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Antimicrobial and anthelmintic activity of *Eulophia herbacea* Lindl. tubers (Family: Orchidaceae)

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Abstract

Traditionally *Eulophia herbacea* Lindl (Orchidaceae) tubers are used in treatment of tumors of scrofulous gland of neck, worms and rheumatism. This study aimed to evaluate antimicrobial and anthelmintic activity of Eulophia extracts. Pet. ether, chloroform, methanol and aqueous extracts of *Eulophia herbacea* Lindl were tested for antimicrobial activity by agar-well diffusion and broth micro dilution method. Gentamycin (for bacteria) and Ketoconazole (for fungi) were used as standard. The anthelmintic activity of the extracts was determined by using earthworm (*Pheretima posthuma*).The antimicrobial activities of the crude extracts were increased with increasing the concentration. Methanol extract was the most effective among all and Antimicrobial, antifungal results of plant extract are compared with standard antibiotic and showed moderate effects. The methanolic extract showed most potent anthelmintic activity and it was superior to standard drug.The result indicates the potential usefulness of tubers of *Eulophia herbacea* Lindl in treatment of helmintiasis and microbial infections, however this claim demands further isolation of active components responsible for significant activity.

1. INTRODUCTION

Plants have been a major source for new drug development [1-6] .Infectious diseases represent a serious public health problem and they remain the leading cause of death throughout the world[7-10]. Currently, the problems of microbial drug resistance and the toxicity effect of several antimicrobial drugs is the greatest challenge to the effective treatment of infections globally [11-13]. Similarly helminthiasis is among the most pervasive infection and a foremost degenerative disease distressing a large proportion of world's population. In developing countries, they pose a large threat to public health and significantly contribute to the prevalence of anemia, eosinophilia, malnutrition, and pneumonia [14]. However, development of resistance in helminthes against conventional anthelmintic is a foremost problem in treatment of helminthes diseases [15-18]. Hence necessitated a search for new drugs from natural source which are provide novel antimicrobial and anthelmintic agents. Furthermore plants have been used as major source of drugs [19, 20].

Eulophia herbacea Lindl (Fam.: Orchidaceae) is commonly known as Kukad-kand [21].It is a terrestrial herbs with fleshy subglobose tuber ,Stem sheathed, leaves linear-lanceolate or elliptic-lanceolate, glabrous, multi-nerved, plicate, 12-30cm x 2.5-8.5cm. Flowers are white, purple-nerved, in racemes.

No extensive work has been recorded previously on this plant. The extract of this plant is used as salep. It has been reported to contain mucilage, starch, aluminous bodies. Traditionally tubers are used in treatment of tumors of scrofulous gland of neck, treat on worms and rheumatism. Other species of Eulophia found to contain few known phenanthrene and a mixture of phytosterols [22].

2. MATERIALS AND METHODS

2.1 Plant material

The root tubers of *Eulophia herbacea* Lindl were collected from hilly area of Toranmal region, Maharashtra, in the month of August 2014. The collection region is subtropical hilly evergreen forest with heavy rainfall and was taxonomically identified by Dr. D.A.Patil (Taxonomist Department of Botany, S.S.V.P.S College of science, Dhule, Maharashtra, India.)

2.2 Preparation, Storage and extraction of plant material

Plant material was washed with the water, followed by 95 % ethanol to prevent microbial contamination and deterioration during drying and storage. Dried tubers were powered by using pulverizes and passed through sieve no 40 and stored in airtight container, protected from light for further use.200 g of dried tubers of *Eulophia herbacea* was successively extracted with different solvents namely pet ether ,chloroform, methanol and aqueous. Finally all the extracts were filtered & concentrated separately under reduced pressure in rotary vacuum evaporator. The dried extracts are then collected and preserved in desiccators.

2.3 Preliminary phytochemical screening

Preliminary phytochemical tests for the presence of alkaloids, saponins, phytosterols, phenolic compounds, flavanoids,tannins, carbohydrates, terpenoids, oils and fats was carried out by the standard protocol [23-25] .

2.4 GC-MS analysis

GC-MS analysis was done by Auto system XL GC+ Perkin Elmer instrument and having NIST Mass spectra library. Instrumental conditions maintain during analysis are, Analyzer Quadruple with prefilter, Mass Range 50-650 amu, Ionization Modes EI 250° C , Positive / Negative Chemical Ionization, Vacuum Pump was Turbo molecular pump 250L/Sec,. Turbo Mass software, Injector temp 250° C, Carrier gas Helium at a flow rate of 0.9 mL min−1, Injection volume 0.5 µL. Components of extract were identified by comparison of their mass spectra and retention indices with those published in the NIST '98 MS computer library.

2.5 Antimicrobial Screening

2.5.1 Samples preparation

Different concentration of extracts (pet. ether, chloroform, methanol and aqueous) were prepared in dimethyl sulfoxide (DMSO) such as 1000-2000 µg/ml .Extracts were then sterilized by filtration through 0.2 µm pore sterile filter syringe and stored as aliquots until it was used[26].For bacteria Gentamycin solution (Genticyn ampoules, 60 mg in 1.5ml manufactured by Nicholas) and For fungi Ketoconazole as standard (Nizral tablet, 200 mg, Johnson) was used. Concentration of standard solutions (gentamycin, ketoconazole) 20µg/ ml was prepared in dimethyl sulfoxide (DMSO).

2.5.2 Microbial strains

The antimicrobial activity were assessed with four bacterial strains- *Bacilus subtilis* NCIM 2250, *Staphylococcus aureus* NCIM 2079 (gram +ve), *Escherichia coli* NCIM 2109, *Pseudomonas aeruginosa* NCIM 2036 (gram-ve) and two fungal cultures, *Aspergillus niger* NCIM 545, *Candida albicans* NCIM 3471 were used for experiment. Culture of both gram positive and gram negative bacterial organism and fungal strains were selected as per Indian pharmacopoeia antibiotic assay.

2.6 Antimicrobial assay

2.6.1 Disc diffusion method

Agar well diffusion assay was used for the determination of zone of inhibition [27, 28]. About 20 ml of sterile Muller Hinton agar medium (Hi-media) for bacteria and Potato dextrose agar (Hi-media) for fungi was poured and allowed to set in empty sterile Petri plates. About 0.1ml of fungal inoculums and bacterial inoculums in respective media were made in Petri plates. The well of 6mm diameters were bored on the agar media using sterile borer and each well was filled with 0.5 ml of plant extracts. The plates containing bacteria were incubated at 37^{o}C for 24 hours and those containing fungi were incubated at 27^{o}C for 48 hours. The positive antimicrobial activity was read by measuring zone of inhibition (in mm) after incubation. All the tests were performed in triplicates.

2.6.2 Minimum inhibitory concentration (MIC) by Micro dilution method

Minimum inhibitory concentration (MIC) was measured by using lowest concentration of extract and standard antibiotics needed to inhibit visible growth of test organisms [29]. This was done by using 96 well micro titer plates. Broth with extract serves as negative control. 150 µl of double strength broth was poured in micro titer plate wells. To the first well 100 µl of the extracts having conc. of 1000 µg/ml was added. Then serial two fold dilution of extract was done up to 31.25µg/ml by transferring 150 µl of first well to the second well and so on. Similarly two fold serial dilutions of standard drugs Gentamycin and Ketoconazole were prepared from 20 - 0.625 µg/ml. Then, to each well 50 µl of bacterial (106 cfu/ml)/fungal (5×105 spores/ ml) suspension was added. Micro titer plates were incubated at 37 0C for 24 hr for bacteria and 27 ^{o} C for 48 hours for fungi. After incubation, wells were observed for visible growth. Each sample was tested in triplicate and the observation was recorded.

2.7 Anthelmintic activity

The anthelmintic activity of the extracts of tubers of *Eulophia herbacea* was determined by using the method of Patra et al 2008 with slight modification. Earthworms (*Pheretima posthuma*) of about 5-7 cm long were used due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. Plant extracts in the conc. 10, 25 and 50 mg/ml were prepared in 1% Tween 80 in normal saline. 1% Tween 80 in normal saline used as control.20 ml suspension was taken into each petri dish. Time required for paralysis and death of animals were noted for each sample. Paralysis was noted when the worms became immobile even in the normal saline solution. Death was concluded when the worms lost their motility followed by fading away of their body colour. Albendazole (Zentel suspension, 400 mg in 10 ml, Glaxo smithkline) was used as standard [30].

2.8

3. RESULTS AND DISCUSSION

Preliminary phytochemical investigation of *Eulophia herbacea* Lindl extracts revealed the presence of alkaloids, saponins, phytosterols, phenolic compounds, flavanoids, tannins, carbohydrates, terpenoids, oils and fats. GCMS analysis was done and components of extract were identified by comparison of their mass spectra and retention indices with those published in the NIST '98 MS computer library.

GC-MS/MS spectrum of Pet. ether extract having peak at *Rt* 29.23**(Fig.1)** represent compound having m/e 414 and fragmentation pattern of this compound was perfectly matched with standard β-sitosterol So it contains phytosterol like β-sitosterol.

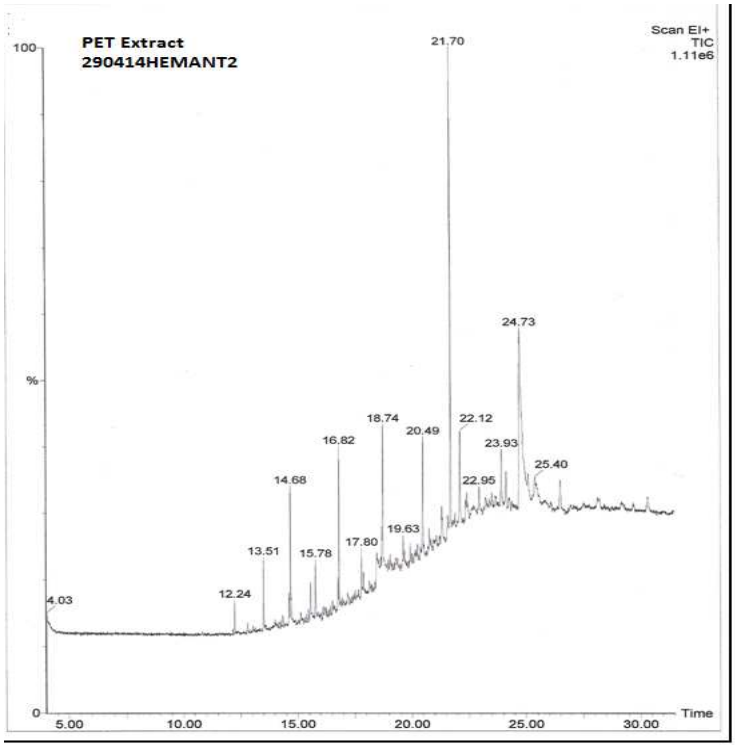

Fig.1. GC MS spectra of pet ether extract of *Eulophia herbacea*

Antimicrobial activity of *Eulophia herbacea* Lindl extracts is shown in **Table 1**. Generally, results showed that the various successive extracts had concentration dependent inhibitory activity against all tested bacteria and fungi. All extract shows maximum activity at concentration of 2000 µg/ml and methanol extract had better activity compare to other extracts and was comparable with standard drugs Gentamycin and Ketoconazole, while aqueous extract were least active.

Table 1: Zone of inhibition by *Eulophia herbacea* extracts

Sample	Conc. (µg/ml)	Diameter of zone of inhibition(mm)					
		Gram +ve bacteria		Gram -ve bacteria		Fungus strains	
		B. subtilis	***S. aureus***	***E. coli.***	***P. aeruginosa***	***C. albicans***	***A. niger***
Pet. ether extract	1000	11.0±0.9*	12.0±1.1*	9.0±0.7*	12.0±0.8*	13.0±1.2*	14.0±0.7*
	1500	15.0±1.4*	13.4±0.9*	11.8±1.1*	16.0±1.0*	15.8±0.9*	17.2±1.5**
	2000	20.0±0.7*	16.0±1.3*	15.0±0.9*	22.0±1.9**	19.0± 0.7*	20.3±1.6**
Chloroform	1000	9.0±0.7*	12.3±1.1*	10.0±0.6*	13.0±1.1*	9.2±0.6*	14.5±1.3*
	1500	15.2±1.5**	14.8±1.1*	14.0±1.1*	16.2±1.3*	13.0± 1.1*	17.8±1.6**
	2000	20.0±1.8**	17.0±1.6**	16.0±1.5**	21.0±1.9**	16 .0±1.5**	21.0±1.6**
Methanol extract	1000	12.5±0.8*	13.0±1.0	08.0±0.6*	15.0±1.3	11.0±0.8*	14.7±1.3
	1500	17.0±1.4**	15.4±1.1*	12.5±1.2*	18.1±1.6**	14.8 ±1.2*	19.2±1.8**
	2000	23.0±1.7**	18.2±1.6**	14.2±1.2*	23.0±2.1**	17. 5±1.6**	23.0±2.0**
Aqueous extract	1000	10.2±0.9*	10.0±0.4*	7.0±0.5*	13.2±0.8*	9.0±0.6*	15.0±1.4*
	1500	13.1±1.2*	11.8±0.9*	11.0±0.9*	17.0±1.5*	13.6±1.2*	18.4±1.5*
	2000	16.0±1.3*	14.0±1.3*	14.0±1.3*	20.0±1.8**	16.0± 1.3*	21.0±1.9**
Gentamycin	20	26.0±2.2**	25.0±2.3**	27.0±2.1**	28. 0±2.4**	-	-
Ketoconazole	20	-	-	-	-	24.0±2.1**	28.0±2.6**

*Values are expressed as mean±SEM (Standard Error Mean) * indicates P < 0.001, one-way ANOVA followed by Dunnet's test as compared to control.*

Pet ether extract had better activity against *E.coli* (15± 0.9mm) and *C.albicans* (19.0±0.7mm) than methanol extract. MIC value of all extracts for tested bacteria's were 500 µg/ml and for fungi 250 µg/ml **(Table 2**).

Table 2: Minimum inhibitory Concentration (MIC) of different extracts of *Eulophia herbacea*

Microorganism Bacteria/fungus	Pet. ether Ext.	Chloroform Ext.	Methanol Ext.	Aqueous Ext.	Gentamycin	Ketokonazole
B.subtilis	500	500	500	500	1.25	-
S.aureus	500	500	500	500	1.25	-
E.coli	500	500	500	500	0.62	-
P.aeruginosa	500	500	500	500	0.62	-
C.albicans	250	250	250	250	-	2.5
A.niger	250	250	250	250	-	1.25

All data expressed as (µg/ml); Results are in triplicate data

Anthelmintic activity of *Eulophia herbacea* Lindl extracts is shown in **Table 3.** It was found that different extract show concentration dependent anthelmintic activity. Methanol extract show maximum anthelmintic activity while aqueous extract had least activity. Methanol, pet. Ether and chloroform extracts at concentration of 50mg/ml showed significant level of anthelmintic activity while aqueous extract (50mg/ml) showed activity comparable with standard drug albendazole.

Table 3: Anthelmintic activity of *Eulophia herbacea* extract

Extract/Standard	Concentration (mg/ml)	Time taken for Paralysis (P) and for death(D) of *Pheretima posthuma* worms in min	
		P	D
Pet. ether Ext.	10	57±0.51*	99±0.51*
	25	36±0.81*	86±2.30*
	50	25±0.93*	51±0.58*
Chloroform Ext.	10	72±0.71*	103±0.71*
	25	38±0.58*	70±0.68*
	50	15±0.58*	28±0.37*
Methanol Ext.	10	55±0.73*	98±0.75*
	25	26±0.51*	52±0.73*
	50	12±0.55*	24±0.66*
Aqueous Ext.	10	98±0.51*	169±0.93*
	25	62±0.66*	111±0.71*
	50	34±0.89*	57±1.2*
Albendazole	10	31±0.58*	62±0.60*

*Values are expressed as mean±SEM (Standard Error Mean) * indicates P < 0.001, one-way ANOVA followed by Dunnet's test as compared to control.*

Now a day's traditional plants are the main sources for isolation of potent drugs. Interest in this area continues and many new potent drugs have been isolated. Many medicinal plant extracts have been known to possess antimicrobial and anthelmentic activity. The extracts of *Eulophia herbacea* Lindl shows promising anthelmintic and moderate antibacterial, antifungal activity. The methanolic extract showed most potent anthelmintic activity, chloroform and pet ether extract exhibit moderate while aqueous extract showed least activity. Results demonstrated that both paralysis and death of worms at higher concentration of 50 mg/ml as compared to reference drug albendazole. Higher concentration of each crude extract produced paralysis much earlier and the time to death was shorter.

The present study indicated that *Eulophia herbacea* tubers possess a good anthelmintic and moderate antimicrobial activity of *Eulophia herbacea* lindl extract hence further fractionation and identification of bioactive compound is required.

4. CONCLUSION

Results obtained from this study found that the various successive extracts had concentration dependent inhibitory activity against all tested bacteria and fungi. All extract had maximum activity at concentration of 2000 µg/ml and methanol extract had better activity compare to other extracts and was comparable with standard drugs Gentamycin and Ketoconazole, while aqueous extract were least active. Similarly methanol extract show maximum anthelmintic activity while aqueous extract had least activity. It is concluded that plant *Eulophia herbacea* Lindl is safe and alternative remedy for treatment of bacterial, fungal infections and helminthiasis.

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