Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of *Isodon* (Schrad. ex Benth.) Spach (Lamiaceae)

Thesis
submitted to the University of Calicut
for the award of the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By

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SEPTEMBER 2015

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CERTIFICATE

This is to certify that the thesis entitled "**Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of *Isodon* (Schrad. ex Benth.) Spach (Lamiaceae)**" submitted to the University of Calicut for the award of the degree of DOCTOR OF PHILOSOPHY, is an authentic record of original research work done by SANDHYA VINCENT NEELAMKAVIL during the period of her study (2012-2015) at the Cell and Molecular Biology Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for the award of any degree/diploma to any candidate of any university.

Dr JOHN E. THOPPIL
Supervising Teacher

DECLARATION

I, SANDHYA VINCENT NEELAMKAVIL, hereby declare that the thesis entitled "**Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of *Isodon* (Schrad. ex Benth.) Spach (Lamiaceae)**" submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY is a record of original research work done by me under the supervision and guidance of Dr John E. Thoppil, Professor, Department of Botany, University of Calicut and that it has not formed the basis for the award of any degree/diploma to any candidate of any university.

Date: **Sandhya Vincent Neelamkavil**

ACKNOWLEDGEMENT

With immense gratitude, I would like to mention all those who have lend a helping hand in carrying out my research work and materializing it into a doctoral thesis.

Dr John E. Thoppil, Professor and Head, Department of Botany, University of Calicut is my research guide without whose supervision I could not have accomplished my research work. His expert guidance, precious advice, timely suggestions, immense patience and ceaseless motivation have helped me to remain focused on my work and bring it to completion. I express my heartfelt thankfulness and deepest sense of gratitude to him for being an inspiration and an excellent supervising guide.

I owe greatly to Dr K. M. Jayaram, Dr M. Sabu and Dr K. V. Mohanan, former Heads, Department of Botany, University of Calicut for providing all the necessary facilities and administrative assistance towards the completion of my research work.

I am obliged to Dr S. Nandakumar, Former Head, Department of Botany, University of Calicut for his suggestions and clarifications regarding my queries.

I place on record my sincere gratitude for the help rendered by Dr A. K. Pradeep, Assistant Professor and Dr P. Sunoj Kumar, Associate Professor,

Department of Botany, University of Calicut in identifying and authenticating the plant materials used for the research work.

I express my deep sense of gratitude to Dr Ramadasan Kuttan, Director and Dr Jose Padikkala, Joint Director, Amala Cancer Research Centre, Thrissur for providing facilities to do a part of my research work.

I gratefully acknowledge Kerala State Council for Science, Technology and Environment (KSCSTE), Thiruvananthapuram for the financial support provided in conducting the research work.

I would like to acknowledge the staff of Biochemistry Division, CEPC Laboratory and Technical Division, Cashew Bhavan, Kollam, Kerala and CARe KERALAM, KINFRA Small Industries Park, Koratty, Thrissur, Kerala for the technical facilities and assistance provided in analytical studies.

A word of appreciation to Mr Prakashan P. M., Librarian, Department of Botany as well as the staff of INFLIBNET, University of Calicut for the help rendered during literature survey.

I am immensely thankful to all my teachers at Department of Botany, University of Calicut, St. Thomas' College, Thrissur and Vimala College, Thrissur with special mention to Dr K. Ignatius Antony, Dr (Sr) Serene, Dr (Late) Jose Marydas and Dr Jose T. Puthur for their inspiration and encouragement throughout the study.

A special word of thanks to all my labmates at Amala Cancer Research Centre, Thrissur especially Lincy Lawrence, Seema Menon and Indu M. S., for

their kind co-operation and scholarly assistance rendered during my research work.

The immeasurable help, co-operation and constructive suggestions extended by my labmates Dr Jija Mathew, Dr Asha Ramachandran, Dr Julie John, Dr Renjana P. K., Mrs. Seema Devi R., Mrs Anjana S., Mrs Sumitha K. V., Mrs Lasi Das, Ms Prajitha V., Ms Neethu Kannan B., Mrs Sinitha K., Mr Aneesh S. and Ms Archana E. R. are thankfully acknowledged.

I am greatly indebted to Mr Alfred Joe for the good will, wholehearted support and motivation rendered to me throughout my research work without which I could not have achieved this endeavour. I also express my sincere thanks to Dr Shahina P. M., Ms. Deepna Latha K. P., other research scholars and M. Sc. friends for the endless support and motivation.

I extend my deep sense of gratitude towards my family members for their love, patience, endurance and untiring support which was the driving force behind the successful completion of my work.

Above all, I thank God Almighty for his blessings, without which I could not have reached to this extent.

Sandhya Vincent Neelamkavil

CONTENTS

ABBREVIATIONS

°C	-	Degree Celsius
µg	-	Microgram
µl	-	Microlitre
µM	-	Micro molar
32D	-	Rat hematopoietic stem cells
A	-	Degree of asymmetry of karyotypes
A_1	-	Intrachromosomal asymmetry index
A_2	-	Interchromosomal asymmetry index
A2780	-	Human ovarian cell line
A549	-	Human lung adenocarcinoma cell line
ABTS	-	2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
A. cepa	-	*Allium cepa*
ACL	-	Average chromosome length
ADP	-	Adenosine diphosphate
AIDS	-	Acquired immunodeficiency syndrome
$AlCl_3$	-	Aluminium chloride
ANOVA	-	Analysis of variance
As K%	-	Karyotype asymmetry index
ASK	-	Human astrocytoma cell line
ATP	-	Adenosine triphosphate
B16-F10	-	Murine melanoma cell line
Bax	-	Bcl-2-associated X protein
Bcap37	-	Human breast cancer cell line
Bcl-2	-	B-cell lymphoma 2
BEL-7402	-	Human hepatic carcinoma cell line
BGC823	-	Human gastric cancer cell line
BIU87	-	Human cystic cancer cell line
b. wt	-	Body weight
c.	-	Circa
C8166	-	Human T-lymphocyte
CA	-	Human liver cancer cell line
CAPAN-1	-	Human pancreatic ductal adenocarcinoma cell line

CAPAN-2	-	Human pancreatic ductal adenocarcinoma cell line
cm	-	Centimetre
CNE	-	Human nasopharyngeal carcinoma cell line
CNE2	-	Nasopharyngeal carcinoma cell line
Conc.	-	Concentrated
CPCSEA	-	Committee for the purpose of control and supervision of experiments on animals
cu. mm	-	Cubic millimeter
DI	-	Disparity index
Dil.	-	Dilute
dL	-	Decilitre
DLA	-	Dalton's lymphoma ascites cell line
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DPPH	-	2,2-diphenyl-2-picrylhydrazyl
DU145	-	Human prostate cancer cell line
DW	-	Dry weight
EAC	-	Ehrlich ascites carcinoma cell line
EC	-	Equivalent concentration
EC1	-	Esophageal cancer cell line
EC109	-	Esophageal cancer cell line
EC9706	-	Esophageal cancer cell line
EDTA	-	Ethylene diamine tetra acetic acid
ESI	-	Electro spray ionization
$FeCl_3.6H_2O$	-	Ferric chloride
Fig.	-	Figure
FRAP	-	Ferric reducing antioxidant power
$FeSO_4 \cdot 7H_2O$	-	Ferrous sulphate
g	-	Gram
GAE	-	Gallic acid equivalent
GC	-	Gas chromatography
GC-MS	-	Gas chromatography-mass spectrometry
GLC-82	-	Human lung adenocarcinoma cell line
H1299	-	Human non-small lung carcinoma cell line
H_2O_2	-	Hydrogen peroxide
H_2SO_4	-	Sulphuric acid

HCl	-	Hydrochloric acid
HCT	-	Human colorectal carcinoma cell line
HCT116	-	Colon cancer cell line
HeLa	-	Human cervical cancer cell line
Hep3B	-	Human hepatoma cell line
HepG2	-	Human hepatocellular liver carcinoma cell line
HL60	-	Human promyelocytic leukemia cell line
HO-8910	-	Human ovarian carcinoma cell line
HOS	-	Human osteosarcoma cell line
HPB-ALL	-	Human T cell leukemia
HPLC	-	High Performance Liquid Chromatography
HS-SPME	-	Headspace solid phase microextraction
HT-29	-	Human colon carcinoma cell line
IAEC	-	Institutional animal ethics committee
IC_{50}	-	Inhibitory concentration at 50%
I. coetsa	-	*Isodon coetsa*
IL-1β	-	Interleukin-1 beta protein
IL-6	-	Interleukin 6
IR	-	Infra red
IMR-32	-	Human neuroblastoma cell line
I. nigrescens	-	*Isodon nigrescens*
I. nilgherricus	-	*Isodon nilgherricus*
K562	-	Human lymphocytic leukemia cell line
KB	-	Human oral epidermoid carcinoma cell line
KB-V	-	Vinblastine-resistant KB cell line tested in the presence (+VLB) or absence (-VLB) of 1μM vinblastine
Kg	-	Kilogram
KH_2PO_4	-	Potassium dihydrogen phosphate
KOH	-	Potassium hydroxide
L1210	-	Murine lymphoma cell line
LC-MS	-	Liquid chromatography-mass spectrometry
LNCaP	-	Hormone-dependent human prostatic cancer cell line
LoVo	-	Human colon adenocarcinoma cell line
LPS	-	Lipopolysaccharide

Lu1	-	Human lung cancer cell line
m	-	Metre
MCF7	-	Human breast cancer cell line
MDA-MB-231	-	Human breast cancer cell line
MDA-MB-453	-	Human breast cancer cell line
ME180	-	Human cervical carcinoma cell line
mg	-	Milligram
min	-	Minute
mmol	-	Mill mole
MKN-28	-	Human gastric cancer cell line
MKN45	-	Human gastric cancer cell line
ml	-	Millilitre
MS	-	Mass spectrometry
NaCN	-	Sodium cyanide
Na_2CO_3	-	Sodium carbonate
Na_2HPO_4	-	Disodium hydrogen phosphate
$NaNO_2$	-	Sodium nitrite
NaOH	-	Sodium hydroxide
NB4	-	Acute promyelocytic leukemia-derived cell line
NBT	-	Nitroblue tetrazolium
NF-κB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nm	-	Nanometre
NMR	-	Nuclear Magnetic Resonance
NO	-	Nitric oxide
OD	-	Optical density
p65	-	Nuclear factor NF-kappa-B p65 subunit
PANC-1	-	Pancreatic carcinoma cell line
PBS	-	Phosphate buffered saline
PC-3	-	Human prostate cancer cell line
PC-9/ZD	-	Lung cancer cell line
PC12	-	Rat pheochromocytoma cell line
QE	-	Quercetin equivalent
QGY-7701	-	Human hepatocellular carcinoma cell line
RAW 264.7	-	Murine macrophage cell line
RCL	-	Range of chromosome length

SD	-	Standard deviation
SDE	-	Simultaneous-distillation extraction
SE	-	Standard error
SGC790	-	Gastric cancer cell line
SGC-7901	-	Human gastric cancer cell line
SHG-44	-	Human glioma cell line
SH-SY5Y	-	Human neuroblastoma cell line
SK-BR-3	-	Human breast cancer cell line
SK-OV-3	-	Human ovarian cell line
SMMC-7721	-	Human hepatoma cell line
SOD	-	Superoxide dismutase
SPC-A-1	-	Lung cancer cell line
SW1990	-	Human pancreatic adenocarcinoma cell line
SW480	-	Human colon adenocarcinoma cell line
SW626	-	Ovarian cancer cell line
T24	-	Human bladder carcinoma cell line
TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric acid reacting substances
Tb	-	Human oral squamous cell carcinoma line
TCL	-	Total chromosome length
TF%	-	Total forma percentage
TNF-α	-	Tumor necrosis factor alpha
TpTz	-	2,4,6-Tripyridyl-s-triazine
U87	-	Human primary glioblastoma cell line
U251	-	Human glioma cell line
U937	-	Human leukemic monocyte lymphoma cell line
VC	-	Variation coefficient
v/v	-	volume/volume
WBC	-	White blood corpuscles
WHO	-	World Health Organization
ZR-75-1	-	Hormone-dependent human breast cancer cell line

Introduction

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

INTRODUCTION

Human beings, the most triumphant creations in the history of earth, are always in the run to conquer the new frontiers of universe. In an attempt to become the master of all trades, man has been a failure in identifying the devastating consequences which underlie unrevealed. This has been clearly established in the field of medical science. Greatest desire of medical world is to control the human body from being attacked and destroyed by diseases. Scientific community ardently works for a world without illness, but in vain. New diseases crop up each day not only from the biological world but also from the synthetic drugs used as cures. Added to this, lifestyle diseases are also establishing which are ignored in the busy schedule. The boom of pharmaceutical industry with the use of synthetic drugs in controlling the microbial world and other diseases are at a setback due to the unexpected reversal of drugs causing diseases. Along with the side effects, the high cost, inadequate supply of drugs to the rising population especially in developing countries as well as development of resistance to drugs and diseases are the hurdles to be surmounted.

Health concerns are the greatest nightmare in human life. Expected illness like accidents, animal bites/stings, injuries, *etc*. can be cured to a certain extent but when the disorders are from within like metabolic errors, genetic disorders, cancer, *etc*., the remedies need to be sorted. The millions of years of life on earth have indirectly taught the bountifulness of nature in protecting life. Nature with its inexplicable resources has always been a savior but the benefits and detriments depend on the way it is exploited. In the fast developing world, instant healers are preferred with the consequences unknowingly accepted. Plant drugs constitute only 25% of the total drugs consumed in developed countries like United States while the contribution is

about 80% in developing countries like India and China (Joy *et al*., 2001). Synthetic drugs in pharmaceutics which are only target specific have the power of restoring health rapidly but the penalty to be paid is far above than expected. Target specificity of drugs is a curse in disguise since it only focuses on the element to be cured leaving aside the associated links which becomes the cause of future tribulations.

From the ancient era, diseases were controlled and cured by drugs prepared from herbal formulations. But, with the advancement of science and research, the use of herbs was surpassed by synthetic and modern medicine. The safety and security concerns are now overpowering the time bound mind which makes man revisit and reutilize the vast garden of naturals. Herbs and herbal formulations have always been associated with traditional medicines commonly used in a particular area. The World Health Organization observes that around 80% of the population in developing countries relies greatly on traditional medicines. Herbalism has its lineage in many countries but the most established are those of Asian origin where ideas of diagnosis and treatment are from Ayurveda, Unani, Siddha (India), Wu-Hsing (China) and Kampo (Japan) systems of medicine (Vogel, 1991; Wong *et al*., 1998; Kanba *et al*., 1998). Herbs, in general, have the ability to kindle the self healing powers of the body and eradicate the underlying causes of illness. The hidden potentials of herbs left unexplored needs to be utilized for attaining new heights in the medical field. The Chinese are one of the ancient groups which explored herbal remedies even well before Indians. They had 350 herbal remedies when the world began developing systematic pharmacopoeias in 3000 BC. China uses traditional medicines in the best possible way by pharmacologically validating and improving herbal medicines and integrating it with formal health care systems (Joy *et al*., 2001). Roughly estimated, about 70,000 plant species are ethno-medicinally used worldwide (Haq *et al*., 2012).

Researchers in pharmaceutics are now in the search of drugs which are cost efficient, have apparently no side effects and less prone to developing resistance. Herbal medicines use plant or plant parts while drugs have the active chemical components isolated. Traditional medicines have the uncharted information regarding thousands of plants with potential curing properties which can be a good directive in the preparation of safe and secure drugs. The prime focus of researchers in herbal drug preparations are on plants which can be identified based on ethno-medicinal use and hence can be sourced easily (Verpoorte *et al*., 2005). Folk medicines thus has its importance in the scientific world where the practitioner has prior knowledge about healing practices, medicines and ideas about body physiology and health preservation based on the cultural norms of particular area (Bakx, 1991; Acharya and Shrivastava, 2008). Moreover, most of the modern medicines in production are primarily based on plants having its origin in folk medicines (Gilani, 2005).

The future of organic compound drug development programmes rely mainly on natural products (Newman and Cragg, 2007) which are essentially procured from traditional folk medicines. The indigenous people have extensive ideas regarding the properties of the plants from the long and intimate association with their floras, experimentations and floral dependence for a living (Schultes, 1994). This imparted knowledge needs to be channelized for the development of efficient drugs. Drug development urges the scientists to search for particular medicinal properties, rather than the holistic approach towards folk literature or known cures, in the aspiration to manipulate synthetic compounds which only delays the application of potential remedies to a more efficacious product. Promotion of folk medicine aims to sort out the discarded knowledge and act as a complement to modern medicine. Biomedical researchers have always employed plants with ethnopharmacological relevance for developing new drugs (Rao *et al*., 2008).

It is noteworthy that the products related to folk medicine accounts for 30% of the top 35 worldwide natural product-based drugs sold (Butler, 2004) and that 80% of the 122 plant-derived drugs used are related to their original ethnopharmacological purposes (Fabricant and Farnsworth, 2001). Understanding the importance of traditional medicines, the US Government had established the 'Office of Alternative Medicine' at the National Institute of Health at Bethesda for a possible integration of traditional systems of medicines such as Chinese, Ayurveda, *etc*. with modern medicines.

Folk medicines need to be thoroughly exploited for the development of phytomedicines. The flora confined to a particular area is the key to folk literature which needs to be conserved and protected as the destruction of environment is a threat to the uninvestigated potential plants which might become extinct unknowingly. In general, it is estimated that 121 major plant drugs of known structure are unique with no synthetic substitutes produced (Joy *et al*., 2001). Thus, cultivation, conservation and preservation are inevitable for which pharmacovigilance is the initial step. Folk medicines for particular cure needs to be identified, standardized, validated and analyzed for its biological properties. An interdisciplinary holistic approach is the need of the hour which can bear fruits in pharmacognosy research.

The members of the family Lamiaceae are one of the most sought after in traditional folk medicine especially by the Chinese and Turkish people for centuries. It has a global distribution with about 240 genera and 7200 species occurring mainly in tropical and temperate regions except Antarctica (Harley, 2012). The characteristic feature of the family includes squarish or quadrangular stem with simple opposite decussate leaves, zygomorphic bilabiate flowers arranged in verticillasters, superior ovary and gynobasic style. The aromatic and culinary herbs of Lamiaceae such as mint, rosemary, sage, savory, marjoram, oregano, thyme and lavender has been widely used,

and it belongs to the most diverse family in terms of ethnomedicine. The medicinal value of the members are due to the volatile oils produced by the external glandular structures (Sarac and Ugur, 2007; Giuliani and Bini, 2008). Volatile oil seems to have significant role in pesticide, pharmaceutical, flavouring, perfumery, fragrance and cosmetic industries (Özkan, 2008). The members being rich in terpenoids, which includes mono-, sesqui-, di- and triterpenes, along with flavonoids and phenolics (Ulubelen *et al*., 2005) possess a number of biological activities like antimicrobial, anti-inflammatory, antioxidant, antiviral, cytotoxic, wound healing, neuroprotective, anticholinesterase (Perry and Howes, 2011) as well as healing quality identified from folk medicine towards various disorders like common cold, throat infections, psoriasis, seborrheic eczema, hemorrhage, menstrual disorders, miscarriage, ulcer, spasm and stomach problems (Topcu and Kusman, 2014). The aforesaid properties makes the family an apt choice for research since it has the hidden potentials to be identified and developed e preparation of efficient drugs towards various ailments.

Isodon (Schrad. ex Benth.) Spach (tribe: Ocimeae Dumort.; subfamily: Nepetoideae) is a cosmopolitan genus of *c.* 100 species in Lamiaceae family (Harley *et al*., 2004) and is widely used in traditional Chinese and Japanese medicine for the treatment of various ailments from ancient times. The distribution of the genus is primarily observed in tropical and subtropical Asia, with a centre of species diversity (*c.* 70%) in the Hengduan Mountain region of south-west China. It also ranges west to Afghanistan and Pakistan further up to central and southern Africa (Yu *et al*., 2014). The genus includes undershrubs, subundershrubs or perennial herbs with paniculate inflorescences composed of many flowered cymes. In general, *Isodon* can be distinguished from other members of tribe Ocimeae based on a combination of morphological characters which include bracteolate cymes, 4/1-bilabiate corolla limb, equally or sub-equally 5-toothed or 3/2-bilabiate calyx and free

filaments inserted at the base of the corolla tube (Li, 1988). None of these characters are unique to *Isodon* but a combination of these features makes the genus stand out from other Lamiaceae members. *Isodon* can be distinguished from its allied genera by its paniculate inflorescence, staminal filaments attached to above the middle of the corolla tube, corolla lip being concave and in the presence of bracteoles in the inflorescence (Zhong *et al*., 2010).

Isodon, formerly called *Rabdosia*, has many of its members being transferred from *Plectranthus* due to morphological and floral differences. *Isodon* differs from *Plectranthus* in having flowers borne in dichotomous cymes produced in the axils of leafy bracts and the calyx with subequal, shortly triangular teeth (Codd, 1968). Moreover, the position of the genus in systematic classification is not yet clarified. Ryding (1993) during the studies on *Rabdosiella* examined the relationship between *Plectranthus*, *Isodon* and *Rabdosiella*, and suggested that *Isodon* was more closely related to subtribe Hyptidinae and was misplaced in subtribe Plectranthinae. But Paton and Ryding (1998) identified a close relationship between *Isodon*, *Hanceola* and *Siphocranion* and placed the genera as *incertae sedis*. Further studies of Harley *et al*. (2003) suggested that *Isodon* forms a branch of polytomy with three other monophyletic clades: subtribe Hyptidinae, subtribe Ociminae and subtribe Plectranthinae. The remaining division of tribe Ocimeae was subtribe Lavandulinae which is sister to other subtribes including *Isodon*. As other elements of the least inclusive clade containing *Isodon* are recognised as subtribes, *Isodon* along with *Hanceola* and *Siphocranion* are recognised as subtribe Hanceolinae. According to Paton *et al*. (2004), tribe Ocimeae is a well supported monophyletic group with a morphological synapomorphy of dorsifixed, synthecous anthers. But the relationships among *Isodon*, Hyptidinae, Plectranthinae and Ociminae could not be resolved. The analysis indicated that only Hyptidinae had a clear non-molecular synapomorphy of a hinged anterior corolla lip and that *Isodon* does not clearly belong to any of

the existing subtribes. Moreover, the close affinities between *Isodon*, *Hanceola* and *Siphocranion* were not supported by the analysis conducted by Zhong *et al*. (2010). The studies could not support the occurrence of a single clear sister taxon for *Isodon*. Neverthless, the *Isodon* species inclusive of *Skapanthus* formed a strongly supported monophyletic lineage separate from the clades containing other genera and subtribes suggesting that *Isodon* represents a distinct subtribe within Ocimeae. Morphological synapomorphies such as the basal insertion of stamens in the corolla tube and the absence of enlarged finger-like abaxial disc lobes further support the need for a new subtribe Isodoninae, sister to Ociminae in addition to Plectranthinae and *Hanceola* along with *Hyptis* clades. Thus, considering the latest studies of Zhong *et al*. (2010), it may be concluded that *Isodon* belongs to the subtribe Isodoninae within the tribe Ocimeae and that the relationship of *Isodon* within the tribe Ocimeae along with the position of subtribe Isodoninae remains unresolved.

Genus *Isodon* is a popular member of Chinese folk medicine with long tradition in the treatment of respiratory and gastrointestinal bacterial infections, inflammations and cancer (Sun *et al*., 2006). The medicinal properties can be attributed to the wide range of biologically active diterpenoids which are the principal secondary metabolite found in the genus. The basic hydrocarbon backbone structures identified in *Isodon* diterpenoids generally include kauranes (the most abundant), abietanes, labdanes, pimaranes, isopimaranes, gibberellanes, clerodanes and atisanes (He *et al*., 2009; Li *et al*., 2010a). The classification of isolated diterpenoids from *Isodon* based on the structure led to the identification of eleven groups with five sub-groups (Sun *et al*., 2006). It involves C-20 non-oxygenated *ent*-kauranes with 212 compounds, C-20 oxygenated *ent*-kauranes with 200 compounds, 6,7-seco-*ent*-kauranes with 105 compounds, 8,9-seco-*ent*-kauranes with two compounds, 8,15-seco-*ent*-kauranes and 15,16-seco-*ent*-kauranes with four

compounds, 7,20-cyclo-*ent*-kauranes and *ent*-kaurane dimers with one compound each, miscellaneous *ent*-kauranes with four diterpenoids, *ent*-gibberellane with one compound, abietanes and *ent*-abietanes with 15 compounds and, other tricyclic and bicyclic diterpenes which include pimarane and kaurane, *ent*-labdanes and *ent*-clerodanes. These diterpenoids act as potential antibacterial, antitumour, anti-inflammatory and anti-feeding agents which imply its significance in drug research.

Several diterpenoids isolated from the genus have been shown to possess varied biological activities. The genus is also extensively used as possible cure for various diseases as far as folk literature is concerned. One of the most significant bioactivity observed is the antitumor activity with low toxicity which has its lineage in folk medicine. Cancer is the most devastating disease engulfing the human race in recent years. Biomedical researchers are desperate to find a drug which can handle the uncontrolled metabolism of cancer cells. In this scenario, *Isodon* has its relevance in being a subject of anticancer research. The extensive use of the genus in folk medicine does not give it authenticity to be used as a cure against any disease. It needs to be scientifically proven since unknown properties can be a boon or bane. Curative nature of drugs can be reversed if the right dose is not identified. In addition to this, proper identification of the plants is also necessary as adulterants may take the toll of life. Thus, for a plant to be developed as an efficacious drug, several parameters need to be tested and verified.

Misidentification of plants has always been a chaos builder in the scientific world. Vernacular names used in different parts of the world for a single plant along with taxonomic disputes over identification based on morphology have been creating dilemma in giving proper identity for the source plants used for various scientific studies. Apart from this, the plants should be tested for its biological activity and the effective dosages need to be

found out. Non-judicious use of any drug, be it natural or synthetic, can be lethal. Folk medicines generally employ crude form of drugs which are believed to possess complementary activity reducing the side effects. The synergistic effect of the crude drug should be thus compared to the activity of individual components for a proper selection of plant form in the preparation of an efficient drug. This is particularly considered, since many individual components are not found to replicate the activity of crude extracts. Phytochemical screening of the genus is also utmost necessary as the components producing the activity can be identified and be cautious about any toxic components present in the extract. Thus, a holistic interdisciplinary approach is essential in the development of a new drug which can in effect control and cure the diseases without any harmful side effects.

Isodon being a genus with considerable taxonomic dispute and intricacy in identifying features, reliable stable characters needs to be studied for ensuring the individuality of its members. Incorrect identification of plants used especially in food and medicines can lead to intoxication in extreme cases. Chromosome number has its own say in furnishing identity of a species. Each species possess a characteristic number of chromosomes which generally varies only in populations that are in a continuous frequent process of evolution and speciation. Apart from this, exceptions to constancy of chromosome number is observed in species with several cytotypes and supernumerary or B chromosomes (Heslop-Harrison and Schwarzacher, 2011; Jones *et al*., 2008). Supernumerary chromosomes are small chromosomes derived from standard chromosomes which have detectable negative effects on phenotype but survive and amplify in number within some plants. In general, chromosomes evolve in the process of fission or fusion accompanied by duplication and inversion of chromosome arms which may or may not result in change in chromosome number (Jones, 1998).

Researchers interested in evolutionary relationships (Guerra, 2008; Levin and Wilson, 1976) predominantly at the generic level (Goldblatt, 2007) had focussed on the number of chromosomes in each taxa. Calculation of chromosome size enables the chromosome number data to complement genome size estimates which correlates well with evolutionary age (Mehra, 1972). Chromosome number data is also a useful tool for plant breeders since variations in it can influence inbreeding depression and the potential for introgression of traits resulting to changes in breeding strategy (Fehr, 1991). Cytogenetics employs number and morphology of chromosomes as the major distinctive characters (Biondo *et al*., 2005) and a comparative analysis helps in establishing the unique features of each species as well as the common features in a group of closely related taxa (Guerra, 1990). Thus, chromosome number and other morphological features can be used as an added parameter in the taxonomic distinction of various species from allied taxa. The chromosome number can be considered as a plesiomorphic characteristic of a large clade or a recurrent trait which arose independently in two or more clades (Guerra, 2008) that needs to be predicted based on various karyotypic characters.

Cytotaxonomy relies on the fact that closely related taxa possess more similar karyotype than less related ones. The chromosomal characters analyzed may represent a continuous (*e.g*. total length of the chromosome set, symmetry indices, nuclear DNA amount) or discontinuous variation (*e.g*. chromosome number, heterochromatic bands, number of rDNA sites) (Guerra, 2012). The entire chromosome complement of a nucleus is considered as the karyotype and it differs in a distinct manner between organisms of defined phylogenetic relationship. The size of metaphase chromosomes varies but the upper and lower tolerance limits are apparently determined by the genome size, chromosome number and karyotype structure (Schubert, 2007). The karyotypic features, in general, are found to be

systematically informative (Stebbins, 1971; Kenton *et al*., 1986; Bernardello and Anderson, 1990). Karyotype represents the genome itself and hence, the parameters like environmental conditions, age, developmental phase, *etc*. do not affect its phenotype at metaphase. Moreover, the changes like polyploidy, chromosome inversions and reciprocal translocations have predictable consequences for the fertility of those individuals. Hence, cytological data can be considered as excellent and unparalleled source of information for taxonomic and evolutionary studies (Guerra, 2012).

Karyosystematics appraises the genetic relationships among species or population and helps to comprehend the way it diverged from each other (Guerra, 2008) as well as supports the taxonomic studies in a genus (Pedro and Delgado-Salinas, 2009). The advent of molecular cytogenetics, phylogenetics and genomics in the last 20 years resulted in detailed studies on chromosome structure and on the mechanisms generating chromosomal changes (Lysak *et al*., 2006; Abrouk *et al*., 2010; Wu and Tanksley, 2010; Heslop-Harrison and Schwarzacher, 2011). The chromosomal changes in combination with phylogenetic trees revealed the recurrent nature of chromosome characteristics (Murray, 2002) and substantiated the need for precise cytological evidence in the interpretation of chromosome data (Dobigny *et al*., 2004). On detailed study, the classical karyotype rearrangement of shift in centromere position was found to be explained as due to pericentromeric inversion, deletion and duplication (Guerra, 2012). The chromosome number, size, lengths of arms, position of centromere, secondary constriction and related parameters thus have its own significance. The relationship between karyotype evolution and related taxa can be inferred from the variations in the number and position of satellites in karyotypes (Rodrigues *et al*., 2014). Hence, a precise karyomorphometric analysis along with chromosome number study of each species can render a helping hand to sort out the issues related to taxonomic disputes in closely allied taxa.

Cancer can be ironically considered as an effective population control mechanism as it is the leading cause of death in economically developed countries and second leading cause of death in developing countries (WHO, 2008). Research on cancer has attained heights as far as molecular basis of the disease is concerned but the progress is not at par when the control and remedial measures are looked upon. Cancer treatment revolves around surgery, radiation therapy, chemotherapy and immunotherapy (DeVita and Rosenberg, 2012) but development of chemo-resistance and radio-resistance hampers the expected outcome. Preventive measures are yet to be apprehended with only the historic development of vaccines against cancers caused by viral infections (DeVita and Rosenberg, 2012). In the current scenario, regimens of cancer have limited survival value especially in advanced stage cancers since the treatment targets the tumors rather than cancer stem cells (Reya *et al*., 2001; Dean *et al*., 2005). Conventional cancer therapy shows lack of efficacy in the long run as it targets the fast growing cell, leaving aside the cancer stem cells. Furthermore, non-specificity of treatments resulting in toxic effects on normal cells and, high resistance to drugs and slow proliferation rate of cancer stem cells (Jones *et al*., 2004) adds fuel to the unproductive treatment.

Efforts are being made to control the disease but factors promoting the diseases and the tissues affected are too complex to be clasped. Traditional therapy is detained by chemo-resistance and radio-resistance (Moltzahn *et al*., 2008) and hence, the disease can be subjugated only by eradicating cancer stem cells. Burden of cancer is enhanced especially in economically developing countries through population aging and growth, along with adoption of cancer associated lifestyle choices which includes smoking, physical inactivity and westernized diets (Jemal *et al*., 2011). Economically developing countries with the toll of its life being taken away by cancer are unable to afford the expenses of cancer therapy especially cancer stem cell

eradication. Folk literature has details regarding herbs used against cancer which needs to be explored, tested and verified. This will provide the economically backward class a ray of hope against the chronic disease at a cheaper rate. Apart from cost, the side effects of drugs and development of resistance will be comparatively less for herbal preparations.

Carcinogenesis is a multistep process which involves initiation level where damage to deoxyribonucleic acid occurs followed by promotion level where the cells proliferate and expand into abnormal cells and finally in the progression stage, malignant cell formation takes place (Klaunig and Kamendulis, 2004). Cancer is constrained from attaining devastating ends commonly by combined treatment with surgery, chemotherapy and radiotherapy. Radiotherapy as well as chemotherapy kills cancer and tumor cells by elevating the levels of oxidants. Radiotherapy induces direct lesions on DNA or biological molecules which deregulate cell division and finally destroy the daughter cells (Baba and Câtoi, 2007). Chemotherapy drugs act by the mechanism of production of reactive oxygen species or free radicals in which chemical substances operate on mitotic cells whereby the antimitotic agents destroy cancer cells. Apart from free radical production, the side effects associated with these drugs include nephrotoxicity, ototoxicity, cardiotoxicity, peripheral neuropathy, *etc*. (Badajatia *et al*., 2010; Fuchs-Tarlovsky, 2013). Cell signaling and bacteriocidal actions of phagocytes are mediated by free radicals and other oxygen species which make it essential for life (Conklin, 2000). But the increased concentrations can be hazardous to the cell components like DNA, protein and cell membrane which can reverse the effect into development of cancer and other diseases (Diplock *et al*., 1998; Valko *et al*., 2007). Thus, free radical formation needs to be under control in biological systems for a healthier lifecycle.

Reactive oxygen species which include singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide are commonly generated from endogenous factors like aerobic respiration or as byproducts of biological reactions and from exogenous factors (Halliwell and Gutteridge, 1990; Wiseman and Halliwell, 1996). Along with this, the oxidant load of the body is elicited by various factors like vigorous exercise which accelerates cellular metabolism, chronic inflammation, infections and other illnesses. The exposure to allergens, presence of leaky gut syndrome and exposure to drugs or toxins such as cigarette smoke, pollution, pesticides and insecticides are also favorable aspects (Sarma *et al*., 2010). Pathogenesis of numerous disorders and pathophysiological processes of cardiovascular diseases, diabetes and cancer are found to be significantly effected by oxidative stress (Evans, 2007). It is the result of imbalance between the production and removal of reactive oxygen species or reactive nitrogen species (Kowluru and Chan, 2007). Reactive oxygen species mediated injury is usually counterbalanced by the endogenous antioxidant defense systems which function by chelating and clearing intracellular oxidation activity and, by accumulation and maintenance of redox equilibrium (De Bont and Van Larebeke, 2004) which on the other hand can result in irreversible modification of cellular viability and functions (Klaunig and Kamendulis, 2004). The endogenous enzymatic antioxidant defenses include superoxide dismutase, glutathione peroxidase and catalase which compensate the oxidative microenvironments by chelating superoxide and various peroxides while the non-enzymatic endogenous antioxidants like vitamins E and C, coenzyme Q, β-carotene and glutathione have the capacity to quench reactive oxygen species activity (Ziech *et al*., 2010). But this is not sufficient for the balanced physiological system of human body which can only be maintained by added antioxidant supplements.

Natural antioxidants are capable of functioning as reducing agents, free radical scavengers, complexes of pro-oxidant metals and quenchers of the formation of singlet oxygen (Pratt, 1992). The overproduction of free radicals as well as reactive oxygen species along with decreased physiological antioxidant capacity is mainly due to the changed lifestyle (López *et al*., 2007) which needs to be surmounted. The endogenous antioxidant systems are augmented with exogenous supplements like fruits, vegetables and grains called as dietary antioxidants (Diplock *et al*., 1998; Bouayed and Bohn, 2010) to cope with the increasing need. *In vitro* and *in vivo* studies on exogenous antioxidants have shown to prevent the types of free radical damage associated with cancer development which supports the fact that dietary antioxidants act as cancer preventing agents (Miranda-Vilela *et al*., 2011). Antioxidants from natural sources are always in demand and if obtained as added advantage with food and medicines is a sweepstake.

Many herbs are found to possess antioxidant compounds which protect cells from reactive oxygen species (Narayanaswamy and Balakrishnan, 2011). Medicinal plants are rich sources of phenols and flavonoids which are found to possess antioxidant activity. This helps in maintaining the balance between oxidants and antioxidants thus combating oxidative stress (Scalbert *et al*., 2005). Cancer chemotherapy is enhanced as well as the side effects are reduced through the dietary supplementation with antioxidants (Conklin, 2000). Antioxidant administration prior to tumor inoculation has depicted tumor growth inhibition (Miranda-Vilela *et al*., 2011) which shows the effectiveness of antioxidant systems. Thus, antioxidants are an indispensable part of life which protects the body from several unfavorable consequences. Causatives of cancer as well as treatment are well complemented by exogenous antioxidants. This emphasizes the fact that antioxidants should be included in the diet of a healthy human being. Herbs used as medicines are generally rich sources of phenols and flavonoids

which are excellent antioxidant suppliers and the side effects are considerably less. This may be the reason behind successful advocation of folk medicines for various diseases. Herbal remedies with anticancer property having additional benefit of providing antioxidants will be the focus in biomedical research which needs to be further exploited for the development of a feasible drug in cancer therapy.

Cell death is a natural phenomenon which balances cell division and is essential for maintaining the normal well being and size of all life forms. The altruistic death of each cell upholds the functionality of every organism (Schulze-Osthoff, 2008). Based on the characteristic morphology, cell death can be distinguished into apoptosis and necrosis (Jurisic and Bumbasirevic, 2008). Apoptosis or programmed cell death eliminates the damaged or unwanted cells of the body mediated through the intrinsic/mitochondrial apoptotic pathway initiated by intracellular apoptotic signal or extrinsic apoptotic pathway initiated by stimulation of the cell surface death receptor by the binding of death ligand (Elmore, 2007; Deep *et al*., 2010). Necrotic cells are morphologically characterized by swelling of cytoplasm, vacuolation, plasma membrane rupture, organelle dilation and moderate condensation of chromatin which educes the damaging inflammatory response while the cells burst spilling the contents (Festjens *et al*., 2006). Cancer cells with its alarming rate of progression needs to be controlled and eliminated and is made possible by the apoptotic and necrotic cell death phenomenon. But the innate immunity is not sufficient enough to overwhelm such devastating stages and needs to be conquered with effective drugs.

Normal growth and development necessitates cell death regulation, abnormalities of which can elicit diseases like cancer, autoimmune syndromes, AIDS, ischemia, liver diseases and neurodegenerative disorders such as Parkinson’s and Alzheimer’s disease (Fischer and Schulze-Osthoff,

2005). Defective apoptosis can be considered as the major driving force behind development and progression of cancer (Amirkhiz *et al*., 2013). Cell mediated cytotoxicity involves two possible complementary cytocidal mechanisms which are the apoptotic mechanism where the genomic DNA is fragmented before cell lysis due to the activation of an autolytic cascade in the target cell by the effector cell and the lytic mechanism where the cell lysis occurs due to the formation of pores in the target cell membrane by the polymerization of the lytic molecules, markedly perforin, secreted by the effector cell into the intercellular space (Schulze-Osthoff, 2008). Prior to lysis, apoptotic cells are rapidly phagocytosed to cause inflammation which is essential for tissue reconstitution in tissue development and after cell damage. Apoptosis characteristically prevent spread or release of DNA from transformed and virus-infected cells but tumor cells escape apoptosis machinery using diverse mechanisms to suppress apoptosis resulting in cancer growth (Sachs and Lotem, 1993). Hence, therapeutic strategies need to be devised for amending life-death mechanism of cells.

Cancer is a disease with complex symptoms and developing mechanisms, the proliferation of which is mainly due to circumventing of the apoptotic pathway. Traditional cytotoxic chemotherapy entails indirect induction of apoptosis but the side effects are alarming and in the long run, tumors develop resistance to cell death (Jordan and Wilson, 2004; Kawabe, 2004; Garber, 2005). Cell cycle modulation is a prerequisite which can be accomplished only from natural products due to its diverse chemical structures and pharmacological properties. The understanding of the cellular growth control mechanism contributes to the exploration and identification of compounds with specific antitumor activity (Kang *et al*., 2000). Plants used as antitumor agents are rarely found in literature since the symptoms of cancer are too complex to be differentiated. The ethnopharmacological use of plants in skin disorders, inflammatory, infectious, parasitic and viral diseases can be

generally investigated since it has the probability of reflecting disease states, weighing significance to cancer or a cancer symptom (Cordell *et al*., 1991; Popoca *et al*., 1998).

Genotoxic assessment of traditional medicines is essential to identify the potential effects on genetic material since negative outcome could cause critical mutations increasing the risks of cancer and other diseases (Celik, 2012). The greatest concern lies with DNA damage without concomitant cytotoxicity than those with simultaneous evident signs of cytotoxicity (Galloway *et al*., 1998; Galloway, 2000). Many plant extracts and active principles are being described and employed as therapeutic agents which needs to be analyzed for potential mutagenic, carcinogenic and antigenotoxic properties. Thus, various experimental systems [*e.g*. membrane systems, plant test systems, cell culture, animal models, humans (clinical trials)] are employed to ensure the relatively safer use of medicinal plants (Celik, 2012). Permeability assays are extensively accepted as measurement of cytotoxicity. Cell viability assays based on alterations of plasma membrane permeability resulting in the consequent release of components into the supernatant which are excluded by viable cells are usually used to identify the toxicity of extracts (Schulze-Osthoff, 2008). Tumor cells depicts uncontrolled proliferation and are exploited in cell line culture in controlled environment (Makari *et al*., 2008) for cytotoxicity screening to identify plant extracts with potent antitumor activity. The multiple mechanisms of cancer to escape regulated growth and to evade apoptosis need to be subjugated. Crude plant extracts with a diverse array of components can be a possible solution rather than isolated compounds due to the unified activity and multiple targets of the constituents (Solowey *et al*., 2014). The various *in vitro* and *in vivo* assays inclusive of cytotoxicity, cell viability as well as antitumor studies using animal models are excellent techniques in identifying elements of herbal remedies with affirmative outcome in cancer therapy.

Biomedical research mainly focuses on natural products obtained from herbs used in folk medicines. The natural products of therapeutic importance are the secondary metabolites produced as a result of various biotic and abiotic stresses from the diverse flora on earth which are encoded to be bioactive and a reservoir of potential drugs (Lamottke *et al*., 2011). Secondary metabolites can be commonly classified into three main groups which includes terpenes (plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (alkaloids and glucosinolates) (Agostini-Costa *et al*., 2012). These bioactive non-essential nutrients in plants possess diverse human health effects of putative chemo-preventive properties as well as intrusion in tumor promotion and progression (Surh, 2003; Kwon *et al*., 2007) which needs to be exploited and utilized in the preparation of efficacious drugs.

Terpenes are the largest and the most diverse class of secondary metabolites (Breitmaier, 2006) and are generally classified as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}) and sesterterpenes (C_{25}) based on the number of building blocks (Wang *et al*., 2005a). Pharmacological studies reveal that terpenes possess immense bioactivity which include antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory, chemotherapeutic and immunomodulatory properties (Frank *et al*., 2002; Friedman *et al*., 2002; Hammer *et al*., 2003; Wagner and Elmadfa, 2003; Paduch *et al*., 2007). Phenols are another class of compounds with excellent antioxidant capacity and are found to possess anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral and anti-inflammatory activities (Owen *et al*., 2000; Veeriah *et al*., 2006; Baidez *et al*., 2007; Han *et al*., 2007). Phenols, in general, include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones and phenolic mixtures,

and other phenylethanoids and phenylpropanoids. The pharmacological activity can be attributed to the antioxidant and free radical scavenging properties as well as due to the function of regulating detoxifying enzymes (Surh, 2003). Flavonoids, commonly formed from the aromatic amino acids phenylalanine and tyrosine, and malonate (Davies, 1995), are found to possess anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties (Middleton, 1998). It can be classified into flavones, flavonones, catechins and anthocyanins based on the molecular structure (Rice-Evans *et al*., 1996). Moreover, flavonoids are able to scavenge a wide range of reactive species (*e.g*. hydroxyl radicals, peroxyl radicals, hypochlorous acid and superoxide radicals) (Hollman and Arts, 2000) and chelate transition metal ions which decreases the ability to promote reactive species formation. It also inhibits bio-molecular damage by peroxynitrite *in vitro*, prevent carcinogen metabolic activation, induce apoptosis by arresting cell cycle, promote differentiation, modulate multidrug resistance and, inhibit proliferation and angiogenic process (Hollman and Arts, 2000; Ahmad *et al*., 2006; Tsuji and Walle, 2006; Shih *et al*., 2007). The quantification of the secondary metabolites is thus essential to identify the hidden therapeutic potential in the plants used in folk medicine which will make a marked difference in pharmaceutical research.

Herbal extracts demand for characterization, standardization and quantification for the safe and effectual use in biomedical research. Separation, detection, mass determination and structure elucidation are some of the competent techniques which could be employed for determining the credibility of a drug. Gas chromatography mass spectrometry (GC-MS) and Liquid chromatography mass spectrometry (LC-MS) are two techniques commonly employed for unequivocal detection of individual components in complex mixtures. The separation power of gas chromatography is coupled with the detection power of mass spectrometry in GC-MS technique where

the production, subsequent separation and identification of charged species occurs based on mass to charge (m/z) ratio. It is an ideal system for the qualitative and quantitative determination of volatile and semi-volatile organic compounds in samples even with low detection limit as meager as nanograms. Readily available software with a library of spectra for unknown compounds helps in the detection of compounds (Sneddon *et al*., 2007). A wide range of organic compounds from small molecule drug metabolites to peptides and proteins can be separated by liquid chromatography while mass spectrometers generate three dimensional data which provide information about molecular weight, structure, identity, quantity and purity of a sample. This allows LC-MS in analyzing and characterizing the constituents and their metabolites in a single run along with high selectivity and sensitivity (Raju *et al*., 2015). Thus, GC-MS and LC-MS aids in the identification of the major volatile, non-volatile and organic components in the extracts which might become the milestones in anticancer research.

Traditional medicines have always been a reservoir of potential drugs left unnoticed and unexplored. Nowadays, focus is on herbal formulations especially in developing nations due to the easier access to raw materials, low cost and negligible side effects along with nominal probability of developing resistance. Multiple components enable multiple mechanisms of resistance and targets which helps the herbal medicines to surmount chemo-resistance to a great extent than single agents (Wang *et al*., 2014). Cancer treatment has passed on from conventional chemotherapy to targeted therapeutics which can be accomplished only with the aid of natural products. Natural products are found to mitigate even the side effects of onco-chemotherapy or radiotherapy (David *et al*., 2015). Clinical testing is always necessary for a safer application but the biological effect cannot always be attributed to the presence of a single active natural product. Traditional preparations of tea, decoction, tincture, *etc*. are complicated mixtures of natural products with

multiple target action (Gertsch, 2011). Hence, studies need to be conducted to identify a drug or an extract to be used as a successful medical blueprint.

The genus *Isodon*, a familiar member in Chinese folk medicine is investigated in the present study for its various bioactivities and phytochemicals. Being a genus under scrutiny for its taxonomic identity, the plants chosen are subjected to karyomorphometric studies to yield data on reliable chromosomal characters which can complement cytotaxonomy. The isolated diterpenoids have been extensively focused by researchers but the crude form most commonly utilized by indigenous people have not been much targeted. In this regard, the biological properties of crude extracts are evaluated. Although the study concentrates on crude forms, the phytochemical components are scrutinized to identify the various elements and their multiple targets along with any potential toxic compound which can be hazardous and a warning for the non-judicious use of plants in preparation of competent drugs.

Thus, the present study of chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of *Isodon* aims to fulfill the following objectives:

- Chromosome imaging and karyomorphometric analysis
- Antioxidant studies using free radical scavenging assays
- *In vitro* cytotoxic and *in vivo* antitumor assays to determine the bioactivity
- Estimation of the total phenolic, flavonoid and terpenoid content
- Phytochemical characterization using GC-MS and LC-MS techniques.

Review of Literature

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

REVIEW OF LITERATURE

Human beings' ardent aspiration for development and modernization has ultimately resulted in the negligence of basic amenities of life. In a fast developing world, time has a new sense which makes man unable to think of the consequences that follow to the future. New and genetically advanced diseases pop up in a matter of few days gap in every nook and corner but, in the false belief of being invulnerable to them, the devastating effects are avoided. Drugs especially synthetics are the instant healers while the mishap lies in diseases caused by drugs. Apart from the diseases caused by microorganisms, the one caused by the side effects and overuse of synthetic drugs also impose a major concern. Thus, attention should not only be on the control of diseases but also on the drug chosen for attaining cure.

Nature has solution to all tribulations stimulated by it and hence the beckon should not be avoided. Natural drugs have always had its place in curing ailments and traditional medicines form a directive in achieving the objective. Developing countries are a reservoir of uncharted bounty of nature which needs to be conserved and exploited for the benefit of mankind. Folk medicines have the hidden secrets of healing diseases but the scientific bases, active moiety as well as the safety of use are always overlooked. As aptly stated, the priority should be given in developing potent drugs through exploration and investigation of traditional medicines and natural products (Umadevi, 2000) rather than brooding over the synthetic products. Fervor of man in conquering all the engrossed diseases can only be achieved with the understanding of the biological activities and associated mechanisms of different plants used in various traditional and folk medicines.

One of the inexplicable members of Chinese folk medicines is the genus *Isodon* which has significant therapeutic role in many diseases but the potential is unexploited as far as commercialization is concerned. Being a rich source of diterpenoids, it has a role in pharmaceutics which can be attained only after scientific analyses and clinical trials. Before making such a big leap, a review of the previous studies conducted and the outcome reached needs to be identified. Henceforth, a chronological description of the earlier works reported have been done based on the current topic which deals with chromosome studies, antioxidant and antitumor activities as well as the phytochemistry of the genus *Isodon*.

The three species chosen for the present study includes *I. coetsa*, *I. nigrescens* and *I. nilgherricus* of which much work has not been reported so far especially from India. The works already conducted sheds light on the biological activities the species possesses. Studies on *Rabdosia coetsa* (Buch.-Ham ex D. Don) Hara (Chen *et al*., 1990) revealed the identification of a new diterpene rabdocoetsin D along with rabdocoetsins B, C and rosthorin A from the ethereal extract of the plant. Three diterpenoids isolated from *R. coetsa*, rabdocoetsins A-C, were found to be inactive against HeLa epithelioid carcinoma cells (Xu and Kubo, 1993). Wang and Wang (1998) isolated eleven compounds from the dried leaves of *R. coetsa*. The compounds included gesneroidin C, daucosterol, ursoilc acid, uvaol, oleanolic acid, 2α,3β-dihydroxy-oleanolic acid, betulinic acid, phytol, phytyl myristate, ethyl 9Z,12Z-dien-palmitate and glyceryl 12Z,15Z-dien-arachidate. Liu *et al*. (2006c) isolated and structure elucidated two abietane diterpenoids *viz*., sincoetsins A and B from the aerial parts of *Isodon coetsa* (Buth-Ham ex D. Don) Hara. Bioassay guided isolation of the extract of *R. coetsa* led to the identification of three compounds *viz*., ethyl caffeate, rosmarinic acid and

methyl rosmarinate which depicted angiotensin-converting enzyme inhibitory activity (Li *et al*., 2008b). Zhao *et al*. (2011b) investigated the chemical constituents in aerial parts of *I. coetsa* and identified twenty three compounds of which many had cytotoxic activity against various cell lines. Xerophilusin B showed significant cytotoxicity against HT-29, BEL-7402 and SKOV-3 cell lines along with other compounds *viz*., phyllostachysin A, rabdoternin E, ponicidin, macrocalin B, rosthorin A, lasiokaurin and oridonin while 1-(5-hydroxy-4-methoxyphenyl)-2-[2,6-dimethoxy-4-(2,3-epoxy-1-hydroxypropyl) -phenyl]-oxetane, 15α-hydroxy-6,7-seco-1α,7:11α,6-diolide-*ent*-kaur-16-en, rabdonervosin B, hebeiabinin, rabdoternins A, B, xerophilusin L, rubescensin C and nervosanin B showed non-significant activity. Other compounds isolated include 2α,3α,24-trihydroxy-11α,12α-epoxy-urs-20(30)-en-28,13β-olide, 2α,3α,24-trihydroxy-12,20(30)-ursadien-28-oic acid, coetsin B, rabdocoetsins A, D and rabdoternin B.

CHROMOSOME ANALYSIS

Authentic identification of plants marks the safety and accuracy of herbal drugs which is very much essential for the credibility of medicines. Karyotype analysis in this sense is a noteworthy method which gives data about number, morphology, ploidy and structure of chromosomes. Chromosomes are inexplicable part of the genetic system and depict great consistency than any other character (Den Hartog *et al*., 1979; Iwatsubo and Naruhashi, 1991b) which makes it an acceptable parameter for species identification. The genus *Isodon*, formerly called *Rabdosia*, and many being transferred from *Plectranthus* have an uncertainty in its members which needs to be sorted out. Previous reports as well as the karyomorphometric data enable to generate clarity in the genus identification.

Perusal of literature revealed that the studies on chromosome counts of *Isodon* had begun from the 1950's and around 28 species and five varieties have yielded results. Although there are more number of species to the credit, most of them are same with synonymised names. The taxa of which the chromosome counts were identified includes *I. umbrosus*, *I. excisus*, *I. rugosus*, *I. japonicus*, *I. ternifolius*, *I. coetsa*, *I. lophanthoides*, *I. nilgherricus*, *I. repens*, *I. scrophularioides*, *I. wightii*, *I. ramosissimus*, *I. effusus*, *I. inflexus*, *I. longitubus*, *I. shikokianus*, *I. trichocarpus*, *I. serra*, *I. albopilosus*, *I. flabelliformis*, *I. forrestii*, *I. gesneroides*, *I. parvifolius*, *I. pleiophyllus*, *I. polystachys*, *I. racemosus*, *I. scoparius, I. weisiensis* along with *I. japonicus* var. *glaucocalyx*, *I. lophanthoides* var. *graciliflorus*, *I. shikokianus* var. *intermedius*, *I. shikokianus* var. *occidentalis* and *I. oreophilus* var. *elongatus*. A consolidated data is provided with the reported chromosome numbers as well as the synonymised names in table 1.

Table 1. Reported chromosome counts in *Isodon*

Sl. No.	Species	Haploid count (n)	Diploid count (2n)	Reference
1	*R. umbrosa* (Maxim.) H. Hara var. *hakusanensis* (Kudô) H. Hara [*I. umbrosus* (Maxim.) H. Hara]	-	24	Wakabayashi, 1973
2	*R. excisa* (Maxim.) H. Hara [*I. excisus* (Maxim.) Kudô]	-	24	Sokolovskaya *et al*.,1986
3	*R. japonica* (Burm. f.) H. Hara var. *glaucocalyx* (Maxim.) H. Hara [*I. japonicus* var. *glaucocalyx* (Maxim.) H. W. Li]	-	24	Sokolovskaya *et al*.,1986
4	*R. rugosa* (Wall. ex Benth.) H. Hara [*I. rugosus* (Wall. ex Benth.) Codd]	12	-	Khatoon and Ali, 1993
5	*R. glaucocalyx* (Maxim.) Prob. [*I. japonicus* var. *glaucocalyx* (Maxim.) H. W. Li]	-	24	Shatalova, 2000; Probatova *et al*., 2000
6	*P. japonicus* (Burm. f.) Koidz [*I. japonicus* (Burm. f.) H. Hara]	-	24	Suzuka, 1950
7	*P. striatus* Benth. [*I. lophanthoides* var. *graciliflorus* (Benth.) H. Hara]	12	-	Mehra and Gill, 1972; Bir and Saggoo, 1981, 1984 Saggoo, 1983; Gill, 1984; Saggoo and Bir, 1986
		-	24	Krishnappa and Basavaraj, 1982

8	*P. ternifolius* D. Don [basionym of *I. ternifolius* (D. Don) Kudô]	12	-	Mehra and Gill, 1972; Gill, 1984
9	*P. rugosus* Wall. ex Benth. [*I. rugosus* (Wall. ex Benth.) Codd]	12	-	Mehra and Gill, 1972; Vij and Kashyap, 1975, 1976; Bir and Saggoo, 1981, 1984; Saggoo, 1983; Gill, 1984
10	*P. coetsa* Buch.-Ham. ex D. Don [basionym of *I. coetsa* (Buch.-Ham. ex D. Don) Kudô]	11	-	Vij and Kashyap, 1975
		12	-	Mehra and Gill, 1972; Vij and Kashyap, 1976; Saggoo, 1983; Bir and Saggoo, 1981, 1984; Gill, 1984; Saggoo and Bir, 1986
11	*P. gerardianus* Benth. [basionym of *I. lophanthoides* (Buch.-Ham. ex D. Don) H. Hara]	12	-	Mehra and Gill, 1972; Saggoo, 1983; Bir and Saggoo, 1981, 1984; Gill, 1984; Saggoo and Bir, 1986

12	*P. maddenii* Benth. ex Hook. f. [*I. coetsa* (Buch.-Ham. ex D. Don) Kudô]	12	-	Bir and Saggoo, 1980, 1984
13	*P. nilgherricus* Benth. [basionym of *I. nilgherricus* (Benth.) H. Hara]	12	-	Cherian and Kuriachan, 1981; Saggoo and Bir, 1982; Saggoo, 1983
		-	24	Cherian and Kuriachan, 1981
14	*P. gerardianus* var. *graciliflorus* (Benth.) Hook. f. [*I. lophanthoides* var. *graciliflorus* (Benth.) H. Hara]	12	-	Saggoo and Bir, 1982, 1986
15	*P. repens* Wall. [*I. repens* (Wall. ex Benth.) Murata]	12	-	Saggoo and Bir, 1982, 1983, 1986; Saggoo, 1983
16	*P. scrophularioides* Wall. ex Benth. [*I. scrophularioides* (Wall. ex Benth.) Murata]	12	-	Saggoo and Bir, 1982, 1983, 1986; Saggoo, 1983
17	*P. wightii* Benth. [*I. wightii* (Benth.) H. Hara]	12	-	Saggoo, 1983; Saggoo and Bir, 1983; Bir and Saggoo, 1985; Thoppil and Jose, 1996a
		-	24	Krishnappa and Basavaraj, 1982; Thoppil and Jose, 1996b

18	*P. macraei* Benth. [*I. coetsa* (Buch.-Ham. ex D. Don) Kudô]	12	-	Saggoo, 1983; Saggoo and Bir, 1983; Bir and Saggoo, 1985
19	*P. stocksii* Hook. f. [*I. lophanthoides* (Buch.-Ham. ex D. Don) H. Hara]	12	-	Bir and Saggoo, 1985; Saggoo and Bir, 1983
20	*I. ramosissimus* (Hook. f.) Codd	-	42	Morton, 1993
21	*I. japonicus* var. *glaucocalyx* (Maxim.) H. W. Li	-	24	Jin and Sha, 2004
22	*I. effusus* (Maxim.) H. Hara	-	24	Yamashiro *et al*., 2005
23	*I. inflexus* (Thunb.) Kudô	-	24	Yamashiro *et al*., 2005
24	*I. japonicus* (Burm. f.) H. Hara	-	24	Yamashiro *et al*., 2005
25	*I. longitubus* (Miq.) Kudô	-	24	Yamashiro *et al*., 2005
26	*I. shikokianus* (Makino) H. Hara	-	24	Yamashiro *et al*., 2005
27	*I. shikokianus* var. *intermedius* (Kudô) Murata	-	24	Yamashiro *et al*., 2005
28	*I. shikokianus* var. *occidentalis* Murata	-	24	Yamashiro *et al*., 2005
29	*I. tricocarpus* (Maxim.) Kudô* (*I. trichocarpus* (Maxim.) Kudô)**	-	24	Yamashiro *et al*., 2005
30	*I. umbrosus* (Maxim.) H. Hara	-	24	Yamashiro *et al*., 2005
31	*I. umbrosus* var. *excisinflexus* (Nakai) K. Asano [*I. umbrosus* (Maxim.) H. Hara]	-	24	Yamashiro *et al*., 2005
32	*I. umbrosus* var. *hakusanensis* (Kudô) K. Asano [*I. umbrosus* (Maxim.) H. Hara]	-	24	Yamashiro *et al*., 2005
33	*I. umbrosus* var. *latifolius* Okuyama [*I. umbrosus* (Maxim.) H. Hara]	-	24	Yamashiro *et al*., 2005

34	*I. umbrosus* var. *leucanthus* (Murata) K. Asano [*I. umbrosus* (Maxim.) H. Hara]	-	24	Yamashiro *et al*., 2005
35	*I. lophanthoides* (Buch.-Ham. ex D. Don) Hara	-	36	Huang, 2011
36	*I. lophanthoides* var. *gerardiana* (Benth.) H. Hara* (*I. lophanthoides* var. *gerardianus* (Benth.) H. Hara)** [*I. lophanthoides* (Buch.-Ham. ex D. Don) H. Hara]	-	24	Huang, 2011
37	*I. lophanthoides* var. *graciliflora* (Benth.) H. Hara* (*I. lophanthoides* var. *graciliflorus* (Benth.) H. Hara)**	-	24	Huang, 2011
38	*I. serra* (Maxim.) Kudô	-	24	Huang, 2011
39	*I. albopilosus* (C. Y. Wu & H. W. Li) H. Hara	-	24	Xiang *et al*., 2014
40	*I. coetsa* (Buch.-Ham. ex D. Don) Kudô	-	24, 48	Xiang *et al*., 2014
41	*I. flabelliformis* (C. Y. Wu) H. Hara	-	24	Xiang *et al*., 2014
42	*I. forrestii* (Diels) Kudô	-	24	Xiang *et al*., 2014
43	*I. gesneroides* (J. Sinclair) H. Hara	-	24	Xiang *et al*., 2014
44	*I. lophanthoides* var. *graciliflorus* (Benth.) H. Hara	-	24	Xiang *et al*., 2014
45	*I. oreophilus* var. *elongatus* (Hand.-Mazz.) A. J. Paton & Ryding	-	24	Xiang *et al*., 2014
46	*I. parvifolius* (Batalin) H. Hara	-	24	Xiang *et al*., 2014
47	*I. pleiophyllus* (Diels) Kudô	-	24	Xiang *et al*., 2014
48	*I. polystachys* (Y. Z. Sun) H. Hara	-	24	Xiang *et al*., 2014
49	*I. racemosus* (Hemsl.) Murata	-	24	Xiang *et al*., 2014
50	*I. scoparius* (C. Y. Wu & H. W. Li) H. Hara	-	24	Xiang *et al*., 2014
51	*I. weisiensis* (C. Y. Wu) H. Hara	-	24	Xiang *et al*., 2014

*cited by the author; **original binomial name

ANTIOXIDANT STUDIES

Diseases have gripped the life of man and plants are looked upon as saviors. Oxidative stress results in the production of free radicals which has inevitable role in various kinds of ailments. Reactive oxygen species causes cellular damage by interaction with biomolecules of the body (Zhu *et al*., 2004) which needs to be subjugated for a healthy life. Mitigation of oxidative stress is made possible with the consumption of antioxidants. Hence, drugs with antioxidant capacity endows with dual benefit. Many herbal anticancer drugs have proved to possess antioxidant abilities which can be exploited for the impartment of improved health conditions. Previous studies reveal the antioxidant potential of the genus *Isodon* and with its established biological properties can be a gain to pharmaceutics.

Several studies have been conducted especially on the antioxidant ability of isolated compounds in *Isodon*. The *ent*-kaurane diterpenoids *viz*., leukamenin E, glaucocalyxin A and wangzaozin A isolated from *I. racemosa* and epinodisinol, epinodosin, rabdosin B, rabdosinate, lasiokaurin and oridonin from *I. japonica* var. *glaucocalyx* were subjected to lipid peroxidation assay, comet assay and thiobarbituric acid reactive substances determination to analyze the antioxidant potential (Liu *et al*., 2006a). The diterpenoids showed concentration dependent protection against DNA damage in human peripheral blood mononuclear cells which was caused by hydrogen peroxide. Inhibition of lipid peroxidation was also concentration dependent with rabdosin B and oridonin showing strongest inhibition followed by lasiokaurin and wangzaozin A. All others had moderate effect with the weakest being rabdosinate. The phenolic compounds *viz*., pedalitin, quercetin, rutin, isoquercitrin and rosmarinic acid isolated from *R. japonica* was subjected to antioxidant studies using DPPH and superoxide scavenging assays (Masuoka *et al*., 2006). All the compounds showed superoxide

scavenging activity with pedalitin being the most potent where it prevented superoxide radical generation by inhibiting xanthine oxidase competitively. Pedalitin and quercetin inhibited superoxide and uric acid formation on xanthine oxidase, while isoquercitrin, rutin and rosmarinic acid inhibited only superoxide formation without inhibiting the enzyme. Thirugnanasampandan *et al*. (2008) isolated melissoidesin, an *ent*-kaurene diterpenoid from *I. wightii*. The antioxidant studies using DPPH free radical scavenging activity was conducted which revealed it to be a promising source as food and in drug preparations.

Decolorized polysaacharide product of *I. lophanthoides* var. *gerardianus* was subjected to antioxidant studies using DPPH assay and the results showed antioxidant activity against DPPH radicals (Wen *et al*., 2011). The antioxidant activity of polyphenols obtained from *R. serra* was studied using DPPH assay and the results showed good radical scavenging activity (Li *et al*., 2012c). The antioxidant activities using DPPH radical scavenging and FRAP assays along with anticancer effects and the regulation of apoptosis-related gene expression of *I. japonicus* was studied by Kyung-A *et al*. (2011). The results revealed that the extract possessed moderately high antioxidant activity as well as inhibited the growth of HepG2 cancer cells *in vitro*, upregulated p53, p21 and Bax gene expression, and downregulated Bcl-2 gene expression. The extraction efficiency of different solvents from *R. serra* roots along with the antioxidant capacity were evaluated by Dong *et al*. (2011). The highest total phenol content was depicted by 60% ethanol extract which also had the strongest antioxidant ability as determined by DPPH, ABTS and Fe^{2+} chelating capacity assays.

The antioxidant capability of *I. rugosus* using DPPH radical scavenging activity was evaluated by Rauf *et al*. (2013) on crude, hexane, chloroform, ethyl acetate, methanol and quercetin extracts. Ethyl acetate and

methanolic extracts revealed good activity which implies the role of the plant in reducing diseases due to free radicals. The antioxidant activity of *I. rugosus* using DPPH assay was also studied by Janbaz *et al*. (2014) which were aimed at giving a scientific validation for the folkloric use of the plant in ameliorating various disease processes. Honey obtained from *I. rugosus* plant was investigated for antioxidant activity using DPPH assay (Zahoor *et al*., 2014). The results showed moderate antioxidant activity which increased with increasing concentrations. The antioxidant potential of eight different solvents *viz*., hexane, acetone, 80% acetone, ethanol, methanol, 80% ethanol, 80% methanol and water extracts of *R. rubescens* was studied using DPPH, ABTS, FRAP assay (Feng and Xu, 2014). The results revealed that 80% acetone extract had the highest antioxidant activity and the lowest for hexane extracts which was in accordance with the total polyphenols and flavonoids present in each solvent extracts. Thirteen phenolic compounds isolated from *I. lophanthoides* var. *graciliflorus* were subjected to antioxidant studies using DPPH and FRAP assay (Zhou *et al*., 2014). The results showed that compounds rosmarinic acid, methyl rosmarinate, clinopodic acid A, salvianolic acid A, nepetoidin B, caffeic acid, vinyl caffeate, danshensu and syringic acid possessed antioxidant ability better than or equivalent to L-ascorbic acid used as control which concludes that the herb is beneficial especially in health aspect. The antioxidant potential of different fractions of the extracts of *I. rugosus* were investigated using DPPH, H_2O_2 and ABTS free radicals scavenging assays (Zeb *et al*., 2014b). The flavonoid, chloroform and ethyl acetate fraction of the extracts showed highest antioxidant potential in DPPH and H_2O_2 assays while the plant samples depicted moderate activity towards ABTS free radical assay. The results had high correlation with the phenols and flavonoids content present in the extract. An abietane diterpenoid, abietic acid was isolated and structure elucidated from the petroleum ether extract of *I. wightii* and tested for antioxidant activity

(Ramnath *et al*., 2015). The compound showed moderate activity against DPPH free radical and hydroxyl radical scavenging assays while the activity was less against metal ion chelation and inhibition of lipid peroxidation.

ANTITUMOR STUDIES

The pharmacological aspects of *Isodon* have long been known especially from the Chinese folk medicines and have always been the target of researchers due to the presence of abundant biologically active constituents particularly diterpenoids. Natural products exhibit promising antitumor effects by induction of cancer cell apoptosis. *In vitro* cytotoxic studies, determination of cell death and *in vivo* studies help in identifying compounds which can be the endpoints of proliferating cancer cells. Chemotherapy drugs are the redeemers of cancer patients but the slow and silent death of cells due to non-specific targets and side effects needs to be surpassed with the help of herbal cancer drugs.

Cytotoxic assays

Cytotoxic screening with *Allium cepa* assay is employed as one of the preliminary markers in anticancer studies. It detects the cytostatic, cytotoxic and mutagenic aspects of plant derived compounds (Kuraś *et al*., 2006) and has high correlation with other test system particularly MIT-217 cell test with mice, rats or humans *in vivo* which enables it to be used as an alternative test material to laboratory animals (Fiskesjö and Levan, 1993). Screening of polar and non-polar fractions of crude extracts of *I. coetsa* for cytotoxicity was assayed using *Allium* test (Neelamkavil and Thoppil, 2014). Chromosome fragments, stickiness, ring chromosomes, chromosome bridges, pulverization, binucleate cell, micronucleus, ball metaphase, chromosome laggards, and shift in microtubule organizing center were the major aberrations observed. The clastogenic nature of non-polar components was clearly portrayed along

with slight increase in mitotic index for certain concentrations and time periods which might be due to the C-mitotic activity of the leaf extract as reported in the studies of Neelamkavil and Thoppil (2013). Apart from increase in mitotic index, the C-mitotic activity of the extract was established by high frequency of C-metaphase, polyploidy, cytostasis and vagrant chromosome aberrations.

Cell viability assays are imperative in cytotoxicity since it gives preliminary idea about tumor susceptibility. Live cells are identified based on the cell membrane integrity (Kroemer *et al*., 2009) and hence infiltration of various dyes in the cytoplasm is the indication to determine the vitality of cells. One of the widely used laboratory assays for cell viability is trypan blue dye exclusion assay where cells with intact membrane exclude the dye. Oridonin isolated from *R. rubescens* was assessed for cell viability against HPB-ALL cell lines using trypan blue dye exclusion method (Liu *et al*., 2006b). The number of viable cells showed gradual decrease with increasing concentration and time durations. Oridonin also depicted significant antiproliferation effect on the cell line by inducing apoptosis and necrosis which makes it a potential antileukemia reagent. The phenolic compounds pedalitin, quercitin and rutin isolated from *R. japonica* were subjected to cell viability assays against murine B16-F10 melanoma cell lines (Nitoda *et al*., 2008). Pedalitin and quercitin showed potent cytotoxicity and cell viability was on a decrease in dose dependent manner while rutin had no activity. The viability of PC12 cells after treatment with 19-hydroxy-1α,6-diacetoxy-6,7-seco-*ent*-kaur-16-en-15-one-7,20-olide, a diterpene called as CBNU06 isolated from *I. japonicus* against beta-amyloid (Aβ) induced cytotoxicity was identified using trypan blue exclusion method (Kim *et al*., 2009). The studies revealed that a dose dependent increase in viable cells could be observed after pretreatment with CBNU06 prior to Aβ treatment.

The cytotoxic activity of an *ent*-kaurane diterpenoid, weisiensin B, isolated from *I. weisiensis* against human BEL-7402 tumor cell line was studied (Wang *et al*., 2011b). Viability of cells was assessed by trypan blue exclusion method and the results showed significant reduction in cell growth in a dose and time dependent manner. Four major *ent*-kaurene diterpenoids *viz*., enmein, nodosin, oridonin and isodonal isolated from *R. japonica* were investigated for cell viability against B16-F10 cell line (Satooka *et al*., 2012). Oridonin showed the most potent cytotoxic activity and the cell viability followed a dose dependent manner. The cytotoxic activity was found to be concentration dependent with the diterpenoids showing decreasing cytotoxicity in the order oridonin = nodosin > isodonal > enmein.

Cytotoxicity is also well determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay where mitochondrial dehydrogenase of intact cells reduces MTT into a purple formazan product (Zhang *et al*., 2007). Sulforhodamine B colorimetric (SRB) assay also identifies cytotoxicity based on cellular protein content which determines cell density (Vichai and Kirtikara, 2006). Most of the isolated components of *Isodon* are subjected to cytotoxicity employing these two assays which are consolidated in table 2 and 3.

Table 2. Compounds from *Isodon* exhibiting cytotoxic activity against human cell lines

Sl. No.	Species	Chemical compound	Human cell line	Reference
1	*R. japonica* (Burm.f.) H. Hara var. *glaucocalyx* (Maxim.) H. Hara	Glaucocalyxin A	HL60, U937, K562, SGC-7901, A549, H1299, U251, SHG-44, HepG2, SMMC-7721, BEL-7402, MDA-MB-231, HeLa	Gao *et al*., 2011
2	*R. weisiensis* C. Y. Wu	Weisiensin B	BEL-7402, HO-8910, SGC-7901, HepG2	Ding *et al*., 2008
3	*I. adenantha* (Diels) Hara	Adenanthins C, F, Weisiensin A	K562	Jiang *et al*., 2002c
4	*I. adenoloma** (*I. adenolomus*)**	Longikaurin F	Lu1, KB, KB-V (+VLB), LNCap, ZR-75-1	Sun *et al*., 1995a
5	*I. adenolomus* (Hand-Mazz.) H. Hara	Isoadenolin O	SMMC-7721, MC7, SW480	Zhao *et al*., 2011a
		Isoadenolin N	MC7, SW480	
6	*I. albopilosus* (C. Y. Wu & H. W. Li) H. Hara	Albopilosin H, Excisanin	HepG2	Huang *et al*., 2005
		Alboatisin B	A549, HT-29, K562	Huang *et al*., 2007b
		Alboatisin A	A549, HT-29	
		Alboatisin C	A549	
7	*I. enanderianus* (Hand.-Mazz.) H. W. Li	6-Epiangustifolin	K562	Na *et al*., 2002
		Enanderianins K, L, P, Rabdocoetsin B, D	K562	Xiang *et al*., 2003

8	*I. eriocalyx* (Dunn.) Hara	Maoecrystal V	HeLa	Li *et al*., 2004
		Eriocalyxin B, Longikaurin E	K562, T-24, ME180, QGY-7701, BIU87	Shen *et al*., 2005
		Eriocalyxin A	K562, T-24, QGY-7701	
		Laxiflorin L, Maoecrystal C	K562	
		Maoecrystal B	ME180	
		Maoecrystal Z	K562, MCF7, A2780	Han *et al*., 2006
		Longikaurin C, Effusanin B, Maoecrystal R	HT-29, BEL-7402, SK-OV-3	Li *et al*., 2010a
		Maoecrystal M	BEL-7402, SK-OV-3	
		3α,6β-dihydroxy-7,17-dioxo-*ent*-abieta-15(16)-ene, Maoecrystal J, Effusanin D, Kamebacetal A, Henryin	HT-29, SK-OV-3	
		Maoesin D	HT-29	
		Eriocasin D, 3β-Acetoxyeriocasin D	SK-OV-3	
		Eriocalyxin B	PANC-1, SW1990, CAPAN-1, CAPAN-2	Li *et al*., 2012b
9	*I. eriocalyx* (Dunn) Hara var. *laxiflora* C.-Y. Wu et H.-W. Li	Laxiflorin C	Lu1, KB, KB-V, LNCaP, ZR-75-1	Sun *et al*., 1995b
		Laxiflorin E, I, Eriocalyxin B, Maoecrystal C	K562, T24	Niu *et al*., 2002a
		Laxiflorin C	K562	
		Laxiflorin L	K562, A549, T24	Niu *et al*., 2002b

		Laxiflorin J, Maoecrystal P	K562, T24	
		Laxiflorin K	K562	
		Laxiflorin C	K562	Niu *et al*., 2002c
		Eriocalyxin B	K562, HL60, A549, MKN, CA	Yang *et al*., 2009
		Maoecrystal U	HL60, SMMC-7721, MCF7, SW480	Wang *et al*., 2012b
10	*I. excisoides* (Sun ex C. H. Hu) C. Y. Wu et H. W. Li)	Kamebacetal-A, Glaucocalyxin-A, Kamebanin, Wangzaozin-A, Leukamenin-E	BEL-7402, HO-8910	Wang *et al*., 2005b
11	*I. gesneroides* (J. Sincl.) Hara	Gesneroidin B	Lu1, KB, KB-V (+VLB), KB-V (-VLB), LNCap, ZR-75-1	Sun *et al*., 1995c
		Gesneroidin C, Dawoensin A	Lu1, KB, LNCap, ZR-75-1	
12	*I. henryi* (Hemsl.) Hara	Minheryin G, Leukamenin F, Excisoidesin, Leukamenin E, Wangzaozin A, Pseurata A, Racemosin A	K562, HepG2	Zhao *et al*., 2009b
		Rabdocoetsin B	HL60, SMMC-7721, A549, MCF7, SW480	Hu *et al*., 2011
		Ponicidin, Macrocalin B	SMMC-7721, MCF7, SW480	
		Rabdoternin E, F	SMMC-7721, MCF7	
		Glaucocalactone	HL60, MCF7	
13	*I. japonica** (*I. japonicus*)**	Lasiokaurin	HL60, A549, HO-8910	Bai *et al*., 2005
		Oridonin, Shikokianin	HL60, A549	

14	*I. japonica* (Burm.f.) Hara var. *galaucocalyx* (maxin) Hara* (*I. japonicus* (Burm.f.) H. Hara var. *glaucocalyx* (Maxim.) H. W. Li)**	Rabdosin B, Oridonin, Epinodosin, Rabdosinate, Lasiokaurin, Epinodosinol	HepG2, GLC-82, HL60	Ding *et al*., 2011
15	*I. japonicus* (Burm.f.) H. Hara	Oridonin	SPC-A-1	Liu *et al*., 2004
		Maoyecrystal 1	K562	Han *et al*., 2004e
		Ponicidin	QGY-7701, HepG2	Zhang *et al*., 2007
16	*I. leucophyllus* (Dunn) Kudô	Lasiocarpanin	K562, Bcap37	Zhao *et al*., 2004b
17	*I. lophanthoides* (Buch.-Ham. ex D. Don) H. Hara var. *graciliflorus* (Benth.) H. Hara	Micranthin B	A549, MCF7, HeLa	Liang *et al*., 2013
		16-Acetoxylsugiol, Graciliflorin E, 15-Hydroxy-20-deoxocarnosol, 3β-Hydroxysempervirol, Abieta-8,11,13-triene-14,19-diol, 6,12,15-Trihydroxy-5,8,11,13-abietatetraen-7-one	A549, MCF7, HeLa	Zhou *et al*., 2013b
18	*I. loxothyrsus* (Hand.-Mazz.) H. Hara	Loxothyrin A, Adenolin B	LNCaP, ZR-75-1	Sun *et al*., 1995a
19	*I. macrophyllus* (Migo) H. Hara	Rabdosin A, Shikokianin, Effusanin A	DU145, LoVo	Zhang *et al*., 2009a
20	*I. megathyrsus* (Diels) H. Hara	Megathyrin B	KB, KB-V	Qiu *et al*., 1998

21	*I. melissoides* (Bentham) H. Hara	Melissoidesins N, O, S, G, Xindongnin B, Dawoensin A	BGC823	Zhao *et al*., 2004a
22	*I. nervosus* (Hemsl.) Kudô	Nervonin B, Weisiensin A	K562, A549, HepG2	Li *et al*., 2008a
		Calcicolins A, B, Adenanthin	K562, HepG2	
		Isodocarpin, Effusanin A	HL60, SMMC-7721, HeLa	Yan *et al*., 2009
		Effusanin E	HL60	
		Rabdonervosin H	HepG2, PC-9/ZD	Gao *et al*., 2013
		Rabdonervosin I	HepG2, CNE2	
23	*I. oresbius* (W. W. Smith) Kudô	Nodosin	K562	Xiang *et al*., 2004b
24	*I. parvifolius* (Batalin) H. Hara	Parvifoline C	K562	Li *et al*., 2006b
		Parvifolines Z, AA	A549, HT-29, K562	Li *et al*., 2006c
		Parvifoline Y	A549, HT-29, K562	Li *et al*., 2006d
		Parvifolines Q, U, V, Lasiocarpanin	A549, HT-29, K562	Li *et al*., 2006e
25	*I. pharicus* (Prain) Hara	Pharicinin B	NB4, SH-SY5Y, MCF7	Zhao *et al*., 2009a
		Pharicinin C	NB4, SH-SY5Y	
		12-*O*-Acetylpseurata B	NB4, SH-SY5Y	Zhao *et al*., 2009c
		17-Methoxydihydropseurata C	NB4, PC-3	
		Pseurata B acetonide, Pseurata C, Glaucocalyxin B	NB4, A549	
		Pseurata A, Glaucocalyxin A, Wangzaozin A	NB4, A549, MCF7	

		Isodomedin, Leukamenin E	NB4	
26	*I. phyllostachys* Kudô	Phyllostachysins D, F, G, Rabdoloxin B, Amethystoidin A	K562	Li *et al*., 2006g
		Xerophilusin B	K562, HepG2	Li *et al*., 2008c
27	*I. pleiophyllus* Kudô	Coetsoidins B, G	Lu1, KB, LNCap, ZR-75-1	Sun *et al*., 1995a
		Coetsoidin A	Lu1, KB, KB-V (+VLB), LNCap, ZR-75-1	
28	*I. pseudo-irrorata* C. Y. Wu	Pseudoirroratin A	Lu1, SW626, LNCaP, KB, HOS	Zhang *et al*., 2002
29	*I. racemosa* (Hemsl.) Hara	Racemosin A, Leukamenin E, Glaucocalyxin A, Wangzaozin A	BEL-7402, HO-8910	Ding *et al*., 2006
		Kamebacetal A	BEL-7402, HO-8910	Wang *et al*., 2010a
30	*I. rosthornii* (Diels) Hara	Ponicidin, Xerophilusin B	HL60, SMMC-7721, A549, MCF7, SW480	Zhan *et al*., 2011
		Macrocalin B	HL60, SMMC-7721, MCF7, SW480	
31	*I. rubescens* (Hemsl.) H. Hara	Rubescensin M	K562	Han *et al*., 2004a
		Bisrubescensin A	A549, HT-29, K562	Huang *et al*., 2006a
		Hebeirubescensins A-E, G, H, Rabdoternins A, F, E, G, Oridonin, Lasiokaurin	A549, HT-29, K562	Huang *et al*., 2006b
		Ponicidin, Oridonin	EC1, U87, A549, MCF7, HeLa	Xie *et al*., 2011
		15α-Hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7α(6,11α)-diolide	EC1, MCF7, HeLa	

		Rabdosin A, Isodocarpin	HL60, SMMC-7721, A549, MCF7, SW480	Liu *et al*., 2012b
		Ponicidin	SMMC-7721, A549, MCF7, SW480	Liu *et al*., 2012c
		Jianshirubesin D	HL60, A549, MCF7, SW480	Liu *et al*., 2012d
		Oridonin	A549, MCF7, SMMC-7721, SW480, HL60	Liu *et al*., 2015
32	*I. rubescens* var. *lushanensis*	Lushanrubescensin H, Ponicidin, Isodonal	K562	Han *et al*., 2003b
		Oridonin, Ponicidin	CA	
		Lushanrubescensin J	K562	Han *et al*., 2005d
		Luanchunins A, B	HL60, SK-BR-3	Zhang *et al*., 2010a
		Lasiokaurin	U937, Jurkat, HL60, K562, SGC790, HepG2	Zhang *et al*., 2010b
33	*I. rubescens* var. *lushiensis*	Ludongnin J	K562, CA, HeLa	Han *et al*., 2003c
		Guidongnin A, Angustifolin, 6-Epiangustifolin, Sculponeatin J, Ludongnins A	K562	
		Isolushinin D, (20*S*)-11β,14β,20-Trihydroxy-7α,20-epoxy-*ent*-kaur-16-en-15-one, (20*S*)-11β,14β-Dihydroxy-20-methoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one, (20*S*)-11β,14β-Dihydroxy-20-ethoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one, Phyllostachysin F	HL60, SMMC-7721, A549, SK-BR-3, PANC-1	Luo *et al*., 2010

		Flexicanlin A	HL60, SMMC-7721, A549, PANC-1	
		Isolushinin H	HL60, SK-BR-3, PANC-1	
		Kamebakaurinin, Phyllostachysin H, Oridonin, Enmenol, Ludongnin A	HL60	
34	*I. rubescens* var. *rubescens*	Xindongnins A-C, F, Melissoidesin G, Dawoensin A, Glabcensin V	K562	Han *et al*., 2004g
		Hebeiabinin E	A549, HT-29, K562	Huang *et al*., 2007a
35	*I. rubescens* var. *taihangensis* Gao et Li	Rubescensins S, T	K562	Han *et al*., 2004b
		Oridonin, Ponicidin, Lasiodonin, Lasiokaurin	K562	Han *et al*., 2005c
		Acetonide of lasiodonin	K562, Bcap37, CA, CNE, BIU87, BGC823, HeLa	
36	*I. scoparius* (C. Y. Wu et H. W. Li) H. Hara	Isoscoparins F, L, Kamebanin	NB4, A549, MCF7, SH-SY5Y	Zhao *et al*., 2009d
		Isoscoparin I, Phyllostachysin D, Excisanins A, K	NB4	
		Rabdoloxin B, Rabdokunmin A	NB4, A549	
		Henryine A	NB4, SH-SY5Y, PC-3	
37	*I. sculponeata** (*I. sculponeatus*)**	Sculponeatin H, Epi-nodosin	K562, T24	Jiang *et al*., 2002a
		Sculponeatin I, Enmein, Macrocalyxoformin B	K562	
38	*I. sculponeatus* (Vaniot) Kudô	Sculponeatin J	K562, T24	Jiang *et al*., 2002b
		Sculponin A	K562, A549, HepG2	Li *et al*., 2007b

		Sculponins D, E, Epinodosin, Sculponeatins A, C, 4α-Hydroperoxy-5-enovatodiolide, 4-Methylene-5*β*-hydroperoxyovatodiolide, Ovatodiolide	K562, A549, HepG2	Li *et al*., 2009b
		Nodosin, Enmein	K562, HepG2	
		Sculponeatins A, C, N, O, R, Epinodosin, Enmein, Isodocarpin, Nodosin, Macrocalyxoformin B	K562, HepG2	Li *et al*., 2010b
		Longirabdolide C	K562	
		Isodocarpin	HL60, SMMC-7721, A549, MCF7, SW480	Jiang *et al*., 2013
		Nodosin, Sculponeatin A	SW480	
		Sculponeatins C, J	HL60, SMMC-7721, A549, MCF7, SW480	Jiang *et al*., 2014a
		Sculponin T	HL60, SMMC-7721, MCF7, SW480	
39	*I. sinuolata* C. Y. Wu et H. W. Li	Trichorabdal A	NB4, A549, SH-SY5Y, PC-3, MCF7	He *et al*., 2009
		Sinuolatin A	NB4, SH-SY5Y, MCF7	
		Macrocalyxin J	NB4, A549, SH-SY5Y	
		Sinuolatin B, Effusanin E	NB4, SH-SY5Y	
		Isodonal	NB4, PC-3	
		Sinuolatin E	SH-SY5Y	

		Maoecrystal A, Nodosin, Rubescensin Q, Enmenin, Oreskaurin B, Wikstroemioidin C	NB4	
40	*I. tenuifolius* (W. W. Smith) Kudô	Tenuifolin I, Inflexarabdonin E	HL60, SMMC-7721, A549, MCF7, SW480	Yang *et al*., 2013
		Adenanthin C	HL60, SMMC-7721, MCF7, SW480	
		Tenuifolin J	HL60, SMMC-7721	
		Isoabietenin C, Tenuifolin M	HL60	
41	*I. weisiensis* C. Y. Wu	Weisiensin B, Kamebanin, Kamebacetal A, Macrocalyxin D	BEL-7402, HO-8910	Ding *et al*., 2005a
		(1α,7α,14β)-1,7,14-Trihydroxy-*ent*-kaur-16-en-15,18-dione, (1α,7α,14β)-1,7,14,18,20-Pentahydroxy-*ent*-kaur-16-en-15-one, (3β,7α,14β)-3,7,14 Tris(acetyloxy)-*ent*-kaur-16-en-15-one	BEL-7402, HO-8910	Ding *et al*., 2005b
		Weisiensin C, Glaucocalyxin A, Kamebanin, Macrocalyxin D, Excisanin K	BEL-7402, HO-8910	Ding *et al*., 2005c
42	*I. wightii* (Bentham) H. Hara	Melissoidesin	A549, IMR-32	Thirugnanasampandan and Jayakumar, 2009
43	*I. wikstroemioides* (Hand.-Mazz.) H. Hara	Wikstroemioidins G, H, M, O-Q, Adenanthin, Isoscoparin L, Excisanin C, Albopilosin A, Phyllostachysin F, Rabdoloxin B	HL60, SMMC-7721, A549, MCF7, SW480	Wu *et al*., 2014
		Macrocalyxin C	HL60, SMMC-7721, MCF7, SW480	

		Isoscoparin E, Rabdokunmins A, B	HL60, SMMC-7721, SW480	
		Wikstroemioidin V	SMMC-7721, MCF7, SW480	
		Wikstroemioidins E, F	SW480	
		Isowikstroemins H, J, Macrocalyxin B	HL60, SMMC-7721, A549, MCF7, SW480	Wu *et al*., 2015
		Isowikstroemins K, L	HL60, SMMC-7721, A549	
44	*I. xerophilus* (C. Y. Wu and H. W. Li) H. Hara	Xerophilusin K, Longikaurin B	K562, HL60, HCT, MKN-28	Hou *et al*., 2001
		Xerophilusins I, J, Enanderianin C	K562, HL60, MKN-28	
		Xerophilusin G	HL60	
		Ponicidin	K562, T24	Li *et al*., 2002b
		Xerophilusin R	K562, T24	Niu *et al*., 2004
		Xerophinoids A, B	K562, NB4, NB4-R2, ME180	Weng *et al*., 2007
		Xerophilusin 1, XI, XIII, Longikaurin B, D, Rosthorin A, Xerophilusin B, D, I, K	K562, MKN45, HepG2	Li *et al*., 2007c
		Xerophilusin N, Phyllostachysin A	K562, MKN45	
		Xerophilusin II, F, Ponicidin	K562, HepG2	
		Hebeirubescensins G and H, Rabdoternin E, Xerophilusin A	K562	
		Maoecrystal I	MKN45	
		Xerophilusin B, Macrocalin B	K562, HL60, A549, MKN, CA	Yang *et al*., 2009

*cited by the author; **original binomial name

Table 3. Compounds from *Isodon* exhibiting non-significant cytotoxic activity against human cell lines

Sl. No.	Species	Chemical compound	Human cell line	Reference
1	*I. adenantha* (Diels) Hara	Adenanthins B, D, E, H, I, L, Adenanthin, Weisiensin A, Nervosanin	K562	Jiang *et al*., 2002c
2	*I. adenoloma** (*I. adenolomus*)**	Longikaurin F	KB-V (-VLB), ASK	Sun *et al*., 1995a
3	*I. adenolomus* (Hand.-Mazz.) H. Hara	16-Hydroxy ferruginol, Hinokiol, Kaurane-3β,16β-diol	HL60, SMMC-7721, A549, MC7, SW480	Zhao *et al*., 2011a
		Isoadenolin N	HL60, A549, SMMC-7721,	
		Isoadenolin O	HL60, A549	
4	*I. albopilosus* (C. Y. Wu & H. W. Li) H. Hara	Albopilosins A-G, I, J, Macrocalyxin C, Rabdokunmin C, Amethystonoic acid, Coetsanoic acid	HepG2	Huang *et al*., 2005
		Alboatisin C	HT-29, K562	Huang *et al*., 2007b
		Alboatisin A	K562	
5	*I. enanderianus* (Hand.-Mazz.) H. W. Li	Enanderinanin F	K562	Na *et al*., 2002
6	*I. eriocalyx* (Dunn) Hara	Maoecrystal V	K562, A549, BGC823, CNE	Li *et al*., 2004
		epi-Maoecrystal N, Eriocalyxin G, Maoecrystal A, D, F, N, L, S, T, X, Y, Rabdosichuanin B, Coetsoidin A, Odonicin, Ternifolin, Trichokaurin,	K562, T-24, ME180, QGY-7701, BIU87	Shen *et al*., 2005

		Wikstroemioidin B, Shikokianidin, Sodoponin		
		Eriocalyxin A	ME180, BIU87	
		Laxiflorin L, Maoecrystal C	T-24, ME180, QGY-7701, BIU87	
		Maoecrystal B	K562, T-24, QGY-7701, BIU87	
		Eriocatisins A-C, E, 3-Acetyleriocasin C, Maoesins A-C, E, F, 3α-Acetoxymaoesin A	HT-29, BEL-7402, SK-OV-3	Li *et al*., 2010a
		Eriocasin D, 3β-Acetoxyeriocasin D	HT-29, BEL-7402	
		Maoesin D, 3α,6β-Dihydroxy-7,17-dioxo-*ent*-abieta-15(16)-ene, Maoecrystal J, Effusanin D, Kamebacetal A, Henryin	BEL-7402, SK-OV-3, BEL-7402	
		Maoecrystal M	HT-29	
7	*I. eriocalyx* (Dunn) Hara var. *laxiflora* C.-Y. Wu et H.-W. Li	Laxiflorin A	Lu1, KB, KB-V, LNCaP, ZR-75-1, ASK	Sun *et al*., 1995b
		Laxiflorin C	ASK	
		Laxiflorin M, Maoecrystal A	K562, A549, T24	Niu *et al*., 2002b
		Laxiflorin K	A549, T24	
		Laxiflorin J, Maoecrystal P	A549	
		Laxiflorins F, G	K562	Niu *et al*., 2002c
		Eriocalyxin B	HCT	Yang *et al*., 2009
		Neolaxiflorins D-H	A549, HL60, MCF7, SMMC-7721, SW480	Wang *et al*., 2012a

		6*β*,13*α*,15*β*-Trihydroxy-16-ene-3*α*,20-epoxy-*ent*-kaur-1,7-dione, 6-Hydroxy-3*α*,20-epoxy-5(6)-ene-*ent*-kaur-1,7,15-trione, 6-Hydroxy-15*β*-acetoxy-3*α*,20-epoxy-16*β*,17-epoxy-5(6)-ene-*ent*-kaur-1,7-dione, 3*α*,17-dihydroxy-15(16)-ene-*ent*-kaur-7-one, Laxiflorin M, Eriocalyxin C, Maoecrystal A	A549, HL60, MCF7, SMMC-7721, SW480	Wang *et al*., 2012b
		Maoecrystal U	A549	
8	*I. gesneroides* (J. Sincl.) Hara	Gesneroidin C, Dawoensin A	KB-V (+VLB), KB-V (-VLB)	Sun *et al*., 1995c
9	*I. henryi* (Hemsl.) Hara	Minheryins A-E	K562	Zhao *et al*., 2009b
		Isodonhenrins A, B, E, 15-α-Hydroxy-6,7-seco-1α,7:11α,6-diolide-20-al-*ent*-kaur-16-ene, Sculponeatin E, Rabdoternin A, B, Rosthorin A, Rabdonervosin B	HL60, SMMC-7721, A549, MCF7, SW480	Hu *et al*., 2011
		Glaucocalactone	SMMC-7721, A549, SW480	
		Rabdoternin E, F	HL60, A549, SW480	
		Ponicidin, Macrocalin B	HL60, A549	
10	*I. japonica** (*I. japonicus*)**	Taihangjaponicain A, Epinodosin, Epinodosinol, 1alpha-O-beta-D-Glucopyranosylenmenol, Lasiodonin, Rabdosichuanin D, Rabdoternin A	HL60, A549, HO-8910	Bai *et al*., 2005

		Oridonin, Shikokianin	HO-8910	
11	*I. japonicus* (Burm.f.) H. Hara	Bisjaponins A, B	K562, HepG2	Yang *et al*., 2008
		Hikiokoshins A-I	L1210, KB	Tanaka *et al*., 2014
12	*I. leucophyllus* (Dunn) Kudô	Baiyecrystal E, Oridonin, Rosthorin A, Phyllostachysin A	K562, Bcap37	Zhao *et al*., 2004b
		Bisleuconins A-D	HT-29, BEL-7402, SK-OV-3	Zhang *et al*., 2011
13	*I. lophanthoides* (Buch.-Ham. ex D. Don) H. Hara var. *graciliflorus* (Benth.) H. Hara	Graciliflorins A-D	A549, MCF7, HeLa	Liang *et al*., 2013
		15-Hydroxy-1-oxosalvibretol, 3α-Hinokiol	A549, MCF7, HeLa	Zhou *et al*., 2013b
14	*I. loxothyrsus* (Hand.-Mazz.) H. Hara	Loxothyrin A, Adenolin B	Lu1, KB, KB-V (+VLB), KB-V (-VLB), ASK	Sun *et al*., 1995a
15	*I. macrophylla* (Migo)* (*I. macrophyllus*)**	Macrophynins E, F	A549, HL60	Qin *et al*., 2007
16	*I. macrophyllus* (Migo) H. Hara	Dayecrystals D-E, Isojaponin A, Lushanrubescensin J, Wikstroemioidin B, Maoyecrystal C, Rabdosin B, Isodonal	DU145, LoVo	Zhang *et al*., 2009a
17	*I. melissoides* (Bentham) H. Hara	Melissoidesins F, E, M, P-R, T, U	BGC823	Zhao *et al*., 2004a
18	*I. nervosus* (Hemsl.) Kudô	Nervonins A , C-J, Nervosanin, Adenanthins E, K, J, Forrestins B, C	K562, A549, HepG2	Li *et al*., 2008a

		Calcicolins A, B, Adenanthin	A549	
		Effusanin E	SMMC-7721, HeLa	Yan *et al*., 2009
		Epinodosinol, Epinodosin, 1-O-β-□-Glucopyranosyl-(2S,3S,4R,5E,9Z)-2-N-(2'-hydroxyl-tetracosanoyl)1,3,4-trihydroxy-5,9-octadienine	HL60, SMMC-7721, HeLa	
		Rabdonervosins D-F	HepG2, CNE2, PC-9/ZD, HeLa, MCF7, HCT116	Wei *et al*., 2011
		Rabdonervosin G, J	HepG2, CNE2, PC-9/ZD, HeLa, HCT116	Gao *et al*., 2013
		Rabdonervosin H	CNE2, HeLa, HCT116	
		Rabdonervosin I	PC-9/ZD, HeLa, HCT116	
19	*I. oresbius* (W. W. Smith) Kudô	Oreskaurin B	K562	Xiang *et al*., 2004b
20	*I. parvifolius* (Batalin) H. Hara	Parvifoline C	A549, HT-29	Li *et al*., 2006a
		Parvifolines D-K, Lasiodonin	A549, HT-29, K562	
		Parvifoline AB	A549, HT-29, K562	Li *et al*., 2006c
		Parvifoline X	A549, HT-29, K562	Li *et al*., 2006d
21	*I. pharicus* (Prain) Hara	Pharicinin B	A549, PC-3	Zhao *et al*., 2009a
		Pharicinin C	A549, PC-3, MCF7	
		3-Epipseurata B, 7-*O*-Acetylpseurata C, 3*β*,12α,15*β*-Trihydroxy-14*β*-	NB4, A549, SH-SY5Y, PC-3, MCF7	Zhao *et al*., 2009c

		acetoxy-*ent*-kaur-16-ene, 12-Deoxyisodomedin, Dihydropseurata F, Pseuratas B, F, Minheryin G		
		12-*O*-Acetylpseurata	A549, PC-3, MCF7	
		17-Methoxydihydropseurata C	A549, SH-SY5Y, MCF7	
		Pseurata B acetonide, Pseurata C, Glaucocalyxin B	SH-SY5Y, PC-3, MCF7	
		Pseurata A, Glaucocalyxin A, Wangzaozin A	SH-SY5Y, PC-3	
		Isodomedin, Leukamenin E	A549, SH-SY5Y, PC-3, MCF7	
		Pharicinins D, E, Pharicinin D acetal	NB4, A549, SH-SY5Y, PC-3, MCF7	Zhao *et al*., 2010
22	*I. phyllostachys* Kudô	Phyllostachysins E, H, Rabdoloxin A, Rabdoinflexin B, Rabdokunmin D, Macrocalyxin E	K562	Li *et al*., 2006g
		Phyllostacins F, G, Enmenol, Phyllostachysin A	K562, HepG2	Li *et al*., 2008c
23	*I. pleiophyllus* Kudô	Coetsoidins B, G	KB-V (+VLB), KB-V (-VLB), ASK	Sun *et al*., 1995a
		Coetsoidin A	KB-V (-VLB), ASK	
24	*I. rosthornii* (Diels) Hara	Isorosthornins A-C, Dihydroponicidin, Rabdonervosin A, B	HL60, SMMC-7721, A549, MCF7, SW480	Zhan *et al*., 2011
		Macrocalin B	A549	

25	*I. rubescens* (Hemsl.) Hara	Rubescensins I-L, P, (3α,14β)-3,18-[(1-Methylethane-1,1-diyl)dioxy]-*ent*-abieta-7,15(17)-diene-14,16-diol, (3α,14β)-14,16-Epoxy-3,18-[(1-methylethane-1,1-diyl)dioxy]-*ent*-abieta-7,15(17)-diene-14,16-diol	K562	Han *et al*., 2004a
		Bisrubescensins B, C	A549, HT-29, K562	Huang *et al*., 2006a
		Hebeirubescensins F, I-L	A549, HT-29, K562	Huang *et al*., 2006b
		15α-Acetoxyl-6,11α-epoxy-6α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7-olide	EC1, U87, A549, MCF7, HeLa	Xie *et al*., 2011
		15α-Hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7α(6,11α)-diolide	K562, A549, U87	
		Ponicidin, Oridonin	K562	
		Jianshirubesins G-I, Parvifoline AA, Rabdoepigibberellolide, Laxiflorins C-E	HL60, SMMC-7721, A549, MCF7, SW480	Liu *et al*., 2012c
		Ponicidin	HL60	
		Jianshirubesins A, B, Epinodosin, Enmein, Rabdosichuanin C, Taibaijaponicains A, Maoyecrystal K, 6β,15α-Dihydroxy-6,7-seco-6,20-epoxy-1α,7-olide-*ent*-kaur-16-ene,	HL60, SMMC-7721, A549, MCF7, SW480	Liu *et al*., 2012b

		Epinodosinol, 6α,15α-Dihydroxy-20-aldehyde-6,7-seco-6,11α-epoxy-*ent*-kaur-16-en-1α,7-olide		
		Jianshirubesin E, Rubescensin W	HL60, SMMC-7721, A549, MCF7, SW480	Liu *et al*., 2012d
		Jianshirubesin D	SMMC-7721	
		Rubesanolides C-E	K562, A549, MCF7	Zou *et al*., 2012
		Kaurines A, B	A549, MCF7, SMMC-7721, SW480, HL60	Liu *et al*., 2015
26	*I. rubescens* var. *lushanensis*	Lushanrubescensin H	Bcap37, BGC823, BIU87, CA, CNE, HeLa	Han *et al*., 2003b
		Isodonal	Bcap37, BGC823, CA, HeLa	
		Oridonin	K562, Bcap37, BGC823, BIU87, CNE, HeLa	
		Ponicidin	Bcap37, BGC823, BIU87, CNE, HeLa	
		Lasiodonin, Isodonoiol	K562, Bcap37	
		Rabdosin B	K562, Bcap37, BGC823, CA, HeLa	
		Luanchunins A, B	SMMC-7721, A549, PANC-1	Zhang *et al*., 2010a
		Rubluanins B-D, Dayecrystal B, Kamebacetal A, Henryin, Kamebakaurin, Oridonin, Isodonoiol, Megathyrin A, Lasiodonin, Epinodosin	U937, Jurkat, HL60, K562, SGC790, HepG2	Zhang *et al*., 2010b

27	*I. rubescens* var. *lushiensis*	Ludongnin F-I, Guidongnins B, C, Ludongnin B	K562	Han *et al*., 2003c
		Isolushinins A, B, F, G, Ludongnin G, Sculponeatin B	HL60, SMMC-7721, A549, SK-BR-3, PANC-1	Luo *et al*., 2010
		Kamebakaurinin, Phyllostachysin H, Oridonin, Enmenol, Ludongnin A	SMMC-7721, A549, SK-BR-3, PANC-1	
		Isolushinin H	SMMC-7721, A549	
		Flexicanlin A	SK-BR-3	
28	*I. rubescens* var. *rubescens*	Hebeiabinins A-D, F, Rubescensins M, J, O, P, I, rabdoternin F, Oridonin	A549, HT-29, K562	Huang *et al*., 2007a
29	*I. rubescens* var. *taihangensis* Gao et Li	Rubescensins Q, R, Wikstroemioidin B, Enmenol, 1-*O*-β-□-Glucopyranosyl-enmenol, Trichokaurin, Acetonide of maoyecrystal F, Rabdoternins A-D	K562	Han *et al*., 2005c
30	*I. scoparius* (C. Y. Wu et H. W. Li) H. Hara	Isoscoparins D, H, Acetonide of rabdoloxin A, Rabdoloxin A, Rabdokunmins C, D, Rabdoinflexin B, Phyllostachysin H, Lasiokaurinol	NB4, A549, PC-3, MCF7, SH-SY5Y	Zhao *et al*., 2009d
		Isoscoparins F, L, Kamebanin	PC-3	
		Isoscoparin I, Phyllostachysin D, Excisanins A, K	A549, PC-3, MCF7, SH-SY5Y	
		Rabdoloxin B, Rabdokunmin A	PC-3, MCF7, SH-SY5Y	
		Henryine A	A549, MCF7	

		Scopariusic acid, Isoscoparin P	A549, HL60, MCF7, SMMC-7721, SW480	Zhou *et al*., 2013a
31	*I. sculponeata** (*I. sculponeatus*)**	Sculponeatin I, Enmein, Macrocalyxoformin B	T24, A549	Jiang *et al*., 2002a
		Sculponeatin H, Epi-nodosin	A549	
32	*I. sculponeatus* (Vaniot) Kudô	Sculponeatin K	K562, T24	Jiang *et al*., 2002b
		Sculponins B, C	K562, A549, HepG2	Li *et al*., 2007b
		Sculponins F-L, Maoyecrystal D, Sculponeatins B, D, Nervosanin B, Enmenol, Ememogin	K562, A549, HepG2	Li *et al*., 2009b
		Nodosin, Enmein	A549	
		Sculponeatins B, P, Q, S, Sculponeatin C diol, Macrocalyxoformin E	K562, HepG2	Li *et al*., 2010b
		Longirabdolide C	HepG2	
		Sculponins M-P, Enmein, Epinodosin, Sculponeatin B, 6β,15α-Dihydroxy-6α,20-epoxy-6,7-seco-*ent*-kaur-16-en-1α,7-olide	HL60, SMMC-7721, A549, MCF7, SW480	Jiang *et al*., 2013
		Nodosin, Sculponeatin A	HL60, SMMC-7721, A549, MCF7	
		Sculponeatins K, Q	HL60, SMMC-7721, A549, MCF7, SW480	Jiang *et al*., 2014a
		Sculponin T	A549	

		Sculponin Y, 1α,6β,7β,15β-Tetrahydroxy-7α,20-epoxy-*ent*-kaur-16-ene, Bisjaponin A, Lushanrubescensin J, *ent*-Kaurane-7α,16β,17-triol, Sculponeatins L, N, *ent*-Abienervonin C, Hebeiabinin B	HL60, SMMC-7721, MCF7, SW480, A549	Jiang *et al*., 2014b
33	*I. sinuolata* C. Y. Wu et H. W. Li	Sinuolatins C, D, Maoecrystal G, Longikaurin E, Lasiodonin, Oreskaurin C, Adenolin A, B, Wikstroemioidin B	NB4, A549, SH-SY5Y, PC-3, MCF7	He *et al*., 2009
		Maoecrystal A, Nodosin, Rubescensin Q, Enmenin, Oreskaurin B, Wikstroemioidin C	A549, SH-SY5Y, PC-3, MCF7	
		Sinuolatin E	NB4, A549, PC-3, MCF7	
		Sinuolatin B, Effusanin E	A549, PC-3, MCF7	
		Sinuolatin A	A549, PC-3	
		Macrocalyxin J	PC-3, MCF7	
		Isodonal	A549, SH-SY5Y, MCF-7	
34	*I. tenuifolius* (W. W. Smith) Kudô	Isoabietenins A, B, Tenuifolins B-H, L, Adenanthin B, E	HL60, SMMC-7721, A549, MCF7, SW480	Yang *et al*., 2013
		Isoabietenin C, Tenuifolin M	SMMC-7721, A549, MCF7, SW480	
		Tenuifolin J	A549, MCF7, SW480	
		Adenanthin C	A549	

35	*I. wikstroemioides* (Hand.-Mazz.) H. Hara	Wikstroemioidins I-L, R, T, U, Nervonins G, E, Adenanthins J, K, Rabdokunmin D, Albopilosin B, 3β,7α,14β,18-tetrahydroxy-*ent*-kaur-16-ene-12,15-dione	HL60, SMMC-7721, A549, MCF7, SW480	Wu *et al*., 2014
		Wikstroemioidins E, F	HL60, SMMC-7721, A549, MCF7	
		Wikstroemioidin V	HL60, A549	
		Isoscoparin E, Rabdokunmins A, B	A549, MCF7	
		Macrocalyxin C	A549	
		Isowikstroemins I, M	HL60, SMMC-7721, A549, MCF7, SW480	Wu *et al*., 2015
		Isowikstroemins K, L	HL60, SMMC-7721, A549	
36	*I. xerophilus* (C. Y. Wu and H. W. Li) H. Hara	Rosthorin A	K562, HL60, HCT, MKN-28	Hou *et al*., 2001
		Xerophilusin G	K562, HCT, MKN-28	
		Xerophilusins I, J, Enanderianin C	HCT	
		Xerophilusin S	K562, T24	Niu *et al*., 2004
		Xerophinoids A, B	T24, BIU87	Weng *et al*., 2007
		Xerophilusins IV, V, VI, X, XII, G, H, Rabdoternins D, C, Macrocalin B, Trichorabdal B, Enanderinanin J	K562, MKN45, HepG2	Li *et al*., 2007c
		Maoecrystal I	K562, HepG2	
		Hebeirubescensins G and H,	MKN45, HepG2	

		Rabdoternin E, Xerophilusin A		
		Xerophilusin II, F, Ponicidin	MKN45	
		Xerophilusin N	HepG2	
		Xerophilusin B, Macrocalin B	HCT	Yang *et al*., 2009
		Xerophilusins XIV–XVI, Hebeiabinin B, Maoyecrystals G, H, Rubescensin I	K562, MKN45, HepG2	Li *et al*., 2011
		Oleanolic acid, Ursolic acid, Maslinic acid, 2*α*-Hydroxyursolic acid, Hederagenin, 3β,23-Dihydroxy-urs-12-en-28-oic acid, Arjunolic acid, Asiatic acid, 2α, 3α,24-Trihydroxyolean-12-en-28-oic acid, 2α,3α,24-Trihydroxyurs-12-en-28-oic acid	K562, MKN45, HepG2	Li *et al*., 2012a

*cited by the author; **original binomial name

Antitumor assays

Antitumor studies *in vivo* allow research on anticancer drugs to soar one step ahead by effectively screening the activities of the cytotoxic compounds. Diterpenoids isolated from *Isodon* was subjected to antitumor studies employing EAC inoculated into mice (Fujita *et al*., 1976). Oridonin, lasiokaurin along with enmein and enmein-3-acetate showed antitumor activity and the structure analysis revealed that α-methylene-cyclopentanone system must be an important active centre of these compounds. Diterpenoids isolated from *Rabdosia* was subjected to cytotoxic studies against HeLa cell lines as well as antitumor activities where the effect on the life span of mice inoculated with EAC cells were taken into account (Fujita *et al*., 1988). Of the compounds studied, eukamenin B, kambebanin, effusanin A and sculponeatin C were the most effective *in vivo*. Fuji *et al*. (1989) investigated the antitumor properties of enmein, oridonin and trichorabdal type of diterpenoids obtained from *R. trichocarpa* against EAC in mice. Highest activity was observed for trichorabdal type which could be attributed to the synergistic increase arising from plural active sites in the molecule. Rabdosianone I and II, two novel bitter diterpenes isolated from *I. japonicus* was subjected to electrophysiological experiments. The results showed that taste responses of chorda tympani nerves in Wistar rats was less for rabdosianone I than quinine (Yamada *et al*., 1999).

Sartippour *et al*. (2005) investigated the inhibitory effects on the *in vitro* proliferation of the human breast cancer cells MDA-MB231 and the umbilical vein endothelial cells which led to the conclusion that *R. rubescens* is a potential agent against breast cancer. Experiments on mice revealed *R. rubescens* extract to suppress xenograft size and the tumor vessel density of breast cancer. Oridonin, a diterpenoid isolated from *R. rubescens*, *I. japonicus* and *I. trichocarpus* was investigated for antitumor activity both

in vitro and *in vivo* against t(8;21) acute myeloid leukemic cells (Zhou *et al*., 2007). The studies revealed that oridonin significantly reduced dissemination of the disease and induced apoptosis of leukemic cells in liver, spleen, and bone marrow of mice and prolonged the life span of C57 mice bearing leukemia cells. It also reduced the tumor growth in nude mice inoculated with t(8;21)-harboring Kasumi-1 cells which suggests that oridonin could be a potent candidate for treatment of human t(8;21) leukemia. The *ent*-kaurene diterpenoid melissoidesin was isolated from *I. wightii* and subjected to anticarcinogenic studies (Thirugnanasampandan and Jayakumar, 2009). Comet assay revealed DNA damage protecting activity of melissoidesin in dose dependent manner while the deoxyribose degradation assay proved the DNA protecting ability was through free radical scavenging. A diterpenoid, excisanin A isolated and purified from *Isodon* 'MacrocalyxinD' was investigated for antiproliferation activity on human Hep3B and MDA-MB-453 cell lines *in vitro* and Hep3B xenograft models *in vivo* (Deng *et al*., 2009). Dose and time dependent inhibition of cell viability was shown by excisanin A with the antiproliferation activity induced by apoptosis. Antitumor activity of the compound on Hep3B xenografts induced mice depicted reduction in tumor growth with negligible toxicity.

Eriocalyxin B, a natural diterpenoid isolated from *I. eriocalyx* var. *laxiflora* was subjected to *in vivo* antitumor activity in murine xenograft B- and T-lymphoma models (Zhang *et al*., 2010c). Subcutaneous inoculation of cell lines into nude mice resulted in tumor growth which was seen reduced in eriocalyxin B treated mice. The antitumor activity of eriocalyxin B isolated from *I. eriocalyx* was tested *in vivo* on human pancreatic tumor CAPAN-2 cells xenografted in BALB/c nude mice (Li *et al*., 2012b). The compound depicted preferential toxicity towards cancer cells and could not reveal significant side effects. The antiproliferation effect could be ascribed to the regulation of cellular apoptosis and cell cycle arrest *via* activation of caspase

and p53 pathways. A natural *ent*-kaurene diterpenoid, longikaurin A, isolated from *Isodon* genus was investigated for antitumor activity against nasopharyngeal carcinoma both *in vitro* and *in vivo* (Zou *et al*., 2013). The results revealed that the inhibition of cell growth was due to apoptosis and cell cycle arrest where longikaurin A depicted S phase arrest at low concentrations and caspase dependent apoptosis at high concentrations. *In vivo* antitumor studies was conducted with nude mice xenograft models and the results showed that longikaurin A significantly reduced the tumor growth caused by poorly differentiated nasopharyngeal carcinoma cell line. Longikaurin A was also isolated from *I. ternifolius* and subjected to antitumor studies against human hepatocellular carcinoma cell lines (Liao *et al*., 2014). The studies showed that longikaurin A inhibited the cell line growth in time and dose dependent manner by inducing cell cycle arrest at G2/M phase and apoptosis. SMMC-7721 cells inoculated in nude mice were used for *in vivo* studies and the compound inhibited the growth of tumor xenografts and showed low levels of toxicity.

For the successful development of any drug, clinical trials are necessary. But, humans being considered more precious than any other organism, the experimentation is practically impossible. Since cancer is an unconquered disease and the toll of life being taken away is devastating, clinical tests in laboratory proven drugs are performed. Clinical efficiency of *R. rubescens* for treatment of esophageal cancer patients was tested by Wang *et al*. (2007). Cancer patients at the initial stage of the disease when treated with *R. rubescens* showed greater survival rate while for those at advanced stage, the effect of chemotherapy was enhanced by the species. Moreover, no side effects were produced by the administration of *Rabdosia* species.

PHYTOCHEMICAL STUDIES

Plants become the ultimate source of curatives in most of the diseases but the specifity is not ensured when crude forms are employed. Active principles need to be identified for the efficacy and accuracy of the drugs. Moreover, many compounds isolated from plants are found to be excellent substitutes for synthetic drugs which demonstrate no side effects (Carson and Riley, 2003). Thus, phytochemical screening and profiling is essential for development of effective herbal formulations.

Preliminary phytochemical profiling on hexane, chloroform, water and methanolic extracts of *I. rugosus* was done by Rauf *et al*. (2013). Maximum components were detected in the polar solvents where water and methanol extract showed the presence of steroids, terpenoids, flavonoids, tannins, saponins, reducing sugars, cardiac glycosides, comurrine, betacyanin and protein/aminoacid while the non-polar solvents had fewer components with chloroform extract revealing steroids, terpenoids, flavonoids, reducing sugars, cardiac glycosides, comurrine and protein/aminoacid. Hexane extract showed the presence of steroids only. Zeb *et al*. (2014a) also conducted a preliminary qualitative phytochemical analysis on the crude extract of *I. rugosus* to detect the presence of secondary metabolites. The extract was found to possess glycosides, alkaloids, tannins, terpenoids, flavonoids, saponins, anthraquinones and oils but sterols could not be identified. The total phenol and flavonoid content present in different fractions of *I. rugosus* extract were also studied (Zeb *et al*., 2014b). The results indicated that chloroform, ethyl acetate and crude methanolic extract had highest phenol and flavonoid contents which correlated well with the antioxidant ability of the extracts.

Fujita *et al*. (1967a) investigated the constituents of the stem of *I. trichocarpus* which showed the presence of ursolic acid, oleanolic acid, enmein and oridonin. Stigmasterol and β-sitosterol was identified using vapor

phase chromatogram. Change in the amount of diterpenoid constituents during the growth period of *I. trichocarpus* was explored by Fujita *et al*. (1972) using GC-MS. Enmein and oridonin depicted similar changes with a marked increase in quantity during June-July months. Trivedi *et al*. (1979) conducted HPLC analysis to determine *Isodon* diterpenoids. The technique led to the separation of four polyoxygenated diterpenes commonly found in *I. japonicus*, *I. trichocarpus*, *I. lasiocarpus* and four *ent*-kaurane diterpenes typically found in *I. kameba*, *I. umbrosus* and *I. shikokianus* var. *intermedius*. HPLC analysis of *I. japonicus* revealed that the major reason for the tannic activity of leaf extracts was rosmarinic acid which was found in fairly large amount (Okuda *et al*., 1986). Isobe *et al*. (1994) isolated ten compounds *viz*., kamebanin, kamebakaurin, pedalitin, 4'-O-methylpedalitin, genkwanin, pilloin, eupatorin, methyl caffeate, β-sitosterol and β-sitosterol glucoside from *R. umbrosa* var. *excisinflexa* (Nakai) Hara. The GC-MS analysis revealed that β-sitosterol and aglycon of β-sitosterol glucoside consisted of stigmasterol and campesterol.

The phytochemical analysis of Chinese herb *R. serra* using GC-MS led to the isolation of a carboxylic acid *viz*., hexadecanoic acid and three steroids *viz*., 24 methylcholesterol, stigmasterol and β sitosterol from the plant (Meng *et al*., 2000). Na (2005) investigated the chemical constituents present in the volatile oil obtained from the aerial parts of *I. eriocalyx* var. *laxiflora* and identified 105 compounds which constituted 85.68% of the total peak area. Han *et al*. (2005a) employed HPLC-ESI-MS-MS to identify the principal components present in *I. rubescens*. Five diterpenoids *viz*., rabdoternin B, rubescensin A, OH-rubescensin B, bis-diterpenoid, effusanin E and five flavonoids *viz*., quercetin-3-*O*-rutinoside, kaempferol-3,7-di-*O*-rhamnoside, quercitrin, isorhamnetin, kaempferol-3-*O*-rhamnoside were the major compounds identified, of which the flavonoids were mainly glycosides which could be related with the anti-inflammatory and antitumor activity of the

plants. Oridonin is a diterpenoid and marker compound extracted from *R. rubescences* (*R. rubescens*) (Xu *et al*., 2006). Determination of oridonin in rat plasma was done by HPLC/ESI-MS which was a rapid and accurate method successfully applied in rats after intravenous administration of oridonin for a preliminary pharmacokinetic study. Isodonis herba, a Japanese dietary supplement and folk medicine was subjected to LC-MS analysis to ensure the chemical identity of the plants used (Maruyama *et al*., 2007). The results revealed that Japanese Isodonis herba originated from *I. japonicus* and *I. trichocarpus*, while Chinese Isodonis herba had its origin from *I. eriocalyx* which was identified from the varying ratios of enmein, oridonin and eriocalyxin B present. The essential oil components in *P. rugosus* Wall. syn. *R. rugosa* Wall. was determined by GC and GC-MS analysis (Tiwari *et al*., 2008). Of the twenty five components identified, the major constituents were of sesquiterpene class. β-caryophyllene, germacrene D, spathulenol and ot-cadinol were the major sesquiterpenes identified while the monoterpene hydrocarbons included p-cymene, γ-terpinene and limonene. Zhou *et al*. (2008) utilized negative electrospray ionization tandem mass spectrometry to identify the fragmentation pathways of *ent*-kaurane diterpenoids in *Isodon*. The results showed that loss of CH_2O or CO_2 was the predominant process associated with 7,20-epoxy and 7,20:14,20-diepoxy subgroup of C-20-oxygenated *ent*-kauranes. Similar studies conducted by Zhou *et al*. (2009) revealed the fragmentation pattern of *ent*-6,7-seco-kaurane diterpenoids in which loss of CH_2O and CO_2 were the predominant process linked with the enmein type in negative ion mode while loss of CO_2 was related to spiro-lactone type in positive ion mode.

The plant *I. nervosa* was subjected to HPLC-ESI-MS method for analysis of bioactive constituents from twenty one different samples (Du *et al*., 2010a). Twenty major components were detected which included 16 diterpenoids *viz*., effusanin A, enmein, lasiodonin, oridonin, epinodosinol,

nervosanin B, isodonoiol, sodoponin, rabdosinate, epinodosin, nodosin, ponicidin, rabdoternin A, enmenol, hebeirubesensin K, lasiokaurin as well as four phenolic acids - protocatechuic aldehyde, salicylic acid, caffeic acid and rosmarinic acid. The variation in the amount of the compounds was mainly due to differences in source of the plant, growth parameters and the plant parts used to prepare the medication. Four diterpenoids *viz*., lasiodonin, oridonin, ponicidin and rabdoternin A were found to be the active components in *I. rubescens* extract. HPLC-MS/MS, a novel method was employed for the validation and analysis of the compounds in rat plasma along with the study of pharmacokinetic parameters (Du *et al*., 2010b). HPLC-MS/MS is a method to simultaneously analyze the chemical components both qualitatively and quantitatively (Du *et al*., 2010c). The concurrent determination of twenty eight major components from twenty one batches of natural and cultured samples of *I. rubescens* was done and the identified components included nineteen diterpenoids (effusanin A, enmein, lasiodonin, epinodosinol, nervosanin B, serrin B, isodonoiol, sodoponin, shikokianidin, rabdosinate, epinodosin, nodosin, ponicidin, rabdoternin A, oridonin, enmenol, hebeirubesensin K, lasiokaurin, lasiokaurinol), six phenolic acids (protocatechuic aldehyde, salicylic acid, ferulic acid, caffeic acid, chlorogenic acid, rosmarinic acid) and three flavonoids (quercetin, isorhamnetin, rutin). Standardization and differentiation of similar samples could be attained through this method. The phytochemical constituents of *I. serra* was determined using liquid chromatography coupled with electrospray ionization mass spectrometry method and it was found to be a reliable quality control technique (Liu *et al*., 2010). Twenty seven major components were identified which included eighteen diterpenoids - sodoponin, effusanin A, enmein, lasiodonin, epinodosinol, oridonin, nervosanin B, serrin B, isodonoiol, shikokianidin, rabdosinate, epinodosin, nodosin, ponicidin, enmenol, lasiokaurin, lasiokaurinol, rabdosin B, six phenolic acids - protocatechuic

aldehyde, salicylic acid, ferulic acid, caffeic acid, chlorogenic acid, rosmarinic acid, and three flavonoids - quercetin, isorhamnetin, rutin. Jin *et al*. (2010) quantified nineteen diterpenoids from *I. amethystoides* using HPLC-ESI-MS/MS method. The compounds included effusanin A, enmein, lasiodonin, oridonin, epinodosinol, nervosanin B, serrin B, isodonoiol, sodoponin, shikokianidin, rabdosinate, epinodosin, nodosin, ponicidin, rabdoternin A, enmenol, hebeirubesensin K, lasiokaurin and lasiokaurinol, and the technique was found to be excellent in selectivity and sensitive towards quantification and identification of low level of compounds.

Du *et al*. (2011) developed LC-MS/MS method for the quantification and identification of seven diterpenoids namely epinodosin, epinodosinol, nodosin, oridonin, lasiokariurinol, lasiokaurin and rabdoternin A in rat plasma. The method was employed to determine the pharmacokinetic parameters of the diterpenoids after single oral administration of *I. serra* extract to rats. He *et al*. (2011) employed semi-preparative high-speed counter-current chromatography for the isolation and purification of oridonin from *I. rubescens*. The isolated compound was analyzed by HPLC and the chemical structure was identified with the help of IR, ^{1}H-NMR and ^{13}C-NMR. GC-MS analysis of extracts of *I. excisa* was done to determine the volatile constituents with low polarity (Liu *et al*., 2012a). The results showed the presence of 111 compounds of which sitosterol and 3-amino-4-pyrazolecarbonitrile were the major constituents. Zhou and Fu (2012) conducted GC-MS analysis to identify the components present in the volatile oil of *I. pubescens* (Hemsl.) C. Y. Wu et Hsuan. Of the 94 compounds identified, 39 were detected by steam distillation and the remaining 55 compounds by microwave-assisted extraction. Lin *et al*. (2013) examined the volatile components present in *R. serra* employing headspace solid phase microextraction (HS-SPME) and simultaneous-distillation extraction (SDE) methods. Alcohols, aldehydes, hydrocarbons, ketones, carboxylic acids, esters

and aromatics were the major compounds detected using HS-SPME of which 1-octen-3-ol and (2E)-hexenal contributed to the aroma of the plant. Compounds with low volatility including fatty acids and esters were identified by SDE method. Du *et al*. (2013) studied the biliary excretion time profile and cumulative excretion of diterpenoids in rat bile after single oral administration of *I. rubescens* extract. HPLC-ESI-MS/MS quantified ten diterpenoids *viz*., effusanin A, lasiodonin, oridonin, epinodosin, nodosin, ponicidin, rabdoternin A, enmenol, lasiokaurin and lasiokaurinol and it was identified to be a specific and sensitive method for excretion study. Yao *et al*. (2013) isolated and purified the compounds present in chloroform and n-butanol fraction of *R. japonica* var. *glaucocalyx* employing column chromatography and preparative HPLC. Eleven compounds were identified which included stigmasterol, stigmas-9(11)-en-3-ol, glaucocalyxin D, kamebakaurin, maslinic acid, corosolic acid, minheryins I, diosmetin, caffeic acid ethylene ester, caffeic acid and vitexin. The essential oil isolated from *I. japonicus* var. *glaucocalyx* was subjected to GC-MS analysis and the results showed the presence of 34 components of which the major components were sesquiterpenoids and monoterpenoids (Liu *et al*., 2014). The major compounds identified were thujone, morillol, caryophyllene oxide, β-caryophyllene, α-terpineol, 1,8-cineole, linalool, Z-caryophyllene and γ-eudesmol. Several other compounds were also isolated and structure elucidated from different species of *Isodon* using NMR and MS analysis which are consolidated in table 4.

Table 4. Phytochemical compounds isolated from *Isodon*

Sl. No.	Species	Chemical compound	Reference
1	*R. eriocalyx* (Dunn) H. Hara	Maoecrystals A-E, eriocalyxins A, B, Neorabdosin, Odonicin, Sodoponin	Li *et al*., 1988
2	*R. lungshengensis* O. Y. Wu et H. W. Li	Lungshengrabdosin, Lushanrubescensin C, Ursolic acid, 2α-Hydroxyursolic acid	Luo *et al*., 1988
3	*R. rosthornii* (Diels) H. Hara	Rosthornins A, B	Xu and Ma, 1989
4	*I. adenantha* (Diels) Hara	Adenanthins G, J, K, Calcicolin B, Forrestin C	Jiang *et al*., 2002c
5	*I. adenanthus* (Diels) Kudô	Adenanthusone	Xiang *et al*., 2004c
6	*I. adenolomus* (Hand.-Mazz.) H. Hara	Isoadenolin M, Rabdoinflexin B, Phyllostachysin H, Rabdokunmin C, 4-*Epi*-henryine	Zhao *et al*., 2011a
7	*I. angustifolius* Kudô var. *glabrescens* (C. Y. Wu & H. W. Li) H. W. Li	Glabcensins Q-U	Zhao *et al*., 1998a
		Glabcensins V-Y	Zhao *et al*., 1999b
8	*I. calcicola* [(Hand.-Mazz.) Hara] var. *subculva* [(Hand.-Mazz) C. Y. Wu et H. W. Li]	Calcicolins A-E, Weisiensin A, Adenanthin, Forrestin C, Nervosanin	Chen *et al*., 1998a
9	*I. enanderianus* (Hand.-Mazz.) H. W. Li	Enanderianins A, B	Wang *et al*., 1998b
		Enanderinanins B, G, H, Macrocalin B, Xerophilusins A, G, Trichorabdals A, B,	Na *et al*., 2002

		Effusin, Angustifolin, Longikaurins D, F, Shikokianin	
		Enanderianins M-O, Rabdocoetsin A, Megathyrins A, B	Xiang *et al*., 2003
10	*I. eriocalyx* (Dunn) Hara	Maoecrystal P, Coetsoidin A	Wang *et al*., 1998a
		Maoecrystal U, W	Shen *et al*., 2005
11	*I. eriocalyx* (Dunn) Hara var. *laxiflora* C.-Y. Wu et H.-W. Li	Laxiflorin B, Eriocalyxin B, Oriodonin, Maeocrystals A, B	Sun *et al*., 1995b
		Eriocalyxin A, Laxiflorins A, D, H, Maoecrystals Q, S, Enmelol	Niu *et al*., 2002a
		3α,20-Epoxy-6β-hydroxy-1,7-dioxo-*ent*-abiet-15(17)-en-16-oic acid, *ent*-Abieta-7,15(17)-diene-3β,16,18-triol	Niu *et al*., 2003
		Eriocalyxin B	Wang *et al*., 2012a
12	*I. excisa** (*I. excisus*)**	Excisanins D, E	Wang *et al*., 1997
13	*I. excisoides* (Sun ex C. H. Hu) C. Y. Wu et H. W. Li	Glaucocalyxin A	Wang *et al*., 2009b
		Taihangexcisoidesins A, B	Zhang *et al*., 2009b
		Taihangexcisoidesins C, D	Wang *et al*., 2010b
		Taihangexcisoidesins C, D Rabdosinatol, Rabdoternin A, Lushanrabescence F, Epinodosin, Xerophilusin B,	He *et al*., 2010

		Wikstroemioidin B, Rabdosichuanin D, (+)-1-Hydroxy-pinoresinol, Vomioflio	
14	*I. excisus* (Max.) Kudô	Excisusin A-E, Inflexarabdonin A, G, I, Inflexin, Inflexanin A, B, Inflexinol	Hong *et al*., 2007
		Inflexin	Ko *et al*., 2010
		Excisusin F, Inflexarabdonin D, E, Corchoionol A, Corchori fatty acid B	Hong *et al*., 2011
15	*I. excisus* var. *coreanus*	Inflexin, Ursolic acid, Ursolic acid 3-O-acetate	Jeong *et al*., 2000
16	*I. flavidus* (Hand.-Mazz.) H. Hara	Flavidusins A, B, Glutinosin, *ent*-Kauran-16β,17-diol, Siegesbeckiol, 16-Hydroxy feruginol, Hinokiol, Maslinnic acid, 5-Hydroxy-7,4'-dimethoxyflavone	Zhao *et al*., 1998b
17	*I. gesneroides* (J. Sincl.) Hara	Gesneroidin A	Sun *et al*., 1995c
		Gesneroidins D-F, Rabyuennane A, 3-Acetylcalcicolin A	Chen *et al*., 1998b
18	*I. glutinosa** (*I. glutinosus*)**	Glutinosin C	Niu *et al*., 2002d
19	*I. glutinosus* (C. Y. Wu & H. W. Li) H. Hara	Isodoglutinosin A, B	Huang *et al*., 1997
20	*I. henryi* (Hemsl.) Kudô	Taibaihenryiins A, B, Shikokianin, Longikaurin D, F, 2*α*-Hydroxyursolic acid, Lasiodin	Li *et al*., 2001

		Taibaihenryiin C, Odonicin, Rabdosianin B, Wikestroemioidin C, Eupafolin	Li *et al*., 2002a
		Rabdosianin B	Li *et al*., 2005
		Hinokiol	Li *et al*., 2007a
		Minheryin F	Zhao *et al*., 2009b
		Minheryins H, I	Zhao *et al*., 2008
		Isodonhenrins C, D, Oridonin	Hu *et al*., 2011
21	*I. inflexus* (Thunb.) Kudô	3α,6β-Dihydroxy-7,17-dioxo-*ent*-abieta-15(16)-ene, Kamebacetal A, Kamebakaurin, Excisanin A, Ursolic acid	Lee *et al*., 2008
		Inflexanin C, D, Inflexuside A, B	Xie *et al*., 2012
22	*I. irrorata* (Forrest.)* (*I. irroratus*)**	Irroratin A	Sun *et al*., 2001
23	*I. japonica* (Burm.f.) Hara* (*I. japonicus*)**	Taibaijaponicains A, B	Li and Tian, 2001
		Taibaijaponicains C, D	Li, 2002
		Maoyecrystal F, Acetonide derivative of maoyecrystal F, Lasiodonin, Maoyerabdosin, Odonicin, Enmenin, Oridonin, beta-Sitosterol, Daucosterol	Zhang *et al*., 2003
		Taihangjaponicain B	Bai *et al*., 2005
		Maoyecrystal B	He *et al*., 2006
		Maoyecrystal L	Di *et al*., 2010

24	*I. japonica* var. *glaucocalyx** (*I. japonicus* var. *glaucocalyx*)**	Glaucocalyxin A	Ma *et al*., 2007
		Glaucocalyxin B	Bai *et al*., 2009
25	*I. japonicus* (Burm.f.) H. Hara	Enmein, Enmein-3-acetate	Fujita *et al*., 1967b
		Enmein, Isodonal, Nodosin, Trichodonin, Epinodosin, Oridonin, Enmein-3-acetate	Kubo *et al*., 1974
		Kamebanin, Kamebacetal A, Kamebakaurin, Excisanin A	Hwang *et al*., 2001
		Isodojaponins A-E, Pseurata C, Longikaurin B, C, D, Effusanin C, Effusanin D, Excisanin B, Lasiokaurin, Megathyrin A, Loxothyrin A	Hong *et al*., 2008
		Isodojaponin D	Lim *et al*., 2010
		Effusanin C	Kim *et al*., 2013
26	*I. leucophyllus* (Dunn) Kudô	11α-Acetoxyeffusanin D, 6-Acetylepinodosinol, 16β-Ethoxymethyleneshikokianin, 16α-Ethoxymethyleneshikokianin	Chen *et al*., 1999
		Baiyecrystal D, Xerophilusin B, Macrocalin B, Rabdoternin A, Phyllostachysin B	Zhao *et al*., 2004b
		Rabdoloxin A	Zhang *et al*., 2011
27	*I. lophanthoides* (Buch.-Ham. ex D. Don) H. Hara	Lophanic acid, 8(17),12,14-Labdatriene-19-oic acid, 11β-Hydroxyisopimara-8,15-diene-3-one	Jiang *et al*., 2000

28	*I. lophanthoides* var. *gerardianus* [Benth.] H. Hara	Gerardianin A, 6,7-Dehydroroyleanone	Lin *et al*., 2008
		Lophanthodin G, 6α-Hydroxyferruginol	Liu *et al*., 2008
29	*I. lophanthoides* var. *graciliflorus* [Benth.] H. Hara	Podocarpa-8,11,13-triene-3α,13-diol	Liang *et al*., 2013
		Graciliflorin F, 15-O-Methylgraciliflorin F	Zhou *et al*., 2013b
		Rosmarinic acid, Methyl rosmarinate, Clinopodic acid A, Salvianolic acid A, Nepetoidin B, Caffeic acid, Vinyl caffeate, Danshensu, Latifolicinin C, Hydroxytyrosol, Procatechuic aldehyde, 3,4-Dihydroxybenzoic acid, Syringic acid	Zhou *et al*., 2014
30	*I. lophanthoids* var. *micranthus** (*I. lophanthoides* var. *micranthus* (C. Y. Wu) H. W. Li)**	Micranthin C, 16-Acetoxy-7α-hydroxyroyleanone, 16-Acetoxy-7α-methoxyroyleanone, Hyptol	Zhao *et al*., 2003b
31	*I. loxothyrsus* (Hand.-Mazz.) H. Hara	3β,13β-Dihydroxy-urs-11-en-28-oic acid, Oleanolic acid, Ursolic acid, 2α-Hydroxy ursolic acid, 2α,19α-Dihydroxy ursolic acid, Rabdoloxin B, Rabdokunmin D	Huang *et al*., 1996b
32	*I. lungshengensis* C. Y. Wu et H. W. Li	Lungshengenins D, F	Jiang *et al*., 1998
33	*I. macrocalyx* Kudô	Macrocalyxins B, F, G, H, Maoyerabdosin, Rabdophyllin H, Ponicidin, Oridonin, Enmenol	Wang *et al*., 1995
34	*I. macrophylla* (Migo) H. Hara	(-)-Lambertic acid, Rubescensin A, Parvifoline E, Lasiodonin, Effusanin E	Qin *et al*., 2007

		Dayecrystal C	Chen *et al*., 2008
35	*I. melissoides* (Benth.) H. Hara	Melissoidesins E-H, Dawoensin A, (+)-l-Hydroxypinoresinol-l-β-□-glucoside, (+)-Fraxiresinol-1-β-□-glucoside	Zhao *et al*., 1999a
		Melissoidesins I-L	Zhao *et al*., 2003a
		Melissoidesins C, D, V, W, Glabcensin W, Melissoidesins A, B, 3α,4α-isopropyliden-β-ionol, 3-hydroxy-4-oxo-β-ionol, Megastigma-7-en-3,5,6,9-tetraol, Blumenol A, Salicylic acid, Syringic acid, Cirsiliol	Zhao *et al*., 2005
36	*I. nervosus* (Hemsl.) Kudô	6β,7β,13α-Trihydroxy-1α-acetoxy-7α,20-epoxy-*ent*-kaur-16-en-15-one, 15β-Hydroxy-6,7-seco-6,11β:6,20-diepoxy-1α,7-olide-*ent*-kaur-16-ene	Yan *et al*., 2008
		6,20,15α-Trihydroxy-6,7-seco-1α,7-olide-*ent*-kaur-16-ene, 7β,12α-Dihydroxy-6β,15β-diacetoxy-7α,20-epoxy-*ent*-kaur-2,16-dien-1-one	Yan *et al*., 2009
		ent-Abienervonins A-C, Hebeiabinin B, *ent*-Isopimanervosides A, B, Urolignoside, Glochidioboside, (-)-Secoisorariciresinol-9'-O-β-□-glucoside, (+)-Pinoresinol-4-O-β-□-glucoside, 8-Hydroxypinoresiol-4'-O-β-□-glucoside, 8-Hydroxypinoresiol-4-O-β-□-glucoside, Daidzein-4'-O-β-□-glucoside	Li *et al*., 2009a

37	*I. oresbius* (W. W. Smith) Kudô	Oresbiusin, Pinostrobin, 5-Hydroxy-7,8-dimethoxyflavanone, Dihydrowogonin, Sakuranetin, Chrysoeriol, Apigenin, Luteolin	Huang *et al*., 1996a
		Oresbiuside	Huang *et al*., 1998
		Oleanolic acid, Ursolic acid, Sodoponin, Astragalin, Quercetin-3-O-glucoside, Oresbiusin A, Rosmarinic acid, Methyl rosmarinate, Neo-angustifolin	Huang *et al*., 1999
		Oreskaurins A, C, Enmenin monoacetate, Effusanin E, Adenolin B, Maoecrystal G, Enmelol, Trichokaurin, Sodoponin, Trichorabdal A, Enmein, Vitexin	Xiang *et al*., 2004b
38	*I. parvifolius* (Batalin) H. Hara	Parvifolines L-N, Rubescensin P, I, J, Enanderianin P, 14β,16-Dihydroxy-3α,18-[(1-methylethane-1,1-diyl)dioxy]-*ent*-abieta-7,15(17)-diene, 8β,13β-Oxidoeperu-14-en-18-oic-acid	Li *et al*., 2006a
		Rabdoternin G, Adenolin E, Lushanrubescensin F, Parvifoliside, Effusanins A, B, E, Taibaihenryiin A, Shikokianin, Maoyecrystal J, Acetonide of lasiodonin	Li *et al*., 2006b
		Phyllostachysin A, Isodonoiol, Epicandicandiol, Xylopic acid, Paulownin, 3',4-O-Dimethylcedrusin, Methyl	Li *et al*., 2006c

		rosmarinate, Cirsimaritin, 5-Hydroxy-4',6,7-trimethoxyflavone, Quercitrin, (3S,5S,6R,9R)-3-hydroxy-5,6-epoxy-β-ionol, Blumenol, Betulinic acid, N-2'-hydroxy-eicosanoyl(henicosanoyl-hexacosanoyl)-4-hydroxy-trans-8-sphingenine	
		Parvifolines O, P, R-T, W, Rosthorin A, Longikaurin B, E, Adenolin D	Li *et al*., 2006e
39	*I. pharicus* (Prain) Hara	Isodopharicin A, B, C	Wang *et al*., 1991
		Bispseurata F, Pharicinin A	Zhao *et al*., 2009a
		3β,14β,15β-Trihydroxy-*ent*-kaur-16-en-12-one, 3α,7α,14*β*,15*β*-Tetrahydroxy-*ent*-kaur-16-en-12-one, 3-Epipseurata B acetonide, 12-Deoxyisodomedin acetonide, Pseurata B acetal, Kamebanin, Dihydropseurata F	Zhao *et al*., 2009c
40	*I. phyllostachys* (Diels) Kudô	Phyllostachysin C, Sculponeatins B, C, Nodosin, Ursolic acid, 2 alpha-hydroxyursolic acid	Hou *et al*., 2000
		Phyllostacins A, B, Irroratin A, Serrin B	Li *et al*., 2006f
		5,7-Dihydroxy-4' hydroxylflavone, Oleanolic acid, Daucosterol	Li *et al*., 2006g
		Phyllostacins H, I, Rosthorin A, Rabdoternin C, Oridonin, Lasiocarpanin, Ponicidin,	Li *et al*., 2008c

		Macrocalin B, Sculponeatin C, Macrocalyxoformin E	
41	*I. pseudo-irrorata* C. Y. Wu	Pseurata A	Zhang *et al*., 2002
42	*I. rubescens* (Hemsl.) H. Hara	Taibairubescensins A, B	Li *et al*., 2000
		Rubescensins N, O, Rabdoternins A-F, Xerophilusin N	Han *et al*., 2003a
		Rabdoternins A-D, Rubescensins C, O, Q, Enmenol, Ponicidin, Macrocalin B, Xerophilusin B, Acetonide of maoyecrystal F, Trichokaurin	Huang *et al*., 2006b
		Rubescensin C, Trichokaurin, Maoyecrystal F, Rabdoternin A, 1α-O-β-D-gluco-pyranosyl enmenol, Acetonide, Rabdonervosin B, Acetonide of rubescensin J	Xie *et al*., 2011
		Rubesanolides A, B	Zou *et al*., 2011
		Jianshirubesin C	Liu *et al*., 2012b
		Jianshirubesin F	Liu *et al*., 2012d
		Kaurine C	Liu *et al*., 2015
43	*I. rubescens* var. *lushanensis*	Lushanrubescensins F, G, I, Rabdoternins A, B, Enmenol, Epinodosin, Inflexusin	Han *et al*., 2003b
		Kamebakaurin	Zhang *et al*., 2010a
		Rubluanin A, 7-Isopropyl-podocarpinol	Zhang *et al*., 2010b

44	*I. rubescens* var. *lushiensis*	Isolushinins C, E, I, J, Guidongnins E, F	Luo *et al*., 2010
45	*I. rubescens* var. *rubescens*	Xindongnins M-O	Han *et al*., 2004c
		Xindongnins D, E, G	Han *et al*., 2004g
		Xindongnins A, B, H-L, Melissoidesins G, Dawoensin A, Glabcensin V	Han *et al*., 2004d
		Xindongnin P	Han *et al*., 2005b
46	*I. rubescens* var. *taihangensis* Z. Y. Gao and Y. R. Li	Rubescensin W	Han *et al*., 2004e
		Rubescensins U, V	Han *et al*., 2004f
47	*I. rugosus* (Wall.) Codd	Rugosinin, Effusanin A, B, E, Lasiokaurin, Oridonin	Abbaskhan *et al*., 2003
48	*I. scoparius* (C. Y. Wu et H. W. Li) H. Hara	Isoscoparins A-C	Xiang *et al*., 2004a
		Isoscoparins E, G, J, K, Phyllostachysin F, G, Macrocalyxin E	Zhao *et al*., 2009d
49	*I. sculponeata** (*I. sculponeatus*)**	Sculponeatins E-G, Epi-nodosinol, Macrocalyxoformins A, 2alpha,3beta-Ursolic acid, 2alpha,3beta,19alpha-Dihydroxy-urs-12-en-28-oic acid, Trihydroxy-urs-12-en-28-oic acid, beta-Sitosterol, Daucosterol, Quercetin, Pedalitin, Rosmarinic acid, Caffeic acid, Ethyl caffeic acid	Jiang *et al*., 2002a
		Sculponeatins A, K, N, O, Sculponeatic acid, Hyptadienic acid	Wang *et al*., 2009a
50	*I. sculponeatus* (Vaniot) Kudô	Sculponins Q-S	Jiang *et al*., 2013

		Sculponins U-X, Z, Wikstroemioidin A, Maoyecrystals G, H	Jiang *et al*., 2014b
		Sculponeatin N	Moritz *et al*., 2014
51	*I. serra* (Maxim.) Hara	Serrins A-C, Isodocarpin, Epinodosin, Nodosin, Carpalasionin, Enmein, Oridonin, Lasiodonin	Zhao *et al*., 2004c
		15α,20β-dihydroxy-6β- methoxy-6,7-seco-6,20-epoxy-1,7-olide-*ent*-kaur-16-ene, 6α,15α-Dihydroxy-20-aldehyde-6,7-seco-6,11α-epoxy-1,7-olide-*ent*-kaur-16-ene	Yan *et al*., 2007
52	*I. tenuifolius* (W. W. Smith) Kudô	Tenuifolin K	Yang *et al*., 2013
53	*I. weisiensis* C. Y. Wu	Excisanin D	Ding *et al*., 2005a
54	*I. wikstroemioides* (Hand.–Mazz.) H. Hara	Pseudoirroratin A	Wu *et al*., 2015
55	*I. xerophilus* (C. Y. Wu et H. W. Li) H. Hara	Xerophilusins L-N, Rabdoternin A, Longikaurin F	Li *et al*., 2002b
		Rabdoternin D, F, Xerophilusin A, B, G, H, K, Macrocalin B, Ponicidin, Longikaurin B, Rosthoriin A, Effusanin A, Rubescensin C, D	Weng *et al*., 2007
		Xerophilusin III, VII, VIII, IX	Li *et al*., 2007c
		Xerophilusins A, B, F, Longikaurin B	Aquila *et al*., 2009
		Pinoresinol, 5,8-Dihydroxy-4',6,7-trimethoxy-flavone-8-O-β-D-glucopyranoside, Patriscabratine, 9,16-Dioxooctadec-10,12,14-trienoic acid	Li *et al*., 2011

*cited by the author; **original binomial name

Lee *et al*. (2001) isolated two new compounds from *I. excisus* of which 3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-methoxyethyl] acrylamide was found to be an inhibitor of etoposide-induced apoptosis in U937 cells while 3-(3,4-dihydroxyphenyl)acrylic acid 1-(3,4-dihydroxyphenyl)-2-methoxycarbonylethyl ester showed weak activity. Melissoidesin G isolated from *I. melissoides* was investigated for *in vitro* antileukemic activity against leukemia cell lines and primary acute myeloid leukemia blast cells (Yu *et al*., 2007). The compound was able to inhibit the proliferation of cell lines and blast cells by induction of apoptosis which disrupted the redox balance, caused perturbation of mitochondria as well as activated caspases. Selective toxicity in combination with malignant cells was also shown by the synergistic apoptotic effects of the compound with As_2O_3 in leukaemia and blast cells but not in normal cells. Weng *et al*. (2007) examined the biological activities of xerophinoids A and B isolated from *I. xerophilus*. The compounds showed cytotoxicity against rat hematopoietic stem cells (32D) but were non-cytotoxic towards human T-lymphocyte cells (C8166). Wang *et al*. (2009b) characterized glaucocalyxin A from the leaves of *I. excisoides* and through surface molecular electrostatic potential technique, identified α-methylene cyclopentanone moiety to play the dominant role in the biological activity. Cytotoxic activity of eight *ent*-kaurane diterpenoids *viz*., leukamenin E, glaucocalyxin A, wangzaozin A, kamebanin, macrocalyxin D, weisiensin B, excisanin K, weisiensic C isolated from *I. excisoides*, *I. weisiensis* and *I. racemosa* were investigated against four human tumor cell lines *viz*., HepG2, Tb, HO-8910 and SGC-7901 (Ding *et al*., 2010). Results showed that weisiensic C and excisanin K depicted non-significant cytotoxicity against all cell lines while weisiensin B had weak cytotoxicity against HO-8910 and SGC-7901 cell lines. All other compounds had significant toxicity against all cell lines except for kamebanin against HO-8910 cells. Han *et al*. (2010) identified eriocalyxin B, a well-known

antitumor natural product, inducing apoptosis from *I. eriocalyx* by means of an analytical high-speed counter-current chromatography and fluorescence-based caspase-3 biosensor detection system which was a novel method for fractionation and identification.

The molecular mechanism involved in the anticancer activity of oridonin isolated from *I. rubescens* against liver cancer cells was analyzed (Wang *et al*., 2011a). Studies confirmed the expression of eight up-regulated proteins, namely heat shock 70 kDa protein 1, stress-induced phosphoprotein 1, serine–threonine kinase receptor-associated protein, trifunctional purine biosynthetic protein adenosine-3, inorganic pyrophosphatase, chromobox protein homolog 1, translationally-controlled tumor protein, glycyl-tRNA synthetase and one down-regulated protein, namely poly(rC)-binding protein 1 after the treatment with oridonin which were identified by proteomic analysis. Jaridonin, a novel *ent*-kaurane diterpenoid was isolated from *I. rubescens* which potently reduced the viabilities of several esophageal cancer cell lines *viz*., EC109, EC9706 and EC1 (Ma *et al*., 2013). The anti-proliferative and apoptotic effects of jaridonin make it a good candidate in chemotherapy against esophageal cancer. The anti-invasive effects of the diterpenoid excisanin A isolated and purified from *Isodon* 'macrocalyxin D' against breast cancer cells along with the underlying molecular mechanism was investigated (Qin *et al*., 2013). The results revealed that suppression of mRNA and protein level expression of matrix metalloproteinase-2 and matrixmetalloproteinase-9 inhibited the invasion and that the integrin β1/FAK/PI3K/AKT/β-catenin signaling pathway was involved in the process which makes it a potential anti-metastatic chemotherapeutic agent in the treatment of breast cancer. The molecular basis of the cellular activity produced by oridonin isolated from *I. rubescens* in leukemia-derived Jurkat cells was studied (Piaz *et al*., 2013). Oridonin was found to bind multifunctional, stress-inducible heat shock protein 70 1A resulting in a

complex formation in Jurkat cells and the binding site on chaperone was also identified. Henryin isolated from *I. rubescens* var. *lushanensis* showed growth inhibitory effects on the colon cancer cells *viz*., SW-480, HT-29 and HCT116 by inhibiting Wnt signaling (Li *et al*., 2013) which makes it a potent candidate in prevention of colorectal cancer. The compounds from *R. serra* were screened for antitumor activity *in vitro* (Chen *et al*., 2014) and the studies revealed that purified compounds from acetidin extraction of the plant had cytotoxic activity against the tumor cells MCF7, BGC-823 and HepG2.

Materials and Methods

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

MATERIALS AND METHODS

Genus *Isodon* extensively used in Chinese and Japanese folk medicines was the source plant for the current study. Three taxa belonging to the genus was investigated for chromosome analysis, antioxidant and antitumor activity as well as phytochemical assays. The protocols adopted and experiments performed are detailed below. All the experiments were tested out at Cell and Molecular Biology Division, Department of Botany, University of Calicut, Kerala, India and also at Amala Cancer Research Centre, Thrissur, Kerala, India. The analyses carried out in the present study were:

1. Chromosome analysis using karyomorphological parameters in three taxa of the genus *Isodon*.
2. Antioxidant studies of methanolic extracts of the three taxa using four assays - DPPH radical scavenging activity, Hydroxyl radical scavenging activity, Superoxide radical scavenging activity and Ferric ion reducing antioxidant power activity.
3. *In vitro* cytotoxic and *in vivo* antitumor studies of methanolic extracts against DLA and EAC cell lines.
4. Estimation of the total phenolic, flavonoid and terpenoid content in the three methanolic extracts.
5. Phytochemical analysis of methanolic extracts employing Gas chromatography - mass spectrometry (GC-MS) and Liquid chromatography - mass spectrometry (LC-MS) techniques.

I. PLANT MATERIALS

The experiments in the current study were carried out using three species of *Isodon viz*., *I. coetsa* (Buch.-Ham. ex D. Don) Kudô, *I. nigrescens* (Benth.) H. Hara and *I. nilgherricus* (Benth.) H. Hara (Plate 1).

The plant specimens were collected from wild populations, at different sites in Munnar coming under the jurisdiction of Idukki district of Kerala, India and authenticated taxonomically by Dr A. K. Pradeep and Dr P. Sunoj Kumar at the Angiosperm Taxonomy Division, University of Calicut, Kerala, India. The voucher specimens were deposited at Calicut University Herbarium (CALI), Kerala, India.

1. ***Isodon coetsa* (Buch.-Ham. ex D. Don) Kudô** (CALI 123726)

   Habit: Perennial herb or undershrub

   Habitat: In open bamboo forests, grasslands, disturbed areas, along paths in evergreen forests at an altitude around 700-2200 m

   Distribution: Western Ghats and Eastern Ghats, Evergreen Forests

   Phenology: Flowering in August-January, Fruiting in October-April

   Distinct character: Thinner leaves, subequal calyx lobes, strongly decurved corolla tube

Stems were woody at base, much branched, hollow, quadrangular growing upto 3 m length. Leaves were membranous to chartaceous, acute apex with base decurrent on petiole, serrate margin and dotted with minute sessile glands. Inflorescence showed axillary and terminal type with several narrow raceme like branches forming a large panicle. Calyx was companulate which were inconspicuously two lipped and with sessile gland outside. Corolla was blue or pale purple coloured and pubescent with sessile glands seen on outer side. Stamens were found to be included in the anterior corolla lip and style which was included or exceeding the corolla lip. Nutlets were revealed to be brown or black in colour producing mucilage when wet or not (Suddee *et al*., 2004).

PLATE 1

Fig. 1 Habit A *Isodon coetsa* (Buch.-Ham. ex D. Don) Kudô, B *Isodon nigrescens* (Benth.) H. Hara, C *Isodon nilgherricus* (Benth.) H. Hara. Insight – flowers of respective plants enlarged

2. ***Isodon nigrescens*** **(Benth.) H. Hara** (CALI 123727)

Habit: Perennial herb

Habitat: Seen on wooded hilltops and upper mountain slopes or in highland rainforests in around 2000-2350 m altitude

Distribution: Western Ghats, Evergreen Forests, Grasslands

Phenology: Flowering in March-April, Fruiting in October

Distinct character: Rugose leaves that are nigrescent when dry

Stems were branched, obtusely quadrangular and dark brown in colour when dry. Cauline leaves were found to be opposite, subsessile with ovate blade, subacuminate apex and broadly cuneate to rotund base. Apart from this, leaves were crenate with chartaceous rugose nature, nigrescent when dry and were dotted with red glands at lower surface. Inflorescence was panicle and terminal, and was composed of remote cymes. The peduncle, pedicels and rachis were found to be densely pubescent. Calyx was campanulate while the fruiting calyx was tubular campanulate and dilated. Corolla was only gradually dilated and white with purple spots on limb. It showed two lipped limb with upper lip reflexed and lower lip suborbicular, concave. Stamens and style were included or subincluded. Nutlets were found to be brown and glabrous (Li, 1988).

3. ***Isodon nilgherricus*** **(Benth.) H. Hara** (CALI 123728)

Habit: Perennial herb

Habitat: Seen on hillsides around 1700 m altitude

Distribution: Western Ghats, Evergreen Forests, Grasslands

Phenology: Flowering and fruiting in October

Distinct character: Conspicuously rugose leaves

Stems were erect, obtusely quadrangular and around 60 cm in height with reddish colour and the internodes were found to be shorter than leaves.

Cauline leaves were opposite, blade orbicular-ovate with acuminate apex and cordate at base, crenulate, chartaceous, rugose and dotted with reddish brown glands beneath. Inflorescence was of panicle type and terminal, composed of spreading many flowered cymes and sessile bracts. The peduncle, pedicels and rachis showed densely villose nature. Calyx was tubular-campanulate while corolla tube was slightly dilated at throat and glabrous with red spots on lips inside. It featured two lipped corolla with upper lip reflexed and lower lip suborbicular, patent. Stamens and style were exserted. Nutlets were brownish in colour and glabrous (Li, 1988).

II. METHODOLOGY

The standard protocols adopted and techniques followed in order to identify the karyomorphology of the chromosomes of the above mentioned plants along with the antioxidant, cytotoxic and antitumor activities as well as the detection of phytochemical components present in the three taxa of *Isodon* are explained here upon.

CHROMOSOME ANALYSIS

Cytology and karyotype analysis are very important in solving taxonomic disputes of closely related species as it gives proof of evolution that might have occurred during the course of time. Chromosome number and morphology are important parameters taken into account in cytotaxonomy. Karyomorphological studies helps in identifying the chromosome number and morphology as well as in developing the karyogram and idiogram of the chromosomes present in different species.

Mitotic squash preparation

Healthy plants collected from wild populations were domesticated and stem cuttings were obtained at the nodes. The nodal stem cuttings were grown in shallow pots filled with sand for the development of primary roots. The

peak mitotic period (09.00-10.00 AM) was identified by trial and error method, and the young, healthy root tips were collected during this favorable mitotic time. Pretreatment of the collected root tips were carried out after washing thoroughly in double distilled water. The cytostatic chemicals were not employed since the plants had inherent ability for the separation of chromosomes as noted down in the studies on C-mitotic effect of *I. coetsa* (Neelamkavil and Thoppil, 2013). Chilled double distilled water with trace amount of saponin to remove the phenols and secondary metabolites were used as the pretreatment solution.

Pretreatment solution was obtained after chilling at 0-5°C for 10 min. Root tips were immersed in the solution and was kept under refrigeration at 12-15°C for 1 hour. The root tips thus obtained was thoroughly washed in double distilled water and fixed in modified Carnoy's fluid prepared with a composition of 1 acetic acid: 2 ethyl alcohol for 1 hour. Improved techniques suggested by Sharma and Sharma (1990) for mitotic squash experiments were pursued.

Root tips after fixation and thorough washing was hydrolyzed in 1N HCl for 15 min. The root tips were washed again with double distilled water which was essential for removal of acid content and was followed by staining in 2% acetocarmine for 3 hours. Microslides were prepared after destaining with 45% acetic acid for 5 min. The slides were scanned for metaphase chromosomes in Olympus Binocular Research microscope CX 21 and photomicrographs were taken with the help of Olympus Camedia C- 4000 Zoom digital compact camera attached to the microscope.

Karyomorphometry

Computer based programs such as Adobe photoshop, AutoCAD and a data based analyzing system (Microsoft Excel) was employed for the

generation of karyograms from photomicrographs. Each chromosome was falsely numbered for identification and loaded to AutoCAD program for karyomorphometric studies. Centromeric position of each chromosome was identified and the arm lengths were measured. The data thus obtained was used for the calculation of centromeric indices. The homologous chromosomes were identified based on the arm ratio and centromeric indices. The classification of chromosomes was done based on the data calculation reported by Abraham and Prasad (1983) (Table 5). Karyograms and idiograms were generated with the help of Photoshop program.

Table 5. Chromosome nomenclature based on centromere location (Abraham and Prasad, 1983)

Nomenclature	Notation	R_1 s/l	R_2 l/s	I_1 100 s/c	I_2 100 l/c
Median	M	1.00	1.00	50.00	50.00
Nearly median	Nm	0.99 to 0.61	1.01 to 1.63	49.99 to 38.01	50.01 to 61.99
Nearly submedian	nsm(-)	0.60 to 0.34	1.64 to 2.99	38.00 to 25.01	62.00 to 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly submedian	nsm(+)	0.32 to 0.23	3.01 to 4.26	24.99 to 18.20	75.01 to 81.80
Nearly subterminal	nst(-)	0.22 to 0.15	4.27 to 6.99	18.19 to 12.51	81.81 to 87.49
Subterminal	ST	0.14	7.00	12.50	87.50
Nearly subterminal	nst(+)	0.13 to 0.07	7.01 to 14.38	12.49 to 5.01	87.51 to 94.99
Nearly terminal	Nt	0.06 to 0.01	14.39 to 19.99	5.00 to 0.01	95.00 to 99.99
Terminal	T	0.00	α	0.00	100.00

The length of the chromosome, position of the centromere and presence/absence of secondary constriction were the parameters based on which the karyotype formula was worked out.

Disparity index (DI) of the chromosomes was calculated from the formula by Mohanty *et al*. (1991) which was

$$\mathrm{DI} = \frac{\text{Longest chromosome} - \text{shortest chromosome}}{\text{Longest chromosome} + \text{shortest chromosome}} \times 100$$

The variation coefficient (VC) among the chromosome complements was determined from the formula by Verma (1980) which was

$$\mathrm{VC} = \frac{\text{Standard Deviation}}{\text{Mean Length of chromosomes}} \times 100$$

The total Forma percentage (TF%) or mean centromeric index value was calculated from the formula by Huziwara (1962) which was

$$\mathrm{TF\%} = \frac{\text{Total sum of short arm length}}{\text{Total sum of chromosome length}} \times 100$$

The karyotype asymmetry index (As K%) was calculated from the formula developed by Arano (1963) which was

$$\text{As K\%} = \frac{\text{Total sum of long arm length}}{\text{Total sum of chromosome length}} \times 100$$

The karyotype asymmetry was also evaluated by two indices developed by Greilhuber and Speta (1976) which were called the Syi index and the Rec index by Venora *et al.* (2002) from the formula which was

$$\mathrm{Syi} = \frac{\text{Mean length of short arms}}{\text{Mean length of long arms}} \times 100$$

$$\mathrm{Rec} = \frac{\sum_{i=1}^{n} \frac{\mathrm{CLi}}{\mathrm{LC}}}{n} \times 100$$

where n = number of analyzed chromosome, CLi = length of each chromosome, LC = longest chromosome.

The intrachromosomal asymmetry index (A_1) and the interchromosomal asymmetry index (A_2) was calculated from the formula by Zarco (1986) which was

$$A_1 = 1 - \left(\frac{\sum_{i=1}^{n} \frac{bi}{Bi}}{n} \right)$$

where bi = the average length for short arms in every homologous chromosome pair or group, Bi = the average length for long arms in every homologous chromosome pair or group, n = the number of homologous chromosome pairs or groups.

$$A_2 = \frac{\text{Standard deviation of chromosome length}}{\text{Mean of chromosome length}}$$

The degree of asymmetry of karyotypes (A) was calculated from the formulas defined by Watanabe *et al.* (1999) which was

$$A = \frac{\sum_{i=1}^{n} \frac{pi - qi}{pi + qi}}{n}$$

where p and q are the lengths of long arm and short arm of the chromosome respectively and n is the haploid chromosome number of the species.

Statistical analysis

The numerical data expressed were calculated based on well spread metaphase plate in triplicate. Each data represented the arithmetic mean ± standard deviation (SD) of three independent experiments. The mean separation and significance of treatments were evaluated based on one-way analysis of variance (ANOVA). The statistical analyses were performed using the computer software SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

ANTIOXIDANT STUDIES

Remedial potential of plants need to be studied for its therapeutic use in the preparation of drugs. Plants with antioxidant ability are the need of the hour as it will scavenge the free radicals which are the major stimulators and reason for the development of many dreadful diseases. The added advantages of antioxidant effect along with biological activity are always the priority of researchers as such plants turn out to be a good candidate in curing ailments.

Methanolic extract preparation

Shade dried aerial parts of the plants were used for the current study in powdered form. Soxhlet apparatus was employed for methanolic extract preparation. Fifty gram of the powdered plant was extracted in 100% methanol for 6 hours. The extract thus formed was collected, filtered and evaporated for the complete removal of methanol in water bath, and was stored in amber coloured bottles at 4°C.

Preparation of drugs

Ten milligram of the dried methanolic extract of each plant was dissolved in 200 µl of dimethyl sulphoxide (DMSO) and made up to 1 ml using distilled water which was used as the stock solution for *in vitro* antioxidant analysis. Dilutions at varying concentrations were made in distilled water from the stock solutions for further studies.

Antioxidant assays

Experiments were conducted in triplicate with varying concentrations of extracts prepared from stock solutions for each species.

DPPH radical scavenging assay

Free radical scavenging activity of methanolic extracts using DPPH (2,2-Diphenyl-1-picrylhydrazyl), a stable free radical soluble in methanol, was determined by the modified method proposed by Çoruh *et al*. (2007). DPPH has an absorption peak at 515 nm and the radical scavenging potential was identified by the degree of discolouration from purple to yellow. Reaction mixture was prepared by mixing varying concentrations of 100 µl of the extract solution in methanol with 1 ml of 100 µM freshly prepared solution of DPPH in methanol. For comparison, Ascorbic acid was taken as the standard. Control was prepared by mixing 1 ml of DPPH solution in methanol with 100 µl methanol. Incubation of the reaction mixture for 20 min at room temperature in the dark was done and the absorbance was measured against methanol which was the reference solution. Reduction in optical density on addition of extract was compared to the control and standard to estimate the antioxidant activity. The percentage of inhibition of DPPH radical was computed as follows:

$$\%\ \text{of inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where $A_{control}$ is the absorbance of the control (DPPH radical solution without test sample) and A_{sample} is the absorbance of the extract.

Hydroxyl radical scavenging assay

The competition between deoxyribose and test compounds for hydroxyl radicals generated from the Fe^{3+}/Ascorbate/EDTA/H_2O_2 system (Fenton reaction) was studied in order to determine the scavenging activity of the extract which was done by thiobarbituric acid reacting substances (TBARS) method. Deoxyribose degraded by hydroxyl radicals resulted in the formation of TBARS (Kunchandy and Rao, 1990). Reaction mixture was

prepared by mixing varying concentration of the extract with 2.8 mM deoxyribose, 0.1 mM ferric chloride, 0.1 mM EDTA, 1.0 mM H_2O_2, 0.1 mM ascorbate and 20 mM KH_2PO_4-KOH buffer at pH 7.4 and was made to a final volume of 1 ml. Control was prepared with all the compounds except the extract. The control and test solutions were incubated for 37^oC for 1 hour. Comparison was done with α-tocopherol as the standard. TBA method proposed by Ohkawa *et al*. (1979) was employed to estimate the TBARS formed. The absorbance value of the control, standard and treatments were determined at 532 nm and compared to calculate the hydroxyl radical scavenging activity. The percentage inhibition of hydroxyl radicals was determined as follows:

$$\% \text{ of inhibition } = \frac{\text{OD of the control} - \text{OD of the treated}}{\text{OD of the control}} \times 100$$

Superoxide radical scavenging assay

The extent to which nitroblue tetrazolium (NBT) is reduced due to the superoxide generation from riboflavin formed the basis of estimation of superoxide (O_2^-) radical scavenging activity of extracts. It was determined by the riboflavin photoreduction method described by Mc Cord and Fridovich (1969). The superoxide radical generated reduces NBT to a blue coloured complex. The reaction mixture was prepared by mixing varying concentrations of the extract along with 3 µg NaCN in 6 µM EDTA, 2 µM riboflavin, 50 µM NBT, 67 mM phosphate buffer (KH_2PO_4-Na_2HPO_4) at pH 7.8 made into a final volume of 3 ml. Control solution was prepared by mixing all the above mentioned compounds except the extracts. Uniform illumination under incandescent lamp was provided for 15 min to the tubes containing the reaction mixture and control to cause the photo reduction of riboflavin. Ascorbic acid was taken as the standard for comparison. Spectrophotometric determination of optical density was carried out before

and after illumination at 530 nm. Comparison of the absorbance value of the test solution, control and standard estimated the antioxidant ability. The percentage inhibition of the superoxide radical generation was evaluated as follows:

$$\%\ \text{of inhibition} = \frac{\text{OD of the control} - \text{OD of the treated}}{\text{OD of the control}} \times 100$$

Ferric ion reducing antioxidant power assay

FRAP assay is based on the reduction capacity of the extract wherein the Fe^{3+}-TpTz complex is reduced to ferrous (Fe^{2+}) form visualised by an intense blue coloured complex. The assay proposed by Benzie and Strain (1996) was followed with modifications by Pulido *et al*. (2000). FRAP reagent was prepared by mixing 2.5 ml of 20 mM 2,4,6-tripyridyl-s-triazine (TpTz) in 40 mM HCl, 2.5 ml of 20 mM ferric chloride ($FeCl_3.6H_2O$) and 25 ml 0.3 M acetate buffer at pH 3.6. Varying concentrations of extract were mixed with FRAP reagent for the preparation of reaction mixture which was made upto 1 ml by the addition of acetate buffer. Reaction mixture without the extract was taken as the control. The contol and test solutions were incubated at 37°C for 15 min and the absorbance was measured at 595 nm. Calibration curve of the standard was prepared using varying concentrations of methanolic solutions of known Fe (II) concentration ranging between 100 and 1000 µmol/L ($FeSO_4 \cdot 7H_2O$). The concentration of antioxidant which has a ferric-TpTz ability equivalent to that of 1 mmol/L $FeSO_4 \cdot 7H_2O$ is referred as equivalent concentration. It was computed as the concentration of antioxidant with an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1 mmoL/L concentration of Fe (II) solution determined from the corresponding regression equation using regression analysis.

Statistical analysis

Each data represented the arithmetic mean ± standard error of means (SEM) of three independent experiments. The mean separation and significance of treatments were evaluated based on one-way analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955). Linear regression analysis was performed to determine the 50% inhibition concentration (IC_{50} value). The statistical analyses were performed using the computer software SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

ANTITUMOR STUDIES

Cancer has become the most dreadful disease left unconquered and uncontrolled in the history of mankind. Although studies are conducted all around the world, success stories are few in number especially when the aspect of preventive measure is concerned. Radiation and chemotherapy has its effect in controlling the disease but the side effects and cost of medicines are devastating. Herbal formulations are the major source of research in anticancer therapy to produce effective, low cost drugs with apparently no side effects. *In vitro* cytotoxic and *in vivo* antitumor assays are efficient methods employed to identify the anticancer potential in plants.

Cytotoxic assays

Primary identification of biological activities of plants is essential for further activity guided studies and assays that determine the medicinal attribute. *In vitro* cytotoxic assays were conducted in triplicate with extracts prepared of varying concentrations from stock solutions for each species.

Determination of cell death

Preliminary cytotoxic screening was conducted with the help of *Allium cepa* test system. Uniform sized bulbs were kept for rooting in moisturized

sterilized sand which was further used for experimental studies. Cell death was determined by staining the control and treated roots of the *Allium* bulbs with 0.25% (w/v) aqueous solution of Evans blue for 15 min (Baker and Mock, 1994). The roots thus obtained were washed thoroughly in distilled water, and batches of ten stained root tips from the control and treated bulbs of equal length (10 mm) were excised and soaked in 3ml of N,N-dimethylformamide for 1h at room temperature. Evans blue eluted was measured as the absorbance at 600 nm spectrophotometrically which evaluated the intensity of cell death.

Cell lines

The two cell lines - Dalton's Lymphoma Ascites tumor cells (DLA) and Ehrlich's Ascites Carcinoma cells (EAC) maintained in Amala Cancer Research Centre, Thrissur were used for the present study. The cell lines were maintained in the ascites fluid of the peritoneal cavity of healthy Swiss albino mice. The aspirated DLA/EAC cells, washed in PBS (pH-7.4), were adjusted to a cell count of 1×10^6 cells/ml with the help of haematocytometer and were injected intraperitonially to develop ascites tumor in mice.

Preparation of drug

The stock solution was prepared by dissolving 10 mg of the extract in 200 µl DMSO and made up to 1 ml with distilled water. Different dilutions were prepared from the stock solution by addition of distilled water for cytotoxic assay**.**

Trypan Blue Exclusion assay

DLA and EAC cell lines maintained in the intraperitoneal cavity of the mice were aspirated aseptically and suspended in 0.1 ml PBS (pH 7.4) at a density of 1×10^6 cells/ml. For the assay, 0.1 ml of the viable suspension was

dispensed in tubes containing varying concentrations of the extract ranging from 100-2000 µg/ml and 0.8 ml PBS attaining to a final volume of 1 ml. Incubation was accomplished at 37°C for 3 hours after which the viability of cells were determined by trypan blue exclusion method (Babu *et al*., 1995). 0.1 ml of trypan blue dye was added to the incubated mixture and, the viable and non-viable cells were counted in a microscopic field by mounting on a haemocytometer. The percentage of cytotoxicity of extract was calculated using the formula:

$$\%\ \text{of Cytotoxicity} = \frac{\text{Number of dead cells}}{\text{Number of total cells}} \times 100$$

Statistical analysis

Each data represented the arithmetic mean ± standard error of means (SEM) of three independent experiments. The mean separation and significance of treatments were evaluated based on one-way analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955). Linear regression analysis was performed to determine the 50% inhibition concentration (IC_{50} value). The statistical analyses were performed using the computer software SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

Antitumor assays

In vivo antitumor studies in mice are essential to confirm the anticancer potential of plants to be used as source for various anticancer drugs. The experiments were conducted in six replicas with high and low doses of extract suspended in distilled water for each species.

Experimental animals

Swiss albino mice within age group of 6-8 weeks of either sex were purchased from Sri Venkateshwara Agencies, Bangalore and maintained at

Amala Cancer Research Centre breeding section under sterilized environmental conditions (22-28ºC, 60-70% relative humidity, 12 h dark/light cycle) and was fed with normal mice chow (Sai Feeds, India) and water *ad libitum*. Mice weighing approximately 20-25 g were used for antitumor study. The experiments on mice were carried out with prior approval of the Institutional Animal Ethics Committee (IAEC) (Lic No. 149/1999) and were accomplished strictly according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Preparation of drug and administration for *in vivo* studies

Preliminary toxicity studies were conducted for each species and the concentration of drugs to be administered were determined. Low and high doses (25 and 50 mg/kg body weight) of the drug were prepared by dissolving the required amount of methanolic extract of each species in distilled water based on the average body weight of the animals for which the drug was provided. The extracts were administered orally to Swiss albino mice in treated groups while the standard drug, cyclophosphamide, was injected intraperitoneally to the mice in standard group.

Evaluation of antitumor activity

DLA induced solid tumor model

The antitumor activity of the three extracts was studied using DLA induced solid tumor model. The experimental Swiss albino mice were divided into eight groups of six animals each. DLA cells were aspirated from the peritoneal cavity of the tumor bearing mice and the solid tumor was induced by injecting 0.1 ml of the cell lines (1×10^6 cells/animal) intramuscularly to the right hand limb of the animals. The control group was induced with solid

tumor only and the standard with cyclophosphamide drug while the treated groups received low and high doses of plant extracts.

Group I	-	Control: Tumor alone
Group II	-	Standard: Cyclophosphamide (25 mg/kg b. wt)
Group III	-	Low dose of *I. coetsa* (25 mg/kg b. wt)
Group IV	-	High dose of *I. coetsa* (50 mg/kg b. wt)
Group V	-	Low dose of *I. nigrescens* (25 mg/kg b. wt)
Group VI	-	High dose of *I. nigrescens* (50 mg/kg b. wt)
Group VII	-	Low dose of *I. nilgherricus* (25 mg/kg b. wt)
Group VIII	-	High dose of *I. nilgherricus* (50 mg/kg b. wt)

Drugs were administered orally for ten consecutive days just after 24 hours of tumor implantation. Diameters of the developing tumors were measured using vernier calliper at fixed interval of three days for 30 days and the tumor volume was calculated using the formula:

$$\text{Tumor volume} = \frac{4}{3}\pi {r_1}^2 \times r_2$$

where r_1 and r_2 represents the radii of the tumor at two different planes (Rajeshkumar *et al*., 2002). Percentage of inhibition of tumor volume was calculated using the formula:

$$\%\ \text{of Inhibition} = \left[\frac{\text{Tumor volume of the control on 30th day} - \text{Tumor volume of the treated on 30th day}}{\text{Tumor volume of the control on 30th day}}\right] \times 100$$

The weights of the animals were also noted at three days interval. Percentage increase in the body weight of the animals was calculated using the formula:

$$\% \text{ Increase in body weight} = \left[\frac{\text{Body weight of animals on respective day– Body weight of animals on 0th day}}{\text{Body weight of animals on 0th day}}\right] \times 100$$

Analysis of haematological parameters

The haematological parameters were analyzed which included total WBC count and haemoglobin to determine the health condition in mice due to tumor induction and administration of the drugs. Blood samples were collected from the caudal vein of the experimental mice into heparinised tube on every third day for 30 days.

Determination of total WBC count

For the estimation of the total leucocyte count, 20 µl of blood was mixed with 380 µl of Turk's diluting fluid. It was immediately mounted on Neubauer counting chamber for counting the number of WBC cells. Turk's fluid lyses RBC and stains WBC. WBC count was calculated from the formula:

$$\text{Total WBC count/cu. mm} = \frac{\text{Number of cells counted} \times \text{Dilution factor} \times \text{Depth of fluid}}{\text{Area counted}}$$

Determination of haemoglobin (Hb) content - Cyanmethaemoglobin method

Haemoglobin in reaction with ferricyanide in Drabkin's fluid forms methaemoglobin, which is converted to cyanmethaemoglobin by cyanide. The haemoglobin count was determined by mixing 20 µl of the blood sample with 5 ml of Drabkin's fluid which was allowed to react for 15 minutes. The optical density was determined at 546 nm and compared with the standard. The standard solution depicts optical density corresponding to 60 mg/dL haemoglobin while the optical density of the sample is directly proportional to the amount of haemoglobin present in blood.

$$\text{Blood Haemoglobin (g/dL)} = \frac{\text{OD of the sample}}{\text{OD of the standard}} \times \text{Concentration of standard}$$

EAC induced ascites tumor model

The antitumor activity of the three extracts was also studied using EAC induced ascites tumor model. The experimental Swiss albino mice were divided into eight groups of six animals each. EAC cells were aspirated from the peritoneal cavity of the tumor bearing mice and the ascites tumor was induced by injecting 0.1 ml of the cell lines (1×10^6 cells/animal) intraperitoneally to the animals. The control group was induced with ascites tumor only and the standard with cyclophosphamide drug while the treated groups received low and high doses of plant extracts.

Group I - Control: Tumor alone

Group II - Standard: Cyclophosphamide (25 mg/kg b. wt)

Group III - Low dose of *I. coetsa* (25 mg/kg b. wt)

Group IV - High dose of *I. coetsa* (50 mg/kg b. wt)

Group V - Low dose of *I. nigrescens* (25 mg/kg b. wt)

Group VI - High dose of *I. nigrescens* (50 mg/kg b. wt)

Group VII - Low dose of *I. nilgherricus* (25 mg/kg b. wt)

Group VIII - High dose of *I. nilgherricus* (50 mg/kg b. wt)

Drugs were administered orally for ten consecutive days just after 24 hours of tumor implantation. Development of ascites tumor was observed and the death pattern of animals due to tumor burden was noted. The percentage of increase in life span was calculated using the formula:

$$\%\ \text{Increase in life span} = \frac{(T - C)}{C} \times 100$$

where T and C are mean survival of treated and control mice respectively (Joy *et al*., 2000).

The weights of the animals were also noted at three days interval. Percentage increase in the body weight of the animals was calculated using the formula:

$$\% \text{ Increase in body weight} = \left[\frac{\text{Body weight of animals on respective day– Body weight of animals on 0th day}}{\text{Body weight of animals on 0th day}}\right] \times 100$$

Statistical analysis

Each data represented the arithmetic mean ± standard error of means (SEM) of three independent experiments. The mean separation and significance of treatments were evaluated based on one-way analysis of variance (ANOVA) followed by Dunnett's test (Dunnett, 1955). The statistical analyses were performed using the computer software SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

PHYTOCHEMICAL STUDIES

In vitro and *in vivo* screening of herbs and herbal formulations are essential for the determination of bioactivity but the driving force behind the biological activities will be mystifying unless the phytochemical profile of the source plants are attributed. Various isolation and separation techniques are employed for the identification of the active principles in plants which may be secondary metabolites or other compounds with excellent target specific properties.

Preparation of methanolic extracts

Aerial parts of the three species collected from wild, were unsoiled and washed thoroughly to remove the external pollutants and shade dried. Methanolic extract was prepared from dried powdered material with the help

of Soxhlet apparatus. Ten gram of the plant material was extracted for 6 hours in 25 ml methanol. The obtained extract was then cooled, filtered and evaporated to dryness in a vacuum evaporator. For analysis, the extract was dissolved in 10 ml methanol (HPLC Grade, Merck) and filtered through 0.45 micron membrane filter to ensure minimum contamination.

Phytochemical assays

Phytochemical assays for the identification of volatile and non-volatile components of the methanolic extracts was employed in the study. Preliminary phytochemical screening, estimation of total flavonoid, phenolic and terpenoid content along with standard methods of gas chromatography coupled with mass spectrometry as well as liquid chromatography with mass spectrometry were used for the qualitative and quantitative determination of the major bioactive constituents.

Preliminary phytochemical screening

Preliminary phytochemical assays were conducted to determine the presence of alkaloids, flavonoids, phenols, saponins, tannins and terpenoids in the methanolic extracts which could act as a flicker of light in identifying the active components present in the plants.

Test for alkaloids (Sofowara, 1993)

Solvent free extract was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. The filtrate was tested for the presence of alkaloids.

Dragendroff's test – To 2ml of the filtrate, 1ml of Dragendroff's reagent was added. Occurrence of orange red or orange brown precipitate indicates the presence of alkaloids.

Mayer's test – To 2ml of the filtrate, 1 ml of Mayer's reagent was added. Formation of dull white/creamy white/buff-coloured precipitate indicates the presence of alkaloids.

Test for anthraquinones (Kumar *et al*., 2007)

Borntrager's test – 50 mg of the solvent free extract was stirred with 1 ml of 10% ferric chloride solution and 1ml of conc. HCl, and heated. The extract was cooled and filtered. The filtrate obtained was shaken well with equal amount of diethyl ether. The ether extract thus obtained was further extorted with strong ammonia. Development of pink or deep red colouration in the aqueous layer indicates the presence of anthraquinones.

Test for flavonoids (Evans and Evans, 2002)

Sovent free extract was dissolved in water and filtered. The filtrate was tested for the presence of flavonoids.

Alkaline reagent test – Test solution was added with 2 ml of 10% aqueous sodium hydroxide which produces a yellow colouration. Few drops of dilute HCl is added which decolourises the yellow colour of the test solution is an indication of the presence of flavonoids.

Test for glycosides (Parekh and Chanda, 2007)

Keller Killiani test – To 2 ml of test solution, added 1 ml of glacial acetic acid and 1 ml of ferric chloride, and mixed well. About 1 ml of conc. H_2SO_4 was added slowly along the sides of the test tube resulting in the formation of two layers. Green-blue colouration in the acetic acid layer at interface indicates the presence of glycosides.

Test for phenols (Kumar *et al*., 2007)

Phenol test – The solvent free extract is spotted on a filter paper and a drop of phoshomolybdic acid reagent is added. Exposure of the spot to ammonia vapours produces blue colouration which indicates the presence of phenols.

Test for saponins (Sofowara, 1993)

One gram of solvent free extract was boiled with 5 ml of distilled water and filtered. The filtrate was further used for saponin analysis.

Froth test – The filtrate was added with 3 ml of distilled water and shaken vigorously for 5 minutes. Froth formed which persists on warming indicates the presence of saponins.

Test for tannins (Evans and Evans, 2002)

About 0.5 g of solvent free extract was stirred with 10 ml of distilled water and filtered. The filtrate was analysed for the presence of tannins.

Ferric chloride test – To 2 ml of the filtrate, few drops of 1% ferric chloride solution was added. Occurrence of blue-black, green or blue-green precipitate indicates the presence of tannins.

Test for steroids (Sofowara, 1993)

Liebermann-Burchard test – To 0.2 g of solvent free extract, added 2 ml of acetic acid. The solution was cooled well in ice and small amount of conc. H_2SO_4 was added carefully. Development of violet to blue or bluish-green colour indicates the presence of steroids.

Test for terpenoids (Edeoga *et al*., 2005)

Salkowski test – To 5 ml of the extract, added 2 ml of chloroform and mixed well. To the solution, added 3 ml of conc. H_2SO_4 carefully to form a layer.

Formation of reddish brown colouration at interface indicates the presence of terpenoids.

Determination of phenolic, flavonoid and terpenoid contents of the extracts

Plants, in general, are abundant sources of flavonoids, phenols, terpenoids and other metabolic compounds. These compounds have more significance in other biological activities rather than in the vital functions of the plants. This can be considered indirectly as a booster to the health and immune system of animals especially human beings since the antioxidant and pharmacological activities of the components present help the biological system of animals to triumph over the dreadful, devastating microorganisms and diseases. Thus, the total amount of flavonoids, phenolics and terpenoids are estimated to identify the biological efficiency of the species studied and in a way to throw light on the active compounds present in it.

Estimation of total phenolic content

The total phenolic content of the plant extracts was determined using Folin–Ciocalteu reagent based assay described by Oueslati *et al*. (2012). Gallic acid was used as the standard for estimation. An aliquot of diluted sample/standard solution of gallic acid was mixed with 0.5 ml of distilled water and 0.125 ml of the Folin–Ciocalteu reagent (1 N) and shaken well. The solution was allowed to stand for 6 min after which 1.25 ml of 7% Na_2CO_3 was added and made to a final volume of 3 ml with distilled water. The reaction mixture thus obtained, was mixed thoroughly, and incubated in dark at ambient temperature for 90 min. The absorbance at 760 nm was recorded against blank, *i.e*., distilled water after incubation. Calibration curve of gallic acid was plotted and the total phenolic content expressed as milligrams of

gallic acid equivalents per gram of dry weight (mg GAE/g DW) was determined using regression equation. Samples were analyzed in triplicates.

Estimation of total flavonoid content

The total flavonoid content of all plant extracts was determined using the aluminium chloride colorimetric method with some modifications (Oueslati *et al*., 2012). Quercetin was used as the standard for estimation. An aliquot of diluted sample/standard solution of quercetin was mixed with 75 µl of $NaNO_2$ solution (7%) for 6 min. The solution thus obtained was added with 0.15 ml $AlCl_3$ (10%). After 5 min, 0.5 ml of 1 M NaOH solution was also added and the final volume was adjusted to 2.5 ml. The absorbance of the mixture, after thorough mixing, was determined at 510 nm against blank *i.e*., methanol. Calibration curve of quercetin was plotted and the total flavonoid content expressed as mg quercetin equivalent per gram of dry weight (mg QE/g DW) was determined using regression equation. Samples were analysed in triplicates.

Estimation of total terpenoid content

The total terpenoid content of the plant extracts was determined based on an assay described by Ghorai *et al*. (2012) with some modifications. Linalool was used as the standard for estimation. An aliquot of the reaction mixture obtained after Salkowski test employed for the qualitative analysis of terpenoids in the extract was transferred to colorimetric cuvette. The absorbance was measured at 538 nm against blank *i.e*., 95% (v/v) methanol. For the standard curve, 200 µl of linalool solution in methanol was added with 1.5 ml chloroform and serial dilutions [dilution level-100 mg/200 µl to 1 mg/200 µl linalool Conc.] were prepared in which total volume of 200 µl was made up by the addition of 95% (v/v) methanol. Calibration curve of linalool was plotted and the total terpenoid content expressed as milligrams of

linalool equivalents per gram of dry weight (mg linalool/g DW) was determined using the regression equation. Samples were analysed in triplicates.

An alternate method described by Ferguson (1956) for the estimation of total terpenoid content was also employed. From the powdered aerial parts of the plant, 100 mg was taken and soaked in alcohol for 24 hours and filtered. The filtrate was then extracted with petroleum ether using a separating funnel. The portion of the ether extract was treated as total terpenoids.

Phytochemical analysis

Analytical techniques are employed for the identification of specific components in the extracts which may be the cause of the biological activities of the plant extracts. Gas chromatography coupled with mass spectrometry techniques are utilized for the analysis of volatile compounds while the liquid chromatography coupled with mass spectrometry technique reveals metabolites that are not volatile and have not been derivatised. Hence, both techniques are adopted for developing an idea about the active compounds which could have resulted in the antioxidant and antitumor activity.

Gas chromatography-mass spectrometry (GC-MS) analysis

Varian model CP-3800 GC interfaced with a Varian Saturn 2200 Ion Trap Mass Spectrometer (ITMS) operating at 70eV and 250˚C equipped with a CP-1177 Split/Splitless capillary injector and Combi PAL autosampler was employed for GC-MS analyses. A cross linked FactorFour capillary column, VF 5ms with 30 m x 0.25 mm ID and 0.25 µm film thickness was utilized. Carrier gas was Helium at a flow rate of 1 ml/min. Injection volume was 1µl. The split ratio was 1:20. The temperature programme for the chromatographic analysis was set at 60^{o}C for 1 min (initial) and then heated up at a rate of

3˚C/min to 280˚C. Run time was 60 min. Quantification was done using percentage peak area calculations and identification of individual components was done with the help of NIST MS search. The relative concentration of each compound in the methanolic extract was quantified based on the peak area integrated by the analysis programme.

Liquid chromatography-mass spectrometry (LC-MS) analysis

The experiment was accomplished using an Agilent 1260 Infinity Bio-inert Quaternary LC equipped with an Agilent 6120 Series Quadrupole MS System and Agilent MassHunter Workstation software. The chromatographic separation was achieved on a reverse-phase Agilent Zorbax-C18 110 Å analytical column (250 x 4.6 mm ID., 5 µm) operated at 40°C. Elution was achieved with a gradient mobile phase consisting of methanol (A) and 0.5 µM ammonium formate (B) (9:91) in water (C) at a flow rate of 1 mL/min. The gradient system used was as follows: Mobile phase C was held constant throughout the run and a linear gradient of B was maintained for 30 min. Mode of injection was ALS (Automatic liquid sampling). Electrospray ionization (ESI) was used as the interface and was operated in positive selected ion monitoring (SIM) mode. The analytes were monitored by tandem-mass spectrometry with positive electrospray ionization. The injection volume was 20 µl. The probe temperature was set at 500 °C and needle voltage was set at 20 V. The cone voltage was set at 50 V for all Selected Ion Monitoring (SIM) scans.

Results

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

RESULTS

The current investigation on the karyomorphology, antioxidant and antitumor activity as well as phytochemical constituents of the three species of the genus *Isodon* used extensively in traditional Chinese folk medicines led to the identification of the following cytological features, bioactivities and phytochemical profiles of the source plants (Plate 1).

CHROMOSOME ANALYSIS

The different cytotypes of each species of *Isodon* was identified based on specific karyomorphological and karyomorphometric data (Plates 2-10; Tables 6-15; Fig. 11). Mitotic squash preparations of the root meristems of the three species led to the disclosure of the karyological identity of the plants. Chromosome number, ploidy and karyomorphology are important parameters in cytotaxonomy and become all the more significant especially in a disputed genus like *Isodon*. This is the first report of the chromosome number of *I. nigrescens* to the cytological world. Though the chromosome counts of *I. coetsa* and *I. nilgherricus* have already been identified, a detailed karyomorphometric data and asymmetry index has been lacking which are the batons to the path in determining the karytotype evolution of each genus. The stability of the karyological characters were confirmed by screening multiple samples from available localities and its subsequent conservation and analysis at Department of Botany, University of Calicut, Kerala, India.

The karyomorphological data and the somatic chromosome numbers of various accessions of *I. coetsa* are detailed in Tables 6-8. The mitotic metaphase chromosome number was revealed to be 2n = 24 along with two variants of 6 and 12 chromosomes (Plates 2-4). The range of chromosome length varied between 1.1100 μm to 0.7055 μm for the normal chromosome

complement. The variant chromosomes had nearly median centromeric positions for all the chromosomes while the normal chromosome count showed nearly median centromeric positions for all except six chromosomes which possessed nearly submedian centromeric position. The karyotype asymmetry was identified based on various asymmetry indices of which the intrachromosomal asymmetry index, interchromosomal asymmetry index and the degree of asymmetry of karyotypes of normal metaphase chromosome count of *I. coetsa* was found to be 0.21, 0.15 and 0.24 respectively. The karyogram and idiogram of the chromosome counts were drawn based on centromeric index and arranged based on size, in decreasing order.

The karyomorphological data and the somatic chromosome numbers of various accessions of *I. nigrescens* are detailed in Tables 9-11. The mitotic metaphase chromosome number was revealed to be 2n = 24 along with two variants of 6 and 12 chromosomes (Plates 5-7). The range of chromosome length varied between 1.0707 μm to 0.6229 μm for the normal chromosome count. The variant chromosomes had nearly median centromeric positions for all chromosomes except one with nearly submedian position for variant number 6 while the normal chromosome count showed nearly median centromeric position for most chromosomes and nearly submedian position only for two chromosomes. The karyotype asymmetry was identified of which the intrachromosomal asymmetry index, interchromosomal asymmetry index and the degree of asymmetry of karyotypes of normal metaphase chromosome count was found to be 0.24, 0.17 and 0.28 respectively. The karyogram and idiogram of the chromosome counts were drawn based on centromeric index and arranged in decreasing size range.

The karyomorphological data and the somatic chromosome numbers of various accessions of *I. nilgherricus* are shown in Tables 12-14. The mitotic metaphase chromosome number was revealed to be 2n = 24 along with two

variants of 6 and 12 chromosomes (Plates 8-10). The range of chromosome length varied between 1.2290 μm to 0.8116 μm for the normal chromosome count. The variant chromosomes had nearly median centromeric positions for all chromosomes while the normal chromosome count showed nearly median centromeric position for all except two chromosomes with nearly submedian position. The karyotype asymmetry was identified of which the intrachromosomal asymmetry index, interchromosomal asymmetry index and the degree of asymmetry of karyotypes of normal metaphase chromosome count was found to be 0.24, 0.12 and 0.28 respectively. The karyogram and idiogram of the chromosome counts were drawn based on centromeric index and arranged in decreasing size range.

Data assessment revealed that all the three species possessed chromosomes with small size of which the reasonably higher values were shown by *I. nilgherricus*. The number of secondary constrictions was in equity for all the species where the variant count of 6 possessed only one chromosome with secondary constriction while chromosome count of 12 had two chromosomes with secondary constriction. The normal chromosome count of 24 depicted a total of four chromosomes with secondary constriction in paired condition. Summarized karyomorphometric data are tabulated in Table 15 and the scatter plot for population dispersion in Fig. 11. Karyogram, idiogram and the various morphometric features of the karyotype as well as the karyotype formula provided a cytological identity to each species of the genus.

Variant chromosome complement of *I. coetsa* with 6 chromosomes

Variant somatic chromosome number	:	6
Karyotype formula	:	6nm
Chromosomes with secondary constriction	:	1
Range of chromosome length (RCL)	:	1.0201 μm to 0.7700 μm
Total chromosome length (TCL)	:	5.2306 μm
Average chromosome length (ACL)	:	0.8718 μm
Disparity index (DI)	:	13.97
Variation coefficient (VC)	:	10.40
TF value (%)	:	43.67
Karyotype asymmetry index (As K%)	:	56.33
Syi index	:	77.52
Rec index	:	85.46

PLATE 2

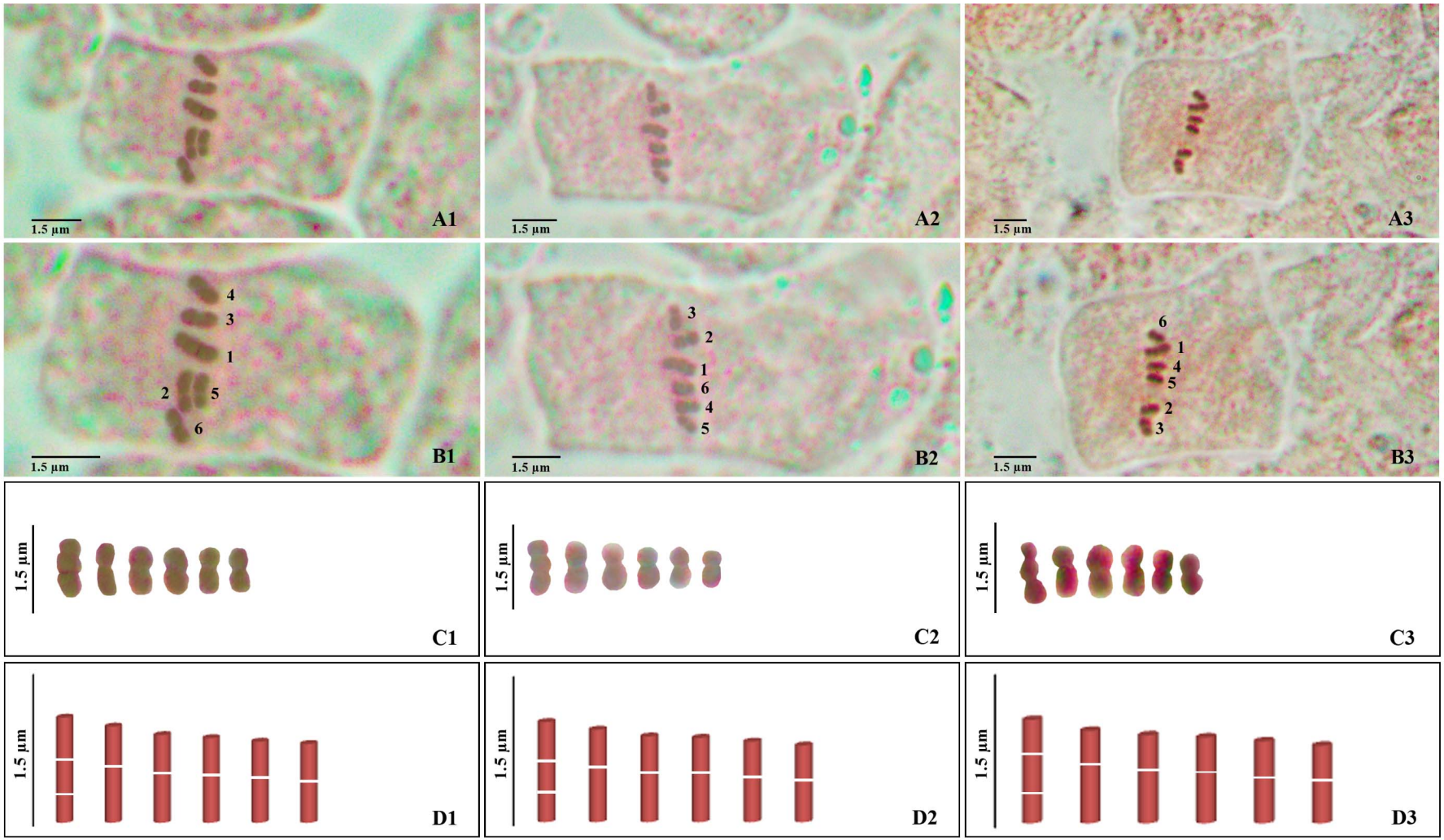


Fig. 2 Variant chromosome complement images of *Isodon coetsa* with 6 chromosomes
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 6 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 6. Detailed karyomorphometric data of *I. coetsa* with 6 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	1.0201 ± 0.016	0.4241 ± 0.008	0.5960 ± 0.008	0.7116 ± 0.005	1.4053 ± 0.009	41.5744 ± 0.163	58.4256 ± 0.163	nm
1	0.9294 ± 0.021	0.4175 ± 0.011	0.5119 ± 0.010	0.8155 ± 0.007	1.2262 ± 0.011	44.9195 ± 0.215	55.0805 ± 0.215	nm
1	0.8640 ± 0.002	0.3907 ± 0.007	0.4733 ± 0.005	0.8255 ± 0.023	1.2114 ± 0.034	45.2199 ± 0.698	54.7801 ± 0.698	nm
1	0.8423 ± 0.006	0.3693 ± 0.006	0.4730 ± 0.011	0.7807 ± 0.029	1.2809 ± 0.049	43.8420 ± 0.923	56.1580 ± 0.923	nm
1	0.8048 ± 0.004	0.3521 ± 0.008	0.4527 ± 0.011	0.7779 ± 0.037	1.2855 ± 0.061	43.7541 ± 1.159	56.2459 ± 1.159	nm
1	0.7700 ± 0.008	0.3305 ± 0.008	0.4396 ± 0.002	0.7518 ± 0.017	1.3301 ± 0.030	42.9159 ± 0.564	57.0841 ± 0.564	nm

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Variant chromosome complement of *I. coetsa* with 12 chromosomes

Variant somatic chromosome number	:	12
Karyotype formula	:	12nm
Chromosomes with secondary constriction	:	2
Range of chromosome length (RCL)	:	1.1154 μm to 0.5778 μm
Total chromosome length (TCL)	:	10.0148 μm
Average chromosome length (ACL)	:	0.8346 μm
Disparity index (DI)	:	31.75
Variation coefficient (VC)	:	19.82
TF value (%)	:	44.97
Karyotype asymmetry index (As K%)	:	55.03
Syi index	:	81.73
Rec index	:	74.82

PLATE 3

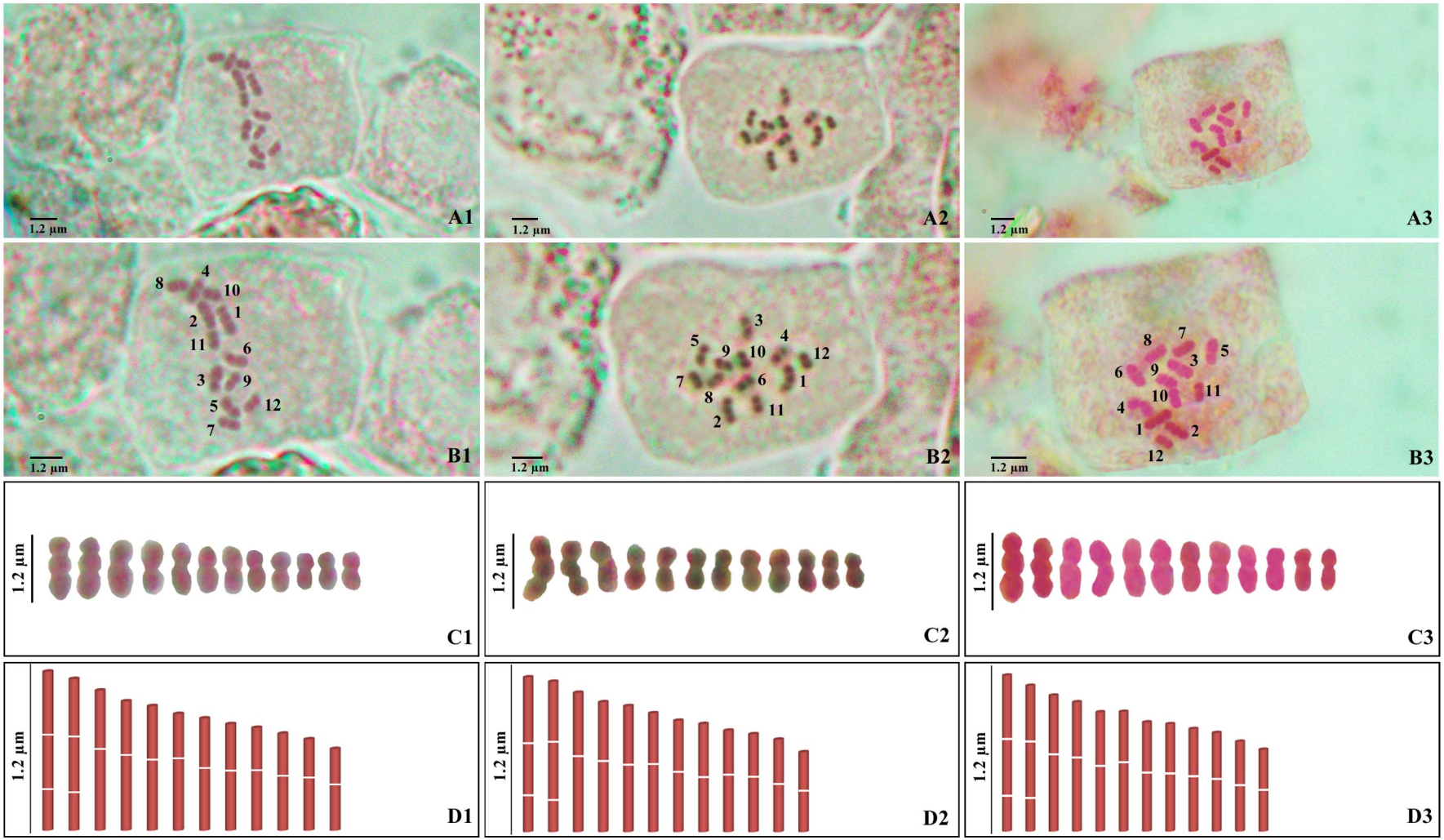


Fig. 3 Variant chromosome complement images of *Isodon coetsa* with 12 chromosomes
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 12 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 7. Detailed karyomorphometric data of *I. coetsa* with 12 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	1.1154 ± 0.017	0.4812 ± 0.015	0.6342 ± 0.014	0.7588 ± 0.035	1.3178 ± 0.059	43.1445 ± 1.110	56.8555 ± 1.110	nm
1*	1.0628 ± 0.019	0.4362 ± 0.009	0.6266 ± 0.014	0.6961 ± 0.018	1.4366 ± 0.037	41.0412 ± 0.622	58.9588 ± 0.622	nm
1	0.9861 ± 0.012	0.4667 ± 0.011	0.5195 ± 0.006	0.8984 ± 0.023	1.1131 ± 0.029	47.3229 ± 0.648	52.6771 ± 0.648	nm
1	0.9222 ± 0.002	0.4379 ± 0.008	0.4844 ± 0.010	0.9040 ± 0.036	1.1062 ± 0.043	47.4789 ± 0.980	52.5210 ± 0.980	nm
1	0.8779 ± 0.022	0.4210 ± 0.016	0.4569 ± 0.007	0.9213 ± 0.025	1.0854 ± 0.030	47.9534 ± 0.686	52.0466 ± 0.686	nm
1	0.8433 ± 0.013	0.3728 ± 0.005	0.4705 ± 0.008	0.7922 ± 0.008	1.2623 ± 0.013	44.2033 ± 0.264	55.7967 ± 0.264	nm
1	0.7900 ± 0.009	0.3704 ± 0.009	0.4196 ± 0.001	0.8827 ± 0.024	1.1329 ± 0.031	46.8838 ± 0.676	53.1162 ± 0.676	nm
1	0.7648 ± 0.005	0.3599 ± 0.007	0.4049 ± 0.006	0.8890 ± 0.029	1.1248 ± 0.036	47.0624 ± 0.798	52.9376 ± 0.798	nm
1	0.7283 ± 0.007	0.3218 ± 0.008	0.4066 ± 0.001	0.7914 ± 0.023	1.2635 ± 0.037	44.1785 ± 0.716	55.8215 ± 0.716	nm
1	0.6967 ± 0.008	0.3086 ± 0.006	0.3881 ± 0.007	0.7951 ± 0.023	1.2577 ± 0.037	44.2924 ± 0.729	55.7076 ± 0.729	nm
1	0.6495 ± 0.007	0.2834 ± 0.005	0.3661 ± 0.006	0.7740 ± 0.020	1.2919 ± 0.034	43.6313 ± 0.641	56.3687 ± 0.641	nm
1	0.5778 ± 0.010	0.2444 ± 0.004	0.3334 ± 0.007	0.7329 ± 0.014	1.3643 ± 0.026	42.2950 ± 0.459	57.7050 ± 0.459	nm

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Normal chromosome complement of *I. coetsa* with 2n = 24 chromosomes

Normal somatic chromosome number	:	24
Karyotype formula	:	18nm + 6nsm(-)
Chromosomes with secondary constriction	:	4
Range of chromosome length (RCL)	:	1.1100 µm to 0.7055 µm
Total chromosome length (TCL)	:	21.1596 µm
Average chromosome length (ACL)	:	0.8817 µm
Disparity index (DI)	:	22.28
Variation coefficient (VC)	:	14.92
TF value (%)	:	43.69
Karyotype asymmetry index (As K%)	:	56.31
Syi index	:	77.58
Rec index	:	80.34
Intrachromosomal asymmetry index (A_1)	:	0.21
Interchromosomal asymmetry index (A_2)	:	0.15
Degree of asymmetry of karyotypes (A)	:	0.24

PLATE 4

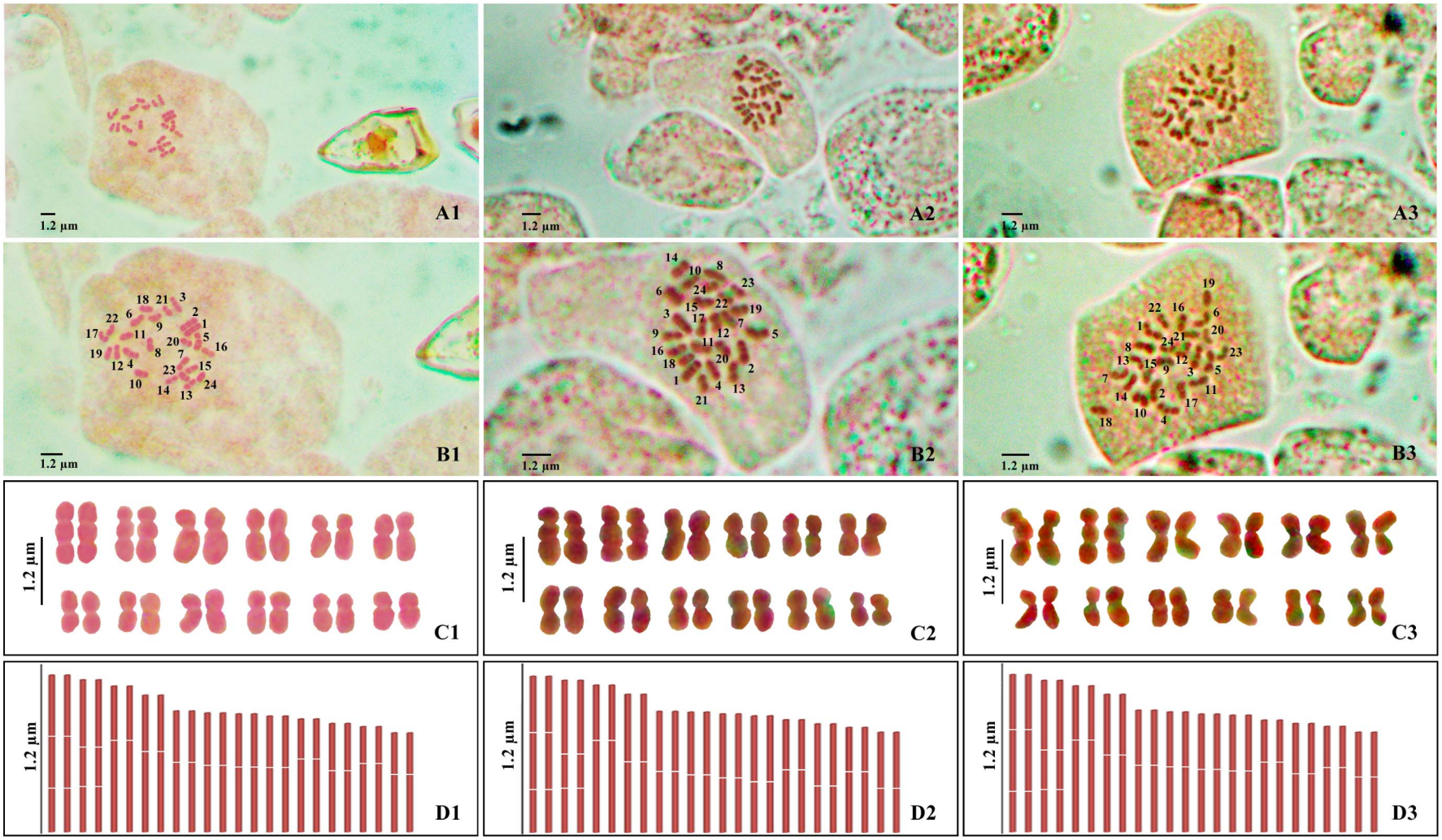


Fig. 4 Normal chromosome complement images of *Isodon coetsa* (2n = 24)

A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 24 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 8. Detailed karyomorphometric data of *I. coetsa* with 2n = 24 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	1.1100 ± 0.007	0.4048 ± 0.004	0.7052 ± 0.007	0.5741 ± 0.009	1.7419 ± 0.029	36.4715 ± 0.379	63.5285 ± 0.379	nsm(-)
2*	1.0757 ± 0.006	0.5086 ± 0.003	0.5670 ± 0.003	0.8970 ± 0.006	1.1148 ± 0.008	47.2854 ± 0.178	52.7146 ± 0.178	nm
2	1.0370 ± 0.003	0.3859 ± 0.003	0.6511 ± 0.003	0.5927 ± 0.006	1.6872 ± 0.016	37.2131 ± 0.232	62.7869 ± 0.232	nsm(-)
2	0.9735 ± 0.001	0.4389 ± 0.007	0.5346 ± 0.006	0.8211 ± 0.023	1.2179 ± 0.035	45.0882 ± 0.702	54.9118 ± 0.702	nm
2	0.8588 ± 0.003	0.4074 ± 0.001	0.4514 ± 0.003	0.9024 ± 0.007	1.1081 ± 0.009	47.4362 ± 0.194	52.5637 ± 0.194	nm
2	0.8482 ± 0.001	0.4058 ± 0.003	0.4424 ± 0.001	0.9173 ± 0.009	1.0902 ± 0.010	47.8425 ± 0.235	52.1575 ± 0.235	nm
2	0.8365 ± 0.003	0.4019 ± 0.001	0.4346 ± 0.004	0.9248 ± 0.010	1.0814 ± 0.012	48.0454 ± 0.285	51.9546 ± 0.285	nm
2	0.8241 ± 0.001	0.3931 ± 0.006	0.4310 ± 0.006	0.9121 ± 0.027	1.0963 ± 0.033	47.7026 ± 0.747	52.2974 ± 0.747	nm
2	0.7964 ± 0.006	0.3213 ± 0.007	0.4751 ± 0.005	0.6762 ± 0.019	1.4789 ± 0.041	40.3399 ± 0.668	59.6601 ± 0.668	nm
2	0.7684 ± 0.003	0.3598 ± 0.006	0.4086 ± 0.009	0.8805 ± 0.035	1.1357 ± 0.046	46.8225 ± 0.100	53.1775 ± 0.100	nm
2	0.7457 ± 0.004	0.2823 ± 0.009	0.4633 ± 0.005	0.6093 ± 0.026	1.6411 ± 0.071	37.8632 ± 0.997	62.1368 ± 0.997	nsm(-)
2	0.7055 ± 0.001	0.3120 ± 0.007	0.3935 ± 0.005	0.7930 ± 0.028	1.2611 ± 0.044	44.2266 ± 0.870	55.7734 ± 0.870	nm

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Variant chromosome complement of *I. nigrescens* with 6 chromosomes

Variant somatic chromosome number	:	6
Karyotype formula	:	5nm + 1nsm(-)
Chromosomes with secondary constriction	:	1
Range of chromosome length (RCL)	:	1.2110 µm to 0.6201 µm
Total chromosome length (TCL)	:	4.8769 µm
Average chromosome length (ACL)	:	0.8128 µm
Disparity index (DI)	:	32.27
Variation coefficient (VC)	:	27.21
TF value (%)	:	43.76
Karyotype asymmetry index (As K%)	:	56.24
Syi index	:	77.81
Rec index	:	67.12

PLATE 5

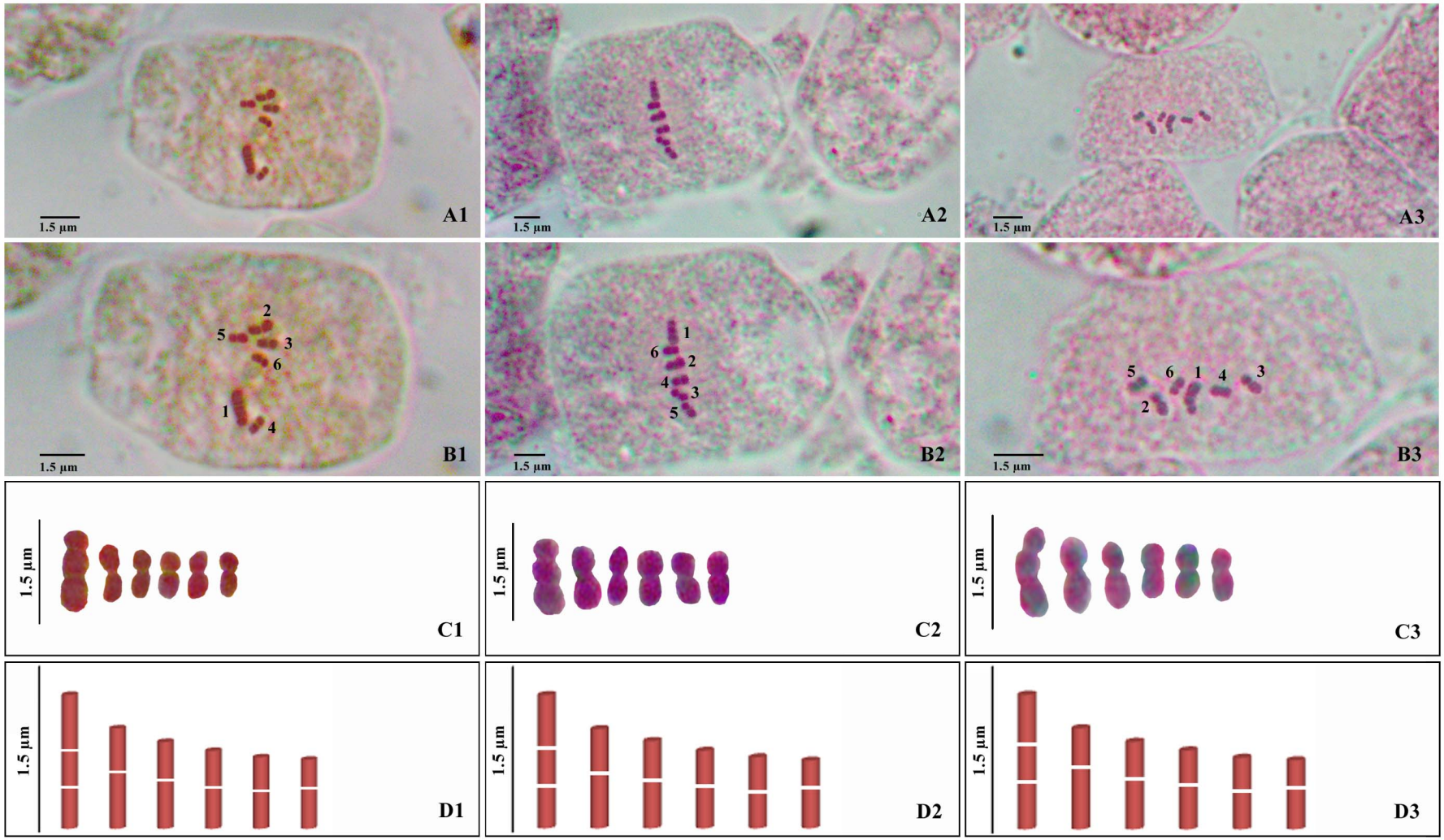


Fig. 5 Variant chromosome complement images of *Isodon nigrescens* with 6 chromosomes
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 6 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 9. Detailed karyomorphometric data of *I. nigrescens* with 6 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	1.2110 ± 0.002	0.5556 ± 0.004	0.6554 ± 0.006	0.8477 ± 0.014	1.1797 ± 0.020	45.8782 ± 0.422	54.1218 ± 0.422	nm
1	0.9063 ± 0.005	0.4154 ± 0.010	0.4909 ± 0.005	0.8463 ± 0.029	1.1817 ± 0.041	45.8364 ± 0.854	54.1636 ± 0.854	nm
1	0.7884 ± 0.004	0.3419 ± 0.005	0.4465 ± 0.002	0.7658 ± 0.013	1.3058 ± 0.022	43.3687 ± 0.422	56.6313 ± 0.422	nm
1	0.7061 ± 0.003	0.3047 ± 0.004	0.4014 ± 0.007	0.7591 ± 0.024	1.3174 ± 0.042	43.1525 ± 0.776	56.8475 ± 0.776	nm
1	0.6450 ± 0.002	0.2874 ± 0.003	0.3576 ± 0.003	0.8035 ± 0.016	1.2445 ± 0.025	44.5530 ± 0.501	55.4470 ± 0.501	nm
1	0.6201 ± 0.005	0.2292 ± 0.008	0.3909 ± 0.006	0.5863 ± 0.029	1.7055 ± 0.083	36.9618 ± 1.137	63.0382 ± 1.137	nsm(-)

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Variant chromosome complement of *I. nigrescens* with 12 chromosomes

Variant somatic chromosome number	:	12
Karyotype formula	:	12nm
Chromosomes with secondary constriction	:	2
Range of chromosome length (RCL)	:	0.9632 µm to 0.5647 µm
Total chromosome length (TCL)	:	8.2203 µm
Average chromosome length (ACL)	:	0.6850 µm
Disparity index (DI)	:	24.02
Variation coefficient (VC)	:	18.82
TF value (%)	:	43.65
Karyotype asymmetry index (As K%)	:	56.35
Syi index	:	77.45
Rec index	:	71.12

PLATE 6

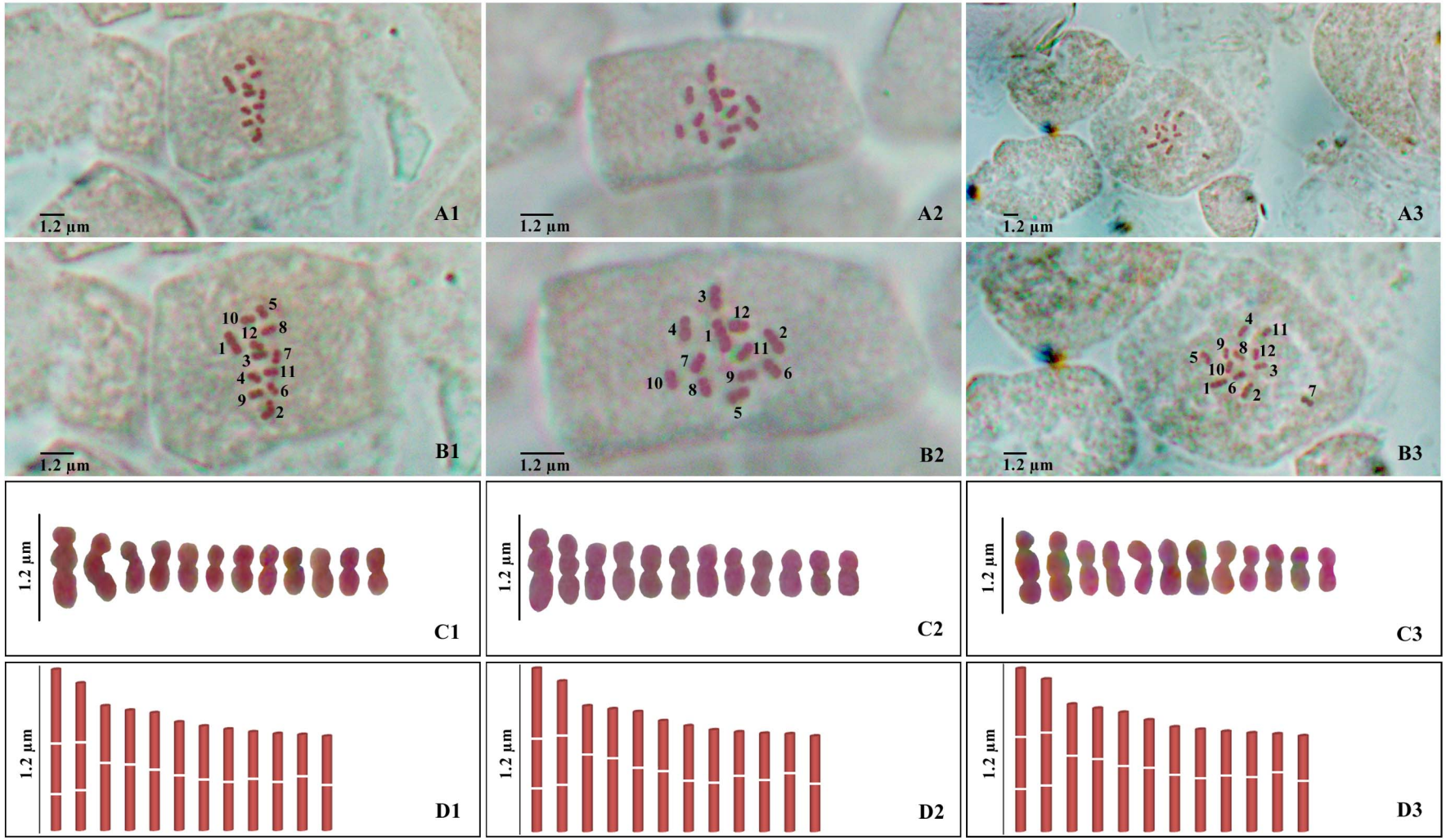


Fig. 6 Variant chromosome complement images of *Isodon nigrescens* with 12 chromosomes
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 12 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 10. Detailed karyomorphometric data of *I. nigrescens* with 12 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	0.9632 ± 0.003	0.4292 ± 0.009	0.5340 ± 0.009	0.8039 ± 0.030	1.2443 ± 0.047	44.5579 ± 0.928	55.4421 ± 0.928	nm
1*	0.8898 ± 0.007	0.3422 ± 0.008	0.5476 ± 0.003	0.6248 ± 0.017	1.6004 ± 0.045	38.4558 ± 0.657	61.5442 ± 0.657	nm
1	0.7467 ± 0.003	0.3244 ± 0.006	0.4223 ± 0.004	0.7681 ± 0.021	1.3019 ± 0.035	43.4425 ± 0.661	56.5574 ± 0.661	nm
1	0.7239 ± 0.003	0.3076 ± 0.002	0.4164 ± 0.002	0.7387 ± 0.007	1.3537 ± 0.013	42.4855 ± 0.241	57.5145 ± 0.241	nm
1	0.7053 ± 0.004	0.3270 ± 0.006	0.3783 ± 0.007	0.8645 ± 0.030	1.1568 ± 0.040	46.3658 ± 0.859	53.6342 ± 0.859	nm
1	0.6543 ± 0.003	0.2914 ± 0.002	0.3629 ± 0.004	0.8028 ± 0.011	1.2456 ± 0.017	44.5310 ± 0.345	55.4689 ± 0.345	nm
1	0.6225 ± 0.006	0.2904 ± 0.010	0.3320 ± 0.005	0.8747 ± 0.041	1.1432 ± 0.053	46.6585 ± 1.154	53.3415 ± 1.154	nm
1	0.6033 ± 0.004	0.2828 ± 0.001	0.3205 ± 0.003	0.8823 ± 0.010	1.1334 ± 0.013	46.8726 ± 0.278	53.1274 ± 0.278	nm
1	0.5900 ± 0.001	0.2547 ± 0.003	0.3353 ± 0.004	0.7596 ± 0.018	1.3164 ± 0.031	43.1695 ± 0.586	56.8305 ± 0.586	nm
1	0.5807 ± 0.001	0.2541 ± 0.002	0.3266 ± 0.001	0.7782 ± 0.008	1.2850 ± 0.014	43.7633 ± 0.268	56.2367 ± 0.268	nm
1	0.5759 ± 0.002	0.2260 ± 0.002	0.3499 ± 0.001	0.6458 ± 0.007	1.5484 ± 0.017	39.2406 ± 0.269	60.7593 ± 0.269	nm
1	0.5647 ± 0.001	0.2582 ± 0.001	0.3066 ± 0.002	0.8421 ± 0.007	1.1875 ± 0.010	45.7148 ± 0.213	54.2852 ± 0.213	nm

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Normal chromosome complement of *I. nigrescens* with 2n = 24 chromosomes

Normal somatic chromosome number	:	24
Karyotype formula	:	22nm + 2nsm(-)
Chromosomes with secondary constriction	:	4
Range of chromosome length (RCL)	:	1.0707 µm to 0.6229 µm
Total chromosome length (TCL)	:	19.2966 µm
Average chromosome length (ACL)	:	0.8040 µm
Disparity index (DI)	:	26.44
Variation coefficient (VC)	:	16.86
TF value (%)	:	42.96
Karyotype asymmetry index (As K%)	:	57.04
Syi index	:	75.31
Rec index	:	75.09
Intrachromosomal asymmetry index (A_1)	:	0.24
Interchromosomal asymmetry index (A_2)	:	0.17
Degree of asymmetry of karyotypes (A)	:	0.28

PLATE 7

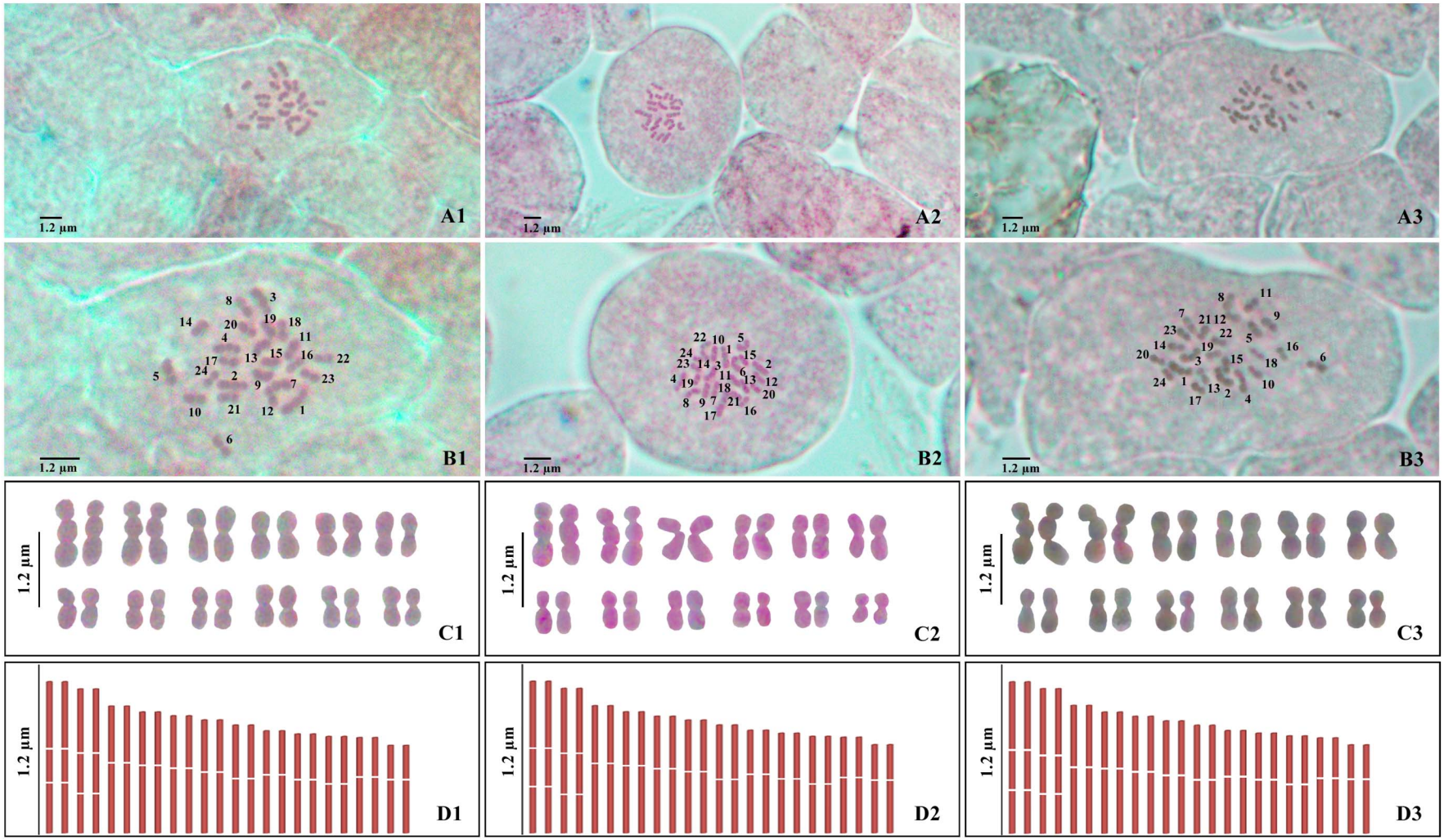


Fig. 7 Normal chromosome complement images of *Isodon nigrescens* (2n = 24)
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 24 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 11. Detailed karyomorphometric data of *I. nigrescens* with 2n = 24 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	1.0707 ± 0.001	0.4482 ± 0.001	0.6225 ± 0.001	0.7200 ± 0.002	1.3890 ± 0.003	41.8592 ± 0.060	58.1408 ± 0.060	nm
2*	1.0207 ± 0.001	0.4255 ± 0.003	0.5951 ± 0.003	0.7150 ± 0.009	1.3986 ± 0.018	41.6917 ± 0.032	58.3083 ± 0.032	nm
2	0.9003 ± 0.003	0.4020 ± 0.004	0.4983 ± 0.001	0.8068 ± 0.010	1.2394 ± 0.015	44.6538 ± 0.294	55.3462 ± 0.294	nm
2	0.8561 ± 0.001	0.3734 ± 0.002	0.4827 ± 0.001	0.7735 ± 0.007	1.2928 ± 0.011	43.6142 ± 0.208	56.3858 ± 0.208	nm
2	0.8269 ± 0.003	0.3599 ± 0.001	0.4671 ± 0.002	0.7705 ± 0.002	1.2979 ± 0.004	43.5182 ± 0.070	56.4818 ± 0.070	nm
2	0.7975 ± 0.004	0.3517 ± 0.001	0.4458 ± 0.003	0.7888 ± 0.003	1.2677 ± 0.005	44.0980 ± 0.104	55.9020 ± 0.104	nm
2	0.7634 ± 0.001	0.3663 ± 0.002	0.3971 ± 0.002	0.9223 ± 0.010	1.0843 ± 0.012	47.9783 ± 0.269	52.0217 ± 0.269	nm
2	0.7250 ± 0.001	0.2866 ± 0.001	0.4384 ± 0.001	0.6537 ± 0.003	1.5297 ± 0.007	39.5310 ± 0.107	60.4690 ± 0.107	nm
2	0.7046 ± 0.003	0.3069 ± 0.003	0.3977 ± 0.001	0.7717 ± 0.007	1.2959 ± 0.012	43.5566 ± 0.229	56.4434 ± 0.229	nm
2	0.6848 ± 0.003	0.3319 ± 0.005	0.3529 ± 0.002	0.9406 ± 0.002	1.0632 ± 0.021	48.4691 ± 0.502	51.5309 ± 0.502	nm
2	0.6754 ± 0.001	0.2670 ± 0.001	0.4084 ± 0.001	0.6537 ± 0.004	1.5298 ± 0.010	39.5291 ± 0.157	60.4708 ± 0.157	nm
2	0.6229 ± 0.002	0.2254 ± 0.003	0.3975 ± 0.002	0.5670 ± 0.010	1.7635 ± 0.030	36.1856 ± 0.400	63.8144 ± 0.400	nsm(-)

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Variant chromosome complement of *I. nilgherricus* with 6 chromosomes

Variant somatic chromosome number	:	6
Karyotype formula	:	6nm
Chromosomes with secondary constriction	:	1
Range of chromosome length (RCL)	:	1.3386 µm to 0.8310 µm
Total chromosome length (TCL)	:	6.3376 µm
Average chromosome length (ACL)	:	1.056 µm
Disparity index (DI)	:	23.39
Variation coefficient (VC)	:	17.12
TF value (%)	:	42.77
Karyotype asymmetry index (As K%)	:	57.23
Syi index	:	74.74
Rec index	:	78.91

PLATE 8

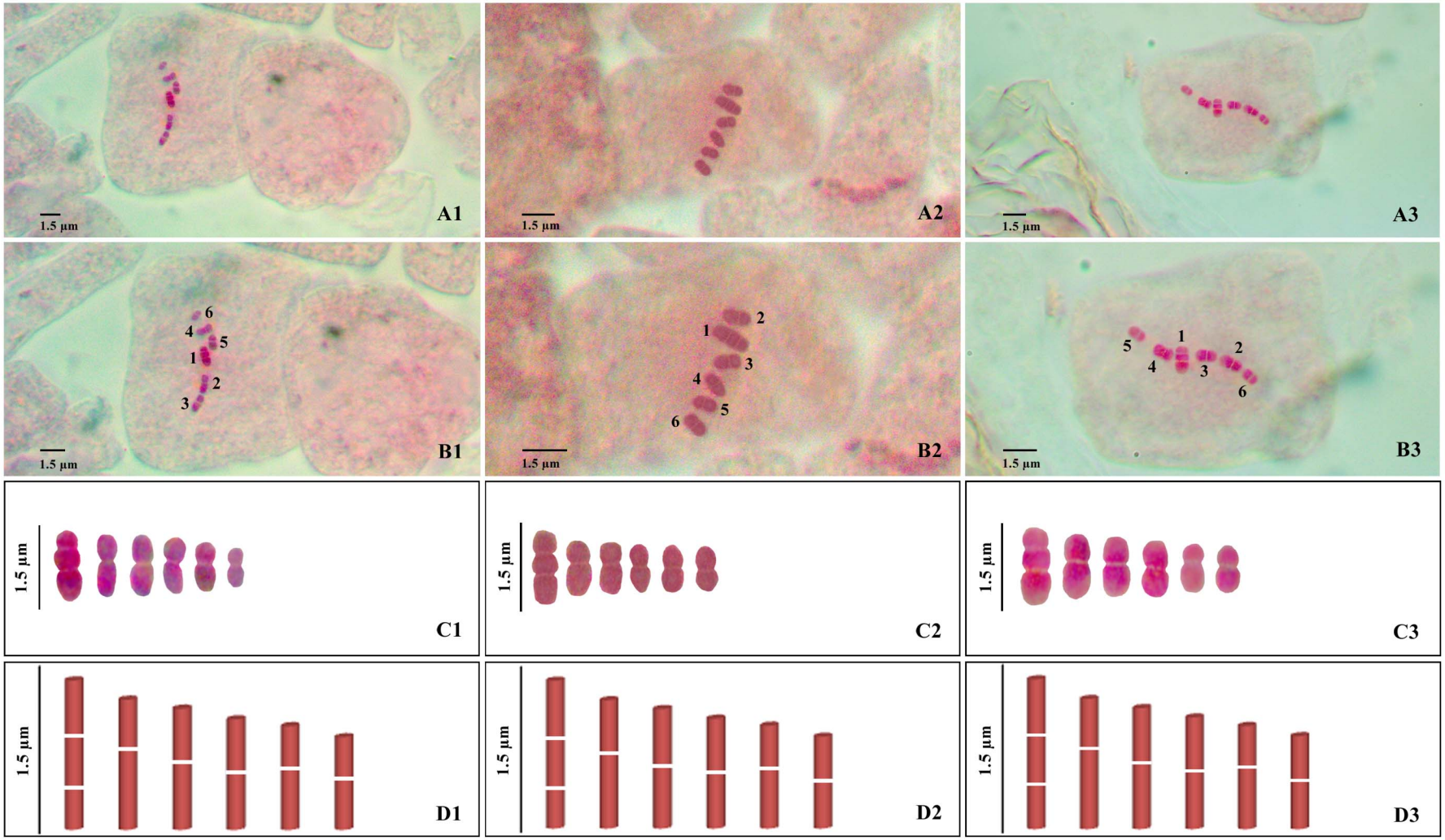


Fig. 8 Variant chromosome complement images of *Isodon nilgherricus* with 6 chromosomes
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 6 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 12. Detailed karyomorphometric data of *I. nilgherricus* with 6 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	1.3386 ± 0.001	0.5743 ± 0.002	0.7644 ± 0.002	0.7513 ± 0.006	1.3310 ± 0.010	42.8995 ± 0.187	57.1005 ± 0.187	nm
1	1.1640 ± 0.003	0.4779 ± 0.001	0.6860 ± 0.002	0.6967 ± 0.002	1.4354 ± 0.004	41.0607 ± 0.067	58.9393 ± 0.067	nm
1	1.0828 ± 0.002	0.4951 ± 0.001	0.5877 ± 0.002	0.8424 ± 0.004	1.1870 ± 0.006	45.7240 ± 0.121	54.2759 ± 0.121	nm
1	0.9943 ± 0.004	0.4563 ± 0.003	0.5381 ± 0.001	0.8480 ± 0.005	1.1793 ± 0.007	45.8867 ± 0.141	54.1133 ± 0.141	nm
1	0.9269 ± 0.004	0.3623 ± 0.001	0.5646 ± 0.003	0.6417 ± 0.002	1.5583 ± 0.005	39.0887 ± 0.076	60.9113 ± 0.076	nm
1	0.8310 ± 0.001	0.3450 ± 0.002	0.4861 ± 0.003	0.7097 ± 0.010	1.4090 ± 0.019	41.5106 ± 0.326	58.4894 ± 0.326	nm

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Variant chromosome complement of *I. nilgherricus* with 12 chromosomes

Variant somatic chromosome number	:	12
Karyotype formula	:	12nm
Chromosomes with secondary constriction	:	2
Range of chromosome length (RCL)	:	1.6215 µm to 0.8246 µm
Total chromosome length (TCL)	:	13.1981 µm
Average chromosome length (ACL)	:	1.0998 µm
Disparity index (DI)	:	32.58
Variation coefficient (VC)	:	21.23
TF value (%)	:	44.82
Karyotype asymmetry index (As K%)	:	55.18
Syi index	:	81.24
Rec index	:	67.83

PLATE 9

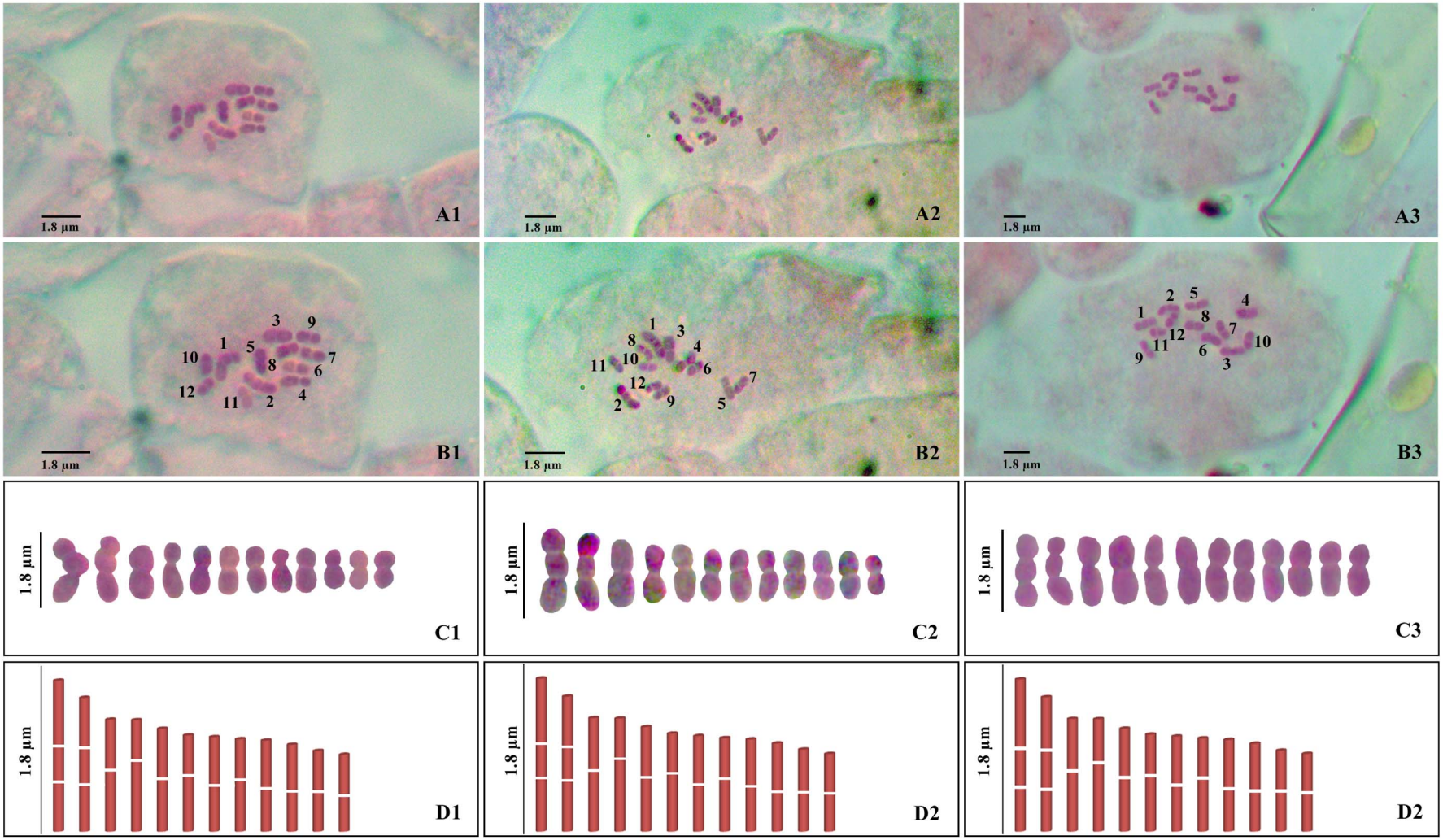


Fig. 9 Variant chromosome complement images of *Isodon nilgherricus* with 12 chromosomes
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 12 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 13. Detailed karyomorphometric data of *I. nilgherricus* with 12 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
1*	1.6215 ± 0.002	0.7651 ± 0.002	0.8565 ± 0.001	0.8933 ± 0.002	1.1195 ± 0.003	47.1817 ± 0.069	52.8183 ± 0.069	nm
1*	1.4331 ± 0.002	0.5885 ± 0.002	0.8445 ± 0.001	0.6969 ± 0.006	1.4350 ± 0.011	41.0681 ± 0.193	58.9319 ± 0.193	nm
1	1.2019 ± 0.001	0.5866 ± 0.002	0.6153 ± 0.001	0.9533 ± 0.003	1.0489 ± 0.003	48.8061 ± 0.074	51.1939 ± 0.074	nm
1	1.1979 ± 0.002	0.4628 ± 0.001	0.7352 ± 0.002	0.6295 ± 0.004	1.5886 ± 0.010	38.6304 ± 0.149	61.3696 ± 0.149	nm
1	1.1029 ± 0.006	0.5267 ± 0.004	0.5762 ± 0.002	0.9140 ± 0.005	1.0940 ± 0.006	47.7543 ± 0.144	52.2457 ± 0.144	nm
1	1.0350 ± 0.003	0.4390 ± 0.001	0.5960 ± 0.002	0.7366 ± 0.002	1.3575 ± 0.004	42.4173 ± 0.071	57.5827 ± 0.071	nm
1	1.0133 ± 0.001	0.4767 ± 0.002	0.5366 ± 0.001	0.8884 ± 0.005	1.1256 ± 0.006	47.0459 ± 0.141	52.9541 ± 0.141	nm
1	0.9916 ± 0.001	0.4153 ± 0.002	0.5763 ± 0.001	0.7206 ± 0.005	1.3877 ± 0.009	41.8818 ± 0.161	58.1182 ± 0.161	nm
1	0.9768 ± 0.001	0.4694 ± 0.001	0.5074 ± 0.001	0.9252 ± 0.002	1.0809 ± 0.003	48.0565 ± 0.061	51.9435 ± 0.061	nm
1	0.9339 ± 0.002	0.4461 ± 0.003	0.4879 ± 0.002	0.9143 ± 0.010	1.0937 ± 0.012	47.7621 ± 0.269	52.2378 ± 0.269	nm
1	0.8656 ± 0.004	0.3834 ± 0.002	0.4822 ± 0.002	0.7952 ± 0.005	1.2576 ± 0.008	44.2947 ± 0.155	55.7053 ± 0.155	nm
1	0.8246 ± 0.003	0.3563 ± 0.003	0.4683 ± 0.001	0.7610 ± 0.008	1.3141 ± 0.013	43.2129 ± 0.249	56.7871 ± 0.249	nm

Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Normal chromosome complement of *I. nilgherricus* with 2n = 24 chromosomes

Normal somatic chromosome number	:	24
Karyotype formula	:	22nm + 2nsm(-)
Chromosomes with secondary constriction	:	4
Range of chromosome length (RCL)	:	1.2290 μm to 0.8116 μm
Total chromosome length (TCL)	:	23.0086 μm
Average chromosome length (ACL)	:	0.9587 μm
Disparity index (DI)	:	20.45
Variation coefficient (VC)	:	17.52
TF value (%)	:	42.84
Karyotype asymmetry index (As K%)	:	57.16
Syi index	:	74.96
Rec index	:	78.01
Intrachromosomal asymmetry index (A_1)	:	0.24
Interchromosomal asymmetry index (A_2)	:	0.12
Degree of asymmetry of karyotypes (A)	:	0.28

PLATE 10

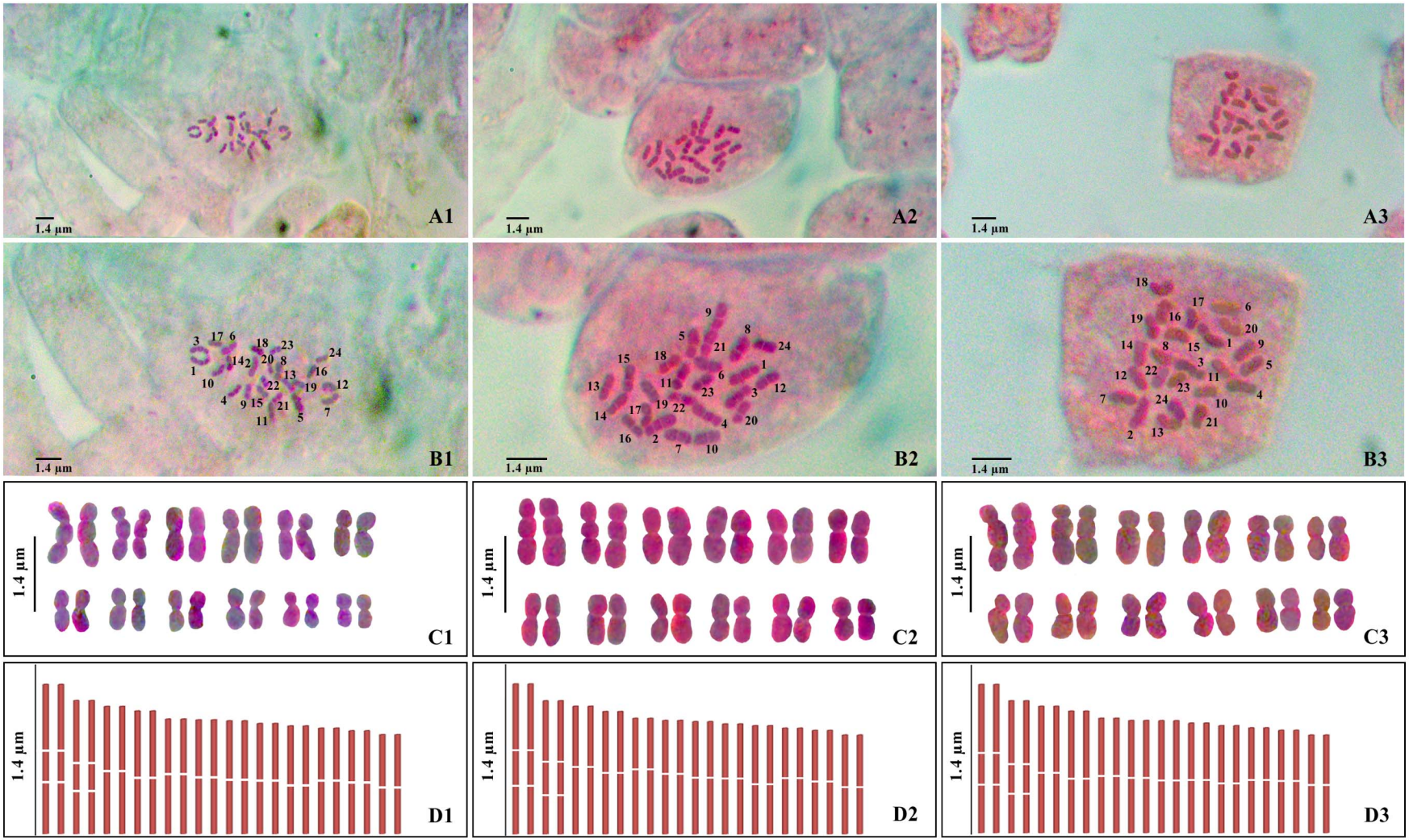


Fig. 10 Normal chromosome complement images of *Isodon nilgherricus* (2n = 24)
A1-A3 Cell at mitotic metaphase, B1-B3 Karyotype with 24 chromosomes, C1-C3 Karyogram, D1-D3 Idiogram

Table 14. Detailed karyomorphometric data of *I. nilgherricus* with 2n = 24 chromosomes

No. of Chr.	c ± SD (µm)	s ± SD (µm)	l ± SD (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c %)	I_2 (l/c %)	Nature of PC
2*	1.2290 ± 0.001	0.4547 ± 0.002	0.7743 ± 0.001	0.5872 ± 0.003	1.7030 ± 0.010	36.9958 ± 0.137	63.0041 ± 0.137	nsm(-)
2*	1.0933 ± 0.002	0.4286 ± 0.001	0.6647 ± 0.001	0.6449 ± 0.002	1.5507 ± 0.005	39.2055 ± 0.071	60.7945 ± 0.071	nm
2	1.0472 ± 0.001	0.4529 ± 0.002	0.5943 ± 0.003	0.7620 ± 0.008	1.3123 ± 0.014	43.2473 ± 0.254	56.7527 ± 0.254	nm
2	1.0073 ± 0.003	0.4848 ± 0.003	0.5225 ± 0.001	0.9278 ± 0.007	1.0778 ± 0.008	48.1287 ± 0.191	51.8713 ± 0.191	nm
2	0.9467 ± 0.003	0.3846 ± 0.002	0.5621 ± 0.001	0.6841 ± 0.002	1.4617 ± 0.005	40.6218 ± 0.082	59.3782 ± 0.082	nm
2	0.9328 ± 0.003	0.4071 ± 0.002	0.5257 ± 0.003	0.7745 ± 0.006	1.2911 ± 0.010	43.6464 ± 0.198	56.3536 ± 0.198	nm
2	0.9267 ± 0.004	0.4141 ± 0.002	0.5126 ± 0.002	0.8079 ± 0.002	1.2378 ± 0.003	44.6874 ± 0.065	55.3126 ± 0.065	nm
2	0.9060 ± 0.004	0.3932 ± 0.001	0.5128 ± 0.003	0.7667 ± 0.003	1.3044 ± 0.005	43.3959 ± 0.093	56.6041 ± 0.093	nm
2	0.8874 ± 0.001	0.4238 ± 0.002	0.4635 ± 0.003	0.9143 ± 0.010	1.0937 ± 0.012	47.7630 ± 0.271	52.2370 ± 0.271	nm
2	0.8683 ± 0.001	0.3568 ± 0.001	0.5116 ± 0.001	0.6974 ± 0.002	1.4339 ± 0.005	41.0864 ± 0.083	58.9136 ± 0.083	nm
2	0.8480 ± 0.002	0.3643 ± 0.001	0.4837 ± 0.002	0.7531 ± 0.005	1.3278 ± 0.008	42.9582 ± 0.154	57.0418 ± 0.154	nm
2	0.8116 ± 0.002	0.3639 ± 0.003	0.4477 ± 0.003	0.8127 ± 0.010	1.2304 ± 0.015	44.8351 ± 0.310	55.1649 ± 0.310	nm

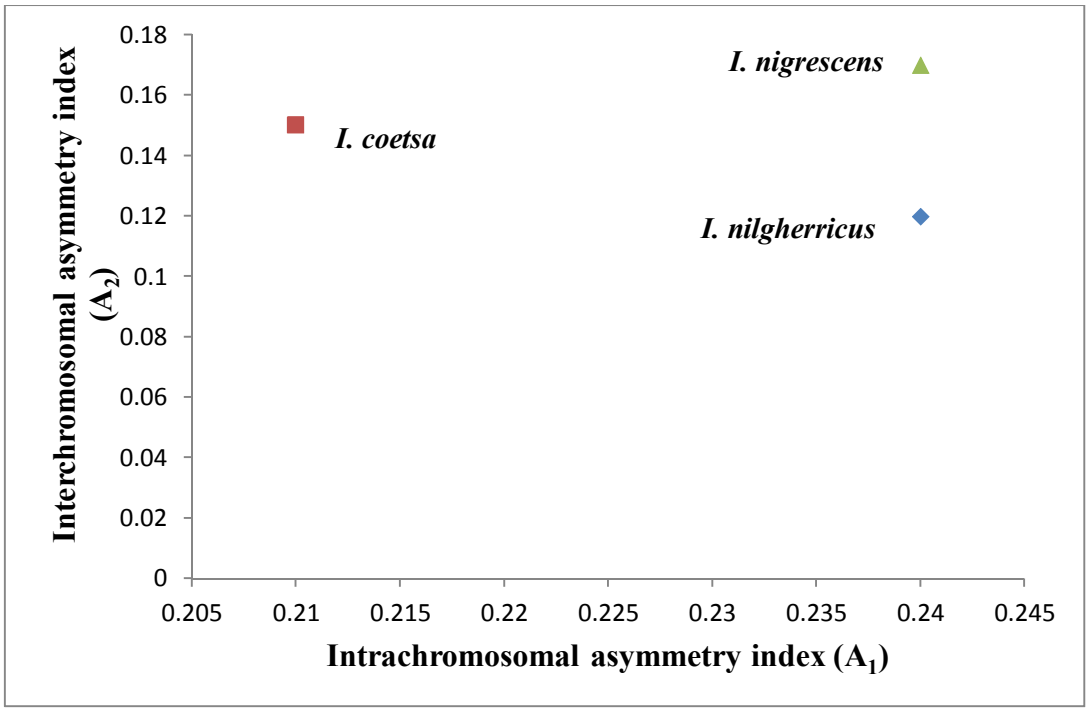
Chr. - Chromosome, c - total length of chromosome, l - long arm length, s - short arm length, R_1 - arm ratio 1, R_2 - arm ratio 2, I_1 - centromeric index 1, I_2 - centromeric index 2, PC - primary constriction, SD - standard deviation
*Chromosomes with secondary constriction

Table 15. Summary of karyomorphometric features of three species of *Isodon*

Karyotype	No. of chr. with sec. constr.	TCL (µm)	ACL (µm)	RCL (µm)	DI	VC	TF%	As K%	Syi	Rec	A_1	A_2	A	Karyotype formula
I. coetsa (Variant = 6)	1	5.2306	0.8718	1.0201 - 0.7700	13.97	10.40	43.67	56.33	77.52	85.46	-	-	-	6nm
I. coetsa (Variant = 12)	2	10.0148	0.8346	1.1154 - 0.5778	31.75	19.82	44.97	55.03	81.73	74.82	-	-	-	12nm
I. coetsa (2n =24)	4	21.1596	0.8817	1.1100 - 0.7055	22.28	14.92	43.69	56.31	77.58	80.34	0.21	0.15	0.24	18nm + 6nsm(-)
I. nigrescens (Variant = 6)	1	4.8769	0.8128	1.2110 - 0.6201	32.27	27.21	43.76	56.24	77.81	67.12	-	-	-	5nm + 1nsm(-)
I. nigrescens (Variant = 12)	2	8.2203	0.6850	0.9632 - 0.5647	24.02	18.82	43.65	56.35	77.45	71.12	-	-	-	12nm
I. nigrescens (2n = 24)	4	19.2966	0.8040	1.0707 - 0.6229	26.44	16.86	42.96	57.04	75.31	75.09	0.24	0.17	0.28	22nm + 2nsm(-)
I. nilgherricus (Variant = 6)	1	6.3376	1.0560	1.3386 - 0.8310	23.39	17.12	42.77	57.23	74.74	78.91	-	-	-	6nm
I. nilgherricus (Variant = 12)	2	13.1981	1.0998	1.6215 - 0.8246	32.58	21.23	44.82	55.18	81.24	67.83	-	-	-	12nm
I. nilgherricus (2n = 24)	4	23.0086	0.9587	1.2290 - 0.8116	20.45	17.52	42.84	57.16	74.96	78.01	0.24	0.12	0.28	22nm + 2nsm(-)

chr. - chromosome, sec. - secondary, constr. - constriction

Fig. 11. Scatter plot of three species of *Isodon* based on karyotype asymmetry indices



ANTIOXIDANT STUDIES

The antioxidant capacity of the methanolic extract of three species of *Isodon* used in folk medicines was evaluated using four different *in vitro* assays. The assays included DPPH radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging and ferric ion reducing antioxidant power activities. Methanolic extract was prepared with the help of Soxhlet apparatus and the total yield percentage of extracts was found to be 42.58% for *I. coetsa*, 37.94% for *I. nigrescens* and 46.60% for *I. nilgherricus*. The extracts showed potent antioxidant activity when compared to standard at varying concentrations (Tables 16-29; Figs 12-24). The IC_{50} value of the extracts for the assays (Table 16) and EC for FRAP assay (Table 29) were also calculated using regression analysis.

DPPH radical scavenging assay

The decrease in absorbance of the DPPH solution in the presence of a hydrogen donating antioxidant enabled the measurement of free radical scavenging activity in DPPH assay. The colour change resulted from the reduction of relatively stable DPPH free radical by hydrogen donation was detected. The methanolic extracts showed considerable free radical scavenging activity when compared to standard. Ascorbic acid was used as the standard which had an IC_{50} value of 5.26 ± 0.01 µg/ml (Table 16) determined from the percentage inhibition ranging between 19.25 ± 0.39 to 85.81 ± 0.81 for a concentration series of 1-10 µg/ml (Table 17; Fig. 12). The antioxidant activity of the extracts increased with increase in concentration. The most efficient free radical scavenger was *I. coetsa* with an IC_{50} value of 9.32 ± 0.11 µg/ml (Table 16) calculated from the percentage inhibition ranging between 16.49 ± 0.15 to 92.52 ± 0.59 for a concentration series of 2.5-25 µg/ml (Table 18; Fig. 13) followed by *I. nilgherricus* with an

IC_{50} value of 11.04 ± 1.80 µg/ml (Table 16) calculated from the percentage inhibition ranging between 44.15 ± 1.66 to 94.79 ± 0.14 for a concentration series of 5-50 µg/ml (Table 20; Fig. 15) and *I. nigrescens* with an IC_{50} value of 22.59 ± 0.26 µg/ml (Table 16) calculated from the percentage inhibition ranging between 12.90 ± 0.33 to 88.50 ± 0.33 for a concentration series of 5-50 µg/ml (Table 19; Fig. 14).

Hydroxyl radical scavenging assay

Degradation of deoxyribose by hydroxyl radicals generated from the Fe^{3+}/Ascorbate/EDTA/H_2O_2 system was measured to determine the scavenging activity of the extracts. The methanolic extracts showed excellent hydroxyl radical scavenging activity when compared to standard. α-tocopherol was used as the standard which had an IC_{50} value of 6.86 ± 0.02 µg/ml (Table 16) determined from the percentage inhibition ranging between 35.55 ± 0.29 to 62.02 ± 0.15 for a concentration series of 2-12 µg/ml (Table 21; Fig. 16). The antioxidant activity of the extracts increased in dose dependent manner. The most efficient hydroxyl radical scavenger was *I. nigrescens* with an IC_{50} value of 1.19 ± 0.01 µg/ml (Table 16) calculated from the percentage inhibition ranging between 20.86 ± 1.51 to 51.23 ± 0.28 for a concentration series of 0.2-1.4 µg/ml (Table 23; Fig. 18) followed by *I. coetsa* with an IC_{50} value of 1.25 ± 0.01 µg/ml (Table 16) calculated from the percentage inhibition ranging between 23.51 ± 0.69 to 51.25 ± 0.21 for a concentration series of 0.2-1.4 µg/ml (Table 22; Fig. 17) and *I. nilgherricus* with an IC_{50} value of 5.29 ± 0.11 µg/ml (Table 16) calculated from the percentage inhibition ranging between 41.57 ± 0.39 to 60.77 ± 0.20 for a concentration series of 2-12 µg/ml (Table 24; Fig. 19).

Superoxide radical scavenging assay

The light induced superoxide generation from riboflavin and the subsequent reduction of nitroblue tetrazolium forms the basis of riboflavin photoreduction method which was employed for the determination of the scavenging capacity of the extracts. The methanolic extracts showed considerable superoxide radical scavenging activity when compared to standard. Ascorbic acid was used as the standard which had an IC_{50} value of 53.35 ± 0.16 µg/ml (Table 16) determined from the percentage inhibition ranging between 23.39 ± 0.33 to 54.41 ± 0.25 for a concentration series of 10-60 µg/ml (Table 25; Fig. 20). The antioxidant activity of the extracts increased with increase in concentration. The most efficient superoxide radical scavenger was *I. coetsa* with an IC_{50} value of 90.01 ± 0.22 µg/ml (Table 16) calculated from the percentage inhibition ranging between 27.65 ± 0.15 to 59.45 ± 0.13 for a concentration series of 20-120 µg/ml (Table 26; Fig. 21) followed by *I. nigrescens* with an IC_{50} value of 108.89 ± 0.22 µg/ml (Table 16) calculated from the percentage inhibition ranging between 0.00 ± 0.00 to 56.77 ± 0.03 for a concentration series of 20-120 µg/ml (Table 27; Fig. 22) and *I. nilgherricus* with an IC_{50} value of 118.83 ± 0.25 µg/ml (Table 16) calculated from the percentage inhibition ranging between 12.14 ± 0.26 to 61.36 ± 0.34 for a concentration series of 20-160 µg/ml (Table 28; Fig. 23).

Ferric ion reducing antioxidant power assay

The methanolic extracts of three species of *Isodon* were analysed for the ferric ion reducing antioxidant capacity. The concentration of antioxidant was measured which showed a reduction ability to ferric-TpTz in terms of $FeSO_4 \cdot 7H_2O$ equivalent employing the standard equation obtained from the linear calibration curve ($y = 5.023x - 0.009$, $R^2 = 1$; Fig. 24). The ferric

reducing activity of 1 µg/ml of the methanolic extracts was equivalent to the reducing power of 12.28 ± 0.40 µmol/ml for *I. coetsa*, 6.98 ± 0.20 µmol/ml for *I. nigrescens* and 6.24 ± 0.13 µmol/ml for *I. nilgherricus* of $FeSO_4 \cdot 7H_2O$ (Table 29). The results revealed that *I. nigrescens* and *I. nilgherricus* have similar FRAP activity while *I. coetsa* showed a two fold decrease.

The antioxidant values obtained provided substantiation for the remarkable activity of the methanolic extracts of the three species of *Isodon* which is clearly evident from the high IC_{50} values of the extracts in scavenging the hydroxyl radicals than the control. All the values were found to be significant at $p < 0.05$ when statistically analysed.

Table 16. *In vitro* antioxidant activities of standards and methanolic extracts of three species of *Isodon*

Extract	IC_{50} ± SE (µg/ml)		
	DPPH radical scavenging assay	**Hydroxyl radical scavenging assay**	**Superoxide radical scavenging assay**
Standard	5.26 ± 0.01^{a}	6.86 ± 0.02^{c}	53.35 ± 0.16^{a}
I. coetsa	9.32 ± 0.11^{b}	1.25 ± 0.01^{a}	90.01 ± 0.22^{b}
I. nigrescens	22.59 ± 0.26^{c}	1.19 ± 0.01^{a}	108.89 ± 0.22^{c}
I. nilgherricus	11.04 ± 1.80^{b}	5.29 ± 0.11^{b}	118.83 ± 0.25^{d}

IC_{50} - Concentration of the samples causing 50% inhibition of radicals
Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 17. Scavenging effects of ascorbic acid (standard) on DPPH radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
1	19.25 ± 0.39[a]
2	27.76 ± 0.84[b]
3	36.84 ± 0.13[c]
4	39.48 ± 0.70[d]
5	45.61 ± 0.19[e]
6	55.72 ± 0.10[f]
7	62.61 ± 0.24[g]
8	68.53 ± 0.83[h]
9	75.58 ± 0.34[i]
10	85.81 ± 0.81[j]

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 18. Scavenging effects of methanolic extract of *I. coetsa* on DPPH radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
2.5	16.49 ± 0.15[a]
5	39.29 ± 1.15[b]
10	58.21 ± 1.38[c]
15	78.65 ± 1.88[d]
20	84.48 ± 0.87[e]
25	92.52 ± 0.59[f]

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 19. Scavenging effects of methanolic extract of *I. nigrescens* on DPPH radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
5	12.90 ± 0.33^{a}
10	34.75 ± 0.70^{b}
20	55.99 ± 0.99^{c}
30	60.08 ± 0.23^{d}
40	77.81 ± 0.09^{e}
50	88.50 ± 0.33^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 20. Scavenging effects of methanolic extract of *I. nilgherricus* on DPPH radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
5	44.15 ± 1.66^{a}
10	68.57 ± 1.76^{b}
15	80.58 ± 0.38^{c}
20	91.41 ± 0.12^{d}
25	$92.02 \pm 0.02^{d,e}$
30	$92.47 \pm 0.10^{d,e}$
40	$93.78 \pm 0.38^{d,e}$
50	94.79 ± 0.14^{e}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Fig. 12. *In vitro* DPPH radical scavenging activity of ascorbic acid (standard)

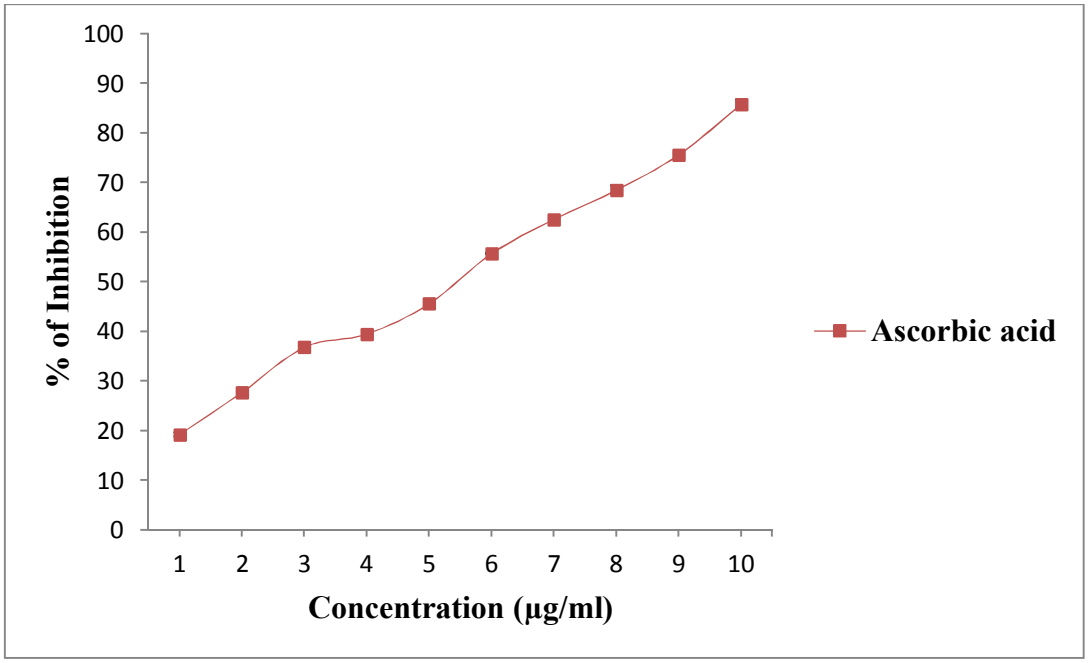


Fig. 13. *In vitro* DPPH radical scavenging activity of methanolic extract of *I. coetsa*

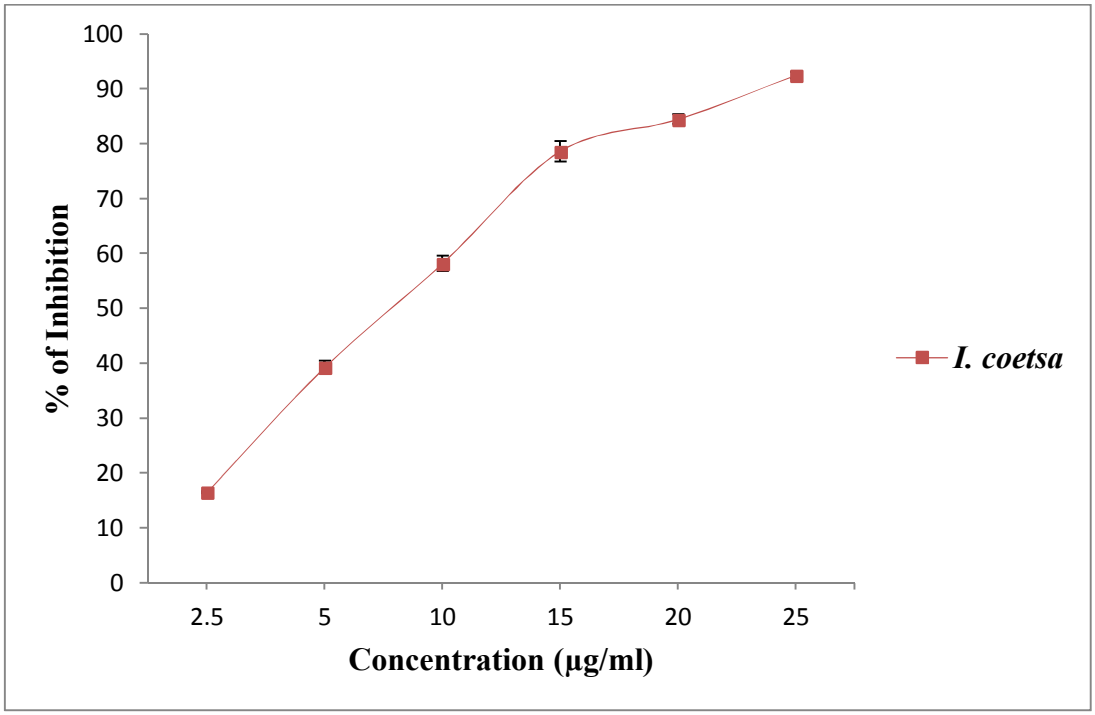


Fig. 14. *In vitro* DPPH radical scavenging activity of methanolic extract of *I. nigrescens*

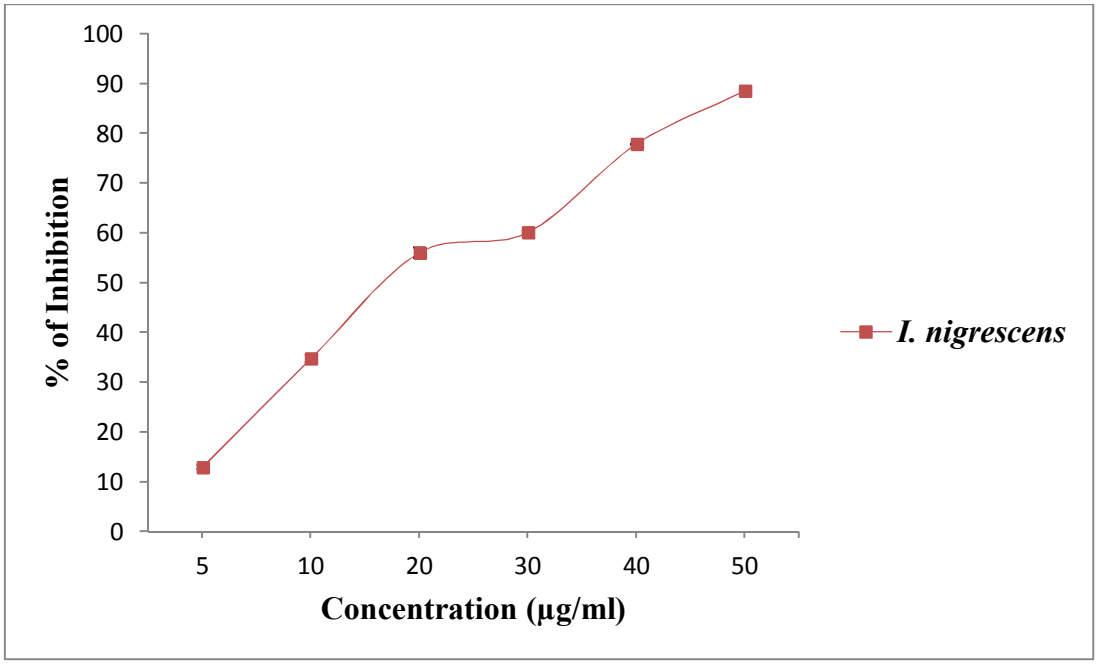


Fig. 15. *In vitro* DPPH radical scavenging activity of methanolic extract of *I. nilgherricus*

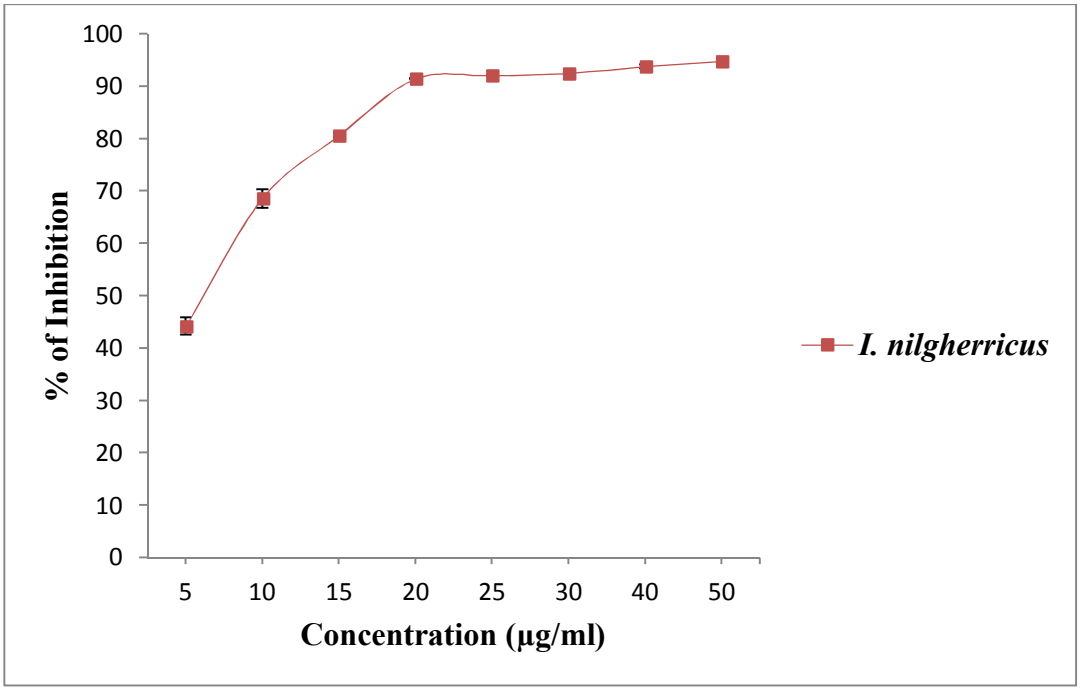


Table 21. Scavenging effects of α-tocopherol (standard) on hydroxyl radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
2	35.55 ± 0.29^{a}
4	42.89 ± 0.12^{b}
6	47.33 ± 0.26^{c}
8	54.94 ± 0.50^{d}
10	59.60 ± 0.18^{e}
12	62.02 ± 0.15^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 22. Scavenging effects of methanolic extract of *I. coetsa* on hydroxyl radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
0.2	23.51 ± 0.69^{a}
0.4	26.44 ± 0.71^{b}
0.6	35.65 ± 0.07^{c}
0.8	41.40 ± 0.42^{d}
1.0	45.33 ± 0.95^{e}
1.2	48.96 ± 0.26^{f}
1.4	51.25 ± 0.21^{g}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 23. Scavenging effects of methanolic extract of *I. nigrescens* on hydroxyl radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
0.2	20.86 ± 1.51^{a}
0.4	31.42 ± 1.15^{b}
0.6	37.65 ± 0.42^{c}
0.8	44.90 ± 0.30^{d}
1.0	47.51 ± 0.62^{e}
1.2	$49.79 \pm 0.11^{e,f}$
1.4	51.23 ± 0.28^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 24. Scavenging effects of methanolic extract of *I. nilgherricus* on hydroxyl radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
2	41.57 ± 0.39^{a}
4	48.75 ± 0.16^{b}
6	52.76 ± 0.20^{c}
8	56.55 ± 0.28^{d}
10	58.46 ± 0.22^{e}
12	60.77 ± 0.20^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Fig. 16. *In vitro* hydroxyl radical scavenging activity of α-tocopherol (standard)

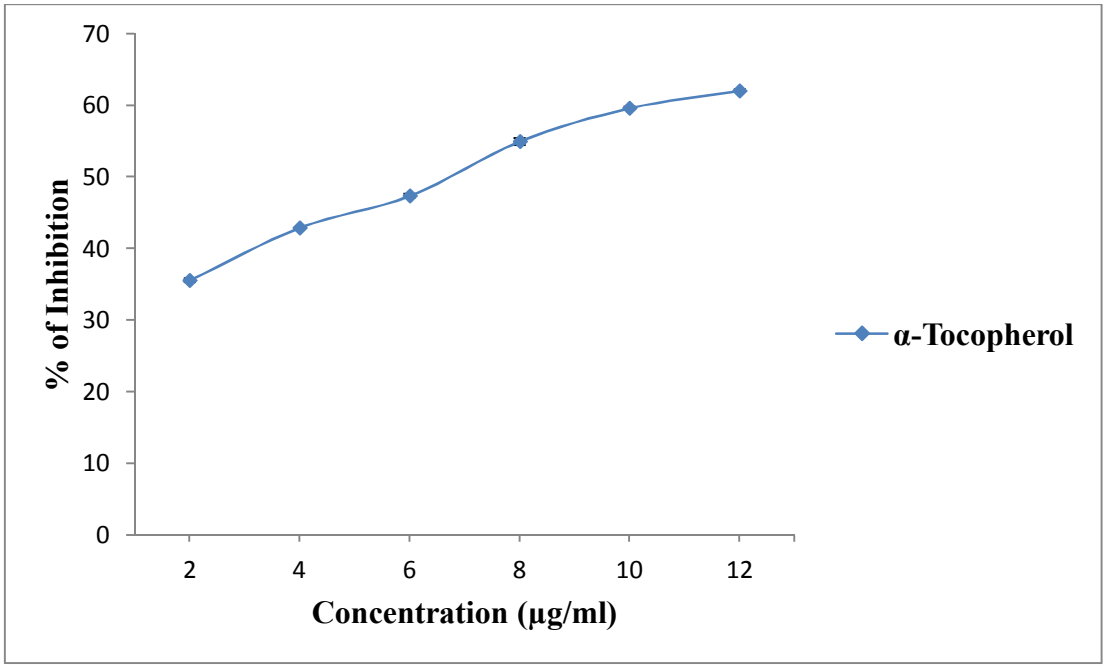


Fig. 17. *In vitro* hydroxyl radical scavenging activity of methanolic extract of *I. coetsa*

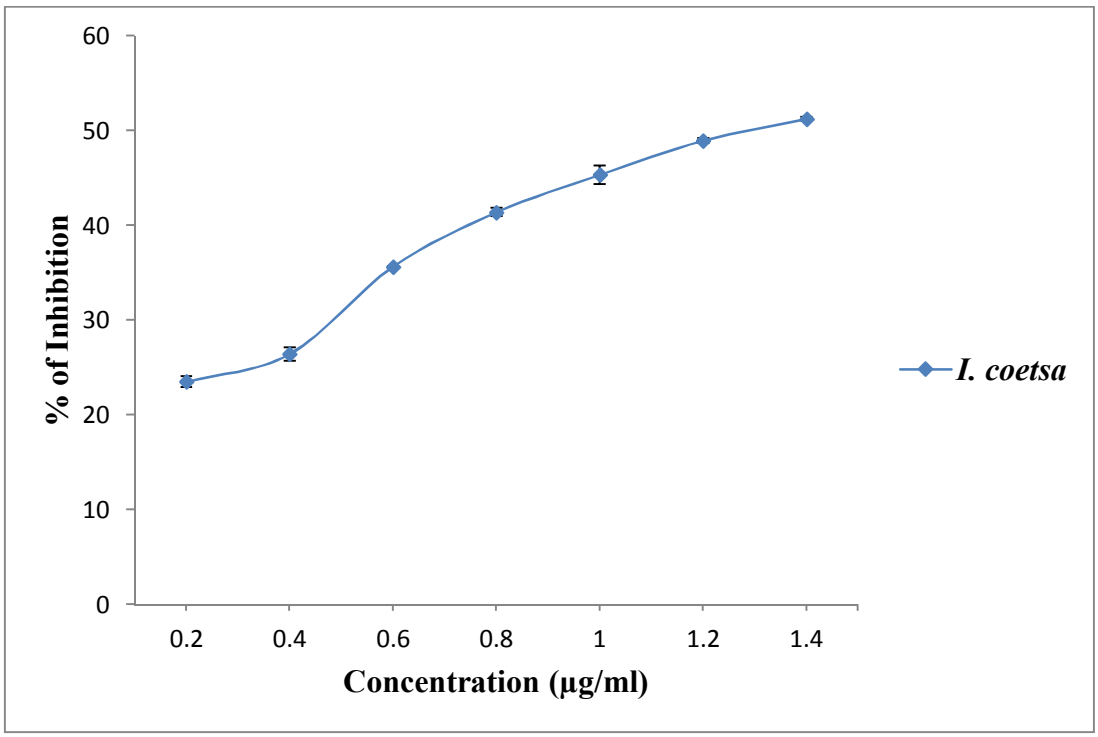


Fig. 18. *In vitro* hydroxyl radical scavenging activity of methanolic extract of *I. nigrescens*

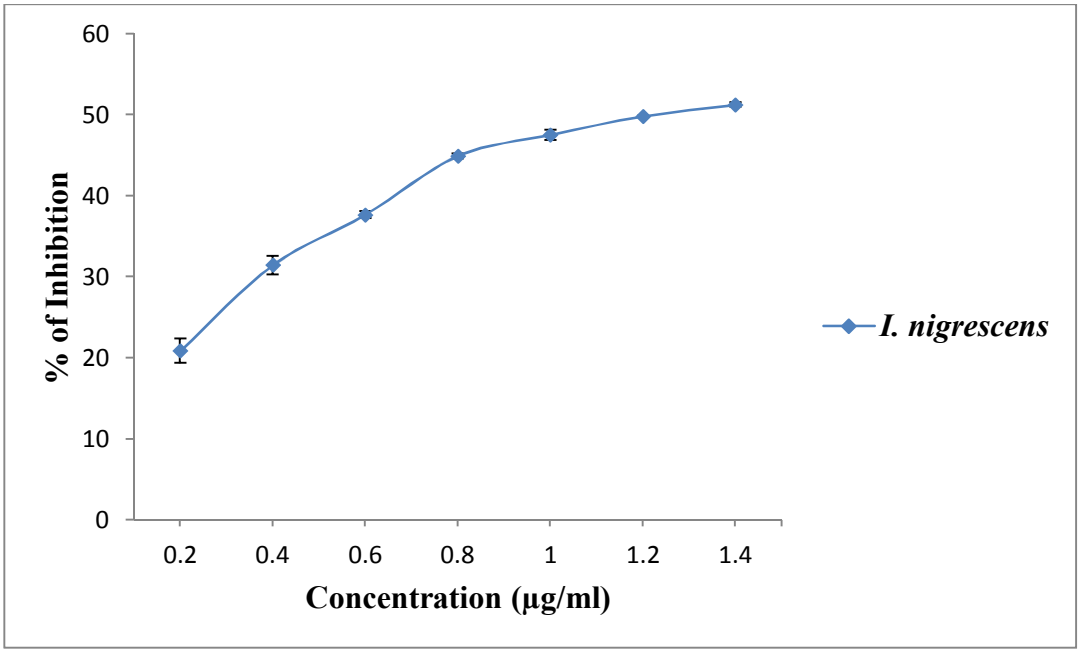


Fig. 19. *In vitro* hydroxyl radical scavenging activity of methanolic extract of *I. nilgherricus*

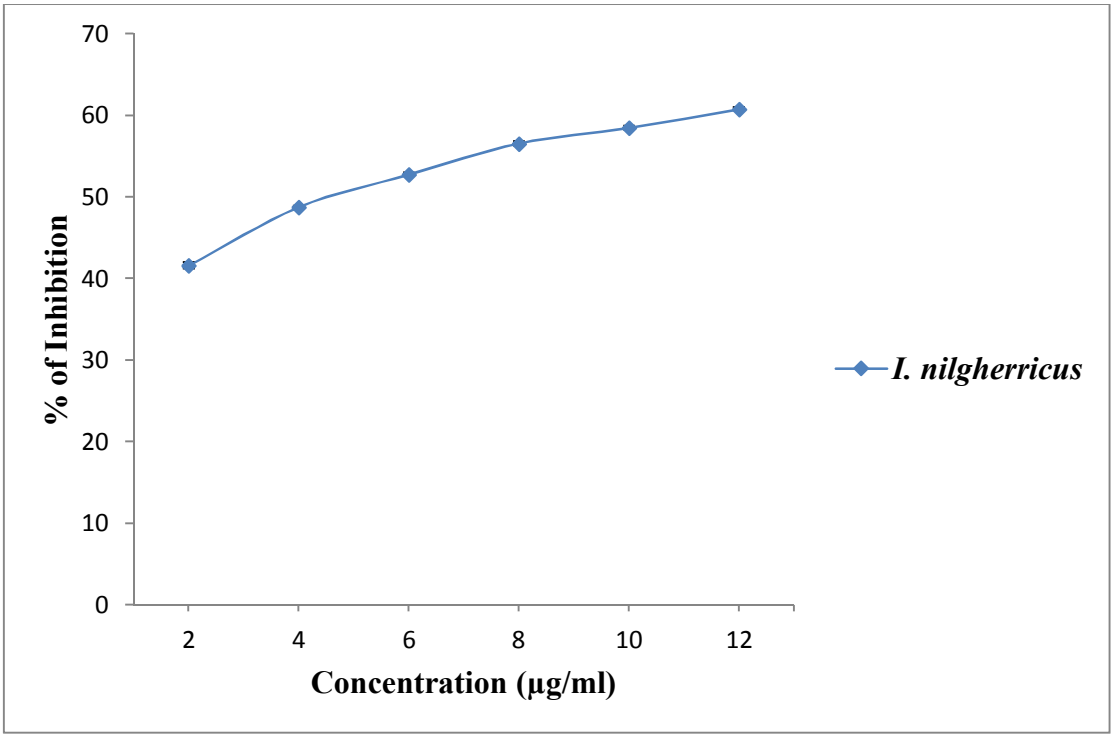


Table 25. Scavenging effects of ascorbic acid (standard) on superoxide radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
10	23.39 ± 0.33^{a}
20	28.80 ± 0.19^{b}
30	34.45 ± 0.23^{c}
40	40.92 ± 0.13^{d}
50	48.55 ± 0.12^{e}
60	54.41 ± 0.25^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 26. Scavenging effects of methanolic extract of *I. coetsa* on superoxide radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
20	27.65 ± 0.15^{a}
40	32.28 ± 0.15^{b}
60	33.66 ± 0.22^{c}
80	45.47 ± 0.10^{d}
100	58.65 ± 0.18^{e}
120	59.45 ± 0.13^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 27. Scavenging effects of methanolic extract of *I. nigrescens* on superoxide radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
20	0.00 ± 0.00^{a}
40	14.97 ± 0.06^{b}
60	21.36 ± 0.21^{c}
80	29.12 ± 0.22^{d}
100	47.65 ± 0.23^{e}
120	56.77 ± 0.03^{f}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 28. Scavenging effects of methanolic extract of *I. nilgherricus* on superoxide radical *in vitro* at different concentrations

Concentrations (µg/ml)	% Inhibition ± SE
20	12.14 ± 0.26^{a}
40	30.99 ± 0.40^{b}
60	37.46 ± 0.26^{c}
80	38.77 ± 0.27^{d}
100	47.45 ± 0.12^{e}
120	49.40 ± 0.18^{f}
140	53.81 ± 0.19^{g}
160	61.36 ± 0.34^{h}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Fig. 20. *In vitro* superoxide radical scavenging activity of ascorbic acid (standard)

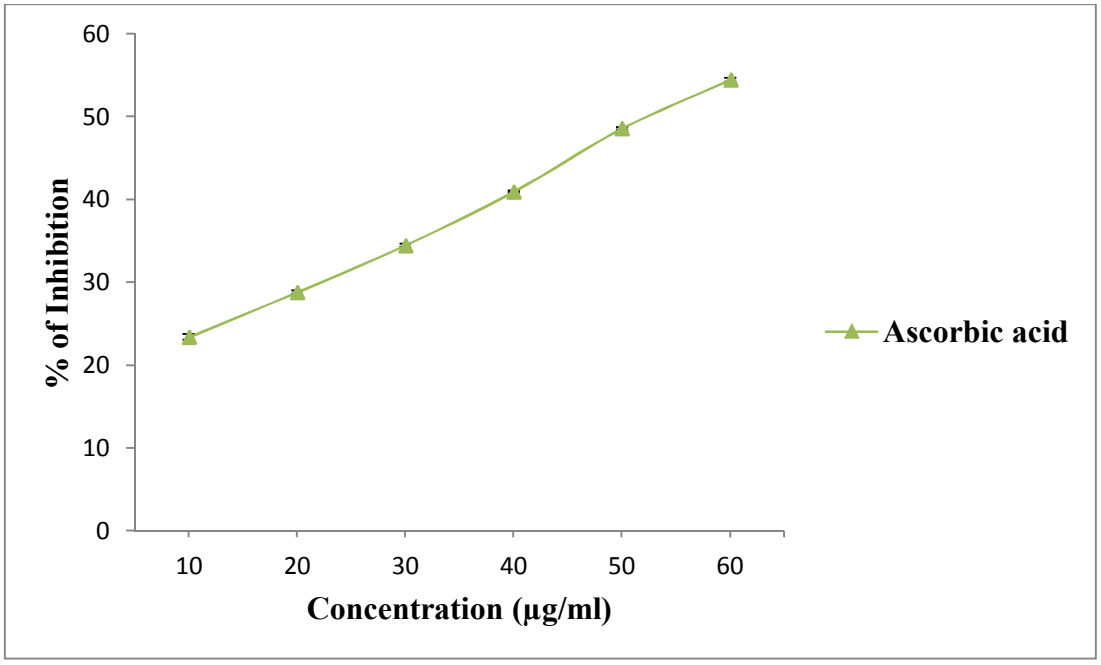


Fig. 21. *In vitro* superoxide radical scavenging activity of methanolic extract of *I. coetsa*

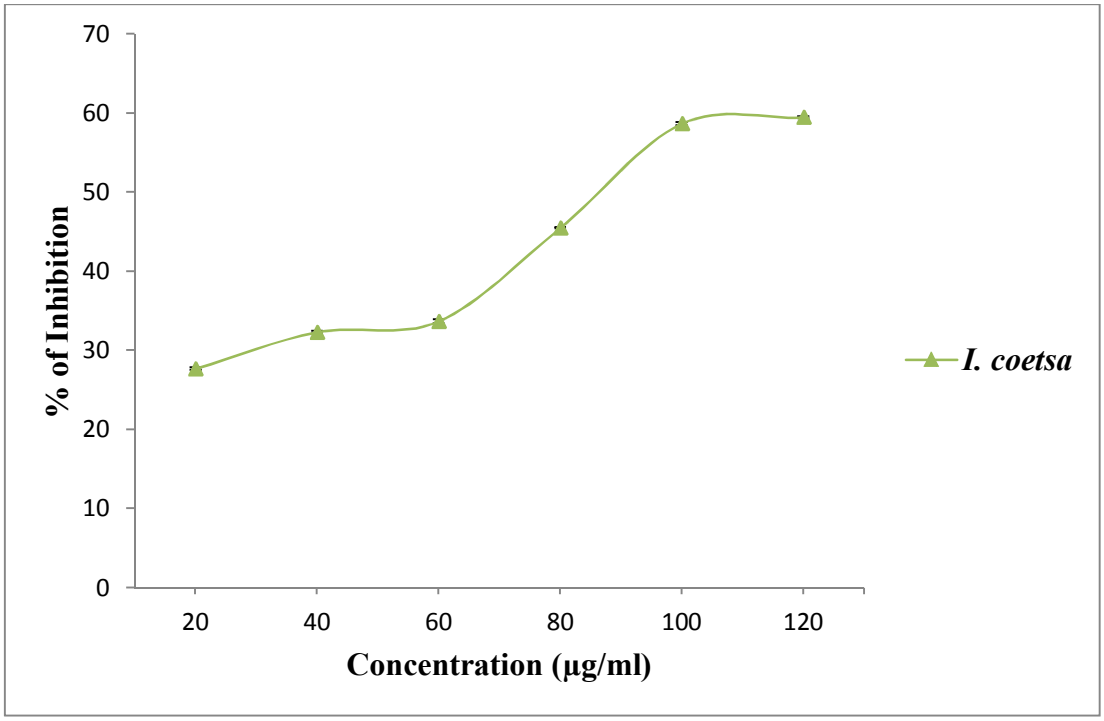


Fig. 22. *In vitro* superoxide radical scavenging activity of methanolic extract of *I. nigrescens*

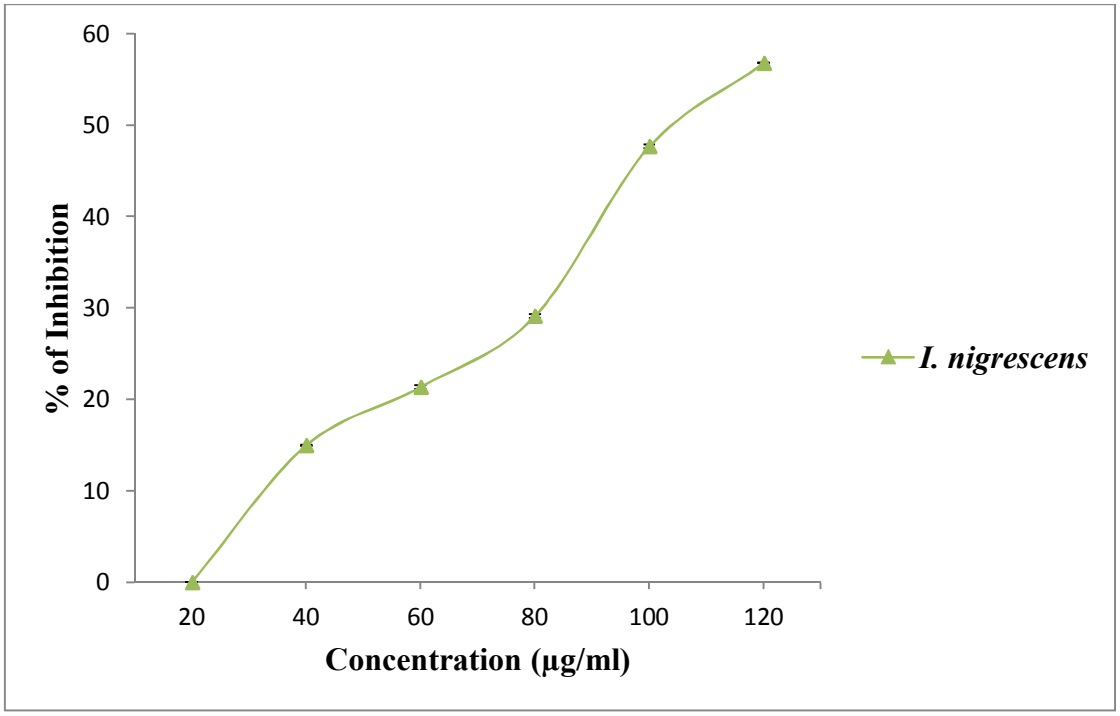


Fig. 23. *In vitro* superoxide radical scavenging activity of methanolic extract of *I. nilgherricus*

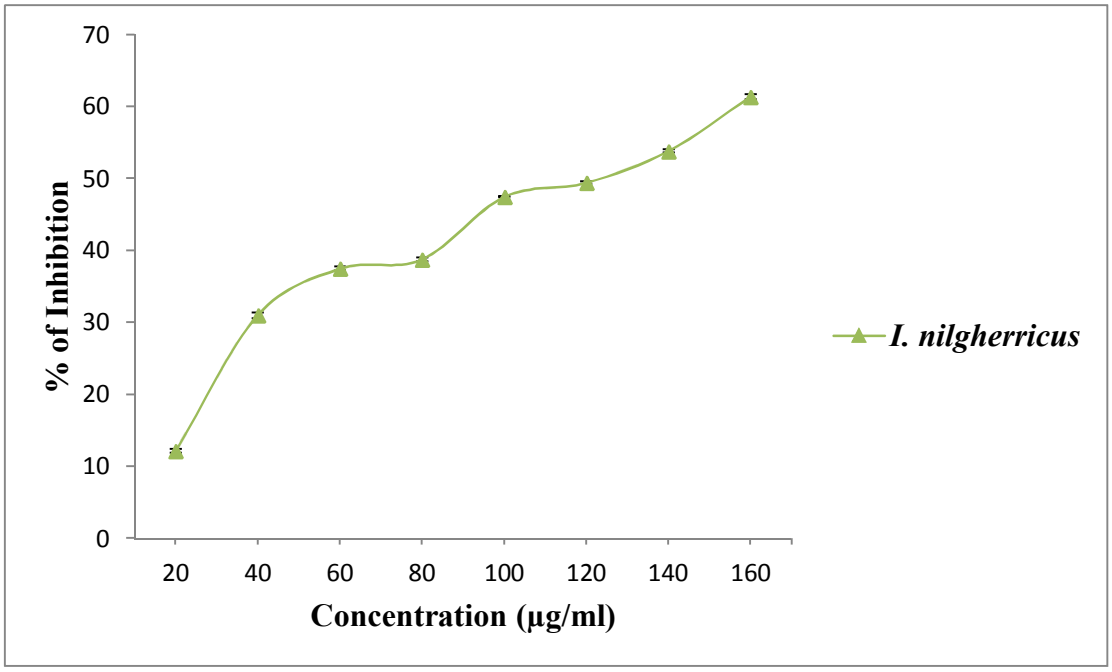


Fig. 24. Standard calibration curve for the determination of ferric ion reducing activity

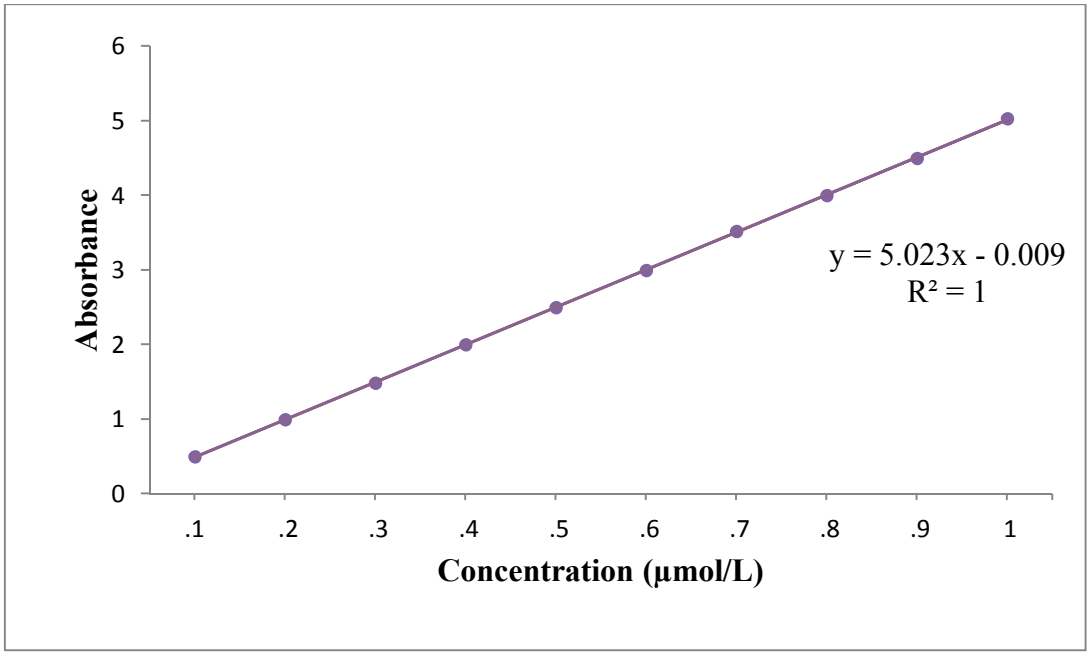


Table 29. Reducing activity of methanolic extracts of three species of *Isodon in vitro* on ferric ion

Concentrations (µg/ml)	EC ± SE (µmol/ml)		
	I. coetsa	*I. nigrescens*	*I. nilgherricus*
0.2	5.91 ± 0.24^{a}	3.78 ± 0.35^{a}	2.79 ± 0.01^{a}
0.4	7.56 ± 0.71^{b}	$4.25 \pm 0.35^{a,b}$	3.45 ± 0.07^{b}
0.6	$8.56 \pm 0.70^{b,c}$	4.71 ± 0.18^{b}	4.18 ± 0.35^{c}
0.8	9.89 ± 0.37^{c}	6.57 ± 0.30^{c}	4.84 ± 0.35^{d}
1.0	12.28 ± 0.40^{d}	6.98 ± 0.20^{c}	6.24 ± 0.13^{e}
1.2	15.53 ± 0.01^{e}	9.75 ± 0.20^{d}	6.57 ± 0.001^{e}

EC - Equivalent concentration with ferric-TPTZ reducing ability expressed as µmoL Fe (II) equivalents/µg extract

Values are expressed as mean ± standard error (SE)

Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

ANTITUMOR STUDIES

Cytotoxic assays

The *in vitro* cytotoxic activity of the methanolic extracts of three species of *Isodon* was determined using two different assays. The assays included Evans blue staining on *A. cepa* test system and Trypan blue exclusion assay employing two cell lines *viz*., Dalton's lymphoma ascites tumor cells (DLA) and Ehrlich's ascites carcinoma cells (EAC).

Cell death determination

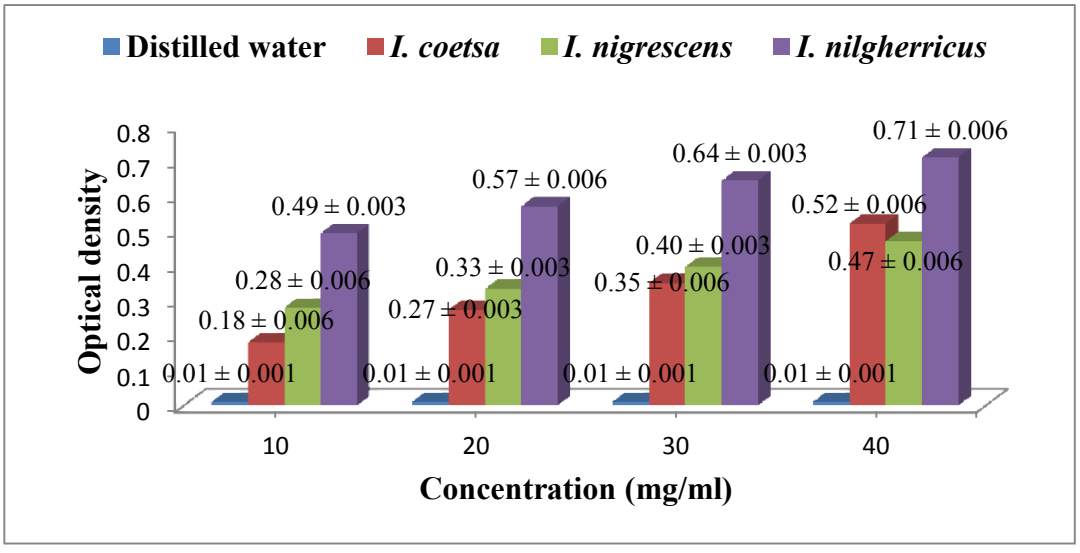
Evans blue staining of the methanolic extract treated *A. cepa* root meristems was a preliminary screening employed to determine the cytotoxic effect of the extract. The Evans blue uptake of the damaged or dead cells in the root meristems were spectrophotometrically determined. The results revealed that the extracts possessed significant cytotoxic activity over a concentration range of 10-40 mg/ml (Table 30; Fig. 25). The cytotoxic activity of the extracts increased in a dose dependent manner. The highest activity was shown by *I. nilgherricus* followed by *I. coetsa* and *I. nigrescens*.

Table 30. Induction of cell death with control and methanolic extracts of three species of *Isodon* in *A. cepa* root tissue

Extract	Concentrations (mg/ml)	Optical density ± SE
Distilled water (Negative control)	-	0.01 ± 0.001^{a}
I. coetsa	10	0.18 ± 0.006^{b}
	20	0.27 ± 0.003^{c}
	30	0.35 ± 0.006^{e}
	40	0.52 ± 0.006^{i}
I. nigrescens	10	0.28 ± 0.006^{c}
	20	0.33 ± 0.003^{d}
	30	0.40 ± 0.003^{f}
	40	0.47 ± 0.006^{g}
I. nilgherricus	10	0.49 ± 0.003^{h}
	20	0.57 ± 0.006^{j}
	30	0.64 ± 0.003^{k}
	40	0.71 ± 0.006^{l}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Fig. 25. Cell death induction in *A. cepa* root tissue by methanolic extracts of three species of *Isodon*



Trypan blue exclusion assay

Cell viability of DLA and EAC cell lines *in vitro* after the treatment with the methanolic extracts of three species of *Isodon* was determined using trypan blue exclusion assay. The extracts showed potent cytotoxic activity at varying concentrations (Tables 32, 33; Figs 26, 27). The IC_{50} value of the extracts for the cytotoxic assay (Table 31) was also calculated using regression analysis. The activity of the extracts increased in concentration dependent manner. The most effective cytotoxic activity against DLA cell line was shown by *I. nilgherricus* with an IC_{50} value of 0.82 ± 0.01 mg/ml followed by *I. coetsa* with an IC_{50} value of 1.21 ± 0.02 mg/ml and *I. nigrescens* with an IC_{50} value of 1.60 ± 0.01 mg/ml determined from the inhibition percentage recorded over a concentration range of 0.2-1.8 mg/ml (Table 32; Fig. 26). The highest cytotoxic activity against EAC cell line was shown by *I. nilgherricus* with an IC_{50} value of 0.89 ± 0.01 mg/ml followed by *I. coetsa* with an IC_{50} value of 1.36 ± 0.01 mg/ml and *I. nigrescens* with an IC_{50} value of 1.51 ± 0.01 mg/ml calculated from the inhibition percentage obtained over a concentration range of 0.2-1.8 mg/ml (Table 33; Fig. 27).

The cytotoxic assays revealed the excellent activity of the methanolic extracts of *Isodon*. All the values were found to be significant at $p < 0.05$ when statistically analysed. A comparative analysis, in general, demarcated the excellent potential of *I. nilgherricus* in *in vitro* cytotoxic assays followed by *I. coetsa* and *I. nigrescens*.

Table 31. *In vitro* cytotoxic activities of methanolic extracts of three species of *Isodon*

Extract	IC_{50} ± SE (mg/ml)	
	DLA	EAC
I. coetsa	1.21 ± 0.02^{b}	1.36 ± 0.01^{b}
I. nigrescens	1.60 ± 0.01^{c}	1.51 ± 0.01^{c}
I. nilgherricus	0.82 ± 0.01^{a}	0.89 ± 0.01^{a}

IC_{50} - Concentration of the samples causing 50% cytotoxicity
Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Table 32. Cytotoxic effects of methanolic extracts of three species of *Isodon* on DLA cell lines *in vitro* at different concentrations

Concentrations (mg/ml)	% Inhibition ± SE		
	I. coetsa	*I. nigrescens*	*I. nilgherricus*
0.2	12.75 ± 0.38[a]	2.57 ± 0.29[a]	17.62 ± 1.93[a]
0.4	27.15 ± 0.98[b]	9.68 ± 0.27[b]	31.15 ± 0.30[b]
0.6	33.82 ± 1.20[c]	14.02 ± 0.12[c]	40.47 ± 0.28[c]
0.8	37.67 ± 0.39[d]	18.27 ± 0.59[d]	51.35 ± 0.34[d]
1.0	42.59 ± 0.23[e]	26.42 ± 0.42[e]	61.14 ± 0.79[e]
1.2	44.47 ± 0.15[e]	32.66 ± 0.50[f]	72.76 ± 0.84[f]
1.4	55.77 ± 1.44[f]	45.19 ± 0.98[g]	77.66 ± 0.67[g]
1.6	64.82 ± 0.17[g]	52.29 ± 0.36[h]	82.81 ± 0.36[h]
1.8	69.24 ± 0.85[h]	57.64 ± 0.60[i]	84.64 ± 0.25[h]

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Fig. 26. *In vitro* cytotoxic activity of methanolic extracts of three species of *Isodon* on DLA cell lines

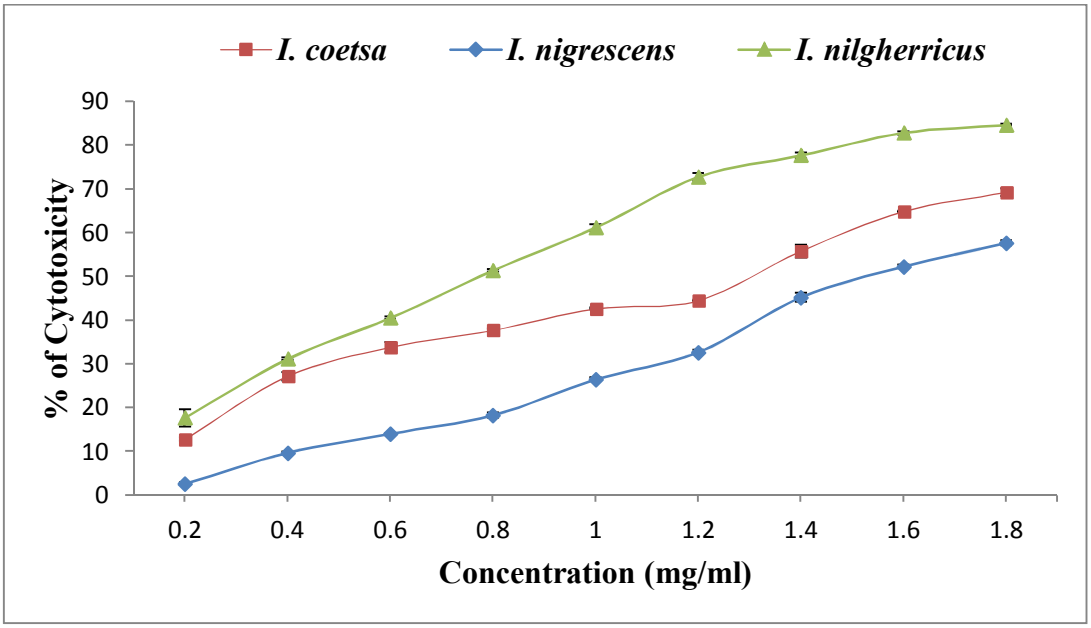
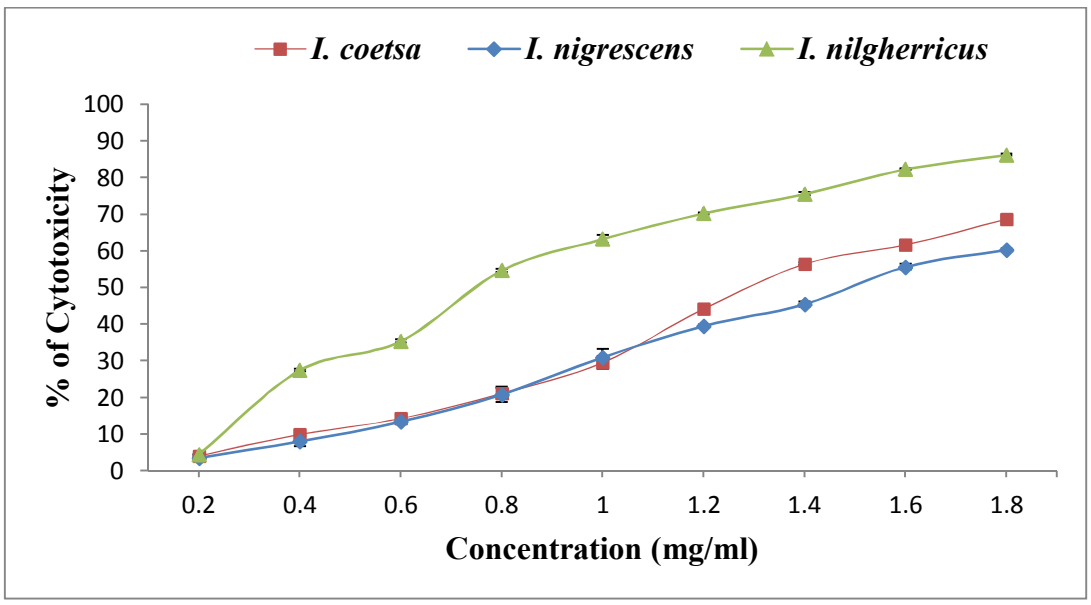


Table 33. Cytotoxic effects of methanolic extracts of three species of *Isodon* on EAC cell lines *in vitro* at different concentrations

Concentrations (mg/ml)	% Inhibition ± SE		
	I. coetsa	*I. nigrescens*	*I. nilgherricus*
0.2	4.08 ± 0.27^{a}	3.41 ± 0.25^{a}	4.37 ± 0.12^{a}
0.4	9.90 ± 0.38^{b}	8.04 ± 1.33^{b}	27.42 ± 0.35^{b}
0.6	14.24 ± 0.85^{c}	13.37 ± 0.41^{c}	35.39 ± 0.42^{c}
0.8	21.08 ± 1.45^{d}	20.80 ± 2.16^{d}	54.67 ± 0.45^{d}
1.0	29.52 ± 1.81^{e}	30.96 ± 2.17^{e}	63.21 ± 1.09^{e}
1.2	44.17 ± 0.01^{f}	39.57 ± 0.23^{f}	70.21 ± 0.32^{f}
1.4	56.45 ± 0.32^{g}	45.46 ± 0.72^{g}	75.57 ± 0.46^{g}
1.6	61.73 ± 0.31^{h}	55.66 ± 0.82^{h}	82.29 ± 0.17^{h}
1.8	68.65 ± 0.71^{i}	60.30 ± 0.33^{i}	86.27 ± 0.25^{i}

Values are expressed as mean ± standard error (SE)
Means within a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Duncan's multiple range test)

Fig. 27. *In vitro* cytotoxic activity of methanolic extracts of three species of *Isodon* on EAC cell lines



Antitumor assays

In vivo antitumor assays are essential for the proper identification of anticancer potential of any source plant used in drug research. The methanolic extracts of three species of *Isodon* was evaluated for antitumor activity based on its effect against DLA induced solid tumor and EAC induced ascites tumor in Swiss albino mice. Methanolic extracts and the standard drug used as control were administered to the animals to determine the effect of antitumor activity. Excellent activity was shown by the extracts against DLA induced solid tumor (Plates 11-13; Tables 34-39; Figs 31-33) while moderate effect was revealed against EAC induced ascites tumor (Plates 14-16; Tables 43-45; Fig. 37).

DLA induced solid tumor model

Solid tumor formation in Swiss albino mice was induced by the intramuscular injection of the DLA cell lines into the right hand limb of the animals. The extracts and standard drug was administered after 24 hours of tumor implantation. The methanolic extracts were provided orally for ten consecutive days while the standard drug cyclophosphamide was injected intraperitoneally. The development of tumor was monitored regularly and tumor volume was measured with the help of vernier calliper at fixed interval of three days for thirty consecutive days. The differences in the body weight of the animals as well as the haemoglobin and WBC content of the animals were also noted to determine the general health of the animals.

Administration of the methanolic extract of *I. coetsa* showed significant reduction in tumor volume in a dose dependent manner (Plate 11; Table 37; Fig. 31). Tumor volume was reduced to almost normal when the mice were administered with 50 mg/kg b. wt of the extract. The percentage inhibition of tumor volume was found to be 95.46 ± 1.03 for 25 mg/kg b. wt

while it was 97.82 ± 0.29 for 50 mg/kg b. wt (Table 34). The body weight of the animals also showed significant change with percentage increase of 4.45 ± 0.21 and 1.73 ± 0.68 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. Haematological parameters when observed showed a slight decrease in haemoglobin content while the WBC content was on an increase than the normal mice (Table 40).

Administration of the methanolic extract of *I. nigrescens* also showed significant reduction in tumor volume in a concentration dependent manner (Plate 12; Table 38; Fig. 32). Tumor volume was reduced to almost normal when the mice were administered with 50 mg/kg b. wt of the extract. The percentage inhibition of tumor volume was found to be 88.90 ± 1.41 for 25 mg/kg b. wt while it was 96.82 ± 0.41 for 50 mg/kg b. wt (Table 35). The body weight of the animals also showed significant change with percentage increase of 5.94 ± 0.31 and 0.78 ± 0.24 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. Haematological parameters when observed showed a slight decrease in haemoglobin content while the WBC content was on an increase with respect to lower dose and it was slightly lesser in the higher dose when compared with the normal mice (Table 41).

Administration of the methanolic extract of *I. nilgherricus* showed significant reduction in tumor volume with increasing concentration (Plate 13; Table 39; Fig. 33). Tumor volume was reduced to almost normal when the mice were administered with 50 mg/kg b. wt of the extract. The percentage inhibition of tumor volume was found to be 73.94 ± 2.36 for 25 mg/kg b. wt while it was 96.19 ± 1.11 for 50 mg/kg b. wt (Table 36). The body weight of the animals also showed significant change with percentage increase of 10.18 ± 0.59 and 5.73 ± 0.59 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. Haematological parameters when observed showed a slight

decrease in haemoglobin content while the WBC content was on an increase than the normal mice (Table 42).

The standard drug cyclophosphamide when administered showed significant reduction in tumor volume and the tumor was almost invisible. But the haematological parameters showed anaemic condition where the Hb count was less when compared to normal but the WBC count was maintained close to normal. Moreover, the weak condition of mice was depicted in peripheral body characters such as the visible effects of hair loss. Mice administered with extracts, although showed a slight anaemic condition, were healthy in overall external features. The antitumor effect was excellent with high doses of the extracts and comparable to standard drug but low doses of *I. nilgherricus* showed non-significant activity and that of *I. coetsa* and *I. nigrescens* revealed low significance. In comparison, the highest antitumor activity against DLA induced solid tumor was shown by *I. coetsa* followed by *I. nigrescens* and *I. nilgherricus*.

Table 34. Effect of methanolic extract of *I. coetsa* on DLA induced solid tumor in mice

Treatment groups	% Increase in body weight ± SE	% Inhibition of tumor volume ± SE
Control (Tumor alone)	13.31 ± 2.04	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	0.39 ± 0.09	97.88 ± 0.67**
I. coetsa (25 mg/kg b. wt)	4.45 ± 0.21	95.46 ± 1.03*
I. coetsa (50 mg/kg b. wt)	1.73 ± 0.68	97.82 ± 0.29**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Table 35. Effect of methanolic extract of *I. nigrescens* on DLA induced solid tumor in mice

Treatment groups	% Increase in body weight ± SE	% Inhibition of tumor volume ± SE
Control (Tumor alone)	21.09 ± 1.12	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	0.83 ± 0.16	95.92 ± 0.54**
I. nigrescens (25 mg/kg b. wt)	5.94 ± 0.31	88.90 ± 1.41*
I. nigrescens (50 mg/kg b. wt)	0.78 ± 0.24	96.82 ± 0.41**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Table 36. Effect of methanolic extract of *I. nilgherricus* on DLA induced solid tumor in mice

Treatment groups	% Increase in body weight ± SE	% Inhibition of tumor volume ± SE
Control (Tumor alone)	27.37 ± 1.64	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	0.43 ± 0.11	98.69 ± 0.78**
I. nilgherricus (25 mg/kg b. wt)	10.18 ± 0.59	73.94 ± 2.36
I. nilgherricus (50 mg/kg b. wt)	5.73 ± 0.59	96.19 ± 1.11**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Table 37. Antitumor activity of methanolic extract of *I. coetsa* on DLA induced solid tumor in mice

Treatment groups	Tumor volume (cm^3) ± SE											% Inhibition ± SE
	0^{th} day	3^{rd} day	6^{th} day	9^{th} day	12^{th} day	15^{th} day	18^{th} day	21^{st} day	24^{th} day	27^{th} day	30^{th} day	
Control (Tumor alone)	0.76 ± 0.14	1.47 ± 0.18	2.90 ± 0.16	5.80 ± 0.76	6.32 ± 0.32	12.27 ± 1.78	14.23 ± 1.97	18.96 ± 2.25	25.32 ± 2.46	24.78 ± 2.72	30.37 ± 3.15	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	0.90 ± 0.06	1.47 ± 0.12	0.99 ± 0.14	0.81 ± 0.09	2.14 ± 0.36	1.31 ± 0.27	1.42 ± 0.18	1.57 ± 0.25	0.84 ± 0.09	0.72 ± 0.21	0.64 ± 0.22	97.88 ± 0.67
I. coetsa (25 mg/kg b. wt)	1.12 ± 0.10	1.70 ± 0.17	1.91 ± 0.20	2.76 ± 0.30	3.54 ± 0.14	2.13 ± 0.19	3.31 ± 0.69	2.51 ± 0.44	2.14 ± 0.38	1.70 ± 0.50	1.38 ± 0.31	95.46 ± 1.03
I. coetsa (50 mg/kg b. wt)	0.33 ± 0.06	0.45 ± 0.04	0.80 ± 0.06	1.42 ± 0.32	2.10 ± 0.14	1.95 ± 0.37	1.24 ± 0.39	1.01 ± 0.29	0.77 ± 0.20	0.46 ± 0.07	0.66 ± 0.09	97.82 ± 0.29

Data represent the mean ± SE (Standard error)

PLATE 11

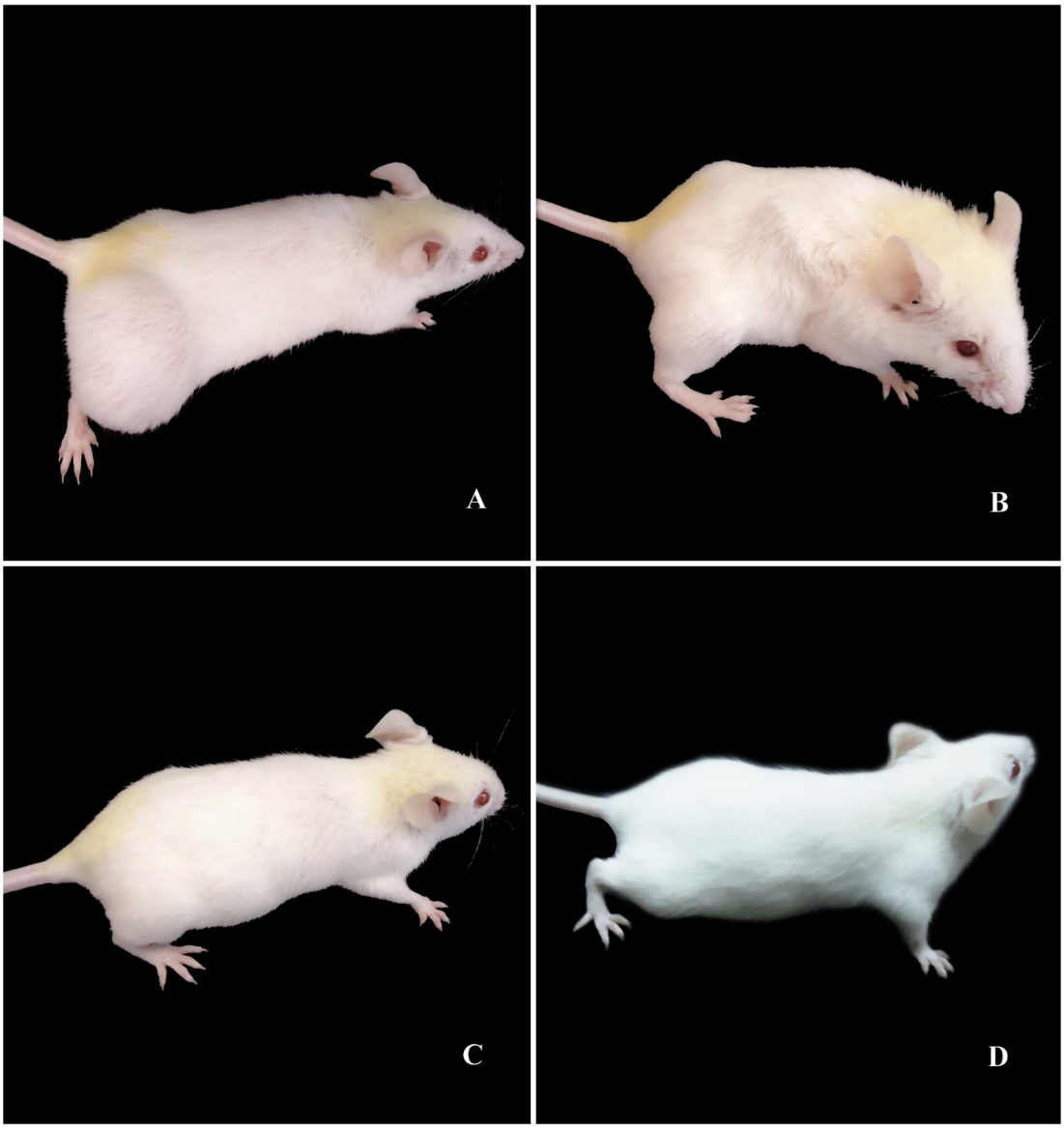


Fig. 28 Antitumor effect of methanolic extract of *Isodon coetsa* on DLA induced solid tumor in mice. A Control, B Standard, C *I. coetsa* (25 mg/kg b. wt), D *I. coetsa* (50 mg/kg b. wt)

Fig. 31. *In vivo* antitumor effect of methanolic extract of *I. coetsa* on DLA induced solid tumor in mice

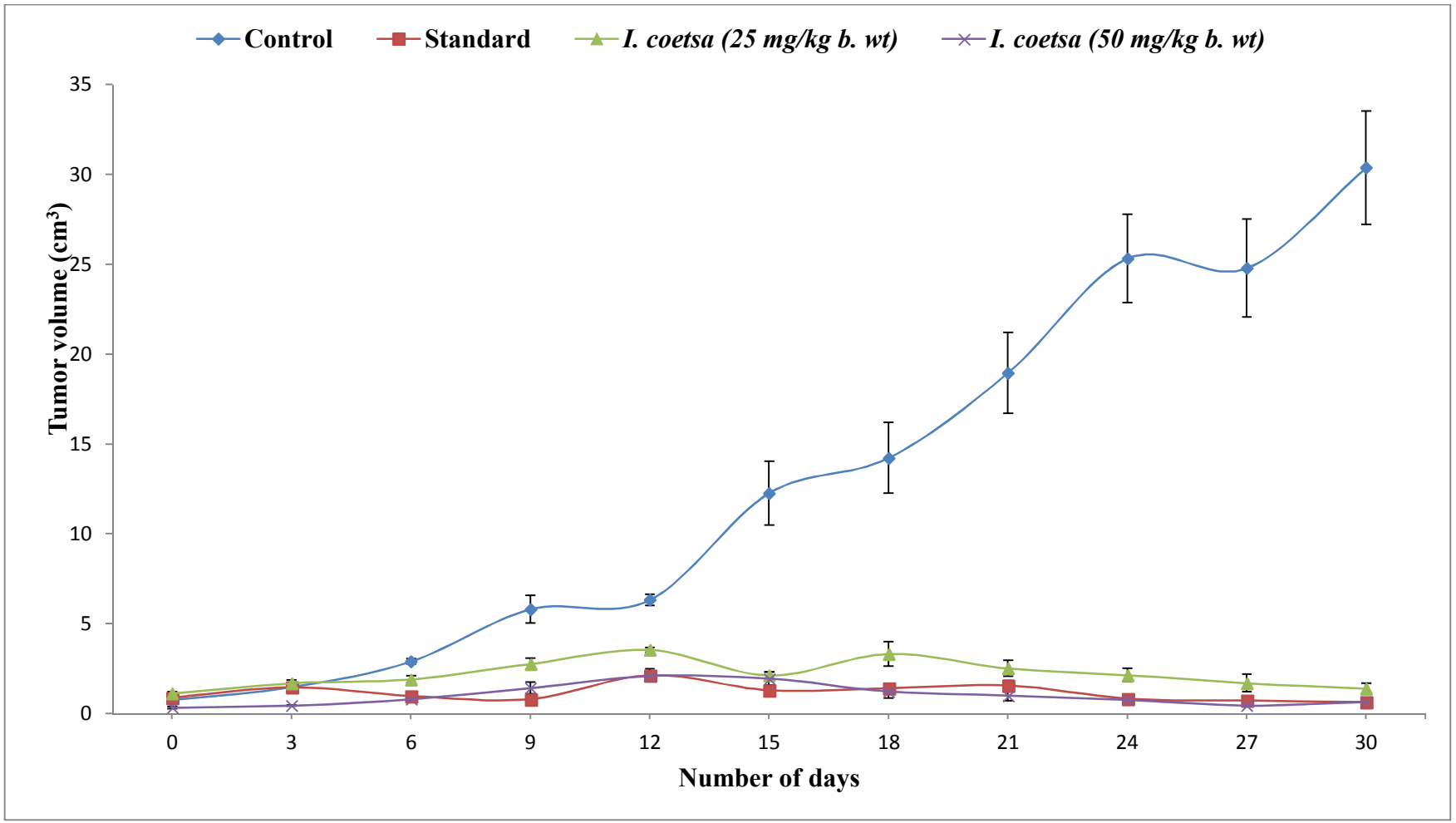


Table 38. Antitumor activity of methanolic extract of *I. nigrescens* on DLA induced solid tumor in mice

Treatment groups	Tumor volume (cm^3) ± SE											% Inhibition ± SE
	0^{th} day	3^{rd} day	6^{th} day	9^{th} day	12^{th} day	15^{th} day	18^{th} day	21^{st} day	24^{th} day	27^{th} day	30^{th} day	
Control (Tumor alone)	1.06 ± 0.18	1.48 ± 0.21	3.59 ± 0.29	8.32 ± 0.68	7.27 ± 0.48	7.63 ± 1.35	8.42 ± 2.49	12.06 ± 2.95	12.00 ± 2.83	13.58 ± 3.03	14.78 ± 3.61	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	0.82 ± 0.11	1.44 ± 0.13	1.51 ± 0.19	1.21 ± 0.10	1.57 ± 0.29	1.65 ± 0.23	1.87 ± 0.41	1.48 ± 0.36	1.05 ± 0.14	0.58 ± 0.20	0.60 ± 0.27	95.92 ± 0.54
I. nigrescens (25 mg/kg b. wt)	1.10 ± 0.11	1.23 ± 0.24	2.05 ± 0.26	2.27 ± 0.20	2.38 ± 0.29	2.35 ± 0.36	3.32 ± 0.36	3.24 ± 1.02	2.80 ± 0.41	2.02 ± 0.23	1.64 ± 0.21	88.90 ± 1.41
I. nigrescens (50 mg/kg b. wt)	0.33 ± 0.01	0.34 ± 0.04	0.69 ± 0.08	1.02 ± 0.15	1.66 ± 0.23	1.45 ± 0.31	0.97 ± 0.29	0.60 ± 0.13	0.51 ± 0.15	0.39 ± 0.08	0.47 ± 0.06	96.82 ± 0.41

Data represent the mean ± SE (Standard error)

PLATE 12

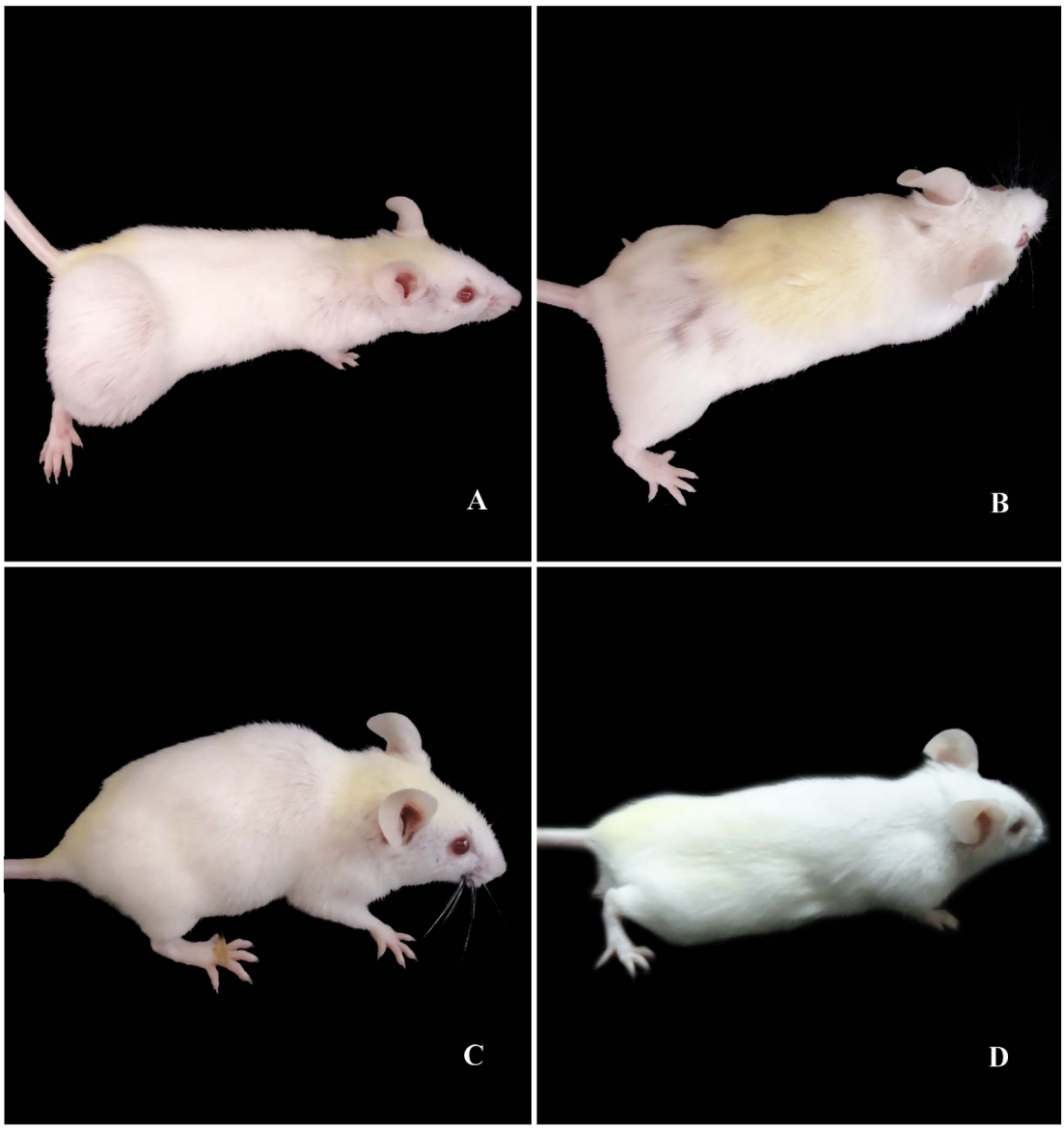


Fig. 29 Antitumor effect of methanolic extract of *Isodon nigrescens* on DLA induced solid tumor in mice. A Control, B Standard, C *I. nigrescens* (25 mg/kg b. wt), D *I. nigrescens* (50 mg/kg b. wt)

Fig. 32. *In vivo* antitumor effect of methanolic extract of *I. nigrescens* on DLA induced solid tumor in mice

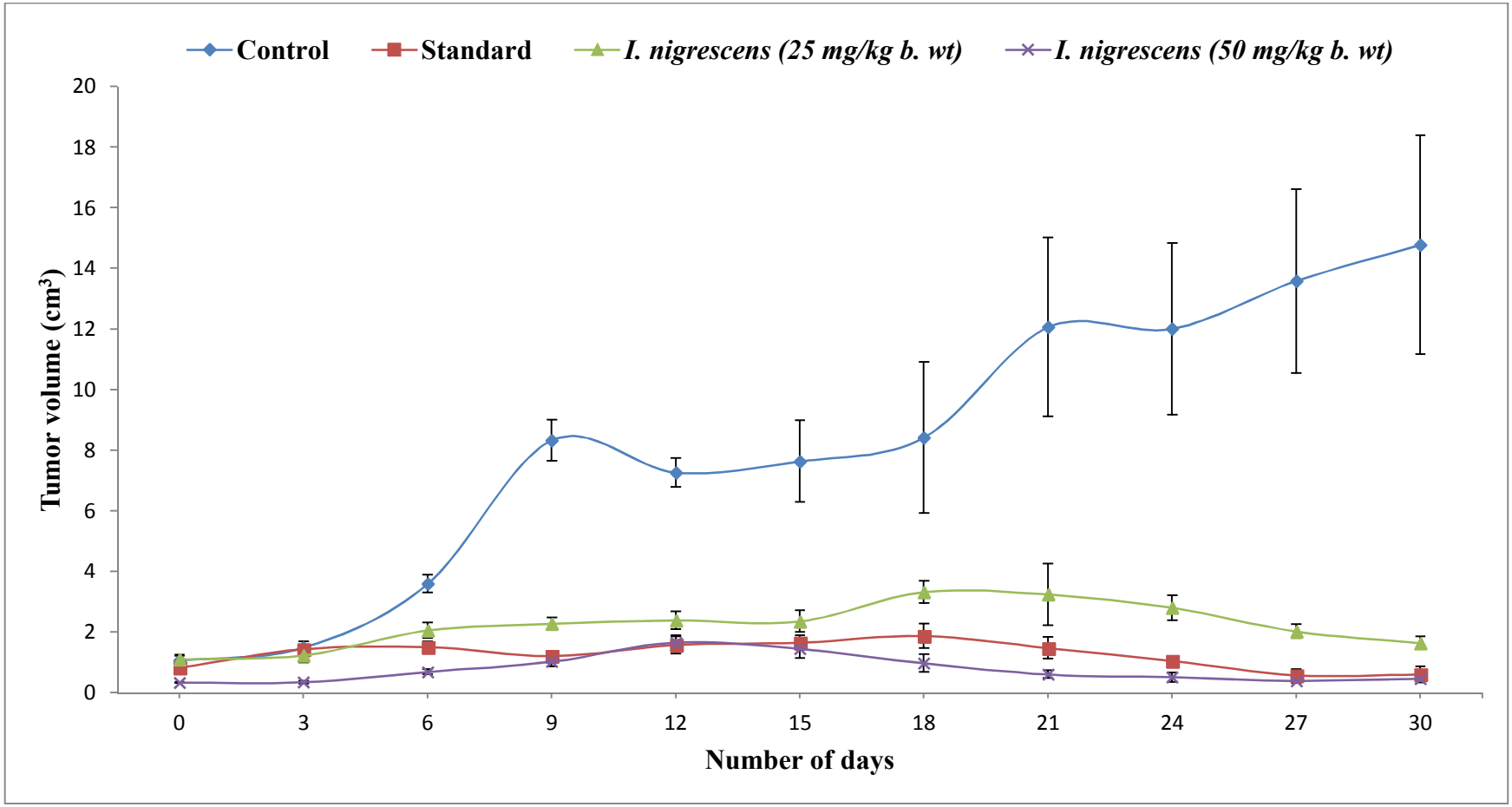


Table 39. Antitumor activity of methanolic extract of *I. nilgherricus* on DLA induced solid tumor in mice

Treatment groups	Tumor volume (cm^3) ± SE											% Inhibition ± SE
	0^{th} day	3^{rd} day	6^{th} day	9^{th} day	12^{th} day	15^{th} day	18^{th} day	21^{st} day	24^{th} day	27^{th} day	30^{th} day	
Control (Tumor alone)	1.09 ± 0.17	1.92 ± 0.24	3.59 ± 0.33	7.25 ± 0.65	6.95 ± 0.31	7.59 ± 1.45	6.71 ± 2.77	7.04 ± 2.14	9.87 ± 2.93	10.54 ± 3.32	12.72 ± 3.17	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	0.64 ± 0.05	1.62 ± 0.17	1.44 ± 0.23	1.15 ± 0.22	1.30 ± 0.28	1.07 ± 0.19	0.56 ± 0.41	0.61 ± 0.43	0.80 ± 0.13	0.24 ± 0.19	0.16 ± 0.29	98.69 ± 0.78
I. nilgherricus (25 mg/kg b. wt)	1.11 ± 0.11	1.74 ± 0.21	3.15 ± 0.67	3.64 ± 0.09	3.89 ± 0.23	4.76 ± 0.94	3.94 ± 0.56	4.95 ± 0.39	3.93 ± 0.49	3.47 ± 0.40	3.31 ± 0.30	73.94 ± 2.36
I. nilgherricus (50 mg/kg b. wt)	0.35 ± 0.03	0.37 ± 0.09	1.13 ± 0.24	1.24 ± 0.15	2.26 ± 0.66	1.40 ± 0.41	1.06 ± 0.51	1.20 ± 0.68	1.08 ± 0.55	0.73 ± 0.32	0.48 ± 0.14	96.19 ± 1.11

Data represent the mean ± SE (Standard error)

PLATE 13

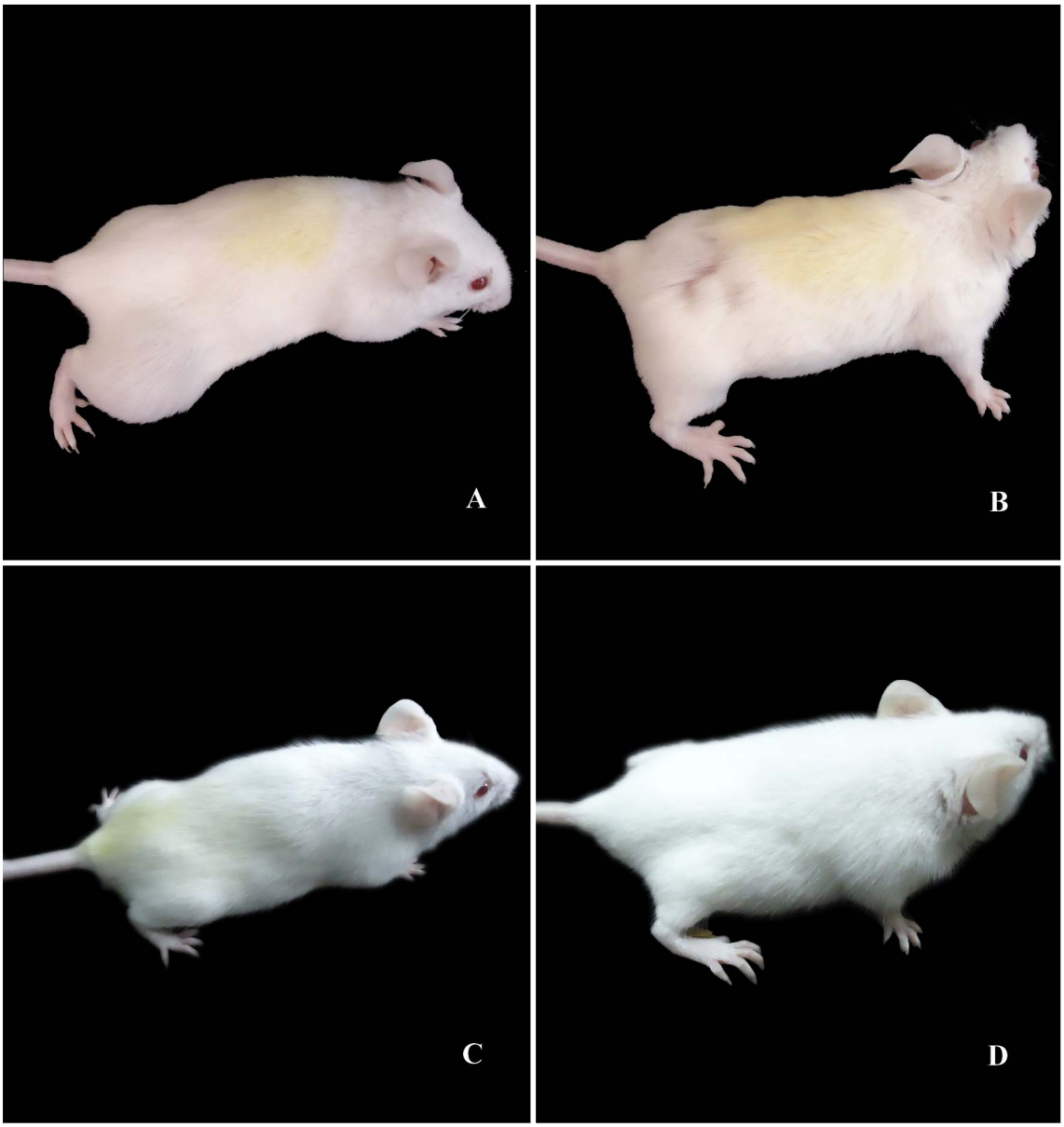


Fig. 30 Antitumor effect of methanolic extract of *Isodon nilgherricus* on DLA induced solid tumor in mice. A Control, B Standard, C *I. nilgherricus* (25 mg/kg b. wt), D *I. nilgherricus* (50 mg/kg b. wt)

Fig. 33. *In vivo* antitumor effect of methanolic extract of *I. nilgherricus* on DLA induced solid tumor in mice

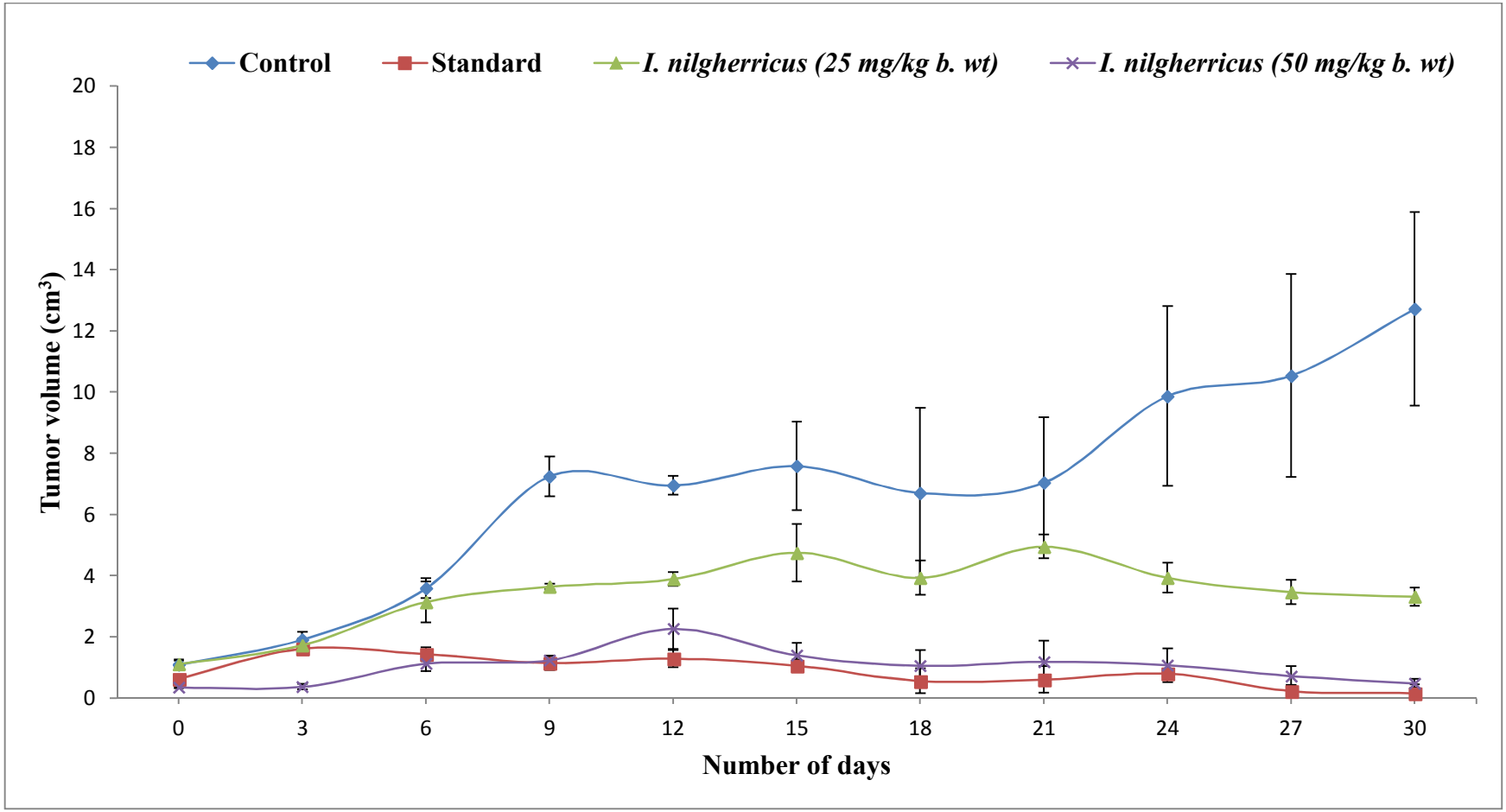


Table 40. Effect of methanolic extract of *I. coetsa* on the haematological parameters of Swiss albino mice with solid tumor

Treatment groups	Haemoglobin (g/dl) ± SE	WBC (10^3 cells/mm^3) ± SE
Normal	13.39 ± 0.28	8.70 ± 0.06
Control (Tumor alone)	7.40 ± 0.17	14.18 ± 0.07
Standard (Cyclophosphamide: 25 mg/kg b. wt)	8.98 ± 0.08*	8.85 ± 0.13**
I. coetsa (25 mg/kg b. wt)	10.95 ± 0.11**	9.97 ± 0.12*
I. coetsa (50 mg/kg b. wt)	11.95 ± 0.15**	9.03 ± 0.18**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Table 41. Effect of methanolic extract of *I. nigrescens* on the haematological parameters of Swiss albino mice with solid tumor

Treatment groups	Haemoglobin (g/dl) ± SE	WBC (10^3 cells/mm^3) ± SE
Normal	13.39 ± 0.12	8.78 ± 0.18
Control (Tumor alone)	7.61 ± 0.13	14.12 ± 0.19
Standard (Cyclophosphamide: 25 mg/kg b. wt)	8.97 ± 0.13*	8.98 ± 0.09**
I. nigrescens (25 mg/kg b. wt)	10.13 ± 0.10**	11.33 ± 0.12
I. nigrescens (50 mg/kg b. wt)	12.03 ± 0.06**	8.70 ± 0.26**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Table 42. Effect of methanolic extract of *I. nilgherricus* on the haematological parameters of Swiss albino mice with solid tumor

Treatment groups	Haemoglobin (g/dl) ± SE	WBC (10^3 cells/mm^3) ± SE
Normal	13.28 ± 0.13	8.67 ± 0.16
Control (Tumor alone)	7.67 ± 0.08	14.27 ± 0.09
Standard (Cyclophosphamide: 25 mg/kg b. wt)	8.91 ± 0.06*	8.82 ± 0.11**
I. nilgherricus (25 mg/kg b. wt)	9.61 ± 0.05*	13.43 ± 0.13
I. nilgherricus (50 mg/kg b. wt)	11.84 ± 0.09**	9.13 ± 0.13**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

EAC induced ascites tumor model

Ascites tumor formation in Swiss albino mice was induced by the injection of the EAC cell lines intraperitoneally to the animals. The extracts and standard drug was administered after 24 hours of tumor implantation. The methanolic extracts were provided orally for ten consecutive days while the standard drug cyclophosphamide was injected intraperitoneally. The development of tumor was monitored regularly and the death pattern of the animals due to tumor burden was observed. The differences in the body weight of the animals were noted at three days interval and the percentage increase in life span was calculated to identify the effect of the extract.

Administration of the methanolic extract of *I. coetsa* showed significant increase in the number of survival days in dose dependent manner (Plate 14; Table 43). The percentage increase in life span was found to be 17.94 ± 3.72 for 25 mg/kg b. wt while it was 53.41 ± 7.55 for 50 mg/kg b. wt (Table 43). The body weight of the animals also showed significant change with percentage increase of 70.86 ± 10.31 and 89.62 ± 5.26 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively.

Administration of the methanolic extract of *I. nigrescens* showed significant increase in the number of survival days with increase in concentration (Plate 15; Table 44). The percentage increase in life span was found to be 33.92 ± 1.55 for 25 mg/kg b. wt while it was 85.87 ± 4.55 for 50 mg/kg b. wt (Table 44). The body weight of the animals also showed significant change with percentage increase of 68.25 ± 17.29 and 121.65 ± 15.31 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively.

Administration of the methanolic extract of *I. nilgherricus* also showed significant increase in the number of survival days with increase in concentration (Plate 16; Table 45). The percentage increase in life span was

found to be 50.10 ± 6.66 for 25 mg/kg b. wt while it was 75.05 ± 6.23 for 50 mg/kg b. wt (Table 45). The body weight of the animals also showed significant change with percentage increase of 107.22 ± 8.09 and 108.31 ± 9.62 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively.

The standard drug cyclophosphamide when administered showed excellent increase in number of survival days in mice harbouring ascites tumor. The percentage increase in body weight was comparatively less for mice group provided with standard drug. The extract administered mice groups had significant increase in body weight but the survival days were comparable only in high dose which showed the endurance of the mice to the tumor due to the effect of the extract. The antitumor effect was moderate with high doses of the extracts but in low dose, only *I. nilgherricus* extract showed significant activity. In comparison, the highest antitumor activity against EAC induced ascites tumor was shown by *I. nilgherricus* followed by *I. nigrescens* and *I. coetsa* (Fig. 37).

Antitumor studies of the methanolic extracts of three species of *Isodon* against DLA and EAC induced tumors revealed that the extracts were more effective against solid tumor rather than ascites model. The extracts showed highly significant activity against solid tumor which were comparable and at par with standard drug cyclophosphamide. The extract treated mice also showed less visible side effects. But the ascites tumor harbouring mice had varying effects of increase in life span and was not at par with standard drug cyclophosphamide. The extracts were shown to increase the toleration capacity of the tumor cells in the body of mice as evident from the prolonged survival days with increase in body weight. All the values were statistically analysed and the significance levels reported. The effect of the methanolic extracts within itself were not comparable as each responds in different manner against different assays. Thus, each extract with its inherent activity can be exploited as a source of potent anticancer drug in pharmaceutical research.

Table 43. Effect of methanolic extract of *I. coetsa* on EAC induced ascites tumor in mice

Treatment groups	% Increase in body weight ± SE	Survival time ± SE (days)	% Increase in lifespan ± SE
Control (Tumor alone)	88.56 ± 3.13	18.67 ± 0.33	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	40.86 ± 4.17	36.67 ± 0.67	96.49 ± 3.51***
I. coetsa (25 mg/kg b. wt)	70.86 ± 10.31	22.00 ± 0.58	17.94 ± 3.72
I. coetsa (50 mg/kg b. wt)	89.62 ± 5.26	28.67 ± 1.76	53.41 ± 7.55*

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Table 44. Effect of methanolic extract of *I. nigrescens* on EAC induced ascites tumor in mice

Treatment groups	% Increase in body weight ± SE	Survival time ± SE (days)	% Increase in lifespan ± SE
Control (Tumor alone)	81.60 ± 2.95	19.33 ± 0.33	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	49.60 ± 1.83	37.00 ± 0.58	91.40 ± 1.67***
I. nigrescens (25 mg/kg b. wt)	68.25 ± 17.29	25.00 ± 0.58	33.92 ± 1.55
I. nigrescens (50 mg/kg b. wt)	121.65 ± 15.31	34.67 ± 0.33	85.87 ± 4.55**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

PLATE 14

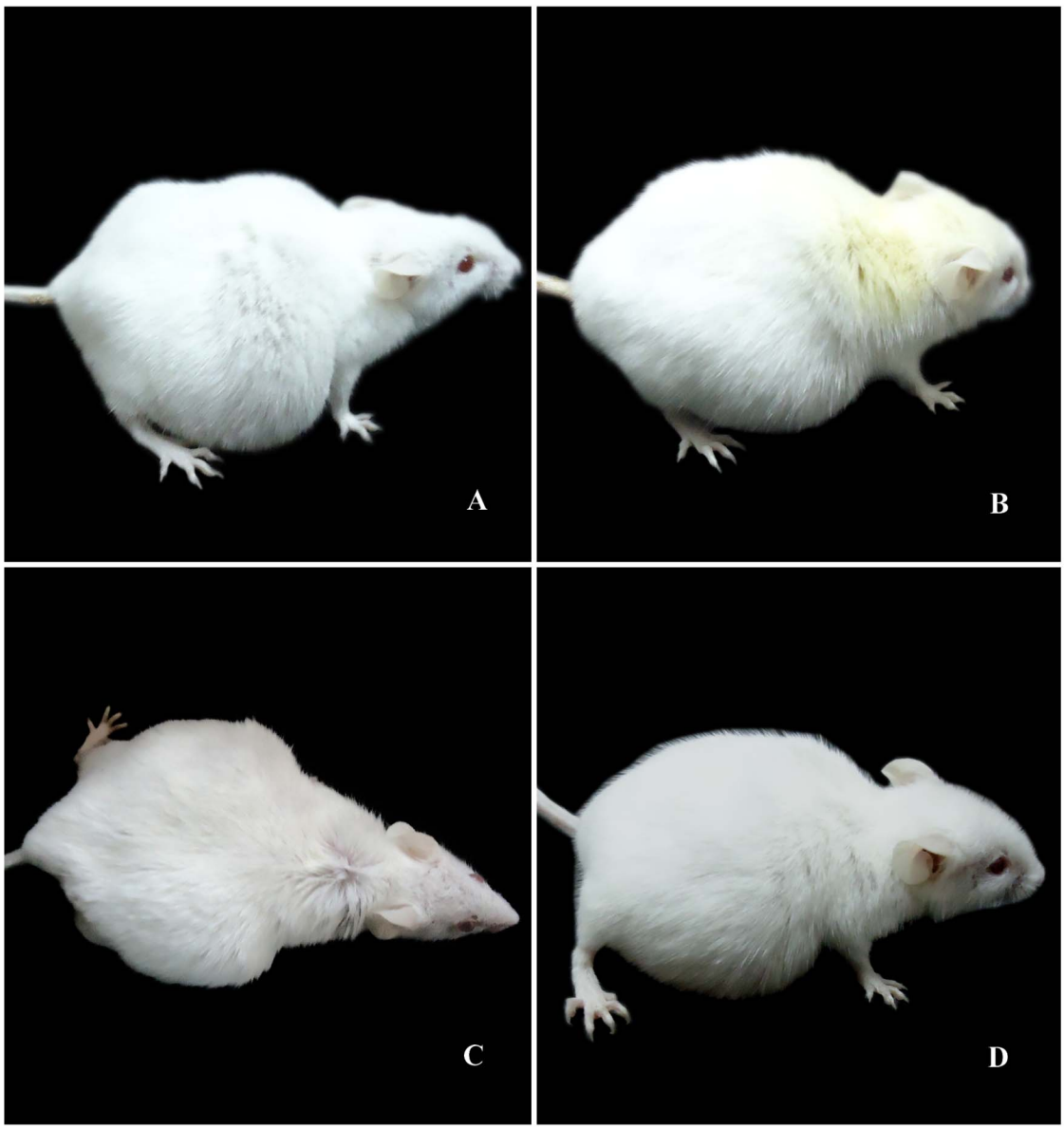


Fig. 34 Antitumor effect of methanolic extract of *Isodon coetsa* on EAC induced solid tumor in mice. A Control, B Standard, C *I. coetsa* (25 mg/kg b. wt), D *I. coetsa* (50 mg/kg b. wt)

PLATE 15

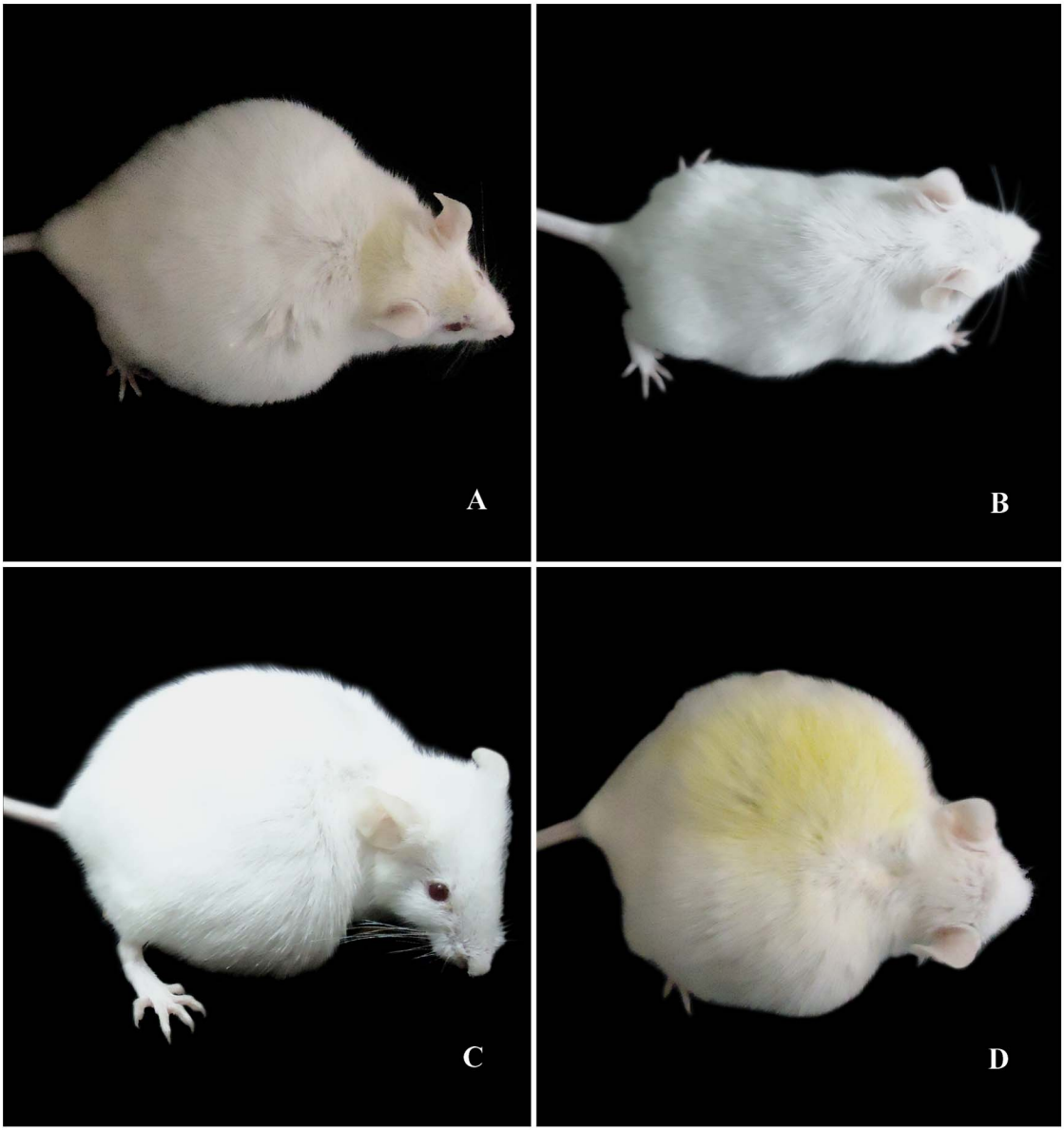


Fig. 35 Antitumor effect of methanolic extract of *Isodon nigrescens* on EAC induced solid tumor in mice. A Control, B Standard, C *I. nigrescens* (25 mg/kg b. wt), D *I. nigrescens* (50 mg/kg b. wt)

PLATE 16

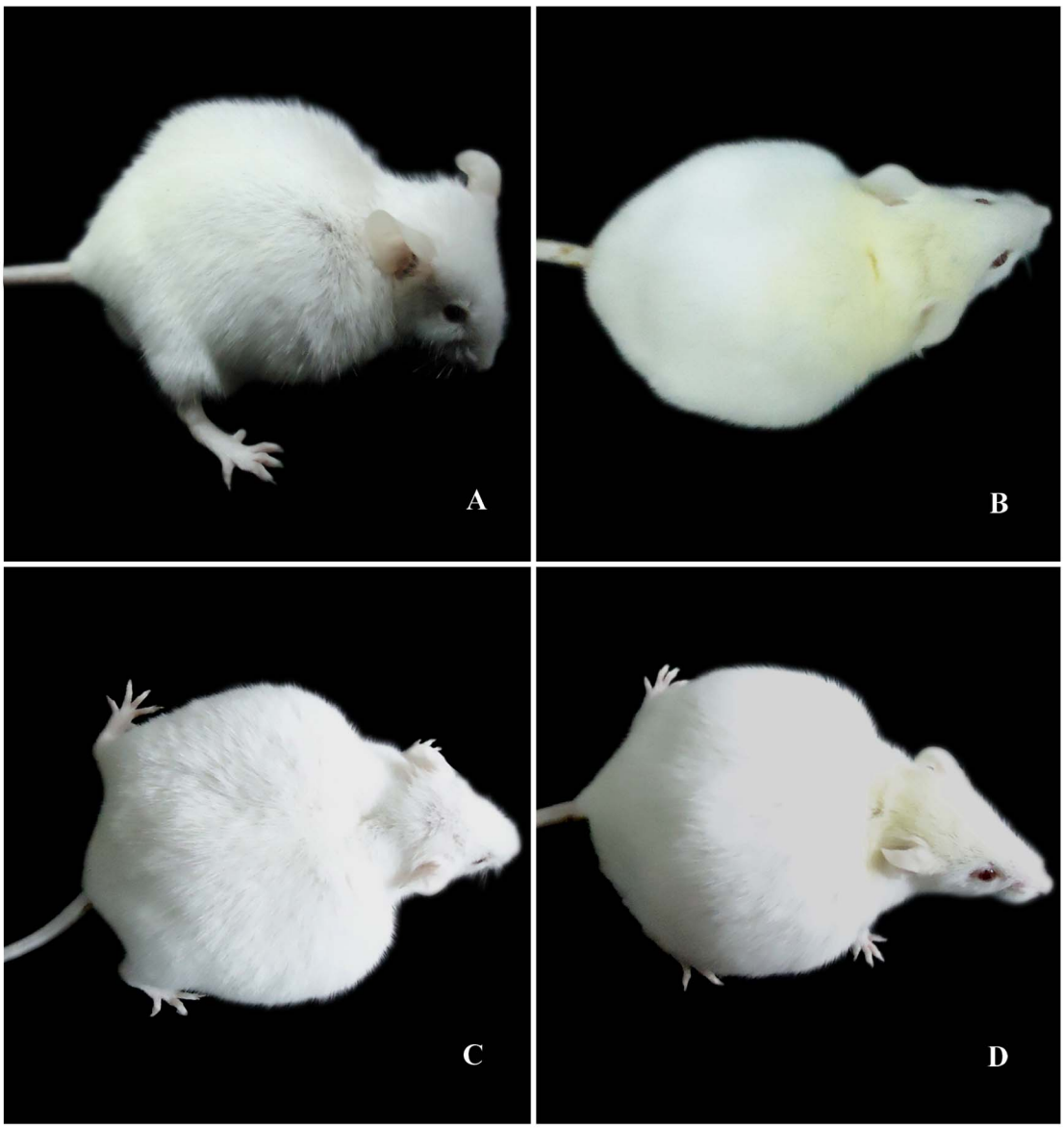


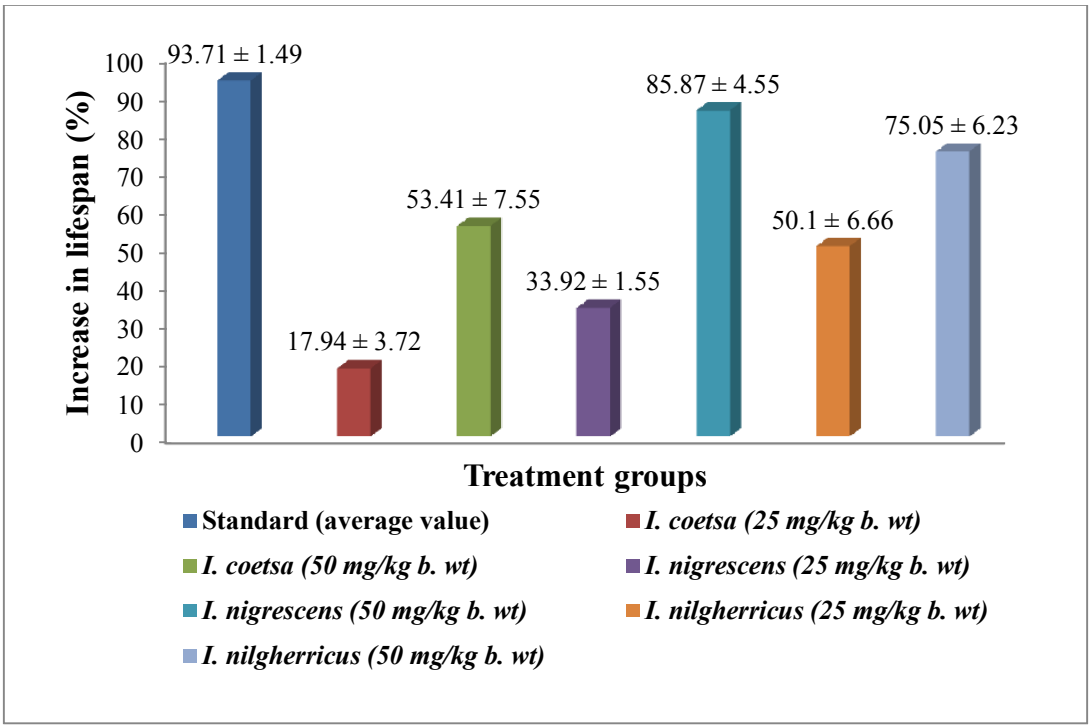
Fig. 36 Antitumor effect of methanolic extract of *Isodon nilgherricus* on EAC induced solid tumor in mice. A Control, B Standard, C *I. nilgherricus* (25 mg/kg b. wt), D *I. nilgherricus* (50 mg/kg b. wt)

Table 45. Effect of methanolic extract of *I. nilgherricus* on EAC induced ascites tumor in mice

Treatment groups	% Increase in body weight ± SE	Survival time ± SE (days)	% Increase in lifespan ± SE
Control (Tumor alone)	83.74 ± 2.64	19.00 ± 0.58	-
Standard (Cyclophosphamide: 25 mg/kg b. wt)	62.33 ± 2.26	36.67 ± 0.33	93.25 ± 4.39***
I. nilgherricus (25 mg/kg b. wt)	107.22 ± 8.09	28.00 ± 1.15	50.10 ± 6.66*
I. nilgherricus (50 mg/kg b. wt)	108.31 ± 9.62	32.67 ± 1.20	75.05 ± 6.23**

Data represent the mean ± SE; *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (One way ANOVA, Dunnett's test)

Fig. 37. *In vivo* antitumor effect of methanolic extracts of three species of *Isodon* on EAC induced ascites tumor in mice



PHYTOCHEMICAL STUDIES

Preliminary phytochemical screening

Preliminary phytochemical screening of methanolic extracts of three species of *Isodon* was conducted to determine the detectable constituents which could have resulted in the bioactivities of the plants. Various assays for the identification of alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins, tannins, steroids and terpenoids were performed. The three methanolic extracts exhibited the presence of detectable amount of flavonoids, phenols, tannins, steroids and terpenoids (Table 46) which could be the major components present in these plants.

Table 46. Preliminary phytochemical screening of methanolic extracts of three species of *Isodon*

Secondary metabolites	Chemical tests	*I. coetsa*	*I. nigrescens*	*I. nilgherricus*
Alkaloids	Dragendroff's test	–	–	–
	Mayer's test	–	–	–
Anthraquinones	Borntrager's test	–	–	–
Flavonoids	Alkaline reagent test	+	+	+
Glycosides	Keller Killiani test	–	–	–
Phenols	Phenol test	+	+	+
Saponins	Froth test	–	–	–
Tannins	Ferric chloride test	+	+	+
Steroids	Liebermann-Burchard test	+	+	+
Terpenoids	Salkowski test	+	+	+

Determination of phenolic, flavonoid and terpenoid contents

The methanolic extracts of three species of *Isodon* were tested for the estimation of the total phenolic, flavonoid and terpenoid content. The phenolic content in terms of gallic acid equivalent, flavonoid content in terms of quercetin equivalent and terpenoid content in terms of linalool equivalent were determined (Table 47) to identify the abundance of these secondary metabolites.

The total phenolic content was determined using the Folin–Ciocalteu reagent based assay. The phenolic content was measured and expressed in terms of gallic acid equivalent employing the standard equation obtained from the linear calibration curve ($y = 0.011x + 0.016$, $R^2 = 0.999$; Fig. 38). The total phenolic content was estimated to be 61.58 ± 0.03 mg GAE/g DW for *I. coetsa*, 33.82 ± 0.05 mg GAE/g DW for *I. nigrescens* and 66.88 ± 0.06 mg GAE/g DW for *I. nilgherricus*. The results revealed that the highest amount of total phenolic content was observed in methanolic extracts of *I. nilgherricus* followed by *I. coetsa* and *I. nigrescens*.

The total flavonoid content was determined using the aluminium chloride colorimetric method. The flavonoid content was measured and expressed in terms of quercetin equivalent employing the standard equation obtained from the linear calibration curve ($y = 0.001x – 0.007$, $R^2 = 0.998$; Fig. 39). The total flavonoid content was estimated to be 144.00 ± 0.58 mg QE/g DW for *I. coetsa*, 152.67 ± 0.88 mg QE/g DW for *I. nigrescens* and 151.67 ± 0.67 mg QE/g DW for *I. nilgherricus*. The results revealed that the methanolic extracts of *I. nigrescens* and *I. nilgherricus* have comparatively equivalent amount of flavonoids while *I. coetsa* possess the lowest quantity.

The total terpenoid content was determined using an assay in which the terpenoid content was measured and expressed in terms of linalool equivalent

employing the standard equation obtained from the linear calibration curve ($y = 0.005x - 0.006$, $R^2 = 0.987$; Fig. 40). The total terpenoid content was estimated to be 221.87 ± 0.67 mg linalool/g DW for *I. coetsa*, 485.20 ± 1.15 mg linalool/g DW for *I. nigrescens* and 269.87 ± 0.67 mg linalool/g DW for *I. nilgherricus*. The results revealed that the highest amount of total terpenoid content was observed in methanolic extracts of *I. nigrescens* followed by *I. nilgherricus* and *I. coetsa*. But the total terpenoid content as determined by the alternate method described by Ferguson (1956) revealed that the highest amount was observed in *I. nilgherricus* with 17% followed by *I. nigrescens* with 7.6% and *I. coetsa* with 3.6%.

The methanolic extracts of the three species of *Isodon* possessed significant phenolic, flavonoid and terpenoid content which could be attributed to the excellent antitumor and antioxidant activity of the extracts. All the values were statistically analyzed and found significant at $p < 0.05$. In general, *I. nilgherricus* can be considered to be a secondary metabolite enriched plant.

Table 47. Total phenolic, flavonoid and terpenoid content in the methanolic extracts of three species of *Isodon*

Extract	Phenolic content (mg GAE/g DW) ± SE	Flavonoid content (mg QE/g DW) ± SE	Terpenoid content (mg linalool/g DW) ± SE
I. coetsa	61.58 ± 0.03^{b}	144.00 ± 0.58^{a}	221.87 ± 0.67^{a}
I. nigrescens	33.82 ± 0.05^{a}	152.67 ± 0.88^{b}	485.20 ± 1.15^{c}
I. nilgherricus	66.88 ± 0.06^{c}	151.67 ± 0.67^{b}	269.87 ± 0.67^{b}

GAE - Gallic acid equivalents; QE - Quercetin equivalents
Values are expressed as mean ± standard error (SE)
Means in a column followed by the same superscript letters are not significantly different ($P < 0.05$, one-way ANOVA, Tukey-Kramer HSD test)

Fig. 38 Standard calibration curve for the determination of total phenolic content

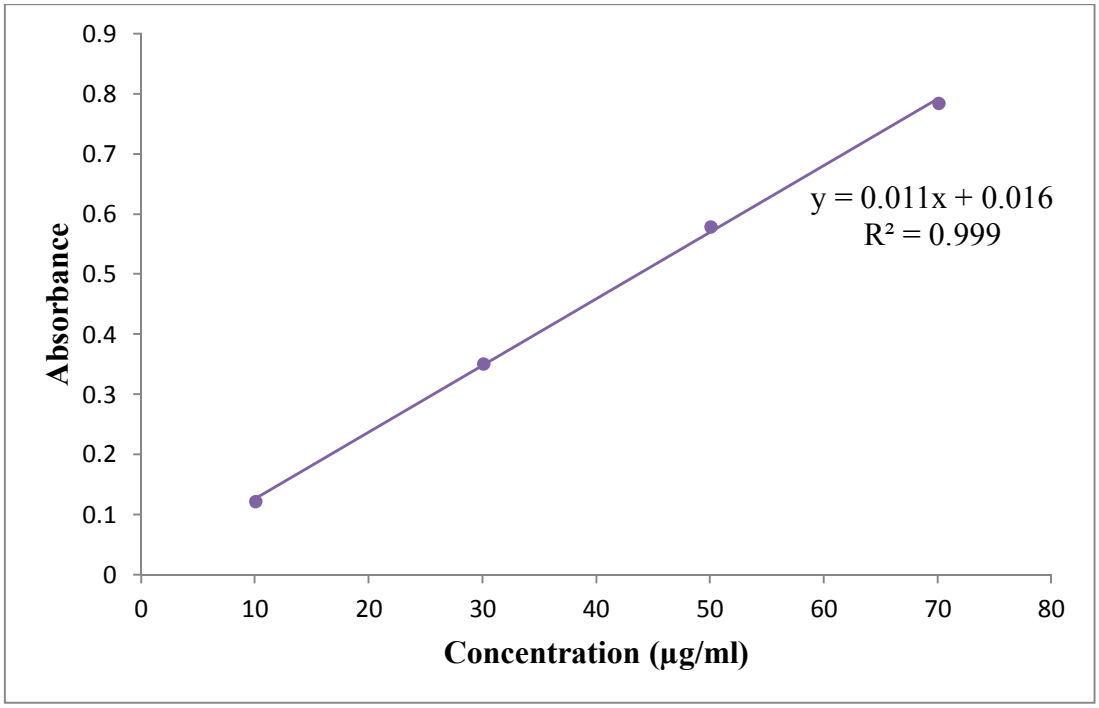


Fig. 39 Standard calibration curve for the determination of total flavonoid content

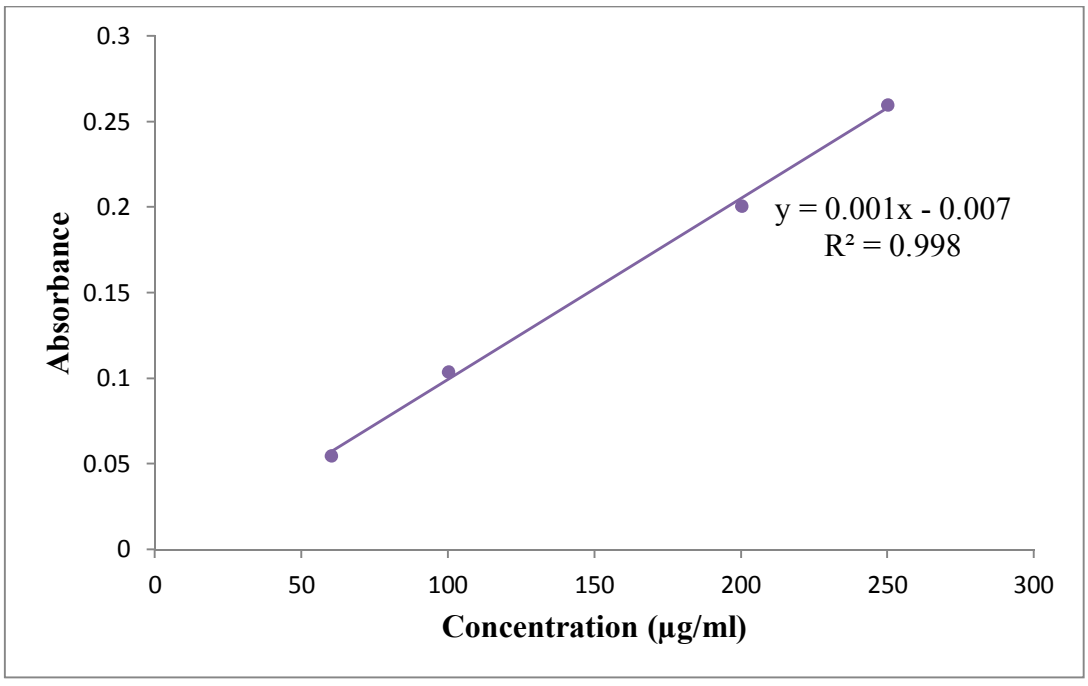
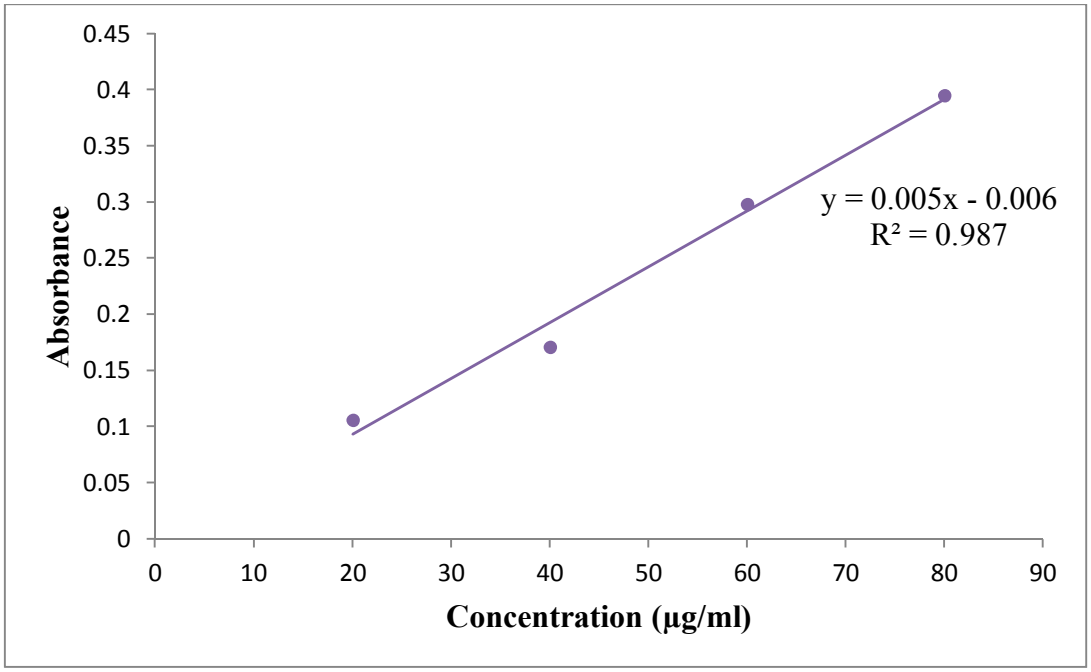


Fig. 40 Standard calibration curve for the determination of total terpenoid content



Gas chromatography-mass spectrometry (GC-MS) analysis

The phytochemical profile of the methanolic extracts of three species of *Isodon* was determined using GC-MS analysis. A wide spectrum of compounds were determined which are consolidated in Table 48. The gas chromatogram obtained for the three species are given in Figs 41-43 and the mass spectrums in Fig. 44. A total of 136 compounds were detected from the three methanolic extracts which were grouped into 14 classes of compounds *viz*., terpenoids, phenols, alkaloids, steroids, flavonoids, fatty acid esters, vitamins, furans, aromatics, alkanes, aldehydes and ketones, alcohols, organic compounds and other miscellaneous compounds (Table 49).

A total of fifty six compounds were identified from the methanolic extract of *I. coetsa*. The major components detected includes 5-methylsalicylaldehyde (13.54%), 1,2,3,4,8,9,10,11-octahydropyrido[2,1-f][1,6]naphthyridin-6-one (9.89%), α-tocopherol (9.28%), phytol (7.64%),

2-naphthyl myristate (6.49%) and 3,5,7-triamino-1-azaadamantane (6.27%). The methanolic extract of *I. nigrescens* revealed the presence of fifty nine components of which the major components detected were phytol (20.87%), α-tocopherol (15.72%), p-vinylguaiacol (9.20%) and sugiol (6.14%). Fifty one compounds were obtained from the methanolic extract of *I. nilgherricus* of which the major components were euphracal (13.20%), D5-dehydro-7-oxo-totarol (12.97%), abieta-6,8,11,13-tetraen-12-yl acetate (10.04%), 1,2-bis(dicyclohexylphosphino)ethane (8.75%), p-vinylguaiacol (7.41%) and α-tocopherol (7.32%).

The composition analysis of the methanolic extracts revealed the predominance of terpenoid class in *I. nigrescens* and *I. nilgherricus* (Table 49). *I. coetsa* depicted a complex mixture of terpenoids, alkaloids, vitamins and several other classes of compounds. The common compounds observed in the three methanolic extracts included 2,2-bipiperidine, dihydroactinidiolide, 3-(1-methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one, 2-methyl-7-octadecyne, phytol, α-tocopherol and γ-sitosterol of which α-tocopherol and phytol were abundantly found (Table 48).

Table 48. Phytochemical constituents detected in the methanolic extracts of three species of *Isodon* identified through GC-MS analysis

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	***I. nigrescens***	***I. nilgherricus***
1	1.972	N,N-Dimethylhydroxylamine	-	0.7748	-
2	2.132	Methoxy phenyl oxime	-	-	0.2043
3	3.142	5-Methyl-4-hexen-3-one	-	0.5077	-
4	3.188	2-Hexen-4-olide	0.5961	-	-
5	3.684	2,3,4-Trichloroacetophenone	-	0.1903	-
6	3.806	Benzeneacetaldehyde	0.7949	-	-
7	3.810	Spiro[2.4]hepta-4,6-diene	-	0.5131	0.1812
8	4.333	3-Acetylthymine	0.3186	0.2505	-
9	4.722	6-Azabicyclo[3.2.1]octane	2.6700	-	-
10	4.849	2-Amino-1-[3-(benzyloxy)-2-fluorophenyl]ethanol	0.2309	-	-
11	4.851	Phenethyl alcohol	-	0.2591	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
12	5.060	Nerolin	0.2213	-	-
13	5.133	2-Methyl-2-thiazolidine carboxylic acid	-	0.2089	-
14	5.142	Bamiphylline	0.4653	-	-
15	5.384	2-Vinylbenzofuran	-	2.0542	-
16	5.396	2-Naphthyl myristate	6.4862	-	-
17	5.940	2,2-Bipiperidine	2.1918	1.7156	0.6138
18	6.106	(2S,4S)-1-(tert-Butoxycarbonyl)-4-methylpyrrolidine-2-carboxylic acid	-	0.1821	-
19	6.118	3,5-Dihydroxy-2-methyl-4H-pyran-4-one	0.2266	-	-
20	6.248	1-tert-Butyl-3-(1-methylcyclohexyl)aziridin-2-one	-	0.0846	-
21	6.969	1-(2,4-Dichlorobenzoyl) piperazine	1.2220	-	-
22	7.003	5-Hydroxymethylfurfural	-	0.3782	-
23	7.043	β-Cyclocitral	-	-	0.0694
24	7.389	1-(2-Methyl-1,3-oxathiolan-2-yl) ethanone	0.8171	-	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
25	8.818	Indolizine	0.3840	-	-
26	9.204	1-(2-Hydroxy-5-methylphenyl)-ethanone	2.2759	-	-
27	9.215	p-Vinylguaiacol	-	9.1982	7.4135
28	9.650	2-Methyl-4-hydroxyacetophenone	3.4767	-	-
29	10.421	Dehydro-ar-ionene	-	0.1314	-
30	11.151	2,5-Octadecadiynoic acid, methyl ester	0.1735	-	-
31	11.497	1-Octyl-2-[7-hydroxy-7-(4,4-dimethyl-2-oxazolin-2-yl)heptyl]-cyclopropene	-	0.2451	-
32	12.192	4,8,8-Trimethyl-4-vinyl-bicyclo[5.2.0]nonane	-	0.2177	-
33	12.196	β-Caryophellene	0.5915	-	-
34	12.579	5-Methylsalicylaldehyde	13.5359	-	2.3580
35	13.143	Elixene	0.3619	-	-
36	13.785	N-Acetyl-veratramine	0.3436	-	-
37	13.876	α-Cubebene	0.2749	-	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
38	14.315	4,11-Selinadiene	0.2435	-	-
39	15.370	Dihydroactinidiolide	0.2834	0.3866	0.1669
40	17.767	11-Hydroxy-11-methyltricyclo[4.3.1.1^{2,5}]undecan-10-one	0.1870	-	-
41	19.033	8-Isopropenyl-1,3,3,7-tetramethyl-bicyclo[5.1.0] oct-5-en-2-one	-	-	0.1421
42	19.034	3,3,5,6,8,8-Hexamethyl- syn-tricyclo[5.1.0.0(2,4)] oct-5-ene	0.4179	-	-
43	19.352	2-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-,(E)- 3-buten-2-ol	0.4116	0.3524	-
44	19.742	3-Oxo-α-ionol	0.5339	-	0.3543
45	19.743	(6R,7E,9R)-9-Hydroxy-4,7-megastigmadien-3-one	-	0.4994	-
46	20.821	3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one	1.7270	0.8198	0.3283
47	21.041	1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-1,3-butanedione	0.3510	-	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
48	21.554	4-[2-Isopropyl-5-methyl-5-(2-methyl-5-oxocyclopentyl) cyclopentenyl]-2-butanone	-	0.5632	0.1349
49	22.796	3,3,6,6-Tetramethylcyclohexa-1,4-diene	-	0.4258	-
50	23.300	(E)-Conipheryl alcohol	0.7628	-	-
51	23.302	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	-	-	0.2069
52	24.310	8-Isopropylidene-bicyclo[4.3.0]nonan-2-one	0.8594	1.6997	-
53	24.847	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	-	0.7837	-
54	24.851	Adipostatin A	-	-	0.3203
55	24.868	3,5,7-Triamino-1-azaadamantane	6.2697	-	-
56	26.019	1S,3R,4S,5R,6S-1-Hydroxy-2,2,3,4,5,6-hexamethyl-8-oxo-7,9-dioxatricyclo [4.2.1.0(3,5)]nonane	-	0.5092	-
57	26.214	2-Methyl-7-octadecyne	1.8509	1.2348	0.4558
58	26.372	Vincadifformine	-	0.5829	0.1658
59	26.755	Methyl indole-3-carboxylate	0.2811	-	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
60	26.876	Phytol	7.6434	20.8691	1.8625
61	26.935	Cinnamic acid, 4-hydroxy-3-methoxy-, methyl ester, acetate	2.4946	-	-
62	27.819	Isoferulic acid	1.4416	-	-
63	27.999	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol	-	-	0.1596
64	28.483	Methyl 13-methylpentadecanoate	0.4255	-	-
65	28.486	Hexadecanoic acid, methyl ester	-	0.3787	0.1255
66	28.489	10,18-Bisnorabieta-8,11,13-triene	-	-	0.1255
67	29.168	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	-	-	2.9264
68	29.378	Estra-1,3,5(10)-trien-17.beta.-ol	0.9572	0.4309	-
69	29.390	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	-	-	0.1401
70	31.099	2(3H)-Quinazolinone	-	0.6104	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
71	31.387	12α-Acetyloxy-5β-chol-3-en-24-oic acid methyl ester	-	-	0.2987
72	32.086	2-Linoleoyl glycerol	0.2150	0.2434	-
73	32.206	1-Heptatriacotanol	-	-	0.1794
74	32.212	Methyl (Z)-5,11,14,17-eicosatetraenoate	-	0.6646	-
75	32.420	1(22),7(16)-Diepoxy-tricyclo[20.8.0.0(7,16)] triacontane	-	-	1.3244
76	33.040	Docosahexaenoic acid 1,2,3-propanetriyl ester	-	0.5371	-
77	33.060	7,10,13-Hexadecatrienoic acid, methyl ester	0.8699	-	-
78	34.234	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	-	-	0.3902
79	34.891	14-Isopropyl-13-desisopropylabieta-8,11,13-triene-7α,13-diol	-	-	2.9596
80	35.010	4-(1,1-Dimethylallyl)-9-methoxy-7H-furo[3,2-g][1]benzopyran-7-one	-	-	0.4551
81	35.020	5,19-Cyclo-5β-androst-6-ene-3,17-dione	-	-	0.0218

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
82	36.359	2',3',4-Trimethoxy-[1,1'-biphenyl]-2-methanol	-	-	0.4368
83	35.383	Pimara-7,15-dien-3-one	-	-	0.2129
84	35.635	β-Carotene	1.1741	0.4637	-
85	35.987	Totara-8,11,13-triene-7-β,13-diol	-	-	0.4553
86	36.264	16-Nitrobicyclo[10.4.0]hexadecan-1-ol-13-one	-	0.1570	-
87	36.474	Totarol	-	-	3.2295
88	36.653	2-Methylthio-4,4,5-trimethyl-5-[(2-methylthio-4,4,-dimethyl-1-pyrrolinidenyl-5)methylene]-1-pyrroline	-	0.1727	-
89	36.863	4,4-Dimethyl-androsta-5,7-diene	-	-	0.6358
90	36.984	trans-4'-Hexyloxy-4-(methylthio) chalcone	-	-	2.4268
91	37.132	Dehydrosqualene	-	-	0.4132
92	37.250	3,6-Dihydro-7-isopropyl-6-[(7-isopropyl-1-methylazulen-4-yl)methyl]-1,4-dimethyl-2-azulenecarbaldehyde	-	-	2.5818
93	37.463	1,4-Dimethyl-8-isopropylidenetricyclo [5.3.0.0(4,10)]decane	-	-	0.9047

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
94	37.526	(3-Isopropyl-5-methylamino-2,4,6-cycloheptatrienylidene)malononitrile	0.2329	-	-
95	37.721	11β-Hydroxy-4-androsten-3,17-dione	0.1453	1.3351	-
96	37.730	1b,6b-Epoxyhexahydrocannabinol acetate	-	0.0951	-
97	38.208	D5-dehydro-7-oxo-totarol	-	-	12.9672
98	38.394	Abieta-6,8,11,13-tetraen-12-yl acetate	-	-	10.0365
99	38.725	Sugiol	-	6.1420	-
100	38.873	Alloisoimperatorin	-	0.5053	-
101	38.955	17-(Methoxyimino)-2-nitroestra-1(10),2,4-trien-3-yl acetate	-	4.0252	-
102	39.426	Glycerol 1,3 dipalmitate	-	0.0708	-
103	39.433	1,2,3,5,6,7-Hexahydro-1,1,5,5-tetramethyl-4,8-bis(3-methylbutyl)-s-indacene	-	-	0.7343
104	39.589	1-(9-Borabicyclo[3.3.1]nonan-9-yl)-3,5-ditert-butylpyrazole	-	0.5752	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
105	39.603	1,1,4,7,7,8-Hexamethyl-1,2,3,5,6,7-hexahydro-s-indacene	-	-	1.6176
106	39.821	14-Isopropylpodocarpa-8,11,13-triene-3α,13-diol	-	1.2746	-
107	40.257	N-Acetylstepharine	-	1.8407	-
108	40.339	Euphracal	-	-	13.1973
109	40.446	1,5,9-Trimethyl-12-(1-methylethyl)-4,8,13-cyclotetradecatriene-1,3-diol	0.8830	-	-
110	40.530	4-Isopropyl-1-phenyl-5,6,7,8,9,10-hexahydrocycloocta[c]pyran-3-one	-	1.8358	-
111	41.531	16-Hydroxymethyleneandrost-5-en-3-ol-17-one	-	-	0.7006
112	41.635	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-en-1-ol	0.6221	-	-
113	41.672	4a(2H)-Phenanthrenecarboxylic acid, 1,3,4,9,10,10a-hexahydro-9-hydroxy-6-methoxy-1,1-dimethyl-7-(1-methylethyl)-, ethyl ester	-	0.7042	-
114	41.718	(1aR)-5β,6β-Bis(benzoyloxy)-1aα,2,5,5a,6,9,10,10aα-octahydro-5aβ-hydroxy-4-(hydroxymethyl)-1,1,7,9α-tetramethyl-1H-2α,8aα-methanocyclopenta[a]cyclopropa[e]cyclodecen-11-one	2.4968	-	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
115	42.256	2-[4-Methyl-6-(2,6,6-trimethylcyclohex-1-enyl) hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	0.4114	-	-
116	42.441	1,2-Bis(dicyclohexylphosphino)ethane	-	-	8.7466
117	43.048	1,2,3,4,8,9,10,11-Octahydropyrido[2,1-f][1,6] naphthyridin-6-one	9.8929	-	-
118	43.192	4,4-Dimethyl-3.beta.-methoxy- (10.beta.,13.beta.)-19,3-epoxyandrosta-5,7-dien-17-one	-	0.2534	-
119	43.286	7-(3-Methoxy-2-oxa-1-oxocyclopent-5-yl)-10-phenyl-8,9-benzodispiro[2.0.2.4]decane	-	-	3.4043
120	43.603	Powelline	-	-	1.1157
121	43.861	8(14)-Dehydro-hexahydroergosterol	1.3447	-	-
122	44.380	2,2'-(Octahydro-2,3-quinoxalinediylidene) bis[1-phenyl-] ethanone	-	1.6934	-
123	44.381	8,8a-Bis(acetyloxy)-2a-[(acetyloxy)methyl]-1,1a,1b,1c,2a,3,3a,6a,6b,7,8,8a-dodecahydro-3,3a,6b-trihydroxy-1,1,5,7-tetramethyl-4H-cyclopropa[5',6']benz[1',2':7,8]azuleno[5,6-b]oxiren-4-one	-	-	0.0534
124	44.548	Anthiaergosta-5,7,9-trien-3-one	-	0.6565	-
125	44.703	2-Amino-4-phenyl-5-p-aminophenylthiazole	-	1.5968	-
126	45.178	Astaxanthin	2.0646	-	-

Sl. No.	Retention time	Constituents	Peak area (%)		
			I. coetsa	*I. nigrescens*	*I. nilgherricus*
127	46.187	17-19-Diacetoxy-4,4-dimethyl-(5.beta.,13.beta.) androst-8-en-3-one	-	2.5692	-
128	48.743	Cycloeucalenol acetate	-	1.9453	-
129	49.275	3β-Acetoxyurs-12-en-28-al	-	3.5798	-
130	50.730	α -Tocopherol	9.2833	15.7170	7.3244
131	53.748	(1aR)-2α,4aβ,9β,9aα-Tetrakis(acetyloxy)-3β-[(acetyloxy)methyl]-1,1aα,1bβ,2,3,4,4a,7aα,7b,8,9,9a-dodecahydro-2β,7bα-dihydroxy-1,1,6,8α-tetra methyl-5H-cyclopropa[3,4]benz[1,2-e]azulen-5-one	-	0.3923	-
132	54.653	4,4-Dimethyl-cholesta-22,24-dien-5-ol	0.3195	-	0.1523
133	54.978	6S-2,3,8,8-Tetramethyltricyclo[5.2.2.0(1,6)]undec-2-ene	0.0491	-	-
134	55.529	9,11-Dimethyltetracyclo[7.3.1.0(2.7).1(7.11)] tetradecane	2.7223	-	2.8337
135	57.044	γ-Sitosterol	2.4729	3.3111	1.7350
136	58.562	Urs-12-en-24-oic acid, 3-oxo-, methyl ester	-	0.5846	-

Table 49. Percentage composition of various class of compounds detected in the methanolic extracts of three species of *Isodon* identified through GC-MS analysis

Sl. No.	Class of compounds	*I. coetsa* (%)	*I. nigrescens* (%)	*I. nilgherricus* (%)
1	Terpenoids	14.63	37.61	49.50
2	Phenols	2.20	9.45	7.62
3	Alkaloids	12.43	4.75	1.89
4	Steroids	4.92	12.63	3.39
5	Flavonoids	-	-	2.43
6	Fatty acid esters	3.96	1.58	0.27
7	Vitamins	9.28	15.72	7.32
8	Furans	-	2.56	-
9	Aromatics	3.86	0.30	-
10	Alkanes	5.39	0.73	17.22
11	Aldehydes and ketones	21.73	6.65	2.69
12	Alcohols	1.48	1.38	0.78
13	Organic compounds	16.80	5.52	5.91
14	Miscellaneous	3.32	1.12	0.98
	Total	100.00	100.00	100.00

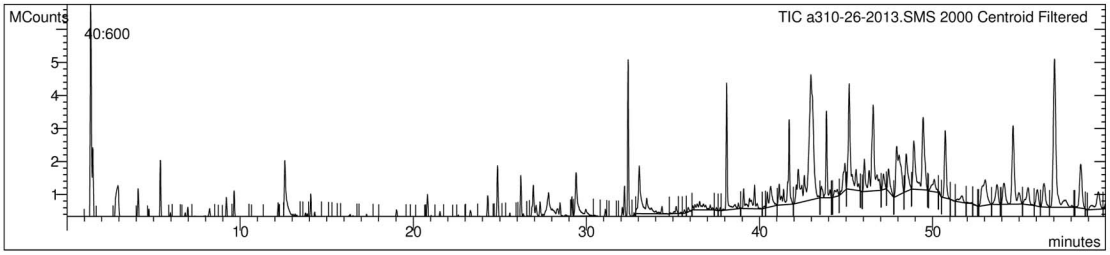


Fig. 41 Gas chromatogram of methanolic extract of *Isodon coetsa*

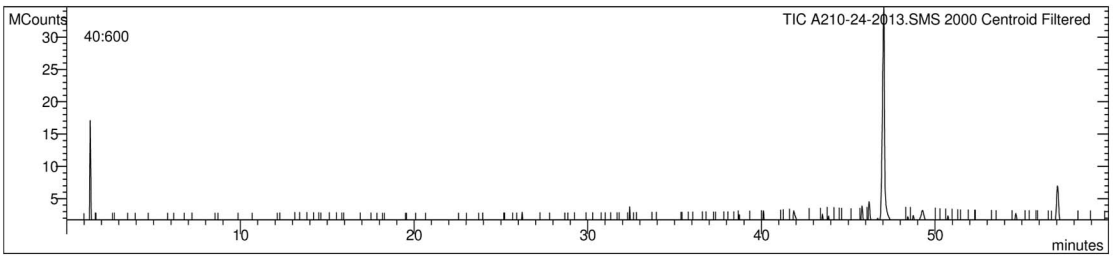


Fig. 42 Gas chromatogram of methanolic extract of *Isodon nigrescens*

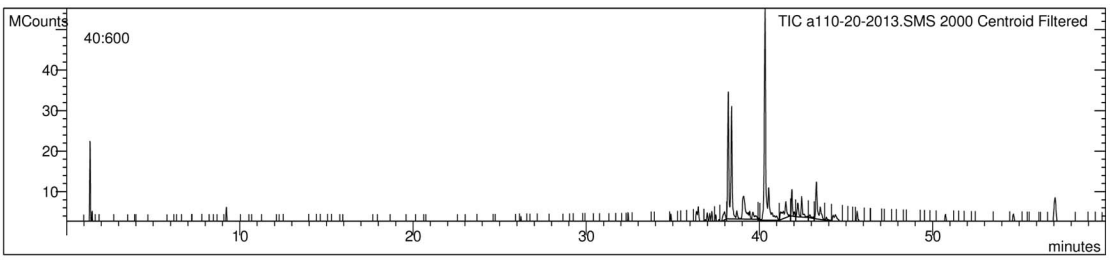


Fig. 43 Gas chromatogram of methanolic extract of *Isodon nilgherricus*

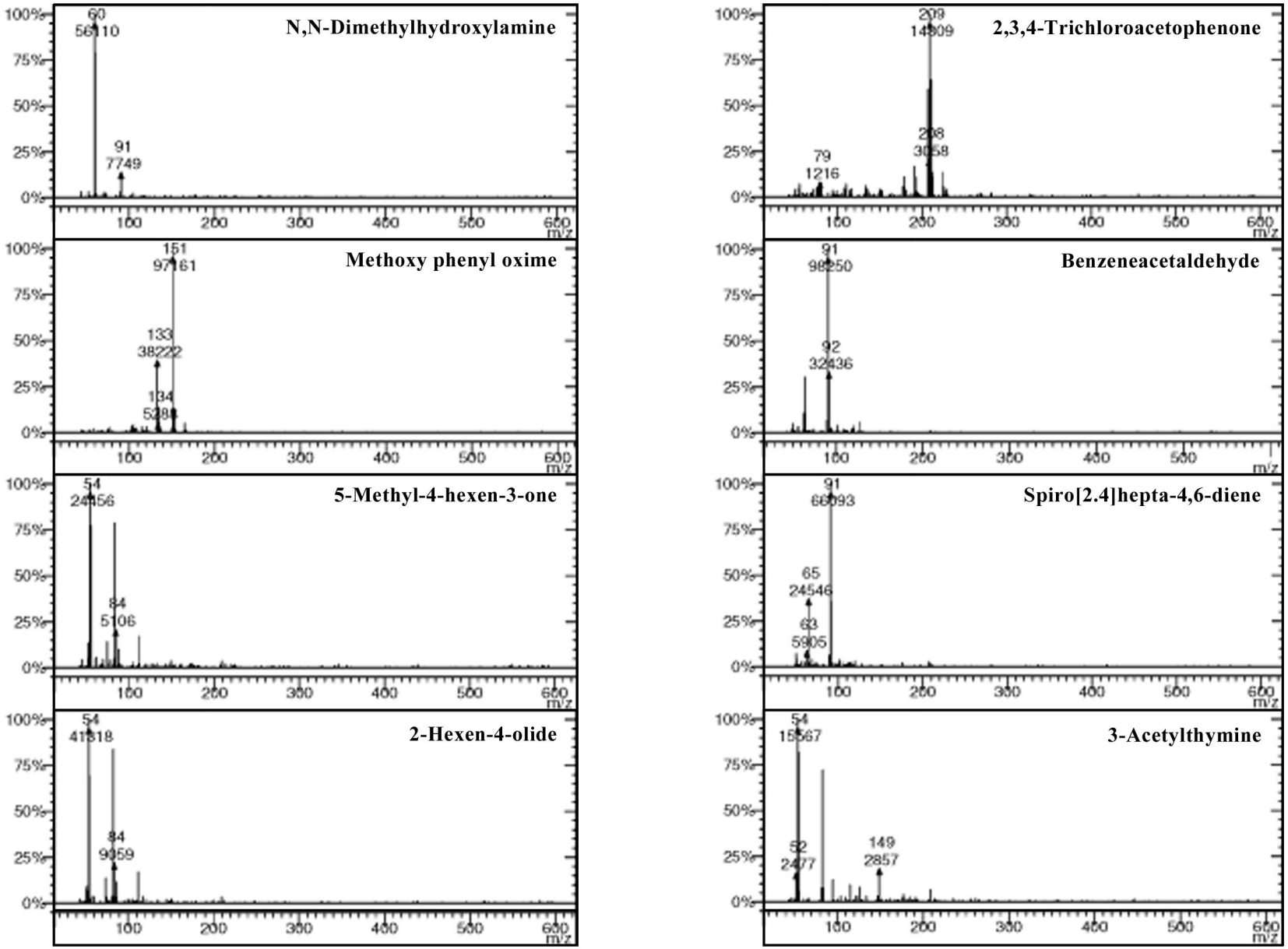


Fig. 44 (i) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

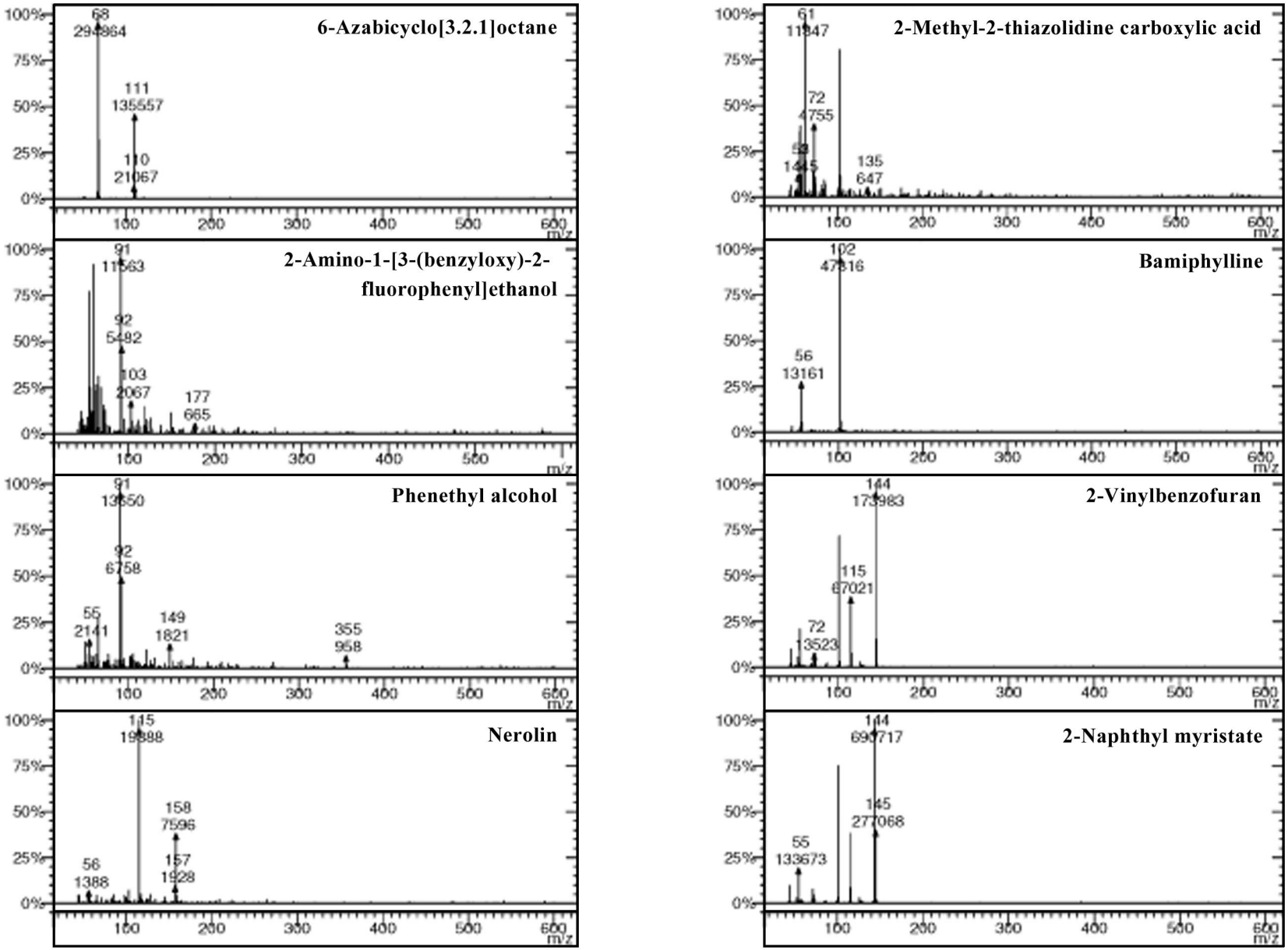


Fig. 44 (ii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

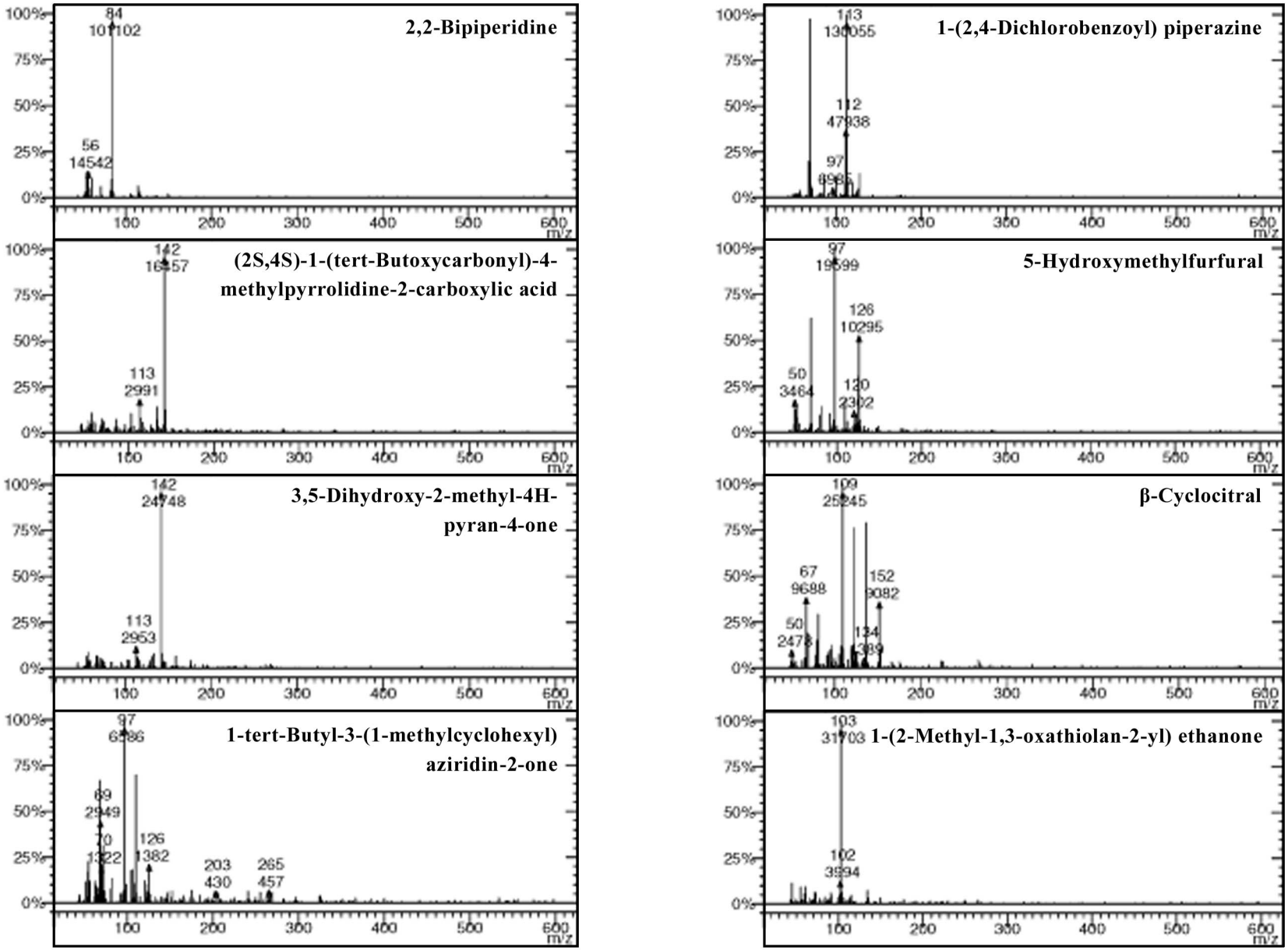


Fig. 44 (iii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

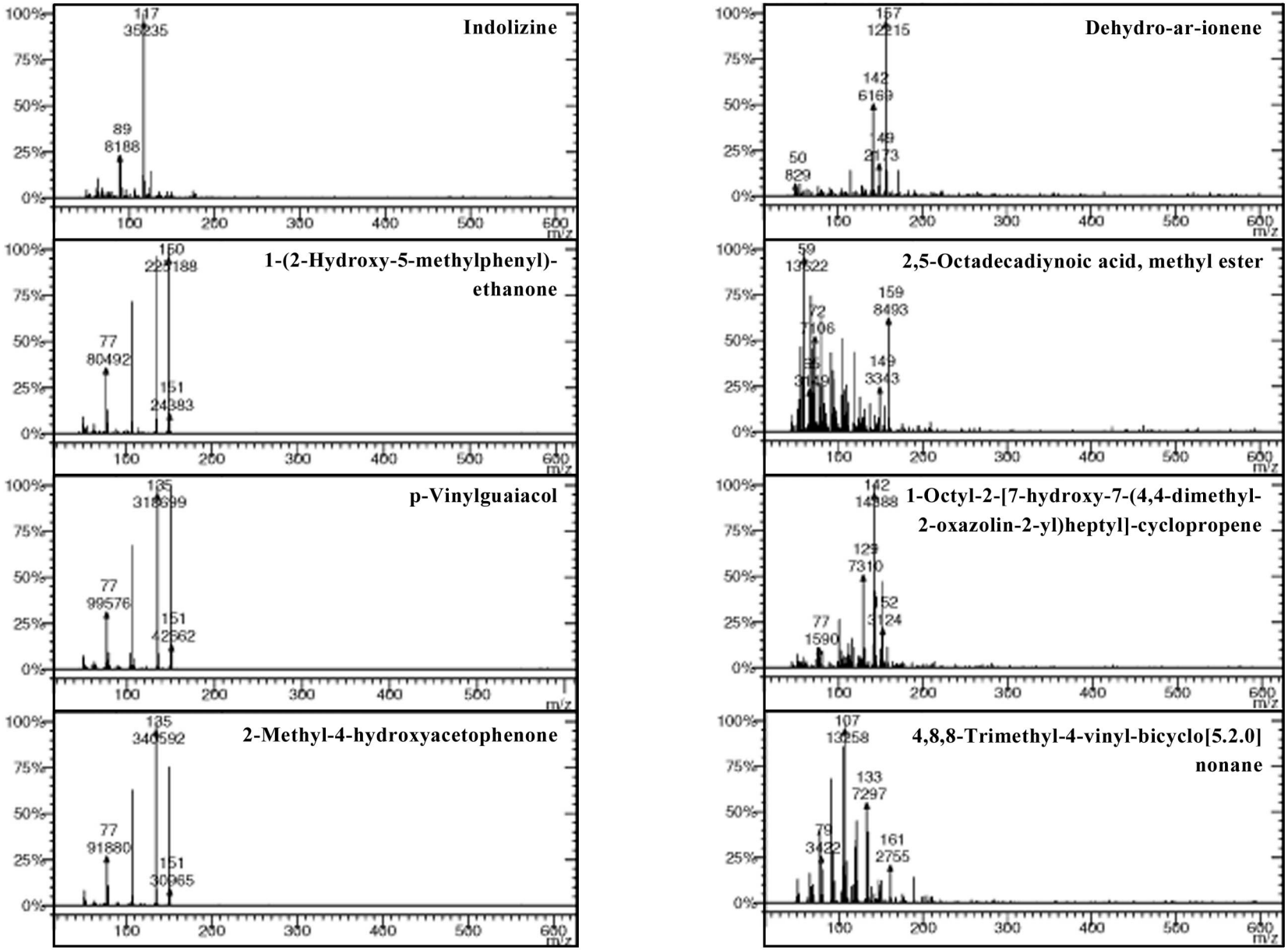


Fig. 44 (iv) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

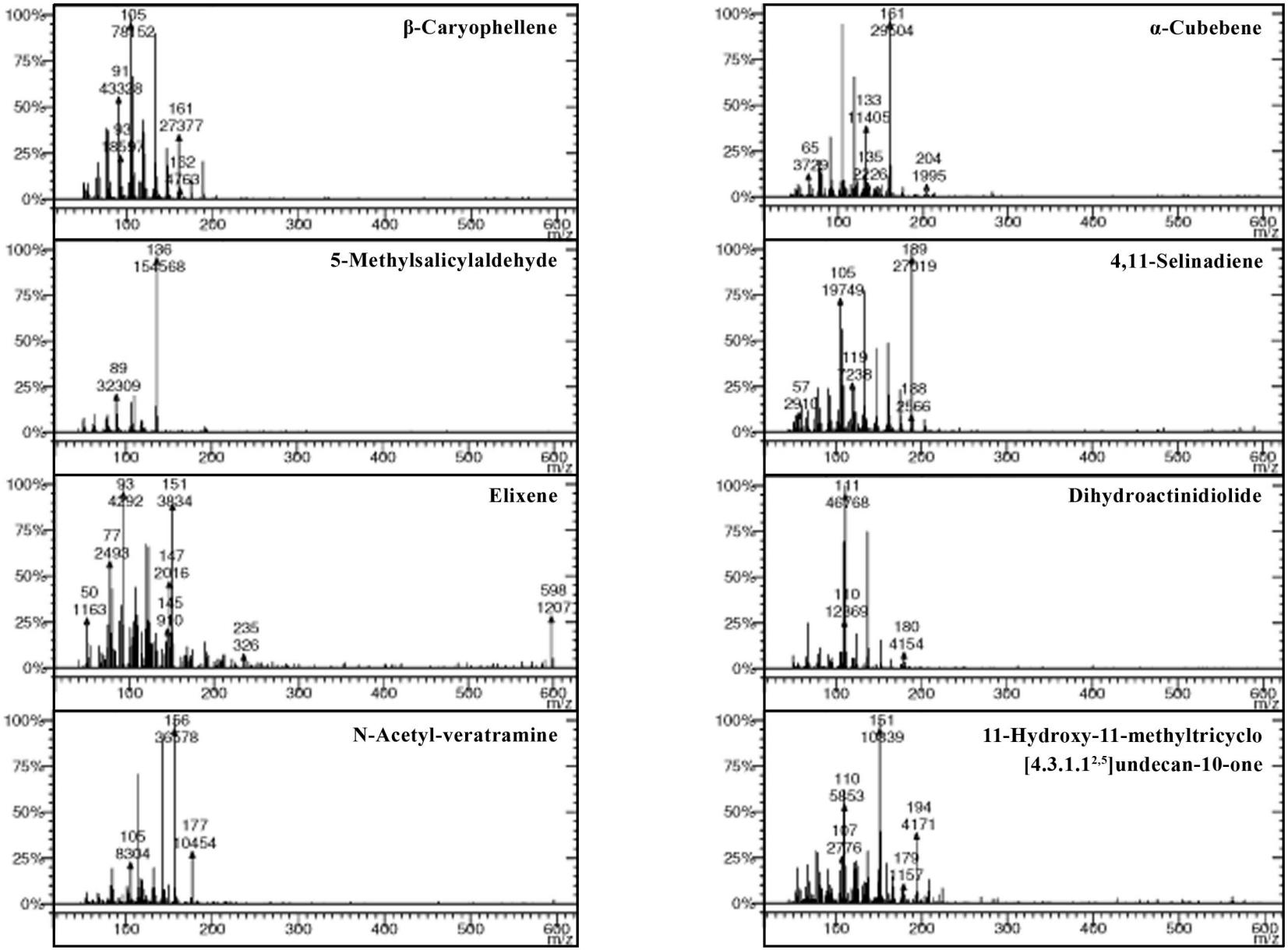


Fig. 44 (v) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

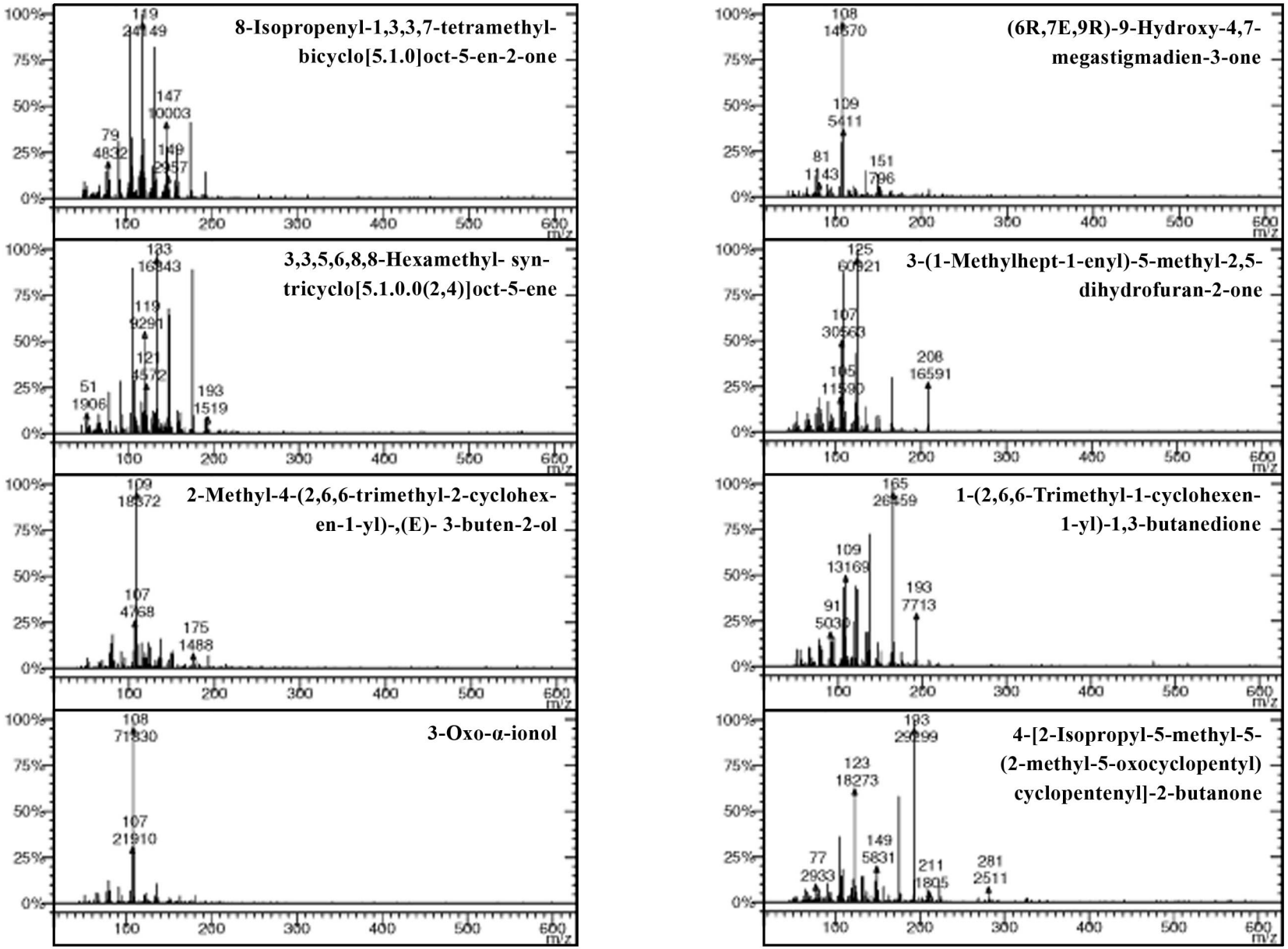


Fig. 44 (vi) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

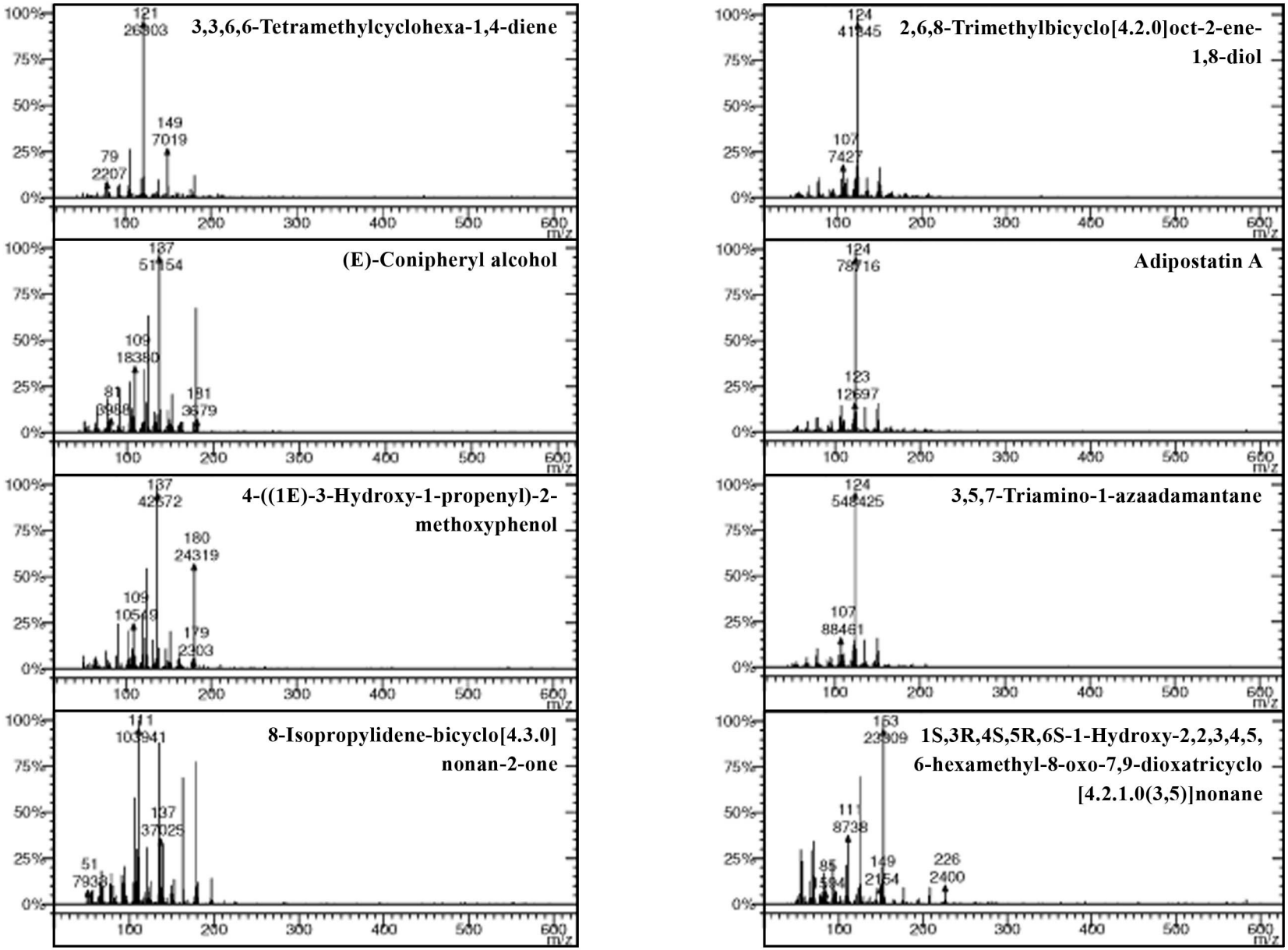


Fig. 44 (vii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

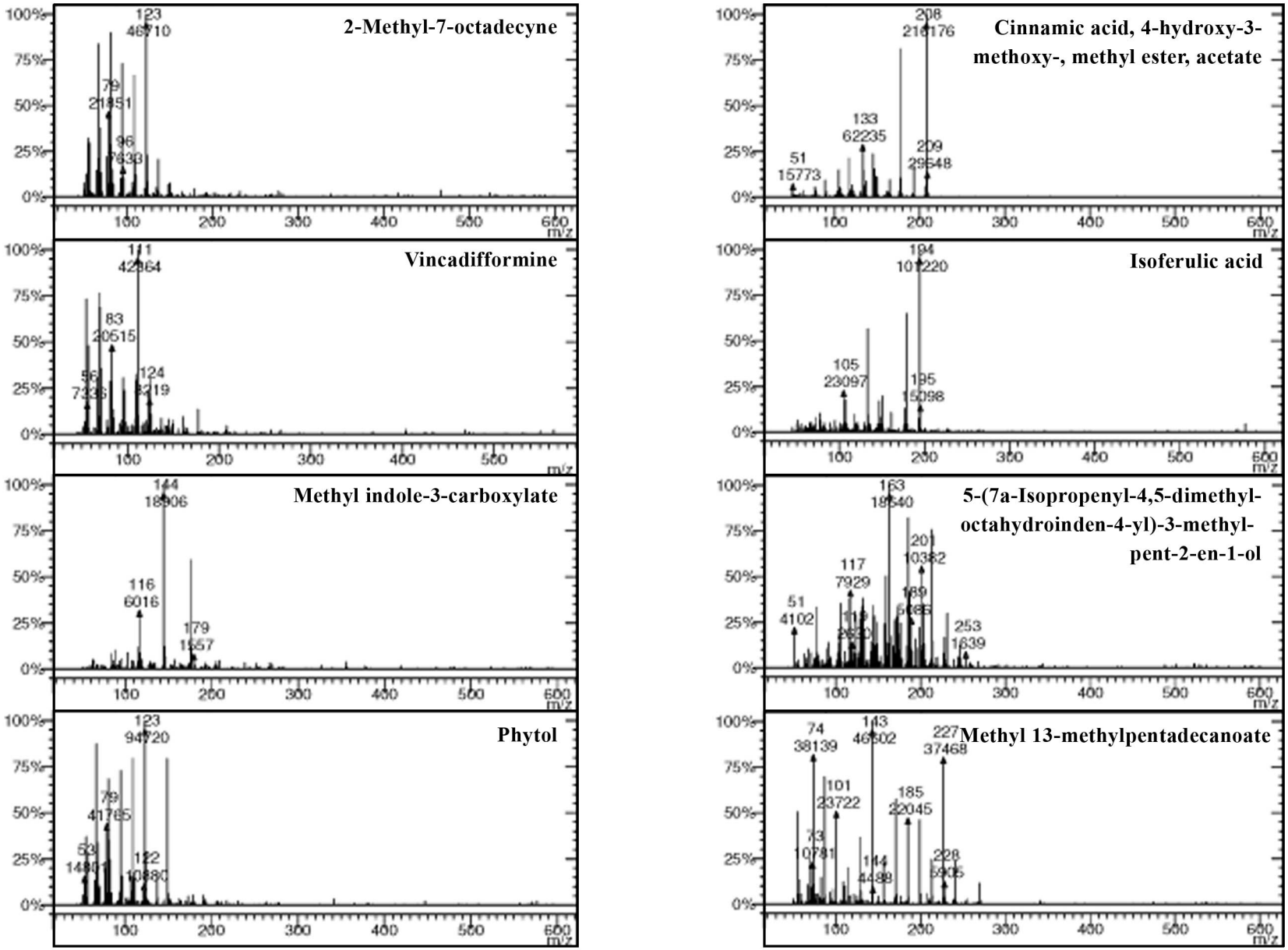


Fig. 44 (viii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

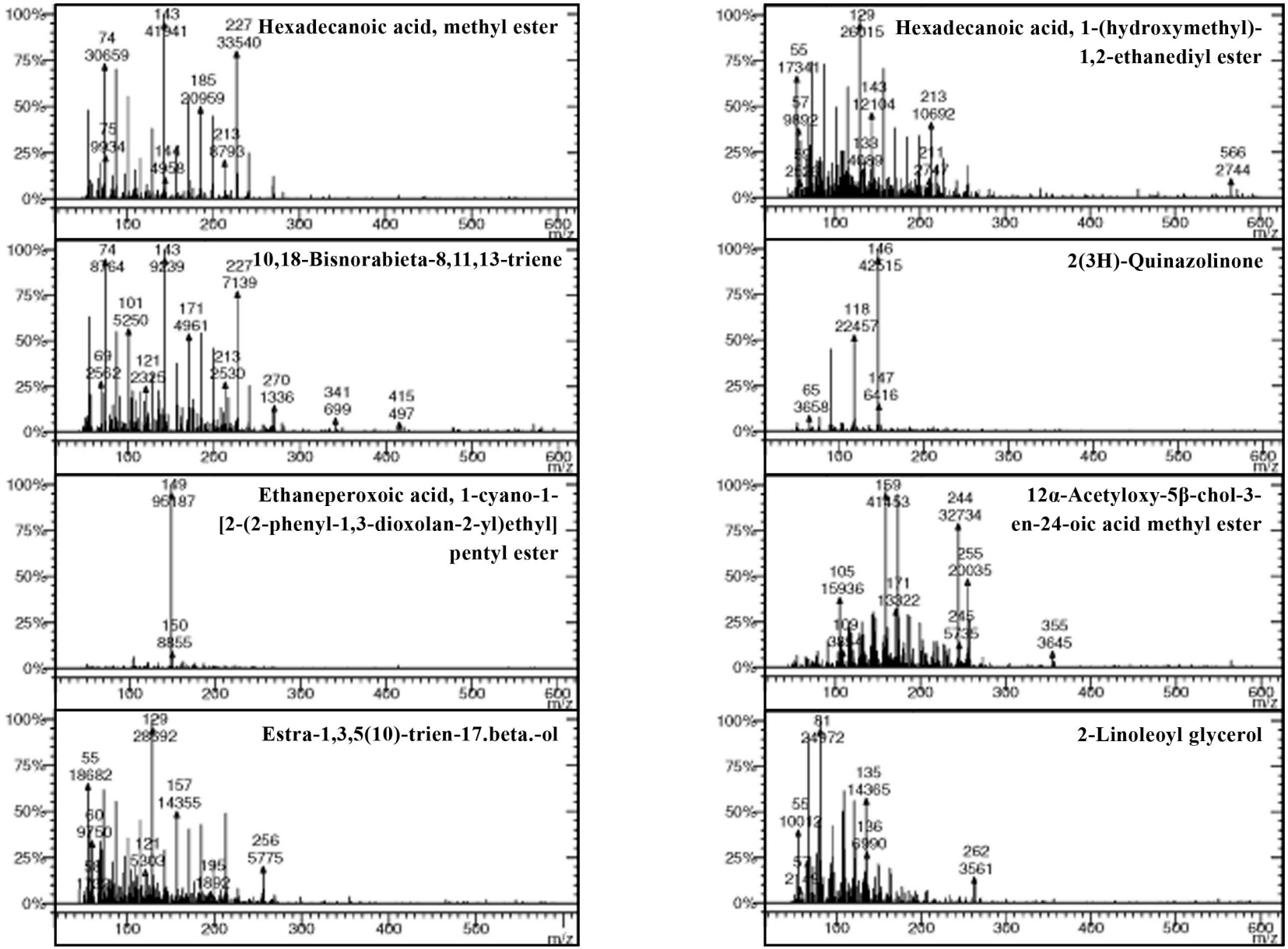


Fig. 44 (ix) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

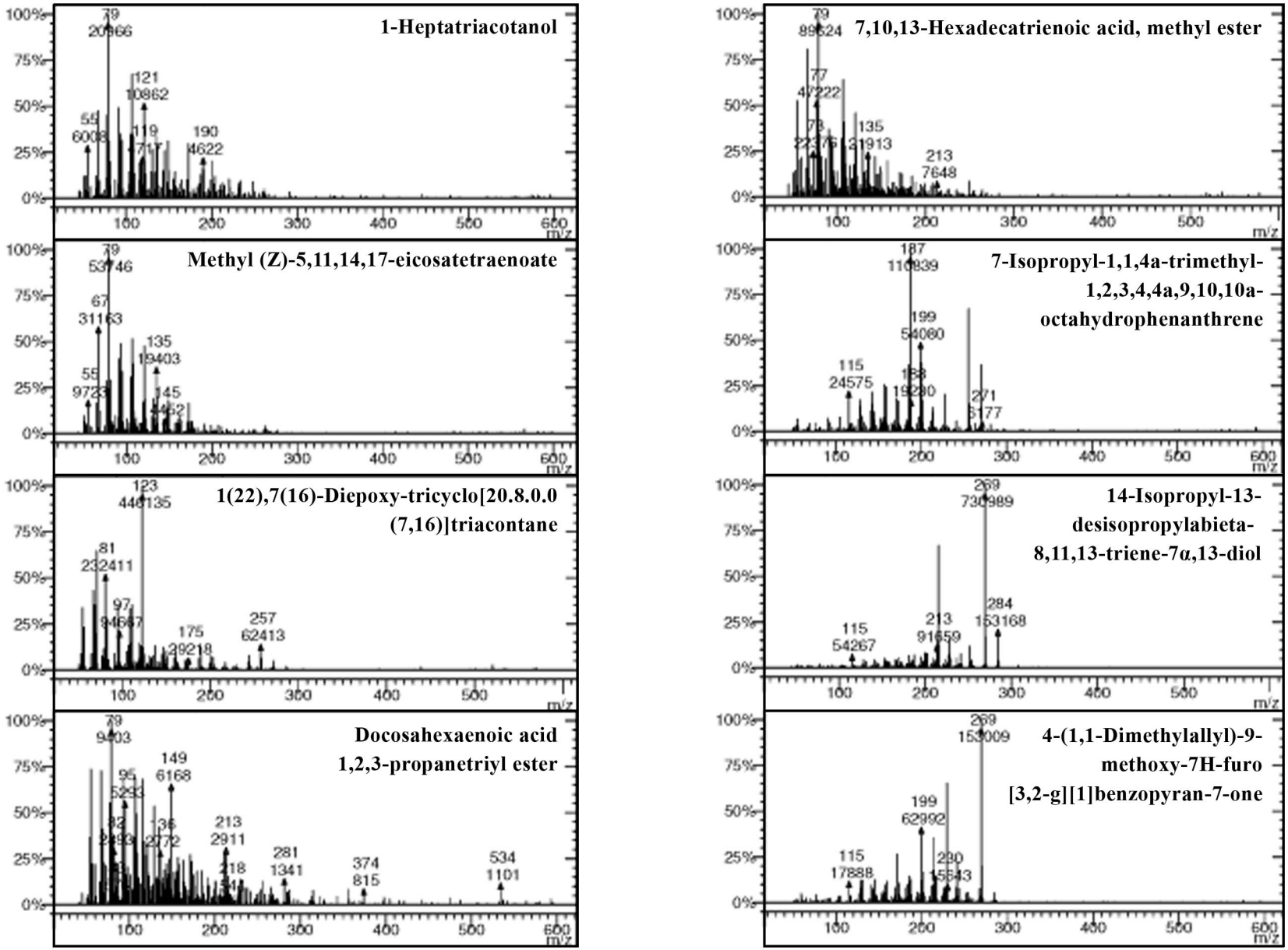


Fig. 44 (x) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

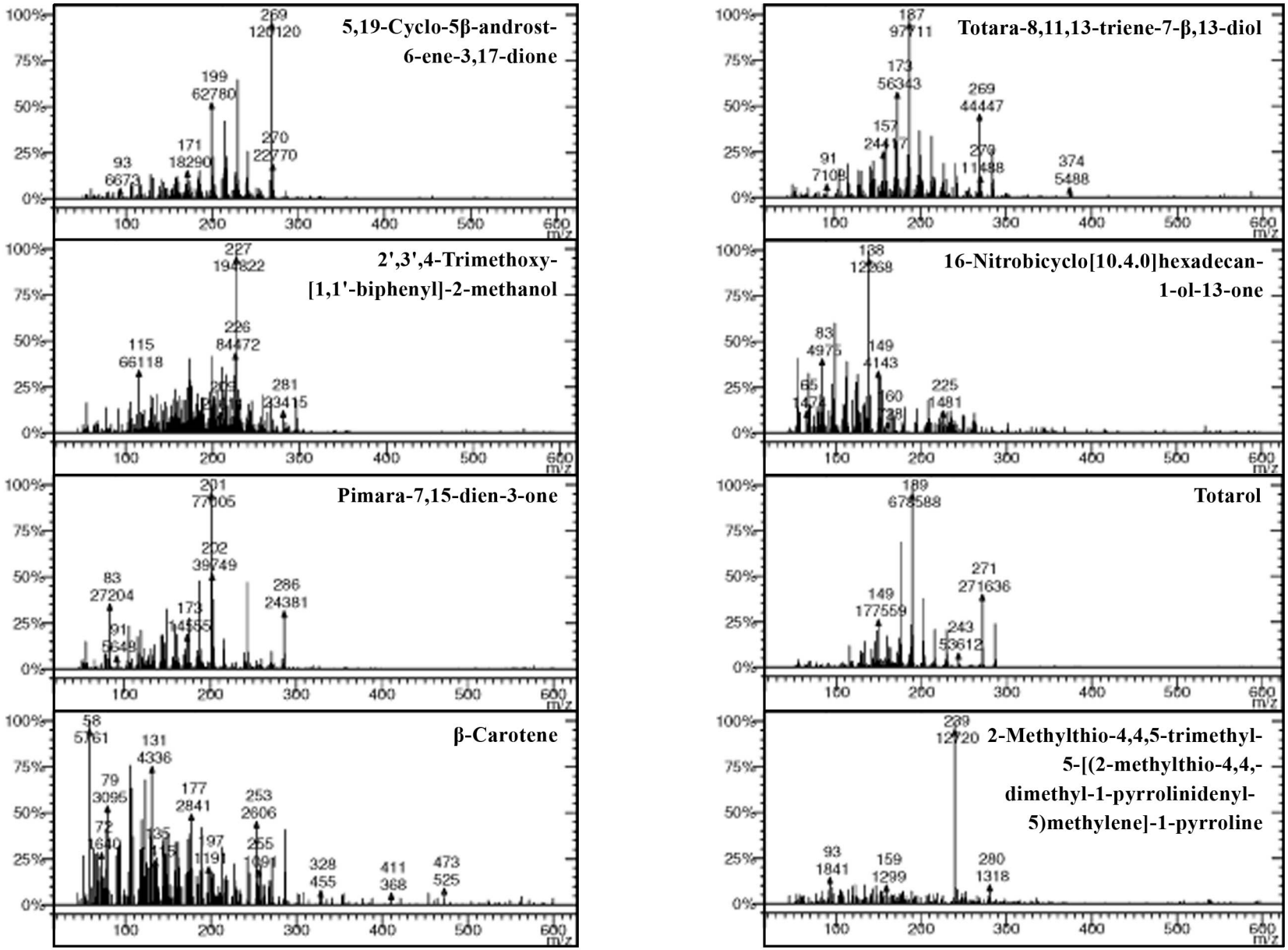


Fig. 44 (xi) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

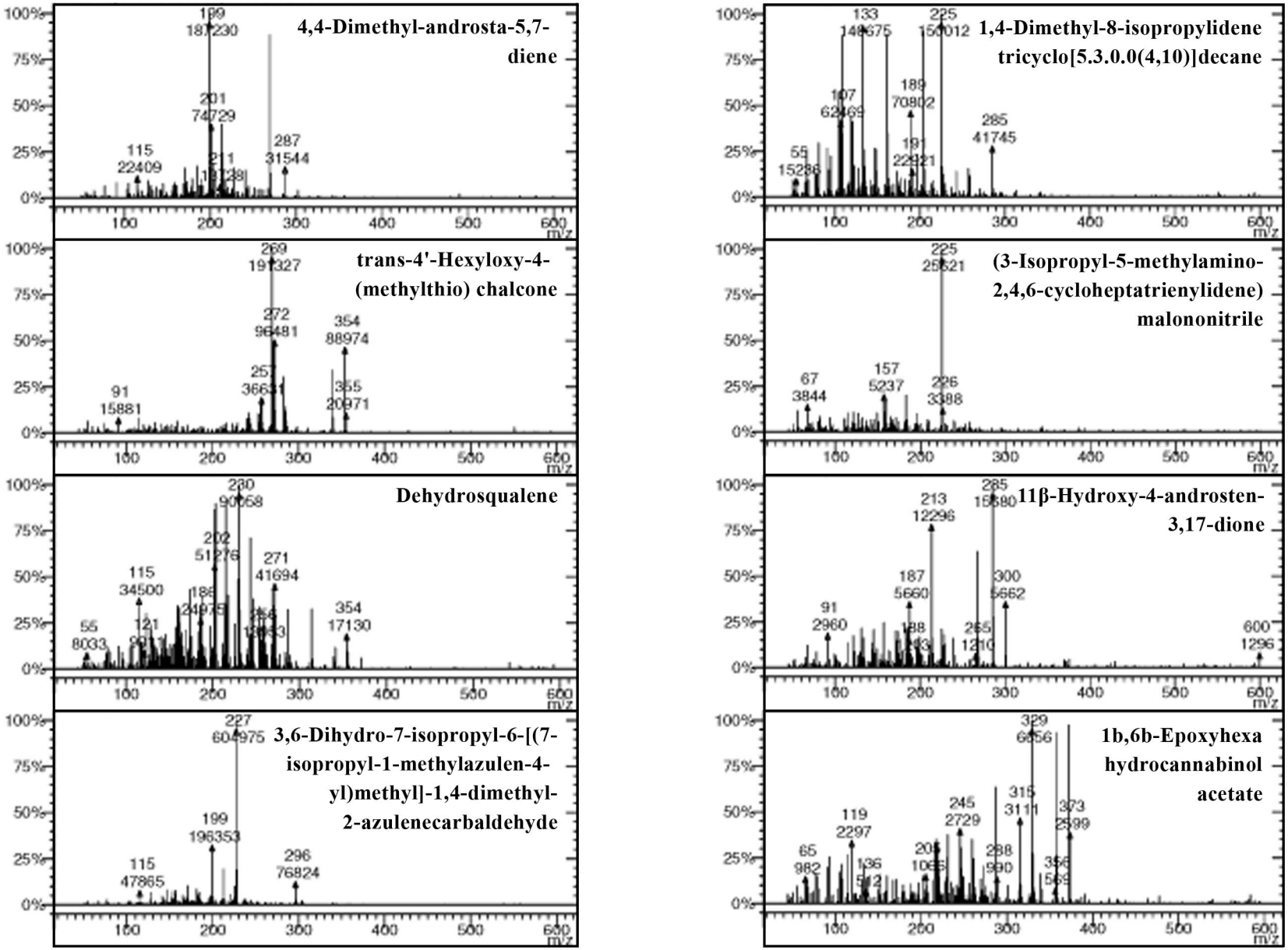


Fig. 44 (xii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

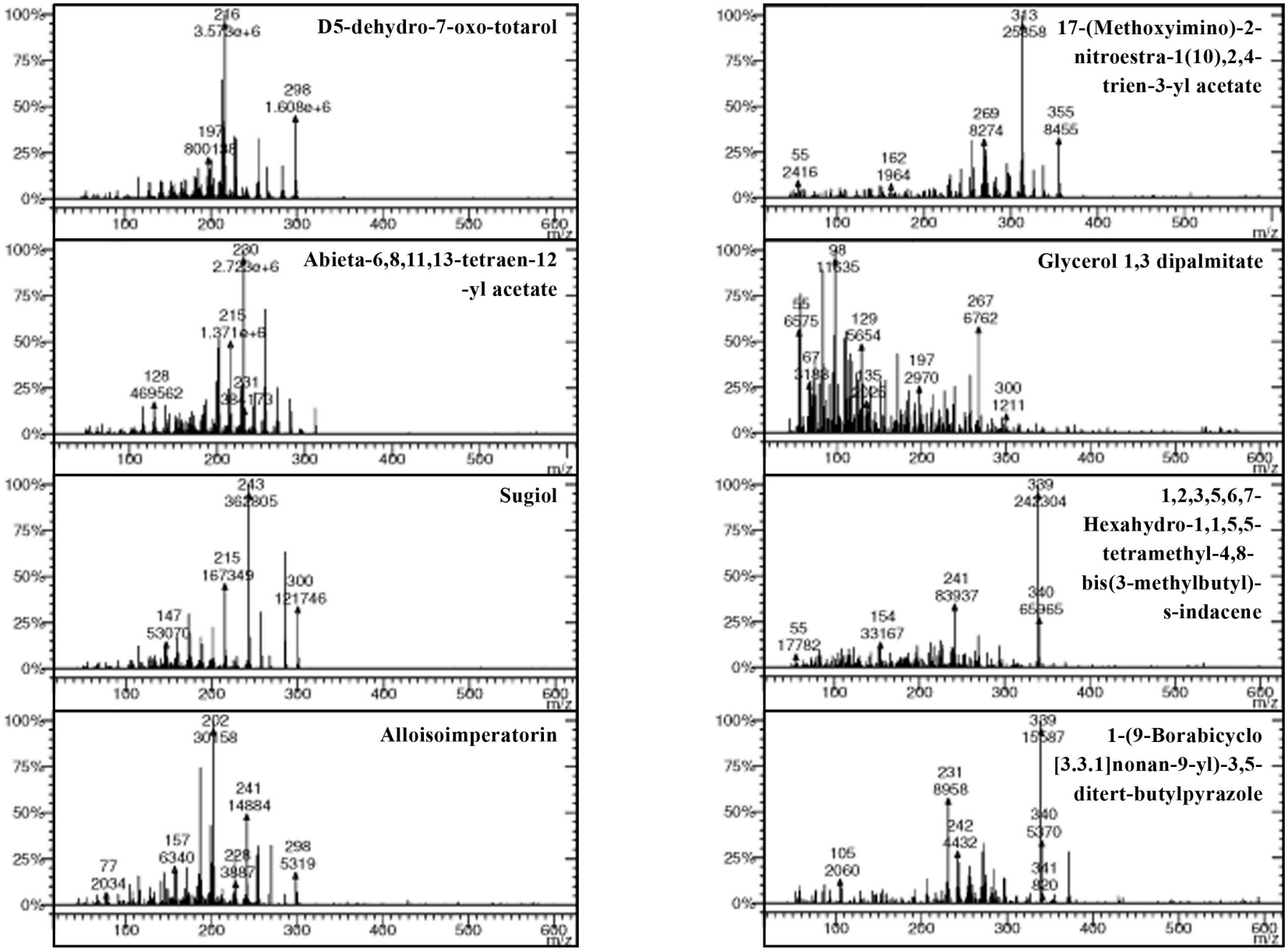


Fig. 44 (xiii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

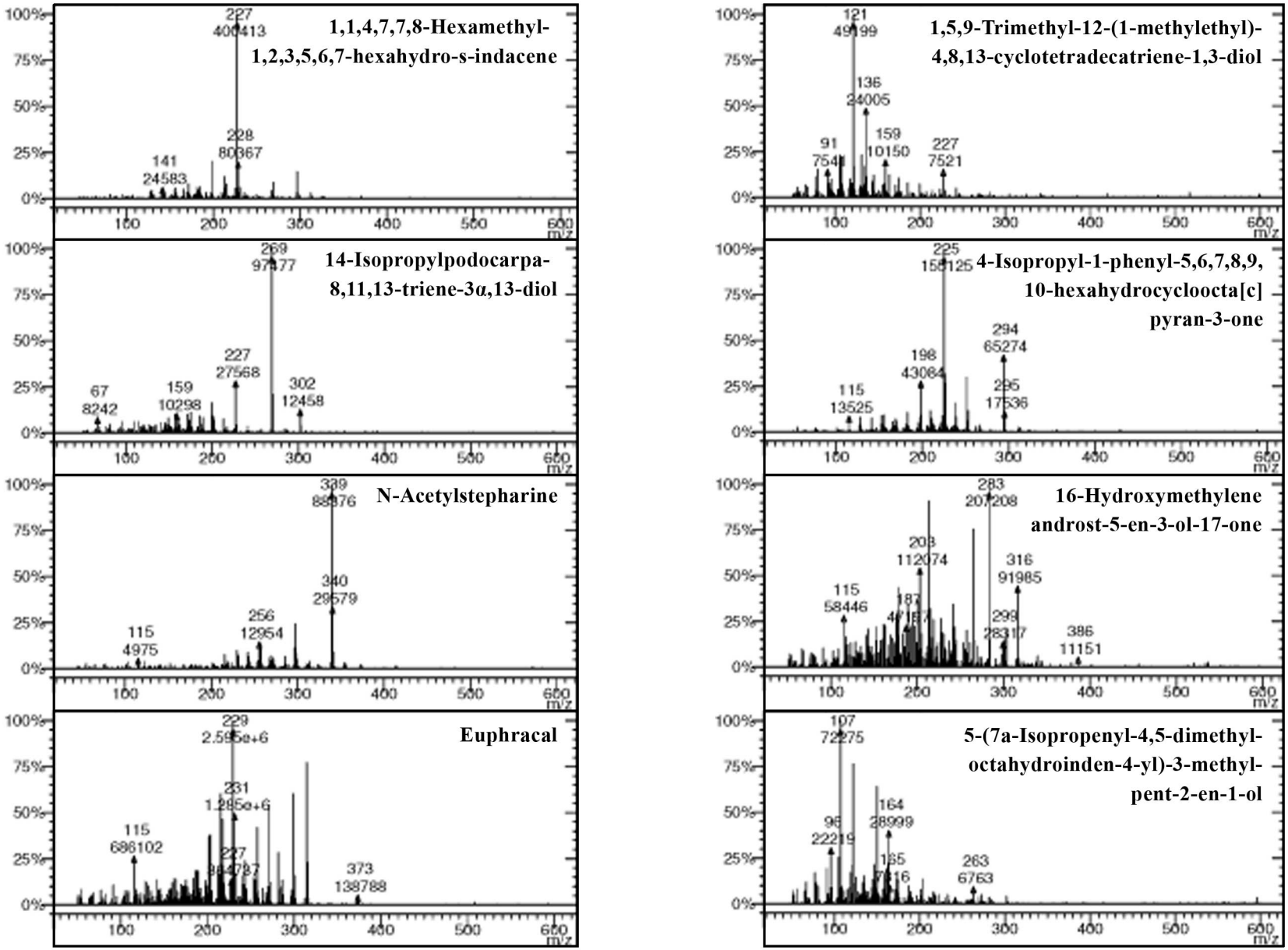


Fig. 44 (xiv) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

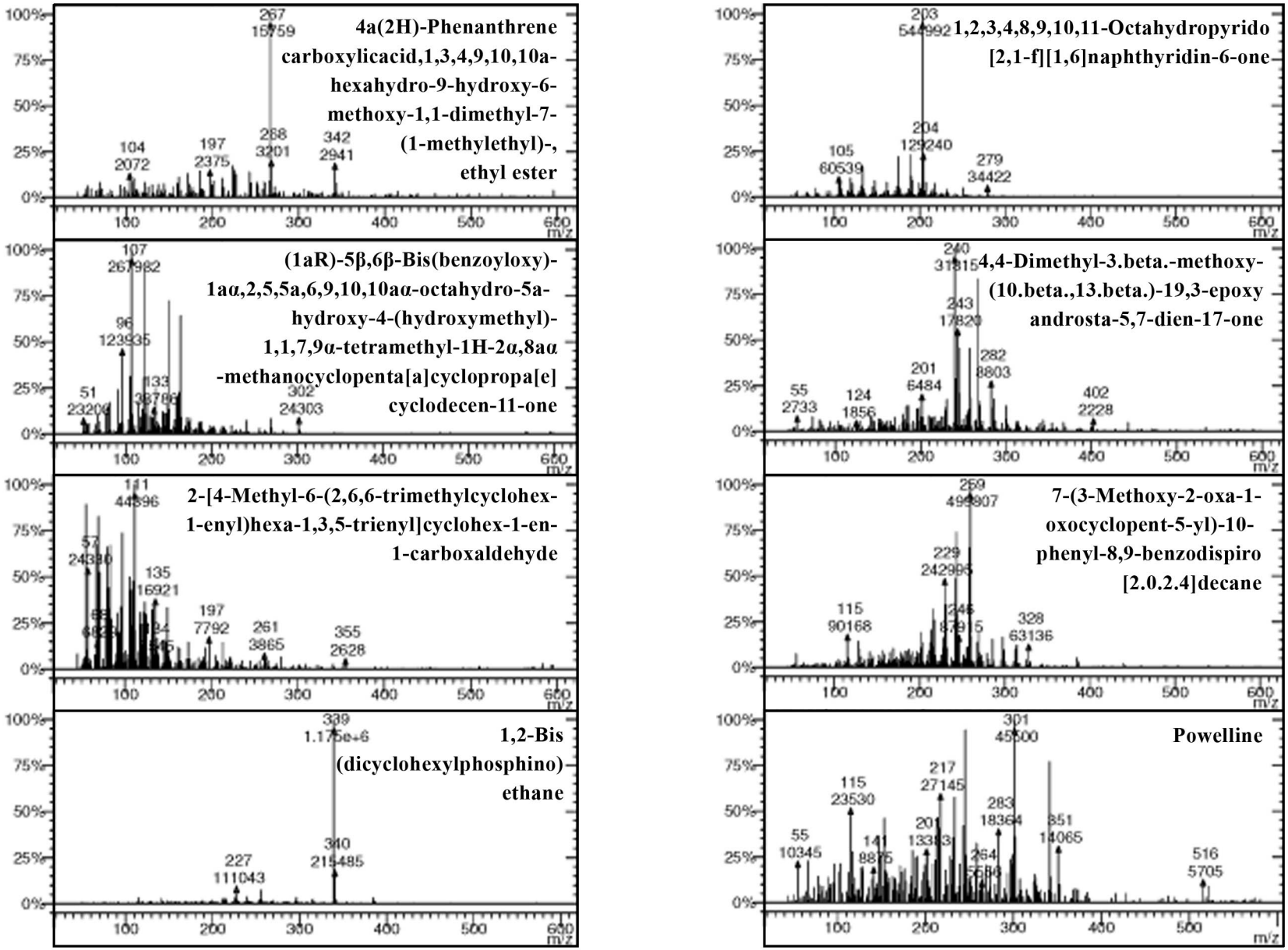


Fig. 44 (xv) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

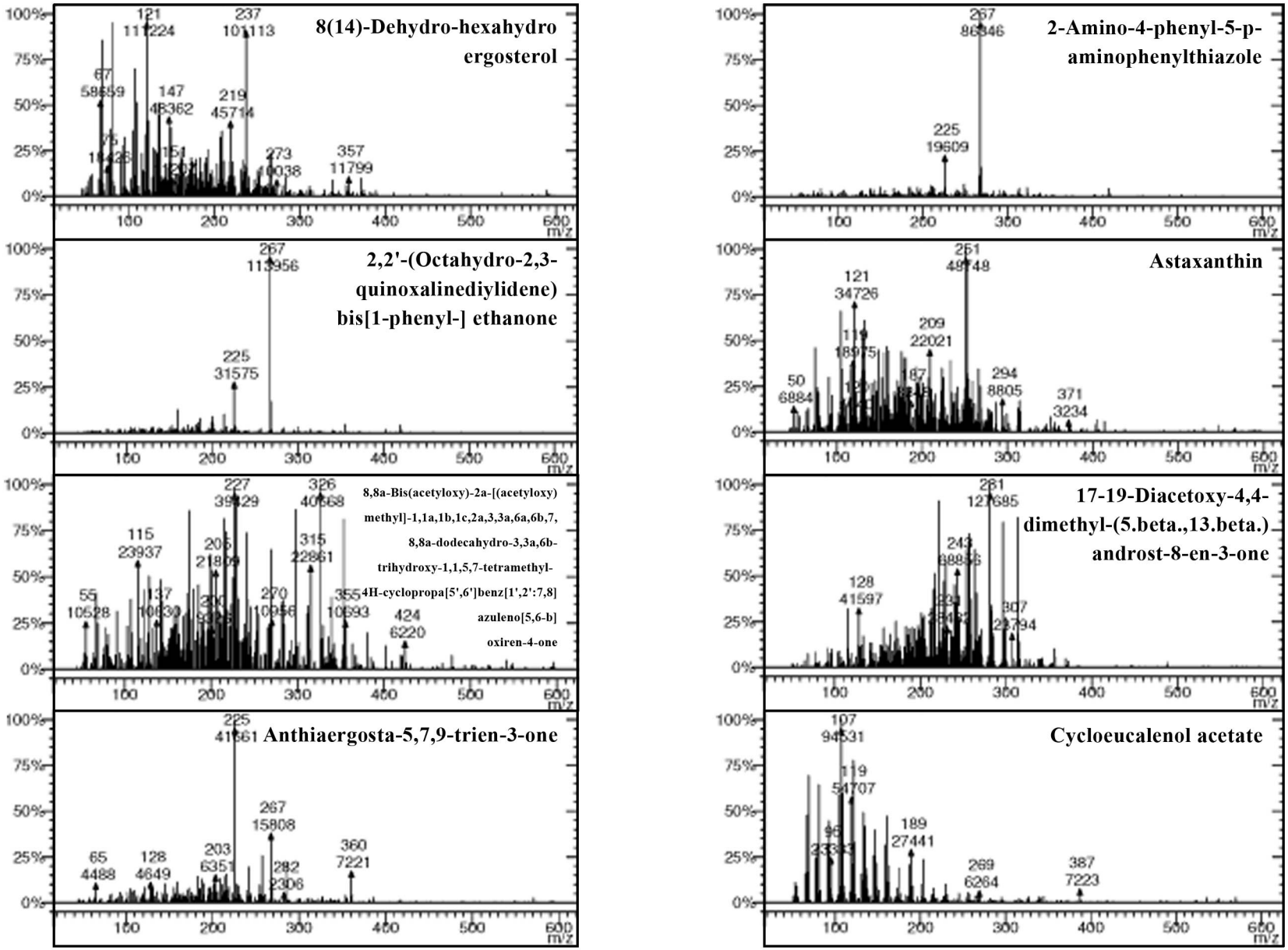


Fig. 44 (xvi) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

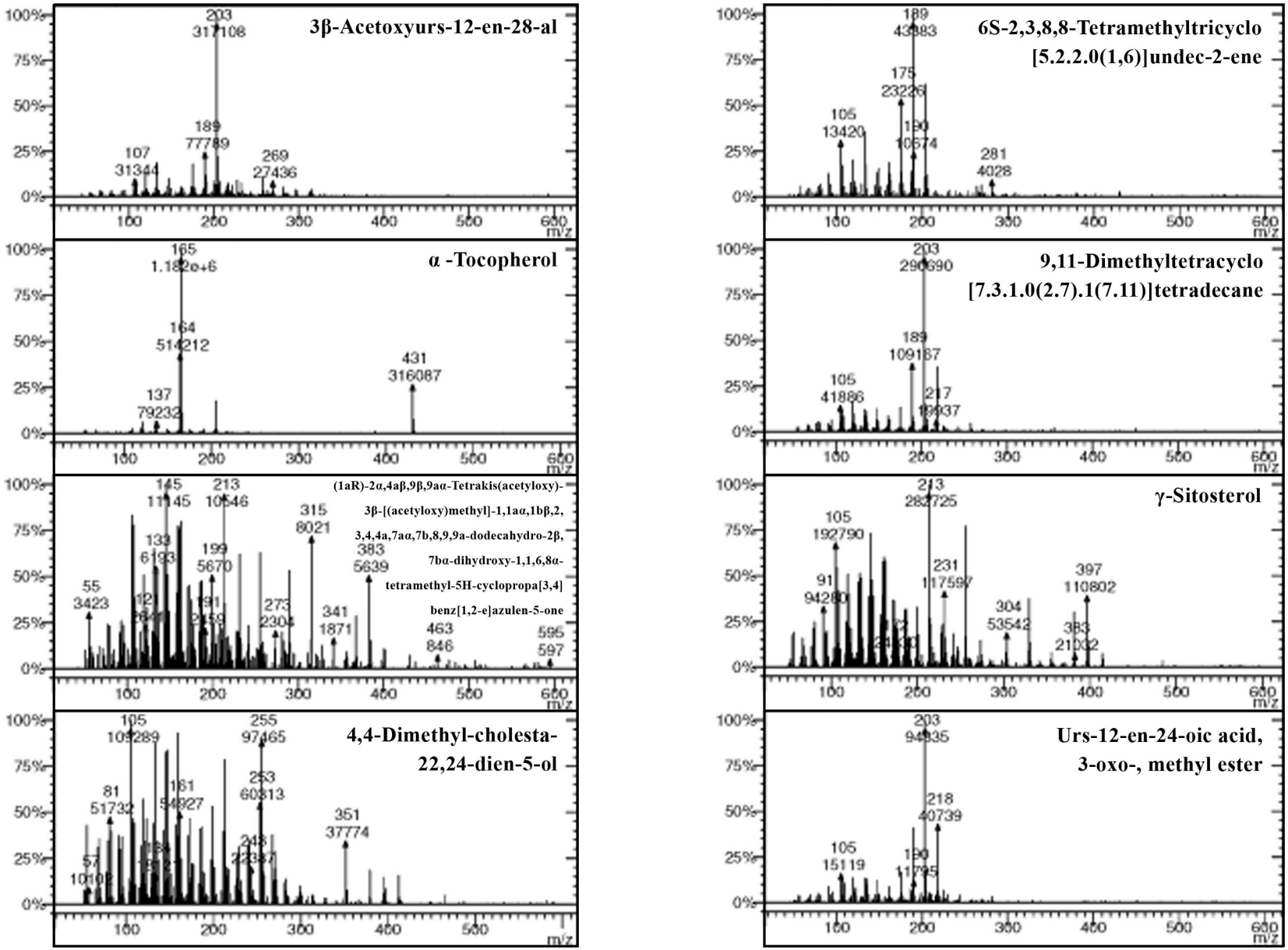


Fig. 44 (xvii) Mass spectra of chemical components detected in the methanolic extracts of three species of *Isodon*

Liquid chromatography-mass spectrometry (LC-MS) analysis

The phytochemical profile of the methanolic extracts of three species of *Isodon* was determined using LC-MS analysis. The compounds identified are consolidated in Table 50 and the liquid chromatograms obtained for the three species are given in Figs 45-47. The major class of compounds detected belonged to sesquiterpenoids. The methanolic extract of *I. coetsa* revealed the presence of two major components *viz*., spathulenol (93.44%) and quercetin (6.56%) while the methanolic extract of *I. nigrescens* identified two components which were α-cadinol (94.42%) and achillin (5.58%). Only a single compound of γ-eudesmol (100%) could be detected from the methanolic extract of *I. nilgherricus*.

The phytochemical profiling of the methanolic extract of three species of *Isodon* thus revealed the predominance of terpene class of compounds through various analyses. Terpenes being an important group with excellent biological properties can be considered as the driving force behind the various bioactivities as well as for the extensive use of the genus in folk medicines.

Table 50. Phytochemical constituents detected in the methanolic extracts of three species of *Isodon* identified through LC-MS analysis

Extract	Retention time	Chemical constituents	Class of compounds	Molecular formula	Molecular mass (g/mol)	Peak area (%)
I. coetsa	3.649	Spathulenol	Sesquiterpenoid	$C_{15}H_{24}O$	220.35	93.44
	4.895	Quercetin	Flavonoid	$C_{15}H_{10}O_7$	302.24	6.56
I. nigrescens	3.669	α-Cadinol	Sesquiterpenoid alcohol	$C_{15}H_{26}O$	222.37	94.42
	4.883	Achillin	Sesquiterpene lactone	$C_{15}H_{18}O_3$	246.30	5.58
I. nilgherricus	3.777	γ-Eudesmol	Sesquiterpenoid	$C_{15}H_{26}O$	222.37	100

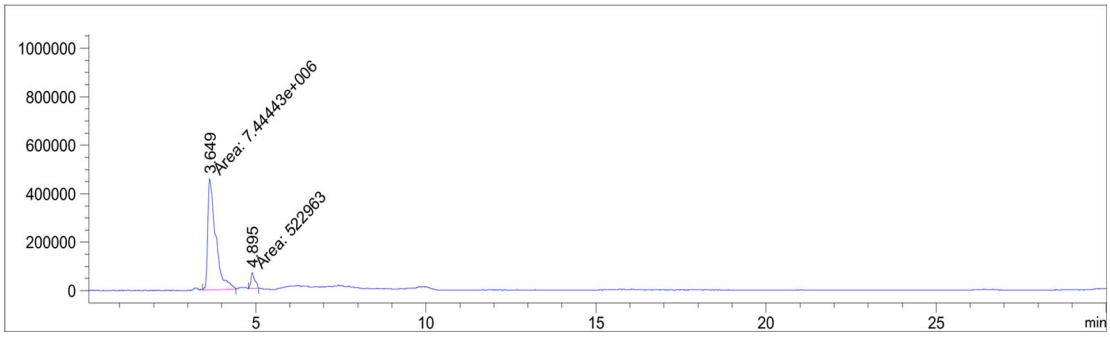


Fig. 45 Liquid chromatogram of methanolic extract of *Isodon coetsa*

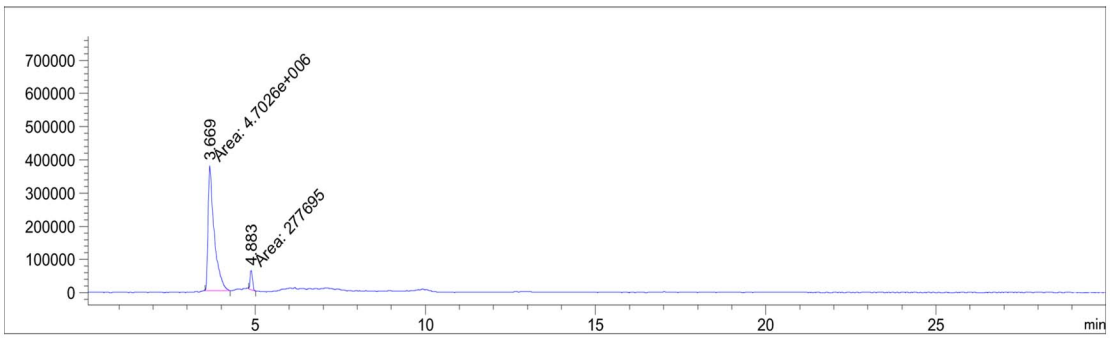


Fig. 46 Liquid chromatogram of methanolic extract of *Isodon nigrescens*

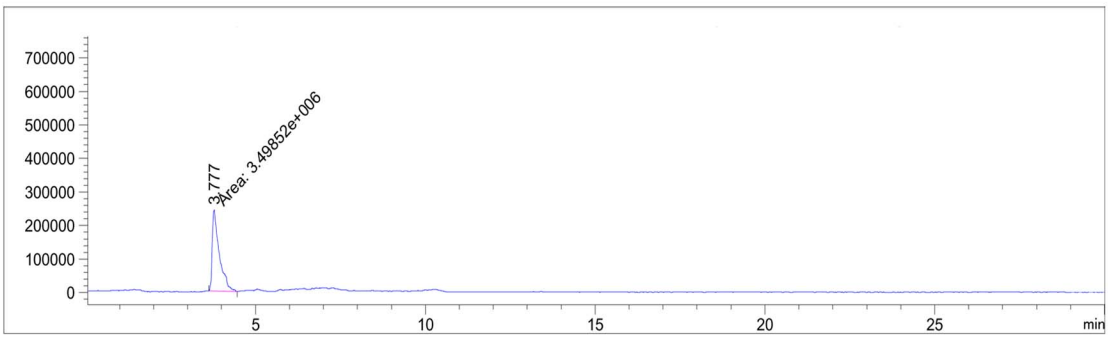


Fig. 47 Liquid chromatogram of methanolic extract of *Isodon nilgherricus*

Discussion

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

DISCUSSION

The genus *Isodon* being a prominent member in traditional Chinese medicine has been extensively used in the treatment of inflammations and cancer. The present study envisages an attempt to explore the bioactive potentials attributed to the three species of the genus (Plate 1) very well described in literature. The taxonomic identity of the plants is at stake and hence a cytological study encompassing the karyomorphology and morphometrics has been endeavored. It is expected to provide a reliable data in cytotaxonomy as well as a directive in defining the path of evolution. Antioxidant, cytotoxic and antitumor studies of the methanolic extracts of the three selected species abundantly available in Kerala has been ventured to determine the potentiality of the plant in being a candidate for commercially feasible drug research. The phytochemical studies employing GC-MS, LC-MS, estimation of various secondary metabolites in the methanolic extracts have also been established which could provide a phytochemical profile as well as a database for compound specific studies in pharmaceutics. The results obtained on the prospect of the genus in the biological world are hereby analysed and discussed.

CHROMOSOME ANALYSIS

Chromosome number, karyomorphometric data as well as the karyogram and idiogram were figured out for the cytogenetic characterization of three species of *Isodon*. Apart from this, the evolutionary significance of the plants was also outlined cytotaxonomically considering the various karyological parameters. The substantial information about a species is more precisely obtained from chromosome number which makes it an important cytotaxonomic parameter (Anil *et al*., 2013). Chromosomes are the storehouse of genetic information which provides a platform for genetic linkage groups,

the structures on which replication, transcription, transmission of the hereditary information as well as whole genome duplication and physical reorganization takes place (Heslop-Harrison and Schwarzacher, 2011). Karyosystematics is regarded as one of the most relevant parameter in the evaluation of the genetic relationship and divergence among species or populations (Guerra, 2008). Cytotaxonomic features have the disadvantage of being lost along with the loss of live specimens. The plants undergoing extinction threat are leaving behind a void in the cytological world with unidentified knowledge of karyotype and its evolutionary pathway. Hence, karyomorphological studies needs to be encouraged for establishing a cytological identity in taxonomic field as well as for the conservation of evolutionary knowledge.

The three species of *Isodon viz*., *I. coetsa*, *I. nigrescens* and *I. nilgherricus* were subjected to chromosome analysis through karyomorphological studies. The chromosome number was found to be 2n = 24 which was in agreement with previous reports (Table 1). It was the first report on the chromosome number of *I. nigrescens* to the world of cytology. A detailed karyomorphometric data of the reported chromosome number of the plant species was not available which was attempted here. The constancy of chromosome number was maintained in the three species but two cytotypes could be noticed with variant numbers of 6 and 12 in the worked out specimens. Chromosome numbers resulting from variations due to polyploidy and dysploidy have significant roles in phylogeny and karyotype evolution while other factors do not possess any evolutionary implication beyond species level (Guerra, 2000). The two variant cytotypes observed cannot be considered as a consequence of either polyploidy which results from multiplication of the haploid set or dysploidy resulting from gradual increase or decrease of the original number. But, with regards to the chromosome number, it can be a result of somatic reduction which implies on

the heterogenous nature of the genus. Though the karyotypic features of the cytotypes are characterized, it can only be employed in establishing the intraspecific variation pertaining to the genus. The abnormal mitotic phenomenon observed can only be a paradigm of chromosome mosaicism which might most probably be heritable since the cytotypes were not of a rare occurrence in the explored species.

Basic chromosome numbers can be defined as one of the haploid numbers observed in a taxon which most parsimoniously elucidates the chromosome variability of the group as well as discloses the clear relationship with the base numbers of closely related taxa (Guerra, 2000). Literature perusal have revealed that *Isodon* possess a basic chromosome number of x = 12 (Xiang *et al*., 2014). The frequency of polyploid chromosome number reported in the genus was considerably low which infers the negligible role of polyploid speciation in the evolutionary history (Zhong *et al*., 2010) and the probable diversification at the diploid level (Yamashiro *et al*., 2005). The karyotypic analysis data acquired from various species of *Isodon* along with three species under study revealed that the secondary basic chromosome number of the genus was $x_2 = 12$ which probably might have originated from the primary base number of $x_1 = 6$ through protoautoploidy. The chromosome counts perceived are in perfect agreement to be the multiples of the secondary basic chromosome number in the genus.

The karyomorphometrical studies in detail have not yet been attempted in the genus due to the small size of the chromosomes as well as unclear centromeres (Jin and Sha, 2004; Yamashiro *et al*., 2005; Huang, 2011; Zhang *et al*., 2012). This is evident from the morphometric details of the karyotypes in the three species wherein the range of chromosome length of each chromosome complement does not exceed 2 µm. The size of metaphase chromosomes varies depending on the genome size, chromosome number and karyotype structure of the investigated species (Schubert, 2007). The ACL of

all the three species ranged between 0.6850 μm - 1.0998 μm which established the small size of chromosomes (Table 15). In comparison, the size of chromosomes showed a decrease in the order of *I. nilgherricus* > *I. coetsa* > *I. nigrescens*.

Isodon coetsa

The normal diploid chromosome count was found to be 2n = 24 with two cytotypes of variant chromosome counts 6 and 12. The cytotype with chromosome number 6 had a single chromosome with secondary constriction and all of them possessed nearly median centromere positions (Plate 2). The chromosome length ranged between 1.0201 μm - 0.7700 μm summing upto a total of 5.2306 μm in length (Table 6, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 13.97, 10.40, 43.67, 56.33, 77.52 and 85.46 which was used to determine the asymmetry index as well as the evolutionary progress of the species. The cytotype with chromosome number 12 had two chromosomes with secondary constriction and all of them possessed nearly median centromere positions (Plate 3). The chromosome length ranged between 1.1154 μm - 0.5778 μm summing upto a total of 10.0148 μm in length (Table 7, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 31.75, 19.82, 44.97, 55.03, 81.73 and 74.82 which was used to determine the asymmetry index as well as the evolutionary progress of the species.

The diploid somatic chromosome number of 2n = 24 had four chromosomes with secondary constriction and the centromeric position was found to be nearly median for eighteen chromosomes and nearly submedian for remaining six chromosomes (Plate 4). The chromosome length ranged between 1.1100 μm - 0.7055 μm summing upto a total of 21.1596 μm in length (Table 8, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 22.28, 14.92, 43.69, 56.31,

77.58 and 80.34 which was used to determine the asymmetry index as well as the evolutionary progress of the species. The asymmetry index of the diploid count was also resolved based on other parameters of A_1, A_2 and A which was found to be 0.21, 0.15 and 0.24 respectively.

Isodon nigrescens

The normal diploid chromosome count was found to be 2n = 24 with two cytotypes showing variant chromosome counts of 6 and 12. The cytotype with chromosome number 6 had a single chromosome with secondary constriction and five of them possessed nearly median and one with nearly submedian centromere positions (Plate 5). The chromosome length ranged between 1.2110 µm - 0.6201 µm summing upto a total of 4.8769 µm in length (Table 9, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 32.27, 27.21, 43.76, 56.24, 77.81 and 67.12 which was used to determine the asymmetry index as well as the evolutionary progress of the species. The cytotype with chromosome number 12 had two chromosomes with secondary constriction and all of them possessed nearly median centromere positions (Plate 6). The chromosome length ranged between 0.9632 µm - 0.5647 µm summing upto a total of 8.2203 µm in length (Table 10, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 24.02, 18.82, 43.65, 56.35, 77.45 and 71.12 which was used to determine the asymmetry index as well as the evolutionary progress of the species.

The diploid somatic chromosome number of 2n = 24 had four chromosomes with secondary constriction and the centromeric position was found to be nearly median for twenty two chromosomes and nearly submedian for remaining two chromosomes (Plate 7). The chromosome length ranged between 1.0707 µm - 0.6229 µm summing upto a total of 19.2966 µm in length (Table 11, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 26.44,

16.86, 42.96, 57.04, 75.31 and 75.09 which was used to determine the asymmetry index as well as the evolutionary progress of the species. The asymmetry index of the diploid count was also resolved based on other parameters of A_1, A_2 and A which was found to be 0.24, 0.17 and 0.28 respectively.

Isodon nilgherricus

The normal diploid chromosome count was found to be 2n = 24 with two cytotypes revealing variant chromosome counts of 6 and 12. The cytotype with chromosome number 6 had a single chromosome with secondary constriction and all of them possessed nearly median centromere positions (Plate 8). The chromosome length ranged between 1.3386 µm - 0.8310 µm summing upto a total of 6.3376 µm in length (Table 12, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 23.39, 17.12, 42.77, 57.23, 74.74 and 78.91 which was used to determine the asymmetry index as well as the evolutionary progress of the species. The cytotype with chromosome number 12 had two chromosomes with secondary constriction and all of them possessed nearly median centromere positions (Plate 9). The chromosome length ranged between 1.6215 µm - 0.8246 µm summing upto a total of 13.1981 µm in length (Table 13, 15). The various parameters of DI, VC, TF%, As K%, Syi and Rec were calculated and were found to be 32.58, 21.23, 44.82, 55.18, 81.24 and 67.83 which was used to determine the asymmetry index as well as the evolutionary progress of the species.

The diploid somatic chromosome number of 2n = 24 had four chromosomes with secondary constriction and the centromeric position was found to be nearly median for twenty two chromosomes and nearly submedian for remaining two chromosomes (Plate 10). The chromosome length ranged between 1.2290 µm - 0.8116 µm summing upto a total of 23.0086 µm in length (Table 14, 15). The various parameters of DI, VC,

TF%, As K%, Syi and Rec were calculated and were found to be 20.45, 17.52, 42.84, 57.16, 74.96 and 78.01 which was used to determine the asymmetry index as well as the evolutionary progress of the species. The asymmetry index of the diploid count was also resolved based on other parameters of A_1, A_2 and A which was found to be 0.24, 0.12 and 0.28 respectively.

Karyotype asymmetry can be considered to be the driving force behind speciation since symmetrical karyotypes represents ancestral condition in evolution (Stebbins, 1971). Parameters determining asymmetry of karyotype can thus be used to interpret the evolutionary advancement of a species and general morphology of chromosomes. The concepts of symmetry and asymmetry, position of centromere and relative chromosome length allows the assessment of chromosome affinities (Lavania and Srivastava, 1992). The divergence and evolution in higher plants have been related to the variations in morphological characters of the genome (Zarco, 1986) resulting in genome size variations. Thus, karyomorphological studies apart from providing identity to each species, helps in inferring the evolutionary advancement which are essential in the field of molecular cytogenetics.

Disparity index (DI) is a parameter employed for determining the asymmetry of karyotype. The variations in the size of the chromosomes can be identified from disparity index. The homogeneity of chromosomes is depicted by low DI value while high value indicates the heterogenous assemblage (Mohanty *et al*., 1991). Based on the DI value, heterogenous assemblage of chromosomes were more prominent in variant cytotype 12 of *I. coetsa* and *I. nilgherricus* as well as variant cytotype 6 of *I. nigrescens* while homogeneity was observed for variant cytotype 6 of *I. coetsa*. All the normal chromosome counts represented intermediary DI values which might be the indication of the occurrence of each species in an evolutionary path. Karyotype heterogeneity when occurring both cytologically as well as

genetically, provide important intimation regarding evolution of plant species (Stebbins, 1958). Variation in chromosome size and its distribution are very well established by variation coefficient (VC) parameter. Low values of VC reflect the homogeneity and primitiveness of the karyotype (Stebbins, 1959). VC represents the same pattern of values as DI which means that the heterogeneity and homogeneity of the species are in perfect correlation based on these parameters. The somatic chromosome count of the three species can hence be considered in the path of evolution due to its intermediary heterogeneous nature both cytologically and genetically.

Eight different parameters were established for evaluating the karyotype asymmetry. Seven quantitative indices *viz*., TF%, Syi, Rec, As K%, A_1-A_2 and A (Table 15) along with a population dispersion plot based on A_1-A_2 parameters (Fig. 11) helped in the exemplification of karyotype asymmetry. Increasing asymmetry depicts reduction in values for TF%, Syi and Rec indices but brings about an increase in As K%, A_1-A_2 and A values (Zuo and Yuan, 2011). The total forma percentage (TF%) is a karyotype asymmetry parameter which provides data to infer about the karyotype affinity in related taxa. It measures the extent of centromeric change in a species. The occurrence of relatively high number of nearly median centromere positions in the three species correlates with the variations in TF% values. Absolute symmetry of karyotype can be attributed to a TF% of 50 which implies median centromeres for all chromosomes while TF% of zero exihibits complete asymmetry with terminal centromeres (Kapoor, 1977). The values of TF% ranging between 42.77% - 44.97% lies well below the half way mark and along with the possession of nearly median and submedian centromere positions in the studied taxa marks the path of evolution of the species from a primitive to advanced one. Moreover, the decrease in TF% results from the loss of DNA from the short arm of chromosomes (Gao *et al*., 2012) which is bound to increase the genetic efficiency of a species. Thus, the

difference in distribution of nucleic acids generates variation in arm ratio as well as asymmetry leading to karyotype evolution.

Karyotype asymmetry of a complement is generally defined by the variation in chromosome length along with variation in centromere position (Levitsky, 1931; Stebbins, 1971). Asymmetry assessment is the most important parameter in karyotype analysis which could be evaluated by the index of karyotype asymmetry known as Syi index and index of chromosomal size resemblance referred as Rec index. The karyotype analysis reveals a reduction in value of indices for most cytotypes. The normal somatic count of the three species had a Syi index of 77.58, 75.31, 74.96 which are low when compared to other values indicating a shift towards asymmetric karyotype. The most symmetric karyotype was observed in the cytotypes especially for variant 12 of *I. coetsa* and *I. nilgherricus*. But Rec index depicted the lower values for cytotype 6 of *I. nigrescens* with 67.12 and cytotype 12 of *I. nilgherricus* with 67.83 which means that individual parameters cannot produce a conclusive remark in karyotype asymmetry studies. Combinations of various parameters are required to identify the karyotype asymmetry as well as evolutionary advancement within a genus.

Variation in the centromere position in a chromosome complement are studied using the five indices namely TF%, Syi, As K%, A_1 and A. Rec and A_2 indices are in particular used to evaluate the variation in chromosome length in a complement (Paszko, 2006). Analysis reveals that As K% index has a perfect negative correlation with the TF% and the Syi indices. As K% are commonly employed to identify the phylogenetic relations between and within the genera. Scrutiny of chromosome morphology revealed that the As K% value was almost similar in the normal chromosome count of *I. nigrescens* and *I. nilgherricus* with 57.04 and 57.16 while *I. coetsa* showed slight variation with 56.31. Higher values of As K% is considered as a sign of increasing asymmetry of karyotype. Thus, all the parameters assessed leaving

behind A_1, A_2 and A points to the fact that the normal chromosome count of 24 when compared to the variant cytotypes has intermediary values in karyotype asymmetry. The median values provide evidence for the role of the species in evolution wherein the three species are in a path of evolutionary advancement. From the data obtained, it can be confirmed that the three species are not in a primitive stage of evolution.

Karyotype asymmetry was quantified and graphically represented with the help of Zarco's asymmetry indices A_1 and A_2 which determines the most asymmetric karyotype within a population. A_1 corresponds to the arm ratio of each pair of homologous chromosomes while A_2 deals with Pearson's coefficient of dispersion related with the different lengths of the chromosomes. High value of A_1 indicates the asymmetry of karyotype while high value of A_2 reflecting the amplitude of chromosome size depicts karyotype symmetry. A value also indicates the degree of asymmetry of karyotypes. Normal chromosome complements of three species of *Isodon* when compared based on A_1-A_2 and A indices revealed that *I. nigrescens* possess the most asymmetric karyotype while *I. coetsa* has the most symmetric one. The karyotype asymmetry of *I. nilgherricus* was intermediate since it had high A_2 value when compared to *I. nigrescens*. The scatter plot based on A_1-A_2 parameters also illustrated the highest degree of asymmetry for *I. nigrescens* and lowest for *I. coetsa* which means that *I. nigrescens* is the most evolved species among the three species studied (Fig. 11). It was also well supported by the small size of chromosomes which is an advanced feature in chromosome morphology.

Primitiveness of karyotypes are expressed by the presence of symmetrical karyotypes, longer chromosomes, median centromere, equal length of chromosome arms and low basic chromosome numbers while the more advanced karyotype features depicts asymmetrical karyotypes, shorter chromosomes, submedian or above centromeres, unequal length of

chromosome arms and higher basic chromosome numbers. The criteria for asymmetry in karyotype are comparatively fulfilled by the genus *Isodon* with high basic chromosome numbers and shorter chromosomes along with nearly median centromeres for majority of chromosomes and unequal arm ratio. The other parameters discussed about the asymmetry of karyotype of *Isodon* including the A_1-A_2 and A indices confirms the advanced status of the chromosomes of the genus in the path of evolution.

Characterization of genomes are possible based on three important parameters *viz*., position of centromere, location of secondary constriction as well as the variation in size between chromosomes in a genome which are easily represented as karyograms and idiograms based on the data obtained during mitotic metaphase (Stace, 2000). These factors help in the establishment of a karyotype for a particular species which can be utilized to develop a cytological identity and statistical data in comparative cytotaxonomy. Moreover, the phylogenetic relationship among flowering plants can be brought to limelight only through karyomorphological studies (Iwatsubo and Naruhashi, 1991a). The genus *Isodon* is one of the poorly investigated taxa as far as karyotype analysis is concerned due to the presence of small chromosomes and unclear centromeres. The selected three species of *Isodon* were analysed for its karyomorphological features and to identify the trend in evolution. The various parameters explored revealed the comparatively primitive symmetric karyotype of *I. coetsa*, advanced asymmetric karyotype of *I. nigrescens* and more or less intermediate status of *I. nilgherricus*. Differences in karyomorphological details between individuals with same chromosome number were observed indicating the ongoing evolutionary process even at the micro level. Hence, the members of the genus can be considered as a population with heterogeneous character and an active partaker in the ongoing process of evolution and speciation.

ANTIOXIDANT STUDIES

Herbal formulations are being exhaustively explored for the preparation of effective drugs in the treatment of various ailments. Curable properties of drugs are surpassed by its side effects, development of resistance and ineffectiveness to supplement the other metabolic activities switched on. Cancer and chemotherapy is one such process where the effectiveness is flattened due to oxidative stress. Cell signalling, energy production as well as bactericidal action of phagocytes are some of the processes employing free radicals and other reactive oxygen species. Thus, reactive oxygen species can be considered essential for life and is being produced as a consequence of mitochondrial respiration in all respiring organisms (Conklin, 2000). The excess of free radical production induces oxidative stress which is extremely harmful to cells. Chronic health problems including cancer, atherosclerosis and aging are found to be associated with the free radical induced peroxidation of membrane lipids and oxidative damage of DNA (Halliwell and Gutteridge, 1999) while the gene transcriptions are influenced by the oxidants, antioxidants and other determinants of the intracellular redox state (Cai *et al*., 2003).

The imbalance in the antioxidant status due to the production of reactive oxygen species as well as defense and repair mechanisms results in the production of oxidative stress in human beings (Antolovich *et al*., 2002). Gutteridge (1994) defined antioxidant to be a compound which significantly inhibits or delays the oxidation of the substrate at low concentrations than the oxidizable substrate. A perfectly balanced antioxidant system is always necessary in building up a healthy environment and maintaining metabolic equilibrium. Two classes of antioxidants can be recognized, in general, which includes primary or chain breaking antioxidants and secondary or preventive antioxidants (Jadhav *et al*., 1996). The primary antioxidants are found in trace amounts affecting initiation or propagation step which either delays or inhibits

the initiation step by reacting with a lipid radical or inhibits the propagation step by reacting with peroxyl or alkoxyl radicals. The secondary antioxidants are compounds which retard the rate of oxidation employing several methods including removal of substrate or singlet oxygen quenching (Frankel and Meyer, 2000). Antioxidants, whether primary or secondary, are necessary for the removal of excessive free radicals produced in the body.

It is well evident that reactive oxygen species play a significant role in the initiation and promotion phases of carcinogenesis (Borek, 2004; Senthilkumar *et al*., 2008). Some of the major reactive oxygen species which produce excessive oxidative stress are free radicals such as superoxide ions (O_2^-), hydroxyl radicals (OH^-), nitric oxide radical (NO), singlet molecular oxygen, peroxynitrite radicals as well as hydrogen peroxide (H_2O_2). The antioxidant defenses of cancer cells are usually overcome by the high level of oxidative stress produced by chemotherapy resulting in reduction of lipid peroxides or halting of cancer cell proliferation and thus ending in antineoplastic activity (Conklin, 2000). Antioxidant systems needs to be provided for the neutralization of the oxidative stress induced.

Anticancer drugs are generally cytotoxic and hence can be active only against proliferating cells. The oxidative stress produced by chemotherapeutic drugs results in the inactivation of cell proliferation and thus becomes ineffective in destroying cancer cells. This may be one of the major reasons behind recurrent cancer growth after anticancer therapy. It is observed that tumor cells in G_0 non-proliferative status are literally unmoved by anticancer drugs resulting in unresponsive state to chemotherapeutic drugs (Conklin, 2000). The circumstances are repeated regarding the case of chemotherapy drugs producing oxidative stress. The proliferating cancer cells enter non-dividing status ending in an insensitive response towards anticancer drugs. Thus, provision of antioxidant systems along with chemotherapeutic

drugs can only be a solution towards maintaining cells in proliferative condition and as a consequence of which it can be destroyed.

Efficiency of anticancer drugs needs to be reinvestigated since oxidative stress stands as a hindrance in the path of fruitful treatment. Antioxidant supplements needs to be provided to overcome the oxidative stress which thereby can forego the mechanism of non-proliferation of cancer cells. Deterrence of damage caused by reactive free radicals to cells and cellular components is renowned as the primary biological responsibility of antioxidants (Masuoka *et al*., 2006). Thus, positive results of antitumor effects from chemotherapeutic drugs can only be achieved through the enhancement with antioxidant systems. Herbal drugs are the warehouse of antioxidant potential and therapeutic value. Natural compounds with excellent capacity to surmount the oxidative stress along with anticancer activity can produce a ray of hope in anticancer research. Plants identified with potential chemotherapeutic value are evaluated for its antioxidant capacity for the development of efficacious drugs.

DPPH radical scavenging assay

The scavenging effects of the methanolic extracts and standard were measured from the bleaching of the purple coloured methanol solution using the stable free radical, 2,2-diphenyl-1-1-picrylhydrazyl (DPPH). It is a substrate free assay and extensively used due to the simplicity and speed of reaction. The antioxidants transfer either an electron or a hydrogen atom to DPPH resulting in the neutralization of the free radical (Yamaguchi *et al*., 1998). The reduced DPPH-H (diphenylhydrazine) molecules were visualised from the colour transition of purple to yellow and was recorded spectrophotometrically. It was observed to be stoichiometric with respect to number of electrons captured. The radical scavenging activity was expressed

as the percentage reduction of the initial DPPH absorbance and the DPPH radicals was quenched by the extracts in dose dependent manner.

The antiradical activity of DPPH was measured on the assumption that inhibition of oxidation results mainly by the detainment of initiating or propagating free radicals in auto oxidation (Antolovich *et al*., 2002). The methanolic extracts of the three species of *Isodon* were found to be effective free radical scavengers when evaluated with the standard (Tables 17-20; Figs 12-15). Ascorbic acid, a good antioxidant was used as the standard which exhibited efficient DPPH radical scavenging activity with an IC_{50} value of 5.26 ± 0.01 µg/ml (Table 16). The antiradical activity of the methanolic extracts were comparable to standard with the highest radical quencher being *I. coetsa* with an IC_{50} value of 9.32 ± 0.11 µg/ml followed by *I. nilgherricus* with an IC_{50} value of 11.04 ± 1.80 µg/ml and *I. nigrescens* with an IC_{50} value of 22.59 ± 0.26 µg/ml (Table 16). The free radical scavengers contribute an electron resulting in the reduction of the absorbance of DPPH radical which was found to be directly proportional to the amount of antioxidants present in the reaction mixture.

Hydroxyl radical scavenging assay

The hydroxyl radical is an exceptionally reactive species which can induce oxidative damage to DNA, lipids and proteins (Spencer *et al*., 1994). Thiobarbituric acid reacting substances (TBARS) method was utilized to determine the radical scavenging of hydroxyl radicals generated by the Fenton reaction. Oxidative degradation of deoxyribose was detected by heating the products with TBA under low pH to develop a pink chromogen (thiobarbituric acid reactive species) which was detected spectrophotometrically (Cheng *et al*., 2003).

In free radical pathology, hydroxyl radical is measured as the most potent antioxidant as well as the major damaging species (Sheeja *et al*., 2011). The hydroxyl radicals were produced by the direct addition of iron (II) salts to the reaction mixture which attacks deoxyribose ultimately resulting in the formation of TBARS. The antioxidant compounds were found to compete with deoxyribose and diminish the chromogen formation in a dose dependant manner (Tables 21-24; Figs 16-19). α-Tocopherol, a good antioxidant was used as the standard which exhibited efficient hydroxyl radical scavenging activity with an IC_{50} value of 6.86 ± 0.02 µg/ml (Table 16). The antioxidant activity of the methanolic extracts were more effective than the standard with the highest radical quencher being *I. nigrescens* with an IC_{50} value of 1.19 ± 0.01 µg/ml followed by *I. coetsa* with an IC_{50} value of 1.25 ± 0.01 µg/ml and *I. nilgherricus* with an IC_{50} value of 5.29 ± 0.11 µg/ml (Table 16). The methanolic extracts of the three species of *Isodon* showed excellent free radical scavenging activity. The hydroxyl radical scavenging potential was found to be efficacious, even higher than that shown by the standard and is in direct correlation with its antioxidant ability.

Superoxide radical scavenging assay

Superoxide radicals are placed under the class of free radicals which are essential in metabolism but can be harmful when converted to other reactive oxygen species. Normal physiological processes results in the production of superoxide radicals in mitochondria which can react with sensitive and critical cellular targets. Superoxide anion radical ($O_2^{\cdot -}$) generation occurs by four-electron reduction of molecular oxygen into water or as a consequence of electron leakage from the electron transport chain in aerobic cells. It is also produced by the activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils since $O_2^{\cdot -}$ generation is imperative in the killing of bacteria by phagocytosis. $O_2^{\cdot -}$ radicals thus formed

are eliminated from the living systems by the enzymes called as superoxide dismutases (SOD) (Halliwell and Gutteridge, 1999; Packer and Glazer, 1990). Superoxide anion radical are at a high risk of getting converted to other harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radical which impair biomolecules causing tissue damages and chronic diseases (Al-Mamun *et al*., 2007).

Superoxide radical scavenging activity of the methanolic extracts was determined by the riboflavin photoreduction method. Superoxides generated by the auto oxidation of riboflavin in presence of light were effectively inhibited by the addition of varying concentrations of extracts as determined from the reduction of NBT (Nitro blue tetrazolium). The methanolic extracts of the three species were found to be effective superoxide radical scavengers when evaluated with the standard (Tables 25-28; Figs 20-23). Ascorbic acid, a good antioxidant was used as the standard which exhibited efficient superoxide radical scavenging activity with an IC_{50} value of 53.35 ± 0.16 µg/ml (Table 16). The antioxidant activity of the methanolic extracts were comparable to the standard with the highest radical quencher being *I. coetsa* with an IC_{50} value of 90.01 ± 0.22 µg/ml followed by *I. nigrescens* with an IC_{50} value of 108.89 ± 0.22 µg/ml and *I. nilgherricus* with an IC_{50} value of 118.83 ± 0.25 µg/ml (Table 16). The radical scavenging activity depicted an increase in antioxidant effect in concentration dependent manner.

Ferric ion reducing antioxidant power assay

The ferric ion reducing antioxidant power assay (FRAP) is a widely suitable method for antioxidant determination *in vitro* and estimates the total antioxidant activity (Antolovich *et al*., 2002). It is a simple and relatively standard assay which is inexpensive and highly reproducible. It involves the

use of antioxidants as reductants in a redox linked colorimetric method and employs an easily reduced oxidant system present in stoichiometric excess. The ferric reducing ability of biological fluids and aqueous solutions of pure compounds are commonly determined by FRAP assay (Pulido *et al*., 2000).

The change in absorbance due to the formation of a blue coloured Fe (II)-tripyridyl triazine compound from the colourless oxidized Fe (III) in acidic medium was determined spectrophotometrically. Fe (III) reduction is an indicator of electron donating activity by the antioxidants. Colour formation represented the reducing ability of the sample owing to the presence of excess Fe (III) and Fe (II) -TpTz being the rate limiting factor (Benzie and Strain, 1996). The ferric ion reducing activity of the methanolic extracts were determined and the values were calculated from the regression equation (Fig. 24) as equivalent concentrations of $FeSO_4.7H_2O$. The ferric reducing activity of 1 µg/ml of the methanolic extracts was equivalent to the reducing power of 12.28 ± 0.40 µmol/ml for *I. coetsa*, 6.98 ± 0.20 µmol/ml for *I. nigrescens* and 6.24 ± 0.13 µmol/ml for *I. nilgherricus* of $FeSO_4 \cdot 7H_2O$ (Table 29). Reducing equivalents were found to be increasing with increasing concentration of the extracts. Higher the equivalent concentration, lower was the reducing capacity. Thus, the highest antioxidant activity was shown by *I. nilgherricus* followed by *I. nigrescens* and *I. coetsa*.

Test for antioxidant activity can be enviable only if the substrate and the conditions can replicate the original situation and the results can be interpreted and quantified with reference to an efficient standard (Antolovich *et al*., 2002). The capacity of the antioxidant in controlling the extent of oxidation is generally measured as the antioxidant activity. The methanolic extracts of three species of *Isodon* when subjected to DPPH, hydroxyl radical, superoxide radical and FRAP assays depicted good antioxidant activities with exceptional effect for hydroxyl radical scavenging assay *i.e*., activity even

greater than the standard. Each extract showed good antioxidant capacity which cannot be compared as a whole since responsive reducing capacity of each varied per assay. Being members of folk medicines, the effect showed by the extracts of plants can be considered as excellent for a positive future in drug research.

Reactive oxygen species are produced in the living systems naturally as part of metabolic processes and are prevented from being deleterious by means of variety of antioxidants such as glutathione, α-tocopherol, ascorbic acid, superoxide dismutase, catalase, peroxidase, *etc*. which are produced within cells (Griffin and Bhagooli, 2004). But the natural antioxidant defense fails when the oxidative stress produced as a consequence of diseases and various curative drugs become irrepressible. The protective enzymes such as superoxide dismutase, catalase and peroxidase are devastated when an excess of free radicals are produced which results in destructive and lethal cellular effects (*e.g*., apoptosis) by oxidation of membrane lipids, cellular proteins, DNA and enzymes, and eventually ending up in the shut down of the cellular respiration process. The reactive oxygen species also influences cell signaling pathways in ways that are yet to be unraveled (Bae *et al*., 1999; Bauer *et al*., 1999). Thus, for the attainment of normal healthy conditions, there is always a demand for antioxidants from natural sources (Rimbach and De Pascual-Teresa, 2005).

Medicinal plants are found to possess antioxidant activity which contributes to the curative properties. The free radical scavenging capacity of natural antioxidant agents have attracted considerable attention (Osawa *et al*., 1990) which on consumption have been renowned to be inversely associated with the morbidity and mortality from degenerative disorders (Gülçin, 2012). Plant based drugs with antioxidant potential needs to be promoted in the treatment of various ailments for a constructive outcome. Antioxidants are

found to scavenge the reactive oxygen species or protect the antioxidant defense mechanisms in the fight against free radicals (Umamaheswari and Chatterjee, 2008). The devastating consequences of oxidative stress are by and large prevented from expression or inhibited by the natural antioxidants commonly found in herbs, herbal formulations and spices.

Natural cancer chemopreventive agents are found to possess antioxidant potential (Senthilkumar *et al*., 2008) with the bioactive phytochemicals in biological antioxidants articulating vital roles in the protection of cells from oxidative stress and diseases like cancer (Alessio *et al*., 2002; Cragg and Newman, 2005). The promotion of natural drugs with anticancer activity as well as antioxidant potential can be an effective formulation in the treatment of the dreadful disease. But, the factors on which the effectiveness of such herbs in tumor treatment depends on are the nature of antioxidants as well as the prevalent conditions of PO_2 in the various tissues (Ferreira and Matsubara, 1997; Borek, 2004). The natural antioxidants which may be herbs or dietary supplements needs to be exhaustively analyzed for identification of its activity against free radical scavenging systems prior to application.

Antioxidant supplements are required for the effectiveness of anticancer therapy. Cancer cells should be prevented from entering G_0 stage for the effective cytotoxic activity of chemotherapeutics. In addition to this, nature of antioxidants as well as the prevalent conditions of PO_2 should be identified for the effective outcome of antioxidant combined chemotherapy. Synergistic activities of antioxidant supplements and anticancer drugs need to be studied for the identification of proper medication in diseases like cancer. Herbs with antioxidant activity as well as anticancer effects are extensively identified from traditional medicines which need to be properly investigated for an efficacious drug. It reduces the burden of dual consumption of

antioxidants and drugs as well as improves the chances of production of complementary activity in ailment of diseases and against various metabolic irregularities. Thus, antioxidant potential of various herbs used as anticancer agents needs to be identified for the formulation of a proper cure against cancer.

The members of the genus *Isodon* are traditionally well known in cancer therapy. The three species belonging to the genus when investigated for the antioxidant potential exhibited significant activity compared to the standards which promotes them as apt candidates in anticancer research. Hence, the plants necessitate to be explored for the activity against the dreadful cancer disease as well as for the identification of the phytochemical compounds present to substantiate its use as anticancer drug in folk medicines.

ANTITUMOR STUDIES

The anticancer potential of the three species of *Isodon* was established using *in vitro* cytotoxic and *in vivo* antitumor assays. Generally, the anticancer drugs are cytotoxic which makes it effective against proliferating cells. Selection of plant extracts with essential antineoplastic properties can only be possible through preliminary data procured from cytotoxicity screening models (Cardellina *et al*., 1999). A crude plant extract proved cytotoxic through *in vitro* assays when replicates the activity in *in vivo* models as antitumor/anticancer effect provides substantial scientific basis for the use of the species in cancer therapy, which had already been advocated by the folk medicine practitioners. Natural products identified in cancer chemotherapeutics from folk medicines are the prime source of biomedical research. It calls for more efficient and time saving scheme when compared with random selection of herbs from any particular geographic area.

Cytotoxic assays

Tumor cells are characterized by the uncontrolled proliferation which needs to be restrained for the survival of the affected organism. Cell death is an important event in the cell cycle which when stimulated at an early stage controls the unproliferative growth of cancer cells. Cell death occurs due to diverse factors and can be classified based on morphological appearance, enzymological criteria, functional aspects or immunological characteristics (Melino, 2001). The two distinct phenomenons *viz*., apoptosis and necrosis are considered to be the core in cell death mechanism. Apoptosis is a specific morphological facet of cell death exemplified by rounding up of the cell, retraction of pseudopodes, pyknosis, chromatin condensation, karyorrhexis, little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing and engulfment by resident phagocytes (*in vivo*) while necrosis is morphologically distinguished by oncosis, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Kroemer *et al*., 2009). Cytotoxic activities are found to illustrate concomitant results with antitumor studies. Hence, cytotoxicity based assays are employed which have the added advantage of simultaneous determination of the mechanisms associated with cell proliferation and cell death.

Cell based screening assays determines the capacity of a plant species in anticancer research. Cytotoxic assays indicting number of dead cells, viability assays determining numbers of live cells, estimation of total number of cells as well as identification of the mechanism of cell death such as apoptosis are the critical aspects in enumerating the anticancer effect of plants or plant based drugs. Viability measurements of cancer cell lines are determined at the end of exposure period and are inevitably followed by induction of apoptosis, reduction in ATP concentration of the cell and destabilization of membrane integrity (Riss, 2005). Loss of membrane

integrity ends up in the failure to synthesize ATP in cells as well as in the destruction of remaining ATP by endogenous ATPases thus causing a sudden and abrupt fall in the amount of ATP levels.

Cell death phenomenon and cytotoxic assays can be generally relied upon for cytotoxicity determination of plant based extracts which was attempted using Evans blue staining technique as well as Trypan blue exclusion assay. Evans blue staining determined the apoptotic cells in *Allium cepa* cells while Trypan blue exclusion assay on DLA and EAC cell lines determined the number of dead cells due to the effect of the extract which exclusively confirms the cytotoxic activity of the plant species on a prelude basis.

Cell death determination

An important pathway of anticancer agents in the inhibition of tumor cells is apoptosis. The apoptotic program of cell death is characterized by certain morphological features, such as loss of membrane asymmetry and detachment, condensation of the cytoplasm and nucleus, DNA fragmentation as well as the poly(ADP-ribose) polymerase degradation (Lazebnik *et al*., 1994). Apoptosis occurs as a consequence of complex signal transduction pathways and ends in gene mediated cell death (Kanadaswami *et al*., 2005). Cytotoxicity can be measured employing Evans blue staining of root meristems since it is a cell death parameter. Evans blue is a non-permeating stain but can penetrate through the plasma membrane of a dead cell and is considered as a marker of plasma membrane degradation and cell death (Peterson *et al*., 2008). *Allium* assays are generally utilized for the detection of cytotoxic and mutagenic activities of several anticancer drugs of plant origin (Kuraś *et al*., 2006). The results obtained from *Allium* tests are commonly found to correlate with the effects shown by mammalian systems and it has an added advantage of the existence of an oxidase enzyme system

which is indispensable for promutagen evaluations (Fiskesjö, 1985). Evans blue staining of *A. cepa* root meristems treated with the extracts of three species of *Isodon* was thus conducted and the cytotoxic effects were identified.

Induction of cell death by the extracts exhibited significant cytotoxic activity in root meristems of *A. cepa* which varied in dose dependent manner. The highest activity was reported for *I. nilgherricus* followed by *I. coetsa* and *I. nigrescens* over a concentration range of 10-40 mg/ml (Table 30; Fig. 25). The results indicated the loss of membrane integrity in plant cells and the consequent increase in cell death which established the potential of the plants as cytotoxic agents. Poly(ADP-ribose) polymerase is a DNA damage sensor which when activated by DNA break, direct the cells to enter repair or programmed cell death based on the severity of damage and amplitude of activation (Briggs and Bent, 2011). Cell death assay based on Evans blue staining indicates the plasma membrane disintegration by the extracts (Baker and Mock, 1994) and has been shown to be a reliable measure of programmed cell death in rice seedlings (Chang *et al*., 2005). Cytotoxic activity of the extracts might have resulted in DNA damage leading to cell death phenomenon which was detected in Evans blue staining. It implies the fact that the extracts of three species of *Isodon* have the ability to destroy cells which can be exploited in the case of cancer cells and developed as excellent anticancer drugs. This was a preliminary assay which needs to be tested in cell lines and *in vivo* models for a more confirmed outcome.

Trypan blue exclusion assay

Cell viability assays are conducted in cancer cell lines to determine the cytotoxic activity of extracts in destroying or inducing cell death mechanisms which can be fruitful in elimination of cancer cells as well as its proliferative growth. *In vitro* viability or proliferation of cells can be determined using

various assays of which the most widely accepted is the permeability assay. Staining damaged cells with a dye and counting the viable cells that exclude the dye forms the basis of permeability assay. It is a quick, inexpensive method and requires only a small fraction of total cells from a cell population (Schulze-Osthoff, 2008). Dye exclusion assay employing Trypan blue is one of the most common permeability assay in which the cell viability is estimated based on cell membrane integity (Jurisic and Bumbasirevic, 2008). The dye, Trypan blue, stains the cells which has a deteriorated or lost plasma membrane integrity and excludes the viable undamaged cells. It helps in the identification of number of dead cells due to the effect of the extracts which determines the depth of cell death in cancer cell lines.

The two cell lines *viz*., Dalton's Lymphoma Ascites tumor cells (DLA) and Ehrlich's Ascites Carcinoma cells (EAC) were subjected to Trypan blue exclusion assay after treating with different concentrations of the methanolic extracts of three species of *Isodon*. DLA is a poorly differentiated malignant tumor occuring as lymphocytes in mouse while EAC is an undifferentiated rapidly growing malignant tumor coming under the class of carcinoma and can be proliferated in all strains of mice (Jayaseelan *et al*., 2012). The cell lines when treated with the three methanolic extracts depicted significant cytotoxic activity which increased in concentration dependent manner (Figs 26, 27). The most effective cytotoxic activity against DLA cell line was shown by *I. nilgherricus* with an IC_{50} value of 0.82 ± 0.01 mg/ml followed by *I. coetsa* with an IC_{50} value of 1.21 ± 0.02 mg/ml and *I. nigrescens* with an IC_{50} value of 1.60 ± 0.01 mg/ml (Table 31). The highest cytotoxic activity against EAC cell line was shown by *I. nilgherricus* with an IC_{50} value of 0.89 ± 0.01 mg/ml followed by *I. coetsa* with an IC_{50} value of 1.36 ± 0.01 mg/ml and *I. nigrescens* with an IC_{50} value of 1.51 ± 0.01 mg/ml (Table 31). The *in vitro* cytotoxicity of the extracts showed same depth of

activity when plant species are compared but the concentrations showed slight variation.

Differential staining cytotoxicity assay based on the concept of cell death of the entire population of tumor cells is the most versatile cell death end points. It has wide applications in specimens where a pure population of highly enriched tumor cells cannot be obtained, in determining solid and haematologic neoplasms as well as in a wide variety of drugs ranging from traditional cytotoxic agents, biological response modifiers, targeted kinase inhibitors and even antivascular agents (Weisenthal, 2011). Exclusion dyes are employed in differential staining where viable cells with intact plasma membranes and dead cells with damaged membranes can be distinguished. Trypan blue is the extensively used assay for staining dead cells which measures cytotoxicity based on the alterations of plasma membrane permeability and the consequent release of components into the supernatant or by the uptake of dyes excluded by viable cells (Schulze-Osthoff, 2008).

Cytotoxic screening using *in vitro* assays on test systems of *A. cepa* and DLA, EAC cell lines revealed the potent efficacy of the methanolic extracts of all the three species of *Isodon*. It is well supported by the cytotoxic activities of isolated compounds and extracts of *Isodon* previously reported in literature (Sun *et al*., 2006). Apoptosis is a common phenomenon of cell death produced in response to various stimuli and is characterized by membrane blebbing, cytoplasmic and nuclear shrinkage, and nuclear DNA fragmentation due to endonuclease activation (Momtazi-Borojeni *et al*., 2013) and it reorganizes the life cycle of a cell. Normal apoptosis is the end point of a healthy cell while defective apoptosis can become the reason for various diseases including cancer. Moreover, the proliferative growth of cancer cells are controlled and destroyed by the same event of apoptosis. Thus, apoptosis is utmost necessary in the well being of an organism. It is an indicator of

tumor diagnosis as well as proliferation which was identified using various assays on treatment with plant derived extracts. The results showed efficacy of drugs in inducing apoptosis *in vitro* which is a sign of the potentiality of the plants. The *in vivo* assays were conducted for validation of the activity which authenticates the use of the plants in folk medicines as well as its caliber in advanced pharmaceutical research.

Antitumor assays

The *in vitro* cytotoxic activity exhibited by the methanolic extracts of three species of *Isodon* prompted the testing of antitumor activity in *in vivo* models. Cancer is the most devastating group of disease ever occurred on earth arising from a variety of identified and unidentified factors, affecting every possible organ and characterized by uncontrolled growth and metastasis. It is a hyper proliferative disorder associated with mechanisms of transformation, proliferation, invasion, sustained angiogenesis and metastasis (George and Kuttan, 2012) and hallmarked by self sufficient growth signals, resistance to growth inhibitory signals as well as apoptosis (Siveen and Kuttan, 2011). The common pathogenic mechanisms linked to the disease are DNA damage, oxidative stress and chronic inflammation (De Flora and Ferguson, 2005) which are more likely to be expressed in persons with genetic backgrounds of familial prevalence or genes associated with certain cancers. Infections from chronic/oncogenic viruses, exposure to carcinogenic chemicals as well as environmental and lifestyle factors such as tobacco smoking, alcohol consumption, excess use of caffeine and other drugs, sunshine, exposure to asbestos coupled with the failure of the immune system to eliminate cancer cells at an early stage are the major causes identified in mammalian cancer (Sakarkar and Deshmukh, 2011).

Immortality of cancer cells promote the uncontrolled growth, invading nearby tissues and ending up in metastasis. The most lethal aspect of

carcinogenesis is metastasis (McNutt, 1995). Chemotherapy, radiotherapy and surgery are accepted treatments of cancer but surgery cannot be a solution in metastatic phase. One of the crucial therapeutic strategies for the disease to be in control is to trigger the cellular suicide pathway and induce tumor cell apoptosis in response to the stimuli from various chemotherapeutic drugs (Su *et al*., 2009). The antineoplastic drugs, in general, follow the mechanisms of destruction of DNA, inhibition of nucleic acid and protein synthesis as well as interfere with the hormonal balance for the control of the disease. The role of chemotherapeutic drugs can thus be considered as to slow down and finally halt the growth and spread of cancer cells (Murali and Kuttan, 2011). Hence, a cancer chemopreventive agent is required to eliminate the damaged or malignant cell *via* cell cycle inhibition or apoptosis and is expected to be less or not at all toxic to normal cells (Srivastava and Gupta, 2006). Herbal formulations with anticancer properties are found to be less toxic which are the focus of biomedical research for the development of effective chemotherapeutic drugs.

Cytotoxic activities of herbs are indictors of the hidden potential of anticancer effects which requires exploitation. The application of herbal medicines as chemotherapeutic drugs involves prior testing with *in vivo* models as well as clinical trials. *In vivo* models include various animal strains which can most technically provide an idea about the effect of drugs since the pathway of cancer has been laid down through studies conducted in different strains of rodent models. A cancer chemotherapy agent can only be developed following a sequence of steps which includes *in vitro* studies, rodent studies, pharmacology studies and toxicology studies (Venkatesh and Lipper, 2000). The toxicology studies for the identification of safety levels of administration of the drug to humans are beyond the echelon of normal *in vivo* assay. Animal models have an established role as an evaluation tool for the prediction of carcinogens and investigation of carcinogenic mechanisms (Corbett *et al*.,

2004) of which mice models are the most attractive due to low cost, ease of handling as well as identified genetic information (Cheon and Orsulic, 2011). A preclinical tumor model can be found to be effective only when the treatment exhibits decreased tumor burden, decreased tumor-associated morbidity, improved quality of life as well as lengthened life span, irrespective of the host species (Hollingshead, 2008). Thus, different tumor models are employed to obtain an authenticated result regarding the efficacy of the herbs used as anticancer drugs in folk medicines.

DLA induced solid tumor model

Dalton's Lymphoma Ascites is a transplantable poorly differentiated malignant tumor growing in both solid and ascitic forms and found in mouse as lymphocytes (Kleinsmith, 2006). Lymphoma affects lymphocytes and the lymphatic system which includes spleen, thymus, liver as well as other lymphatic tissues (Kalaiselvi *et al*., 2012). DLA induced solid tumor model of Swiss albino mice was treated with the methanolic extracts of three species of *Isodon* and compared with the mice treated with standard drug cyclophosphamide (Plates 11-13). The changes in the tumor volume, body weight as well as the haematological parameters of each group of mice were monitored to identify the effectiveness of the administered drug.

Methanolic extracts of the three species of *Isodon* exhibited significant reduction in tumor volume with the highest antitumor activity shown by *I. coetsa* followed by *I. nigrescens* and *I. nilgherricus* (Tables 37-39; Figs 31-33). The percentage inhibition of tumor volume of *I. coetsa* was found to be 95.46 ± 1.03 for 25 mg/kg b. wt and 97.82 ± 0.29 for 50 mg/kg b. wt (Table 34) while the body weight of the animals showed a percentage increase of 4.45 ± 0.21 and 1.73 ± 0.68 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. The percentage inhibition of tumor volume of *I. nigrescens* was found to be 88.90 ± 1.41 for 25 mg/kg b. wt and 96.82 ± 0.41 for 50 mg/kg

b. wt (Table 35) while the body weight of the animals depicted a percentage increase of 5.94 ± 0.31 and 0.78 ± 0.24 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. The percentage inhibition of tumor volume of *I. nilgherricus* was found to be 73.94 ± 2.36 for 25 mg/kg b. wt and 96.19 ± 1.11 for 50 mg/kg b. wt (Table 36) while the body weight of the animals showed a percentage increase of 10.18 ± 0.59 and 5.73 ± 0.59 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. The variations could be observed in the haematological parameters also which illustrated the general health condition of the animals.

Efficacy of preclinical studies in mice is easily detected by the measurement of tumor burden and by figuring out the volume from the measured values (Schuh, 2004). Monitoring of the tumor volume along with the increase/decrease in body weight helps in the determination of the progression and decline of developed disease. The mice induced with tumor alone showed excellent growth which made the animal difficult in mobility since the tumor was injected and developed on the hind limb. Chemotherapy drug, cyclophosphamide which belongs to the class of alkylating agents, was used as the standard drug for comparison. The mice administered with the standard drug showed excellent activity with the tumor burden almost reduced, and externally the hind limbs were observed normal. The percentage increase in body weight was very less which can account for the side effects of the standard when discussed along with haematological parameters. The high doses of the extracts *i.e.*, 50 mg/kg b. wt also showed excellent activity in comparison with the standard. The tumor volume was found to increase in subsequent days of DLA induction but was found to decrease gradually on latter days which were revealed during the medieval period through increase in body weight. Low doses of the extracts *i.e.*, 25 mg/kg b. wt also showed significant activity but was comparatively less for *I. nigrescens* and *I. nilgherricus*. The efficacy of *I. coetsa* was at par with the standard drug for

both low and high doses while the three extracts at high doses at times had activity greater than the standard.

Tumor development influences haematological parameters especially haemoglobin and WBC content and antitumor activity is generally evaluated by the restoration of the normal count compared to control. Cancer chemotherapy is always back stamped by myelosuppression and anaemia (Marklund *et al*., 1982; Price and Greenfield, 1958) of which anaemia is an associated trait of cancer (De Vita *et al*., 1993). Reduction in RBC or hemoglobin content occurs in cancer patients due to iron deficiency or haemolytic or other myelopathic conditions (Hoagland, 1982). Elevation in WBC levels is also observed as a result of haematopoitic systems (Bansal *et al*., 2005) which account for the search for better chemotherapeutic drugs. Administration of the standard drug cyclophosphamide was effective in reducing the tumor volume but the mice were found unhealthy. Symptoms of hair loss were noticed along with a reduction in haemoglobin count. The WBC count was found to be normal which depicts that the mice were recovering from the disease but anaemic. Methanolic extracts treated mice were healthier than those treated with standard drug in that the haemoglobin count was found to be gradually restored. Normal haemoglobin count could not be maintained in any of the treated mice while the low dose *i.e*., 25 mg/kg b. wt extracts treated mice had comparatively increased number of WBC (Tables 40-42). This calls for the low activity shown by the three extracts in inhibiting tumor growth. Thus, it can be concluded that the three species of *Isodon* possess significant antitumor activity especially at higher concentrations of 50 mg/kg b. wt and the side effects observed when weighed against the health conditions of mice are negligible with the highest efficacy illustrated by methanolic extracts was for *I. coetsa*.

EAC induced ascites tumor model

Ehrlich Ascites Carcinoma is a rapidly proliferating, undifferentiated carcinoma with aggressive behavior (Segura *et al*., 2000). It is originally hyperdiploid with high transplantable capacity, shorter lifespan, cent percent malignancy and is devoid of tumor specific transplantation antigen (Kaleoğlu and İşli, 1977). It grows in all strains of mice and occurs both in ascites or solid form. Being undifferentiated with rapid growth rate, EAC are most sensitive to chemotherapy and has resemblance with human tumors (Ozaslan *et al*., 2007). Tumor implantation is characterized by local inflammatory reaction, increased vascular permeability resulting in intense oedema formation, cellular migration as well as progressive ascitic fluid formation (Fecchio *et al*., 1990). The nutritional requirements of the tumor cells are fulfilled by the ascitic fluid and are essential for tumor growth (Shimizu *et al*., 2004). EAC induced ascites tumor model of Swiss albino mice was treated with the methanolic extracts of three species of *Isodon* and compared with the mice treated with standard drug cyclophosphamide (Plates 14-16). The increase in lifespan and body weight of each group of mice was monitored to identify the effectiveness of the administered drug.

Methanolic extracts of the three species of *Isodon* exhibited moderate increase in life span with the highest antitumor activity shown by *I. nilgherricus* followed by *I. nigrescens* and *I. coetsa* (Fig. 37). The percentage increase in life span of *I. coetsa* was found to be 17.94 ± 3.72 for 25 mg/kg b. wt and 53.41 ± 7.55 for 50 mg/kg b. wt (Table 43) while the body weight of the animals showed percentage increase of 70.86 ± 10.31 and 89.62 ± 5.26 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. The percentage increase in life span of *I. nigrescens* was found to be 33.92 ± 1.55 for 25 mg/kg b. wt and 85.87 ± 4.55 for 50 mg/kg b. wt (Table 44) while the body weight of the animals depicted percentage increase of

68.25 ± 17.29 and 121.65 ± 15.31 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. The percentage increase in life span of *I. nilgherricus* was found to be 50.10 ± 6.66 for 25 mg/kg b. wt and 75.05 ± 6.23 for 50 mg/kg b. wt (Table 45) while the body weight of the animals showed percentage increase of 107.22 ± 8.09 and 108.31 ± 9.62 for 25 mg/kg b. wt and 50 mg/kg b. wt respectively. The increase in number of survival days illustrated the efficacy of the extracts.

Prolongation of the life span of mice is a reliable criterion for determining the antitumor activity of the extracts (Clarkson and Burchenal, 1965) since in EAC induced tumor, the death of host animal ensues due to the pressure exerted by the tumor volume and/or the devastating effects of the tumor (Aktaş, 1996; Altun, 1996; Öner, 1985). Tumor alone induced mice had very low mean survival days while the life span was comparatively higher for cyclophosphamide treated mice. The extracts showed moderate activity for high doses *i.e*., 50 mg/kg b. wt while the activity shown by low doses of 25 mg/kg b. wt was not much significant. Only *I. nilgherricus* demonstrated good activity for both low and high doses while the activity of low dose of *I. coetsa* was non-significant. The percentage increase of life span for high dose of *I. nigrescens* pointed that it was the only species which had moderately comparable effect with the standard drug. Percentage increase in body weight was very much less compared to control for the standard drug while the extracts showed extreme values. The mice administered with the extracts were found to endure the tumor burden and prolonged its life. Enhanced body weight was due to the increased ascitic fluid which was the source of nutrition of tumor cells. It refers to the unproliferative tumor growth whose nutritional necessity was fulfilled by the ascitic fluid as noticed from the increased body weights (Prasad and Giri, 1994). Moreover, it is reported that the cell viability of the EAC cells do not decrease significantly when it transits from proliferative to plateau phase (Schmidt *et al*., 1991). Hence,

from the data obtained, it can be concluded that the methanolic extracts of *Isodon* possessed only moderate effect chiefly for higher concentration of 50 mg/kg b. wt with significant activity for *I. nigrescens*.

Efficacy studies involving several subtypes are required to determine the extent of activity of the extracts. The therapeutic interventions in tumor models accounts for discovery, target validation and *in vivo* pharmacology as well as examination of therapeutic efficacy, safety and interactions (Schuh, 2004). Clinical evaluation has innate problems of practicability but the therapeutic efficiency of the drugs can provide room for anticancer research through *in vitro* and preclinical studies. Mice models are excellent mediators to define antitumor immune response as well as to identify the disease progression during various therapies. Herbs and herbal formulations were found to enhance the activity of the immune cells of the body thereby promoting the production of cytokines including interleukin, interferon, tumor necrosis factor and colony stimulating factor which enables the body to fight cancer as well as surmount the toxic side effects (Sakarkar and Deshmukh, 2011). The three species of *Isodon* have been proved to be good anticancer agents with reduced side effects and efficiency to combat oxidative stress through the established activity during antioxidant and antitumor studies.

Literature perusal revealed that several species of *Isodon* have reports of clinical efficiency in antitumor studies (Fujita *et al*., 1976; Zou *et al*., 2013). Different phytochemical compounds have been isolated from the genus, majority of which belong to diterpenoids and have shown cytotoxic activity against various cell lines (Tables 2, 3). The antitumor activity have been reported for some of the isolated compounds of enmein, oridonin, lasiokaurin *etc*. (Fujita *et al*., 1976; Zou *et al*., 2013) which attributes the biological activity to various active groups in the basic structure. Presence of α-methylene cyclopentanone system as well as α, β-unsaturated ketone moiety

in the isolated compounds is considered to be responsible for the excellent antitumor activity (Fujita *et al*., 1976; Fujita and Node, 1984; Sun *et al*., 2006). The three species of *Isodon* investigated reported excellent antioxidant activities along with *in vitro* and *in vivo* antitumor potential. The affirmative outcome of the studies conducted prompts for the phytochemical scrutiny of the compounds present in various species which could be a lending hand in reaching out the applicability of an efficacious drug in chemotherapy.

PHYTOCHEMICAL STUDIES

Traditional medicines have always been the shelter for biomedical research whenever a complicated unidentified new disease prop up in the revolutionizing world. From the advent of history, all cultures and civilizations depend on herbal medicine for a successful life due to its effectiveness, affordability, availability, low toxicity and acceptability (Akharaiyi and Boboye, 2010). But, a sophisticated knowledge and scientific authenticity is lacking which makes the modern world stay away from folk medicines. This results in the loss of valuable information of folkloric medicines which might be the reservoirs of curatives of diseases which modern man is incapable of holding off. Biological studies as well as phytochemical validation are necessary for the practical application of an herbal drug in therapeutic medicine. The three species of *Isodon* investigated was proved for their biological properties, revealing anticancer potential through various studies conducted. The phytochemical components need to be identified for the validation of the plants as well as to identify the safety levels for application as a drug. Hence, preliminary phytochemical screening, estimation of flavonoids, phenolics and terpenoids as well as GC-MS and LC-MS analyses were conducted to create a phytochemical profile of the three species.

Serene exploration of the natural products are the need of the hour for the preservation of biodiversity as well as development of efficient herbal pharmaceutics. The use of natural drugs that can reverse, suppress or prevent carcinogenic progression is the major focus in cancer chemoprevention (Tsao *et al*., 2004). Secondary metabolites are important bioactive natural products which are the reservoir of major drug leads that necessitate to be utilized efficiently to develop a successful pharmaceutical profile. The natural compounds isolated from plants have established roles as anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, antiviral and many other activities (Cai *et al*., 2004; Miliauskas *et al*., 2004). Plant derived compounds of magnitude in pharmaceutics include alkaloids, phenols, flavonoids, terpenoids *etc*. having excellent potential as antioxidants and anticancer agents are being focused to obtain a directive in the phytochemistry of the genus.

Preliminary phytochemical screening

Preliminary phytochemical screening led to the identification of detectable amounts of flavonoids, phenols, tannins, steroids and terpenoids in all the three species of *Isodon* (Table 46) which are reported to be compounds with significant bioactive potential in literature. The classes of compounds identified were focused to determine the efficiency of three species of *Isodon* since the biological activity reported showed relevance for further research. The amount of flavonoids, phenolics and terpenoids present in the methanolic extracts were detected for identifying the potentiality of the three species of *Isodon* as source plants.

Determination of phenolic, flavonoid and terpenoid contents

Phenolics are the class of compounds distinguished with one or more aromatic rings associated with one or more hydroxyl groups. Plant phenolics

include phenolic acids, flavonoids, tannins as well as stilbenes and lignans (Dai and Mumper, 2010). In plant tissues, phenolics are distributed non-uniformly with insoluble phenolics at the cell walls and the soluble phenolics within the plant cell vacuoles (Wink, 1997). Phenolics are known to possess significant antioxidant and anticancer potential both *in vitro* and *in vivo*. The mechanism of antioxidant properties of phenolics are mediated either by scavenging radical species such as reactive oxygen species/reactive nitrogen species or by suppressing the free radical formation via inhibition of some enzymes/chelating trace metals involved in free radical production or by upregulating/protecting antioxidant defense (Cotelle, 2001) while the mechanism of phenolics in inhibition of carcinogenesis and tumor growth involves either modification of the redox status or interference with the basic cellular functions (Kampa *et al*., 2007). The chemopreventive effect of polyphenols involves estrogenic/antiestrogenic activity, antiproliferation, induction of cell cycle arrest or apoptosis, prevention of oxidation, induction of detoxification enzymes, regulation of the host immune system, anti-inflammatory activity as well as the changes in cellular signaling (García-Lafuente *et al*., 2009). The antioxidant and chemopreventive effect if utilized can be a boon and rejuvenation in anticancer therapy.

The total phenolic content of the methanolic extract of three species of *Isodon* was estimated using the Folin–Ciocalteu reagent based assay in which gallic acid was used as the standard. The assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes [possibly $\{PMoW_{11}O_{40}\}^{4-}$] (Singleton and Rossi, 1965; Singleton *et al*., 1999). The phenolic content was measured and expressed in terms of gallic acid equivalent using the standard equation obtained from the linear calibration curve ($y = 0.011x + 0.016$, $R^2 = 0.999$; Fig. 38). The total phenolic

content was estimated to be 61.58 ± 0.03 mg GAE/g DW for *I. coetsa*, 33.82 ± 0.05 mg GAE/g DW for *I. nigrescens* and 66.88 ± 0.06 mg GAE/g DW for *I. nilgherricus* (Table 47).

The three species of *Isodon* possessed fair amount of total phenolic content in terms of gallic acid equivalent in the order *I. nilgherricus* > *I. coetsa* > *I. nigrescens*. Phenolics are found to elicit multifaceted cellular mechanism for production of anticancer effects through regulation of growth factor-receptor interactions as well as cell signaling cascades which includes kinases and transcription factors that determine the expression of genes involved in cell cycle arrest, cell survival and apoptosis or programmed cell death (Wahle *et al*., 2010). Phenolics also enhance the immune system of the body and attenuate the adhesiveness and invasiveness of cancer cells for lowering the metastatic potential. The biological activities and mechanisms reported for phenolics ascertain its role in living systems. It helps in maintaining a healthy balanced system and more importantly has significant role in preventing cancer cells from reaching metastatic ends. Presence of phenolics in the three extracts thus affirms the antitumor potential shown in the present study and asserts itself to the world of cancer chemotherapeutics for potential exploitation.

Flavonoids are a major group of secondary metabolites with more than 4000 polyphenolic compounds commonly found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine (Nijveldt *et al*., 2001). Characterized by a common phenylbenzopyrone structure (C6-C3-C6), it is grouped into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols based on the saturation level as well as opening of the central pyran ring (Middleton and Kandaswami, 1993; Harborne and Williams, 2000). Several *in vitro* and *in vivo* studies conducted have elucidated the effectiveness and mechanism of action of flavonoids in combating cancer and tumor progression.

Anticarcinogenic effects of flavonoids are expressed through the inhibition of protein kinases, angiogenesis, interactions with phase I and phase II metabolizing enzymes as well as with the cascade of immunological events which have significant roles in the promotion and proliferation of cancer (Ren *et al*., 2003; Batra and Sharma, 2013). Flavonoids can thus be considered to have a significant role in cancer chemoprevention which can enhance the immune system against cancer. Intake of flavonoids as food or as supplements can help in the promotion of health status of a diseased individual to normal level.

The total flavonoid content of the methanolic extracts of three species of *Isodon* was estimated using the aluminium chloride colorimetric method in which quercetin was used as the standard. The sample fractions after reaction with sodium nitrite developed coloured flavonoid-aluminium complex using aluminium chloride in alkaline medium (Abu Bakar *et al*., 2009). The principle involved is that $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols and also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Bag *et al*., 2015). The flavonoid content was measured and expressed in terms of quercetin equivalent using the standard equation obtained from the linear calibration curve ($y = 0.001x - 0.007$, $R^2 = 0.998$; Fig. 39). The total flavonoid content was estimated to be 144.00 ± 0.58 mg QE/g DW for *I. coetsa*, 152.67 ± 0.88 mg QE/g DW for *I. nigrescens* and 151.67 ± 0.67 mg QE/g DW for *I. nilgherricus* (Table 47).

The three species of *Isodon* possessed high amount of total flavonoid content in terms of quercetin equivalent in the order *I. nigrescens* > *I. nilgherricus* > *I. coetsa*. Flavonoids are polyphenolic compounds which are free radical scavengers with significant antioxidant properties (Dewick, 2002). *In vitro* and *in vivo* studies have also revealed the anticancer

mechanism of flavonoids in combination or individually through carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation as well as reversal of multidrug resistance (Ren *et al*., 2003). Hence, one of the major classes of compounds which enabled the three species of *Isodon* to depict the significant cytotoxic and antitumor potential as well as antioxidant activity in the present study can be considered to be the flavonoids. It calls for the exploitation of these species as potential chemopreventive agents in human therapeutics as well as boosters for a better well being.

Terpenes are the largest and the oldest group of natural products which are biosynthetically constructed from isoprene (2-methylbutadiene) units (Ruzicka, 1953; Gershenzon and Kreis, 1999). Bioactivities of terpenoids include prevention and therapy of diseases like cancer as well as antimicrobial, antifungal, antiparasitic, antiviral, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory, and immunomodulatory properties (Wagner and Elmadfa, 2003; Sultana and Ata, 2008; Rabi and Bishayee, 2009; Shah *et al*., 2009). The antioxidant capabilities of terpene classes are also well known (Grassmann, 2005). The master regulator of immune responses is the NF-κB system which is an ancient signaling pathway in the host defense of multicellular organisms and has a significant role in anti-apoptotic signaling and the development of cellular resistance against apoptosis (Karin and Greten, 2005; Dutta *et al*., 2006). Several terpenoids have been found to possess capacity to inhibit nuclear factor-κB signaling which has a major role in the pathogenesis of inflammatory diseases and cancer (Salminen *et al*., 2008). The excellent antioxidant activity as well as anticancer potential expressed through inhibition of signaling pathways emphasizes the importance and requirement of exploitation of terpenoids in cancer therapy.

The total terpenoid content of the methanolic extract of three species of *Isodon* was estimated using an assay in which linalool was used as the standard. The terpenoid content was measured and expressed in terms of linalool equivalent using the standard equation obtained from the linear calibration curve ($y = 0.005x - 0.006$, $R^2 = 0.987$; Fig. 40). The total terpenoid content was estimated to be 221.87 ± 0.67 mg linalool/g DW for *I. coetsa*, 485.20 ± 1.15 mg linalool/g DW for *I. nigrescens* and 269.87 ± 0.67 mg linalool/g DW for *I. nilgherricus* (Table 47).

The three species of *Isodon* possessed high amount of total terpenoid content in terms of linalool equivalent in the order *I. nigrescens* > *I. nilgherricus* > *I. coetsa*. Different terpenoids are found to possess a wide spectrum of activities such as reduction in oxidative stress, suppression of inflammation, induction of apoptosis, regulation of cell cycle, inhibition of cell proliferation and also modulation of multiple signal transduction pathways (Thoppil and Bishayee, 2011). All plant organs possess terpenoid synthases which are expressed either constitutively or *via* induction during biotic stress (Tholl, 2006). The biological systems of terpenes are complex than phenolics and flavonoids, and own specific mechanism of action for each group of terpenes. Sesquiterpenoids are found to be excellent inhibitors of NF-κB signaling while the diterpenoid and triterpenoid class have potent inhibitors but not equivalent to sesquiterpenoids (Salminen *et al*., 2008). Terpenoid class when present in plants holds excellent potential in therapeutics. The high content of terpenoids in the three species of *Isodon* also figured out the therapeutic multifaceted potential in the plants along with the antioxidant and anticancer activities expressed in the biological assays.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis is an ideal technique employing a combination of the principle separation technique of gas chromatography with the best

identification technique of mass spectrometry. The analysis involves qualitative and quantitative determination of volatile and semi-volatile compounds (Karthishwaran *et al*., 2012). The methanolic extracts of three species of *Isodon* were subjected to GC-MS analysis for the determination of the various compounds present (Figs 41-44). A total of 136 compounds were detected from the three species (Table 48) which were grouped into 14 classes of compounds *viz*., terpenoids, phenols, alkaloids, steroids, flavonoids, fatty acid esters, vitamins, furans, aromatics, alkanes, aldehydes and ketones, alcohols, organic compounds and other miscellaneous compounds (Table 49).

Fifty six compounds were identified from the methanolic extract of *I. coetsa* which included 5-methylsalicylaldehyde (13.54%), 1,2,3,4,8,9,10,11-octahydropyrido[2,1-f][1,6]naphthyridin-6-one (9.89%), α-tocopherol (9.28%), phytol (7.64%), 2-naphthyl myristate (6.49%) and 3,5,7-triamino-1-azaadamantane (6.27%) as the major components. The methanolic extract of *I. nigrescens* revealed the presence of fifty nine components of which the major components detected were phytol (20.87%), α-tocopherol (15.72%), p-vinylguaiacol (9.20%) and sugiol (6.14%). Fifty one compounds were obtained from the methanolic extract of *I. nilgherricus* of which the major components were euphracal (13.20%), D5-dehydro-7-oxo-totarol (12.97%), abieta-6,8,11,13-tetraen-12-yl acetate (10.04%), 1,2-bis (dicyclohexylphosphino) ethane (8.75%), p-vinylguaiacol (7.41%) and α-tocopherol (7.32%). The composition analysis revealed the predominance of terpenoid class in *I. nigrescens* and *I. nilgherricus* while *I. coetsa* depicted a complex mixture of terpenoids, alkaloids, vitamins and several other classes of compounds. The common compounds observed in the three methanolic extracts included 2,2-bipiperidine, dihydroactinidiolide, 3-(1-methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one, 2-methyl-7-octadecyne, phytol, α-tocopherol and γ-sitosterol of which α-tocopherol and phytol were abundantly found.

The compounds identified from the extracts have proven biological potential which could be the reason for the excellent activity of the three species in antitumor studies. β-Caryophyllene, a sesquiterpene compound was found to be an apoptosis inducing agent with antiproliferative activity selectively against tumor cell lines but not on normal cells (Amiel *et al*., 2012). Isoferulic acid belonging to the class of phenolic acids has reported antioxidant and anti-inflammatory effects (Sakai *et al*., 1999; Wang *et al*., 2011c). The chemopreventive and antioxidant activities of β-carotene have long been under biomedical research for efficacy studies (Bendich and Olson, 1989). The antioxidant and anti-inflammatory activities of the aromatic aldehyde, benzeneacetaldehyde have been reported (Kochi *et al*., 1980). Astaxanthin belongs to the group of carotenoids and have established roles as antioxidants and anticancer agents (Ambati, *et al*., 2014). α-Tocopherol is an excellent *in vitro* antioxidant with prominent role in cell signaling systems (Azzi, 2007). γ-Sitosterol is a phytosterol with significant antioxidant activity (Raman *et al*., 2012). Antioxidant capabilities of hexadecanoic acid, methyl ester belonging to the class of fatty acid esters have been acknowledged (Hema *et al*., 2011). 5-Hydroxymethylfurfural has been studied extensively to identify its potential in antioxidant activity (Kim *et al*., 2011). Furanocoumarin and alloisoimperatorin exhibited potent antioxidant activities (Piao *et al*., 2004). An abietane type diterpenoid, sugiol showed significant anti-inflammatory activity when analyzed (Chao *et al*., 2005). The potential antioxidant capacity of cycloeucalenol acetate, a triterpenoid has been evaluated (Kandasamy *et al*., 2014). The diterpene alcohol, phytol and the triterpene squalene have been identified to possess cancer preventive property (Sermakkani and Thangapandian, 2012). Anticancer and antitumor properties of methoxy phenyl oxime have been established (Dahpour *et al*., 2012). The diterpene, totarol was known to possess antioxidant activity (Haraguchi *et al*., 1996). Perusal of literature revealed that the several compounds present in

major and minor amounts possessed known and unknown biological activities.

The significant antioxidant, cytotoxicity and antitumor activities of the three species of *Isodon* cannot be thus attributed to the effect of a single major compound. All the compounds with reported and unidentified potentials have resulted in the cumulative effect of the extracts. The synergistic actions of the chemical compounds at multiple targets are the rationale behind beneficial effects of phytomedicines (Adwan *et al*., 2006). Different extracts exert the additive effects of the chemical components present in them resulting in efficacy against various ailments. It is observed that folk medicines have more curative role in many more diseases with fewer side effects than modern medicine due to these collective properties.

Liquid chromatography-mass spectrometry (LC-MS) analysis

The methanolic extracts of three species of *Isodon* were subjected to LC-MS analysis for the determination of the various compounds present (Figs 45-47). Liquid chromatography enables the separation of a wide range of non-volatiles as well as organic compounds while the mass spectrometer provides valuable information about the molecular weight, structure, identity, quantity and purity of a sample which adds to the specificity of quantitative and qualitative analyses. The major class of compounds detected from the extracts belonged to the sesquiterpenoid group (Table 50). The methanolic extract of *I. coetsa* revealed the presence of two major components *viz*., spathulenol (93.44%) and quercetin (6.56%) while the methanolic extract of *I. nigrescens* identified two components which were α-cadinol (94.42%) and achillin (5.58%). Only a single compound of γ-eudesmol (100%) could be detected from the methanolic extract of *I. nilgherricus*.

Spathulenol belongs to the class of sesquiterpenoids with potent immunomodulatory effect. It also depicts the capacity to inhibit proliferation in the lymphocytes as well as the ability to induce apoptosis possibly through a caspase-3 independent pathway (Ziaei *et al*., 2011). Moreover, it has evident activity to be a potent candidate in combination chemotherapy of multidrug resistant cancer disease (Martins *et al*., 2010). Quercetin is a widely distributed flavonoid with immense biological activities which include antioxidant, antigenotoxic, vasodilating effects as well as chemopreventive and anticancer properties (Kook *et al*., 2008). The various mechanisms associated with antitumor effects include inhibition of enzymes that activate carcinogens, modification of signal transduction pathways and interactions with receptors and proteins (Moon *et al*., 2006). α-cadinol is a sesquiterpenoid alcohol with significant biological importance. It was found to be one of the major component of essential oils *Taiwania cryptomerioides* and *Machilus mushaensis* from Taiwan which depicted significant antioxidant and *in vitro* cytotoxic activity respectively (Ho *et al*., 2012; Su and Ho, 2013). Achillin is a sesquiterpene lactone occurring as plant metabolite and is a nitric oxide synthase activator. Other biological properties of the compound are yet to be reported. γ-eudesmol is a sesquiterpenoid which is a major component of the oil of *Cryptomeria japonica* which showed remarkable inhibition of IL-1β, IL-6 and TNF-α in enzyme immunoassay (Yoon *et al*., 2009). The detected compounds have reported biological properties of antioxidant and anticancer activities which could be the driving force behind the antitumor effect shown in *in vitro* and *in vivo* studies by the methanolic extracts of the three species.

The phytochemical review of *Isodon* ends up in the identification of the prominent class of compounds in the genus to be terpenes especially diterpenes with excellent biological activity (Table 4). The present study also points to the fact that the three species of *Isodon* evaluated showed abundance of the terpene class. The isolated diterpenoids from different species of *Isodon*

have extensively shown potent antitumor activities with low toxicity and hence are promising candidates as anticancer drugs (Sun *et al*., 2006). The classification of diterpenes on a chemotaxonomic evaluation basis revealed that *Isodon* exclusively included kauranes and fujinanes along with abietanes and 8,9-secokauranes (Alvarenga *et al*., 2001). The different classes of terpenes isolated and identified with biological activities stresses the relevance of the genus in pharmaceutics. Literature perusal revealed the isolation and structure elucidation of several compounds with or without cytotoxic and antitumor activity (Tables 2, 3). Although research based on isolated compounds have been going on for several decades, potent antitumor drug is far from application with a few to name *i.e*., oridonin, ponicidin *etc*. which are expecting for *in vivo* clinical studies. But the plants have extensive application in traditional medicines which can be attributed to the collective activity of all the compounds present. The laboratory set up of *in vitro* and *in vivo* studies also produced excellent results which emphasize the fact that crude drugs of *Isodon* can be a better candidate than isolated compounds in anticancer research.

Cancer has been and is the focus of biomedical research since therapeutic cancer targets have not led to successful new therapies. Although the disease has conquered and triumphed over human beings reaching devastating ends, the treatment of malignances have been discouragingly low. Several strategies have been developed but the side effects and ineffectiveness in the long run are the challenges to be faced off. Introduction of new drugs against cancer has the major setback of development of resistance. Drug resistance is the major cause of treatment failure in metastatic cancer which can develop through increased and decreased drug influx, drug inactivation, alterations in drug target, processing of drug-induced damage and evasion of apoptosis (Longley and Johnston, 2005). The multi-mechanisms of drug resistance in chemotherapy are a target to be subjugated which has not been

possible for isolated compounds with single mechanism of activity, resistance and target. Crude herbal drugs have multiple components with multi-targeted activity which can possibly overcome the chemoresistance and become effective therapeutic drugs (Wang *et al*., 2014). Cancer disease has multiple mechanisms to escape regulated growth and avoid apoptosis which makes it irrepressible. Studies reveal that the therapeutic effects of plants are due to the complex additive/synergistic and/or antagonistic activity of the multiple compounds rather than a single constituent (Liu, 2003; Karna *et al*., 2012). Thus, crude extracts with multiple intracellular targets necessitates to be focused rather than isolated compounds for a practical constructive end product.

The three species of *Isodon* chosen for the current study showed significant effects as antioxidants, cytotoxicants and with antitumor activity which can be attributed to the combined action of the multiple components present in it. The individual capabilities of each species cannot be compared since each extract showed significant activity but not in a progressive sequential order. The extracts showed significant free radical scavenging activity with excellent results in hydroxyl radical scavenging assay. The enhanced activity shown in hydroxyl scavenging than the standard α-tocopherol can be attributed to the cumulative effects of the components along with the high amount of α-tocopherol present in the extracts. Anticancer drugs with inherent antioxidant capability can mitigate the oxidative stress produced due to the metabolic irregularities arising from the disease. Cytotoxic and antitumor effects were also noticeable with extracts showing exceptional results in *in vivo* antitumor activity against DLA induced solid tumor mice models. The phytochemical screening emphasized the importance of focus on multiple components with prominence of terpenoids and flavonoids which have proven biological properties. Polyphenols and terpenoids have anticancer property and the mechanism of action can be as

cancer-blocking agents, preventing initiation of the carcinogenic process and as cancer-suppressing agents, inhibiting cancer promotion and progression (Maurya *et al*., 2011). Cancer prevention is promoted by the combinational use of various antioxidants (Nishino *et al*., 2004). The antioxidant capability of the different components of the extracts can thus be of advantage in cancer therapy. Plant derived drugs are the prototypes from which more effective and less toxic medicines are developed (Aswar *et al*., 2008). Ancient herbal medicines due to the presence of wide variety of components have the possibility to produce synergistic activities or can buffer toxic effects (Gavamukulya *et al*., 2014). Diseases have complex mechanisms which target various end points. Healing properties of compounds need to produce an additive effect which can cure the disturbed metabolism completely. Thus, the multiple components act as synergists for the therapeutic effects as well as antagonists for the side effects of the active components and other toxic components in the crude preparation which can produce a comprehensive outcome in the control of the disease (Koul *et al*., 2005). Hence, it can be concluded that the crude extracts have more efficacy than targeted single molecules since the major and minor components present in the plants have synergistic activity which can modulate the mechanism of action of each compound and when collectively focused and applied can produce excellent results in pharmaceutical research.

The highlights of the present investigations are:

- The chromosome complements of three species of the genus *Isodon viz*., *I. coetsa* (2n = 24), *I. nigrescens* (2n =24) and *I. nilgherricus* (2n = 24) commonly used in traditional Chinese medicine were established. It is the first report on the chromosome number of *I. nigrescens*. The secondary basic chromosome number of the genus was identified to be $x_2 = 12$.

- Variant cytotypes of 6 and 12 were recognised in the three species which calls for chromosome mosaicism in the genus. The most advanced karyotype features in evolution was documented for *I. nigrescens*.

- The karyomorphometric features of total chromosome length, average chromosome length, range of chromosome length, nature of primary constriction, number of chromosomes with secondary constriction, disparity index, variation coefficient, total forma percentage, karyotype asymmetry index, Syi index, Rec index, intrachromosomal asymmetry index, interchromosomal asymmetry index and degree of asymmetry of karyotypes of the three taxa exhibited variations. The karyotype formulae of three taxa were also established.

- Percentage yield of the three species in methanolic extract preparation was measured and was found to be 42.58% for *I. coetsa*, 37.94% for *I. nigrescens* and 46.60% for *I. nilgherricus*

- The antioxidant activities of the methanolic extracts of three species were assessed using various *in vitro* free radical scavenging assay systems. The methanolic extracts showed significant, but varying degrees of activities in all the assays.

- The different antioxidant assays used included DPPH radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging and ferric ion reducing antioxidant power activities which revealed that the three species could very well inhibit the various free radicals. The extracts showed exceptional activity greater than the standard in hydroxyl radical scavenging assay.

- *In vitro* cytotoxic screening of the three species using Evans blue staining of *A. cepa* root meristems were conducted which revealed their cytotoxic potential.
- The cytotoxic activity of the methanolic extracts *in vitro* against DLA and EAC cell lines using Trypan blue exclusion assay were also determined and the three species depicted significant activity.
- The methanolic extracts of three species were screened for antitumor activity against DLA induced solid tumor and EAC induced ascites tumor models in Swiss Albino mice. Tumor volume in DLA models were reduced significantly and the extracts showed excellent results at par with the standard drug administered. A statistically significant increase in life span was observed for the three species in EAC models of antitumor study.
- Phytochemical analysis of the methanolic extracts using GC-MS and LC-MS techniques as well as estimation of phenolics, flavonoids and terpenoids were conducted which revealed the chemical profile and complexity of the three species.
- Preliminary phytochemical screening resulted in the identification of detectable amounts of flavonoids, phenols, tannins, steroids and terpenoids in the methanolic extracts of the three species.
- The total phenolic, flavonoid and terpenoid content of the methanolic extracts were determined which revealed terpenoids to be the abundant class of secondary metabolite. The three species showed significantly high amount of the three metabolites.
- GC-MS analysis of the methanolic extracts of the three species revealed 136 compounds which were grouped into 14 classes of compounds *viz*., terpenoids, phenols, alkaloids, steroids, flavonoids,

fatty acid esters, vitamins, furans, aromatics, alkanes, aldehydes and ketones, alcohols, organic compounds and other miscellaneous compounds. A total of fifty six compounds for *I. coetsa*, fifty nine compounds for *I. nigrescens* and fifty one compounds for *I. nilgherricus* were detected. The phytochemical profile revealed terpenoids to be the prominent class in the extracts.

- LC-MS analysis of the extracts identified biologically active phytochemicals in the methanolic extracts of which the majority of the compounds belonged to the class of sesquiterpenoids.

The three species of *Isodon* chosen for the current study established significant activities in the various studies conducted. The karyomorphometric studies revealed the chromosome identity of the plants along with its evolutionary status. The studies envisaged identified *I. nigrescens* to be the most evolved member of the three while *I. coetsa* was the most primitive. The antioxidant, antitumor and phytochemical characterization of the plants produced a new insight in development of an anticancer drug in chemotherapy. The three species depicted significant activity and the most contributing secondary metabolite was the terpenoid class. The biological activities could be attributed to the synergistic mechanism of multiple components rather than a single major component which establishes the success of the genus in crude formulations. The activities of each species varied indefinitely but on the whole *I. nilgherricus* was found be the most efficacious herb of the three taxa. The results obtained affirm the established role of the genus *Isodon* in traditional Chinese medicines as curatives of various ailments.

Biomedical research is a never ending phenomenon until a clinically successful and a pharmaceutically feasible drug is developed. Since the activities identified can only be attributed to the synergistic effects of the

multiple components in the extracts, research on isolated components and specific targeted activity needs to be slowed down. Chemoresistance of drugs and complex mechanisms associated with cancer proliferation needs to be subjugated for the disease to be down casted. Thus, mechanism of action of crude drugs in combating the disease along with the multiple targets activated due to the components in the extracts needs to be focussed as a future goal of continuing research in anticancer therapy.

Summary

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

SUMMARY

World has changed from the naive level to a highly efficient advanced system which symbolizes modernization. Expertise in particular fields with specificity of targets are the most sought after attributes for being a success which are being slowed down by the devastating effects of diseases capable of overpowering the human ability to exterminate them. Traditional medicines are the reservoir of drugs which have annihilating capacity against a whole lot of diseases. Scientific validation of the herbal formulations furnish a ray of hope in curing the otherwise unrepressed diseases. Herbs needs to be exploited for developing efficient drugs which can be only made possible after sufficient *in vitro* studies and substantiation through clinical trials employing animals and human beings. Thorough investigation of the pharmacological effects as well as the phytochemical constituents provides a picture of curable diseases which could be targeted by a particular herb. Diseases like cancer affect particular organs and have unfocussed negative aspects of drug resistance, initiation of metabolic disorders as well as promotion of the disease through oxidative stress induction. Drugs which can mitigate these effects are necessary for a fruitful treatment and the glimpses of such efficient therapy are noticed in crude formulations used by indigenous people.

Lamiaceae with its plethora of biologically active secondary metabolites is one of the prominent members in traditional medicines. Hence, drug research never leaves a chance of exploiting any of the lamiaceous members. Literature perusal identified the importance of the genus *Isodon* in being the source plant for several ailments including inflammations and cancer. The diterpenoid rich genus is a strong contingent in anticancer therapy with low toxicity. Less explored species of *Isodon coetsa*, *I. nigrescens* and

I. nilgherricus abundantly found in the high ranges of Kerala state was thus chosen for the current investigation. The potential of the plants in being a candidate for the establishment of an efficient anticancer drug is being focused.

Misidentification is the major cause of low acceptability of herbal formulations. Plants need to be identified properly for enabling efficacy in action. Chromosome analysis and deduction of karyomorphometric details can provide a cytological identity to each species which helps in sorting out a taxonomically disputed genus. Antioxidant studies are conducted to identify the capability of the plants in extenuating oxidative stress arising out of the disease as well as to act as a supplement in increasing the efficacy of the drug. Anticancer potential can be examined through *in vitro* cytotoxic and *in vivo* antitumor studies which endorse the possibility of developing a chemotherapeutic drug after clinical trials. Phytochemical profiling is essential for the scrutinizing of the components producing the biological effects as well as the toxicity levels. Apart from this, the various assays conducted can provide information about the synergistic effects as well as the reduced side effects of herbal formulations. Hence, the study revolves around establishing a cytological identity, biological aspects and phytochemical profiling of the three selected species of *Isodon*.

Methodology

Chromosome analyses of the plants were carried out based on the observations in the mitotic metaphase stage of chromosomes. Mitotic squash experiments on the young healthy root tips obtained from the nodal stem cuttings during the peak mitotic period was conducted using improved techniques. Photomicrographs taken were used for karyogram generation with the aid of computer-based programs such as Adobe Photoshop, AutoCAD and Microsoft Excel. The arm ratio and centromeric indices were calculated from

the karyomorphometric details which led to the identification of homologous chromosomes. Karyotypic formula was deduced based on the length of the chromosome, position of the centromere and presence/absence of secondary constriction. Various parameters of disparity index, variation coefficient, total forma percentage, karyotype asymmetry index, Syi index, Rec index, intrachromosomal asymmetry index, interchromosomal asymmetry index and degree of asymmetry of karyotypes were also calculated which gave an insight about the karyotype evolution.

Methanolic extracts of three species of *Isodon* was subjected to antioxidant and antitumor studies. The efficiency of the extracts in free radical scavenging was examined employing four *in vitro* assays *viz*., DPPH radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging and ferric ion reducing antioxidant power activities. *In vitro* cytotoxic activities was determined using Evans blue staining of methanolic extract treated *Allium cepa* root meristems to identify the intensity of cell death and Trypan blue exclusion assay on extract treated DLA and EAC cell lines to recognize the cell viability. The methanolic extracts were also screened for *in vivo* antitumor activity against DLA induced solid tumor and EAC induced ascites tumor in Swiss albino mice. Various assays conducted established the biological efficiency of the extracts.

The chemical constituents responsible for the various activities of the extracts were investigated using different techniques. The preliminary phytochemical screening and estimation of total phenolic, flavonoid and terpenoid content led to the determination of the prominent classes of compounds in the extracts. Phytochemical profile of the plants was also developed based on the results obtained from GC-MS and LC-MS techniques which identified individual components essential for scrutinizing the synergistic toxicity levels and biological activity of the extracts.

Upshots

Detailed karyomorphological study of the three species led to the identification of the chromosome count and other cytological parameters. It is the first report of the normal chromosome count of *I. nigrescens* and the karyomorphometric data of the three species. Normal chromosome complement was found to be 2n = 24 for all the three species with two variant cytotypes of 6 and 12. The secondary basic chromosome number could be identified which was $x_2 = 12$ arising out of protoautoploidy from the primary basic chromosome number. Homologous chromosomes, karyotypic formula and various other parameters were deduced which gave a cytological identity to each species. The chromosomes were found to be small in size with the total length of each chromosome falling below 2 μm. Various asymmetry indices and the degree of asymmetry of karyotypes were calculated, which depicted *I. nigrescens* to possess the highest asymmetry of karyotype. From the evolutionary point of view, the small size of chromosomes and asymmetry of karyotype recognizes *I. nigrescens* to be the most evolved and *I. coetsa* to be the primitive one, among the three taxa studied.

Methanolic extracts prepared had high percentage of total yield with 42.58% for *I. coetsa*, 37.94% for *I. nigrescens* and 46.60% for *I. nilgherricus* which was used for further analysis. Antioxidant activity was studied using various assays and the methanolic extracts showed significant effect in comparison with the standard. The most efficient DPPH radical scavenger was *I. coetsa* while the hydroxyl scavenging activity was high for *I. nigrescens*. But all the three species were excellent hydroxyl radical scavengers since the activities shown were extremely higher than that of the compared standard, α-tocopherol. *I. coetsa* also depicted the highest efficiency of superoxide free radical scavenging while FRAP assay revealed the efficiency for both *I. nigrescens* and *I. nilgherricus*. The mixed response

in antioxidant assays revealed the effectiveness of all the three extracts as good antioxidant supplements.

Cytotoxic assays conducted on *A. cepa* root meristems identified *I. nilgherricus* to be the highest inducer of cell death phenomenon. Cell viability assays against DLA and EAC cell lines also revealed the effectiveness of *I. nilgherricus* methanolic extracts. The other two species also showed significant effects. The antitumor assays on mice models established the anticancer potential of the three species. Though *I. coetsa* depicted high potential of activity on DLA induced solid tumor, administration of the high concentration (50 mg/kg b. wt) of all the three species produced significant activity at par with the standard drug cyclophosphamide with visually no side effects. In EAC induced ascites tumor model, significant effects were shown by high doses of the extracts with highest percentage of increase in life span was shown by *I. nilgherricus*. The antitumor studies revealed that all the three species have anticancer potential since each extract produced statistically significant results in all the assays.

Phytochemical screening was essential for the determination of the agents causing the multitude of activities in the extracts. Preliminary phytochemical screening provided an idea about the major compounds *viz*., flavonoids, phenols, tannins, steroids and terpenoids which could induce the various activities. Assays employed to determine the total phenolic, flavonoid and terpenoid contents identified the plants to be rich source of secondary metabolites. Terpenoid class was found to be the abundant group among the three which has already been established for excellent biological activities. GC-MS analysis identified 136 components from the extracts of the three taxa, which could be grouped into terpenoids, phenols, alkaloids, steroids, flavonoids, fatty acid esters, vitamins, furans, aromatics, alkanes,

aldehydes and ketones, alcohols, organic compounds and other miscellaneous compounds. Terpenoid richness was noticed with many biologically significant constituents in each species. α-Tocopherol and phytol were abundantly found in the methanolic extracts which have proven potential of excellent antioxidant and cancer preventive properties. LC-MS analysis also revealed the sesquiterpenoid abundance of the extracts which affirms the role of terpenes in producing significant biological activities.

Conclusion

The advancement of modern medicine is being slowly surpassed by the herbal renaissance due to the need for a healthier outcome devoid of side effects and triggering of new metabolic disorders as well as cost effectiveness. Herbal drugs have conquered the markets due to the alarming rate of diseases caused by synthetic drugs and the ineffectiveness of modern medicine following long term use. Crude formulations have its own say in pharmaceutical industry but require to be scientifically established. The various assays conducted thus provide data on biological significance of the three species of *Isodon* which is a well known genus in Chinese folk medicines.

Cytological identity of the three species along with the recognition of the evolutionary status is an asset in solving taxonomic dispute where other consistent parameters hold off. Antioxidant efficacy analyzed the ability of the plants in reducing and neutralizing oxidative stress which is a major challenge in cancer disease. Cytotoxic and antitumor assays confirmed the anticancer potential of the extracts and promoted the genus to be an excellent candidate in curing cancer disease but only after clinical trials. Phytochemical analysis revealed the individual components present in the extract with a magnitude of proven activities. The secondary metabolite richness and activities reported, identified the synergistic effect of the various components

present rather than the efficacy of an individual constituent. Crude extracts have escaped the negative effects of drug resistance and side effects in response to the additive effects of multiple components which makes them effective in folk medicines. Various components in a single extract have the capability of counterbalancing the multiple targets activated by the mechanism of action of the drugs and diseases. Current investigation of methanolic extracts also revealed the same effects which could be comprehended from the varying degrees of activity shown by the plants in different assays. Although *I. nilgherricus* can, in general, be considered the potent anticancer species of the three, other plants have equivalent activities which could be sometimes more efficient in clinical trials.

Studies conducted ensure the efficacy of the three species in anticancer treatment and assures the role as medicinal herb in folk medicines. Activity of the extracts can be attributed to the multitude of components present in the extract which promotes inhibition of dysregulation in metabolic functions as well as the development of drug resistance. Oxidative stress induction can be played down with the help of antioxidant rich medicinal herbs which also promotes the effectiveness of the drugs. Synergistic activity produced due to the combination of various components present in the extract results in fruitful multiple targeted chemotherapy. The three species chosen have all the capabilities discussed which ascertain the scientific basis of the use of the genus in folk medicine as well as its potential exploitation in pharmaceutical field. Prior to application, the extracts needs to be investigated for the mechanism of action as well as the synergisms involved for ending up on a positive note in the course of production of a feasible drug.

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Appendices

Sandhya Vincent Neelamkavil “Chromosome analysis, antioxidant, antitumor and phytochemical studies in some species of Isodon (Schrad. ex Benth.) Spach (Lamiaceae) ” Thesis. Department of Botany, University of Calicut, 2015

Appendix 1

Acetate Buffer

Sodium acetate : 3.1 g

Glacial acetic acid : 16 ml

Dissolve in 1000 ml double distilled water and pH adjusted to 3.6

Appendix 2

Acetocarmine

Carmine : 2 g

Acetic acid : 45 ml

Dissolve in 55 ml distilled water by boiling for 4 hours and filter

Appendix 3

Drabkin's Fluid

$K_3Fe(CN)_6$: 200 mg

KCN : 50 mg

KH_2PO_4 : 140 mg

Non-ionic detergent : 1 ml

Dissolve the chemicals in 1000 ml distilled water and then add the detergent, stir gently to obtain a clear reagent solution in pale yellow colour

Appendix 4

Evan's Blue Stain

Evan's Blue : 0.25 g

Dissolve in 100 ml distilled water

Appendix 5

KH_2PO_4 - KOH buffer

KH_2PO4 : 0.272 g

KOH : 0.112 g

Dissolve each in 100 ml double distilled water separately. Mix both the solutions and pH adjusted to 7.4

Appendix 6

Phosphate Buffered Saline (PBS)

NaCl	:	4 g
KCl	:	0.1 g
$Na_2HPO_4.2H_2O$	:	0.72 g
KH_2PO_4	:	0.1 g

Dissolve in 500 ml double distilled water and pH adjusted to 7.4

Appendix 7

Phosphate Buffer

KH_2PO_4	:	916 mg
Na_2HPO_4	:	950 mg

Dissolve each in 100 ml double distilled water separately. Mix 8.5 ml of KH_2PO_4 solution and 91.5 ml of Na_2HPO_4 solution and pH adjusted to 7.8

Appendix 8

Trypan Blue Dye

Trypan blue	:	100 mg
Saline (0.9%)	:	100 ml

Stir for 3 hours and filter

Appendix 9

Turk's Fluid

Glacial acetic acid	:	2 ml
Crystal violet (1%)	:	1 ml

Dissolve in 97 ml distilled water, stir overnight and filter

Appendix 10

Certificate of approval of Institutional Animal Ethical Committee

Appendix 11

Published research articles

1. Sandhya Vincent Neelamkavil and John Ernest Thoppil (2013) C-mitotic effect of aqueous extracts of *Isodon coetsa* (Buch.-Ham. ex D. Don) Kudo (Lamiaceae). Nucleus 56 (2): 117-122.

2. Sandhya Vincent Neelamkavil and John Ernest Thoppil (2014) Toxicological evaluation of polar and non-polar components of *Isodon coetsa* (Lamiaceae). Turkish Journal of Botany 38: 252-257.

3. Sumitha, K. V., Prajitha, V., Sandhya, V. N., Anjana, S. and Thoppil, J. E. (2014) Potential larvicidal principles in *Eryngium foetidum* L. (Apiaceae), an omnipresent weed, effective against *Aedes albopictus* Skuse. Journal of Essential Oil Bearing Plants 17 (6): 1279-1286.

4. Sandhya Vincent Neelamkavil and John E. Thoppil (2015) Genotoxic assessment of Calcium hypochlorite and *Strychnos potatorum* Linn. seeds - two commonly used water purifying agents. Reviews on Environmental Health 30 (1): 19-23.

5. Sandhya Vincent Neelamkavil and John E. Thoppil. Chromosome complements of *Isodon* (Family: Lamiaceae – Subfamily: Nepetoideae): Trends in karyotype evolution. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences (Accepted).