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Antifungal metabolites of *bulbophyllum nilegiriensis* wt. from western ghats of karnataka, India

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ABSTRACT

Bulbophyllum nilegiriensis Wt., an orchid of ethno medical significance, from Western Ghats of Karnataka was studied for antifungal and phytochemical properties. The phytochemical constituent analysis showed different phytoconstituents in different extracts. Petroleum ether, alcohol and chloroform extract showed alkaloid, steroid and terpinoids. Flavonoids were present in chloroform and alcohol extracts. Tannins were observed in petroleum ether and chloroform extract. Tannins and Flavonoids were present in chloroform extract and absent in petroleum ether extracts. In vitro antifungal efficacy of all the four extracts was tested by well in agar method. Both yeasts and filamentous fungi were inhibited with zone of inhibition upto 26 mm. Candida albicans was significantly inhibited in all four tested extracts followed by Candida kruseii and Fussarium oxysporium. Saccharomyces cerevisiae was unaffected by all the extracts. A zone of 20mm was observed for Cryptococcus neoformens only in water extracts. The purity of the chloroform extract was assessed by Column Chromatography, HPLC and UV absorption studies with a single peak and absorption of 257nm.

KEYWORDS

Orchid;
Antifungal;
Bulbophyllum nilegiriensis Wt.,
well in agar;
Western Ghats.

INTRODUCTION

India possesses rich floristic wealth and diversified genetic recourses of medicinal plants. Orchidaceae members constitute the vital herbal medicines of India and China. Orchidaceae is known for its beautiful flowers. *Bulbophyllum nilegiriensis* Wt. (Orchidaceae) is a fleshy native Indian epiphyte reputed to cure stomach ache in traditional Indian medicine. It is a robust epiphytic or lithophytic herb with slender, creeping rhizome. Pseudobulb is conical-ovoid, yellowish green, fleshy with terminal leaf. (Rao, 1998).

The genus *Bulbophyllum*, belonging to the Orchidaceae consists of about 1000 species found in Asia, America and Africa. It contains mainly phenanthrenes and bibenzyls. *B. odoratissimum* (J.E. Smith) Lind is widely distributed in China, Nepal, Bhutan, India, Burma, Thailand, Laos and Vietnam it is used in folk medicine to treat tuberculosis, chronic inflammation and investigation on the compounds from *B. odoratissimum* have revealed the presence of phenanthrone, lignin, flavonoids, bibenzyls, phenolic glycosides, aldehydes and acids (Chen *et al*, 2007). *Bulbophyllum nilegiriensis*.Wt is distributed in

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Shimoga, South Canara and Kodagu districts of Karanataka and also in northern districts of Tamilnadu and Kerala.

The present study concentrated on the antifungal metabolites of *Bulbophyllum nilegiriensis* Wt, and its pharmacological constituents as little work has been done on these parameters. Plant products are also a source of very potent and powerful drugs that have stood the test of times and modern chemistry has not been able to replace most of them. Plant based drugs constitute a major share of all the officially recognized systems of health in India, China viz., Ayurveda, yoga, unani, siddha, homeopathy and naturopathy, except allopathy[1]. In many countries including India and china, thousands of tribal communities still use folk here medicinal plants for the cure of various diseases. It has been confirmed by WHO, that traditional medicines, based largely on different species of plants and animals serve the health needs of large number of people; especially for millions of people in the vast rural areas of developing countries[2]. Serving to the peoples all over the world, Phytochemicals of medicinal plants serve a lead compounds is drug discovery and design. Medicinal plants are rich source of novel drugs that forms the ingredients is traditional systems of medicine, modern medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs[5]. The orchids are one of the largest groups of Angisperms belonging to the family Orchidaceae. They occur in diverse habitat conditions of our country. There are about 20,000 species grouped about 800 genera distributed all over the world except polar region and dry deserts. India is one of the richest orchid habitats with about 2500 species in 167 genera represented in six sub families, 17 tribes and 30 sub tribes. Various phytogeographical situation accompanied by the variation in elevation, temperature, rain fall and humidity have contributed to the rich diversity of orchids in India. The orchids have been described as the 'Royal family' and contain their own specialized characters such as intriguing flowers, exciting colours, varied shapes and great diversity of growth habitats would certainly agree that they are rather special plants[6].

MATERIALS AND METHODS

Preparation and extraction of plant material

The whole plant, except roots, was collected from forest trees of Shimoga and South Canara districts of Karnataka in March-April 2008. The plant was identified by at the Department of Botany, Sahyadri Science College, Shimoga by referring literature. A fresh specimen weighing 750 g was surface sterilized in 70% ethanol with two drops of surfactant, tween 20 for 1 minute and washed with sterile distilled water for 2-3 times. The air dried specimen was then cut into 5 mm pieces aseptically and shade dried aseptically for 30 days in laboratory. The dried plant material was powdered with help of blender.

250 g powder was subjected to soxhlet extraction using Petroleum Ether, Chloroform, Ethyl Alcohol and Water as solvent, the crude extract was obtained by removing the solvent using flash rotary evaporator, similarly water extracts were prepared by hot water extraction. The dried crude extracts were stored in dry air tight glass vials.

Preliminary phytochemicals screening

Phytochemical tests were carried out for the solvent extracts. Petroleum ether, chloro form. Ethanol and ageous extracts were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents. Standard procedures were followed to identify the described by sofowara (1993), Harborne (1973) and Brindha *et al*., (1982)[3].

1. **Test for Terpenoids** 5 ml of the extract was mixed with 2 ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown coloration in the interface showed the presence of terpenoids.
2 **Test for flavonoids** 5 ml of the diluted ammonia solution a portion of the aqueous extract was added, followed by addition of concentrated suphuric acid. Appearance of yellow coloration indicates the presence of flavonoids.
3. **Test for Reducing Sugars** 2 ml of test solution was added with a 2 ml Fehling's reagent. A (or) B. and 2 ml of water formation of reddish orange colour indicates the presence of reducing sugar.
4. **Test for Phenols** 2 ml of test solution in alcohol was added with one drop of neutral ferric chloride 5% solution. Formation of intense blue colour indicates the presence of phenols.

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5. **Test for Catechins** 2 ml of test solution in alcohol was added with Ehrich reagent and a few drops of concentrated HCl formation of pink colour indicate the presence of catechins.
6. **Test for saponins** 2 ml of test solution was added with H2O and shacked formation of foamy eather indicates the presence of saponins.
7. **Test for Tannins** ml of test solution was added with H2O and head acetate. Formation of while precipitate indicates the presence of tannins.
8. **Test for Anthroquinone** 2 ml of test solution was added with magnesium acetate. Formation of pink colour indicates the presence of Anthroquinones.
9. **Test for Quinine** 1 ml of extract, 1 ml of concentrated sulphuric acid was added and was allowed to and for some time to develop colour. Development of red colour shows the presence of quinine.
10. **Test for Coumarin** 1 ml of extract, 1 ml of 10% NaOH was added and was allowed to stand for some time development of yellow colour shows the presence of coumarin.
11. **Test for Glycosides** 1 ml of the extract, 1 ml of alpha napthol was added to which chloroform was added along the sides and it was looked for the development of colour and the result was recorded. Development of Violet colour indicates the presence of glycosides.
12. **Test for Carbohydrate** Aqueous or alcoholic solution of substance was added with 10% aqueous solution of alpha Napthol shaken and added concentrates sulphuric acid along the side of the side of the tube. Violet ring at the Junction of two liquids shows presence of Carbohydrates.
13. **Test for Sugar** 0.5 ml of the Filtrate. 0.5 ml Benedict's reagent was added. The mixture was heated on boiling water both for 2 minutes. A characterises of red coloured precipitate shows presence of sugar.

In-vitro Antifungal Assay

Sample preparation

10% of extracts was prepared by dissolving all four extracts separately in DMSO, which was used for the antibacterial activity.

Antifungal Activity

The extracts were tested for the antifungal activity. The fungal strains employed in the biological assays were

Test Organisms

Yeasts – *Saccharomyces cervesiae* – NCIM 3095, *Candida albicans* – NCIM 3100, *Cryptococcus neoformans* -– NCIM 3541, *Candida kruseii* – NCIM 3129, *Candida lipolytica* -– NCIM 3472.

Filamentous fungi - *Aspergillus niger* – NCIM 572, *Aspergillus pumilis* – NCIM 2108, *Aspergillus wentii* – NCIM 651, *phyto* pathogens *Fusarium* species, *Curvularia* species and skin pathogens *Trichophyton cutaneum* – NCIM 3326, *The* cultures were procured from NCIM, Pune.

Determination of antifungal activity

Agar well diffusion method was followed but nutrient medium used was Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA). The Agar plates were swabbed (Sterile Cotton Swabs) with 72 h old broth culture of the respective fungi. A sterile cork borer was used to place four wells, each measuring 8 mm in diameter, in each of the plates, about 0.1 ml each of 50, 25, 12.5 and 6.25 mg/ml of concentrated test samples with DMSO was added into the wells using sterilized dropping micro pipettes and allowed for diffusion at room temperature for 2h. The plates were incubated at 28æ%C for 48-72 h. Diameter of the inhibition zones was recorded. The experiment was repeated thrice and the average values were calculated for antifungal activity. Similarly standard antibiotics antifungal assay was done.

RESULTS

Preliminary phytochemical analysis of various solvent extracts such as ethanol and aqueous extracts are recorded. Petroleum ether extract contain alkaloid and steroid. Chloroform extract contain alkaloid, steroid, flavonid and triterpinoid. Alkaloid, steroid, tannin and triterpinoid were present in ethanol extract. Where as aqueous extract contain only alkloids. Except chloroform extract flavonoid was absent in rest of extracts.

These solvent extracts are subjected to antifungal assay, the inhibition zone is tabulated (TABLE 1), the value ranging from 10-26mm. Aqueous extract inhibits all fungal organism tested except *Saccharomyces*

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TABLE 1 : Antifungal activity of *Bulbophyllum nilegiriensis* Wt.

Sl.No.	Test Organisms	Zone of Inhibition in mm				
		Pet.Ether	Chloroform	Ethanol	Water	Control (Solvent)
	Saccharomyces cerevisiae	--	--	--	--	00
	Curvularia Sp	14	14	15	12	00
	Trichophyton cutanium	11	14	16	10	00
	Aspergillus niger	13	14	10	14	00
	Aspergillus pumilus	11	12	--	14	00
	Fussarium oxysporium	14	21	16	14	00
	Candida lipolytica	16	15 19(Less growth)	14	16	00
	Candida albicans	24	23	26	25	00
	Candida kruseii	20	19	19	20	00
	Cryptococcus neoformens	--	--	--	20	00

cerevisiae, which showed resistance towards all four extract tested. *Cryptococcus neoformens* was inhibited only by aqueous extract, other three extracts showed no inhibition zone. Petroleum ether extract and chloroform extract showed similar inhibition efficacy (11-24mm), except *Saccharomysis cerevisiae* and *Cryptococcus neoformens*. Ethanol extract has showed high degree of inhibition except *Saccharomyces cerevisiae, Cryptococcus neoformens* and *Aspergillus pumilis*. Inhibition zone ranging from14-26mm. *Candida* species were inhibited by all four extracts to maximum extent 23-26mm. The ethanol extract showed 26mm inhibition towards *Candida albicans*. The filamentous fungi also showed considerable degree of inhibition 11-21mm. *Curvularia*, *Trichophyton cutanium*, *Aspergillus niger* were inhibited by all four extract tested. *Aspergillus pumilis* did not showed inhibition towards ethanol extract, *Fusarium oxysporium* was inhibited by all four extract tested, chloroform extract showed maximum inhibition of 21mm. The standard antifungal antibiotics 10mg/ml concentration moderately inhibited the test isolates. When compared with all the four solvent extracts (TABLE 2).

TABLE 2 : Antifungal Activity of Standard Antibiotics

Test organism	Antifungal	
	Griseofulvin	Flucanazole
C.kruseii	0	10
C.albicans	0	0
S.cervesiae	12	14
C.lipolytica	0	25
C.neoformens	0	28
A.niger	17	0
A.pumilis	16	0
Fusarium	15	12
Curvularia	16	0
T.cutaneum	23	0

DISCUSSION

Kalaiarasan A *et al* (2012)[10] enumerated antimicrobial activity of *Bulbophyllu kaitense* and performed antibacterial and antifungal assay. The present investigation showed the promising results of *Bulbophyllum neilghirensis* Wt. antifungal property. The extract showed inhibitory effect on human pathogenic fungi like *Candida albicans,Trichophyton cutanium* (11-26mm). These extracts are also effective against phytopathogenic fungi like *Curvularia* Sp and *Fussarium oxysporium* (14-21mm). Similar study was conducted on *Acanthephippium bicolor* by Shanmugavalli *et al* (2009)[7]. Since plant grow in abundance and they are widely distributed. This plant can be used in organic fungicide preparation. The present work emphasized on antifungal potential of Bulbophyllum *neilgherrense* Wt in solvent extracts, where the pseudobulbs were collected in summer and winter and assessed for their antifungal potential. Antifungal activity of pseudobulb remained same, even though collected in different seasons, confirmed the same results.

Kala S and Senthilkumar.S(2010)[8] studied antibacterial activity of *Acanthephippium bicolor*, Lindley,

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tested 35 bacteria and found out that, seasonal change does not influence on the antimicrobial property of plant.

Priya K and Krishnaveni C (2005)[9] tested different solvent extracts of *Bulbophyllum neilgherrense* Wt against bacterial isolates, where it had shown moderate activity, while the present investigation clearly showed presence of best antifungal principles, from same species of orchid. Since the inhibited fungi includes human pathogenic yeasts, dermatophytes and plant pathoges, the extraction of active fraction from both ethanol and aqueous medium may provide a novel antifungal agent from this orchid. This can be exploited in scientific medicine.

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