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STUDY OF ANTIMICROBIAL ACTIVITY OF *ORCHIS LATIFOLIA*

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ABSTRACT

Orchis latifolia belonging to the family Orchidaceae is extensively used in traditional Indian medicine against a wide spectrum of ailments including dysentery, diarrhoea, chronic fever, wounds, burns, fractures and general weakness. The present study relates to the bioactive extracts from *O. latifolia* against multidrug resistant (MDR) bacteria and *Candida albicans*. Methanolic extract of *O. latifolia* was prepared and subsequently partitioned with various solvents. The extracts and fractions were evaluated for antimicrobial activities to identify the most active fractions. The bioactive fractions were studied by thin layer chromatography and direct bioautography. The *n*-Hex fraction was identified as most active against MDR clinical isolates. The EtOAc fraction showed maximum activity against *C. albicans*. Phytochemical analysis of the active fractions demonstrated the presence of flavanoids, steroids and tannins. The antimicrobial activity of *O. latifolia* might be attributed due to the presence of alkaloids, flavanoids, steroids and tannins. The bioautography of these two active fractions exhibited the presence of few important chemical constituents which could serve as a promising lead against MDR target drug discovery.

KEYWORDS: **Bioautography, *Candida albicans*, MDR clinical bacterial isolates, *Orchis latifolia***

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INTRODUCTION

Orchids are one of the largest and most diverse groups among the angiosperms in the Plant Kingdom. They are usually cultivated for ornamental purposes and hence are widely known for their economic importance [1]. Phytochemically, some orchids have been reported to contain alkaloids, triterpenoids, flavonoids and stilbenoids. A large number of orchids have been empirically used for treatment of different diseases as diuretic, anti-rheumatic, anti-inflammatory, anti-carcinogenic, hypoglycaemic, antimicrobial, anticonvulsive, relaxation, neuro-protective, and antivirus agents [2]. The plant *O. latifolia* grows in wet meadows and marshes in rich soils. Since the time immemorial, this species is used in various Indian medicine system including Ayurveda, Siddha and Unani, and traditional system of medicine called Amchi system of medicine. Amchi system is principally based on Tibetian system of medicine, prevailed in cold desert region of Ladakh. In amchi system it is widely used to cure dysentery, diarrhoea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures and general weakness, particularly in debilitated women after delivery and to increase regenerative fluids [3]. Tubers of this plant are found to be rich in starch, mucilage, sugar, phosphate, chloride and a glucoside-loroglossin [4]. The aqueous extract of this plant rich in phytosterols and aqueous extract has been evaluated for its efficacy against streptozotocin and alloxan induced sexual dysfunction[5]. Further, ethanolic extract of *O. latifolia* showed activity against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* [6]**.** However, no such systematic studies have been carried out with this plant extract against human opportunistic pathogen *C. albicans.* The main aim of this study is to focus on the medicinal potential of *O. latifolia* against MDR bacteria and human opportunistic pathogen *C. albicans. Candida albicans* a diploid fungus that grows both as yeast and filamentous cells is a causal agent of opportunistic oral and genital infections in humans. It has become one of the leading causes of opportunistic fungal infections in immunocompromised individuals, including AIDS patients, transplant recipients, and cancer patients[7]. Additionally, *Candida* is also a causative agent for a range of mucosal infections such as oral thrush and vaginitis. Most *Candida* infections are routinely treated with topical antifungal drugs, such as clotrimazole, miconazole, nystatin and tioconazole, or oral drugs, such as fluconazole and amphotericin B. However, widespread and overuse of these antibiotics have led to development of resistance against these drugs. Similarly multiple drug resistance amongst bacterial population has become common nowadays due to the indiscriminate use of commercial antimicrobial drugs that are commonly used during the treatment of infectious diseases[8]. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance in microbes of medicinal importance, there is a constant need for new and effective therapeutic agents. Bioprospecting for novel antimicrobials has been possible due to the combined efforts of ethnopharmacologists, botanists, microbiologists, and natural-products chemists. A large number of terrestrial plants possessing a wide range of secondary metabolites including tannins, terpenoids, alkaloids, and flavonoids have been investigated for antimicrobial activity. However, orchids of Himalayan region have not been exploited fully for their medicinal use. Hence, we thought of investigating *O. latifolia,* a high altitude orchidaceae plant for anticandidal activity for the first time. Orchids are one of the largest and most diverse groups among the angiosperms in the Plant Kingdom. They are usually cultivated for ornamental purposes and hence are widely known for their economic

importance [3]. Phytochemically, some orchids have been reported to contain alkaloids, triterpenoids, flavonoids and stilbenoids. A large number of orchids have been empirically used for treatment of different diseases as diuretic, anti-rheumatic, anti-inflammatory, anti-carcinogenic, hypoglycaemic, antimicrobial, anticonvulsive, relaxation, neuro-protective, and antivirus agents [4]. The plant *O. latifolia* grows in wet meadows and marshes in rich soils. Since the time immemorial, this species is used in various Indian medicine system including Ayurveda, Siddha and Unani, and traditional system of medicine called Amchi system of medicine. Amchi system is principally based on Tibetian system of medicine, prevailed in cold desert region of Ladakh. In amchi system it is widely used to cure dysentery, diarrhoea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures and general weakness, particularly in debilitated women after delivery and to increase regenerative fluids [5]. Tubers of this plant are found to be rich in starch, mucilage, sugar, phosphate, chloride and a glucoside - loroglossin [6]. The aqueous extract of this plant rich in phytosterols and aqueous extract has been evaluated for its efficacy against streptozotocin and alloxan induced sexual dysfunction[7]. Further, ethanolic extract of *O. latifolia* showed activity against *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*[8]**.** However, no such systematic studies have been carried out with this plant extract against human opportunistic pathogen *C. albicans.* The main aim of this study is to focus on the medicinal potential of *O. latifolia* against MDR bacteria and human opportunistic pathogen *C. albicans.*

MATERIALS AND METHODS

(i) Plant material collection and extraction

Dried bark of *O. latifolia* was procured from an authorised vendor of Delhi. The material was confirmed by Dr. M.P. Sharma, Department of Botany, Hamdard University, New Delhi and voucher specimen was deposited in the herbarium of Amity Institute of Biotechnology, Amity University, Uttar Pradesh, Noida, India. 500 g of the plant material was extracted with MeOH:Water (9:1) at room temperature. The concentrated methanol extract of the plant was then partitioned with *n*-hexane (*n*-Hex), dichloromethane (DCM), ethylacetate (EtOAc) and aqueous (Aq) fractions [9] respectively. The fractions were concentrated under reduced pressure and temperature below 50^0C and subsequently they were evaluated for antibacterial and anticandidal activity.

(ii) Phytochemical analysis of the fractions

Detailed phytochemical analysis was performed with *n*-Hex, DCM, EtOAc and Aq fractions of methanolic extract of *O. latifolia* to test for the presence of various phytochemicals as described by Rajesh *et al* [10]. Flavanoids, steroids, alkaloids and tannins were detected by NaOH/HCl test, Salkowsi's reaction, Dragendroff's reaction and ferric chloride test respectively. Additional tests were carried out for check the presence of reducing sugars, cardiac glycosides, anthraquinones, triterpenoids and phlobatannins.

(iii) Bacterial and Fungal strains

The five different MDR bacterial clinical isolates including *Escherichia coli, Staphylococcus aureus, Enterococcus sp., Acinetobacter sp*. and *Serratia sp.* were obtained from Dr. Kumardeep Dutta Choudhary, Department of Medical Oncology, Rajiv Gandhi Cancer Research Institute, Delhi, India with their respective antibiotic resistance profiles (Table 1).

Table 1
Antibiotic resistance profiles of MDR clinical isolates

Antibiotics	*E. coli*	*S. aureus*	*Enterrococcus* sp.	*Serratia* sp.	*Acinetobacter* sp.
Amikacin	S	S	R	S	R
Ampicillin	R	-	-	R	-
Ciprofloxacin	R	S	R	R	R
Ceftriaxone	R	S	R	R	-
Chloramphenicol	R	-	-	R	-
Gentamicin	S	S	R	R	R
Imepenem	S	S	R	S	R
Levofloxacin	R	S	R	R	-
Meropenem	S	S	R	S	R
Nalidixic acid	R	-	-	-	-
Nitrofurantoin	S	-	-	-	-
Norfloxacin	R	-	-	-	-
Ofloxacin	R	S	R	R	-
Piperacillin	R	S	R	S	R
Vancomycin	-	S	R	-	-
Tobramycin	R	-	-	R	R

(R): Resistant; (S): Sensitive

Standard isolates *Staphylococcus aureus* (MTCC 96) and *Escherichia coli* (MTCC 443) were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. All bacterial strains were revived in nutrient broth for antibacterial assay. Standard human opportunistic pathogen *Candida albicans* (MTCC 227) was also obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India and revived and maintained on Sabarouds Dextrose broth for anticandidal assays.

(iv) Determination of antibacterial activity

The antibacterial activity of the plant fractions was determined in accordance with the agar-well diffusion method described by Jhon J Rojas *et al* [11] and Kareem *et al* [12].In brief the nutrient agar media plates were seeded with the test organism and open wells of 7 mm diameter were bored with a sterile cork borer. The wells were filled with 50 µL of the plant extract prepared to a final concentration of 1mg/mL in DMSO and water having DMSO concentrations not more than 2 %. Sterilised distilled water was taken as the negative control. Standard antibiotic disc of gentamicin (30 µg) was used as positive control. The plates were incubated at 37 ^{0}C for 24 h and observed for development of zones of inhibition around the wells. The diameters of the circular zone of inhibition were measured. The experiments were performed in triplicate and the antibacterial activity was expressed as mean of inhibition with standard deviation.

(v) Thin Layer Chromatography and Direct Bioautography (TLC-DB)

The *n*-Hex and EtOAc fractions exhibiting significant antibacterial potential against *S. aureus* and *E. coli* were analyzed using TLC-DB. TLC profiling was performed in accordance with the methods described by Das Talukdar *et al* [13]. 10 µL of 1mg/mL of these fractions were loaded on pre-coated silica gel plates (TLC-grade; Merck India; 60 F_{254}). The plates for *n*-Hex and EtOAc fractions were developed with 30:70 EtOAc: *n*-Hex and 70:30 EtOAc: *n*-Hex solvent systems respectively. TLC plate of each fraction was run in triplicate. TLC chromatogram A for *n*-Hex and TLC chromatogram B for EtOAc fractions were visualized in UV light at 254 nm and the fluorescent bands were marked. TLC plates of *n*-Hex fraction after development with above mentioned solvent system were subjected to bioautography for MDR *E. coli* (A1) and *S. aureus* (A2). Similarly, TLC chromatograms B1 and B2 were used for bioautography assay of EtOAc fraction against *E. coli* and *S. aureus* respectively. The bacterial suspension of *E. coli* and *S. aureus* were sprayed on respective chromatograms until wet. The plates are then kept for incubation at 37^{0}C for 24 h in a humid environment for the bacteria to multiply on the

plates. Subsequently, the plates are sprayed with 2.5 mg/mL 2, 3, 5 – triphenyl tetrazolium chloride (TTC) and kept in incubation at 37^0C again for 4-5 h. White zones against pink background indicated the presence of antibacterial compounds in the particular zone of the chromatogram.

(vi) Determination of anticandidal activity

The anticandidal assay was performed using agar well diffusion technique as described by Najafi and Nejad [2]. SDA media plates were prepared and inoculated with 100 µL of *Candida* suspension and spread with a sterilised glass spreader. The plated were allowed to dry and a 7 mm sterile cork borer was used to bore wells in the agar medium. The wells were filled with 50µL of 1mg/mL of each fraction. Sterilised distilled water was used as a negative control. The plates were incubated at 37°C for 24 hrs and observed for the presence or absence of zone of inhibition around the wells. The experiments were performed in duplicate and the anticandidal activity was expressed as mean of inhibition with standard deviation

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RESULTS

(i) Phytochemical analysis of the fractions

The qualitative analysis of the methanolic extract and fractions of the plant is presented in Table 2.

Table 2
Phytochemical screening of methanol extract and fractions of the plant Orchis latifolia as described by Rajesh et al [10].

Group of chemical constituents	Methanol extract	*n*-Hex Fraction	DCM Fraction	EtOAc Fraction	Aq. Fraction
Alkaloids	+	+	+	-	+
Flavonoids	+	+	+	+	-
Steroid	+	+	+	+	-
Tannins	+	+	+	+	+
Reducing Sugars	+	-	-	-	+
Cardiac Glycosides	+	-	-	-	+
Triterpenoids	+	-	-	-	+
Anthraquinones	-	-	-	-	+
Phlobatanins	-	-	-	-	-

(+): Presence; (-): Absence

The results show the presence of alkaloids, flavonoids, steroids and tannins in the bioactive *n*-Hex and EtOAc fractions.

(ii) Antibacterial activity

The results obtained showed that the bark extracts of *O. latifolia* have bactericidal effects on MDR clinical isolates (Table 3).

Table 3

Antibacterial activity of the methanolic extract and plant fractions of Orchis latifolia expressed as zone of inhibition in mm (Mean ± SD of three assays)

Bacteria	*n*-Hex*	DCM*	EtOAc*	Aq*
E. coli	21±1.2	12±0.4	17±0.8	10±0.3
S. aureus	16±0.3	10±0.2	16±0.4	11±0.6
Enterococcus sp.	14±0.5	10±0.4	10±0.6	-
Serratia sp.	-	-	-	-
Acinetobacter sp.	-	-	-	-

Inhibition zone in mm includes diameter of the borer (7mm).

**** 50 µL of 1 mg/mL of the extracts were poured into 7 mm diameter agar wells and zone of inhibition diameter was noted after incubation at 37° C for 24 hours.***

(-): No inhibition

The largest zone of inhibition (21±1.2 mm) was demonstrated by the *n*-Hex fraction against *E. coli* while the value dropped to 12±0.4 mm and 17±0.8 mm for DCM and EtOAc fractions respectively when tested against the same organism. However, the aqueous extract was not as effective against *E. coli* as compared to organic solvent fractions even though slight antibacterial activity was observed. The *n*-Hex fraction also inhibited the growth of *S. aureus* with an inhibition zone 16±0.3 mm which was same as that for ethyl acetate fraction also. The *Enterococcus sp.* was least inhibited by the plant fractions. However, *Serratia sp.* and *Acinetobacter sp.* were not inhibited by any fraction of the plant extract. The negative control plate did not demonstrate inhibition on the tested bacterial isolates. Further, standard antibiotic gentamicin produced significantly larger inhibition zones against the tested bacteria.

(iii) Thin Layer Chromatography and Direct Bioautography (TLC-DB)

The UV analysis of the chromatogram of the *n*-Hex fraction showed the presence of at least seven different compounds which may be responsible for the antibacterial activity (Figure1). Similarly, EtOAc fraction showed the presence of at least nine classes of compounds (Figure 2).

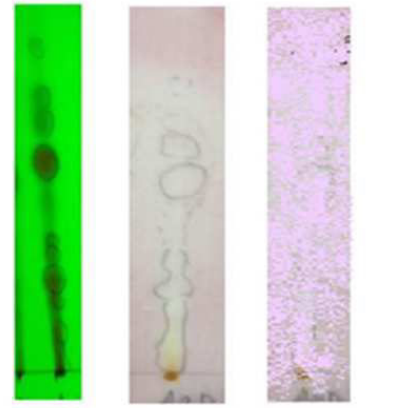

Figure 2

TLC chromatogram (Plate B) and Bioautograms (Plates B1 and B2) for EtOAc fraction against E. coli and S. aureus respectively. 10µL of sample was loaded on TLC-grade; Merck India; 60 F_{254} and developed with 30:70 n-Hex:EtOAc solvent system. White zones against pink background indicated the presence of antibacterial compounds in the particular zone of the chromatogram.

The appearance of white areas against a red-pink background on the chromatograms denotes inhibition of growth of the bacteria due to presence of compound(s) that inhibit their growth. Actively growing micro organisms have the ability to reduce TTC to a pink-red colour. In the presence of active plant compounds on the chromatograms, the growth of the organism is inhibited. All the UV visible spots for *n*-Hex and EtOAc fractions observed on TLC chromatogram A2 and B2 were found to be active against *S. aureus*. On the contrary, only UV visible spots of *n*-Hex fraction on chromatogram A1 were able to inhibit *E. coli*. (Figures 1 and 2).

(iv) Anticandidal activity

The results of anticandidal activity of various fractions of methanolic extract of *O. latifolia* are summarized in Table 4.

Table 4

Anticandidal activity of the methanolic extract and plant fractions of Orchis latifolia expressed as zone of inhibition in mm (Mean ± SD of three assays) against Candida albicans

Fraction	*n*-Hex*	DCM*	EtOAc*	Aq*
Diameter of zone of inhibition(in mms)	9±0.3	-	23± 0.1	-

Inhibition zone in mm includes diameter of the borer (7mm).

**** 50 µL of 1 mg/mL of the extracts were poured into 7 mm diameter agar wells and zone of inhibition diameter was noted after incubation at 37° for 24 hours.***

(-): No inhibition

Strongest anticandidal activity was demonstrated by the EtOAc fraction followed by the *n*-Hex fraction of the extract.

DISCUSSION

Phytochemical analysis of methanolic extract showed the presence of various classes of compounds including flavonoids, saponins, cardiac glycosides, tannins, triterpenes etc. However, the active fractions *n*-Hex showed the presence of only steroids, alkaloids, flavanoids and tannins. Additionally, EtOAc fraction showed the presence of flavonoids, steroids and tannins. Previous works have reported role of flavonoids and phenolic compounds in exhibiting antibacterial activity of the plant extracts [14]. The observed wide range of antimicrobial properties for the methanol extract and fractions can be explained by the presence of various groups of potentially active classes of secondary metabolites. The results of antibacterial assays by agar well diffusion method clearly demonstrated the efficacy of *n*-Hex against MDR bacteria that were obtained from clinical samples. This could be because of the presence of substances like alkaloids and flavonoids as detailed by phytochemical analysis, but this does not exclude the fact that and the observed antibacterial activity could be a result of the combined effect of all the detected components in the *n*-Hex fraction. TLC profiling of all plant fractions gives an idea about the presence of various phytochemicals. Since strong antibacterial activity was demonstrated by the *n*-Hex and EtOAc fractions of the plant extract, these fractions were subjected to TLC followed by bioautography. Bioautograpghy has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied. TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture[15]. Direct bioautography was performed against *E. coli* and *S. aureus* and the *n*-Hex fraction compounds showed very prominent zones of growth inhibition being consistent with agar well diffusion test results and phytochemical tests performed on the fraction (Figure 1).

Figure 1

TLC chromatogram (Plate A) and Bioautograms (Plates A1 and A2) for n-Hex fraction against E. coli and S. aureus respectively. 10µL of sample was loaded on TLC-grade; Merck India; 60 F_{254} and developed with 70:30 n-Hex:EtOAc solvent system. White zones against pink background indicated the presence of antibacterial compounds in the particular zone of the chromatogram.

It was interesting to note that the EtOAc fraction of the plant exhibited antimicrobial activity against *E. coli* and *S. aureus* when assayed by agar well diffusion method but no antimicrobial effect was observed for this fraction against *E. coli* in direct bioautography assay. The observed result may be due to either evaporation of active components or lower concentrations of the compounds. Also, synergism also might play an important role in extracts that were active when antibacterial activity of the fractions was determined, but when separated did not exhibit the same activity. Similar results were reported in a previous study on fennel oil [16]. The results of anticandidal assay demonstrated the efficacy of EtOAc fraction of the extract in inhibiting the growth of human opportunistic pathogen *C. albicans*.

CONCLUSION

The results obtained from this study reveal that *O. latifolia* may be useful in the development of antimicrobial phytomedicines and these results thus hold a promising future in discovery of more novel drugs against MDR target microorganisms

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