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Phytochemical constituents and *in vitro* trematocidal activity of *Blechnum orientale* Linn. against *Gastrothylax crumenifer*

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Abstract

The aim of this study is to assess the phytochemical constituents in *Blechnum orientale* Linn., column purification, gas chromotography-mass spectrometry (GC-MS) analysis and evaluate the *in vitro* antitrematocidal efficacy against *Gastrothylax crumenifer*. Qualitative analysis of phytoconstituents (n=13), using solvents (aqueous, acetone, chloroform, ethanol and petroleum ether) showed ethanolic extract performed well to express chemical constituents in ferns, with strong positivity in (n=7) phytoconstituents. Quantitative phytochemistry of total terpenoids, total tannin, total phenol and total flavonoids showed highest level of total terpenoids content (79.0 mg/g). Column chromatography was performed, using standard protocols where fraction VI showed highest antioxidant activity (64.4 %) when compared to standard BHT (98.3%). GC-MS analysis performed, showed phytol isomer, a diterpenoids and quercetin 7´3´,4´ trimethoxy, a flavonoids possess potent anthelmintic property. Best eluted fraction under column chromatography under *in vitro* incubation study using Hedon-Fleig solution (25 ml) (pH 7.0) against *Gastrothylax crumenifer* amphistomes using test extracts (1 mg, 2 mg, 3 mg, 4 mg and 5 mg concentration, respectively), oxyclozanide as positive control @ 1% concentration (0.25 g/25 ml) and negative control showed fern extract with highest concentration (5 mg), had significant and intense trematocidal activity rather than oxyclozanide and taken lesser time to kill the trematodes. However, the study confirmed that drug concentration is inversely proportional to time taken to kill the worms and are holding better drug efficacy against *Gastrothylax crumenifer*.

Key words: Phytochemistry, gas chromatography-mass spectrometry, *in vitro* trematocidal, *Blechnum orientale* Linn., *Gastrothylax crumenifer*

1. Introduction

Pteridophytes are vascular cryptogams (ferns and fern-allies), shade loving plants, form the dominant vegetation on the earth. Fraser Jenkins (2008) calculated the Indian pteridophytic flora includes 1100 species of fern and fern allies. Among these, 337 species are categorized as threatened or endangered. More than 300 species of ferns and fern allies are reported from the Western Ghats, south India, of which 45 ferns are categorized as rare and endangered (Manickam and Irudayaraj, 1992). There is an urgent need for the conservation of pteridophytes in the Western Ghats region (Maridass and Raju, 2010). Pteridophytes have an important role in the ecosystem and had various economic uses which had to be investigated in detail. Pteridophytes are unfortunately ignored group of plants (Annie and Kumaresan, 2010; Vashishta *et al.*, 2012), had significant medicinal property and are traditionally used by ethnic communities. Most of the vascular cryptogams are in threatened list and some are in endangered status in the Red Data book of IUCN. The limited knowledge of these medicinal plants for disease control and their weed habitat make these ferns to be destroyed by human. Nature had provide in such a way that various secondary metabolites of pteridophytes had adaptability mechanism to their environment under varied climatic conditions (Bennett and Wallsgrove, 2006). Swain (1977) stated that these metabolites are polyphenols, flavonoids, terpenoids, steroids, quinones, alkaloids and polysaccharides that engaged with the color, flavor and aroma of plants. These functional metabolites have properties which prevent and cure various diseases as well as ageing in mammals including humans. Ferns are expected to have many useful secondary metabolites than other plants. Ferns were reported to have many useful phytochemicals (secondary metabolites) such as flavonoids, steroids, alkaloids, phenols, triterpenoid compounds, varieties of amino acids and fatty acids (Zeng-fu *et al.*, 2008). They also have some unique secondary metabolites which have not been discovered in higher plants (Zhao *et al.*, 2007; Shinozaki *et al.*, 2008). Traditional medicinal property of ferns are reported for the cure of various ailments, ascarid infection (May, 1978; Wu, 1990; Benjamin and Manickam, 2007), antioxidant property (Ding *et al.*, 2008; Shin and Lee, 2010), antimicrobial (Parihar and Bohra, 2002),

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antiviral (McCutcheon *et al.,* 1995), anti-inflammatory (Liu *et al*., 1998; Punzon *et al.,* 2003), antitumor (Konoshima *et al.,* 1996) and anti-HIV (Mizushina *et al.,* 1998) and so on.

Blechnum orientale Linn. used in this study, though commonly available ferns, also called as Hard ferns, had significant medicinal property and are used for urinary bladder complaints, polynesia, diaphoretic and anthelmintic (Benjamin and Manickam, 2007). The ferns are used as folklore medical practice by ethnic communities of Western Ghats, Kani tribes. There is no applied research study on anthelmintic potential of these ferns and the study focussed to lime light the *in vitro* antitrematocidal property of these ferns and are supported with relevant literature stating the anthelmintic property of various other ferns (Blakemore *et al.,* 1964; Singh, 2003; Kalpana Devi *et al.,* 2015). However, the detailed study on antitrematocidal status of *B.orientale* Linn. has not been reported so far. Wink (2012) stated that medicinal chemists have synthesized a number of drugs which can be used against many but by for not all endoparasites. It is a well known factor that parasitism develop resistance against anthelmintic drug, used frequently and there attribute a challenge developed among modern researchers to overcome anthelmintic resistance. The cost factor also play an important role in poor economic developed countries like India, where the farmer cannot meet out the treatment cost to deworm all animals. An alternative to synthetic drugs is the search for antiparasitic plant extracts or secondary metabolites, derived from them. Natural products still plays an important role in therapy and also need frequent change in drug chosen or a combination of more drugs (Subramoniam, 2014), used to overcome resistance. Therefore, the aim of the present study was to analyse the chemical composition, using GC-MS analysis to ascertain the antitrematocidal activity of *B.orientale* Linn.

2. Material and Methods

2.1 Plant collection and identification

Blechnum orientale Linn. (Blechnaceae), collected from Kodhayar hills, Western Ghats, Tamil Nadu, India and the herbarium prepared was identified at Scott Christian College, Nagercoil, Kanyakumari District with different floras (Beddome, 1868-74; Manickam and Irudayaraj, 1992) and a sample specimen (No. SPCH 1004) was preserved in A.V.V.M Sri Pushpam College, Thanjavur, Tamil Nadu, India.

2.2 Preparation of the plant extract

One gram of shade dried powder of whole plant, *B. orientale* Linn. was extracted with 20 ml ethanol, 75% acetone, chloroform, aqueous and petroleum ether (Merck, extra pure) for 1 min, using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature as stated (Samundeeswari *et al.,* 2013). The sample was then filtered through Whatman No. 1 paper in a Buchner funnel and evaporated under vacuum in a rotavator at 40°C to a constant weight and then dissolved in respective solvents. The dissolving rate of the crude extracts was approximately 100%. The solution was stored at 18°C under room temperature until use.

2.3 Qualitative phytochemical screening of plant extract

The phytochemical screening was assessed as per standard method (Brinda *et al.,*1981; Savithramma *et al.,* 2011). Phytochemical screening was performed, using aqueous, acetone, chloroform, ethanol and petroleum ether solvents to identify the major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins and steroids. General reactions in these analyses revealed the presence or absence of these compounds in the leaf extracts tested.

2.4 Quantitative phytochemical analysis

Total terpenoid content in the extracts were assessed by standard method of Ferguson (1956). One gram of plant powder was taken separately and soaked in alcohol for 24 h. Then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoid. Tannins content in the extracts was estimated as described by Fagbemi *et al.* (2005). The sample extracts (1 ml) were mixed with Folin-Ciocalteau's reagent (0.5 ml), followed by the addition of saturated sodium carbonate (Na_2CO_3) solution (1 ml) and distilled water (8 ml). The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm, using UV-Visible Spectrophotometer and was expressed as µg tannic acid equivalent (TAE) per gram of the sample. Total phenolic content in the extracts was determined by the Folin-Ciocalteau colorimetric method (Slinkard and Singleton, 1956). For the analysis, 0.5 ml of aliquot of sample was added to 0.5 ml of Folin-Ciocalteau reagent (0.5 N) and the contents of the flask were mixed thoroughly. Later 2.5 ml of sodium carbonate (2%) was added, and the mixture was allowed to stand for 30 min. after mixing. The absorbance was measured at 760 nm in a UV-Visible Spectrophotometer and were expressed as mg gallic acid equivalents (GAE)/g extract. Total flavonoid content in plant extracts was determined by the aluminium chloride colorimetric method (Mervat *et al*., 2009). 0.5 ml of plant extracts at a concentration of 1mg / ml was taken and the volume was made up to 3 ml with methanol. Then 0.1 ml $AlCl_3$ (10%), 0.1ml of potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance was recorded at 415 nm after 30 min. of incubation. A standard calibration plot was generated at 415 nm, using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.

2.5 Column chromatography

2.5.1 *Extraction of active compounds for antioxidant potential*

The concentrated extract in aqueous extract was separated and analysed by column chromatography technique. The crude extract (5 gm) was triturated with 90% methanol. The prepared solution was then fractionated successively, using solvents of increasing polarity, such as n-hexane (HX : 820 mg), carbon tetrachloride (CT: 550 mg) and chloroform (CF: 665 mg). All the three fractions were evaporated to dryness by using rotary evaporator at low temperature of 39^0C and kept in air tight containers for further analysis. Column chromatography was performed, using chloroform soluble materials of methanolic extract by gel permeation, using silica gel and was packed with Sephadex (LH-20) and soaked with a ration of n-hexane:dichloro methane:methanol (2:5:1) for at least 12 h. The column was then eluted with the same solvent mixture and finally the column was washed with dichloromethane and methanol mixtures of increasing polarity

2.6 Gas chromotography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed in separated fraction of fern extract, having high antioxidant potential (Fraction VI) under column

chromatography. Based on the antioxidant potential, the most effective fraction of the methanolic extracts of the fern (F6) was further used for the identification of bioactive constituents by GC-MS analysis. The Trace GC Ultra and DSQII model MS from Thermo Fisher Scientific Limited, were engaged for analysis. The instrument was set as follows:

Injector port temperature set to 250ºC, interface temperature set as 250ºC, and source kept at 200ºC. The oven temperature programmed as a variable, 70ºC for 2 min, 150ºC @ 8ºC/min, up to 260ºC @ 10ºC/min. Split ratio set as 1:50 and the injector used was splitless mode. The MS was set to scan from 50 to 650 Da. The source was maintained at 200ºC and < 40 motor vacuum pressure. The ionization energy was -70eV. The MS was also having inbuilt pre-filter which reduced the neutral particles. The data system has two inbuilt libraries, NIST4 and WILEY9 for searching and matching the spectrum. Only those compounds with spectral fit values equal to or greater than 700 were considered positive identification.

2.7 *In vitro* trematocidal activity

2.7.1 *Collection of worms*

Adult live and healthy amphistomes were collected from the rumen of infected sheeps, goats and cattles slaughtered at Orathanadu, Thanjavur and Pattukottai abbatoir. Adult live worms were washed thoroughly in physiological saline and maintained in Hedon-Fleig solution for the *in vitro* maintenance of amphistomes studied (Veerakumari, 1996). Collected worms species were identified, based on morphology (Soulsby, 1982) and identified as *Gastrothylax crumenifer* which have been taken for this *in vitro* incubation study.

2.7.2 *In vitro incubation study*

In vitro incubation study against *Gastrothylax crumenifer* was performed, using twenty-five milliliter of Hedon-Fleig salt solution, containing various concentrations (1 mg, 2 mg, 3 mg, 4 mg and 5 mg, respectively) of fraction VI extracts; oxyclozanide as positive control @ 1% concentration (0.25 g/25 ml) and negative control, H-F salt solution alone distributed to air tight containers. Twenty five amphistomes were incubated in 25 ml of each concentration of fern extracts (test group), positive control and negative control. The motility of the parasites was observed visually at a regular time interval of 0 min, 10 min, 15 min, 30 min, 1 h and 2 h, respectively. A score index was made for the motility criteria, using the following criteria (Jiraunkoorskul *et al.*, 2005).

Active : Score 3 - Moving whole body

Moderately active : Score 2 - Moving only parts of the body

Sluggish : Score 1 - Immobile but alive

Dead : Score 0 - Immobile

3. Results

Qualitative phytochemical analysis stated out of 5 solvent extracts, ethanol extract performed well to show positivity of phyto-constituents than other 4 solvent extracts (Table 1). The quantitative estimation of the phytochemicals have shown that *B.orientale* Linn. contained higher amount of terpenoid (79.0 ± 0.84 mg/g), followed by with total tannin (35.0 ± 0.24 mg TAE/g), total flavonoid (8.25 ± 0.41 mg QE/g) and total phenol (1.30 ± 0.71) mg GAE/g) (Figure 1). Among the various fractions of fern extracts eluted, fraction VI exhibited high antioxidant potential (64.4%) under purification and separation, using column chromatography technique (Figure 2). The GC-MS chromatogram and mass spectrum of 11 chemical compounds were mentioned in (Table 2, Figure 3). *In vitro* anthelmintic drug trials against amphistomiasis showed significant action at higher concentration (5 mg) of fraction VI eluent, showing significant trematocidal acitivty (Table 3, Figure 4).

Table 1: Qualitative phytochemical analysis

Phytochemicals	***Blechnum orientale* Linn.**				
	Aqueous	Ethanol	Chloroform	Petroleum ether	Acetone
Tannins	-	++	+	+	++
Saponins	++	++	+	+	-
Quinones	++	++	-	-	++
Terpenoids	+	++	+	-	+
Steroids	+	++	+	-	++
Flavonoids	++	++	+	+	+
Phenol	++	++	+	+	+
Alkaloids	-	+	+	+	+
Glycosides	+	+	-	-	+
Cardiac glycosides	-	+	-	-	+
Coumarins	++	+	-	-	+
Antho cyanin	-	-	-	-	-
Beta cyanin	+	+	-	+	+

++ Strong Positive; + Positive ; – Negative

Table 2: Medicinal property of chemical constituents under GC-MS analysis

RT	Compound name	Nature	Medicinal property
7.81	Dodecane (CAS)	Iodophore compound	Antimicrobial
16.03	1,2,3-Propanetricarboxylic acid, 2-hydroxy-, triethyl ester (CAS)	Fatty acid derivative	Antiulcer, anti-inflammatory
22.26	Dibutyl phthalate1, 2-Benzene dicarboxylic acid, dibutyl ester (CAS)	Plasticizer compound	Antifouling, antimicrobial
25.16	2-Hexadecen-1-ol, 3,7,11, 15-tetramethyl, [R-[R*,R*-(E)]]- (CAS) phytol isomer, Lucenin 2, Quercetin 7'3'4' - Trimethoxy	Diterpenoids Flavonoids Triterpenoids	Antioxidant, antimicrobial, anthelmintic, anticancer, anti-inflammatory, diuretic and antidiabetic
29.69	Hexanedioic acid, mono (2-ethylhexyl) ester	Saturated fatty acid	Antioxidant, hypocholesterolemic, nematicidal, pesticidal, lubricant, haemolytic, antiandrogenic and flavouring property
33.38	3-benzoyl-4-methyl-6-ethyl-2(1H)-Pyridone	Polycylcic aromatic compounds	Antioxidant, antimicrobial

Table 3 : *In vitro* trematocidal activity of *Blechnum orientale* Linn. against *Gastrothylax crumenifer*

Conc/	0 min				10 min				15 min				30 min				1 h				2 h			
Score	3	2	1	0	3	2	1	0	3	2	1	0	3	2	1	0	3	2	1	0	3	2	1	0
1 mg/l	25	0	0	0	25	0	0	0	25	0	0	0	20	1	0	4	15	0	3	7	12	0	5	8
2 mg/l	25	0	0	0	25	0	0	0	24	0	0	1	17	1	1	6	7	3	2	13	4	4	7	10
3 mg/l	25	0	0	0	25	0	0	0	0	20	3	2	0	6	11	8	0	4	5	16	0	0	3	22
4 mg/l	25	0	0	0	0	8	4	13	0	4	1	20	0	0	0	25	-	-	-	-	-	-	-	-
5 mg/l	25	0	0	0	0	1	2	22	0	0	0	25	-	-	-	-	-	-	-	-	-	-	-	-
PC	25	0	0	0	0	0	0	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NC	25	0	0	0	25	0	0	0	25	0	0	0	25	0	0	0	25	0	0	0	25	0	0	0

PC - Oxyclozanide; NC - Hedon-Fleig salt solution; Score 0 - Immobile; Score - Immobile but alive; Score 2 - Moving only parts of body; Score 3 - Moving whole body

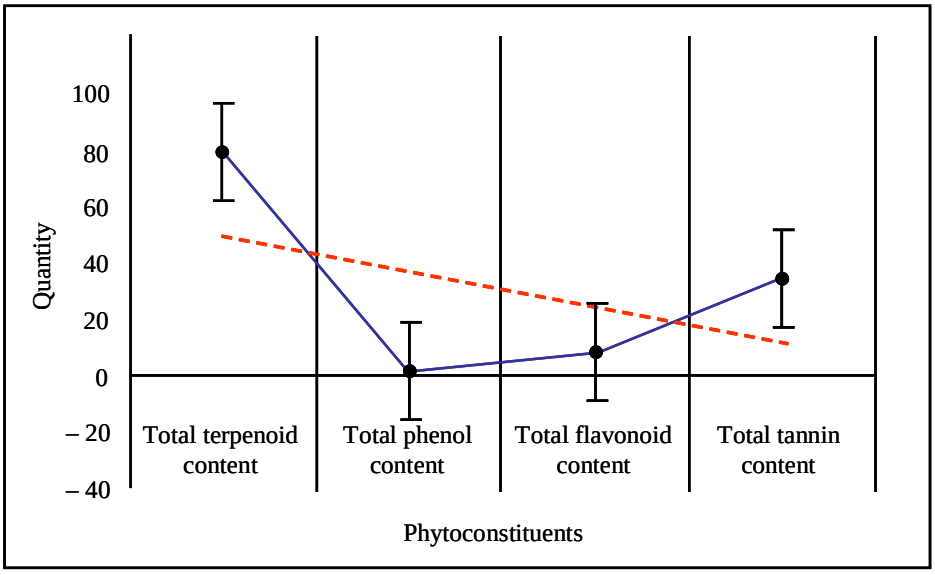

Figure 1: Quantitative phytochemical analysis

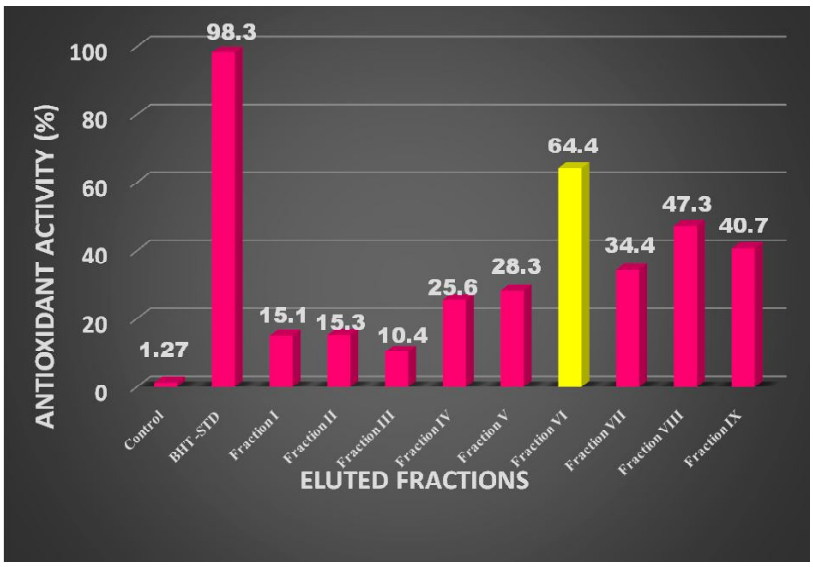

Figure 2: Comparative analysis of various fraction eluted under column chromatograph of *B. orientale* Linn.

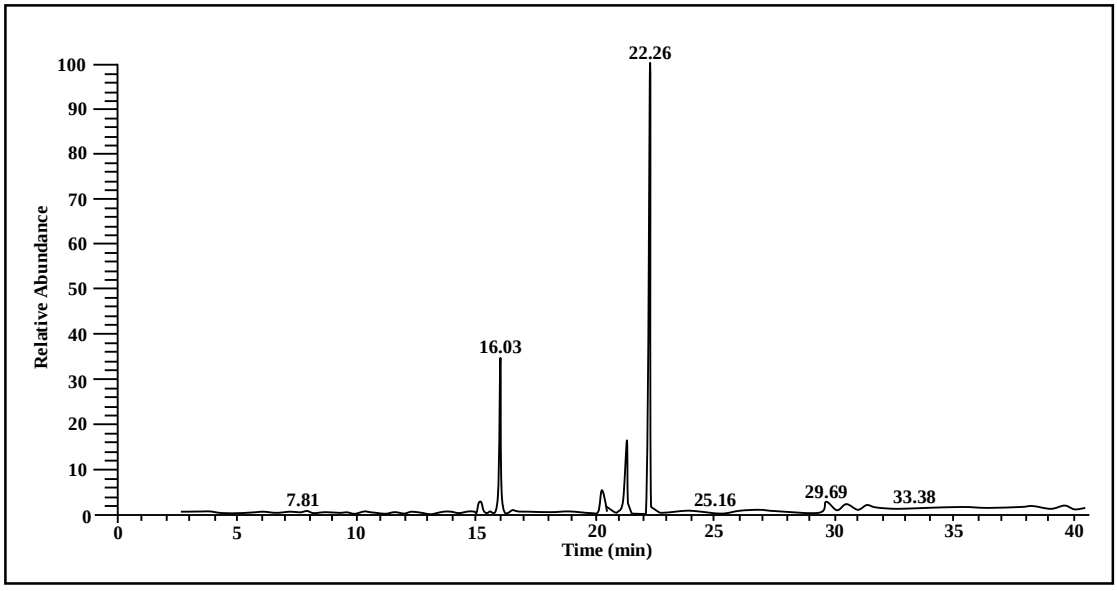

Figure 3: Gas chromatography-mass spectrometry analysis

4. Discussion

Examination for evidences of phytoconstituents showed ethanol extract performed well to express the phytochemicals in the fern studied. Tannin, phenol, terpenoid, flavonoid, steroids, quinones and saponins showed strong positivity in ethanol fern extract which was the best solvent to express phytoconstituents in this study. Mithraja *et al.* (2012) performed phytochemical screening with acetone, benzene, chloroform, ethanol, petroleum ether and aqueous extracts of whole plants of various pteridophytic plants, revealed that the presence or absence of the phytoconstituents depend upon the solvent medium, used for extraction and the physiological property of individual taxa. The present study on the phytochemical analysis of *B.orientale* Linn. was in confirmation with the study of (Mithraja *et al.*, 2012), stated that tannin containing drugs are used in medicine as astringent and have been found to possess antiviral, antibacterial and antiparasitic effects for possible therapeutic applications. Tannin was present in the acetone and ethanolic extract of all the fern under study.

Kumudhavalli and Jaykar (2012) evaluated the petroleum ether, chloroform, acetone, ethanol and aqueous extracts of the fern, *Hemionitis arifolia* (Burm.) Moore, for preliminary phytochemical screening. Our study was subjected for quantifying *B.orientale* Linn. Gracelin *et al.* (2013) conducted qualitative and quantitative phytochemical analysis in five Pteris fern species. Qualitative

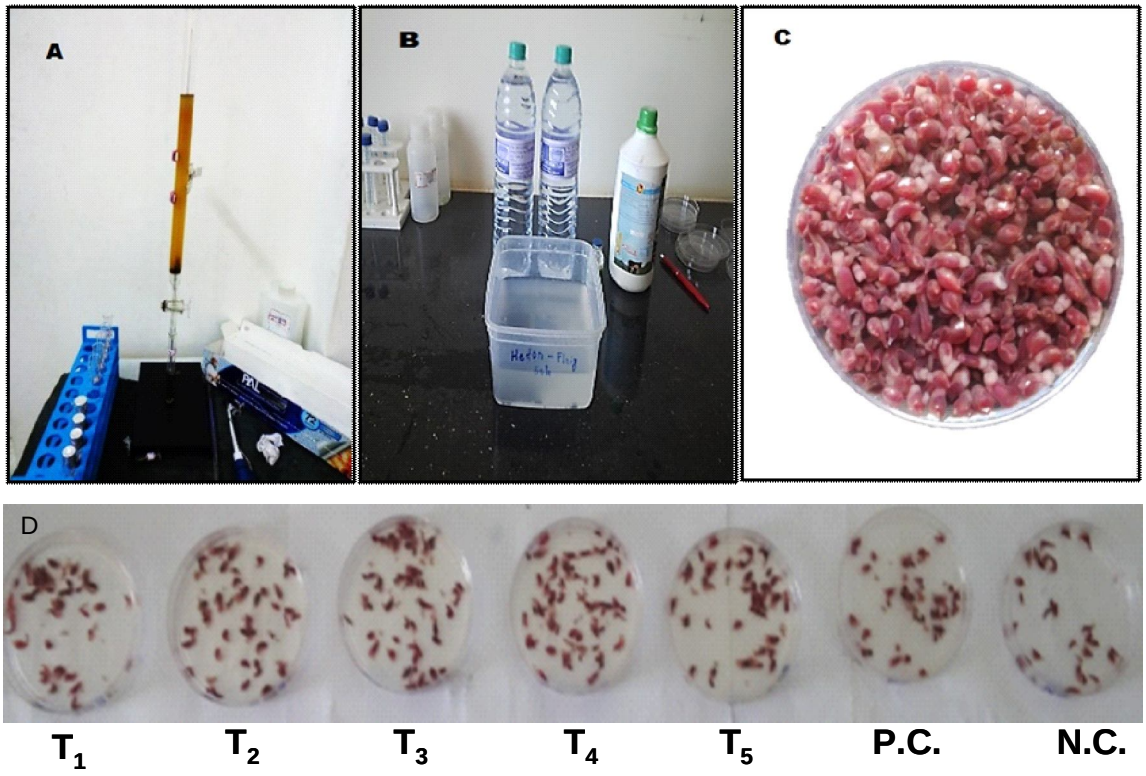

Figure 4: *In vitro* incubation study of trematodes: (A) Column chromatography, (B) H-F salt solution, (C) Collected amphistomes, and (D) *In vitro* trials in *Blechnum orientale* Linn.

analysis of methanol extract exhibited positivity for 10 phytochemical tests. The study involves analysis of ethanolic, petroleum ether, acetone, chloroform and aqueous extract of *B. orientale* Linn., showed ethanolic extract performed well to exhibit. The quantitative analysis of Pteris species showed highest value of flavonoids, then followed by alkaloids and phenolic compounds. The amount of tannin and saponin was very low in the fern extract. However, our quantitative study showed terpenoid content was highest and flavonoid content was least in *B.orientale* Linn.

The results of the phytochemical screening and quantitative estimation of the chemical constituents of plant sample have indicated high content of terpenoids, total tannin, total phenol and flavonoids. The abundance of flavonoids which are hydroxylated phenolics substances might be responsible for their therapeutic effectiveness against wide array of microorganisms, probably due to their ability to complex with extracellular and soluble proteins and to complex with the bacterial cell wall (Cowan, 1999). Quercetin 7, 3´, 4´trimethoxy, a potent flavonoid (polyphenolic group) and phytol isomer, a potent diterpenoids present in these fern extract under GC-MS analysis might be attributed to significant antioxidant potential and antitrematocidal activity. These findings provide quantitative estimation of the phytochemicals as well as mineral element analysis which are important in understanding the pharmacological and/or toxicological actions of medicinal plants. Further work on extraction and purification of active constituents should be of interest (Aliyu *et al.*, 2008).

Various studies on *in vitro* and *in vivo* antitrematocidal activity of medicinal plants, *viz*., alcoholic extract of *Allium sativum* and *Piper longum* (Singh *et al.*, 2015), alcoholic extract *of Balanites aegyptica* (Swarnakar *et al.*, 2015), bark of *Prosopis cineraria* (Manigandan and Veerakumari, 2015), plumbagin on newly excysted and 4 weeks old juvenile parasites of *Fasciola gigantica* (Natcha *et al.*, 2014), plumbagin on motility, survival and tegument structure of *Paramphistomum cervi* (Naruwan *et al.*, 2013). Similarly, *in vivo* study on natural fasciolosis infected cattle (Shokier *et al.*, 2013), has been reported. The present study is first report on pteridophytic plants, having *in vitro* antitrematocidal efficacy. The drug action of pteridophytes is particularly attributed to target predilection sites of trematodes, teguments with varied changes in tegumental enzymes and hinder the metabolic action of trematodes, ultimately causes death of worms. In the present study, the death of worms happened due to the similar mechanism but varied to time duration and concentration. Higher concentration (5 mg) had active metabolites at higher conecentration and cause intense and immediate death within short period of time. However, lower concentration (1 mg) need more time to kill the worm. But the mechanism of drug action is similar on the tegumental surface of worm. Kamaraj and Rahuman (2011) studied the *in vitro* larvicidal and ovicidal activity of leaf and seed extracts of yet another Solanum species, *i.e.*, *Solanum torvum* on nematode, *H. contortus*. At the maximum concentration tested (50 mg/kg), a 100 % inhibition of egg hatching and larval development was recorded for an ethyl acetate extract of the plant. The extract also showed its antiparasitic effects on some hematophagous parasites of cattle and goat and also against a digenean fluke of sheep; namely, *Paramphistomum cervi*. Many previous studies have assigned the antiparasitic effects of medicinal plants to these alkaloids (Athanasiadou and Kyriazakis, 2004). However, the exclusive secondary metabolite responsible for antioxidant and antitrematodal activity should be scrutinized further subjecting for LC-MS analysis.

5. Conclusion

In conclusion, the ethanol extracts of *B.orientale* Linn. showed very high terpenoids, total phenolic and total tannin content and are potent antioxidants. Quercetin 7, 3´, 4´ trimethoxy, a potent flavonoid (polyphenolic group), phytol isomer, a potent diterpenoids present in these fern extract under GC-MS analysis, showed evidences of anthelmintic activity and are used as potent antitrematocidal drugs in these studies. However, further investigative work is warranted to isolated the secondary metabolite, essential for *in vivo* trials. These plants could be a good source of natural antioxidants and antitrematocidal drugs.

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Conflict of interest

We declare that we have no conflict of interest.

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