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Research Article

EVALUATION OF PHENOLIC COMPOUNDS, FLAVONOIDS AND ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF METHANOLIC AND AQUEOUS LEAVES AND BRANCHES OF *DAPHNE GNIDIUM L.*

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ABSTRACT

Daphne gnidium L. (Thymeleaceae) are evergreen shrubs native to Asia, Europe, and North Africa. Various species of *Daphne* are used in several folk medicines to treat gonorrhoea and cutaneous affections, rheumatoid arthritis, wound healings, malaria and anti-inflammations. The study was designed to evaluate the phenolic, flavonoid contents and antioxidant and antimicrobial activities of methanolic and aqueous leaves and branches of *Daphne gnidium*. Antioxidant activity was determined by DPPH free radical scavenging capacity and the reducing power activity, the antimicrobial activity was tested with three bacterial strain and one fungi including yeast (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC27853, *Bacillus subtilis* ATCC6633 and *Candida albicans* ATCC1024). Our results showed that the aqueous and methanolic leaves extract reported a considerable free radicals scavenging activity and reducing effect, because the extracts are richness on polyphenols and flavonoids.

Keywords: *Daphne gnidium*; Antioxidant; Antimicrobial; Phenolic and flavonoid contents

INTRODUCTION

Plants are potential sources of natural antioxidants, and certain species are particularly significant because they may be used for the production of raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits [1]. There is an upsurge in demand of plant materials containing phenolic as they retard oxidative degradation of lipids and thereby improving quality and nutritional value of food [2-4].

Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species [5,6]. Polyphenol compounds are widely studied for their antioxidant properties, although the term antioxidant has a broad range of meanings. For the purposes of

this review, antioxidant activity refers to both the ability of polyphenol compounds to prevent damage from reactive oxygen species (ROS) (such as through radical scavenging) or to prevent generation of these species (by binding iron). As described in the title, the primary focus will be on polyphenol–iron interactions as a mechanism of antioxidant activity [7].

Daphne gnidium is an evergreen shrub that grows in the Mediterranean area and can grow to a height of 2 m [8]. In folk medicine the infusion of the leaves is used as hypoglycemic [9] and to treat skin diseases [10,11]. This plant is also used in traditional textile dyeing [12]. The main objectives of this study were to determine the phenolic, antioxidant and antimicrobial activities of *Daphne gnidium* extracts.

MATERIALS AND METHODS

Plant material

The random sampling were used during the harvesting, the areal parts of *Daphne gnidium* L., were harvested from Beni Fouda north East of Setif determined by Dr. Nouioua Wafa.

Preparation of aqueous extracts

The method for preparing aqueous extracts from the dried plant has been already described by [13]. Briefly, dried plant material (10 g) was stirred in 100 ml of distilled water for 15 min at 90°C followed by rapid filtration through four layers of gauze and then by a more delicate filtration through Whatman filter paper #1. The resulting filtrate evaporated to dryness under vacuum. The powder was stored at –10°C until required

Preparation of methanol extract

The areal parts were powdered and macerated in 80% methanol for 24, 48 and 72 hours, at the laboratory temperature (1:10 w/v, 10 g of dried herb). After maceration, the extracts were collected, filtered and evaporated to dryness under vacuum [14]. The dry extract was stored at a temperature of -18°C for later use.

Determination of Total Phenolic Content

For total polyphenol determination, the Foline Ciocalteu method was used [15]. The samples (0.2 mL) were mixed with 1 mL of the Folin-Ciocalteu reagent previously diluted with 10 mL of deionized water. The solutions were allowed to stand for 4 minutes at 25°C before 0.2 mL of a saturated sodium carbonate solution (75 mg/mL) was added. The mixed solutions were allowed to stand for another 120 minutes before the absorbance were measured at 765 nm. Gallic acid was used as a standard for the calibration curve. The total phenolic compounds content was expressed as mg equivalent of Gallic acid per gram of extract (mg EAG/GE).

Determination of total flavonoids content

The flavonoids content in crude extract were estimated by the Aluminium chloride solution according to the method described by [16]. Briefly, 1 mL of the methanol solution of the extract was added to 1 mL of 2% AlCl₃ in methanol. After 10 minutes, the absorbance was determined at 430 nm. Quercetin was used as a standard. Results were expressed as mg equivalent. Quercetin per gram of extract (mg EQ/GE).

DPPH assay

The donation capacity of extract was measured by bleaching of the purple-coloured solution of 1, 1-

diphenyl-2-picrylhydrazyl radical (DPPH) according to the mentioned method [17]. One millilitre of the extract at different concentrations was added to 0.5 mL of a DPPH-methanol solution. The mixtures were shaken vigorously and left standing at the laboratory temperature for 30 minutes in the dark. The absorbance of the resulting solutions were measured at 517 nm. The antiradical activity was expressed as IC₅₀ (micrograms per millilitre). The ability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH \text{ scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where:

A₀: the absorbance of the control at 30 minutes

A₁: is the absorbance of the sample at 30 minutes. BHT was used as standard [18].

Reducing power

The reducing power was determined according to the method of Oyaizu [19]. The extract (2.5 mL) was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 10 mg/mL potassium ferricyanide. The mixtures were incubated at 50°C for 20 minutes; after cooling, 2.5 mL of 100 mg/mL trichloroacetic acid were added and the mixtures were centrifuged at 200g for 10 minutes. The upper layer (5 mL) was mixed with 5mL of deionized water and 1 mL of 1 mg/mL ferric chloride, and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. EC₅₀ value (mg extract/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. BHA was used as standard [20].

Antimicrobial activity

Bacteria Strains were obtained from the American Type Culture Collection: (*Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC6633 and *Candida albicans* ATCC1024). Muller Hinton agar was used for bacteria culture and Sabouraud for yeast.

Anti-bacterial activity

Agar disc diffusion method was employed for the determination of antibacterial activities of *Daphne gnidium* extracts [21,22]. Briefly, a suspension of the tested microorganism (10⁸ CFU/mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µL (100 mg/mL) of the extract and placed on the inoculated plates. These plates were incubated at 37°C for 24 hours. Gentamicin (10 µg/disc) was used as a standard and dimethylsulfoxide DMSO as a control.

The antibacterial activity was determined by measuring of inhibition zone diameters (mm) and was evaluated according the parameters suggested by Wayne P [22]:

- <9 mm, inactive;
- 9–12 mm, less active;
- 13–18 mm, active;
- >18 mm, very active.

Antifungal activity

The antifungal activity was tested by disc diffusion method with modifications [21]. *Candida albicans* ATCC1024 suspension was obtained in physiological saline 0.9% from a culture in Sabouraud (incubated before 24 hours at 37°C), adjusted to 10⁵ CFU/mL.

One hundred microliter of the suspension was placed over agar in Petri dishes and dispersed. Then, sterile paper discs (6 mm diameter) were placed on agar to load 10 µL (100 mg/mL) of each sample. Amphotericin 100 µg was used as standard and dimethylsulfoxide DMSO as control. Inhibition zones were determined after incubation at 27°C for 48 hours.

Statistical analysis

Results were expressed as mean ± standard deviation in triplicates. Data was statistically analysed using t test of Student as primary test followed by Fisher test with the criterion of P <0.05 to determine whether there were any significant differences between methanol extract of *C. africanum* and standards, using Graphpad prism 5 Demo Software.

RESULTS AND DISCUSSION

Metabolite composition (phenolic and flavonoid contents) is the important factor for determination of biological activities such as anticancer, antiviral, anti-inflammation, and antimicrobial of plant extracts. [23-25]. The results of yield and Total phenolic and total flavonoid contents of methanolic and aqueous leaves and branches of *Daphne gnidium* extracts are shown in Table 1.

Plant materials	Extracts	Yield%	Polyphenols (mg EAG/GE)	Flavonoids (mg EQ/GE.)
Leaves	Methanolic	10.2%	426.46 ± 6.00	56.09 ± 0.22
	Aqueous	5.2%	194.55 ± 5.41	29.74 ± 7.53
Branches	Methanolic	9.6%	278.76 ± 3.33	40.93 ± 5.91
	Aqueous	13.4%	277.61 ± 2.9	42.89 ± 2.31

Table 1: Yield, polyphenols and flavonoids quantification of of methanolic and aqueous leaves and branches of *Daphne gnidium*.

From this results, it is easily to conclude that the highest total polyphenol and flavonoid contents were found in methanolic leaves (426.46 ± 6.00 mg GAE/g and 56.09 ± 0.22 mg QE/g, respectively). Phenolic compounds are ubiquitous in plants. Flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignins, are important in the plant for normal growth development and defense against infection and injury. These compounds are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity [26].

DPPH scavenging

The DPPH assay has been widely used to determine the free radical scavenging activity of various plant extracts. The IC₅₀ for DPPH scavenging activity in various extract methanolic and aqueous leaves and branches of *Daphne gnidium* are shown in Table 2. The IC₅₀ of *Daphne* extracts increased in the order of aqueous leaves extract (16.71 ± 0.82 mg/mL), methanol leaves extract (42.27 ± 4.76 mg/mL), methanolic branches extract (69.09 ± 5.51 mg/mL) and aqueous branches

extract (77.51 ± 8.21 mg/mL), respectively. Flavonoids and phenolic acids are classified as mixed antioxidants [27-29] (because they are able to donate protons to free radicals, and are still capable of preventing the formation of reactive oxygen species (ROS) either by the inhibition of enzymes involved in the process, and by chelating metal traces involved in their production [27,28]).

Plant materials	Extracts	IC ₅₀ (µg/mL)
Leaves	Methanolic	$42.27 \pm 4.76^{**}$
	Aqueous	$16.71 \pm 0.82^{***}$
Branches	Methanolic	$69.09 \pm 5.51^*$
	Aqueous	77.51 ± 8.21
BHA		4.47 ± 0.37
***: highly significant difference; ** very significant difference; * significant difference with P < 0,001		

Table 2: IC₅₀ of standard, methanolic, and aqueous leaves and branches of *Daphne gnidium* for the DPPH test.

Reducing power assays

Reducing power assays are often used as an indicator of electron–donating activity, which are an important mechanism of antioxidant compounds, especially phenolics. Therefore, potassium ferricyanide was applied to evaluate reducing power potentials of *Daphne* extracts. (Table 3). Reducing power of extracts and standard compound (BHT) decreased in the following order: BHT>aqueous leaves extract>methanol leaves extract>methanol branche extract>aqueous branches extract, with absorbance of ($7,91 \pm 0,12$, 32.62 ± 1.12 , 32.62 ± 1.12 , 68.09 ± 3.62 and 89.31 ± 2.21 mg/mL) respectively. Reducing power of a compound set referred to its electron transfer capacity in a redox reaction, leading to the conversion of free radicals in less reactive or inert products. However, in addition to stabilizing the radical cation, the buffer systems by controlling the ratio of the protonated or deprotonated states of the antioxidants could result in an induced antioxidant activity [29]. Phenolic compounds are also reported to be effective hydrogen donors, making them very good antioxidants [30].

Plant materials	Extracts	IC ₅₀ (µg/mL)
Leaves	Methanolic	$32.62 \pm 1.12^*$
	Aqueous	$32.62 \pm 1.12^{**}$
Branches	Methanolic	68.09 ± 3.62
	Aqueous	89.31 ± 2.21
BHT		$7,91 \pm 0,12$
***: Highly significant difference; ** very significant difference; * significant difference with P < 0.001		

Table 3: IC₅₀ values of of methanolic and aqueous leaves and branches of *Daphne gnidium* for reducing power test.

The results of antimicrobial activity screening are showed in Table 4 of methanolic and aqueous leaves and branches of *Daphne gnidium*.

Plant materials	Extracts	<i>Escherichia coli</i> ATCC 25922	<i>Pseudomonas aeruginosa</i> ATCC27853	<i>Bacillus subtilis</i> ATCC6633	<i>Candida albicans</i> ATCC1024
Leaves	Methanolic	8 ± 0,12	7 ± .23	7.56 ± 1.52	10,12 ± 2.11
	Aqueous	7 ± 036	7,5 ± 2.01	7 ± 2.15	9 ± 3.04
Branches	Methanolic	9 ± 014	8.28 ± 2.14	7.5 ± 0.54	8 ± 012
	Aqueous	6.75 ± 2.15	7 ± 3.12	7.25 ± 1.56	9.25 ± 2.14
Standard		18,50 ± 0,41	18,53 ± 0,41	23,83 ± 0,62	15,58 ± 0,12
Control		NI	NI	NI	NI

Table 4: Antimicrobial activity of standards and of methanolic and aqueous leaves and branches of *Daphne gnidium*.

The antibacterial activity of flavonoids has been increasingly documented and many research groups have identified the chemical structures endowed with anti-bacterial activity. Flavonoids can inhibit bacterial growth using different mechanisms including the inhibition of nucleic acid synthesis, particularly flavonoids with bring hydroxylation [31,32].

CONCLUSION

The results of this study showed that the methanolic and aqueous leaves and branches of *Daphne gnidium* have considerable amounts of phenolic and flavonoid compounds. Both dried showed the higher radical scavenging activity of DPPH and reduction power. The antioxidant activity may be as a result of the presence of different molecules or substances no determined in this study which are present in the extracts.

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