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Anti-Microbial Activity of Chemical Constituents of *Hygrophila auriculata* (K. Schum)

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The Chloroform extract of flowers of *Hygrophila auriculata* (K. Schum) Heine (Syn. *Asteracantha longifolia* Nees. Acanthaceae) was used to isolate the chemical constitutes present in the plant and the isolated constitutions were tested against antimicrobial activity. The antimicrobial activity of the isolated compounds were performed with the help of three test organism known as *Aspergillus, Alternaria altarnata* and *Penicillium.* Different concentration perimeters studies were carried out.

Keywords : *Hygrophila auriculata* (K.Schum), *Asteracantha longifolia* Nees. Antimicrobial activity etc.

INTRODUCTION

Folk medicine in the form of Herbal and Natural products have been used for centuries in every culture throughout the world. The researchers and medical professionals have shown increased interest in this field as they recognize the true health benefits of these remedies. "Let food be your medicine and let medicine be your food" was advised by the father of medicine, Hippocrates, over two millennia ago. The World Health Organization estimated that about 80% of the people in developing countries rely on medicinal plants for the treatment of various ailments. In India, Ayurvedic system which was evolved over 5,000 year ago is still in practice. The Rig Veda and Atharvana Veda have included more than 700 medicinal prescriptions (Mninh, 1966). There has been an increasing interest in the study of traditional medicines and their popularity is increasing day by day in different parts of the world for the last few decades (Prashant *et al.,* 2008). Natural products in general, and plants in medicinal particular, are believed to be an important source of new chemical substances with potential therapeutic efficacy. *Hygrophila auriculata* (K. Schum) Heine (synonym: *Asteracantha longifolia* Nees, Barleria *auriculata* Schum, *Barleria longifolia* linn) Acanthaceae, is a widely distributed herb commonly found in moist places of banks of tanks, ditches and paddy fields throughout India and described in ayurvedic literature as Ikshura, Ikshugandha, and Kokilasha "having eyes like the Kokila or Indian Cuckoo." The plant is widely distributed throughout India, Srilanka, Burma, Malaysia and Nepal. It is classified in Ayurvedic system as seethaveeryam, mathuravipaka and used for the treatment of premeham (Diabetes), athisaram (Dysentery) etc., (Nadkarni, 1978 and Chopra and Chopra 1996). The whole plant, roots, seeds, and ashes of the plant are extensively used system in traditional of medicine for various ailments like rheumatism, inflammation, jaundice, hepatic obstruction, pain, urinary infections, edema and gout (Mishra 1986, Shailjan *et al.,* 2002). The Plant is known to possess antibacterial (Vlietnick *et al.,* 1995), hypoglycemic (Fernanoda *et al.,* 1991), antitumor (Manjumdar *et al.,* 1997 and Ahemd *et al.,* 2001), hepatoprotective (Singh and Honda 1995, Hewawassam *et al.,* 2003), and anti-nociceptive properties (Shanmugasundram 2005). The earlier studies on the plant have revealed the presence of anthraquionones in aerial parts (Rao *et al.,* 1999). Since past several years, there has been a frightening increase in fungal infections and allergies due fungal aeroallergens (Crameri *et al.,* 2014). Various fungal species including *Alternaria alternate, Aspergillus niger* (Person *et al.,* 2010) and *Penicillum* spp (Lyratozopolous *et al.,* 2002) are known as common aeroallergens causing diseases in respiratory tract (Fukutomi *et al.*, 2015, Teresa *et al.*, 2015). These aeroallergens are gradually becoming resistant to broad spectrum antibiotics and other antifungal agents used for their treatment and simultaneously leading to chronic conditions in the patients (Rai and Saraf 2009). Keeping in the view the present report deals with the isolation and structure elucidation of chemical constituents from the flowers of *Hygrophila auriculata* along with their antifungal activity.

MATERIAL AND METHODS

Plant Material : The plant *H. auriculata* was collected from Berhampur (Orissa) during the month of May and authenticated through Regional Research Laboratory Bhubaneswar. A voucher specimen has been deposited at the department of Natural Products in the same institute. The flower was plucked dried under shade and coarsely powdered.

Preparation of extract and fraction : Dried powdered aerial parts of *H. auriculata* (500gm) were defatted by extracting exhaustively with petroleum ether in a Soxhlet apparatus. The

defatted material was then extracted with methanol in similar manner. The methanol extract (4.5 g) of flowers of *H. auriculata* was suspended uniformly in water and extracted with Chloroform and later with ethyl acetate. Both the extracted portions were concentrated under reduced pressure. All the extracts were subjected to *in-vitro* phytochemical screening (Khandelwal 2002).

Phytochemical Screening : All the extracts were subjected to preliminary phytochemical screening (Wagner and Bladt 1996, Parashar and Singh 1965) and it was observed that the petroleum ether, chloroform, ethyl acetate and methanolic extracts show presence of alkaloids (Parashar and Singh 1965) while flavonoids, and Iridoid glycosides were not found in the present species although in other species of *Hygrophila* they were reported. The chloroform extract was subjected to column chromatography using silica gel (60-120 mesh size), and eluted with petroleum ether, Chloroform, ethyl acetate in gradient fashion. The following five compounds were mainly obtained from the faction eluted with chloroform.

Compound 1 isolated from petroleum ether and crystallized as light orange coloured flakes, with m.p. 175-76°C showed a single spot on TLC and analyzed for $C_{15}H_{10}O_2$. The compound shows pink colour with aqueous NaOH and positive to Borntrager reaction. It was identified as tectoquinone by mixed melting point and spectral data.

Compound 2 crystallized from hexane-ethyl acetate, m.p. 258-60°C, $[\alpha]_D$ +76.25° (c-0.13 $CHCl_3$). Violet colour in Lieberman Burch reaction and purple color with Conc. H_2SO_4. The compound was confirmed to be Arnidiol by mixed melting point and spectral data.

Compound 3 Hexane-chloroform fraction after successive re-column chromatography produce yellow crystals, with m.p. 210-12°C. It gave pink colour with aq. Alkali and violet colour with conc. H_2SO_4. It has been identified as 3, 8-Dihydroxy-4methoxy-2-methylanthraquinone based on matching the spectral data with authentic sample.

Compound 4 Hexane chloroform fraction in the ratio (2:8) produce orange-red needless with m.p. 210-12°C. Pink coloration was obtained when treated with aq. Alkali while violet colour was obtained on treatment with conc. H_2SO_4. The compound was found to be 1, 3, 5-Trihydroxy-4-methoxy-2-methylanthraquinone by comparison of its spectral data with authentic sample.

Compound 5 was obtained by crystallization from chloroform and the m.p was 256-58°. It gave dark pink colour with alkali and blue colour with conc. H_2SO_4. The compound was identified as 1, 3, 4-Trihydroxy-5 (or 8) methoxy -2-methylanthraquinone by comparison of its spectral properties with authentic sample.

Fungal strains : The anti-fungal activity was examined in the plant. Initially, some important strains of fungal aeroallergens including *Penicillum* sp., *Aspergillus niger*, and *Alternaria alternate* were used in the present study. Test organisms were obtained from fungal germ plasma collection center, Department of Biological Science, R. D. University, Jabalpur. The cultures were revived by inoculating potato-dextrose broth incubated at 28ºC for 7 day. After 7 day of incubation the concentration of spore in the suspension was adjusted to $2x10^7$ spores per ml and re-inoculated on PDA plates. The PDA plates were incubated at 28ºC for 7 day and used as inoculum for the study.

Determination of antifungal activity : As compounds 1 & 2 are not common for these particular plants *i.e., H. auriculata,* further studies not perform for above two compounds. Out of isolated compounds only compounds 3, 4 & 5 were selected for determination of antimicrobial activity. We dissolved compound 3 in 95% of 1 ml butenol and 4 & 5 were dissolved in 1 ml of 95% ethanol and added to Potato-Dextrose -Agar (PDA) media to make final concentration of these chemicals 0.001, 0.01 and 0.1%. Pure PDA media was used as control and PDA supplemented with 0.1% ethanol and butenol were used as placebo. In SET 1, petriplates were prepared by pouring 15 ml of each of above media in triplicate. All Petriplates were inoculated with 6 mm disc cut from 7 day old growing culture in other Petridis with PDA. In SET 2, petriplates were inoculated with spore suspension prepared by harvesting the spore from the culture grown on PDA at 28ºC for seven days followed by pouring the 15 ml of PDA media. Inoculated plates were incubated at 28ºC ± 1ºC for a week and observed daily. Colony diameter increased in SET 1 and number of colonies appeared in SET 2 was recorded. Fertility of cultures was determined by counting number of spores per ml using haemocytometer.

RESULTS, DISCUSSION AND CONCLUSION

The present study was an effort to investigate and validate the pharmaceutical and ethno botanical studies conducted with regard to phytochemical constituents and studying the anti-fungal activity of *H. auriculata*. After defatting with petroleum ether, the methanolic extract of the aerial part of *H. auriculata* was suspended in H_2O and later extracted with different solvent like, Chloroform, ethyl acetate, and *n-Butanol*. All the extracts were tested to find out the presence of Iridoid glycosides. The chloroform extract was collected and finally subjected to column chromatography using silica gel (60-120 mesh size), and eluted with petroleum ether, Chloroform, ethyl acetate in gradient fashion. Analytical studies were made on the five pure compounds obtained mainly from the faction eluted with chloroform and the results are as follows:

Compound 1 gave positive tests for anthraquionones and showed [M]+ ion peak m/z 222 and analyzed for molecular formula $C_{15}H_{10}O_2$. Its spectral data suggested the compound to be 2-methylanthraquinone (tectoquinone) which has been reported in *Tectona grandis* and in many other plants (Klobb, 1903). The identification was confirmed by direct comparison with an authentic sample.

Fig. 1 Tectoquinone

Fig. 2 Arnidiol

Fig. 3 (3, 8-Dihydroxy-4-methoxy-2-methylanthraquione)

Fig. 4 (1, 3, 5-Trihydroxy 4-methoxy-2-ethylanthraquione)

Fig. 5 (1, 3, 4-Trihydroxy5 (or8)-methoxy-2-methylanthraquione)

Compound 2 gave positive LB reaction. Its molecular ion peak appears at 442 consistent with formula $C_{30}H_{50}O_2$ and identified as Arnidiol which was first isolated from *Arnica montana* and *Tussilago farfara* (Santer and Stevenson 1962, Rao, *et al.,* 1984).

Compound 3 showed $[M]^+$ ion peak at m/z 284 and analyzed for the formula $C_{16}H_{12}O_5$. Its IR spectrum shows band at 3430 cm^{-1}, (OH), 1682, 1626 (carbonyl). The NMR spectrum also shows characteristic peaks for a para OH and methoxy group. The compound may be dihydroxy monomehtoxy monomethyl anthraquinone, rest of the peaks were confirmed through literature.

Compound 4 displayed $[M]^+$ speak at m/z 300, consistent with the formula $C_{16}H_{12}O_6$. A band at 3430 (OH), 1602cm^{-1} appears in the IR spectrum of the compound. The ^1H NMR spectrum showed signals for perihydroxyls, β-OH, a methoxy and a methyl group indicating it could be a trihydroxy monomehtoxy monomethyl anthraquinone. The other peaks were confirmed through literature and the compound was

Table 1 : Antifungal Assay (compound 3).

S.No.	Name of Test Fungi	Conc. of Chemical 1 (%)	No. of fungal colonies appeared (after 3 day incubation) Mean ±SEM	Diameter of Fungal Colony (after 5 day incubation) Mean ±SEM (cm)	No. of spores / ml (after 5 day incubation) Mean ±SEM
1.	*Aspergillus* sp	0.001	18 ± 1.1	4.9 ± 0.05	3.6×10^2
		0.01	11 ± 0.6	4.3 ± 0.28	0.22×10^2
		0.1	06 ± 0.7	3.2 ± 0.02	Sterile
		Control	25 ± 0.99	7.8 ± 0.06	2.2×10^4
		Placebo	25 ± 1.15	7.8 ± 0.05	2.2×10^4
2.	*Alternaria alternata*	0.001	12 ± 0.9	4.1 ± 0.03	0.6×10^2
		0.01	09 ± 0.5	3.2 ± 0.04	Sterile
		0.1	04 ± 0.5	2.6 ± 0.04	Sterile
		Control	21 ± 1.15	7.6 ± 0.03	2.2×10^2
		Placebo	20 ± 1.1	7.6 ± 0.02	2.2×10^2
3.	*Penicillum* sp.	0.001	12 ± 0.57	5.4 ± 0.06	3.6×10^2
		0.01	05 ± 0.6	4.3 ± 0.05	0.2×10^4
		0.1	02 ± 0.5	2.6 ± 0.07	Sterile
		Control	26 ± 1.15	7.8 ± 0.06	2.2×10^4
		Placebo	28 ± 1.1	7.8 ± 0.04	2.3×10^4

Data presented is average of three replicates

Table 2 : Antifungal Assay (Compound 4).

S.No.	Name of Test Fungi	Conc. of Chemical 1 (%)	No of fungal colonies appeared (after 3 day incubation) Mean ±SEM	Diameter of Fungal Colony (after 5 day incubation) (cm) Mean ±SEM	No. of spores / ml (after 5 day incubation)
1.	*Aspergillus* sp	0.001	12 ± 0.7	5.3 ± 0.02	4.2×10^2
		0.01	07 ± 0.5	4.6 ± 0.04	0.3×10^2
		0.1	02 ± 0.5	3.2 ± 0.06	Sterile
		Control	25 ± 0.99	7.8 ± 0.08	2.2×10^4
		Placebo	25 ± 1.17	7.8 ± 0.05	2.2×10^4
2.	*Alternaria alternata*	0.001	06 ± 0.57	4.3 ± 0.04	0.6×10^2
		0.01	04 ± 0.5	3.8 ± 0.04	Sterile
		0.1	03 ± 0.5	2.5 ± 0.03	Sterile
		Control	21 ± 0.6	7.6 ± 0.08	2.2×10^2
		Placebo	20 ± 0.5	7.6 ± 0.15	2.2×10^2
3.	*Penicillum* sp.	0.001	11 ± 0.99	5.8 ± 0.07	2.8×10^2
		0.01	07 ± 0.5	4.5 ± 0.05	0.2×10^4
		0.1	02 ± 0.57	2.9 ± 0.04	Sterile
		Control	26 ± 0.5	7.8 ± 0.03	2.2×10^4
		Placebo	28 ± 0.9	7.8 ± 0.06	2.3×10^4

Data presented is average of three replicates

Table 3 : Antifungal Assay (Compound 5).

S.No.	Name of Test Fungi	Conc. of Chemical 1 (%)	No of fungal colonies appeared (after 3 day incubation) Mean ±SEM	Diameter of Fungal Colony (after 5 day incubation) (cm) Mean ±SEM	No. of spores / ml (after 5 day incubation)
1.	*Aspergillus* sp	0.001	15 ± 0.57	5.1 ± 0.07	3.9×10^2
		0.01	07 ± 0.9	4.5 ± 0.03	0.2×10^2
		0.1	03 ± 0.5	3.6 ± 0.08	Sterile
		Control	25 ± 1.15	7.8 ± 0.05	2.2×10^4
		Placebo	25 ± 0.99	7.8 ± 0.08	2.2×10^4
2.	Alternaria alternata	0.001	20 ± 0.9	4.4 ± 0.09	0.2×10^2
		0.01	09 ± 0.5	3.6 ± 0.02	Sterile
		0.1	05 ± 0.9	2.8 ± 0.05	Sterile
		Control	21 ± 0.99	7.6 ± 0.03	2.2×10^2
		Placebo	20 ± 0.9	7.6 ± 0.07	2.2×10^2
3.	*Penicillum* sp.	0.001	19 ± 0.57	5.5 ± 0.04	3.2×10^2
		0.01	13 ± 0.9	4.8 ± 0.06	0.25×10^4
		0.1	06 ± 0.57	3.2 ± 0.06	Sterile
		Control	26 ± 1.3	7.8 ± 0.07	2.2×10^4
		Placebo	28 ± 1.15	7.8 ± 0.04	2.3×10^4

Data presented is average of three replicates

identified as 1, 3, 5 - trihydroxy - 4 - methoxy - 2 - methyl Anthraquinone which was earlier reported from *Ventilago calculate* (Caron and Brassard 1993) and also synthesized.

Compound 5 also showed $[M]^+$ peak at m/z 300, consistent with molecular formula $C_{16}H_{12}O_6$. It was assumed that this compound may be an isomer of 4. Form the available literature, the compound has been identified as 1, 3, 4-trihydroxy-5 (or 8)-methoxy-2- methyl anthraquinone.

It is found that in case of test organism *Aspergillus,* compound 04 shows test effective where in 0.1% concentration shows 02 colonies, in compound 3 it is 06 and compounds 5, it is 03. In 0.001% concentration it is 12 in compound 4, 18 in compound 3 and 15 in compound 6. In case of diameter of colony in compound 3 lowest concentration shows 3.2cm, while in highest concentration it is 4.9cm; whereas compound 4 & 5 it is 5.3cm & 3.2cm and 5.1cm & 3.6cm respectively. In case of number of spores/mL is concerned compound 3 shows number 3.6×10^2, whereas compound 4 & 5 shows 4.2×10^2 and 3.9×10^2 respectively. In all the cases in highest concentration only are found sterile hyphal. In case of test organism *Alternaria altarnata*, number of colonies found in different concentration against three compounds as follows. In compound 3 04 & 12 nos; compounds 4, 03 & 06 in compound 3 it is 5 & 20. In case of diameter of colony, compound 3 shows 2.6cm & 4.1cm; whereas 2.5cm & 4.3cm and 2.8cm & 4.4cm shows respectively by compound 4 & compound 5. In case of third perimeter spores/mL compound 3 shows 0.6×10^2 whereas compound 4 & 5 0.6×10^2 & 0.2×10^2 respectively. In all these cases highest hyphal. In test organism *Penicillium* again number of colonies shows in lowest concentration are 04, 03 & 05 respectively in compound 3,4 & 5. In highest concentration it is 12, 11 & 19 respectively. In case of diameter of colony compound 3 shows 2.6cm & 5.4cm whereas 2.9cm & 5.8cm and 3.2 cm & 5.5cm shows by compound 4 & compound 5 respectively. Regarding spores/ mL compound 3 shows 3.6×10^2 whereas compound 4 & compound 5 shows 2.8×10^2 and 3.2×10^2 respectively. In all the cases highest concentration shows only sterile hyphal. Observing all the above comparison we can comments that compound 4 *i.e.,* 1, 3, 5-trihydroxy-4-methoxy-2-methyl anthraquinone more effective as antimicrobial compound isolated from *H. auriculata*.

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