

Evaluation Of Phytochemical and Antioxidant Activities of *Daphne Gnidium*

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Abstract. This study aimed to quantify the phenolic and flavonoid contents of *Daphne gnidium* leaf extracts and evaluate their antioxidant properties. *Daphne gnidium*, a species from the *Thymelaeaceae* family, was extracted using methanol and ethyl acetate as solvents through two methods: sonication and Soxhlet extraction. The polyphenol and flavonoid contents were determined using spectrophotometric assays and were found to vary significantly depending on the extraction method. The sonicated extracts yielded between 67.57 and 70.12 μg gallic acid equivalents (GAE) per 100 mg of dry weight, while Soxhlet extracts ranged from 68.53 to 87.11 μg GAE/100 mg. Similarly, total flavonoid content ranged from 3.63 to 4.93 μg quercetin equivalents (QE) per 100 mg in sonicated extracts, and from 4.14 to 4.72 μg QE/100 mg in Soxhlet extracts. The antioxidant activity was evaluated using the DPPH radical scavenging assay and the β -carotene bleaching method. The extracts showed strong antioxidant potential, with IC_{50} values in the DPPH assay ranging from 0.42 to 1.60 $\mu\text{g}/\text{mL}$, and an IC_{50} of 0.815 ± 0.007 mg/mL in the β -carotene bleaching assay. Notably, the ethyl acetate extract obtained via Soxhlet and the methanolic extract obtained via sonication exhibited the highest antioxidant activities. Also, the two extracts tested, the Soxhlet extraction by ethyl acetate and the methanolic extract by sonication gave the most active sample of *Daphne gnidium* extracts with are more active than the reference extract. **Keywords:** *Daphne gnidium*, Phenolic compounds, Flavonoids, Antioxidant activity, Sonication and Soxhlet

1 Introduction

Reactive oxygen species (ROS) can induce lipid peroxidation in food products during processing and storage, leading to the deterioration of food quality and safety. One of the most effective strategies to eliminate or reduce ROS activity is the use of antioxidant compounds, which can interrupt free radical chain reactions. However, the use of commonly employed synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has raised health concerns due to their potential adverse effects on human health [1-3]. In contrast, numerous medicinal plants and their bioactive

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constituents have been reported to possess significant antioxidant activity, offering a promising natural alternative [4, 5].

Northern Morocco is renowned for its rich diversity of medicinal plants, many of which are endemic to the region. These plants are particularly notable for their high content of phenolic and flavonoid compounds, which contribute to their pronounced antibacterial and antioxidant activities [6-8].

Daphne gnidium L., a species belonging to the family *Thymelaeaceae*, is a medicinal plant native to the Mediterranean basin, including regions of Northern Africa, Southern Europe, and the Middle East [9]. It is well known for its wide range of pharmacological properties, attributed to its rich composition of bioactive compounds. Previous studies have demonstrated that *Daphne gnidium* exhibits antioxidant, antibacterial, anti-inflammatory, antitumor, antiproliferative, and pro-apoptotic activities [9–20]. In Morocco, this plant is commonly used in traditional medicine and frequently cited in ethnobotanical surveys. Despite its widespread use, there is a lack of comprehensive pharmacological and phytochemical studies conducted locally to validate its traditional applications. In particular, *Daphne gnidium* holds a significant place in the ethnomedical practices of the Al-Hoceima province.

In this context, our study aimed to evaluate the total phenolic content and compare the antioxidant activity of methanol and ethyl acetate extracts obtained from the leaves of *Daphne gnidium*, a plant collected in the Al-Hoceima province of northern Morocco.

2 Materials and methods

2.1 Plant material

The plant samples were collected in February 2023 from the Al Hoceima province in northern Morocco and were authenticated at the former National Institute of Medicinal and Aromatic Plants. The collected material was dried using hot air at 40°C for 48 hours, after which the leaves were ground into a fine powder.

2.2 Soxhlet extraction

Soxhlet extraction was performed following the protocol described by Vongsak et al. (2013), with minor modifications [21]. A total of 160 g of the powdered plant material was placed into a cellulose cartridge, which was fixed onto the main chamber of the Soxhlet apparatus and topped with a condenser. The extraction was carried out using 2 L of ethyl acetate placed in the flask. The process continued for approximately one week, until the solvent in the siphon tube became increasingly clear, indicating exhaustive extraction. The remaining plant residue was subsequently re-extracted with 2 L of methanol, following the same procedure, until the solvent again appeared clear.

2.3 Sonication extraction

Ultrasound-assisted extraction was performed using a CY-500 sonicator (JP Selecta S.A., Spain) operating at a power of 500 W and a frequency of 20 kHz [22]. A total of 25 g of powdered plant material was mixed with 250 mL of solvent in a sonicator flask. The probe was immersed approximately 4 cm into the mixture, and ultrasound was applied for 45 minutes using an intermittent cycle of 3 seconds on and 1 second off, resulting in a total treatment time of 60 minutes.

2.4 Qualitative phytochemistry

Qualitative characterization tests are based either on the formation of insoluble complexes using precipitation reactions, or on the formation of colored complexes using coloring reactions.

2.4.1 Polyphenol Revelation

Flavonoid Detection: To detect flavonoids, mix 5 mL of the alcoholic plant extract with a few drops of concentrated hydrochloric acid (HCl). Then add approximately 0.5 g of magnesium turnings. The formation of a pink or red coloration confirms the presence of flavonoids [23].

Tannin Detection: To test for tannins, add one drop of 2% ferric chloride (FeCl₃) in alcohol to 2 mL of the alcoholic plant extract. The development of a blue-black or green coloration indicates the presence of polyphenolic compounds such as tannins. Gallic acid serves as a positive control, exhibiting a characteristic color change for comparison [24]

2.5 Quantitative analysis

2.5.1 Dosage of total polyphenols and total flavonoids

The total polyphenol content (TPC) was estimated using the Folin–Ciocalteu (FC) assay, a widely accepted method for routine analysis [25]. Briefly, all samples and standard gallic acid were dissolved in 50% (v/v) aqueous methanol. TPC was calculated from a gallic acid standard curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method. TFC was calculated from a quercetin standard curve and expressed as milligrams of quercetin equivalents (QE) per gram of extract.

2.6 Antioxidant activity

2.6.1 Trapping of the free radical DPPH

The antioxidant activity of the plant extracts was assessed using the DPPH method [26], with slight modifications, based on their hydrogen-donating or radical-scavenging abilities. Briefly, 2.5 mL of each extract was mixed with 2.5 mL of a methanolic DPPH solution in test tubes [27]. The mixtures were incubated in the dark at 30 °C for 30 minutes. Following incubation, absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ Anti-radical activity} = [(Abs_control\ 517 - Abs_sample\ 517) / Abs\ control\ 517] \times 100$$

Abs_control_517: Absorbance of DPPH solution at 517 nm without extract or standard.

Abs_sample_517: Absorbance at 517 nm with extract or standard.

The antioxidant activity was expressed as the IC₅₀ value, representing the concentration of extract needed to reduce the initial DPPH radical concentration by 50%. A lower IC₅₀ value reflects greater radical scavenging activity.

2.6.2 β -Carotene Bleaching Test

In this assay, antioxidants in the plant extracts inhibit the oxidative degradation of β -carotene by neutralizing free radicals. This action prevents the bleaching of β -carotene, indicating antioxidant activity [38].

To prepare the emulsion, 2 mg of β -carotene were dissolved in 10 mL of chloroform. From this solution, 1 mL was mixed with 200 mg of Tween 20 and 20 μ L of ascorbic acid. The chloroform was then evaporated under reduced pressure. Next, 100 mL of diluted hydrogen peroxide were added, and the mixture was stirred vigorously to form a stable emulsion.

For the assay, 0.1 mL of each plant extract or the standard antioxidant, butylated hydroxytoluene (BHT), at various concentrations, was added to 2 mL of the prepared β -carotene/linoleic acid emulsion. The samples were incubated at 50 °C for 120 minutes, and absorbance was measured at 470 nm. A control was prepared in the same way, substituting the extract with the solvent used in extraction.

The antioxidant activity (AA) was calculated as a percentage of β -carotene bleaching inhibition using the following formula:

$$\text{Percentage of inhibition (\%)} = [(A_{E(120)} - A_{T(120)}) / (A_{T(0)} - A_{T(120)})] \times 100$$

$A_{E(120)}$: Absorbance of the experimental group (E) at 120 minutes.

$A_{T(120)}$: Absorbance of the treatment/control group (T) at 120 minutes.

$A_{T(0)}$: Absorbance of the treatment/control group at 0 minutes.

3 Results and discussion

3.1 Comparative Analysis of *Daphne gnidium* Yield Using Two Extraction Methods

Daphne gnidium was extracted using two different methods Soxhlet extraction and ultrasound-assisted extraction, in order to compare their extraction yields. Results are shown in the Table below.

Table 1. Extract Yields of *Daphne gnidium* Using Soxhlet and Ultrasound-Assisted Extraction Methods

Plant material	Methods	Extract	Yield %
<i>Daphne gnidium</i>	Soxhlet	Ethyl acetate	6.94
		Methanol	29.76
	sonication	Ethyl acetate	15.2
		Methanol	10.52

We observe that, among the two extracts obtained by Soxhlet extraction, the methanolic extract presents the highest yield (29.76 %), followed by the extract in ethyl acetate with a yield of (6.94 %).

In Soxhlet extraction, the yield generally increases with the polarity of the solvent. This is because polar solvents are more effective at dissolving a wide range of polar phytochemicals present in plant material. In contrast, during sonication extraction, the highest yield was observed with ethyl acetate. This may be due to the mechanical effects of ultrasound, which disrupt plant cell walls and enhance the release of intracellular compounds. Ethyl acetate, being moderately polar, can effectively solubilize both polar and non-polar compounds, making it particularly effective in sonication. Furthermore, when plant material undergoes successive extractions with different solvents, the overall yield typically decreases with each step due to the progressive depletion of extractable

compounds. These observations underscore the importance of solvent polarity in determining extraction efficiency, particularly in Soxhlet extraction.

3.2 Qualitative phytochemistry

The result of our phytochemical screening, carried out on the five plants, is summarized in the following table.

Table 2. Results of Qualitative Phytochemical Revelation of the Plants Studied

Plant	Flavonoids	Tannins
<i>Daphne gnidium</i>	++	+++

++: indicates presence (moderate or slight),

+++: indicates higher presence or strong indication.

Qualitative phytochemical analysis of *Daphne gnidium* has shown that the plant is particularly rich in polyphenolic compounds, especially flavonoids and tannins, with tannins being more prevalent. These compounds are among the most common secondary metabolites found in plants [29]. The relative abundance of flavonoids and tannins can vary due to a range of factors, including geographical and environmental conditions such as light exposure, rainfall, topography, and soil composition. Seasonal variation and the timing of harvest also play significant roles. Furthermore, differences may arise from the plant's genetic makeup, the specific part of the plant being analyzed, the types of phytochemicals present, and the extraction techniques used [30–32].

3.3 Total polyphenols and flavonoids

The results of total polyphenol and flavonoid contents in the four crude extracts are presented in Table 3. Total polyphenol content was determined using a linear calibration curve based on gallic acid ($y = ax + b$) over a concentration range of 0 to 100 $\mu\text{g/mL}$. Flavonoid content was expressed in milligrams of quercetin equivalents per gram of extract (mg QE/g extract) [33].

Table 3. Total Polyphenol and Flavonoid Contents in *Daphne gnidium* Extracts Obtained by Soxhlet and Sonication Methods Using Ethyl Acetate and Methanol.

Extraction method	Extract	Total phenols μg EAG/100 mg	Total flavonoids $\mu\text{g EQ/100 mg}$
Soxhlet	Ethyl acetate	87.11 ± 0.01	4.72 ± 0.02
	Methanol	68.53 ± 0.04	4.14 ± 0.06
Sonication	Ethyl acetate	70.12 ± 0.02	4.69 ± 0.08
	Methanol	67.57 ± 0.01	3.63 ± 0.09

As shown in Table 3, the total polyphenol content in the methanolic extracts of *Daphne gnidium* obtained by Soxhlet extraction ($68.53 \pm 0.04 \mu\text{g GAE/100 mg}$ extract) and ultrasound-assisted extraction ($67.57 \pm 0.01 \mu\text{g GAE/100 mg}$ extract) was relatively low compared to that of the ethyl acetate extracts obtained by Soxhlet ($87.11 \pm 0.01 \mu\text{g GAE/100 mg}$ extract) and sonication ($70.12 \pm 0.02 \mu\text{g GAE/100 mg}$ extract). These findings suggest that *Daphne gnidium* extracts are generally rich in polyphenols and that ethyl acetate is a more effective solvent than methanol for polyphenol extraction, regardless of the extraction method used.

For comparison, D. Amini et al. (2015) [34] reported a significantly higher polyphenol content of (10.52 ± 0.87 mg GAE/g extract), which could be attributed to variations in extraction protocols, plant origin, or environmental conditions. Regarding flavonoid content, the methanolic extracts obtained by Soxhlet (4.14 ± 0.06 μ g QE/100 mg extract) and sonication (3.63 ± 0.09 μ g QE/100 mg extract) showed relatively low values. The ethyl acetate extracts exhibited slightly higher flavonoid contents with Soxhlet (4.72 ± 0.02 μ g QE/100 mg extract) and sonication (4.69 ± 0.08 μ g QE/100 mg extract). In contrast, Bouyahya et al. (2016) [35] reported a much higher flavonoid content of (17.68 ± 1.23 mg QE/g extract) using ethanol as the extraction solvent via maceration, emphasizing the strong influence of solvent polarity and extraction technique on the yield of flavonoids.

3.4 Antioxidant activity

3.4.1 Trapping of the free radical DPPH

The antiradical activity of the different extracts of *Daphne gnidium*, as well as the standard (BHT) at different concentrations ranging from (200 μ g/ml to 6.25 μ g/ml), are measured by following the appearance of the yellow color of (DPPH) reduced at 517 nm. The results are shown in the following figure.

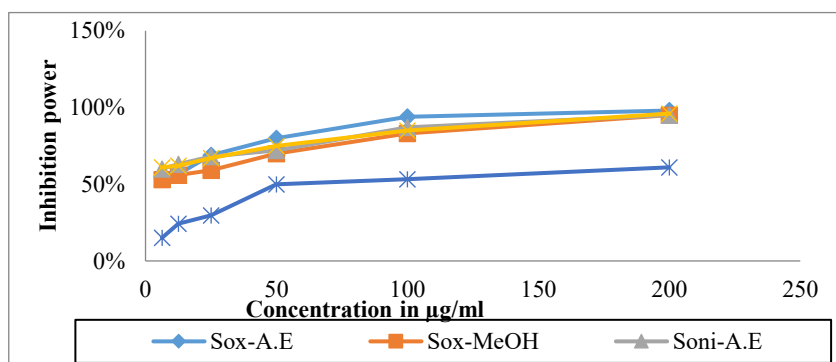


Fig. 1. Antioxidant activity of *Daphne gnidium* extracts obtained by sonication and Soxhlet extraction methods.

The results demonstrate that all *Daphne gnidium* extracts exhibit antiradical activity, with the percentage of DPPH inhibition increasing proportionally with extract concentration. Notably, extracts obtained by ultrasound-assisted extraction showed stronger radical scavenging activity than those obtained by Soxhlet extraction. Furthermore, all *Daphne gnidium* extracts outperformed the synthetic antioxidant BHT under the tested conditions. The observed antiradical activity is likely attributable to the presence of various phenolic compounds, which are widely recognized as key constituents in plants responsible for primary antioxidant or free radical scavenging effects [36]. Both methanolic and ethyl acetate extracts demonstrated significant antioxidant potential.

To quantify this activity, the IC_{50} values the concentrations required to reduce the initial DPPH concentration by 50% were determined for each extract. Since IC_{50} is inversely related to antioxidant power, a lower IC_{50} indicates higher radical scavenging capacity. The IC_{50} values of the tested extracts are presented in the following figure.

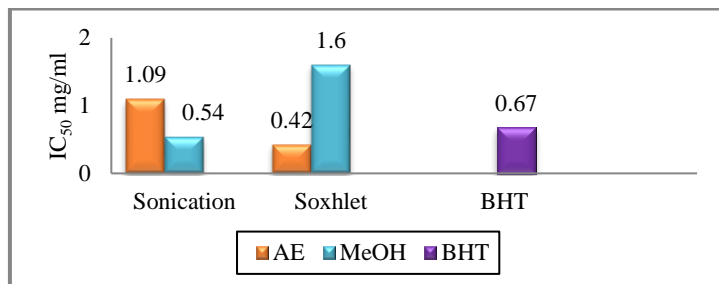


Fig. 2. IC₅₀ values of *Daphne gnidium* extracts obtained by sonication and Soxhlet extraction methods

Among the four extracts tested, the ethyl acetate extract obtained by Soxhlet extraction exhibited the strongest antiradical activity, with an IC₅₀ value of 0.42 ± 0.01 mg/mL. This was followed by the methanolic extract obtained by ultrasound-assisted extraction, which showed an IC₅₀ value of 0.54 ± 0.04 mg/mL. Both extracts demonstrated greater antioxidant activity than the reference standard BHT, which had an IC₅₀ value of 0.68 ± 0.06 mg/mL.

3.4.2 β -Carotene Bleaching Test

The discoloration inhibition percentages and IC₅₀ values results of β -carotene solution of methanolic extracts of *Daphne gnidium* as well as BHT are shown in the following Table:

Table 4. Inhibition percentages and IC₅₀ values of methanolic extracts of *Daphne gnidium* and BHT at a concentration of 2 mg/mL in the β -carotene bleaching assay.

Sample	Inhibition (%) at 2 mg/mL	IC ₅₀ (mg/mL)
<i>Daphne gnidium</i> (Methanol)	76.19 ± 2.73	0.815 ± 0.007
BHT (Standard)	76.2 ± 3.2	0.923 ± 0.050

The methanolic extract of *Daphne gnidium* exhibited a significant antioxidant effect in the β -carotene-linoleic acid bleaching assay, showing an inhibition percentage of (76.19 ± 2.73) % at a concentration of 2 mg/mL. At the same concentration, the standard antioxidant BHT displayed a comparable inhibition rate of (76.2 ± 3.2) %. Notably, the IC₅₀ value of the methanolic extract 0.815 ± 0.007 mg/mL was lower than that of BHT 0.923 ± 0.050 mg/mL, indicating slightly higher antioxidant potency under the experimental conditions.

These findings suggest that the inhibition of β -carotene discoloration by the *Daphne gnidium* methanolic extract may be attributed to the presence of apolar phenolic compounds. These compounds are known to effectively inhibit the oxidation of linoleic acid in lipid-in-water emulsions, such as the β -carotene-linoleic acid system. In this model, apolar antioxidants preferentially localize at the lipid–water interface, where they scavenge lipid peroxy radicals and prevent oxidative degradation of β -carotene [37].

Previous review studies have reported a strong positive correlation between total phenolic content and antioxidant activity in plant extracts. Consistent with these findings, the antioxidant potential observed in the present study is closely associated with the polyphenol and flavonoid contents of the *Daphne gnidium* extracts [38, 39].

4 Conclusion

The extraction yield of *Daphne gnidium* was found to vary based on both the extraction method (Soxhlet or sonication) and the polarity of the solvent used. Phytochemical analysis of the extracts revealed the presence of polyphenols and flavonoids, with particularly high

concentrations of these compounds observed in the ethyl acetate and methanolic extracts. The plant's antioxidant potential appears to be closely linked to its polyphenol and flavonoid content, as demonstrated by significant activity in both DPPH radical scavenging and β -carotene bleaching inhibition assays. Furthermore, an ethnobotanical survey conducted as part of the study highlighted several traditional uses of *Daphne gnidium*. Locals mix its leaves with henna to prevent hair loss and enhance hair shine, while the seeds are used traditionally as a fish poison.

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