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Preliminary Phytochemical Analysis & In Vitro Bioactivity against Clinical Pathogens of Medicinally important Orchid of *Rhynchostylis retusa* Blume.

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ABSTRACT

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 were to determine photochemical constitutes and antibacterial activity of medicinal orchid *Rhynchostylis retusa*. Hexane, Chloroform and methanol extracts of this orchid were used to determine antibacterial activity against ten clinical pathogenic bacteria. A quantitative photochemical analysis was performed for the detection of Alkaloids, tannins, Flavoniods, Flavones, Flavonones, Glycosides, Phenols, Saponins, coumarines etc. The present study will be successful to identify antibacterial 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. Among these three extracts, chloroform and methonolic extracts shows highest zone of inhibition than the hexane extracts.

Keywords: *Rhynchostylis retusa*, Agar Well Diffusion method, Chloroform extract, Antibacterial activity.

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INTRODUCTION

Medicinal plants are distributed throughout the world and widely used in everyday life as part of folk medicinal remedies. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plants in Hindu culture is founds in "Rigveda" which is said to have been written between 4500-1600 B.C. and is supposed to the oldest repository of human knowledge. In Ayurveda, the foundation of medicinal science of Hindu culture, in its eight division deals with specific properties of drugs and various aspects of science of life and the act of healing[1]. In recent times, there has been increased in antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains that are multi resistant[2]. The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality[3]. Consequently, this had led to the search for more effective antimicrobial agents among materials of plant origin, with the aim of discovery potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs[4]. Herbal medicine is still the mainstay of about 75-80% of the whole population, and the major part of traditional therapy involves the use of plant extract and their active constituents[5]. There is a growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity[6]. There are many reports on the presence of antimicrobial components in various plants[7,8].

Orchidaceae family is one of the largest, most diverse and most important categories of botanically and commercially significant flowering plants with 20,000-30,000 species[9]. Orchids are well known for their beauty and its medicinal use[10]. *Rhynchostylis retusa* is a monopodial epiphytic species that grows in the broad-leaved forests along the lower Himalayan ranges[11]. It Is an epiphytic herbaceous orchid commonly seen in the forest of Western Ghats[12]. It can tolerate a wide range of temperature from 3°C -31°C[13]. This plant leaf juice and Aerial roots were also used in ear pain and cleaning[14,15]. It also used as emollient, throat inflammation etc[16]. In Bangladesh, kurigram District, peoples were used this plant leaves to cure Rheumatic pain[17]. *Rhynchostylis retusa* roots were used to cure malarial fewer[18]. The whole plant preparations were used to cure blood dysentery, Tuberculosis, epilepsy, menstrual disorders, fewer, gout, Asthma, rheumatism etc[19]. The purpose of the study was to investigate Preliminary Phytochemical Analysis and antimicrobial 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 and antimicrobial compounds.

MATERIALS AND METHODS

The plant parts (leaves) and Whole plant of *Rhynchostylis retusa* was collected from the forest of Chinthapalli. The specimen was identified with the help of regional floras[20,21] and the vocher specimen was deposited at Andhra University Herbarium(AU), Viskhapatnam, Andhrapradesh, 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. The crude sample was subjected to antibacterial screening against the pathogenic bacteria. Various concentrations of plant extracts (500, 250, 100 mg/ml) were dissolved in DMSO (Di methyl sulphoxide).

Collection of microorganisms:

The microbial strains Viz., *E.coli* (isolated), *Proteus vulgaris* (isolated), *Xanthomonas* sps.(isolaed*), Pseudomonas mirabilis (*isolated*), Pseudomonas aerosinosa* (isolated), *Klebsella oxytoca* (isolated), *Staphylococcus aureus* (isolated), *Staphylococcus epidermidis* (isolated), *Staphylococcus mitis* (MTCC 2696) and *Staphylococcus anginosus* (MTCC 1929) were used and these organisms obtained from Visakha Eye Hospital(Isolated strains), Visahkapatnam, Andhrapradesh, India and the Microbial Type Culture Collection centre, Institution of Microbial Technology (IMTECH), Chandigarh, India.

Antibacterial Assay:-

Antibacterial activity of extracts was determined by well diffusion method on nutrient agar medium. Nutrient agar medium[22] was prepared, sterilized and 0.2 ml of 24 hrs broth culture was mixed in the nutrient agar medium and poured in petriplates. After solidifying wells (6mm diameter) are made in nutrient agar plates using cork borer[23,24,25]. Different concentrations (500, 250, 100 mg/ml) of different solvent extracts (Hexane, Chloroform and Methanol) were poured in wells and incubated at 37^{0} C for 24 hrs.

Phytochemical screening:

The plant extract was soaked in methanol solvent and incubated for 48 hrs and then filtrated using Whattmann No.1 filter paper to obtained methanol plant extraction. Phytochemical analysis was carried out using Methanol plant extract using standard methods[26-28].

Identification Tests for Phytochemical Constituents:-

The tests were performed to find out the presence of active chemical constituents such as alkaloids, terpenes, flavones, flavonoids, steroids, reducing sugars, proteins, aminoacids, carbohydrates, tannins, anthraquinones, glycosides, cardiac glycosides by the following procedure. Phytochemical analysis was carried out for all the extracts using standard methods.

Alkaloids:-

Extracts were dissolved individually in diluted hydrochloric acid. The resulting acidic solution was tested for alkaloids by adding Mayer's reagent, Wagner's reagent, Dragendroff reagent and Hager's reagent. The formation of a faint turbidity or precipitation on the addition of the above reagents indicates the presence of alkaloids.

Flavanoids:-

2-3ml of plant extract was dissolved in 50% of methanol and warmed then add a piece of magnesium ribbon and 1ml of concentrated Hcl. Red or yellow coloration of the solution indicates the presence of flavanoids.

Steroids:-

1ml of extract dissolved in 1ml of acetic anhydride, 1ml of chloroform and 1ml of Conc. Hcl separately. Formation of green colour indicates the presence of steroids.

Triterpenoids:-

To 1 ml of extract tin bit and thionyl chloride were added. Appearance pink colour indicates the presence triterpenoids.

Coumarins:-

To 1ml of plant extract, 1ml of 10%NaOH was added. The formation of yellow colour indicates the presence of coumarins.

Cardiac glycosides:-

To the plant extract few ml of glacial acetic acid, ferric chloride and conc.H_2SO_4 were added. Green colour indicates the presence of cardiac glycosides.

Terpenes:-

To the plant extract few ml of chloroform was added, filtered. To the filtrate few drops of acetic anhydride and H_2SO_4 were added. The colour changing from blue to green indicates the presence of terpenes.

Anthraquinones:-

Benzene extract was taken to this 5ml of 10% ammonia was added .Pink, red or violet colour indicates the presence of anthraquinones.

Phlobtannins:-

Plant extract was dissolved in distilled water. The filtrate was boiled with 2% HCl. Red precipitate indicates the presence of phlobtannins.

Quinones:-

To 1ml of extract 1ml of conc.H_2SO_4 were added, formation of red colour indicates the presence of quinones.

Flavanones:-

To few ml plant extract, 10% of few drops of NaOH was added yellow colour indicates the presence of flavanones.

Anthocyanins:-

To the plant extract 10% NaOH was added, blue colour indicates the presence of anthocyanins.

Proteins:-

Biuret test:-

To few ml of plant extract, 1ml of 40% NaOH solution and 2ml of 1%$CuSO_4$ were added. Violet colour indicates the presence of proteins.

Xanthoproetic test:-

To few ml extract 1ml of conc.HNO_3 was added. White precipitate was observed boiled and cooled. Then 20% of NaOH or NH_3 was added. Presence of orange colour indicates the presence of aromatic amino acid.

Tannic acid:-

To few ml of plant extract 10% of tannic acid was added. White precipitate indicates the presence of proteins.

Carbohydrates:-

Molisch's test:-

To few ml of plant extract 1ml of alpha-Napthol solution and conc.H_2SO_4 was added along the walls of the test tube. Purple to reddish violet colour at the junction of the two layers indicates the presence of carbohydrates.

Fehling's test:-

Equal volumes of Fehling's-A & B were added. On heating the formation of brick red precipitate indicates the presence of carbohydrates.

Benedict's test:-

To 5 ml of Benedict's reagent few ml of plant extract was added and boiled for 2min, cooled. The formation of red precipitate indicates the presence of carbohydrates.

Aminoacids:-

2 drops of Ninhydrin Reagent was added to the plant extract. Purple colour indicates the presence of amino acids.

Glycosides:-

The extract was mixed with a little amount of Anthrone on watch glass and 1drop of conc.H_2SO_4 was added and made to fine paste and boiled gently on water bath. Presence of glycosides shows dark green coloration.

RESULTS AND DISCUSSION:-

Preliminary phytochemical screening of *Rhynchostylis retusa* revealed the presence of tannins, alkaloids, Steroids, Terpenes, Flavanoids, Triterpenoids, Coumarins, Flavones, Flavanones, Anthocyanins, Quinones, and Carbohydrates (Table-1).

Table – 1 Phytochemical screening of methanol extract of *Rhynchostylis retusa*

S.No.	Phyto constituents	Whole plant extract
1	Tannins	+
2	Alkaloids	+
3	Saponins	-
4	Cardiac glycosides	-
5	Steroids	+
6	Terpenes	+
7	**F**lavanoids	+
8	Phlobtannins	-
9	Anthraquinones	-
10	Triterpenoids	+
11	Coumarins	+
12	Flavones	+
13	Flavanones	+
14	Anthocyanins	+
15	Phenols	-
16	Glycoside	-
17	Quinones	+
18	Carbohydrates	+
19	Aminoacids	+
20	Proteins	+

(+) – Positive , (-) – Negative

Alkoloids are formed as metabolic byproducts and have been reported to be responsible for the antibacterial activity[29]. Steroids and triterpenoids are known for anti-inflammatory, lipolytic and anti-cholesteremic activities[30]. Flavonoids have been referred to as nature's biological response modifiers, because of their inheritant ability to modify the bodies reaction to allergies and they showed their anti-allergic, anti inflammatory, antimicrobial and anticancer activities[31]. Flavonoids are hydroxylated phenolic substance known to synthesized by plants in response to

microbial infection and it should not be surprising that they have been found invitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cellwalls[32]. It has also been observed and accepted that the medicinal value of plant lies in the bioactive phyto components present in the plants[33].

In the present investigation, the active phyto compounds of Rhychostylis retusa was studied and further the antimicrobial activity of the plant extract was also tested against ten potential clinical pathogenic bacteria namely *E.coli, Proteus vulgaris, Xanthomonas* sps., *Pseudomonas mirabilis, Pseudomonas aerosinosa, Klebsella oxytoca, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus mitis, Staphylococcus anginosus* at different concentrations of the plant extracts(500, 250, 100 mg/ml) to understand the most effective activity(Table-2).

Table - 2 Antimicrobial activity of leaf extract of Rhychostylis retusa

S.No.	Name of organism	Zone of inhibition(in mm)								
		Hexane extract			Chloroform extract			Methanol		
		mg/ml			mg/ml			mg/ml		
		500	250	100	500	250	100	500	250	100
1	*E.coli*	-	-	-	14	13	13	12	12	11
2	*Proteus vulgaris*	8	7	7	19	14	13	17	13	11
3	*Xanthomonas sps.*	7	7	7	11	9	8	14	13	11
4	*Pseudomonas mirabilis*	-	-	-	11	10	8	13	11	9
5	*Pseudomonas aerosinosa*	7	-	-	14	10	8	16	13	9
6	*Klebsella oxytoca*	-	-	-	11	10	10	11	9	7
7	*Staphylococcus aureus*	-	-	-	14	13	13	12	10	8
8	*Staphylococcus epidermidis*	-	-	-	13	10	9	17	15	13
9	*Staphylococcus mitis*	-	-	-	11	9	8	12	10	8
10	*Staphylococcus anginosus*	-	-	-	13	11	9	14	13	11

- No Activity

Chloroform Extract shows maximum zone of inhibition was obtained against Proteus vulgaris at a concentration of 500 mg/ml(Figure 1). The chloroform extract was more effective on test pathogens showed significant inhibition zones ranged from 7-19 mm. Hexane extract of this plant shows least zone of inhibition (7-8 mm). Hexane extract doesn't show any antibacterial activity against these test pathogens except *Proteus vulgaris , Xanthomonas* sps. **,** *Pseudomonas aerosinosa***.** *Rhynchostylis retusa* chloroform extract shows more effective on test organisms than the methanol and hexane extracts. The results of antibacterial assay of three concentrations (100, 250,500 mg/ml) of different solvent plant extracts like chloroform and methanol exhibited most effective antibacterial activity .

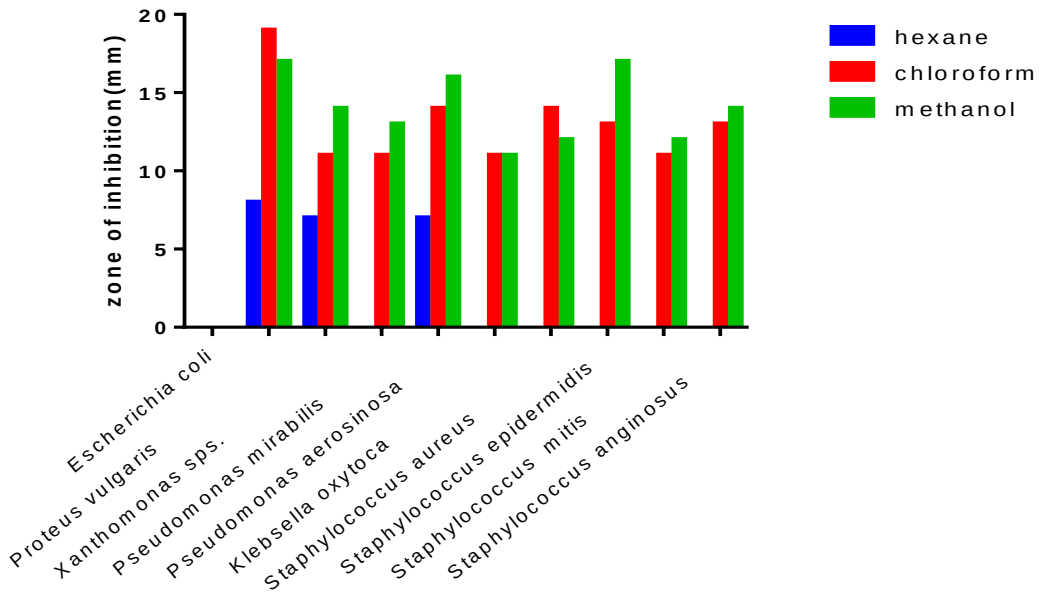

Figure 1: Bar chart showing results of antibacterial activity against ten pathogenic bacteria of hexane, chloroform and methanol (500 mg/ml conc.) of Rhychostylis retusa.

CONCLUSION:

In conclusion, different solvent extracts of this plant showed promising antimicrobial activity against all the selected clinical pathogens. It is very necessary to introduce new and biologically safe and active drugs eco-friendly in nature and effective as microbial agents. Usually medicinal orchid plants contain several phytochemical components, which are very much necessary to control the growth of the microorganisms. From the above studies, it is concluded that the traditional plants may represent new sources of antimicrobials with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine and the knowledge about the botanical preparation of traditional sources of medicinal plants can be extended for future investigation into the field of pharmacology, phytochemistry, ethanobotany and drug discovery.

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