore be promoted as good source for natural antioxidants. Formulations can be prepared using these plants for human welfare especially in the field of functional foods and neutraceuticals.

4.4 *IN VITRO* CYTOTOXICITY OF SELECTED *PIPER* SPECIES AND SELECTED BLACK PEPPER VARIETIES ON CANCER CELL LINE 'CaSki'

The search for natural sources which possess effectiveness against cancer increases tremendously due to concern about the safety and side effects of synthetic drugs. Plant is one of the best sources for such natural high value compounds (Ali *et al.*, 2014). In this context, different extracts and essential oils of selected *Piper* species were subjected to *in vitro* cytotoxicity analysis against cervical cancer cell line CaSki. *In vitro* cytotoxicity was checked on cervical cancer cell line CaSki since it is one of the least studied cancer cell line among the selected *Piper* species

4.4.1 *In vitro* cytotoxicity of sequential extracts

Sequential extracts (n-hexane, chloroform, methanol and water) of high yielding black pepper varieties (Sreekara, Subhakara, IISR Malabar Excel, IISR Thevam, Panchami and Panniyur-1) and *Piper* species (Wild *P. nigrum*, *P. longum*, *P. chaba* and *P. colubrinum*) used for *in vitro* antioxidant potential was subjected to *in vitro* cytotoxicity on cervical cancer cell line CaSki by MTT assay as per section 3.2.9.

MTT assay was performed using all extracts with a mass range of 25-100 µg for three time intervals *viz.* 24, 48 and 72 hrs. All the extracts showed cytotoxicity (%) in a dose-dependent and time-dependent manner. The results are given in Table 4.27. Among all extracts tested, chloroform extracts of all samples and hexane extract of *P. colubrinum* expressed more cytotoxicity. The amount of extract required for 50% toxicity to CaSki (IC_{50}) was calculated for chloroform extracts of black pepper varieties and wild *P. nigrum* and they have categorized by Duncan's multiple range test (DMRT) to screen black pepper chloroform extract with highest cytotoxicity. Result showed that chloroform extract of IISR Malabar Excel showed lowest IC_{50} value and thus highest cytotoxicity to CaSki for all the three time intervals (Table 4.28). Thus, chloroform extract of IISR Malabar Excel, *P. longum, P. chaba, P. colubrinum* and hexane extract of *P. colubrinum* were screened as potent cytotoxic extracts. All the potential extracts along with synthetic anticancer drug Doxorubicin were again put for MTT assay with a mass range of 5-100 µg for three time intervals (24, 48 and 72 hrs) and their IC_{50} was calculated (Table 4.29). Results showed more cytotoxicity with more extract and more time of exposure with CaSki. Chloroform extract of *P. longum* and *P. colubrinum* were found to be highly toxic to CaSki for all the three time intervals. Chloroform extract of IISR Malabar excel was toxic to CaSki for 24, 48 and 72 hrs. Hexane extract of *P. colubrinum* showed almost similar toxicity to CaSki as that of chloroform extract of IISR Malabar Excel. *P. chaba* was less toxic in 24 hrs and 48 hrs but highly toxic in 72 hrs. The IC_{50} value for certain extracts and Doxorubicin were beyond the adopted mass range. So, further experiment has to be performed to find out their exact IC_{50} values.

Table 4.27. *In vitro* cytotoxicity (%) of all the sequential extracts by MTT assay

Sample	Extract (µg)	Sequential extracts											
		Hexane			Chloroform			Methanol			Water		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Sreekara	25	4.01	15.98	28.65	33.12	39.80	47.70	8.83	11.24	35.17	2.95	8.46	17.4
	50	8.54	21.34	32.45	44.91	47.01	64.09	11.4	19.81	40.54	7.28	20.5	33.3
	100	12.9	27.54	36.54	59.44	65.25	67.08	24.4	32.51	43.84	11.2	26.3	34.9
Subhakara	25	3.88	11.40	35.71	33.11	40.29	48.32	5.44	18.94	38.91	1.22	9.46	21.0
	50	7.64	21.95	37.95	44.24	46.24	62.41	5.95	22.46	40.69	6.42	21.6	37.3
	100	10.2	33.01	39.99	63.26	66.81	71.58	8.48	34.50	44.01	9.64	32.5	39.6
Panniyur-1	25	3.45	12.58	22.54	15.75	32.82	44.41	2.44	12.48	28.03	2.88	12.9	20.7
	50	4.35	19.64	27.56	23.54	48.41	61.63	2.99	18.56	29.06	3.12	22.5	26.8
	100	7.54	28.70	31.99	61.09	62.42	66.85	3.96	22.21	34.24	4.49	29.7	31.6
IISR Thevam	25	16.9	21.56	27.01	40.34	43.54	48.26	16.4	19.01	32.91	15.2	20.0	26.1
	50	28.9	34.52	36.46	47.87	61.39	70.69	19.4	27.56	34.63	27.8	32.5	35.3
	100	32.9	36.85	37.42	55.55	66.19	72.32	28.2	33.54	37.51	32.0	34.4	37.4
IISR Malabar Excel	25	20.0	24.59	26.99	37.82	46.11	49.99	23.6	24.01	27.98	19.5	23.6	25.6
	50	25.7	31.56	38.54	46.41	61.71	71.48	24.1	26.55	39.67	24.0	30.1	37.7
	100	30.5	37.01	41.02	61.57	63.36	72.41	25.0	31.54	43.59	28.9	36.9	40.9

Sample	Extract (µg)	Sequential extracts											
		Hexane			Chloroform			Methanol			Water		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Panchami	25	20.9	25.01	27.05	38.05	46.01	49.97	21.2	28.44	31.57	20.1	22.5	24.9
	50	25.9	28.94	30.09	42.12	56.76	64.19	29.0	32.56	37.56	24.9	26.9	29.5
	100	30.6	31.95	34.95	59.51	58.41	66.66	31.7	36.42	40.98	26.8	29.9	33.9
P. nigrum (wild)	25	2.54	9.01	13.99	15.75	32.82	40.41	2.41	9.56	14.55	1.98	8.41	13.5
	50	4.01	11.54	17.68	22.01	46.41	58.02	3.01	12.54	18.96	2.96	10.5	16.5
	100	7.01	20.01	24.99	61.09	62.42	66.85	3.85	16.85	24.56	3.79	14.6	23.1
P. chaba	25	4.41	14.85	24.99	37.90	43.85	64.63	8.22	21.06	25.42	3.42	13.1	24.6
	50	9.56	24.56	32.98	39.42	45.65	68.25	9.01	26.58	32.99	8.54	22.6	32.9
	100	12.5	29.99	37.11	40.63	50.01	70.30	14.8	31.55	37.54	9.97	29.5	36.9
P. longum	25	16.6	18.11	22.07	63.85	68.37	73.84	6.01	16.55	36.84	15.4	17.6	21.1
	50	18.9	31.99	36.54	63.99	70.04	75.47	8.1	24.54	37.19	18.1	30.0	35.3
	100	22.7	32.57	36.98	64.16	72.01	78.67	10.1	31.01	40.21	22.2	31.1	35.4
P. colubrinum	25	40.1	44.42	49.99	55.86	65.99	75.46	25.1	27.11	30.74	21.4	24.0	29.9
	50	48.4	57.15	62.41	59.99	66.73	78.52	27.9	29.97	35.02	23.4	29.8	32.6
	100	61.6	57.94	63.50	64.65	72.04	79.66	29.1	32.57	41.33	25.4	31.0	32.8

Table 4.28. Variability for *in vitro* cytotoxicity (IC_{50}-µg) of chloroform extracts of selected black pepper varieties and wild *P. nigrum* by MTT assay

Sample	Time intervals		
	24 hrs	**48 hrs**	**72 hrs**
Sreekara	64^{c}	58^{a}	28^{c}
Subhakara	64^{c}	60^{a}	28^{c}
IISR Malabar Excel	60^{c}	30^{c}	25^{d}
Panchami	72^{b}	32^{c}	26^{cd}
Panniyur-1	84^{a}	52^{b}	32^{b}
IISR Thevam	60^{c}	32^{c}	26^{cd}
P. nigrum (wild)	86^{a}	58^{a}	36^{a}

Table 4.29. *In vitro* cytotoxicity (IC_{50}-µg) of potential extracts and Doxorubicin by MTT assay

Sample	Time intervals		
	24 hrs	**48 hrs**	**72 hrs**
IISR Malabar Excel (Chloroform extract)	60.00	30.00	25.00
P. chaba (Chloroform extract)	>100	100.0	<5
P. longum (Chloroform extract)	12.00	5.2.00	<5
P. colubrinum (Chloroform extract)	12.00	<5	<5
P. colubrinum (Hexane extract)	56.00	32.00	25.00
Doxorubicin	<5	<5	<5

Even though there are reports on anticancer activity of these *Piper* species, the reports for their effect on cerivical cancer is scanty. The

cytotoxicity of different *P. nigrum* extracts have been analyzed against Human promyelocytic leukemia cells (HL-60) by Lim *et al*. (2009) and revealed that petroleum ether (IC_{50}-11.2 µg/mL) and chloroform extracts (IC_{50} -9.8 µg/mL) were bioactive against HL-60 cell line but the ethyl acetate extract had no activity. The alcoholic and water extracts of black pepper have been tested against esophageal squamous cell carcinoma (TE-13) and found that aqueous extract was more potent than ethanolic extract (Dwivedi *et al*., 2011)**.**

The methanolic and dichloromethane extracts from black pepper was checked for cytotoxicity against different breast cancer cell lines (MCF-7, MDA-MB-468, MDA-MB-231 and MCF-12A) by MTT assay for 72 hrs. Methanolic extract showed highest cytotoxicity for MCF-7 (IC_{50}-20.25±0.01 µg/mL) and MDA-MB-231 (IC_{50}-22.37±2.31 µg/mL) whereas dichloromethane extract showed highest cytotoxicity for MDA-MB-468 (IC_{50}-7.94±4.52 µg/mL) and MCF-12A (IC_{50}-35.65±0.27 µg/mL) (Sriwiriyajan *et al*., 2014).

The hot ethyl acetate and cold hexane: water (1:1) extracts of *P. longum* were tested for *in vitro* cytotoxicity (%) against leukaemic cell line K562. The extracts showed low but dose-dependent cytotoxicity and the antitumor activity was found to be higher for hot ethyl acetate (10% at 200 µg/mL, 13% at 400 µg/mL, 21% at 800 µg/mL) than cold hexane: water (2% at 200 µg/mL, 5% at 400 µg/mL, and 9% at 800 µg/mL) extracts (Joy *et al*., 2010). The anticancer activity of ethyl acetate, methanol and water extracts of *P. longum* fruits were checked against lung epithelial adenocarcinoma cell line HCC-827. The *in vitro* cytotoxicity of these extracts, expressed as decrease in viable cell count compared to control, showed that all the extracts had a dose-dependent antitumor activity and methanol extract

is more effective ($1.14x10^5$, $0.9 x10^5$ and $0.5 x10^5$ for 24 hrs and $1.1 x10^5$, $0.9 x10^5$, $0.46 x10^5$ for 48 hrs at concentrations of 10, 50 and 100 µg/mL respectively) against HCC-827 (Sawhney *et al*., 2011).

Jacob *et al*. (2012) reported potent cytotoxicity for silver nanoparticles synthesized using *P. longum* against HEp-2 cell line as 94.02% at 500 µg/mL concentration. Reddy *et al*. (2014) revealed that the silver nanoparticles synthesized using aqueous extract of *P. longum* fruit had potent cytotoxic effect on breast cancer cell lines, MCF-7 with an IC_{50} value of 67µg/mL for 24 hrs by the MTT assay.

The anticancer efficacy of *P. longum* has also been reported by researchers against oral cancer, colon cancer, Dalton's lymphoma ascites and Ehrlich ascites carcinoma cells (Sunila & Kuttan, 2004; Senthil *et al*., 2007; Ovadje *et al*., 2014).

The ethanol and water extracts of *P. chaba* fruit collected from Thailand have been subjected to cytotoxicity studies against human breast adenocarcinoma cell line (MCF7) at an exposure time of 72 hrs and result showed that ethanolic extract is more cytotoxic (IC_{50}-35.17±1.91 µg/mL) than water extract (IC_{50}- >100 µg/mL) (Sakpakdeejaroen & Itharat, 2009). *In vitro* cytotoxicity of crude ethanol extract of *P. chaba* fruit has been evaluated against human cholangiocarcinoma (CL-6), human laryngeal (Hep-2) and human hepatocarcinoma (HepG2) cell lines. The extract showed potent activity against CL-6 (IC_{50}-40.74 µg/mL), HepG2 (IC_{50}-68.09±22.58 µg/mL) and Hep-2 (IC_{50}-18.93±5.03 µg/mL) cell lines (Mahavorasirikul *et al*., 2010).

Ruangnoo *et al*. (2012) investigated *in vitro* cytotoxicity of ethanolic extract of *P. chaba* fruits on large lung carcinoma cell line COR-L23,

cervical cancer cell line Hela and liver cancer cell line HepG2 and result revealed more cytotoxicity to COR-L23 (IC_{50} value of 15.82 µg/mL) than other three cell lines. Plengsuriyakarn *et al*. (2012) reported *in vitro* cytotoxicity of crude ethanolic extract of *P. chaba* fruit against cholangiocarcinoma cell line CL-6.

4.4.2 *In vitro* cytotoxicity of essential oils

The essential oils (black pepper variety IISR Malabar Excel, *P. longum* and *P. chaba*) used for *in vitro* antioxidant potential were also subjected to *in vitro* cytotoxicity analysis. Among black pepper varieties, the essential oil from IISR Malabar Excel was selected for *in vitro* cytotoxicity study since its chloroform extract was already screened for highest *in vitro* cytotoxicity on CaSki cell line (section 4.4.1).

In vitro cytotoxicity was tested on cervical cancer cell line CaSki by MTT assay as per section 3.2.9. MTT analysis was performed using 25% of selected essential oils for three time intervals *viz.* 24, 48 and 72 hrs. All the essential oils showed very good cytotoxicity (%) in a time-dependent manner. That is, cytotoxicity of the selected oils increased as time of exposure of essential oils to CaSki increased (Fig. 4.28). In the case of 24 hrs, *P. nigrum* and *P. chaba* essential oils showed high cytotoxicity (61.64 and 65.61% respectively) whereas *P. longum* (53.42%) was comparatively low. But when the time of exposure exceeded to 48 hrs, *P. nigrum* essential oil showed highest (71.92%) whereas *P. chaba* essential oil showed lowest (65.63%) cytotoxicity to CaSki cells. When the time of exposure reached to 72 hrs, there was no significant difference among selected essential oils for their toxicity to CaSki (78.52 to 84.04%). By considering the three time intervals, essential oil from black pepper (IISR Malabar Excel) sowed more toxicity to CaSki cell lines.

In vitro cytotoxicity of the crude essential oils from selected *Piper* species were not studied much in human cancer cell lines compared to their solvent extracts.

Black pepper essential oil has been reported for anti proliferative effect on murine leukemia and human mouth epidermal carcinoma cell lines (Manosroi *et al*., 2006). *In vitro* cytotoxicity of black pepper oil in combination with methanolic extract of *Terminalia arjuna* and volatile oil of cumin has been reported against lung cancer cell line A-549 (Pingili *et al*., 2012). However, Yen *et al*. (2012) reported that essential oil from black pepper is not cytotoxic at the concentration of 200 µg/mL for different cancer cell lines include Human liver (Hep 3B and Hep G2), lung (A549) and breast (MCF-7 and MDA-MB-231) cancer cell lines. However, information is scanty regarding the anticancer activity of essential oil from *P. longum* and *P. chaba*. From the available literature, it can be concluded that this is the first report regarding *in vitro* cytotoxicity of essential oil extracted from black pepper (IISR Malabar Excel), *P. longum* and *P. chaba* fruits on cervical cancer cell line CaSki.

The medicinal properties of essential oils from aromatic plants are mainly contributed by their active constituents. The chemical constituents of selected essential oils were already discussed in sections 4.1.1.3.1.2 and 4.1.2.3.1.2. Thus, the *in vitro* cytotoxicity shown by these essential oils may be due to the major constituents or due to cumulative action of major and minor constituents.

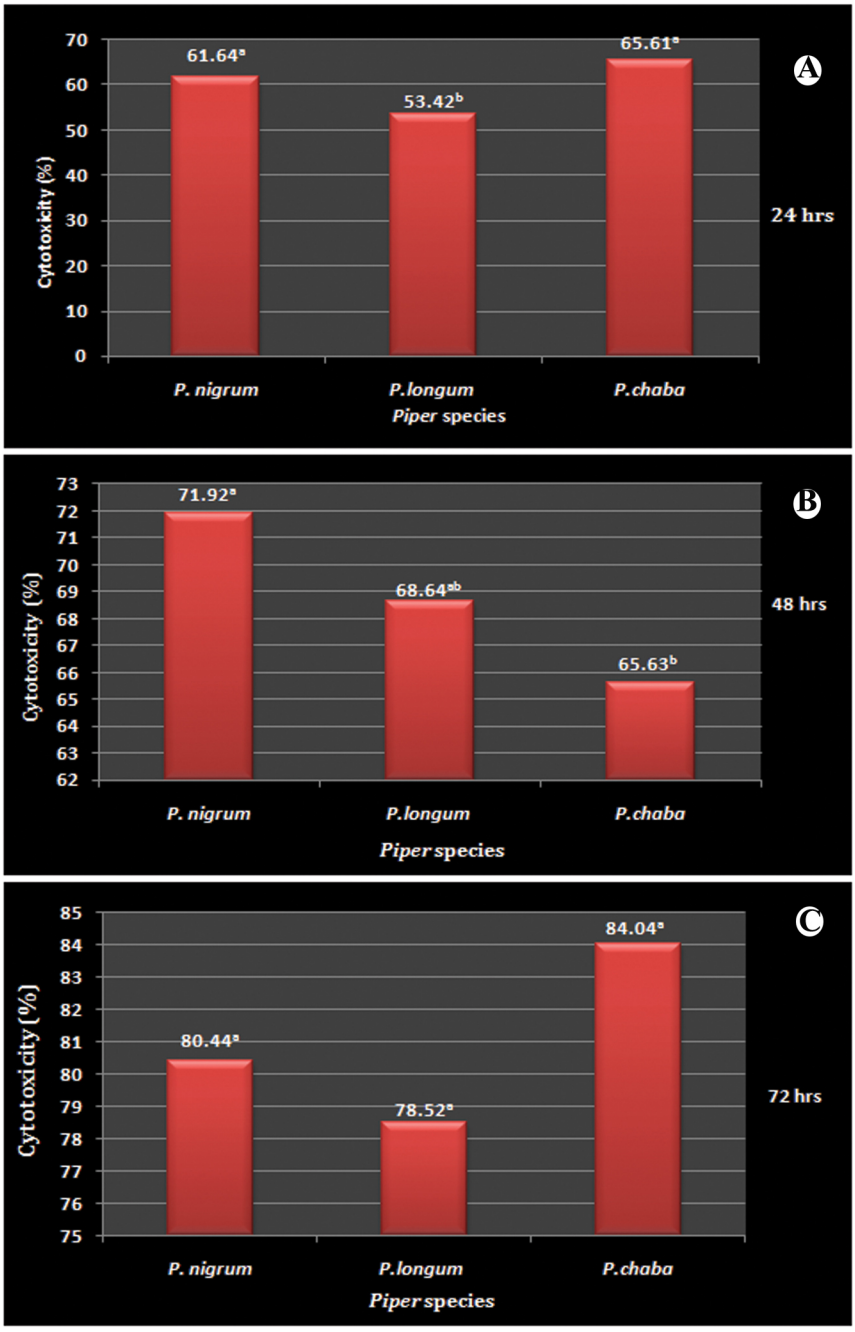

Fig. 4.28. *In vitro* cytotoxicity (%) of selected essential oils by MTT assay. A) 24 hrs, B) 48 hrs, C) 72 hrs

4.5 *In vitro* antioxidant activity, cytotoxicity and chemoprofiling of essential oil from *P. colubrinum* leaves

In vitro antioxidant activity and cytotoxicity of essential oil from *P. colubrinum* fruits could not be conducted since the essential oil recovery from *P. colubrinum* fruit is very low (section 4.1.1.3.1.1) and also due to the difficulty to get bulk quantity of fruits at a time for essential oil extraction. However, to know about antioxidant and cytotoxic effects of essential oil of this underexplored crop, fresh leaves of *P.colubrinum* was subjected to essential oil extraction. The extracted oil was evaluated for its *in vitro* antioxidant activity, cytotoxicity and also for its volatile constituents.

Recovery (yield) of P. colubrinum leaf oil

The collected leaves of *P. colubrinum* was subjected to essential oil extraction by hydrodistillation using Clevenger trap method (section 3.2.3.1.1) and the extracted oil was quantified as 0.1%.

In vitro antioxidant activity of essential oil of P. colubrinum leaves

The extracted *P. colubrinum* leaf oil was subjected to antioxidant activity studies using 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity, phosphomolybdenum method, ferric reducing power method and ferrous chelating activity. DPPH assay was performed as described in section 3.2.8.1 and expressed in terms of its IC_{50} value. The result showed that *P. colubrinum* leaf oil is a potent DPPH radical scavenger with very low IC_{50} value of 0.029 mg/mL (29 µg/mL). It also showed total antioxidant activity of 0.43 M AAE/g of oil (430 mM AAE/g of oil) by phosphomolybdenum method, which was performed as explained in section 3.2.8.2 and ferric

reducing power of 195.4 mM AAE/g of oil by FRP method (section 3.2.8.3). However, it was noted that the ferrous chelating ability checked for *P. colubrinum* leaf oil according to section 3.2.8.4 was found to be low (6.7 mg EDTA/g of oil). The antioxidant activity of *P. colubrinum* leaf oil is illustrated in Fig. 4.29.

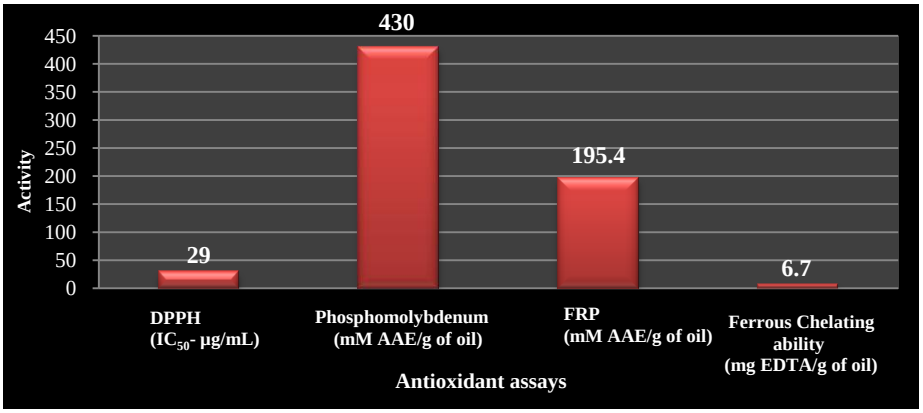

Fig. 4.29. Antioxidant potential of *P. colubrinum* leaf oil by adopted methods

In vitro cytotoxicity of P. colubrinum leaf oil

In vitro cytotoxicity of *P. colubrinum* leaf oil was also tested on cervical cancer cell line CaSki by MTT assay, as per section 3.2.9. MTT analysis was performed using 25% of *P. colubrinum* leaf oil for three time intervals *viz.* 24, 48 and 72 hrs. The sample showed good cytotoxicity (%) for 24 hrs (42.24%), 48 hrs (70.74%) and 72 hrs (84.43%). This indicated that, cytotoxicity of this oil increased as its time of exposure to CaSki increased (Fig. 4.30).

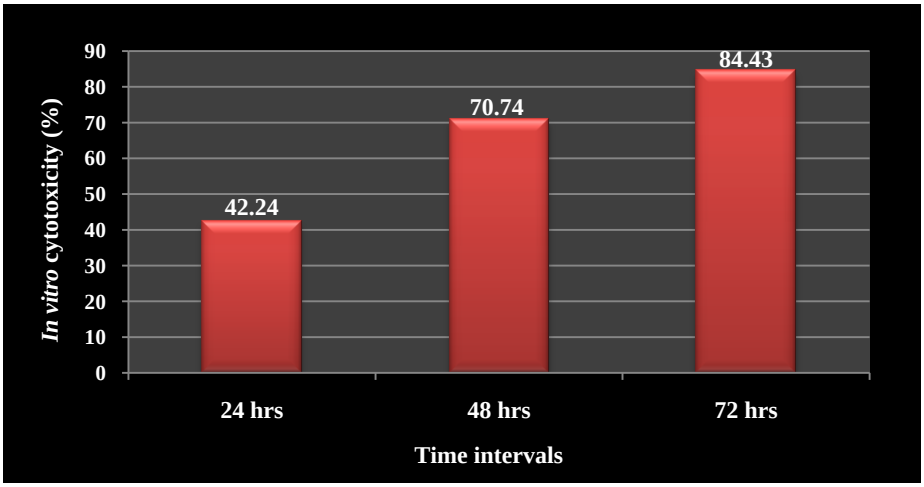

Fig. 4.30. *In vitro* cytotoxicity (%) of *P. colubrinum* leaf oil by MTT assay

Chemoprofiling of P. colubrinum leaf oil

Therapeutic potential of essential oils are mainly contributed by their active constituents. These properties may be due to major constituents or due to the presence of other constituents in minor quantity or the result of their cumulative action (Politeo *et al.,* 2006). So, identification of oil constituents is very crucial. The chemical constituents of *P. colubrinum* leaf oil were thus identified by GC-MS (section 3.2.3.1.2) and the oil profile is given in Table 4.30. A total of 30 compounds comprised of 93.27% of total essential oil were identified by GC-MS. The result indicated the presence of high amount of sesquiterpenes and miscellaneous constituents in *P. colubrinum* leaf oil. However, monoterpenes and aliphatic compounds are less in this oil. The miscellaneous constituents mainly include phenyl propanoids like methyl eugenol (23.6%) and chavibetol (8.21%). Methyl eugenol was the major compound identified from *P. colubrinum* leaf oil

followed by eugenyl acetate, β-caryophyllene, germacrene B, chavibetol, etc. Beta-elemene, germacrene D, δ-cadinene, etc. were also identified from P. *colubrinum* leaf oil. The GC-MS chromatogram for this essential oil profiling is given in Fig. 4.31. Presence of high phenyl propanoids may be the reason for high DPPH radical scavenging activity of *P. colubrinum* leaf oil since phenolics are the major phytoconstituents with antioxidant activity (especially radical scavenging ability), although they are not the only ones (Balasundram *et al*., 2006).

The study thus revealed that *P. colubrinum* leaf oil is a good source of phytoconstituents especially phenolic compounds and thus showed good antioxidant activity and cytotoxicity on CaSki cell lines. Based on available reports, this is the first report regarding chemical composition, *in vitro* antioxidant activity and cytotoxicity on cervical cancer (CaSki cell line) of leaf oil from *P. colubrinum* cultivated in India.

Table 4.30. Chemoprofiling of *P. colubrinum* leaf oil by GC-MS

Compound	**% Composition**	***RI**
α-Pinene	0.10	936
β-Pinene	0.20	979
β-Myrcene	0.08	993
D-Limonene	0.17	1032
Z-Ocimene	0.10	1041
E-Ocimene	1.63	1053
β-Linalool	0.02	1105
2,6-Dimethyl-3,5,7-octatriene-2-ol, ,E,E-	0.31	1214
Chavibetol	8.21	1374

Compound	% Composition	*RI
α-Copaene	0.48	1383
β- Elemene	9.23	1392
Methyl eugenol	23.6	1428
β-Caryophyllene	9.88	1435
α-Humulene	1.05	1464
Alloaromadendrene	0.24	1471
γ-Muurolene	0.69	1487
Germacrene D	4.91	1495
β-Selinene	0.80	1499
Germacrene B	9.50	1512
8-Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	0.72	1518
γ-Cadinene	0.58	1524
δ-Cadinene	2.59	1534
Eugenyl acetate	10.2	1546
cis-Nerolidol	1.21	1573
Spathulenol	0.93	1580
Globulol	1.95	1599
Epiglobulol	1.31	1607
Rosifoliol	0.68	1615
α-Cadinol	1.27	1656
Phytol	0.63	-
Total (Nos.)	30	
Total (%)	93.27	

*Retention Indices

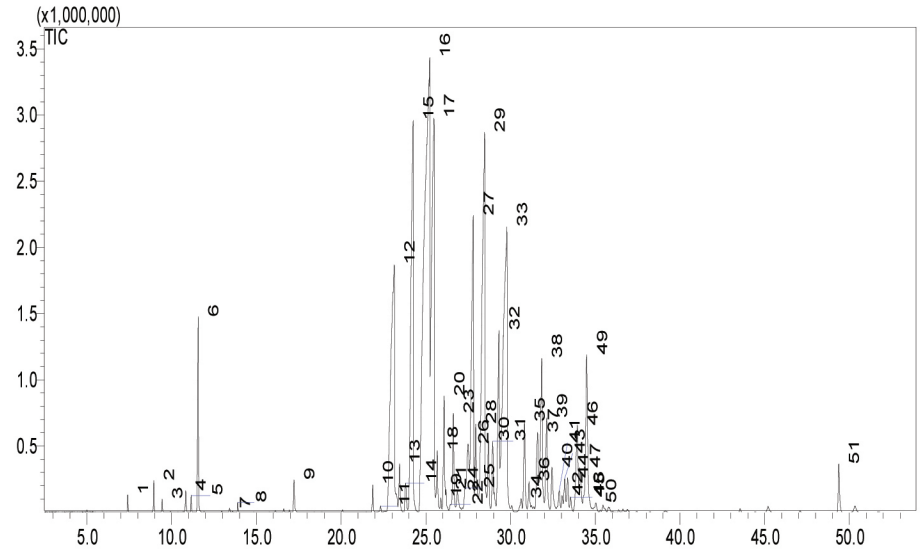

Fig. 4.31. GC-MS chromatogram for essential oil constituents of *P. colubrinum* leaf**.** X-axis represents the retention time (Rt) and Y-axis represents the peak intensity (mV)

4.6 Preliminary phytochemical analysis of screened extracts

Essential oil constituents which impart *in vitro* antioxidant activity and cytotoxicity were identified by GC-MS. However the class of compounds which contributes to high *in vitro* antioxidant activity and cytotoxicity of screened sequential extracts need highly sophisticated instruments like LC-MS-Q-TOF and NMR for identification. In the case of *in vitro* antioxidant activity, methanol extract of IISR Malabar Excel and chloroform extract of *P. colubrinum* were found to be superior. The methanol extract of Panchami also showed high antioxidant activity. In the case of *in vitro* cytotoxicity, chloroform extract of *P. colubrinum*, IISR Malabar Excel, *P. longum*, *P. chaba* and hexane extract of *P. colubrinum* showed high activities. By considering both *in vitro* antioxidant activity and cytotoxicity,

chloroform extract of *P. colubrinum* was found to be more active than other extracts. These screened extracts were tested preliminary for the presence of constituents which may contribute to their high activities. These extracts were tested for alkaloids, flavones, flavonols, phenolics, steroids/triterpenes, saponins, fixed oils and fats, carbohydrates and protein (section 3.2.10) and results are shown in Table 4.31. Alkaloids, phenolics and steroids/triterpenes were present in all the extracts whereas flavonols were present in all the methanol extracts as well as chloroform extract of *P. colubrinum*. Flavones were present in all samples except hexane extract of *P. colubrinum* whereas saponins were present only in methanolic extracts. Fixed oils and fats were present only in hexane extract of *P. colubrinum.* Carbohydrates were present in all methanol extracts and chloroform extract of *P. longum* and *P. chaba.* None of the sample has given positive result for protein.

Table 4.31. Preliminary phytochemical analysis of screened extracts

Phytochemicals Tested	**Screened extracts**						
	MM	**MP**	**CC**	**CM**	**CL**	**CH**	**HC**
Alkaloids	+	+	+	+	+	+	+
Flavones	+	+	+	+	+	+	-
Flavonols	+	+	+	-	-	-	-
Phenolics	+	+	+	+	+	+	+
Steroids/triterpenes	+	+	+	+	+	+	+
Saponins	+	+	-	-	-	-	-
Fixed oils and fats	-	-	-	-	-	-	+
Carbohydrates	+	+	-	-	+	+	-
Protein	-	-	-	-	-	-	-

MM=Methanol extract of IISR Malabar Excel, MP=Methanol extract of Panchami, CC=Chloroform extract of *P. colubrinum*, CM=Chloroform extract of IISR Malabar Excel, CL=Chloroform extract of *P. longum*, CH=Chloroform extract of *P. chaba*, HC=Hexane extract of *P. colubrinum* (+) for presence; (-) for absence

Preliminary phytochemical analysis of crude ethanolic extract of *P. nigrum* has been conducted by Nahak & Sahu (2011) and revealed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, reducing sugar and anthraquinones. Barua *et al*. (2014) reported the presence of terpenoids, alkaloids, diterpenes, flavonoids, steroids, tannin, glycoside and saponin in chloroform extract and the presence of terpenoids, alkaloids, diterpenes, flavonoids and steroids in aqueous extracts of *P. longum* through preliminary phytochemical screening.

Based on this qualitative analysis, it is found that, the potent extracts are good source of various phytochemicals. Hence, further studies can be performed for the identification of bioactive constituents, determination of their efficacy by *in vivo* studies and the demonstration of their safety and effectiveness in clinical trials.

4.7 Identification of possible compounds from potential extract screened for both *in vitro* antioxidant activity and cytotoxicity

By considering both *in vitro* antioxidant activity and cytotoxicity (on CaSki cell line) performed for the selected *Piper* species, chloroform extract of *P. colubrinum* was found to be more active than other extracts (section 4.3.2 and 4.4.1). The preliminary phytochemical analysis (section 4.6) of this extract revealed that it is a potent source of high value compounds. Thus, it was further subjected to LC-MS analysis as per section 3.2.11 and its possible compounds were identified. UV chromatogram at 254 nm and Total Ion chromatogram (TIC) for this extract is illustrated in Fig. 4.32. Twelve compounds were identified and these include phenolics and alkaloids. Ferulic acid, rosamarinic acid, salicylic acid, kaempferol-5-glucoside, 5-methoxysalicylic acid, apigenin-7-galactoside, kaempferide-3-

glucoside, luteolin, kaempferol, apigenin and scutellarein-4'-methyl ether were the phenolic compounds whereas piperlonguminine was the alkaloid compound indentified from this extract. The result is given in Table 4.32.

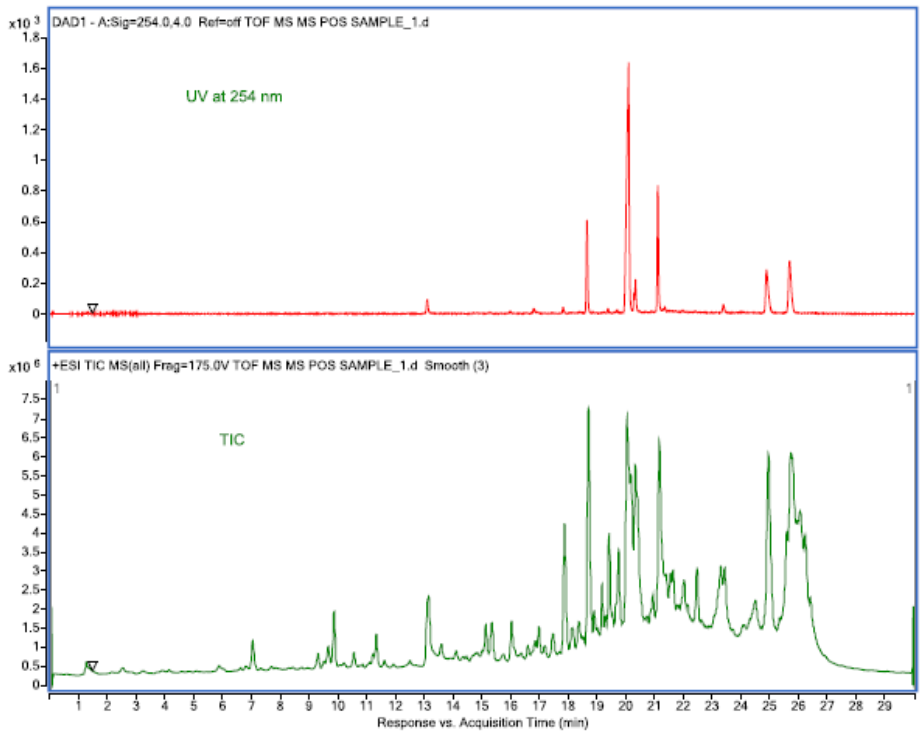

Fig.4.32. LC-MS analysis: UV chromatogram at 254 nm and Total Ion chromatogram (TIC) for chloroform extract of *P. colubrinum*

Table 4.32: Possible compounds identified from chloroform extract of *P. colubrinum* by LC-MS

Compound	**Molecular formula**	**Ionization mode**
Ferulic acid	$C_{10}H_{10}O_4$	ESI positive/negative
Rosamarinic acid	$C_{18}H_{16}O_8$	ESI positive/negative
Salicylic acid	$C_7H_6O_3$	ESI positive
Kaempferol-5-glucoside	$C_{21}H_{20}O_{11}$	ESI negative
5-Methoxysalicylic acid	$C_8H_8O_4$	ESI negative
Apigenin-7-galactoside	$C_{21}H_{20}O_{10}$	ESI positive/negative
Kaempferide-3-glucoside	$C_{22}H_{22}O_{11}$	ESI positive/negative
Scutellarein-4'-methyl ether	$C_{16}H_{12}O_6$	ESI positive/negative
Luteolin	$C_{15}H_{10}O_6$	ESI positive
Kaempferol	$C_{15}H_{10}O_6$	ESI negative
Apigenin	$C_{15}H_{10}O_5$	ESI negative
Piperlonguminine	$C_{16}H_{19}NO_3$	ESI positive

Thus, the potency of chloroform extract of *P. colubrinum* on *in vitro* antioxidant activity and cytotoxicity on CaSki cell line might be the reflection of the ability of these compounds individually or due to possible synergism between them. Hence, further studies can be performed for determination of their efficacy by *in vivo* studies and the demonstration of their safety and effectiveness in clinical trials.

Chapter 5

Summary and Conclusion

Piper is the most representative genus among Piperaceae family. The *Piper* species have great economical, commercial and medicinal importance. However, many of the members of this genus are still underexplored for their diverse therapeutic potential and chemical profile. Thus, *Piper* species have assumed great significance in the field of biological research and hence, four medicinally valued *Piper* species viz., *Piper nigrum* L., *Piper longum* L., *Piper chaba* Hunter and *Piper colubrinum* Link. were selected for the proposed study.

P. nigrum (Black Pepper) is the most important and most widely consumed spice in the world. Black pepper of commerce is the dried and matured berries of *P. nigrum*. It provides physiological benefits and prevent chronic ailment in addition to the fundamental nutrition. *P. longum* (Long Pepper) and *P. chaba* (Java long pepper) are mainly cultivated for its fruit and are the major components of traditional systems of medicines due to their diverse medicinal potential. *P. colubrinum* is an exotic *Piper* species known for its resistance to plant pathogens like *Phytophthora capsici*, *Radopholus similis* and *Meloidogyne incognita*.

Detailed study is lacking with regard to chemical and pharmacological diversity among black pepper varieties. Likewise, systematic study for intrinsic quality of black pepper in relation to different locations has not been observed. Furthermore, information is scanty regarding the correlation between chemical constituents of black pepper berries. Even though high value compounds with significant importance in human life has been identified from *P. longum,* many of its phytochemicals and therapeutic potential are still unexplored.

Likewise, *P. chaba* has not received a deserved pharmacological importance due to the dearth in the information regarding their detailed chemical profile and medicinal values. On the phytochemical and medicinal point of view, the studies on *P. colubrinum* are also scanty. Oxidative stress and related diseases, mainly cancer, are dangerous to human health. In the modern research, there is great inducement to discover antioxidant and anticancer compounds from plants due to their medicinal potential and fewer side-effects compared to synthetic drugs. However, many of the plants are still underexplored with regard to their chemical profile, antioxidant and anticancer properties. *P. longum, P. chaba* and *P. colubrinum* are few among such plant species which require much more attention in these aspects.

By considering the above mentioned information, the present study was undertaken with following objectives

- Variability in physico-chemical and biochemical profile among selected *Piper* species and also among selected black pepper varieties.
- Variability in physico-chemical and biochemical profile of black pepper variety Panniyur-1 in relation to different locations.
- Antioxidant potential of selected *Piper* species and selected black pepper varieties.
- *In vitro* cytotoxicity of selected *Piper* species and selected black pepper varieties on cancer cell line CaSki.

The present study with aforementioned objectives can be summarized as follows:

Variability in physico-chemical and biochemical profile among selected *Piper* species

Variability in physico-chemical and biochemical constituents were analyzed among four *Piper* species (wild *P. nigrum, P. longum, P. chaba* and *P. colubrinum*) and good variability was observed. Variability was found to be significant for almost all constituents. Important finding was that, *P. colubrinum* is distinct from other *Piper* species for its piperine, essential oil, oleoresin, total phenol, total flavonoid, reducing sugars, starch, total protein, total ash and acid insoluble ash. It also contained high P, Mg, Mn and Zn contents. Essential oil constituents were also identified from these *Piper* species by GC-MS analysis. Wild *P. nigrum* contained high amount of monoterpenes (α-thujene, α-and β-pinene, δ-3 carene, D-limonene, etc.) followed by sesquiterpenes (β-caryophyllene, α-humulene, caryophyllene oxide, etc). Delta-3 carene and D-limonene were the major monoterpenes whereas β-caryophyllene was the major sesquitepene identified from wild *P. nigrum* essential oil sample. The aliphatic compounds predominated in *P. longum* essential oil followed by monoterpenes. n-Pentadecane was the major aliphatic compound whereas β-pinene was the major monoterpene identified from this oil. In the case of *P. chaba* fruit oil, sesquiterpenes were predominated followed by aliphatic compounds. Beta-caryophyllene was identified as major sesquiterpene whereas 1-pentadecene and n-heptadecane were identified as major aliphatic compounds from this sample. In *P. colubrinum* fruit oil, the major contribution was given by sesquiterpenes especially α-muurolol and β-caryophyllene. These

Piper species were also subjected to clustering study (average linkage cluster analysis) based on their biochemical features and the result revealed that *P. colubrinum* is distinct, whereas, *P. longum* and *P. chaba* are closely related species. The study thus revealed that, the selected *Piper* species are distinctly different for various constituents studied.

This is found to be the first report on detailed physico-chemical and biochemical analysis of *P. chaba* and *P. colubrinum* fruits. Likewise, the study also contributes to the scarce knowledge regarding the cluster analysis of these *Piper* species based on their biochemical composition.

Variability in physico-chemical and biochemical profile among selected black pepper varieties

Variability in physico-chemical and biochemical constituents of different varieties (Panniyur-1, Panniyur-5, Sreekara, Subhakara, Panchami, IISR Malabar Excel, IISR Sakthi, IISR Girimunda and IISR Thevam) of black pepper collected from same location (IISR Experimental Farm, Peruvannamuzhi, Kozhikode, Kerala) was also examined. The result revealed significant variability for constituents among black pepper varieties. Variability was profound in total ash, acid insoluble ash, total carbohydrate, reducing sugars, starch, crude fibre, total protein, essential oil, piperine, total phenol, oleoresin, Fe, Zn, Cu and Mn. It is noted that the black pepper variety Panchami is superior in quality attributes of black pepper *viz.,* essential oil, oleoresin and piperine. It is also high for total carbohydrate, crude fibre, total ash, acid insoluble ash and minerals like P, K, Ca, Fe, Cu and Zn. On the other hand, the black pepper variety IISR Malabar

Excel is found to be highest for medicinal value attributes *viz.,* total phenol and total flavonoid. It is also found to be high for quality parameters *viz.,* oleoresin and piperine and also for crude fibre, N and Ca contents. The black pepper variety Panniyur-1 is also found to be high for piperine, total protein, total free amino acid, crude fibre, total fat and Mn contents. Alpha thujene, α- and β-pinene, sabinene, myrcene, D-limonene and α-phellandrene were the major monoterpenes identified from the essential oils of black pepper varieties and of which, D-limonene predominated. Major sesquiterpene identified from these samples was β-caryophyllene. Good varietal variability was observed for essential oil constituents. Variability was profound in α-thujene, α-pinene, β-pinene, sabinene, myrcene, δ-3-carene, α-phellandrene, D-limonene, α-terpinolene, β-linalool, 4-terpineol, δ-elemene, β-elemene, β-caryophyllene and caryophyllene oxide. Based on their biochemical constituents, the black pepper varieties were clustered into different groups using average linkage cluster analysis and resultant dendrogram clearly showed the closely related and distant black pepper varieties in relation to biochemical data. Thus, the present study established variability in intrinsic quality of black pepper with respect to varieties.

Fatty acid profiling of selected *Piper* species

Matured and dried fruits/berries of *P. nigrum* (variety Panniyur-1), *P. longum, P. chaba* and *P. colubrinum* were subjected to fatty acid profiling by GC-MS. Linoleic acid was the major fatty acid in black pepper followed by oleic and palmitic acids. Stearic, myristic, lauric and palmitoleic acids were the other fatty acids identified from black pepper sample. In the case of *P. longum,* palmitic acid was the dominant fatty acid followed by linoleic and linolenic acids. Stearic

and behenic acids were also identified from *P. longum.* Palmitic acid was the predominant fatty acid in *P. chaba* also. Linoleic, oleic, lauric, myristic and stearic acids were also identified from *P. chaba*. Unlike other samples, *P. colubrinum* had oleic acid as the major fatty acid followed by palmitic and linoleic acids. Other fatty acids identified in this sample include lauric, myristic, stearic and arachidic acids. Thus, the result indicated that the selected *Piper* species is a good source of fatty acids which shown to possess various applications and importance to human health. This is also a flash to the scarce knowledge regarding fatty acid profiling of *P. chaba* and *P. colubrinum* fruits.

Phenolic profiling of selected *Piper* species

Matured and dried fruits/berries of *P. nigrum* (variety Panniyur-1), *P. longum*, *P. chaba* and *P. colubrinum* were subjected to phenolic profiling using LC-MS and HPLC. The identified phenolic compounds mainly include phenolic acids and flavonoids. Thirteen compounds were identified from black pepper which mainly includes hydroxybenzoic acids (salicylic acid, syringic acid, protocatechuic acid, 4-hydroxybenzoic acid, gentisic acid and vanillic acid), hydroxycinnamic acids (caffeic acid, ferulic acid and 4-coumaric acid) and flavonoids (luteolin-8-C-glucoside and apigenin). Guaiacol and 4-hydroxymandelic acid were also identified from black pepper sample. Among the six compounds identified in *P. longum,* three belongs to hydroxybenzoic acid (salicylic acid, 2-pyrocatechuic acid and gentisic acid) and the other three from hydroxycinnamic acid (4-coumaric acid, caffeic acid and ferulic acid) category. Seven compounds were identified from *P. chaba,* which comprises hydroxybenzoic acids (salicylic acid and protocatechuic acid),

hydroxycinnamic acids (caffeic acid and ferulic acid) and other compounds like salicylaldehyde, caffeic aldehyde and 5-methoxysalicylic acid. No flavonoids were identified from *P. longum* and *P. chaba.* In the case of *P. colubrinum,* ten compounds were identified and majority was flavonoids (kaempferol-5-glucoside, apigenin-7-galactoside, 5,7,2',5'-tetrahydroxy flavanone, kaempferide-3-glucoside and scutellarein-4'-methyl ether). Hydroxybenzoic acids (protocatechuic acid, 2-pyrocatechuic acid and salicylic acid), phenolic aldehyde (vanillin) and other compounds like 5-methoxysalicylic acid were also identified from *P. colubrinum.* However, no hydroxycinnamic acids were identified from *P. colubrinum* sample. Salicylic acid was the only phenolic compound identified from all the four species. In addition, HPLC analysis confirmed the presence of salicylic acid, 4-hydroxybenzoic acid, syringic acid, ferulic acid, 4-coumaric and caffeic acid in *P. nigrum*, salicylic acid, 4-coumaric acid, caffeic acid and ferulic acid in *P. longum,* salicylic acid, caffeic acid and ferulic acid in *P. chaba* and salicylic acid in *P. colubrinum.* The compound 4-hydroxymandelic acid from black pepper berries, salicylic acid, 4-coumaric acid and 2-pyrocatechuic acid from *P. longum* fruit*,* salicylaldehyde, salicylic acid, caffeic aldehyde, 5-methoxysalicylic acid and ferulic acid from *P.chaba* fruit and 2-pyrocatechuic acid*,* salicylic acid*,* 5-methoxysalicylic acid*,* 5,7,2',5'-tetrahydroxy flavanone, kaempferide-3-glucoside, vanillin and scutellarein-4'-methyl ether from *P. colubrinum* fruit are reported for the first time.

Even though LC-MS-Q-TOF showed the presence of these phenolic compounds, their molecular structure can be established further by additional techniques like NMR and IR. Some of these identified

compounds are underexplored for their medicinal values or well studied compounds are still unknown for certain medicinal properties. Thus, these findings may be a lead to researchers in the pharmacological field to extend their work on this natural phytochemicals.

Variability in physico-chemical and biochemical profile of black pepper variety Panniyur-1 in relation to different locations

The matured and dried berries of black pepper variety, Panniyur-1, collected from eleven locations of India (Kasaragod, Chelavoor, Peruvannamuzhi, Panniyur, Ambalavayal, Pampadumpara, Appangala, Mudigere, Pechiparai, Thadiankudisai and Dapoli), were subjected to variability studies for physico-chemical and biochemical constituents. A significant location wise variability was obtained for both primary and secondary metabolites and also for physico-chemical and mineral parameters. Variability was profound in essential oil, oleoresin, piperine, total phenol, crude fibre, starch, reducing sugars, bulk density and also in minerals like P, Fe, Cu and Mn. It is noted that Panniyur-1 sample from Kasaragod is found to be superior for quality parameters like piperine and oleoresin, other secondary metabolites like total phenol and total flavonoid, primary metabolites like total protein, total fat and crude fibre, physico-chemical constituents like total ash content as well as minerals like P, K, Ca, Mg, Fe and Mn. In addition, Peruvannamuzhi sample also showed high primary metabolites (total protein, total free amino acid, total fat and crude fibre), secondary metabolites (essential oil, oleoresin, total phenol and piperine) and N content. A total of 49 constituents were also identified from essential oil extracted from these Panniyur-1 black pepper samples. Many of these oil constituents, especially α-thujene, α- and β-pinene, sabinene,

D-limonene, 4-terpineol, α-copaene, δ- and β-elemene, β-caryophyllene, α-humulene, β-bisabolene, caryophyllene oxide and α-cadinol showed variability in relation to location. Apart from location wise variation, a clear altitudinal variation was also observed in the case of β-caryophyllene and total phenol. These two constituents were low at high elevations (>500 m MSL) and high at plains. Similarly, monoterpenes like thujene, α-pinene and sabinene were relatively high at higher altitudes compared to plains. It is also observed that, the aroma profile of Panniyur-1 essential oil at higher altitudes literally or more matches with reported desired aroma qualities. Thus, the present study established variability in intrinsic quality of black pepper with respect to locations and thus, the study assumes a great commercial significance.

The study also revealed a significant correlation between many constituents of Panniyur-1 from different locations. Total phenol, essential oil, piperine, oleoresin, crude fibre, total fat, total protein and total free amino acid showed positive correlation with each other and negative correlation with bulk density and starch. Bulk density showed positive correlation with starch and negative correlation with all other constituents. The correlation study also revealed a significant and strong positive correlation for total ash with P, K, Ca, Mg, Fe and Mn and thus proved the relationship between total ash and mineral contents of the sample. The correlation between biochemical and mineral contents of Panniyur-1 samples in relation to location was also studied and significant positive correlations were obtained for N with essential oil, P with total protein and piperine, Mg with total protein and Fe with total protein, total free amino acid and piperine.

Panniyur-1 samples from different locations were also clustered by adopting average cluster linkage analysis, using their biochemical data. The resultant dendrogram clearly established that the samples with similar biochemical constituents were closely clustered and thus samples from Kasaragod and Peruvannamuzhi were clustered together and found to be distinct from other samples.

This is the first report regarding location wise variation and correlation among constituents of Panniyur-1 black pepper berries. This is also the first report on biochemical clustering of Panniyur-1 black pepper berries in relation to location.

The physico-chemical properties of Panniyur-1 black pepper growing soil and the effect of soil parameters on the above mentioned Panniyur-1 berry constituents, in relation to location were also analyzed. Soil samples were collected from Panniyur-1 black pepper growing plot of Appangala, Thadiankudisai, Pampadumpara, Panniyur, Dapoli, Ambalavayal, Peruvannamuzhi, Chelavoor and Pechiparai at the same period of berry collection and the soil parameters (pH, electrical conductivity, organic carbon, available N, available P, exchangeable K, Ca, Mg and available Fe, Cu, Zn and Mn) were analyzed. The results revealed a significant variability in soil parameters in relation to location. Variability was profound in soil electrical conductivity, P, K, Ca, Mg and Cu contents. The influence of soil parameters on various constituents of Panniyur-1 black pepper berries from different locations was also established by performing correlation between soil and berry nutrients/minerals and also between soil nutrients and berry biochemical constituents. Soil N showed significant positive correlation with berry N whereas soil P with berry K and berry Fe. Likewise, soil K showed significant positive

correlation with berry K as well as soil Ca showed significant positive correlation with berry Ca. On the other hand, soil Zn showed significant negative correlation with berry Zn. Soil Mn showed significant positive correlation with berry Mn and significant negative correlation with berry N. Soil N showed a significant negative correlation with reducing sugar content of panniyur-1 black pepper berries whereas soil Ca with berry oleoresin content. Soil Mn was in significant positive correlation with total carbohydrate, starch and negative correlation with total free amino acid, total fat, essential oil, piperine and oleoresin contents. The soil analysis thus established the variability in soil parameters and their effect on biochemical and mineral constituents of Panniyur-1 black pepper berries in relation to location.

In vitro antioxidant activity and cytotoxicity of sequential extracts and essential oils from selected *Piper* species

The *in vitro* antioxidant activity and cytotoxicity of sequential extracts (n-hexane, chloroform, methanol and water) from matured fruits/berries of *Piper* species (wild *P. nigrum*, *P. longum*, *P. chaba* and *P. colubrinum*) and six black pepper varieties (Sreekara, Subhakara, IISR Malabar Excel, IISR Thevam, Panchami and Panniyur-1) were performed. Methanol and chloroform extracts showed high antioxidant activity than hexane and water extracts. Among black pepper varieties, methanol extract of IISR Malabar Excel followed by that of Panchami and among *Piper* species, chloroform extract of *P. colubrinum* expressed high antioxidant activity. Significant positive correlation between total phenol and antioxidant activity was noted for methanol and chloroform extracts. *In vitro* cytotoxicity of the extracts was tested on cervical cancer cell line

CaSki by MTT assay. Result showed more cytotoxicity with more extract and increased time of exposure with CaSki. Chloroform extract of all the samples and hexane extract of *P. colubrinum* showed high cytotoxicity on CaSki cell lines. Among potential extracts, chloroform extract of *P. longum* and *P. colubrinum* were found to be highly toxic to CaSki. Considering three time intervals, chloroform extract of IISR Malabar Excel was more toxic to CaSki than other black pepper varieties. By considering both *in vitro* antioxidant activity and cytotoxicity, chloroform extract of *P. colubrinum* is found to be more active than other extracts. To the best of our knowledge, this is the first report regarding *in vitro* antioxidant activity and cytotoxicity on CaSki, for sequential extracts of *P. colubrinum* fruits. This is also the first report on variability in antioxidant activity and cytotoxicity (on CaSki) of sequential extracts from black pepper varieties selected for the study.

Essential oil extracted from black pepper (IISR Malabar Excel), *P. longum* and *P. chaba* also showed antioxidant activity with good variability among species. Black pepper oil was superior for antioxidant activity by phosphomolybdenum method, ferric reducing power method and ferrous chelating ability whereas *P. longum* oil showed highest DPPH radical scavenging ability. *In vitro* cytotoxicity of selected essential oils was also tested on cervical cancer cell line CaSki using MTT assay at three time intervals. Results showed a time-dependent increase in the cytotoxicity of essential oils on CaSki cell lines. Based on the cytotoxicity at three time intervals, black pepper essential oil was more toxic to CaSki cell lines.

The *in vitro* antioxidant activity and cytotoxicity of *P. colubrinum* leaf oil was also studied. The sample showed good antioxidant activity

especially DPPH radical scavenging ability. It also showed good cytotoxicity to CaSki cell line for three time intervals. The chemical constituents that impart to these potential effects of *P. colubrinum* leaf oil were also identified by GC-MS. The result indicated the presence of high amount of sesquiterpenes and phenyl propanoids. Methyl eugenol was the major compound identified from *P. colubrinum* leaf oil.

This is the first report regarding the *in vitro* antioxidant activity and cytotoxicity (on CaSki) of *P. colubrinum* leaf oil and the *in vitro* cytotoxicity of essential oil extracted from black pepper (IISR Malabar Excel), *P. longum* and *P. chaba* fruits on cervical cancer cell line CaSki.

Thus, the present study suggested that the selected *Piper* species could be a potential source of antioxidant and anticancer agents and could have greater importance in pharmaceutical field to prevent oxidative stress and related degenerative diseases like cancer.

Preliminary phytochemical analysis of screened extracts

The sequential extracts that screened for high *in vitro* antioxidant activity and cytotoxicity were tested preliminary for the presence of constituents, which may contribute to their high activities. These extracts were tested for alkaloids, flavones, flavonols, phenolics, steroids/triterpenes, saponins, fixed oils and fats, carbohydrates and protein. The results revealed that the potent extracts are good source of various phytochemicals.

Identification of possible compounds from potential extract screened for both *in vitro* antioxidant activity and cytotoxicity

By considering both *in vitro* antioxidant activity and cytotoxicity (on CaSki cell line) performed for the selected *Piper* species, chloroform extract of *P. colubrinum* was found to be more active than other extracts. The preliminary phytochemical analysis of this extract revealed that it is a potent source of high value constituents. Thus, this extract was further subjected to LC-MS analysis and possible compounds contribute to these high activities were identified. Ferulic acid, rosamarinic acid, salicylic acid, kaempferol-5-glucoside, 5-methoxysalicylic acid, apigenin-7-galactoside, kaempferide-3-glucoside, luteolin, kaempferol, apigenin and scutellarein-4'-methyl ether were the phenolic compounds whereas piperlonguminine was the alkaloid compound indentified from this extract.

The salient findings of the study are:

- The variability studies and average linkage cluster analysis of *P. nigrum* (wild), *P. longum*. *P. chaba* and *P. colubrinum* revealed that *P. colubrinum* is distinct, whereas, *P. longum* and *P. chaba* are closely related species.

- Various high value constituents including alkaloids, terpenoids, phenolics and fatty acids are identified from the *Piper* species selected for the study.

- The study clearly established the variability in physico-chemical and biochemical constituents of black pepper in relation to different varieties. These varieties were also clustered based on their biochemical data and the resultant

dendrogram clearly showed the closely related and distant black pepper varieties.

- The study established variability in intrinsic quality of black pepper variety Panniyur-1 in relation to location.

- Altitudinal variation is observed for total phenol and β-caryophyllene contents of Panniyur-1 black pepper berries. These two constituents were low at high elevations (>500 m MSL) and high at plains. Likewise, monoterpenes like thujene, α-pinene and sabinene were relatively high at higher altitudes compared to plains.

- The aroma profile of Panniyur-1 essential oil at higher altitudes very much matches with reported desired aroma qualities.

- The average linkage cluster analysis of Panniyur-1 samples from different locations revealed that, samples from Kasaragod and Peruvannamuzhi are found to be biochemically distinct from samples from other locations.

- The study revealed the correlation between different constituents of Panniyur-1 black pepper berries in relation to location.

- Methanol extract of black pepper variety IISR Malabar Excel and chloroform extract of *P. colubrinum* are found to be superior for *in vitro* antioxidant activity. The methanol extract of Panchami also showed high antioxidant activity.

- Significant positive correlation between total phenol and antioxidant activity is obtained for methanol and chloroform extracts.

- Chloroform extract of black pepper variety IISR Malabar Excel, *P. colubrinum*, *P. longum*, *P. chaba* and hexane extract of *P. colubrinum* are screened as potential extracts with high *in vitro* cytotoxicity on cervical cancer cell line CaSki.

- Chloroform extract of *P. colubrinum* is found to be more active than other extracts with respect to *in vitro* antioxidant activity and cytotoxicity on CaSki cell line. The possible compounds which contribute to these high activities were identified and that include phenolic and alkaloid compounds.

- The essential oil extracted from the selected *Piper* species in the present study also showed *in vitro* antioxidant activity and cytotoxicity (on CaSki cell line).

- The selected *Piper* species in the present study is thus a good source of antioxidant and anticancer agents and could have greater importance in pharmaceutical field to prevent oxidative stress and related degenerative diseases like cancer.

Future perspectives

- ❖ Identification of bioactive constituents from all the extracts screened for high antioxidant activity and cytotoxicity (on CaSki cell line), determination of their efficacy by *in vivo* studies and the demonstration of their safety and effectiveness in clinical trials.

Since research is endless, further studies may reveal many more therapeutic properties and further more novel compounds from these plants and the present study might be a flash to the future research, especially in the field of phytochemistry and pharmacology.

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Appendix

APPENDIX

Preparation of Reagents

Reagent	Method of preparation
Alkaline copper tartarate (Reducing sugars estimation)	A) Dissolved 2.5 g anhydrous sodium carbonate, 2.0 g sodium bicarbonate, 2.5 g potassium sodium tartarate and 20 g anhydrous sodium sulphate in 80 mL distilled water and made up to 100 mL. B) Dissolved 15 g copper sulphate in a small volume of distilled water. Added one drop of H_2SO_4 and made up to 100 mL. Mixed 4.0 mL of B and 96 mL of A before use.
Arsenomolybdate reagent (Reducing sugars estimation)	Dissolved 2.5 g ammonium molybdate in 45 mL distilled water. Added 2.5 mL H_2SO_4 and mixed well. Then added 0.3 g disodium hydrogen arsenate which dissolved in 25 mL distilled water. Mixed well and incubated at 37^{o}C for 24-48 hrs.
Anthrone reagent (Starch estimation)	Dissolved 200 mg anthrone in 100 mL of ice-cold H_2SO_4 (95%).
Alkaline copper solution (Protein estimation)	Reagent A: 2% sodium carbonate in 0.1N NaOH. Reagent B: $CuSO_4.5H_2O$ (0.5%) in 1% potassium sodium tartarate. Reagent C: Mixed 50 mL of A and 1.0 mL of B before use.
Ninhydrin-glycerol buffer	A) Citrate buffer (0.5M; pH 5.5)

(Amino acid estimation)	1) Citric acid 1M (10.505 g in 50 mL distilled water). 2) Sodium citrate. 2 H_2O 1M (14.706 g in 50 mL distilled water). Mixed 13.7 mL of 1 and 36.3 mL of 2 and made up to 100 mL with distilled water. B) 1% ninhydrin in citrate buffer (0.5M; pH 5.5) C) Glycerol AR Mixed 0.4 mL of A, 1.0 mL of B and 2.4 mL of C just before use.
Catalytic mixture (Plant nitrogen analysis)	$CuSO_4.5H_2O$ (20 g), K_2SO_4 (100 g) and Selenium (1.0 g) were mixed and ground into fine powder.
Boric acid-double indicator solution (Nitrogen analysis)	Dissolved 40 g of boric acid in 1.8 L of boiled deionised water. Then added 40 mL indicator solution (prepared by dissolving 0.495 g bromocresol green and 0.33 g of methyl red in 500 mL of ethanol) and made up the volume to 2.0 L with distilled water and adjust the pH of the solution to 4.2-4.3 using 1M NaOH.
Diacid mixture (HNO_3 : $HClO_4$; 9:4) for wet oxidation of plant tissues	Took 1.8 L of conc. HNO_3 in a 3.0 L bottle and added 800 mL conc. perchloric acid. Mixed well and stored.
HNO_3-Vanado Molybdate reagent (Plant Phosphorus analysis)	Dissolved 25 g ammonium molybdate in 400 mL distilled water (solution A). Dissolved 1.25 g ammonium meta vanadate in 300 mL boiling water, cooled and added 250 mL conc. HNO_3 and cooled again to room temperature (solution B). Added solution A to B. Mixed well and made up to 1.0 L.

Ferrous ammonium sulphate hexahydrate solution (Mohr's salt) (Soil organic carbon analysis)	Dissolved 197 g of $(NH_4)_2$ Fe $(SO_4)_2.6H_2O$ in distilled water and 15 mL of conc. H_2SO_4 was added to it. Cooled the solution and diluted to 1.0 L.
O-phenanthroline-ferrous complex (Ferroin) (Soil organic carbon analysis)	Dissolved 14.85 g of *o*-phenanthroline monohydrate and 6.95 g of ferrous sulphate heptahydrate in distilled water and made up the volume to 1.0 L.
Bray-1 reagent (Soil phosphorus analysis)	Added 15 mL of 1N ammonium fluoride and 25 mL of 0.5N HCl to 460 mL of distilled water.
Colouring reagent (Soil phosphorus analysis)	Dissolved 12 g of ammonium paramolybdate in 250 mL of distilled water. Dissolved 0.2908 g of potassium antimony tartarate in 100 mL of distilled water. Added these dissolved reagents to 1.0 L of 5N H_2SO_4. Mixed well and diluted to 2.0 L with distilled water (Reagent A). Dissolved 1.056 g of ascorbic acid in 200 mL of reagent A and mixed well.
Extracting reagent for soil K, Ca and Mg	Diluted 570 mL of glacial acetic acid to 5.0 L with pure water. Then, added 690 mL of conc. ammonium hydroxide. Adjusted the pH to 7.0 after proper mixing and diluted to a volume of 10.0 L with pure water.
Diethylene Triamine Penta acetic acid (DTPA) extracting solution for soil Fe, Cu, Zn and Mn	Dissolved 149.2 g triethanolamine, 19.67 g DTPA and 14.7 g calcium chloride dihydrate in approximately 200 mL distilled water. Diluted to approximately 9.9 L and adjusted to pH 7.3 and diluted to 10.0 L.

Phosphomolybdenum reagent (Antioxidant activity by Phosphomolybdenum method)	28 mM disodium hydrogen phosphate (0.196 g) and 4 mM ammonium molybdate (0.248 g) were dissolved in 25 mL distilled water and 1.62 mL of 0.6M H_2SO_4 was added drop wise to it and made up to 50 mL with distilled water.
Phosphate buffer (0.2M; pH 6.6) (Antioxidant activity by FRP method)	A) 0.2M sodium dihydrogen phosphate (2.3996 g in 100 mL distilled water). B) 0.2M disodium hydrogen phosphate (2.8396 g in 100 mL distilled water). Mixed 63 mL of A and 37 mL of B.
Phosphate Buffered Saline (*in vitro* cytotoxicity)	Sodium chloride (8 g), Potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g) and dihydrogen potassium phosphate (0.24 g) were dissolved in triple distilled water and made up the volume to 1.0 L and pH was adjusted to 7.2.
PBS-EDTA (*in vitro* cytotoxicity)	0.2 g of EDTA was dissolved in 1.0 L PBS and pH was adjusted to 7.2.
Trypsin EDTA (*in vitro* cytotoxicity)	0.25 g of trypsin was dissolved in 1.0 L PBS-EDTA. The solution was filter sterilized using syringe driven filter unit of 0.22 µm pore size and stored at -20^{o}C.
Dulbecco's Modified Eagle's Medium (DMEM) (*in vitro* cytotoxicity)	The autoclaved triple distilled water was added to a mixing container. 13.55 g of DMEM powder was added to it with gentle stirring at room temperature. 3.75 g of sodium bicarbonate and 1.95 g of HEPES buffer was added to the medium and it was diluted to the desired volume with autoclaved distilled water. 10 mL of

	penicillin-streptomycin-fungizone (antibiotic-antimycotic) mixture was added. Mixed well and medium was filtered under vacuum suction using 0.22 µm filter unit. Immediately after filtering, the medium was transferred to a sterile container and stored at 4^{o}C.
MTT solution (*in vitro* cytotoxicity)	A stock MTT solution was prepared by weighing 7.5 mg MTT in 1.5 mL Phosphate Buffered Saline. A working standard was prepared by dissolving 1.4 mL of stock in 5.6 mL of 10% DMEM medium.
MTT lysis buffer (*in vitro* cytotoxicity)	10 g SDS was dissolved in 25 mL of dimethyl formamide and 25 mL of distilled water and kept at room temperature.
Fehling's solution A (Fehling's Test)	Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 mL.
Fehling's solution B (Fehling's Test)	Potassium sodium tartarate (173 g) and sodium hydroxide (50 g) were dissolved in distilled water and made up to 500 mL.
Benedict's reagent (Benedict's Test)	Anhydrous sodium carbonate (100 g), sodium citrate (173 g) and copper (II) sulphate pentahydrate (17.3 g) were dissolved in distilled water and made up to 1.0 L.
Millon's reagent (Millon's test)	Mercury (1g) was dissolved in 9 mL of fuming nitric acid. When the reaction was completed, equal volume of distilled water was added.

Publications

LIST OF PUBLICATIONS

Research Articles (Peer Reviewed)

- **Sruthi D** and Zachariah T J 2016 Phenolic profiling of selected *Piper* species by Liquid Chromatography-Mass spectrometry (LC-MS). *Journal of Spices and Aromatic Crops* (Accepted).
- **Sruthi D** and Zachariah T J 2016 *In vitro* antioxidant activity and cytotoxicity of sequential extracts from selected black pepper (*Piper nigrum* L.) varieties and *Piper* species. *International Food Research Journal* (Accepted).
- **Sruthi D** and Zachariah T J 2015 Chemo profiling, *in vitro* antioxidant activity and cytotoxicity of essential oil from selected *Piper* species. *International Journal of Advances in Pharmaceutical Research* **6** (09): 284-95.
- **Sruthi D**, Zachariah T J, Leela N K and Jayarajan K 2013 Correlation between chemical profiles of black pepper (*Piper nigrum* L.) var. Panniyur-1 collected from different locations. *Journal of Medicinal Plants Research* **7** (31): 2349-57.

Popular Article

- **Sruthi D** and Zachariah T J 2014 Intrinsic quality of black pepper (*Piper nigrum* L.) cultivars. *Cutting Edge* **2** (9): 20-26.

Abstracts in Seminars/Symposia:

- **Sruthi D** and Zachariah T J 2014 *In vitro* antioxidant activity and cytotoxicity of sequential extracts from selected black pepper (*Piper nigrum* L.) varieties and *Piper* species. In: International Symposium on Plantation Crops held at Calicut, Kerala on 10-12 December 2014:183p **(Oral Presentation).**
- **Sruthi D** and Zachariah T J 2013 Evaluation of essential oil profiles of selected wild *Piper* species. In: Symposium on Spices, Medicinal and Aromatic Crops (SYMSAC-VII) held at Madikeri, Karnataka on 27-29 November 2013:200p **(Poster Presentation).**
- **Sruthi D** and Zachariah T J 2012 Varietal evaluation of black pepper (*Piper nigrum* L.) berries for its physicochemical, biochemical and nutritional constituents. In: First National Biodiversity Congress held at Thiruvananthapuram, Kerala on 27-30 December 2012:135p **(Poster Presentation).**