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ORIGINAL RESEARCH ARTICLE

Phytochemical screening and evaluation of antioxidant activity of daphne gnidium in Morocco (taounate province)

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ABSTRACT

This study aims to investigate the biological properties of Daphne gnidium leaf extracts obtained through different extraction methods. Specifically, it will assess how variations in extraction techniques influence the chemical composition and potential pharmacological activities of the resulting extracts. By analyzing antioxidant, effects, the research seeks to provide deeper insight into the therapeutic potential of this plant species and identify the most efficient extraction approach for maximizing its bioactive compounds. Three types of extracts methanolic, hexanoic, and ethyl acetate were prepared using Soxhlet and sonication techniques, alongside an aqueous extract, to enable a comparative analysis of the extraction efficiency. Quantitative colorimetric analyses revealed a high content of total flavonoids and phenolic compounds across all extracts. Antioxidant activity was assessed using the DPPH free radical scavenging assay, demonstrating notable efficacy for all samples. The results indicated that antioxidant potential varied depending on the extraction method and solvent. For Soxhlet extraction, the methanolic extract exhibited stronger activity (IC_{50} = 0.51 ± 0.005 mg/mL) than the ethyl acetate extract (IC_{50} = 1.06 ± 0.001 mg/mL). In contrast, for sonication, the ethyl acetate extract showed superior antioxidant capacity (IC_{50} = 0.44 ± 0.001 mg/mL) compared to the methanolic extract (IC_{50} = 1.8 ± 0.005 mg/mL). These findings highlight the influence of extraction technique and solvent choice on the phytochemical yield and biological activity of Daphne Gnidium.

Keywords: daphne gnidium; flavonoids; antioxidant activity; polyphenols; soxhlet extraction

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1. Introduction

The increasing concerns over the potential toxicological effects of synthetic antioxidant molecules have prompted a growing interest in identifying alternative natural sources of antioxidants[1,2]. Among these, polyphenols naturally occurring compounds widely distributed across the plant kingdom have garnered significant attention due to their well-documented health-promoting properties[3,4]. As natural antioxidants, polyphenols play a pivotal role in the prevention and management of various chronic diseases, including cancer, cardiovascular disorders, and inflammatory conditions[4,5]. Furthermore, these compounds are extensively utilized as functional additives in the food and cosmetic industries[6]. Consequently, considerable scientific efforts have been directed toward the extraction, quantification, and characterization of polyphenolic compounds from a variety of agricultural, horticultural, and medicinal plants[7].

Daphne Gnidium, a member of the Thymelaeaceae family, is a plant of notable ethnopharmacological relevance, particularly in the

Arab world. Its medicinal use, which dates back centuries, spans regions from the Near East to the Middle East. In Morocco, it is predominantly cultivated in the northern regions. Traditionally, D. gnidium has been used in topical preparations for hair care promoting growth, softening, and cleansing as well as a potent purgative for treating constipation[8]. Numerous studies have highlighted its broad spectrum of biological activities, including antioxidant, antibacterial, and antifungal properties[9,10].

Despite its established traditional applications and its presence in Moroccan flora, limited research has been conducted on the pharmacological potential of *Daphne Gnidium* leaves. Therefore, the aim of the present study was to evaluate the antioxidant activity of leaf extracts obtained using different extraction methods[11,12].

2. Materials and methods

2.1. Plant material

The plant material used in this study consisted of Daphne gnidium leaves collected in December 2021 from the Karia Ba Mohamed area in the Taounate province, within the Fes-Meknes region of Morocco. Botanical identification was performed at the laboratory, where a voucher specimen was deposited for reference[13,14]. The harvested leaves were air-dried at ambient temperature (25–30 °C) in a shaded area of the laboratory for two weeks. After drying, the leaves the plant part selected for analysis were ground into a fine powder using a mechanical grinder and stored in airtight containers until further use[15,16].

2.2. Reagents and solvents

The following reagents were employed in this study: 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, aluminum trichloride ($AlCl_3$), and quercetin. Methanol, hexane, and ethyl acetate were used as solvents for extraction and analysis purposes.

2.3. Absorbance Measurement

Absorbance readings were conducted using a UV-Visible spectrophotometer (BTS-350 model).

2.4. Methods

2.4.1. Preparation of alcoholic extracts

A total of 40 g of finely ground, dried Daphne gnidium leaves was subjected to extraction using Soxhlet apparatus with three different solvents: hexane, methanol, and ethyl acetate[17,18]. The extraction process was carried out under reflux for 6 hours, until the plant material was decolorized[19]. The resulting extracts were concentrated to dryness using a rotary evaporator equipped with a vacuum pump and subsequently stored at 5 °C until further analysis[20].

2.4.2. Extraction by sonication

The ultrasound-assisted extraction (UAE) technique was employed to extract bioactive compounds from *Daphne gnidium* leaves, allowing for a comparative evaluation with Soxhlet extraction. For each solvent (**hexane, ethyl acetate,** and **methanol**), a **separate and fresh batch of 25 g** of finely ground plant material was used. The plant material was mixed with **200 mL of solvent** in a sonication flask. The ultrasonic probe was immersed approximately 3 cm into the mixture, and sonication was carried out for 40 minutes using a pulsed mode (3 seconds ON, 1 second OFF) at an amplitude of 40%[21].

The sonication settings were configured as follows:

- Total duration: 00H40min00s
- Pulse mode: 3 seconds ON / 1 second OFF
- Amplitude: 40%

Following sonication, the mixture was filtered through filter paper. The remaining plant residue was successively extracted with 250 mL of ethyl acetate and then with 250 mL of methanol, under the same ultrasonic conditions. All obtained extracts were concentrated to dryness under reduced pressure using a rotary evaporator. The resulting dry extracts were weighed and stored at 4 °C until further analysis[22].

2.4.3. Preparation of the aqueous extract

The aqueous extract of Daphne Gnidium L. was prepared by heating 25 g of powdered plant material under reflux in 200 mL of distilled water for 8 hours. Following decantation, the mixture was filtered through Whatman filter paper. The resulting filtrate was centrifuged at 3900 rpm for 10 minutes to remove suspended solids. The supernatant was then subjected to a second filtration to eliminate residual particles[23,24].

The clear extract was transferred into a Petri dish and placed in an oven at 40 °C for evaporation until a dry residue was obtained. The final extract was stored in a desiccator until further use[25].

The extraction yield was calculated using the following equation:

$$\textbf{Yield}\ (\%) = \frac{\boldsymbol{m_{exp}}}{\mathbf{m_{th}}} \times \mathbf{100}$$

where:

m_{exp} is the experimental mass of the extract obtained (g),

m_{th} is the initial mass of the plant material used (g),

Yield is expressed as a percentage (%)

2.5. Dosage of total polyphenols

The total polyphenol content of the various *Daphne Gnidium* extracts was determined using the Folin–Ciocalteu colorimetric method as described by Singleton et al and modified by Li et al[26,27]. In brief, 200 µL of each plant extract or gallic acid standard solution was mixed with 800 µL of sodium carbonate solution (7.5%). After 5 minutes of vortexing, 1 mL of Folin–Ciocalteu reagent, previously diluted 1:10 with distilled water, was added. The mixture was incubated at room temperature for 2 hours. Absorbance was then measured at 765 nm against a blank solution containing all reagents except the extract[28,29].

All measurements were conducted in triplicate. Total polyphenol content was quantified using a standard calibration curve constructed with gallic acid (Y = ax + b). The results are expressed as micrograms of gallic acid equivalents per milligram of extract (µg GAE/mg extract)[30].

2.6. Dosage of flavonoids

The total flavonoid content of *Daphne gnidium* extracts was determined using the aluminum chloride colorimetric method, as described by Bahorun et al[31]. Briefly, 1 mL of each extract or standard solution (prepared in methanol) was mixed with 1 mL of 2% aluminum chloride ($AlCl_3$) solution in methanol. After 15 minutes of incubation at room temperature, the absorbance was measured at 430 nm using a UV–Visible spectrophotometer, against a blank containing $AlCl_3$ in methanol without sample.

Quercetin was used as a reference compound to construct the calibration curve by measuring absorbance at different concentrations. All measurements were performed in triplicate. Flavonoid concentrations in the plant extracts were calculated based on the standard curve and expressed as micrograms of quercetin equivalent per milligram of extract (µg QE/mg extract)[32].

2.7. Evaluation of the antioxidant activity by the DPPH test

The antioxidant activity of Daphne gnidium extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as described by Lopes-Lutz et al with slight modifications[33].

A series of extract concentrations (40, 120, 250, 300, and 350 µg/mL) were prepared in methanol. In each test tube, 2.5 mL of the extract solution or standard antioxidant (ascorbic acid) was mixed with 1 mL of a 0.1 mM DPPH methanolic solution. The reaction mixtures were incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm against a blank containing pure methanol[34].

A negative control consisted of 1 mL of the DPPH solution mixed with 2.5 mL of methanol. The radical scavenging activity was expressed as a percentage of inhibition (% I) using the following equation:

$$\%I = \left(\frac{\mathbf{AbsC - AbsE}}{\mathbf{AbsC}}\right)\boldsymbol{x100}$$

Where:

$Abs_{(C)}$: Absorbance of the negative control

$Abs_{(E)}$: Absorbance of the test sample (extract or standard)

3. Results

3.1. Results

3.1.1. Extraction yields

Soxhlet extraction

Soxhlet extraction of *Daphne gnidium* was performed using three different solvents—hexane (**Table 1**), ethyl acetate, and methanol to enable a comparative assessment between Soxhlet extraction and ultrasonic-assisted extraction (sonication) (**Figure 1**).

Table 1. Extraction Yields of *Daphne gnidium* Using the Soxhlet Method.

plant material	Extract	Yield%
Daphne gniduim	Hexane	5.0875
	Ethyl acetate	7.9275
	Methanol	30.7662

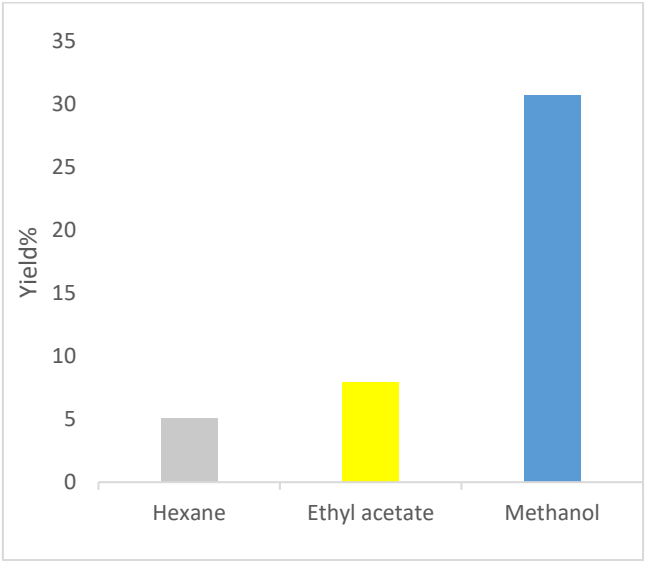

Figure 1. Extraction Yield of Daphne gnidium by Soxhlet Method.

Among all the extracts obtained, the methanolic extract exhibited the highest yield (30.7662%), followed by the ethyl acetate extract (7.9275%) and the hexane extract (5.0875%). These results indicate that extraction yield increases with the polarity of the solvent used in the Soxhlet extraction process[36].

Sonication extraction

Ultrasonic-assisted extraction of *Daphne gnidium* was performed using three different solvents hexane, ethyl acetate, and methanol while maintaining a constant amount of plant material (**Table 2**).

Table 2. Extraction Yields of Daphne gnidium Using the Sonication Method.

Plant material	Extract	Yield%
Daphne gniduim	Hexane	4.22
	Ethyl acetate	17.2
	Methanol	11.52

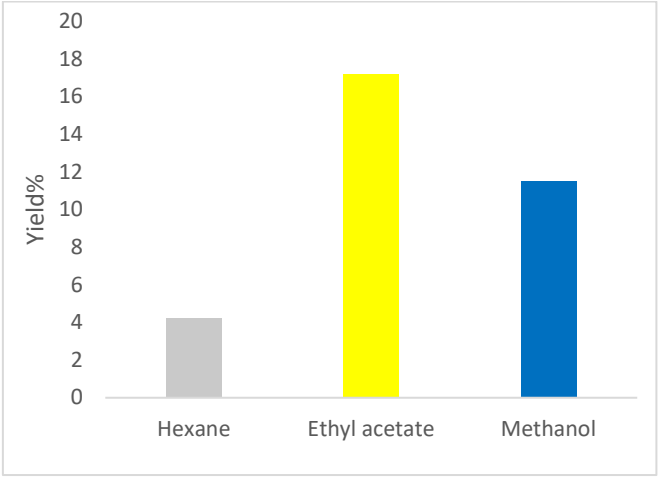

Figure 2. Extraction Yield of Daphne gnidium Using Sonication.

Among the solvents tested, ethyl acetate yielded the highest extraction efficiency (17.2%) (**Figure 2**), while hexane produced the lowest (4.22%). Comparison of Extraction Yields Obtained by Different Methods for *Daphne gnidium.*

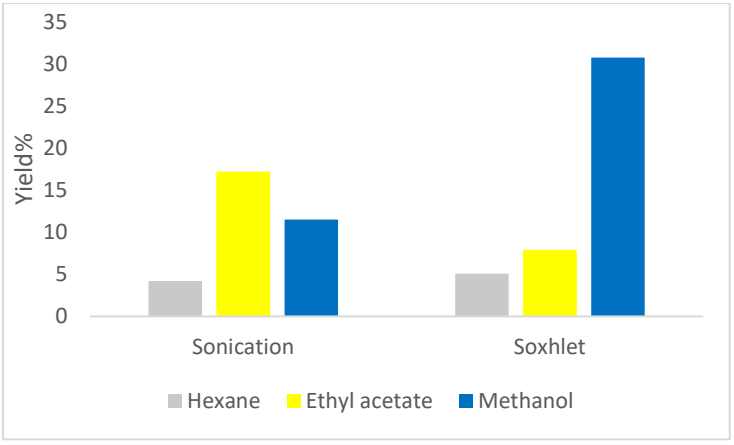

Figure 3. Extraction Yields of Daphne gnidium Obtained by Two Different Methods.

The highest yield obtained by Soxhlet extraction was with methanol (30.76%) (**Figure 3**), whereas for sonication extraction, ethyl acetate provided the best yield (17.2%).

As previously noted, the yield from Soxhlet extraction increased with solvent polarity. In the case of sonication, the superior yield with ethyl acetate can be attributed to the cavitation effect, which disrupts plant cell walls and facilitates the release of active compounds. Additionally, successive extractions of the same plant material with different solvents resulted in a decrease in yield with each subsequent extraction.

3.1.2. Dosage of polyphenols

The calibration curve was constructed using gallic acid as the standard. The phenolic content of each extract was then quantified based on this curve, with results expressed as micrograms of gallic acid equivalents per milligram of extract (µg GAE/mg). The calibration curve demonstrated a strong linear correlation, with a coefficient of correlation (R^2) of 0.99 (**Figure 4**), and was defined by the equation: $y = 0.014x + 0.019$ ($R^2 = 0.990$), where x represents the polyphenol concentration and y the absorbance[37].

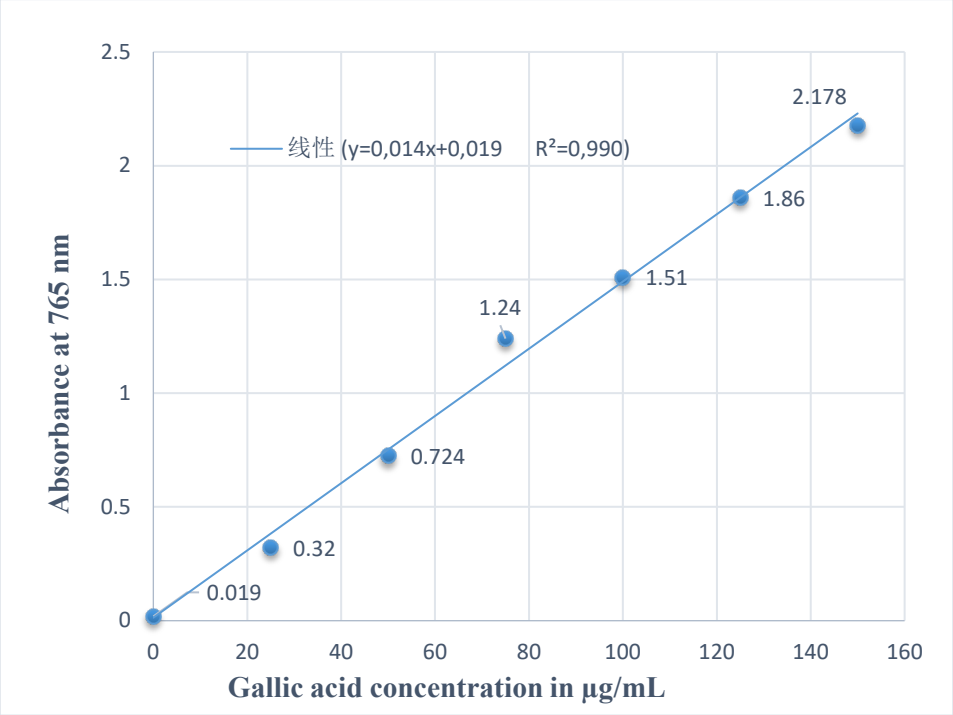

Figure 4. Calibration Curve of Gallic Acid (GA).

3.1.3. Dosage of flavonoids

The calibration curve was generated using various concentrations of quercetin as the standard. Flavonoid content in each extract was quantified based on this curve and expressed as micrograms of quercetin equivalents per milligram of extract (µg QE/mg). The calibration curve exhibited a strong linear relationship, with a correlation coefficient (R^2) of 0.998 (**Figure 5**), described by the equation: $y = 0.034x + 0.010$, where x represents the flavonoid concentration and y the absorbance[38].

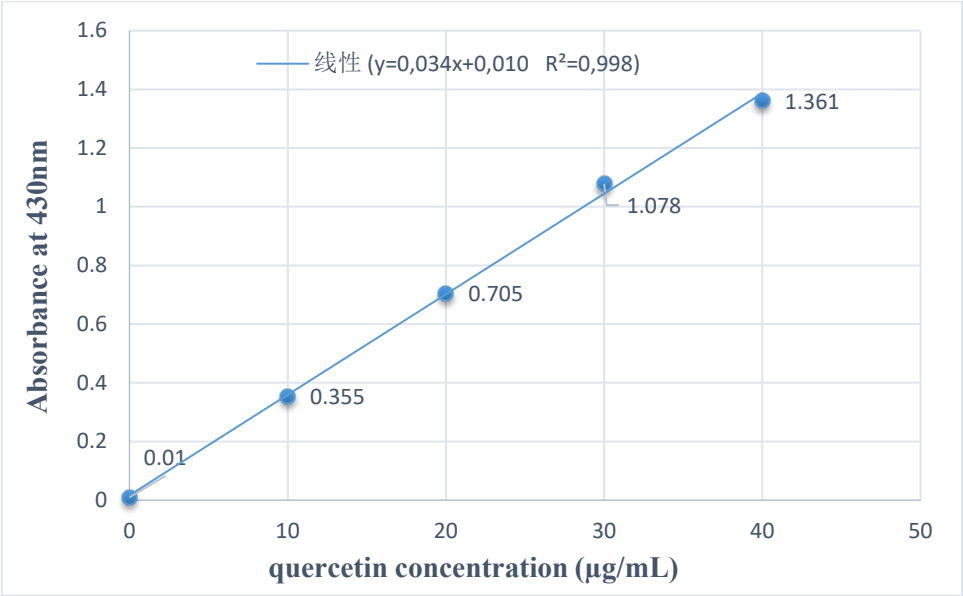

Figure 5. Calibration Curve of Quercetin for Flavonoid Quantification.

The results (**Table 3**) obtained are presented below:

Table 3. Quantity of polyphenols and flavonoids in Daphne gnidium extracts for the two extraction methods.

Method extraction	Extract	Total phenols µg(EAG)/100mg	Total flavonoid µg(EQ)/100mg
Soxhlet	Ethyl acetate	88.21±0.001	4.92±0.002
	Methanol	68.73±0.04	4.24±0.006
Sonication	Ethyl acetate	70.42±0.02	4.79±0.008
	Methanol	67.37±0.01	3.53±0.0009

Note: *The values are expressed in micrograms of gallic acid equivalents (µg GAE) or quercetin equivalents (µg QE) per 100 mg of extract.*

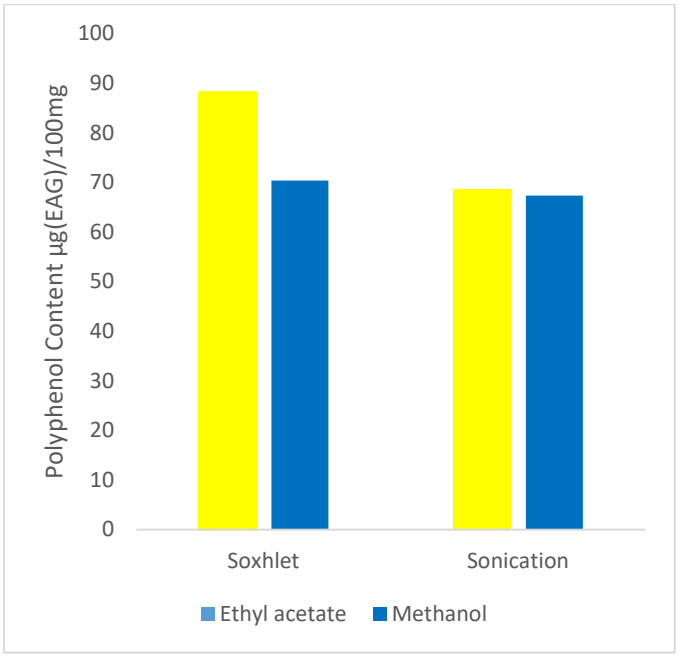

Figure 6. Polyphenol Content of Daphne gnidium Extracts Obtained by Sonication and Soxhlet Methods.

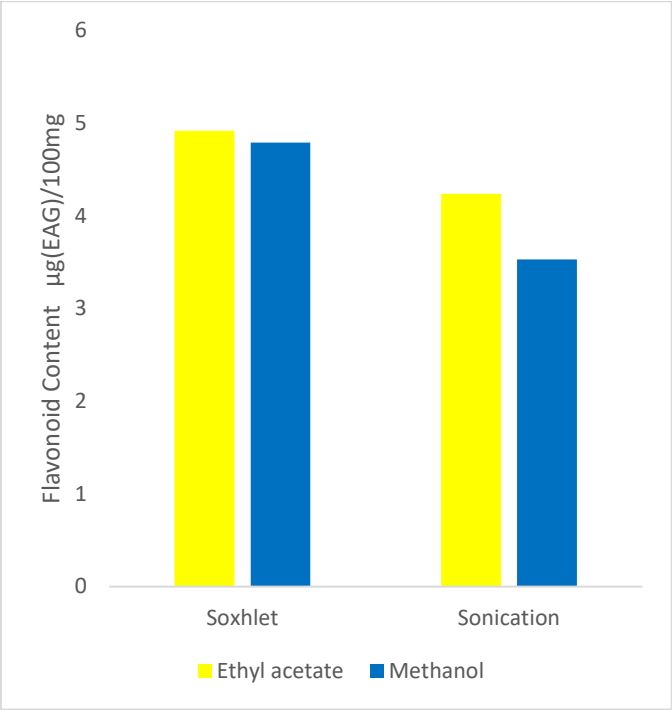

Figure 7. Flavonoid Content of Extracts obtained by Sonication and Soxhlet Methods.

As shown in **Figure 6**, the total polyphenol content of Daphne gnidium methanolic extracts was relatively low for both Soxhlet (68.73 µg GAE/100 mg) and sonication (67.37 µg GAE/100 mg) methods, compared to the ethyl acetate extracts obtained by Soxhlet (88.21 µg GAE/100 mg) and sonication (70.42 µg GAE/100 mg). These results indicate that ethyl acetate extracts consistently contained higher levels of total polyphenols than methanol extracts, regardless of the extraction method used.

As shown in **Figure 7**, the flavonoid content of Daphne gnidium methanolic extracts was relatively low for both Soxhlet (4.24 µg QE/mg) and sonication (3.53 µg QE/mg) methods, compared to the ethyl acetate extracts obtained by Soxhlet (4.92 µg QE/mg) and by sonication (4.79 µg QE/mg). The highest flavonoid levels were observed in the ethyl acetate extracts, regardless of the extraction technique employed.

3.1.4. Antioxidant activity

The percentage inhibition of the DPPH radical by Daphne gnidium extracts and by butylated hydroxytoluene (BHT), used as the reference antioxidant, at various concentrations is summarized in **Table 4**.

Antioxidant activity was assessed for both Daphne gnidium extracts and BHT across a concentration range of 6.25 to 200 µg/mL[39].

The results of the percentage inhibition of the DPPH radical by the plant extracts as well as BHT at different concentrations are presented in **Table 4**.

Table 4. DPPH Radical Scavenging Activity of *Daphne gnidium* Extracts and BHT.

	Soxhlet extraction		**Sonication extraction**		
Concentration µg/mL	SoxAE	SoxMeOH	SoniAE	SoniMeOH	BHT
200	96%	95%	95.2%	96%	61%
100	92%	83%	87%	85%	53.13%
50	80%	71%	72%	75%	49.77%
25	67%	59%	68%	67%	29.33%

	Soxhlet extraction		Sonication extraction		
12.5	55.50%	56.5%	63%	62%	24.14%
6.25	54.10%	52%	60%	61%	15.02%

Table 4. (*Continued*)

The results demonstrated that the four extracts reduce the concentration of free radicals and that the percentage of inhibition increases with increasing concentration. It is also observed that the reducing power of all the extracts of D. gnidium, both by sonication and by Soxhlet, is greater than that of the reference (**Figure 8**).

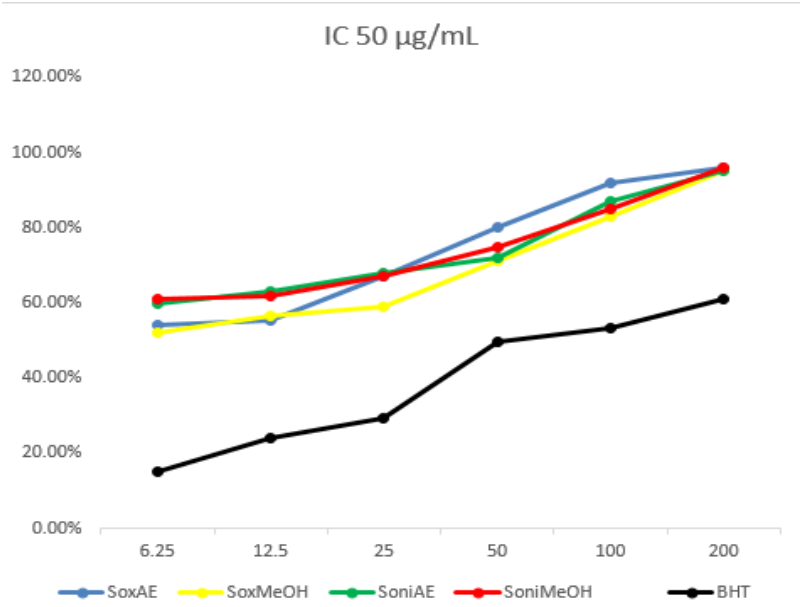

Figure 8. Antioxidant Activity of Daphne gnidium Extracts and BHT as a Function of Concentration Based on the DPPH Assay.

The results from the DPPH assay enabled the determination of the half-maximal inhibitory concentration (IC_{50}) for each sample. IC_{50} values were calculated graphically using linear regression of the inhibition percentage plotted against the sample concentrations. Since IC_{50} is inversely related to antioxidant activity, lower IC_{50} values correspond to higher antioxidant capacities. **Figure 9** presents the IC_{50} values for the tested extracts alongside that of BHT.

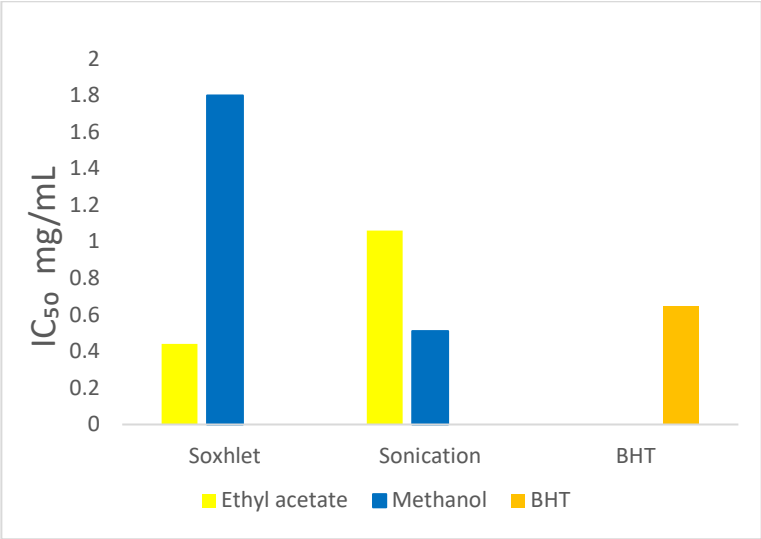

Figure 9. IC_{50} Values of Daphne gnidium Extracts Obtained by Sonication and Soxhlet Methods.

The results presented in **Figure 9** were compared to the standard antioxidant BHT and ranked in decreasing order of antioxidant capacity as follows: ethyl acetate extract obtained by Soxhlet (Ex AE Soxhlet) > methanol extract obtained by sonication (Ex MeOH Ultrasound) > BHT > ethyl acetate extract by sonication (Ex AE Ultrasound) > methanol extract by Soxhlet (Ex MeOH Soxhlet). Both extracts exhibited higher antioxidant activity than the reference compound. Notably, the extraction method influenced the antioxidant efficacy:

- Sonication: The methanol extract (IC_{50} = 0.51 ± 0.005 mg/mL) demonstrated greater activity than the ethyl acetate extract (IC_{50} = 1.06 ± 0.001 mg/mL).
- Soxhlet: The ethyl acetate extract (IC_{50} = 0.44 ± 0.001 mg/mL) showed superior activity compared to the methanol extract (IC_{50} = 1.8 ± 0.005 mg/mL).
- Standard (BHT): The reference antioxidant BHT exhibited a moderate activity with an $IC_{50} = 0.65 \pm 0.002$ *mg/mL*, which was lower than both the Soxhlet ethyl acetate and the sonicated methanol extracts.
- These findings suggest that the chemical composition and consequently the antioxidant activity of the extracts vary depending on the extraction technique employed.

4. Discussion

The quantitative analysis of total phenolic and flavonoid contents using colorimetric methods revealed that *Daphne gnidium* extracts are relatively rich in these bioactive compounds. As shown in **Figure 6**, the total polyphenol content of the methanolic extracts obtained via Soxhlet (68.73 µg GAE/100 mg) and sonication (67.37 µg GAE/100 mg) was lower than that of the ethyl acetate extracts obtained by Soxhlet (88.21 µg GAE/100 mg) and sonication (70.42 µg GAE/100 mg). This indicates that, for both extraction methods, ethyl acetate yielded a higher concentration of total polyphenols than methanol.

A similar trend was observed for flavonoid content. The methanolic extracts obtained via Soxhlet (4.24 µg QE/mg) and sonication (3.53 µg QE/mg) contained lower amounts of flavonoids compared to the ethyl acetate extracts obtained by Soxhlet (4.92 µg QE/mg) and sonication (4.79 µg QE/mg).

These findings contrast with previous results reported by **Süntar et al,** who observed significantly higher total phenolic contents in crude and ethyl acetate extracts of *Daphne oleoides* aerial parts—117.45 ± 1.25 mg GAE/g and 221.00 ± 1.01 mg GAE/g, respectively[40]. The discrepancies between our findings and those from *D. oleoides* may be attributed to species-specific (genotypic) differences between *D. gnidium* and *D. oleoides*, as well as environmental.

Factors such as geographic location, climate, soil fertility, biotic stress, and harvest period. These parameters are known to influence the biosynthesis of secondary metabolites, including phenolic compounds[41].

Regarding antioxidant activity assessed using the DPPH assay, all extracts demonstrated notable radical scavenging capacity. The efficacy of the antioxidant activity was clearly influenced by the extraction method. For sonication, the methanolic extract exhibited higher antioxidant activity (IC_{50} = 0.51 ± 0.005 mg/mL) than the ethyl acetate extract (IC_{50} = 1.06 ± 0.001 mg/mL). In contrast, Soxhlet extraction yielded the most potent activity with ethyl acetate (IC_{50} = 0.44 ± 0.001 mg/mL), which was superior to that of the methanolic extract (IC_{50} = 1.8 ± 0.005 mg/mL).

These observations are consistent with previous studies (Zhang et al; Sharma and Bhat), which reported a positive correlation between total phenolic content and DPPH radical scavenging activity. Furthermore, the current study underscores the importance of both the extraction technique and geographic origin of plant material in determining the chemical composition and bioactivity of *D. gnidium* extracts[42,43].

5. Conclusion and future works

This study aimed to investigate the phytochemical profile and antioxidant activity of *Daphne gnidium* extracts collected from the Karia Ba Mohamed (Taounate) region. By comparing two extraction techniques—Soxhlet and ultrasonic-assisted extraction—using the same solvents, our findings highlight that the chemical composition and biological activity of plant extracts are significantly influenced by the extraction method employed.

All extracts, regardless of the solvent or technique used, demonstrated notable antioxidant activity. A strong correlation was observed between the total polyphenol and flavonoid content and the antioxidant potential of the extracts, confirming the role of these compounds in radical scavenging activity.

The promising results obtained encourage further investigation. Future studies should focus on:

- The detailed characterization and quantification of polyphenolic compounds using advanced chromatographic and spectrometric techniques (e.g., HPLC, GC-MS);
- The separate analysis of each extract to isolate and identify individual bioactive constituents;
- The evaluation of antioxidant and vasodilatory activities of isolated polyphenols, both individually and in combination, to assess potential synergistic effects;
- Toxicological assessments to evaluate the safety profile of the extracts for potential therapeutic or nutraceutical applications.

These future directions will contribute to a deeper understanding of the therapeutic potential of *Daphne gnidium* and support its possible use in health-related fields.

Conflict of interest

The authors declare no conflict of interest.

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