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ANTIBACTERIAL AND ANTIMYCOBACTERIAL ACTIVITY OF MEDICINAL ORCHID OF ARUNACHAL PRADESH

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Pleione maculata, Multi drug resistant (MDR), antibacterial activity, Antimycobacterial activity

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ABSTRACT: Orchidaceae, one of the largest families of plant kingdom, possesses high aesthetic, medicinal and economic value. However, medicinal properties of orchids has not been investigated to its fullest potential. Hence, an orchid of Arunachal Pradesh, *Pleione maculata*, which has not been studied from pharmacological and phytochemical view point so far, is selected for the study. The present study was conducted for antibacterial and antimycobacterial activity by bioassay guided fractionation. The whole plant material was divided into diethyl ether (Et_2O), *n*-butanol (*n*-BuOH) and aqueous (Aq) fractions. The antibacterial activity was evaluated by agar well diffusion method against three Gram negative and two Gram positive multi drug resistant clinical isolates. The antimycobacterial activity was performed by Colorimetric redox indicator assay against H37Rv strain of *Mycobacterium tuberculosis*. The screening result identified Et_2O fraction as the most active fraction showing significant zone of inhibition against *E.coli* and *Staphylococcus aureus sp*. and moderate antimycobacterial activity (MIC value 104.16 µg/mL) against H37Rv strain of *Mycobacterium tuberculosis*. Phytochemical screening of the fractions revealed the presence of triterpenes, phlobatannins, alkaloids and cardiac glycosides in the most active Et_2O fraction. Isolation from the active fraction could lead to interesting compounds. Thus bioassay guided fractionation, successfully divided the phytochemicals and biological activity in a particular fraction, making it suitable for commercial use in herbal formulations.

INTRODUCTION: According to world health organization report, about 25% of the preserved human medicines are derived from plants and 80% people depend on traditional system of medicine [1]. Traditional Chinese Medicine uses orchids extensively for combating various ailments. Out of the thousands species, about 50 orchids for example, *Dendrobium nobile*, *Gastrodia elata*, *Bletilla striata*, *etc* are used in various Chinese herbal medications [2].

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In India, total number of orchids present is around 1229, out of which 523 species are reported from Sikkim and 620 species are reported from Arunachal Pradesh [3]. Approximately 50 to 55 varieties of orchids are reported to be widely used in different systems of medicine, and few orchids like *Vanda roxburghii*, *Orchis latifolia*, *Dendrobium nobile* have been well documented for their proven medicinal values [4].

The major chemical constituents reported from orchid species are alkaloids, terpenoids, flavonoids and stilbenoids [5]. The most important orchid, *Vanilla planifolia* has very high commercial importance as a source of Vanillin, a flavoring agent in food [6]. The tubers and pseudobulbs of several orchids like *Orchis latifolia*, *Orchis mascula*, *Cymbidium aloifolium*, *Zeuxine*

strateumatica, and some species of Dendrobium, Eulophia and Habenaria are used as a restorative and for various other diseases [7]. Pre-clinical studies of some orchids established antimicrobial, antioxidant, hepatoprotective, anti-inflammatory, anti-arthritic and wound healing properties [8]. Many such orchids extracts are being widely used by local medical practitioners. However, in most of the cases systematic inclusive investigations involving chemists and biologists have not been organized.

Pleione maculata, belongs to family Orchidaceae, is an epiphyte which is majorly distributed in mountains and foothills of the Himalayas, India (Arunachal Pradesh, Sikkim and other North-eastern parts), Tibet, Nepal, Bhutan and China. The species are well adapted to cold temperatures and even frost [9]. They grow in well-drained habitats and on rocks covered with moss at altitudes of 600 - 4,200 m and blooms in September-November [10]. Arunachal Pradesh and Sikkim, known as the paradise of orchids, have the largest number of orchidsin India. The traditional knowledge of the local tribes provides valuable information about their use as food, medicine and also its conservation [11]. Approximately 79% of the total population of this state is constituted by tribals. The major tribal dialects Monpa, Nishi, and Khamti [12] are well respected in the society for their knowledge. The pseudobulbs of *Pleione maculata* is reported to be used in liver infection, stomachache and many other common ailments [13]. As the plant has not been investigated by right scientific parameters, we conducted phytochemical profiling and evaluation of antibacterial and antimycobacterial activity.

MATERIALS AND METHODS:

Plant Material Collection and Extraction: *Pleione maculata* was procured from the foot hills of Tipi & Khellong district of Arunachal Pradesh. The voucher specimen (AUUP/AIB/2015/04) was deposited in the herbarium of Amity Institute of Biotechnology, Amity University, Noida, India. One kilogram of the whole plant was extracted with MeOH: H_2O (9:1) for five consecutive days. The extract was decanted and the residual plant material was further extracted with water [14]. The organic extract was concentrated under reduced pressure and was fractionated into Diethyl ether (Et_2O), *n*-Butanol (*n*-BuOH) fractions and aqueous (Aq) residue successively. Concentrated fractions of the plant was subjected to phytochemical investigations and evaluated for antibacterial activity against five MDR clinical isolates and antimycobacterial activity against H37Rv strain of *Mycobacterium tuberculosis.*

Phytochemical Analysis: Detailed phytochemical analysis was performed with Et_2O, *n*-BuOH and Aq fractions of methanolic extract of *P. maculata* to test for the presence of various phytochemicals as described by Sawant and Ghodghate [15]. Flavonoids, steroids, alkaloids and tannins were detected by NaOH/HCl test, Salkowski's reaction, Dragendroff's reaction and ferric chloride test respectively. Additional tests were carried out to check the presence of reducing sugars, cardiac glycosides, anthraquinones, triterpenoids and phlobatannins.

Bacterial Strains: The five different MDR bacterial clinical isolates including *Escherichia coli* (2461), *Staphylococcus aureus* (2413), *Enterococcus sp.* (2449), *Acinetobacter sp*. (2457) and *Serratia sp*.(2442) were obtained from Dr. Kumardeep Dutta Choudhary, Department of Medical Oncology, Rajiv Gandhi Cancer Research Institute, Delhi, India. All bacterial strains were revived in nutrient broth to evaluate antibacterial activity. *Mycobacterium tuberculosis* strain H37Rv was obtained as a gift from Dr. V.M. Katoch, National JALMA Institute of Leprosy and other Mycobacterial Diseases, Agra, India and antimycobacterial work was carried out in AIIMS, Delhi.

Determination of Antibacterial Activity: The antibacterial activity of the plant fractions was determined in accordance with the agar-well diffusion method [16]. 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 30µL of the plant extract prepared to a final concentration of 1mg/mL in DMSO and water having DMSO concentrations not more than 2% sterilized distilled water was taken as the negative control. Standard antibiotic disc of tetracycline (30µg) was used as positive control. The plates were incubated at 37 °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.

Colorimetric Redox Indictor Assay (CRI Assay): The pan sensitive strain of *M. tuberculosis* H37Rv TMC-102 (sensitive to streptomycin, isoniazide, rifampicin, ethambutol and pyrazinamide) was considered for the study. Briefly, the bacterium at log phase of growth (approximately 12 days), was transferred to a sterile vial containing glass beads and 8 mL of sterile 0.85% saline solution. The bacterial suspension was disaggregated by agitation and was allowed to stand for 15 min at room temperature. Turbidity of the suspension Was compared with 1 McFarland tube standard and was adjusted with 7H9 broth to obtain a bacterial concentration of 3×10^8 CFU/mL. The working solution at 1:20 dilution of the suspension, in Middlebrook 7H9 broth was evaluated by Colorimetric Redox Indicator Assay (CRI assay) [17].

The extracts were dissolved in DMSO and were diluted appropriately to obtain final sample concentrations in the range of 100-500 µg/mL. The bacterial suspension at a concentration of 1.5×10^8 cells/mL was added to each well where, media along with bacterial suspension was considered as positive control and 0.5% DMSO with media and bacterial suspension were used as DMSO control. Sterile water was added to all perimeter wells to avoid evaporation. The microplates were incubated for 5 - 7 days at 37 °C in an incubator followed by addition of 25 µL of resazurin (0.02% w/v) dye to each well. Then, the plates were re-incubated at 37°C for 24 h for color development. The minimum inhibitory concentration (MIC) was defined as the lowest drug/extract concentration that prevented color change of resazurin reagent from blue to pink. Blue color is interpreted as no mycobacterial growth and pink color as growth occurrence [18]. All the experiments were carried out in duplicate with three independent experiments.

Cell Cytotoxicity Assay: The cell cytotoxicity was performed by MTT assay as described by Kakad and Dhembare [19]. In brief, fibroblast cells obtained from chick embryo were cultured in DMEM medium supplemented with Fetal Bovine Serum (FBS) and gentamicin. The cells suspension (2 mL) was treated with sample solution at MIC concentration and twice the concentration of MIC. The microtitre plate was incubated aseptically in CO_2 incubator for 24 hours at 37 ^{0}C. After incubation, cells were disaggregated using trypsin (0.25%) and cell viability (%) was calculated.

Statistical analysis: All experiments were carried out in triplicates and the results were expressed as mean ± SD values wherever applicable.

RESULTS:

Phytochemical Screening: The qualitative analysis of the methanolic extract and fractions of the plant revealed extensive presence of triterpenes, phlobatannins, alkaloids and cardiac glycosides in Et_2O fraction of *P. maculata*. In contrast, the *n*-BuOH and Aq fractions showed the presence of flavonoids and tannins, cardiac glycosides and phlobatannins (**Table 1**). Reducing sugars was detected in aqueous residue which was absent in Et_2O and *n*-BuOH fractions.

TABLE 1: PHYTOCHEMICAL SCREENING OF THE FRACTIONS OBTAINED FROM THE METHANOLIC EXTRACT OF *P. MACULATA*

Class of Phytochemicals/ Fractions	Et_2O	*n*-BuOH	Aq
Alkaloids	+++	++	-
Flavonoids	++	++	++
Steroid	+	++	-
Tannins	+	++	+
Reducing Sugars	-	-	++
Cardiac Glycosides	+++	++	+
Triterpenoids	+++	++	+
Anthraquinones	-	-	-
Phlobatanins	++++	+++	+

"+++" indicates highly present, "++" indicates moderately present,"+"indicates present in traces, "-" indicates Absence

Flavanoids, steriods, alkaloids and tannins were detected by NaOH-HCl test, Salkowski's reaction, dragendorff reaction and ferric chloride test respectively.

Additional tests were carried out anthraqinones and terpenoids (Sawant and Godghate, 2013)

Antibacterial Activity: The results obtained for antibacterial activity by agar well diffusion assay showed that the extracts of *P. maculata* have bactericidal effects on MDR clinical isolates (**Table 2**, **Fig. 1**). The largest zone of inhibition (24.1 ± 0.3 and 20.7 ± 0.5 mm) was demonstrated by the Et_2O fraction against *E. coli* and *S. aureus*

sp. while in case of *n*-BuOH fraction the zone of inhibition was 18.7 ± 0.7 mm and 17.1 ± 0.6 mm against *S. aureus* and *Serratia sp.* respectively. However, the aqueous extract was not as effective against any bacterial pathogens as compared to organic solvent fractions even though slight antibacterial activity was observed. The Et_2O fraction also inhibited the growth of *Serratia sp.* and *Enterococcus* with an inhibition zone 15.2 ± 0.5 mm and 13.0 ± 0.5 mm. The *Acinetobacter sp.* was least inhibited by the plant fractions. Further, standard antibiotic tetracycline produced significantly larger inhibition zones against the tested bacteria

TABLE 2: ANTIBACTERIAL ACTIVITY OF *P. MACULATA* FRACTIONS AGAINST FIVE MDR CLINICAL ISOLATES

MDR bacterial isolates	Et_2O	*n*-BuOH	Aqueous	Positive control (Tetracycline)
E. coli (2461)	24.1± 0.3	11.1± 0.3	10.3±0.4	21.79±0.58
Acinetobacter sp. (2457)	10.6± 0.9	12.1± 0.6	9.2±0.3	25.33±0.5
Serratia sp. (2442)	15.2±0.5	17.1±0.6	9.7±0.1	22.33±0.57
S. aureus (2413)	20.7± 0.5	18.7± 0.7	8.0±0.2	25.76±0.58
Enterococcus sp. (2449)	13± 0.5	12.1± 0.6	NI	20.5± 1

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

* 30 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

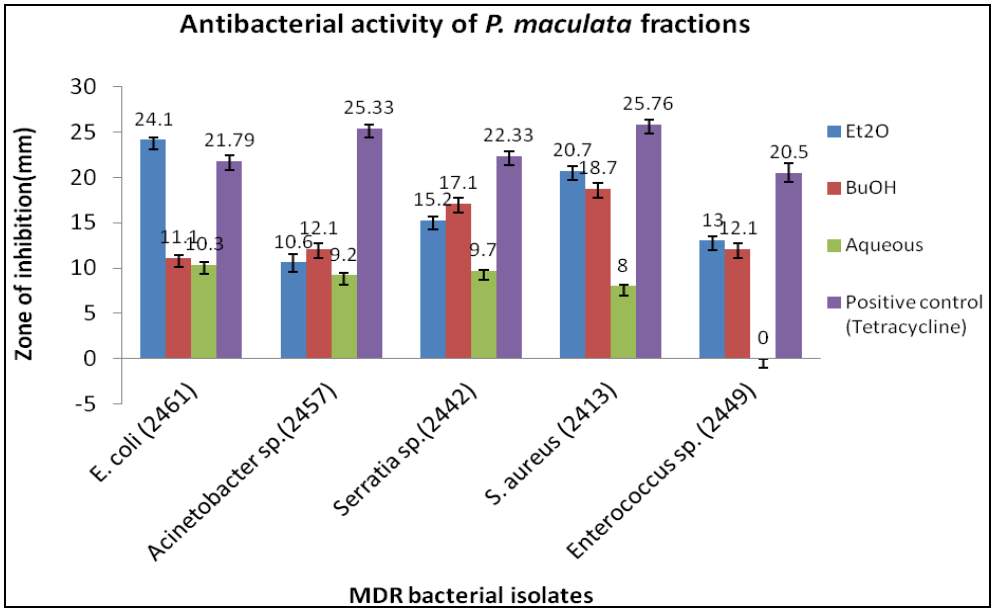

FIG. 1: BAR GRAPH DEPICTING ANTIBACTERIAL ACTIVITY OF THREE FRACTIONS (Et_2O, *n*-BuOH and Aq) OF *P. MACULATA* AGAINST FIVE MDR BACTERIAL CLINICAL ISOLATES

Antimycobacterial Activity: The antimycobacterial activity of the fractions was determined by colorimetric redox indicator assay and the results were expressed as MIC values (**Table 3**, **Fig. 2**). Analysis of the results showed that Et_2O fraction of *P. maculata* exhibited moderate antimycobacterial activity against H37Rv strains with MIC 104.16 µg/mL followed by *n*-BuOH fraction with MIC value 166.66 µg/mL respectively. However, the aqueous residue of the plant did not show any activity against *Mycobacterium tuberculosis* strain, where Rifampicin was used as a positive control.

TABLE 3: MINIMUM INHIBITORY CONCENTRATION (MIC in µg/ml) OF THE ANTIMYCOBACTERIAL ACTIVITY OF CRUDE EXTRACTS/FRACTIONS OF *P. MACULATA* AGAINST H37Rv STRAIN

S. no.	Extract/ Fraction	MIC values against H37Rv (µg/mL)
1	Et_2O	104.16
2	*n*-BuOH	166.66
3	Aq ext	1000
	PC-Rifampicin	0.8

PC- Positive control (Rifampicin)

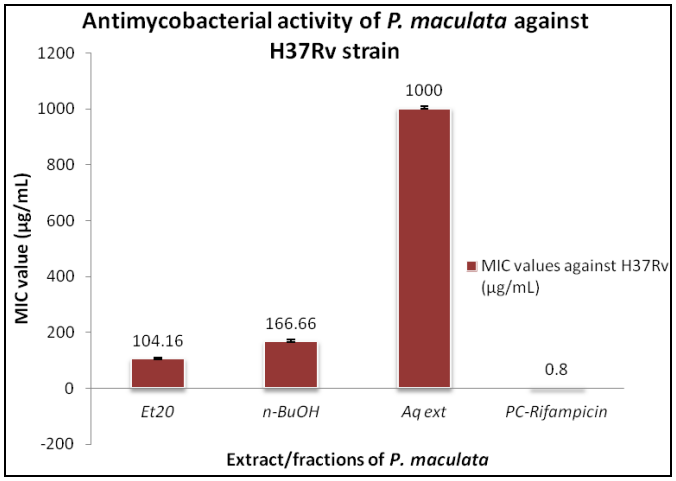

FIG. 2: ANTIMYCOBACTERIAL ACTIVITY BY COLORIMETRIC REDOX ASSAY DEPICTING MIC VALUE OF FRACTIONS OF *P. MACULATA* AGAINST H37Rv STRAIN OF *M. TUBERCULOSIS*

DISCUSSION: Investigating antibacterial and antimycobacterial effects of *Pleione maculata* involved comparison of its fractions with commercially available antibiotics like tetracycline (agar-well diffusion assay) and rifampicin (CRI assay).The result of antibacterial activity against MDR clinical isolates demonstrated activity of the extract against both gram positive and gram negative bacteria which could be an indication of the broad spectrum of activity and thus can be used to source antibiotic substances for herbal drug formulation or drug development that can be used in the control of these bacterial infections.

The antimycobacterial result indicated the potential effect of Et_2O fraction, though activity was found to be moderate. It is well documented that extracts possessing antimycobacterial activity with MIC < 100 µg/mL are considered as potent while, 100 - 625 µg/mL are considered as moderate [20]. Though, *in vitro* biological activities, not necessarily be transposed to clinical trials, but early *in vitro* testing specifically aims to demonstrate safety, which helps investigators to determine whether a drug candidate possesses scientific merit for further investigation. Better performance demonstrated by the non polar fractions might be involving the lipophilic constituents, which causes disturbance of lipid fraction of plasma membrane, leading to a loss of permeability and leakage of intracellular materials [21].

Overall, the result of this study showed the presence of some phytochemicals such as alkaloids, steroids, tannins and cardiac glycosides. Various plant secondary metabolites like flavonoids, cardiac glycosides, tannins, triterpenes and alkaloids have been reported to possess antibacterial and antimycobacterial activities and fractions can be explained by the presence of various groups of potentially active classes of these secondary metabolites [22].

CONCLUSION: The significant antibacterial potential of Et_2O fraction of *P. maculata* has been demonstrated by zone of inhibition assay against clinical isolates of *E. coli* and *S.aureus sp.* and *n*-BuOH fraction showed moderate activity against *S. aureus* and *Serratia sp.* The moderate antimycobacterial activity of Et_2O fraction of *P. maculate* has been demonstrated by colorimetric based assay in our study. Cell cytotoxicity measured at MIC (CRI assay) and twice the concentration of MIC showed that cytotoxicity was well within the permissible limit *i.e.*, 7%. The results provide scientific evidence to support the folk medicinal utilization of these plants for the treatment of various ailments. These results may offer new possibilities in the therapy of pathological conditions. Further phytochemical studies are required to isolate bioactive molecules from *P. maculata*.

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CONFLICT OF INTEREST: The authors declare no conflict of Interest.

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