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Antioxidant and antibacterial activities of ethanolic extract of therapeutically important orchid, *Herminiumlanceum*(Thunb. ex Sw.) Vuijk

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ABSTRACT

Dependency of people on natural source of drugs goes on increasing on entire globe. We are reporting the antioxidant and antibacterial activity of ethanolic extract of plant sample of *Herminiumlanceum*. Efficiency of plant sample extract of different dilution (0.5-10mg/l) for having free radical scavenging potential was evaluated, where tuber collected from micropropagated plant had shown maximum antioxidant activities. Extract of plant samples were also screened to assess their antibacterial activity againstGram positive: *Mycobacterium hassiacum*(3849^T), *Corynebacteriumpollutisoli* (VDS11^T), *Fictibacillushalophilus*(AS8^T), Gram negative: *Sphingopyxisflava*(R11H^T), *Pontibactermucosus*(PB3^T), *Algoriphagusroseus*(W29^T), *Novosphingobiumlindaniclasticum*(LE124^T) bacteria, where tested plant sample extract have shown excellent broad spectrum antibacterial activity.

KEYWORDS: Antioxidant, Antibacterial, *In vitro*, *In vivo*, Tubers.

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1. INTRODUCTION

Plants are the oldest but rich source of bioactive chemical compound having pharmacological or toxicological influence on human beings[1]. These effective compound is synthesized within the plants in form of secondary metabolites such as phenolics, alkaloids, flavonoids, steroide, gylycosides, terpenoid etc. which may have tissue or organ specific properties[2]. Since ancient period medicinal orchids are being used for treatment of several microbial infectious disorders. Mostly tubers of these are use for medicinal purpose. However, actual therapeutic merit of these plants is yet to be explored in a scientific way. Many of the orchids are facing the extreme risk of extinction due to overexploitation and habitat destruction[3].

Herminiumlanceum (Thunb. ex Swartz) Vuijk,commonly known as Jalya[4], is a terrestrial, tuberous herb[5, 6] and found growing on grassy hill slopes in association with other orchids, such as *Satyrium, Brachycorythis, Cephalanther*and *Platenthera* at elevations of about 1500-3000 m in subtropical zones[6]. The plant has an excellent medicinal value, as an extract of the plant is prescribed in cases of urinal disorder[7]. About 5 teaspoons of decoction made from the stem and leaves are often recommended to cure diabetes, fever and bleeding[8].

In fact, there are various reports are available revealing antimicrobial and antioxidant properties of different medicinal plants, however, this plant has not been explored scientifically for its antimicrobial and antioxidant activities. Hence the current investigation was initiated for the screening of various plant samples of *Herminiumlanceum* to determine antioxidant and antibacterial activities for the first time.

2. MATERIALS AND METHODS

2.1 Collection of plant material: *In vivo*leaves and tubers of *Herminiumlanceum* were collected from Dhanaulti, Uttarakhand, India (Latitude: 78°12’02.82”E, Longitude: 30°25’42.91”N, Altitude: 2378m), during the first week of September, while micropropagated plant samples were obtained from Mitra's medium following the protocol of Singh and Babbar (2016)[9]. The botanical identity of the plants was authenticated at the Botanical Survey of India, Dehradun (Uttarakhand) and the herbarium specimen of one of the collected plants was deposited in the Delhi University herbarium (DUH, Voucher No.13740).

2.2 Preparation of ethanolic crude plant extracts

Leaves and tubers of *in vivo* plants while tuber of micropropagated plants was dried for 48 hr in an oven maintained at 50°C. Completely dried plant samples were then pulverized in liquid nitrogen.100 and 200 mg powder of each sample were weighed individually and added to 5ml of

ethanol contained in Falcon tubes (50 ml) and incubated for 12 hr at 200 rpm on a rotary shaker maintained at 25°C.The tubes were then centrifuged at 10,000g for 20 min and the supernatant was filtered through Millipore filters (0.22µm). The filtrate containing plant extracts to be analyzed were stored at 4°C till further use.

2.3 Test Microorganisms

The following Gram-positive and Gram-negative bacteria were used for conducting experiments. Gram positive bacteria:*Corynebacteriumpollutisoli, Fictibacillushalophilus*and *Mycobacterium hassiacum;* Gram negative bacteria:*AlgoriphagusroseusNovosphingobiumlindaniclasticum,Pontibactermucosus*and*Sphingopyxis flava*; these bacterial cultures were obtained from Department of Zoology, University of Delhi, India.

2.4 Preparation of growth media

Luria Bertani(LB) agar media was made for bacterial cultures. pH of LB agar medium was adjusted to 7.5 with 0.11NaOH or 0.1N HCl. Approximately 25ml of the medium was poured into a sterile petridish (90mm, Hi Media, India). Master plates of every culture were also maintained during subculture.

2.5 DPPH Radical Scavenging Assay

Antioxidant activities of *in vivo* and *in vitro* tuber and leaves and leaves collected from the natural habitat were determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay by the method of Brand-Williams *et al.* (1995)[10] with slight modification.The stock solution of the reagent, DPPH was prepared by dissolving 24 mg of it in 100mL of methanol and stored at -23°C until use for the experiments. 1ml from the stock of DPPH was added to the same volume of ethanolic plant extract of varying concentrations (0.5, 1, 5 and 10mg/ml). 1ml DPPH and 1ml methanol solution mixer was used as a control. The resulting mixture was shaken vigorously with vortex and kept in dark for 30m at room temperature. Absorbance of the reacting mixture was registered with the spectrophotometer at 593nm.The role of ethanolic plant extract for their antioxidant activities was expressed as the percentage of inhibition and was calculated using the following formula:

$$\% \text{ radical scavenging activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

2.6 Screening of plant sample for their antibacterial activities

Screening of different concentration(0, 20 and 40mg/l) of crude ethanolic extract of *in vitro* and *in vivo* tuber and *in vivo* leaves were conducted for their antibacterial activities using "disc diffusion technique" described by Dougharie*t al.* (2008)[11].

In this method bacterial suspension was prepared in Luria bertani(LB)broth and one hundred fifty microlitre of the prepared bacterial suspension was poured on the surface of UV sterilized LB medium. They were spread thoroughly with the help of L shaped spreader (Hi media, India) for uniform growth of bacteria.

Thereafter, extract soaked test disc(6mm sterile disc, Hi media, India) and standard disc were placed aseptically on to the surface of the solidified LB agar medium previously seeded with particular bacterial species. Cultures plates were incubated at their best optimum growth condition. All petriplates of gram negative bacteria except *Mycobacterium hassiacum* were incubated inverted at 37 ± 2°C, whereas all plates having grams negative bacteria including one gram positive bacteria were kept at 28 ± 2 °C. Both strains of bacteria were incubated for 48 hr.

Antibacterial activities of the plant extract were calculated by measuring clear zone of inhibition(including diameter of the disc) in mm around each disc and mean diameter was recorded.

2.7 Experiment design and data analysis: All experiments were repeated twice and the results were analyzed using one way ANOVA test and significant difference between each treatment evaluated by the Duncan's multiple range test at P= 0.05 using SPSS (version 22) software package. The values of one parameter followed by same superscript(s) in each of the tables are not significantly different. The cultures were photographed by using Canon camera (PC1234, DC 7.4v), Japan after each observation.

3. RESULTS

3.1 Scavenging activities of in vitro and in vivo tubers and in vivoleaves extract towards DPPH free-radical

The antioxidant activity of *in vitro* tuber, *in vivo* tubers and leaves extract of different concentration (0.5-10mg/l) was determined by using the DPPH method. The free radical scavenging activity of different plant sample was recorded increasing in a dose dependent manner.

Maximum antioxidant activity was observed in *in vitro* tuber extract (61.66%) at the higher concentration (10mg/ml) followed by extract of *in vivo* leaves (53.47%) at similar dose of extract. Ethanolic extract of tuber collected from its natural habitat had the least(52.34%) DPPH free radical scavenging potential at the higher 10mg/ml of extract(Table 1).

Table 1: Antioxidant activities of *in vitro* and *in vivo* plant samples in *Herminiumlanceum*

Plant samples	Conc. of extracts (mg/ml)			
	(0.5)	**(1)**	**(5)**	**(10)**
In vitro tubers	26.54 ± 0.22^{i}	29.96 ± 0.20^{h}	41.46 ± 0.11^{d}	$\mathbf{61.66 \pm 0.19^{a}}$
In vivo leaves	29.97 ± 0.12^{h}	35.72 ± 0.15^{g}	37.12 ± 0.20^{e}	53.47 ± 0.19^{b}
In vivo tubers	22.76 ± 0.20^{k}	24.33 ± 0.22^{j}	36.47 ± 0.18^{f}	52.34 ± 0.24^{c}

Mean values in each column followed by the same superscript are not significantly different (P = 0.05) based on Duncan's multiple range test (DMRT).

3.2 Antibacterial activity of in vitro plant extract

Ethanolic extract of *in vitro* roots was tested against three gram positive and four gram(-) bacteria. The maximum antibacterial activity was registered against *Novosphingobiumlindaniclasticum*(72.74mm^2) at higher concentration (40mg/l) followed by *Pontibactermucosus* (67.25mm^2), however extracts were least effective against *Corynebacteriumpollutisoli*(22.50mm^2) at lower concentration(20mg/l) (Table 2).

Table 2: Antibacterial activities of *in vitro* tuber extracts against gram(+) and gram(-) bacteria using the disc diffusion assay.

TestOrganisms	***In vitro* tuber**		
	Area of inhibition zone (mm^2)		
	Cntrl	**20mg/ml**	**40mg/ml**
Gram positive			
Mycobacterium hassiacum	0.00 ± 0.00^{d}	26.43 ± 4.45^{c}	40.3 ± 4.98^{bc}
Corynebacteriumpollutisoli	0.00 ± 0.00^{d}	22.50 ± 7.26^{cd}	$35.85 \pm 8.17b^{c}$
Fictibacillushalophilus	3.40 ± 3.41^{d}	35.33 ± 0.00^{bc}	51.81 ± 16.50^{ab}
Gram negative			
Sphingopyxisflava	0.00 ± 0.00^{d}	36.90 ± 14.93^{bc}	40.30 ± 4.98^{bc}
Pontibactermucosus	3.40 ± 3.41^{d}	55.74 ± 5.50^{ab}	67.25 ± 9.99^{a}
Novosphingobiumlindaniclasticum	0.00 ± 0.00^{d}	45.27 ± 4.98^{bc}	72.74 ± 6.03^{a}

Mean values in each column followed by the same superscript are not significantly different(P =0.05) based on Duncan's multiple range test (DMRT).

3.3 Antibacterial activity of in vivo plant extract

Ethanolic extract of root collected from its natural habitat was tested against three gram positive and four gram(-) bacteria. The maximum antibacterial activity was recorded against

Fictibacillushalophilus(97.86mm^2) at higher concentration (40mg/l) followed by *Sphingopyxisflava* (56.78mm^2), however, extracts were less effective against *Corynebacteriumpollutisoli*(18.58mm^2) and *Mycobacterium hassiacum* (21.98mm^2) (Table 3).

Table 3: Antibacterial activities of *in vivo* tuber extracts against gram(+) and gram(-) bacteria using disc diffusion method.

Test Organisms	*In vivo* tuber		
	Area of inhibition zone (mm^2)		
	Cntrl	20mg/ml	40mg/ml
Gram positive			
Mycobacterium hassiacum	0.00 ±0.00^{e}	21.98 ±0.00^{de}	26.43 ±4.45^{cde}
Corynebacteriumpollutisoli	0.00 ±0.00^{e}	18.58 ±8.38^{de}	26.43 ±4.45^{cde}
Fictibacillushalophilus	0.00 ±0.00^{e}	61.23 ±5.50^{b}	97.86 ±6.55^{a}
Gram negative			
Sphingopyxisflava	0.00 ±0.00^{e}	50.76±9.08^{bc}	56.78 ±14.66^{b}
Pontibactermucosus	0.00 ±0.00^{e}	22.50 ±7.26^{de}	41.34 ±13.28^{bcd}
Novosphingobiumlindaniclasticum	0.00 ±0.00^{e}	35.85 ±8.17^{bcd}	55.74 ±5.50^{b}

Mean values in each column followed by the same superscript are not significantly different (P = 0.05) based on Duncan's multiple range test (DMRT).

4. DISCUSSION AND CONCLUSION

DPPH assay is the technique to evaluate the antioxidant potential of plant extract. Donation of hydrogen group to free radicals by antioxidant make the species non toxic and therefore reduces lipid oxidation[12].In the present study, % radical scavenging activities of the crude extract of different plant samples were tested and compared with one another. Antioxidant activities of plant extract of different dilution (0.5-10mg/ml) was recorded increasing in a dose dependent manner. Tuber extract of *in vitro* regenerated plants at higher dose (10mg/ml) had maximum antioxidant potential than rest of the sample tested. These results are in parallel with the observation of Sahu*et al.* (2013)[13], where ethanolic extract of various leafy vegetables had shown optimum free radical scavenging activities. In their study, they used ethanol, methanol and hexane as a solvent for preparing plant extract and scoring % of antioxidant activities was maximum at higher concentration, whereas least was in lower level. Similarly in the present study, least antioxidant activities was detected in *in vivo* tuber at lower concentration(0.5mg/ml).

In the present era, resources of medicinal plant are abundant, but these are dwindling fast due to anthropogeic activities [14]. The ethnomedicinal merit of any medicinal plant relies on the its bioactive phytocomponents[15]. Antibacterial activities of micropropagted and *in vivo*ethanolic tuber

extract was tested against three gram positive and four gram (-)bacteria. It has been revealed from the result that plant extracts were active against both types of strain by forming varying degree of zone of inhibition. Such antibacterial activities of the extract might be due to presence of simply general metabolic toxins or antibiotic compound. *In vitro*ethanolictuber extract was more effective against gram negative bacteria than a gram positive. Such difference in the response of plant extract may be owing to single layered cell wall in gram positive, whereas multilayered structure in gram negative[16]. The results of the present investigation are in conformitywith the report of Srinivasan*et al.*(2001)[17], where out of extract of thirty-six plant, only four were active against gram positive bacteria. However, most of the previous reports of the various investigators have also concluded that gram negative were not susceptible against plant extract[18-20]. Interestingly, zone of inhibition for in *in vivo* tuber extract was comparatively maximum against gram positive than gram negative strains. These results lie in tune with the finding of Alzoreky and Nakahara (2003)[21], where extracts of some edible plants was proved most effective against gram positive than gram negative bacteria. Effectiveness of tested sample against both gram(+) and gram(-) bacterial is a clear indication for the presence of broad spectrum antibiotic compound in plant, which may lead to discovery of new class of chemical.

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