Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env.Pharmacol. Life Sci., Vol 8 [12] November 2019 :161-167
©2019 Academy for Environment and Life Sciences, India
Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.876
Universal Impact Factor 0.9804
NAAS Rating 4.95

ORIGINAL ARTICLE **OPEN ACCESS**

Antimicrobial potential of *Delphinium denudatum* (Wall Ex Hook & Thom)

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ABSTRACT
Plants produce a diverse array of secondary metabolites, many of which are known to possess antimicrobial potential. The present study was carried out with an objective to investigate the antimicrobial potential of different plant parts of Delphinium denudatum. Antimicrobial potential of D.denudatum plant parts (stem, root, leaf) extracts were tested against three groups of microorganisms, bacteria(Gram positive and Gram negative bacteria), actinobacteria and fungi. Plant extracts were prepared in seven different solvents (methanol, ethanol, ethyl acetate, acetone, hexane, chloroform and water) according to their polarity. The qualitative estimation of antimicrobial activity was performed following plate assays through disk diffusion methodwhile the quantitative estimations were based on minimum inhibitory concentration (MIC). All the plant parts showed antibacterial activity, maximum being in case of stem. Among different solvents, ethanolic stem extract showed highest antibacterial activity(15.33±0.11mm) against S. marcescens and aqueous leaf extract showed highest antiactinobacterial activity (21.0 ± 0.07mm) against Nocardia tenirefensis. Antifungal activity, that was tested against 5 fungal species, was absent in all the plant parts extracts. Values obtained for MIC varied with respect to different solvents. This is a preliminary study showing the antibacterial potential of D. denudatum with respect to the importance of solvent selection in harvesting antimicrobial metabolites.
KEY WORDS: Delphinium denudatum, antibacterial, antiactinobacterial, antifungal, MIC

Received 01.09.2019 Revised 23.10.2019 Accepted 05.11.2019

INTRODUCTION

ports on antibiotic resistance phenomenon and development of its side effects due to consumption of microbe derived antibiotics are huge in literature. This suggests the need for alternate sources for combating the infectious diseases[1]. In this perspective, plant based antimicrobials (derived from medicinal plants, in particular) are increasingly receiving attention for harnessing their potential in production of antimicrobial substances, as safer source of antibiotics. Crude extracts and essential oils of medicinal plants possess bioactive compounds, often with antimicrobial and antioxidant properties[2]. Antimicrobial compounds are used in various areas such as, pharmaceutical, neutraceuticals, textiles, dairy products, cosmetics and personal care products[3].

Delphinium denudatum is a critically endangered Himalayan perennial herb (Local name- *Jadwar*; Family-*Ranunculaceae*). The generic name of this plant is derived from a Greek word, which means Dolphin. It is found in the Westernand North Western Himalaya, from Kashmir to Kumaun at an altitudinal range from 1400-3600 m, amsl. It occurs in grassy and rocky moist slopes, open grasslands and margins of fields[4]. It has been recommended for treatment of paralysis, epilepsy, insanity, migraine, numbness, tremors, infantile convulsions, aconite poisoning, snake bite, scorpion sting, opium addiction, arthritis, cardiac weakness, palpitation, rheumatism, all kinds of pain, leucoderma and for improving skin complexion [5].

Selection of solvent system in obtaining the plant extracts is important indetermination of any biological activity including production of antimicrobials. Reports are available on the importance of selection of suitable solvents in assessing antimicrobial potential of medicinal plants [3,6,7]. The present study aims on selection of solvent system in production of antimicrobial metabolites from various plant parts of *D. denudatum*. The antimicrobial potential of *D. denudatum* plant parts is investigated with respect to three major groups of microorganisms (bacteria, actinobacteria and fungi) using seven different solvents according to their polarity.

MATERIAL AND METHODS

Study site and Sample collection

The fresh and healthy root, stem, and leaves of *D. denudatum* were collected from Sainj Valley of Kullu District in Himachal Pradesh ($31^0$45.881' N to $31^0$76.897' Nlatitudes and $77^0$19.031' E to $77^0$33.747' E longitudes) during the months May and June, brought to the laboratory, air dried, converted into fine powder, and stored at 4-8 °C.

Extraction

Root, leaf, and stem were extracted (separately) in seven solvents (methanol, ethanol, acetone, chloroform, ethyl acetate, hexane, and water separately) taking in a ratio of 1:5 (dry powder: solvent). The mouth of conical flask was sealed with para-film. Samples were macerated in a rotary shaker (Remi) at 160 rpm for 48 h.

Test microorganisms

Bacteria**:** 2 Gram +ve=*Bacillus subtilis*(NRRLB-30408) and *B. megaterium*(MCC3124); 3 Gram -ve=*Pseudomonas chlororaphis*(MCC2693), *Escherichia coli,* and *Serratia marcescens*(MTCC4822).

Actinobacteria: *Nocardiatenirefensis*(MCC2012)

Fungi: *Paecilomyces variotii*(ITCC3710), *Aspergillus niger*(ITCC2546), *Fusarium oxysporum* (ITCC4219), *F.solani*(ITCC 5017), and *Trametes hirsuta*(MTCC11397).

These test microorganisms were taken from the microbial culture collection, established in the Microbiology Lab of the Institute (GBPNIHESD). Accession numbers shown in the parentheses have been allocated by the National/International depositories: NRRL (Northern Regional Research Laboratory, Agricultural Research Service Patent Culture Collection, United States Department of Agriculture,Illinois; MCC (Microbial Culture Collection, National Centre for Cell Science, Pune, India); MTCC (Microbial Type Culture Collection and GeneBank, Institute of Microbial Technology, Chandigarh, India); ITCC (Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India).

Bioassays for determination of antimicrobial potential of *D. denudatum* extracts

Plate based bioassays

For qualitative estimation of antimicrobial potential of *D. denudatum* extracts, agar plate based bioassays were performed using disc diffusion method. Bacterial and actinobacterial culture suspensions were prepared in tryptone yeast extract (TYE) agar while fungal culture suspensions were prepared in potato dextrose (PD) agar. 100 µl of all the test microorganisms (separately) were spread uniformlyon the respective agar surface (TYE agar plates for bacteria andactinobacteria, and PD agar plates for fungus) with the help of a glass spreader. Sterilized 5 mm filter paper (Whatman No. 1) discs were placed over the agar surface with the help of sterile forceps.15 µl of extract was loaded over the agar disc. The plates were then incubated at 25 ºC. The results were recorded measuring the zone of inhibition (mm) after 24 h for bacteria and 120 h for actinobacteria and fungi. All the experiments were performed in triplicates.

Quantitative estimation/ Minimum inhibitory concentration (MIC)

MIC was determined following Clinical and Laboratory Standard Institute Methodology [8]. Bacterial andactinobacterial culture suspensions were prepared in TYE broth. For determination of MIC, 1 ml extract was diluted using different concentration ranging from 100 to 1000 µg/ml, 1 ml test microorganism and 8 ml broth was taken in sterile test tube, and then incubated at 25 °C for 24 h for bacteria and 120 h for actinobacteria. Control was prepared in two sets; one containing broth medium and test microorganism while the other containing broth medium and extract. After 24 h, the MIC values were recorded on the basis of the lowest concentration showing absence of growth in the tubes. The test was further confirmed by plating on TYE agar medium.

Statistical analysis

Data was expressed as the means ± standard errors (SE) from experiments, performed in triplicates. Statistical significance was determined using student's t-test. A p value <0.05 was considered as significant.

RESULTS AND DISCUSSION

Extract yield

Figure 1 shows the yield of different plant parts of *D.denudatum* (root, stem, and leaf) extracted in different solvents according to their polarity (water> methanol> ethanol> acetone>ethyl acetate> chloroform> hexane) following maceration. The extraction yield was recorded highest in root, followed by leaf and stem, respectively. Methanol was found to be the best solvent for obtaining highest yield from all the plant parts of *D. denudatum*. Extract yield was recorded lowest in hexane (root, stem) and chloroform (leaf). Extract yields were found to be higher in polar solvents in comparison to nonpolar. These results coincide with several previous reports[9,7]. Higher extract yield in polar solvents can be attributed to their high di electric constant which is known to be responsible for solubility of bioactive compounds[3]. The polarity of the solvent influence the extraction yield that, in turn, plays a crucial role in increasing the solubility of phytochemical compounds[10]. This result indicates toward the role of solvent system in obtaining the extract yield of *D. denudatum*.

Qualitative test (Plate based bioassays)

Root, stem, and leaf extracts of *D. denudatum* showed antimicrobial activity against two groups of microorganisms, bacteria and actinobacteria. Zone developed due to inhibition of bacterial and actinobacterial species due to production of antimicrobial metabolites of *D. denudatum* are shown in Figure 2. All the extracts, exceptaqueous, inhibited the growth of bacteria, both Gram +ve and Gram -ve. Antimicrobial activity, recorded in different plant parts, was in the order: stem> leaf> root. Maximum inhibition was recorded in case of *S. marcescens*(15.33± 0.11 mm) in stem ethanolic extract, followed by stem ethyl acetate extract (11.00 ± 0.28 mm) against *P. chlororaphis*(Table 1). In comparative assessment with respect to the bacteria, *E. coli, B. megaterium*, and *B. subtilis*, leaf acetone extract (6.67 ± 0.20 mm), root hexane extract (8.33±0.36mm) and stem ethanolic extract (7.00 ± 0.35 mm) were the most effective extracts, respectively (Figure3). Similar results have been recorded in species of *Delphinium* namely *D. uncinatum*[11].

Aqueous extracts of leaf, root, and stem showed strong inhibition of the actinobacterial species, *N. tenirefensis* in an order: leaf (21.0 ± 0.07 mm)>root (17.33 ± 0.08mm)> stem (16.33 ± 0.04mm). None of the extracts, prepared in different solvents, showed antiactinobacterial activity. This indicates toward capability of water as potential solvent fordetection of actinobacterial compound(s) (Table 1 and Figure 3). This is likely to be the first reporton inhibition of an actinobacterial species by plant extracts of *D. denudatum*.

None of the extracts showed antifungal activity against the test fungi namely *Paecilomyces variotii*, *Aspergillus niger, Fusarium oxysporum*, *F. solani* and *Trametes hirsuta*. Similar observation (absence of antifungal activity) was reported from another species of *Delphinium*, *D.formosum*[12].However, *D.denudatum* has been reported for the presence of antifungal diterpenoid[13].

Quantitative estimation (MIC)

In the MIC experiments, out of seven solvents, acetone, ethanol, and methanol extracts showed strong inhibition of bacterial species. The most affected group was actinobacteria which was supported by the low MIC values of water extracts of leaf, stem, and root (Table 2). Significant variations in MIC recorded in all the extracts demonstrated the role of selection of solvent as well as the type of test microorganisms.

Table 1. Antimicrobial activity of *D. denudatum*plant extracts

Zone of Inhibition (mm)								
Root								
Microorganism		Methanol	Ethanol	Acetone	Ethyl acetate	Water	Hexane	Chloroform
Gram(+)ve	*B. subtilis*	3.00±0.12	2.33±0.11	6.67±0.20	4.00±0.10	ND	5.33±0.11	2.33±0.11
	B. megaterium	3.33±0.23	3.23±0.14	6.63±0.15	4.33 ± 0.12	ND	8.33±0.36	2.67±0.04
Gram(-)ve	*S. marcescens*	4.67±0.11	9.33±0.18	3.00 ± 0.07	ND	ND	6.33±0.11	2.0±0.07
	P. chlororaphis	5.12±0.12	5.67±0.11	3.33± 0.11	5.67 ± 0.25	ND	7.33±0.11	11±0.28
	E. coli	3.33±0.04	4.13±0.25	2.33± 0.04	2.33 ± 0.08	ND	3.33±0.04	4.67±0.15
Actinobacteria	*N. tenirefensis*	ND	ND	ND	ND	17.33 ± 0.08	ND	ND

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Stem								
Gram(+)ve	*B. subtilis*	2.33±0.11	7.00 ± 0.35	5.33 ± 0.15	1.33 ± 0.04	ND	6.12±0.19	2.00±0.07
	B. megaterium	ND	4.00± 0.14	ND	2.33 ± 0.08	ND	3.67±0.11	4.67±0.08
Gram(-)ve	*S. marcescens*	2.61±0.08	15.33± 0.11	2.67 ± 0.04	1.67 ± 0.11	ND	ND	1.33±0.04
	P. chlororaphis	3.53±0.09	6.00± 0.07	2.67 ± 0.11	11.00 ± 0.28	ND	5.67±0.11	3.33±0.11
	E. coli	1.67±0.10	5.33±0.08	1.33 ± 0.04	4.67 ± 0.15	ND	5.03± 0.19	ND
Actinobacteria	*N. tenirefensis*	ND	ND	ND	ND	16.33 ± 0.04	ND	ND
Leaf								
Gram(+)ve	*B. subtilis*	1.67± 0.04	3.00± 0.07	6.00± 0.12	1.67 ± 0.04	ND	4.33±0.11	2.00±0.07
	B. megaterium	ND	5.67 ± 0.35	2.00± 0.07	1.67 ± 0.04	ND	1.67±0.08	3.00±0.07
Gram(-)ve	*S. marcescens*	8.00±0.51	6.33 ± 0.36	2.00± 0.12	2.33 ± 0.16	ND	3.33±0.04	3.67±0.18
	P. chlororaphis	9.00± 0.07	5.33 ± 0.20	3.00± 0.24	11.00 ± 0.07	ND	2.67±0.08	3.33±0.08
	E. coli	3.33± 0.04	6.67 ± 0.08	6.67 ± 0.20	2.00± 0.07	ND	4.67±0.23	4.33±0.15
Actinobacteria	*N. tenirefensis*	ND	ND	ND	ND	21.0 ± 0.07	ND	ND

(ND = activity not detected)

Table 2. Minimum inhibitory concentration (MIC) of *D.denudatum* plant extracts

MIC ug/ml								
Root								
Microorganism		**Methanol**	**Ethanol**	**Acetone**	**Ethyl acetate**	**Water**	**Hexane**	**Chloroform**
Gram(+)ve	*B. subtilis*	500	700	400	800	ND	700	900
	B. megaterium	400	500	400	700	ND	600	800
Gram(-)ve	*S. marcescens*	400	300	500	ND	ND	600	900
	P. chlororaphis	300	500	600	500	ND	600	300
	E. coli	500	600	900	900	ND	900	700
Actinobacteria	*N. tenirefensis*	ND	ND	ND	ND	300	ND	ND
Stem								
Gram(+)ve	*B. subtilis*	700	400	400	800	ND	600	900
	B. megaterium	ND	600	ND	700	ND	700	800
Gram(-)ve	*S. marcescens*	900	300	700	800	ND	ND	900
	P. chlororaphis	500	200	700	300	ND	600	900
	E. coli	800	500	800	700	ND	600	ND
Actinobacteria	*N. tenirefensis*	ND	ND	ND	ND	300	ND	ND
Leaf								
Gram(+)ve	*B. subtilis*	800	600	400	800	ND	800	900
	B. megaterium	ND	500	700	800	ND	900	800
Gram(-)ve	*S. marcescens*	500	300	700	700	ND	800	800
	P. chlororaphis	500	500	700	300	ND	800	700
	E. coli	700	500	500	700	ND	700	600
Actinobacteria	*N. tenirefensis*	ND	ND	ND	ND	200	ND	ND

(ND=activity not detected)

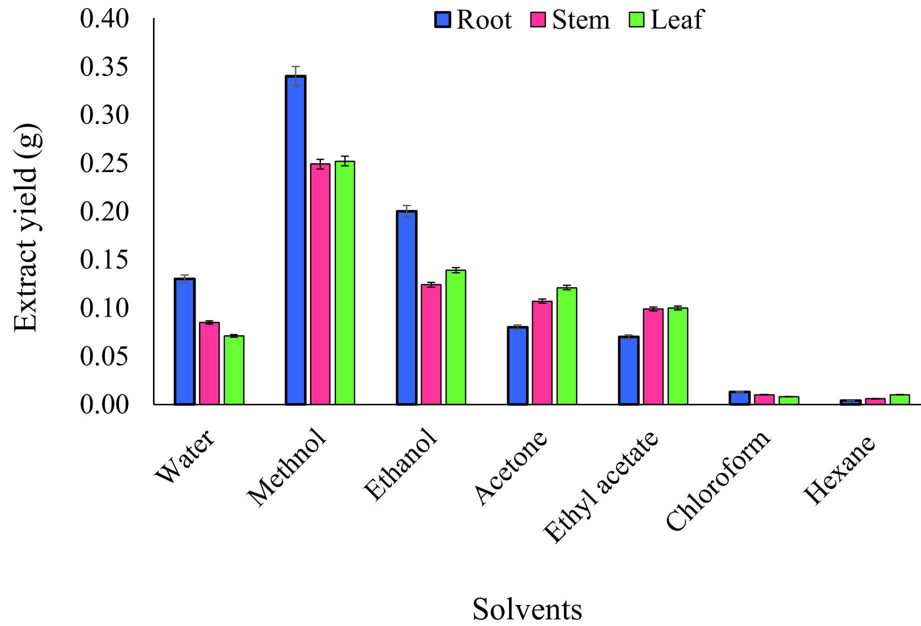

Figure 1. Extract yield of root, stem, and leaf of *D.denudatum*

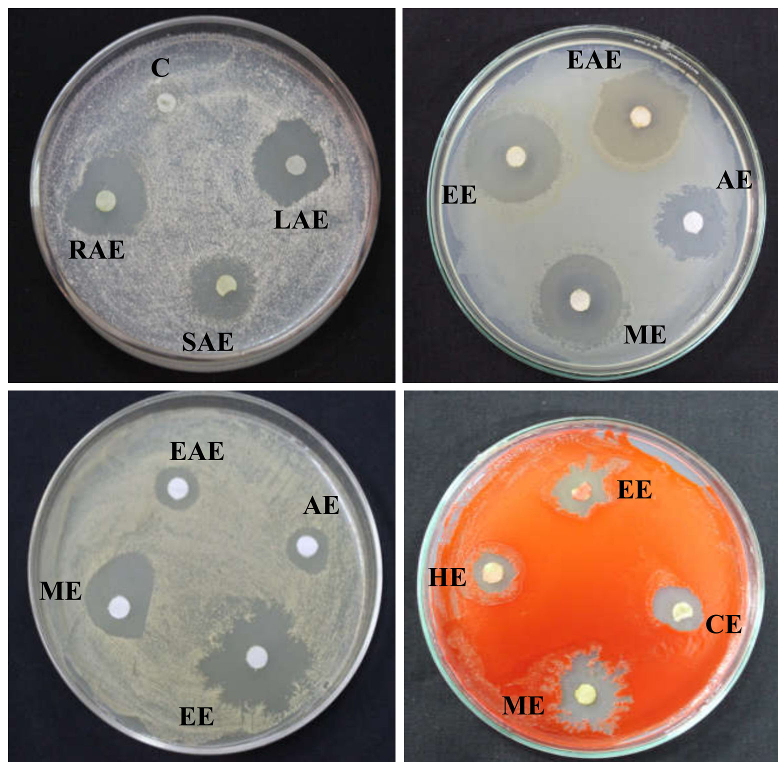

Figure 2. Antimicrobial activity of *D.denudatum*:(A) Antiactinobacterial activity (B) Antibacterial activity. C= Control, EAE= Ethyl acetate extract, ME= Methanolic extract, EE= Ethanolicextract, AE= Acetone extract, CE= Chloroform extract, HE= Hexane extract, LAE= Leaf aqueous extract, SAE=Stem aqueous extract, and RAE= Root aqueous extract

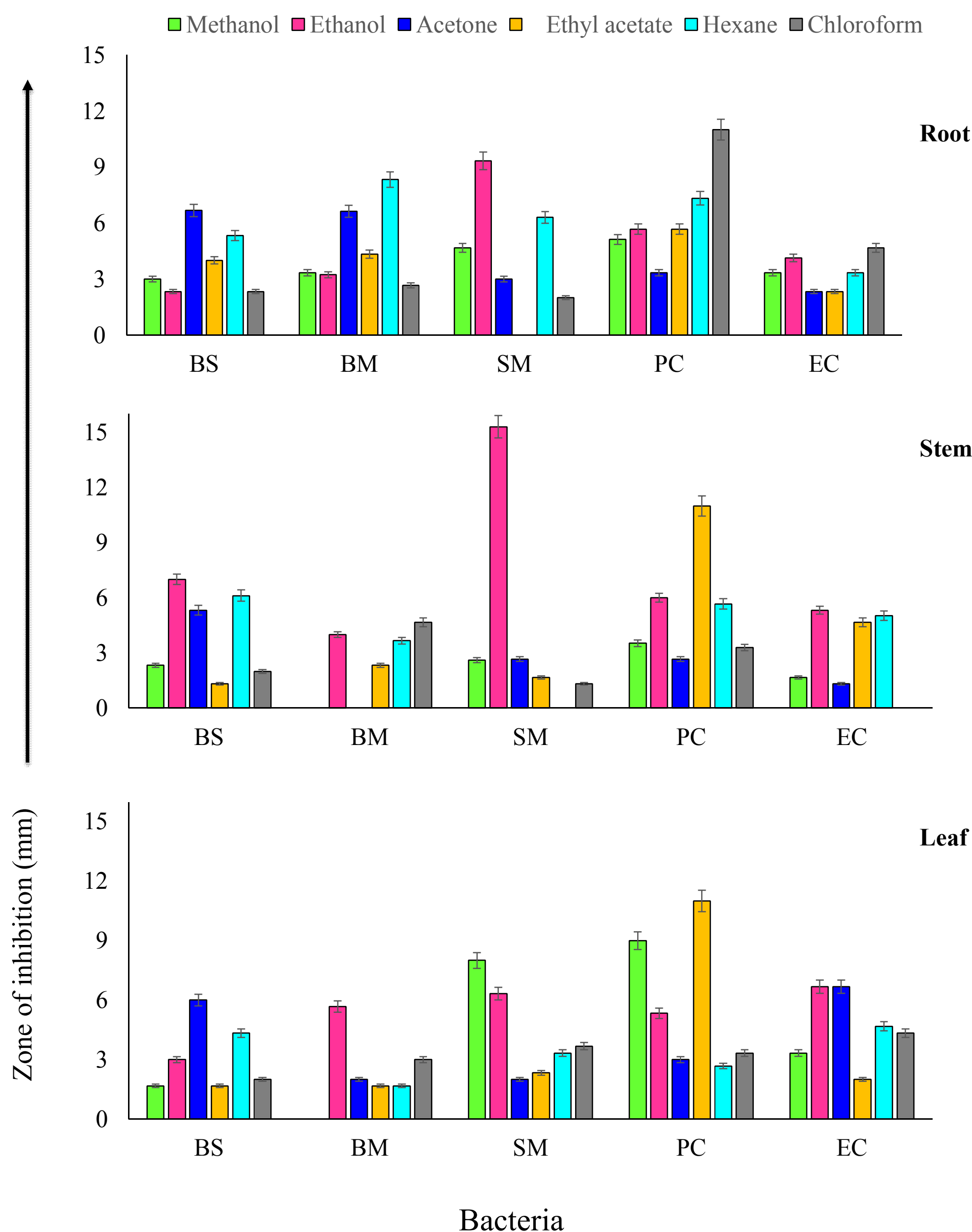

Figure3. Antibacterial activity of leaf, stem, and root of *D.denudatum*: BS=*B.subtilis*,BM=*B.megaterium*, EC=*E.coli*,SM=*S. marcescens*, PC=*P.chlororaphis*

CONCLUSION

On the basis of results obtained, it is concluded that the ethanolic extracts of stem, root, and leaf of *D.denudatum* possessed good antibacterial activity. Besides, aqueous extracts of plant extracts were inhibitory for actinobacteria only, without showing any inhibition of bacteria and fungi. The present study justify the use of *D. denudatum* in the traditional system of the medicine in treating infectious diseases. The experiments performed in the present study are at preliminary stage. These observations shall provide a base for conducting detailed investigations in view of the effect of geographical and climatic conditions on plants in production of secondary metabolites, including antimicrobials.

ACKNOWLEDGEMENT

Authors are grateful to Director GBPINHESD for extending the facilities and Funding agency Himachal Pradesh Power Corporation limited, Sarabai, Distt-Kullu, H.P., for financial assistance.

CONFLICT OF INTEREST STATEMENT

We have no conflict of interest

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CITATION OF THIS ARTICLE

K Kumari, P Adhikari, A Pandey, S.S. Samant and Veena Pande. Antimicrobial potential of *Delphinium denudatum* (Wall Ex Hook & Thom). Bull. Env.Pharmacol. Life Sci., Vol 8 [12] November 2019: 161-167