

Isolation, Characterization and Cytotoxic Potential of *Desmodium oojeinensis* (Roxb) H. Ohashi: A Threatened Medicinal Plant

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ABSTRACT

Desmodium oojeinensis, which belongs to the family Fabaceae, is an endangered plant native to the Himalayan and sub-Himalayan tract. The current work aimed to carry out physicochemical, phytochemical screening, isolation, and *in vitro* cytotoxic activity using MCF-7 and A-549 cell lines by using the sulforhodamine B method. The physicochemical parameters tested were found to comply with pharmacopoeial limits. The extract's phytochemical screening revealed triterpenoids, alkaloids, anthraquinone glycosides, flavonoids, and carbohydrates. Four phytoconstituents belonging to the class of triterpenoids, viz. betulin, betulinic acid, 16-hydroxybetulin, and lupeol, were isolated from the ethanolic extract by column chromatography. The ethanolic extract showed moderate cytotoxic activity on the human lung cancer cell line A-549 at a concentration of 80 µg/ml and growth inhibition of 81.5%. The extract failed to hinder the growth of the human breast cancer cell line MCF-7.

INTRODUCTION

Medicinal plants have proven to be potent allies since time immemorial by curbing various health ailments. Although synthetic drugs have substituted herbal healing to a certain level, the resurgence and attentiveness of herbal medicines are returning (Acharya and Shrivastava, 2008). Medicinal plants are affordable and ecofriendly, but possess (Tridevi, 2008) fewer side effects than synthetic drugs. Among the modern drugs in use today, about 40% are of natural origin. Approximately 60% of anti-cancer remedies and 75% of drugs for infectious diseases are natural or derivatives (Samuelsson and Bohlin, 2009). Among the numerous plants available in nature, few of them have attracted scientists' interest to investigate cancer treatment. Phytoconstituents have played a crucial role in developing leads, which are proved to be clinically valuable in treating neoplasms (Shah B *et al.*, 2010).

Desmodium oojeinensis (Roxb) H. Ohashi is a medicinal plant that belongs to the family Fabaceae. It is commonly known as sandan, Tinsa in Hindi, and Ratha in Sanskrit. It is a deciduous tree

that is distributed among the Himalayan tracks up to an altitude of 1,500 mts and spread across the whole of northern and central India. Traditionally, it is used for various ailments, such as an astringent, stimulant, anti-inflammatory, urinary astringent, acrid anthelmintic, cooling, and rejuvenating (Kirtikar and Basu, 2006; Nadkarni, 1976). Scientifically, the extract from various parts of the *D. oojeinensis* plant were screened for anti-inflammatory, analgesic, antispasmodic, hepatoprotective, wound healing, and antimicrobial activities (Khare, 2004; Mandrekar *et al.*, 2014; Sahu and Roy, 2009; Sahu *et al.*, 2008, 2009). Phytoconstituents, namely genistin, kempferol, lupeol, butuline, hydroxy lupeol, and isoflavones, like dalvergioidin, homoferreirin, and Eugenie, from various parts of the plant have been reported (Balakrishna *et al.*, 1962; Ghosh and Dutta, 1965; Mukherjee *et al.*, 1963).

The current work aimed to accomplish physicochemical, phytochemical screening, isolation, and *in vitro* cytotoxic activity using MCF-7 and A-549 cell lines by using the sulforhodamine B (SRB) method.

MATERIALS AND METHODS

Collection and authentication

The fresh bark of the stem was collected from fully grown *D. oojeinensis* (Roxb) H. Ohashi plants from Chitradurga, Karnataka, India. It was authenticated by Dr. K. Gopalkrishna

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Bhat, Department of Botany, Poornaprajna College, Udupi. The archived specimen is banked at Goa College of Pharmacy (GCP/Ph.cog 2019/P001) for future reference.

Physicochemical parameters

Various physicochemical parameters, such as swelling index, moisture content, foaming index, ash values, and extractive values, were determined as per the World Health Organization's guidelines (Anonymous, 1998).

Extraction and preparation of extract

Coarsely powdered 400 g of air-dried stem bark was extracted with ethyl alcohol (95%) for a period of 3 days at room temperature. The ethanolic layer was decanted off. The operation was repeated thrice. By the rotary vacuum evaporator, the solvent was recovered, and the residue was evaporated and concentrated to a syrupy consistency and then dissipated to dryness (4.25% w/v).

Preliminary phytochemical screening

Preliminary phytochemical studies were carried out on the ethanolic extract of the bark of the stem of *D. oojeinensis* to check for the presence of chemical constituents like alkaloids, carbohydrates, glycosides, flavonoids, triterpenoids, steroids, tannins, phenolic compounds, proteins, resins, starch, etc. (Khandelwal, 2010; Kokate *et al.*, 2006; Shah and Seth, 2010).

ISOLATION OF PHYTOCONSTITUENTS

15 g ethanolic extract was dissolved in ethanol and mixed with 15 g silica gel (#60–120) for adsorption. The adsorbed sample was loaded on a column previously packed with 250 g of silica gel (#60–120) using Pet. ether. The column was eluted with different solvent systems in increasing order of their polarity, starting with Pet. ether (60–80) 100%, followed by Pet. ether: CHCl₃-graded mixtures (95:5, 90:10, 70:30, 50:50, 30:70, and 10:90). This was followed by CHCl₃ 100% and then CHCl₃:EtOAc graded mixtures, (95:5, 90:10, 70:30, 50:50, 30:70, and 10:90). Finally, the column was eluted with EtOAc and then with, EtOAc:MeOH-graded mixtures (99:1, 98:2, 95:5, 90:10, 85:5, and 80:20). The elutions were collected and observed by TLC (silica gel GF 254, visualization under UV 254 and 366), and then identical elutes were combined, concentrated, and processed further to obtain the chemical constituents. The fraction obtained by elution with chloroform (100%) was washed with n-Hexane to afford a white amorphous powder DO 1 (45 mg). The fraction obtained by elution with CHCl₃:EtOAc (70:30) resulted in a white amorphous powder DO 2 (48 mg). The fraction eluted with EtOAc (100%) was recrystallized with MeOH to obtain buff-colored crystals DO 3 (90 mg). EtOAc:MeOH (95:5) eluent gave white crystals DO 4 (40 mg). The MP, IR, NMR, and mass spectroscopic analysis of the isolated compounds were carried out and the structures were identified with the reported values.

In vitro cytotoxic activity

In vitro cytotoxic activity of the ethanolic extract of *D. oojeinensis* was executed by SRB assay. The sample was prepared in dimethyl sulfoxide (DMSO) to obtain the concentration of 100 µg/ml. Human breast cancer cell lines, MCF-7, and human lung cancer cell line, A-549, were seeded at a density of 10³ per

well in a 96-well Petri plate. The plates were incubated after the addition of samples at concentrations of 10, 20, 40, and 80 µg/ml and incubated at 37°C for 48 hours in a 5% CO₂ incubator. The assay was ceased by the addition of cold trichloroacetic acid (TCA). 30% w/w TCA (50 µl) was used to root the cells *in situ* and incubated for 60 minutes at 4°C. The supernatant was rejected, and the plates were rinsed with tap water, and allowed to air-dry. All the wells were supplemented with 50 µl SRB solution (0.4% w/w in 1% acetic acid). The plates were incubated for 20 minutes at 27°C. 1% w/w acetic acid was used to remove unbound dye, and the plates were dried. The bound stain was eluted with 10 µM Trizma base and absorbance was taken at 540 nm. The values were recorded in triplicate and compared with the standard adriamycin (10–80 µg). Growth (%) was calculated on the plate-by-plate basis for tested wells compared to control wells. Growth (%) was articulated using the following formula: Average absorbance of test wells × 100 / Average absorbance of control wells (Houghton *et al.*, 2007; Lamkanfi *et al.*, 2008).

Statistical analysis

Mean ± standard deviation (S.D.) was used to express the values.

RESULTS AND DISCUSSION

The results of physicochemical and phytochemical investigations are depicted in Tables 1 and 2, respectively. The physicochemical investigation of the powdered bark of the stem of *D. oojeinensis* was found to be within the limits of pharmacopoeial standards. A preliminary phytochemical investigation of the ethanolic extract disclosed the existence of alkaloids, carbohydrates, flavonoids, triterpenoids, steroids, tannins, phenolic compounds, and anthraquinone glycosides.

Betulin (DO 1) was isolated as white amorphous powder that was analyzed for the molecular formula C₃₀H₅₀O₂ by ESIMS *m/z* 442.5 [M⁺]. The mp was 255–256°C, IR spectrum showed bands (cm⁻¹) at 3,387.43 (br, OH), 2,942.21 (C-H str in CH₃), 1,453.44 (C-H deformation in germinal dimethyl), 1,033.40 (C-O str in 2° alcohol). The analysis of ¹H NMR (400 MHz, DMSO, δ, ppm): 4.64 (2H, s, H-29b), 4.53 (2H, s, H-29a), 3.75–3.76 (2H, d, *J* = 5.44 Hz, H-28), 3.07–3.10 (1H, m, H-3), 2.95–3.00 (1H, m, H-19), 2.37–2.38 (2H, t, *J* = 5.32 Hz, H-15), 1.84–1.93 (6H, m, H-16,21,22), 1.63 (3H, s, H-30), 1.45–1.61 (8H, m, H-1,2,6,13,18), 1.23–1.27 (3H, t, *J* = 5.04 Hz, H-7,9), 1.05–1.17 (4H, m, H-11 & 12), 0.98 (3H, s, H-26), 0.93 (3H, s, H-27), 0.88 (3H, s, H-23), 0.77 (3H, s, H-25), 0.72 (3H, s, H-24), 0.61–0.66 (1H, t, H-5); C¹³ NMR (400 MHz, DMSO, δ ppm): 39.32 (C1), 18.72 (C2), 79.15 (C3), 39.52 (C4), 54.83 (C5), 17.92 (C6), 36.63 (C7), 40.40(C8), 49.81 (C9), 38.26 (C10), 24.73 (C11), 26.60 (C12), 38.44 (C13), 42.14 (C14), 27.99 (C15), 29.29 (C16), 47.34 (C17), 47.30 (C18), 48.14 (C19), 150.17 (C20), 28.95 (C21), 33.78 (C22), 27.09 (C23), 15.62 (C24), 15.83 (C25), 15.64 (C26), 14.43 (C27), 57.97 (C28), 109.43 (C29), 20.32 (C30).

Betulinic acid (DO 2) was isolated as white amorphous powder that was analyzed for the molecular formula C₃₀H₄₈O₃ by ESIMS *m/z* 456.5 [M⁺]. The mp was 317–318°C, IR spectrum showed bands (cm⁻¹) at 3,399.10 (br, OH), 2,950.70 (C-H str in CH₃), 1,714.21(C=O str in acid), 1,451.40 cm⁻¹ (C-H deformation in germinal dimethyl), 1,023.20 cm⁻¹ (C-O str in 2° alcohol). The

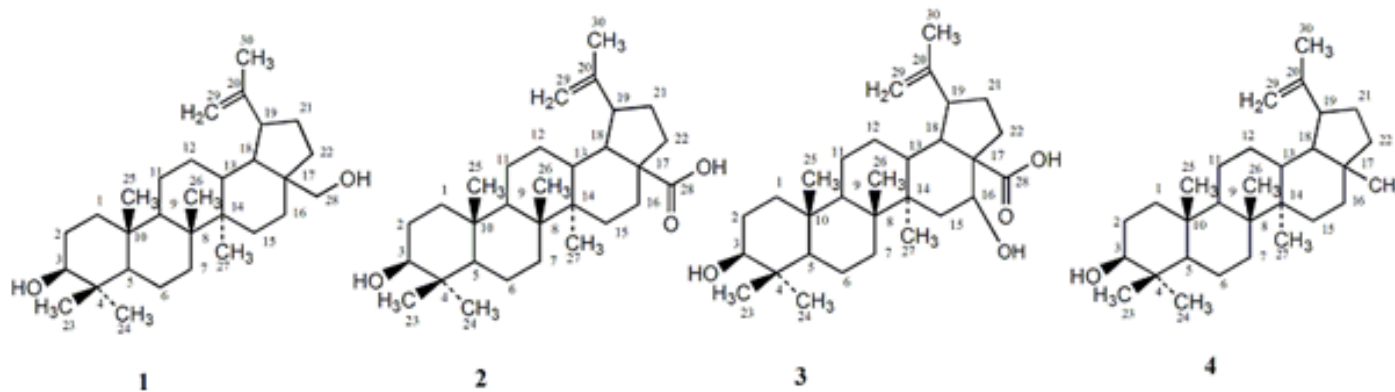


Figure 1. Structures of isolated phytoconstituents: betuline (**DO 1**) [1], betulinic acid (**DO 2**) [2], 16-hydroxybetulinic acid (**DO 3**) [3], and lupeol (**DO 4**) [4].

analysis of ^1H NMR (400 MHz, DMSO, δ , ppm): 4.66 (2H, s, H-29b), 4.59 (2H, s, H-29a), 3.50–3.56 (2H, m, H-3,19), 2.37–2.43 (3H, m, H-13,15), 1.75 (3H, s, H-30), 1.48–1.57 (7H, m, H-2,18,21,22), 1.34–1.45 (6H, t, $J = 6.92$ Hz, H-6,7,16), 1.15–1.26 (5H, m, H-9,11 & 12), 1.11 (3H, s, H-23), 1.09 (3H, s, H-26), 1.05 (3H, s, H-27), 1.02 (3H, s, H-24), 0.97 (3H, s, H-25), 0.72–0.78 (3H, m, H-1,5); ^{13}C NMR (400 MHz, DMSO, δ , ppm): 39.04 (C1), 27.39 (C2), 78.49 (C3), 39.11 (C4), 55.57 (C5), 18.55 (C6), 33.78 (C7), 40.44 (C8), 50.10 (C9), 38.44 (C10), 21.07 (C11), 24.73 (C12), 38.78 (C13), 42.17 (C14), 29.92 (C15), 32.32 (C16), 47.37 (C17), 48.14 (C18), 49.47 (C19), 150.15 (C20), 30.28 (C21), 38.26 (C22), 27.95 (C23), 16.32 (C24), 16.51 (C25), 16.77 (C26), 15.75 (C27), 180.87 (C28), 108.48 (C29), 19.12 (C30).

16-Hydroxybetulinic acid (DO 3) was isolated as buff-colored crystals that were analyzed for the molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_4$ by ESIMS m/z 472.5 $[\text{M}^+]$. The mp was 271–273°C, IR spectrum showed bands (cm^{-1}) at 3,351.40 (br, OH), 2,952.47 (C-H str in CH_3), 1,712.71 (C=O str in acid), 1,451.41 cm^{-1} (C-H deformation in geminal dimethyl), 1,026.20 cm^{-1} (C-O str in 2° alcohol). The analysis of ^1H NMR (400 MHz, DMSO, δ , ppm): 4.71 (2H, s, H-29b), 4.60 (2H, s, H-29a), 4.18–4.22 (1H, dd, $J = 5.2, 6.36$ Hz, H-16), 3.49–3.55 (2H, m, H-3,19), 2.35–2.45 (1H, m, H-13), 1.811–1.83 (2H, d, $J = 7.32$ Hz, H-15), 1.77 (3H, s, H-30), 1.51–1.61 (7H, m, H-2,18,21,22), 1.36–1.49 (6H, m, H-1,6,7), 1.15–1.26 (5H, m, H-9,11 & 12), 1.11 (3H, s, H-23), 1.08 (3H, s, H-26), 1.04 (3H, s, H-27), 1.00 (3H, s, H-24), 0.87 (3H, s, H-25), 0.74–0.76 (1H, t, $J = 9.24$ Hz, H-5); ^{13}C NMR (400 MHz, DMSO, δ , ppm): 39.07 (C1), 27.39 (C2), 78.55 (C3), 39.15 (C4), 55.43 (C5), 18.54 (C6), 34.26 (C7), 40.45 (C8), 50.11 (C9), 37.38 (C10), 21.10 (C11), 24.73 (C12), 38.26 (C13), 42.17 (C14), 29.90 (C15), 79.67 (C16), 49.17, (C17), 49.54 (C18), 49.73 (C19), 150.55 (C20), 30.27 (C21), 37.00 (C22), 27.96 (C23), 16.31 (C24), 16.51 (C25), 16.75 (C26), 15.72 (C27), 180.57 (C28), 109.08 (C29), 19.10 (C30).

Lupeol (DO 4) was isolated as white crystals that were analyzed for the molecular formula $\text{C}_{30}\text{H}_{50}\text{O}$ by ESIMS m/z 426.4 $[\text{M}^+]$. The mp was 210–212°C, IR spectrum showed bands (cm^{-1}) at 3,374.46 (br, OH), 2,934.35 (C-H str in CH_3), 1,635.57 (C=C str), 1,463.74 cm^{-1} (C-H deformation in geminal dimethyl), 1,034.49 cm^{-1} (C-O str in 2° alcohol). The analysis of ^1H NMR (400 MHz, DMSO, δ , ppm): 4.65 (2H, s, H-29b), 4.57 (2H, s,

H-29a), 3.07–3.10 (1H, m, H-3), 2.95–3.00 (1H, m, H-19), 2.35–2.38 (2H, t, $J = 4.08$ Hz, H-15), 1.83–1.91 (2H, m, H-21), 1.67 (3H, s, H-30), 1.50–1.62 (8H, m, H-1,2,6,13,18), 1.23–1.37 (7H, m, H-7,9,16,22), 1.06–1.17 (4H, m, H-11,12), 1.02 (3H, s, H-27), 0.95 (3H, s, H-26), 0.92 (3H, s, H-25), 0.81 (3H, s, H-24), 0.77 (3H, s, H-23), 0.75 (3H, s, H-28), 0.61–0.62 (1H, t, $J = 3.52$ Hz, H-5); ^{13}C NMR (400 MHz, DMSO, δ , ppm): 38.01 (C1), 27.47 (C2), 79.09 (C3), 38.78 (C4), 55.30 (C5), 18.33 (C6), 33.78 (C7), 40.16 (C8), 50.40 (C9), 37.71 (C10), 21.07 (C11), 25.13 (C12), 37.92 (C13), 42.84 (C14), 27.39 (C15), 35.58 (C16), 48.25 (C17), 48.14 (C18), 48.03 (C19), 150.00 (C20), 28.52 (C21), 40.00 (C22), 28.11 (C23), 15.32 (C24), 16.30 (C25), 15.85 (C26), 14.52 (C27), 18.01 (C28), 109.04 (C29), 19.47 (C30).

In-vitro cytotoxic study

The cytotoxic study results on human lung cancer cell lines, A-549, and human breast cancer cell lines, MCF-7, are given in Tables 3 and 4. The result indicates moderate cytotoxic activity against A-549 cell lines at 80 $\mu\text{g}/\text{ml}$ with growth inhibition of 81.5% when compared to the standard adriamycin with percentage growth inhibition of <10 $\mu\text{g}/\text{ml}$. The extract failed to show *in vitro* cytotoxic activity against MCF-7 cell lines. The majority of pentacyclic triterpenoids have proved to possess cytotoxic activity. Betulin and betulinic acid exhibit effective anti-cancer activity by activating the mitochondrial apoptosis pathway in tumor cells (Hordyjewska *et al.*, 2018). Chemical investigation led to the isolation of terpenoids having lupine moiety, isolated from the extract of *D. oojeinensis*, which may be responsible for moderate cytotoxic activity against human lung cancer cell lines A-549.

CONCLUSION

The current research work on the bark of the stem of *D. oojeinensis* was successfully explored. The physicochemical parameters tested were found to be within the pharmacopoeial limits. Chemical examination of the extract was directed to the separation of four triterpenoids, namely betulin, betulinic acid, 16-hydroxybetulinic acid, and lupeol. Based on a literature survey, betulin and betulinic acid exhibited significant cytotoxic activity. The ethanolic extract showed moderate cytotoxic activity in A-549. The activity might be endorsed due to betulin and betulinic acid, which have been isolated from *D. oojeinensis*. Apoptosis

and *in vivo* studies will further potentiate its claim as an effective cytotoxic agent in lung cancer treatment.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve the use of animals or human subjects.

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