Screening of in vitro *antitrematodal activities of compounds and secondary metabolites isolated from selected Pteridophytes*

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Summary
Four ferns *Blechnum orientale* Linn. (BO), *Dicranopteris linearis* (Burm.f) Underw. (DL), *Marattia fraxinea* Sm. (MF), and *Microlepia speluncae* (L.) Moore (MS) were extracted in varied combination of organic solvents followed by the preparation of eluates and isolation of secondary metabolites using chromatography on a glass column with silica gel as the fixed phase. The chemical components were identified using HPTLC and GC-MS analysis. The *in vitro* anti-trematodal activities of these eluates and compounds were evaluated against the sheep trematode worm *Gastrothylax crumenifer* (Plagiorrchiida: Gastrothylacidae) at increasing concentrations (1 to 5 mg/mL), to find the relative motility (RM) values for 0 to 60 min of incubation as reflection of paralysis and death of the worms. Hedon-Fleig salt solution was used as negative control and Oxyclozanide® 1% as standard control. *In vitro* incubation study showed DL and MS extracts had strong trematodicidal activity. BO extract (5 mg/mL) produced moderate trematodicidal activity and MF (5 mg/mL) showed the least trematodicidal activity. Phytochemicals analysis revealed that the ferns are a potential source of trematodicidal compounds such as phytol isomers fern-8-ene and fern-9(11)-ene (terpenoid derivative), quercetin 7,3´,4´-trimethoxy (flavonoid derivative), etc., which offer scope for a more elaborate study for exploitation of ferns for human welfare.

Introduction

Pteridophytes are primitive vascular cryptogamic plants which occupy a systematic position between the lower non-seed bearing and the higher seed-bearing plants. They are generally a much neglected group of plants (Madhusoodanan *et al.* 2001). Ferns are represented by about 305 genera, comprising more than 12,000 species all over the world. About 70 families, including 191 genera and 1,000 species presumably occur in India. In the Southern Western Ghats region the current status of pteridophytes is 272 species belonging to 95 genera and 34 families. Most of the pteridophytes are listed among endemic and rare species. The pteridophytes are little known for use since they are not as easily available as the flowering plants. Therefore, economic and medicinal values of higher plants are being exhaustively investigated where as the pteridophytes have been largely ignored (Vashishta *et al.* 2012). Ethnobotanical approach to human and veterinary medicines is an emerging trend in the treatment of chronic diseases such as skin diseases, tumours, parasitic infections, etc. The drugs/therapies in this approach are least toxic and highly cost-effective. However, there is little research in respect of the pteridophytic medicinal plants, which are on the decline and are being delimited due to habitat displacement, natural calamities, soil erosion, etc., and many of these plants are now in the list of threatened species, and some are in the list of endangered species in the Red Data Book of International Union for Conservation of Nature (IUCN). There has been little effort to conserve them.

The ferns were once popular in folk medicine especially practiced by the tribal communities (Manickam and Irudayaraj 1992). Since these are smaller traditions of Indian systems of medicine the uses have not been recorded, as the result of which there is only fewer literature pertaining to application/ utility of pteridophytes against endoparasitic

infections and their antioxidant potential. Around 300 B.C., Theophrastus recommended oil extract of ferns to expel internal parasites (Amritpal Singh 2011). *Blechnum orientale* Linn. (Blechnaceae) is used ethnomedicinally for the treatment of various skin diseases, stomach pain, urinary bladder complaints and sterilization of women (Lai *et al.* 2010). Rhizomes of various shield ferns (e.g., *Polystichum* and *Dryopteris*) have been used as a cure for some intestinal worm infections since the 18th century. *Cyathea manniana* Hook., (Cyatheaceae) from East Africa, has been used by the Chagga and German troops as an anthelmintic during the first World War. However, the pteridophytes find their use in the ancient systems of medicine, viz., Homoeopathy, Ayurveda, and Unani, for worm infections.

Helminthiasis is the condition resulting from round worm, tape worm and/or flat worm infections, especially in ruminants, and is one of the major prevalent diseases around the world, particularly the tropical countries such as India, Pakistan, Bangladesh, Sri Lanka, etc (Soulsby 1982). The worm infection is managed using anthelmintics but problems have emerged with their uses, especially i) development of resistance in the helminth parasites to the various anthelmintic compounds, ii) accumulation of residues of the compounds in the host, and iii) toxicity issues (Waller and Prichard 1985). In general, the recognition of antigenic complexity of the parasites has caused a slow-down of vaccine development. The frequent use of anthelmintics over many years has inevitably led to the development of resistance to the varied anthelmintics used. It has been documented that helminths are most probably resistant to all three broad spectrum families of anthelmintics viz., benzimidazole, imidazothiazole and ivermectin. The medicinal plants in use for endoparasitic infection, on the other hand, produce fewer unanticipated side effects and apparently do not trigger parasitic chemo-resistance. Under these circumstances investigation of alternatives, such as medicinal plants, to commercially available anthelmintics is being emphasized. For centuries, medicinal plants have been used to combat parasitic infections, and in many parts of the world they are still in use. The use of medicinal plants for prevention and/or therapy of gastro-intestinal parasitic infections, in fact, originated as an aspect of ethno-veterinary medicine. But, there are some problems concerning the use of herbal medicine the most important being lack of scientific evaluation and/or validation. The most effective approach to do such evaluation/validation is ethnobotanical approach, which assumes that indigenous uses of plants indicate the presence of biologically active compounds in them.

Chromatographic techniques have significant place in natural products chemistry and contribute greatly to the discovery of novel compounds of pharmaceutical and biomedical importance. Column chromatographic technique is especially useful in the isolation of bioactive compounds. The compounds can further be purified using high performance liquid chromatography (HPLC), followed by nuclear magnetic resonance (NMR) spectral analyses. At present there are innumerable products in the market which inflict adverse side effects on one's health. Therefore, the use of secondary metabolites of plant origin could be an advantage and an effective solution to narrow down the use of unhealthy products. In the past, plant or microbial extracts, in crude or partially purified form, were the only sources of medication available for the treatment of human and animal diseases. The effect of a drug in human body is due to an interaction of the drug with the biological molecules. This opened up new vistas in pharmacology, whereupon isolated chemicals, rather than extracts, are practiced for treatment of diseases. Therefore, the objective of this study has been to fractionate and isolate the biologically active secondary metabolites present in the ferns for *in vitro* anti-trematodal activity. Phytochemical investigation of the ferns has been conducted only to a limited extent as compared to the higher plants. Reporting the compounds in ferns responsible for the anti-trematodal activity may be first of its kind in the field of ethnobotany. The phytochemical analysis of plants present in the wild and controlled endoparasitic drug trials along with contemporary knowledge of parasite control strategies may offer new opportunities for effective and economical control of parasitic diseases. Applied to veterinary medicine, this would improve the economic conditions of the farmers who hold the livestock and, thus, improve the economic status of the country. In this study we investigated extracts of four species of ferns for *in vitro* trematodicidal property using *Gastrothylax crumenifer* (Creplin, 1847) as the model trematode parasite, and attempt was also made to isolate the compounds with the potential therapeutic property.

Materials and methods

The study was carried out during the period from October, 2014 to October, 2016, in the Department of Botany and Microbiology, A. Veeraiya Vandayar Memorial (A.V.V.M.), Sri Pushpam College, Poondi, Thanjavur District, Tamil Nadu, India. Four species of ferns, *Blechnum orientale* Linn. (BO) (Polypodiopsida, Blenchnaceae), *Dicranopteris linearis* (Burm.f) Underw. (DL) (Polypodiopsida, Gleicheniaceae), *Marattia fraxinea* (Sm.) (MF) (Polypodiopsida, Marattiaceae) and *Microlepia speluncae* (L.) (MS) (Polypodiopsida, Dennstaedtiaceae) were collected from Kothayar hills (Latitude, 8° 31´ 24.42´´ N, Longitude, 77° 21´

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11.40´´ E and altitude 1,250 m a.s.l.), a portion of the southern-most part of Western Ghats, the peninsular Kanyakumari District.

Identification of ferns and preparation of extracts

Specimens of the selected ferns were species-identified based on report of different flora (Manickam and Irudayaraj 1992, Beddome 1868-1874). The voucher specimens of *Blechnum orientale* Linn. (SPCH 1004), *Dicranopteris linearis* (Burm.f) Underw. (SPCH 1005), *Marattia fraxinea* (Sm.) (SPCH 1006) and *Microlepia speluncae* (L.) (SPCH 1007) were deposited at the herbarium of A.V.V.M. Sri Pushpam College. Extracts were prepared as per the method already described (Kalpana Devi *et al.* 2015). Briefly, 100 g of the dry powder of each of the four ferns was extracted successively (1:10, w/v) in petroleum ether (Polarity Index = 0.1) (b.p., 60-80 °C), chloroform (Polarity Index = 4.1), acetone (Polarity Index = 5.1) and ethanol (Polarity Index = 5.1) by cold maceration for 24 hours. The extracts were filtered through Whatman #1 filter paper and dried in IKA-RV 10 digital rotary evaporator until constant dry weight of each extract. The extracts were stored aseptically at 4 °C until further use. In two more separate batches, the powdered plant material was extracted by cold maceration for 48 hours using sterile distilled water (Polarity Index = 10.2) and ethanol (Polarity Index = 5.1) in a ratio of 1:10 (w/v). The mixture was filtered and centrifuged at 3,500 rpm for 20 minutes. The supernatant was filtered through Whatman #1 filter paper followed by 0.2 µm membrane filter. The extract thus obtained was evaporated to dryness in IKA-RV 10 digital rotary evaporator and preserved aseptically at 4 °C until further use.

Phytochemical screening

Phytochemical screening of the fern extracts was carried out using different solvents based on increasing polarity index viz., non-polar to most polar solvents successively (petroleum ether, chloroform, acetone, ethanol and water) to identify the major natural chemical groups (Harborne 1998) such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, quinones, glycosides, cardiac glycosides, coumarins, steroids, anthocyanin and beta-cyanin. General reactions in these analyses revealed the presence or absence of the respective compounds in the fern extracts. Total flavonoid content in the fern extracts was determined by aluminium chloride colorimetric method (Mervat *et al.* 2009), total phenolic content by Folin-Ciocalteau colorimetric method (Slinkard and Singleton 1956), tannin content by the method of Fagbemi and colleagues (Fagbemi *et al.* 2005), and total terpenoid content by the method described in Ferguson (Ferguson 1956).

Extraction using column chromatography

In order to isolate the bioactive compounds from the crude extracts, the latter were further fractionated using column chromatography, with silica gel G (40 g, 60-120 #, LOBA Chemie Pvt. Ltd.) as the stationary phase (https://www.shodhganga.inflibnet.ac.in). The dried crude extract was loaded onto a glass column (60 x 3.0 cm) packed with silica gel G. The extract was dissolved in a minimal quantity of ethanol and adsorbed on to silica gel G. When the sample had adsorbed to the silica gel, a small amount of sand was poured to cover sample. The mobile phase was poured continuously to the top of the column by aid of a funnel. The bottom outlet of the column was opened. The eluates (fractions) were collected in separate test tubes. The column was eluted with solvents of different polarities, first eluted with petroleum ether (100%), followed by graded mixtures of petroleum ether 80% and chloroform 20%, chloroform 100%, graded mixtures of chloroform 80% and acetone 20%, acetone 100%, graded mixtures of acetone 80% and ethyl acetate 20%, ethyl acetate 100%, graded mixtures of ethyl acetate 80% and ethanol 20%, and ethanol 100%, in that order. The test tubes were changed after 10 mL of each fraction was collected, and thereafter all fractions were analyzed. The eluted components were monitored using acetone, chloroform, ethanol (4:4:1, v/v/v) as the mobile phase, and visualized under UV light as well as after derivatization with anisaldehyde sulfuric acid reagent followed by heating in oven for 15 min at 80 °C. The fractions were collected and the solvent was removed, then dried, weighed and analyzed.

High Performance Thin Layer Chromatography (HPTLC)

HPTLC studies were carried out using ethanolic extracts of the four ferns, where one hundred milligrams of four extracts were dissolved in 1 ml of ethanol. A number of solvent systems were tried, for further extraction of specific classes of chemical compounds. Ethyl acetate- butanone-formic acid- water (5:3:1:1, v/v/v/v) was the most satisfactory solvent for flavonoids; toluene, acetone, formic acid (4.5:4.5:1, v/v/v) proved to be good for phenols; toluene-ethyl acetate- formic acid-methanol (3:3:0.8:0.2, v/v/v/v) did well for tannins and n-hexane, ethylacetate (7.2:2.9, v/v) proved to be good for terpenoids. Chromatography was performed on silica gel 60 F254 TLC precoated plates using Hamilton syringe and CAMAG

Linomat 5 instrument. 2 µL of standard solution and 2 µL of the test solution (extract) were loaded as 5 mm band length in the 4″ × 10″ glass plates at the distance of 10 mm from the edge of the plates, with the help of a CAMAG Liwomat 5 sample applicator. After the application of sample, the chromatogram was developed in Twin trough glass chamber (10 × 10 cm) saturated with previously equilibrated mobile phase for 30 min. The chromatographic conditions were previously optimized to achieve the best resolution and peak shape. The air-dried plates were viewed in ultraviolet radiation to mid-day light. The chromatograms were scanned by densitometer at 420 nm after spraying with specific reagents like Dragendorff's reagent for terpenoids, ethanolic aluminium chloride for flavonoids, sodium carbonate solution and Folin-Ciocalteu reagent for phenols and ferric chloride reagent for tannins, respectively. Phytol, quercetin, resorcinol, catechol, glycallic acid, hydroquinone and tannic acid were used as the reference standards. The plates were kept in photo-documentation chamber (CAMAG Reprostar 3) and images were captured in white light, UV 254 nm and UV 366 nm. After derivatization, the plates were fixed in scanner stage (CAMAG TLC Scanner 3) and scanning was done at UV 366 nm, 254 nm and 500 nm. The Peak table, Peak display and Peak densitogram were recorded. The retention factors (Rf) and % area were calculated by the WinCats software. The TLC was run under laboratory conditions. The spots were quantified using a CAMAG TLC scanner model 3 equipped with CAMAG WinCats Software.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The ethanolic extract of the ferns was further used for identification of bioactive compounds by GC-MS analysis. The Trace GC Ultra and DSQII model MS from Thermo Fisher Scientific Limited was engaged for the analysis (Renuka 2014). The instrument was set as follows, injector port temperature set to 250 °C; interface temperature set to 250 °C; and source kept at 200 °C. The oven temperature was programmed as a variable, 70 °C for 2 min, 150 °C at 8 °C/min, up to 260 °C at 10 °C/min. Split ratio was set as 1.50 and the injector used was splitless mode. The DB-35 MS non-polar column was used, the dimensions of which were 0.25 mm OD × 0.25 µm ID × 30 m long, procured from Agilent Co., USA. Helium was used as the carrier gas at 1 mL/min. The MS was set to scan from 50 to 650 Da. The source was maintained at 200 ºC and < 40 motor vacuum pressure. The ionization energy was - 70 eV. The MS was also having an inbuilt pre-filter which reduced the neutral particles. The data system had two in-built libraries, NIST4 and WILEY9, each containing more than five million references, for searching and matching the spectrum. Only those compounds which had spectral fit values equal to or greater than 700 were considered positive identification.

In vitro trematodicidal activity

Collection of worms

Adult live amphistomes were collected from the rumen of infected Kilakarsal sheep Breed, 10 months old slaughtered at Orathanadu and Pattukottai abbatoir of Thanjavur District, Tamilnadu, washed thoroughly in physiological saline and maintained in Hedon-Fleig (HF) salt solution (pH 7.0), an ideal medium for *in vitro* maintenance of the amphistomes (Veerakumari 1996). *In vitro* incubation was performed within 1 h of collection. The worms were identified based on morphology (Soulsby 1982).

In vitro incubation study

Twenty five milliliters of HF salt solution containing increasing concentrations (1, 2, 3, 4 and 5 mg/mL) of the four fern extracts in ethanol, standard control (oxyclozanide 1%, i.e., 0.25 g/25 mL) and negative control (only HF salt solution) were individually distributed to petri-dishes. Twenty five amphistomes were incubated in each of the petri-dishes. The motility of the parasites was observed visually at 5, 15, 30 and 60 min intervals. The motility response of the parasites was categorized as very active (+++), moderately active (++), sluggish (+) and dead (-). A score index was made for the motility criteria as follows (Jiraunkoorskul *et al.* 2005).

Very active - Score 3 – Moving the whole body

Moderately active – Score 2 – Moving only parts of the body

Sluggish – Score 1 – Immobile but alive

Dead – Score 0

Statistical analysis

The efficacies of the tested drugs against adult *G. crumenifer* were inferred based on calculated Relative Motility (RM) values using the formula as below (Kiuchi *et al.* 1987).

$$\text{RM Value} = \frac{\text{MI test} \times 100}{\text{MI control}} \quad \text{Motility Index (MI)} = \frac{\sum nN}{N}$$

n = motility score, N = number of flukes with the score of n.

Relative motility assay was conducted for estimation and comparison of mean mortality and drug concentration (Saowakon *et al.* 2009, Saowakon *et al.* 2013).

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Results

Qualitative and quantitative phytochemistry

Qualitative phytochemical analysis of extracts of the four fern plants, using the respective solvents, revealed that ethanol extracted out most of the phytochemicals present in BO and DL. Acetone did the best in respect of MF and MS. Among the 13 classes of phytochemicals studied, anthocyanin was absent in the extracts of all four ferns. Glycosides were absent in MF and MS extracts. Selected secondary metabolites which might contribute to the anthelmintic property (total flavonoids, total phenol, total tannin and total terpenoids) were quantitatively analyzed. The highest amount of total phenol (28.27 ± 0.18 mg gallic acid equivalent/g) was present in DL extracts. Terpenoids and flavonoids were present in the highest concentrations in MS [terpenoids, 136.78 ± 1.32 mg/g; flavonoids (quercetin), 19.00 ± 0.58 mg/g] extracts. Total tannin content was the highest in BO (34.93 ± 0.41 mg tannic acid equivalent/g) extracts. To sum up, DL and MS extracts contain higher amounts of terpenoids, tannins, phenols and flavonoids as compared to BO and MF (Table I).

Column chromatographic fractions

A total of 9 fractions were separated as eluents from BO extracts 13 each from DL and MF extracts and 16 from MS extracts under column chromatography (Figure 1). Among the eluted fractions, fraction VI (64.4%) in BO extracts, fraction V (79.5%) in DL extracts, fraction IV (74.01%) in MF extracts and fraction IX (89.6%) in MS extracts showed the highest antioxidant value under column chromatography when compared to the standard butylated hydroxytoluene (BHT). The column-separated fractions of MS showed the highest antioxidant activity, followed by DL, MF and BO extracts.

High performance thin layer chromatography

The results of HPTLC analysis of terpenoid, phenol, flavonoid and tannin profiles for the four fern extracts are presented in Figure 2. The chromatogram using finger print profile for UV derivatization was scanned under UV 254 nm/ UV 366 nm/visible light. The peak table, peak display and peak densitogram are presented in Figure 3. The HPTLC results for terpenoids were compared using standard phytol. Similarly, four standards were used for analysis of phenols (S2.1-resorcinol, S2.2-catechol, S2.3-glycallic acid; S2.4-hydroquinone). Flavonoids were determined using quercetin as the standard. Tannin profile was determined using tannic acid as the standard. The bands of BO, DL and MS were matching with the standard phytol as similar to the case of UV 366 nm. No band was noticed in MF extracts. This could be correlated with the peak densitogram which depicted the peak in Rf values of BO, DL and MS, matching the phytol standard. The most prominent peak in Rf value was present in MS extract. The HPTLC analysis of phenols revealed that the bands of the four fern extracts were matching the four standards (S2.1, S2.2, S2.3 and S2.4) used under UV 254 nm and the densitogram showing the peak in Rf values of the four fern extracts which were matching the standards used. The HPTLC

Table I. *Percentage yield, total terpenoids, total phenolics, total flavonoids and tannin content of various whole fern extracts.*

Sample	Percentage yield (w/w)				Total terpenoids (mg/g)				Total phenolics (mg GAE/g)				Total flavonoids (mg QE/g)				Tannins (mg TAE/g)			
	BO	DL	MF	MS	BO	DL	MF	MS	BO	DL	MF	MS	BO	DL	MF	MS	BO	DL	MF	MS
PE	3.23	6.67	3.05	4.08	-	6.32 ± 0.24	1.41 ± 0.26	3.23 ± 0.17	1.11 ± 0.29	2.44 ± 0.22	0.97 ± 0.03	2.74 ± 0.13	0.74 ± 0.14	-	-	-	1.10 ± 0.05	-	-	-
CH	4.45	6.97	2.60	5.28	1.22 ± 0.13	-	2.17 ± 0.09	7.70 ± 0.17	2.28 ± 0.36	1.89 ± 0.20	0.70 ± 0.05	2.13 ± 0.08	0.71 ± 0.15	-	-	-	0.77 ± 0.15	-	-	1.50 ± 0.25
AC	30.77	34.75	32.75	40.00	73.02 ± 0.10	90.87 ± 0.47	20.58 ± 0.42	138.18 ± 0.40	0.82 ± 0.12	20.75 ± 0.38	6.43 ± 0.23	18.40 ± 0.25	7.38 ± 0.19	7.72 ± 0.13	3.13 ± 0.07	21.23 ± 0.12	14.28 ± 0.17	18.42 ± 0.30	1.30 ± 0.15	8.13 ± 0.35
ET	33.81	39.60	31.97	38.71	79.05 ± 0.33	97.66 ± 1.08	16.83 ± 0.83	136.78 ± 1.32	1.43 ± 0.16	28.27 ± 0.18	5.27 ± 0.13	17.23 ± 0.38	8.30 ± 0.36	8.33 ± 0.17	1.25 ± 0.14	19.00 ± 0.58	34.93 ± 0.41	30.27 ± 0.27	7.41 ± 0.26	19.97 ± 0.26
WA	12.08	13.18	9.60	10.03	6.28 ± 0.21	8.17 ± 0.09	-	-	0.69 ± 0.16	17.15 ± 0.15	3.45 ± 0.26	11.26 ± 0.14	4.72 ± 0.11	5.51 ± 0.26	1.15 ± 0.10	16.44 ± 0.17	-	-	-	2.88 ± 0.48

Values were obtained in triplicates and represented as mean ± SE.
PE = petroleum ether; CH = chloroform; AC = acetone; ET = ethanol; WA = water; BO = *B. orientale*; DL = *D. linearis*; MF = *M. fraxinea*; MS = *M. speluncae*.

analysis for flavonoids showed the band width of fern extracts BO, DL and MS to match the standard quercetin. The densitogram peak in Rf value was in accordance with the chromatogram studied under UV 254 nm. HPTLC analysis of tannin profile as compared to standard tannic acid revealed close matching of band width in DL and BO extracts against the standard tannic acid and partial matching with MS extracts. However, for MF extract the band was negligible and not matching, and was in accordance with the Rf value under densitogram graphical representation.

Gas chromatography - mass spectrometry analysis

The bioactive compounds present in the ethanolic extract of the four ferns were identified by GC-MS analysis (Figure 4). Six compounds were detected in the extract of BO, seven in DL, eight in MF and ten in MS. The spectra of the compounds were matched with Wiley 9.0 and National Institute of Standards and Technology Libraries. The major compounds detected were 1,2-benzenedicarboxylic acid and 1,2,3-propanetricarboxylic acid 2-hydroxy-triethyl ester (CAS). The compounds responsible for anthelmintic activity in BO extract were quercetin 7´,3,´4´- trimethoxy, a flavonoid derivative (polyphenols group) (0.13%) and phytol isomer, a diterpenoid (0.53%). In DL extract, the compounds for anthelmintic activity were phytol, a diterpenoid (0.37%), quercetin 7´,3,´4´-trimethoxy, a flavonoid derivative (polyphenols group) (0.20%), and fern-8-ene, a triterpenoid (0.40%). Similarly, in MF extract the compounds responsible for anthelmintic activity were phytol (0.44%), xanthorrhizol, a terpenoid (0.69%), and fern-9(11)-ene, a fernane type triterpenoid (2.83%). In the case of MS extract the compounds responsible for anthelmintic activity were phytol (5.67%), quercetin 7´,3,´4´-trimethoxy (0.23%), and fern-8-ene, a triterpenoid (2.55%).

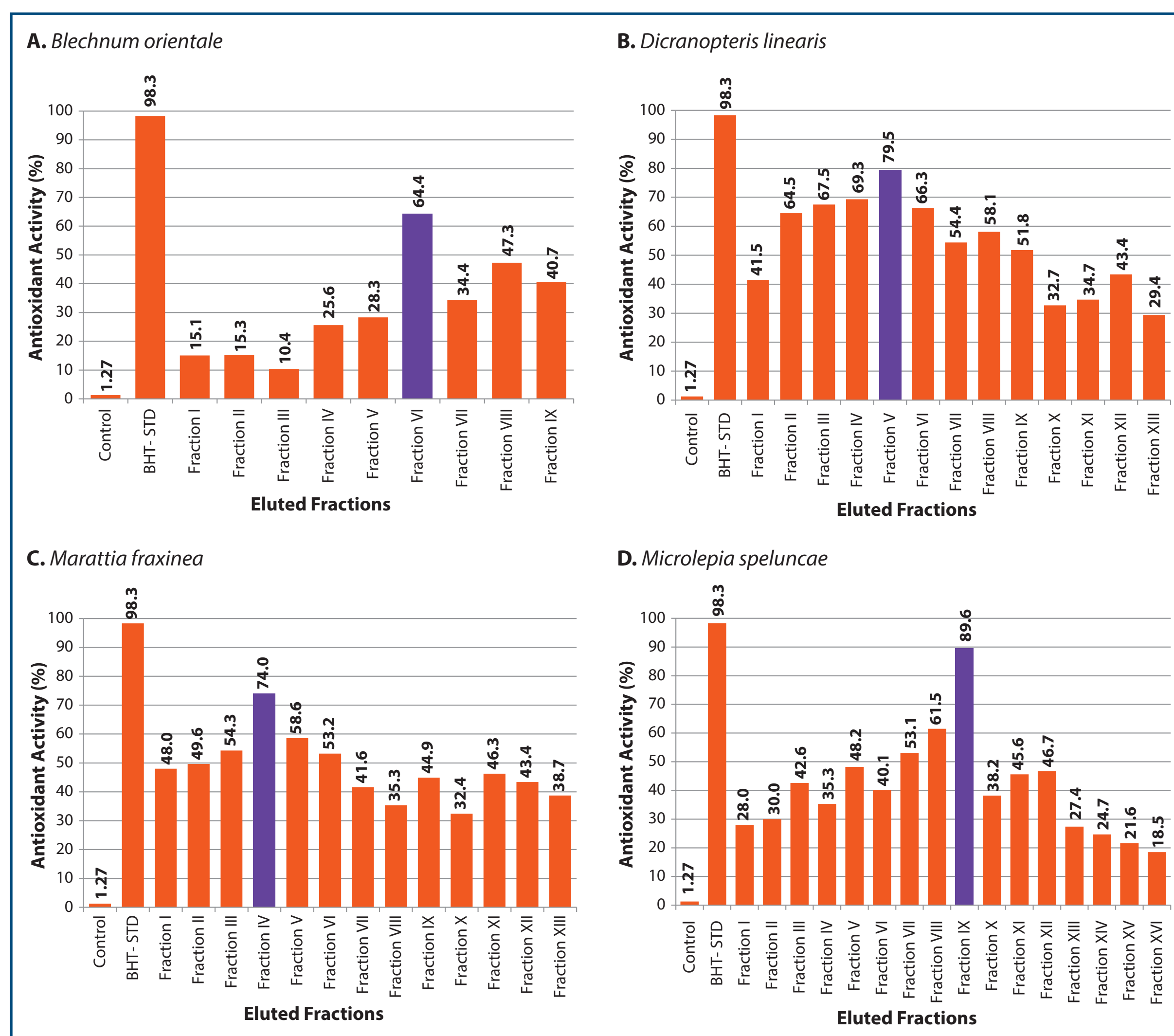

Figure 1. *Comparative analysis of various fractions of fern extracts eluted from column chromatography.*

In vitro trematodicidal property of the pteridophyte extracts

In vitro incubation study of BO extract (Table II) showed that 5 mg/mL concentration would kill all the worms at 10 min incubation, the same as the standard control (Oxyclozanide). On the other hand 2, 3, 4 mg/mL concentrations caused only partial mortality even at 60 min incubation. When incubated in the extract at 1 mg/mL concentration, there was no mortality during the entire 60 min incubation. DL extract (Table III) also, at 5 mg/mL concentration, produced drug effect comparable to the standard, killing all flukes at 10 min incubation; at the lesser concentrations (1,2,3,4 mg/mL) the extracts were equally effective killing all flukes at varied incubation times at rates proportional to drug concentration in a manner dose-dependent. Extract of MF (Table IV) revealed poor efficacy at 5 mg/mL concentration it took almost 60 min incubation to kill all the flukes,

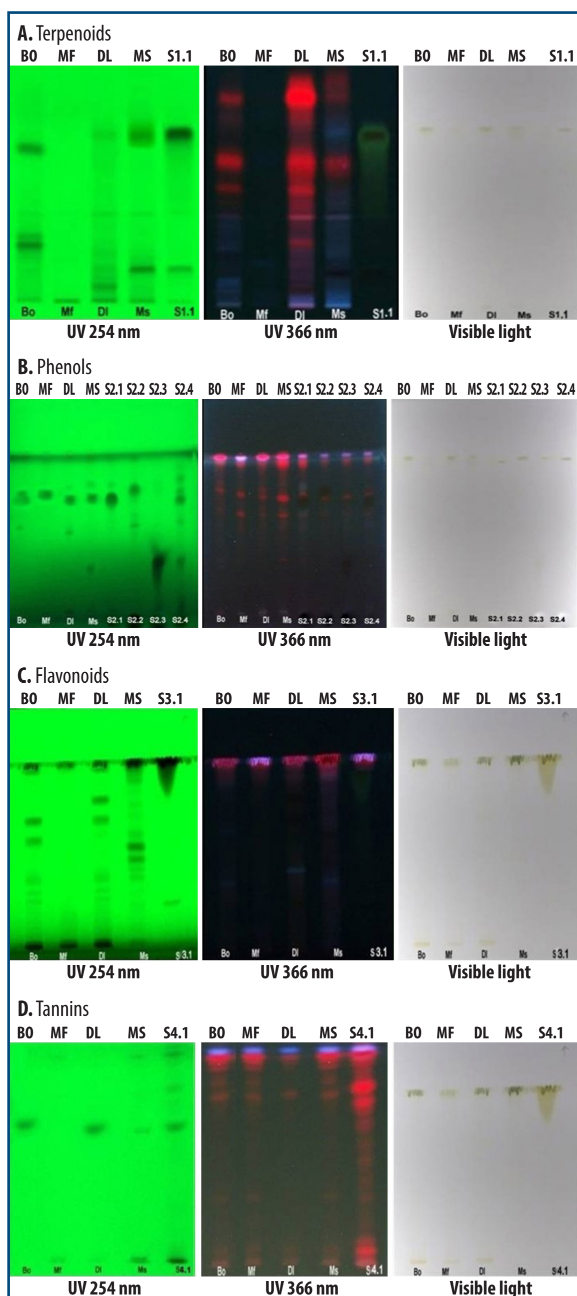

Figure 2. *High performance thin layer chromatography chromatogram using finger printing profile.* BO = *B. orientale;* DL = *D. linearis;* MF = *M. fraxinea;* MS = *M. speluncae;* S1.1 = Standard (Phytol)*;* S2.1 = Standard (Resorcinol)*;* S2.2 = Standard (Catechol)*;* S2.3 = Standard (Glycallic acid)*;* S2.4 = Standard (Hydroquinone)*;* S3.1 = Standard (Quercetin)*;* S4.1 = Standard (Tannic acid).

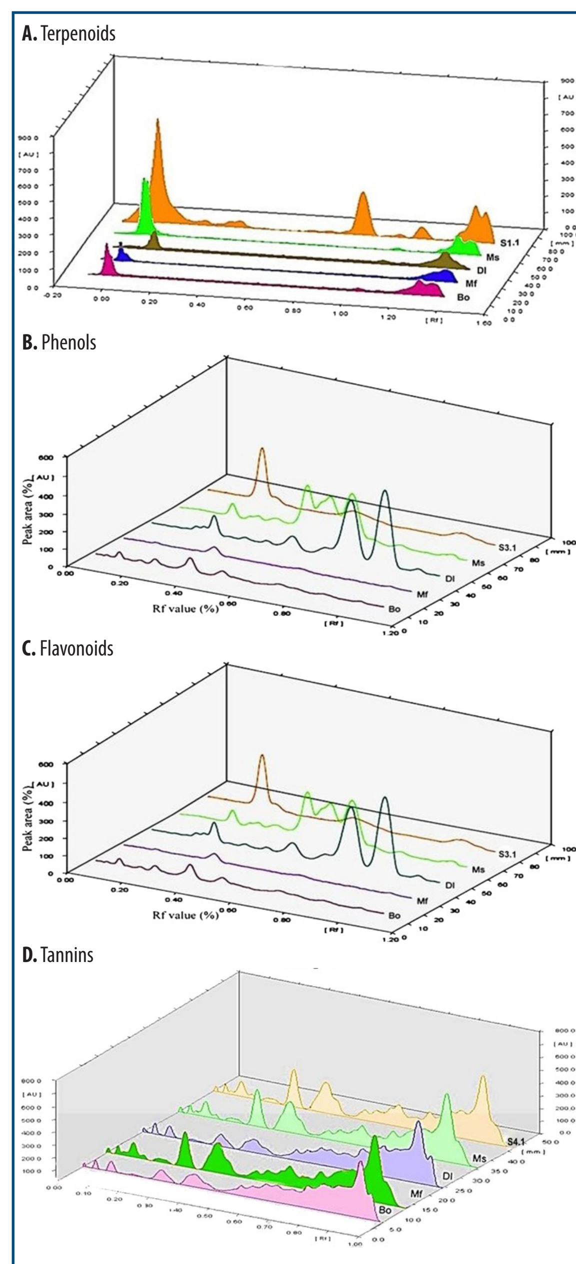

Figure 3. *Densitograms of high performance thin layer chromatograms (HPTLC).* BO = *B. orientale;* DL = *D. linearis;* MF = *M. fraxinea;* MS = *M. speluncae;* S1.1 = Standard (Phytol)*;* S2.1 = Standard (Resorcinol)*;* S2.2 = Standard (Catechol)*;* S2.3 = Standard (Glycallic acid)*;* S2.4 = Standard (Hydroquinone)*;* S3.1 = Standard (Quercetin)*;* S4.1 = Standard (Tannic acid).

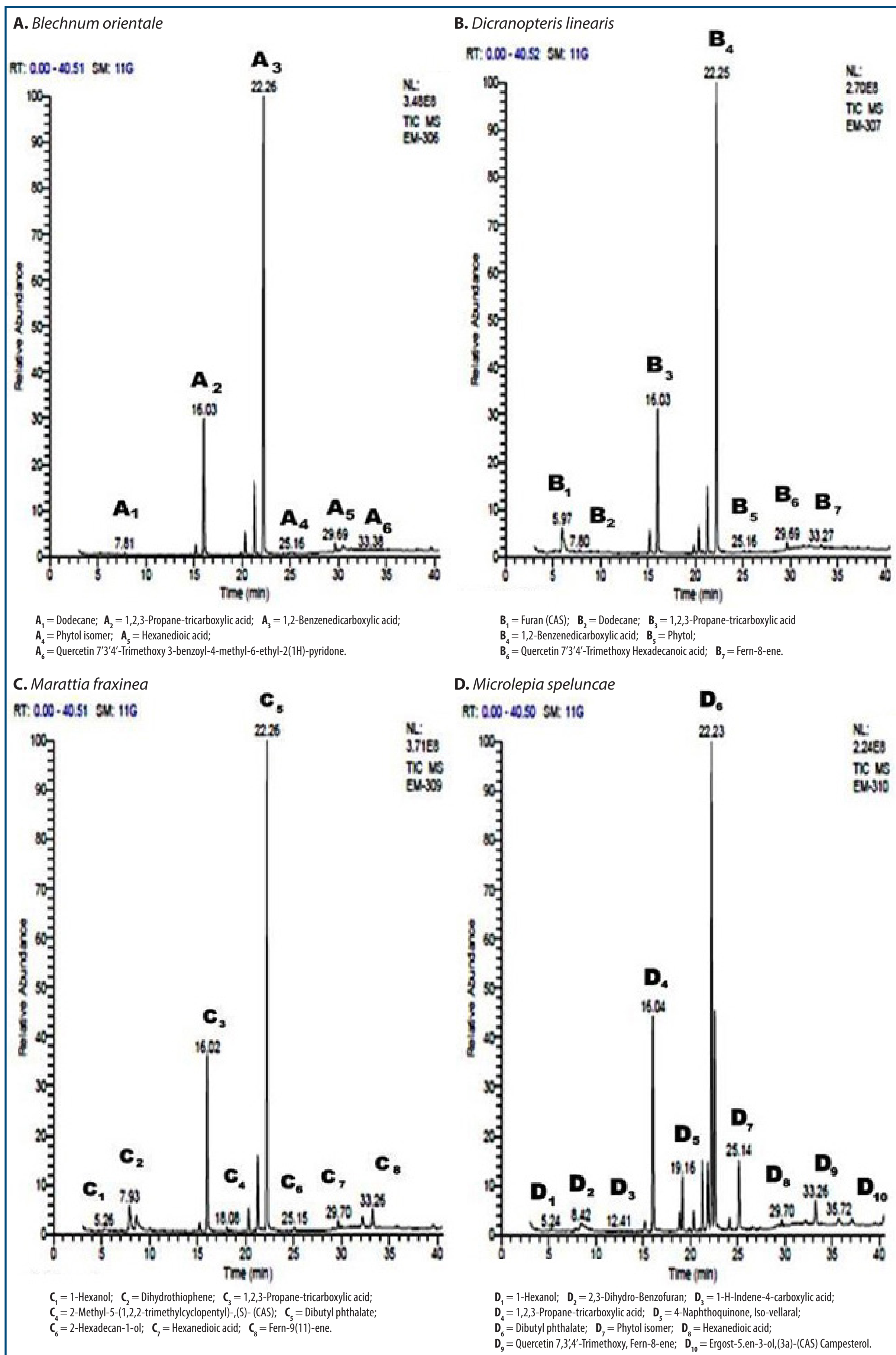

Figure 4. *Gas chromatographic and mass spectrometric analysis of pteridophytes.*

Table II. *Motility index and relative motility values of* G. crumenifer *on various time points of exposure to* B. orientale *extract.*

Conc (mg/L)	0 min			10 min			15 min			30 min			60 min		
	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD
	MI	RM		MI	RM		MI	RM		MI	RM		MI	RM	
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
0 (NC)	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
1	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
	3.00	1.00		3.00	1.00		3.00	1.00		2.16	0.72		1.16	0.39	
2	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.04	0.68	0.74 ± 0.045	1.92	0.64	0.56 ± 0.083
	3.00	1.00		3.00	1.00		3.00	1.00		2.48	0.83		1.92	0.64	
	3.00	1.00		3.00	1.00		2.88	0.96		2.16	0.72		1.16	0.39	
3	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	1.60	0.53	0.82 ± 0.143	2.04	0.68	0.69 ± 0.013	1.92	0.64	0.45 ± 0.099
	3.00	1.00		3.00	1.00		2.88	0.96		2.04	0.68		0.92	0.31	
	3.00	1.00		3.00	1.00		1.72	0.57		0.92	0.31		0.52	0.17	
4	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	1.60	0.53	0.58 ± 0.032	0.84	0.28	0.27 ± 0.026	0.64	0.21	0.18 ± 0.015
	3.00	1.00		3.00	1.00		1.92	0.64		0.88	0.22		0.48	0.16	
	3.00	1.00		0.80	0.28		0.36	0.12		0.00	0.00		0.00	0.00	
5	3.00	1.00	1.00 ± 0.00	0.76	0.25	0.25 ±0.017	0.40	0.13	0.12 ± 0.003	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.88	0.22		0.36	0.12		0.00	0.00		0.00	0.00	
	3.00	1.00		0.16	0.05		0.00	0.00		0.00	0.00		0.00	0.00	
OXY (SC)	3.00	1.00	1.00 ± 0.00	0.12	0.04	0.04 ± 0.003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.12	0.04		0.00	0.00		0.00	0.00		0.00	0.00	

NC = Negative control; SC = Standard control; MI = Motility index; RM = Relative motility.

Table III. *Motility index and relative motility values of* G. crumenifer *on various time points of exposure to* D. linearis *extract.*

Conc (mg/L)	0 min			10 min			15 min			30 min			60 min		
	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD
	MI	RM		MI	RM		MI	RM		MI	RM		MI	RM	
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
0 (NC)	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
	3.00	1.00		2.28	0.76		1.96	0.65		1.40	0.47		0.00	0.00	
1	3.00	1.00	1.00 ± 0.00	2.12	0.71	0.75 ± 0.019	1.84	0.61	0.62 ± 0.018	1.44	0.48	0.48 ± 0.058	0.00	0.00	0.00
	3.00	1.00		2.32	0.77		1.76	0.59		1.48	0.49		0.00	0.00	
	3.00	1.00		1.92	0.64		1.44	0.48		0.92	0.31		0.00	0.00	
2	3.00	1.00	1.00 ± 0.00	1.92	0.64	0.62 ± 0.017	1.40	0.47	0.48 ± 0.006	0.80	0.27	0.28 ± 0.018	0.00	0.00	0.00
	3.00	1.00		1.76	0.59		1.48	0.49		0.76	0.25		0.00	0.00	
	3.00	1.00		1.48	0.49		0.72	0.24		0.00	0.00		0.00	0.00	
3	3.00	1.00	1.00 ± 0.00	1.40	0.47	0.49 ± 0.012	0.44	0.15	0.21 ± 0.032	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		1,52	0.51		0.76	0.25		0.00	0.00		0.00	0.00	
	3.00	1.00		0.40	0.13		0.00	0.00		0.00	0.00		0.00	0.00	
4	3.00	1.00	1.00 ± 0.00	0.28	0.09	0.12 ± 0.013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.40	0.13		0.00	0.00		0.00	0.00		0.00	0.00	
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
5	3.00	1.00	1.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
OXY (SC)	3.00	1.00	1.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	

NC = Negative control; SC = Standard control; MI = Motility index; RM = Relative motility.

Table IV. *Motility index and relative motility values of* G. crumenifer *on various time modes of exposure to* M. fraxinea *extract.*

Conc (mg/L)	0 min			10 min			15 min			30 min			60 min		
	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD
	MI	RM		MI	RM		MI	RM		MI	RM		MI	RM	
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
0 (NC)	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
	3.00	1.00		3.00	1.00		2.96	0.99		1.96	0.65		1.16	0.39	
1	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.88	0.96	0.97 ± 0.009	1.88	0.63	0.64 ± 0.007	1.28	0.43	0.41 ± 0.012
	3.00	1.00		3.00	1.00		2.92	0.97		1.96	0.65		1.20	0.40	
	3.00	1.00		3.00	1.00		2.84	0.95		1.56	0.52		1.04	0.35	
2	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.80	0.93	0.92 ± 0.024	1.56	0.52	0.51 ± 0.013	0.96	0.32	0.33 ± 0.009
	3.00	1.00		3.00	1.00		2.60	0.87		1.44	0.48		1.00	0.33	
	3.00	1.00		3.00	1.00		2.76	0.92		1.16	0.39		0.60	0.20	
3	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	2.60	0.87	0.88 ± 0.021	1.08	0.36	0.37 ± 0.007	0.48	0.16	0.19 ± 0.015
	3.00	1.00		3.00	1.00		2.56	0.85		1.12	0.37		0.64	0.21	
	3.00	1.00		1.84	0.61		1.32	0.44		0.56	0.19		0.20	0.07	
4	3.00	1.00	1.00 ± 0.00	1.72	0.57	0.59 ± 0.011	1.28	0.43	0.43 ± 0.003	0.52	0.17	0.17 ± 0.007	0.20	0.07	0.06 ± 0.007
	3.00	1.00		1.76	0.59		1.28	0.43		0.48	0.16		0.16	0.05	
	3.00	1.00		1.44	0.48		0.84	0.28		0.12	0.04		0.00	0.00	
5	3.00	1.00	1.00 ± 0.00	1.32	0.44	0.46 ± 0.012	0.76	0.25	0.25 ± 0.017	0.08	0.03	0.04 ± 0.006	0.00	0.00	0.00
	3.00	1.00		1.40	0.47		0.88	0.22		0.16	0.05		0.00	0.00	
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
OXY (SC)	3.00	1.00	1.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	

NC = Negative control; SC = Standard control; MI = Motility index; RM = Relative motility.

Table V. *Motility index and relative motility values of* G. crumenifer *on various time modes of exposure to* M. speluncae *extract.*

Conc (mg/L)	0 min			10 min			15 min			30 min			60 min		
	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD	Replicates		Mean RM ± SD
	MI	RM		MI	RM		MI	RM		MI	RM		MI	RM	
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
0 (NC)	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00	3.00	1.00	1.00 ± 0.00
	3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00		3.00	1.00	
	3.00	1.00		2.28	0.76		2.00	0.67		1.40	0.47		0.00	0.00	
1	3.00	1.00	1.00 ± 0.00	2.14	0.71	0.72 ± 0.021	1.80	0.60	0.63 ± 0.021	1.20	0.40	0.44 ± 0.020	0.00	0.00	0.00
	3.00	1.00		2.06	0.69		1.86	0.62		1.32	0.44		0.00	0.00	
	3.00	1.00		1.92	0.64		1.44	0.48		0.92	0.31		0.00	0.00	
2	3.00	1.00	1.00 ± 0.00	1.76	0.59	0.61± 0.015	1.34	0.45	0.47 ± 0.009	0.94	0.31	0.31 ± 0.00	0.00	0.00	0.00
	3.00	1.00		1.80	0.60		1.44	0.48		0.92	0.31		0.00	0.00	
	3.00	1.00		1.48	0.49		0.72	0.24		0.00	0.00		0.00	0.00	
3	3.00	1.00	1.00 ± 0.00	1.54	0.51	0.51 ± 0.012	0.60	0.20	0.22 ± 0.012	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		1.60	0.53		0.62	0.21		0.00	0.00		0.00	0.00	
	3.00	1.00		0.52	0.17		0.00	0.00		0.00	0.00		0.00	0.00	
4	3.00	1.00	1.00 ± 0.00	0.52	0.17	0.17 ± 0.003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.48	0.16		0.00	0.00		0.00	0.00		0.00	0.00	
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
5	3.00	1.00	1.00 ± 0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	3.00	1.00		0.20	0.07		0.00	0.00		0.00	0.00		0.00	0.00	
OXY (SC)	3.00	1.00	1.00 ± 0.00	0.12	0.04	0.05 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3.00	1.00		0.12	0.04		0.00	0.00		0.00	0.00		0.00	0.00	

NC = Negative control; SC = Standard control; MI = Motility index; RM = Relative motility.

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as compared to the standard which killed all flukes at 10 min or less incubation, and at the lower concentrations (1,2,3,4 mg/mL) the extract was not effective at all. The efficacy of extract of MS (Table V) was almost equivalent to that of DL extract, killing all flukes at 10 min or less at 5 mg/mL concentration. The efficacies of all other drug concentrations were proportionate to the durations of incubation, lesser the concentration more the time.

Discussion

Phytochemical analysis

The first outcome of this study is understanding of efficiency of the five solvents, water, ethyl acetate, chloroform, petroleum ether and acetone, in the decreasing order of polarity index, used for extraction of the phytochemical classes wherein alcohol as the general organic solvent extracts out all phytochemicals from all the four ferns, followed by acetone, ethyl acetate, water, chloroform and petroleum ether, in that order. Looking at the number of chemical classes or extent of extraction, ethanol performed well with respect to BO and DL whereas acetone performed well in the case of MF and MS. This differential efficiency of extraction greatly depends on polarity of the solvent, solubility of the phytochemical of interest, and the physiological/biochemical status of the fern species reflecting on number and variety of phytochemicals. This has been the pointer of many earlier studies of phytochemical analysis of ferns. For e.g., phytochemical screening of acetone, benzene, chloroform, ethanol, petroleum ether and aqueous extracts of whole plants of *Blechnum orientale, Ceratopteris thalictroides, Drymoglossum heterophyllum, Dicranopteris linearis, Hemionitis arifolia, Lindsaea ensifolia, Nephrolepis multiflora, Pityrogramma calomelanos*, and *Pteris confuse*, and leaves and rhizomes of *Drynaria quercifolia*, in all 66 extracts, revealed presence or absence of specific classes of compounds (Mithraja *et al.* 2012a). Exclusive studies of *D. quercifolia* adopting extraction in all these media, though with emphasis on antimicrobial (Mithraja *et al.* 2012b) and anti-arthritic (Saravanan *et al.* 2013) applications, also support this inference. Phytochemical analysis of the ferns *Actinopteris radiata, Drynaria quercifolia, Dryopteris cochleata* and *Pityrogramma calomelanos* indicated that ethanol was the most efficient solvent with strong positivity for the major phytochemicals tested (Kalpana Devi *et al.* 2014). Qualitative and quantitative phytochemical analysis in *Pteris argyreae, Pteris confusa, Pteris vittata, Pteris biaurita* and *Pteris multiaurita* revealed methanol extract to prove to be positive for most phytochemicals of interest (De Britto *et al.* 2012, Gracelin *et al.* 2013). Thus, alcohol (ethanol/methanol) is proven as the best solvent for fern species to extract out the chemical compounds as a crude mixture.

The abundance of chemical classes in our ferns is in the order MS > DL > BO > MF. All four pytochemical classes, selectively analyzed quantitatively, are present in all four fern species studied- terpenoids and flavonoids forming the predominant chemicals in MS, phenol in DL, and terpenoid and tannin in BO and MS. However, all four classes are the lowest in MF, indicating that MF would be poorly rewarding as a plant of pharmacological importance whereas the other three ferns would prove to be good (Lacaille-Dubois and Wagner 2000). Irrespective of species, terpenoids are chemicals of greatest abundance, followed by tannins, flavonoids and phenols, in that order. An earlier study of quantitative analysis of the extract of *Pteris* species showed flavonoid content to be the highest followed by alkaloids and phenolic compounds (Gracelin *et al.* 2013). Terpenoids, also known as isoprenoids, are the most numerous and structurally diverse natural products found in plants. Several studies, *in vitro*, preclinical and clinical, have confirmed that this class of compounds possesses a wide array of very important pharmacological properties (Ludwiczuk *et al.* 2017). Saponins, as a group, include compounds that are glycosylated steroids, triterpenoids and steroidal alkaloids. Many saponins are known antimicrobials, inhibit moulds, and protect plants from insect attack. Saponins may constitute an aspect of defence systems of plants and, hence, they are phytoanticipins or phytoprotectants (Ludwiczuk *et al.* 2017). These structurally diverse compounds have also been observed to kill protozoans and helminths, to be antioxidants and also act as antifungals and antivirals (Takechi *et al.* 1999). Intake of a small quantity of the correct kind of tannin may be beneficial to human health by affecting the metabolic enzymes, immunomodulation or other functions (Chung *et al.* 1998). Phenolic compounds also possess a diverse range of beneficial biological activities (Huang and Cai 2010). Flavonoids are directly associated with human dietary ingredients and health. Prevention and cure of diseases using phytochemicals, especially flavonoids, are well known (Kumar and Pandey 2013). Ferns, available in plenty and have little if any economic value, are herein proven to be a rich source of terpenoids, tannins, phenolic compounds and flavonoids which can be applied in human and animal health.

HPTLC analysis of the four fern extracts showed evidence of matching with respective standards, phytol for terpenoids, tannic acid for tannins, quercetin for flavonoids and S1-resorcinol, S2-catechol, S3-glycallic acid and S4-hydroquinone for phenols. It was a noteworthy feature that terpenoids, phenols, flavonoids, and tannins

contribute to anti-trematodal and antioxidant drug action either independently of each other or in a combined effect of phytoconstituents in the pteridophytes. The important phytoconstituents (terpenoids, phenols, flavonoids, and tannins) under quantitative phytochemical analysis were confirmed and proved to be effective trematodicidal and antioxidant compounds

Flavonoids are pigments present in almost all terrestrial plants with a high spectrum of positive effects on plant and mammalian cells in relation to therapeutic applications. Their ability to inhibit specific enzymes to stimulate some hormones and neurotransmitters, and to scavenge free radicals, has been established. It is well known that some flavonoids inhibit or kill many bacterial strains. Chemical analyses of plant extracts which showed high anthelmintic activity revealed the presence of flavonoids, along with other classes of phytochemicals. Although toxicity of most isolated flavonoids to animal cells is very low (Middleon *et al.* 2000), several ubiquitous flavonoids such as genistein, kaemferol, rutin, quercetin, etc., produced deleterious effects on selected species of parasitic helminths. It is possible that different developmental stages (larva, juvenile or adult) of helminths might be differently susceptibile to selected flavonoids (Hrckova and Velebny 2013). The phenolic diketone curcumin and the flavonoid kaempferol exerted a strong adulticidal effect on *Schistosoma mansoni*, but no activity against nematodes was demonstrated. Quercetin, a flavonoid that belongs to polyphenolic derivatives, possesses both antioxidant and anti-parasitic activities (Dupuy *et al.* 2003). Kaempferol (flavonol) and its three derivatives were isolated from two plant species of *Styrax camporum* and *Styrax pohlii* (Braguine *et al.* 2012), and examined *in vitro*. Kaempferol was most effective in separating *S. mansoni* male and female couples and killing adult worms at a concentration of 100 µg/mL. The selective toxicity of the flavones quercetin, chrysin, and 3-hydroxyflavone toward the several cancer cell lines but not normal mammalian cells is worth mentioning (Pilatova *et al.* 2010). Phenolic compounds interfere with energy generation metabolism by uncoupling the oxidative phosphorylation and also interfere with the glycoprotein of the cell surface of the parasites and cause death. Polyphenols are potent antioxidants and very good anthelmintics. Both phenols and terpenoids are promising anthelmintics; the mechanism of action of these phytoterapeutics is multi-targeted and would act against the helminth parasites through diverse spectra of action (Mukherjee *et al.* 2016). They would also prevent and promote apoptosis and disturb redox status and therefore are potent antioxidants. It was indicated that the presence of terpenoids (Phytol and Fern-8-ene), and flavonoids (Quercetin 7,3´,4´-trimethoxy) in the fern extracts would account for action as antioxidant and anthelmintic.

Tannins interfere with energy metabolism of worms by uncoupling oxidative phosphorylation or they bind to free proteins of the gastrointestinal tract of the host animal or glycoprotein on the cuticles of the worms and lead to death (Patel *et al.* 2010). Alkaloids might act on central nervous system and cause paralysis. Steroidal alkaloids and oligoglycosides are present in alkaloids, which might suppress transfer of sucrose from stomach to small intestine. Alkaloids act as antioxidants capable of reducing nitrate generation which would interfere in local homeostasis that is essential for the embryonic development of helminths. Saponins affect permeability of the cell membrane of parasites and cause vacuolization and disintegration of teguments (Melzig *et al.* 2001). Isoflavonones inhibit the enzymes of glycolysis and glycogenolysis and disrupt Ca2+ homeostasis and NO activity in the parasites (Stepek *et al.* 2006). In the present study, the phytochemical components under HPTLC analysis provided substantial evidence of presence of terpenoids, phenols, tannins and flavonoids in the ferns which leads to understanding of the pharmacological and/or toxicological actions of the plants of interest (Mukherjee *et al.* 2007).

Plant secondary metabolites such as saponins, alkaloids, non-protein amino acids, tannins and other polyphenols, that are present on our ferns, must be responsible for the antiparasitic effect. There is a report connecting anthelmintic and free radical scavenging potentials of *Glinus oppositifolius* associating with the polyphenols (Bhaskar *et al.* 2012). Terpenoids are also secondary plant metabolites that hold both antioxidant and anthelmintic properties (Grassmann 2005). Deriving thereupon, the phenols and terpenoids present in our fern extracts correlate with the antioxidant potential and anthelmintic activity.

GC-MS analysis revealed the presence of 6 compounds in BO, 7 in DL, 8 in MF and 10 in MS extracts. These compounds belong to different classes and so would possess varied biological activities. The major groups of compounds include terpenoids, flavonoids (polyphenols) and fatty acid derivatives. The secondary metabolites that would be responsible for the anthelmintic activity in BO would be Quercetin 7´,3,´4´ trimethoxy, a flavonoid derivative (polyphenols group), and Phytol isomer, a diterpenoid. In DL the compounds with anthelmintic activity would be Phytol, Quercetin 7´,3,´4´-trimethoxy, and Fern-8-ene, a triterpenoid. The compounds in MF that are responsible for

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anthelmintic action would be Phytol, Xanthorrhizol, a terpenoid, and fern-9(11)-ene, a fernane type triterpenoid. The compounds in MS responsible for anthelmintic action would be Phytol, Quercetin 7´,3,´4´-trimethoxy, and fern-8-ene.

Hexadecanoic acid dioctyl ester (CAS), one of the major compounds detected through GC-MS analysis, is a palmitic acid derivative. It is known for various biological functions such as antioxidant, flavouring agent, pesticide, lubricant, antiandrogenic, haemolytic, alpha reductase inhibitor, anti-inflammatory, hypocholesterolemic, nematicide, insectifuge and anti-bacterial (Cowan 1999). The abundant flavonoids might be responsible for their therapeutic effectiveness against a wide array of microorganisms, probably due to their ability to complex with extracellular and soluble proteins and to complex with the bacterial cell wall. Flavonoids and other phenolic compounds are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity, and anthelmintic activity (Talukdar *et al*. 2011). Quercetin 7, 3´, 4´ trimethoxy present in these ferns, as revealed in the GC-MS analysis, might be attributed with significant antioxidant property (Lee *et al*. 2005). Phytol also would possess antioxidant property (Dukes).

Recently, *in vitro* larvicidal and ovicidal activity of leaf and seed extracts of *Solanum torvum* was discovered. It is antiparasitic to some hematophagous parasites of cattle and goat and also against a digenean fluke of sheep, *Paramphistomum cervi*. Several studies have assigned antiparasitic effect of medicinal plants to the alkaloids (Athanasiadou and Kyriazakis 2004). Similarly, anthelmintic effect *in vitro* of tropical plants *Lysimachia ramosa, Olea europaea,* and *Satureja khuzestanic* has been reported (Challam *et al.* 2010). Adult trematode *Fasciolopsis buski*, nematode *Ascaris suum* and cestode *Raillietina echinobothrida* were exposed to alcoholic extract of *Lysimachia ramosa* Wall. The treated parasites were completely inactivated through loss of motility/flaccid paralysis and deformity of the surface architecture, ultimately leading to death. The pharmacologically active components responsible for these effects are triterpenoids, saponins, organic acids, and flavones, from different species of the genus *Lysimachia*. The terpenoids, phenols, fatty acids and flavonoids revealed in the GC-MS analysis could be responsible for the anti-parasitic action and antioxidant property. These form a good combination of secondary metabolites showing efficient action against trematodes. This is to be viewed in the background that ingredients in human food, such as coconut, onion, garlic, fig, date, pineapple, chicory, etc., possess high anthelmintic potential against intestinal nematodes, cestodes, and trematodes. The active compounds were extracted into either chloroform, water, or polyethylene glycol/propylene carbonate (PEG/PC) and were examined on the cestode models *Hymenolepis diminuta, Hymenolepis microstoma, Taenia taeniaeformis*, and the trematode models *Fasciola hepatica* and *Echinostoma caproni*; most of the extracts were anthelmintic *in vivo* (Abdel-Ghaffar *et al.* 2011). Treatment of infected animals with a combination of onion and coconut extracts in PEG/PC eliminated all cestodes. The same composition of extracts was effective against *E. caproni* but failed to kill the liver fluke *F. hepatica* in the final hosts. Validation of antiparasitic activities of compounds obtained from plant extracts requires standardization of methodologies and this issue is discussed in a review, wherein the authors focussed on the strengths and weaknesses of the existing methodologies used in the control and also discussed issues like the seasonal variability of the plant composition and how this can influence their antiparasitic properties (Athanasiadou *et al.* 2001). Therefore, it is important to isolate and identify the phytochemicals and decipher their mechanisms of action in the parasites with respect to unique molecular targets and physiological pathways.

Essential oils are volatile, natural, complex odoriferous secondary metabolites produced by aromatic plants. Essential oils being very complex mixtures can contain 20-60 components at quite different concentrations. They are characterized by 2-3 major compounds at fairly high concentrations (20-70%) compared to others components that are present only in trace amounts. The major compounds are terpenes (e.g., geraniol, carvacrol, thymol, cymene, sabinene, alpha-pinene, betapinene, citronellol, sesquiterpene farnesol) or terpenoids (menthol, ascaridole), and the other aromatic and aliphatic compounds, characterized by low molecular weight (e.g., cinnamyl alcohol, eugenol, safrole). In general, the many compounds determine the biological properties of the particular essential oil and it is likely that they interact with several targets in the cells. The tegument of cestodes and trematodes is morphologically and physiologically different from the cuticule of nematodes. Therefore, the essential oils are likely to pass through the layers of tegument. Oils of spices, such as *Nigella sativa* (black cumin), *Allium sativum* (garlic) and *Piper longum* (Indian long pepper) have been many a times shown to be trematodicidal, based on studies in *Schistosoma mansoni* and *Fasciola gigantica* (Mahmoud *et al.* 2002, Singh *et al.* 2009). This provides support for our inference that the oils of the ferns in this study also would possess trematodicidal property.

In vitro anti-trematodal property of the pteridophytes

Various active components of plant extracts have been tested for anthelmintic activity wherein it has been shown that tegument of trematodes and cestodes represents a potential target site since the small molecules could be adsorbed on to it (Athanasiadou and Kyriazakis 2004). The secondary metabolites, especially plant alkaloids, act on adult stage of flukes producing anti-trematodal effects in cattle, sheep and goat. Several studies have shown that extracts of botanical foods and spices possess high anthelmintic potential against intestinal nematodes, cestodes and trematodes (Abdel-Ghaffar *et al.* 2011). Our study shows the ferns to be effective in dealing with flukes of ruminants.

Anthelmintics are drugs that cause adverse effects on the helminth parasites by affecting vital activities such as feeding, neuromuscular transmission, ion exchange or the tegument (Shokier *et al.* 2013). The normal phytochemicals present in the plant extracts act in manner similar to the mechanisms exhibited by conventional anthelmintics. The saponins interact with the cell membranes, causing changes in the cell membranes, and subsequent changes in the cell wall (Radwan *et al.* 2012). Tannins have the capacity to bind to proteins and impair vital processes like feeding, and reproduction of the parasite and disrupt the integrity of the cuticle (Priya *et al.* 2014). The condensed tannins interact with proline-rich proteins on cuticle that will interfere with feeding, motility and other key metabolic processes like ex-sheathment and moulting (Bachaya *et al.* 2009). The ferns in our study not only demonstrated this property, but they also caused early death of worms. Thus, this study reveals that fern extracts are promising *in vitro* anthelmintics. These plants are known to be rich in alkaloids, phenols, terpenoids, flavonoids, glycosides, tannins, etc. (Hossain *et al.* 2012). The anthelmintic property could be attributed to these bioactive compounds individually or in combination. The *in vitro* trematodicidal and antioxidant properties as found in this study might be attributed due to the action of the abundant fern secondary metabolites.

Classes of secondary metabolites such as alkaloids and flavonoids are considered the source of chemical compounds responsible for wide therapeutic activities of several medicinal plants (Wink 2012). Anthelmintic activity of plant extracts on larvae and adults of gastrointestinal nematodes is attributed to the capacity of tannins to bind to proteins and operate via several mechanisms (Athanasiadou *et al.* 2001). Anthelmintic activity of *Fumaria parviflora* might be due to alkaloids which have the ability to intercalate with replicating DNA (Maqbool *et al.* 2004). Transcuticular diffusion is a common means of entry of non-nutrient and non-electrolyte substances into nematode, cestode and trematode parasites as opposed to oral ingestion (Alvarez *et al.* 2007). Possible explanation for the good anthelmintic activity of ethanolic extract could be easier transcuticular absorption of ethanolic extract into the body. Our study has revealed for the first time, to the best of our knowledge, broad anthelmintic activity of all four ferns relative to the efficacy of oxyclozanide, a salicylanilide anthelmintic. Naturally occurring combinations of plant compounds in crude extracts would act synergistically and often produce appreciable antimicrobial activity compared to purified individual compounds. Such plant-based treatments could be made part of an integrated management strategy for control of helminths in developing countries. Further studies are required to isolate and identify the active compound contained in the crude extracts so as to establish the mechanism of action.

The present study used Oxyclozanide, which by mechanism of action uncouples oxidative phosphorylation, as the standard drug and compared the response in *G. crumenifer*, as the trematode model, and compared the effect of treatment with extracts of the four selected ferns. It was found that the worm succumbed to death following complete paralysis at all concentrations tested. But 100% mortality (RM Value 0) was accomplished at the highest concentration with less incubation time, effect comparable to that of the standard control. It was proved that DL and MS extracts produce the strongest trematodicidal action, at the highest concentration, 5 mg/mL (RM value 0), killing all the worms in the group at 10 min incubation time. However, BO extracts produced only moderate trematodicidal action at the same dose since all worms in the group were killed at 30 min incubation time. MF produced the least trematodicidal action whereupon all worms in the group were killed at this concentration at 60 min incubation time. Overall, the *in vitro* incubation study for anti-trematodal property of the ferns revealed DL and MS extracts to produce the strongest trematodicidal action followed by BO and MF extracts. Looking at the statistical interpretation for assessment of MI and RM value obtained at 5 mg/mL concentration, the RM value 0 (100% mortality) was achieved at 30 min for BO, 10 min for DL, 60 min for MF and 10 min for MS. The statistical analysis further revealed the action of fern extracts as dose-dependent and directly proportional to the dose. Among the four trials of fern extracts, the standard control (Oxyclozanide) produced 100% mortality (RM value 0) at 10 min incubation time.

A drug, which would produce adverse effects in a parasite, sparing the host, is a potential anthelmintic drug. Anthelmintic drugs impair the vital activities

of the worm and, consequently, lead to death of the worm. Embarking upon drug discovery/treatment requires a thorough knowledge of life cycle of the parasite, and its physiology, biochemistry and surface morphology/composition (tegument or cuticle). Surface of flatworms, mouth of trematodes and gut of nematodes are the key role players in drug absorption/uptake. However, no single drug available today is useful for the treatment or prevention of both nematode and trematode infections in humans. There are also differences in susceptibility of worm species to individual chemical derivates in the same class.

Conclusions

Herein we have demonstrated and compared the trematodicidal action of four species of ferns collected from the wild and substantiated the antiparasitic medicinal property of Pteridophytes as practiced in folklore medicine. The study leads to the conclusion that ferns are a potential source of novel anthelmintics for medicinal practice. Nevertheless, the conclusion is based on an *in vitro* study, so the outcome must be corroborated with an *in vivo* controlled array of drug discovery research. The study suggests fern-8-ene, fern-9(11)-ene, Phytol, Quercetin 7´,3´,4´ trimethoxy and Xanthorrhizol as the potential plant secondary metabolites to look single chemical compounds for exploration in *in vivo* study. To sum up, this controlled array of *in vitro* drug research has expounded that ferns could be a potent source of trematodicidal antibiotics.

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