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RESEARCH ARTICLE

Antifungal and Cytotoxic activities of medicinal important orchid *Rhynchostylis retusa* Blume

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Abstract

Medicinal plants are distributed throughout the world and widely used in everyday life as part of folk medicinal remedies. *Rhynchostylis retusa* is a medicinal orchid, used to cure blood dysentery, Tuberculosis, epilepsy, menstrual disorders, fewer, gout, Asthma, rheumatism, Malarial fewer etc. The purpose of this study was to determine cytotoxic activity and antifungal activity of medicinal orchid *Rhynchostylis retusa*. Hexane, Chloroform and methanol extracts of this orchid were used to determine antifungal activity against six phytopathogens. Among these three extracts, chloroform and methonolic extracts shows highest zone of inhibition than the hexane extract. Various solvent extracts of *Rhynchostylis retusa* was screened by the brine shrimp lethality assay and found Methanol extract showed significant toxicity to the brine shrimp and exhibited potent activity with LC_{50}. The present study will be successful to identify antifungal and cytotoxic activity which could be further exploited for isolation and characterization of the novel phytochemicals in the treatment of infectious diseases especially in light of the emergence of drug resistant microorganisms and the need to produce more effective antimicrobial agents. Present study could be useful in the search for new antitumor compounds from the Indian flora.

Introduction

India possesses rich floristic wealth and diversified genetic resources of medicinal plants. Approximately 20% of the world plants have been submitted to pharmacological or biological test, it could be concluded that Natural products from plant origin are important sources to discover new leads with economical and pharmaceutical importance and great possibilities to be developed drugs[1]. The use of plants and plant extracts and pure compounds isolated from natural sources provided the foundation of modern pharmaceutical compounds[2]. Several studies have been conducted in the past three decades that focused on the antimicrobial properties of herbs, spices and their derivatives such as essential oils, extracts and decotions[3]. There are several reports on the presence of antimicrobial compounds in various plants[4, 5,6,7,8]. It is an evident that the use of plants for various diseases since from Vedic period. Documentation of the Ayurvedic system recorded by Susruta and Charaka dates from about 1000 BC[9]. The commercial availability of inexpensive brine shrimp eggs, the low cost and easy of performing the assay make brine shrimp lethality assay, a very useful bench top method[10]. A number of studies have demonstrated the use of the brine shrimp assay to screen the cytotoxicity of plant extracts[11, 12, 13]. Lethality assay has been successfully to biomonitor the isolation of Cytotoxic[14], antimalarial[15], insectisidal[16] and antifeedent[17] compounds from plant extracts.

Rhynchostylis retusa is a monopodial epiphytic species that grows in the broad-leaved forests along the lower Himalayan ranges[18]. It is an epiphytic herbaceous orchid commonly seen in the forest of Western Ghats[19]. It can tolerate a wide range of temperature from 3°C - 31°C[20]. This plant leaf juice and Aerial roots were

also used in ear pain and cleaning[21,22]. It also used as emollient, throat inflammation etc[23]. In Bangladesh, kurigram District, peoples were used this plant leaves to cure Rheumatic pain[24]. *Rhynchostylis retusa* roots were used to cure malarial fewer[25]. The whole plant preparations were used to cure blood dysentery, Tuberculosis, epilepsy, menstrual disorders, fewer, gout, Asthma, rheumatism etc[26]. The purpose of this study was to investigate antifungal activity and cytotoxic properties of *Rhynchostylis retusa*. In this paper we report the results of such studies in order to orient future investigations towards the finding of new, potent and safe bioactive anticancer and antimicrobial compounds.

Materials and Methods:

The whole plant of *Rhynchostylis retusa* was collected from the forest of Chinthapalli. The specimen was identified with the help of regional floras[27, 28] and the voucher specimen was deposited at Andhra University Herbarium (AU), Visakhapatnam, Andhra Pradesh, India.

Preparation of plant extracts:

The collected leaves were shade dried, powered and extracted with hexane, chloroform and methanol using soxhlet apparatus for 8 hours. The extracts were filtered and filtrates were concentrated under reduced pressure at 40^{o} C using a rotaflash evaporator. Various concentrations of plant extracts (500, 250, 100 mg/ml) were dissolved in DMSO (Di methyl sulphoxide). The crude sample was subjected to antifungal screening against the phytopathogens and the brine shrimp lethality assay.

Collection of microorganisms:

The microbial strains Viz., *Macrophomina phaseolina(*MTCC 257) , *Sclerotium rolfsii*(MTCC 2465), *Fusarium oxysporium*(MTCC 2087), *Altarnaria altarnata*(MTCC 2723), *Rhizoctonia solani*(MTCC 4634), *Fusarium solani*(MTCC 350) were used and these organisms obtained from Microbial Type Culture Collection centre, Institution of Microbial Technology (IMTECH), Chandigarh, India.

Antifungal activity:-

Antifungal activity of extracts was determined by well diffusion method on Potato Dextrose Agar (PDA) medium. PDA medium[29] was prepared, sterilized and 0.2 ml of 24 hrs broth culture was mixed in the PDA agar medium and poured in petriplates. After solidifying wells (6mm diameter) are made in agar plates using cork borer[30, 31, 32]. Different concentrations (500, 250, 100 mg/ml) of different solvent extracts (hexane, chloroform and methanol) were poured in wells and incubated at 30^{0} C for 72 hrs. The antifungal activity was evaluated by measuring the zone of inhibition around the well. The experiment was done in triplicate and the mean diameter of the inhibition zone was calculated[33].

Brine Shrimp Lethality bioassay:

Brine Shrimp Lethality bioassay was carried out to investigate the cytotoxicity of extracts of medicinal plants of India. Brine shrimp (Artemia saline) were hatched using Brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial sea water (prepared using sea salt 38 g l^{-1} and adjusted to pH 8.5 using 1 N NaOH) under constant aeration for 48 h. after hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of plant extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 hr under the light and surviving larvae were counted. Experiments were conducted along with control[34,35]

Statistic analysis:

The percentage lethality was calculated from the Mean survival larvae of extracts treated tubes and controls. LC_{50} values were obtained by best-fit line method.

Table - 1. Antifungal activity of leaf extract of *Rhynchostylis retusa*

S.No.	Name of organism	Zone of inhibition(in mm)								
		Hexane extract mg/ml			Cholorofom extract mg/ml			Methanol extract mg/ml		
		500	250	100	500	250	100	500	250	100
1	*Macrophomina phaseolina*	12	10	10	14	13	13	10	9	9
2	*Sclerotium rolfsii*	11	10	9	14	13	12	7	7	7
3	*Fusarium oxysporium*	11	10	8	14	12	10	7	-	-
4	*Altarnaria altarnata*	-	-	-	8	7	-	8	8	7
5	*Rhizoctonia solani*	11	9	8	8	7	7	9	8	7
6	*Fusarium solani*	-	-	-	11	9	8	12	11	11

The maximum zone of inhibition was obtained for *Macrophomina phaseolina* at a concentration of 500 mg/ml (Fig 1). The chloroform extract was more effective on test pathogens showed significant inhibition zones ranged from 7-14 mm. Hexane extract doesn't show any antifungal activity against these test pathogens except *Macrophomina phaseolina, Sclerotium rolfsii, Fusarium oxysporium and Rhizoctonia solani*. *Rhynchostylis retusa* chloroform extract shows more effective on test organisms than the methanol and hexane extracts. The results of antifungal assay of three concentrations (100, 250,500 mg/ml) of different solvent plant extracts like chloroform and methanol exhibited most effective antifungal activity in Table - 1. *Rhynchostylis retusa* contains several phytochemical compounds, which are very much necessary to control the growth of microorganisms.

Fig 1. Bar chart showing results of antifungal activity against six phytopathogens of hexane, chloroform and methanol (500 mg/ml conc.) of Rhynchostylis retusa .

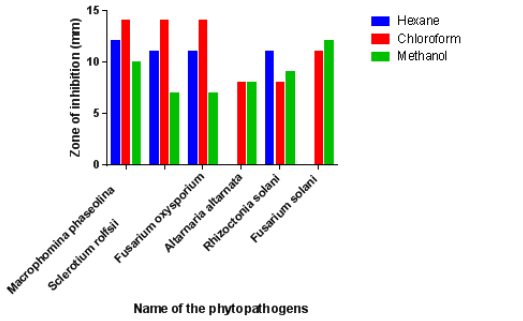
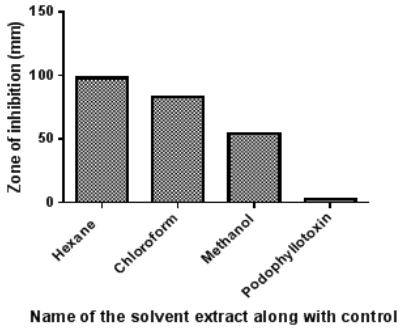


Table 2. LC_{50} values of different solvent extracts of *Rhynchostylis retusa*

S.No.	Name of the solvent extract	LC_{50}, 24 h
1	Hexane	86.41
2	Chloroform	30.50
3	Methanol	18.52
4	Podophyllotoxin	2.82

Fig 2. Differnt solvent extracts of Rhychostylis retusa LC_{50}



The degree of lethality was found to be directly proportional to the concentration of the extract. The LC_{50} values of the brine shrimp obtained for extracts of this plant and that of the positive control, Podophylotoxin, have been presented in Table 2 and Fig 2. In the evaluation for general toxicity using brine shrimp, maximum mortalities took place at a concentration of 1000 µg/ml where as: least mortality was at 10 µg/ml concentration and the lethality ($LC_{50)}$ $_{values}$ were 86.41, 30.50 and 18.52 µg/ml respectively (Table 2). The LC_{50} values of plant extracts (24 h) were obtained by a plot of percentage of the shrimps killed against the concentrations of the extracts and the best-fit line was obtained from the data by means of regression analysis. LC_{50} was obtained from the best-fit line method. This method allows the use of smaller quantity of the extracts and permits larger number of samples and dilutions within shorter time than using the original test vials[36]. Further clinical studies need to be carried out for isolation of potential chemicals, which can augment the cancer fighting ability of modern medicine.

Conclusion:

From the preliminary screening, we have identified that the Methanol extract out of 3 different solvent extracts screened for toxicity against brine shrimp had less LC_{50} values less than 100 µg ml^{-1} in interesting and lend supporting to the traditional use of this plant extract. Although the brine shrimp lethality assay is rather inadequate the elucidation of the mechanism of action, it is very useful to assess the bioactivity of the plant extract. The brine shrimp lethality assay results suggest that the plant can be a promising source of anticancer compound. Based on the possible relationship between brine shrimp lethality and plant bioactivity, this work could serve for further ehanobotanical and phytochemical research. From this study, suggesting a need to isolate and evaluate active constituents responsible for the exhibited biological activities.

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