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Investigation on the Phytochemical Analysis and Biological Activity of *Daphne papyracea* Wall. ex Steud. Leaf Extracts from Kumaun Himalaya

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Abstract: The aim of the present research is to examine the leaf extracts of *D. papyracea* Wall. ex Steud. for their phytochemicals and biological activity. Antimicrobial activities were determined by the disc diffusion method against gram-negative and positive bacterial strains. Qualitative estimations were done for the presence of various phytochemical classes. Quantitative determination of total phenolics, total flavonoids, and two *in vitro* antioxidant activities (DPPH and FRAP) was carried out using spectrophotometric methods. Phytochemical analysis unveiled the presence of cardiac glycosides, coumarins, and flavonoids in DM, DE, and DC extracts, but saponins were absent in all leaf extracts of *D.papyracea*. Antimicrobial activity tests revealed that only ethyl acetate extract (DEE) showed positive results at a zone of 14mm against the MTCC-1522 strain. The highest phenolic content was detected in hexane leaf extract (39.69±0.11 mg GAE/g), while the highest flavonoid content was found in methanolic leaf extract, i.e., 131.8±0.15 mg QE/g. The radical scavenging activity measurement was expressed in terms of IC_{50}, and the methanolic extract exhibited the highest scavenging activity (60.50±0.01µg/mL). The highest reducing power (163.29±0.01 mg AA/g) was shown by the methanolic leaf extract. This study showed that *D.papyracea* is serviceable and may have a future in the pharmaceutical and cosmetic industries. Phytochemicals found in this also have virtuous efficacy.

Keywords: antioxidant; spectophotometric; antimicrobial; cardiac glycosides; coumarins.

1. Introduction

Daphne papyracea Wall. ex Steud. belongs to the family Thymeleaceae, is an evergreen shrub that grows at an elevation zone of 1600-2500m in the Himalayan region. The genus *Daphne* is scattered in East Asia- the Himalayas from Pakistan to Central Nepal [1,2], including 44 families with approximately 500 herbal species. The bark/fruits are used as a dye-yielding natural material. China is the primary center of the evolution of this genus. It is a much-branched, erect, evergreen shrub with smooth grey bark, dull green narrow lanceolate to oblanceolate leathery leaves, and scented white or greenish-white flowers [2]. The shrub grows up to a maximum height of 3-4m and a diameter of 2m [3].

The plant flowers from November to April and the fruits are fleshy, at first orange, then turn to deep red when fully ripe. Because of the existence of many species and subspecies in the genus Daphne, the taxonomy is very complex and complicated. The species prefers growing

gregariously in the understorey of both coniferous and broad-leaved forests and prefers medium (loamy) and heavy (clay) soils, requires well-drained soil and prefers acid, neutral, and basic (alkaline) soils [4].

Different parts of the *Daphne* plant contain specific bioactive metabolites, which can potentially be used in pharmaceutical, cosmetic, and food industries[5]. Interestingly, diterpenoids from *Daphne* species exhibit potent anti-cancer activities against various types of cancers both *in vitro* and *in vivo* [6-8].

Daphne species (Thymelaeaceae) have shown a variety of pharmacological actions. The antileukemic activity showed on P-388 lymphocytic cells in mice from merezin obtained from *D. mezereum* [9]; odorocin is a nematocidal compound from *D.odora* [10], and daphnetin 8-glucoside from *D. acuminata* [11]possessed cardiotoxic activity. The roots of *D.genkwa* [12] have been used against chistosomiasis, and flavonoids from *D. papyracea* [13]have shown sedative and hypotensive effects [14]. *D. mucornata* has been used to cure skin, allergies, and cancer [15]. The leaf and flower of *D. sericea* have been used as traditional remedies for treating hemorrhoids [16]. *Daphne gnidium* L. bark has been used as a diuretic to treat toothache and hepatitis [17].

In many areas of the world like Asia, Europe, and Africa, other species of *Daphne* are used to treat several ailments, such as bruises, rheumatisms, diarrhea, inflammations, cancer, sore throat, indolent ulcers, snake bites, laryngitis, malaria, fever and apoplexy [6].

The interest in the plants of the *Daphne* genus is increasing because of the numerous beneficial medicinal properties of these plants that can be of potential clinical significance [5]. Previous literature on the traditional and economic uses of *D.papyracea*reported the ethanol-medicinal importance of the plant [18]. Marpa *et al*. reported that the leaf and root of the plant are used for fiber and fodder [19]. Traditionally, *D. papyracea* has been acknowledged to cure intestinal complaints [19], high blood pressure [20], and skin diseases [21].

As far as we are aware, there is no published report on the phytochemistry and biological activities of *D. papyracea.* Therefore, the present study assessed the phytochemical, antioxidant, and antibacterial activity of *D. papyracea* collected from Kumaun Himalaya.

2. Materials and Methods

2.1. Collection of plant material and preparation of extract.

The required plant part (leaf) was collected randomly from the Nainital catchment area at the altitude of 2,279 m a.s.l. in February 2021. The identity of the collected specimens was authenticated by (Dr. G.C Joshi, Ex Scientist in charge RARI CCRAS, Ranikhet). The voucher specimen was deposited in the Botany department, D. S. B. Campus, Nainital. The freshly collected *D.papyracea* leaves were washed and allowed to dry naturally, i. e., under shade, at room temperature, and powdered. After completion of the drying process, the material was ground in a grinder, and the powder was kept in an appropriately labeled plastic bottle.

Ten grams of the dried powder were taken in a flask, and 100 mL of hexane was added, incubated at 37°C for 5 days, and then filtered using Whatman No.1 filter paper. The extracted solvent was kept for drying, and the remaining filtered powder was re-extracted/ fractionated with other solvents (methanol; DME, ethyl acetate; DEE, chloroform; DCE, and hexane; DHE). A hundred mg/mL final stock of each extract was prepared by dissolving the obtained extract in 25% DMSO (in case of methanol extract) and isopropanol (in case of ethyl acetate, chloroform, hexane extract) Upadhyay *et al*. [22]. The dried crude extract was 1.238g for

methanol extract, 0.255 g for chloroform extract, 0.331 g for ethyl acetate extract, and 0.297g for hexane extract.

2.2. Qualitative phytochemical analysis.

Qualitative analysis of a major group of secondary metabolites such as saponins, flavonoids, coumarins, and cardiac glycoside was carried out as described below:

2.2.1. Test for coumarins.

Two milliliters of each extract were treated with 3 mL of 10% NaOH. The presence of coumarins is indicated by the formation of yellow color.[23].

2.2.2. Test for saponins (foam test).

Two milliliters of each extract were taken in a test tube, and 6 mL of distilled water was added to it. The mixture was then shaken vigorously. The persistence of foam was observed, indicating the presence of saponins [23].

2.2.3. Test for flavonoids (alkaline reagent test).

Two milliliters of the extract were treated with a few drops of 1N NaOH solution and observed the formation of intense yellow color. This yellow color becomes colorless with the addition of dilute hydrochloric acid, showing the presence of flavonoids Shah *et al*. [24].

2.2.4. Test for cardiac glycosides.

Five milliliters of each solvent extract was mixed with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This solution was further under layered with 1mL conc. H_2SO_4. Browning on the interface indicated a deoxy sugar characteristic of cardenolides. A violet ring might appear below the brown ring, whereas a greenish ring in the acetic acid layer might form gradually throughout the thin layer (Khatak *et al*. [25].

2.3. Quantitative analysis.

2.3.1. Total phenolic content.

Total phenolic content is a quantitative assay that lays out a rapid, subtle, and easy way of measuring the antioxidant capacity of biological samples and is estimated by Folin-Ciocalteu's phenol reagent (FCR) method following Upadhyay *et al*. [26] with little modification. Different concentrations (1, 2, 4, 9, 18, 35, 75, 150, 300 mg/mL) of standard and test samples were taken and prepared in different solvents (methanol, hexane, ethylacetate, and chloroform) from the stock solution. 0.5mL of each concentration was mixed with 0.2 mL of FCR and 0.5 mL Na_2CO_3 (20%). After incubation of 30 minutes at room temperature, at 750 nm, the absorbance was measured against blank. From the calibration curve of a standard gallic acid solution, the total phenolic content concentration was calculated as mg gallic acid equivalent/g extract.

2.3.2. Total flavonoid content.

To assess total flavonoid content (TFC), the aluminum trichloride ($AlCl_3$) method was used. Different concentrations (4, 9, 18, 35, 75, 150, and 300 µg/mL) of standard and test samples were prepared in different solvents from the stock solution. Half milliliter of each concentration was mixed with 0.2 mL of $AlCl_3$, 0.5mL $NaNO_3$ (20%). By letting the solution at room temperature for 30 min, ultimately, the absorbance was measured at 506 nm by spectrophotometer. The concentration of total flavonoid content was calculated as mg quercetin acid equivalent/g extract from the calibration curve of the standard solution of quercetin acid following the methodology of Garg and Garg [27].

2.4. Antibacterial assay.

Antibacterial assay of the extracts of *D. papyracea* was performed by disc diffusion method in nutrient agar (NA) plates against one Gram-negative (*Aeromonas salmonicida*; MTCC-1522) and two gram-positive bacteria (*Bacillus subtilis*; 441 and *Streptococcus mutans*; 497).The test organisms were inoculated in nutrient broth (NB) and incubated overnight at 37°C to adjust the turbidity to 0.5 McFarland standards giving a final inoculum of 1.5×108 CFU/mL. NA plates were plated with 100 µL of microbial culture broth. Discs of 6mm were bored in the inoculated media with the help of a sterile cork-borer (6mm). Each well was filled with different concentrations of extracts that were added to the disc. Ampicillin (1mg /mL) was taken as positive control while DMSO and diol propanol as negative/solvent control. It was allowed to diffuse for 30 minutes at room temperature and incubated for 18-24hours at 37°C. After incubation, a clear zone around the well was observed for the tested compounds, measured in mm.

2.5. Antioxidant activity.

2.5.1. DPPH (1,1-Diphenyl -2-picrylhydrazyl) free radical scavenging assay.

The radical scavenging activity of leaf extracts was determined by using DPPH assay, according to Chang *et al*. [28]. Ascorbic acid (1mg/mL in methanol) was used as a reference. 1, 1 Diphenyl 2- picryl hydrazyl is a stable (in powder form) free radical with red color which turns yellow when scavenged. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Each plant extracts (Methanol, hexane, ethyl acetate and chloroform) sample stock solution (1mg/mL) was diluted in final concentration of (5, 10, 50, 100, 150, 200, 250, 300 µg/mL). At room temperature in the dark condition, the reaction mixture was incubated for 20 min, and then the optical densities were measured at 593 nm using UV–Vis spectrophotometer. All the determinations were performed in triplicate. The % radical scavenging activity of the plant extracts was calculated using the following formula,

$$\% \text{ RSA} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs control}} \times 100$$

2.5.2. Ferric Reducing Antioxidant Power (FRAP).

FRAP (Ferric ion reducing the ability of plasma) assay was conducted to determine the reducing power of *D.papyracea* as described by Benzie and Strain [29] with few modifications.

The present assay is a typical ET-based method that measures the reduction of ferric ion (Fe^{3+})-ligand complex to the intensely blue-colored ferrous (Fe^{2+}) complex by antioxidants in an acidic medium. Briefly, the FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 mM TPTZ (prepared in 40 mM HCl), and 20 mM ferric chloride in a ratio of 10:1:1. Different extracts (methanol, chloroform, hexane, ethyl acetate) were prepared by integrating2 g of leaf extract in 10 mL of absolute methanol. 200 µL of the extract was mixed with 6 mL FRAP reagent, diluted in final concentrations (10, 50, 100, 200, 300 µg/mL), and kept in the dark for 30 min. Thereafter the optical densities of the solution were measured at 593 nm using a UV-Vis spectrophotometer, and reducing capacity was determined as mg AA/g.

3. Results and Discussion

3.1. Phytochemical screening.

Phytochemicals are non-nutritive plant chemicals possessing a high degree of preventive properties against various diseases. Table 1 shows the results of the preliminary phytochemical screening carried out on the leaf extract of *D. papyracea.* The diterpenoids are considered representative components of the genus *Daphne*[30]. The present results showed the presence of some secondary metabolites, i.e., cardiac glycosides, flavonoids, and coumarins, in selected extracts. But the saponins were absent in all the *D. papyracea* extracts (Table1).

Table 1. Phytochemical analysis of *D. papyracea*

Test	ME	EE	CE	HE
Cardiac glycoside	+++	++	+	-
Coumarins	++++	++	++	-
Flavonoids	+++	++	++	-
Saponins	-	-	-	-

(+): Present; (++, +++, ++++): Highly present, ME: Methanol extract, EE: Ethyl acetate extract, CE: Chloroform extract, HE: Hexane extract

Ulubulen *et al*. evaluated the phytochemical composition of a Soxhlet ethanolic extract from *D. sericea* collected in Turkey and reported the presence of only flavonoid compounds in the extract[31]. According to Ayhaan *et al.* [14], flavonoids and coumarins were present in *D. gniioides,* while Amira *et al.* [32] delineate the appearance of tannins, flavonoids, and coumarins in a similar plant. Rassol *et al*. reported that in ethyl acetate extract, three compounds (cardiac glycosides, flavonoids, and coumarins) were present in *D. mucornata* [33]. Sovrlić *et al*. [34] also described the presence of coumarins and flavonoids in the leaf and twig extracts of *D. alpina* and similar compounds seen in the preliminary phytochemical screening of the leaf and twig of *D. malyana* observed by Jusković *et al*. [35]. More than 150 compounds consisting of various classes, including coumarins, flavonoids, lignans, diterpenes, and miscellaneous ingredients, among which coumarins and flavonoids were the two major classes, were identified from phytochemical screening on *D. giraldii* [36-39].

3.2. Antibacterial activity of the extract.

The leaf extracts of *D. papyracea* were also examined for antibacterial activity against three standard strains of bacteria. For positive control, ampicillin was taken. The results revealed that antibacterial activity was exhibited by methanol, hexane, and chloroform extracts at a very high concentration range. Antibacterial activity was observed only in ethyl acetate

extract against the MTCC-1522 strain. The zone of inhibition was measured to be 9 mm, 14 mm, 16 mm at a concentration range of 20-80 µg/mL Table 2 and Fig. 1.

Table 2. Zone of inhibition (mm) of ethyl acetate extracts of *D. papyracea* and standard.

Concentration (µg/µL)	Ampicilin (Standard) (mm)	Ethyl acetate extract (mm)
5	19	-
15	24	-
20	26	9
25	29	-
30	-	-
40	-	14
50	-	-
60	-	-
70	-	-
80	-	16

The ethyl acetate extract inhibited the growth of *Aeromonas salmonicida* with the MIC value of 10µg/µL. The isopropanol was taken as a negative control. The chloroform and methanol extracts of *D. alpina* showed a comparatively higher MIC value (31.25 µg/mL) against *S. aureus*, *K. pneumoniae*, *E. coli*, *P. vulgaris*, *C. albicans*, and *A. niger* [34]. In the methanol extract of *D. cneorum* leaves, the highest susceptibility was exhibited by *P. vulgaris* ATCC 13315 (MIC = 15.62 µg/mL), as reported by Manojlović *et al*. [40].

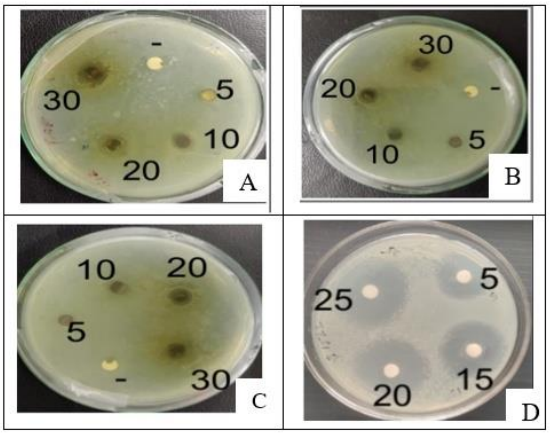

Figure 1. (a, b, c) Antibacterial activity of ethyl acetate extract of *D. papyracea* leaf at different concentrations (5, 10, 20, 30 µg/mL) against MTCC-1522; (d) Positive control is ampicillin at different concentration (5, 15, 20, 25 µg/mL).

3.3.Quantitative assays and antioxidant activity.

Plant polyphenols are a significant group of compounds acting as primary antioxidants; therefore, it is legitimate to examine the total phenolic content (TPC) in the plant extract. TPC (mg/mL) in the study was determined using Folin-Ciocalteu's phenol reagent and a calibration curve of the standard was drawn to evaluate TPC ($Y = 10.01x - 0.911$; $R^2 = 0.947$; Figure 2).

Table 3 summarizes that the total phenolic compound in *D. papyracea* wasranging from 25.49±0.08 and 39.69±0.11 mg GAE/g. The highest phenolic content was found in hexane leaf extract (39.69±0.11 mg GAE/g), followed by ethyl acetate (33.08±1.81 mg GAE/g), methanolic (27.31±0.21 mg GAE/g) and the lowest phenolic content was found in chloroform extract (25.49±0.08 mg GAE/g) of *D. papyracea* (Table 3).

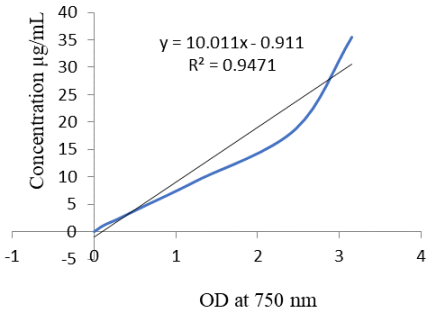

Figure 2. Standard curve of gallic acid for total phenolic content, OD: Optical density.

The TPC in the methanolic and chloroform leaf extracts of *D. alpina* was observed to be 85.88±0.97 mg GAE/g and 78.98±0.67 mg GAE/g as reported by Sovrlic *et al*. [34]. Manojlovic *et al*. [41] observed that the methanol extract (77.45±0.43 mg GA/g) and the chloroform leaf extract (76.56±0.89 mg GA/g) of *D. blagayana*were found to have a higher amount of TPC than present findings. Similarly, Manojlovic *et al*. reported that the phenolic content in the methanolic leaf extract of *D. cneorum* was found to be 76.45±0.79 mg GA/g[40].

The total flavonoid content was assessed using an aluminum chloride reagent and calculated from quercetin's calibration curve (Y = 64.79x - 47.92; R² = 0.826; Figure 3).

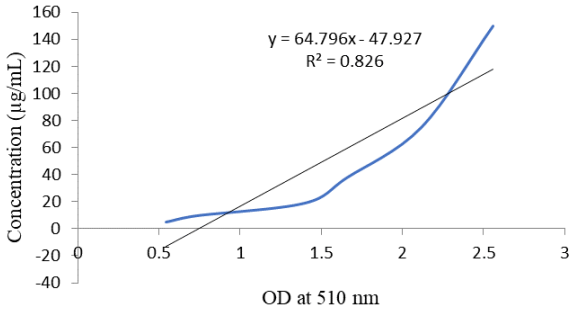

Figure 3. Standard curve of quercetin for total flavonoid content, OD: Optical density.

The highest flavonoid content was observed in the methanol leaf extract (131.8±0.15 mg QE/g), followed by ethyl acetate (21.16±0.08 mg QE/g), chloroform (15.33±0.03 mg QE/g) and the lowest flavonoid content was found in hexane extract (11.79±0.08 mg QE/g) of *D. papyracea* (Table 3). The most abundant flavonol, which has a good antioxidant property, is quercetin, which has all the right structural features for free radical scavenging activity [42]. The TFC in the methanolic and chloroform leaf extracts of *D. alpina* was 32.65±0.89 mg RU/g and 28.09±0.85 mg RU/g [34]. Similar results were reported by Manojlovic *et al*. [40], with total flavonoid content of 24.67±0.35 mg RU/g in the methanolic leaf extracts of *D. cneorum.* Manojlovic *et al*. also reported that the methanolic and chloroform leaf extracts of *D. blagayana* contained 27.95±0.88 mg RU/g and26.79±0.35 mg RU/g TFC [41].

The antioxidant ability and radical scavenging properties of plants are related to their medicinal values. DPPH scavenging activity (IC_{50} values) of *D. papyracea* leaf extract in different solvents is shown in Table 3. The methanolic extract showed the highest antioxidant activity (60.50±0.01µg/mL), followed by ethyl acetate (96.49±0.005 µg/mL), hexane extracts (121.90±0.03 µg/mL), while the chloroform extract had the minimum radical scavenging activity (142.24±0.005 µg/mL). The results of FRAP assay of *D. papyracea* leaf extract are shown in Table 3.

Table 3. DPPH assay, FRAP assay, Phenolic and Flavonoid content of leaf extract of *D. papyracea.*

***D.papyracea* extract**	**DPPH Assay IC_{50} (µg/mL)**	**FRAP Assay (mg AA/g)**	**Total phenolic content (mg GA/g)**	**Total flavonoid content (mg QE/g)**
Methanol	60.50±0.01	163.29±0.01	27.31±0.21	131.8±0.15
Chloroform	142.24±0.004	42.43±0.01	25.49±0.08	15.33±0.03
Ethyl acetate	96.49±0.005	62.24±0.008	33.08±1.81	21.16±0.08
Hexane	121.90±0.03	24.75±0.04	39.69±0.11	11.79±0.08

AA: Ascorbic acid, GA: Gallic acid, QE: Quercetin

The methanolic leaf extract showed the highest reducing power (163.29±0.01 mg AA/g) followed by ethyl acetate (62.24±0.008 mg AA/g), chloroform (42.43±0.01 mg AA/g) extracts. The least reducing power was found in hexane extract (24.75±0.04 mg AA/g). The trend confirmed the dependency of FRAP activity on the flavonoid content of *D. papyracea*. In a previous study, Manojlovic *et al*. reported that the methanolic leaves extract of *D. cneorum* showed good scavenging activity (22.79±0.94 µg/mL) against DPPH assay[40].The scavenging effect on DPPH radical was observed to be higher in methanolic extract (20.95±0.99 µg/mL) than in the chloroform leaf extract (25.24±0.15 µg/mL) of *D. blagayana*[41]. The chloroform (25.45±0.89 µg/mL) and methanol (23.15±1.05 µg/mL) leaf extracts of *D. alpina* showed more antioxidant activity than our results [34]. *D. mucornata* showed good scavenging activity (0.5 ±0.06µg/mL) against DPPH radical [43]

4. Conclusions

Researches on unexplored and new plant species provide a very significant source of plant antioxidant and bring light to new natural products in the pharmaceutical and food industry for their everyday battle with reactive oxygen species. From the overall scenario, it is concluded that *D.papyracea*was found to have phytochemicals such as coumarins, flavonoids, and cardiac glycosides but completely lacks saponins in all the extracts. Out of all secondary metabolites, coumarins were found to be abundant. To treat congestive heart failure and cardiac arrhythmia, cardiac glycosides have been used. Also, the present analysis sheds light on the antimicrobial activity of *D.papyracea*. The phenolic and flavonoid content in *D. papyracea* leaf extracts makes it medicinally important. The present study indicated good scavenging activity in FRAP and DPPH radical scavenging assays. In this sense, we can conclude that *D.papyracea* has a smattering potential to serve society as it provides promising chemicals like coumarins. Further study is needed to examine the exact active principle responsible for this biological activity.

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Conflicts of Interest

The authors declare no conflict of interest.

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