PHARMACOLOGICAL PROSPECTS OF *KAEMPFERIA ROTUNDA* L., *LAGENANDRA TOXICARIA* DALZ., AND THEIR ENDOPHYTES WITH SPECIAL EMPHASIS ON ANTHELMINTIC ACTIVITY

Thesis submitted in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Faculty of Science

University of Calicut

By

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2022

DECLARATION

I, PRAVEEN K., hereby declare that the thesis entitled **"PHARMACOLOGICAL PROSPECTS OF *KAEMPFERIA ROTUNDA* L., *LAGENANDRA TOXICARIA* DALZ., AND THEIR ENDOPHYTES WITH SPECIAL EMPHASIS ON ANTHELMINTIC ACTIVITY"** is based on the original work carried out by me at Department of Zoology, Christ College (Autonomous), Irinjalakuda, Thrissur, Kerala, under the guidance of Dr. Leyon Varghese, Assistant Professor in Zoology, Christ College (Autonomous), Irinjalakuda, Thrissur, Kerala. The thesis has been subjected to plagiarism check and no part thereof has been presented for the award of any other degree, diploma or other similar titles of any University.

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This is to certify that **Mr. PRAVEEN K.** has completed the research work for the full period prescribed under the Ph.D. ordinance of the University of Calicut. This thesis **"PHARMACOLOGICAL PROSPECTS OF *KAEMPFERIA ROTUNDA* L., *LAGENANDRA TOXICARIA* DALZ., AND THEIR ENDOPHYTES WITH SPECIAL EMPHASIS ON ANTHELMINTIC ACTIVITY"** embodies the results of his investigations conducted during the period at which he worked as a research scholar in this college. I recommend the thesis to be submitted for the evaluation for the award of the degree of Doctor of Philosophy in Zoology by the University of Calicut.

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No one who achieves success does so without acknowledging the help of others.

The wise and confident acknowledge this help with gratitude

: Alfred North Whitehead

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Abstract

ABSTRACT

The documented use of plants for medicinal purposes dates back over 5000 years and it is assessed that 70-80% of people worldwide rely on herbal medicine to meet their primary health care needs. Helminth infections are among the serious infections distressing the huge proportion of the world's population. In developing countries, they pose a large threat to human health and contribute to the prevalence of under nourishment, anemia, eosinophilia, etc. Along with humans, helminths also affect millions of livestock resulting in huge economic loss in domestic and farmyard animals. An anthelmintic agent should have a broad spectrum of action, a high percentage of cure with a single therapeutic dose, free from toxicity to the host and should be cost-effective. Unfortunately, most common drugs like piperazine salts have been shown to have side effects like nausea, intestinal disturbances and dizziness. Another important defect with synthetic drugs is the resistance developed by most parasites. Hence, it has become an important objective to discover non-resistance developing novel anthelmintic agents preferably from natural sources. Similarly, there has been a rising alertness towards the development of novel antibacterial drugs from diverse sources to fight against bacteria and bacterial resistance. The exciting synthetic compounds from the golden era such as tetracyclines, aminoglycosides, cephalosporins and macrolides are losing their potential against many pathogenic bacteria nowadays. Plants are considered as the single largest source for the potential bioactive compounds in traditional medicines. About 25% of novel drugs tested for clinical use are from plants. *Kaempferia rotunda* L. used in the present study is an aromatic herb that comes under the Family Zingiberaceae. This family contains perennial rhizomatous herbs that include economically important spices, like, ginger (*Zingiber officinale* L.), turmeric

(*Curcuma longa* L.), galangal (*Kaempferia galangal* L.) and black cardamom (*Amomum subulatum* Roxb). The second plant, *Lagenandra toxicaria* Dalz. is a semi-aquatic herb that belongs to the Family Araceae, and is an important ingredient in folklore treatment modalities. Its rhizomes and roots were also reported to show several pharmacological potentials.

Heavy metal accumulation is one of the single most important matters that concerns the indiscriminate use of plant parts for different ailments. Therefore a wet digestion followed by atomic absorption spectroscopy (AAS) was used to estimate arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) in the rhizomes of *K. rotunda* and *L. toxicaria*. The amount of As, Hg and Cd in the *K. rotunda* rhizome was below the detection limit (0.05 mg/kg) by AAS. The amount of Pb in the *K. rotunda* rhizome was in permissible limits. In the case of *L. toxicaria* rhizome, the presence of As, Hg and Pb were in permissible limits and the concentration of Cd was below the detection limit for human use. Though the results do not exclude the hyper accumulation properties of these plants, the extracts we made for further bioassays will be safe from any metal interferences. Further, these plants, when grown in non-polluted environments, are expected to have no metal accumulation in them.

Solvent extraction of the rhizome was made using ethyl acetate, ethanol and water for analyzing their phytochemical constituents and bioactivity. The qualitative phytochemical screening methods analysed the presence of various secondary metabolites such as alkaloids, polyphenols, tannins, flavonoids, terpenoids, saponins and glycosides in the ethyl acetate, ethanol and water extracts of *K. rotunda* (KrEA, KrOH, KrWT) and *L. toxicaria* (LtEA, LtOH, LtWT). The results showed that phenolic classes of secondary metabolites were abundant in all the three extracts of

both plants. Flavonoids were also present in all the six extracts tested whereas alkaloids, saponins and terpenoids were mostly absent in all extracts. The quantitative estimation of total phenol content (TPC) showed a high amount of phenol content in the KrEA (41 ± 0.18 mg GAE/g) and KrOH (35 ± 0.43 mg GAE/g) extracts. In the case of *L. toxicaria*, LtEA extract showed high phenolic content with a TPC value of 32.30 ± 0.72 mg GAE/g. Total Flavonoid Content (TFC) of plant rhizome extracts was estimated using the aluminum chloride colorimetric method. For *K. rotunda*, high amount of flavonoid content was observed with KrEA (30.24 ± 0.11 mg QCE/g) and KrOH (28.32 ± 0.65mg QCE/g) extracts. Similarly, the LtEA extract of *L. toxicaria* showed the presence of considerable amount of flavonoids compared to the LtOH and LtWT. GC-MS analysis of rhizome extracts reported the presence of several volatile compounds that were present in ≥ 1% abundance. The HPLC mediated polyphenol profiling showed the presence of high amounts of myricetin (5061.75 ± 0.004 µg/g), quercetin (3431.02 ± 0.022 µg/g) and ellagic acid (1678.2 ± 0.047 µg/g) with KrEA extract. Similarly, KrOH extract also showed the presence of high amounts of quercetin (4559.02 ± 0.002 µg/g), ellagic acid (1582.45 ± 0.024 µg/g), kaempferol (1292.17 ± 0.004 µg/g) and myricetin (1193.25 ± 0.042 µg/g). In the case of *L. toxicaria* extracts, among the nine polyphenols identified in LtEA, myricetin (6344.52 ± 0.008 µg/g) was present in high amounts and others were present in trace amounts.

The agar disk diffusion assay was used to evaluate the antibacterial potentials of ethyl acetate, ethanol and water extracts of *K. rotunda* and *L. toxicaria* against different bacterial strains tested. Here, KrEA showed pronounced inhibitory potential against five out of seven bacterial strains. At the highest test concentration (1mg/disk), KrEA induced a zone of inhibition of 26.46 ± 0.85 mm and 23 ± 0.63

mm against the Gram-positive bacteria *E. faecalis* and *S. aureus*. KrOH extract also showed strong activity against Gram-positive bacteria *E. faecalis*, *B. cereus* and *S. aureus*. Same time *S. flexneri* and *S. marcescens* strains were resistant against all the tested rhizome extracts of *K. rotunda*. In the case of *L. toxicaria* extracts, majority of the tested bacterial strains showed resistance against LtEA, LtOH and LtWT extracts. Comparatively, LtEA showed noticeable activity against Gram-positive and Gram-negative bacteria used in this study. Overall, the Gram-positive bacteria showed more susceptibility to the rhizome extract treatment compared to Gram-negative strains, and it was the ethyl acetate extract that showed stronger antibacterial activity.

Different *in vitro* models executed in the present study verified anthelmintic effects *K. rotunda* and *L. toxicaria* against plant parasitic as well as animal parasitic helminths. Root-knot nematode, *M. incognita*, was one among the highly susceptible helminth worms against KrEA, with highest mortality (71.22 ± 4.1 percent) obtained after 24h of treatment with 8 mg/ml of extract. KrOH extract at this concentration showed a mortality of 62.11 ± 1.16 percent and KrWT was least effective against the *M. incognita*. In the case of LtEA *L. toxicaria* extracts, an apparent increase in the mortality of *M. incognita* was observed with the progress of time. The mortality rates of juvenile *M. incognita* (J2) increased in accordance with the increase in time and concentration of extracts. The nematicidal assay also showed that the KrEA and KrOH extracts were effective against another plant parasitic burrowing nematode *R. similis*. Both the extracts showed similar nematicidal effects against the second-stage juveniles of *R. similis*. Highest mortality of 57.56 ± 5 and 53.11 ± 1.20 percent was obtained after 24 h of treatment in 8 mg/ml of KrEA and KrOH extracts respectively. On the other hand, KrWT showed the least activity against this parasite

also. Similarly, the LtEA showed strong activity against *R. similis* whereas the LtWT extract showed the lowest nematicidal potential. The maximum mortality obtained at the highest tested concentration (8 mg/ml) was 61.12 ± 1 percent for LtEA extract and the lowest was 12.78 ± 0.77 percent for LtWT extract.

The anthelmintic activity of the extracts were further evaluated against several animal parasites such as *Haemonchus contortus, Fischoederius cobboldi*, *Syphacia obvelata* and *Hymenolepis nana.* The egg hatch assay with *H. contortus* showed a dose-dependent activity for KrEA and KrOH extracts compared to KrWT. Similarly, in the larval paralysis assay using 3rd instar *H. contortus* larvae, the KrEA extract induced 93.97 ± 1.07 percent paralysis at 10 mg/ml concentration. This was followed by KrOH extract, with a percent paralysis of 90.18 ± 1.26. In the case of *L. toxicaria* extracts, all the tested concentrations of LtEA and LtOH extracts significantly ($P<0.001$) inhibited the egg hatch of *H. contortus*. Likewise, the 3rd instar *H. contortus* larvae was highly susceptible with LtEA and LtOH extracts ($P < 0.001$) induced larval paralysis. Here also LtWT showed very poor potential when compared with the other two extracts. A similar result was obtained in the *in vitro* assays using the trematode parasite, *F. cobboldi*. Treatment for 30 minutes in the highest tested concentration (25 mg/ml), the fluke mortality was 17.65% and 8.94% respectively for KrEA and KrOH extracts. Interestingly the mortality significantly increased to 100% after 1 h of treatment in KrEA extract. On the other hand, it took 2 h to obtain 100% mortality with KrOH extract. The KrEA and KrOH extract-treated flukes exhibited deleterious changes to the surface syncytium, tegument, and the underlying structures of treated flukes. In the case of *L. toxicaria* 12.5 mg/ml and 25 mg/ml of LtEA extract-treated flukes showed surface syncytium degeneration, alterations to the muscle tissue and detachment from the basement

membrane. Similarly, LtEA and LtOH extracts of *L. toxicaria* significantly reduced the survival rate of *F. cobboldi*. Post 1.5 h after treatment in the highest tested concentration (25 mg/ml) of LtEA and LtOH extracts, the mortality of flukes reached 100%. The flukes treated in 25 mg/ml of LtOH extract showed severe tegument and surface syncytium degenerations. On the other hand, no observable alterations to the teguments or muscle tissue of *F. cobboldi* were observed when treated using LtWT extract.

The 14-days acute toxicity and 28-days sub-acute toxicity studies fixed the safe, non-toxic *in vivo* doses of KrEA and KrOH extracts. Safe doses were used to test the *in vivo* anthelmintic efficacy of KrEA and KrOH extracts against the nematode, *Syphacia obvelata* (pinworm) and cestode, *Hymenolepis nana* (tapeworm). KrEA extract at 400 mg/kg b.wt significantly reduced the number of *S. obvelata* eggs from 62.6 ± 9.57 to 18.8 ± 3.89 and 4.4 ± 0.74 eggs respectively after 8 and 12 days of treatment. A lower dose of KrEA (200 mg/kg b.wt) also effectively reduced the number of *S. obvelata* eggs from 51.8 ± 3.16 to 30.8 ± 3.54 by 8 days and to 15.4 ± 2.31 by 12 days of treatment. KrOH extract at 200 and 100 mg/kg b.wt doses also induced considerable reduction in the pinworm eggs during the 15 days treatment. KrEA extract at 400 mg/kg b.wt. induced significant reduction in the EPG (eggs per gram) value of *H. nana*. The initial EPG was 43.8 ± 4.81 reduced respectively to 14.2 ± 1.2 and 4.8 ± 2.03 after 8 and 12 days of treatment.

Endophytic fungi from *K. rotunda* were isolated and evaluated for their antimicrobial and anthelmintic activities after identification based on the morphological and molecular taxonomic studies using ribotyping targets, such as large-subunit rDNA gene (D1–D2) and internal transcribed spacer (ITS) region. A total of three endophytic fungi were isolated to pure culture from rhizomes of *K.*

rotunda. Interestingly all the three isolates that showed repeated appearance in the rhizomes of the plant exhibited the characteristic features of the genus *Aspergillus.* Of the three isolates from *K. rotunda*, KMPR02 and KMPRH1 inhibited five out of seven bacterial strains. Considerable activity was observed against Gram-positive strains compared to Gram-negative bacteria. The results were consistent as the rhizome extracts of *K. rotunda* also exhibited prominent inhibitory potential against the tested Gram-positive bacterial strains. Nematicidal activity of the endophytes were also evaluated and among the endophytic fungal isolates KMPR02 and KMPRH1 showed a concentration-dependent inhibition to egg hatch and induced larval paralysis to the 3rd instar *H. contortus* larvae. HPLC mediated polyphenol profiling of these extracts revealed the presence of many phenolic molecules common in both the crude rhizome extract and the endophyte extracts of the *K. rotunda*. So an *in silico* docking study of these compounds was performed against helminthic tubulin protein. All the seven tested phenolic ligand molecules showed noticeable binding affinity against tubulin dimer. Among these the highest binding energy was exhibited by myricetin, quercetin, ellagic acid, kaempferol and apigenin. Furthermore, except chlorogenic acid and gallic acid, all other molecules showed higher binding affinity to tubulin (α+β) than the reference anthelmintic drug albendazole.

This is the first report on the anthelmintic potentials of *L. toxicaria* and *K. rotunda*. We were also able to isolate three endophytic fungi into pure cultures from the rhizomes. Interestingly, the endophytes also showed similar bioactivity as their host plant. It is noteworthy that endophytic fungi, among the novel biotypes, are in the top priorities now, which would make drastic changes to the medicinal, pharmaceutical, and agriculture industries in the near future. The strong bioactivity

of the extracts can be attributed to the presence of a high amount of secondary metabolites in it. The TPC, TFC and HPLC studies highlighted the presence of different phenolic metabolites in the ethyl acetate and ethanol rhizome extracts of both plants and the endophytes. We presume that the individual and/or synergistic action of these molecules might have resulted in the observed bioactivity.

Keywords: Anthelmintic, Antimicrobial, *Kaempferia rotunda*, *Lagenandra toxicaria*, Phytochemicals, Rhizome extract.

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Abbreviations

AAS	-	Atomic absorption spectroscopy
AC	-	Acetabulum
ALB	-	Albendazole
ANOVA	-	Analysis of Variance
ALP	-	Alkaline phosphatase
As	-	Arsenic
BDL	-	Below detection lomit
BLAST	-	Basic Local Alignment Search Tool
BL	-	Blebs
Bm	-	Basement membrane
B.WT.	-	Body weight
Cd	-	Cadmium
DG	-	Deep grooves
DMSO	-	Dimethyl sulphoxide
EC	-	Effective concentration
ECR	-	Egg count reduction
EHA	-	Egg hatch assay
EPG	-	Eggs per gram
ER	-	Erosion
FECR	-	Fecal egg count reduction
GC-MS	-	Gas chromatography–mass spectrometry
Hb	-	Hemoglobin
HDL	-	High-density lipoprotein
Hg	-	Mercury
HPLC	-	High performance liquid chromatography
ITS	-	Internal transcribed spacer
KrEA	-	*Kaempferia rotunda* ethyl acetate
KrOH	-	*Kaempferia rotunda* ethanol
KrWT	-	*Kaempferia rotunda* water
LCL	-	Lower confidence limit
LDL	-	Low-density lipoprotein
LM	-	Light microscope
LPA	-	Larval paralysis assay
LtEA	-	*Lagenandra toxicaria* ethyl acetate
LtOH	-	*Lagenandra toxicaria* ethanol

LtWT	-	*Lagenandra toxicaria* water
MCV	-	Mean corpuscular volume
MCHC	-	Mean corpuscular hemoglobin concentration
Mu	-	Muscle
ND	-	Not detected
OS	-	Oral sucker
Pb	-	Lead
PCV	-	Packed cell volume
PDA	-	Potato dextrose agar
PDB	-	Potato dextrose broth
PL	-	Permissible limit
PLT	-	Platelet
PS	-	Posterior sucker
RBC	-	Red blood cells
rDNA	-	Ribosomal DNA
SD	-	Standard Deviation
SEM	-	Standard error of the mean
SGOT	-	Serum glutamic-oxaloacetic transaminase
SGPT	-	Serum glutamic pyruvic acid transaminase
SS	-	Surface syncytium
Ssd	-	Surface syncytium degeneration
SW	-	Swollen
TC	-	Total count
Td	-	Tegument degeneration
TP	-	Total Protein
Ts	-	Tegument sloughing
TFC	-	Total flavonoid content
TPC	-	Total phenol content
UCL	-	Upper confidence limit
VC	-	Vehicle control
VLDL	-	Very low-density lipoprotein
WHO	-	World Health Organization

GENERAL INTRODUCTION

In tropical and subtropical developing countries the gastrointestinal nematode infection by *Haemonchus contortus* remains one of the chief causes for the decreased production in small ruminants (Perry & Randolph 1999; Jabbar et al. 2006). Furthermore, this most prevalent and pathogenic gastrointestinal helminth parasite is the major reason for anaemia, weight loss and mortality in young infected ruminants (Newton et al. 1999; Raza et al. 2009). The haemonchosis induced by *H. contortus* contributed to about 27% of weight reduction and 29% of reduction in the milk production in sheeps and goats (Qamar et al. 2011).

As an outcome of frequent transfer of experimental animals between laboratories and researchers, the nematode parasites like *Syphacia obvelata* and *Aspiculuris tetraptera* commonly known as pinworms are comparatively common in experimental rats and mice (Kozan et al. 2006). Even though the *S. obvelata* infection normally does not induce any clinical symptoms in normal mice, heavy worm loads have been observed with rectal prolapse, intussusception, and intestinal impaction (Flynn et al. 1989).

Hymenolepiasis is a parasitic disease caused by different cestodes from the genus *Hymenolepis*. The rat tapeworm, *Hymenolepis diminuta* and mice tapeworm *Hymenolepis nana* are considered as chief causative organisms (Leder et al. 2013). This parasitic infection has got high pervasiveness in people among the tropical and subtropical areas of the world (Kline et al. 2013). The *H. nana* infection is relatively more common among children and most of the infections remain self-limited and pass asymptomatically. However, certain severe cases develop symptoms such as vomiting, abdominal pain and diarrhea (Kandi et al. 2019).

Paramphistome, commonly known as rumen flukes of the super family Paramphistomoidea are the chief agents that cause paramphistomosis (Horak 1971; Wang et al. 2006; Sanabria & Romero 2008; Anuracpreeda et al. 2012). Chronic ulcerative ruminitis and anemia are caused by adult rumen flukes of Paramphistomoidea (Rolfe et al. 1991; Anuracpreeda et al. 2013). Paramphistomes spend their early stages in the small intestine of the host and eventually migrate up to the abomasums towards the rumen and spend their adult stage at the rumen (Sanabria & Romero 2008). Same time, the immature parasites cause severe acute gastroenteritis, incomplete digestion, dehydration and death, mainly in young animals (Ilha et al. 2005; Khan et al. 2008). Furthermore, paramphistomosis in domesticated animals cause huge monetary loss with escalated morbidity and mortality rates in the livestock industry (Gupta et al. 1978; Hanna et al. 1988; Tariq et al. 2008; Anuracpreeda et al. 2015). Many previous studies documented the occurrence of paramphistomosis from a digenetic trematode parasite *Fischoederius cobboldi* of Gastrothylacidae Family (Chethanon et al. 1985; Anuracpreeda et al. 2012).

To minimize the economic losses due to the above described helminth infections, commercial anthelmintic agents have been hired. The use of such synthetic anthelmintic drugs considerably increased livestock production worldwide (Jabar et al. 2006). However, the uncontrolled use of these synthetic chemicals developed significant resistance in parasites (Waller 1997; Jabar et al. 2006). Bithionolsulfoxide has been considered an effective drug for the treatment of paramphistomosis in cattle, sheep and goats for many years (Rolfe and Boray, 1988). However, the escalated resistance of parasitic flukes to this anthelmintic drug diluted the control measures taken against the rumen flukes (Prasitirat et al. 1997).

Many corticosteroid drugs have been prescribed for the treatment of different disease conditions. The immunosuppressive side effects of these steroid molecules are considered dangerous when it comes to parasitic infections. Oliver (1962) documented that the steroid cortisone can help protect intestinal helminth parasites to evade host defenses. Similarly, Moss (1972) also highlighted the immunomodulation associated with corticosteroid treatment and this might alter the microenvironment favourable for parasitic worms. Alongside, in developing countries and underdeveloped countries, the increased cost of chemical products represents an additional hindrance for the use of synthetic chemicals as anthelmintic agents (Hounzangbe-Adote et al. 2005).

Antibiotic chemotherapy has made noticeable advancements since the use of antibiotic drugs in 1950s. According to Finch (2010), for antibiotic chemotherapy initially, alkaloids such as quinine and emetine were used against pathogenic bacteria. After that synthetic compounds entered in the battle against microbes. It was in 1982 with the discovery of penicillin by Sir Alexander Fleming, the antibiotic era has started. Many effective antibiotics entered the market with the optimistic thought of overruling infectious pathogens and associated diseases in near future. However, the scenario has changed when the bacteria started showing resistance against many of the synthetic antibiotics. Many pathogenic bacteria showed significant resistance against the rousing synthetic antibiotics such as tetracyclines, aminoglycosides, cephalosporins and macrolides (Mayers et al. 2009). Bacteria exhibited various resistance mechanisms such as horizontal gene transfer, mutations that induce vertical evolution, regulation of efflux pumps, enzyme inactivation, covalent alteration of antibiotic compounds, and formation of resistant biofilms against the antibiotic compounds (Sommer et al. 2017). The frequent waves of

emerging and re-emerging diseases, the resistance of bacteria against standard drugs, frequent non-target effects of synthetic chemicals, etc., made antimicrobial therapy using synthetic chemicals difficult. In this scenario that less resistant, environmentally safe drugs become the need of the hour.

The increased resistance and side effects associated with popular synthetic chemicals invited the interest of the scientific community to search for relatively less resistant and environmentally safe bioactive molecules from natural sources. Traditional medicine practitioners have been using herbal medicines for treating various diseases. Nowadays the modern medicinal research also focuses on exploring bioactive compounds or extracts from herbal and other related sources. An alkaloid named morphine extracted from plant *Papaver somniferum* was the first herbal product commercially marketed (Dias et al. 2012). Following that many effective compounds were isolated from plants and are available as potent drugs. Some of the important bioactive compounds isolated from plants are vincristrine and vinblastine from *Catharanthus roseus*, digitoxin and digoxin from *Digitalis furfura*, alkaloids such as morphine and codeine from *Papaver somniferum*, atropine from *Atropa belladonna, paclitaxel from Taxus brevifolia* (Rates 2001; Atanasov et al. 2015).

The consideration of plant-derived drugs as a sustainable alternative to conventional chemotherapy invited the interest of the scientific community in studying their anti-parasitic properties also elevated as documented by various studies. Some of the different plants claimed by traditional practitioners such as *Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *Morinda lucida*, *Carica papaya Albizia gummifera*, *Hedera helix*, *Coriandrum sativum*, *Plectranthus punctatus* have been screened and studied for their anthelmintic potentials (Eguale et al. 2006,

2007a, 2007b; Tadesse et al. 2009). Some of these tested plant extracts have shown significant anthelmintic potential; for instance dose-dependent significant potential of alcoholic extracts of four tropical plants *Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *Morinda lucida* and *Carica papaya* against different life cycle stages *of H. contortus* was reported by Hounzangbe-Adote et al. (2005). The aqueous and hydroalcoholic extracts of *Hedera helix* induced significant inhibition to the *in vitro* egg hatching of *H. contortus* this study also documented 39% faecal egg count reduction of *H. contortus* eggs in the *in vivo* study using artificially infected sheep (Eguale et al., 2007b). Likewise, the efficacy of plants extracts was explored and proved against the trematode flukes (Tandon et al. 1997; Anuracpreeda et al. 2015; Yamson et al. 2019) and cestode parasites (Lin et al. 2014; Gogoi & Yadav 2016; Bayoumy et al. 2020). The traditional use of medicinal plants plays an important role to overrule the growing concern of resistance and also the non-target toxicity of the currently available synthetic antibiotics (Ali et al. 2001). Among the novel antibacterial drugs, approved during the period 1981–2006, approximately 69% originated from natural products (Newman 2008). Hence the discovery of medicinal plant extracts and compounds with significant antimicrobial potential will expand the collection of available antibiotics (Zaidan et al. 2005). Many previous studies reported the significant antibacterial potential of plant extracts and plant-derived compounds against pathogenic bacteria. Ali et al. (2001) screened the ethanolic extracts of 20 selected traditional medicinal plant species against a series of Gram-positive and Gram-negative bacteria and proved the variable degrees of antibacterial potential exhibited by 14 out of 20 species tested. Similarly, Ibrahim & Kebede (2020) documented the significant antibacterial potential of methanol extracts of *Azadirachta indica*, *Lepidium sativum* and *Moringa oleifera*, against the

bacteria *Streptococcus agalactiae*, *Salmonella typhi*, *Shigella boydii* and *Staphylococcus aureus*.

Most of the bioactive principles from phyto origins have been produced as secondary metabolites within the plants. Compounds from important secondary metabolite classes such as phenolics, terpenoids and alkaloids have been reported for a wide range of bioactivities. The phenolic class includes polyphenols, phenylpropanoids, flavonoids, quinones and tannins known for their *in vitro* and *in vivo* bioactivities such as antioxidant, antimicrobial, anthelmintic, anticancer, anti-inflammatory, antiviral, antifungal and insecticidal (Cushnie & Lamb, 2005, Lin et al. 2005; Dai & Mumper 2010; Cushnie & Lamb 2011; Bazh & El-Bahy 2013; Wong et al. 2014; Cushnie et al. 2014).

Plants enter a mutualistic relationship with an infinite number of microbes, commonly represented as endophytes (Schulz et al. 2002; Strobel et al. 2004). They inhabit different internal tissues symbiotically and spend a major part of their life period within the host plant tissues without causing damages. It is estimated that about one million endophytic species are present in the plant kingdom (Fouda et al. 2015). Endophytic fungi are considered as a huge repository of bioactive compounds that can be effectively altered to produce required novel analogs for various therapeutic purposes. The bioactive metabolite production in the endophytes can be highly regulated by the physiology and chemistry of host plants (Kusari et al. 2012). As part of co-evolution, certain endophytes were reported to produce bioactive metabolites and other important chemicals that are the same or similar to the plants they inhabit (Jia et al. 2016). For instance, the production of plant growth-regulating hormones such as indole acetic acid and gibberellic acid by certain endophytic fungal groups were reported (Rai et al. 2014). Regarding bioactive metabolite

production, many studies have reported the capability of endophytic fungi to produce compounds having high therapeutic value as antibacterial, antifungal, anticancer, cytotoxic and antiviral agents (Jalgaonwala et al. 2017). The important anticancer and cytotoxic compounds such as paclitaxel, phodophyllotoxin, camptothecin, vincristine, vinblastine, vinleunosine, and vinrosidine were reported to be produced by various endophytic fungi (Flores-Bustamante et al. 2010; Zaiyou et al. 2017; Puri et al. 2006; Amna et al. 2006; Shweta et al. 2013; Johnson et al. 1963; Noble 1965). Likewise, significant antibacterial activity was exhibited by the crude extracts or specific compounds isolated from various endophytic fungi. The *Alternaria alternata* isolated from the leaves of *Azadirachta indica* showed potent antimicrobial properties against a series of Gram-negative and Gram-positive bacteria (Chatterjee et al. 2019). The compound pseurotin A, isolated from *Penicillium janczewskii* of the plant *Prumnopity sandina* exhibited signifivcant antibacterial activity against plant pathogenic bacteria *Pseudomonas syringae* and *Erwinia carotovora* (Schmeda-Hirschmann et al. 2008). The *Aspergillus* sp. CY725 isolated from the plant *Cynodon dactylon* produced bioactive molecules such as monomethylsulochrin, helvolic acid, 3β-hydroxy-5α, 8α-epidioxy-ergosta-6, 22-diene, and ergosterol. These molecules showed prominent antibacterial activity against *Helicobacter pylori, Sarcinalutea* and *Staphylococcus aureus* (Li et al. 2005). Similarly, the *Aspergillus versicolor* from the brown alga *Sargassum thunbergii* produced antimicrobial compounds 6, 8-di-O-methylaverufin, brevianamide M and 6-O-methylaverufin that showed antimicrobial potential against the Gram-negative *E. coli* and Gram-positive *S. aureus* (Miao et al. 2012). The anthelmintic compounds such as peptaibols isolated from endophytic fungal order Hypocreales showed significant anthelmintic activity against the *Haemonchus*

contortus (L3) larvae (Ayers et al. 2012). Likewise, Bechem et al. (2018) reported the nematicidal potential of endophytic fungal isolates from banana and plantain cultivars against the plant parasitic nematodes *Radopholus similis* and *Pratylenchus coffeae*.

The Zingiberaceae family is comprised of economically and medicinally important spices such as ginger (*Zingiber officinale*), turmeric (*Curcuma longa*.), galangal (*Kaempferia galanga*) and black cardamom (*Amomum subulatum*) (Stevenson et al. 2007). Some common plant species from this family have been extensively used as condiment for flavoring owing to the presence of high amounts of flavor compounds (Chane-Ming et al. 2002; Naik et al. 2004; Pino et al. 2004). Various species from this family are regularly prescribed by traditional healers for treating stomach-ache, carminative, diarrhea, and dysentery (Tewtrakul & Subhadhirasakul 2007). Also, some plant species from Zingiberaceae family showed the presence of several compounds of medicinal interest owing to their antioxidant, antibacterial and antifungal potential (Masuda et al. 2004; Wilson et al. 2005). *Kaempferia*, is an important genus under the family Zingiberaceae. This perennial member of Zingiberaceae is generally cultivated in India, Indonesia, Bangladesh and other parts of south east Asia (Atun et al. 2013).The genus consists of about 60 species of plants (Swapana et al. 2018) that have been regularly utilized as medicines, spices and as ornamentals (Pancharoen et al. 2000).

Kaempferia rotunda L., belongs to the Family Zingiberaceae is a fragrant aromatic herb with tuberous rhizome. *K. rotunda* extracts are known to contain diverse secondary metabolites such as flavonoids, flavonols, stigmasterol, chalcones, quercetin, β-sitosterol, syringic acid, and protocatechuic acid (Sulianti & Chairul 2005; Stevenson et al. 2007; Diastuti et al. 2020). Several recent studies have also

endorsed the fact that *K. rotunda* possesses a wide range of pharmaceutical activities such as antibacterial, anti-proliferative, anti-mutagenic, and antioxidant activity (Priya et al. 2008; Atun et al. 2013; Kabir et al. 2013).

The Family Araceae is considered as one of the highly diverse families with about 105 genera and 3,300 species all over the world (Mayo et al. 1997; Croat 1998; Ribeiro et al. 1999; Coelho 2000; Vargas 2002) in which approximately 800 species from Araceae have been considered as edible, medicinal and ornamental (Pedralli 2002). The genus *Lagenandra* under the family Araceae consists of about 15 different species of which majority are observed in Srilanka, India and Bangladesh (Sivadasan et al. 2001). The plants under the genus *Lagenandra* are perennial and all species are ecologically similar, and they are commonly observed along streams and rivers in forest and also in plantations, irrigation ditches and canals near rice fields (Sivadasan et al. 2001). A few previous studies have reported the ornamental and medicinal properties of different species under this genus (Chopra 1982; Selvakumari & de Britto 2007; Sasidharan & Padikkala 2012).

The *Lagenandra toxicaria* Dalz., a semi-aquatic herb that comes under the Family Araceae, is an important ingredient in the folklore treatment modalities. It is used in the preparations of ointments for skin itch, renal and bilious complaints (Chopra et al. 1994). The *L. toxicaria* rhizomes and roots were also reported to be diuretic, carminative, tonic, and also used for wound healing. Its rhizomes have also shown insecticidal and antimicrobial properties (Sivarajan 1994; Selvakumari 2004).

Keeping the above-mentioned in view, the present study was designed with the following objectives:

1. Solvent extraction of *K. rotunda* and *L. toxicaria* rhizomes and phytochemical characterization of the extracts.
2. Evaluation of the antibacterial potential of rhizome extracts of *K. rotunda* and *L. toxicaria*.
3. Evaluation of anthelmintic potential of rhizome extracts *K. rotunda* and *L. toxicaria* using *in vitro* systems.
4. Acute and Sub-acute toxicity studies of bioactive extracts in Swiss albino mice.
5. Evaluation of anthelmintic potential of bioactive rhizome extracts using non-toxic concentrations in the *in vivo* systems.
6. Isolation and identification of endophytic fungi from the rhizomes of *K. rotunda* and to explore its bioactivity.

REVIEW OF LITERATURE

Anthelmintic resistance in parasites

The helminth parasites developed variable resistance towards almost all types of anthelmintic drugs available in the market and the rise of drug resistance remains a major obstacle for the treatment of parasitic diseases in animals (Geary 2012; Srivastava & Misra-Bhattacharya 2015; Taman & Azab 2014;). Raza et al. (2016a) reported an alarming fact that the helminth resistance towards a recent drug, monepantel, occurred within about four years after the introduction of the drug into market. The helminth resistance mechanisms can be broadly classified into two types such as pharmacodynamic resistance and pharmacokinetic resistance (Stuchlikovaa et al. 2018). In pharmacodynamic resistance mechanisms, the parasite exhibits decrease in the number or target molecules or alterations in their structure. The β-tubulin protein was reported to be an important target of benzimidazole kind of anthelmintics. Against these drugs, the parasite genome exhibits single nucleotide polymorphism (SNPs) at β-tubulin isotype-1 gene, altering the 3D structure of the β-tubulin protein thereby making it incapable to bind with benzimidazole drugs (Chaudhry et al. 2015; Lubega & Prichard 1991). Same time, the pharmacokinetic mechanisms work by reduced drug uptake, up regulated drug inactivation and quicker efflux of drugs. This ultimately reduces the concentration of the drug molecules and the effects associated with active binding of drugs (Fernando et al. 2016; Matouskova et al. 2016; Raza et al. 2016b). Here, the regulated expression of different xenobiotic-metabolizing enzymes aids the pharmacokinetic drug resistance mechanisms in helminth parasites (Brophy et al. 2012; Matouskova et al. 2016). For example, *Haemonchus contortus* metabolized the standard anthelmintic drug, albendazole in to ABZ-sulfoxide, ABZ-N-glucosides and another important drug flubendazole was metabolized in to different glucosides (Vokral et al. 2012; Vokral

et al. 2013). The non-target side effects caused by the synthetic anthelmintics to the environment and other unrelated organisms also exhibit strong hindrance for the treatment of helminth infections. The eco toxicological data documents the important danger associated with the existing uses of these synthetic drugs have been reported after the exposure of different aquatic organisms to benzimidazole drugs (Wagil et al. 2015). Furthermore, the biotransformation of the synthetic anthelmintic molecules like albendazole in host organism could cause oxidative stress that develops reactive oxygen species (ROS), reactive nitrogen species (RNS), malondialdehyde (MDA) and the liver cell isoenzymes (LDH5), which ultimately leads to severe cell damage (Dimitrijevic et al. 2012).

Traditional medicinal plants as anthelmintic drugs

During recent decades, the appearance of chemo resistance in helminth parasites against the well-known synthetic anthelmintics has amplified the importance of screening novel natural anthelmintic compounds from herbal sources (Sebai et al. 2020). Traditional practices have been widely used to detain the infections caused by helminth parasites for ages. The plants and plant derived metabolites have been considered as anthelmintic agents against a wide range of parasites. Many Ethno pharmacological studies scientifically validated the potential of herbals against different life cycle stages of helminth parasites. The *in vitro* analysis of the alcoholic rhizome extracts of *Kaempferia galangal*, *Alpinia galangal*, *Andrographis paniculata*, *Lippia nodiflora*, *Tephrosia purpurea*, *Zingiber zerumbeth*, *Pollia serzogonian* and leaf extract of *Moringa citrifolia* were tested effective against *Ascaris lumbricoides* (Raj 1975). The plants from the genus *Artemisia* were reported to show promising anthelmintic activities. The study by Hammond et al. (1997) proved the efficacy of *Artemisia* extracts against the

nematodes *Ascaris suum* and *Toxocara* sp. Similarly, the efficacy of *Artemisia absinthium* extracts against the larvae of *H. contortus* was also proved (Tariq et al. 2008). Likewise a survey in Italy revealed the copious use of *A. absinthium* and other plant species such as *Ruta graveolens*, *Cucurbita maxima* and *Allium sativum* for the treatment against the helminth parasites of livestock animals (Guarrera 1999). In another anthelmintic study by Eguale & Giday (2009), they estimated the effects of *Jatropha curcas* and *Chenopodium ambrosioides* extracts on the eggs and adults of *H. contortus*. The traditional medicinal plants from Africa, including *Terminalia avicennioides, Anogeissus leiocarpus*, *Cassia occidentalis, Annona senegalensis, Aloe barteri* and *Diospyros mespiliformis* was estimated by Ibrahim et al. (1984) for their anthelmintic effect using *in vivo* systems. Likewise the root and bark extracts of *Abrus precatorius* and *Elephantorrhiza goetzei* respectively showed noticeable *in vitro* efficacy against the cestode, *Hymenolepis diminuta* (Molgaard et al. 2001). Previously, Pessoa et al. (2002) showed significant ovicidal potential of essentials oils and a chief compound eugenol, isolated from *Ocimum gratissimum* against *H. contortus* and suggested the possibility of developing *O. gratissimum* essential oil as a drug for treating helminthiasis of small ruminants. Earlier, Hordegen (2003) studied the efficacy of different plant extracts against the helminth parasites *Trichostrongylus colubriformis* and *H. contortus* and proved the significant inhibitory potential of *Fumaria parviflora* extracts. The *in vitro* anthelmintic study against the free living nematode, *Caenorhabditis elegans,* showed the effectiveness of *B. grandiflora* extracts and the compound betulinic acid isolated from it (Enwerem et al. 2001). Similarly, the effectiveness of aqueous extract of *Mangifera indica* and the compound mangiferin against *Trichinella spiralis* infections in mice was established (Garcia et al. 2003). The extracts from *Xylopia aethiopica*, a

traditional medicinal plant of Nigeria showed anthelmintic potential against the hook worm of rodents, *Nippostrongylus brasiliensis* (Suleiman et al. 2005). Earlier, Kozan et al. (2006) showed *in vivo* anthelmintic potential of herbal extracts from *Mentha longifolia, Jasminum fruticans, Zea mays, Juniperus oxycedrus Juniperus drupacea, Pinus nigra, Plantago lanceolata* and *Citrullus lanatus,* against mice pinworms, *Syphacia obvelata* and *Aspiculuris tetraptera*. The effectiveness of the leaf extracts from *Tephrosia vogelii* and *Vernonia amygdalina* was evaluated and found effective against *Ascaridia galli* (Siamba et al. 2007). Likewise, the *Carica papaya* extracts was reported to be active against a series of helminth parasites including *Trichostrongylus tenuis*, *A. galli* and *Heterakis gallinarum* (Ameen et al. 2012). The *in vitro* testing of alcoholic extracts of fruits of *Piper longum* and *Allium sativum* bulbs showed prominent paralyzing effects against the flukes, *Fasciola gigantica* and *Gigantocotyle explanatum* (Singh et al. 2007). Earlier, Lalchhandama (2009) tested and proved the anthelmintic potential of *Acacia caesia* bark extract on the avian gastrointestinal parasitic tapeworm *Raillietina echinobothrida* by inducing trans- tegumental alterations. The *in vitro* anthelmintic study of *Coriandrum sativum* extracts showed strong efficacy against the eggs and larvae of *H. contortus* by significantly reducing the faecal egg count and worm counts (Eguale et al. 2007a). Similarly, in an *in vivo* study, *Azadirachta indica* seed extracts showed significant potential against *H. contortus* and *Trichostrongylus* sp (Chagas et al. 2008; Iqbal et al. 2010). Furthermore, a previous study compared the effectiveness of extracts from *Aristolochia* sp to the synthetic anthelmintic drug febendazole, and proved more effective than this marketed drug against *H. contortus* (Mini et al. 2013). Maggiore et al. (2012) showed the anthelmintic properties of essential oil from *M. pulegium* against *Echinococcus granulosus*. Recently, Sebai et al. (2020) showed the *in vitro*

and *in vivo* anthelmintic potential of the hydro-ethanolic extract of *M. pulegium* against *H. contortus*.

Traditional medicinal plants as anthelmintic drugs in India

The use of traditional plants as medicines in India dates back to 3500-1800 BC wherein the use of plant based drugs for various curatives was mentioned in Rig-Veda (Tandon et al. 2011). Nevertheless, the wide acquaintance in this area has been transferred through different folklore practices available in different societies (Prakash & Mehrotra 1987). In the case of anthelmintic practices, review through the literatures showed that many previous studies employed the use of earthworm, *Pheritima posthuma* for testing the anthelmintic potential of plant based oils or extracts (Dixit & Varma 1975; Banerjee & Nigam 1978; Agarwal et al. 1979; Girgune et al. 1979; Mishra et al. 1979; Mehta et al. 1981; Garg & Kasera 1982; Nanda et al. 1987; Siddiqui & Garg 1990; Garg & Siddiqui 1992). The essential oils from *Piper betle* showed *in vitro* anthelmintic potential against earthworms (Ali & Mehta 1970). Similarly another study highlighted the significant anthelmintic potential of the extracts from *Cymbopogon nardus* over *C. citratus* and *Zanthoxylum alatum* against *P. posthuma* (Kokate & Varma 1971). These studies were executed purely based on the easy availability and morphological similarity exhibited by the earthworms with the roundworms, though they do not share any anatomical or physiological similarities with parasitic roundworms (Tandon et al. 2011). Alongside many *in vitro* and *in vivo* anthelmintic studies employed the use of actual parasitic helminths from nematode, cestode and trematode groups as experimental models. Many studies were designed based on the local availability of these parasites (Tandon et al. 2011). The important parasitic species that have been used for *in vitro* anthelmintic studies includes *H. contortus*, *Ascaridia galli*, *Ascaris*

suum, *Trichinella spiralis*, *Hymenolepis diminuta*, *Taenia* sp., *Raillietina echinobothrida*, *Fasciolopsis buski*, *Fasicola hepatica*, *Gastrothylax crumenifer*, and *Paramphistomum* sp. and for *in vivo* anthelmintic studies, the *H. contortus*, *H. diminuta* or mixed species of gastrointestinal helminth parasites were generally used (Akhtar et al. 2000; Tangpu et al. 2004; Tangpu et al. 2006; Yadav & Tangpu 2006; Mali & Mehta 2008). Garg & Atal (1963) studied the anthelmintic potential of a proteolytic enzyme called as calotropain, isolated from the latex of *Calotropis procera* against the helminth parasites *Oesophagostomum columbianum* of the family Strongylidae and *Bunostomum trigonocephalum* of the family Ancylostomatidae commonly observed in the sheeps. Gaind et al. (1964) reported the anthelmintic effects of triphala, a mixture of aqueous extracts of *Chebulic myrobalans*, *Emblic myrobalans* and *Beleric myrobalans*. They also reported that the synergistic effects of triphala showed higher anthelmintic potential compared to the activities of individual extracts. The extracts of the plants *Embelia ribes, Ananas sativus*, *Mucuna prurita* and *Melia azedarach* was reported to show significant anthelmintic potential against *Paramphistomum cervi* and *Taenia canina* (Neogi et al. 1963). The significant anthelmintic properties of the water extracts of the *Carica papaya* seeds against the parasitic roundworms *Ascaridia galli* and *Ascaris lumbricoides* were proven previously (Dhar et al. 1965). Another study proved the high susceptibility of a series of parasitic helminths such as *A. lumbricoides*, *F. buski*, *Moniezia expansa*, and *H. diminuta* when treated in the aqueous extract of *Cucurbita Mexicana* seeds (Shrivastava & Singh 1967). Similarly, *Alangium lamarckii* root bark extract exhibited noticeable activity against the hookworms of dogs and poultry animals (Dubey & Gupta 1968). Another study showed the susceptibility of *H. contortus* from goats when treated with the extracts of

Calotropis gigantea, *Cucurbita pepo*, *Momordica charantia*, *Juglans regia*, *Scindapsus offcinalis* and *Musa paradisaca* (Sharma et al. 1971). The alcohol extracts of *Zingiber officinale* rhizomes, *Helleborus niger* stem and *Agati grandiflora*, *Carum copticum* and *M. indica* seed extracts showed reasonable potential against *A. lumbricoides* (Kalesaraj 1974). Similarly, Kalesaraj (1975) showed the anthelmintic potential of *Zingiber zerumbet* against human parasitic roundworm *A. lumbricoides*. Furthermore, Palasonin, an active compound from *Butea monosperma, also* showed promising *in vitro* anthelmintic potential against *A. lumbricoides* (Lal et al. 1978). In another study by Dixit & Varma (1975) the rhizome oils of *Hedychium coronarium* and *H. spicatum* exhibited promising activity against earthworms and tapeworms compared to the piperazine phosphate. Likewise higher anti-tapeworm activity was exhibited by the essential oils of *Cinnamomum tamala* and *Boswellia serrata* than piperazine citrate in *in vitro* assays (Girgune et al. 1978). Similarly, the essential oils from a series of plants including *Inula racemosa*, *Cyperus rotundus*, *Gardenia lucida*, *Pistacia integerrima*, *Randia dumetorum* and *Litsea chinensis* showed noticeable activity against cestodes and earthworms (Girgune et al. 1979; Mishra et al. 1979). In another study, the *Ascaridia galli* showed high susceptibility to the extracts of *Momordica charantia*, *Carica papaya*, *Butea frondosa* and *Sapindus trifoliatus* (Lal et al. 1978). In another anthelmintic study, the condensed tannins extracted from *Danish legumes* showed strong *in vitro* nematicidal properties (Kahiya et al. 1999). Likewise, the *Ocimum sanctum* essential oils also showed *in vitro* anthelmintic potential against the free living nematode, *Caenorhabditis elegans* (Asha et al. 2001). Similarly, Roy & Tandon, (1996) reported the susceptibility of *Artyfechinostomum sufrartyfex* and *Fasciolopsis buski* on *in vitro* treatment with ethanol root-tuber extract of *Flemingia*

vestita. The *F. vestita* treated helminth showed extensive tegumental alterations. Similarly, root-tuber extract of *F. vestita* also showed potential anthelmintic activity against a series of nematode, cestode and trematode helminth parasites including, *A. lumbricoides*, *Heterakis gallinarum*, *Ascaris suum*, *R. echinobothrida*, *Paramphistomum* sp. (Tandon et al. 1997). Furthermore, Taylor et al. (1998) showed that genistein was the bio-active compound in the *F. vestita* extracts that induced paralysis and tegumental alterations to the tested helminth parasites. Das et al. (2004) validated the use of dried rhizomes of *Stephania glabra* and aerial roots of *Trichosanthes multiloba* against *A. galli*, *F. buski*, *Ancylostoma ceylanicum*, *H. gallinarum*, *R. echinobothrida*, and *A. suum*. The activity of compounds, genistein from *F. vestita* and tetrahydropalmatine from *S. glabra* was tested and proved effective against *Raillietina* sp (Das et al. 2009). Another study validated the use of some plants which were traditionally used to cure intestinal parasitic infections caused by cestodes. The study proved significant anthelmintic potential of *Houttuynia cordata* and *Psidium guajava* leaf extracts and *Lasia spinosa* stalk extract against *R. echinobothrida* (Temjenmongla & Yadav 2005). Furthermore the *L. spinosa* leaf extract was proved effective against *H. diminuta* and *Trichinella spiralis* (Temjenmongla & Yadav 2006; Yadav & Temjenmongla 2012). The study of Challam et al. (2010) showed the *in vitro* potential of *Lysimachia ramosa* against *F. buski*, *A. suum* and *R. echinobothrida*. Their study revealed serious surface damage to the treated parasites. Similarly, *Oroxylum indicum* stem bark extract showed strong inhibitory potential against the juveniles and adults of *H. diminuta* and proved more effective than the standard drug praziquantel (Deori & Yadav 2016).

Anthelmintic compounds of plant origin

The compounds from alkaloid class such as vinblastine and colchicine showed anthelmintic activity against *Schistosoma mansoni* in the *in vitro* conditions (Bogitsh 1977). A previous study reported that gingerol, a bio-active compound isolated from *Zingiber officinale,* completely reduced the miracidia of *S. mansoni* and cercariae of *B. glabrata* in infected mice (Adewunmi et al. 1990). The flavanone, pinocembrin showed very promising activity against infective eggs of *Ascaridia galli* and newly excysted *Fasciola hepatica* (Del Rayo-Camacho et al. 1991). Likewise the palasonin, an active compound isolated from the seeds of *Butea frondosa,* showed anthelmintic potential against *A. galli* by seriously affecting the energy metabolism of the parasites (Kumar et al. 1995). In another study, Kermanshai et al. (2001) reported the *in vitro* and *in vivo* anthelmintic potential of benzyl isothiocyanate and cysteine proteases from papaya seed extract against the rodent gastrointestinal nematodes such as *Trichuris muris*, *Heligmosomoides polygyrus,* and *Protospirura muricola*. Similarly, Molan et al. (2003b) proved the anthelmintic potential of flavan-3-ol gallates against the infective larvae of *Trichostrongylus colubriformis*. The active compound genistein isolated from the plant *F. vestita* was reported to show promising anthelmintic potential against different helminth parasites. Souza et al. (2008) isolated bis-tetrahydrofuran acetogenin from the seeds of *Annona squamosa*. This compound showed noticeable activity against the *H. contortus* eggs. The activity of genistein and tetrahydropalmatine from *S. glabra* was tested and proved effective against *Raillietina* sp (Das et al. 2009). Likewise, the treatment of *R. echinobothrida* with genistein, induced severe tegumental alterations and loss of motility to the flukes (Tandon et al. 1997**)**. Another study by da Silva et al. (2008) proved the promising

anthelmintic activity of biochanin A, an isoflavone against the nematode pinworm, *Aspiculuris tetraptera* in mice. Likewise, Vijaya & Yadav (2014) reported the efficacy of biochanin A against *H. diminuta* and compared the activity with the standard drug praziquantel. The biochanin A showed more effectiveness than the synthetic drug praziquantel used in the same concentration. A study by Lakshmi et al. (2010) exhibited the potent anthelmintic activity of the compounds flavones, naringin, naringenin, rutin, hesperetin and chrysin against the filarial parasite *Brugia malayi*. Here the naringenin, flavones and hesperetin showed prominent anthelmintic potential compared to other tested compounds. Similarly, Ayers et al. (2010) showed the *in vitro* and *in vivo* anthelmintic potential of herbal compounds such as linoleic acid (fatty acid), roselipins and aurantiogliocladin against *H. contortus* in *in vitro* conditionand *Heligmosomoides polygyrus* in the *in vivo* environment. Lin et al. (2010) in their study showed dose dependent activity of hexahydrocurcumin, gingerol and shogaol against the rat lungworm, *Angiostrongylus cantonensis.* The study of Leite et al. (2011) showed the efficacy of an alkaloid, epiisopiloturina extracted from *Pilocarpus microphyllus* against *S. mansoni*. By altering worm cuticles, the compound induced significant mortality to the parasites. A study by Sissouma et al. (2011) showed the *in vitro* anthelmintic potential of 3-(3-Arylpropenoyl) imidazopyridine, a phenolic compound against *H. contortus*. Another compound, piplartine, exhibited strong time and concentration dependent activity against *S. mansoni* (de Moraes et al. 2012). Additionally, the microscopic examination of piplartine treated parasites showed severe alterations to the teguments and tubercles compared to the parasites in the untreated control group. Keiser et al. (2012) showed the *in vitro* anthelmintic efficacy of the compound mangostin extracted from *Garcinia mangostana*, against different helminth parasites

such as *Fasciola hepatica*, *Echinostoma caproni*, and *Schistosoma mansoni*. Similarly, Kozan et al. (2013) studied the nematicidal effects of luteolin-7-b-oglucopyranoside and quercetin-3-O-b-glucopyranoside, isolated from *Vicia pannonica* against *Trichostrongylus* larvae. Another study by Dasgupta et al. (2013) evaluated the *in vitro* anthelmintic potential of the active compound virosecurinine from *Securing* virosa. Their study showed prominent anthelmintic activity against *R. echinobothrida* causing severe structural alterations to the teguments of the helminth parasite. Several studies reported the effects of tannins against the helminth parasites. Molan & Faraj (2010) evaluated the efficacy of condensed tannins against the nematode, *Teladorsagia circumcincta*. Their results showed that the treatment of tannins severely affected the egg hatch and larval motility of the tested parasites. Studies of Waller & Thamsborg (2004) showed the potential of condensed tannins extracted from the plants showed significant efficacy against the gastrointestinal helminth parasites. The inclusion of tannins of phyto origin in the poultry diet significantly reduced the faecal egg counts of *Trichostrongylus colubriformis* (Butter et al. 2000) and *H. contortus* (Max et al. 2005). The *in vitro* treatment of plant derived condensed tannins against the nematode *A. suum* showed strong anthelmintic potential by causing huge mortality to the 3rd instar larvae (Williams et al. 2014). Furthermore, treatment of condensed tannins on different life cycle stages of swine fluke *Oesophagostomum dentatum,* showed high susceptibility of free-living larvae compared to moderate susceptibility of the infective stages (Williams et al. 2014). A compound named α-viniferin isolated from *Carex baccans* was tested effective against *R. echinobothrida*. The active compound induced severe distortions and degenerations to the teguments, altered cellular organelles, crumbling of microtriches and disorganization of muscle bundles (Giri & Roy 2014). Likewise, a

similar study explored the effects of the compound, resveratrol from grapes, berries and peanuts against *R. echinobothrida*. This study results showed that the resveratrol induced apoptosis in helminth parasites leading to severe nuclear damages and death of the organisms (Giri & Roy 2014).

Phenolic class of secondary metabolites as anthelmintic drugs

The anthelmintic potential of herbal extracts is accredited to the synergic effect of many secondary metabolites including tannins, flavonoids and other phenolic compounds (Klongsiriwet et al. 2015). Furthermore, alongside their anthelmintic potential, phenolic compounds exhibited a noticeable ability to reduce oxidative stress through the production of non-enzymatic antioxidants (Lingua et al. 2016). Many previous studies stated the important role of the phenolic class of secondary metabolites for the observed anthelmintic property exhibited by herbal extracts. According to Klongsiriwet et al. (2015) the anthelmintic potential of plant extracts can be attributed to the synergic effect of phenolic class of secondary metabolites such as flavonoids, tannins and other polyphenol compounds. The role of flavonoids and condensed tannins isolated from plants were tested and proved against helminth parasites (Hoste et al. 2006; Akkari et al. 2008; Paria et al. 2012). Many previous study results showed that flavonoids alone or in combination with condensed tannins exhibits significant anthelmintic potential against different helminth parasites (Athanasiadou et al. 2001; Azando et al. 2011; Klongsiriwet et al. 2015). Furthermore, dose dependent anthelmintic potential of phenolic compounds were demonstrated in both *in vitro* (Hoste et al. 2006; Molan et al. 2003a) and *in vivo* studies (Hoste et al. 2006; Terrill et al. 2009). In another study, Akkari et al. (2016) showed that the compound Kaempferol isolated from the flowers of *Capparis spinosa* showed anthelmintic property. In their study higher anthelmintic

potential of flower bud extracts of *Capparis spinosa* against the eggs and adults of *H. contortus* could be attributed to the presence of high concentrations of active phenolic class of metabolites. Likewise, Lasisi & Kareem (2011) reported the anthelmintic potential of quercetin isolated from the stem bark extract of *Bridelia ferruginea*. Recently, Sebai et al. (2020) also isolated the same compound from the extracts of *M. pulegium* that exhibited significant activity against *H. contortus*. Ferulic acid, another phenolic metabolite isolated from *Hibiscus mutabilis, showed* a significant filaricidal potential against *Setaria cervi* (Saini et al. 2012).

Mode of action of anthelmintic agents

Regarding the mechanism of action of anthelmintic agents, the tegument or cuticle seems to be an important target. Both synthetic and natural drugs were reported to alter the morphology of tegumental regions (Alvarez et al. 2006; Mehlhorn et al. 1983). Considering this point many researchers extend their anthelmintic study to evaluate the mode of action of synthetic chemicals or herbal extracts using microscopic techniques, especially scanning electron microscopy (SEM) seems to be an important tool in investigating the alterations of teguments (Tandon et al. 2011). A study that explored the potential of the tuber extracts of *F. vestita* against *A. suum* under *in vitro* conditions also showed wrinkle and crack formations on lips and body cuticle of the treated helminth parasites compared to control group (Yadav et al. 1992). Similarly, vacuolization and pit formation was observed when *F. buski* treated with *Artyfechinostomum sufrartyfex* extracts (Roy & Tandon 1996). Likewise, the treatment of *R. echinobothrida* with genistein, the active compound of *F. vestita,* induced severe tegumental alterations and loss of motility to the flukes (Tandon et al. 1997). In a similar study the *in vitro* treatment of

Stephania glabra extract on *R. echinobothrida* also showed observable changes to the tegument area (Tandon et al. 2004).

Antimicrobial resistance in pathogenic bacteria

Antimicrobial resistance, including the resistance exhibited by bacteria, fungi, viruses and protozoans, intimidates the capability to treat various infectious diseases across the world (Mcewen & Collignon 2018). The projected annual deaths worldwide due to antimicrobial resistance will reach about 10 million by the year 2050 (O'Neill 2016). Certain bacterial strains are naturally resistant to one or more classes of antibiotics. Whereas certain others initially show susceptibility to the antibacterial agents obtain resistance eventually (Tenover 2006). Hence, before developing an antibiotic drug one should have proper knowledge regarding the resistance mechanisms exhibited by bacteria (Walsh 2000). Pathogenic bacteria exhibit resistance to standard antimicrobial drugs mainly through three mechanisms: target site modifications (Spratt 1994), enzymatic inactivation of antimicrobials and by the activation of efflux pumps (Davies 1994). A number of pathogenic bacteria develop resistance by modifying the targets of antimicrobial agents. This modification results in the reduced affinity of the ligand (antibiotics) to its binding site (Lambert 2005; Sibanda & Okoh 2007). Different enzyme induced modifications are common among several pathogenic bacterial strains. The 50S ribosome of the *Streptococcus* sp. is the major target for synthetic antibacterial molecules such as macrolides, lincosamide and streptogramin B. However, methylation of N6 amino group of an adenine nucleotide in 23S rRNA induced conformational changes in the binding sites for these antibiotics and made them inactive against the bacteria (Kataja et al. 1998; Seppala et al. 1998). The enzymatic inactivation of antimicrobial agents was reported to be chiefly through the

production of certain hydrolytic enzymes and transferases (Wright, 2005). The genes that code for resistant enzymes are generally observed on bacterial plasmids or other mobile genetic elements (Sibanda & Okoh, 2007). The resistance of *S. aureus* and *S. pneumonia* towards β-lactams by the production of β-lactamases was reported earlier by Golemi-Kotra et al. (2003). The β-lactamases hydrolyse the amide bonds of the β-lactam ring present in the antibiotic and thereby making it inactive (Wilke et al. 2005). Similarly, the resistance to aminoglycosides was induced by the enzymatic process such as acetylation and phosphorylation of specific sites (Over et al. 2001). The bacteria equally make use of antibiotic efflux mechanisms alongside the aforementioned methods. The multi-drug resistant efflux pumps are ubiquitous proteins observed in both Gram-positive and Gram-negative bacterial strains (Akama et al. 2005). The bacteria produce the efflux pump proteins by expressing the house-keeping genes present on the bacterial genome (Lomovskaya & Bostian 2006). Many studies reported that the pathogenic bacteria exhibit the efflux mechanism against almost all synthetic antibiotics studied till date (Gill et al. 1999; Lin et al. 2002). The majority of the bacterial efflux proteins work non-specifically by utilizing high energy molecules available in the cell. They recognize and exclude a wide range of antibiotic molecules without any special preferences (Kumar & Schweizer 2005). Furthermore, this resistance mechanism allows the pathogenic strains of bacteria to survive even in the elevated antibiotic concentrations (Marquez 2005). Earlier, Lomovskaya & Bostian (2006) reported that the dominant resistance exhibited by the Gram-negative strains was due to the combinatorial effects of their membrane barrier and efflux proteins. The NorA protein characterized by the Gram-positive bacterium, *S. aureus,* isone of the best studied chromosome encoded pumps observed with Gram-positive strains (Hooper, 2005). This NorA proteinconfers

resistance to chief antibiotic molecules such as chloramphenicol and fluoroquinolone (Hooper, 2005). Another efflux protein, PmrA is a chromosome encoded multidrug resistant pump that provides resistance to the *S. pneumonia* like that of NorA protein in *S. aureus* (Kohler et al. 1999). AcrAB-TolC is a multidrug resistant efflux pump observed with Gram-negative bacteria. The Gram-negative *Escherichia coli*, reported to use AcrAB-TolC to expel an extensive range of antibacterial compounds (Touze et al., 2004).

Plants as sources of new antimicrobials and resistance modifying agents

The use of plants as the source of medicine has been documented by the extensive history of medical records from different civilizations across the globe (Chassagne et al. 2020). The oldest written record dates back to 2600 BC and was obtained from the clay tablets in Mesopotamia that explained the use of oils from the Mediterranean cypress tree (*Cupressus sempervirens* L.) and opium poppy (*Papaver somniferum* L.) for treating different diseases (Cragg & Newman 2005). Today, in various parts of the world, especially in developing countries about 70 to 95% of people rely on plant based formulations to meet their primary healthcare needs. Also, many countries have adapted and implemented new regulations to incorporate herbal medicines into mainstream healthcare practices (RBGWillis 2017). Based on the data published by Medicinal Plant Names Services (MPNS), about 28,187 species (~7.5% of all plant species on Earth) are recorded as being used medicinally (MNPS 2020), but approximately 4,478 species were only scientifically validated (RBGWillis 2017). In the case of the number of plant species evaluated for antibacterial potential, countries such as South Africa, Cameroon, Brazil, India, and Iran occupy the top five ranks (Chassagne et al. 2020). Plants have been traditionally used as a source of novel drug molecules, as plant derived extracts or compounds

have made huge donations to human health and welfare (Iwu et al. 1999). Owing to their traditional use as medicines for various infectious diseases, searches have been extended to isolate crude extracts or compounds with promising antimicrobial potential from plants (Betoni et al. 2006). To overrule the scarcity of new antibacterial agents and resistance towards the present antibiotics, many workers explored plants for novel, less resistant, and environmentally safe antimicrobials (Chassagne et al. 2020). And many of the plant derived extracts and compounds showed promising results in overruling the antibiotic resistance in pathogenic bacteria (Rossiter et al. 2017). Undeniably, plants represent the storehouse of bioactive secondary metabolites to fight against various pests and pathogens before they induce serious damages (Chassagne et al. 2020). Plant secondary metabolites help protect plants from biotic as well as abiotic stresses. They also act as a tool for communication with various invertebrates and microorganisms. So without these chemicals plant development and sustainability would be seriously affected (Kessler & Kalske 2018; Wink 2020). The secondary metabolites of plants are generally classified into one of three broad classes namely, phenolics, alkaloids and terpenoids. Many compounds from these classes act individually or in combination with other secondary metabolites from the same class or different classes. The combinatorial effects produced by these bioactive compounds are called synergy. The plant secondary metabolites acts synergistically by targeting multiple receptors, facilitating the transport towards specific targets, developing immunity from degradation and also modifying the microbe's resistance mechanism (Gilbert & Alves 2003). Previous studies reported that the synergistic effects of plant crude extracts were lost when the extracts were divided into different fractions (Abreu et al. 2017; Inui et al. 2012). Furthermore, there has been a developing notion in this

research field that the utilization of single active compounds is not sufficient to entangle microbial infection and overrule the microbial resistance. Hence, synergy and other related interactions of compounds must be evaluated precisely (Caesar & Cech 2019). Alongside the study of synergy within the crude extracts of plants, recent innovations also focuses on finding the herbal compounds that exhibits synergy with existing standard antibiotic drugs more precisely a compound or compounds that acts as resistance-modifying agents for use against the resistant bacterial strains (Abreu et al. 2012; Abreu et al. 2017; Dettweiler et al. 2020).

Phenolic compounds as antibacterial agents

The phenolic class of secondary metabolites include a diverse range of bioactive natural compounds that have been extensively utilized for medical purposes (Khameneh et al. 2019). The bioactive flavonoids, tannins and several other phenylpropanoids come under this class of secondary metabolites. Many previous studies reported the role of these bioactive compounds to develop significant activity against many of the resistant bacterial strains through various mechanisms (Farhadi et al. 2019; Gorniak et al. 2019; Ramezani et al. 2004). The phenolic compound resveratrol was reported to show a significant inhibitory potential against the efflux pumps of many bacteria. The CmeABC efflux pump of *Campylobacter jejuni* and another efflux pump of *M. smegmatis* were inhibited by resveratrol (Lechner et al. 2008; Klancnik et al. 2017). When Ferreira et al. (2014) studied the efflux pump inhibitory potential of resveratrol against the *Arcobacter butzleri* and *Arcobacter cryaerophilus*, an increased accumulation of ethidium bromide was observed. Another compound, baicalein, a flavone isolated from the roots of *Thymus vulgaris*, *Scutellaria baicalensis*, and *Scutellaria lateriflora* showed prominent antibacterial potential against a series of bacterial strains (Lu et al. 2011).

Furthermore, the baicalein was reported to restore the efficacy of β-lactam antibiotics, ciprofloxacin and tetracycline against MRSA strains through inhibition of the efflux pump NorA (Chan et al. 2011). Fujita et al. (2005) reported the synergistic effects of baicalein and the standard drug tetracycline against *E. coli*. Another isoflavone molecule biochanin A was reported to inhibit the efflux pump, NorA of MRSA (Zou et al. 2014). Similarly, the efflux pump inhibitory potential of biochanin A was reported against *Mycobacterium* strains (Cannalire et al. 2017; Lechner et al. 2008). Morel et al. (2003) reported the activity of isoflavonoids and flavonolignans on the NorA efflux pumps. The NorA efflux pump of *S. aureus* was inhibited by the flavonolignan compound silybin from the medicinal plant *Silybum marianum* and isoflavonoid compounds biochanin A, genistein, and orobol from *Lupinus argenteus* (Morel et al. 2003; Stermitz et al. 2001). Interestingly, the hybridization of many known antibiotics with the flavonoid compounds were reported to enhance the inhibitory potential on the efflux pumps and also deregulates the resistance mechanisms in bacteria (Xiao 2014). The bioactive flavonoid molecule kaempferol isolated from plant parts showed potent activity against the MRSA and fluconazole-resistant *Candida albicans* (Randhawa et al. 2016; Shao et al. 2016). Additionally, inhibition of the MRSA efflux pump by kaempferol was similar to the mechanism exhibited by the control drug verapamil. Furthermore, the kaempferol rhamnoside a glycoside derived from the kaempferol of natural origin showed significantly upregulated the potential of the antibiotic ciprofloxacin against activity against the NorA overexpressed *S. aureus* bacteria (Holler et al. 2012a). The bacterial NorA efflux pump inhibition by 4,6-Dihydroxy-3,5-dimethyl-2-methoxychalcone, a chalcone isolated from *Dalea versicolor,* was reported by Belofsky et al. (2004). Similarly, Holler et al. (2012b) screened 117 chalcones and

published the potential inhibition of bacterial efflux pump by two synthetic chalcones 4-phenoxy-4′-dimethylaminoethoxychalcone and 4-dimethylamino-4′-dimethylaminoethoxychalcone. The epigallocatechin gallates (EGCG), another category of polyphenols, were also reported to inhibit the NorA efflux pump of MRSA strains (Gibbons et al. 2004). Alongside the inhibition to efflux pumps certain other phenolic compounds make use of different modes of action against the pathogenic bacterial strains (Farhadi et al. 2019). The synthetic aminocoumarin molecule novobiocin was reported to inhibit the enzyme DNA gyrase of bacteria. This inhibition stalls the DNA replication process in the bacteria (Anderle et al. 2008). Likewise the natural phenolic compounds also inhibit the DNA replication process. The green tea tannin, EGCG, was reported to inhibit the ATP binding site of the bacterial DNA gyrase enzyme (Gradisar et al. 2007). Along with the bacterial DNA gyrase inhibition, the EGCG showed different mechanisms of action. As mentioned before the EGCG targets bacterial efflux pumps and also their chromosomal penicillinase enzyme (Khameneh et al. 2019). Another tannin, chebulinic acid extracted from the plant *Terminalia chebula* showed potent inhibition to the DNA gyrase of quinolone resistant *M. tuberculosis* effectively when screened using *in silico* methods (Patel et al. 2015). Natural anthraquinones also target the bacterial replication system by inhibiting the DNA gyrase enzyme. In a study by Duan et al. (2014) a semisynthetic natural anthraquinone derivative called haloemodin showed strong inhibition to the DNA gyrases of MRSA and vancomycin-resistant *Enterococcus faecium*. Wu et al. (2016) screened a novel polyphenol compound 3-p-trans-coumaroyl-2-hydroxyquinic acid (CHQA), isolated from the plant *Cedrus deodara* against eleven food-borne pathogenic microbes and proved effective. This compound induced severe cytoplasmic membrane damage to

the susceptible bacteria. Likewise the hydroxycinnamic acid category of phenolic molecules such as caffeic acid, ferulic acid and p-coumaric acid are natural compounds that show potent antibacterial activity by disrupting the membranes of the target microorganisms. Here, the p-coumaric acid being highly lipophilic was reported to possess a significant membrane disrupting potential compared to other compounds under hydroxycinnamic acid category (Campos et al. 2009). Similarly the previously mentioned flavonoid compound Kaempferol showed strong activity against the Gram-negative bacterium *E. coli* by reducing the membrane fluidity and thereby rigidifying the *E. coli* membrane (Wu et al. 2013). The *in silico* screening of flavanones such as taxifolin, eriodictyol and naringenin showed potent inhibition to the *E. faecalis* KAS III enzyme. Same time the *in vitro* studies showed only moderate activity against *E. faecalis* (Jeong et al. 2009). Similarly, another flavonol, 3,6-dihydroxyflavone was reported to inhibit the KAS III and I enzymes of *E. coli* (Farhadi et al. 2019). These results indicate the potential role of flavonoids in inhibiting the crucial enzymes responsible for the development of bacterial membranes. curcumin, a well-established phenol compound isolated from the turmeric plants, exhibited significant activity against *E. coli* and *S. aureus* by causing serious damages to the cell membranes (Tyagi et al. 2015). The bacterial enzyme d-Alanine:d-alanine ligase is critical for the biosynthesis of peptidoglycan layer. Apigenin and quercetin, two important flavonoids inhibit the critical enzyme d-Alanine:d-alanine ligase of *H. pylori* and *E. coli*. Here the quercetin showed higher potential compared to apigenin (Wu et al. 2008). A prenylated flavonoid molecule sophoraflavanone B showed noticeable activity against the MRSA by altering the peptidoglycan layer of tested bacteria (Mun et al. 2014). Certain other published data showed that important enzymes such as urease, dihydrofolate

reductase, and sortase of bacteria were considered as important targets of some of the polyphenols (Navarro-Martínez et al. 2005; Maresso et al. 2008; Xiao et al. 2013). These previous results clearly highlight the importance of using phenolic class of secondary metabolites against a wide range of bacteria. The synergistic activity of crude extracts having polyphenol compounds make bacteria more susceptible and relatively less resistant.

Family Zingiberaceae

The family Zingiberaceae consists of perennial herbs with a modified fleshy stem called rhizome that occurs below the soil. The Zingiberaceae family is comprised of economically and medicinally important spices such as ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), galangal (*Kaempferia galanga*) and black cardamom (*Amomum subulatum*) (Stevenson et al. 2007). Some common plant species from this family have been extensively used as condiments for flavoring owing to the presence of high amounts of flavor compounds (Chane-Ming et al. 2002; Naik et al. 2004; Pino et al. 2004). Some plant species are reported to contain compounds of medicinal interestowing to their antioxidant, antibacterial and antifungal potential (Masuda et al. 2004; Wilson et al. 2005). Alongside many species from this family are regularly prescribed by traditional medical practitioners for treating stomach-ache, carminative, diarrhea, and dysentery (Tewtrakul & Subhadhirasakul 2006). Antimicrobial potential of the plants from Zingiberaceae against different pathogenic microbes was reported by many previous studies. The antimicrobial activity of *Curcuma longa* extracts was reported against a series of bacteria such as *Bacillus cereus*, *Candida albicans*, *Escherichia coli*,*Helicobacter pylori*, *Listeria monocytogenes*,*Salmonella* sp. and *Staphylococcus aureus* (Thongson et al. 2004; Mahady et al. 2005; Sacchetti et al. 2005; Jagannath &

Radhika 2006). Similarly, the antimicrobial activity of *Alpinia galanga* extract against *Candida albicans*, *Cryptococcus neoformans*, *Entamoeba histolytica*, *Giardia intestinalis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* was reported in different studies (Haraguchi et al. 1996; Sawangjaroen et al. 2005; Voravuthikunchai et al. 2005; Phongpaichitet al. 2006; Sawangjaroen et al. 2006). Some other studies reported the antimicrobial potential of *Zingiber officinale* extracts against *Aspergillus niger*, *Bacillus cereus*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Listeria monocytogenes*,*Salmonella* sp., *Staphylococcus aureus* and *Streptococcus pyogenes* (Akoachere et al. 2002; Alzoreky & Nakahara 2003; Konning et al. 2004). In the case of antioxidant activity, the strong antioxidant potential of *Alpinia galanga* extract was attributed to the presence of curcuminoids and other phenolics in the extracts (Siripongvutikorn et al. 2005). Furthermore, the reducing power, lipoxygenase inhibition, scavenging of free radicals, scavenging of superoxide anions and Fe^{2+} chelating activities of *Alpinia galanga* extract have been reported (Juntachote & Berghofer 2005). Murakami et al. (1995) reported the effective inhibition potential of *Alpinia galangal* extracts against digestive tract tumor formation. Similarly the anti-inflammatory and antioxidant potential of *C. longa* and *Z. officinale* were reported (Nakatani 2000). Furthermore, the potential of *Z. officinale* to relieve nausea (Ernst & Pittler 2000) reduce digestive problems (Gupta & Sharma 2001) lower cholesterol (Bhandari et al. 1998) and prevent seizures (Minami et al. 2000) has been documented.

Genus *Kaempferia*

The genus *Kaempferia* of Zingiberaceae consists of about 60 species of plants (Swapana et al. 2018) that have been regularly used as medicines, spices and as ornamentals (Pancharoen et al. 2000). The name of the genus honours the well-

known German naturalist and physician Engelbert Kaempfer (Kumar et al. 2013). The genus *Kaempferia* can be broadly classified into two groups namely *K. galanga* group and the *K. rotunda* group based on the time of appearance of inflorescences (Phokham et al. 2013). The "spring-blooming" of *K. rotunda* group occurs during the end of March to early May. The inflorescences of *K. galanga* group appear mostly during August to September (Phokham et al. 2013). Regarding the distribution, the genus *Kaempferia* enjoys wide distribution in tropics, especially in India, China, Bangladesh, Thailand, Malaysia, Vietnam, Myanmar, Philippines, Indonesia, Laos and Cambodia (Pham et al. 2021). The ethno medicinal extracts from different plants under this genus have been used in traditional treatment systems for the cure against wound infections, malaria, diabetes, urticarial, herpes, allergic infections, inflammation etc. (Boonsombat et al. 2017). The *Kaempferia galanga* is considered one among the most common species used in the traditional treatment system in Asian countries. The leaf extract is used to treat fungal skin infection caused by *Tinea versicolor*. The flower extracts for treating eye diseases, the dried rhizome extracts for cardiac issues and neuronal problems, the stem extracts for dyspepsia and menstrual stimulation (Nopporncharoenkul et al. 2017; Saensouk et al. 2019). In China, the extracts from this species have been traditionally used to treat hypertension, abdominal pain, pectoral pain, toothache, inflammation, dyspepsia and coughs. Also the rhizome essential oils were reported effective for indigestion, abdominal problems, headache etc. (Saensouk et al. 2019). In Malaysia, the rhizome extracts of this species were reported to treat hypertension, ulcer and asthma related problems (Tuan & Trong 2017). In Vietnam, the alcohol extracts of rhizome were reported to be used for relief from muscle pain, abdominal pain, rheumatism, headache, cold treatment, joint pain, tooth pain, cough

expectorant and also for poor digestion (Chawengrum et al. 2018). In India, the *K. galanga* commonly known as 'kacholam' has been used as an important ingredient in about 59 Ayurvedic formulations such as *Dasamulariṣṭam*, *Kaccoradi curṇa*, *Valiya rasnadi kaṣhayam,Valiya Narayaṇa tailam*, *Asana eladi thailam* etc for treatment against various infections and diseases (Kumar et al. 2013; Ali et al. 2018). Alongside the highlighted medicinal use, this species is also utilized in cosmetics, perfumery and also as spices in Indian cuisines (Preetha et al. 2016). The methanol extract of *K. parviflora* exhibited potential toxicity against human cholangiocarcinoma cells (Leardkamolkarn et al. 2009). Similarly, the methanol extract also induced apoptosis in HL-60 cancer cells (Ratana et al. 2008). Panduratin A isolated from *K. pandurata* showed significant cytotoxicity on human epidermis KB cancer cells (Yanti Lee et al. 2009) and pancreatic cancer cells Pan-1 (Nwet 2008).

Kaempferia rotunda

Commonly known as Malan-kua, Chengazhi, Chengazhineer kizhangu (Malayalam), Bhuichampaka (Sanskrit), Bhuchampa (Hindi), and Blackhorm (English) and commonly known as peacock ginger is a perennial aromatic herb with a tuberous rhizome that are short, stout aromatic 2.5-3.5 x 2 cm; roots stout, fleshy, normally terminating in ovoid shape, 2-5 x 0.5 cm, yellow-white tubers. Leafy shoots are 50 cm high. Leaves few, radical, erect; lamina, 15-30 x 5-12 cm, oblong-lanceolate, with acute base and gradually acuminate apex, purple beneath, mottled green above, upper surface glabrous, lower surface densely covered with very short hairs; petiole 6-8 cm long; logule small, 2-4 mm long, hairy. Inflorescence appearing before the leaves, shortly peduncled, enclosed within greenish-purple, narrow sheaths; 4-12 flowered. Normally 1-2 flowers open at the same time. Calyx

5-6 cm long, unilaterally split, light violetish, sparsely hairy. Corolla tube longer than calyx, 6-7 cm long, slender, obliquely funnel-shaped towards the mouth; lobes white. Labellum 5.5-6 x 2-2.5 cm, broadly ovate, deeply divided into 2 suborbicular lobes, lilac with deep violet in the centre, with many radiating violet lines. Lateral staminodes 5-5.5 x 1.6 cm, ovate elliptic, tip acuminate, white with a violet tinge towards the margin. Filament short, erect, 5 mm long, upper half light violet. Ovary 5 x 3 mm, tricarpellary, with many ovules on axile placenta, glabrous. Fruiting is not common.

Distributed throughout India and cultivated in countries like Indonesia, Vietnam and Malaysia for medicinal purposes. The *K. rotunda* was initially introduced as an ornamental plant because of the presence of a prominent flower with white and purple colour. The spikes are radical, appearing before the leaves (Lim 2016).The leaves and rhizomes of this plant are cooked as vegetables, and also used as spices. The dried rhizome powder is available in Indonesian markets. This powder has been utilized for several traditional treatments (Lotulung et al. 2008). Likewise the folklore traditional medicinal practices reported the use of rhizome extracts to treat abdominal pain and the whole plant extracts to reduce fever. The ayurvedic formulation consisting of *K. rotunda* extracts as chief constituent was reported to treat atopic dermatitis and other skin diseases (Lotulung et al. 2008). Furthermore, a formulation with skin lightening potential contains *K. rotunda* extracts as a chief constituent and this regulates the melanin formation (Lotulung et al. 2008). In Vietnam, the rhizome extract was reported for treating abdominal pain, menstrual disorder and dysmenorrhea. Similarly in Malaysia, the *K. rotunda* rhizome is used for treating abdominal pain, viral infections, wound healing etc. (Jagadish et al. 2016; Karmakar et al. 2016). A previous study by Atun et al. (2013)

showed the preventive effects of *K. rotunda* rhizome extracts and the isolated flavone compounds against *in vivo* chromosome fragmentation. The results of their study were attributed to the abundance of antioxidant compounds in the extracts. Furthermore the study also highlighted the antioxidant, antimutagenic, and chemo preventive effects of the methanol rhizome extracts. A previous report by Pai et al. (1970) showed the presence crotepoxide, quercetin, chalcones, flavonols, syringic acid, protocatechuic, β– sitosterols, stigmasterol, and some hydrocarbons in the *K. rotunda* extracts. Crotepoxide, one of the main constituents of *K. rotunda* extracts, was reported effective for the prevention of tumor growth (Kupchun et al. 1969). The flower extract contains the compound benzyl benzoate that acts to treat scabies and is used presently to formulate ointments. The tuberous rhizome part has been used for malignancy and has potential blood clotting and antioxidant effects. Regarding the bioactive compounds, most of the bioactive compounds have been segregated from the tuberous rhizome part of the plant. In the main rhizome part, the benzyl benzoate was reported as the most abundant constituent (69.7%), whereas n-pentadecane prevailed mainly in the lateral parts (53.8%). Camphene, α-pinene, bornyl acetate and heptadecane are other chief constituents however the contribution of mono- and sesquiterpenes to the essential oil of *K. rotunda* was observed in negligible amounts (Woerdenbag et al. 2004). Likewise, Stevenson et al. (2007) showed that the methanol extract of *K. rotunda* rhizomes contained polyoxygenated cyclohexane derivatives and a triacylated derivative of salicin that assist killing the armyworm (*Spodoptera littoralis*) proving it's insecticidal potential.

Family Araceae

The family Araceae is considered as one of the highly diverse families with about 105 genera and 3,300 species worldwide (Mayo et al. 1997; Croat 1998;

Ribeiro et al. 1999; Coelho 2000; Vargas 2002). According to Croat et al. (1998) the great diversity of species under the Araceae has been observed in tropical America. Brazil is considered one of the important countries having the largest variety of Araceae, with 30 genera and 700 species (Mayo et al. 1997). About 800 species of plants from Araceae have been reported as edible, medicinal and ornamental. Most of the species from Araceae family are considered as ornamental plants in which the *Anthurium* Schott, *Philodendron* Schott, *Dieffenbachia* Schott, *Monstera* Adams and *Zantedeschia* Spreng are few important genera (Pedralli 2002). Many species are also considered as food. Approximately 10% of the world population consumes the rhizomes of *Colocasia esculenta* as food (Pedralli 2002). Alongside the utilization for ornamental and food purposes the plants from the Araceae family also possess medicinal importance. It was reported that in the Amazon region, many plants from the family Araceae have been utilized for traditional medicinal purposes, including the treatment of malaria and related fevers and the most important species are from the genus *Philodendron* Schott (Milliken et al. 1997a; Kvist et al. 2006). The whole plant extracts of *P. stratiotes* have been used in Togo to treat malaria and associated fevers (Lahitte et al. 1998; Kyei et al. 2012). The dichloromethane fraction of the ethanol extract of the stems of *Montrichardia linifera* showed significant antiplasmodial potential against *Plasmodium falciparum*. Whereas the ethanol and hexane extracts were found inactive against the Dd2 strain of *P. falciparum* (Amarante et al. 2011). Lekana-Douki et al. (2011) reported the capacity of dichloromethane root extracts of *Culcasia lancifolia* to inhibit the growth of FCB and W2 strains of *P. falciparum*. The aqueous and ethanol extracts of the leaves of *Pistia stratiotes* exhibited antiarthritic and antipyretic effects in formalin-induced arthritis and LPS-induced fever in Sprague-Dawley rats (Kyei et al. 2012). On the

other hand some plant species are used only for treating the symptoms associated with malaria such as heavy fevers and headaches. The extracts of *P.linnaei* were reported to treat the symptoms associated with malaria (Grenand et al. 1987). Likewise, the *Spathiphyllum floribundum* inflorescences are utilized for treating headaches (Croat 1994). The use of plant extracts from Araceae for curing wounds, stings, skin infections and insect bites were reported by Bown (2000). The use of another species *Amorphophallus konjac* was recorded for the treatment of wounds, bruises, tumors, and skin issues in China (Liu et al. 1998). The plant *Acorus calamus* has been used as appetite stimulant, carminative and antidiarrhoeal in countries like India, Egypt and China (Milne and Milne 1967). Similarly, the antibacterial and antifungal potentials of different species of Araceae including *Amorphophallus bequaertii* (Tshibangu et al. 2002) and *Sauromatum guttatum* (Khan et al. 2006) were reported. Singh et al. (2000) isolated andidentified 39 compounds from the *Homalomena aromatica* oils through HPLC and GC-MS analysis. Among the compounds identified the linalool showed potent antifungal potential against *Curvularia pallescens*, *Fusarium graminearum* and *Aspergillus niger*. Similarly, three new hydroperoxysterols isolated from another species *Xanthosoma robustum*, showed significant antibacterial potential against *Bacillus subtilis*, *Escherichia coli* and *Micrococcus luteus* (Kato et al. 1996). Some pharmacological studies also reported the anti- inflammatory and anticancer properties of plant extracts from the Araceae family. The *Philodendron guttiferum* extracts showed anti- inflammatory, antiviral activities (El-Seedi et al. 2001). Segura et al. (1998) tested the anti-inflammatory potential of water, ethanol, and dichloromethane extracts of *Anthurium cerrocampanense* using croton oil-induced mouse ear oedema test and proved the significant anti- inflammatory activity of dichloromethane extract. Bown

(2000) reported the use of*Pinellia ternata* extracts in Chinese traditional medicine to treat breast and stomach cancer and also leukaemia. The fresh extracts of *Typhonium divaricatum* showed significant effects on human cancer cell lines cultured from leukaemia, colon, and melanoma sources (Neoh 1996). Another species *Arisaema tortuosum*, commonly called as Himalayan Cobra lily, was reported to show the presence of a lectin that showed potent *in vitro* anticancer properties against human cancer cell lines (Dhuna et al. 2005). Another study by Tan et al. (2005) proved that the hexane extracts of *Epipremnum pinnatum* has the potential to induce non-apoptotic programmed cell death to T-47D breast tumor cells.

Genus *Lagenandra*

The genus *Lagenandra* consists of about 15 different species of which the majority are observed in Srilanka, India and Bangladesh (Sivadasan et al. 2001). The plants under the genus *Lagenandra* are perennial and all species are ecologically similar, and they are commonly observed along streams and rivers in forest and plantations but also in irrigation ditches and canals near rice fields. In the wet season they remain submerged and exhibit an entomophilous type of pollination (Sivadasan et al. 2001). Also, many species of *Lagenandra* are cultivated as ornamentals (Cook 1996). A very few studies are documented regarding the bioactive potentials of the extracts or compounds from the genus *Lagenandra*. Selvakumari & de Britto (2007) showed the antibacterial potential of *L. ovata* rhizome oil against a series of Gram-positive and Gram-negative bacteria. The Ayurvedic formulation 'Drakshadhi' is generally prepared with the rhizome of *K. rotunda* (Chengazhi) as a chief ingredient. This formulation has been prescribed for fever, burning sensation, vomiting, fainting, fatigue and stomach issues (Chopra 1982). When *K. rotunda* becomes

scarce, the rhizomes of *L. toxicaria* are used as substitutes in this formulation (Sasidharan & Padikkala 2012).

Lagenandra toxicaria

It is a procumbent or erect semi-aquatic evergreen rhizomatous herb; rhizome ca. 3 to 4 cm diameter. Leaves dimensions 30-40 x 10-15 cm, oblong-acuminate, pinnately parallel, involute venation**;** cylindrical petiole ca. 40-47 cm long, Inflorescence with a short peduncle of ca. 2 cm long. Spathe ca. 13-15 cm long, divided into a basal tube like portion, a middle broad limb and a terminal tapering candidate end; light pinkish cream in colour, smooth outer area; tube ca. 2 cm long, approximately 1.2 cm diameter, purplish having dark vertical ridges Inside; limb ca. 2-3 cm long, 1-1.5 cm diameter, laterally compressed and irregularly oriented with 6 cm long terminal caudation. Spadix is a very small car. 1.8 cm long with basal pistillate portion followed by a slender barren interstice of ca. 3 mm long, a staminate portion, and terminating into a short barren appendix. Fruit a more or less fleshy capsule; dehiscence by 3-4 longitudinal splits from the base upwards at maturity; seeds ca. 1.6 mm long, ovoid-ellipsoid, slightly bent, longitudinally ridged. The plant is used in the preparations of ointments for skin itch, renal and bilious complaints (Chopra et al. 1994). The *L. toxicaria* rhizomes and roots were also reported to be diuretic, carminative and also used for wound healing. Its rhizomes have also shown insecticidal and antimicrobial properties (Sivarajan & Indira 1994; Selvakumari & De Britto 2004). The rhizome oil has been shown to possess significant antibacterial potential against the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumonia* and also against the Gram-positive bacterium, *Staphylococcus aureus* (Selvakumari & De Britto 2004). Furthermore, the *in vitro* effectiveness of the rhizome oil was correlated with that of the standard drug

chloramphenicol (Selvakumari & De Britto 2004). The insecticidal potential of *L. toxicaria* extracts was documented previously (Kirtikar & Basu 1918).The rhizome oil of *L. toxicaria* as well as possesses insecticidal and germicidal properties (Selvakumari 2014). The filter paper impregnation technique evaluated the insecticidal potential of *L. toxicaria* rhizome oils against the ubiquitous storage pest *Tribolium castaneum* and obtained an LC_{50} of 0.069% within 24h treatment period (Selvakumari 2014).

Endophytic fungi

Plants often enter a mutualistic relationship with microorganisms, commonly called endophytes. They colonize inside the host tissues without causing any harm to the host plants (Schulz et al. 2002; Strobel et al. 2004). The plant provides shelter to these microbes, in return they help maintain the physiological and ecological roles of the host plants (Kaul et al. 2012; Rabiey et al. 2019; Wang et al. 2019). Importantly, the endophytes prevent the invasion of pathogenic microbes by producing bioactive metabolites. Alongside, the endophytic microbes upregulate the plant growth by preventing different biotic and abiotic stresses (Eid et al. 2019; Khare et al. 2018; Rabiey et al. 2019). Here, this mutual interaction is regulated by genes of both mutualists and controlled by different environmental factors (Moricca & Ragazzi, 2008). About one million endophytic species are estimated to be present in the plant kingdom (Fouda et al. 2015). Among the endophytic microbes, the fungal endophytes seem to be more efficient in the production of pharmacological active metabolites with antibacterial, antifungal, anticancer and antiviral properties (Jalgaonwala et al. 2017). It is also relevant to mention the capacity of a few endophytic fungal groups to produce the same growth hormones produced by their host plants (Rai et al. 2014). As a part of coevolution, these endophytic fungi

developed the capacity to synthesize biologically active metabolites that are identical to their hosts (Zhao et al. 2011; Jia et al. 2016).

Endophytic fungi as antibacterial agent

The world health organization (WHO) reports that antibiotic resistance by pathogenic bacteria ruins the human health and related health care system by imposing severe financial burden (WHO 2020). Over the past couple of years many novel antibiotic molecules entered the market. However, many reports later confirmed the resistance of microbes emerging against all these molecules (Manganyi et al. 2020). Huge resistance against standard synthetic antimicrobial drugs highlighted the importance of using less resistant and environmentally safe natural antibacterial molecules (Aharwal et al. 2016). This search for less resistant and environment friendly molecules opened the doors towards relatively less explored endophytic fungal research and many studies already reported the capability of endophytic fungi to overrule the invasion of pathogenic microbes by the production of secondary metabolites (Khare et al. 2018, Pavithra et al. 2019; Fadiji et al. 2020). The fungal secondary metabolites having antimicrobial properties have been classified under different classes, such as phenolic class of metabolites, terpenoid class of metabolites, alkaloid class of metabolites and peptides (Tirsit, 2018).

The antimicrobial compounds from Ascomycetes

Ascomycetes are one of the largest groups of fungi with great therapeutic importance. Some genera of this huge phylum are vigorous producers of diverse bioactive metabolites. The well-known antibacterial molecules such as penicillin and cephalosporins were isolated from fungi that belong to this group (Deshmukh et al. 2014). The genus *Aspergillus* classified under Trichocomaceae family of order

Eurotiales and class Eurotiomycetes under the division Ascomycota (Geiser et al. 2006; Krishnakumar et al. 2021) contains a ubiquitous group of fungi having pathological and therapeutic importance (Vadlapudi et al. 2017). The *Aspergillus* sp. CY725 isolated from the plant *Cynodon dactylon* was reported to produce antibacterial molecules such as monomethyl sulochan, helvolic acid, 3β-hydroxy-5α, 8α-epidioxy-ergosta-6, 22-diene, and ergosterol. These molecules showed prominent antibacterial activity against the bacteria *Helicobacter pylori*. Additionally, helvolic acid showed inhibitory potential against *Sarcina lutea* and *Staphylococcus aureus* (Li et al. 2005). Mixed culture of endophytic *Aspergillus* strains FSY-01 and FSW-02 produced antibacterial compounds aspergicin and neo aspergillic acid. The aspergicin and neo aspergillic acid showed prominent activity against *Staphylococcus epidermidis*, *S. aureus*, *Escherichia coli*, *Bacillus subtilis*, *Bacillus dysenteriae* and *Bacillus proteus* (Zhu et al. 2011*). Aspergillus* sp., isolated from the mangrove plant *Bruguiera gymnorrhiza* produced dihydroisocoumarin derivatives aspergillumarins A and B. Both exhibited low to moderate activity against *B. subtilis* and *S. aureus* (Li et al. 2012). The *Aspergillus versicolor* from the brown alga *Sargassum thunbergii* produced antimicrobial compounds 6, 8-di-O-methylaverufin, brevianamide M and 6-O-methylaverufin that showed activities against *E. coli* and *S. aureus* (Miao et al. 2012). Similarly, siderin and isorhodoptilometrin-1-Me ether isolated from *A. versicolor* of the green alga *Halimeda opuntia* induced antimicrobial potential *against B. subtilis*, *S. aureus* and *Bacillus cereus* (Hawas et al. 2012). The metabolites yicathin B and C from the endophytic fungus *Aspergillus wentii* PT-1 isolated from the alga *Gymnogongrus flabelliformis* showed antibacterial activity against *E. coli* and *S. aureus* (Sun et al. 2013). The alkaloid class of metabolites pseurotin A and fumigaclavine C produced by the endophytic

fungus *Aspergillus* sp. EJC08 isolated from *Bauhinia guianensis* showed conspicuous activity against *Pseudomonas aeruginosa*, *B. subtilis*, *S. aureus* and *E. coli*. (Pinheiro et al. 2013). The *Aspergillus* sp. (ZJ-68) isolated from the mangrove plants was reported to produce phenylpropanoid derivatives that possess antimicrobial potential (Cai et al. 2019). A novel cyclic pentapeptide, malformin E, along with 13 known cyclic dipeptides, were isolated from the endophytic fungus *Aspergillus tamarii* from *Ficus carica*. The malformin E showed conspicuous antimicrobial potential against *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* (Ma, et al. 2016). Earlier, Ogbole et al. (2017) reported the antibacterial potential of the crude extracts of endophytic fungus *A. tamarii* isolated from the plant *Lycoperdon umbrinum*. The *A. tamarii* showed considerable activity against *E. coli*, *S. typhi*, *S. aureus* and *B. subtilis*. Fusapyridon A isolated from *Fusarium* sps. from the plant *Maackia chinensis* showed activity against *P. aeruginosa* and *S. aureus* (Tsuchinari et al. 2007). Beauvericin isolated from the endophyte *Fusarium redolens* Dzf2, inhabitant of *Dioscorea zingiberensis* showed potent activity against *Pseudomonas lachrymans*, *Staphylococcus haemolyticus*, *B. subtilis*, *X. vesicatoria*, *E. coli* and *Agrobacterium tumefaciens* (Wu et al. 2010). Similarly, the beauvercin produced by the fungus *Fusarium oxysporum* inhabitant of *Cinnamomum kanehirae* showed conspicuous activity against MRSA and *B. subtilis* (Wang et al. 2011). Antibacterial compounds such as javanicin, dihydronaphthalenone, 3-O-methylfusarubin and 5-Hydroxy-3- Methoxydihydrofusarubin A were isolated from *Fusarium* sp. BCC14842 from bamboo leaf (Kornsakulkarn et al. 2011). The *Fusarium* sps. isolated from mangroves produced Fusaric acid which exhibited potent antibacterial activity against *Mycobacterium* tuberculosis H37Rv and *M. bovis* (Pan et al. 2011). The plant *Ficus carica* reported to the storehouse of a potent endophytic fungus

Fusarium solani that produced several antibiotic compounds such as gliotoxin, helvolic acid, fumitremorgin B, fumitremorgin C, Bis-N-norgliovietin and bisdethiobis (methylthio) gliotoxin. All of these compounds showed significant activity against *P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus* (Zhang et al. 2012). The mixed culture of the bacterium *B. subtilis* and the endophytic fungus *Fusarium tricinctum* isolated from the plant *Aristolochia paucinervis* produced the antimicrobial compounds enniatins A1, enniatins B1 and lateropyrone. These compounds exhibited antibacterial potential against the bacteria *S. aureus*, *S. pneumoniae* and *E. faecalis* (Ola et al. 2013). A benzophenanthridine alkaloid, Sanguinarine isolated from the fungus *Fusarium proliferatum* of the plant *Macleaya cordata* showed potent antibacterial activity against a series of pathogenic bacterial strains (Wang et al. 2014).

Penicillium sps are filamentous fungi that are commonly observed in soil habitats and plants as endophytes (Visagie et al. 2014). The *Penicillium* sps. have already been considered as being effective producers of bioactive metabolites such as terpenoids, steroids, quinones, polyketides, alkaloids, esters, peptides and other unidentified metabolites (Petit et al. 2009; Gao et al. 2010; Zhelifonova et al. 2010; Nicoletti et al. 2014;), that have significant antimicrobial potentials (Yu et al. 2010). The compound pseurotin A, isolated from *Penicillium janczewskii* of the plant *Prumnopitys andina* showed considerable antibacterial potential against plant pathogenic bacteria *Pseudomonas syringae* and *Erwinia carotovora* (Schmeda-Hirschmann et al. 2008). The compounds erythritol and emodin isolated from the fungus *Penicillium citrinum* of the plant *Bruguiera gymnorrhiza* exhibited conspicuous inhibition to the growth of *B. subtilis* (Li et al. 2010). Another fungus, *Penicillium chrysogenum* from the red alga was reported to produce two

antibacterial compounds namely, conidiogenol and conidiogenone B. Here the conidiogenone B showed high activity against a series of bacteria including MRSA, *S. epidermidis*, *Pseudomonas fluorescens* and *P. aeruginosa*. Whereas the conidiogenol showed activity against *S. epidermidis* and *P. fluorescens* (Gao et al. 2011). Similarly, the *Penicillium chrysogenum* isolated from the leaves of *Solanum mauritianum* showed significant activity against *Mycobacterium bovis* and *Mycobacterium smegmatis* (Pelo et al. 2020). According to Lai et al. (2013) the endophytic fungus *Penicillium citrinum* isolated from *Ocimum tenuiflorum* produced three antibacterial compounds, alternariol, citrinin and perinadine A. these compounds exhibited moderate inhibition against *S. aureus*. Malhadas et al. (2017) evaluated the antimicrobial potential of endophytic fungus *Penicillium commune*, *Penicillium canescens* and *Alternaria alternata* isolated from the plant *Olea europaea*. Their study results confirmed strong inhibitory potential of *P. commune* and *A. alternata* against Gram-positive bacterium *B. cereus*. The *A. alternata* also showed good activity against *B. subtilis*. Against the Gram-negative *E. coli*, *P. commune* and *P. canescens* showed considerable inhibition.

Previous studies reported the strong antibacterial potential of the extracts isolated from the endophytic fungus *Alternaria alternata* inhabiting coffee plants, silver trumpet tree and sweet wormwood against a series of bacteria (Fernandes et al. 2009; Sadananda et al. 2011; Qadri et al. 2013). Similarly, *A. alternata* isolated from the leaves of neem plant (*Azadirachta indica*) showed antimicrobial properties against a series of Gram-negative and Gram-positive bacteria (Chatterjee et al. 2019). Similarly, *Alternaria* sp. were also isolated from *Pelargonium sidoides*. The Linoleic acid (9,12-octadecadienoic acid (Z,Z)) and cyclodecasiloxane isolated from this *Alternaria* sp. showed noteworthy potential against many food-borne bacteria,

including *E. coli*, *B. cereus*, *Enterococcusgallinarum* and *Enterococcus faecium* with a zone of inhibition of 2-12 mm (Manganyi at al. 2019). The secondary metabolites isolated from the endophytic *Penicillium* sp. exhibited significant inhibition against the Gram-positive bacterium *E. faecalis* (de Miranda et al. 2019). The endophytic fungus, *Trichoderma asperellum* isolated from *Panax notoginseng* produced antibacterial molecules halobacillin and Cyclopeptides PF1022F. These compounds showed potent activity against *E. faecium* and *S. aureus* (Ding et al. 2012). The *Nigrospora* sps. MA75, from mangrove plant *Pongamia pinnata* produced compounds such as 2,3-Didehydro-19α-hydroxy-14-epicochlioquinone B, griseophenone C, 3,6,8-Trihydroxy-1- methylxanthone and Tetrahydrobostrycin. Here 2,3-Didehydro-19α-hydroxy-14- epicochlioquinone B showed potent activity against MRSA, *P. aeruginosa*, *E. coli*, *S. epidermidis* and *P. fluorescens*. Likewise, the compound griseophenone C, showed strong activity against MRSA, *P.* aeruginosa, *E. coli* and *P. fluorescens*. The tetrahydrobostrycin showed significant activity against *E. coli* and MRSA. Whereas 3,6,8-Trihydroxy-1- methylxanthone showed inhibition against *S. epidermidis* only (Shang et al. 2012). Similarly, Wang et al. (2013b) reported the presence of antimicrobial compounds 4-Deoxybostrycin and nigrosporin in the *Nigrospora* sps. from mangrove plants. These compounds showed high activity against *M. tuberculosis*.

The endophytic fungi from the genus *Pestalotiopsis* were reported to produce diverse antimicrobial molecules. For example, the ambuic acid isolated from *Pestalotiopsis* sps showed noticeable activity against *S. aureus* (Ding et al. 2009). Similarly, pestalotiopen A isolated from Pestalotiopsis sps. Of *Rhizophora mucronata* exhibited reasonable antibacterial activity against *Enterococcus faecalis* (Hemberger et al. 2013). A phenolic compound (4-(2, 4, 7-trioxa-bicyclo [4.1.0]

heptan-3-yl) phenol) produced by *Pestalotiopsis mangiferae* isolated from *Mangifera indica* showed prominent activity against *B. subtilis*, *Klebsiella pneumoniae*, *E. coli*, *Micrococcus luteus* and *P aeruginosa*. Another antibiotic compound pestalone from *Pestalotia* sps. Isolated from brown alga *Rosenvingea* sps. exhibits strong activity against MRSA (Cueto et al. 2001). Endophytic fungi from genus Phomopsis also exhibit noticeable activity against different microbes. The dicerandrol A, B and C isolated from the endophytic fungi *Phomopsis longicolla* of *Dicerandra frutescens* showed significant inhibition against *B. subtilis* and *S. aureus* (Wagenaar & Clardy 2001). Also, dicerandrol C isolated from *P. longicolla* strain of the seaweed *Bostrychia radicans* showed significant inhibition against *S. aureus* and *Staphylococcus saprophyticus* (Erbert et al. 2012). Another compound, phomoxanthone isolated from *Phomopsis* sp. of Costus sp. exhibited moderate activity against *Bacillus megaterium* (Elsaesser et al. 2005). Similarly, ethers like phomosines A, B and C isolated from *Phomopsis* sp. from the plants *Teucrium scorodonia* and *Ligustrum vulgare* showed inhibition against *B. megaterium* and *E. coli* (Krohn et al. 2011). The *Phomopsis* sp. from *Notobasis syriaca* produces diverse antibacterial compounds such as 6β-trihydroxycyclohex-2-en, phomosine K, (+)-epiepoxydon, 2-hydroxymethyl-4β, 5α, (+)-epoxydon monoacetate and (−)-phyllostine. Here the (−)-phyllostine, 2-hydroxymethyl-4β, 5α, 6β-trihydroxycyclohex-2-en, (+)-epoxydon monoacetate and (+)- epiepoxydon showed antibacterial activities against *B. megaterium* and *E. coli*. Whereas the phomosine K showed strong inhibition against *Legionella pneumophila* alongside *E. coli* and *B. megaterium* (Hussain et al. 2011). The *Phomopsis* sps from *Santolina chamaecyparissus* produces compounds like phomopsinone B and C which -were

reported to have moderate inhibition against the bacteria *B. megaterium* and *E. Coli* (Hussain et al. 2012).

Another genus *Phoma* also produces diverse metabolites with antibacterial potential. The antibiotic phomol isolated from *Phoma* sps. of *Erythrina cristagalli*, a medicinal plant, was reported to be active against *Corynebacterium insidiosum* and *Arthrobacter citreus* (Weber et al. 2004). Another compound phomodione from *Phoma* sps. of*Saurauia scaberrinae* showed activity against *S. aureus* (Hoffman et al. 2008). Qin et al. (2010) previously reported the antimicrobial potential of a series of compounds such as 2-chloro-6-(hydroxymethyl) benzene-1,4-diol, epoxydine B, (4R, 5R,6S)-6-acetoxy-4,5-dihydroxy-2-(hydroxymethyl) cyclohex-2-en-1-one and epoxydon from *Phoma* sps isolated from *Salsola oppositifolia* against *B. megaterium* and *E. coli*. Similarly, antimicrobial compounds (+)-flavipucine and (−)-flavipucine isolated from *Phoma* sps. of*S. oppositifolia* were reported to induce effective inhibition against Gram positive and Gram-negative bacteria such as *S. aureus*, *B. subtilis*, and *E. coli* (Loesgen et al. 2011). Piperine, originally isolated from the plant *Piper longum,* was identified and extracted from the endophytic fungus *Periconia* sps. from the same plant. Piperine showed sturdy antibacterial activity against *M. smegmatis* and *M. tuberculosis* (Verma et al. 2011). The endophytic fungi *Periconia siamensis* isolated from *Thysanoleana latifolia* reported to produce antibacterial compounds that showed activity against *Listeria monocytogenes*, *Bacillus* cereus, MRSA, *E. coli* and *P. aeroginosa* (Bhilabutra et al. 2007). The endophytic genus *Ulocladium* produced effective compounds ophiobolins T and P. These compounds showed moderate antibacterial potential against MRSA and *B. subtilis* (Wang et al. 2013a). The *Chloridium* sp. isolated from *Azadirachta indica* produced the compound javanicin that was reported to show

significant potential against *P. aeruginosa* and *P. fluorescens* (Khrawar et al. 2009). The two endophytic isolates *Epicoccum nigrum* VD021 and *E. nigrum* VD022 from *Vaccinium dunalianum* showed significant inhibition against *S. aureus*, *B. subtilis*, *L. monocytogenes*, *S. bacteria* and *P. vulgaris* (Tong et al. 2018)

Endophytic fungi as anthelmintic agents

A few studies have established the anthelmintic potential of endophytic extracts against helminth parasites. According to Hallmann & Sikora (1996), the endophytic fungi might have produced inhibiting toxins or compete for space with these tested parasitic nematodes and this ultimately results in the significant reduction of nematode infestations. The peptaibols isolated from the fungal order Hypocreales showed significant anthelmintic activity against the infective stage of *Haemonchus contortus* (L3) (Thirumalachar & Antiamoebin 1968; Schiell et al. 2001; Ayers et al. 2012). The nematicidal potential of endophytic fungal isolates from banana and plantain cultivars was studied against the nematodes *Radopholus similis* and *Pratylenchus coffeae* and showed the efficacy of isolates such as MB2, GN4 and BT1 against the tested nematodes and able to kill 100% *of R. similis* and *P. coffeae* within 12h of treatment (Bechem et al. 2018). In another study, the endophytic fungi, *Daldinia eschscholtzii* (BPEF73) isolated from black pepper showed promising activity against the burrowing nematode *R. similis* (Sreeja et al. 2015). An interesting study documented the significant potential of endophytic *Aspergillus* sp. 2XA5 and *Selenosporella* sp. MR26 against the juveniles (J2) of *M. incognita* (Gamboa-Angulo et al. 2015). Furthermore, the promising nematotoxic effect of both fungal groups in their culture filtrates and methanol extracts of mycelia were attributed to the presence of metabolites with polar nature. Similarly, the culture filtrate from endophytic *Nigrospora* sp., also exhibited significant

activity against the juveniles of the root knot nematodes, *M. incognita* (Amin 2013). Some other studies documented the effects of non-pathogenic *F. oxysporum* isolates as biocontrol agents for *R. similis* in bananas (Niere et al. 1999; Pocasangre 2000). The endophytic fungal isolates *Fusarium oxysporum* and *F. diversisporum* from the tissues of banana, and tomato, when tested for their inhibitory potential against *R. similis*, the root penetration of *R. similis* got decreased to about 41% post 15 days after inoculation (Vu et al. 2006).

MATERIALS AND METHODS

A) Materials

1. Chemicals & Reagents

Acetic anhydride	: MERCK, India
Alkaline phosphatase	: Agappe diagnostics Ltd., India
Ammonia	: Sigma Aldrich, USA
Albendazole	: Sigma Aldrich, USA
Aluminum chloride	: MERCK, India
Amoxicillin	: Sigma Aldrich, USA
Arsenic	: MERCK, Germany
Bilirubin	: Agappe diagnostics Ltd., India
Bromine water	: NICE, India
Bouin's fixative solution	: Hi-Media, India
Cadmiun	: MERCK, Germany
Carbosulfan	: MERCK, India
Cholesterol (Total)	: Agappe Diagnostics Ltd., India
Chloroform	: NICE, India
Creatinine	: Euro Diagnostic Systems Pvt. Ltd., Chennai
Crystal violet	: Sigma Aldrich, USA
Dimethyl sulfoxide (DMSO)	: MERCK, India
Drabkin's reagent	: Agappe Diagnostics Ltd., India
Dragendroff's reagent	: NICE, India
Mayer's reagent	: NICE, India
Ethyl acetate	: MERCK, India
Ferric chloride	: MERCK, India
Folin-Ciocalteau reagent	: Sigma Aldrich, USA
Gallic acid	: Sigma Aldrich, USA
Glacial acetic acid	: Nice, India

Haemoglobin (Hb)	: Agappe Diagnostics Ltd., India
Haemotoxylin and eosin (H & E)	: Hi-Media, India
Hydrochloric acid	: MERCK, India
Lactophenol cotton blue	: Hi-Media, India
Lead	: MERCK, Germany
Lead acetate solution	: MERCK, India
Leishman's stain	: Hi-Media, India
Lugol's iodine solution	: Hi-Media, India
Mercury	: MERCK, Germany
Methanol	: Sigma Aldrich, USA
Mueller-Hinton Agar	: Hi-Media, India
Nitric acid	: MERCK, India
Olive oil	: NICE, India
Potassium acetate	: MERCK, India
Potato dextrose agar	: Hi-Media, India
Potato Dextrose Broth	: Hi-Media, India
Propylene glycol	: MERCK, India
Quercetin	: Sigma Aldrich, USA
SGOT	: Agappe diagnostics Ltd., India
SGPT	: Agappe diagnostics Ltd., India
Sodium carbonate	: Nice, India
Sodium chloride	: Hi-Media, India
Sodium hydroxide	: Nice, India
Sodium hypochlorite	: MERCK, India
Sodium sulphate	: MERCK, India
Sulphuric acid	: MERCK, India
Total protein	: Agappe diagnostics Ltd., India

Triglycerides	: Agappe Diagnostics Ltd., India
Urea	: Euro Diagnostic Systems Pvt. Ltd., Chennai
Wagner's reagent	: NICE, India
Xylene	: Hi-Media, India

2. Instruments

Accelerated solvent extractor	: Thermoscientific (Dionex ASE 150), USA
Atomic absorption spectroscopy	: Shimatzu (AA-7000), Japan
Centrifuge	: REMI (column oven CTO-20A), India
Electronic weighing balance	: ShimatzuCoorporation Ltd, India
GC-MS analyser	: Agilent (7890 A GC system fitted with a DB 5MS column), USA
Gel documentation system	: Bio-Rad Laboratories,USA
Hatching chambers	: Rotek Instruments Pvt. Ltd., India
HPLC	: Shimadzu, Japan
Hot air oven	: Rotek Instruments Pvt. Ltd., India
Light microscope	: Leica (DM 500) German Radicle, Ambala
Magnetic stirrer	: Spinit, Tarsons Products Pvt. Ltd., Kolkata
PCR thermal cycler	: Agilent (Sure Cycler), USA
PCR thermal cycler	: Applied Biosystems (Gene Amp PCR System 9700), USA
Rotary evaporator	: Profilab (KNF Rotary evaporator RC 600), Germany
Sequence Analyzer	: Applied Biosystems (ABI3730xl Genetic Analyzer), USA
Shaker incubator	: Labline, India
Spectrophotometer	: Agilent (Carry 60 UV vis), USA
Stereo zoom microscope	: Leica (M205 C) German Radicle, Ambala
Sterile laminar air flow	: Labline, India

UV transilluminator	: Genei Labs Pvt. Ltd., India.
Vortex mixer	: Rotek Instruments Pvt. Ltd., India
Water bath	: Rotek Instruments Pvt. Ltd., India

3. Plant material

3.1 *Kaempferia rotunda* L.

3.1.1 Taxonomic classification

Scientific name	:	*Kaempferia rotunda* L.
Kingdom	:	Plantae
Phylum	:	Tracheophyta
Class	:	Liliopsida
Order	:	Zingiberales
Family	:	Zingiberaceae
Genus	:	*Kaempferia*
Species	:	*Kaempferia rotunda* L.
Common Names	:	Malan-kua, Chengazhi, Chengazhineerkizhangu (Malayalam)
Habitat	:	Cultivated

Figure 1: Images of *Kaempferia rotunda* L.

3.2 *Lagenandra toxicaria* Dalz..

3.2.1 Taxonomic classification

Scientific name	:	*Lagenandra toxicaria* Dalz..
Kingdom	:	Plantae
Phylum	:	Tracheophyta
Class	:	Liliopsida
Order	:	Alismatales
Family	:	Araceae
Genus	:	*Lagenandra*
Species	:	*Lagenandra toxicaria* Dalz
Common Names	:	Andavazha, Neerchengazhi (Vernacular)
Habitat	:	Along streams

Figure 2: Images of *Lagenandra toxicaria* Dalz.

4. Animals

Swiss albino mice (20–30 g) were used as the animal model for *in vivo* studies. They were purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala. Animals were housed under controlled conditions of temperature (22- 28º C) and humidity (60-70%) and were provided with standard mice chow (Sai Durga feeds, Bangalore) and water *ad libitum*. The animals were housed in polypropylene cages with paddy husk bedding and top grill of stainless steel with facilities for providing food and water. All the experiments were carried out with prior permission from Institutional Animal Ethics Committee, Amala Cancer Research Centre (Approval No. ACRC/IAEC/21(1)-P1 dated 17/03/2021) according to the rules and regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

B) Methods

1.0 Collection and identification of plant material

The *K. rotunda* and *L. toxicaria* were respectively collected from the Irinjalakuda and Chalakudy areas of Thrissur District, Kerala, India. The morphological identification of plants was executed by Dr. A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut, Kerala, India. The voucher specimens were kept for future references (*L. toxicaria* 7001 and *K. rotunda* 7002).

2.0 Extraction of plant materials

The rhizomes were dried individually using a hot air oven with temperature set at 50^0 C. The dried rhizomes were powdered using a mixer grinder. The powdered rhizome was weighed (15g) and extracted separately in 250 ml of solvents such as ethyl acetate, ethanol and water of increasing polarity for about 15 minutes

using an accelerated solvent extractor (Dionex ASE 150, Thermoscientific). The extracts were evaporated to dryness using a rotary evaporator (KNF Rotary evaporator RC 600) and the resulting crude extracts were stored in amber coloured bottles under refrigeration (4^0 C) until further studies. The percentage yield (w/v) of individual extracts was calculated. Prior to the start of experiments, the extracts were dissolved in DMSO or propylene glycol for *in vitro* and *in vivo* studies respectively.

3.0 Screening for phytochemicals and metals

3.1 Qualitative phytochemical screening

The Qualitative phytochemical screening of various rhizome extracts of *K. rotunda* and *L. toxicaria* was executed to identify the presence of important secondary metabolites. The concentrated crude extracts were used for the screening of alkaloids, polyphenols, tannins, flavonoids, terpenoids, saponins, glycosides, steroids and coumarins according to the standard procedures (Sofowora 1996; Trease & Evans 1989; Harborne 1999).

3.1.1 Test for alkaloids

About 0.2 g of the extract was boiled with 5 ml of 1% hydrochloric acid on a steam bath for approximately 5 minutes. The boiled mixture was allowed to cool and filtered into 3 tubes labelled 1, 2 and 3. Each tube was treated separately with 2 drops of freshly prepared Dragendroff's, Mayer's and Wagner's reagents respectively. Appearance of brown to reddish brown precipitate with Dragendroff's test, creamy white precipitate with Mayer's test and reddish brown precipitate with Wagner's test indicates the presence of alkaloids.

3.1.2 Test for polyphenols

a) Ferric chloride test: To the test tube containing 1 ml of rhizome extracts in water, an equal volume of ferric chloride was added and observed for the formation of deep

blue colour. Formation of deep blue colour indicates the presence of polyphenols in the extracts.

b) Lead acetate Test: To the rhizome extracts, 10% lead acetate solution was added and observed for the formation of white precipitate. Formation of white precipitate indicates the presence of polyphenols in the extracts.

3.1.3 Test for tannins

About 0.5 g of the crude rhizome extract was added 5 ml of 45% ethanol and boiled in a water bath for about 5 minutes. The mixture was cooled and filtered. To 1 ml of the filtrate added 3 drops of lead acetate solution. Formation of a gelatinous precipitate indicates the presence of tannins. To another 1 ml of the filtrate 0.5 ml of bromine water was added and observed for a pale brown coloured precipitate. This also indicates the presence of tannins in the extracts.

3.1.4 Test for flavonoids

a) Ferric chloride test: About 0.5 g of the crude rhizome extract was boiled with water and filtered. To 1 ml of this filtrate added two drops of ferric chloride solution. Appearance of bluish green or violet colours indicates the presence of flavonoids in the extracts.

b) Sulphuric acid test: Formation of yellow colour while adding concentrated sulphuric acid to the extract indicates the presence of flavonoids.

3.1.5 Test for terpenoids

Salkowski test: About 2 ml of the rhizome extracts was treated with acetic anhydride and concentrated sulphuric acid. The formation of a bluish green ring indicates the presence of terpenoids.

3.1.6 Test for saponins

About 0.1 g of the rhizome extract was boiled with 5 ml of distilled water for 2-3 minutes. The solution was filtered. To 1 ml of the filtrate added 2 drops of olive oil and shaken for a minute to observe the formation of emulsion. Alongside, 1 ml of the filtrate was diluted using 4 ml of distilled water and shaken for a minute to observe the formation of stable froth. Formation of emulsion and stable froth indicates the presence of saponins in the extracts.

3.1.7 Test for glycosides

Borntrager's test: the rhizome extracts were treated with dilute HCL and incubated for about 1 hour in a water bath. This mixture was initially treated with chloroform and mixed thoroughly for the chloroform layer to separate. The chloroform layer was then treated with equal volume of dilute ammonia solution. Formation of pink colour during the addition of ammonia indicates the presence of glycosides in the extracts.

3.1.8 Test for steroids

a) Salkowski test: About 2 ml of the rhizome extracts was treated with acetic anhydride and concentrated sulphuric acid. The formation of a brownish red colour indicates the presence of steroids in the extracts.

b) Liebermann Buchard test: Initially 1 ml of acetic anhydride was added to equal volume of chloroform and cooled to 0°C using a refrigerator. To this mixture concentrated sulphuric acid and 1 ml of rhizome extracts were respectively added. Formation of green, red or orange colour that changes with time indicates the presence of steroids in the extracts.

3.1.9 Test for coumarins

To about 2 ml of rhizome extract taken in a test tube was mixed with 3 ml of 10% NaOH solution and incubated for 4-5 minutes in a water bath. Post incubation the test tube was examined under UV light for the presence of yellow fluorescence. The yellow fluorescence indicates the presence of coumarins in the extracts.

3.2 Quantitative phytochemical screening

3.2.1 GC-MS analysis for identification of volatile compounds

GC-MS analysis of crude rhizome extracts of *K. rotunda* and *L. toxicaria* was performed on an Agilent 7890 A GC system fitted with a DB 5MS column (30 m x 0.250mm Diameter x 0.25 Micro Meter Thickness). Ultra-pure Helium (99.99%) was employed as carrier gas at a constant linear velocity of 1ml/min. The sample volume of 3 µl was injected by maintaining the injector temperature at 280 °C in split mode. The ionizing energy was 80Ev. The oven temperature was programmed from 40^0 C (hold time of 5 min) to 280^0 C at a rate of 5^0 C/min. The crude sample was dissolved in 1ml of specific solvent, then injected in GC after further dilution (100 microlitre in 900 microliter particular solvent and filtered through 0.2µm nylon syringe filter). The constituents in the crude extract were expressed as a percentage by peak area. The identification of chemical compounds in the crude extract was executed according to the GC retention time. The mass spectra obtained were compared and matched with those standards available in the database of National Institute of Standards and Technology (NIST).

3.2.2 Quantification of Total Phenolic Content (TPC)

The TPC was estimated using Folin-Ciocalteau spectrophotometric technique proposed by Singleton & Rossi (1965). Briefly, 0.1 ml of the rhizome extracts was mixed with 3.9 ml of distilled water and 0.5 ml reagent (Folin-

Ciocalteau). The tubes were then kept intact for 3 minutes. After that, 2 ml of 20% sodium carbonate solution was added to the tubes and incubated for 1 minute in a boiling water bath. The absorbance of the solution was measured at 650 nm (Agilent Carry 60 UV vis) against blank prepared without Folin-Ciocalteau reagent. The gallic acid was used as standard and the results were expressed as mg of gallic acid equivalents (mg GAE/g) of the extract.

3.2.3 Estimation of Total Flavonoid Content (TFC)

The TFC of plant rhizome extracts was estimated using the aluminum chloride colorimetric method previously described by Chang et al. (2002) with minor changes. Briefly, the rhizome extracts were diluted using methanol to a concentration of 100 mg/ml. The diluted rhizome extracts were then mixed with 0.1 ml of 0.1 mM potassium acetate solution and 0.1 ml of 10% (w/v) aluminum chloride solution. The mixed solution was incubated for 30 minutes at room temperature. Same procedure was repeated with quercetin (QCE). Post incubation, the absorbance was measured at 415 nm using Agilent Carry 60 UV vis spectrophotometer. The TFC was calculated from a calibration curve and data was expressed as milligram of quercetin equivalents (mg QCE/g) of extract.

3.2.4 Polyphenol profiling and Quantification

Polyphenol profiling and quantification was executed using the high-performance liquid chromatography (HPLC) method proposed by Rodriguez-Delgado et al. (2001) with minor modifications. The analysis was performed using a prominence UFLC system (Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µl volume and a diode array detector (SPD-M20A). The solutions of *K. rotunda* and *L. toxicaria* rhizome

extracts and the polyphenol reference compounds (1 mg/ml) were filtered using 0.45 µl PTFE filter; and injected about 20 µl into the HPLC system. Retention times of standard peaks were used to compare and identify the sample peaks.

3.3 Heavy metal screening

Determination of heavy metals was done following to the guidelines of the Association of Official Agricultural Chemists (AOAC 1995). The wet digestion followed by atomic absorption spectroscopy (AAS) was used for the estimation of arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) in the rhizomes of *K. rotunda* and *L. toxicaria* (Meena et al. 2010). The standards of As, Hg, Cd and Pb were maintained for instrument calibration and estimation of heavy metal contents. A series of dilutions from 1 to 10 ppm of working solutions were prepared from the 1000 ppm standard stock solution (Merck, Germany). After 2-3 h of charring process the samples were subjected for 5 h treatment in Muffle furnace at 500°C for ashing. The samples post ashing process were cooled and subjected for concentrated acid digestion (digested with HCl and HNO_3 taken in 1:1 proportion). The acid digested sample solutions were made up to 100 ml using deionized water. The solutions were filtered using Whatman 40 filter paper and subjected for atomic absorption spectroscopy in AA-7000 (Shimatzu) system. Blanks were prepared in similar method and concentrations of As, Hg, Cd and Pb were determined from the absorbance values.

4.0 Antibacterial potential of rhizome extracts

4.1 Bacterial Strains

Seven strains of bacteria used in this study were from MTCC. *Staphylococcus aureus* (MTCC 3160), *Enterococcus faecalis* (MTCC 3159), and

Bacillus cereus (MTCC 430) were the three Gram-positive strains used in the study. Whereas *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 661), *Shigella flexneri* (MTCC 9543) and *Serratia marcescens* (MTCC 2645) were the four Gram-negative strains used.

4.2 Agar disk diffusion assay

The antibacterial activity of the *K. rotunda* (KrEA, KrOH and KrWT) and *L. toxicaria* (LtEA, LtOH and LtWT) rhizome extracts was tested using an agar disc diffusion assay (Bauer et al. 1966). The collected crude extract was weighed and finally dissolved in 1 % DMSO and diluted to four different concentrations such as 12.5µg/µl, 25µg/µl, 50µg/µl, and 100µg/µl for assay. A sterile paper disc (6 mm diameter, Whatman no. 1) was impregnated with 10µl of 1% DMSO dissolved rhizome extracts using a micropipette and kept under a laminar hood for 30 min to dryness. The air-dried paper discs containing 0.125, 0.25, 0.5 and 1 mg crude extract were used to test the activity against the bacterial strains. The bacterial suspension (100 µl) from overnight broth culture, adjusted to contain 1×10^8 CFU/ml of bacteria was spread by a sterile glass rod onto the surface of solidified Mueller-Hinton Agar Petri plates. The paper discs containing different concentrations of crude rhizome extract were placed on the surface of the Mueller-Hinton Agar medium seeded with test bacterium in separate Petri plates. The paper disc dried after impregnating with only 1 % DMSO of the same volume was considered as vehicle control. The standard antibiotic amoxicillin (10 µg/disc) served as positive control. The plates were incubated at 35 ± 2^0 C for 24 h and the degree of sensitivity was determined by measuring the zone of bacterial growth inhibition. Each test was replicated thrice.

5. Anthelmintic activity against helminth parasites of plants

5.1 Preparation of nematode inoculum

5.1.1 *M. incognita*

The extraction of nematode eggs was executed according to the method proposed by Hussey and Barker (1973) with slight modifications. To begin initial *M. incognita* cultures, a single egg mass was surface-sterilized in 1% sodium hypochlorite for about 1 minute. This was followed by washing in sterile distilled water and inoculated into a pot containing 3 weeks old tomato plant grown in sterilized soil. The temperature was maintained at 25 ± 5^0C and humidity at 70%. In this condition, the plants were allowed to grow for about 3 months. Post 3 months growth, the root-knot nematode eggs were extracted from the roots of these previously infested old tomato plants (*Solanum lycopersicon*L.). The eggs collected in a mesh sieve were incubated at 28^0C in hatching chambers. The second-stage juvenile *M. incognita* (J2) with a maximum age of 48 h after hatching were taken for nematicidal studies.

5.1.2 *R. similis*

The *R. similis* was extracted according to the maceration and filtration technique proposed Southey (1986) with minor modifications. The nematodes were extracted from the roots of black pepper (*Piper nigrum*) which were previously infested. Following that the *R. Similis* were cultured in carrot disks. The second stage juveniles of *R. similis* for the mortality test were collected from the culture maintained on carrot disks. The carrot disks were chopped into smaller pieces for the recovery of juveniles into the sterile water.

5.2 Mortality test of nematode larvae

The test was conducted in 6 well microtiter plates. In the assay, 100 juvenile nematodes in 0.5 ml water were placed in each well. Serial concentration of each plant extract in total volume of 0.5 ml in 0.5% DMSO was added to make concentrations of 8, 4, 2, 1 and 0.5 mg/ml together with water containing the worms. Negative and positive controls were established with 0.5% DMSO and 0.1% carbosulfan respectively. Each extract concentration was replicated 6 times and the experiment was performed thrice. Nematode mortality was assessed after 24 h, 48 h and 72 h of exposure using a stereomicroscope. The toxicity of the rhizome extracts was assessed as the mean percentage of the dead nematodes. Dead nematodes were scored based on a complete lack of motion. The nematodes were poked with a needle when their status remains uncertain (Cayrol et al. 1989).

6.0 Anthelmintic activity against helminth parasites of animals

6.1 *In vitro* anthelmintic activity against *Haemonchus contortus*

The in vitro anthelmintic capacities of rhizome extracts against animal parasitic nematodes were evaluated using egg hatch assay and larval motility inhibition assay. Fresh *H. contortus* ova and 3rd instar larvae were used to study *in vitro* egg hatch assay and larval motility inhibition assay respectively.

6.1.1 Faecal egg floatation

Primarily, faecal pellets were collected from the rectum of a naturally infected donor goat (Getachew et al. 2012). For faecal floatation, eggs were extracted by the method proposed by Coles et al. (1992). About 3g of faeces was taken in a container. To this, about 42 ml of water was added and soak for few minutes for the faeces to get soften. Using a mortar and pestle the solution was homogenised until the faecal pellets got broken. The homogenised solution was then

poured through a 100 mesh (0.15 mm aperture) 20 cm diameter sieve into a beaker. This solution was added to 15 ml centrifuge tubes and centrifuged at 2000 rpm for 2 min on a bench top centrifuge (REMI). The supernatant was gently removed. Then the tubes were agitated to loosen the sediment and to this normal saline was added and made up to 15 ml. mixed well and centrifuged at 2000 rpm for 2 minutes. Post centrifugation, the tubes were kept undisturbed for 3 minutes. After that, aspirated 1 – 2 ml of the supernatant from the top layer of the solution. To this distilled water was added and centrifuged again for 2 minutes at 2000 rpm. Finally the supernatant was removed and the remaining part was mixed well and checked in a light microscope (40X magnification) for the presence of helminth eggs. From the results, EPG of faeces were determined by Mc Master method (Coles al. 1992).

6.1.2 Egg hatch assay (EHA)

The egg hatch assay was conducted according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al. 1992) with minor modifications. In the assay, approximately 100 *H. contortus* ova in 0.5 ml distilled water were placed in six-well plates with serial dilutions (1.25, 2.5, 5 and 10 mg/ml) of the rhizome extracts. Albendazole at concentrations 6.25, 12.5, 25, 50 µg/ml served as positive control while 1% DMSO served as negative control. Further, the treated and control multi well plates were incubated for 48 h at 27°C. Six replicates were kept for each concentration. Post 48 h incubation, hatching was stopped forcefully by the addition of Lugol's iodine solution. A dissection microscope (40X magnification) was used to count the unhatched eggs and hatched larvae (dead or alive).

6.1.3 Larval paralysis assay (LPA)

The assay was based on the method previously described by Varady and Corba (1999) with modifications. Faecal samples from the rectum of naturally infected goat were collected and cultured for 7 days at 27°C to obtain third instar *H. contortus* larvae. Post 7 days the larvae were collected and stored at 4°C. Approximately 100 motile larvae of third instar (L3) *H. contortus* collected in 100 µL water were used for larval motility inhibition assay in 96-well microtitre plates. To this equal volume of *K. rotunda* rhizome extracts in dilutions 1.25, 2.5, 5 and 10mg/ml were added in triplicates. Albendazole (6.25, 12.5, 25, 50 µg/ml) and 1% DMSO served as positive and negative controls respectively. All samples were then incubated for 24 h at room temperature. The loss of motility of the larvae after 24 h of treatment was recorded. Percent larvae found non-motile/ dead were counted under a dissection microscope at 40X magnification and the results were postulated as mean percent paralysis.

6.2 *In vitro* adulticidal assay against *Fischoederius cobboldi*

6.2.1 Collection of adult flukes

Adult *F. cobboldi* were freshly collected from the rumens of infected cattle killed for consumption in a local slaughter house. The flukes were initially washed several times in 0.85% NaCl solution as per Anuracpreeda et al. (2015), the healthy ones that exhibited active motility were selected and immediately used for experiments.

6.2.2 *In vitro* anthelmintic activity

The adulticidal assay of the plant extracts was conducted on *F. cobboldi* according to the method proposed by Anuracpreeda et al. 2015 with minor

modifications. The various concentrations of test extract were prepared by dissolving in 0.1% DMSO. Adult flukes were randomly selected and 25 flukes each for all concentrations, positive and vehicle controls were used for the study. Group 1 served as the vehicle control (0.1% DMSO); group 2 with 1 mg/ml of albendazole (ABZ) was served as positive control; groups 3-5 were treated respectively with ethyl acetate (KrEA), ethanol (KrOH) and water extracts (KrWT) of various doses (3.125, 6.25, 12.5 and 25 mg/ml). Three replicates were kept for each group. The *F. cobboldi* in culture were incubated with CO_2 for 24h at 37^0 C and were observed under a stereo zoom microscope for motility at 0, 0.5, 1, 1.5, and 2 h of incubation times. The time required for total inactiveness or paralysis followed by the death of fluke was recorded, and the tegumental changes were examined under a light microscope.

6.2.3 Stereo zoom microscope study for morphological evaluation

For studying the morphological changes to flukes' post-treatment, stereo zoom microscope analysis was done. *Fischoederius cobboldi* treated in different concentrations of extracts and vehicle controls were studied using Leica M205 C stereo zoom microscope. Images were taken by Leica DMC4500 digital camera attached to Leica M205 C stereomicroscope with the software package LAS, version 4.3.0.

6.2.4 Light Microscope study

The fluke specimens were prepared for histology examination by the method proposed by Anuracpreeda et al. (2013). Dead *F. cobboldi* from each group were fixed in Bouin's fixative solution for 12 h, and then was transferred to 10% formalin for further histological processing. Sections of the tissues were taken and stained

using hematoxylin and eosin and observed for abnormalities and photographed under a light microscope (Leica DM 500).

7.0 Toxicity and *in vivo* anthelmintic activity

7.1 Oral acute toxicity

The oral acute toxicity study of KrEA and KrOH extracts was evaluated strictly following the guidelines of OECD (OECD, 2001). Fifteen Swiss albino female mice having 25 – 30 g weight were separated into three groups (n=5). Group 1: Vehicle control (1% propylene glycol); Group 2: Single dose of 2000 mg/kg body weight of KrEA dissolved in 1% propylene glycol; Group 3: Single dose of 2000 mg/kg body weight of KrOH dissolved in 1% propylene glycol. Post administration of single dose, the mice were observed for death or any visible sign of toxicity periodically during the initial 24 h and twice daily for 14 days thereafter. All animals were euthanized following 14 days of study to examine the changes in internal organs. In the absence of mortality, $1/5^{th}$ and $1/10^{th}$ of oral acute dosage was respectively taken as high and low dose for further studies. In the presence of mortality, the acute oral toxicity experiment was repeated using lower doses.

7.2 Oral Sub-acute toxicity

The oral sub-acute toxicity estimation of KrEA and KrOH extracts were executed following the guidelines of OECD 407.After oral toxicity studies, the sub-acute doses of KrEA (200 and 400 mg/kg b.wt) and KrOH (100 and 200 mg/kg b.wt) were fixed. Fifty Swiss albino mice weighing around 25 – 30 g were divided into ten groups (n=5). Group 1a: Vehicle control female mice received 1% propylene glycol; Group 2a: Female mice received a daily dose of 200 mg/kg b.wt of KrEA dissolved in 1% propylene glycol; Group 3a: Female mice received a daily dose of 400 mg/kg b.w of KrEA dissolved in 1% propylene glycol; Group 4a:

Female mice received a daily dose 100 mg/kg b.wt of KrOH dissolved in 1% propylene glycol; Group 5a: Female mice received a daily dose 200 mg/kg b.wt of KrOH dissolved in 1% propylene glycol; Group 1b: Vehicle control male mice received 1% propylene glycol; Group 2b: Male mice received a daily dose of 200 mg/kg b.wt of KrEA dissolved in 1% propylene glycol; Group 3b: Male mice received a daily dose of 400 mg/kg b.wt of KrEA dissolved in 1% propylene glycol; Group 4b: Male mice received a daily dose 100 mg/kg b.wt of KrOH dissolved in 1% propylene glycol; Group 5b: Male mice received a daily dose 200 mg/kg b.wt of KrOH dissolved in 1% propylene glycol. The mice were dosed by oral gavage for 28 days. On day 29, the mice were sacrificed and blood samples were collected via cardiac puncture for biochemical and haematological analyses.

7.2.1 Relative organ weight

Post sacrifice nine vital organs, such as heart, brain, liver, stomach, intestine, lungs, kidney, spleen, testes and ovary from test and control groups were dissected and washed in 0.9% saline. Using electronic weighing balance, the weight of the aforementioned organs was obtained and from this relative organ weight was calculated (Peter et al. 2018). The following formula was used to calculate relative organ weight:

$$\text{Relative organ weight} = \frac{\text{Organ weight}}{\text{Body weight}} \text{x } 100$$

7.2.2 Determination of haematological parameters

7.2.2.1 Estimation of haemoglobin (Hb)

A method previously described by Drabkin and Austin (1935) was used for determining the haemoglobin content in the blood.

Principle

The haemoglobin in the blood initially reacts with potassium ferrocyanide and gets oxidised into methaemoglobin, which finally reacts with potassium cyanide to form a stable product, cyanmethaemoglobin. This cyanmethaemoglobin has an absorbance maximum at 546 nm and its absorbance is directly proportional to the concentration of Hb present in the blood.

Procedure

Approximately 20 µL of heparinized blood was mixed with 5 ml of Drabkin's reagent followed by 5 min incubation at room temperature. Later, absorbance of the reaction mixture was measured at 546 nm. Similarly, the absorbance of the standard in 60 mg/dl concentration (delivered with the reagent kit) was also measured alongside the sample.

The haemoglobin content was estimated using the formula:

$$\text{Haemoglobin } \left(\frac{\text{g}}{\text{dl}}\right) = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{251}{1000} \times 60$$

7.2.2.2 Estimation of red blood cells (RBC)

A method previously described by Cheesbrough and McArthur (1976) was used for calculating the RBC count in the blood.

Principle

The blood sample was diluted 200 times using an RBC diluting fluid (Hayem's solution: Mercuric chloride (0.25 g), Sodium sulphate (2.50 g) and Sodium chloride (0.50 g)dissolved in 100 ml distilled water). This help fixes the RBC and prevents haemolysis. Post dilution, the RBC count was estimated using a haemocytometer and the values are represented as number of RBC/cu mm of whole blood.

Procedure

Using an RBC pipette, the blood was drawn up to the mark of 0.5 and diluted up to the mark of 101 using RBC diluting fluid. The solution was mixed well by rotating the RBC pipette between palms for few minutes. The Neubauer counting chamber was charged with 4-5 drops of diluted blood from the RBC pipette deprived of air bubbles and allowed to settle down for 3 -4 minutes. The red blood cells were counted using a light microscope (40X magnification).

The RBC count was estimated using the formula:

$$\text{RBC count (cells/mm}^3) = \frac{\text{Number of cells counted x dilution factor}}{\text{Depth factor x area counted}}$$

Here,

Dilution factor = 200

Depth of counting chamber = 0.1mm

Area counted = 5/25

So, total RBC count = Number of cells × 10,000/mm^3

7.2.2.3 Estimation of total white blood cell count (TC)

A method previously described by Cheesbrough and McArthur (1976) was used for calculating the total leukocyte count in the blood.

Principle

Treatment of blood using Turk's fluid (Glacial acetic acid (2 ml) and Crystal violet (1 g/ 100 ml) dissolved and made up to100 ml in distilled water. This solution wasstirred using a magnetic stirrer overnight, filtered and used) results in the lysis of RBCs (The acetic acid present in the Turk's fluid lyse the enucleated red blood cells only) leaving the WBCs intact and stained with crystal violet in the fluid.

Procedure

Using a WBC pipette, the blood was drawn up to the mark of 0.5 and diluted up to the mark of 11 using Turk's fluid. The solution was mixed well by rotating the WBC pipette between palms and kept it for few minutes to allow the cells to settle. The Neubauer counting chamber was charged with few drops of diluted blood from the WBC pipette deprived of air bubbles and allowed to settle down for 3-4 minutes. The white blood cells in the four corner squares of the counting chamber were counted using a light microscope (10X magnification).

The total WBC count was estimated using the formula:

$$\text{Total WBC count (cells/mm}^3) = \text{Number of cells counted } \times 50$$

7.2.2.4 Estimation of differential leucocyte count (DC)

The differential count of leucocytes was determined according to the method previously proposed by Wintrobe and Greer (2009).

Principle

Leishman stain was used to stain the blood smear in order to calculate the percent of different types of leucocytes present. Different types of leucocytes present can be easily distinguished by observing the shape of nucleus, size of cell and presence or absence of granules. Post Leishman staining the neutrophil appear as a purple-coloured nuclei and pink cytoplasm. Same time eosinophil has pink cytoplasm and purple coloured nucleus with dumbbell shape. Whereas the basophils appear with purple-coloured nucleus with dark blue granules. Monocytes appear as a purple-coloured horseshoe shaped nucleus and pink cytoplasm. Similarly, lymphocytes have dark blue circular nucleus with light blue cytoplasm. Platelets appear as violet-coloured granules.

Procedure

Initially, a thin blood smear was prepared using a drop of blood and air dried. The blood smear was then stained using Leishman stain (0.15 g/100 ml methanol) and kept for 2-3 min. To dilute the stain an equal amount of distilled water was added and kept for 2-3 min. Excess stain on the slides were removed by washing and air dried. The WBC counts were obtained by moving the slides in a zig-zag fashion under a light microscope at 40X magnification.

7.2.2.5 Estimation of platelet count (PLT)

The estimation of platelet count was done according to the method previously proposed Cheesbrough and McArthur (1976).

Principle

The isotonic balance of the 1% ammonium oxalate solution (dilution fluid) exclusively lyses the erythrocytes leaving the platelets intact. Later, with the help of Neubauer counting chamber and a light microscope the cells were estimated.

Procedure

Platelet counting procedure was similar to the method previously explained while counting the RBCs. Here also RBC pipette was used and 1:200 dilution of blood was prepared by adding 1% ammonium oxalate (dilution fluid). The solution was then mixed mildly for about 2 minutes and added to the Neubauer's counting chamber and observed under 40X magnification of a light microscope.

The platelet count was estimated using the following formula:

$$\text{Number of platelets per mm}^3 = \text{N} \times 10000$$

Where N = Total number of cells counted

7.2.2.6 Estimation of packed cell volume (PCV)

The Packed cell volume was estimated by the method previously proposed by Wintrobe and Greer (2009)

Principle

The centrifugation of anticoagulated blood in Wintrobe Micro haematocrit tube (Wintrobe tube) at a moderate speed result in the sedimentation of erythrocytes at the bottom of the tube. This sedimented part can be considered as packed cell volume also known as haematocrit.

Procedure

The anti-coagulated blood was filled in the Wintrobe tube with the help of a Pasteur pipette. The tubes were subjected to centrifugation at 2500 g for about 30 minutes. The length of erythrocyte cell column was measured and the percentage of erythrocyte cell column to the total volume of blood sample was estimated.

7.2.2.7 Estimation of erythrocyte sedimentation rate (ESR)

Westergren tube method (Westergren 1957) was used to calculate the erythrocyte sedimentation rate.

Principle

The anti-coagulated blood taken in Westergren's tube was kept undisturbed in a vertical position. The level of RBCs was noted initially (0 hour) and post 1 hour of the study. The ESR was expressed as the distance of travelled by the RBC column (mm/hr).

Procedure

Here, 1.6 ml of venous blood was mixed with 0.4 ml 3.8% sodium citrate solution and mixed thoroughly. The Westergren's E.S.R. tube was filled with this solution up to the level marked as zero and kept in vertical position. Reading was

taken after 30 minutes, 1 hour and 2 hours after filling the Westergren's E.S.R. tube. The ESR value of RBCs was expressed as distance travelled (mm) per 1 hour.

7.2.3 Liver function analysis

The blood collected through heart puncture was transferred to Eppendorf tubes and centrifuged at 5000 rpm for 10 minutes for the separation of serum. Collected serum was used to analyse the liver function marker enzymes such as SGOT, SGPT and ALP. Alongside, the amount of bilirubin and total protein was also estimated.

7.2.3.1 Serum glutamate oxaloacetic transaminase (SGOT)

The Serum glutamate oxaloacetic transaminase activity was estimated according to the method previously described (Thefeld et al.1974; Bergmeyer et al. 1976).

Principle

The SGOT execute transamination reaction in which the L-aspartate and α-ketoglutarate gets converted to oxaloacetate and L-glutamate. In the presence of malate dehydrogenase, the oxaloacetate reacts with $NADH.H^+$ to form L-malate and NAD^+. The decrease in absorbance at 340 nm can be spectrophotometrically determined and it is directly proportional to the activity of enzyme.

Procedure

The SGOT reagent kit were purchased from Agappe Diagnostics Ltd. The kit consists of two reagents, namely reagent 1 (R1) and reagent 2 (R2). Here, the R1 comprises of Tris buffer having pH 7.8 (88 mmol/l), L-Aspartate (260 mmol/l), MDH (>900 U/L) and LDH (>1500 U/L). Similarly, the R2 contains, NADPH (0.24 mmol/L) and α-ketoglutarate (12 mmol/l). Mixing 4 volumes of R1 and 1 volume of

R2 results in a solution called as working reagent. This remains stable for about a month if kept at 2 - 8ºC. During assay 1000 µl of working reagent and
100 µl of serum samples were added, mixed thoroughly and incubated for 1 min at 37 °C. Post incubation, variation in absorbance was measured at 340nm keeping 1minute interval for 3 minutes.

The SGOT activity was estimated using the formula

$$\text{SGOT activity (U/L)} = (\Delta OD/\text{min}) \times 1745$$

7.2.3.2 Serum glutamate pyruvate transaminase (SGPT)

The Serum glutamate pyruvate transaminase activity was estimated according to the method previously described (Thefeld et al.1974)

Principle

The SGPT execute transamination reaction in which the L-alanine and α-ketoglutarate gets converted to pyruvate and L-glutamate. In the presence of lactate dehydrogenase, the pyruvate reacts with $NADH.H^+$ to form lactate and NAD^+. The decrease in absorbance at 340 nm can be spectrophotometrically determined and it is directly proportional to the activity of enzyme.

Procedure

The SGPT reagent kit was purchased from Agappe Diagnostics Ltd. The kit consists of two reagents, namely reagent 1 (R1) and reagent 2 (R2). Here, the R1 comprises of Tris buffer having (110 mmol/l), L- alanine (600 mmol/l) and LDH (>1500 U/l). Similarly, the R2 contains, NADH (0.24 mmol/l) and α-ketoglutarate (16 mmol/l). Mixing 4 volumes of R1 and 1 volume of R2 results in a solution called as working reagent. This remains stable for about a month if kept at 2 - 8ºC. During assay 1000 µl of working reagent and 100 µl of serum samples were added,

mixed thoroughly and incubated for 1 min at 37 °C. Post incubation, variation in absorbance was measured at 340 nm keeping 1minute interval for 3 minutes.

The SGPT activity was estimated using the formula

$$\text{SGPT activity (U/L)} = (\Delta\text{OD/ min}) \times 1746$$

7.2.3.3 Alkaline phosphatase (ALP) activity

The alkaline phosphatase activity was determined according to the method previously proposed by Schlebusch et al. (1974).

Principle

The ALP enzyme converts p-Nitrophenyl phosphate in to p-Nitrophenol and inorganic phosphate at pH 10.4. The change in absorbance at 405 nm can be spectrophotometrically determined and it is directly proportional to the activity of enzyme.

Procedure

The ALP reagent kit was purchased from Agappe Diagnostics Ltd. The kit consists of two reagents, namely reagent 1 (R1) and reagent 2 (R2). Here, the R1 comprises of diethanolamine buffer having pH 10.2 (125 mmol/l) and magnesium chloride (0.625 mmol/l). Similarly, the R2 contains, p-nitrophenyl phosphate (50 mmol/l). Mixing 4 volumes of R1 and 1 volume of R2 results in a solution called as working reagent. During assay 1000 µl of working reagent and 20 µl of serum samples were added, mixed thoroughly and incubated for 1 min at 37 °C. Post incubation, variation in absorbance was measured at 405 nm keeping 1minute interval for 3 minutes.

The ALP activity was estimated using the formula

$$\text{ALP activity (U/L)} = (\Delta\text{OD/ min}) \times 2750$$

7.2.3.4 Total Bilirubin

The total bilirubin was estimated according to the method previously proposed by Walters & Gerarde (1970).

Principle

The reaction of sulfanilic acid and sodium nitrite results in the formation of diazotized sulfanilic acid. This diazotized sulfanilic acid reacts with bilirubin in the presence of TAB (diazo reagent) to form azobilirubin, a pink coloured complex. The OD is measured at 546 nm.

Procedure

To 50 µl of serum, 20 µl activator total and 1 ml bilirubin reagent (28.9 mmol/l of sulfanilic acid and 9 mmol/l of TAB) was added, mixed thoroughly and incubated at room temperature for 5 minutes. Aforementioned solution without serum was kept as blank. Post incubation, the absorbance was measured at 546 nm.

The total bilirubin in the sample was estimated using the formula:

$$\text{Total bilirubin concentration (mg/dl)} = \text{OD of the test} - \text{OD of blank} \times 25$$

7.2.4 Renal function

Commercially available diagnostic kits were used to test the renal function by analysing the levels of urea and creatinine in the serum.

7.2.4.1 Quantification of serum urea

Urea levels in the serum was estimated according to the method previously proposed by Young et al. (1975).

Principle

The urease enzyme converts urea into carbon dioxide and ammonia. Here, ammonium reacts with α-ketoglutarate and NADH to form glutamate and NAD^+.

The oxidation of NADH to NAD^+ is proportional to the concentration of urea present in the sample. The decrease in absorbance is measured at 340 nm.

Procedure

The diagnostic kit was purchased from Euro Diagnostic Systems Pvt. Ltd., Chennai, India. Initially, the working reagent was prepared by mixing 4 volumes BUN reagent and 1 volume of urease. After that, 1 ml of working reagent and 10 µl of serum was mixed well at 37ºC and absorbance was measured at 340 nm. The initial absorbance was obtained after 30s (A1) and final absorbance after 60s (A2). The change in absorbance (ΔA) was obtained by subtracting A2-A1. The same experiment was repeated with standard delivered with the assay kit.

The concentration of urea present in the sample was calculated using the formula:

$$\text{Urea (mg/dl)} = \left(\frac{\Delta AT}{\Delta AS}\right) \text{x } 50$$

Where, ΔAT and ΔAS represents change in the absorbance of test and standard respectively. Also 50 was the concentration of standard.

7.2.4.2 Estimation of serum creatinine

The creatinine level in the serum was determined using Jaffe's kinetic method with slight modification (Bones & Tausky 1945; Toro & Ackermann 1975).

Principle

In alkaline condition, the picric acid gets converted to sodium picrate. This sodium picrate reacts with creatinine in the sample to form an orange-coloured creatinine picrate complex. This complex shows maximum absorbance at the wavelength 520 nm.

Procedure

The diagnostic kit for testing creatinine was purchased from Euro Diagnostic Systems Pvt. Ltd., Chennai, India. The kit contains two reagents; R1 - picric acid reagent and R2 - alkaline buffer. The working reagent was prepared by mixing equal volume of R1 and R2. After that, 1 ml of working reagent and 50 µl of serum was mixed well and absorbance was measured at 520 nm. The initial absorbance was obtained after 30s (A0) and final absorbance after 90s (A1). The change in absorbance (ΔA) was obtained by subtracting A1-A0. The same experiment was repeated with creatinine aqueous standard delivered with the assay kit.

The concentration of creatinine present in the sample was calculated using the formula:

$$\text{Creatinine (mg/dl)} = \left(\frac{\Delta AT}{\Delta AS}\right) \text{x } 2$$

Where, ΔAT and ΔAS represents change in the absorbance of test and standard respectively. Also 2 was the concentration of standard.

7.2.5 Lipid profile

7.2.5.1 Estimation of total cholesterol

CHOD-POD method was used to determine the total cholesterol in the serum (Young, 1997).

Principle

Cholesterol esters in serum are hydrolysed to free cholesterol by the enzyme, cholesterol esterase. Later another enzyme, cholesterol oxidase oxidises the free cholesterol into H_2O_2 and 4-cholesterol-3-one which in the presence of peroxidase couples with 4-aminoantipyridine and phenol to yield a red quinone. This is generally measured at 505 nm.

Procedure

The cholesterol reagent kit contains phenol (26 mmol/l), cholesterol oxidase (300 U/L), pipes (90 mmol/l, pH 6.9), cholesterol esterase (100 U/L), 4-aminophenazone (0.4 mmol/l) and peroxidase (650 U/L). 1 ml of the reagent was mixed with 10 µl of serum. This solution was mixed well and incubated for 5 min at 37º C. Post incubation the absorbance of the solution was measure at 505 nm. The same experiment was repeated with standard delivered with the assay kit.

The total cholesterol in the sample was calculated using the formula:

$$\text{Total cholesterol (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \text{x } 200$$

7.2.5.2 Triglycerides

The triglycerides present in the serum were estimated by GPO-POD method using diagnostic kit purchased from by Euro diagnostic systems, Chennai, India.

Principle:

Incubation of lipoprotein lipase with serum triglycerides results in the formation of glycerol and fatty acids. The glycerol in the next step gets phosphorylated by glycerol kinase into glycerol-3-phosphate. The glycerol phosphate dehydrogenase converts glycerol-3-phosphate formed in the previous step into dihydroxyacetone phosphate (DHAP) and H_2O_2. The peroxidase enzyme mediates the reaction of H_2O_2 with 4-aminophenazone (4-AP) and p-chlorophenol to form a red coloured compound quinone. The absorbance of the final compound is measured at 505 nm. The intensity of quinone is directly proportional to the amount of triglycerides in the sample.

Procedure

Approximately 1 ml of reagent in the diagnostic kit was mixed with 10 µL of serum and incubated for about 5 minutes at 20º C. Post incubation, the absorbance was obtained at 505 nm. The same experiment was repeated with standard delivered with the assay kit. (The reagent consists of 2 mmol/l p-chlorophenol, 50 mmol/l GOOD (pH 6.3), 0.1 mmol/l 4-aminophenazone, 150000 U/L lipoprotein lipase, 3500 U/L glycerol 3-kinase, 500 U/L glycerol kinase and 0.1 mmol/l ATP).

The total triglycerides in the sample were calculated using the formula:

$$\text{Total triglycerides (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \text{x } 200$$

7.2.5.3 HDL cholesterol (HDLc)

The concentration of HDL cholesterol in serum was estimated according to the enzymatic photometric method previously proposed by Naito (1985). For the assay commercial HDL kit was purchased from Euro Diagnostic Systems Pvt. Ltd., Chennai, India.

Principle

The detergent present in the kit solubilize the HDLc in the serum. The released HDLc then reacts with the enzymes such as cholesterol esterase and cholesterol oxidase and finally with chromogens to develop a color which can be measured colorimetric method. The intensity of color develops would be proportional to the amount of HDLc in the sample. Sametime the non-HDL lipoproteins and chylomicrons are absorbed by the detergents. So that their inhibition is over ruled.

Procedure

To three test tubes labelled as, sample, calibrator and blank 450 µL of R1 solution was added. Following that, 10 µl of serum and calibrator (lyophilized human serum, con 39.2 mg/dl) were added to the tubes labelled as sample and calibrator respectively. The solutions were mixed thoroughly and incubated at 37^0C for 5 minutes. After incubation, 150 µl of R2 solution was added to all test tubes, mixed thoroughly and incubated at 37^0C for 5 minutes. The absorbance was measured at 600 nm against the reagent blank. The R1 solution contains, <1000 U/L cholesterol oxidase and <1 mMDSBmT. The R2 solution contains, <1 mM 4-aminoantipyrine, <1500 U/L cholesterol esterase, <1300 U/L peroxidase <3000 U/L ascorbic oxidase and <2% detergent.

The HDL cholesterol in the sample was calculated using the formula:

$$\text{HDLc(mg/dl)} = \left(\frac{\Delta\text{A sample}}{\Delta\text{A calibrator}}\right) \text{x concentration of calibrator}$$

7.2.5.4 LDL cholesterol (LDLc)

The LDL cholesterol in the serum was estimated using Friedewald's formula (Friedewald et al. 1972)

$$\text{LDLc (mg/dl)} = \text{Total Cholesterol} - \text{HDLc} - (\text{Triglycerides}/5)$$

7.2.5.5 VLDL cholesterol (VLDLc)

VLDL cholesterol in the serum was estimated using the formula given below:

$$\text{VLDLc (mg/dl)} = \text{Triglycerides (mg/dl)}/5$$

7.2.6 Serum electrolytes

7.2.6.1 Determination of sodium and potassium in the blood

Principle

Flame photometry method is employed for the estimation of sodium and potassium (Deal 1954). The photodetector in the flame photometer detects a specific frequency of light produced when an alkali metal in a solution in aerosol form is aspirated into a low temperature flame. The light emitted during the process is directly proportional to the alkali concentration.

Procedure

Initially sodium (1000 mEq/l) and potassium (100 mEq/l) stock solutions were prepared. From the stock solution, mixed working standards such as S1 (120/2 mEq/l), S2 (140/4 mEq/l) and S3 (160/6 mEq/l) were prepared. After that 100 µl of serum (test) and working standards (S1, S2 and S3) were added to different tubes. About 10 ml of distilled water was also added to all tubes, mixed well and subjected for flame photometry. The concentration of the sample was estimated from the calibration curve.

7.2.6.2 Determination of chloride ion

The estimation of chloride ion was using electrolyte diagnostic reagent kit and photometer equipped system (Schoenfeld & Lewellen 1964; Levinson 1976).

Principle

The chloride ions react with a solution containing mercuric thiocyanate and ferric nitrate to form red coloured ferric thiocyanate and mercuric chloride. The intensity of the red color formed during the reaction is directly proportional to the amount of chloride ion present in the serum.

Procedure

The serum (10 µl) was mixed with the reagent (1 ml) in the diagnostic kit and the absorbance was measured at 505 nm against the blank in a Photometer 4010

system. Similarly, a standard solution was also prepared using chloride provided with the diagnostic kit.

The concentration of chloride ions in the serum was calculated using the following formula:

$$\text{Chloride (mmol/l)} = \text{Absorbance of sample} / \text{Absorbance of standard} \times 100$$

7.2.7 Histopathology study

Post experimental duration, the animals were sacrificed using a CO_2 chamber. After that, nine organs, such as heart, brain, liver, stomach, intestine, lungs, kidney, spleen, testes and ovary from test and control groups were excised using a sterile blade and washed with normal saline (0.9% NaCl) for the removal of unwanted debris. Tissues were then fixed in 10% formalin solution and dehydrated using increasing grades of alcohol and were cleared in xylene. Paraffin-embedded tissue samples were subjected for microtome sectioning to obtain 3-4 μm thick sections. The sections after deparaffinization were stained in haemotoxylin and eosin (H & E) stain. Post staining the sections was examined under a microscope (Leica DM 500) and microscopic images were obtained using the camera mounted on it.

7.3 *In vivo* anthelmintic activity

Before the start of in vivo anthelmintic study, natural infections in the mice were identified using standard procedures (Coles et al. 1992; Sueta et al. 2002; Kozan et al. 2006).

7.3.1 Faecal egg floatation

For faecal egg floatation a modified McMaster method proposed by Coles et al. (1992) was used. About 3g of faeces was taken in a container. To this, about 42

ml of water was added and soak for few minutes for the faeces to get soften. Using a mortar and pestle the solution was homogenised until the faecal pellets got broken. The homogenised solution was then poured through a 100 mesh (0.15 mm aperture) 20 cm diameter sieve into a beaker. This solution was added to 15 ml centrifuge tubes and centrifuged at 2000 rpm for 2 min on a bench top centrifuge (REMI). The supernatant was gently removed. Then the tubes were agitated to loosen the sediment and to this normal saline was added and made up to 15 ml. mixed well and centrifuged at 2000 rpm for 2 minutes. Post centrifugation, the tubes were kept undisturbed for 3minutes. After that, aspirated 1 – 2 ml of the supernatant from the top layer of the solution. To this distilled water was added and centrifuged again for 2 minutes at 2000 rpm. Finally the supernatant was removed and the remaining part was mixed well and checked in a light microscope (40X magnification) for the presence of helminth eggs. From the results, eggs per gram (EPG) of faeces were calculated.

7.3.2 Perianal cellophane tape method

Perianal cellophane tape method was employed for the detection of pinworm eggs (Sueta et al. 2002; Kozan et al. 2006). A single piece of cellophane tape with varied length of 25 mm to 30 mmh was gently applied to the anal region of the experimental mice. For observations of pinworm eggs, the tapes were removed and attached to standard microscope slides (25mm× 75 mm) and counted using a light microscope (40X magnification).

7.3.3 *In vivo* anthelmintic treatment

Thirty male mice having natural infections were divided into 6treatment groups of five animals each on the basis of nematode and cestode counts (mean ± SEM). Each groups received different treatment as follows.

Group 1: Two doses of KrEA at 400 mg/kg body weight in one week interval

Group 2: Two doses of KrEA at 200 mg/kg body weight in one week interval

Group 3: Two doses of KrOH at 200 mg/kg body weight in one week interval

Group 4: Two doses of KrOH at 100 mg/kg body weight in one week interval

Group 5: Vehicle control (Drug replaced by suitable volumes of the dosing vehicle)

Group 6: Single dose of Albendazole at 20 mg/kg body weight

For faecal sample collection the mice were isolated individually in separate cages on day zero (pre-treatment) and on days 4, 8, 12 and 15 (post-treatment). The faecal pellets were collected, weighed and then subjected to centrifugal floatation for the collection of fresh eggs. For the identification of *S. obvelata* (pinworm) eggs perianal cellophane tape method with minor modifications was used on day zero (pre-treatment) and on days 4, 8, 12 and 15 (post-treatment). On day 15, mice were sacrificed, and the gastrointestinal tract was removed and washed using normal saline. Percent egg count reduction (ECR %) in both faecal centrifugal floatation (FECR) and cellophane tape (ECR) was calculated using the modified formula previously described by Lone et al. (2012):

$$\text{ECR } \% = \frac{\text{Pre treatment egg count} - \text{Post treatment egg count}}{\text{Pre treatment egg count}} \times 100$$

8.0 Isolation, identification and bio-active potential of endophytic fungi

8.1 Isolation and morphological identification of fungal endophytes

Isolation of endophytic fungi were done according to the method described previously by Ezra et al. (2004). Rhizomes of *L. toxicaria* and *K. rotunda* were used for the isolation of endophytic fungi. Tissues were washed separately in running water for 10 minutes to remove debris and finally washed with double distilled

water to minimize the microbial load from the sample surface. Thin sections of plant parts (approximately 2–3cm in length) were then subjected to surface sterilization using0.5% sodium hypochlorite for 3 minutes followed by 70% ethanol for 2 minutes and finally rinsing in sterile distilled water for removing alcohol traces. Tissues were then dried under the sterile laminar air flow. The surface treatment was done adopting the methodology of Petrini et al. (1992), and the effectiveness of surface sterilization was checked according to the method of Schulz et al. (1993). The outer tissue layers were removed using sterile scalpel and the internal tissues were cut into smaller pieces of 0.5–1 cm and plated individually in Petri dishes containing potato dextrose agar, to which 0.1% antibiotic solution (stock) was previously added. The potato dextrose agar (PDA) plates were incubated at 25°C for 4 weeks under dark and checked every 24 hours for any fungal growth. Fungal hyphae emerging out of the plant tissues were sub-cultured several times to obtain the pure culture. Isolation procedure was repeated for 10 times to confirm the presence of the same endophyte. Only those endophytic fungi that have been obtained during repeated isolation were further taken for morphological and molecular identification. The phenotypic study was based on the culture characteristics and morphology of the spores. Morphological changes such as growth rate, color, color variation over different time periods, upside and down side color of the colonies, and surface texture were observed. Also, microscopic features such as the mycelium, conidiophores, conidia were studied using lactophenol cotton blue staining methods (Gaddeyya et al. 2012).

8.2 DNA isolation, amplification and sequencing of rDNA ITS gene sequences

DNA isolation, PCR amplification and sequencing were according to the method proposed by White et al. (1990).

8.2.1 DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel)

Briefly 100 mg of the fungal mycelium were crushed using liquid nitrogen and the powdered tissue was transferred to a micro centrifuge tube. Precisely 400µl of PL1buffer was added and vortexed for 1 minute. Following that 10µl of RNaseA solution was added and mixed well. Post incubation at 65ºC for 10 minutes, the lysate was transferred transferred to a Nucleospin filter and centrifuged at 11000 x g for about 2 minutes. Following that 400µl of PC buffer was added and mixed thoroughly. The mixture was then transferred to a Nucleospin Plant II column and centrifuged for 1 minute and the flow through liquid was discarded. After that 400µl of PW1 buffer was added and centrifuged at 11000 x g for 1 minute and flow though liquid was discarded. Then 700 µl PW2 was added and centrifuged at 11000 x g and flow through liquid was discarded. After that Finally 200 µl of PW2 was again added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. Now the column was transferred to a new 1.7 ml tube and 50 µl of PE buffer was added and incubated at 65ºC for 5 minutes. The column was then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4ºC until further use.
Post isolation, the quality and quantity of the DNA was estimated using a 0.8 % agarose gel electrophoresis and visualized in a UV transilluminator (Genei). The good quality DNA obtained was further amplified using PCR analysis.

8.2.2 Amplification of internal transcribed spacer (ITS) regions of rDNA using PCR

The ITS segments of the rDNA was amplified using universal primers ITS-1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4R (5'-TCCTCCGCTTATTGATATGC-3'). The components used for the PCR amplification were, 19 µl sterile water, 2.5 µl Taq buffer, 0.5 µl dNTP mix, 0.5 µl

Taq polymerase, 0.5 µl each of forward & reverse primers and 1.5 µl DNA. The amplification was carried out in a PCR thermal cycler, Agilent Sure Cycler 8800 (Agilent Technologies, USA). The amplification profile of PCR reaction consists of 2 minutes at 94° C, 35 cycles of 30 seconds at 94° C, 1 minute at 55°C and 1 minute 30 seconds at 72° C, and lastly 8 minutes at 72° C.

Post amplification, Agarose Gel electrophoresis was used to check the PCR products. The 2-log DNA ladder (NEB) was used as molecular standard and the gels were visualized in a UV transilluminator (Genei) captured under UV light in a Gel documentation system (Bio-Rad).

The amplified PCR products were subjected for ExoSAP-IT Treatment for the removal of unwanted nucleotides and primers and thereby reducing the interference. ExoSAP-IT (GE Healthcare) kit consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer. About 5 µl of amplified product was mixed with 0.5 µl of ExoSAP-IT and incubated at 37ºC for 15 minutes followed by enzyme inactivation at 85º C for 5 minutes. The amplified DNA was stored at -20° C for further use.

8.2.3 Sequencing using BigDye Terminator v3.1

The Exosap treated PCR product was sequenced using the same primers used fro PCR reaction. Sequencing reaction was done in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequencing PCR mix consisted of 6.6µL D/W, 1.9 µl 5X sequencing buffer, 0.3 µl each of forward and reverse primers, 0.2 µl sequencing mix and 1 µl Exosap treated PCR product. The PCR amplification profile for sequencing is described below:

8.2.4 PCR amplification profile for sequencing

96° C	-	2min	
96° C	-	30sec	} 30 cycles
50° C	-	40sec	
60° C	-	4min	
4° C	-	∞	

8.2.5 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v 5.1. The amplified sequences were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) program in the NCBI GenBank (www.ncbi. nlm.nih.gov) DNA database for identifying the fungal strains. The ITS sequence obtained for each strain was submitted to the GenBank.

8.3 DNA isolation, amplification and sequencing of rDNA D1/D2 gene sequences

Approximately 0.001 g of mycelium was ground with mini grinder using 75 µl of STE extraction buffer (320 mM Sucrose, 10 mM Tris-Cl, 20 mM EDTA, 75 mM NaCl and 2.5ml of 20% SDS) along with 5mg of Polyvinyl pyrrolidone and 0.1g of silica powder, incubated at 65°C for 10 minutes. The sample was centrifuged at 13,000 rpm for 10 minutes. To the supernatant, an equal volume of chloroform: isoamyl alcohol was added and repeated the centrifugation. To the aqueous layer, added 2/3 volume of isopropanol and centrifuged at 13,000 rpm for 10 min. The pellet was washed with 70% ethanol by centrifuging and the pellet was dried, dissolved in 50 µl TE buffer.

The D1/D2 region was amplified by PCR from fungal genomic DNA using PCR universal primers: DR - 5'-GGTCCGTGTTTCAAGACGG-3' and

DF- 5'-ACCCGCTGAACTTAAGC-3' in the standard PCR reaction. The PCR reaction was as follows: 5 minutes at 95^0 C, 30 seconds at 48^0 C and 45 seconds at 72^0 C, with a final elongation period of 10 minutes at 72^0 C. After amplification, products were purified by using column purification method (Thermo Scientific USA). The concentration of the purified DNA was determined andwas subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer (Applied Biosystems, USA).Each nucleic acid sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequence at the 3 'and 5' ends (considering peak and Quality Values for each base) using the sequence analysis tools. The edited sequences were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) program in the NCBI GenBank (www.ncbi.nlm.nih.gov) DNA database for identifying the fungal strains.

8.4 Fermentation and solvent extraction of endophytes

The isolated colonies were cultured in 200 ml of Potato Dextrose Broth (PDB) at 25° C for 2 weeks with constant shaking in a shaker incubator (Labline shaking incubator). These fermented broths were homogenized using 10% methanol and then repeatedly extracted with the same volume of ethyl acetate. The solvent extracts were then combined and evaporated to dryness using a rotary evaporator (KNF Rotary evaporator RC 600) giving a final yield of about 0.8%–1.1% (Gond et al. 2012).

9.0 The *in silico* studies

9.1 Docking Studies Using AutoDockVina

Docking studies were conducted using the open source program AutoDockVina designed and developed by Dr. Oleg Trott (Trott & Olson 2010).

Vina is the docking software and can be run with the help of AutoDock tools (ADT), PyMol visualisation software and Discovery studio.

9.2 Protein and ligand preparation for docking

Docking between a receptor and ligand requires their chemical structures available in certain formats. The protein three dimensional structure derived from crystallographic techniques were downloaded in .pdb format from RSC Protein Data Bank. Similarly the drugs (also referred to as ligands) were downloaded in .sdf or .mol formats from PubChem database or ZINC database.

The PDB ID of the following proteins were collected from the crystallography data of RCSB-PDB. For the docking experiment to be effective the 3D structure of the protein to be docked should have good resolution, so the search was narrowed down to high resolution entries of RCSB-PDB.

1) Protein name – 1FJG (PDB-ID) 3.0 A^0 - Resolution

2) Protein name – 1FJG (PDB-ID) 3.0 A^0 - Resolution

The downloaded .pdb files were then visualized and prepared for docking. The protein/receptor needs to be prepared for performing docking experiments using the PyMol visualization software. The major changes done to the protein using command prompt of PyMol include removal of water molecules, removal of unwanted bound ligands other than the co-factors, selection of polypeptide chains functionally relevant to the receptor-ligand interaction as in the case of a protein complex and addition of hydrogen atoms. In the meantime, Open Babel software was used to convert the ligand .sdf file or .mol file that was downloaded from PubChem into .pdb format. Once the .pdb files of both protein and ligand were

available, they were converted into .pdbqt format and saved using AutoDock Tools 1.5.6 (ADT MGL tools).

9.3 Finding the active site of the protein

The center x, y, z coordinates of the 'active site' of the receptor were obtained by uploading the receptor .pdb file in Biovia's Discovery Studio Visualizer. The active site consists of amino acid residues that form temporary bonds with the substrate (binding site) and residues that catalyse a reaction of that substrate (catalytic site). Prior knowledge about the binding site before the docking process significantly increases the docking efficiency. Biovia's Discovery Studio helps to define binding sites of the receptor from the receptors cavities. It requires a receptor to be defined and then the largest binding site will be displayed. Even though it displays all possible sites, the first one shown is usually the most visible and favourable one for docking.

9.4 Generation of vinaconfig file

AutoDock requires pre-calculated grid maps; this grid stores the potential energy arising from the interaction with the receptor and must surround the region of interest that is the active site of the receptor (Forli et al. 2016). Grid box parameters considered were coordinates of the centre of box x,y,z as obtained earlier through Discovery Studio and size x,y,z. The input file comprising details such as the receptor.pdbqt, ligand.pdbqt, output.pdbqt and grid coordinates of centre of box x,y,z, values and a default value of size x,y,z (25,25,25) were composed.

9.5 Docking

Docking was performed between the receptor and ligand using Autodockvina and the results were obtained as 'affinity' also known as 'the docking

score' expressed in kcal/mol. Docking by AutoDockVina is an approach where the receptor is kept rigid and the ligand is flexible. Orientation or geometry of the ligand inside the binding site is defined as 'POSE'. Algorithm searches are done to look through such different poses and to arrive at the preferred orientation. Scoring functions are employed to finally predict the strength of association or binding affinity between the receptor and ligand. At the end of docking, results are depicted as binding energy/affinity for each pose in kcal/mol with rmsd values (root mean square deviation). The first pose with the least binding energy and zero rmsd value is taken into account.

9.6 Analysis of Docking output using Discovery Studio Visualiser

The docking outputs were then analysed using Biovia's Discovery Studio and 2D images of receptor ligand interaction were saved as separate files. The ligand-receptor binding interactions can also be visualised and recorded as 3D images. The docking calculations may then be validated by redocking with ligands that were found co crystallized with the receptor structures shown in the RCSB-PDB website.

CHAPTER 1

Phytochemical screening of *Kaempferia rotunda* and *Lagenandra toxicaria* rhizome extracts

Introduction

Traditional medicine can be defined as a combination of diverse health practices, knowledge applied individually or in combination to maintain the wellness. The huge number of people on this planet still dependent on traditional medicinal plants and plant derived materials for their everyday health solutions (Gurib-Fakim 2006). Based on WHO reports, about 80% of the world's population, mainly those of developing and under developed countries, depend on plant- based chemicals for their health care needs (Eloff 1998). In developing countries, the plant-based traditional medicine treatments are very high (Salim 2008). The traditional medicine practises is widespread in various Asian countries such as China, India, Japan, Pakistan, Sri Lanka and Thailand (DaSilva & Hoareau 1999). It was estimated that about one quarter of all medicinal prescriptions comprises of substances extracted from plants or synthetic analogs of plant–derived molecules (Eloff 1998).

Plant based traditional medical treatments have been widely executed for thousands of years by people of China, India, and several other countries (Kear 2006). The connection between medicinal plants of ethno medical usage and synthetic medicines derived from those plants was reported by Fabricant et al. (2001). Their study exposed that the 88 single molecules isolated from 72 medicinal plants have been introduced into modern medicinal practices, in which, many molecules have the same or a similar therapeutic role as they have in ethno medicine (Fabricant et al. 2001).

Plant derived secondary metabolites are generally considered as bioactive non-nutrient chemicals, commonly observed in vegetables, fruits, grains etc., whose intake considerably reduces the risk of chronic diseases (Sathishkumar & Bhaskar

2014). They have unique role as pharmaceuticals, food additives and fine chemicals (Sathishkumar & Bhaskar 2014). Previous studies have shown that many plants are rich repository of antioxidant molecules including, vitamins A, C, E, and phenolic class of secondary metabolites such as flavonoids, tannins, and lignins (Suffredini 2004). Phenolics, consists of a unique class of secondary metabolites that are synthezied via the primary metabolic pathways. Plants synthesize these molecules during their normal development. In plants, phenolics exhibits different roles such as phytoalexins, antioxidants and UV protectant, contributors to plant pigmentation, antifeedants and attractants for pollinators (Naczk and Shahidi 2006). Phenolics and other shikimic acid pathway derived molecules have raised particular interest in pharmacology because of their effective role as antioxidant, anti-inflammatory, immunomodulatory, cardioprotective and anticarcinogenic properties (Laparra & sans 2010).

Different extraction methods such as maceration, soxhlet extraction, supercritical fluid extraction, sub critical water extraction and ultrasound mediated extraction have been used for the recovery of secondary metabolites from plants. Nevertheless, the yield and collection of desired molecules not only depends on the method of extraction but also on the nature of solvent used (Do et al. 2014). For the extraction of polyphenol class of secondary metabolites, polar solvents are commonly used (Do et al. 2014). Ethanol has been considered as a good solvent for the extraction of polyphenols. For extracting flavonoid class of metabolites from tea, aqueous ethanol extract performed better than aqueous methanol (Wang and Helliwell 2001). The methanol extract showed more efficiency in extraction of lower molecularweight polyphenols, however other polar solvents such as aqueous

acetone is good for extracting polyphenols with higher molecular weight (Dai & Mumper 2010).

K. rotunda used in the present study is indigenous to south-east Asia and cultivated for different medicinal purposes (Woerdenbag et al. 2004). Plant parts, mainly rhizome, are traditionally used for treating abdominal pain, dysentery, cold, obesity, and diarrhea in humans (Heinrich 2003). Another plant *L. toxicaria* that have not been mentioned in the Ayurvedic literature such as Ayurvedic Pharmacopeia, and is reported to be an adulterant for *K. rotunda* in many places (Sereena et al. 2011). On the contrary there are also reports showing the plant has several bioactive properties (Selvakumari 2014).

The current chapter discussed the extraction and phytochemical screening of the *K. rotunda* and *L. toxicaria* rhizome extracts. The rhizome parts extracted in ethyl acetate, ethanol and water were subjected for both qualitative and quantitative assessment for it's phytoconstituents, especially phenolic molecules. The GC-MS assay screened the volatile components in the extracts whereas the HPLC mediated polyphenol profiling evaluated the presence of 13 bioactive phenolic compounds in the rhizome extracts.

Methodology

Plant collection and authentication

The detailed methodology for the collection and authentication of *K. rotunda* and *L. toxicaria* plants is described in the Methods section 1.0.

Determination of heavy metal content in the rhizome

Determination of heavy metals was done following to the guidelines of the Association of Official Agricultural Chemists (AOAC 1995). The wet digestion

followed by atomic absorption spectroscopy (AAS) was used for the estimation of arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) in the rhizomes of *K. rotunda* and *L. toxicaria* (Meena et al. 2010). Methods section 3.3 describes the detailed methodology for estimating these heavy metals**.** The results obtained were compared with the permissible limits of heavy metals set by Ayush (Ayush 2005) for medicinal plants in India.

Extraction of plant material

The rhizome of *K. rotunda* and *L. toxicaria* were extracted in ethyl acetate, ethanol and water using an accelerated solvent extractor (Dionex ASE 150, Thermoscientific) as per the procedure mentioned in Methods section 2.0. Thereafter, each extract was evaporated to dryness using a rotary evaporator and the resulting crude extracts were used for the analysis.

Qualitative Phytochemical Screening

Qualitative phytochemical screening of various extracts of *K. rotunda* and *L. toxicaria* rhizomes was conducted to identify the presence of important secondary metabolites such as alkaloids, polyphenols, tannins, flavonoids, terpenoids, saponins and glycosides according to the standard procedures (Sofowora 1996; Trease & Evans 1989; Harborne 1999). Detailed procedure of qualitative phytochemical screening is described in Methods section 3.1.

Gas chromatography - mass spectrometric analysis (GC-MS)

GC-MS analysis of *K. rotunda* and *L. toxicaria* were done according to the standard procedures explained in Methods section 3.2.1. The identification of peaks was done by comparing with the retention times of standards, and the mass spectra obtained were compared with the data available in NIST libraries.

Quantification of Total Phenolic Content (TPC)

The TPC was estimated using Folin-Ciocalteau spectrophotometric technique proposed by Singleton and Rossi (1965). A detailed methodology for the estimation of TPC is described in Methods section 3.2.2.

Estimation of Total Flavonoid Content (TFC)

The TFC of plant rhizome extracts was estimated using the aluminum chloride colorimetric method previously described by Chang et al. (2002) with minor changes. A detailed methodology for the estimation of TFC is described in Methods section 3.2.3.

Polyphenol profiling and quantification using HPLC

Polyphenol profiling and quantification was executed using the high-performance liquid chromatography (HPLC) method as proposed by Rodriguez-Delgado et al. (2001) with minor modifications. A detailed method for estimating polyphenol molecules is described in the Methods section 3.2.4.

Results

Determination of heavy metal content in the rhizome

The concentration of heavy metals such as As, Hg, Cd and Pb were estimated and are depicted in table 1.1. The concentration of As, Hg and Cd in the *K. rotunda* rhizome was below the detection limit (0.05 mg/kg). Same time, the concentration of Pb in *K. rotunda* rhizome was 2.16 ± 0.49 mg/kg. In the case of *L. toxicaria* rhizome, the assay showed the presence of As, Hg and Pb in permissible limits with Pb being the highest (4.38 ± 1.13). The concentration of Cd was below the detection limit (Table 1.1).

Extraction and phytochemical screening

Three solvents were selected for extraction with different relative polarity levels. Among the three solvents, ethyl acetate is the least polar, ethanol on a higher scale, and water being the most polar. Ethyl acetate was giving the lowest yield of extraction and aqueous extraction was giving the highest yield (Table 1.2).

Qualitative phytochemical analysis showed the presence of broad variety of secondary metabolites in all the six extracts as depicted in table 1.3. Among these bioactive molecules identified, phenolic classes of secondary metabolites were abundantly present in the extracts. Flavonoids were present in all the six extracts tested. KrEA and KrOH extracts of *K. rotunda* showed dominance of polyphenolic class of secondary metabolites. Among *L. toxicaria* extracts, high amount of phenolic content was present in the LtEA extract compared to LtOH and LtWt extracts.

Estimation of Total Phenol Content (TPC)

The quantitative estimation of total phenol content shows high amount of phenols in the KrEA (41 ± 0.18 mg GAE/g) and KrOH (35 ± 0.43 mg GAE/g) extracts of *K. rotunda* (Table 1.4). In the case of *L. toxicaria*, the LtEA extract showed high phenolic content with a TPC value of 32.30 ± 0.72 mg GAE/g and LtWT showed the lowest TPC value (2.12 ± 1.54 mg GAE/g).

Estimation of Total Flavonoid Content (TFC)

The total flavonoid content in the rhizome extracts of *K. rotunda* and *L. toxicaria* were estimated quantitatively and reported in Table 1.4. High amount of flavonoid content was observed with KrEA (30.24 ± 0.11 mg QCE /g) and KrOH (28.32 ± 0.65 mg QCE /g) extracts of *K. rotunda*. Similarly, LtEA extract showed the presence of considerable amount of flavonoids compared to the LtOH and

LtWT. Furthermore, the water extract of *L. toxicaria* (LtWT) showed lowest TFC (1.05 ± 0.18 mg QCE /g).

GC-MS analysis

The GC–MS chromatogram of *K. rotunda* and *L. toxicaria* extracts were analyzed by relating to the known compounds present in the NIST library. The compounds detected in ≥ 1% (area %) in the rhizome extracts of *K. rotunda* by GC-MS analysis are listed in the order of their abundance (Table 1.5, 1.6 and 1.7). Also, the GC–MS chromatogram of KrEA, KrOH and KrWT extracts are provided (Figure 1.1). In KrEA extract, 37 compounds were detected, that show ≥ 1% abundance. The most dominant of all the detected compounds from KrEA was methyl 16-methyl-heptadecanoate (11.99%), 1-hydroxymethyl-5,8,9-endo-10-exo-tetramethyltricyclo [6.3.0.0(5,11)] undecane (8.27%), 4-hydroxy-.beta.-ionone (6.14%) and methyl benzoate (5.24%) (Table 1.5). KrWT extract showed the presence of 32 compounds in which hexadecanoic acid, methyl ester (8.3%), propanoic acid, 2-hydroxy-, methylester, (.+/-.)- (8.09%), L-Ascorbyl 2,6-Dipalmitate (7.46%), and ethane, 1-(benzylthio)-2-(2-chloroethylthio)- (7.23%) were most dominant (Table 1.7). KrOH extract showed the presence of 21 compounds with ≥ 1% abundance. Here, dimethyldi(3-ethylphenoxy) silane (9.23%), and spiro[4.4]non-3-en-2-one, 4-methyl-3-(1H-tetrazol-5-yl)-1-oxa- (5.18%)and 6-ethyl-2,3,5,7-tetrahydroxy 1,4-Naphthoquinone(4.19%) were abundant (Table 1.6).

Figure 1.2 showed the GC-MS chromatogram of LtEA, LtOH and LtWT extracts. The GC-MS analysis of LtWT and LtEA extracts showed the presence of 19 (Table 1.10) and 17 (Table 1.8) compounds respectively that has ≥ 1% abundance. LtOH extract had only 9 volatile compounds that exhibited ≥ 1% abundance (Table 1.9). In LtEA extract, pyrimidine-4,6 (3H,5H)-dione, 2-butylthio-

(12.13 %), 11-dodecen-1-ol trifluoroacetate (9.95%), n-hexadecanoic acid (8.91%), tetradecanoic acid (6.91 %) and stigmasta-5,22-dien-3-ol (5.12%) were most abundant. Compounds such as 1,2-benzenedicarboxylic acid (31.54%), 1,5-pentanediol(22.88%), hexadecanoic acid (13.89%) and 9,12-octadecadienoic acid (8.94%) were abundant in the LtOH extract. Same time, the LtWT extract showed the abundance of oleic Acid (12.8%), methyl 10-trans,12-cis-octadecadieonate (7.69%), cholesta-3,5-diene (5.39%) and ergosta-4,6,22-trien-3.beta.-ol (5.16%).

Polyphenol profiling and quantification using HPLC

Qualitative phytochemical characterization showed the presence of phenolic content and the semi quantitative assay also revealed abundance of TPC and TFC. Therefore, the polyphenol content in the extracts were quantified using high-performance liquid chromatography method. Thirteen standard reference compounds (1 mg/mL concentration) suchas (1) myricetin, (2) catechol, (3) quercetin, (4) caffeic acid, (5) ferulic acid, (6) p-coumaric acid, (7) syringic acid, (8) ellagic acid, (9) gallic acid, (10) cinnamic acid, (11) chlorogenic acid, (12) kaempferol and (13) apigenin were used for estimation of samples. Retention time was used for the identification of molecules. The phenolic compounds identified during the assay were reconfirmed through spiking with the individual standards. KrEA extract contains high amounts of myricetin (5061.75 ± 0.004 µg/g), quercetin (3431.02 ± 0.022 µg/g) and ellagic acid (1678.2 ± 0.047 µg/g). KrOH extract showed high amounts of quercetin (4559.02 ± 0.002 µg/g), ellagic acid (1582.45 ± 0.024 µg/g), kaempferol (1292.17 ± 0.004 µg/g) and myricetin (1193.25 ± 0.042 µg/g), whereas KrWT extract showed the presence of ellagic acid (997.27 ± 0.056 µg/g) and catechol (56.34± 0.002 µg/g) only (Table 1.11). The combined chromatogram of *K. rotunda* extracts are shown in figure 1.3. Among the nine

polyphenols identified in LtEA extract, myricetin (6344.52 ± 0.008 µg/g) was present in high amounts. This was followed by gallic acid (4626.28± 0.016 µg/g). The LtOH extracts showed high amounts of myricetin (2580.5 ± 0.067) and ellagic acid (2046.04 ± 0.009). On the other hand, phenolic profile of LtWT only showed the presence of a single compound, catechol in trace amounts (Table 1.12). Figure1.4 showed the combined chromatogram of *L. toxicaria* rhizome extracts.

Discussion

Along with the development of pharmacologically effective drugs from the natural sources like plants, it is necessary to address their safety and toxicity properly. The rising incidences of heavy metal toxicity and related negative effects associated with plants and plant derived drugs are against the widespread misunderstanding that the plants are totally safe (Ernst 2002). Plants are considered as an important link in the transmission of toxic heavy metals from the contaminated sites into the humans (Dghaim et al. 2015). Since the heavy metals have considerable damaging effects to humans and other animals it is necessary to analyse the heavy metal content in the plants or plant parts that are intended for therapeutic purposes. In the current study the concentration of toxic metals in the powdered rhizome of both plants were studied. Among the four toxic metals tested, the *K. rotunda* rhizome showed trace of lead well within the permissible range set by Ayush (Ayush 2005). In the case of *L. toxicaria*, heavy metals like arsenic, mercury and lead were detected in the rhizome powder. Nevertheless, the concentration of these toxic metals was too low when compared to the maximum permissible limit allotted by Ayush (2005) for herbal medicines.

Solvents selected for the extraction of phytochemicals from herbal origins are selected based on the polarity of the solute of interest (Wong & Kittis 2006). A

solvent having similar polarity of the solute will dissolve the solute properly (Altemimi et al. 2017). Scientists make use of multiple solvents for the extraction of bioactive secondary metabolites from the plant parts. Also, they preferably use dried powders of plant parts to extract phytochemicals in order to eliminate the interference of water in the plant material (Altemimi et al., 2017). Studies of Sathishkumar & Bhaskar (2014) proved that for the extraction of phytochemicals, methanol and ethanol extracts were best. In the present study polar solvents such as ethyl acetate, ethanol and water were used for extracting secondary metabolites from the rhizomes of *K.rotunda* and *L. toxicaria*. In both plants, maximum yield was obtained with water extracts (highly polar). This was followed by ethanol extracts. Least yield in both cases was reported with ethyl acetate extracts. The preliminary phytochemical analysis showed the occurrence of different metabolites including phenolics, flavonoids, tannins, alkaloids.

The GC-MS analysis showed the presence of several volatile compounds in the rhizome extracts of both plants. The compounds that show ≥ 1% abundance were only listed in the current study. In the case of *K. rotunda*, the KrEA extract showed the presence of 37 compounds that exhibited ≥ 1% abundance. Compounds such as methyl isostearate, 1-hydroxymethyl-5,8,9-endo-10-exo-tetramethyltricyclo [6.3.0.0 (5,11)]undecane, 4-hydroxy-.beta.-ionone, methyl benzoate and benzyl benzoate showed abundance in the KrEA extract. Same time, KrOH extract only showed the presence 21 volatile compounds that comes in the aforesaid abundance range. Here, dimethyl di (3-ethylphenoxy) silane, spiro [4.4]non-3-en-2-one, 4-methyl-3-(1H-tetrazol-5-yl)-1-oxa-,6-ethyl-2,3,5,7-tetrahydroxy 1,4-naphthoquinone were present in high amounts. Like, KrEA extract the KrOH extract also showed the presence of benzyl benzoate in reasonable amounts (1.55%). Previously, Woerdenbag et al.

(2004) reported the benzyl benzoate as an abundant constituent of *K. rotunda* rhizome extracts. Alongside, their study also reported the presence of similar compounds detected in our study such as methyl benzoate and undecane in the *K. rotunda* rhizome extracts. In the current study, the KrWT extract showed the presence of 32 compounds. Here, hexadecanoic acid, methyl ester, propanoic acid, 2-hydroxy-, methylester, (.+/-.)-, L-ascorbyl 2,6-dipalmitate, ethane, 1-(benzylthio)-2-(2-chloroethylthio)-, heptadecanoic acid, 15-methyl-, methyl ester, 9-octadecenoic acid, methyl ester,(E)-, 1,2-benzenedicarboxylic acid, diisooctyl ester were present in high amounts. Our results are in agreement with previous study that reported the petroleum ether extract of *K. rotunda* rhizomes showed the presence of compounds, n-dodecane, hexadecane, stearaldehyde, dodecanoic acid, kauren-ol in high amounts (Sereena et al., 2011). Some previous studies also reported the presence of many cyclohexane derivatives from the rhizome extracts of *K. Rotunda* (Pancharoen et al. 1996; Marco-Contelles et al. 2004; Stevenson et al. 2007). In the case of *L. toxicaria* rhizomes, the LtWT extract showed the presence of 19 compounds with ≥ 1% abundance. Similarly, the LtEA extract showed the presence of 17 volatile compounds in the aforementioned abundance range, whereas the LtOH extract only exhibited the presence of 9 volatile compounds that exhibited ≥ 1% abundance. Among the volatile molecules present in the *L. toxicaria* extracts, the oleic acid was commonly observed in the three extracts tested. Also, the LtEA extract showed the presence of dibutyl phthalate in 3.74% and the LtOH extract showed the presence of terephthalic acid (2.58%). These results are in total agreement with a previous study that reported the presence of diethyl phthalate, dioctyl phthalate, oleic acid in the methanol extract of *L. toxicaria* rhizome (Selvakumari & de Britto, 2008)

The GC-MS analysis in the present study also revealed the presence of several fatty acids and fatty acid derivatives of therapeutic importance. The KrEA extract showed high amounts of hexadecanoic acid, methyl ester, 9-octadecenoic acid, methyl ester and n-hexadecanoic acid. The fatty acid, hexadecanoic acid was also present in the KrOH extract. Likewise, KrWT extract showed the presence of hexadecanoic acid, methyl ester, propanoic acid, 2-hydroxy-, methylester, (.+/-.)-, heptadecanoic acid, 15-methyl-, methyl ester, 9-octadecenoic acid, methyl ester,(E)-, 10-octadecenoic acid, methyl ester, and oleic acid in high amounts. In the case of *L. toxicaria* extracts, the LtEA showed the presence of high amounts of fatty acids such as n-hexadecanoic acid, tetradecanoic acid and oleic acid. Similarly, LtOH extract exhibited hexadecanoic acid, 9,12-octadecadienoic acid, cis-13-octadecenoic acid. Likewise, oleic acid and 9-octadecenoic acid, methyl ester was abundant in the LtWT extract. Earlier, Barhoi et al. (2021) reported n-Hexadecanoic acid among the important bioactive compounds identified in the GC-MS analysis of *Moringa oleifera* leaves. Various pharmacological properties of fatty acids like palmitic acid, palmitoleic acid, dodecanoic acid, tetradecanoic acid and octadecanoic acid, palmitic acid was described earlier (Rahuman et al. 2000; Niu et al. 2008; Abubakar & Majinda 2016; Weimann et al. 2018).

Phenolics are among the important secondary metabolites synthesized by plants during their development (Sathishkumar & Bhaskar 2014). In plants, phenolics have various roles such as phytoalexins, attractants for pollinators, some phenolic molecules act as antifeedants and certain other contributes to plant pigmentation. Also, majority of the phenolic class of molecules act as antioxidants and protective agents against harmful UV radiations (Naczk & Shahidi 2006). The antioxidant potential of a plant material is usually attributed to the polyphenol

compounds present in it (Arun et al. 2017). The preliminary phytochemical analysis of the current study showed the presence of high amount of phenolic compounds in the ethyl acetate and ethanol extracts of *L. toxicaria* and *K. rotunda* rhizomes. In the present study, TPC and TFC contents was estimated and high amount of phenolic and flavonoid content was estimated in the ethyl acetate and ethanol extracts of both the plants. Water extracts showed least phenol content, especially with *L. toxicaria*. The current study results that showed that the effects of solvents on TPC and TFC of both plants are similar to some previous reports. According to Koffi et al. (2010) the ethanolic extracts from Ivorian plants showed higher number of phenolic compounds compared to acetone, methanol and water extracts. Do et al. (2014), reported similar results during their study on the TPC and TFC content of *Limnophila aromatica*. The highest TPC and TFC in their case was obtained with 100% ethanol extract, followed by 100% acetone extract. Similarly, the water extract showed the lowest TPC and TFC. One previous study showed the presence of phenolic class of secondary metabolites in the form of pink and yellowish patches with the dried powder of the *L. toxicaria* rhizome (Selvakumari 2014). The HPLC mediated polyphenol profiling quantified the amount of thirteen important phenolics present in the plant rhizome extracts. In the case of *L. toxicaria*, maximum phenolic compounds were identified with the LtEA extracts (nine phenolic compounds). This was followed by LtOH with four phenolic compounds. The water extract (LtWT) only showed the presence of catechol in trace amounts. In the case of *K. rotunda*, KrEA and KrOH extracts showed maximum number of phenolic compounds. Meanwhile, KrWT extract showed relatively low amount of phytoconstituents. Pai et al. (1970) screened the methanol extract of K. rotunda and revealed the presence of flavonoids, crotepoxide, chalcones, quercetin, flavonols, β-sitosterols,

stigmasterol, syringic acid, protocatechuic acid, and some hydrocarbon compounds. The results of HPLC mediated phytochemical analysis in the present study were in confirmation with the earlier studies. The study results showed the presence of nine and eight polyphenol compounds, KrEA and KrOH extracts respectively. Among this high amount of quercetin, myricetin, and ellagic acid was present in both extracts. Even though the KrWT extract showed the presence of only two phenolic compounds such as ellagic acid and catechol, the concentration of ellagic acid was very high. Previously, Atun et al. (2013) isolated three known flavanones, namely 5-hydroxy-7-methoxyflavanone (1), 7-hydroxy-5-methoxyflavanone (2), and 5,7-dihydroxyflavanone (3) from *K. rotunda* rhizomes**.** Lotulung et al. (2008) showed the presence of compounds 2'-hydroxy-4, 4', 6'-trimethoxy chalcone and crotepoxide in methanolic extract of *K. rotunda*.

Table 1.1: Concentration range of heavy metals (mg/kg) in the rhizome samples of the plants.			
Heavy metal	*Kaempferia rotunda* rhizome	*Lagenandra toxicaria* rhizome	PL according to Ayush 2005 for medicinal plants in India
Arsenic	BDL	0.14 ± 0.11	3.0 mg/kg
Mercury	BDL	0.09 ± 0.34	1.0 mg/kg
Cadmium	BDL	BDL	0.3 mg/kg
Lead	2.16 ± 0.49	4.38 ± 1.13	10.0 mg/kg
Values are expressed as mean ± SEM; BDL : Below detection limit; PL: Permissible limit.			

Table 1.2: The type of solvent, coding and percent yield of extraction for the plants				
Name of the Plant	Plant part	Solvent	Code	% Yield
Kaempferia rotunda	Rhizome	Ethyl Acetate	KrEA	4.5 ± 1.13
		Ethanol	KrOH	9.7 ± 1.13
		Aqueous	KrWT	14.6 ± 1.13
Lagenandra toxicaria	Rhizome	Ethyl Acetate	LtEA	3.4 ± 0.21
		Ethanol	LtOH	8.12 ± 0.51
		Aqueous	LtWT	15± 1.13
Values are expressed as mean ± SEM				

Table 1.3: Qualitative phytochemical analysis of the extracts						
Type of Phytochemicals	*Kaempferia rotunda*			*Lagenandra toxicaria*		
	KrEA	KrOH	KrWT	LtEA	LtOH	LtWT
Alkaloids	-	-	-	-	+	+
Flavonoids	+	+	+	+	+	+
Polyphenols	+	+	+	+	+	+
Tannins	+	+	+	+	+	-
Saponins	-	-	-	-	-	-
Terpenoids	+	+	-	+	-	-
Glycosides	+	+	-	+	+	-
Steroids	-	-	-	+	+	+
Coumarins	+	+	-	+	+	+
+ Present, - Absent						

Table 1.4: Total phenol content (TPC) and total flavonoid content (TFC)			
Name of the Plant	Extract	TPC (mg GAE/g)	TFC (mg QCE/g)
K. rotunda	KrEA	41 ± 0.18	30.24 ± 0.11
	KrOH	35 ± 0.43	28.32 ± 0.65
	KrWT	6.47 ± 1.28	4.18 ± 0.55
L. toxicaria	LtEA	32.30 ± 0.72	25.84 ± 0.36
	LtOH	26. 08 ± 1.12	12.17 ± 0.64
	LtWT	2.12 ± 1.54	1.05 ± 0.18
Values are expressed as mean ± SEM			

Table 1.5: Phytochemical constituents of KrEA extract by GC-MS analysis			
SL.No.	RT	Area%	Compound
1	57.348	11.99	Methyl 16-methyl-heptadecanoate
2	64.788	8.27	1-Hydroxymethyl-5,8,9-endo-10-exo-tetramethyltricyclo[6.3.0.0(5,11)]undecane
3	63.089	6.14	4-Hydroxy-.beta.-ionone
4	16.341	5.24	Methyl benzoate
5	46.873	4.04	Benzyl Benzoate
6	52.524	3.62	Hexadecanoic acid, methyl ester
7	56.624	3.02	Methyl Linoleate
8	64.51	2.55	Silane, dimethyl (3-ethylphenoxy)-
9	59.382	2.47	4,5,6,7-tetramethyl-Benzo[1,2,5]-thiadiazole
10	57.587	2.45	Pyridine, 4-(propylthio)
11	60.962	2.45	[1,1'-Bicyclohexyl]-3,3'-dione,2,2'-dimethyl-5,5'-bis(1-methylethenyl)-, sterioisomer
12	56.816	2.04	9-Octadecenoic acid, methyl ester,
13	56.91	1.9	Pregna-3,5-dien-20-one
14	60.276	1.78	Dimethyl 2,2'-diaminobiphenyl-4,4'-dicarboxylate
15	61.331	1.76	Cyclohexanone, 2-methyl-6-(4-nitro-1-oxobutyl)-
16	60.443	1.72	2,3-Dimethyl-8-oxo-non-2-enal
17	59.754	1.7	1-Carbomethoxy-1,2,5,5-tetramethyl-cis-decalin(1R,2S,4as,8as)
18	54.492	1.55	1H-Naphtho[2,1-b]pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3R-(3.alpha.,4a.beta.,6a.alpha.,10a.beta.,10b.alpha.)]-
19	61.612	1.54	5-(4-Methoxy-phenylcarbamoyl)-1H-imidazole-4-carboxylic acid, ethylester
20	54.601	1.52	n-Hexadecanoic acid
21	61.193	1.49	Androstan-17-ol,4,4-dimethyl-,(5 alpha, 17beta.)-
22	61.935	1.43	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol
23	58.289	1.42	11-Dodecen-1-ol trifluoroacetate
24	66.088	1.37	Benzeneacetaldehyde, alpha-(diphenylmethylene)-4-methoxy-
25	57.844	1.36	6H-[1]Benzopyrano[4,3-b][1,2]dioxolo[4,5-g]quinolin-6-one
26	58.59	1.36	p-Tolylpentamethyl-disiloxane
27	53.173	1.33	5-(3-Methyl butyl)-2-pyridine carboxylic acid
28	57.075	1.33	9(11)-Dehydrotestosterone

29	62.032	1.3	4-Methoxycarbonyl-2-methoxyphenyl isothiocyanate
30	64.304	1.29	11-Dimethyl-1,2,3,4-tetrahydro-1,4-methanophenazine 1-carboxylic acid, methyl ester
31	60.603	1.28	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-
32	53.728	1.26	Phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-
33	61.85	1.25	2-Amino-5,6-dimethylbenzothiazole
34	54.239	1.16	Ethyl 14-methyl-hexadecanoate
35	57.423	1.13	3-Methoxy-D-homoestra-1,3,5(10)-trien-17a-one (8.beta., 9.alpha., 14.alpha.)
36	56.022	1.08	Wogonin
37	62.436	1.06	1,3a-Azulenedimethanol, 1,2,3,3a,4,7,8,8a-octahydro-1-[5-hydroxy-3-(hydroxyme)

Table 1.6: Phytochemical constituents of KrOH extract by GC-MS analysis

SL.No.	RT	Area%	Compound
1	64.628	9.23	dimethyldi(3-ethylphenoxy) silane
2	57.222	5.18	Spiro[4.4]non-3-en-2-one, 4-methyl-3-(1H-tetrazol-5-yl)-1-oxa-
3	64.73	4.19	6-ethyl-2,3,5,7-tetrahydroxy 1,4-Naphthoquinone
4	54.266	3.27	Hexadecanoic acid, ethyl ester
5	62.998	3.02	2-Adamantanecarboxylic acid, 4,8-dioxo-
6	60.895	2.19	24-Norcholane, 23-[2-methyl-1-(1-methylethyl)cyclopropyl]-, (5.alpha.)-
7	46.729	1.55	Benzyl Benzoate
8	59.326	1.48	2-Buten-1-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-
9	66.26	1.48	Gamma-Sitosterol
10	60.234	1.43	Estra-1,3,5(10)-trien-17-one, 3-hydroxy-15-methoxy-, (15.beta.)-
11	57.945	1.35	9,12-Octadecadienoic acid, ethyl ester
12	66.327	1.32	Beta-Sitosterol
13	66.028	1.31	Androstenediol diacetate
14	57.511	1.19	Pyridine-2,4-diol, 3,5,6-trimethyl
15	64.391	1.15	1H-Trindene, 2,3,4,5,6,7,8,9-octahydro-1,1,4,4,9,9-hexamethyl-
16	61.14	1.12	3,4,5,8-Tetramethyl-1-phenyl-4,5,6,8-tetrahydropyrazolo[3,4-b][1,4]diazepin-7(1H)-one
17	61.543	1.1	5-(4-Methoxy-phenylcarbamoyl)-1H-Imidazole-4-carboxylic acid, ethyl ester

18	57.322	1.08	1-methoxy-4-methyl-Estra-1,3,5(10)-trien-17-one,
19	64.258	1.05	1-Phenyl-3,5,6,8-tetramethyl-7-oxo-6,7(8H)-dihydropyrazolo(3,4-b)(1,4)diazepine
20	61.267	1.03	Stigmasterol
21	58.081	1.01	9,12-Octadecadienoic acid (Z,Z)-

Table 1.7: Phytochemical constituents of KrWT extract by GC-MS analysis

SL.No.	RT	Area%	Compound
1	52.413	8.3	Hexadecanoic acid, methyl ester
2	6.048	8.09	Propanoic acid, 2-hydroxy-, methylester, (.+/-.)-
3	54.01	7.46	L-Ascorbyl 2,6-Dipalmitate
4	5.233	7.23	Ethane, 1-(benzylthio)-2-(2-chloroethylthio)-
5	57.244	4.7	Heptadecanoic acid, 15-methyl-, methyl ester
6	56.639	4.37	9-Octadecenoic acid, methyl ester,(E)-
7	64.463	4.28	1,2-Benzenedicarboxylic acid, diisooctyl ester
8	72.606	3.72	Stigmast-5-en-3-ol, oleate
9	56.794	3.35	10-Octadecenoic acid, methyl ester
10	69.676	2.93	Cholest-5-en-3-ol (3.beta.)-, propanoate
11	16.272	2.79	Benzoic acid, methyl ester
12	64.558	2.76	Sambucoin
13	58	2.64	Oleic Acid
14	71.6	2.42	Stigmasta-5,22-dien-3-ol, acetate,(3.beta.)-
15	60.143	2.08	alpha-Hydroxystearic acid
16	33.776	1.87	Cycloheptasiloxane, tetradecamethyl-
17	56.468	1.78	9,12-Octadecadienoic acid (Z,Z)-,methyl ester
18	34.696	1.72	Nonadecane
19	67.524	1.71	Heneicosanoic acid, 18-propyl-, methyl ester
20	26.282	1.65	Cyclohexasiloxane, dodecamethyl-
21	60.513	1.58	Hexacosanoic acid
22	67.415	1.52	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane
23	61.097	1.5	l-(+)-Ascorbic acid 2,6-dihexadecanoate
24	17.374	1.49	Cyclopentasiloxane, decamethyl-
25	62.871	1.27	5-Butyl-6-hexyloctahydro-1H-indene
26	62.764	1.24	1H-Indene, 5-butyl-6-hexyloctahydro-

27	69.212	1.22	Cyclononasiloxane, octadecamethyl-
28	63.596	1.21	(1S,2E,4S,5R,7E,11E)-Cembra-2,7,11-trien-4,5-diol
29	61.155	1.16	3,7,11,15-Tetramethyl-hexadecanol,
30	68.643	1.16	1,22-Docosanediol
31	73.001	1	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-
32	68.961	0.78	i-Propyl 11,12-methylene-octadecanoate

Table 1.8: Phytochemical constituents of LtEA extract by GC-MS analysis

SL.No.	RT	Area%	Compound
1	8.29	12.13	Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-
2	58.262	9.95	11-Dodecen-1-ol trifluoroacetate
3	54.85	8.91	n-Hexadecanoic acid
4	54.206	6.91	Tetradecanoic acid
5	71.644	5.12	Stigmasta-5,22-dien-3-ol
6	63.594	4.21	1-Naphthalenol, 1,2,3,4-tetrahydro -, acetate
7	58.63	3.76	Oleic Acid
8	53.149	3.74	Dibutyl phthalate
9	65.631	3.11	Pregnenolone
10	12.723	2.64	Silane, (2-ethoxyethoxy)trimethyl-
11	66.259	2.59	3-Butylthiophene 1,1-dioxide
12	58.742	2.5	Estra-1,3,5(10)-trien-17.beta.-ol
13	72.615	2	Stigmastan-3,5-diene
14	66.325	1.98	2-Butyl(dimethyl)silyloxybutane
15	48.211	1.43	Heneicosane
16	49.519	1.36	Bicyclo[3.1.1]heptane
17	68.643	1.23	2,6,10,14-Hexadecatetraenoic acid

Table 1.9: Phytochemical constituents of LtOH extract by GC-MS analysis			
SL.No.	RT	Area%	Compound
1	64.609	31.54	1,2-Benzenedicarboxylic acid
2	6.517	22.88	1,5-Pentanediol
3	54.288	13.89	Hexadecanoic acid
4	57.964	8.94	9,12-Octadecadienoic acid
5	58.083	4.75	cis-13-Octadecenoic acid
6	54.413	3.46	l-(+)-Ascorbic acid 2,6-dihexadecanoate
7	71.635	3.18	Stigmasta-5,22-dien-3-ol
8	58.646	2.7	Methyl 17-methyl-octadecanoate
9	67.678	2.58	Terephthalic acid
10	68.826	2.31	Spinasterone
11	58.209	1.86	Oleic Acid
12	72.618	1.11	Stigmastan-3,5-diene

Table 1.10: Phytochemical constituents of LtWT extract by GC-MS analysis			
SL.No.	RT	Area%	Compound
1	58.146	12.8	Oleic Acid
2	58.515	7.69	Methyl 10-trans,12-cis-octadecadieonate
3	69.664	5.39	Cholesta-3,5-diene
4	72.593	5.16	Ergosta-4,6,22-trien-3.beta.-ol
5	56.635	3.61	9-Octadecenoic acid (Z)-, methyl ester
6	71.145	3.58	1H-Isoindole
7	70.955	3.16	1-Eicosene
8	9.936	3.01	Methoxyphenyl-oxime
9	56.784	2.18	9-Octadecenoic acid, methyl ester
10	57.277	1.95	Octadecanoic acid, methyl ester
11	71.566	1.75	Stigmasteryl tosylate
12	58.731	1.62	Octadec-9-enoic acid
13	71.025	1.58	i-Propyl 9,12-octadecenadienoate
14	71.973	1.58	Stigmasta-5,22-dien-3-ol, acetate
15	70.067	1.26	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)
16	5.64	1.21	Isopropoxycarbamic acid, ethyl ester

17	58.339	1.13	trans-13-Octadecenoic acid
18	58.859	1.1	cis-13-Octadecenoic acid
19	72.213	1.03	Pyridine-3-carboxamide

Table 1.11 : Estimation and quantification of polyphenols in KrEA, KrOH and KrWT extracts of *K. rotunda* L. rhizome

SL. No	Standards	Retention times	KrEA (µg/g)	KrOH (µg/g)	KrWT (µg/g)
1	Gallic acid	7.724	ND	ND	ND
2	Catechol	12.773	23.33 ± 0.003	ND	56.34 ± 0.002
3	Chlorogenic acid	23.807	ND	268.98 ± 0.004	ND
4	Caffeic acid	25.349	199.8 ± 0.025	174.79 ± 0.007	ND
5	Syringic acid	26.298	ND	ND	ND
6	P-Coumaric acid	27.567	133.1 ± 0.063	ND	ND
7	Ferulic acid	28.629	77.09 ± 0.005	ND	ND
8	Ellagic acid	31.155	1678.2 ± 0.047	1582.45 ± 0.024	997.27 ± 0.056
9	Myricetin	31.814	5061.75 ± 0.004	1193.25 ± 0.042	ND
10	Cinnamic acid	33.329	878.27 ± 0.014	228.36 ± 0.063	ND
11	Quercetin	34.531	3431.02 ± 0.022	4559.02 ± 0.002	ND
12	Kaempferol	37.586	436.46 ± 0.00	1292.17 ± 0.004	ND
13	Apigenin	38.322	ND	859.95 ± 0.012	ND

ND : Not Detected

Table 1.12: Estimation and quantification of polyphenols in LtEA, LtOH and LtWT extracts of *L. toxicaria* Dalz. rhizome

SL. No	Standards	Retention times	LtEA (µg/g)	LtOH (µg/g)	LtWT(µg/g)
1	Gallic acid	7.724	4626.28± 0.016	ND	ND
2	Catechol	12.773	94.4 ± 0.038	43.71± 0.021	13.44 ± 0.073
3	Chlorogenic acid	ND	ND	ND	ND
4	Caffeic acid	25.349	44.99 ± 0.029	ND	ND
5	Syringic acid	26.298	8.72± 0.034	ND	ND
6	p-Coumaric acid	27.567	9.96 ± 0002	ND	ND
7	Ferulic acid	28.629	60.83 ± 0.023	ND	ND
8	Ellagic acid	31.155	911.75 ± 0.011	2046.04 ± 0.009	ND
9	Myricetin	31.814	6344.52 ± 0.008	2580.5 ± 0.067	ND
10	Cinnamic acid	33.329	355.12 ± 0.014	9.258 ± 0.029	ND
11	Quercetin	ND	ND	ND	ND
12	Kaempferol	ND	ND	ND	ND
13	Apigenin	ND	ND	ND	ND

ND : Not Detected

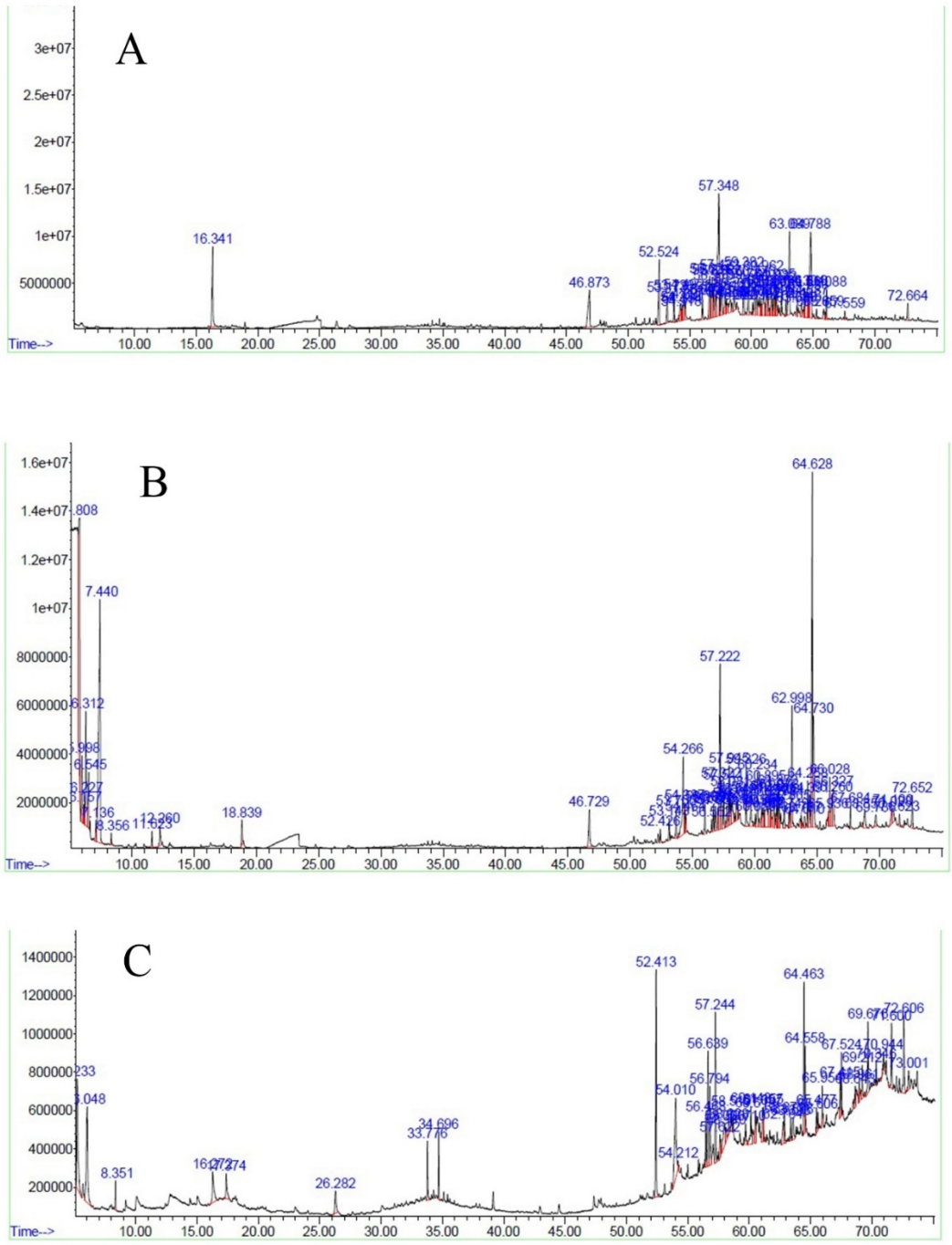

Figure 1.1 : GC-MS chromatogram of A) KrEA, B) KrOH, C) KrWT extracts of *K. rotunda*

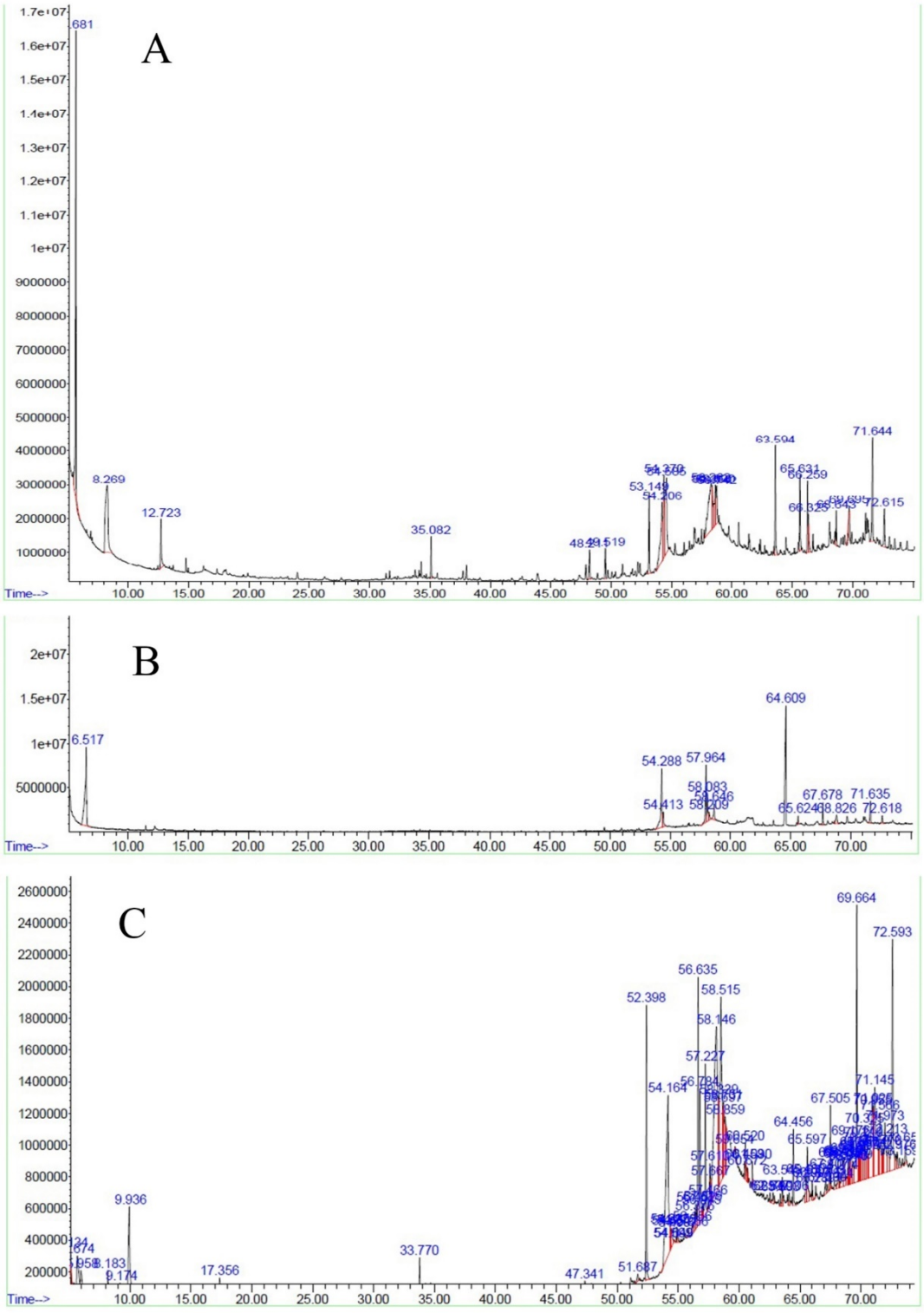

Figure 1.2 : GC-MS chromatogram of A) LtEA, B) LtOH, C) LtWT extracts of *L. toxicaria*

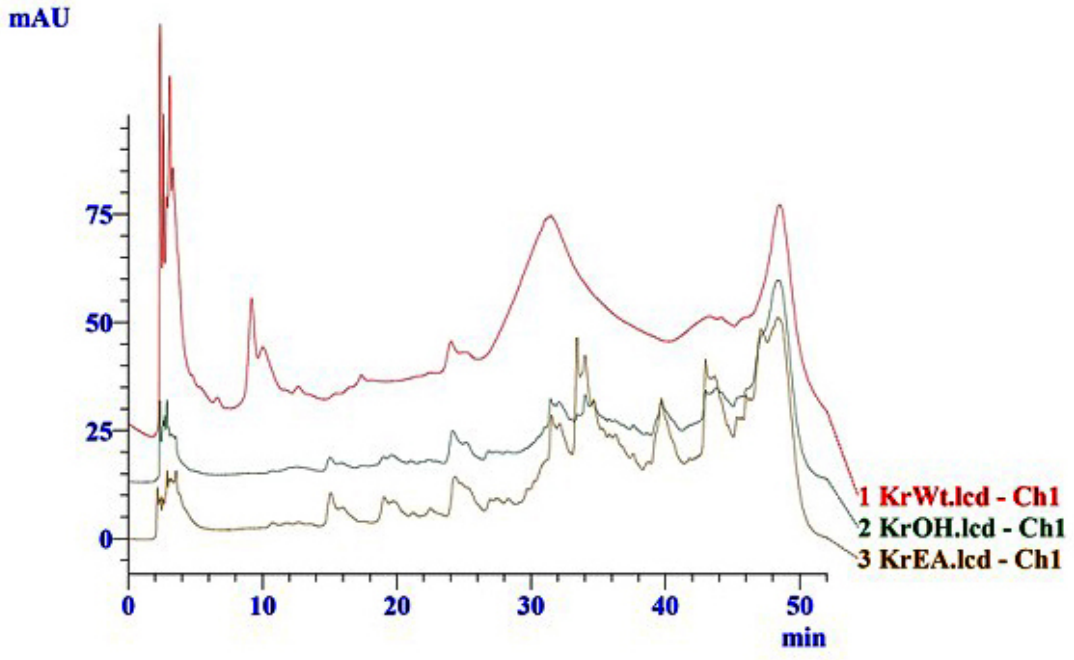

Figure 1.3: Combined HPLC chromatogram of KrEA, KrOH and KrWT extracts of *K. rotunda*.

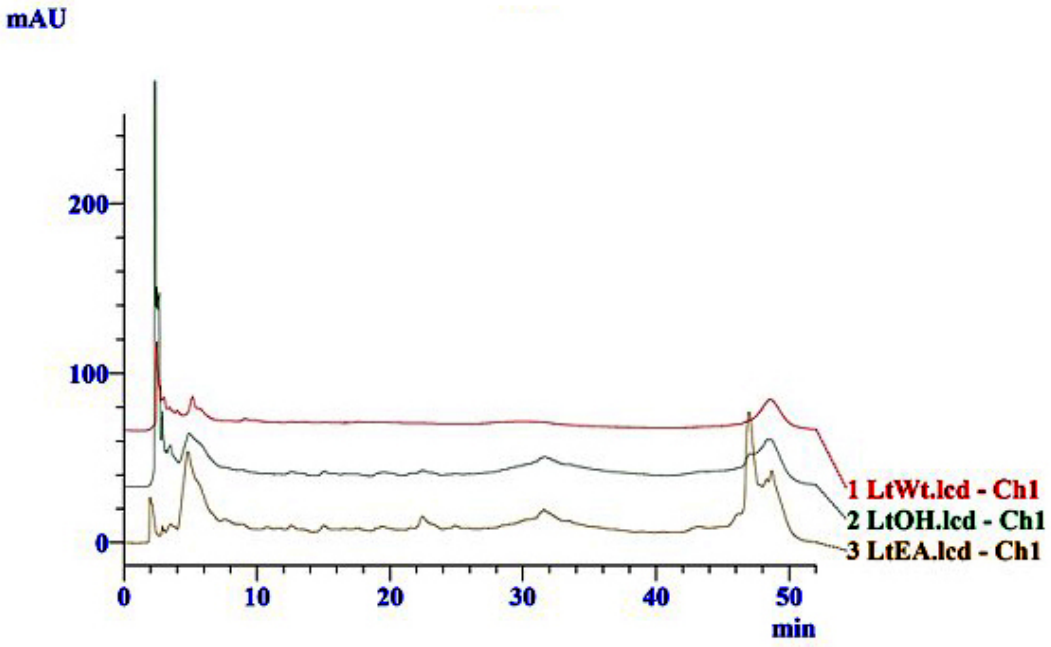

Figure 1.4: Combined HPLC chromatogram of LtEA, LtOH and LtWT extracts of *L. toxicaria*.

CHAPTER 2

Antibacterial potential of *Kaempferia rotunda* and *Lagenandra toxicaria* rhizome extracts

Introduction

In recent times, there has been a rising awareness towards the development of novel antimicrobial drugs from diverse sources to fight against bacteria and bacterial resistance (Balouiri et al. 2016). The exciting synthetic compounds from the golden era such as tetracyclines, aminoglycosides, cephalosporins and macrolides are losing their potential against many pathogenic microbes nowadays (Mayers et al. 2009). The increased resistance to a bacteriostatic or bactericidal compound is explained based on a high probability of therapeutic failure against microbe (Jhanji et al. 2021). Many previous studies have reported several resistance mechanisms such as horizontal gene transfer, mutations that induce vertical evolution, regulation of efflux pumps, enzyme inactivation, covalent alteration of antibiotic compounds, and formation of resistant biofilms against antibiotic compounds (Sommer et al. 2017). Hence, it has become an important objective to discover relatively less resistant novel antibiotic molecules from natural sources. About 25% of novel drugs tested for clinical use are from plants (Ullah 2014). The antibiotics from plant and endophytic microbial sources occupy the key part of the antimicrobials discovered from natural sources (Berdy 2005). The plants and other related environmentally safe sources have been considered as huge repository of diverse volatile and non-volatile secondary metabolites with prominent antimicrobial potential (Runyoro et al. 2006; Mabona et al. 2013; Nazzaro et al. 2013). The antimicrobial potential of various classes of secondary metabolites like phenols and alkaloids through a diverse mode of action is a matter of current research (Jhanji et al. 2021). Along with that the synergistic effects of certain plant derived compounds in combination with synthetic antibiotics was also reported (Hemaiswarya et al. 2008).

Many approaches have been used to screen the *in vitro* antimicrobial potential of a crude extract or a purified compound. However, the most basic and highly recommended *in vitro* antimicrobial screening method is agar disc diffusion assay (Balouiri et al. 2016). Agar disc diffusion assay initially developed in 1940s (Heatley 1944) had been in use for regular antimicrobial susceptibility testing for many years. Currently, many modified and accepted standards of agar disc diffusion are published by the Clinical and Laboratory Standards Institute (CLSI 2012) for antimicrobial susceptibility testing. The advantages such as simplicity and low cost, have provided universal acceptance to this technique to screen antimicrobial susceptibility of crude extracts, plant derived compounds, essential oils and even synthetic drugs (De Billerbeck et al. 2007; Das et al. 2010; Konate et al. 2012). In the present study the *in vitro* antibacterial effects of the crude rhizome extracts (ethyl acetate, ethanol and water) of *K. rotunda* and *L. toxicaria* were evaluated against seven pathogenic bacterial strains using disc diffusion assay.

Methodology

Collection and extraction of plant material

A detailed outline of the procedures for collecting and extracting *K. rotunda* and *L. toxicaria* rhizomes are provided in the Methods section 1 and 2.

Agar disk diffusion assay

The antibacterial activity of the *K. rotunda* (KrEA, KrOH and KrWT) and *L. Toxicaria* (LtEA, LtOH and LtWT) rhizome extracts was tested using an agar disc diffusion assay (Bauer et al. 1966) against seven pathogenic bacterial strains (*S.aureus*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *B. cereus*, *S. flexneri* and *S. marcescens*). Methods section 4.2 contains detailed information about the agar disk diffusion assay.

Statistical analysis

The data were represented as mean ± SEM. The data were compared using ANOVA followed by Bonferroni post-hoc test using GraphPad Prism version 5.00.

Results

Antibacterial potential of *K. rotunda* by disk diffusion assay

Table 2.1, 2.2 and 2.3 respectively represents the disk diffusion assay results of KrEA, KrOH and KrWT extracts. Among the three extracts tested, the KrEA and KrOH showed prominent inhibitory potential against five out of seven bacterial strains tested. It includes both Gram-positive and Gram-negative strains. Here the concentration depended rise in the antibacterial activity was evident. Compared to the Gram-negative bacterial strains the KrEA showed significant activity against the tested Gram -positive bacteria. At the highest test concentration (1 mg/disk) KrEA induced a zone of inhibition of 26.46 ± 0.85 mm and 23 ± 0.63 mm against the Gram-positive bacteria *E. faecalis* and *S. aureus* (Table 2.1). Further, KrEA extract showed considerable activity against Gram-negative *E. coli* and *K. pneumonia* with zone of inhibition ranging 19± 0.18 and 17.11 ± 0.22 mm respectively. The KrOH extract also showed significant activity against Gram-positive bacteria *E. faecalis*, *B. cereus* and *S. aureus*. Against *E. faecalis* the zone of inhibition was 33.1 ± 0.4 mm at the highest test concentration (Table 2.2). Alongside, noticeable activity of KrOH extract against Gram-negative *E. coli* and *K. pneumonia* was also observed. Among the three *K. rotunda* rhizome extracts, the KrWT extract was least active against the tested bacteria. Even at the highest test concentration, it showed moderate activity against the tested bacterial strains (Table 2.3). Among the Gram-positive bacterial strains tested, the *S. aureus* and *B. cereus* seems to be highly susceptible. In the case of Gram-negative bacterial strains, the *E. coli* was highly susceptible to these

rhizome extracts. Same time the *S. flexneri* and *S. marcescens* strains showed high resistance against the *K. rotunda* rhizome extracts. All the extracts failed to inhibit the growth of these Gram-negative bacteria.

The ANOVA followed by Bonferroni post-hoc test analysed the difference between vehicle control and treatment groups. KrOH extract showed significant ($p<0.05$) antibacterial potential against majority of the tested bacteria. However, KrEA extract at the lowest test concentration (0.125 mg/disk) did not show significant ($p>0.05$) antibacterial activity against the Gram-positive *E. faecalis* and Gram-negative bacterial strains *S. flexneri* and *S. marcescens* compared to the vehicle control. However at test concentrations above 0.125 mg/disk, except *S. flexneri* and *S. marcescens* all other tested bacterial strains were significantly inhibited. On the other hand, KrWT extract at the lowest test concentration (0.125 mg/disk), showed significant potential against the Gram-negative bacterium, *K. pneumonia*.

Antibacterial potential of *L. toxicaria* by disk diffusion assay

The disk diffusion assay showed majority of the tested bacterial strains are resistant to *L. toxicaria* extracts. Comparatively the LtEA showed a noticeable activity and at the highest test concentration, it induced a zone of inhibition of 20.81 ± 0.23 mm against *S. aureus* and was followed by *E. coli* (17.11 ± 0.19 mm) (Table 2.4). The LtEA extract also showed moderate inhibition to *K. pneumonia* (14.21 ± 0.34 mm) and *B. cereus* (11.46 ±0.93 mm). In the case of LtOH extract, noticeable activity was observed against *S. aureus* with zone of inhibition of 18.61 ± 0.81 mm at a concentration of 1 mg/disk (Table 2.5).

The differentiation among vehicle control and treatments were analysed using ANOVA followed by Bonferroni post-hoc test. The LtEA extract at

concentrations 0.5 mg/disk and 1 mg/disk showed significant difference ($P<0.001$) against *S. aureus*, *B. cereus*, *E. coli* and *K. pneumonia* compared to vehicle control. Similarly, LtOH extract at 1 mg/disk showed significant activity ($P<0.001$) against *S. aureus*, *E. coli* and *K. pneumonia*. On the other hand, the LtWT extract at the highest test concentration (1 mg/disk) showed considerable activity only against the Gram-positive bacterium, *S. aureus* (Table 2.6).

Discussion

Increasing numbers of testified cases of bacterial infections and growing frequency of drug-resistant microbes has drawn the attention of the scientific and pharmaceutical communities towards exploring the potential antibacterial activity of plant-derived bioactive extracts and compounds (Savoia 2012). The current study explored the antibacterial potential of rhizome extracts (ethyl acetate, ethanol and water) from *K. rotunda* and *L. toxicaria* against seven bacterial strains included both Gram-positive and Gram-negative strains.

For *K. rotunda*, the disk diffusion assay clearly resulted the dominance of KrEA and KrOH extracts against the Gram-positive bacteria tested. The *S. aureus* and *B. cereus* were more susceptible among these. Alongside, the KrEA and KrOH extracts at the highest test concentration (1 mg/disk) exhibited significant activity against *E. faecalis*. Eventhough the resistance developed by the Gram-negative bacteria tested in the present study was generally high, the KrEA and KrOH extracts showed conspicuous inhibition against the *E. coli* and *K. pneumonia*. These results were in agreement with previous reports that showed the potential of ethyl acetate extract of *K. rotunda* rhizomes against *S. aureus* and *E. coli* (Kumar et al. 2015; Malahayati et al. 2018). Among the three extracts of *L. toxicaria*, LtEA showed conspicuous inhibitory potential against *S. aureus* and *E. coli*, with the zone of

inhibition at 20.81 ± 0.23 mm and 17.11 ± 0.19 mm respectively at the highest test concentration (1 mg/disk). LtOH also showed considerable antibacterial activity at the highest test concentration against the *S. aureus* with 18.61 ± 0.81 mm inhibition zone. Our results are in confirmation with a previous study that reported significant antibacterial activity of n-hexane extract of *L. toxicaria* against the Gram-positive *S. aureus* (Anusha et al. 2019). Similarly, the methanol oil fractions of *Lagenandra ovate* was reported to be active against *S. aureus*, *E. coli* and *K. pneumoniae* (Selvakumari and de Britto 2007). The present study results also emphasize the incapability of the *L. toxicaria* water extract to prevent the tested bacterial strains. At the highest test concentration, the LtWT exhibited low inhibitory potential against the Gram-positive bacterium *S. aureus*. All other bacteria showed strong resistance against this extract. The significant antibacterial activity of ethyl acetate and ethanol extracts over water extract of the same plant part is attributed to the solubility of the bio active metabolites in different solvents. Ekpo and Etim (2009) also reported significantly higher antibacterial potential of ethanolic extract of *Sida acuta* than the aqueous extract against *S. aureus, Bacillus subtilis, Pseudomonas aeruginosa*.

The present study also emphasized the huge resistance of two Gram-negative bacterial strains *S. flexneri* and *S. marcescens* against the rhizome extracts of both plants. None of the rhizome extracts were active against these two Gram-negative bacteria. These results were in agreement with the work of Kabir et al. (2011), who reported the incapability of the lectins from *K. rotunda* to prevent the growth of two *Shigella* species. In general, Gram-positive bacteria tested in the present study were more susceptible than the Gram-negative strains used. Several other previous studies also showed the dominant antibacterial potential of plant extracts against Gram-

positive bacterial strains compared to Gram-negative strains. Previously Peixoto et al. (2011) reported strong antibacterial activity of aqueous and ethanolic extracts of *Moringa oleifera* leaf against Gram-positive strains *E. faecalis* and *S. aureus* compared to the Gram-negative strains (*Salmonella*, *E. coli*, *P. aeruginosa*, *A. caviae* and *V. parahaemolyticus*). Similarly, Kudi et al. (1999) reported strong antibacterial activity of crude extracts from traditional medicinal plants of Nigeria against Gram-positive bacteria compared to Gram-negative strains. Likewise, Ali et al. (2001) showed the prominent effects of traditional medicinal plant extracts against Gram-positive bacteria over Gram-negative strains. Thus, the relatively low performance of *K. rotunda* and *L. toxicaria* rhizome extracts against majority of the tested Gram-negative bacteria in the current study were not startling as it was already reported that the Gram-negative bacteria are more resistant than Gram-positive bacteria (Martin 1995; Paz et al. 1995; Vlietincket al. 1995).

The difference in the antibacterial potential of rhizome extracts is attributed to the presence of different bio active compounds. Strong inhibitory effects of plant phenolic metabolites against bacteria, fungi, protozoa and viruses were reported in many previous studies. The flavonoid molecules were reported to induce antimicrobial potential through several modes, that includes disruption of bio membrane, prohibiting nucleic acid and cell envelope synthesis, inhibition to biofilm formation, down regulating the bacterial efflux pumps, inhibition of important enzymes such as ATP synthase and NADH-cytochrome C reductase (Salehi et al. 2018; Gorniak et al. 2019). In the present study, presence of high amounts of potent phenolic and flavonoid compounds such as myricetin, quercetin and ellagic acid in the ethyl acetate and ethanol extracts of *K. rotunda* and *L. toxicaria* might have facilitated the antibacterial activities of rhizome extracts. Myricetin was reported to

inhibit several replication enzymes of *E. coli* including DNA and RNA polymerases (Ono et al. 1990), DnaB helicase (Griep et al. 2007) and DNA gyrase (Wu et al. 2013). Earlier Cetin-Karaca and Newman (2015) reported significant antimicrobial potential of myricetin against a series of bacteria including *Salmonella enteritidis*, *Salmonella cholerasuis*, *Salmonella paratyphi*, and *E. coli*. Furthermore many previous studies have reported significant antibacterial activity of myricetin against a series of Gram-positive and Gram-negative bacteria such as *Pseudomonas aeruginosa*, *S. aureus*, *E. coli*, *Proteus mirabilis*, *K. pneumoniae*, *Salmonella typhi*, *Vibrio cholera* (DeSouza and Wahidullah 2010; Gendaram et al. 2011; Nitulescu et al. 2017,Jayaraman et al. 2010). Similarly, another flavonoid quercetin also exhibited many bio activities including antibacterial, antiviral, anti-inflammatory, antioxidant, gastro protective, and immune-modulatory effects (Anand et al. 2016; Massi et al. 2017; Wu et al. 2008). Earlier Bozic et al. (2012) reported the activity of quercetin against *E. coli*, *Salmonellae enterica* and *Listeria monocytogenes* and Jaisinghani (2017) reported activity against *S. aureus* and *P. aeruginosa* (Jaisinghani 2017). Many previous reports are also available regarding the antibacterial potential of the polyphenol, ellagic acid. In a previous study, De et al. (2018) reported the inhibition of ellagic acid against 55 strains of *Helicobacter pylori*. Machado et al. (2003) reported the antibacterial potential of ellagi tannins isolated from pomegranate against both MRSA and MSSA strains (Machado et al. 2003). In addition, Panichayupakaranant et al. (2010) determined strong *in vitro* inhibitory potential of the standardised pomegranate rind extract containing high amounts of ellagic acid against *Propionibacterium acnes*, *S. aureus* and *Staphylococcus epidermidis*. Furthermore, presence of antibacterial compounds such as octadecanoic acid, n-hexadecanoic and myristic acid were reported in the GC-MS analysis. These

compounds might have facilitated the antibacterial potential of the rhizome extracts. The results from the current study highlights the potential of KrEA and KrOH extracts of *K. rotunda* and LtEA extract of *L. toxicaria* against different bacterial strains. However, further studies are necessary before determining its effects in *in vivo* conditions before promoting to veterinary or medicinal use.

Table 2.1: Antibacterial activity of different concentrations of KrEA extract							
Concentration	*S. aureus*	*E. coli*	*K. pneumonia*	*S. flexneri*	*S. marcescens*	*B. cereus*	*E. faecalis*
0.125 mg	6.33 ± 1.14	8.24 ± 0.62	6.28 ± 0.24	0	0	7.26 ± 0.97	0
0.25 mg	14.21 ± 1.14	9.25 ± 0.71	13.74 ± 0.47	0	0	12.46 ± 0.84	6.33 ± 0.76
0.5 mg	19.24 ± 0.98	15.80 ± 0.74	14.11 ± 0.89	0	0	16.15 ± 0.52	13.52 ± 1.13
1 mg	23.18 ± 0.63	19.21 ± 0.18	17.11 ± 0.22	0	0	20.26 ± 0.28	26.46 ± 0.85
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin 10µg	23.6 ± 0.84	25.8 ± 0.88	22.26 ± 0.37	15.18 ± 0.98	15.84 ± 0.41	25.94 ± 0.16	28.11 ± 0.65
Values are expressed as mean ± SEM of the zone dimension in mm.							

Table 2.2: Antibacterial activity of different concentrations of KrOH extract							
Concentration	*S. aureus*	*E. coli*	*K. pneumonia*	*S. flexneri*	*S. marcescens*	*B. cereus*	*E. faecalis*
0.125 mg	10.28 ± 0.87	6.11 ± 0.69	6.34 ± 0.55	0	0	10.44 ± 0.28	11.4 ± 0.87
0.25 mg	14.87 ± 0.57	10.27 ± 0.23	9.46 ± 0.54	0	0	14.24 ± 0.88	21.3 ± 0.65
0.5 mg	18.87 ± 0.13	18.36 ± 0.48	15 ± 0.71	0	0	16.18 ± 0.21	24.38 ± 0.98
1 mg	20.8 ± 0.57	19.3 ± 0.47	18.6 ± 0.14	0	0	21.24 ± 0.18	33.1 ± 0.4
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin 10µg	22.42 ± 0.14	25.3 ± 0.61	22.1 ± 0.17	14.24 ± 0.55	14.08 ± 0.86	27.94 ± 0.73	28.51 ± 1.14
Values are expressed as mean ± SEM of the zone dimension in mm.							

Table 2.3: Antibacterial activity of different concentrations of KrWT extract							
Concentration	*S. aureus*	*E. coli*	*K. pneumonia*	*S. flexneri*	*S. marcescens*	*B. cereus*	*E. faecalis*
0.125 mg	6.16 ± 0.81	0	0	0	0	0	0
0.25 mg	6.21 ± 0.81	8.34 ± 0.44	6.11 ± 0.16	0	0	7 ± 0.22	0.00 ± 0.00
0.5 mg	11.41 ± 0.37	10 ± 0.80	7 ± 0.26	0	0	8.16 ± 0.11	0.00 ± 0.00
1 mg	13.46 ± 1.40	13.22 ± 0.50	11.74 ± 0.67	0	0	14.28 ± 0.86	12.31 ± 1.29
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin 10µg	21.8 ± 0.44	24.31 ± 0.18	23.24± 0.17	15.22 ± 0.93	16.13 ± 0.96	23.94 ± 0.16	28.11 ± 0.65
Values are expressed as mean ± SEM of the zone dimension in mm							

Table 2.4: Antibacterial activity of different concentrations of LtEA extract							
Concentration	*S. aureus*	*E. coli*	*K. pneumonia*	*S. flexneri*	*S. marcescens*	*B. cereus*	*E. faecalis*
0.125 mg	0	0	0	0	0	0	0
0.25 mg	0	0	0	0	0	0	0
0.5 mg	13.12 ± 0.45	11.56 ± 0.84	9 ± 0.66	0	0	0	0
1 mg	20.81 ± 0.23	17.11 ± 0.19	14.21 ± 0.34	0	0	11.46 ±0.93	0
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin 10µg	25.2 ± 0.53	24.31 ± 0.62	23.46 ± 0.71	14.21 ± 0.88	13..64 ± 0.46	25.4 ± 0.22	27.16 ± 0.75
Values are expressed as mean ± SEM of the zone dimension in mm.							

Table 2.5: Antibacterial activity of different concentrations of LtOH extract

Concentration	*S. aureus*	*E. coli*	*K. pneumonia*	*S. flexneri*	*S. marcescens*	*B. cereus*	*E. faecalis*
0.125 mg	0	0	0	0	0	0	0
0.25 mg	0	0	0	0	0	0	0
0.5 mg	0	0	0	0	0	0	0
1 mg	18.61 ± 0.81	14.33 ± 0.63	12.54 ± 0.28	0	0	0	0
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin 10µg	25.2 ± 0.53	24.31 ± 0.62	23.46 ± 0.71	14.21 ± 0.88	13..64 ± 0.46	25.4 ± 0.22	27.16 ± 0.75
Values are expressed as mean ± SEM of the zone dimension in mm.							

Table 2.6: Antibacterial activity of different concentrations of LtWT extract

Concentration	*S. aureus*	*E. coli*	*K. pneumonia*	*S. flexneri*	*S. marcescens*	*B. cereus*	*E. faecalis*
0.125 mg	0	0	0	0	0	0	0
0.25 mg	0	0	0	0	0	0	0
0.5 mg	0	0	0	0	0	0	0
1 mg	7.28 ± 0.19	0	0	0	0	0	0
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin 10µg	25.2 ± 0.53	24.31 ± 0.62	23.46 ± 0.71	14.21 ± 0.88	13..64 ± 0.46	25.4 ± 0.22	27.16 ± 0.75
Values are expressed as mean ± SEM of the zone dimension in mm.							

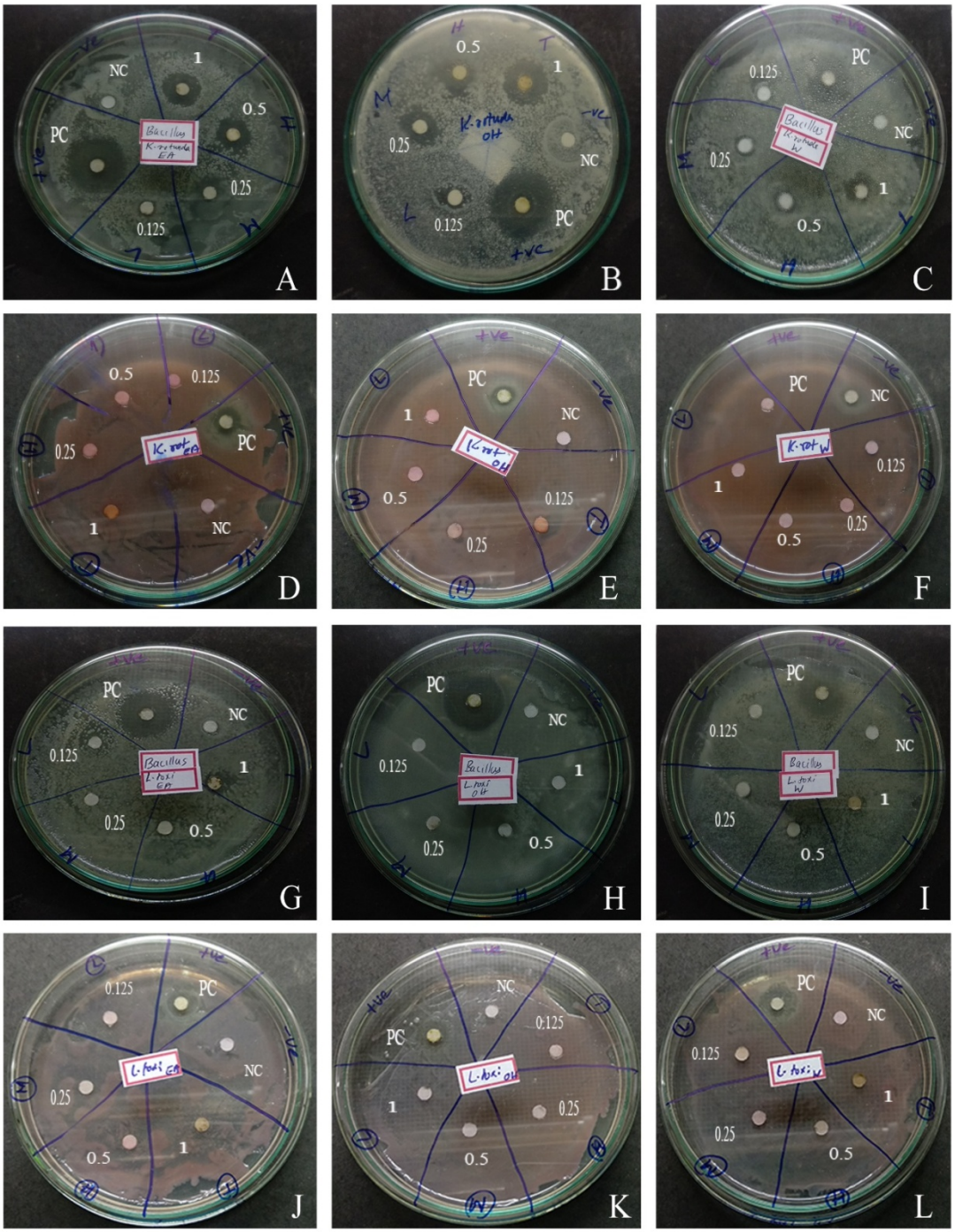

Figure 2.1: Inhibition zones developed against *B. subtilis* (A, B, C) and *S. marcescens* (D, E, F) treated using discs containing *K. rotunda* extracts. *B. subtilis* (G, H, I) and *S. marcescens* (J, K, L) treated using disc containing *L. toxicaria* extracts. PC: Positive control; NC: Negative control. Column 1 – Ethyl acetate extracts, 2 – Ethanol extracts and 3- Water extracts.

CHAPTER 3

In vitro anthelmintic activity of *Kaempferia rotunda* and *Lagenandra toxicaria* against helminth parasites of plants

Introduction

Meloidogyne incognita (Kofoid and White) Chitwood (Tylenchida: Heteroderidae), commonly known as root-knot nematode, is a major plant-parasitic nematode species affecting many annual and perennial crop production. The quality and quantity of *M. incognita* affected crop gets reduced drastically (Wiratno et al. 2009). They have a wide range of hosts and cause serious problems for many crops, causing yield losses of up to 30% under field conditions (Jain et al. 1994). Infected plants show symptoms such as root galling, stunting and nutrient deficiency (Siddiqui et al. 2001). This endo-parasitic nematode penetrates the growing tips of roots and forages the vascular tissues. *M. incognita* infested roots undergo morphological changes including galled or swollen appearance associates with frequent cracking and splitting (Seenivasan & Senthilnathan 2018). Since the nematode infection uplifts the destruction of root tissues, it ultimately reduces the water and mineral uptake, leading to reduced plant growth and yield (Davide & Marasigan 1985; Jonathan & Rajendran 1998).

Another devastating plant parasitic nematode is the burrowing nematode, *Radopholus similis*. Since it parasitizes on about 250 species of plants, it is sorted among the top ten important plant parasitic helminths in the tropics (Haegeman et al. 2010). The well-known 'toppling disease' to banana is caused by this burrowing nematode (Gowen et al. 2005). They induce severe destruction to the root and corm tissues leading to reduced water and nutrient uptake (Stanton 1994; Sarah 2000). Additionally, the *R. similis* also affects black pepper cultivation causing slow decline to the crop (Sreeja et al. 2016).

Various controlling methods have been adopted to prevent/reduce *M. incognita* and *R. similis* infections. Chief and foremost among them is the usage of

synthetic chemicals as nematicidal agent (Seenivasan 2017). Many synthetic pesticides have been widely used for controlling general pests and parasites since 1950s (Taniwiryono et al. 2007). Although these synthetic chemicals have been very successful in down regulating these agents, they have also been found to have certain environmental downsides (Sill 1982). Many of the plant parasitic nematodes are controlled through synthetic chemical nematicides. This raises concern because the occurrence of residues of these poisonous chemicals in the vegetables that are cultivated for fresh consumption could lead to negative effects in the human health (Elbadri et al. 2008). Alongside high costs and unpredictable outputs of synthetic nematicides have augmented the importance of alternative, safe methods for managing plant parasitic nematodes (Viaene et al. 1998).

Numerous plant species are reported to be resistant to parasitic nematodes, plant pathogens, and insect pests (Elbadri et al. 2008). Among these some important reports include marigolds (Tagets spp.), rattlebox (*Crotalaria spectabilis*), chrysanthemums (*Chrysanthemum* spp.), garlic (*Allium sativum*), cinnamon (*Cinnamomumverum*), and neem (*Azardiractaindica*) (Duke 1990; Lee et al. 2001; Satti et al. 2003; Park et al. 2005; Satti et al. 2006; Kong et al. 2007). In this scenario that the herbal products provide a practical solution to the non-targeted effects and environmental issues caused by synthetic pesticides (Kim et al. 2005). Many plants effectively resist the parasite infection through the production of secondary metabolites. These plant derived chemicals are generally non-persistent under field conditions as they are readily transformed by light, oxygen and microorganisms into non-toxic/fewer toxic products (Ujvary 2001). The random isolation, identification, and studying the nematicidal capacity of such compounds may leads to the discovery of novel pesticides with relatively less non-targeted

effects. The objective of the present study was to test the nematicidal potentials of ethyl acetate, ethanol and water extracts of *L. toxicaria* and *K. rotunda* against the root-knot nematode, *M. incognita* and burrowing nematode, *R. similis.*

Materials and methods

Collection and extraction of plant material

The Methods section 1 and 2 provide detailed information on the collection and extraction of plant materials. Three solvents such as ethyl acetate, ethanol and water were used for the extraction of phytochemicals from the rhizomes of both plants. The stock was made by dissolving 100 mg of this crude extract in 1000 µl DMSO (1%). From the stock solution four different test concentrations (8, 4, 2, 1 and 0.5 mg/ml) were prepared by serial dilution.

Preparation of nematode inoculum

M. incognita

The extraction of nematode eggs was executed according to the method proposed by Hussey & Barker (1973) with slight modifications. Figure 3.1 showed the preparation of nematode inoculum of *M. incognita*. The second-stage juvenile *M. incognita* (J2) with a maximum age of 48 h post hatching were taken for the mortality test. Detailed methodology is provided in Methods section 5.1.1.

R. similis

The *R. similis* was extracted according to the maceration and filtration technique proposed Southey (1986) with minor modifications. The nematodes were extracted from the roots of black pepper (*Piper nigrum*) which were previously infested. Figure 3.1 showed the preparation of nematode inoculum of *R. similis*. The second stage juveniles of *R. similis* for the mortality test were collected from the

culture maintained on carrot disks by washing with sterile water. Detailed methodology is provided in Methods section 5.1.2.

Mortality test of nematode larvae

The test was conducted in 6 well microtiter plates. In the assay, 100 juvenile nematodes in 0.5 ml water were placed in each well. Serial concentration of each plant extract in total volume of 0.5 ml in 1 % DMSO was added to make concentrations of 8, 4, 2, 1 and 0.5 mg/ml together with water containing the worms. Detailed methodology is available in Methods section 5.2.

Statistical analysis

The data collected were expressed as mean ± SEM. The survival data was analysed using Kaplan-Meier survival analysis in Graph Pad Prism software version 5. The Log-rank (Mantel-Cox test) compared the survival curves. The extract concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) mortality to the juvenile nematodes was calculated using probit analysis in SPSS version 24.0.

Results

The *in vitro* model reported in the present study verified nematicidal effects of ethyl acetate, ethanol and water extracts of *K. rotunda* and *L. toxicaria* rhizomes against the juveniles of plant parasitic nematodes *M. incognita* and *R. similis*.

Bioactivity against *M. incognita*

The Kaplan-Meier curves shows a significant decrease in the survival rate of *M. incognita* treated in majority of the concentrations of KrEA, KrOH and KrWT extracts when compared with vehicle control (Figure 3.2). Only KrWT at the lowest tested concentration (0.5 mg/ml) did not induce significant mortality ($p=0.333$) compared to the vehicle control. Among the *K. rotunda* rhizome extracts, KrEA extract exhibited pronounced mortality against the root-knot nematode *M. incognita*.

A maximum mean mortality of 71.22 ± 4.1 percent was obtained after 24 h of incubation in 8 mg/ml of KrEA extract. Same time KrOH extract at 8 mg/ml concentration shows a mean percent mortality of 62.11 ± 1.16. Among the three extracts tested, the water extract (KrWT) was least effective against the *M. incognita* (Figure 3.2). Incubation of *M. incognita* in 8 mg/ml concentration of KrWT extract for 24 h induced a mean percent mortality of 18.5 ± 2.53 only. Alongside a concentration and time dependent significant increase in mortality was evident with almost all treatments up to 48 h. The mortality rates induced by KrEA at concentrations 0.5, 1, 2, 4 and 8 mg/ml following 24 h of exposure were 11.66 ± 1.63, 25.44 ± 2.1, 43.22 ± 1.92, 54 ± 3.83, and 71.22 ± 4.13 percent respectively. These were significantly increased to 36.44± 1.86, 64.77 ± 1.58, 80.55 ± 1.16, 89.44 ± 0.66, and 96 ± 0.5 percent respectively during 48 h of exposure time. At the above-mentioned concentrations, the mortality caused by KrOH extract following 24 h of exposure were 11 ± 2.19, 19.55 ± 2.23, 28.33 ± 2.33, 48.78 ± 2.16, and 62.11 ± 1.16 percent respectively. Here also after 48 h the percent mortality rates significantly increased to 18.22 ± 0.89, 39.44 ± 0.81, 51.66 ± 0.66, 78.33 ± 1.66, and 89.88 ± 0.38 percent respectively. Interestingly the mortality rates did not show significant increase during the 48 h – 72 h interval in any of the tested extracts.

The 24 h EC_{50} and EC_{90} values were calculated to determine the toxicity of *K. rotunda* extracts against the root-knot nematode, *M. incognita*. The data obtained by calculating the EC_{50} and EC_{90} values post 24 h also revealed the high toxicity of KrEA and KrOH extracts against the root-knot nematode. The KrEA extract showed the lowest EC_{50} value (3.10 mg/ml) during the initial 24 h (Table 3.2). This was followed by KrOH extract that showed 24 h EC_{50} of 4.64 mg/ml. Same time the 24 h EC_{90} values of both the KrEA and KrOH extracts were greater than the maximum

concentration tested (> 8 mg/ml). KrWT was less toxic to the nematode as the EC_{50} values were beyond the maximum concentration tested (> 8 mg/ml).

In the case of *L. toxicaria* extracts, apparent increase in the mortality of nematodes was observed with the progress of time. The mortality rates of juvenile *M. incognita* (J2) increased in accordance with the increase in time and concentration of *L. toxicaria* extracts up to 48 h (Figure 3.3). Among the three extracts tested, LtEA exhibited pronounced mortality to the juveniles of *M. incognita*. The mortality rates of LtEA at concentrations 0.5, 1, 2, 4 and 8 mg/ml following 24 h of exposure were 9.56 ± 1.3, 19.44 ± 0.67, 26.33 ± 2.88, 36.67 ± 1.65, and 48.44 ± 1.88 percent respectively. This were significantly increased to 27.33 ± 1.33, 51.11 ± 1.29, 59.67 ± 1.68, 89.56 ± 1.99, and 92.22 ± 0.95 percent respectively after 48 h of exposure. Same time the water extract of *L. toxicaria* (LtWT) showed least mortality with an average percent mortality of 15.75 ± 2 and 21.11 ± 1.99 at highest tested concentration (8 mg/ml) following 24 h and 48 h respectively. Also, the LtWT at lower test concentrations (0.5 mg/ml and 1 mg/ml) did not show significant mortality ($p>0.05$) to the juvenile parasites compared to the vehicle control group. The mortality of ethanol extract (LtOH) was found to be intermediate with highest mortality of 80.78 ± 1.39 percent at 8 mg/ml after 48 h of exposure (Figure 3.3).

The initial 24 h EC_{50} values of all the three *L. toxicaria* rhizome extracts against the plant parasitic nematode *M. incognita* exceeded the maximum tested concentration limit (> 8 mg/ml). Therefor the 48 h EC_{50} and EC_{90} values were calculated using probit analysis to determine the toxicity of *L. toxicaria* extracts. Here also the data obtained by calculating the EC_{50} and EC_{90} values post 48 h also revealed the high toxicity of LtEA extract followed by LtOH extract. The 48 h EC_{50}

and EC_{90} values of LtEA were 1.076 mg/ml and 4.98 mg/ml respectively. The LtWT was less toxic to the nematode as the EC_{50} and EC_{90} values were not reached (> 8 mg/ml) even after 48 h of treatment (Table 3.2).

A broad-spectrum synthetic pesticide, carbosulfan in 0.1% and 1 % DMSO were used as positive and negative controls respectively. In the case of carbosulfan treated groups, approximately 100% mortality was observed following 24 h time period. Same time no mortality was observed to the worms treated with 1% DMSO even after 72 h time period.

Bioactivity against *R. similis*

The nematicidal activity of ethyl acetate, ethanol and water extracts of *K. rotunda* rhizome extracts against the second stage juveniles of *R. similis* are shown in figure 3.4. The results showed that the KrEA and KrOH extracts were effective against the second stage juveniles of *R. similis*. On the other hand KrWT extract showed least nematicidal potential. Also, at lower test concentrations (0.5 and 1 mg/ml) of KrWT extract the mortality rate compared to vehicle control was insignificant ($p>0.05$). In the case of *K. rotunda* extracts, KrEA and KrOH extracts showed almost similar nematicidal effects against the burrowing plant parasitic nematodes. A maximum mortality of 57.56 ± 5 and 53.11 ± 1.20 percent was obtained after 24 h of incubation in 8 mg/ml of KrEA and KrOH extracts respectively. Same time the KrWT extract exhibited the lowest nematicidal potential with a mean percent mortality of 15.56 ± 0.68 at 8 mg/ml after 24 h of incubation. A concentration and time dependent increase in mortality was observed with KrEA and KrOH extracts.

The 24 h EC_{50} and EC_{90} values were calculated to determine the toxicity of *K. rotunda* extracts against the burrowing nematode, *R. similis*. The data obtained by

calculating the EC_{50} values after 24 h showed the toxicity of KrEA and KrOH extracts against the *R. similis*. The KrEA extract showed the lowest EC_{50} value (5.352 mg/ml) during the initial 24 h (Table 3.3). This was followed by KrOH extract with a 24 h EC_{50} of 6.823 mg/ml. On the other hand, the 24 h EC_{50} value of KrWT extract was found to be > 8 mg/ml. In the case of 24 h EC_{90} values, all the three extracts exceeded the maximum test concentration (> 8 mg/ml) limit.

In the case of *L. toxicaria*, among the three extracts tested, the LtEA showed prominent activity against *R. similis* after 24 h and 48 h treatment. This was followed by the LtOH extract. Same time the LtWT extract showed the lowest nematicidal potential (Figure 3.5). The Log-rank (Mantel-Cox test) compared the survival curves of extracts with the vehicle control. All test concentrations of LtEA showed significant activity ($p<0.05$) when compared to vehicle control group. Same time majority of the test concentrations of LtWT extract did not elicit significant mortality ($p>0.05$) to the parasites. Furthermore LtOH at higher test concentrations (8, 4 and 2 mg/ml) showed significance compared to the vehicle control. The maximum mortality obtained at the highest test concentration (8 mg/ml) was 61.12 ± 1 percent for LtEA extract and the lowest was 12.78 ± 0.77 percent for LtWT extract. The LtEA extract showed a significant time and concentration dependent increase in mortality from 24 h to 48 h time interval. Same time after 48 h none of the extracts showed significant increase in mortality.

Here also, the initial 24 h EC_{50} values of all the three extracts of *L. toxicaria* exceeded the maximum test concentration used in the current study (> 8 mg/ml). The 48 h EC_{50} and EC_{90} values were calculated using probit analysis to determine the toxicity of *L. toxicaria* rhizome extracts against *R. similis*. The 48 h EC_{50} showed strong toxicity of LtEA and LtOH extracts (Table 3.4), whereas the 48 h

EC_{50} value of LtWT extract was greater than the highest concentration of the extract used in the present study. Similarly, the EC_{90} values of all extracts showed higher values than 8 mg/ml.

Discussion

In the present study, among the six extracts tested, the ethyl acetate and ethanol extracts showed better activity against the juveniles of root-knot nematode *M. incognita* and burrowing nematode *R. similis*. Same time, water extracts of both plants failed to induce noticeable mortality when compared with the other extracts and 0.1% carbosulfan (positive control). The least bioactivity of water extracts of both plants in the present study is also in line with the previous report of Ben-Daniel et al. (2001) in which the organic solvent extracts of plant materials were found to be more toxic to the J2s of *Meloidogyne* sps than water extracts. Superior efficacy of n-hexane extract of *Fumaria parviflora* against *M. incognita* compared to chloroform, ethyl acetate and methanol extracts of the same plant were reported by Naz et al. (2013). Similarly, Abid et al. (1997) reported the huge mortality induced by the crude ethanolic extracts of *F. indica* against the J2s of *Meloidogyne javanica*. These results completely correlate with the present study result that different solvent extracts of the same plant species vary in their anthelmintic potentials. The probable reason for the observed differences between water, ethanol and ethyl acetate extracts could be due to discrepancy in the solubility of active metabolites in these solvents (Eloff 1998).

Probit analysis was used to find out the extract concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) mortality to the juvenile *M. incognita*. In the case of *K. rotunda* rhizome extracts, the KrEA extract showed lowest24h EC_{50} value compared to KrOH and KrWT extracts. Similarly, the LtEA extract of *L. toxicaria*

showed lowest 48h EC_{50} value than LtOH and LtWT extracts. Many previous studies calculated the EC_{50} values to confirm the effects of plant extracts against the root-knot nematode, *M. incognita*. Zaidat et al. (2020) in their study calculated 32h EC_{50} values of plant extracts against *M. incognita*. Another study calculated the EC_{50} to confirm the lethal effect of humic acid on *M. incognita* J2 (Seenivasan & Senthilnathan 2018). Similarly, Zasada et al. (2006) calculated the EC_{50} of the velvet bean extracts to study the survival rate of *M. incognita* J2.

The results were similar against the burrowing nematode *R. similis*. Here also the ethyl acetate and ethanol extracts of both plants exhibited reasonable mortality. Same time the water extracts of both plants showed limited potential against *R. similis*. Especially, the LtWT extract almost failed to develop inhibition at the lowest test concentrations. The results of probit analysis substantiate the results obtained in the mortality studies. The LtEA showed the lowest EC_{50} value and higher activity against *R. similis* compared to other extracts. On the other hand the EC_{50} value of LtWT extract exceeded the maximum concentration used in the current study. This highlights the inefficiency of LtWT extract against the burrowing nematodes. Very few previous studies reported the nematicidal effects of plant extracts or environmentally safe products against *R. similis*. Previously, Bartholomew et al. (2014) reported the application of phytochemicals from the plants *Azadirachta indica* and *Allium sativum* extracts against *R. similis* was effective and comparable to the synthetic nematicide, ethoprophos. Another study reported the nematicidal activity of metabolites from endophytic fungi isolated from black pepper against *R. similis* (Sreeja et al. 2015). Similarly, Mendoza et al. (2008) reported the potential of antagonistic bacterial strains that reduced survival of *R. similis* by 41% over the control groups

The mechanism of the anthelmintic action of crude extracts of *K. rotunda* and *L. toxicaria* is not yet clear, still it is assumed that the compounds in the crude extracts may be acting singly or in synergy for the observed anthelmintic action. The synergistic deed of diverse metabolites in each rhizome extract may vary, which could elucidate the differential anthelmintic potential of the six rhizome extracts of two plants. Polyphenols were among the major secondary metabolites extracted in our study. The ethyl acetate and ethanol extracts of *L. toxicaria* showed high amount of phenolics compared to water extract. It was earlier reported that the phenolic metabolites like tannins in plant extracts have anthelmintic activity on their own ascribed to physical astringent action on different nematodes (Athanasiadou et al. 2001). Previously Bizimenyera et al. (2006) reported that individual compounds isolated from the extracts exhibited less activity than the crude extracts. The current study highlighted the possibility of using the ethyl acetate and ethanol rhizome extracts of *K. rotunda* and *L. toxicaria* as an environment safe remedy against the plant parasitic nematodes *M. incognita* and *R. similis*. Nevertheless more studies are required to elucidate the 1) mechanisms of action of these crude extracts on the tested nematodes, 2) chemical nature of the active compounds responsible for the observed activity before considering the extracts for further applications.

Table 3.1: EC_{50} and EC_{90} values for *M. incognita* after 24 h of exposure to *K.rotunda* extracts using probit analysis		
Extracts	EC_{50}(mg/ml) (LCL - UCL)	EC_{90}(mg/ml) (LCL - UCL)
KrEA	3.10 (2.550-3.894)	> 8
KrOH	4.64 (3.677-6.303)	> 8
KrWT	> 8	> 8
LCL: Lower confidence limit; UCL: Upper confidence limit		

Table 3.2: EC_{50} and EC_{90} values for *M. incognita* after 48 h of exposure to *L. toxicaria* extracts using probit analysis		
Extracts	EC_{50}(mg/ml) (LCL - UCL)	EC_{90}(mg/ml) (LCL - UCL)
LtEA	1.092 (0.626-1.601)	5.892 (3.535-17.644)
LtOH	2.140 (1.775-2.592)	> 8
LtWT	> 8	> 8
LCL: Lower confidence limit; UCL: Upper confidence limit		

Table 3.3: EC_{50} and EC_{90} values for *R. similis* after 24 h of exposure to *K. rotunda* extracts using probit analysis		
Extracts	EC_{50}(mg/ml) (LCL - UCL)	EC_{90}(mg/ml) (LCL - UCL)
KrEA	5.352 (4.217-7.377)	> 8
KrOH	6.823 (5.372-9.475)	> 8
KrWT	> 8	> 8
LCL: Lower confidence limit; UCL: Upper confidence limit		

Table 3.4: EC_{50} and EC_{90} values for *R. similis* after 48 h of exposure to *L. toxicaria* extracts using probit analysis		
Extracts	EC_{50}(mg/ml) (LCL - UCL)	EC_{90}(mg/ml) (LCL - UCL)
LtEA	3.960 (3.029-5.667)	> 8
LtOH	6.367 (4.896-9.207)	> 8
LtWT	> 8	> 8
LCL: Lower confidence limit; UCL: Upper confidence limit		

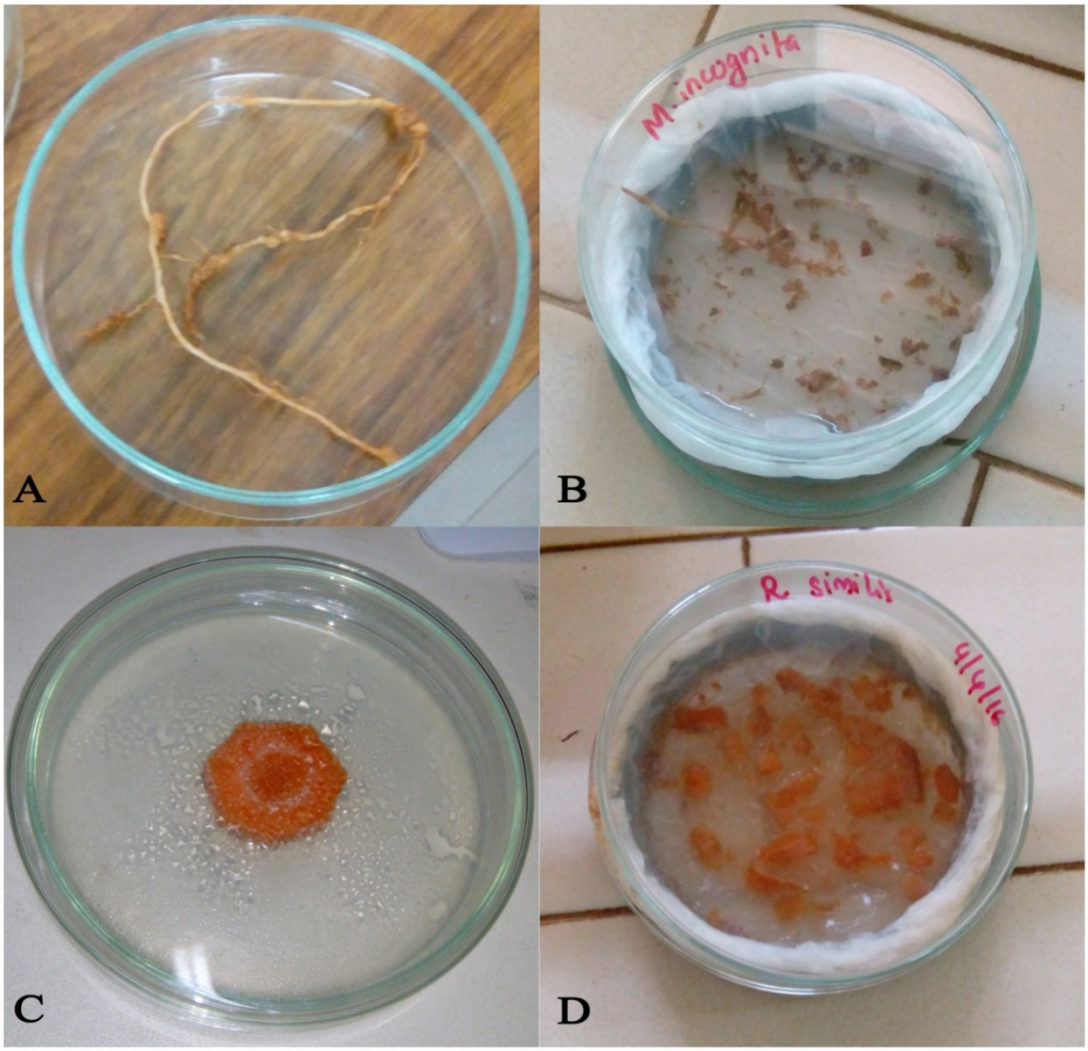

Figure 3.1: Nematode inoculum preparation for *M. incognita* (A & B) and *R. similis* (C & D)

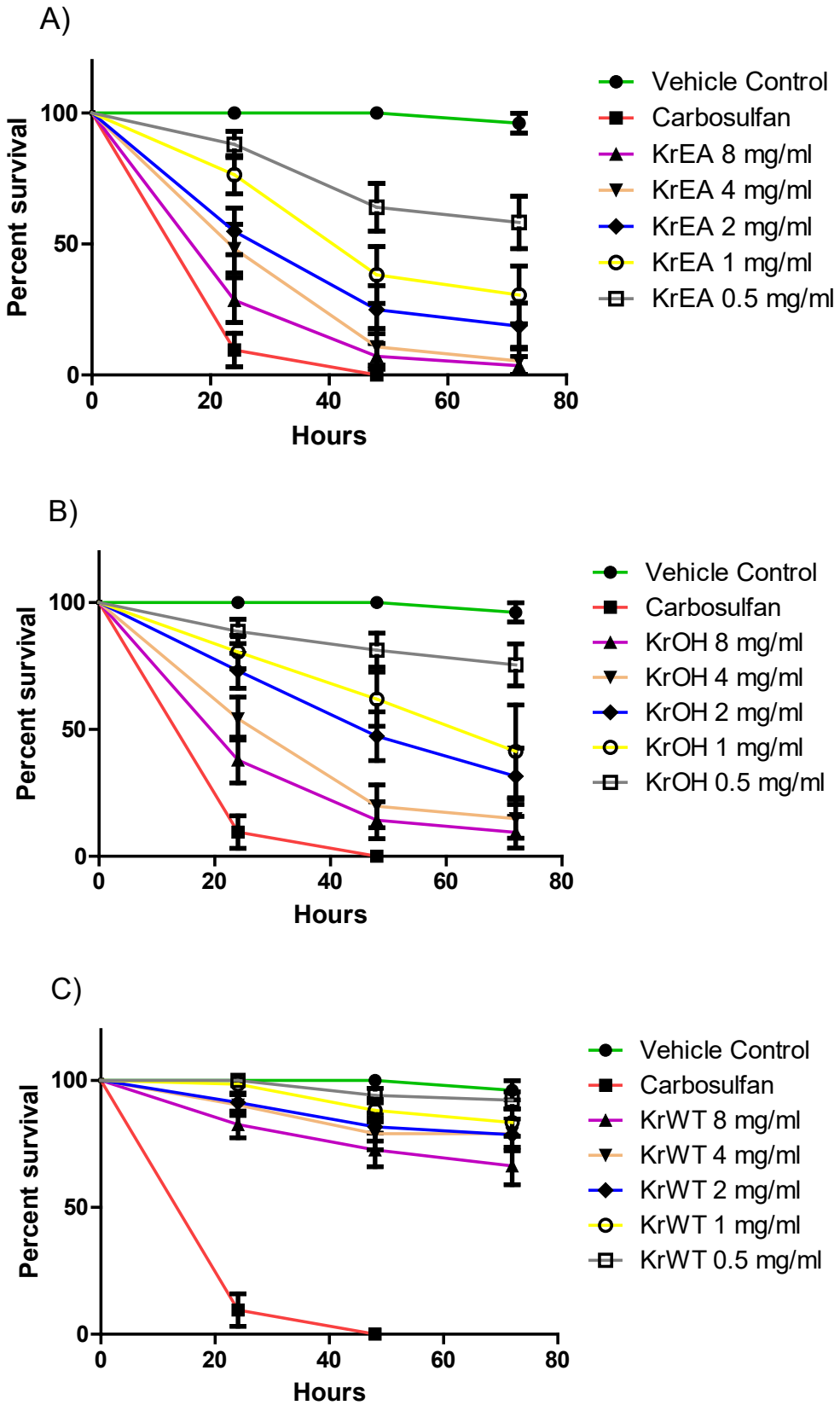

Figure 3.2: Kaplan-Meier survival curves of *M. incognita* treated in (A) KrEA, (B) KrOH and (C) KrWT extracts at various doses and time

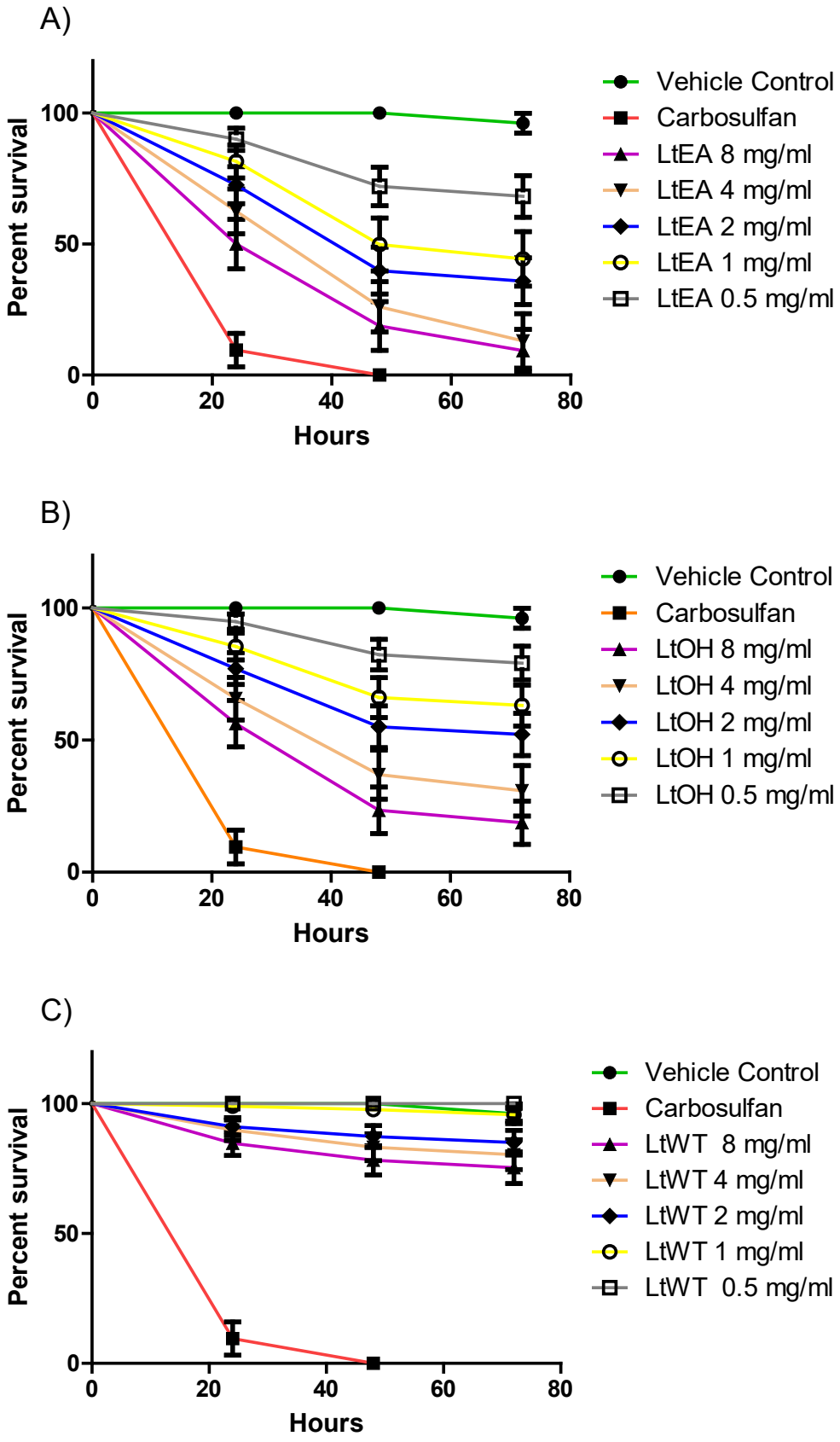

Figure 3.3: Kaplan-Meier survival curves of *M. incognita* treated in (A) LtEA, (B) LtOH and (C) LtWT extracts at various doses and time

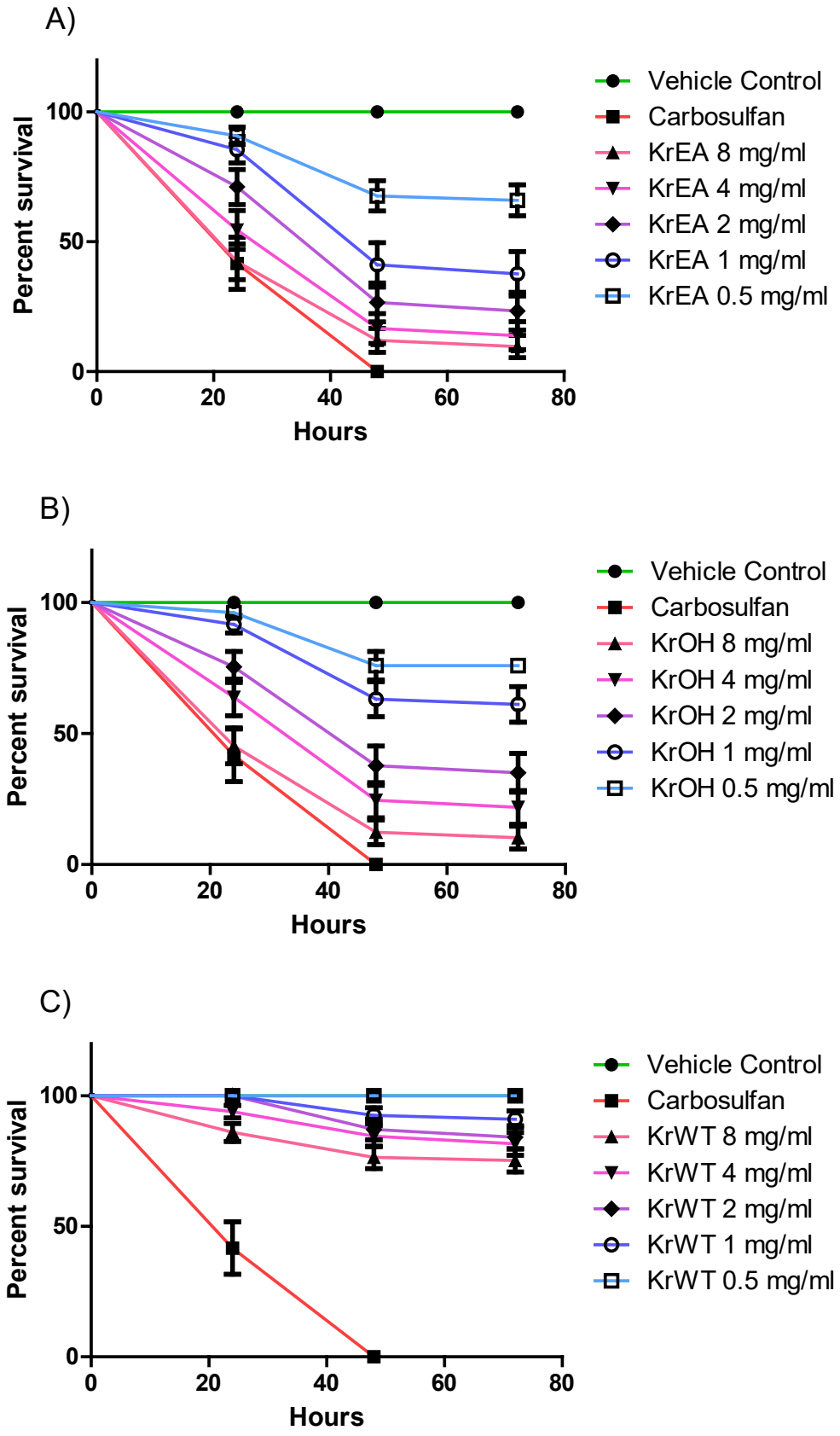

Figure 3.4: Kaplan-Meier survival curves of *R, similis* treated in (A) KrEA, (B) KrOH and (C) KrWT extracts at various doses and time

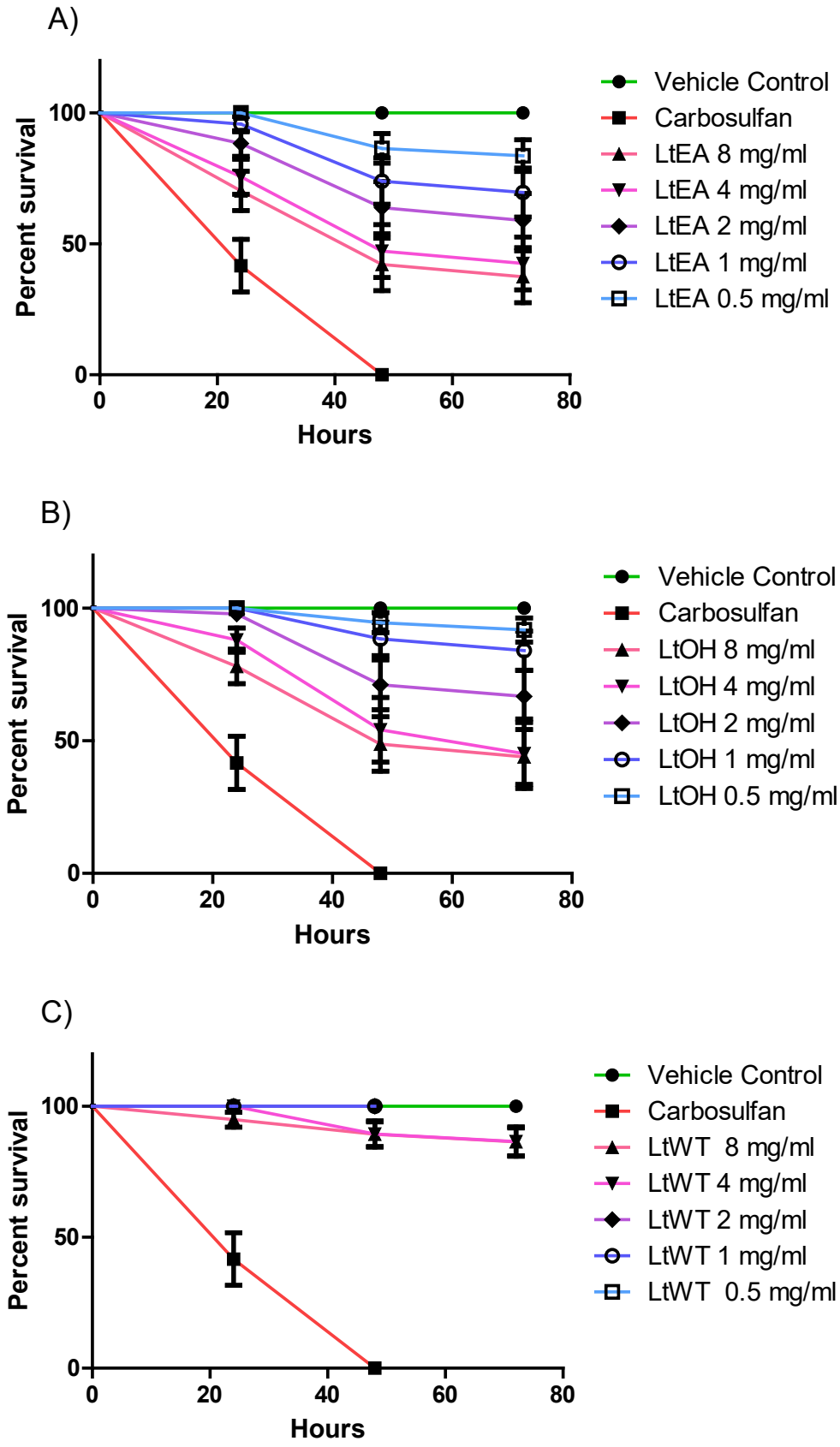

Figure 3.5: Kaplan-Meier survival curves of *R. similis* treated in (A) LtEA, (B) LtOH and (C) LtWT extracts at various doses and time

CHAPTER 4

In vitro anthelmintic activity of *Kaempferia rotunda and Lagenandra toxicaria* against helminth parasites of animals

Introduction

Helminth infection remains one of the serious issues affecting the livestock production throughout the world. The fatalities and production loss associated with gastrointestinal helminth infections to ruminants remained unaddressed in tropical developing countries like India (Dhar et al. 1982; Getachew et al. 2012). This age-old problem seems to be unaltered even after the introduction of synthetic anthelminthic drugs (Van Wyk et al. 1997). The nematode parasite, *Haemonchus contortus* is one among the important helminth parasites that exhibits high prevalence and pathogenicity to animals, especially ruminants (Hounzangbe-Adote et al. 2005; Davuluiri et al. 2019). Compared to other parasitic nematodes, *H. contortus* is proficient of causing acute disease and huge fatality to almost all ruminants (Kamaraj et al. 2010). Haemonchosis, a serious disease caused by *H. contortus* to ruminant animals induces anaemia, anorexia, reduced growth, and ultimately leads to the death of the hosts (Waller & Thamsborg 2004; Guo et al. 2016).

Fischoederius cobboldi is a digenetic trematode parasite of the family Gastrothylacidae. They are considered as one of the important agents causing paramphistomosis in livestock, mainly cattle and sheep throughout the world. Ruminants like cattle, goat, sheep and water buffaloes are infected by rumen flukes. The life cycle involves a definitive host (DH) and an intermediate host (IH). Mammals are the definitive host, and the infection occurs when DH ingest metacercaria passively. The flukes remain in the small intestine for a while and move to the rumen when it reaches the adult stage (Sanguankiat et al. 2016). The disease adversely affects nutrition conversion and milk production. This also

induces weight loss that ultimately leads to decreased productivity, resulting in huge economic losses in many countries (Choubisa & Jaroli 2013).

Helminth infections are generally controlled using the recurrent and premeditated use of synthetic anthelmintics (Davuluiri et al. 2019). However, the development of highly resistive parasitic populations has down the pan of many of the known anthelmintic compounds. It was reported previously that varying degree of resistance among parasitic nematodes has been observed against all categories of anthelmintics available in the market (Stuchlikova et al. 2018). The resistance towards a recent anthelmintic drug, monepantel, which has befallen within four years of the drug first being introduced, is heavily alarming (Raza et al. 2016 a). The extensive rise in the administration of these synthetic molecules is attributed to the increased drug pressure toward the selection of resistance alleles in parasites (Geary 2012). Alongside, the development of adverse effects like allergic reactions, hypersensitive and immunosuppressant actions on the non-targeted organisms (Nawaz et al. 2014) highlights the risk of using these chemicals. The biotransformation of anthelmintic molecules like albendazole has been reported to increase the production of reactive oxygen species and reactive nitrogen species and thereby inducing oxidative stress followed by severe damage to the host cells (Dimitrijev et al. 2012; Sebai et al. 2020). Furthermore, the accumulation of residues in milk and meat and their adverse effects on the environment trigger the attention of scientific community to search for novel anthelmintics which are environmentally safe (Santos et al. 2017). The worldwide studies on medicinal plants have enlighten the possibility of using relatively safe plant derived pharmacologically active metabolites against the parasitic nematodes. In this situation, much importance has been dedicated to the use of plant extracts, especially traditional medicinal plant

derived metabolites as environmentally safe and sustainable alternative control against parasitic nematodes (Waller 1999; Davuluiri et al. 2019). As previously reported in many studies, the anthelmintic potential of herbal extracts is ascribed to the synergic effect of secondary metabolites, especially phenolic class of secondary metabolites (Klongsiriwet et al. 2015; Sebai et al. 2020).

Methodology

Collection and Extraction of Plant Material

The Methods section 1 and 2 provide detailed information on the collection and extraction of plant materials. Three solvents namely ethyl acetate, ethanol and water were used for the extraction of phytoconstituents from the rhizomes of both plants.

In vitro anthelmintic activity of *K. rotunda* and *L. toxicaria* rhizome extracts

The *in vitro* anthelmintic activity of rhizome extracts of *K. rotunda* and *L. toxicaria* against the nematode *Haemonchus contortus* and trematode fluke *Fischoederius cobboldi* were evaluated. The *in vitro* study was divided into:

1. Egg hatch assay (EHA) and larval paralysis assay (LPA) against *H. contortus*.
2. Adulticidal assay against *F. cobboldi*.

Egg hatch assay (EHA)

Primarily, faecal pellets were collected from the rectum of a naturally infected donor goat (Getachew et al. 2012). The faecal pellets were then subjected for the centrifugal floatation (Coles et al. 1992) for the collection of fresh eggs. Further, egg hatch assay was conducted according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al. 1992)

with minor modifications. Methods section 6.1.1 and 6.1.2 provided details of the faecal egg floatation and egg hatch inhibition assay.

Larval paralysis assay (LPA)

The assay was based on the method previously described by Varady & Corba (1999) with modifications. Faecal samples from the rectum of naturally infected goat were collected and cultured for 7 days at 27°C to obtain third instar *H. contortus* larvae. Different concentrations of the rhizome extracts were tested against the 3rd instar *H. contortus* larva. Methods section 6.1.3 describes the larval paralysis assay in detail.

***In vitro* adulticidal assay**

Collection of adult flukes

Adult *F. cobboldi* were freshly collected from the rumens of infected cattle killed for consumption in a local slaughter house. The flukes were initially washed several times in 0.85% NaCl solution Anuracpreeda et al. (2015). The healthy ones that exhibited active motility were selected and immediately used for experiments.

***In vitro* assay**

The adulticidal assay of the plant extracts was conducted on *F. cobboldi* according to the method proposed by Anuracpreeda et al. 2016 with minor modifications. Various concentrations of test extract were prepared by dissolving in 0.1% DMSO. Adult flukes were randomly selected and 25 flukes each for all concentrations, positive and vehicle controls were used for the study. Detailed methodology for the adulticidal assay is provided in Methods section 6.2.2.

Stereo zoom microscope analysis

For studying the morphological changes to flukes' post-treatment, stereo microscope analysis was done. *F. cobboldi* treated in different concentrations of

extracts and controls were studied using Leica M205 C stereomicroscope. Also, images were taken by Leica DMC4500 digital camera attached to Leica M205 C stereomicroscope with the software package LAS, version 4.3.0.

Light Microscope study

The fluke specimens were prepared for histology examination by the method proposed by Anuracpreeda et al. (2013). Dead *F. cobboldi* from each group were fixed in Bouin's fixative solution for 12 h, and then was transferred to 10% formalin for further histological processing. Sections of the tissues were taken and stained using hematoxylin and eosin and observed for abnormalities and photographed under a light microscope (Leica TCM 400). The amphistomes fixed in 10% formalin were also sectioned and subjected to Masson's trichrome staining to observe changes in the connective tissues.

Statistical Analysis

The differences in mean of different readings between extracts treated and vehicle control were determined using one-way ANOVA in Graph pad Prism software version 5. The survival data was analysed using Kaplan-Meier survival analysis in Graph Pad Prism software version 5. The Log-rank (Mantel-Cox) test compared the survival curves. The effective concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) inhibition to nematode egg hatch, L3 larval paralysis and fluke mortality was calculated using probit analysis in SPSS software version 24.

Results

Egg hatch assay

The EHA showed a dose dependent activity of KrEA and KrOH extracts at all the tested concentrations compared to 1% DMSO, the vehicle control (Table 4.1). KrWT extract showed the lowest egg hatch inhibition. At the higher tested

concentration (10 mg/ml), KrEA and KrOH induced mean percent egg hatch inhibition of 90.96 ± 0.87 and 85.64 ± 1 respectively. These results are comparable to the egg hatch inhibition potential of the standard drug, albendazole against *H. contortus* (Table 4.2). Same time, the KrWT extract at higher test dose (10 mg/ml) showed mean percent egg hatch inhibition of 28.18 ± 1.10 only (Table 4.1). No significant difference in the egg hatch inhibition potential ($P>0.05$) of KrEA and KrOH extracts was observed at concentrations 1.25 and 2.5 mg/ml, whereas at 5mg/ml concentration, a significant ($P<0.001$) difference in the egg hatch inhibition potential of both extracts was evident. Table 4.3 showed effective concentrations required for inducing 50 percent and 90 percent (EC_{50} and EC_{90}) inhibition to egg hatch of *H. contortus*. Of all three extracts tested, KrEA and KrOH induced 50 percent egg hatch inhibition at lower concentrations, 2.127 and 2.320 mg/ml respectively. Same time the EC_{50} value of KrWT exceeded the maximum tested concentration (>10 mg/ml).

In the case of *L. toxicaria* extracts, all the tested concentrations of LtEA and LtOH extracts significantly ($P < 0.001$) inhibited the egg hatch of *H. contortus*. Also, a concertation dependent increase in the egg hatch inhibition was evident. Among the three extracts tested, the LtWT showed very poor inhibitory potential when compared with the other two extracts. Furthermore, the lower concentration of LtWT (0.625 mg/ml) showed no significance ($P>0.05$) in preventing the egg hatch of *H. contortus* eggs (Table 4.4). The effective concentrations of *L. toxicaria* extracts required for inducing 50 percent and 90 percent (EC_{50} and EC_{90}) egg hatch inhibition was also calculated. The LtEA extract showed the lowest EC_{50} value of 2.48 mg/ml. This was followed by LtOH extract with 3.35 mg/ml. Nevertheless, the EC_{90} values of both extracts exceeded the maximum tested concentration of the

present study. In the case of LtWT extract, both the EC_{50} and EC_{90}values exceeded the maximum tested concentration used (Table 4.3).

Larval paralysis assay

Table 4.5 shows the percent paralysis of 3rd instar *H. contortus* larvae post treatment in *K. rotunda* rhizome extracts. At the higher tested concentration of 10 mg/ml, the KrEA extract induced 93.97 ± 1.07 percent paralysis. This was followed by KrOH extract, with a percent paralysis of 90.18 ± 1.26. Similar to the egg hatch inhibition assay results, the least activity against 3rd instar *H. contortus* larvae was exhibited by the water extract (KrWT). Significant difference ($P<0.001$) in the activity of KrEA and KrWT extract was evident at all tested doses. Sametime, KrEA and KrOH extracts exhibited significant difference in the activity at concentrations 2.5 ($P<0.001$), 1.25 ($P<0.001$) and 0.125 mg/ml ($P < 0.05$). Likewise different doses of standard drug, albendazole also exhibited significant ($P < 0.001$) mortality to the 3rd instar *H. contortus* larvae (Table 4.2). Effective concentrations required for inducing 50 and 90 percent inhibition to larval motility calculated by probit analysis are depicted in table 4.3. The KrEA extract showed the lowest EC_{50} and EC_{90} values, i.e., 1.60 and 6.82 mg/ml respectively. This was followed by KrOH extract. Similar to the egg hatch inhibition, here also the EC_{50} and EC_{90} values of the water extract (KrWT) exceeded the maximum concentration used in the present study.

Table 4.6 showsthe activity of *L. toxicaria* rhizome extracts against the 3rd instar *H. contortus* larvae. Different concentrations of LtEA and LtOH extracts significantly ($P < 0.001$) induced the larval paralysis of *H. contortus*. Here also, a concertation dependent increase in the larval paralysis was evident. On the other hand, lower concentrations of the LtWT extract failed to act against the tested larvae. The effective concentrations of *L. toxicaria* extracts required for inducing 50

percent and 90 percent (EC_{50} and EC_{90}) paralysis was obtained through probit assay. The LtEA extract showed the lowest EC_{50} value of 3.28mg/ml. this was followed by LtOH extract with 6.25 mg/ml. However, the EC_{90} values of both extracts exceeded the maximum tested concentration of the present study. In the case of LtWT extract, both the EC_{50} and EC_{90} values exceeded the maximum tested concentration used (Table 4.3).

***In vitro* adulticidal study**

Figure 4.3 shows the Kaplan-Meier survival data of *F. cobboldi* treated in different concentration of *K. rotunda* rhizome extracts. In the case of KrEA and KrOH extracts, a concentration-dependent decrease in survival rate was observed. Treatment for 30 minutes in the highest test concentration (25 mg/ml), the average mortality of flukes was 17.65% and 8.94% respectively for KrEA and KrOH extracts. Interestingly the mortality significantly increased to 100% after 1 h of treatment in KrEA extract. On the other hand, it took 2 h for obtaining 100% mortality with KrOH extract. Among the three extracts tested, KrWT caused least mortality to the flukes. 1 h post-treatment, in the highest test concentration (25 mg/ml), the mortality was zero. We could obtain average mortality of 10% after 1.5 h post-treatment and the average mortality increased to 50% after 2 h of incubation. The ABZ (1 mg/ml) treated flukes initially showed vigorous motility. After 1 h of incubation the flukes started showing decreased motility. Approximately 16% of flukes were still alive in ABZ treated samples after 2 h of treatment. All flukes treated with vehicle control remained active throughout the experimental period. The Log-rank (Mantel-Cox) test compared the significance of test survival curves with vehicle control. The 25, 12.5 and 6.25 mg/ml concentrations of KrEA and KrOH showed high significance ($P \leq 0.0001$) when compared with vehicle control.

On the other hand, the Log-rank (Mantel-Cox) test of KrOH extract at 3.125 mg/ml concentration showed no significance ($p>0.05$) when compared with vehicle control. In the case of KrWT extract only 25 mg/ml showed significant difference from the vehicle control ($p<0.05$). All other concentrations were insignificant.

The data obtained by calculating the EC_{50} and EC_{90} values post 2 h of exposure to *K. rotunda* extracts also revealed the high toxicity of KrEA and KrOH extracts against the treated flukes. The KrEA extract showed the lowest EC_{50} value (5.22 mg/ml) for the 2 h treatment period. This was followed by KrOH extract that showed EC_{50} of 6.77 mg/ml. The KrWT was relatively less toxic to the trematode as the EC_{50} values were higher than KrEA and KrOH extracts (Table 4.7).

The Kaplan-Meier survival data of *F. cobboldi* treated with different concentration of *L. toxicaria* rhizome extracts are showed in Figure 4.4. Different concentrations of LtEA and LtOH extracts significantly reduced the survival rate of *F. cobboldi*. Post 1.5 h after treatment in the highest tested concentration (25 mg/ml) of LtEA and LtOH extracts, the mortality of flukes reached 100%. Also, significant decrease in the survival rate was observed in the lower concentrations of LtEA extract treated flukes. Same time the LtWT extract did not showed significant activity against the tested trematode flukes. A maximum mortality observed in this case was 24% after 2 h post-treatment in LtWT at the highest tested concentration. The Log-rank (Mantel-Cox) test compared the significance of the survival curves. Here, 25 and 12.5 mg/ml concentrations of LtEA and LtOH showed high significance ($P \leq 0.0001$) when compared with vehicle control. At the lowest tested concentration (3.125 mg/ml) both LtEA and LtOH extracts failed to show significant effects on the survival of flukes ($P> 0.05$). Also, all the tested concentrations of

LtWT extract failed to show significant mortality (P> 0.05) to the trematode flukes when compared with the vehicle control.

The data obtained by calculating the EC_{50} and EC_{90} values post 2 h of exposure to *L. toxicaria* extracts also revealed the noticeable activity of LtEA and LtOH extracts against the *F. cobboldi*. The LtEA and LtOH extracts showed the lowest EC_{50} values of 5.99 and 6.63 mg/ml respectively during the 2 h treatment period (Table4.8). Same time EC_{50} and EC_{90} values of LtWT extract exceeded the maximum test concentration used in the current study.

Light Microscope study

The histological changes to the test group, vehicle controls, and standard controls were studied under a light microscope (LM). The KrEA and KrOH extract treated flukes exhibited significant concentration dependent changes to the surface syncytium, tegument, and the underlying structures. After 2 h of incubation in vehicle control (0.1 % DMSO), all parasites appeared normal with intact surface syncytium, tegumental folds, grooves and underlying muscles. The *F. cobboldi* incubated for 1.5 h in 1 mg/ml of ABZ (positive control) exhibited mild degeneration to surface syncytium. Whereas the underlying muscle appears normal. Following 2 h treatment in positive control (1 mg/ml), severe degeneration to the surface syncytium, teguments followed by detachment from the basement membrane was observed (Figure 4.5).

The *F. cobboldi* treated with 6.25 mg/ml and 12.5 mg/ml of KrEA extract, exhibited surface syncytium degeneration, while the underlying muscle tissue appears more or less intact. Increase in concentration and duration considerably increased the degeneration of teguments and associated structures. Incubation of flukes in 12.5 mg/ml and 25 mg/ml of KrEA extract respectively for 2 h and for 1 h

induced severe surface syncytium degeneration, and tegument degeneration. Flukes treated in 12.5 mg/ml of KrOH extract for 1.5 h and 2 h also showed significant degeneration to the surface syncytium and teguments. Mass degeneration of the tegument and detachment from the basement membrane was observed when flukes treated for 1.5 h in 25 mg/ml of KrOH extract. Same time treatment in KrWT extract (25 mg/ml) for 2 h showed least changes to the surface morphology. In this case the tegumental folds and grooves, muscles and basement membrane appeared more or less intact (Figure 4.6).

Histopathology study of *F. cobboldi* treated with 12.5 mg/ml and 25 mg/ml of LtEA extracts, showed surface syncytium degeneration, alterations to the muscle tissue and detachment from the basement membrane (Figure 4.7). Similarly, flukes treated in 25 mg/ml of LtOH extract also showed severe tegument and surface syncytium degenerations. On the other hand, no observable alterations to the teguments or muscle tissue of *F. cobboldi* were observed when treated using different concentrations of LtWT extract (Figure 4.7).

Stereo zoom microscope study for morphological evaluation

The Leica M205 C stereomicroscope images of *F. cobboldi* after treatment in different concentrations of *K. rotunda* rhizome extracts showed prominent morphological alterations compared to vehicle control treated samples. Two hours post-treatment in vehicle control (0.1% DMSO) showed intact oral sucker and acetabulum. No alterations were observed on the ventral or dorsal surface of the flukes. Incubation in ABZ (1 mg/ml) for 1 h showed no prominent morphological alterations. Whereas 1.5 h post-treatment in 1 mg/ml of ABZ induced changes to the acetabulum and mild tegumental erosion could also be observed. However, the

tegumental degeneration and tegumental erosion associated with tegumental sloughing increased after 2 h of incubation (Figure 4.8).

Evident morphological changes were observed with the KrEA and KrOH treated flukes. Degradation of outer surface; color and size change throughout the whole body; Swellings and other alterations to the surface architecture could also be observed. Serious tegumental sloughing and degeneration were observed with KrEA and KrOH extract treated samples. Extensive erosion of membrane surface which leads to irregular lesions was also observed at the ventral surface of KrEA extract (12.5 mg/ml) treated samples. The KrOH extract treated samples (12.5 mg/ml conc.) showed irregular swollen tegumental regions with deep grooves and blebs. However, specimens treated with KrWT extracts did not show many variations in their morphology compared to the vehicle control. No tegumental alterations were observed in KrWT extract treated samples (Figure 4.9). Also, the anterior and posterior sucker regions were intact. The vehicle control group also showed intact tegumental regions with highly oriented anterior and posterior sucker regions.

In the case of *L. toxicaria* extracts, the LtEA and LtOH extracts induced observable damages to the external surface of the treated flukes. The flukes treated in higher doses of LtEA and LtOH extracts showed severe tegumental alterations. Also, the LtEA (25 mg/ml conc.) treated flukes showed noticeable changes to the posterior sucker region. Sametime, LtWT extract treated flukes showed no surface damage and the anterior and posterior suckers remained intact (Figure 4.10).

Discussion

Increased resistance of helminth parasites against synthetic anthelmintic drugs invites the search for novel herbal molecules which are relatively less resistant and environmentally safe. Many previous studies have reported the anthelmintic

potential of herbal extracts against *H. contortus* eggs and L3 larvae. (Kamaraj et al. 2010; Lone et al. 2012; Irum et al. 2015; Davuluri et al. 2019; Nwosu et al. 2021). The *in vitro* anthelmintic study exposed a dose-dependent nematicidal effect of ethyl acetate (KrEA) and ethanol (KrOH) rhizome extracts of *K. rotunda* against the *H. contortus* eggs and L3 larvae. Unveiling significant anthelmintic potential against different life cycle stages of a parasite is of utmost importance since this severely reduces the likelihood of resistance in the nematode parasites (Hounzangbe-Adote et al. 2005). The KrEA showed significant egg hatch inhibition and L3 larval mortality with an EC_{50} of 2.127 and 1.605 mg/ml respectively. Similarly, the KrOH extract showed an EC_{50} of 2.320 mg/ml for egg hatch inhibition and 2.371 mg/ml for L3 larval mortality. On the other hand, greater EC_{50} values (>10) obtained with the water extract (KrWT) indicate the lower anthelmintic potential of water extract (KrWT). Among the three rhizome extracts of *L. toxicaria*, the LtEA and LtOH extracts showed promising potential against the nematode eggs and larvae. Whereas the LtWT exhibited neglible activity against the *H. contortus*. The probit analysis showed the efficacy of LtEA extract against *H. contortus* eggs and larvae with low EC_{50} values of 2.48 and 3.28 mg/ml respectively. Sametime, the EC_{50}values calculated for LtWT extract against both egg and larvae were beyond the maximum tested concentration used in the current study. The presence of different bio-active metabolites in the ethyl acetate and ethanol extracts compared to the water extracts might have acted singly, or in combination, against the different life cycle stages *H. contortus*.

Amphistomes including *Fischoederius* genus, commonly referred as ‘stomach’ or ‘rumen’ flukes because of the localization of these flukes in the stomach of ruminants, are digenetic trematodes distinguished by the presence of an

oral sucker and the position of the ventral sucker or acetabulum at the posterior end of the body (Tandon et al. 2014). The rapid spread of resistance to synthetic anthelmintic drugs such as oxyclozanide, triclabendazole, paved the way for the search and discovery of new trematocidal drugs (Keiser & Utzinger 2005) that are safe to the host animals. Here we study the *in vitro* anthelmintic potentials of *K. rotunda* and *L. toxicaria* rhizome extracts against the adult fluke, *F. cobboldi*. The results clearly showed the capability of ethyl acetate and ethanol rhizome extracts of both plants showed noticeable activity against the trematode flukes. Here the *K. rotunda* ethyl acetate extract (KrEA) was comparatively more active to cause significant mortality to adult flukes in a short span of time. Studies on closely related trematode species also showed similar results when treated with crude plant extracts. Treatment of 750-1000 µg/ml crude extract of *Artocarpus lakoocha* showed complete immobilization and death of another trematode *Fasciola gigantica* in twelve-to-twenty-four-hour time (Saowakon et al. 2009). According to Tandon et al. (1997), treatment of *Paramphistomum* sp., with 500 µg/ml of *Flemingia vestita* extract caused its death after 12 h. The broad-spectrum anthelmintic drug ABZ, were used as a positive control (Halton 2004; Hossain et al. 2012; Anuracpreeda et al. (2016) for our anthelmintic studies against trematode parasites at a concentration of 1 mg/ml. Our results are consistent with the earlier observations (Hossain et al. 2012; Anuracpreeda et al. 2016) where 1 mg/ml ABZ treated flukes required more time to show reduced motility.

The present study showed important alterations to the tegumental regions including severe degeneration and sloughing followed by extensive erosion when treated with the extracts. The tegumental surface exhibits high corrugation and transverse folds alternating with grooves and are without spines. The ventral surface

has more complex corrugations and invaginations than those of the dorsal surface of the body (Anuracpreeda et al. 2012). The morphology and histology studies showed that the tegument is the most affected region when treated with ethyl acetate and ethanol extracts of the rhizomes. Same time water extracts showed no prominent tegumental alterations to *F. cobboldi*. The tegumental region of parasites is important in that they help evade many of the host defenses effectively. It plays a chief role in protecting the flukes from host enzymes and immune responses. Additionally, it supports the internal organs, helps maintain the absorption and exchange of nutritive and waste products, maintains osmoregulation and perceives sensory stimuli (Meaney et al. 2004; Anuracpreeda et al. 2015, 2016).

The mechanistic aspect by which the phytochemical constituents exert this action is not fully clear. Also, the multicomponent extracts may have multiple targets (Hrckova & Velebny 2013). The tegumental desquamation and alterations to the underlying musculature to the *F. cobboldi* in the present study show similarity to that investigated in adult *P. explanatum* treated with methanol extract of *B. malabaricum* (Hossain et al. 2012), *G. crumenifer* treated with *D. linearis* extracts (Rajesh et al. 2016) and *F. cobboldi* treated with *T. catappa* crude extracts (Anuracpreeda et al. 2016). The treated trematodes in our case exhibited irregular swollen regions with deep grooves and blebs which according to Stitt & Fairweather (1993) could be considered as an adaptive response of the parasite to withstand a stressful condition, including the flukes attempt for repairing the damaged areas. Skuce et al. (1987) reported that the swellings on the surface could be considered as the after-effects of disruptions to the ion pumps at the apical plasma membrane that results in osmotic imbalance. Drastic degradation of the tegumental surface by plant extracts was visible in our microscopy studies. Degeneration of the surface layer

could help the drugs, penetrate deeper into the muscle cells, and cause motility reduction and death to the flukes (Rajesh et al. 2016). Regional specific differences were also evident when treated with extracts. The ventral surface is the most affected compared to the dorsal surface. Alongside, ingestion of the extract by the flukes might have resulted in the alterations to the swollen appearance and formation of irregular blebs on sucker regions. The biopsy studies revealed a smooth (less corrugated) outer body cuticle in the treated animals. Further, there was collagen loss in tissues of treated trematodes. To produce structurally ordered cuticle, interactions must take place between collagens. Triple helices must be formed by interaction between collagen monomers for developing the final polymerized macromolecular structure (Johnstone 1994). The condensed tannins have high affinity for proteins and bind with them to alter its physical and chemical properties (Hoste et al. 2006). It may thus be one of the reasons for altered cuticle structure seen in the sections of amphistomes treated with ethyl acetate extracts of the plants. The loss of this important protein interaction can lead to the cuticular damage and hence, mortality of amphistomes. Hence, the immediate onset of mortality in the ethyl acetate and ethanol extracts treatment is indicative of the multi targeted effect whereby, some components change the tegumental structure facilitating easy entry of other molecules to the interior tissues to cause its mortality.

Table 4.1: Mean percent egg hatch inhibition of *H. contortus* eggs treated in different concentrations of KrEA, KrOH and KrWT extracts.			
Concentration mg/ml	KrEA	KrOH	KrWT
10	90.96 ± 0.87**	85.64 ± 1.04**	28.18 ± 1.10**
5	82.2 ± 1.23**	72.09 ± 2.36**	28.68 ± 0.94**
2.5	54.96 ± 0.91**	51.38 ± 1.01**	19.18 ± 1.67**
1.25	31.44 ± 1.07**	31.5 ± 1.92**	9.21 ± 1.97*
0.625	11.34 ± 1.56**	10.19 ± 1.87**	2.88 ± 1.3^{NS}
DMSO (1%)	1.74 ± 0.87	1.74 ± 0.87	1.74 ± 0.87
Data are expressed as mean ± SEM. **P < 0.001; *P < 0.01 when the treated group compared to untreated control; NS: Not significant.			

Table 4.2: Mean percent egg hatch inhibition and L3 larval paralysis of *H. contortus* treated in different concentrations of standard drug albendazole		
Concentration µg/ml	Egg hatch inhibition	Larval paralysis
50	93.78 ± 3.16	94.51 ± 0.34
25	92.5 ± 2.77	93.66 ± 3.15
12.5	83.66 ± 3.82	85± 4.99
6.25	77.66 ± 1.68	80 ± 1.64
Data are expressed as mean ± SEM		

Table 4.3 : EC_{50} and EC_{90} values in mg/ml (LCL-UCL) of *H. contortus* egg hatch inhibition				
	Egg hatch inhibiton		Larval motility inhibition	
Extract used	EC_{50}	EC_{90}	EC_{50}	EC_{90}
KrEA	2.12(1.853-2.431)	8.12 (6.586-10.651)	1.60(1.370-1.854)	6.82(5.493-9.065)
KrOH	2.32 (2.005-2.677)	> 10	2.37(2.085-2.693)	8.26(6.782-10.623)
KrWT	> 10	> 10	> 10	> 10
LtEA	2.48(2.07-2.96)	> 10	3.28 (2.03-5.83)	> 10
LtOH	3.35 (2.86-3.98)	> 10	6.25 (5.01-8.33)	> 10
LtWT	> 10	> 10	> 10	> 10
LCL: Lower confidence limit; UCL: Upper confidence limit				

Table 4.4: Mean percent egg hatch inhibition of *H. contortus* eggs treated in different concentrations of LtEA, LtOH and LtWT extracts			
Concentration mg/ml	LtEA	LtOH	LtWT
10	80.13 ± 1.14**	82.98 ± 0.92**	13.55 ± 1.63**
5	71.93 ± 1.79**	58.98 ± 0.79**	12.19 ± 1.24**
2.5	51.53 ± 0.77**	41.06 ± 1.85**	10.04 ± 1.25**
1.25	29.23 ± 2.67**	18.6 ± 1.45**	8.41 ± 2.38*
0.625	18.39 ± 2.18**	13.05 ± 1.53**	1.3 ± 1.1 ^{NS}
DMSO (1%)	1.74 ± 0.87	1.74 ± 0.87	1.74 ± 0.87
Data are expressed as mean ± SEM. **P < 0.001; *P < 0.01 when the treated group compared to untreated control; NS: Not significant.			

Table 4.5 : Mean percent larval paralysis of *H. contortus* L3 larvae treated in different concentrations of KrEA, KrOH and KrWT extracts			
Concentration mg/ml	KrEA	KrOH	KrWT
10	93.96 ± 1.07**	90.18 ± 1.26**	35.11 ± 1.04**
5	84.09 ± 1.74**	82.8 ± 1.3**	26.64 ± 0.43**
2.5	65.97 ± 0.72**	54.45 ± 1.04**	13.81 ± 2.77**
1.25	44.6 ± 0.94**	19.86 ± 1.66**	8.23 ± 2.83*
0.625	17.6 ± 0.98**	10.17 ± 3**	1.13 ± 0.77^{NS}
DMSO (1%)	0.84 ± 0.44	0.84 ± 0.44	0.84 ± 0.44
Data are expressed as mean ± SEM. **P < 0.001; *P < 0.01when the treated group compared to untreated control; NS: Not significant.			

Table 4.6: Mean percent larval paralysis of *H. contortus* L3 larvae treated in different concentrations of LtEA, LtOH and LtWT extracts			
Concentration mg/ml	LtEA	LtOH	LtWT
10	80.85 ± 1.8**	62.36 ± 3.13**	9.5 ± 0.42*
5	75.44 ± 2.1**	44.33 ± 2.26**	5.83 ± 0.7^{NS}
2.5	34.29 ± 1.67**	29.55 ± 0.42**	2 ± 0.68 ^{NS}
1.25	13.93 ± 1.38**	10.7 ± 1.84**	1.33 ± 0.8 ^{NS}
0.625	10.61 ± 0.47**	10.27 ± 0.45**	0 ^{NS}
DMSO (1%)	0.84 ± 0.44	0.84 ± 0.44	0.84 ± 0.44
Data are expressed as mean ± SEM. **P < 0.001; *P < 0.01when the treated group compared to untreated control; NS: Not significant.			

Table 4.7: EC_{50} and EC_{90} values for *F. cobboldi* after 2 h of exposure to *K.rotunda* extracts using probit analysis.		
Extracts	EC_{50}(mg/ml) (LCL - UCL)	EC_{90}(mg/ml) (LCL - UCL)
KrEA	5.22 (3.79-6.67)	15.105 (11.04-27.14)
KrOH	6.77 (4.92-8.88)	23.31 (15.99-48.32)
KrWT	16.2 (10.10-50.42)	>25
LCL: Lower confidence limit; UCL: Upper confidence limit		

Table 4.8: EC_{50} and EC_{90} values for *F. cobboldi* after 2 h of exposure to *L. toxicaria* extracts using probit analysis.		
Extracts	EC_{50}(mg/ml) (LCL - UCL)	EC_{90}(mg/ml) (LCL - UCL)
LtEA	5.99 (4.21-6.98)	17.96 (13.24-34.33)
LtOH	6.63 (4.75-8.72)	23.39 (15.97-49.34)
LtWT	>25	>25
LCL: Lower confidence limit; UCL: Upper confidence limit		

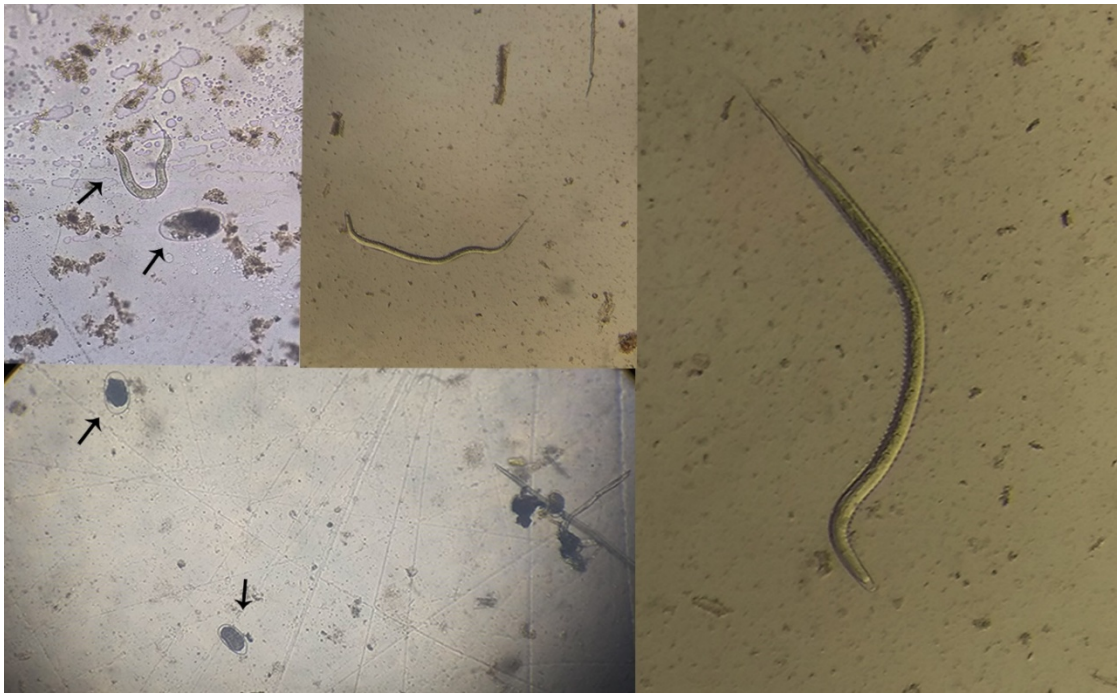

Figure 4.1: *H. contortus* eggs and larvae isolated from the infected goat (10X and 40X magnifications).

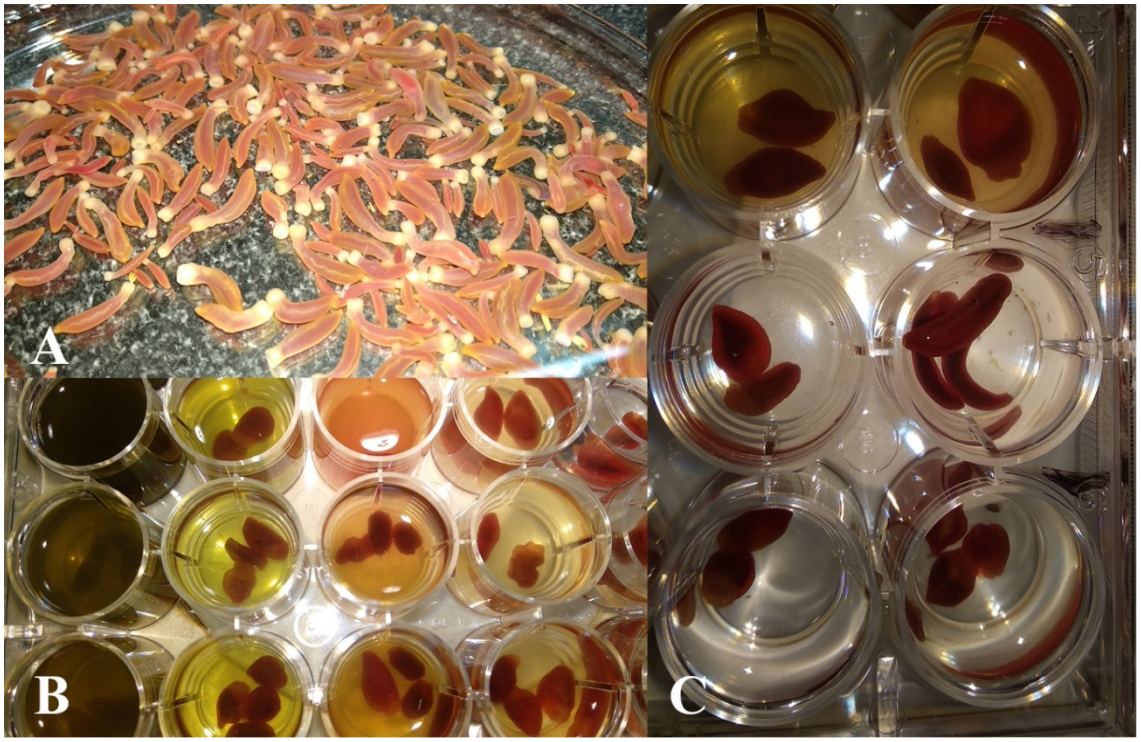

Figure 4.2: A) *F. cobboldi* isolated from the rumen of cattle; (B & C) Treatment of *F. cobboldi* in different concentrations of rhizome extracts.

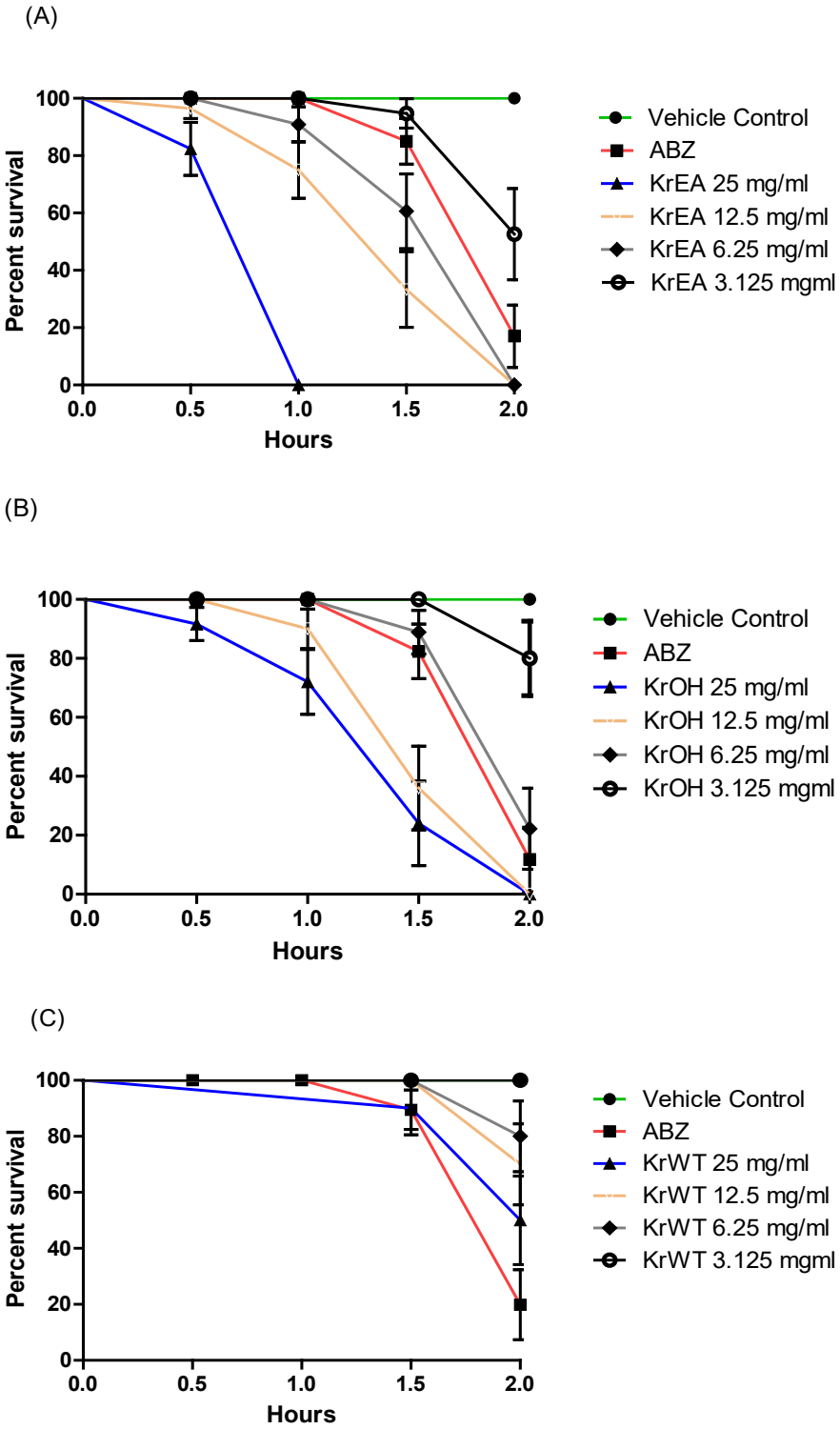

Figure 4.3: Kaplan-Meier survival curves of adult *F. cobboldi* treated in (A) KrEA, (B) KrOH and (C) KrWT extracts at various doses and for different time periods

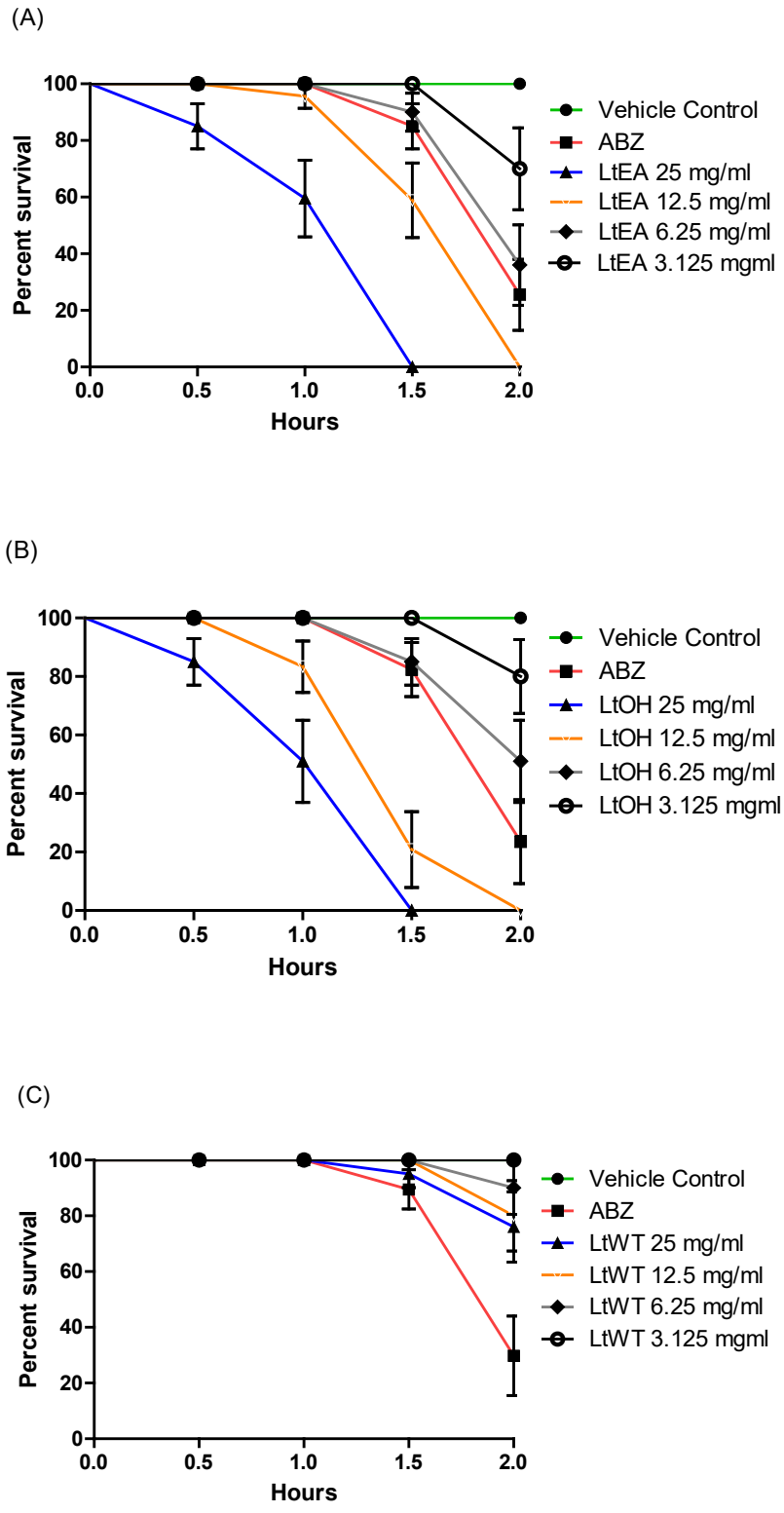

Figure 4.4: Kaplan-Meier survival curves of adult *F. cobboldi* treated in (A) LtEA, (B) LtOH and (C) LtWT extracts at various doses and for different time periods

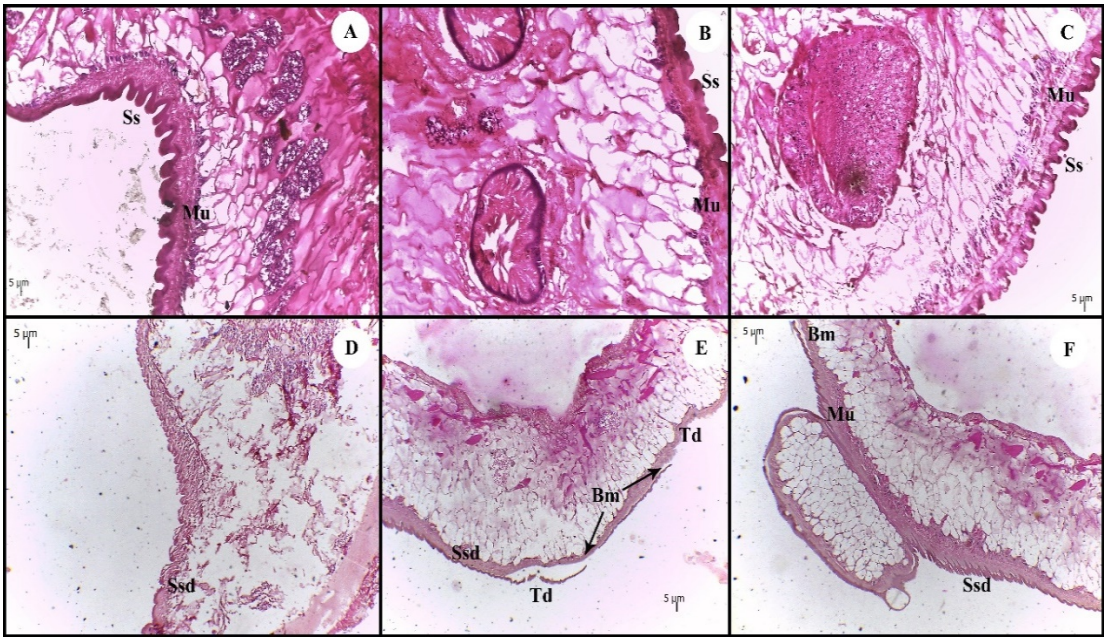

Figure 4.5: Histopathology study under light microscope (10X and 40X magnifications) of *F. cobboldi* incubated in vehicle control (A-C) and positive control (D-F). Treatment of *F. cobboldi* for 1 h (A), 1.5 h (B) and 2 h (C) in 1 % DMSO (Vehicle control) showing intact surface syncytium (Ss) and underlying muscle tissue (Mu). *F. cobboldi* incubated for 1.5 h in 1 mg/ml of ABZ (positive control) showing (D) mild degeneration of surface syncytium (Ssd). Whereas the underlying muscle appears normal. (E and F) Following 2 h treatment in ABZ (1 mg/ml), appearance of severe degeneration to surface syncytium (Ssd) and teguments (Td) followed by detachment from the basement membrane (Bm).

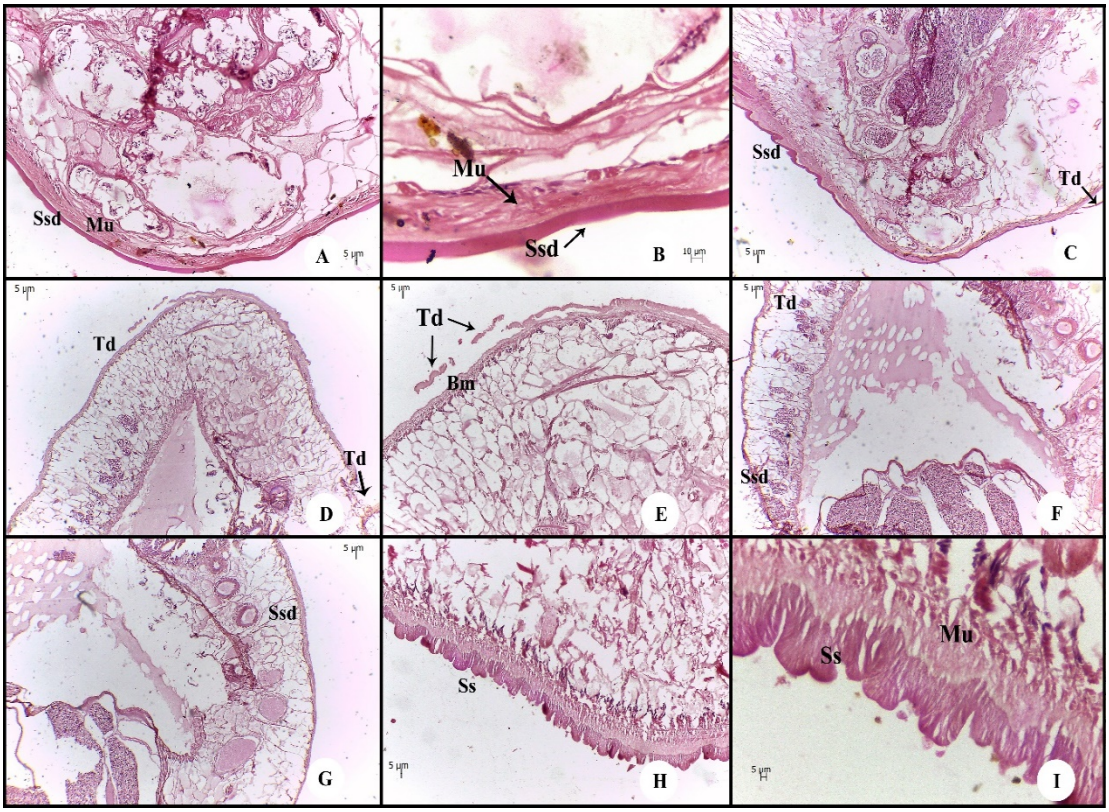

Figure 4.6: Histopathology study under light microscope (10X and 40X magnifications) of *F. cobboldi* treated in KrEA (A-D), KrOH (E-G), and KrWT (H and I) extracts. (A and B) *F. cobboldi* treated with 6.25 mg/ml (A) and 12.5 mg/ml (B) of KrEA extract, showing surface syncytium degeneration (Ssd), while the underlying muscle tissue (Mu) appears more or less intact; (C and D) Following 2h incubation in 12.5 mg/ml (C) and 1 h incubation in 25 mg/ml (D) of KrEA extract showing severe surface syncytium degeneration (Ssd), tegument degeneration (Td) and slight changes to underlying muscle structure; (E) Flukes treated with 25 mg/ml of KrOH extract for 1.5 h showing mass degeneration of teguments (Td) and detachment from the basement membrane (Bm); (F and G) Treatment in 12.5 mg/ml of KrOH extract for 1.5 h (F) and 2 h (G) exhibits alteration to the tegument regions with degeneration to the surface syncytium (Ssd) and teguments (Td); (H and I) Flukes incubated in 25 mg/ml (H) and 12.5 mg/ml (I) of KrWT for 2h showing intact Surface syncytium (Ss) and underlying muscle tissues (Mu).

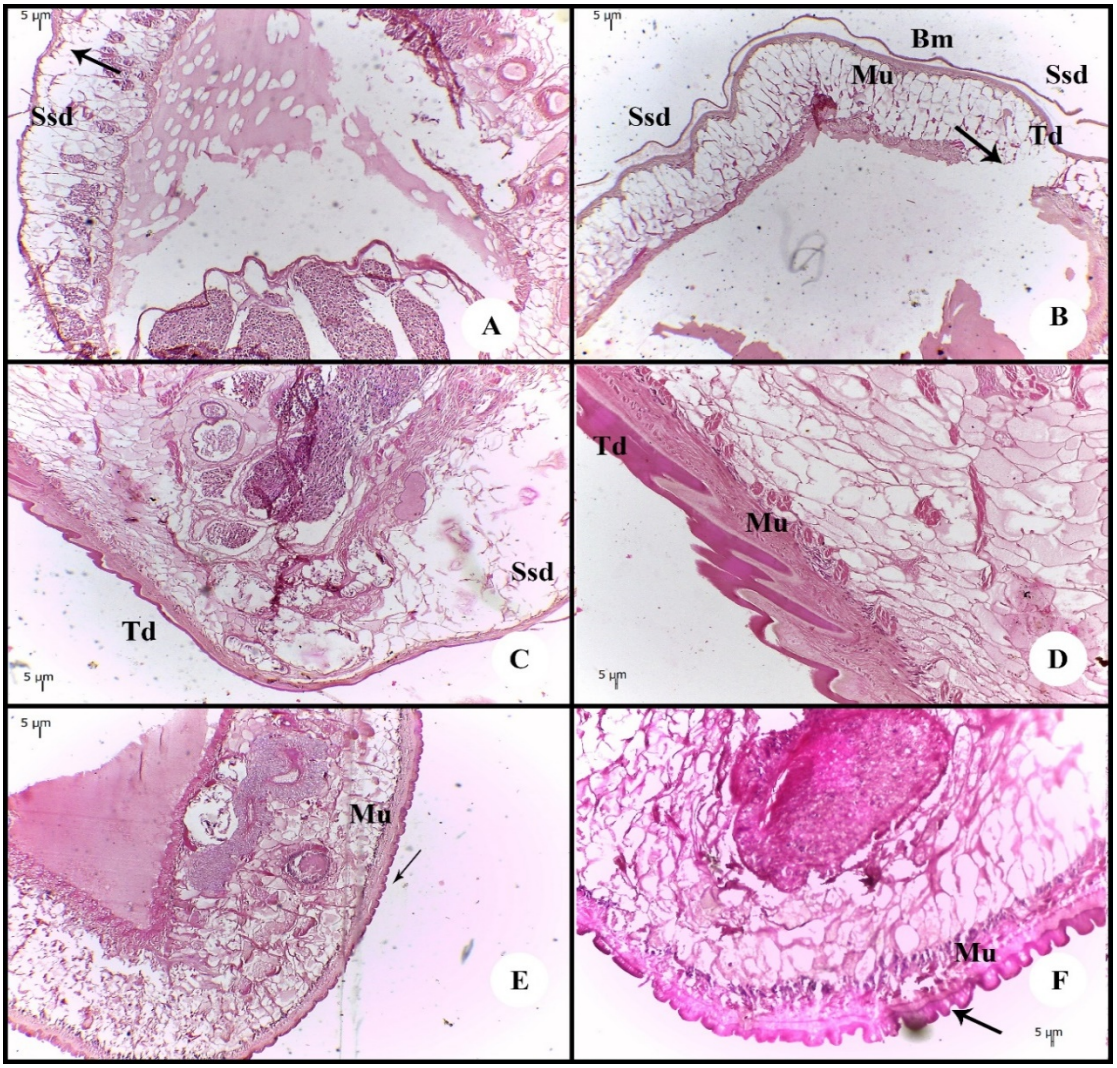

Figure 4.7: Histopathology study under light microscope (10X and 40X magnifications) of *F. cobboldi* incubated in LtEA (A&B), LtOH (C&D), and LtWT (E&F) extracts. (A and B) *F. cobboldi* treated with 12.5 mg/ml (A) and 25 mg/ml (B) of LtEA extract, showing surface syncytium degeneration (Ssd), defective muscle tissue (Mu) and severe tegument degeneration (Td) and detachment from basement membrane (Bm) following 2 h incubation; (C &D) Flukes treated in 25 mg/ml of LtOH extract showing severe tegument degeneration and surface syncytium degeneration (Ssd) post 2h incubation; (E&F) Treatment of *F. cobboldi* in 12.5 (E) and 25 mg/ml (F) of LtWT extract after 2 h incubation showing intact teguments and underlying muscle tissues (Mu).

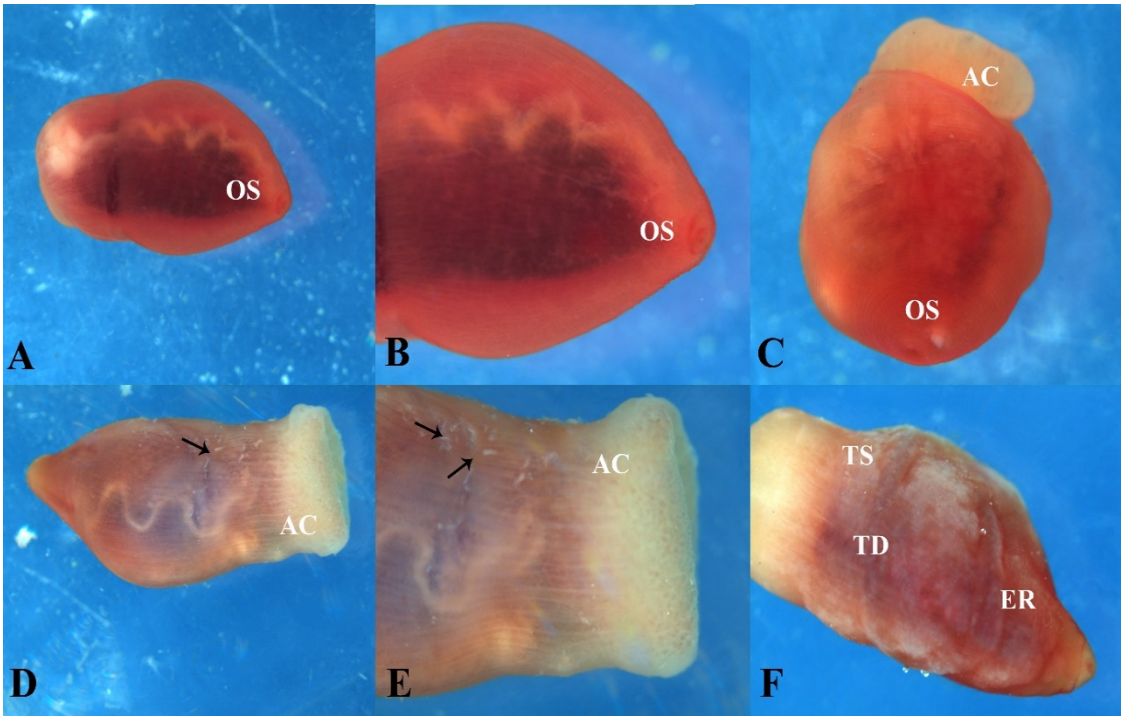

Figure 4.8: Stereo zoom microscope images of the adult *F. cobboldi* treated with vehicle control and positive controls. (A-C) Two hours post-treatment in vehicle control showing intact oral sucker (OS) and acetabulum (AC). (D-E) treatment with ABZ (1 mg/ml) for 1.5 hours showing changes to the acetabulum (AC) and tegumental erosion. (F) Heavy tegumental degeneration (TD) and tegumental erosion (ER) associated with tegumental sloughing (TS) after 2 hours of treatment with in ABZ (1 mg/ml).

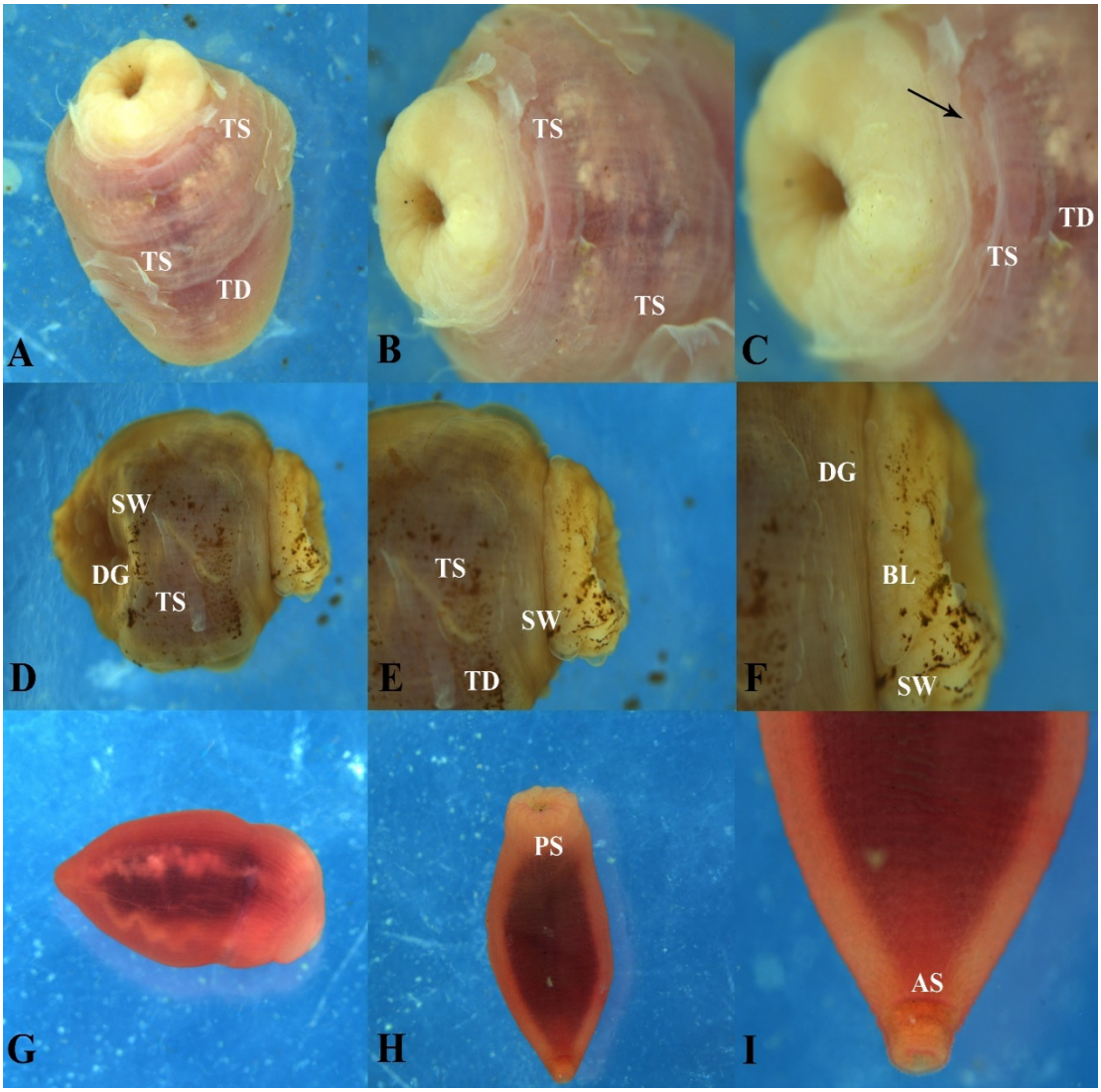

Figure 4.9: Stereo zoom microscope images of the adult *F. cobboldi* treated with ethyl acetate (KrEA), ethanol (KrOH) and water extracts (KrWT) of *K. rotunda* for 2h (12.5 mg/ml conc). (A–C) KrEA treated samples showing severe tegumental sloughing (TS) followed by tegumental degeneration (TD) throughout the body surface. Extensive erosion which leads to irregular lesions were also observed at ventral surface. (D-F) KrOH treated samples show irregular swollen tegumental regions (SW) with deep grooves (DG) and blebs (BL) around posterior sucker regions. Severe tegumental degeneration (TD) associated with tegumental sloughing (TS). (G-I) KrWT treated samples show intact posterior and anterior suckers (PS & AS) with negligible tegumental degeneration.

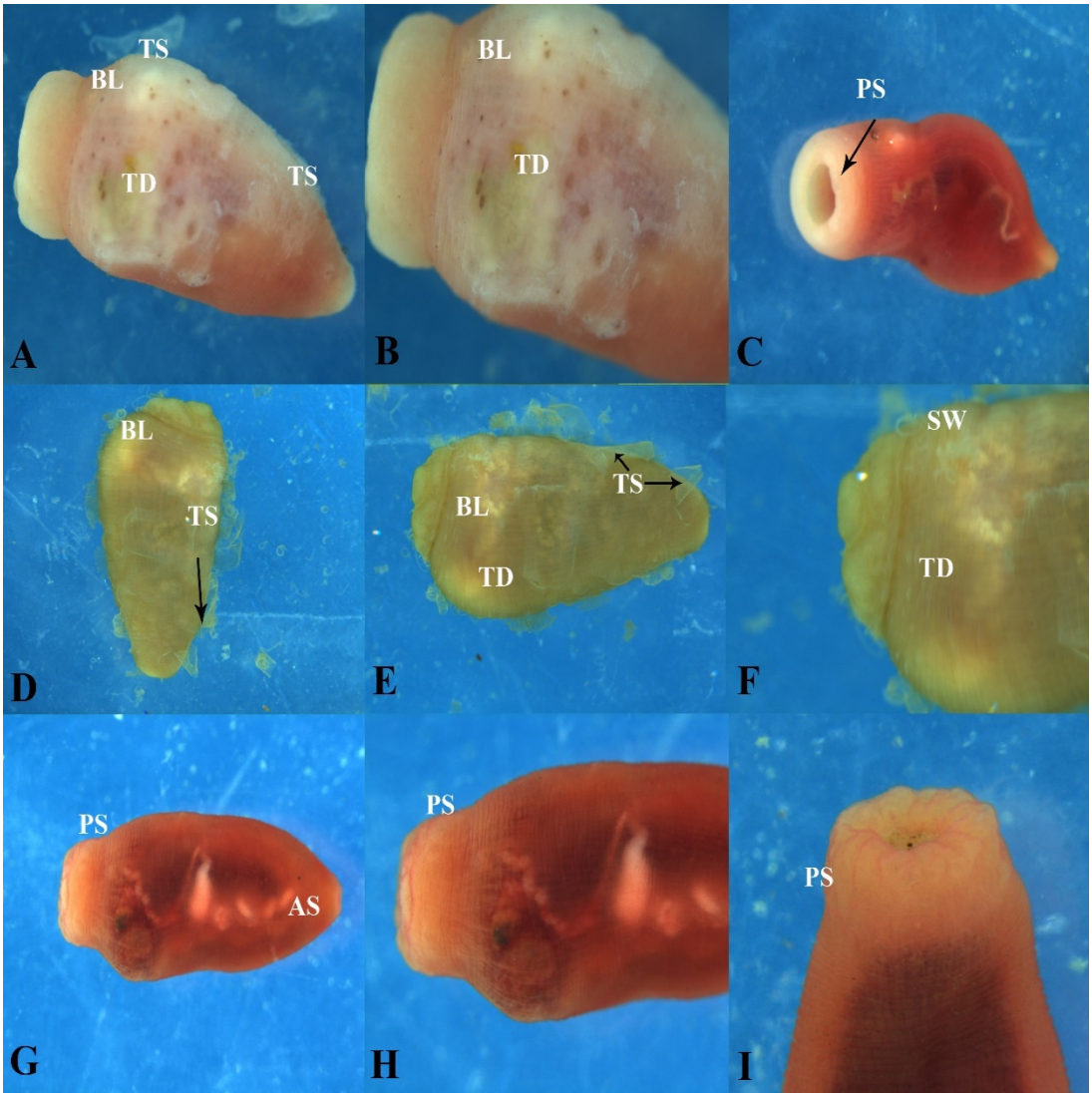

Figure 4.10: Stereo zoom microscope images of the adult *F. cobboldi* treated with ethyl acetate (LtEA), ethanol (LtOH) and water (LtWT) extracts of *L. toxicaria* for 2 h (25 mg/ml conc.). (A–C) LtEA extract treated samples showing severe tegumental sloughing (TS) followed by tegumental degeneration (TD) and associated bleb formation (BL) on the body surface. (D–E) LtOH treated samples showing severe tegumental sloughing (TS) followed by tegumental degeneration (TD) throughout the body surface with irregular blebs (BL). (G–I) LtWT extract treated flukes showing intact posterior and anterior suckers.

CHAPTER 5

Safety evaluation and *in vivo* anthelmintic activity of KrEA and KrOH extracts of *Kaempferia rotunda* rhizomes

Introduction

K. rotunda used in the current study belongs to the family Zingiberaceae. Many plants classified under the family Zingiberaceae are regularly used as spices and condiments in variety of cuisines in Indian sub-continent (Sirirugsa 1999). The fragrant rhizomes of different plants from this family are reported to contain huge deposits of diverse secondary metabolites of therapeutic importance (Nag et al. 2018). Nevertheless, before considering the plant derived natural extracts as medicine, it is inevitable to evaluate the toxicity levels and determine safe *in vivo* doses for consumption. Many previous studies reported severe toxicity and adverse side effects of pharmacologically active concentrated extracts (Ertekin et al. 2005; Koduru et al. 2006). The KRL, a lectin isolated from *K. rotunda* showed cytotoxic effects against brine shrimp nauplii (Kabir et al. 2011; Islam et al. 2019). Transmethylcinnamate isolated from the rhizomes of *A. malaccensis*, another plant from Zingiberaceae family also showed significant toxicity against brine shrimp (Primahana et al. 2015). So, there is germane need to evaluate the toxicity of plant extracts or plant derived compounds on animal models and fix the safe therapeutic doses before further applications.

Due to regular, transfer of laboratory animals like mice between research laboratories, pinworm parasites like *Syphacia obvelata* is commonly observed in the perianal region of the experimental mice (Kozan et al. 2006). The *S. obvelata* and other pin worm infections generally does not develop clinical symptoms in laboratory animals. However, heavy worm loads may lead to rectal prolapse, intestinal impaction and intussusception in the affected animals (Flynn et al. 1989).

Hymenolepiasis is another parasitic infection mainly affecting children. This disease becomes severe among the immune compromised patients if remains

untreated (Bayoumy et al. 2020). *Hymenolepis nana* also known as dwarf tapeworm is a cestode helminth parasite that causes hymenolepiasis (Leder et al. 2013). Previous reports by Kline et al. (2013) stated the high prevalence of this cestode parasites in tropical and subtropical regions of the world. The entire life-cycle of this intestinal cestode parasite takes place in the bowel of infected animal and the infection persists for years if left untreated (Parvathi & Karemungikar 2011). This parasite is indeed the only cestode that completes its life-cycle without an intermediate host (Hosseinzadeh et al. 2016). Generally, the *H. nana* infections are self-limited and pass without any symptoms. Nevertheless, heavy parasite load develops common symptoms like abdominal pain, diarrhea, and vomiting in the host (Kandi et al. 2019). Laboratory animals like mice and rats commonly exhibits the presence of tapeworms from the genus *Hymenolepis* (Bayoumy et al. 2020).

The current chapter details the safety evaluation and *in vivo* anthelmintic potential of ethyl acetate (KrEA) and ethanol (KrOH) rhizome extracts of *K. rotunda*. Initially 14 days oral acute toxicity study using female Swiss albino mice was executed. After that, 28 days oral sub-acute toxicity studies using both sexes of mice were executed following OECD guidelines. Following that safe doses were used to study the *in vivo* anthelmintic potential against natural helminth infections.

Methodology

Collection and Extraction of Plant Material

The Methods section 1 and 2 provide detailed information on the collection and extraction of plant materials. For *in vivo* animal experiments different doses of KrEA and KrOH extracts of *K. rotunda* rhizomes were prepared in 1% propylene glycol. Also, the 1% propylene glycol alone treated mice were considered as vehicle control group.

Animals

Female and male Swiss albino mice (25-30 g) were purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur. All animal experiments were conducted according to the Institutional Animal Ethics Committee (IAEC) and thoroughly followed the guidelines of CPCSEA, Govt. of India.

Toxicity Study

Oral acute toxicity

The oral acute toxicity study of KrEA and KrOH extracts was evaluated strictly following the guidelines of OECD 423 (OECD, 2001). Detailed methodology is available in Methods section 7.1.

Oral Sub-acute toxicity

The oral sub-acute toxicity estimation of KrEA and KrOH extracts were executed following the guidelines of OECD 407 (OECD 2008). Detailed methodology is available in Methods section 7.2.

Anthelmintic study

Before the start of *in vivo* anthelmintic study, natural infections in the mice were identified using standard procedures. Detailed methods are provided in Methods section 7.3.1 and 7.3.2.

Thirty male mice having natural infections were divided into 6 treatment groups of five animals each on the basis of nematode and cestode counts (mean ± SEM). Each groups received different treatment as follows.

Group 1: Two doses of KrEA at 400 mg/kg body weight in one week interval

Group 2: Two doses of KrEA at 200 mg/kg body weight in one week interval

Group 3: Two doses of KrOH at 200 mg/kg body weight in one week interval

Group 4: Two doses of KrOH at 100 mg/kg body weight in one week interval

Group 5: Vehicle control (Drug replaced by suitable volumes of the dosing vehicle)

Group 6: Single dose of albendazole at 20 mg/kg body weight

For faecal sample collection the mice were isolated individually in separate cages on day zero (pre-treatment) and on days 4, 8, 12 and 15 (post-treatment). The faecal pellets were collected, weighed and then subjected to centrifugal floatation for the collection of fresh eggs (Coles et al. 1992). For the identification of *S. obvelata* (pinworm) eggs perianal cellophane tape method (Sueta et al. 2002; Kozan et al. 2006) with minor modifications was used on day zero (pre-treatment) and on days 4, 8, 12 and 15 (post-treatment). On day 15, mice were sacrificed, and the gastrointestinal tract was removed and washed using normal saline. Percent egg count reduction (ECR %) in both faecal centrifugal floatation (FECR) and cellophane tape (ECR) was calculated using the modified formula previously described by Lone et al. (2012):

$$\text{ECR \%} = \frac{\text{Pre treatment egg count} - \text{Post treatment egg count}}{\text{Pre treatment egg count}} \times 100$$

Statistical analysis

The differences in mean between extracts treated and vehicle control were determined using one-way ANOVA in Graph pad Prism software version 5. The differences between the means were considered significant at $p<0.05$ level.

Results

Oral Acute toxicity

The acute toxicity studies showed no lethal effects or toxicity signs in the female mice treated with 2000 mg/kg b.wt of KrEA extract. The body weight, water

and food intake of the treated animals showed no significant difference ($P > 0.05$) with that of vehicle controls (Figure 5.1). Same time mortality (1 death) was observed in the 2000 mg/kg b.wt of KrOH treated group of animals. Also, significant difference in the body weight, water and food intake was observed ($P < 0.05$). So, 14 days oral acute toxicity study of KrOH extract was repeated using 1000 mg/kg b.wt and the treated animals showed no toxicity signs or mortality during the study period (Figure 5.2).

Oral Sub-acute toxicity

Figure 5.3 shows the effect of high doses of KrEA (400 mg/kg b.wt) and KrOH (200 mg/kg b.wt) extracts and vehicle treated on the organs and the body of female and male Swiss albino mice post 28 days of administration. No significant changes were observed in the appearance of organs and the body of extract treated animals when compared with the control group animals.

Food and water consumption

The oral sub-acute toxicity of KrEA and KrOH extracts was estimated in the 28 days study. Figure 5.4 shows the changes in the food intake of female and male mice during 28 days treatment period. KrOH 200 mg/kg b.wt treated female mice showed significant ($P<0.01$) difference in the food intake during the initial week of study when compared with vehicle control. No other extract treated groups showed significant changes in the food intake compared to vehicle control. Also, no significant difference ($P>0.05$) in the food intake of KrEA and KrOH extracts treated male mice were observed compared with vehicle control. Similarly, figure 5.5 shows changes in the water consumption of female and male mice during 28 days treatment period. In female mice, during the third week of study a significant reduction in the water intake ($P<0.05$) of KrEA 200 mg/kg b.wt, KrEA 400 mg/kg

b.wt, KrOH 100 mg/kg b.wt and KrOH 200 mg/kg b.wt (P<0.001) was observed. In male mice, KrEA 200 treated showed significant decrease (P < 0.01) in the water intake during the first and second week. Similarly, KrOH 200 treatment also showed significant decrease (P<0.001) in the water intake during the first week of treatment. It was interesting to note that all these changes in the food and water consumption was reversed and all the animals were having similar consumption as that of the vehicle control in following weeks.

Body weight

No significant changes in the body weight were observed in female mice with different treatments when compared to the vehicle control. Whereas the male mice treated using KrOH 200 mg/kg b.wt showed noticeable decrease in the average body weight during initial days of treatment. i.e., observations on day 3 (P<0.001) and day 6 (P<0.01) showed significant decrease in the body weight but later recovered normally (Figure 5.6).

Haematological parameters

The haematological parameters of the female mice showed significant (p <0.05) decrease in TC with 100 and 200 mg/kg b.wt of KrOH extract treatment. Whereas changes in other haematological parameters were not significant compared to the vehicle control group (Table 5.1). Similarly, the treated male mice, also showed significant decrease in TC at 400 mg/kg b.wt of KrEA extract (p <0.001) and 200 mg/kg b.wt of KrOH extract (p <0.05). Even though the calculated TC values of treated mice showed significant difference from the control group, the values were still in the normal range for rodents. Here also changes in other haematological parameters remained insignificant when compared to the vehicle control group (Table 5.1).

Biochemical Parameters

In both female and male extract treated mice, biochemical parameters like SGOT, SGPT and ALP values were not significantly ($P > 0.05$) different compared to the vehicle control during the 28 days study period (Table 5.2). Similarly, in the case of blood urea and creatinine levels, the male mice did not show any changes whereas female mice treated using 200 mg/kg b.wt of KrOH extract showed slight decrease ($P < 0.05$) in the blood urea level (Table 5.3). The calculated values of serum triglycerides, total cholesterol, HDL, LDL and VLDL of treated animals (both female and male mice) did not showed significant variation from the vehicle control group (Table 5.4). Also, the estimated values were within the normal range for rodents. In the case of serum electrolytes such as sodium, potassium, bicarbonate and chloride levels no significant variations were observed between treated male and female mice and control group mice (Table 5.5).

Relative organ weight

The relative organ weight of liver, kidney, heart, lungs, spleen, stomach, intestine, brain, ovary/testis of treated and control animals were estimated (Table 5.6). KrOH 100 mg/kg b.wt and KrOH 200 mg/kg b.wt treated female showed significant ($p < 0.05$) change in the relative organ weight of liver when compared to the vehicle controls. Likewise, the KrOH 200 mg/kg b.wt showed noticeable change in the relative weight of kidney. On the other hand, the KrOH and KrEA treated male mice did not show significant change in the relative organ weight of other organs measured (Table 5.6).

Histopathology study

Histopathological examination showed no distinguising architectural changes in the tested organs such as a) liver, b) kidney, c) heart d) lungs, e)spleen, f)

stomach, g) intestine, h) brain, i) ovary, j) testis in the extracts treated animals compared to the animals in the control group. The hepatocytes appears normal and exhibits normal portal triads and hepatic veins, the sinusoidal spaces and kupffer cells remain unaltered. In the case of kidney, normal glomeruli and renal tubules were observed and the overall architechture remained same as that of the control group. The examination of heart, lungs, spleen, stomach, intestine and brain sections of treated mice showed normal appearance. The sections of lung showed normal bronchioles and alveoli. The normal appearance of lymphoid tissue and lymphoid follicles were evident with the spleen sections. Also, the stomach and intestine showed normal appearance of mucosal and submucosal layers. Furthermore, usual appearance of astrocytes and glial cells were observed with brain. Study of testis and ovary sections also did not show architectural difference from the control group. The sections of ovary showed normal graffian follicles with unaltered follicular cells. Similarly, testis showed normal seminiferous tubules with proper spermatogenesis (Figure 5.7 & 5.8).

Identification of natural infections

The cellophane tape method and centrifugal flotation methods were used for the identification of natural infections in the laboratory Swiss albino mice (both male and female) used in the study. The nematode, *S. obvelata* (pinworm) was identified using cellophane tape method (Figure 5.9). Centrifugal flotation assay showed dominance of the cestode, *H. nana* (tapeworm) (Figure 5.9).

Anthelmintic activity

The KrEA extract at 400 mg/kg b.wt dose significantly reduced the mean number of *S. obvelata* eggs from 62.6 ± 9.57 to 18.8 ± 3.89 and further to 4.4 ± 0.74 eggs respectively after 8- and 12-days post-treatment (Figure 5.10). Likewise, the

KrEA extract at 200 mg/kg b.wt dose also effectively reduced the mean number of *S. obvelata* eggs from 51.8 ± 3.16 eggs to 30.8 ± 3.54 and 15.4 ± 2.31 eggs respectively after 8- and 12-days post-treatment. Similarly, KrOH extract at 200 and 100 mg/kg b.wt doses also induced considerable reduction in the pinworm eggs (*S. obvelata*) during the 15 days treatment. The *S. obvelata* egg count reduction (ECR) data calculated during different days are represented in table 5.7. In the case of KrEA extract at 400 mg/kg b.wt dose the ECR reached 92.97% and 100% after 12- and 15-days post-treatment respectively. Same time, mice treated with 200 mg/kg b.wt dose of KrOH extract showed a maximum of 80.21% ECR after 15 days post-treatment (Table 5.7).

Against the cestode *H. nana*, the highest dose of KrEA extract, i.e., 400 mg/kg b.wt induced a significant reduction in the EPG (eggs per gram). Here, the initial EPG i.e., 43.8 ± 4.81 reduced to 14.2 ± 1.2 and further to 4.8 ± 2.03 respectively after 8- and 12-days after treatment (Figure 5.11). The FECR value of KrEA extract (400 mg/kg b.wt) treated mice increased to 100% after15 days treatment period. On the other hand, the maximum FECR obtained with the mice treated in the highest dose of KrOH, i.e., 200 mg/kg b.wt was 63.7% after 15 days post-treatment (Table 5.8).

In the present study, 1% propylene glycol served the role of dosing vehicle. In this case, the mean number of *S. obvelata* increased from 58.4 ± 12.46 to 68 ± 9.66 after 15 days post-treatment (Figure 5.10). Whereas in the case of *H. nana*, no noticeable decrease in the EPG was observed after 15 days post-administration of the dosing vehicle (Figure 5.11). It is also revealed that albendazole (20 mg/kg b.wt) treatment effectively reduced the mean number of *S. obvelata* eggs and EPG of *H.*

nana. Here the egg count of both *S. obvelata* and *H. nana* was reduced to zero after 8 days post-treatment.

Discussion

The increased complexity and variations in the herbal products highlight the importance of validating their safety and effectiveness (Shin et al. 2011). Generally, the safety and effectiveness of herbal drugs have been evaluated using acute and sub-acute toxicity studies in laboratory animals, especially rodents (Liju et al. 2013). In the current study, OECD guidelines were followed to perform acute and sub-acuteoral toxicity studies in Swiss albino mice. The acute toxicity studies using 2000 mg/kg b.wt of KrEA extract did not show mortality during 14 days treatment period. Also, no toxic signs such as behavioural changes, hair loss, weight loss, and decrease in food and water consumption were observed throughout the study period. The decrease in the body weight of treated animals during the administration of drugs can be considered as one of the important signs of toxicity **(**Tofovic & Jackson 1999; Teo et al. 2002). The present study results clearly suggests that the tested mice tolerated KrEA extract up to a dose of 2000 mg/kg b.wt. Also, the lethal dose to induce 50 percent mortality (LD_{50}) to the tested animals was higher than the aforementioned acute oral dose delivered to the experimental mice. Following acute toxicity studies, the sub-acute toxicity was evaluated by taking the $1/10^{th}$ and $1/5^{th}$ of 2000 mg/kg b.wt as low and high doses respectively. The sub-acute doses of KrEA extract also did not elicit significant toxic changes to the tested mice compared to the control group. The body weight, food and water intake appear normal. In the current study, the relative organ weights of important vital organs of the tested mice appear normal and no significant change in the relative organ weight of tested mice compared to the control group was observed. The haematological and biochemical

parameters of KrEA and KrOH treated animals remained unaltered during the 28 days sub-acute toxicity period. The results suggests that the KrEA and KrOH extracts in the tested concentrations did not adversely affect the functions of blood, liver and kidney. Furthermore, the histopathological examination of the vital organs showed normal architecture compared to the control group.

Sametime, acute oral toxicity study using 2000 mg/kg b.wt of KrOH extract showed toxicity and mortality among the treated animals. The results are contrary to one of the previous studies that reported the limit test dose of 2000 mg/kg b.wt of *K. rotunda* ethanol extract showed no mortality to Wistar rats during 14 days acute oral toxicity study (Sini et al. 2014). However in our study, no toxicity was observed when we repeated the acute toxicity study using 1000 mg/kg b.wt of KrOH extract. Furthermore, the sub-acute oral administration of KrOH extract at $1/10^{th}$ and $1/5^{th}$ of 1000 mg/kg b.wt (100 and 200 mg/kg b.wt respectively) for 28 days study period did not elicit any relevant toxic symptoms on tested animals. Also, many of the changes in haematological and biochemical parameters were insignificant compared to the control group. Even though, the urea level of 200 mg/kg b.wt dose of KrOH extract treated mice showed some alterations, the observed values were still within the normal range. Likewise, the histopathological results did not show any distinguishable alterations or damages to the organs of treated mice. In conclusion, 14 days acute oral toxicity studies and 28 days sub-acute toxicity studies of the KrEA and KrOH extracts in the aforementioned doses are relatively safe and suitable for further use in *in vivo* animal studies.

The present *in vivo* anthelmintic study focused on natural helminth infections (both nematode and cestode) of laboratory Swiss albino mice. After acute and sub-acute toxicity studies, we evaluated the effect of different concentrations of KrEA

(400 and 200 mg/kg b.wt) and KrOH (200 and 100 mg/kg b.wt) against the nematode, *S. obvelata* and cestode *H. nana*. Pre-treatment centrifugal floatation study showed the presence of the cestode, *H. nana* (tape worm) in the faecal pellets of the mice. Similarly, screening using cellophane tape method before treatment confirmed the presence of the nematode *S. obvelata* (pin worm) on the perianal region of the mice. Earlier Kozan et al. (2006) used cellophane tape trials for the identification of *Syphacia obvelata* natural infections and centrifugal flotation method for the identification of natural infections of *Aspiculuris tetraptera* in mice. Similarly, Gogoi & Yadav (2016) adopted centrifugal floatation and cellophane tape methods respectively for screening the natural infections of *Hymenolepis diminuta* and *S. obvelata* in mice.

The *in vivo* study results clearly highlight the capability of ethyl acetate and ethanol extracts of *K. rotunda* rhizome against the helminth parasites *H. nana* and *S. obvelata*. Comparatively the KrEA at high dose (400 mg/kg b.wt) showed conspicuous reduction in the eggs of both *H. nana* and *S. obvelata*. The ECR of *S. obvelata* and the FECR of *H. nana* reached 100% after 15 days post-treatment in KrEA at 400 mg/kg b.wt Also, the KrEA extract at 200 mg/kg b.wt induced 93.05% ECR of *S. obvelata* eggs and 78.82% FECR of *H. nana* after 15 days post-treatment. Similarly, the KrOH at 200 and 100mg/kg b.wt concentrations showed significant reductions in the egg count of *S. obvelata*. On the other hand, KrOH extract at 100 mg/kg b.wt concentration was less effective against the cestode, *H. nana*. This is indeed the first report on the *in vivo* anthelmintic potential of *K. rotunda* rhizome extracts against helminth parasites. Previously Kozan et al. (2006) reported anthelmintic activity of ethanol extracts of *Citrillus lanatus, Juniperus drupacea* (fruit)*, Juniperus oxcycedrus* and *Plantago lanceolata* against pinworms, *Syphacia*

obvelata and *Aspiculuris tetraptera*, in mice. Likewise, Hosseinzadeh et al. (2016) in their *in vivo* anthelmintic study testified 100 percent efficacy of *Coriandrum sativum* alcoholic extracts at 500 and 700 mg/kg b.wt doses against *H. nana*. Earlier reports regarding the *in vivo* anthelmintic capability of plant parts extracted in ethyl acetate was very rare. Standard anthelmintic drug, albendazole (20 mg/kg b.wt) was used as positive control in the current study. A single dose of albendazole in 20 mg/kg b.wt reduced the egg counts of *S. obvelata* and faecal egg counts of *H. nana* to 100% after 8 days post-treatment. These results are in agreement with the previous work of Gogoi & Yadav (2016) in which they reported 96.1 percent reduction in *S. obvelata* counts during 9 to 11 days post-treatment in 20 mg/kg b.wt of albendazole. Similarly, Eguale et al. (2007b) reported significant FECR ($p < 0.001$) of *H. contortus* in the faecal samples of male Menz sheep after 2 days post-treatment in 3.8 mg/kg b.wt of albendazole.

Table 5.1: Hematological parameters of Swiss albino mice after sub-acute oral administration of KrEA and KrOH extracts

	Treatment	Hb (g/dl)	RBC (×106/mm3)	PLT (×105/mm3)	TC (cells/mm3)	MCV	MCH	MCHC	PCV	N	L	E
Female	KrEA 200	14.7 ± 0.56	8.65 ± 0.07	3.20 ± 1.2	9120 ± 311.12	68 ± 2.1	17.2 ± 0.7	30.5 ±0.7	43 ± 1.41	36.8 ± 0.00	58 ± 0.7	4.5 ± 0.7
	KrEA 400	15 ± 0	8.90 ± 0.28	3.3 ± 1.34	8900 ± 282.84***	70 ± 2.12	16.5 ± 0.7	31 ±0.00	44.1 ± 0.00	42.8 ±2.82	59.2 ± 2.82	5 ±0.00
	KrOH 100	12.4 ± 0.28	6.4 ± 0.00	3.8 ± 1.27	8600 ± 264.21	76 ±1.41	19.5 ± 0.7	35.6 ± 0.00	35 ± 0.7	41 ± 0.7	58.4 ± 1.41	3 ± 0.7
	KrOH 200	15.6 ± 0.14	8.05 ± 0.21	4.1 ± 0.77	8714 ± 429.92**	77 ±2.82	18.2 ± 0.7	36 ± 0.00	40 ± 0.7	44.10 ± 1.41	60 ± 0.7	4.5 ± 0.7
	V. Control	13.3 ± 0.28	7.4 ± 0.34	2.9 ± 3.48	9200 ± 424.26	73 ± 2.12	18 ± 0.00	31.5 ± 2.12	42.5 ± 2.1	41 ± 1.41	57.50 ± 2	4 ± 0.70
Male	KrEA 200	14.05 ± 0.49	7.35 ± 0.07	2.9 ± 2.26	9392 ± 392.44	56±1.41	19± 0.00	34± 0.00	41± 1.41	33.6± 0.70	87.50± 0.7	3±1.41
	KrEA 400	15.75 ± 0.07	8.15 ± 0.07	3.6 ± 4.52	8211 ± 564.27**	55±0.89	19± 0.7	35.5± 0.7	44.5± 0.14	32.7± 1.28	71.2 ± 2.82	2± 2.11
	KrOH 100	15.9 ± 0.14	8.3 ± 0.14	3.6 ± 2.61	9505 ± 431.33	54.5± 0.28	18.5 ± 0.7	35.5± 0.9	45± 0.00	28.71± 0.00	79.5± 1.41	4±1.86
	KrOH 200	14.8 ± 0.84	7.6 ± 0.14	3.8 ± 1.21	8950 ± 353.55*	61.31 ± 2.82	19.5± 0.91	35.5±2.12	42± 0.00	40.8± 1.41	87±2.86	2± 0.00
	V. Control	14.5 ± 0.28	8.5 ± 0.13	3.1 ± 0	9500 ± 10.24	57.50 ± 4.94	17± 0.00	29.50± 2.12	49±4.24	31.5±4.67	66.50± 1.09	2±1.41

Data are expressed as mean ± SD; Significant in relation to vehicle control at *p < 0.05, **p < 0.01, ***p < 0.001.

Table 5.2: Levels of liver function markers of Swiss albino mice after sub-acute oral administration of KrEA and KrOH extracts

	Treatment	SGOT (IU/L)	SGPT (IU/L)	ALP (U/L)	TP (g/dl)	Albumin	Globulin	TB (mg/dl)
Female	KrEA 200	146 ± 11.11	66 ± 2.82	92.5 ± 8.38	6.15 ± 0.67	3.30 ± 0.14	2.85 ± 0.2	0.30 ± 0.00
	KrEA 400	141.5 ± 6.26	59.5 ± 9.19	98.5 ± 0.70	6.25 ± 0.19	3.35 ± 0.17	2.90 ± 0.00	0.40 ± 0.00
	KrOH 100	134.5 ± 13.43	53 ± 2.82*	100.7 ± 14.14	5.95 ± 1.18	3.20 ± 0.14	2.75 ± 0.24	0.20 ± 0.00
	KrOH 200	132 ± 8.18	63.5 ± 0.70	106.5 ± 6.87	6.25 ± 0.21	3.35 ± 0.07	3.40 ± 0.84	0.20 ± 0.14
	V. Control	148.5 ± 17.78	73.5 ± 6.36	89.5 ± 21.92	6.60 ± 0.34	3.55 ± 0.00	4.05 ± 0.28	0.25 ± 0.07
Male	KrEA 200	139.5 ± 5.96	73.5 ± 0.70	95.7 ± 2.82	6.55 ± 1.28	3.35 ± 0.14	3.20 ± 0.00	0.20 ± 0.00
	KrEA 400	145 ± 11	64 ± 15.55	105.5 ± 3.53	6.65 ± 0.35	3.45 ± 0.21	3.20 ± 0.28	0.30 ± 0.00
	KrOH 100	105.5 ± 61.51	66.5 ± 0.70	98.44 ± 7.77	6.20 ± 0.56	3.20 ± 0.14	3 ± 0.42	0.20 ± 0.14
	KrOH 200	134 ± 18.28	63 ± 1.41	110.7 ± 12.02	5.80 ± 0.21	3.20 ± 0.07	2.60 ± 0.84	0.25 ± 0.21
	V. Control	130.5 ± 12.44	60.5 ± 4.94	90.5 ± 4.94	7.85 ± 0.18	3.50 ± 0.00	4.35 ± 1.17	0.30 ± 0.00

Data are expressed as mean ± SD. Significant in relation to vehicle control at *P < 0.05.

Table 5.3: Kidney function analysis of Swiss albino mice after sub-acute oral administration of KrEA and KrOH extracts				
Treatment	Female		Male	
	Urea (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
KrEA 200	46 ± 5.65	0.5 ± 0.021	48.5 ± 4.94	0.51 ± 0.042
KrEA 400	43 ± 2.82	0.52 ± 0.01	47 ± 1.41	0.52 ± 0.02
KrOH 100	42 ± 2.82	0.485 ± 0.04	33.5 ± 0.70	0.52 ± 0.05
KrOH 200	33 ± 1.41*	0.54 ± 0.02	47.5 ± 6.36	0.5 ± 0.01
Vehicle control	41 ± 0.70	0.51 ± 0.024	43.1 ± 16.26	0.56 ± 0.07
Data are expressed as mean ± SD. Significant in relation to vehicle control at *P < 0.05.				

Table 5.4 : Blood lipid profile of Swiss albino mice after sub-acute oral administration of KrEA and KrOH extracts						
	Treatment	Triglycerides (mg/dL))	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL	VLDL (mg/dL)
Female	KrEA 200	136.21 ± 5.81	88.57± 0.49	38 ± 1.41	23.57 ± 4.08	27 ± 0.70
	KrEA 400	136 ± 5.12	82.94± 0.86	35 ± 2.12	21.7 ± 5.14	26.24 ± 1.21
	KrOH 100	140.20 ± 3.58	87.74± 2.74	31.50 ± 1.41	28.20 ± 2.24	28.04 ± 1.41
	KrOH 200	141.2 ± 5.86	92.2± 1.27	33.5 ± 0.7	28.7 ± 6.36	30 ± 0.07
	V. Control	131 ± 4.27	86± 0.64	33.80 ± 0.7	26 ± 3.28	26.20 ± 0.00
Male	KrEA 200	136.37± 3.88	91.86± 2.43	34.26± 0.00	30.33± 10.6	27.27 ± 0.24
	KrEA 400	138.29± 5.69	90.78± 1.19	34.78± 0.70	29± 8.48	27 ± 0.45
	KrOH 100	140.21± 3.35	98± 2.48	33.45± 0.70	35.41± 12.72	29.14 ±1.41
	KrOH 200	144.78± 6.81	95.04± 0.98	29.37± 0.14	34.89± 3.53	30.78 ± 1.72
	V. Control	144.28± 6.76	87.27± 1.13	31.87± 0.00	28.27± 5.74	27.16 ± 1.32
Data are expressed as mean ± SD.						

Table 5.5: Serum electrolytes level of Swiss albino mice after sub-acute oral administration of KrEA and KrOH extracts					
	Treatment	Sodium (mmol/l)	Potassium (mmol/l)	Bicarbonate (mmol/l)	Chloride (mmol/l)
Female	KrEA 200	148.5 ± 2.12	5.6 ± 0.42	26 ± 1.41	105 ± 1.41
	KrEA 400	146.5 ± 1.75	5.9 ± 1.41	27.5 ± 2.12	107.5 ± 3.25
	KrOH 100	144.28 ± 4.1	6.3 ± 2.27	27.5 ± 1.55	108.5 ± 3.56
	KrOH 200	140.24 ± 2.16	6.8 ± 0.99	23.50 ± 1.98	104 ± 2.34
	V. Control	149.5 ± 3.26	4.3 ± 0.64	25.5 ± 0.55	105.5 ± 1.83
Male	KrEA 200	145.1 ± 3.22	5.5 ± 2.43	25.5 ± 0.70	107.5 ± 3.6
	KrEA 400	148.20 ± 1.69	4.8 ± 2.5	25.3 ± 0.89	108.5 ± 2.48
	KrOH 100	147.9 ± 1.5	6.1 ± 1.48	28.45 ± 2.34	108 ± 1.41
	KrOH 200	149.40 ± 1.81	6.5 ± 1.34	25.8 ± 0.28	106.5 ± 2.12
	V. Control	144.6 ± 2.12	5 ± 0.42	24.5 ± 0.86	105.5 ± 2.83
Data are expressed as mean ± SD.					

Table 5.6: Relative organ weight of Swiss albino mice after sub-acute oral administration of KrEA and KrOH extracts										
	Treatment	Liver	Kidney	Heart	Lungs	Spleen	Stomach	Intestine	Brain	Ovary/Testis
Female	KrEA 200	5.04 ± 0.16	0.97 ± 0.15	0.45 ± 0.08	0.60 ± 0.16	0.41 ± 0.12	0.85± 0.05	5.26± 0.14	1.23± 0.05	0.39 ± 0.11
	KrEA 400	4.86 ± 0.35	1.08 ± 0.23	0.47 ± 0.14	0.57 ± 0.27	0.36 ± 0.02	0.88± 0.04	5.05± 0.22	1.14±0.03	0.32 ± 0.26
	KrOH 100	4.78 ±0.18*	1.17 ± 0.22	0.46 ± 0.27	0.64 ± 0.18	0.46 ± 0.12	1.01± 0.07	5.25± 0.35	1.16± 0.00	0.34 ± 0.12
	KrOH 200	4.7 ± 0.18*	1.50 ± 0.27*	0.47 ± 0.31	0.77 ± 0.43	0.45 ± 0.18	0.81± 0.03	4.55± 0.15	1.21±0.02	0.36 ± 0.12
	V. Control	5.06 ± 0.129	1.21 ± 0.489	0.48 ± 0.164	0.58 ± 089	0.40 ± 0.035	0.84± 0.13	5.15± 0.28	1.17± 0.16	0.38 ± 0.34
Male	KrEA 200	5.31± 0.10	1.48± 0.25	0.48± 0.01	0.68± 0.21	0.47± 0.43	0.79±0.02	4.99±0.11	1.02±0.03	0.59± 0.22
	KrEA 400	4.54± 0.7	1.59± 0.45	0.49± 0.00	0.54± 0.41	0.45± 0.65	0.84±0.04	4.92±0.22	1.13±0.01	0.62± 0.06
	KrOH 100	5.21± 0.35	1.41± 0.24	0.49± 0.12	0.60± 0.44	0.38± 0.35	0.70± 0.03	4.93± 0.21	1.05± 0.02	0.49± 0.23
	KrOH 200	5.28± 0.15	1.71± 0.23	0.49± 0.11	0.61± 0.32	0.42± 0.02	0.97±0.05	4.8±0.3	1.09± 0.06	0.58± 0.16
	V. Control	4.94± 0.23	1.23± 0.52	0.49± 1.06	0.58± 0.06	0.49± 0.31	0.91± 0.25	5.34±0.85	1.36±0.3	0.60± 0.10
Data are expressed as mean ± SD; Significant in relation to vehicle control at *p < 0.05.										

Table 5.7: Percent ECR of *S.obvelata* eggs after treatment in different concentrations of *K. rotunda* rhizome extracts				
Treatment	Percent ECR in different days			
	4	8	12	15
KrEA 400 mg/kg b.wt.	6.7	69.96	92.97	100
KrEA200 mg/kg b.wt.	7.72	40.54	70.27	93.05
KrOH 200 mg/kg b.wt.	-3.75	6.14	46.52	80.21
KrOH 100 mg/kg b.wt.	-6	8	42.66	78.33
Albendazole 20 mg/kg b.wt.	92.15	100	100	100
ECR: Egg count reduction; b.wt: body weight				

Table 5.8: Percent FECR of *H. nana* eggs after treatment in different concentrations of *K. rotunda* rhizome extracts.				
Treatment	Percent FECR in different days			
	4	8	12	15
KrEA 400 mg/kg b.wt.	43.37	67.57	89.04	100
KrEA200 mg/kg b.wt.	10.36	30.18	49.54	78.82
KrOH 200 mg/kg b.wt.	11.19	29.34	46.71	63.7
KrOH 100 mg/kg b.wt.	4.95	13.63	24.79	41.32
Albendazole 20 mg/kg b.wt.	97.24	100	100	100
FECR: Faecal egg count reduction; b.wt: body weight				

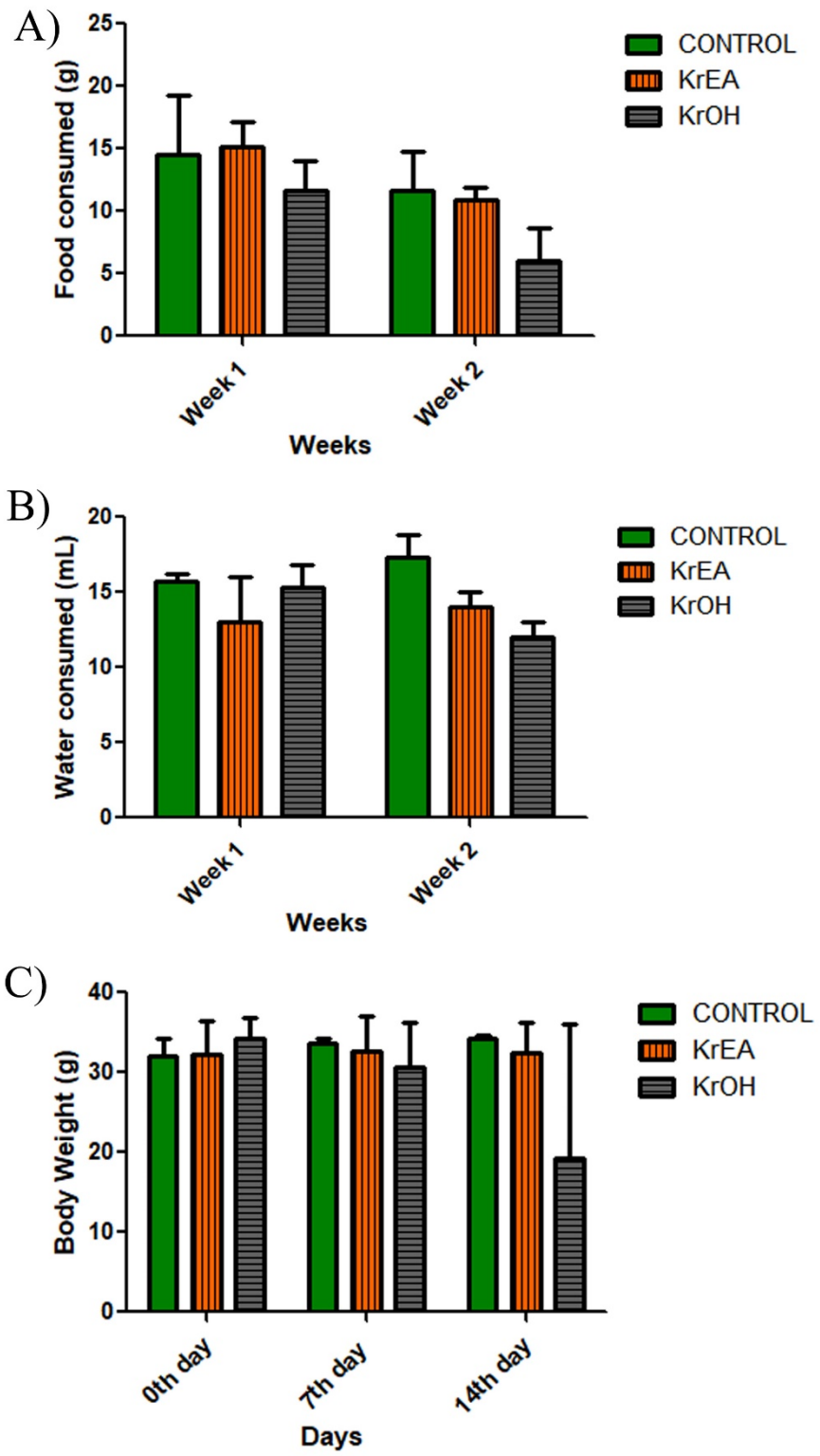

Figure 5.1: A) Food intake, B) Water intake & C) Body weight of female Swiss albino mice during 14 days acute oral administration of KrEA and KrOH rhizome extracts at 2000 mg/kg b.wt. Values are presented as mean ± SD.

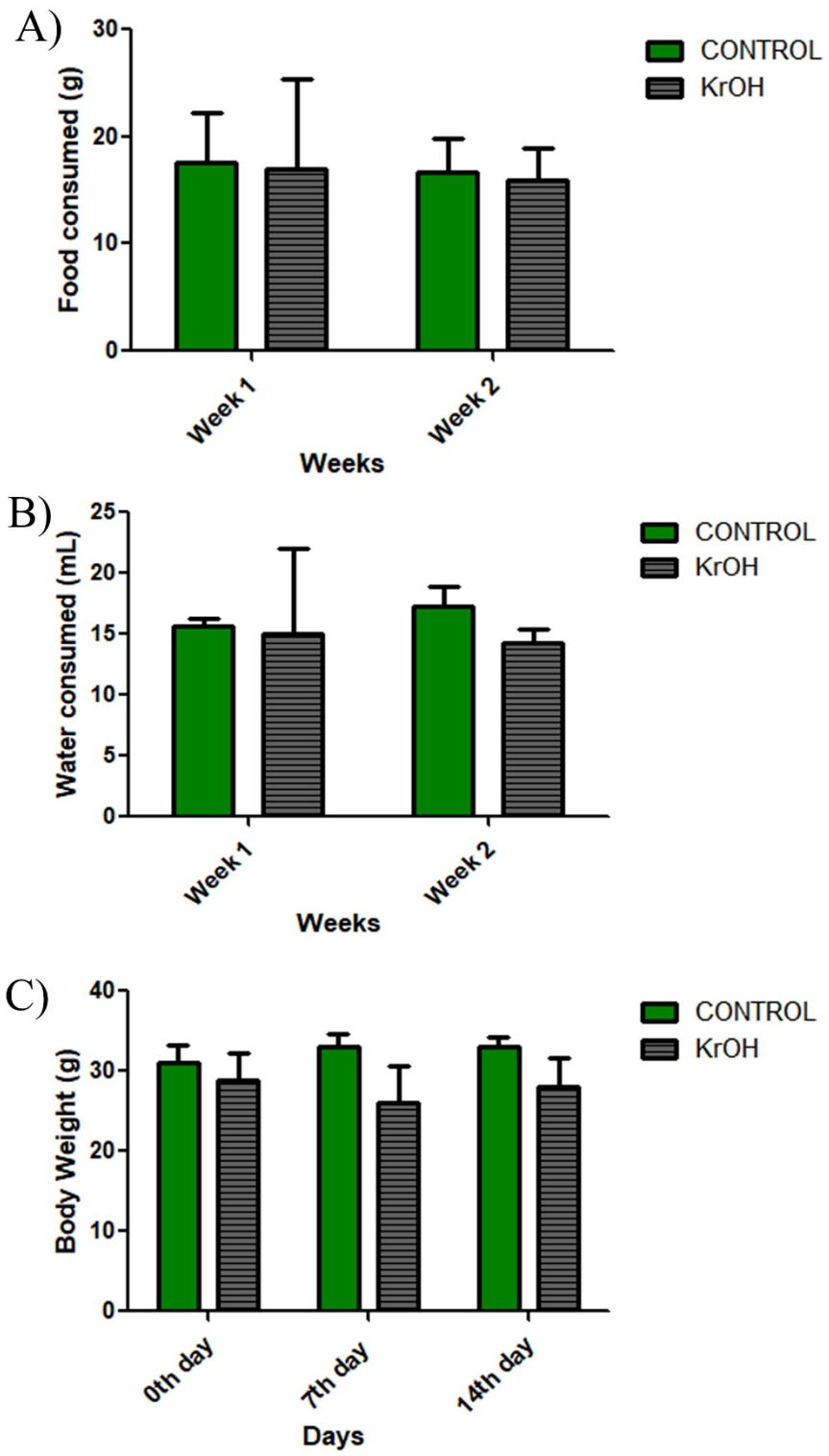

Figure 5.2: A) Food intake, B) Water intake & C) Body weight of female Swiss albino mice during 14 days acute oral administration of KrOH rhizome extract at 1000 mg/kg b.wt. Values are presented as mean ± SD.

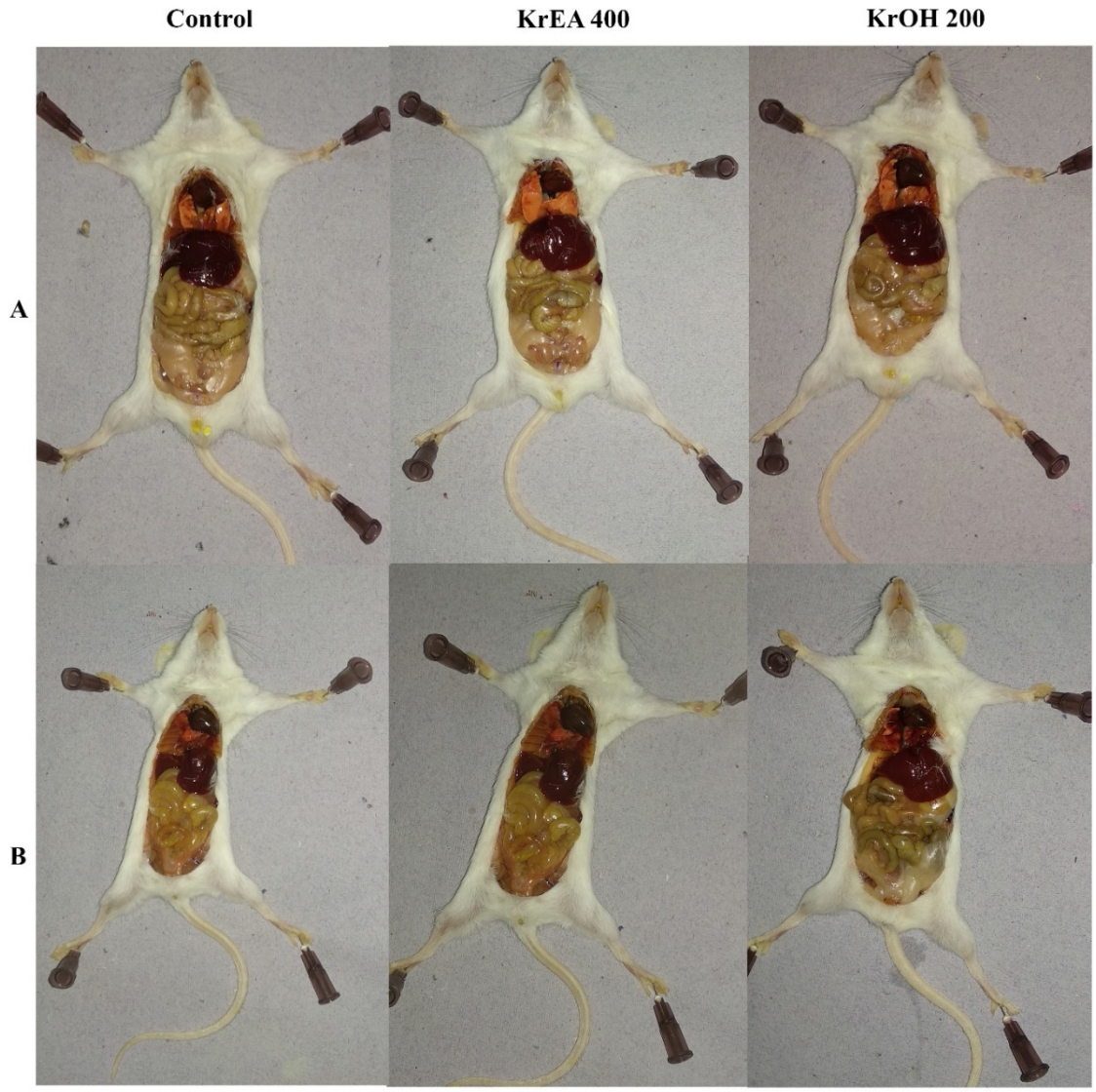

Figure 5.3: Effect of KrEA (400 mg/b.wt) and KrOH (200 mg/b.wt) rhizome extracts on the organs and the body of treated Swiss albino mice post 28 days administration. A) Male and B) Female.

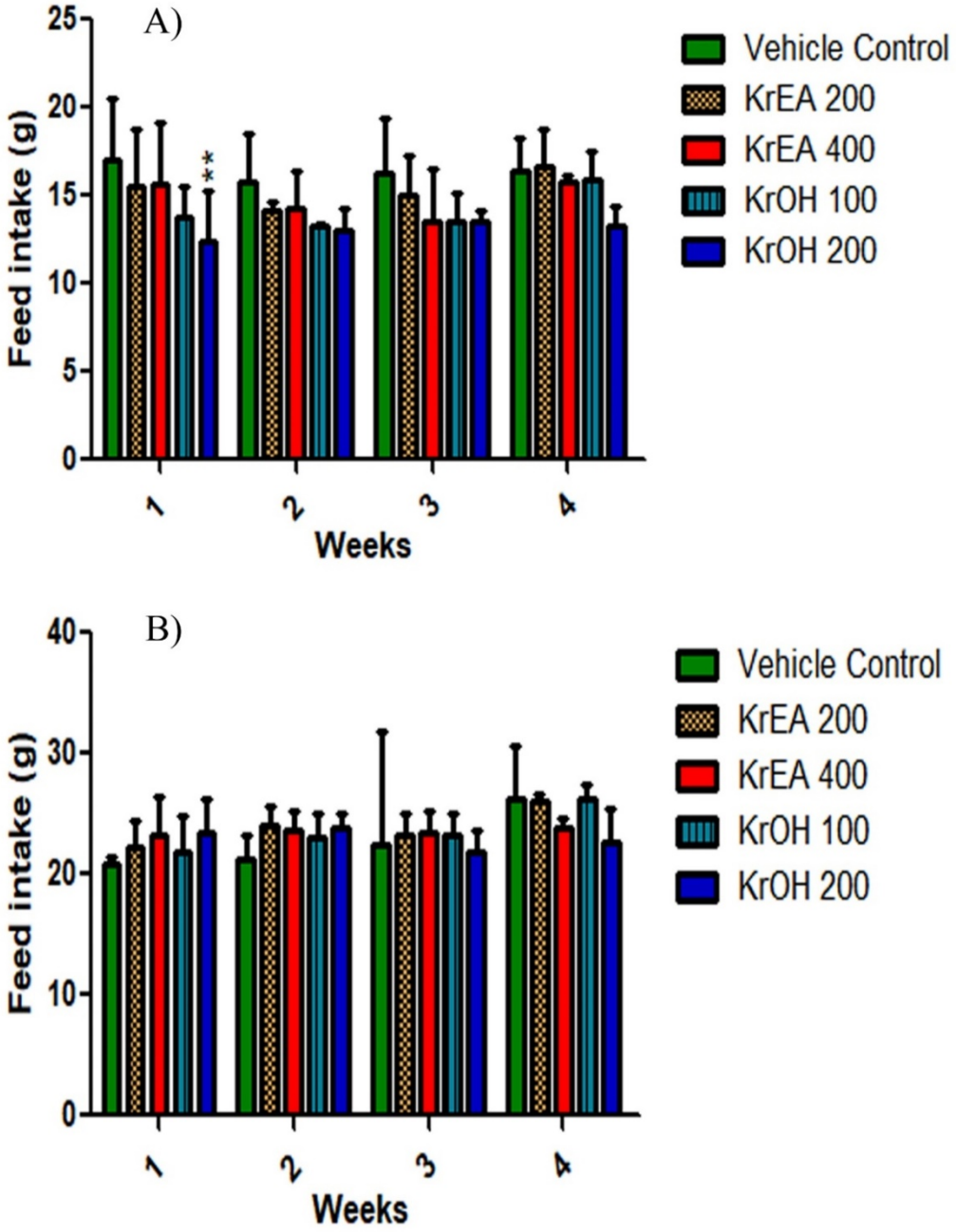

Figure 5.4: Food intake of A) female & B) male Swiss albino mice during 28 days sub-acute oral administration of KrEA and KrOH rhizome extracts. Values are presented as mean ± SD. Significant in relation to the vehicle control at **P < 0.01.

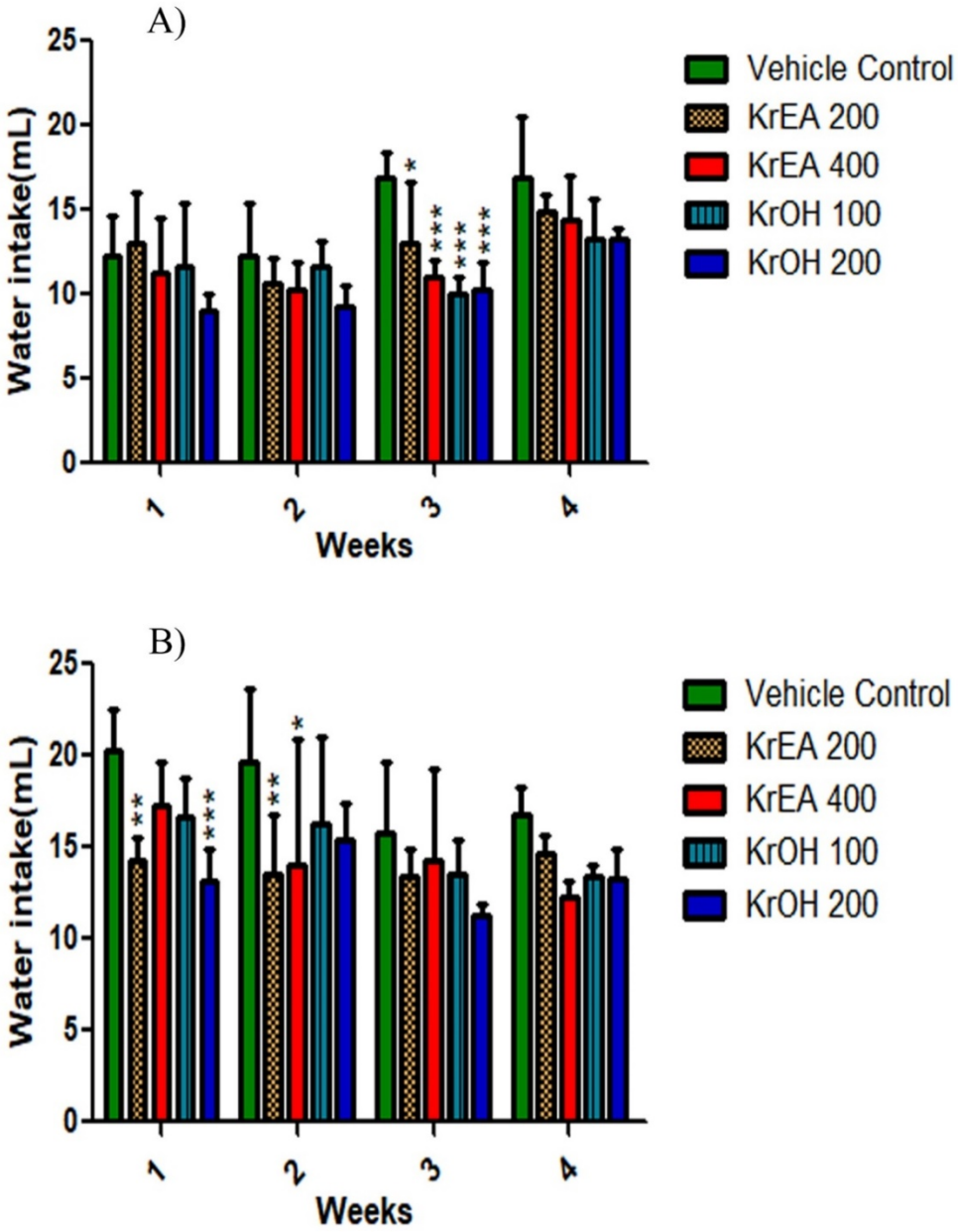

Figure 5.5: Water intake of A) female & B) male Swiss albino mice during 28 days sub-acute oral administration of KrEA and KrOH rhizome extracts. Values are presented as mean ± SD. Significant in relation to the vehicle control at *P < 0.05, ** P < 0.01, *** P < 0.001.

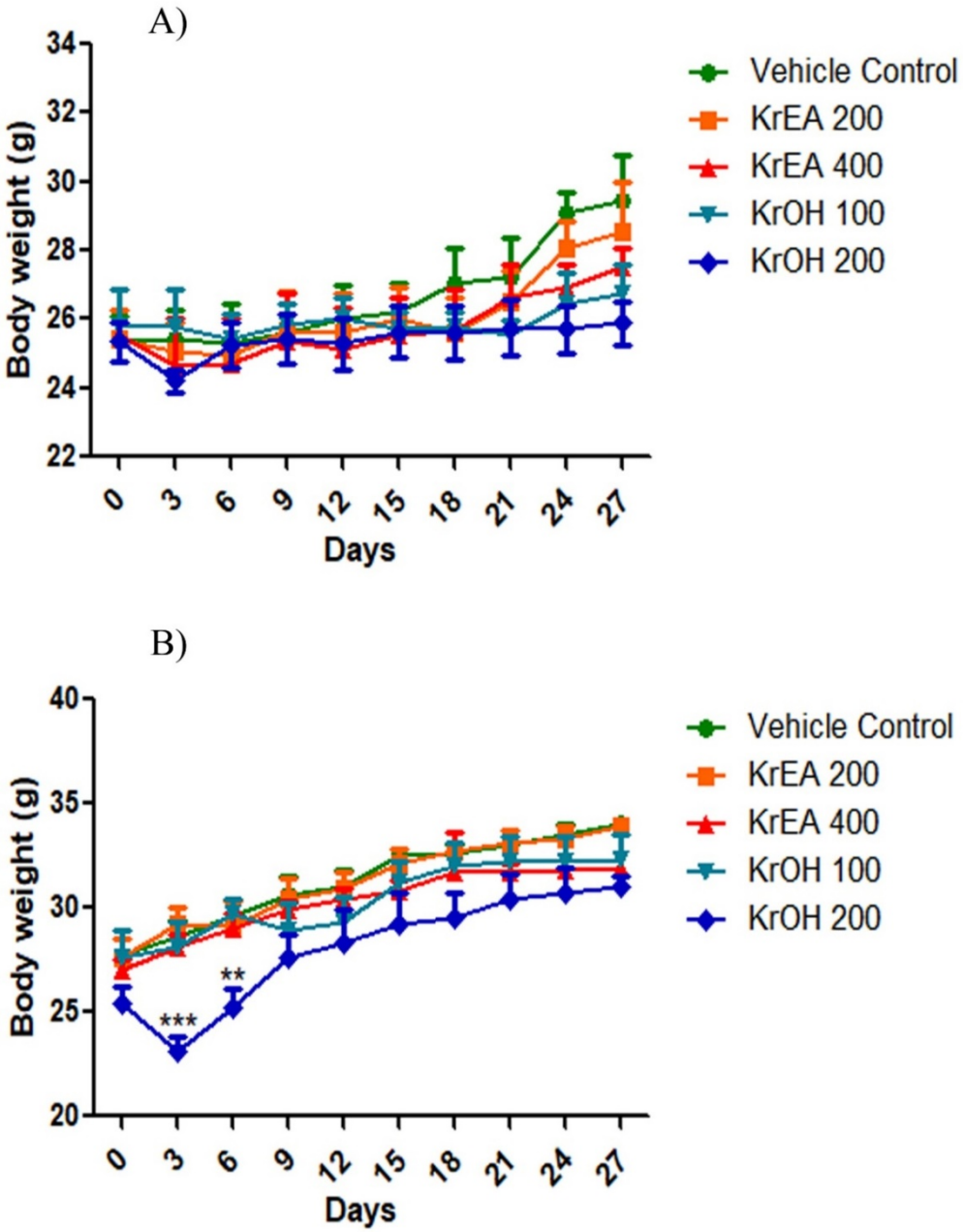

Figure 5.6: Body weight of A) female & B) male Swiss albino mice during 28 days sub-acute oral administration of KrEA and KrOH rhizome extracts. The body weights of the animals were recorded every three days during the study period. Values are presented as mean ± SD. Significant in relation to the vehicle control at **P < 0.01, *** P < 0.001.

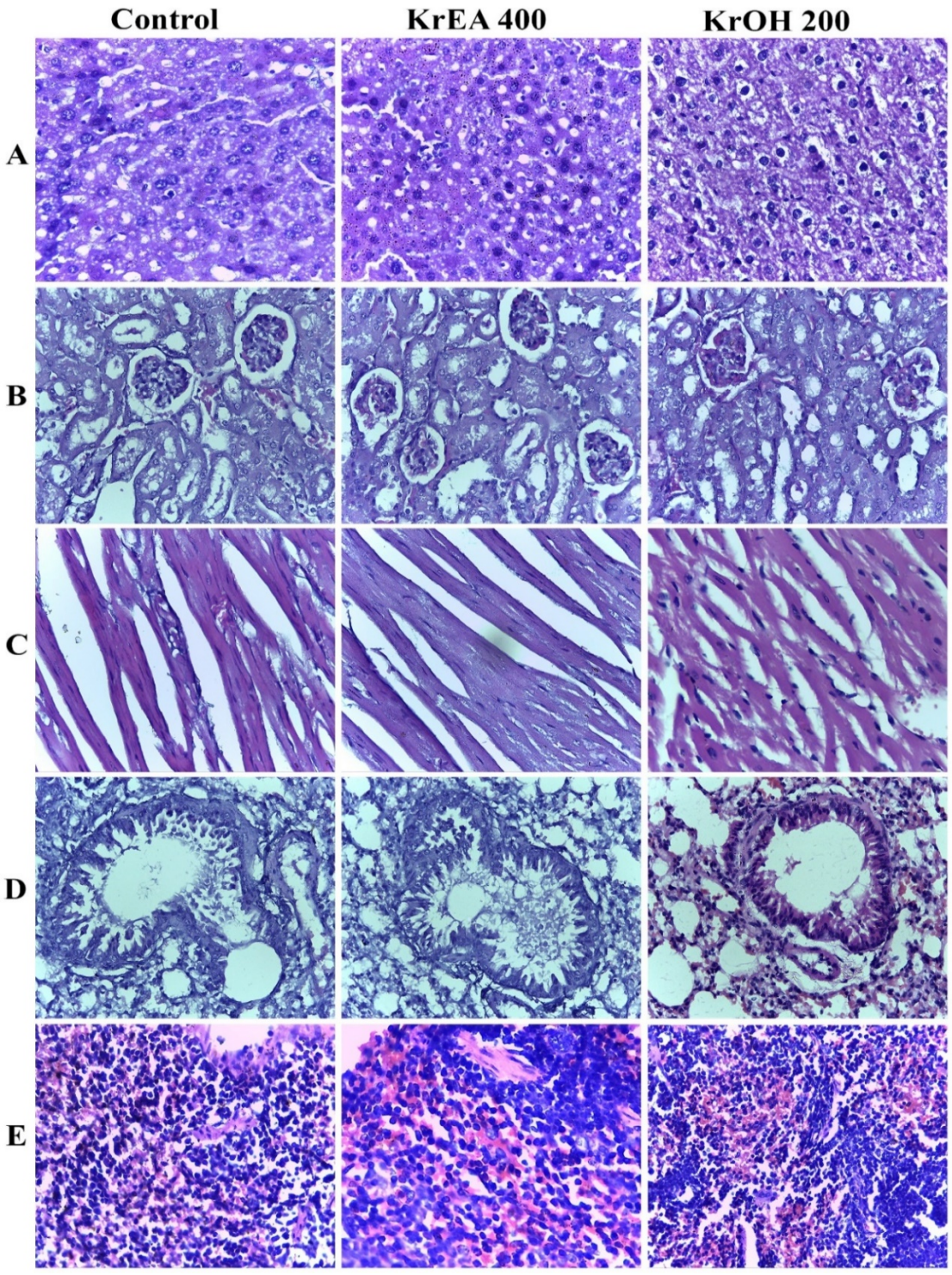

Figure 5.7: Histopathological examination (40X magnification) of vital organs of Swiss albino mice post 28 days treatment in KrEA and KrOH rhizome extracts. Representative microscopic images showing (A) Liver, (B) Kidney, (C) Heart, (D) Lungs and (E) Spleen stained using hematoxylin and eosin stain (H&E).

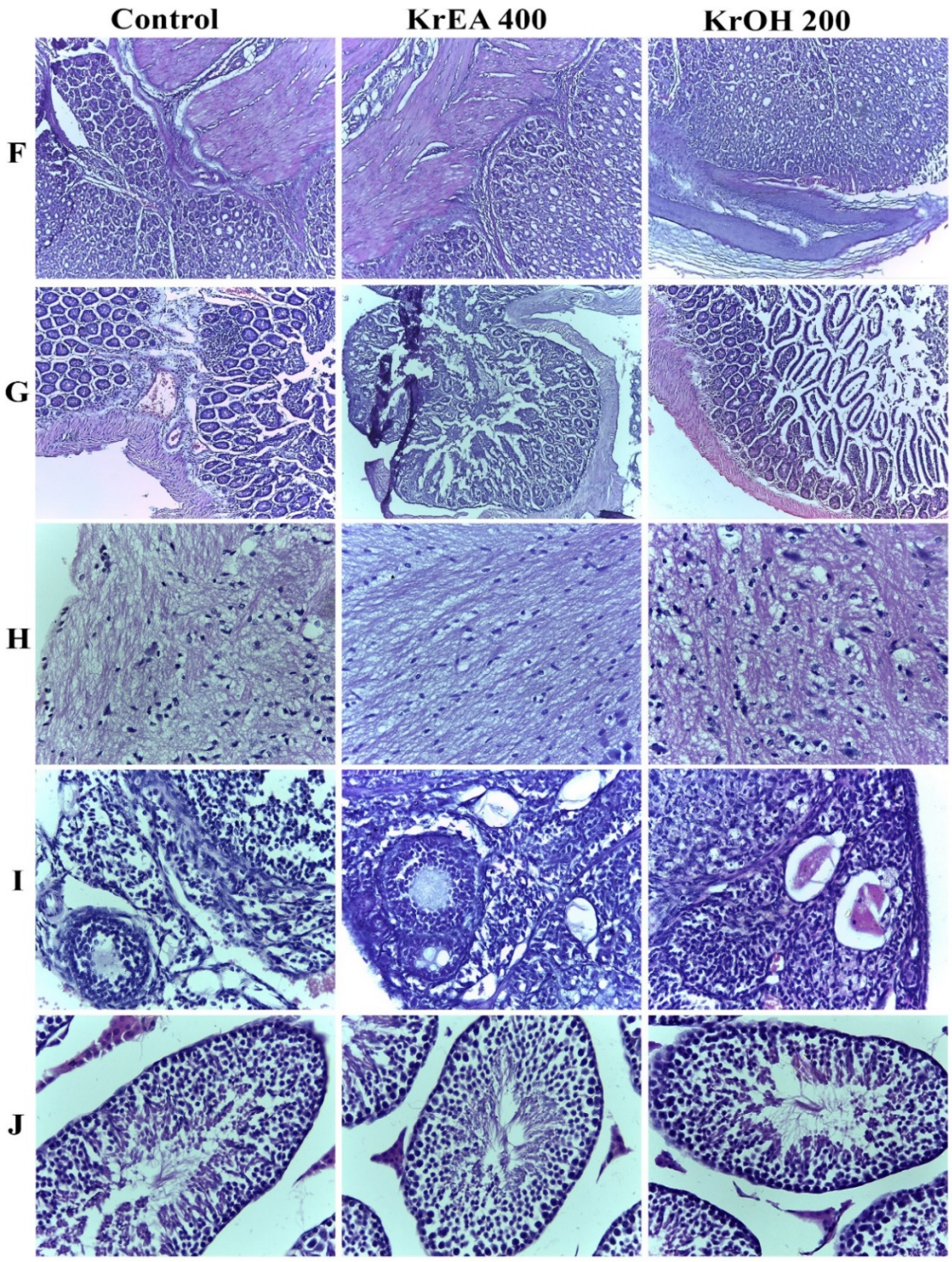

Figure 5.8: Histopathological examination (40X magnification) of vital organs of Swiss albino mice post 28 days treatment in KrEA and KrOH rhizome extracts. Representative microscopic images showing (F) Stomach, (G) Intestine, (H) Brain, (I) Ovary and (J) Testis stained using hematoxylin and eosin stain (H&E).

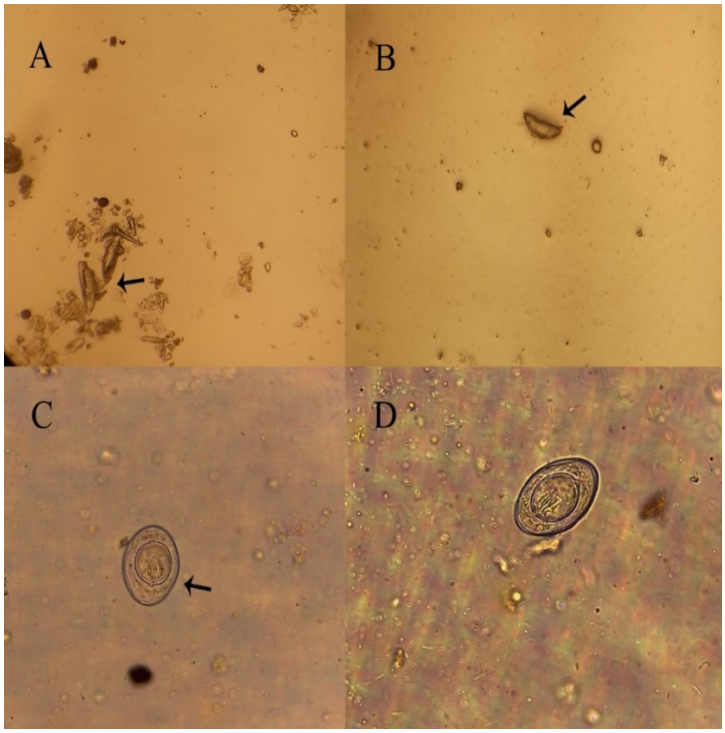

Figure 5.9: Microscopic images(40X magnification) of the nematode, *S. obvelata* (A&B) and the cestode, *H. nana* (C & D) eggs.

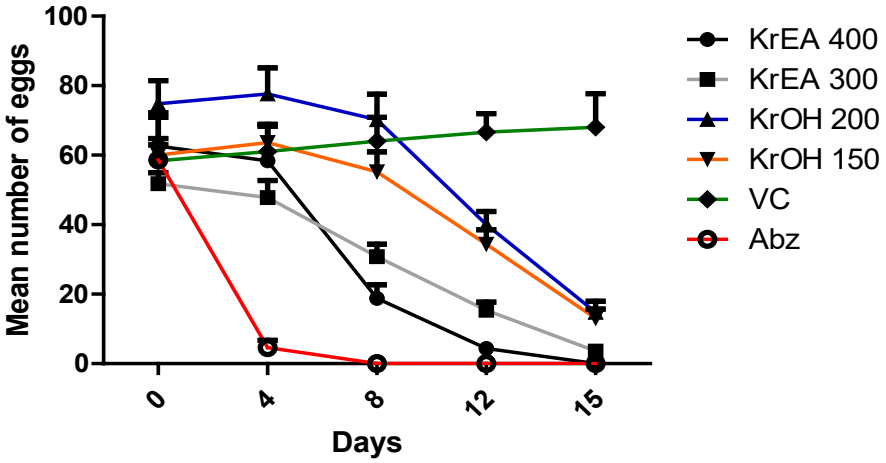

Figure 5.10: *In vivo* anthelmintic potential of *K. rotunda* rhizome extracts against the pin worm, *S. obvelata* in mice. Mean number of *S. obvelata* eggs on different days after the administration of different concentrations ethyl acetate (KrEA 400 and KrEA 200 mg/kg b.wt) and ethanol (KrOH 200 and KrEA 100 mg/kg b.wt) extracts of *K. rotunda*rhizome; 20 mg/kg b.wt albendazole (Abz) as positive control; 1% propylene glycol as vehicle control (VC). Data represented as mean ± SEM. SEM: Standard error of the mean.

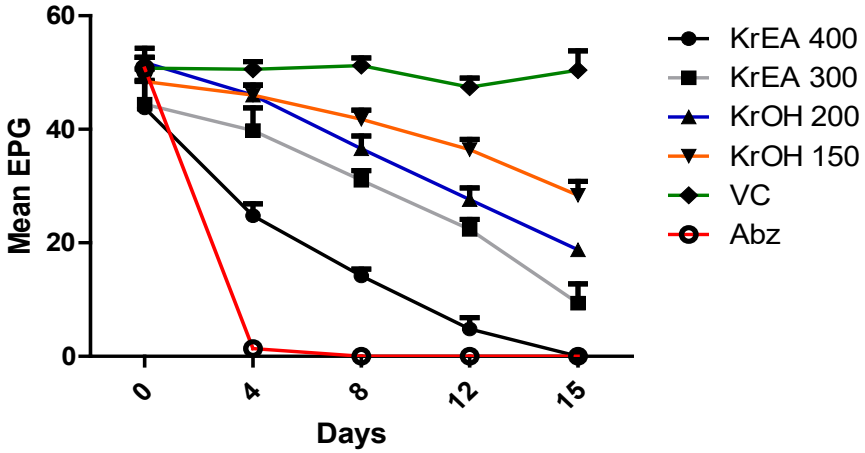

Figure 5.11: *In vivo* anthelmintic potential of *K. rotunda* rhizome extracts against the tape worm, *H. nana* in mice. Mean EPG faeces of *H. nana* on different days after the administration of different concentrations ethyl acetate (KrEA 400 and KrEA 200 mg/kg b.wt) and ethanol (KrOH 200 and KrEA 100 mg/kg b.wt) extracts of *K. rotunda* rhizome; 20 mg/kg b.wt albendazole (Abz) as positive control; 1% propylene glycol as vehicle control (VC). Data represented as mean ± SEM. EPG: Eggs per gram of faeces, SEM: Standard error of the mean.

CHAPTER 6

Antibacterial and anthelmintic potential of endophytes isolated from *Kaempferia rotunda*

Introduction

Plants do not live alone as single entities rather closely associated with the microorganisms that reside both externally and, especially with those living internally (Hardoim et al. 2015). Multiple microbial communities such as bacteria, archaea, and fungi reside inside healthy plant tissues without producing any prominent disease or clinical symptoms in their host can be considered as endophytes (Andrews & Hirano 2012). They devote their entire life or a part of the life cycle in the symplast or apoplast region of healthy plant tissues (Andrews & Hirano 2012; Gond et al. 2012). Endophytes contribute to the overall growth, development, fitness, and diversification of plants (Hardoim et al. 2015). They provide enhanced competitive abilities, increased resistance to herbivores, pathogens, and various abiotic stresses that negatively affect the health and survival of their host (Singh et al. 2011). Endophytes are now considered as a repository of novel bioactive natural compounds. There are several recent studies which illustrate the importance of endophytes as a new reserve of antibacterial and other bioactive molecules (Xie et al. 2018; Wu et al. 2018). Over 8,600 bioactive metabolites of fungal origin have been described and it was also reported that they are able to make the same secondary metabolites as the host plant itself (Verma 2009; Singh et al. 2011).

Methodology

Collection and identification of plant material

Plant materials were collected and identified according to the methods outlined in Methods section 1.0.

Isolation and morphological identification of fungal endophytes

Isolation of endophytic fungi was done according to the method described previously by Ezra et al. (2004). Methods section 8.1 explains the detailed procedure

of endophytic fungal isolation from the rhizome of *K. rotunda*. Only those endophytic fungi that have been obtained during repeated isolation were further taken for morphological and molecular identification.

Identification of the endophytes using molecular taxonomic approach

The clone purified fungal cultures were used for molecular taxonomic studies. Ribotyping targets, especially the large-subunit rDNA gene (D1–D2) and Internal Transcribed Spacer (ITS) region were used for the molecular identification of isolated fungi. Methods sections 8.2 and 8.3 provide detailed information on the isolation, amplification, and sequencing of fungal genomes.

Fermentation and solvent extraction of endophytes

The isolated colonies were cultured in Potato Dextrose Broth (PDB) for 2 weeks. These fermented broths were then repeatedly extracted with the same volume of ethyl acetate. The solvent extracts were then combined and evaporated to dryness by a rotary evaporator (KNF Rotary evaporator RC 600) giving a final yield of about 0.8%–1.1% (Gond et al. 2012). The detailed methodology for the solvent extraction is described in Methods section 8.4.

Qualitative phytochemical screening

The concentrated crude extracts were used for the screening of alkaloids, polyphenols, tannins, flavonoids, terpenoids, saponins and glycosides according to the standard procedures (Trease & Evans 1989; Sofowora 1993; Harborne 1999). Detailed procedure of qualitative phytochemical screening is described in Methods section 3.1.

Quantification of Total Phenolic Content (TPC)

The TPC was estimated using Folin-Ciocalteau spectrophotometric technique proposed by Singleton and Rossi (1965). The estimation of TPC is described in Methods section 3.2.2.

Estimation of Total Flavonoid Content (TFC)

The TFC of endophytic fungal extracts was estimated using the aluminium chloride colorimetric method previously described by Chang et al. (2002) with minor changes. The estimation of TFC is described in Methods section 3.2.3.

HPLC Mediated Polyphenol Profiling of the Extracts

Polyphenol profiling and quantification was executed using the high-performance liquid chromatography (HPLC). Methods section 3.2.4 includes a detailed description of the methodology for the estimation of polyphenols.

Antibacterial Studies

Agar disk diffusion assay

The isolated endophytic fungi were evaluated for their antibacterial activity against seven strains of bacteria mentioned earlier. The disk diffusion assay was performed according to the method described in Methods section4.2.

Anthelmintic Studies

***In vitro* egg hatch assay and larval paralysis assays**

Egg hatch assay (EHA)

Faecal pellets were collected from the rectum of a naturally infected donor goat (Getachew et al. 2012) were then subjected for the centrifugal floatation (Kozan et al. 2006) for the collection of fresh eggs. Methods section 6.1.1 and 6.1.2 provided details of the faecal egg floatation and egg hatch inhibition assays.

Larval paralysis assay (LPA)

The LPA inhibition assay was based on the method previously described by Varady & Corba (1999) with modifications. Methods section 6.1.3 describes the larval paralysis assay in detail.

The *in silico* studies

The *in silico* docking studies of various polyphenols such as quercetin, myrcetin, kaempferol, ellagic acid, apigenin, chlorogenic acid and gallic acid that are commonly observed in plant rhizomes and bioactive endophytic fungal extracts were performed against different tubulin targets of nematodes. The 3D structures of bioactive polyphenols such as quercetin, myrcetin, kaempferol, ellagic acid, apigenin, chlorogenic acid and gallic acid were retrieved from PUBCHEM. The .pdbqt files were prepared using the computational MGL tool and molecular docking studies were carried out by using the AutodockVina 1.5.7. A detailed description of methodology is provided in Methods section 9.0.

Statistical Analysis

The differences in mean between extracts treated and vehicle control were determined using one-way ANOVA in Graph pad Prism software version 5. The effective concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) mortality to the nematode eggs and L3 larvae was calculated using using probit analysis in SPSS software version 24.

Results

Isolation and Identification of Endophytic Fungi

Rhizomes of *K. rotunda* L. were aseptically processed to investigate the presence of endophytic fungi. A total of three endophytic fungi were isolated to pure culture from the rhizomes of *K. rotunda* (Table 6.1). The culture characteristics and

lactophenol cotton blue staining followed by microscopic observations were used for morphological identification of the endophytic fungal isolates. Interestingly all the three isolates that showed repeated appearance in the rhizome exhibited features of the genus *Aspergillus*.

The KMPRO1 isolate appears pale grey coloured with numerous radial lines on PDA medium. The conidiophore ends up with globular heads. In KMPRO2, the conidiophores were heavy walled, pale green coloured, coarsely roughened and approximately 1 mm in length. Also, the phialides appeared biseriate. The KMPRH1 showed copious aerial mycelia with ash black appearance on PDA media. The distal ends of conidiophores were marked by the formation of round structure, called as the conidiophore head. The spherical conidia grew from these conidiophore head (Figure 6.1).

Molecular sequencing method followed by NCBI blast analysis was also employed for the identification of fungal isolates. Based on the D1/D2 region-PCR analysis, the nucleotide sequence of the fungal culture KMPRO2 showed 100% homology with *Aspergillus flavus* (Table 6.1). Therefore, by combined analysis of the fungal morphological characters according to Genera of Hyphomycetes (Carmichael et al. 1980), the strain KMPRO2 is identified as *A. flavus* (Gen Bank accession number MW684712). Similarly, another gene target, ITS was used for the identification of the rest of the endophytes isolated. The KMPRO1 and KMPRH1 isolated from *K. rotunda* rhizomes did not show 100% similarity with any of the available sequences in the GenBank. However, KMPRO1 isolate showed 91% sequence similarity with *Aspergillus assiutensis* and KMPRH1 isolate showed 90% sequence similarity with *Aspergillus tubingenesis*.

KMPR02 D1/D2 gene sequence (576bp):

GTAGGTGAACCTGCGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAAC
CTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCC
GCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCT
GATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGG
ATCTCTTGGTTCCGGTCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATT
GCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCC
GGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTG
GGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGT
CCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTT
GCCGAACGCAAATCAATCTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGC
TGAACTTAAGCATATC

KMPR1-ITS gene sequence (604bp):

ATTGATATGCTTAAGTTCAGCGGGCATCCCTACCTGATCCGAGGTCAATCTGAGAA
GATTGGGGGTCGAGGCAAGCCCCGGCCGGGCCCATAGAGCGGGTGACAGAGCCCCA
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CGCGGCCCAACAACCAGCGGGGCTGGAGGGGAGAAATGACGCTCGGACAGGCATGC
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KMPR2-ITS gene sequence (512bp):

TCTTTGGCGGAACCACCCATCCGTGTCTATTATAACCTGTTGCTTCGGCGGGCACG
CCGCTTGTCGGCCGCCGGGGGGGCGCCTTTGCCACCCGGGCCCGTGCCCGCCGGAG
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AAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCT
GTGTAGGATCGGCTCAATAAGCCGAGGATCATATCAATCAGCGGAGCAGCAGTCAA
TAAATCGA

Qualitative phytochemical screening

Qualitative phytochemical screening showed the presence of different plant secondary metabolites in the tested endophyte fungal extracts (Table 6.2). Phenolic

and flavonoid classes of secondary metabolites were present in all the tested extracts. Saponins were altogether absent in all the endophytic extracts tested.

Estimation of Total Phenol Content (TPC) and Total Flavonoid Content (TFC)

The extracts from KMPRH1 showed high TPC and TFC values. This was followed by KMPRO2 (Table 6.3). Same time low phenol and flavonoid content were observed with the extracts of KMPRO1.

HPLC Mediated Polyphenol Profiling of the Extracts

Thirteen standard reference polyphenolic compounds; gallic acid, catechol, chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, myricetin, cinnamic acid, quercetin, kaempferol, and apigenin were initially analyzed by HPLC at 1 mg/ml concentration. Retention time was noted and later used for the identification of these molecules in the extracts. The phenolic compounds detected were reconfirmed and quantified by spiking with the individual standards. The KMPRO2 from *K. rotunda* showed the presence of highest polyphenolic abundance. It showed high amounts of myricetin, kaempferol, syringic acid, ellagic acid, ferulic acid, P-coumaric acid and caffeic acid. The quantity of myricetin, ellagic acid and syringic acid was also found high in the KMPRH1 isolate of *K. rotunda*. Comparatively the KMPRO1 showed low polyphenolic abundance (Table 6.4).The chromatograms of different endophytic fungal extracts from *K. rotunda* were showed in figure 6.2.

Antibacterial Activity of Endophytic Fungi

Culture extracts of all the three endophytes from *K. rotunda* were tested for antibacterial activity by disc diffusion assay against seven strains of bacteria (*E. coli*, *S. aureus*, *K. pneumoniae*, *S. flexneri*, *S. marcescens, B. cereus*, and *E. faecalis*). The fungal isolates from *K. rotunda*, KMPR02 and KMPRH1 extracts inhibited five out

of seven bacterial strains. Also, prominent activity was observed against Gram-positive strains (Table 6.5). In general, two Gram-negative bacterial strains *S. flexneri* and *S. marcescens* showed strong resistance against the fungal extracts. None of the tested extracts were effective against these Gram-negative bacteria. On the other hand, *S. aureus* was found to be most susceptible and was found to be inhibited by majority of the endophytic fungal extracts (Table 6.5).

Anthelmintic Activity

The anthelmintic potential of endophytic fungi isolated from the rhizomes of *K. rotunda* was studied using egg hatch inhibition assay and larval paralysis assay. Table 6.6 shows the results of the egg hatch inhibition assay. Among the endophytic fungal isolates from *K. rotunda*, KMPR02 and KMPRH1 showed a concentration dependent inhibition to the egg hatch. At the highest tested concentration (10 mg/ml), the KMPR02 and KMPRH1 showed 91.6 ± 0.36 and 88.49 ± 1.57 percent egg hatch inhibition respectively.

Probit analysis calculated the effective concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) mortality to the treated eggs (Table 6.8). Among the isolates from *K. rotunda*, KMPRO2 and KMPRH1 showed low EC_{50} values. Whereas that of KMPRO1 exceeded the maximum tested concentration used in the current study.

The *In vitro* larval paralysis assay envisaged the potential of extracts from endophytic fungi in causing paralysis to the 3rdinstar *H. contortus* larvae. The isolates from *K. rotunda* were found effective against the *H. contortus* larvae with a maximum paralysis of 93.89 ± 0.11 and 92.39 ± 0.88 percent respectively, with the extracts of KMPRH1 and KMPRO2 isolates at 10 mg/ml concentration. The mortality induced by these two fungal extracts was statistically significant at all the tested concentrations when compared with vehicle control (Table 6.7). Crude extracts of KMPRH1 and

KMPRO2 induced 50% lethality to the *H. contortus* larvae at 2.14 and 2.29 mg/ml respectively, within 24 hours treatment (Table 6.8).

Molecular Docking Studies

The *in silico* molecular docking results of major phenolic compounds and the standard drug albendazole on to different nematode targets are shown in table 6.9 and depicted in figure 6.3. The docking of all the seven phenolic ligand molecules showed noticeable binding affinity against different tubulin targets. Among these the highest binding energy was exhibited by myricetin, quercetin, ellagic acid, kaempferol and apigenin (Table 6.9). It was also interesting to note that the affinity was higher when the combined α-β subunits was used as the target.

Discussion

Many previous studies have reported the occurrence of fungal endophytes of medicinal importance residing inside different regions of plants (Gouda et al. 2016; Nisa et al. 2020). The present study was carried out to isolate, identify, and evaluate the antibacterial and nematicidal potentials of endophytic fungi from *K. rotunda* L. rhizomes. We have isolated three species of endophytic fungi that belongs to the genus *Aspergillus*. KMPRO2 isolated from *K. rotunda* was identified as *A. flavus*. Also, KMPRO1 and KMPRH1 isolates showed 91% and 90% homology with *A. assiutensis* and *A. tubingenesis* respectively.

Aspergillus is a ubiquitous genus that consists of various fungal species having pathological as well as therapeutic importance (Vadlapudi et al. 2017). Different species under the genus *Aspergillus* have been reported in many previous studies as fermentation agents, producers of beneficial enzymes and organic acids, antibacterial and antifungal agents (Brook 1994; Oxenboll 1994; Rolinson 1998; Karaffa & Kubicek 2003; Abarca et al. 2004; Samson et al. 2014). Many efforts have been made

regarding the effective use of the fungal genus *Aspergillus* in controlling different plant pathogenic fungi (Aukkasarakul et al. 2014; Szilagyiet al. 2012). The KMPR02 (*A. flavus*) and KMPRH1 (*Aspergillus* sps.) isolates shows reasonable inhibition against both Gram-positive and Gram-negative bacterial strains. Same time, majority of the tested bacterial strains exhibited resistance against KMPRO1 (*Aspergillus* sps.) isolate. The present study also highlighted the high resistance of two Gram-negative bacterial strains *S. flexneri* and *S. marcescens* against endophytic fungal extracts from both plants. None of the endophytic fungal extracts were active against these two Gram-negative bacteria.

Studies on the use of endophytic fungal metabolites against animal parasitic nematodes are very rare. Hence, we decided to extend our studies on the nematicidal activity of the endophytic fungal extracts against the eggs and 3rd instar larvae of *H. contortus*. Our study clearly envisages the potentials of the ethyl acetate extracts of *A. flavus* and KMPRH1 (*Aspergillus* sps.) isolated from the *K. rotunda* rhizomes in causing significant egg hatch inhibition and larval paralysis activities. A concentration-dependent increase in the death toll of nematode larvae was observable with these tested extracts. The other fungal isolate, KMPRO1 showed negligible activity against the *H. contortus*.

Phenols and terpenoids are indeed considered as safe alternatives to commercial anthelmintic drugs and several studies have explained the importance of plant derived flavonoids against worms (Mukherjee et al. 2016). Flavonoids such as genistein, quercetin, and kaempferol induced high mortality to nematodes and trematodes in several earlier studies (Braguine et al. 2012; Rajesh et al. 2016). In the present study, the preliminary qualitative screening and estimation of TPC and TFC showed reasonable amounts of secondary metabolites especially phenolic class of

metabolites in the fungal extracts. In order to identify and characterize the polyphenolic compounds of fungal extracts, we took the advantage of HPLC analysis. The HPLC studies of our extracts confirmed the presence of at least six different phenolic compounds in a considerable quantity. It was further revealed that the pattern of presence of these molecules was comparable between the rhizome and its endophytes. Myricetin was the most abundant molecule in the rhizome extracts and the endophyte extracts. *Kaempferia rotunda* rhizome, which is known for its aroma, was having higher polyphenolic content so also its endophyte isolates. Out of the nine polyphenolic molecules detected, four of them having more than 0.5 mg per gram of the extract were identified in the rhizome and endophyte extracts.

Microtubules are involved in diverse cell functions including cell division, cell motility and cytoplasmic transport in eukaryotic cells. Beta-tubulin protein, the monomer of microtubules in nematode parasites, seems to be a lucrative drug target for anthelmintic drugs including our reference molecule, albendazole. Albendazole preferably binds to the colchicine-sensitive site of tubulin (tubulin β2 chain) of nematodes and inhibits the polymerization and formation of microtubules. The *in silico* molecular docking studies performed using AutoDockVina software was consistent with these previous reports. Further studies showed strong binding efficiency of several of the phenolic compounds detected in our extracts against the colchicine-sensitive site of β tubulin. Myricetin, quercetin, ellagic acid, kaempferol, apigenin, chlorogenic acid and gallic acid were present in both rhizome extracts and endophytic fungal extracts. Among these ligand molecules, except chlorogenic acid and gallic acid, all other molecules showed higher binding affinity to tubulin (α+β) than the reference anthelmintic drug albendazole.

Table 6.1 : Endophytic fungi isolated from the rhizomes of *K. rotunda*							
SI. No	Host plant	Plant part used for isolation	Isolated endophytic fungi	Identification according to D1/D2 gene sequences	Identification according to ITS gene sequences	Gen Bank Deposit name	Gen Bank Accession number
5	*K. rotunda*	Rhizome	KMPRO1	NI	*Aspergillus* sps	KMPR1	OL655392
6	*K. rotunda*	Rhizome	KMPRO2	*Aspergillus flavus*	-	KMPRO2	MW684712
7	*K. rotunda*	Rhizome	KMPRH1	NI	*Aspergillus* sps	KMPR2	OL655393
NI: Not Identified							

Table 6.2: Qualitative phytochemical analysis of the fungal extracts			
Type of Phytochemicals	***Kaempferia rotunda***		
	KMPRO1	KMPRO2	KMPRH1
Alkaloids	-	+	+
Flavonoids	+	+	+
Polyphenols	+	+	+
Tannins	+	+	+
Saponins	-	-	-
Terpenoids	+	-	+
Glycosides	+	+	-
Steroids	-	+	+
Coumarins	+	+	+
+ Present, -absent			

Table 6.3: Total phenol content (TPC) and total flavonoid content (TFC) of the fungal extracts			
Name of the Plant	Endophytic fungi	TPC (mg GAE/g)	TFC (mg QCE/g)
K. rotunda	KMPRO1	4.78 ± 2.14	3.21 ± 1.21
	KMPRO2	30. 14 ± 0.28	29.16 ± 0.55
	KMPRH1	32.47 ± 1.15	31.34 ± 1.18

Table 6.4 : Estimation and quantification of polyphenols in the endophytic fungal extracts of *K. rotunda* rhizomes

Sl No	Phytochemical standards	KMPRO1	KMPRO2	KMPRH1
1	Catechol	ND	ND	ND
2	Chlorogenic acid	ND	ND	297.647
3	Caffeic acid	116.567	1000.64	123.706
4	Syringic acid	641.067	3470.21	2541.38
5	P-Coumaric acid	404.5	1340.12	127.588
6	Ferulic acid	115.267	1960.21	207.559
7	Ellagic acid	444.533	3215.93	2800.76
8	Myricetin	ND	3897.1	2911.18
9	Cinnamic acid	165.233	ND	160
10	Quercetin	ND	ND	ND
11	Kaempferol	ND	3714.14	ND
12	Apigenin	1024.23	ND	ND
13	Gallic acid	ND	ND	94.268

Values are amount of the polyphenolic molecule in µg per gram of the extract; ND: Not Detected.

Table 6.5 : Antibacterial activity of endophytic fungal extracts against different bacteria							
Endophytic fungi	The diameter (in mm) of zone of inhibition						
	S. aureus	*E.coli*	*K. pneumoniae*	*S. flexneri*	*S.marcescens*	*B. cereus*	*E. faecalis*
KMPRO1	9.16 ± 0.65	0	0	0	0	0	8.11 ± 0.08
KMPRO2	13.17 ± 0.47	13.83 ± 0.79	6 ± 0.44	0	0	9.33 ± 0.1	10 ± 0.22
KMPRH1	15.16 ± 0.30	9.5 ± 0.42	6 ± 0.90	0	0	7 ± 0.84	8.54 ± 0.88
DMSO (1%)	0	0	0	0	0	0	0
Amoxicillin (10µg/disc)	28 ± 0.24	31.15 ± 0.63	8 ± 0.88	26 ± 0.13	24 ± 0.11	21 ± 0.34	21 ± 0.26
Values are mean ± SEM.							

Table 6.6 : Mean percent egg hatch inhibition of *H. contortus* eggs treated in different concentrations of endophytic fungal extracts			
Concentration (mg/ml)	Endophytic fungi		
	KMPRO1	KMPR02	KMPRH1
10	5.89 ± 0.16*	91.16 ± 0.36**	88.49 ± 1.57**
5	2.63 ± 0.28^{ns}	78.73 ± 2.23**	70.21 ± 1.11**
2.5	0^{ns}	56.41 ± 2.55**	52.15 ± 2.25**
1.25	0^{ns}	21.92 ± 0.97**	16.79 ± 1.13**
Albendazole (50 µg/ml)	99 ± 1.18	100 ± 0	98.5 ± 0.19
DMSO (1%)	0	0.35 ± 0.18	0
Values are expressed as mean ± SEM. *p < 0.05 and **p < 0.001, when compared to control (1% DMSO); ns: non-significant, where, p>0.05.			

Table 6.7 : Mean percent larval paralysis of *H. contortus* L3 larvae treated in different concentrations of endophytic fungal extracts			
Concentration (mg/ml)	Endophytic fungi		
	KMPRO1	KMPR02	KMPRH1
10	4.6 ± 1.45*	92.39 ± 0.88**	93.89 ± 0.11**
5	3.5 ± 1.14^{ns}	76.18 ± 0.21**	78.61 ± 0.87**
2.5	0^{ns}	58.31 ± 0.28**	53.89 ± 0.63**
1.25	0^{ns}	24.81 ± 0.0.56**	31.56 ± 0.33**
Albendazole	93± 0.66	100 ± 0	100 ± 0
DMSO (1%)	0.81 ± 0.45	0.86 ± 0.50	0.75 ± 0.81
Values are expressed as mean ± SEM. *p < 0.05 and **p < 0.001, when compared to control (1% DMSO); ns: non-significant, where, p>0.05.			

Table 6.8: EC_{50} and EC_{90} values (mg/ml) of endophytic fungi from *K. rotunda* rhizomes against *H. contortus* egg and L3 larvae.

	Egg hatch inhibition		Larval paralysis assay	
Extract used	EC_{50} (LCL-UCL)	EC_{90} (LCL-UCL)	EC_{50} (LCL-UCL)	EC_{90} (LCL-UCL)
KMPRO1	> 10	> 10	> 10	> 10
KMPRO2	2.39 (2.04 – 2.74)	8.3 (6.76 – 11)	2.29 (1.94 – 2.65)	8.26 (6.77 – 11.31)
KMPRH1	2.82 (2.43 – 3.24)	≥ 10 (8.15 – 13.88)	2.14 (1.80 – 2.49)	8.13 (6.53 – 11.06)

EC: Effective concentration; LCL: Lower confidence limit; UCL: Upper confidence limit

Table 6.9: Binding affinity (Kcal/mol) of phenolic compounds and albendazole on various tubulin (PDB ID: 6E88) targets of nematodes.

Polyphenols from extracts + Standard Drug (Albendazole)	Tubulin(α +β)	Tubulin α 1A chain	Tubulin β2 chain
Quercetin	-8.7	-7.1	-7.1
Myrcetin	-9.2	-7.1	-7.1
Kaempferol	-8.3	-7.0	-6.4
Ellagic acid	-8.8	-6.3	-6.2
Apigenin	-8.3	-6.8	-6.5
Chlorogenic acid	-5.7	-6.0	-6.7
Gallic Acid	-6.4	-6.4	-7.0
Albendazole	-7.4	-5.9	-6.6

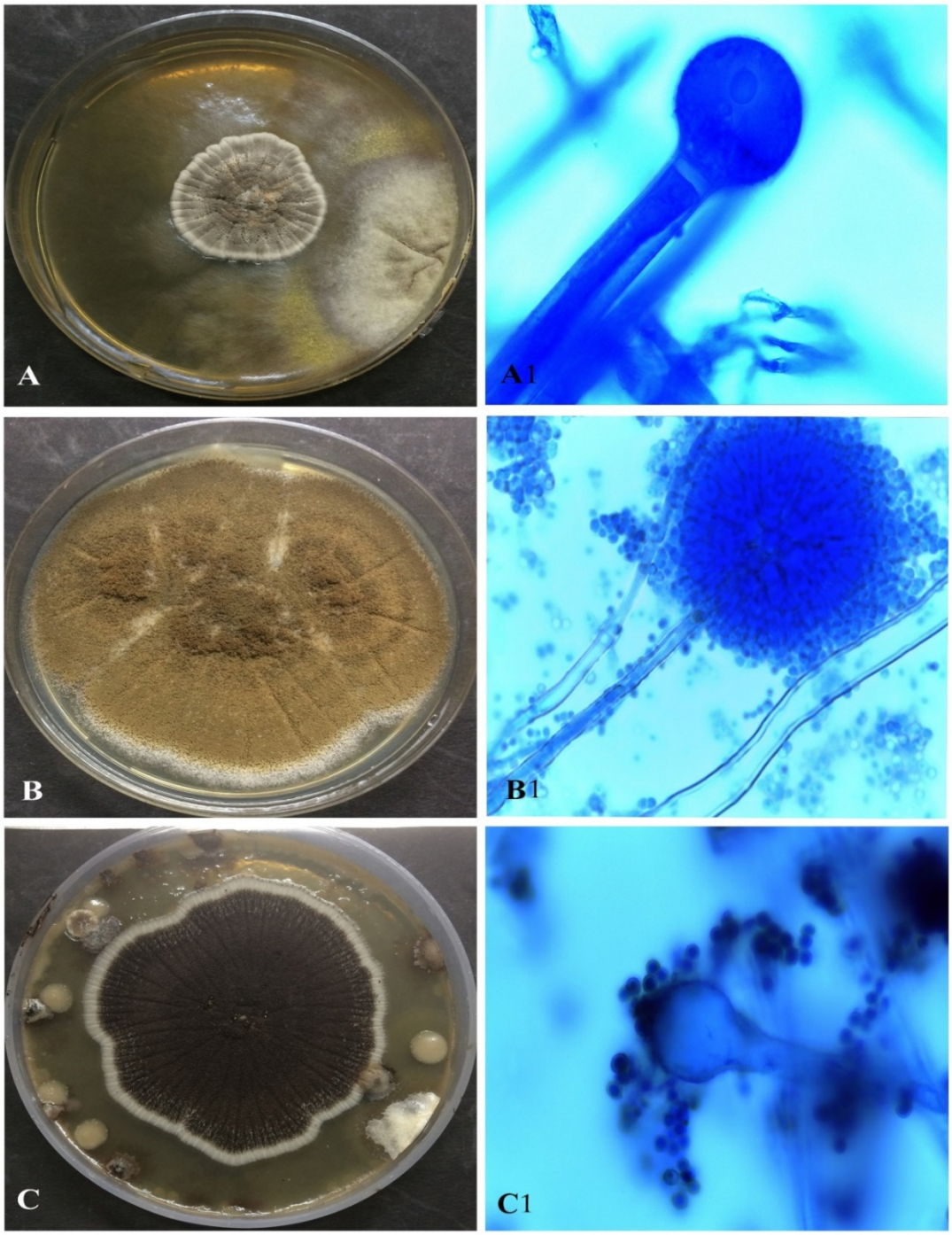

Figure 6.1: Culture plates showing the growth of endophytic fungi A) KMPRO1, B) KMPRO2 and C) KMPRH1 isolated from the rhizomes of *K. rotunda* in PDA. Lactophenol cotton blue stained microscopic images (40X magnification) of conidiophores and conidia of A1) KMPRO1, B1) KMPRO2 and C1) KMPRH1 isolated from the rhizomes of *K. rotunda*.

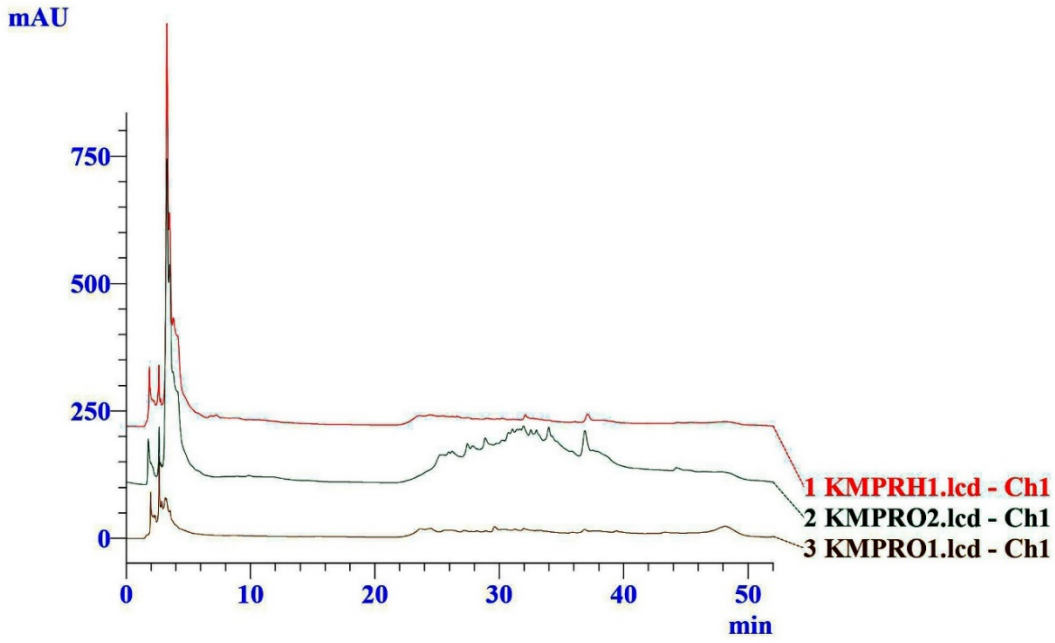

Figure 6.2: Combined HPLC chromatogram of different endophytic fungal extracts from *K. rotunda*

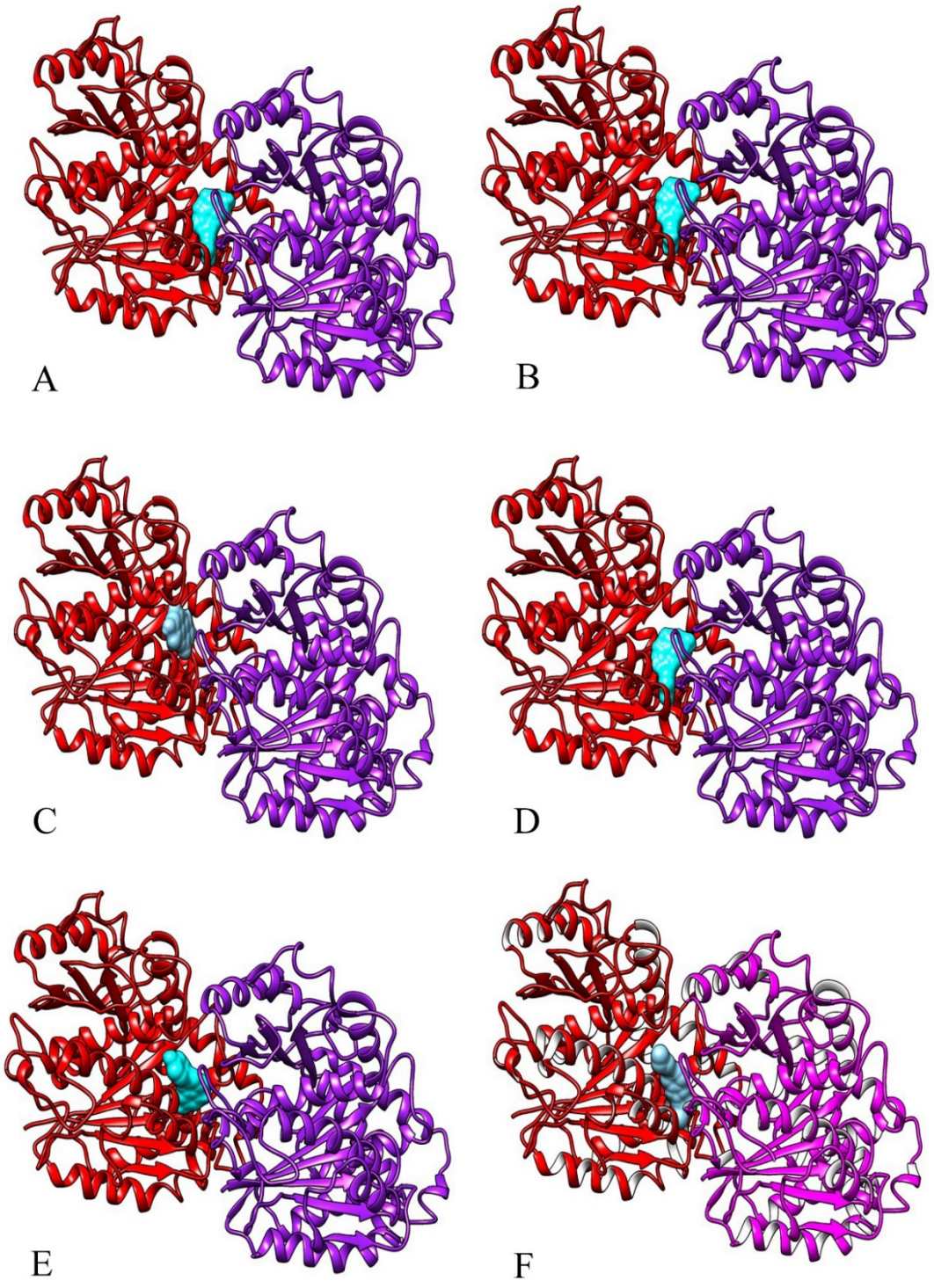

Figure 6.3: Three-dimensional structure of alpha beta tubulin dimers bound with the ligands a) quercetin, b) myrcetin, c) ellagic acid, d) kaempferol e) apigenin and f) albendazole (standard drug) to the colchicine binding site. The ligands are shown in cyan colour.

SUMMARY & CONCLUSION

In the recurrent search by the medical and pharmaceutical industry for novel products, plants seem to be the precious storehouse of bioactive metabolites that have the potential to be developed into new drugs. Populations around the world, especially in developing countries depend on herbal formulations to meet their health care needs. However, most of the herbal formulations in the traditional system are not validated properly. The broad objective of the current study was to test and scientifically validate the bioactive potentials of two herbs, *Kaempferia rotunda* and *Lagenandra toxicaria*. We have evaluated the antibacterial and anthelmintic potentials of six different solvent extracts from these plants using in vitro systems. In general the ethyl acetate extracts were found to be more bioactive than the others. Since organic solvent extracts of *K. rotunda* were eliciting stronger activity towards diverse bacteria and animal parasites, we have also evaluated its potential using *in vivo* systems. We have also isolated endophytic fungi from the rhizomes of *K. rotunda* and assessed the *in vitro* antibacterial and anthelmintic capabilities of their extracts expecting a large scale bioreactor production of the bioactive fraction rather than using the wild collected plants for better reproducibility and sustainability.

The antibacterial potential of organic solvent extracts of the rhizome was relatively high against the Gram-positive bacterial strains. For *K. rotunda*, the disk diffusion assay clearly showed the potential of KrEA and KrOH extracts against the Gram-positive strains, especially *S. aureus* and *B. cereus*. Even though the resistance developed by the Gram-negative bacterial strains in the present study were relatively high, the KrEA and KrOH extracts showed visible inhibition against the strains *E. coli* and *K. pneumonia*. The organic solvent extracts of *L. toxicaria* rhizome (LtEA and LtOH) also showed conspicuous inhibitory potential against some of the bacteria such as *S. aureus*, even though the inhibitory potential was comparatively less than that of

K. rotunda. In general the Gram-positive bacterium, *S. aureus* showed high susceptibility to the rhizome extracts and on the other hand, the Gram-negative *S. flexneri* and *S. marcescens* showed noticeable resistance. It is noteworthy that even though there are conflicting reports on *L. toxicaria, K. rotunda* is widely accepted as a medicinal plant and is used extensively in managing several infectious diseases.

The anthelmintic potential of rhizome extracts was assessed against parasitic nematodes, cestodes and trematodes. The *in vitro* studies signified the anthelmintic efficacy of ethyl acetate and ethanol extracts of *K. rotunda* and *L. toxicaria* against plant-parasitic nematodes, animal parasitic nematodes and animal parasitic trematode flukes. Here also the water extract showed negligible potential against the tested species of parasites. Initially the anthelmintic potential of the extracts were evaluated against plant parasitic nematodes. Unlike the results of antibacterial assays, organic solvent extracts of *L. toxicaria* were giving the most prominent activity. This indeed is expected from a plant species who has a swampy natural habitat where the worm load is expected to be too high. Further field based studies can be conducted for a successful translation of these findings to the agricultural fields.

When it come to the animal parasitic helminth worms, among the six extracts tested, the ethyl acetate (KrEA) and ethanol (KrOH) extracts of *K. rotunda* showed relatively high efficacy against both nematodes and trematode flukes in the *in vitro* condition. Hence we further extended the study to signify the *in vivo* anthelmintic potential of these two extracts. After safety evaluation using acute and sub-acute assays, non-toxic doses were used to treat natural helminth infections of laboratory mice. The results were exciting that the nematode and cestode infections in the KrEA and KrOH extracts treated mice reduced considerably when compared to the control group. These results are correlated with the presence of high amounts of bioactive

metabolites, especially phenolic compounds such as myricetin, quercetin, apigenin, kaempferol, ellagic acid etc., identified in the organic solvent extracts than in the water extract. The beta-tubulin protein, the monomer of microtubules in nematode parasites, seems to be a lucrative drug target for anthelmintic drugs including our reference molecule, albendazole. Comparative *in silico* molecular docking studies of selected polyphenols identified in these rhizome extracts with the standard anthelmintic drug, albendazole proved that phenolic compounds such as myricetin, quercetin, apigenin, kaempferol and ellagic acid have higher binding affinity to the tubulin targets of parasites, even greater than that of albendazole. These molecules can therefore be selected as good alternatives to the synthetic molecule for targeting various helminth infections.

Further we extended our study for the isolation and exploration of bioactivities of endophytic fungi from the rhizomes of *K. rotunda*. We have isolated three endophytic fungi into pure cultures from the rhizomes. The isolated endophytic fungi that showed repeated appearance belongs to the therapeutically important genus *Aspergillus*. The culture characteristic, microscopic observations and molecular approach substantiate this finding. The present results provide a solid platform for the development of novel anthelmintic agents from endophytic fungi of *K. rotunda*. Endophytic fungi, among the novel biotypes, are in the top priorities now, which would make drastic changes to the medicinal, pharmaceutical, and agriculture industries in the near future. We presume that the individual and/or synergistic action of polyphenols might have resulted in the observed bioactivity. Even though the *in silico* study results provide certain information regarding the probable mechanism of action of the phenolic secondary metabolites present in the extracts, to further justify the results more research is required to identify the specific target and mode of action

of bio active molecules. We hope a greater understanding of the mechanism of action of the bioactive molecules can further regulate the bacterial and helminth control strategies positively.

RECOMMENDATIONS

The current study mainly focused on exploring the *in silico, in vitro* and *in vivo* anthelmintic potential of *K. rotunda* and *L. toxicaria* rhizome extracts. Alongside, we also studied the antibacterial potential of the same extracts against various Gram-positive and Gram-negative bacterial strains. The study endorses the following recommendations for the effective development of anthelmintic and antibacterial drugs from the aforementioned natural sources.

Toxicity analysis

- The toxicity levels of ethyl acetate and ethanol extracts of *K. rotunda* were studied by acute as well as sub-acute analysis. The toxicity study conducted *in vivo*, is significant in that it recommends a safe dose of the drug for further use in the *in vivo* system.
- A chronic toxicity analysis is recommended to undertake so that the non-toxic nature of these plant products are further explored in different animal models such as rat and rabbit.

Anthelmintic study

- Exploring the mechanism of action of these potent bio extracts must be the chief priority of future study. Even though the *in silico* docking studies of chief phenolic constituents against tubulin targets of helminth worms were done in the present study, more targets need to be explored before considering them as treatment aid against a wide range of helminth parasites.
- The *in vitro* studies must be expanded with different life cycle stages of more parasitic species to further confirm their potency.

- The *in vitro* study using phenolic compounds such as myricetin, quercetin, ellagic acid, kaempferol and apigenin must be done separately against the different life cycle stages of helminth parasites to compare the effects of these compounds against these parasites.

- Current *in vivo* anthelmintic studies highlighted the potential of non-toxic concentrations of KrEA and KrOH extracts in inhibiting the nematode and cestode parasite infection in Swiss albino mice. So that these non-toxic concentrations should be further explored to study their potential to control the natural helminth infections in different animal models such as rats, rabbits or cattle.

Antibacterial study

- *In vivo* antibacterial potential of those extracts that showed activity in the *in vitro* system must be explored in animal models to further confirm the effectiveness of rhizome and endophytic fungal extracts against tested bacterial strains.

- Knowing the mechanistic aspect of these extracts on bacterial systems also needs to be explored.

Endophyte culture for bioreactor

- As a part of coevolution, many of the endophytic fungal groups have acquired the capacity to synthesize bioactive molecules that are the same or similar to their hosts. Harvesting these molecules from endophyte culture would considerably reduce the over exploitation of plants and thereby will do less harm to the ecosystem.

- In order to overcome the issues related to low yield of the phenolic molecules, further standardization of culture techniques are required. Incorporation of new strategies in upscaling culture would further improve their yield and will be economically viable.

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PUBLICATIONS

List of Publications

Research Publications

1. Krishnakumar P, Varghese L. Nematicidal activity of Lagenandra toxicaria Dalz and Kaempferia rotunda L. rhizome extracts against plant-parasitic nematodes, Meloidogyne incognita (Kofoid and White) Chitwood and Radopholous similis Cobb. Indian Phytopathology (Springer). https://doi.org/10.1007/s42360-022-00527-3

2. Krishnakumar P, Varghese M, Joe MG, Rajagopal A, Varghese L. Identification and bioactivities of endophytic fungi from *Lagenandra toxicaria* Dalz. and *Kaempferia rotunda* L. Journal of Applied Biology & Biotechnology. 2021; 9(04): 117-12. 10.7324/JABB.2021.9416

3. Krishnakumar P, Neethu CB, Sruthi P, Vardhanan S. Phytotoxicity and Anaerobic Sludge Co-Digestion Inhibition of Coconut Husk (*Cocos nucifera* (L.) (Palmae) Leachate: Photo-Oxidative Remediation. Pollution Research. 2019;38(2):301-305. http://www.envirobiotechjournals.com/article_abstract.php?aid=9567&iid=274&jid=4

4. Krishnakumar P, Menon M, Rajagopal A, Varghese L. Trematocidal activity of certain plant species against rumen fluke *Fischoederius cobboldi*. Journal of Medicinal Plants Research. (*In press*)

Book Chapter

1. "Antimicrobial compounds from endophytic fungi with special emphasis on Ascomycetes". 2021. Advances in Sustainable Bioprospecting Methods (Volume -1). Bright sky publications. Page No: 21-38. ISBN : 978-93-92804-22-9. DOI: https://doi.org/10.22271/bs.book.40

Seminar Proceedings

1. "Comparative study on moth diversity at two different habitats of Thrissur District Kerala". 2018. Faunal Diversity and Recent Trends in Animal Taxonomy. –Christ College Irinjalakuda.

Research paper presentations in National & International Seminar/Webinar

1. "Antifungal activity of *Myristica fragrance* fruit extract" National Seminar on 'Progress and Prospects of Biotechnology' (2016), Dept. of Biotechnology, St. Joseph's College, Irinjalakuda.

2. "Evaluation of antibacterial and antifungal activities of *Kaempferia rotunda* L. extracts and its fungal endophytes" National Seminar on Recent Trends in Microbiology (2017). Dept. of Life Sciences, University of Calicut

3. Comparative study on moth diversity at two different habitats of Thrissur District Kerala. National Seminar on "Faunal diversity and Recent Trends in Animal Taxonomy" (2017). Department of Zoology, Christ College.

4. "Nematicidal activity of *Kaempferia rotunda* L. rhizome extracts against free-living nematode *Caenorhabditis elegans*" National seminar on "Advanced functional materials for energy production and medicinal applications"(2018). Organized by the Department of Chemistry, Christ College, Irinjalakuda.

5. *"Lagenandra toxicaria* Dalzell: an appraisal of its anthelmintic properties" International Seminar on "Deliberation on Translation of Basic Scientific Insights into Affordable Healthcare Products" conducted on behalf of 8th

Annual Meeting of Indian Academy of Biomedical Sciences held at CSIR-NIIST Thiruvananthapuram, Kerala on 25-27th February 2019.

6. *"Lagenandra toxicaria* Dalz: An exploration through its phytoremediation potentials" International Conference on Recent Biotechnological Innovation in Aquaculture (RBIA 2020). Organized by Dept. of Zoology, Bharathiar University, Coimbatore in association with ICAR-National Bureau of Fish Genetic Resources.

7. *"In vitro* study of trematocidal potential of *Kaempferia rotunda* L. against the ruminant parasite, *Fischoederius cobboldi"* International Webinar and Symposium on "Trends in Modern Biology " (2021). Organized by Dept. of Zoology, University of Calicut.