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Phytochemical profiling, antibacterial, antifungal and antioxidant evaluation of *Acrotrema arnottianum* Wight - An ethnomedicinal plant

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ABSTRACT

Acrotrema arnottianum Wight (Dilleniaceae) is a perennial herb that grows in damp, shaded areas. It is native to the Western Ghats of Kerala and Tamil Nadu, where it is used in traditional medicine by tribal people, including the Malavedans of Kerala, to treat baldness and hair loss. Histochemical localisation of stem, petiole and midrib was performed to determine the localisation of flavonoids, phenols, alkaloids and tannins. The results indicated the presence of flavonoids and phenols. GC-MS analysis of ethanol extract revealed the presence of metabolites such as Linoleic acid, ethyl oleate, campesterol, tetradecanoic acid, Hexadecanoic acid, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, squalene and α-Tocopherol. GC-MS analysis of ethyl acetate extract showed the presence of α-Tocopherol-β-D-Mannoside, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, squalene, diphenols, phytol, eugenol and β-sitosterols. The antibacterial study against *Staphylococcus aureus* showed that ethyl acetate extract is more potent in inhibiting the growth (17±0.18) while the antifungal study against *Candida albicans* showed that ethyl acetate has a significant effect in inhibiting the growth (16±0.23), in dosage dependent manner. The antioxidant study revealed that ethanol extract has a higher IC_{50} value of 36.02±0.27. This study indicates that *Acrotrema arnottianum* is a source of pharmaceutically active secondary metabolite compounds.

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INTRODUCTION

Secondary metabolites are certain compounds that are produced inside the cell which are not necessary for an organism or cell to live, but they play a crucial role in interactions of the cell or an organism with its environmental changes. These compounds often get involved in an organism's responses against biotic or abiotic stresses (Pagare *et al.*, 2015). Secondary metabolites that are present in several plants have been used by humans for treating illness, infections, health disorders and against pests have been recorded from time immemorial. Many of these compounds have now been exploited by different industries such as the agrochemical, pharmaceutical, food industries, and even perfume and other aroma sector industries. The crude drugs that are residing in these plants have been utilized for treating several infections and have provided a promising future to mankind. Still now, mankind is searching for more and more promising drugs to further improving and improvising their current knowledge in modern treatment methods (Umashankar, 2020).

The Western Ghats is a mountain range where the region contains a wide variety of flora and fauna, of which many are endemic to the region. The tribal communities residing here use several herbal medicines for their ailments which are collected from these regions. These plants feature several secondary metabolites that could be highly effective medicines. The range of these critically important endemic and endangered ethnomedical plants would be negatively impacted by environmental degradation and climate change.

The *Acrotrema* genus is a group of herbaceous, dicot plants belonging to the family Dilleniaceae with many endemic species are distributed to South Asia, found in India, Sri Lanka, Myanmar, Malaysia and Thailand (Dickson, 1971). *Acrotrema arnottianum* Wight (Figure 1a), is an endangered species, which is a small herb with a woody rhizome, a rosette of leaves which are broad having trichomes all over the body, young leaves are crimson in colour and yellow flowers in axillary raceme inflorescence (Kumari *et al.*, 2009). The follicles are

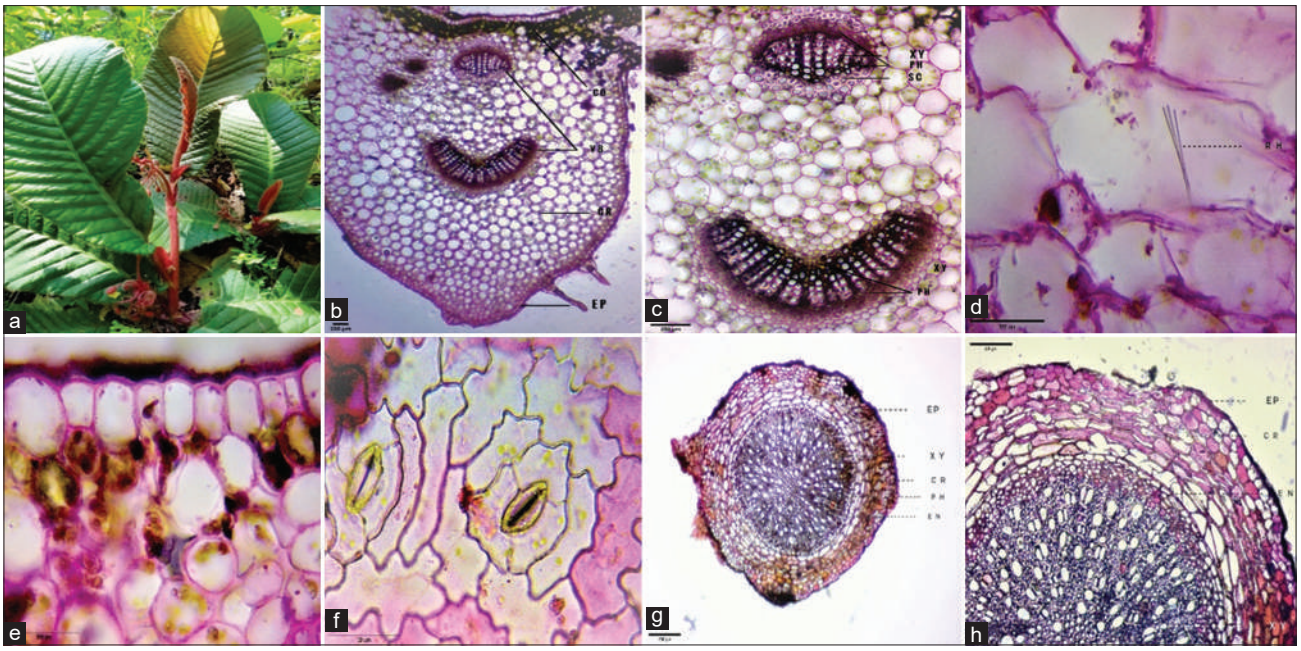

Figure 1: Anatomical sections of leaf midrib and root of *Acrotrema arnottianum*. a) The whole plant specimen, b) T.S of midrib, c) enlarged portion of vascular bundles in midrib, d) parenchyma cells containing raphides, e) collenchyma cells seen below the epidermis layer, f) anisocytic stomata, g) T.S of root and h) enlarged portion of root

ovoid and each follicle contain a single seed which is arillate. The plant is endemic to the southern Western Ghats, mostly in Kerala and Tamil Nadu and is endangered due to the loss of environment and deforestation (Mohanan & Henry, 1994). The plant grows in moist, shady places of deciduous and evergreen forests with moderate sunlight. The medicinal properties of this plant include headache relief, prevents hair fall and baldness and is used by Malavedan tribes of Kerala (Kumari *et al.*, 2009).

Histochemical localisation is crucial for understanding the distribution of biologically active compounds, which can inform both medicinal applications and plant biology research. The GC-MS analysis was utilized to identify the presence of phytoactive compounds that impart the medicinal properties of the plant while antimicrobial and antioxidant studies will determine the effectiveness of this plant against pathogens and oxidative stress. This study aims to determine the presence of secondary metabolites through histochemical localisation and GC-MS analysis using ethanol and ethyl acetate extracts along with antibacterial, antifungal and antioxidant analysis to determine the medicinal value of this endemic plant.

MATERIALS AND METHODS

Acrotrema arnottianum plants were collected from the forest regions near Palode, Thiruvananthapuram, Kerala (8°44′53.758″ N, 77°1′49.717″ E) and was maintained in the Botanical Garden of Sanatana Dharma College, Alappuzha, Kerala, India. The plant was identified, and voucher specimen were deposited in the herbarium at Botanical Survey of India (BSI), Coimbatore with accession no. MH 178216.

Histochemical Localisation of Midrib, Stem and Petiole

Fresh leaves and stems of the plant were taken for histochemical analysis. Sections were taken on the midrib because of its large size, thick and fleshy midveins which are prominent. The mid ribs, petiole, stem and root sections were taken using a sharp razor blade. Then the sections were stained using safranin and were taken for localisation tests. Wagner's reagent was used for the detection of alkaloids (Furr *et al.*, 1981; Pratiwi *et al.*, 2020). A drop of Wagner's reagent is dropped on the thin section and if alkaloids were present, the tissues will develop a brownish red colour appearance. The presence of phenolic compounds was detected using Ferric chloride ($FeCl_3$) and sodium carbonate (Na_2CO_3). The ferric chloride is dusted on the thin section, and several flakes of sodium carbonate were added to the section. A positive reaction would show black or blackish green colour (Johansen, 1940). The treatment with $FeCl_3$ is done to test the presence of tannins. A positive result would show a bluish black colour (Savithramma *et al.*, 2014). A drop of Sodium hydroxide (NaOH) solution is placed on the thin section of the sample to determine the presence of flavonoids and the development of a yellow colour determines the presence of flavonoids.

GC-MS Analysis

The healthy young whole plants were collected, washed to remove any dirt residue and air-dried at room temperature. Then the dried plants were grounded to fine powder and taken to carry out the analysis. The analysis used for the identification of metabolites in the study is the GC-MS technique using ethanol and ethyl acetate extracts. The extracts were prepared using the Soxhlet extraction method. The GC-MS analysis (instrument

model-7890 A GC with 5975 C with triple axis detector, column – DB 5 MS 30 m × 0.25 mm diameter × 0.25 μm thickness) was conducted by injecting 2 μL of the sample in splitless mode. Helium gas was used as the carrier at a flow rate of 1 mL/min and the process was carried out in EI (electron impact) mode with 70 ev ionisation energy and the temperature for the injector was maintained constantly at 280 °C. The compounds were identified by comparing the retention time with the spectral data from the available mass spectral database (NIST - 08 SPECTRAL DATA).

Antioxidant Activity

The antioxidant properties of ethanol, methanol and ethyl acetate extracts were analysed using the DPPH free radical scavenging assay. The reaction mixtures were incubated at 37 °C for 30 minutes and the absorbance of each mixture was measured spectrometrically at 516 nm. The measurements were taken in triplicate. DPPH activity was calculated using the equation:

$$\text{DPPH scavenging activity } (\%) = \left(\frac{Ac - A}{A}\right) \times 100$$

Where Ac is the absorbance of the control and A is the absorbance of the extracts tested, and the results were shown as IC_{50} values.

Antimicrobial Study

The antimicrobial properties of ethanol, methanol and ethyl acetate extracts were determined using the Agar Well Diffusion method by testing the activities in *Candida albicans* and *Staphylococcus aureus* against the control drugs Streptomycin and Nystatin respectively by recording the inhibition zone using a ruler. The studies were done thrice, and the results were recorded.

Statistical Analysis

Data from the antioxidant and antimicrobial study were replicated thrice and the results were reported as the mean±SD (standard deviation).

RESULTS

The transverse section (TS) of the midrib region (Figure 1b & c) depicts an epidermal layer with thick cuticle, followed by several collenchymatous layers (Figure 1e). The epidermis has a thick cuticle, the stomata are anisocytic (Figure 1f) and trichomes are seen at the epidermis. The cortex is made of parenchymatous cells, and some cells contain raphides (Figure 1d). Two vascular bundles are present, one is crescent shaped seen at the centre and a smaller vascular bundle is present above it. Xylem is seen surrounded by phloem in each vascular bundle. Sclerenchymatous bundle sheath is seen at the upper vascular bundle. The TS of root (Figure 1g & h) constitutes an outer cuticle layer followed by epidermis. The cortex is composed of parenchymatic cells, some having raphides and starch grains. The cortex is followed by a thin layer of endodermis. Xylem is seen at the central portion and pith is absent.

Histochemical Localisation

Fresh leaves and stems of the plant were taken for histochemical analysis. The tests were conducted for the localization of flavonoids, phenols alkaloids, and tannins (Table 1). The stained sections were treated with various chemicals including NaOH, $FeCl_3$, Wagner's reagent and Na_2CO_3. The sections treated with Wagner's reagent does not show brownish red colour implies a negative result for alkaloids. Test using NaOH shows the presence of flavonoids near vascular bundles in the midrib; vascular bundle and cortex of the petiole and scattered around the cortical region in stem sections. Treatment with FeCl3 shows the presence of phenol near the vascular bundles and the parenchyma cells in all three sections. Test for Tannins showed a negative result (Figure 2).

GC-MS Analysis

GC-MS analysis using ethanol extract revealed the presence of compounds such as Linoleic acid (3.14%), Hexadecanoic acid (3.24%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (5.85%), 2-propanone,1,1- dimethoxy (5.49%), Squalene (16.42%), campesterol (7.06%), α-tocopherol (4.65%), octadecanoic acid (3.63%) ethyl oleate (2.45%), phytol (4.27%) and tetradecanoic acid (1.57%) in the plant while the GC-MS analysis using ethyl acetate extract revealed the presence of toluene (41.75%), 2-octa decoxy ethanol (1.05%), phytol (1.42%), diphenols (1.05%), squalene (13.61%), α-Tocopherol-β-D-Mannoside (3.57%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (11.61%), β-sitosterols (9.4%) and eugenol (1.34%) were detected. The GC-MS profiling patterns of ethanol and ethyl acetate extracts are represented in Figures 3 and 4 and the Retention Time (RT), active principal molecular weight, molecular formula, peak area % are placed in Tables 2 and 3 respectively along with the structure of some important compounds are provided in Figures 5 and 6.

The antibacterial activity was tested against *Staphylococcus aureus*. Out of the three extracts, ethyl acetate and methanol extract showed a significant antibacterial property. Ethyl acetate extract showed an inhibitory zone of 17±0.18 at 80% concentration and methanol extract showed an inhibitory zone of 15±0.15, both active against the pathogen in a dosage-dependent manner. Ethanol extract has shown an inhibitory zone of 12±0.36 at 80% concentrations and has no effect in

Table 1: Showing the response of sections towards different reagents for histochemical localisation

Metabolic group	Reagents	Leaf Midrib	Leaf Petiole	Stem
Flavonoid	NaOH	+	+	+
Phenol	Ferric chloride+sodium carbonate	+	+	+
Alkaloid	Wagner's reagent	-	-	-
Tannin	Ferric chloride	-	-	-

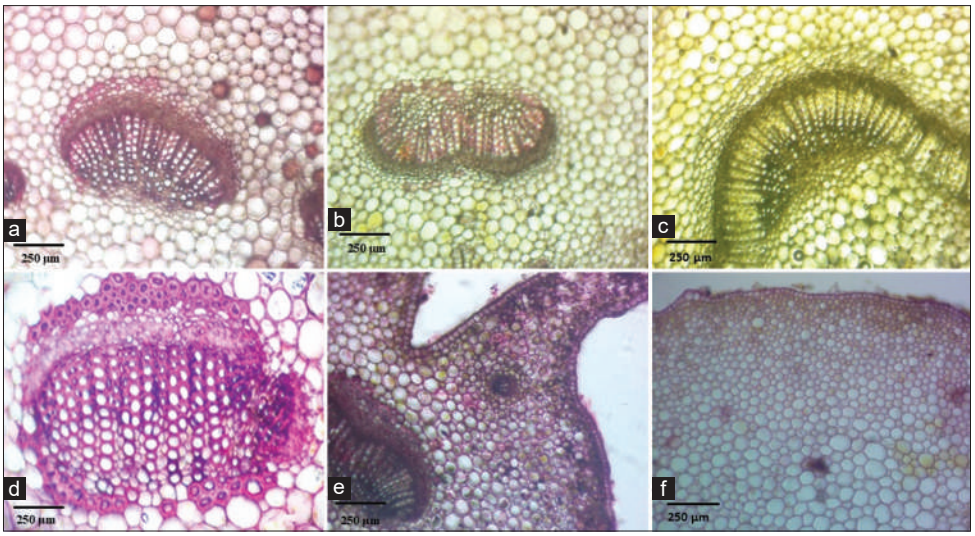

Figure 2: Histochemical localisation of midrib, petiole and stem: (a-c) showing the presence of phenol in the vascular bundle of midrib, petiole and stem respectively and (d-f) showing the presence of flavonoids at the vascular bundles of midrib, cortex and vascular bundle region of petiole and cortical region of stem

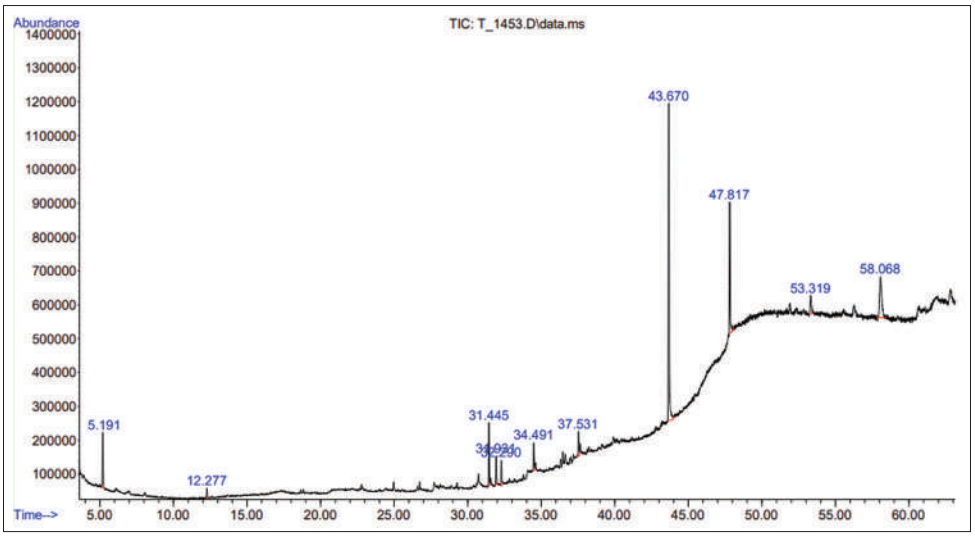

Figure 3: The chromatogram of GC-MS analysis using ethanol extract of *A. arnottianum*. Height of the peak defines the abundance of the compound

lower doses. Antifungal activity was tested against *Candida albicans*. Of the three extracts, only the ethyl acetate extract showed a response with an inhibitory zone of 16±0.23 at 80% concentration in a dosage-dependent manner. Antibacterial and antifungal activities of the extracts are represented in Figures 7, 8 and Table 4.

Antioxidant study using DPPH assay has shown that ethanol extract has got higher IC_{50} value of 36.02±0.27 followed by ethyl acetate 20.69±0.15 and the least activity was observed in methanol extract 13.71±0.12 as represented in Figure 9 and Table 5.

DISCUSSION

The anatomical sections done on midrib, stomata and roots of *Acrotrema arnottianum* shows that the result was allied to the earlier study (Kumari *et al.*, 2009). The present study investigated the histochemical localisation of leaf midrib, petiole and stem of A. *arnottianum* and this is the first report in this plant. Flavonoids show a positive result, in the midrib, petiole and stem, it is located near to vascular bundles and seen scattered throughout the cortex cells. Test for phenols showed positive response with a blackish green colouration very prominently seen in vascular bundles of leaf midrib, petiole and stem. Tests for alkaloids and tannins showed a negative response indicating their absence in the specimen concerned. The study showed that flavonoids and phenols are rich in these plant parts that can be utilised for further studies.

Plant metabolites play a major role in pharmacognostic and therapeutic studies in developing novel drugs and treatment protocols. GC-MS analysis has identified several bioactive compounds with potential biological effects. Linoleic acid is an

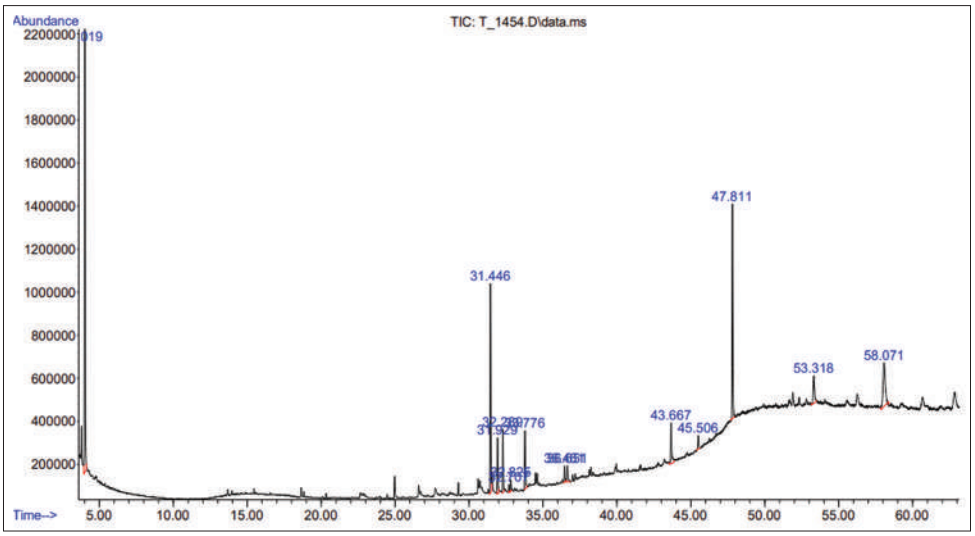

Figure 4: Showing the chromatogram of GC-MS analysis using ethyl acetate extract of *A. arnottianum*. Height of the peak defines the abundance of the compound

Table 2: List of compounds detected in GC-MS analysis of ethanol extract

S. No.	Name of compound	Formula	RT(min)	MW	Area %
1	2-propanone, 1,1-dimethoxy	$C_5H_{10}O_3$	5.192	118	5.494
2	1,1-diethoxy-3-methylbutane	$C_9H_{20}O_2$	12.269	160	1.248
3	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	31.451	296	5.855
4	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	34.493	284	3.243
5	Linoleic acid, ethyl ester	$C_{20}H_{36}O_2$	37.534	308	3.143
6	Squalene	$C_{30}H_{50}$	47.813	410	16.425
7	Campesterol	$C_{28}H_{48}O$	24.589	400	7.06
8	α-Tocopherol	$C_{29}H_{50}O_2$	22.54	430	4.65
9	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	14.454	312	3.63
10	Ethyl oleate	$C_{20}H_{38}O_2$	14.32	310	2.45
11	Phytol	$C_{20}H_{40}O$	14.02	296	4.27
12	Tetradecanoic acid	$C_{16}H_{32}O_2$	11.718	256	1.57

Note: RT=Retention Time; MW=Molecular weight

Table 3: List of compounds detected in GC-MS analysis of ethyl acetate extract

S. No.	Name of compound	Formula	RT	MW	Area %
1	Toluene	C_7H_8	4.029	92	41.75
2	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	31.452	296	11.61
3	2-Octa decoxy ethanol	$C_{20}H_{42}O_2$	36.464	314	1.053
5	4,4′-((p-phenylene) diisopropylidene) diphenol	$C_{24}H_{26}O_2$	45.511	346	1.056
6	Squalene	$C_{30}H_{50}$	47.814	410	13.617
7	α-Tocopherol-β-D-Mannoside	$C_{35}H_{60}O_7$	53.327	592	3.578
8	β-Sitosterol	$C_{29}H_{50}O$	58.076	414	9.461
9	Eugenol	$C_{10}H_{12}O_2$	6.68	164	1.34

Note: RT=Retention Time; MW=Molecular weight

essential fatty acid and its use has been associated with decreased risk for atherosclerosis, headaches and hypercholesterolemia (Mercola & D'Adamo, 2023). 3,7,11,15-Tetramethyl-2-hexadecen-ol is a triterpene alcohol possessing antimicrobial properties (Selvan & Velavan, 2015). Hexadecanoic acid, a saturated fatty acid is an inhibitor of phospholipase A (2) thereby having an anti-inflammatory response (Aparna *et al.*, 2012). Squalene belongs to an isoprenoid group that possesses anticancer, antioxidant, cytoprotective and immune stimulating effects (Das *et al.*, 2003). Squalene plays a key role in keeping skin from oxidative damages due to free radicals. Squalene is used as an additive in topical creams as a drug carrier for delivering to the target site (Fox, 2009). Phenolic compounds have various activities such as antioxidant, anti-inflammatory, anti-mutagenic, apoptosis inducing and anti-cancer properties (Huang *et al.*, 2009). Campesterol is a sterol that exhibits anti-inflammatory, antibacterial and antifungal activities along with chemopreventive effects against breast, lung and prostate cancers by inhibiting angiogenesis (Choi *et al.*, 2007). Eugenol is a phenolic monoterpenoid possessing anti-inflammatory, neuroprotective, anti-cancer, analgesic, antimicrobial and antioxidant along with cardiovascular protection (Nisar *et al.*,

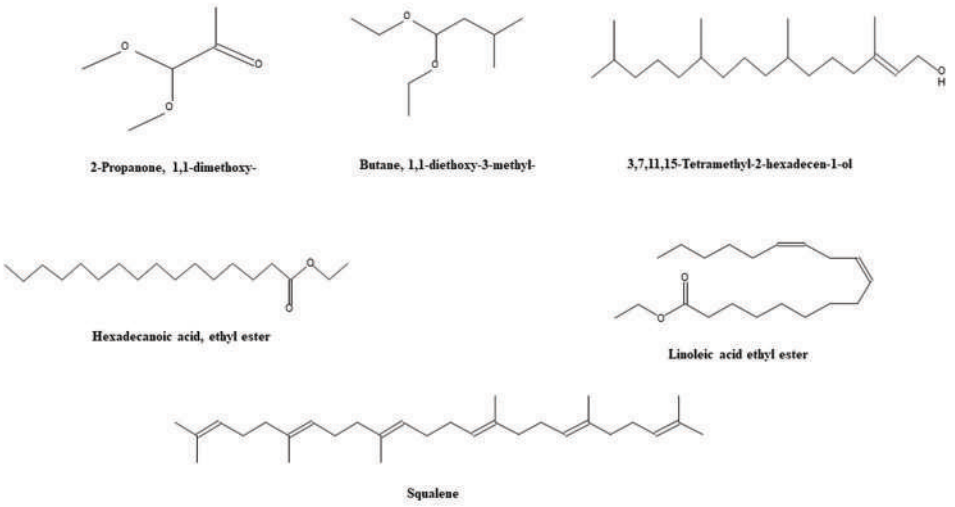

Figure 5: Chemical structure of listed metabolites from ethanol extract of *Acrotrema arnottianum*

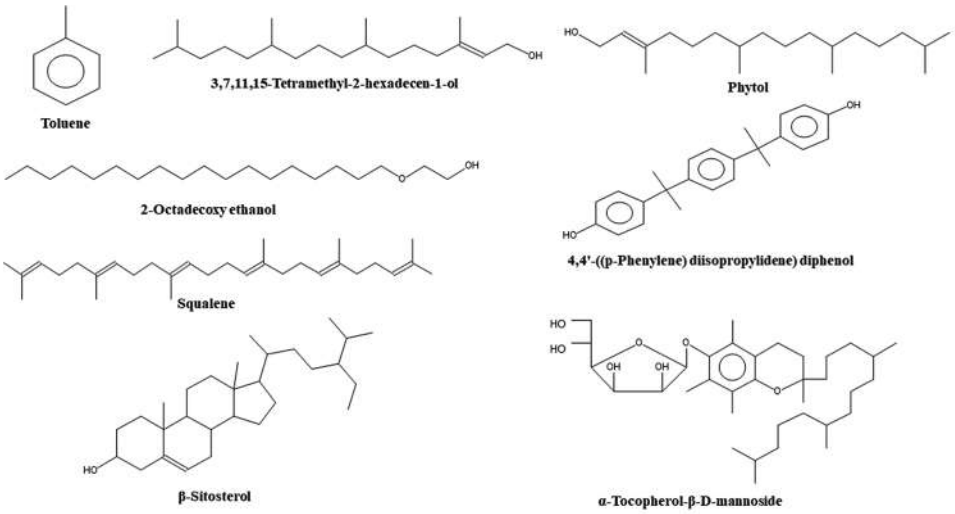

Figure 6: Chemical structure of listed metabolites from ethyl acetate extract of *Acrotrema arnottianum*

Table 4: Showing the zone of inhibition of three extracts at varying concentrations against *Candida albicans* and *Staphylococcus aureus*

Concentration	Methanol extract	Ethanol extract	Ethyl acetate extract
	Candida albicans		
Solvent control	10	11	9
80%	-	-	16±0.23
50%	-	-	13±0.15
25%	-	-	-
Streptomycin 1000 ppm (Standard drug)	25	25	25
	Staphylococcus aureus		
Solvent control	11	10	-
80%	15±0.15	12±0.36	17±0.18
50%	13±0.17	-	14±0.1
25%	11±0.2	-	11±0.14
Nystatin 1000 ppm (Standard drug)	16	16	16

The values represented are mean±SD

Table 5: Showing the antioxidant activity(IC_{50}) of ethanol, methanol and ethyl acetate extracts of *A. arnottianum*

Extracts	IC_{50}±SD
Ethanol	36.02±0.27
Methanol	13.71±0.12
Ethyl acetate	20.69±0.15

The values represented are mean±SD

2021). β-sitosterol is a phytosterol possessing antioxidant, anti-cancer, anti-diabetic, anti-microbial, anti-inflammatory, antipyretic, immune modulation and anti-arthritic activities (Rashed, 2020). Another study suggests that the plant possesses triterpenoids like Lupeol, Lupeol acetate, flavonoids like kaempferol and quercetin, steroids like β-sitosterol (Lima *et al.*, 2014). Early study on phytochemical profiling of this plant revealed that the plant is rich in proteins, amino acids and vitamins, of which vitamin A and E showed an abundance and vitamin C was lower. Presence of major phenolic acids like gallic acid, syringic acid, rutin, vanillate, and caffeic acid along

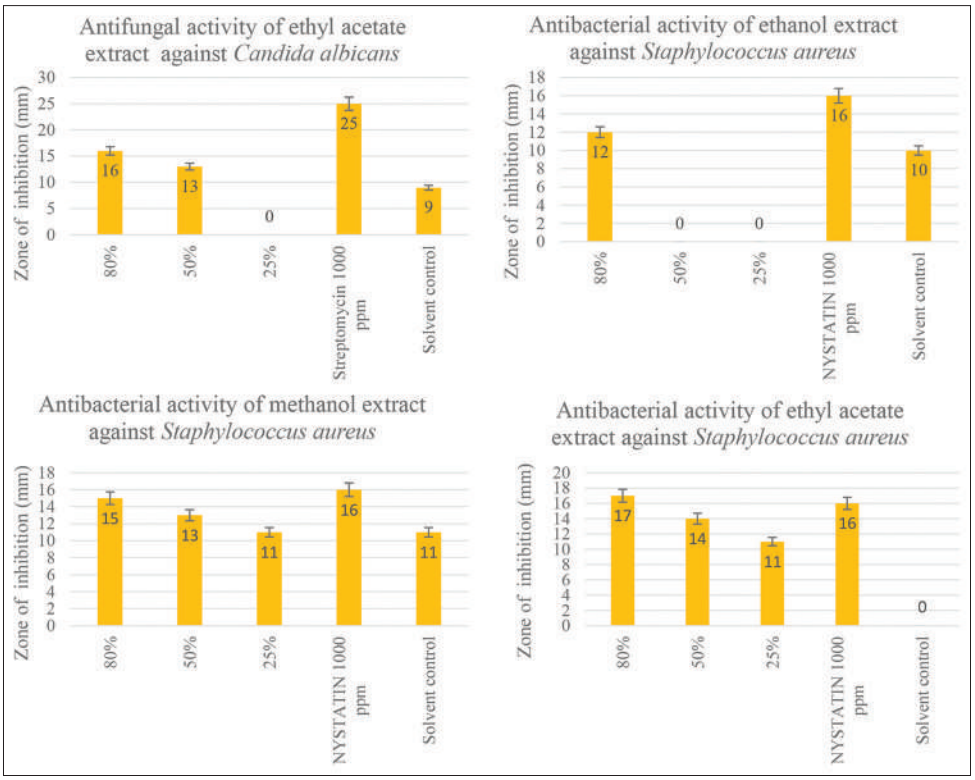

Figure 7: Showing antifungal and antibacterial activity of ethanol, methanol and ethyl acetate extracts of *A. arnottianum*

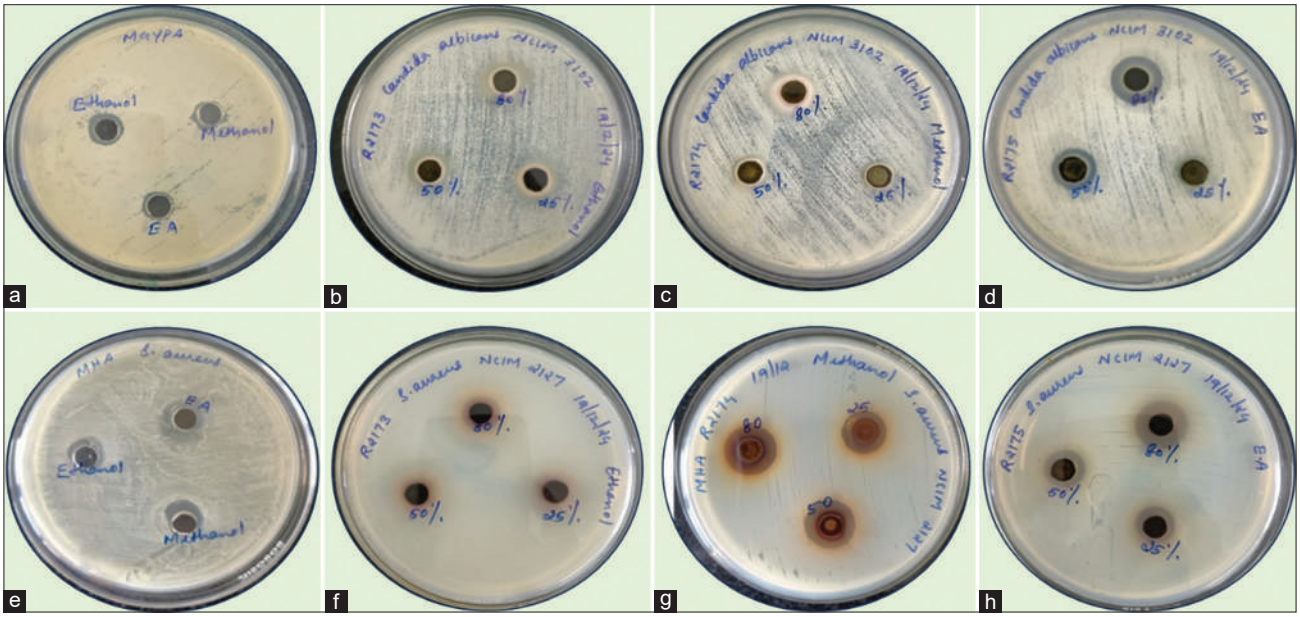

Figure 8: a) Antifungal activity of ethanol, methanol and ethyl acetate solvents against *Candida albicans*, (b-d) Antifungal activity of ethanol, methanol and ethyl acetate extracts respectively, e) Antibacterial activity of ethanol, methanol and ethyl acetate solvents against *Staphylococcus aureus*, (f-h) Antibacterial activity of ethanol, methanol and ethyl acetate extracts respectively

with flavonoids were reported (Kumar *et al.*, 2018). Study using Hexane extract of the whole plant confirmed the presence of several triterpenoids like lupeol, lupeol acetate, steroids like Beta sitosterols while methanol extract confirmed the presence of flavonoids like kaempferol and quercetin (Mathew & George, 2006), the presence of betulin and betulinic acid in the plant (Dan & Dan, 1980) and the presence of ombuin: a type of quercetin is also reported (Gurni & Kubitzki, 1981). The ethanol extract exhibits higher antioxidant activity may be due to the presence of bioactive metabolites in higher proportions compared to ethyl acetate and methanol extracts. Antimicrobial study against *C. albicans* and *S. aureus* suggest that ethyl acetate

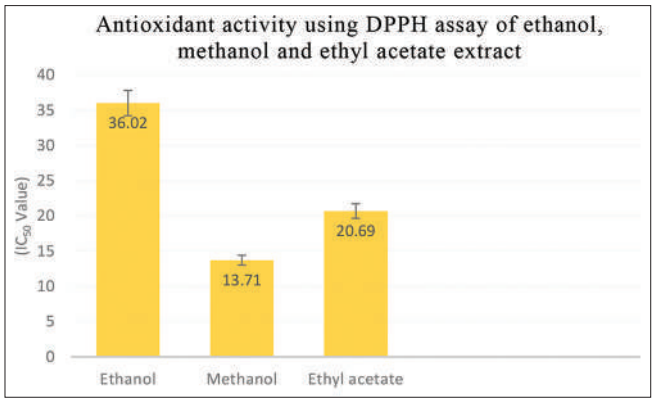

Figure 9: Showing antioxidant activity of ethanol, methanol and ethyl acetate extracts of *A. arnottianum*

extract is effective in controlling microbial proliferation. Another study done on the roots of this genus has showed significant Acetylcholinesterase (AChE) inhibitory activity (Somat *et al.*, 2019) may be due to the presence of these bioactive components These studies suggest the potential medicinal properties of this endemic plant and the promising results will provide leads for further research on A. *arnottianum* Wight.

CONCLUSION

The study ascertained the presence of pharmaceutically potent phytochemicals in the plant parts of A. *arnottianum* Wight along with the antimicrobial and antioxidant study give a promising outlook for this ethnomedicinal plant.

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