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In vitro cytotoxicity screening, phytochemical profile and heavy metal analysis of different extracts of *Acrostichum heterophyllum* L.

Meera George and V S Josekumar*

Department of Zoology, Mar Ivanios College (Autonomous), Thiruvananthapuram–695015, Kerala, India

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The present study was carried out to identify the phytochemical profile, heavy metal content and cytotoxicity of the epiphytic fern, *Acrostichum heterophyllum* L., which is a herb used in many traditional and tribal systems of medicine. Preliminary scientific evaluation towards the possibility of this species as a potential bioactive agent is attempted here. Three different extracts in hexane, ethyl acetate and methanol were prepared sequentially and obtained a percentage yield of 2.714, 3.82 and 8.058, respectively. Phytochemical screening revealed presence of flavonoids, alkaloids, sugars, glycosides, phenolics and tannins as major components of ethyl acetate and methanol extracts. All the heavy metals analyzed were found to be below the permissible limits. MTT assay using L929 cell line identified IC_{50} value of hexane, ethyl acetate and methanolic extracts as 254.18, 266.99 and 191.93 µg/mL, respectively. High IC_{50} values reported in the present study suggests low toxicity of the extracts. The brine shrimp lethality assay also revealed the nontoxic nature of the extracts upto concentrations of 1000 µg/mL. The results indicate the suitability of *A. heterophyllum* as herbal a drug resource.

Keywords: *Acrostichum heterophyllum* L., *Drymoglossum heterophyllum* (L.) C. Chr., Heavy metal, *In vitro* cytotoxicity, L929 cell line, Phytochemical analysis.

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Introduction

Plant derived drugs have contributed to a great extent towards human health and the process of drug discovery. Since ancient times, all cultures have depended fully or partially on herbal medicines due to their affordability, effectiveness, availability, low toxicity and acceptability[1]. People consider herbal drugs as natural and therefore safe. But less than 10 % of herbal products in the world market are truly standardized to known active components and strict quality control measures are not always diligently adhered to[2]. It is necessary that a plant with traditionally known bioactive properties be analysed for its phytochemical constituents and possible toxicities before it is further progressed in the drug discovery pipeline.

Plants are rich sources of phytochemical compounds such as terpenoids, phenolic acids, lignins, tannins, flavonoids, quinones, coumarins, alkaloids, amines, vitamins, and other metabolites. Many of these compounds are proven as antioxidant, anti-inflammatory, antiatherosclerotic, antitumour, antimutagenic, anticarcinogenic, antibacterial and antiviral agents[3,4]. Phytochemical screening of a plant helps to identify the nature of bioactive compounds present in it. Toxicity screening can reveal some of the risks associated with the use of herbs, avoiding potential harmful effects when used as medicine. The brine shrimp lethality assay is an accepted procedure for preliminary assessment of toxicity[5]. *In vitro* screening on non malignant normal cell lines is also followed to evaluate the cytotoxic nature of herbal preparations[6]. For getting desirable therapeutic benefits, quality of these herbal products must be ensured in terms of toxic metal contamination. Analysis of the heavy metal profile of herbal raw material may be useful to identify the possible heavy metal contamination before proceeding to bioactivity studies[7].

Acrostichum heterophyllum L. syn. *Drymoglossum heterophyllum* (L.) C. Chr. is a fern of the family Pteridaceae found as an epiphyte on barks of old tree trunks. In tribal medicine, it is used in the form of poultice on fractured bones after setting up the bones. Antifungal and antibacterial properties of related species *D. piloselloides* have also been reported[8]. Ethanolic and aqueous extracts of this plant was

*Correspondent author
Email: vsjosekumar@gmail.com
Phone: 91-471-2531053
Fax: 91-471-2530023

found to have minimal antibacterial and antifungal effects. *A. heterophyllum* is used for the treatment of jaundice by the Kani tribes of Kerala[9,10]. It is also used as a cooling agent and in the treatment of swellings and sprains[10]. This species is referred in indigenous medicine of Sri Lanka to prepare medicated oil treatment and leaves are used as a styptic to arrest capillary haemorrhages and eczema[11].

Scientific studies on the compatibility of *A. heterophyllum* as herbal drug, its heavy metal content and cytotoxicity is lacking. Accumulation of heavy metals in herbal sources is a serious issue in quality control of herbal drugs. Hence, the present study was undertaken to identify the phytochemical profile, screen cytotoxic nature of different extracts and heavy metal of *A. heterophyllum* to assess its suitability as a herbal drug.

Materials and Methods

Collection and extraction of plant material

Fresh plant material was collected from local sources and authenticated by Dr. G. Valsaladevi, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala and a voucher specimen (No. KUBH 5926) was deposited in the herbarium of the same department. The leaves were washed with distilled water, dried in a hot air oven (40^{o} C) and powdered. The leaf powder was sequentially extracted in hexane (DHE), ethyl acetate (DEE) and methanol (DME) in a soxhlet apparatus in the order of their increasing polarity. Extraction was continued until the solvent in the sample holder became colourless. The temperature was set not to exceed the boiling point of the solvent. Each extract was concentrated in a rotary vacuum evaporator and the concentrated extracts were stored below 0^{o} C till further analysis. Percentage yield was calculated using the formula,

% yield = [dry weight of the extract/dry weight of leaf sample] × 100

Phytochemical Screening

The phytochemical analysis of hexane, ethyl acetate and methanolic extracts were carried out, using standard procedures to identify the constituents as described by Harborne[12].

Heavy metal analysis

Leaf powder (1 g) was transferred to a vial to which 5 mL of concentrated HNO_3 was added and kept for digestion until a clear solution was obtained. The digested solution was cooled and made to final volume of 50 mL with Mili-Q water. Sample solutions were then filtered through Whatman no. 40 filter paper. The digested samples were then used for metal analysis[13] using atomic absorption spectroscopic facility (Thermo Scientific iCE 3500) at the Central Soil Analytical Laboratory, Thiruvananthapuram.

In vitro cytotoxicity study on L929 cell line

Material

L929 normal mouse fibroblast cell line was obtained from NCCS, Pune. Dulbecco's modified eagle medium (DMEM), EDTA, DMSO and Trypsin were purchased from Hi Media, Mumbai. Foetal Bovine Serum was purchased from Invitrogen, India.

Cell line and culture conditions

Cells were maintained in DMEM supplemented with 10 % FBS at 37 °C in a humidified atmosphere with 5 % CO_2 in a CO_2 incubator (New Brunswick Scientific, Eppendorf, Germany). Cells were cultured in healthy conditions; exponentially growing cells were used for cytotoxicity studies.

MTT assay

Cytotoxicity was assessed by standard MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay[14,15]. L929 cells were seeded, in triplicates, in 96-well microplates (5000 cells/well) and kept at 37 °C with 5 % CO_2 in the incubator. After 24 h, the cells were treated with different doses of extracts at a final concentration of 6.25, 12.5, 25, 50 and 100 µg/mL prepared in 0.1 % DMSO. No plant extract was added to negative controls, but the same amount of 0.1 % DMSO was used to eliminate any intervening effects. The percentage difference in cell viability at all doses of extracts was compared with control after 24 h of incubation.

Brine shrimp lethality assay

Brine shrimp cysts were obtained as a gift from Central Marine Fisheries Research Institute, Vizhinjam unit, Thiruvananthapuram. The cysts were hatched in well aerated filtered sea water under illumination from a tungston lamp. The nauplii were hatched within 24-30 h at 30-35 °C. The extracts were first dissolved in 0.1 % DMSO and diluted with seawater for toxicity studies. The toxicity was tested at varying concentrations of extracts from 5 to 1000 µg/mL. Ten nauplii were used in each test. Seawater was used as culture medium. Three replications were done for each concentration. Negative controls without plant extracts but with the same amount of 0.1 %

DMSO in the medium were also kept. After 24 h, survivors were counted using a stereo microscope. The percentage of mortality in each dose was calculated and compared with control. LC_{50} value greater than 1000 µg/mL is considered non-toxic[16].

Statistical analysis

Experiments were done in triplicates and the results are reported as mean ± standard deviation. Best fit linear regression analysis was carried out using MS Excel 2007.

Results and Discussion

The present study reports the phytochemical profile and cytotoxicity screening of *A. heterophyllum.* The extraction was done successively with hexane, ethyl acetate and methanol. The yield of extraction hexane, ethyl acetate and methanol was 2.714, 3.82 and 8.058%, respectively.

The nature of the phytochemicals present in the plant determines the percentage yield in different solvents. Presence of more quantities of polar compounds will result in a higher yield in polar solvents like methanol[17,18]. Many authors have reported that the extraction yield increases with increasing polarity of solvents[19-21]. Phytochemical analysis of the plant revealed presence of phenolic compounds and tannins in both ethyl acetate and methanol extracts suggesting that plant may have higher quantities of polar phytochemicals than hexane soluble non polar compounds.

Phytochemical analysis

The therapeutic potential of a herbal drug is determined by the presence of bioactive compounds like flavonoids, alkaloids, phenolic compounds, glycosidess, etc. Table 1 gives major phytochemical groups present in each extract. Hexane fraction contained oils, fats and glycosides. Ethyl acetate fraction contained flavonoids, alkaloids, sugars, phenolics and tannins. Methanol fraction contained alkaloids, flavonoids, saponins, sugars, phenolics and tannins. Phytochemical profile of *A. heterophyllum* obtained in the present study was similar to an earlier observations[22]. However, in the earlier study, extraction was done with different solvents like acetone, benzene, chloroform, water, ethanol and petroleum ether.

Table 1—Preliminary phytochemical profile of hexane (DHE), ethyl acetate (DEE) and methanolic (DME) extracts of *A. heterophyllum*

Phytochemical	DHE	DEE	DME
Sugars	−	+	++
Cardiac glycosides	+	−	+
Proteins	−	−	+
Amino acids	−	−	−
Flavonoids	−	+	+
Phenolics and tannins	_	++	++
Alkaloids	_	+	+
Saponins	_	_	++
Oils and fats	+	_	_

++ = higher presence, + = moderate presence,−= absence

Identification of phytochemicals present in a plant helps us to verify bioactivities that it possesses. Many studies have identified antioxidant ability of phytochemicals that can scavenge free radicals; anti-bacterial and anti-viral properties; gene regulatory role regulating oncogenes as well as tumor suppressor genes; cell cycle regulatory properties inducing cell cycle arrest and apoptosis; modulation of enzyme activities in detoxification, oxidation and reduction; immunomodulatory properties and regulatory roles in hormone-dependent carcinogenesis[23-29]. *A. heterophyllum* is a herb used in many tribal and traditional medicines for treatment of various diseases[9-11]. Identification of the phytochemicals like phenolic compounds, tannins, glycosides and saponins help to validate its medicinal use as a curative agent in various pathological conditions.

***In vitro* cytotoxicity study**

Effect of the three different extracts of *A. heterophyllum* on the viability of normal mouse fibroblast cells (L929) is given in Fig. 1. Only upto 30 % inhibition was observed in all the three cases. More than 70 % of cells were viable in all the three extracts at the highest tested concentration of 100 µg/mL.

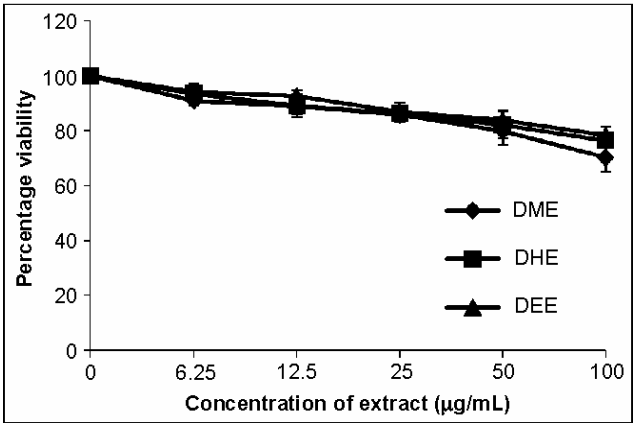

Fig. 1—Cytotoxic effects of different extracts of *A. heterophyllum* on normal mouse fibroblast cell line (L929). Each point represents the mean±SD of three independent experiments.

IC_{50} values of the different extracts were calculated from the best fit linear regression line plotted with percentage viability of cells against different tested concentrations (Fig. 2-4). The IC_{50} value of DHE, DEE and DME were calculated as 254.18, 266.99 and 191.93 µg/mL, respectively. L929 cells have been used by many researchers to assess the toxicity of plant extracts[30,31] and other biomaterials[32,33]. All the three extracts screened in the present study were shown to have high IC_{50} values. Higher IC_{50} values indicate the lesser toxicity of the material. Therefore the three extracts can be considered very less toxic to normal cells *in vitro*. There has been considerable interest in using basal cytotoxicity data to predict the acute effects of compounds *in vivo*. If a compound is acutely toxic, that may disturb the intrinsic functions of cells. Basal cytotoxicity should be considered as a starting point in an integrated assessment of potential *in vivo* toxicity[34].

Brine shrimp lethality assay

Lethality assay using brine shrimp helps to understand the toxicity level of the extracts. The extracts were shown to be non toxic to the brine shrimp nauplii even at a concentration of 1000 µg/mL. The result is in concordance with the very low toxicity observed in the case of L929 cells. Therefore, the plant can be considered as a non toxic candidate for further pharmacological evaluations as suggested in similar observations[16]. Studies have demonstrated a positive correlation between the brine shrimp lethality and oral lethality test in mice in medicinal plant research[35]. Therefore, the brine shrimp lethality assay of *A. heterophyllum* the can be considered as an ideal preliminary screening for toxicity *in vivo*.

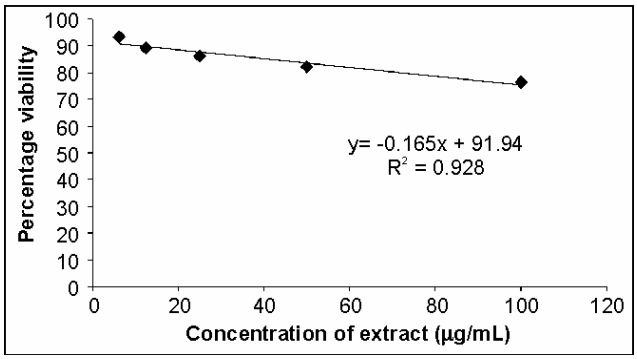

Fig. 2—Best fit linear regression line of percentage viability of L929 cells treated with different concentrations of hexane extract of *A. heterophyllum*.

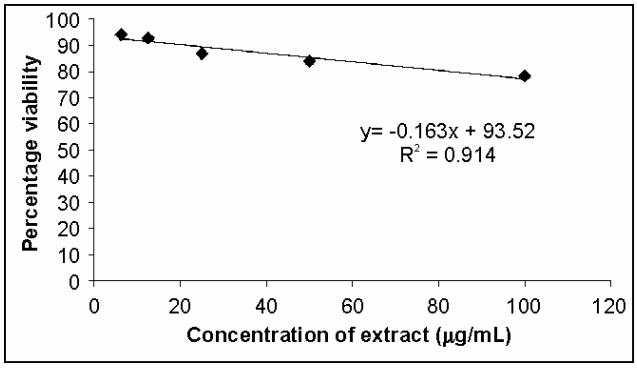

Fig. 3—Best fit linear regression line of percentage viability of L929 cells treated with different concentrations of ethyl acetate extract of *A. heterophyllum*.

Heavy metal analysis

The concentrations of various heavy metals analyzed are given in Fig. 5. The recommended daily allowance of some of the metals[36] is given in Table 2. All the heavy metals analysed were found to be below the permissible upper limits. Heavy metal profile of the plant shows that it does not contain any of the heavy metals in harmful levels.

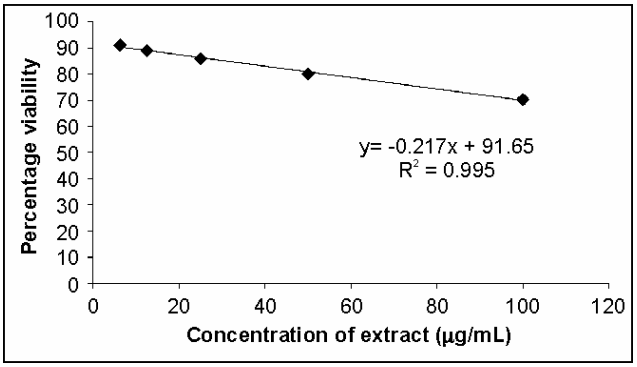

Fig. 4—Best fit linear regression line of percentage cell viability of L929 cells treated with different concentrations of methanol extract of *A. heterophyllum*.

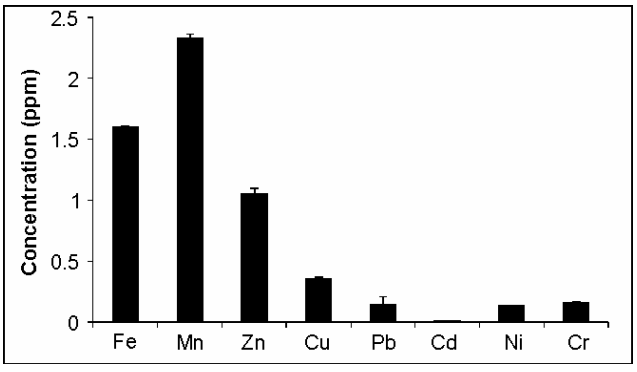

Fig. 5—Heavy metal concentration in the leaves of *A. heterophyllum*. Each point represents the mean±SD of three independent experiments.

Table 2 — Concentrations of different heavy metals present in the leaves of *A. heterophyllum* compared with RDA & permissible upper limits

Metal	Concentration (ppm)	RDA for adults (mg)[36]	Upper limit (ppm)[38]
Fe	1.59 ± 0.007	8 - 18	
Mn	2.33 ± 0.035	1.0 -5.0	
Zn	1.058 ± 0.039	15	
Cu	0.355 ± 0.013	1.0 – 3.0	
Pb	0.149 ± 0.058	-	10
Cd	0.008 ± 0.0005	-	0.3
Ni	0.132 ± 0.002	0.13 – 0.4	
Cr	0.159 ± 0.003	-	

RDA = required daily allowance.
Values presented as mean ± SD of three independent experiments

WHO recommends that medicinal plants that form the raw materials for the finished products may be checked for the presence of heavy metals[37]. It also regulates maximum permissible limits of toxic metals like cadmium and lead. Heavy metals such as iron, chromium, copper, zinc, manganese and nickel are essential metals since they play an important role in biological systems[38]. They become harmful only when organisms are exposed continuously to higher levels beyond the permissible upper limits. All the essential elements studied in the present context are found within the permissible limits. Lead and cadmium are non-essential trace elements having functions neither in human body nor in plants. They can induce various toxic effects in humans. WHO prescribed limit for Pb contents in herbal medicine is 10 ppm while the dietary intake limit is 3 mg/week. The maximum acceptable concentration for cadmium is 0.3 ppm[37]. Lead and cadmium were detected at very low concentrations in the present sample and therefore this plant can be considered suitable for further studies as a herbal drug resource.

Conclusion

A. heterophyllum is a rich source of some major classes of bioactive phytochemicals. Both the toxicity evaluation and heavy metal analysis helped to rule out the possibility of toxicity when using this plant resource. The present investigation suggests and ascertains the quality of this plant for further studies as a potential herbal therapeutic and biomodulatory agent.

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