Advances in Zoology and Botany 11(3): 151-157, 2023
DOI: 10.13189/azb.2023.110301 http://www.hrpub.org

GC-MS Analysis and Antibacterial Effect of Methanol Extract of *Pterobryopsis Pilifolia* (Dixon) Magil

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Received July 4, 2022; Revised November 7, 2022; Accepted December 22, 2022

Cite This Paper in the Following Citation Styles

(a): *[1] Meenu Mathew, Abraham Mathew , "GC-MS Analysis and Antibacterial Effect of Methanol Extract of Pterobryopsis Pilifolia (Dixon) Magil," Advances in Zoology and Botany, Vol. 11, No. 3, pp. 151 - 157, 2023. DOI: 10.13189/azb.2023.110301.*

(b): *Meenu Mathew, Abraham Mathew (2023). GC-MS Analysis and Antibacterial Effect of Methanol Extract of Pterobryopsis Pilifolia (Dixon) Magil. Advances in Zoology and Botany, 11(3), 151 - 157. DOI: 10.13189/azb.2023.110301.*

Abstract The aim of the study was to serially extract phytochemicals from *Pterobryopsis pilifolia* and to determine the antibacterial effect of the methanolic extract. Identification of volatile compounds from the methanolic extract was performed to study the possible compounds that offer antibacterial activity. *Pterobryopsis pilifolia* collected from Idukki district, Kerala, India was used in the present study. Serial extraction of phytochemicals from the bryophyte was done using Soxhlet apparatus with petroleum ether, followed by ethyl acetate and then methanol as solvent. The methanolic extract was used in the present study. The extract was filtered and concentrated using rotary evaporator. Antibacterial activity was tested against ten standard strains of bacteria. Methanolic extract inhibited all the ten tested strains that include both Gram positive and Gram negative bacteria. The highest zone of inhibition noted was against *Xanthomonas campestris* with an inhibition zone of 27 mm ± 1 mm. GC-MS analysis of the extract revealed the presence of several volatile compounds including fatty acids and other bioactive compounds. *Pterobryopsis pilifolia* is usually seen intact without any signs of disease, insects or animals feeding on them. The presence of these compounds might be the reason for the resistance of the taxa against diseases or pests. Studies on these compounds thus can pay the way for elucidating mechanism for pest and disease resistance in plants. The compounds can be used as antifeedant spray that can be applied to crop plants, thereby reducing the effect of harmful chemical pesticides.

Keywords Bryophyte, Soxhlet Extraction, *Pterobryopsis Pilifolia,* Methanol, GC-MS

1. Introduction

Plants form an important source of chemicals and is extensively used in medicines from prehistoric times. Unlike microbes that produce or secrete few chemicals, plants offer an array of chemicals with diverse properties and functions which if properly evaluated can have a profound effect on society. This includes new medicines, bio pesticides, biofuels, biopolymers etc.

Angiosperms dominate in terms of plant richness and biomass and hence is widely used for bioprospecting studies. Bryophytes are the second largest group of terrestrial plants, but are least investigated owing to lesser plant biomass and lack of proper identification techniques. According to Sabovljevic *et al*. [1] the taxa diversity of bryophyte is over 24,000. Botanical Survey of India identifies 2562 taxa of bryophytes in the country with endemism of 2 genera and 168 species of liverworts, 19 species of hornworts and 547 species of mosses plants (http://www.bsienvis.nic.in/Database/Bryophytes_22589.aspx). The number of RET species of bryophyte is also high in India with 133 species (53 species of liverworts, 2 species of hornworts and 78 species of mosses) considered as rare and 14 species as endangered [2].

Bryophytes have several ethno-botanical uses. Taxa which are widely used in medicine include *Riccardia,*

Plagiochasma appendiculatum, Reboulia hemisphaerica, Conocepahlum conicum, Herbertus, Frullania tamarisci, Frullania ericoides, Marchantia polymorpha, Marchantia convoluta, Marchantia palmata, Marchantia paleacea, Dumortiera hirsuta, Pallavicinia, Plagiochila, Riccia, Targionia hypophylla, Philonotis fontana, Bryum argentum, Rhodobryum giganteum, Rhodobryum roseum, Leucobryum, Ditrichum, Fissidens, Funaria, Entodon, Taxiphyllum, Aerobryum, Mnium, Plagiomnium, Polytrichum, Pogonatum, Octoblepharum, Barbula, Hyophila, Sphagnum [3]. Recent studies indicate bryophytes to be an abundant source of plant chemicals. More than 2200 chemical molecules are reported from bryophytes and their number is escalating with recent interest in the taxa and advanced phytochemical screening techniques [4]. Large number of these compounds are novel to phytochemistry and reveal bioactivities like antibacterial, antifungal, antiviral, anticancerous, antioxidant, piscicidal, larvicidal, antifeedant, cytotoxic, plant growth inhibitory and antiobesity activities [5]. Bryophytes remain to be a low choice for herbivores, larvae or insects. This is due to the enormous number of chemicals that offer biochemical defence, many might be new to already identified phytochemicals. Western Ghats being species rich can offer novel phytochemicals that can enhance the economic value of bryophytes.

Pterobryopsis is a moss genus coming under the family Pterobryaceae. There are 52 accepted species of *Pterobryopsis* (http://www.theplantlist.org/). *Pterobryopsis pilifolia* is a corticolous taxa with black rhizomatous primary stem closely appressed to the bark. Secondary stem is often drooping or erect. Leaves broad and plicate, apex slightly dentate. Specimens collected for identification seemed to be healthy with no signs of disease or herbivore attack. This may be attributed to the antimicrobial and antifeedant chemicals present in the taxa. Chemicals from dried and powdered *Pterobryopsis pilifolia* were sequentially extracted using petroleum ether, ethyl acetate and methanol as solvent. The present study deals with methanolic extract. The extract was tested for antibacterial activity and was subjected to GC-MS analysis to identify the volatile compounds present.

2. Materials and Methods

2.1. Collection and Identification of Specimen

The taxa *Pterobryopsis pilifolia* (Dixon) Magil, was collected during March 2020 from Idukki District, Kerala. The plant was collected in sterile glass bottles and brought to the lab and identified based on gametophytic characters.

2.2. Extraction of Phytochemicals

The gametophytic plant material was cleaned and washed to remove soil particles using running tap water and then dried under shade. The dried thallus was powdered. 30 g of powdered thallus was taken in soxhlet extraction apparatus and phytochemicals were serially extracted with petroleum ether, ethyl acetate and methanol. The methanol extract was filtered using filter paper and then the extract was concentrated using rotary evaporator. The extract was stored under refrigeration.

2.3. Antibacterial Activity

Ten strains of bacteria- Escherichia coli (ATCC 10799), Salmonella typhimurium (ATCC 23564), Pseudomonas aeruginosa (ATCC 19154), Bacillus subtilis (ATCC 6051), Xanthomonas campestris (ATCC 29497), Streptococcus lactis (ATCC 11454), Serratia marcescens (ATCC 13880), Shigella flexneri (ATCC 12022), Klebsiella pneumoniae (ATCC 9621), Proteus vulgaris (NCIM 5266), were used in the present study. The antibacterial activity of extracts was screened using disc diffusion agar method. Bacterial strains were inoculated in sterile nutrient broth and after 14 hrs of incubation, using a sterile swab, the bacterial lawn was created in nutrient agar plate. Sterile discs soaked in extract were air dried and then placed on the bacterial lawn. Sterile discs immersed in pure solvent and then air-dried were placed as control. Antibiotic vancomycin was used as positive control. The plates were incubated in inverted condition at 30 °C for 18 hrs and antibacterial activity was measured as zone of clearance that showed no visible bacterial growth. Each treatment was done in triplicates.

2.4. GC MS Analysis

The methanol extract was centrifuged and the supernatant was subjected to GC MS analysis. Agilent-made GC MS was used (GC 7890A with MS 5975). Peaks along with retention time were searched with NIST library to identify the volatile components.

3. Result and Discussion

Gametophyte plant was collected from bark of large trees. Plant was light green and leafy. Stem was dark brown, wiry, twisted and creeping. The branches were either erect on pendant. Leaves were seen crowded and were ovate-cordate and cucullate, costa being small. Based on the morphological and anatomical characters, the plant was identified as *Pterobryopsis pilifolia* (Figure 1).

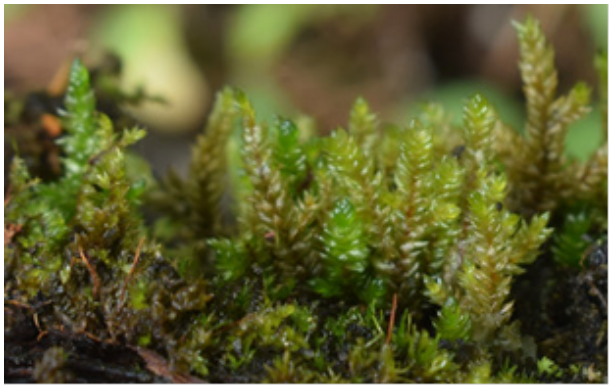

Figure 1. *Pterobryopsis pilifolia* in natural habitat

Methanol extract of *Pterobryopsis pilifolia* was dried in a hot air oven and weighed 0.35 g. The methanol fraction exhibited antibacterial activity against all the tested bacteria (Table 1). Maximum zone of inhibition was noted against *Xanthomonas campestris* (Figure 2).

Both gram positive and gram negative bacteria got inhibited with methanol extract. Veljic *et al*. [6], studied antimicrobial activity of methanolic extract of mosses from Serbia against few bacteria by disc diffusion method. *Staphylococcus aureus* was inhibited by *Pleurozium schreberi*, *Homalothecium philippeanum* and *Anomodon attenuatus. Staphylococcus epidermidis* got inhibited by *Rhytidium rugosum*, *Hylocomium splendens*, *Dicranum scoparium* and *Leucobryum glaucum*. Only *Leucobryum glaucum* extract inhibited the growth of *Micrococcus flavus*, *Homalothecium philippeanum* inhibited *Escherichia coli*, *Palustriella commutata* inhibited *Salmonella typhimurium* and none of the moss plant extract could inhibit *Enterobacter cloacae.*

Aruna and Krishnappa [7], reported chloroform extract of *Pogonatum microstomum* to be superior to petroleum ether and methanol extract against bacteria. Chloroform extract showed a zone of inhibition of 18 mm against *Streptomyces pneumoniae* and *Klebsiella pneumonia*e. Antifungal activity was seen with petroleum ether and chloroform extract of *Pogonatum microstomum*, while methanol extract of same had no antifungal activity.

Table 1. Effect of methanol extract on bacteria

Bacteria	Methanol extract Zone of inhibition in mm
Escherichia coli	17 ± 2
Salmonella typhimurium	13 ± 3
Pseudomonas aeruginosa	23 ± 1
Bacillus subtilis	19 ± 0
Xanthomonas campestris	27 ± 1
Proteus vulgaris	20 ± 2
Shigella flexneri	20 ± 4
Streptococcus lactis	08 ± 3
Serratia marcescens	09 ± 2
Klebsiella pneumoniae	16 ± 2

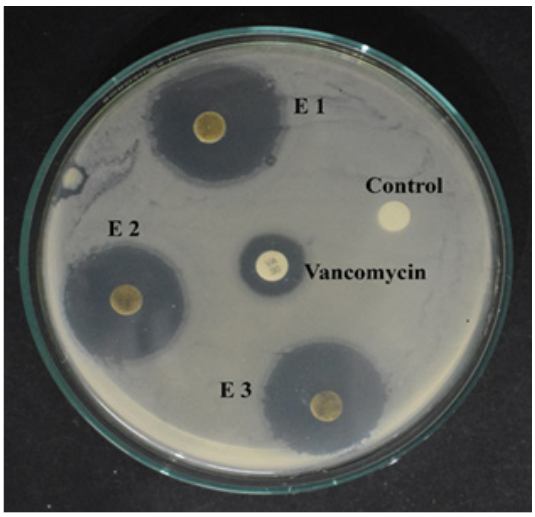

Figure 2. Antibacterial activity of methanol extract against *Xanthomonas campestris*

GC MS analysis revealed presence of several volatile compounds. Some of these compounds have potential bioactivities and are also detected in several higher plants. 21 compounds were identified in methanol extract. Percentage peak area, retention time, compound name and toxicological data (https://pubchem.ncbi.nlm.nih.gov/) are given in Table 2.

Table 2. GC MS data of methanolic extract of *Pterobryopsis pilifolia*

Peak No	Retention time (Min)	Peak Area (%)	Most probable compounds	NCBI PUBCHEM Toxicological data
2	2.288	1.18	1-Heptyn-4-ol	Information not available
3	2.779	2.91	Glyceraldehyde	Information not available
4	3.307	0.79	Cycloserine	Skin and eye irritant, acute toxicity id swallowed or inhaled
5	3.521	3.92	1,3-Dihydroxyacetone dimer	Skin, eye and respiratory irritation
6	5.055	0.5	2-Hydroxyethyl acetate	Eye irritation and eye damage
7	7.231	0.21	Melamine	Reproductive toxicity affecting fertility and unborn child, DNA damage
8	9.03	2.66	Diglycerol	Skin, eye and respiratory irritation
9	10.917	0.31	gamma-Heptalactone	Low concern
10	11.244	0.38	Hydroxymethylfurfural	Skin, eye and respiratory irritation, harmful to aquatic animals
11	11.697	0.69	Monoacetin	Acute toxicity if swallowed
12	12.703	0.84	Nonyl 2-acetyloxyacetate	Information not available
13	14.263	0.37	3-hydroxy-3-methylcaproic acid	Skin, eye and respiratory irritation, specific target organ toxicity
14	17.232	1.83	1,4,2,5 Cyclohexanetetrol	Information not available
15	20.238	7.33	Stearic acid	Skin, eye and respiratory irritation, specific target organ toxicity
16	20.955	1.96	2,3-Epoxybutane	Skin, eye and respiratory irritation and damage, specific target organ toxicity, germ cell mutagen
19	23.634	2	Butyl palmitate	Information not available
20	28.515	1.21	Phytol	Skin irritation, hazardous to aquatic environment
21	30.641	2.21	linoleic acid	Skin, eye and respiratory irritation
22	31.106	1.44	linolenic acid	Skin sensitivity
23	31.471	1.92	trans-Oleic acid	Skin, eye and respiratory irritation, specific target organ toxicity
24	32.188	1.41	Palmitic acid	Skin, eye and respiratory irritation, harmful to aquatic system

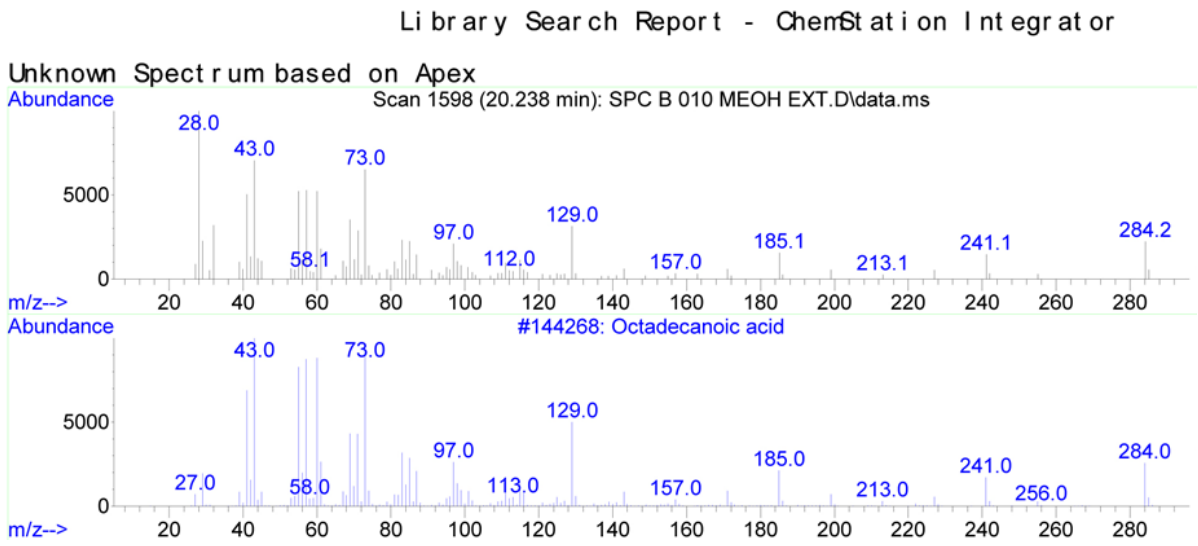

Figure 3. Chromatogram of Octadecanoic acid

Fatty acids and fatty acid esters form major chemical constituents in the methanolic extract (16.31%). This includes several essential fatty acids like tans-oleic acid (1.92%), linolenic acid (1.44%), linoleic acid (2.21%), butyl palmitate (2.0%), stearic acid (7.33%) and palmitic acid (1.41%). The volatile fatty acid belonging to caproates (3-hydroxy-3-methyl-hexanoic acid) constitute 0.37% peak area. The chromatogram of the most abundant chemical (stearic acid or octadecanoic acid) and the corresponding NIST chromatogram is shown in Figure 3.

Fatty acids are considered to be essential for metabolism and growth. However excessive concentrations of same is reported to have several negative impacts on invertebrates. Mcfarlane and Henneberry, reported the inhibitory effect of fatty acids and their methyl esters in the growth of Gryllodes sigillatus. The fatty acids and their methyl esters could get absorbed through external body wall. Lauric acid, stearic acid, myristic acid and behenic acid and methyl esters of palmitic acid, myristic acid, stearic acid and oleic acid could inhibit growth of G. sigillatus [8]. Yawjen and Biechen [9] reported cutback in Aphis gossypii population in cucumber by spraying hexanoic acid. Mixture of fatty acids like octanoic acid, nonanoic acid and decanoic acids (C8 to C10) is used as a repellent against biting and non-biting flies and ticks [10]. Spraying of caprylic acid could kill more than 90% of black chrysanthemum aphids, while lauric acid and myristic acid were powerful against green apple aphid. When sprayed over aphids, fatty acids caused sudden and complete paralysis of aphids [11].

Several bryophytes show typical pungency and odour due to the presence of volatile compounds. 3-hydroxy-3-methyl-hexanoic acid present in Pterobryopsis pilifolia is a major component in human sweat malodour and is reported to have a cheesy and rancid odor [12].

Phytol (1.21%), a diterpene is found in the methanolic extract. Phytol and its derivatives have several biological activities like anxiolytic, antioxidant, anti-inflammatory, antimicrobial, cytotoxicity, autophagy induction, apoptosis induction, immune modulation etc [13]. Saha and Bandyopadhyay [14] reported strong activity of phytol against Bacillus licheniformis. Fish feed supplemented with phytol could significantly reduce mortality of gold fish Carassius auratus and has low toxicity to lower vertebrates.

Moraes et al. [15], performed antischistosomal assay by incorporating phytol in the culture media. The effect of drug was monitored by observing changes on worm motor activity, death of worms, and changes in pairing and egg production. With 25 µg/ml, paired worms got separated, 50 µg/ml female worms died and with 100 µg/ml, all worms died within 24 hr. Phytol also significantly reduced egg production. Every table must have a unique title placed at the top. Titles should be clear and concise, and they should not be complete sentences.

Methanolic extract showed the presence of cycloserine with a percentage peak area of 0.79. Cycloserine, an analog of D-Alanine inhibits alanine racemase and alanine ligase enzymes, both are essential for peptidoglycan synthesis. Thus cycloserine acts as a broad spectrum antibiotic and show either bacteriostatic or bactericidal properties [16]. It can also inhibit synthesis of mycoside-C, an important peptidoglycolipid seen in the cell wall of Mycobacterium [17]. Another possible antibacterial agent present in the methanolic extract was hydroxymethylfurfural (0.38%). It is a common ingredient in honey and is considered as compound arising from the degradation of sugars like fructose at lower pH. At lower concentration of honey and varying concentration of HMF, maximum antibacterial activity was noted with honey containing highest concentration of HMF and inhibited Staphylococcus aureus, E. coli and Bacillus subtilis. However honey with any tested concentration of HMF had no inhibitory effect on Pseudomonas aeruginosa [18]. Makarewicz et al. [19], studied the effect of honey arising from different floral origins on bacteria. Highest HMF content was seen in Eucalyptus honey. However it showed no inhibitory effect on Bacillus subtilis and Pseudomonas putida, but its inhibitory effect on other strains like E. coli, Micrococcus luteus, Proteus myxofaciens was equal to or more than those honey from sunflower, thyme, lavender, coriander and lime.

1,3-Dihydroxypropan-2-one dimer, a dioxane group of compound, is the second abundant volatile molecule in the methanolic extract of Pterobryopsis pilifolia. This compound is common in taxa like Moringa oleifera [20] and in Lomandra leucocephala, with female flowers having almost twice the content than the male flowers [21]. Lactones are considered as signalling molecules in quorum sensing by microbes. Gamma-Lactone is considered as an endogenously produced quorum sensing molecule that regulates growth and secondary metabolite production in Aspergillus nidulans. Higher concentrations of this molecule can reduce lag phase by 3 hrs and was found to enhance penicillin production by A. nidulans to 31.9% compared to control [22]. Lactones are derivatives of lipid metabolism and contribute to aroma and flavour of fruits, vegetables, meat, milk, butter, wine etc. Gamma hepta-lactone is reported to be synthesized by Fusarium poae in malt broth medium under aeration [23]. Gamma lactone catabolic pathway is a mechanism that can protect crop plants by disrupting the quorum sensing communication [24]. The presence of lactones in Pterobryopsis pilifolia thus disrupts the pathway and induces lactone degradation pathway resulting in developing resistance to various bacterial and fungal pathogens.

Melamine, a triazine compound is found in the methanolic extract of P. pilifolia (peak area of 0.21). It is considered as a toxic chemical that can cause several problems in human health. Studies by Guan et al. [25] reveal that increase in melamine concentration in infant food can increase the incidence of urolithiasis. Presence of

melamine in food caused crystalluria in sheep [26], nephrotoxicity in young broiler chickens [27], and decrease in cell proliferation and increased apoptosis in rodent testis [28]. Melamine is a carcinogen and can cause urinary tract cancer [29].

4. Conclusions

In the present investigation, several phytochemicals toxic to insects, and higher animals were detected. Bioactivities of many of them are not yet studied. Presence of these chemicals especially phthalates at high concentration can be the reason for reluctance of animals in feeding bryophytes, specifically *Pterobryopsis pilifolia*. This accounts to addition of knowledge to the field of bryophytes which remain neglected due to low availability of biomass. Further studies are to be conducted, on the use of antifeedant compounds from bryophytes as feeding deterrents, especially on the persistence and cytotoxicity towards human cells. The compounds can be novel and hence have economic value towards developing eco-friendly feeding deterrents.

Acknowledgments

The First author is thankful to CSIR for providing grant in aid as SRF for the present work. The authors thank DST-FIST for providing infrastructure facilities for carrying out the work. We are also thankful to Synthite Industries Pvt Ltd for their valuable suggestions regarding GC MS analysis.

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