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RESEARCH ARTICLE

Bioactive constituent of *Delphinium denudatum* Wall. and their Antioxidant efficacy

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Abstract

Delphinium denudatum known as Jadwar, is critically enlarged Himalayan herbs, which was once commonest species. It is distinguished by its relatively small blue or violet color and it is one of the important drugs used as indigenous medicine in India, especially in Unani medicine system. A number of studies have been done on its phytochemical and pharmacological properties. Bioactive constituent were isolated from the petroleum ether-soluble fraction of root of *Delphinium denudatum* and their structures were elucidated as β-Sitosterol based on mass and nuclear magnetic resonance (NMR) spectroscopy. Antioxidant activity of β-Sitosterol was evaluated through DPPH radical scavenging method and it revealed that β-Sitosterol was shown to trap free radicals in a concentration dependent manner as high as 65.02% using 160 µg/mL.

Keywords: *Delphinium denudatum*, pharmacological properties, β-Sitosterol, antioxidant activity, DPPH, NMR.

Introduction

Delphinium denudatum Wall. is extensively found in the western Himalayas and Kashmir at altitudes of 8000-12000 feet, especially on grassy sicpes (Anonymous, 1952; Nadkarni, 1976; Khorey and Katrak, 1985). A number of compounds have been isolated from roots of *D. denudatum*. Diterpenoid alkaloids like condelphine, isotalatizidine, denudatine, talatizidine, vilmorrianonymouse, panicutine, 3-hydroxy-2-methyl-4H-pyran-4-one, acetylhetero-phyllisine have been reported (Rastogi and Mehrotra, 1993; Rahman *et al.*, 1997). It is one of the important drugs used as indigenous medicine in India especially in Unani medicine (Zafar *et al.*, 2003; Qudsia and Jafri, 2006). Its vernacular name is Jadwar. The other natural products reported from this plant are sterols which are campesterol, stigmasterol, and β-sitosterol and fatty acids (Asif *et al.*, 1981).

β-Sitosterol are important bioactive component of medicinal plant extract exhibiting various pharmacological properties such as antiinflammatory, anti-pyretic, antiarthritic, antiulcer, insulin releasing and oestrogenic effects and inhibition of spermatogenesis. β-Sitosterol is mainly known and used for its cholesterol lowering property. The present study was attempted to evaluate the antioxidant activity of the isolated compound from *D. denudatum* and to compare their antioxidant efficacy through DPPH methods.

Materials and methods

Collection of plant rhizome sample: Rhizome (root) of *Delphinium denudatum* (Tamil-Nirbasi) was purchased from khari baoli market, Old Delhi, India. The sample was authenticated by Prof. P. Jayaraman, Ph.D, Director, Plant Anatomy Research Center (PARC), Chennai, Tamil Nadu.

Preparation of crude extract of D. denudatum rhizome: The root sample (*Delphinium denudatum*-rhizome) was washed well and shade-dried. Rhizome were chopped into small pieces and grounded to a fine powder by using pulverizer. The solvent petroleum ether was used for the crude extracts preparation. The powered root material was packed in the filter paper and placed gently in the soxhlet apparatus. The extraction was carried out for 15 cycles at 50°C. The crude extract obtained was further concentrated by using rotary evaporator and used for the further studies.

Isolation of β-Sitosterol from D. denudatum rhizome: The petroleum ether extract of *D. denudatum* root was subjected to column chromatography on silica gel (60-120 mesh size), which was eluted with hexane, hexane-ethyl acetate, ethyl acetate mixtures in increasing order of polarity to yield fine major fractions namely Fraction A (hexane), Fraction B (hexane-ethyl acetate; 95:5), Fraction C (hexane-ethyl acetate; 90:10), Fraction D (hexane-ethyl acetate; 85:15) and Fraction E (ethyl acetate). The eluted fractions were analyzed for the presence of the sterol. The individual fraction were spotted on the TLC plates using capillary tube and separated using hexane/ethyl acetate (7:3) mobile phase. The plates were developed by dipping them in methanolic H_2SO_4 reagent for few sec and are placed in the hot air oven at 60°C for 5 min.

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Appearance of pink color bands on the TLC plates indicates the zones of steroidal nucleus. The fraction B obtained from hexane-ethyl acetate (95:5) were purified by chromatography using silica gel (200-400 mesh size) eluting with hexane-ethyl acetate (90:10) to obtain two sub-fractions F1 and F2. The sub-fraction were treated with methanolic H_2SO_4 reagent, the appearance of pink color band on the F1 sub-fraction on the TLC plates indicates the presence of sterol. The structural details of the isolated compound obtained from the fraction B (sub-fraction F1) were analyzed based on the elemental analysis and spectroscopic evidence (UV-Vis spectroscopy, Infra Red spectroscopy, 1H NMR and 13C NMR spectroscopy and GC-Mass spectroscopy).

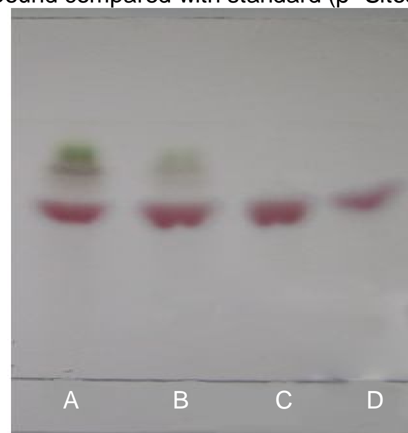
DPPH radical scavenging activity: Free radical scavenging activity of extracts was determined using the 1, 1-diphenyl-2 picrylhydrazyl (DPPH) method according to Saha *et al.* (2004). The activity of scavenging (%) was calculated using the following equation:

DPPH radical scavenging (%) = [(ODcontrol-ODsample)/ ODcontrol] x 100

Results

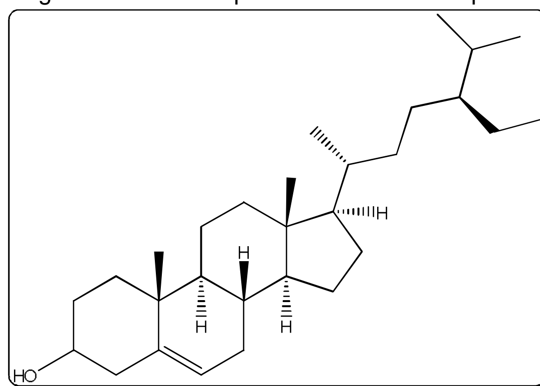
From the positive test for steroids by methanolic H_2SO_4 reagent, it is assumed to be a fraction B which showed clear band with less impurities and pink color band indicating the presence of sterol constituents. Fraction B was further purified to yield sub-fraction F1 by column chromatography using the same solvent system hexane: ethyl acetate (90:10) and finally obtained pure single compound. The purity of the isolated compound was analyzed by thin layer chromatography (Fig. 1). The spot on the TLC chromatogram developed with hexane-ethyl acetate gave a positive result in Libermann-Burrchad test and methanolic H_2SO_4 reagent indicating a steroidal substance. Preliminary identification of the compound was based on the comparison of authentic standard compound. The melting point of the isolated compound was found to 136.2°C. The mass spectra showed a molecular ion peak at m/z 414 $[M+H]^+$ which corresponded to the molecular formula $C_{29}H_{50}O$. The other prominent fragments showed at m/z 399, 396, 303, 273 and 255. IR (KBr) spectrum indicated the presence of OH- stretching, CH-stretching, C=C stretching and C-O-C stretching. The peak assigned 3400 cm^{-1} due to –OH stretching frequency of β- Sitosterol compound. The peak at 2899 cm^{-1} shows the presence of –CH stretching frequency. The C=C stretching shows at 1679 cm^{-1}, 1460 cm^{-1} and C-O-C stretching frequency attributed to 1041cm^{-1}. The ^{1}H NMR of this compound exhibited methyl signals at 0.74 ppm (H-18), 0.83 (H-26), 0.87 (H-27, 29), 1.00 (H-19), 1.02 (H-21). This compound has revealed one proton multiplet at 3.19 (1H, m, C-3H) the position and multiplicity of the steroid nucleus. The proton 5.2 (1H, t, J=6Hz, C-6H) was evident for steroidal skeleton. This compound has shown 29 carbon signal including six methyl, eleven methylene and three quaternary carbons with a hydroxyl group (Fig. 2).

Fig. 1. TLC chromatogram of fraction B, fraction F_1 and isolated compound compared with standard (β- Sitosterol).



A-Fraction B eluted with hexane; ethyl acetate (95: 5); B-Sub-fraction F_1 eluted with hexane; ethyl acetate (90: 10); C-Isolated compound; D-Standard (β-Sitosterol).

Fig. 2. Structural depiction of isolated compound.



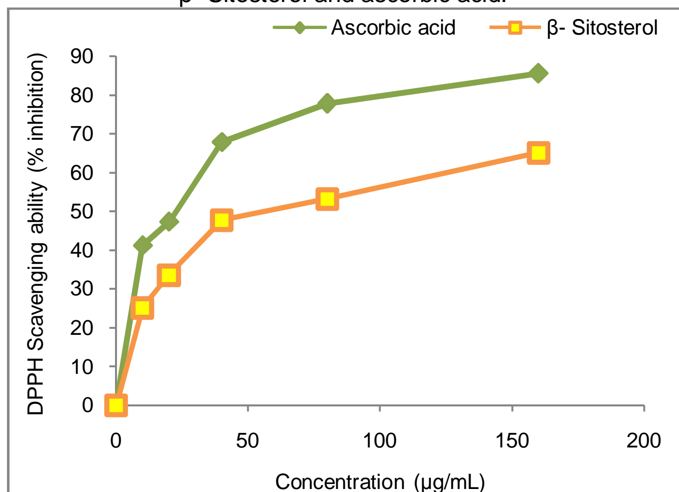
Mol. Wt	:	414
Mol. Formula	:	$C_{29}H_{50}O$
Melting point	:	136.2˚C
UV λ_{max}	:	253 nm (EtOH)
FTIR analysis	:	3400 cm^{-1} (OH- stretching), 2899 cm^{-1} (CH-stretching), 1679 cm^{-1}, 1460 cm^{-1} C=C stretching, 1041 cm^{-1} C-O-C stretching.
Mass spectra	:	m/z: 414 (2.73%, M^+, $C_{20}H_{50}O$), 399 (12.5%, M^+-CH_3), 396 (62%, M^+ H_2O), 381 (18%, M^+ CH, HO), 320 (0.90%), 303 (1.73%), 273 (3.64%), 255 (11.5%)
^{1}HNMR	:	($CDCl_3$, 500 MHz) 0.74 ppm (3H, S, C-18H), 0.81 (H-26), 0.83 (H-27, 29), 1.00 (H-19), 1.02 (3H, S, C-21H), 3.19 (1H, m, C-3H), 5.2 (1H, t, J=6Hz, C-6H).
^{13}C NMR	:	($CDCl_3$, 500 MHz).

^{13}C NMR has given signal at 140.09 and 121.16 ppm for C5=C6 double bond respectively, the carbons of alkenes conjugated are at 140.09 ppm (C5) and 121.16 ppm (C6) which was confirmed from the ^{13}C NMR (Table 1).

Table 1. Structure and molecular weight elucidation of ^{13}C NMR.

Position	Group	Obtained (ppm)
1	CH_2	37.00
2	CH_2	31.09
3	CH	70.89
4	CH_2	42.03
5	C	140.09
6	CH	121.16
7	CH_2	31.89
8	CH	30.20
9	CH	50.17
10	C	36.51
11	CH_2	21.2
12	CH_2	39.69
13	C	42.54
14	CH	55.54
15	CH_2	23.33
16	CH_2	27.78
17	CH	55.28
18	CH_3	11.86
19	CH_3	19.30
20	CH	35.98
21	CH_3	18.09
22	CH_2	33.88
23	CH_3	25.89
24	CH	46.00
25	CH	29.09
26	CH_3	19.78
27	CH_3	18.09
28	CH_2	22.83
29	CH_3	11.98

Fig. 3. DPPH radical scavenging ability of β- Sitosterol and ascorbic acid.



70.89 for C3 β-hydroxyl group 19.30 and 11.86 for angular methyl carbon atoms for C19 and C18 respectively. The free radical scavenging ability of β-Sitosterol was determined by DPPH method and it was compared with ascorbic acid as a standard at various concentrations (Fig. 3). Our results showed a concentration dependent effect that reached a DPPH radical inhibition as high as 65.02 % with 160 µg/mL of β-Sitosterol.

Discussion

Plants contain many bioactive compounds that are nonnutritive plant chemicals rendering defense mechanism to plants. These compound also posses properties that could act as antimicrobial, antioxidant and anticancer agents so the isolation and study of the groups of compound may help to demonstrate their specific properties. Chromatographic separation of the hexane: ethyl acetate fraction F_1 (90:10) over a silica gel G (200-400) mesh size was used to isolate pure compounds that gave positive Liebermann-Burchard test and 10% methanolic sulfuric acid reagent was specific for steroids. Spraying of 10% methanolic sulfuric acid reagent on TLC plate developed the pink color bands indicates the presence of sterol and similar reports were documented by Wagner and Bladt (1996) and Fernand (2003).

The identification of the isolated compound showed a melting point (136.2) and mass (414) and the values were comparable to the standard compound of β-Sitosterol. Furthermore UV, IR, ^{1}H and ^{13}C-NMR data revealed the presence of β- Sitosterol. The spectroscopic evidence obtained in the present study was comparable to the earlier reports. Spectroscopic technique is one of the most powerful and analytical tool for the qualitative and quantitative analysis of bioactive compound (Pateh *et al*., 2009; Muhit *et al*., 2010; Patra *et al*., 2010; Kamboj and Saluja, 2011). β-Sitosterol can scavenge the radicals generated by DPPH method by donating their hydrogen atom to quell the free radicals, indicating that β-Sitosterol has potential antioxidant properties. Furthermore, other reports have shown a significant free radical scavenging capacity of β-Sitosterol, when the compound was examined in plant extracts (Takeoka and Dao, 2003). Yoshida and NiKi (2003) reported antioxidant effects of the β-Sitosterol, stigmasterol and camppesterol, against lipid peroxidation.

Conclusion

The results of the present holistic approach towards the isolation and identification of the antioxidant potential of β-Sitosterol from the extract of *Delphinium denudatum* showed a high activity toward DPPH free radical scavenging assay.

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