Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(11); 1828-1832

ISSN: 0975-4873

Research Article

A Profiling of Anti-Tumour Potential of Sterols in the Mangrove Fern *Acrostichum aureum*.

Anitta Thomas[1], Prashob Peter K J[1*], N Chandramohanakumar[1,2]

[1]*Department of Chemical Oceanography, Cochin University of Science and Technology, Cochin 682016, India*
[2]*Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology Cochin 682016, India*

Available Online: 15th November, 2016

ABSTRACT

This study designates on the anti-tumour potential of phytosterol compositions present in the leaves of mangrove fern *Acrostichum* aureum of Cochin estuary. Very little work has been reported so far about mangrove fern species *Acrostichum aureum* worldwide. In the present study five phytosterols have been identified in the leaves of mangrove fern *Acrostichum aureum*, and are reported here for the first time. Stigmasterol, γ-sitosterol, campesterol, cycloartanol and 24-methylene cycloartanol are the components detected and these compounds were identified and confirmed by comparison of the obtained mass spectra with the published data. Further, in silico prediction of cytotoxicity for tumour cell lines using Cell Line Cytotoxicity Predictor (CLC-Pred) highlighted their potential to suppress adenocarcinoma, carcinoma, and mesothelioma. This study highlights the relevance of processing *Acrostichum aureum* for biological active sterols to suppress the proliferation of cancer cells in stomach, lungs, and pleura.

Keywords: Phytosterols, *Acrostichum aureum*, Anti-tumor, PAAS, Mangrove Fern

INTRODUCTION

Mangrove fern species *Acrostichum aureum* L., (Family: *Pteridaceae*) is a large terrestrial plant observed in flooded areas during rainy seasons and at high tides as mangrove associates and its status is common in Kerala [1]. Whole plant is used as an anthelmintic and styptic, also used as a worm remedy and as an astringent in haemorrhage[2]. This mangrove Fern occurs worldwide in mangrove swamps, salt marshes, canal margins, and low hammocks. It is widely distributed throughout South Florida[3] Brazil, South & West Mexico, Guyanas, Central America, Colombia, Venezuela, Ecuador, Paraguay, Barbados, Trinidad, South china, Taiwan, Japan, North Australia, India, Sri Lanka and Bangladesh[4].

In most of these countries extracts from this species are used as herbal medicine to treat various human ailments. In Fiji, people used it to treat asthma, constipation, elephantiasis, febrifuge, and chest pain[5]. In Bangladesh, preparations from rhizomes and leaves of *A. aureum* are used to treat wounds, peptic ulcers and boils[6]. In China, the rhizomes are used to treat worm infections[6]. The crude extract of a Japanese *A. aureum* specimen is reported to possess anti-oxidant, tyrosinase inhibiting activity[7]. In Costa Rica, its leaves are used as emollients[8].

In vitro and *in vivo* investigations revealed cytotoxic, anti-inflammatory, analgesic and antioxidant activity of extracts from this species. Ethanolic extracts of *A. aureum* showed anti-fertility activity in rats[9]. The cytotoxic effect of water and methanol extracts from a Bangladeshi specimen of *A. aureum* on gastric, colon and breast cancer cells were studied[10]. The anti-inflammatory activity of ethanolic (95%) crude extract of the roots of *A. aureum* was reported in varied carrageenan-induced inflammation rat models[11]. Ethanol extract of *A. aureum* possesses analgesic activity and *in vitro* antioxidant activity which was found to be significantly effective in scavenging DPPH (EC50 =41.95µg/mL)[12].

The extensive literature survey reveals the potential of *A. aureum* as a source of bioactive compounds with diverse pharmacological functions. The whole plant contains glycosides, saponins, steroids and fronds[13]. The active phyto-constituents 2-butanone, ponasterone, pterosterone, kaempferol and quercetin were isolated from ethanolic extract of *A. aureum*[14]. Patriscabratine and tetracosane were isolated from the Bangladesh mangrove fern, *A. aureum* and evaluated their cytotoxic activity against different cancer cell lines[15]. The sesquiterpene, (2S, 3S)-sulfated pterosin C, isolated from methanolic extract of the aerial part of same fern exerts an apoptotic effect on AGS cells within 24 h of treatment[10]. Phyto-sterols can be effectively used in developing safe therapeutics[16], however, in this regard not much is known about the biologically active sterols present in this species. These phyto-chemical screening studies needs to be carried out on *A. aureum* in order to flourish their practical clinical applications, which can be used for the welfare of the mankind. The present study deals with isolation and characterization of sterols from its leaves and predicating their anti-tumour activity using PAAS (Prediction of Activity Spectra for Substances) technology[17].

**Author for Correspondence: prashobpeter@cusat.ac.in*

MATERIALS AND METHODS

The leaf samples of mangrove fern *A. aureum* (Fig. 1) were collected from the Malippuram, Vypin which is a patchy mangrove area in the heart of Cochin. Malipuram is comes under Elankunnapuzha Panchayath of Vypin taluk in Ernakulum District of Kerala State, India. Vypin is the largest single stretch of mangroves in Kerala and Malippuram is a one of the aqua tourism centre, fish farm and Mangrove Park in Vypin Island. It extents between latitude $10^0$12'18" N and longitude $76^0$12'93" E Kerala. Perhaps, this is the only site in Kerala where mangroves are situated right along the accreting seacoast. However, a lot of developmental pressure is threatening the existence of these mangroves.

Extraction and Isolation of Sterols

The method described by Harvey (1994) was selected for the study[18]. The leaf samples collected were coarsely chopped, minced, freeze dried and powdered. 25 g of this powdered sample is mixed with 100 mL dichloromethane (DCM) and methanol (2:1 ratio) followed by soxhlet extraction for 18 h. The extraction was repeated for three times and the combined extracts were evaporated in rotor under reduced pressure. The extracted residue was subjected to mild alkaline hydrolysis (saponification) using 6%w/v KOH in methanol and refluxed for 6 hours at 70ºC. NaCl solution (5%) is added to aqueous alcoholic phase to facilitate the breaking of emulsion. The extraction was repeated for three times and the extracts combined. The above steps ensure the removal of carboxylic acids. The extract was dried over anhydrous Sodium sulphate and was again evaporated in rotor under reduced pressure. Last traces of the solvent were removed by passing dry N_2 gas. (Harvey, 1994)

Purification and derivatization of Sterols Fractions

Fractionation of total lipid and non-polar lipid is most usually achieved by column chromatography (CC) on silica gel or on alumina[19]. Sterols were eluted using 15% ethyl acetate in hexane as eluent. The sterol fractions were then eluted from silica gel and transferred into ethyl acetate. The sterol mixture obtained was evaporated under reduced pressure and dried using N_2 gas. Prior to GC-MS characterization, sterol fraction was converted to trimethylsilyl derivatives (analyte) by reaction with N, O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and pyridine for 3 h at 70˚C.

Characterization of Sterols by GC-MS

Characterization of analyte was done using GC (Perkin Elmer, Clarus 680 Gas Chromatograph), equipped with non-polar HP ultra-double-fused silica capillary column (30 m, 0.32 mm internal diameter, 0.25µm film thickness), coupled to MS (Perkin Elmer, Clarus 600T Mass Spectrometer) detector probe. To elute the molecules from analyte a 49.45 minute three-step temperature program was used. Helium was used as carrier gas with flow rate of 20:1. Molecules eluted from the GC column are bombarded with electrons at 70 eV to Fragment ions. These ions reaching the Mass Analyzer were scanned from 50 to 600 m/z with a scan time of 1.50 s to give a Mass spectrum to provide structural information. Full data acquisition was obtained with the use of MS turbo mass version 5.3.2. Compound identification in the GC-MS analysis was performed using retention time and interpretation of the mass spectra (molecular ion [$M^{+\cdot}$], base peak and main fragments) in comparison with mass spectra of isolated compounds and mass spectrometric fragments described in MS library.

In silico prediction of cytotoxicity

In this investigation, prediction of bioactivity of identified sterols is based on PASS (Prediction of Activity Spectra for Substances) technology (http://www.way2drug.com/PASSonline) and the training set made on the basis of data on cytotoxicity recovered from ChEMBLdb (version 19) (https://www.ebi.ac.uk/chembldb/). Pa (probability "to be active") values greater than 0.5 is reported in this work.

RESULT AND DISCUSSION

The results of the GC-MS analysis of the sterol fraction in mangrove fern A. *aureum* are furnished in Table -1 and Table 2.

Campesterol (24-methylcholest-5-en-3β-ol) was identified from GC retention characteristics and mass spectral characteristics of authentic standard and published data at the retention time 26.167min. The molecular ion was identified at *m/z* 400 [M^+] which corresponds to the molecular formula $C_{28}H_{48}O$ and the characteristic base peak was identified at *m/z* 107. In MS spectrum the characteristic fragment ions occurred at *m/z* 382, 385, 367, 315, 289, 273, 255, 145, 107, 95, 91, 81, 55, 43, 41 which are characteristics of 3β-hydroxy-Δ^5-sterols. Two fragmentation patterns *m/z* 315, 289 are most apparent only in free 3β-hydroxy-Δ^5-sterols with a saturated side chain and these fragmentations are due to the losses of either 85 a.m.u. or 111 a.m.u., respectively. The fragment peak *m/z* 385, 382 and 367 indicates the loss of Me, H_2O and M-Me-H_2O respectively. The experimental data supports the proposed structure of this compound. The fragment ion formed at *m/z* 357 is due to the loss of terminal isopropyl group of the side chain, C_3H_7 (43 a.m.u.) fragment to yield ions corresponding $[M\text{-}C3H7]^+$ ie, $[M\text{-}43]^+$ ion. The saturated side chain is lost to give an $[M\text{-}SC]^+$ ion at *mlz* 273 comprising the ring carbons and further the loss of ROH can then yield the $[M\text{-}SC\text{-}ROH]^+$ ion at *mlz* 255.

GC–MS spectrum tentatively identified stigmasterol at the retention time 26.948min. The molecular ion was identified at *m/z* 412 [M^+] which corresponds to the molecular formula $C_{29}H_{48}O$ and the characteristic base peak was identified at *m/z* 55. The other characteristics fragmentation peaks were at *m/z* 394, 379, 369, 327, 301,300, 271, 255, 213, 199, 159, 145, 133, 105, 83, 81, 69 which are characteristics of 3β-hydroxy-Δ^5-sterols. Two fragmentation patterns *m/z* 327, 301 are most apparent only in free 3β-hydroxy-Δ^5-sterols with a saturated side chain, involve rather complex cleavages across the A- and B-rings. These fragmentations are due to the losses of either 85 a.m.u. or 111 a.m.u., respectively. The fragment peak *m/z* 255 indicates the loss of M-side chain-H_2O and fragment peak *m/z* 213

Figure1: Image of leaf samples of mangrove fern *A. aureum* collected from Malippuram.

Table 1: Major diagnostic fragments used to identify the phytosterols in *A. aureum* as per GC-MS result.

Diagnostic fragments	Compounds				
	1	2	3	4	5
$[M^{+\cdot}]$	400	412	414	426	440
M-15	385				
M-18	382	394	396	408	422
M-Sch-18		255	255		
M-Sch	273		273		
M-18-15	367	379	381	393	407
M-43 (C_3H_7)	357				
M-SC-ROH	255				
M-85	315	327	329		
M-111	289	301	303		

Table 2: List of phytosterols identified in *A. aureum*.

S.No	Compound Name	Composition	MW	Retention time (min)
1	Campesterol	$C_{28}H_{48}O$	400	26.167
2	Stigmasterol	$C_{29}H_{48}O$	412	26.948
3	γ-Sitosterol	$C_{29}H_{50}O$	414	28.498
4	Cycloartanol	$C_{30}H_{50}O$	426	31.259
5	24-methylene cycloartanol	$C_{31}H_{52}O$	440	33.570

indicates that the sterol is a monoene. A molecular ion which is normally observed in the mass spectrum of all free sterol is $[M-18]^+$ due to the loss of 3β-hydroxy group as water. Here the characteristic ion is observed at *m/z* 394. The $[M-H_2O-Me]^+$ ion is observed at *m/z* 379. γ-Sitosterol was identified at the retention time 28.498min with molecular ion at *m/z* 414 $[M]^+$ which corresponds to the molecular formula $C_{29}H_{50}O$ and the characteristic base peak was identified at *m/z* 107. In MS spectrum the characteristic fragment ions occurred at *m/z* 399, 396, 381, 329, 303, 273, 255, 231, 213, 199, 159, 145, 133, 105, 81, 69 which are characteristics of 3β-hydroxy-Δ^5-sterols. Two fragmentation patterns *m/z* 329, 303 are most apparent only in free 3β-hydroxy-Δ^5-sterols with a saturated side chain, involve rather complex cleavages across the A- and B-rings. These fragmentations are due to the losses of either 85 a.m.u. or 111 a.m.u., respectively. The fragment peak *m/z* 273 and 255 indicates the loss of M-side chain and M-side chain-H_2O respectively. The experimental data supports the proposed structure of this compound. The loss of 3β-hydroxy group as water $[M-H_2O]^+$ gives the fragment peak at *m/z* 396 and $[M-H_2O-Me]^+$ ion is observed at *m/z* 381.

At the retention time 31.259 min, GC–MS was comparable to Cycloartenol. It is the starting point for the synthesis of almost all plant steroids, making them chemically distinct from the steroids of fungi and animals which are instead produced from lanosterol. The molecular ion was identified at *m/z* 426 $[M^+]$ which corresponds to the molecular formula $C_{30}H_{50}O$ and the characteristic base peak was identified at *m/z* 69. In MS spectrum the other characteristic fragment ions occurred at *m/z* 408, 393, 366, 339, 313, 286, 271, 159, 145, 105, 81, 69 which are characteristics of 3β-hydroxy sterols. The fragment peak *m/z* 408 and 393 indicates the loss of M- H_2O and M-Me-H_2O respectively. The ion at *mlz* 339 is for the unsubstituted Δ^{24}-side chain. The experimental data supports the proposed structure of this compound.

On the basis of GC–MS spectrum, 9, 19-Cyclo-9β-lanostan-3β-ol, 24-methylene was identified at the retention time 33.570 min which is having molecular ion peak at *m/z* 440 $[M^+]$. It corresponds to the molecular formula $C_{31}H_{52}O$ and the characteristic base peak was identified at *m/z* 55. In MS spectrum the other characteristic fragment ions occurred at *m/z* 422, 407, 353, 315, 300, 273, 145, 105, 81, 69 which are characteristics of 3β-hydroxy sterols. The fragment peak *m/z* 422 and 407 indicates the loss of M- H_2O and M-Me-H_2O respectively. It is similar to the above discussed structure which is having a methylene group at the 24th position instead of a Δ^{24}-side chain. The fragment peak *m/z* 353 indicates 24-methylene side chain. The *mlz* 286 ion is typical of compounds with an unsubstituted side chain.

Results of CLC-Pred (Cell Line Cytotoxicity Predictor) for in silico prediction of cytotoxic effect of phytosterols against non-transformed and cancer cell lines based on their corresponding structural formula is shown in Table 3. These results highlighted the potential of these sterols against adenocarcinoma, followed by carcinoma and mesothelioma. A Pa vale, 0.816 against Stomach adenocarcinoma cells (MKN 74) was obtained for cycloartanol, was followed by 0.746 for 24-methylene cycloartanol, 0.707 for campesterol, 0.684 for stigmasterol and 0.638 for γ-sitosterol. Another significant activity observed was against Gastric carcinoma cells (MKN 7), affecting the stomach was shown by all sterols expect γ-sitosterol. Cycloartanol was also active against Gastric epithelial carcinoma cells

Table 3: Cancer cell line prediction results for phytosterols present in *A. aureum.*

Compound	Tumor cell lines				
	DMS-114	MKN-7	MKN-74	NCI-H2052	MKN-28
Campesterol	-	+	++	-	-
Stigmasterol	-	+	+	-	-
γ-Sitosterol	-	-	+	-	-
Cycloartanol	-	+	+++	+	+
24-methylene cycloartanol	+	+	++		

Pa<0.5 is -, Pa values in between 0.5-0.7 is +, Pa values in between 0.7-0.8 is ++ and Pa >0.8 is +++
DMS-114: Lung carcinoma cells, MKN-7: Gastric carcinoma cells, MKN-74: Stomach adenocarcinoma cells, NCI-H2052: Epithelioid mesothelioma cells, MKN-28: Gastric epithelial carcinoma cells

(MKN-28) along with Epithelioid mesothelioma cells (NCI-H2052) a mesothelioma tumour type affecting the pleura tissue. 24-methylene cycloartanol showed significant activity against Lung carcinoma cells (DMS-114), whereas, activity of other sterols were insignificant.
There is enough evidence from *in vitro* and *in vivo* studies to indicate that compounds identified in the sterol fraction from *A. aureum* are therapeutically significant. Young men with arteriosclerotic heart disease when treated with β-sitosterol, significant reductions in serum cholesterol and β-lipoprotein lipid along with a lesser reduction in total lipid and a slight rise in α-lipoprotein lipid have been observed[20]. Plant sterols such as β-sitosterol in food supplements neutralize the proliferative changes associated with carcinogenesis and reduce the risk from colon carcinogens[21]. Growth inhibition of HT-29 human colon cancer cell along with significant changes in the composition of membrane sphingomyelin, phosphatidylserine, and phosphatidylinositol where observed when treated with β-Sitosterol[22]. Clinical studies have confirmed the efficacy of sitosterol in lowering plasma LDLC concentrations[23], as cholesterol and fat are implicated as dietary factors enhancing the risk of carcinogenesis. Stigmasterol isolated from *Butea monosperma* was reported to posses thyroid inhibitory, antiperoxidative and hypoglycemic effects[24]. The potential of stigma sterol to suppress pro-inflammatory and matrix degradation mediators involved in osteoarthritis-induced cartilage degradation were reported by Gabay co-workers in 2010[25]. Oxidized derivatives of stigmasterol demonstrated apoptotic effects against U937 human monocytic cell line[26]. These observations along with the results of our work highlight the relevance of phytosterols in *A. Aureum* for developing safe anti-tumour therapeutic agents.

CONCLUSION

Due to various health benefits of phytosterols in the field of cardio vascular diseases, the market for phytosterol particularly sitosterol, stigmasterol and campesterol are rising especially in European countries. Based on *in vitro* and *in vivo* data phytosterols have been given the GRAS (Generally Recognized as Safe) status in the U.S. Their use in the food supplements have been approved by both FDA and EU Scientific Committee on Food (SFC). Considering these facts and based on our results *A. aureum* if properly managed could be developed as potent source of stigmasterol, γ-sitosterol, campesterol, cycloartanol and 24-methylene cycloartanol. The bioactivities of these sterols are well known and hence the presence of these phyto-components in *A. Aureum* promotes the economical worth and mangrove reforestation. As no work has been done so far on *A. aureum* from Kerala coast, this work is useful to make interest towards this species which will be helpful in rising new formulations with additional therapeutic and economical worth.

ACKNOWLEDGEMENTS

We thank for the chemicals, facilities, and financial aid, provided by the Department of Chemical Oceanography and Inter University Centre for Development of Marine Biotechnology, Cochin University of Science and Technology, to complete the research work.

REFERENCE

1. Easa PS. Biodiversity documentation for Kerala. Part 5. Pteridophytes. *KFRI handbook*, 2003.
2. Chopra RN, Nayer SL, Chopra IC. Glossary of Indian medicinal plants: 3rd Council of Scientific and Industrial Research, New Delhi, 1992, 7-246.
3. Adams DC, Tomlinson PB. *Acrostichum* in Florida. American Fern Journal 1979; 69(2): 42-46.
4. Medina E, Cuevas E, Popp M, Lugo AE. Soil salinity, sun exposure, and growth of A. aureum, the mangrove fern. Botanical Gazette 1990; 1: 41-49.
5. Coambie RC, Ash J. Fijian Medicinal Plants; CSIRO Publishing: Melbourne, 1994.
6. Momtaz MM. Encyclopedia of Flora and Fauna of Bangladesh; Asiatic Society of Bangladesh: Dhaka, Vol. 5, 2008, 246.
7. Lai HY, Lim YY, Tan SP. Antioxidative, tyrosinase inhibiting and antibacterial activities of leaf extracts from medicinal ferns. Bioscience Biotechnology and Biochemistry 2009; 73(6): 1362-1366.
8. Natural Resources Conservation Service, Plants Database. Natural Resources Conservation Service, United States Department of Agriculture (USDA), Washington DC (http://plants.usda.gov), 2010.
9. Dhar JD, Setty BS, Lakshmi V, Bhakuni DS. Post-coital antifertility activity of the marine plant, Achrostichum aureum L. in rat. The Indian Journal of Medical Research 1992; 96: 150-152.
10. Uddin SJ, Jason TL, Beattie KD, Grice ID, Tiralongo E. (2 S, 3 S)-Sulfated Pterosin C, a cytotoxic sesquiterpene from the Bangladeshi Mangrove Fern *A.*

aureum. Journal of Natural Products 2011; 74(9): 2010-2013.
11. Hossain H, Jahan AI, Nimmi I, Hossain A, Kawsar H. Anti-inflammatory activity of the ethanolic extract of A. aureum (Linn.) root. Bangladesh Pharmaceutical Journal 2011; 14(2): 107-109.
12. Khan SA, Md. Hossain A, Panthi S, Md. Asadujjaman, Hossin A. Assessment of antioxidant and analgesic activity of *A. aureum* Linn. (Family-Pteridaceae). Pharmacol Online, 2013; 1:166-171.
13. Burkill HM. The useful plants of tropical Africa. Royal Botanic Gardens, Kew. 2nd Edition, Families AD, 1, 1985, 479.
14. Mei W. Zeng Y, Ding Z. Dai H. Isolation and identification of the chemical constituents from Mangrove plant *Acrostichum aureum*. Chinese Academy of Tropical Agriculture Sciences 2006; 16: 46-48.
15. Uddin SJ, Grice D, Tiralongo E. Evaluation of cytotoxic activity of patriscabratine, tetracosane and various flavonoids isolated from the Bangladeshi medicinal plant A. aureum. Pharmaceutical Biology 2012; 50(10): 1276-1280.
16. Kala KJ, Prashob PKJ, Chandramohanakumar N. Cyto-toxic potential of fucosterol isolated from Turbinaria Conoides against Dalton's Lymphoma Ascites. International Journal of Pharmacognosy and Phytochemical Research 2015; 7(6): 1217-1221.
17. PAAS (Prediction of Activity Spectra for Substances) technology (http://www.way2drug.com/PASSonline).
18. Harvey HR. Fatty acids and sterols as source markers of organic matter in sediments of the North Carolina continental slope. Deep Sea Research Part II: Topical Studies in Oceanography 1994; 41(4): 783-796.
19. Goad J, Akihisa T. Analysis of sterols. Springer Science & Business Media 2012.
20. Farquhar JW, Smith RE, Dempsey ME. The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease. Circulation 1956; 14(1): 77-82.
21. Nair PP, Turjman N, Kessie G, Calkins B, Goodman GT, Davidovitz H, Nimmagadda, G. Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer. Dietary cholesterol, beta-sitosterol, and stigmasterol. The American Journal of Clinical Nutrition 1984; 40(4): 927-930.
22. Awad AB, Chen YC, Fink CS, Hennessey T. beta-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids. Anticancer Research 1995; 16(5A): 2797-2804.
23. Fernandez ML, Vega-López, S. Efficacy and safety of sitosterol in the management of blood cholesterol levels. Cardiovascular Drug Reviews 2005; 23(1): 57-70.
24. Panda S, Jafri M, Kar A, Meheta BK. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from Butea monosperma. Fitoterapia 2009; 80(2): 123-126.
25. Gabay O, Sanchez C, Salvat C, Chevy F, Breton M., Nourissat G, Wolf C, Jacques C. Berenbaum F. Stigmasterol: a phytosterol with potential anti osteoarthritic properties. Osteoarthritis and Cartilage 2010; 18(1): 106-116.
26. O'Callaghan, YC, Foley DA, O'Connell NM, McCarthy FO, Maguire AR, O'brien NM. Cytotoxic and apoptotic effects of the oxidized derivatives of stigmasterol in the U937 human monocytic cell line. Journal of Agricultural and Food Chemistry 2010; 58(19):10793-10798.