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Research Article

Phytochemistry and Biological Evaluation of *Daphne gnidium* L. Butanol Extract

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ABSTRACT

Background: *Daphne gnidium* L. (Thymelaeaceae) has been used in the Mediterranean basin to treat skin diseases, rheumatism and toothache .**Objective:** the aim of this study was to evaluate the biological activities of butanol extract from the *D. gnidium* leaves. **Methods:** A quantitative analysis by high-performance liquid chromatography with a diode array detector (HPLC-DAD) was performed. The antioxidant activities were evaluated by using three different assays: 2,2-diphenyl-l-picrylhydrazyl assay (DPPH assay), deoxyribose degradation assay and Cellular Antioxidant Activity (CAA) assay. The butanol extract was investigated for anti-inflammatory and analgesic activities in animal models. In addition, its effect on the production of NO and lysosomal activity *in vivo* was assessed. **Results:** The HPLC-DAD analysis showed the presence of daphnetin. The butanol extract had a remarkable antioxidant activity in the different systems tested. Furthermore, it has an anti-inflammatory effect by inhibiting the xylene-induced ear edema and reduced the number of abdominal constrictions in mice indicating analgesic effect. It also was found to inhibit (NO) production and lysosomal activity *in vivo*. **Conclusion:** These findings support the daphne use in traditional medicine for its analgesic and anti-inflammatory activities. Further investigations to elucidate its mechanism of action are required.

Key words: Analgesic, Anti-inflammatory, Antioxidant, Daphne *gnidium*, Lysosmal activity, Nitric oxide production.

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INTRODUCTION

Free radicals are highly reactive and may be responsible of autoimmune diseases such as arthritis and atherosclerosis.[1-2]They are known as reactive oxygen species (ROS) and reactive nitrogen species (RNS).

ROS are playing an important role in the progression of inflammatory response. At high levels, their generation by neutrophils and macrophages cause tissue injury and even neoplastic transformation. Furthermore, others cells show enhanced production of pro-inflammatory cytokines (IL-1, TNF-α etc.) and produce ROS to extend inflammation.[3]

Pathological pain is also associated to inflammatory reactions in the peripheral and central nervous systems. [4]

In order to relieve pain and reduce inflammation, non-steroidal anti-inflammatory drugs (NSAIDs) have been used worldwide. However, these products have many side effects like gastric ulcer and renal damage.[5] Therefore, developing alternative products which possess antioxidant, anti-inflammatory and analgesic activities with fewer adverse effects becomes a necessity. In this context, natural molecules obtained from plants acquire growing interest. [6]

Daphne gnidium L. has been used to treat wound [7] and the bark has been used as a diuretic and to treat toothache and hepatitis. [8-9]

Recently, antioxidant, anti-genotoxic, anti-tumoral, and immunomodulatory effects of extracts from *D. gnidium* L. has been evaluated.[10, 11, 12]

This study aimed to evaluate biological activities such as antioxidant, anti-inflammatory, analgesic, and NO inhibition and lysomal activity of butanol extract from the leaves of *D. gnidium* L. Phytochemical composition of this extract has been also analyzed.

MATERIALS AND METHODS

Animals

Balb/c mice (6–8 week-old, 20–30 g) were acclimatized to laboratory conditions for one week prior to treatment. The temperature in the animal room was maintained between 25 ± 2°C with a relative humidity of 30–70% and illumination cycle was set to 12 h light and 12 h dark. Standard laboratory pelleted feed was given to mice. The protocol was approved by the Ethics Committee of the University Hospital Fattouma-Bourguiba of Monastir, Tunisia.

Plant extract preparation

The leaves of *D. gnidium* L. were dried and pulverized into fine powder. The organic extract was prepared using cold maceration in methanol during a week with continuous stirring. After filtration and evaporation of solvent, the residue was dissolved in water. Successive liquid-liquid extractions were realized with chloroform, ethyl acetate and butanol.

Preliminary phytochemical analysis

Qualitative phytochemical analysis of butanol extract was determined as per standard methods described by Harbone. [13]

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QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Estimation of Total Phenolic content

Total phenolic content was conducted using the Folin-Ciocalteu reagent according to the method described by Lin et Tang.[14]One hundred microliters of plant extract was placed into test tube followed by 2mL of 2% Na2CO3. After 2 minutes of incubation at room temperature in the dark, 100 µL of 50% Folin's reagent were added to the mixture. The absorbance was measured at 720 nm after 30 minutes and total phenolic content was calculated with a gallic acid standard. The values were expressed as gallic acid equivalents (GAE).

Estimation of flavonoids content

Total flavonoids content was determined by aluminium chloride colorimetric assay with slight modification according to Djeridane *et al.*[15] Two hundred fifty microliters of plant extract prepared in methanol and 750 µL of 2% AlCl3 were placed into test tube. The absorbance was measured at 430 after 10 minutes of incubation in the dark.Total flavonoids content was calculated with a quercetin standard .The values were expressed as quercetin equivalents (QE).

HPLC-DAD analysis

Chromatographic analysis was carried out on a HPLC-DAD(*Shimadzu)* fitted with a Shim-pack VP-ODS C18 column (150mm L×4.6 mmi.d.5µm). A binary gradient composed of solvent A (acide formique/eau, 2/98, v/v) and solvent B (methanol/acide formique/eau 80/2/18, v/v/v) as follows: 98% A (2 min), 90% A (10 min), 85% A (10 min), 75% A (13 min), 60% A (7 min), 55% A (2 min) was used. The flow rate and the detection wavelength were 0.8 mL/min and 254 nm, respectively.The daphnetin standard sample was used at 1mg/mL.

ANTIOXIDANT ACTIVITY

DPPH radical-scavenging activity

The antiradical activity of butanol extract was determined using the s 2,2-diphenyl-1- picrylhydrazyl radical (DPPH).[16]A solution of DPPH in ethanol(23.6µg/mL) was added to a range of solutions at different concentrations of butanol extract (1 µg/mL, 10 µg/mL, 100 µg/mL, and 200 µg/mL). Then, the mixture was kept in the dark for 30 min at room temperature and the absorbance was measured at 517 nm against blank (ethanol).Scavenging activity was calculated as:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Where, $A_{control}$: absorbance of the control and A_{sample} : absorbance of the extract or standard. The antioxidant activity was expressed as the inhibitory concentration (IC50) value in µg / mL. It corresponds to 50% inhibition on DPPH.

Hydroxyl radical-scavenging activity

The hydroxyl (•OH) radical-scavenging activity of butanol extract was determined as established by Halliwell *et al.*[17], with some modifications. Briefly, samples were dissolved in ethanol and brought to 400 µl in phosphate buffer (10 mM, pH 7.4). Then, 100µL deoxyribose (28 mM), 100 µL EDTA (20 mM), 100 µL $FeCl_3$ (0.2 mM) and 100 µL H_2O_2 (14.2 mM) were mixed. The reaction was initiated by adding 100 µL ascorbate (0.5 mM). After incubation at room temperature for one hour at 37 °C, the antioxidant activity was measured by estimating acid-maloaldehyde formationby deoxyribose degradation caused by hydroxyl radicals usingthe thiobarbituric acid method by Aruoma. [18]

Cellular antioxidant activity (CAA)

The method of Wolfe and Liuwas used to evaluate the Cellular Antioxidant Activity (CAA) of the extract.[19] Cells were seeded in a 96-well microplate at a density of 510^5/well for splenocytes and $6x10^4$/well for macrophages in a total volume of 100 µl of Phosphate-Buffered Saline (PBS). Then, 10 µl of each concentration of the extract and 5 µL of 2', 7'-dichlorofluorecin diacetate (DCFH-DA, 25µM) were added. After 1 hour of incubation in the dark, 600 µM of 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) dissolved in 100 µl of PBS were added to the cells. A fluorescence reading (Excitation/ Emission = 485/530 nm) was taken using a fluorimeter (Multidetection microplate Reader FL x 800) for one hour, every five minutes. Each plate contains 3 control wells and 3 blank wells. The control contains cells treated with (DCFH-DA) and oxidant (ABAP) while the blank contains cells with PBS without oxidant.

The antioxidant activity was expressed in CAA unit. The area under the curve of fluorescence versus time was integrated to calculate the CAA value at each concentration of the extract as follows:

$$\text{CAA unit} = 100 - (\int SA / \int CA) \times 100$$

Where ∫SA is the integrated area under the sample fluorescence versus time curve and ∫CA is the integrated area from the control curve.

Nitric oxide production by mice peritoneal macrophages

Griess assay was employed to evaluate the NO production by mice peritoneal macrophages.[20]One hundred microliters of the macrophage at a concentration of 6×10^6 cells/mL were seeded in 96 well plates for 24 h in 5 % CO_2. Then, cells were treated by butanol extract (1 µg/mL and 100 µg/mL) and its major component (daphnetin) (1µM, 100µM).

Macrophages were seeded in 96-well plates at a concentration of 2×10^6 cells/mL and incubated for 2 h in 5% CO_2 in air; then non-adherent cells were removed by washing with after 48 h of incubation, nitrite was measured by adding 100 µL of Griess reagent (1 % sulfanilamide and 0.1 % naphthylenediamine in 5 % phosphoric acid) to 100µLcell-culture supernatant. The Absorbance was measured at 577 nm in a microplate reader (Thermo Scientific, Vantaa, Finland).

The percentage NO inhibition was determined according to the formula below.

$$\textbf{NO inhibition (\%)} = 100 \times ((1-(A_{sample}/A_{control}))$$

Where, $A_{control}$: absorbance of the control and A_{sample} : absorbance of the extract or standard.

Lysomal enzyme activity

Lysosomal enzyme activity (reflected by acid phosphatase (AP) activity) in peritoneal macrophages was performed with p-nitrophenyl-derivatized monosaccharide substrates as described by Nasr-Bouzaiene *et al.* [21], with some modification. Released p-nitrophenol was measured by spectrophotometry at 405 nm. The percentage of lysosomal enzyme activity was calculated as below:

Lysosomal enzyme activity (%) = 100 × (OD sample – OD control)/ OD control.

Where, OD_{sample}: Optical density of sample and $OD_{control}$: Optic density of control.

Xylene-induced ear edema assay

The anti-inflammatory activity was carried out according to the method described by Hosseinzadeh *et al.* [22] with some modifications.

Mice were divided into three groups (n=6). Control group received by intraperitoneal administration normal water (no treatment), second group(positive control) received dexamethasone (15 mg/kg, i.p.) and the third group received butanol extract (50 and 100 mg/kg, i.p.).Thirty minutes later, 30 µL of xylene was applied to the anterior and posterior

surfaces of the right ear. The left ear was considered as control. After 2h, the thickness of the ear was measured using a digital caliper. The degree of ear swelling was expressed as an increase in ear thickness in mm.

Acetic acid-induced writhing assay

The analgesic activity was studied as described by Shibata *et al.*[23] Three groups of six mice were formed: Control group received normal water (1 mL/100g, i.p.), positive group received acetyl salicylic acid (100 mg / kg, i.p.) and third group received extract (50, 100 mg/kg, i.p.).

Thirty minutes later, mice received 0.75% acetic acid (at a *dose* of 0.1 mL/10 g, i.p.) was administered intraperitoneally. Afterfive minutes, the number of writhes produced was counted for 15 min. The percentage of analgesic activity was calculated as follows:

$$\textbf{Inhibition percentage (\%)} = (1-(N_t/N))*100$$

Where N is the average number of stretching of control animals per group and N_t is the average number of stretching of treated animals per group.

RESULTS

Preliminary phytochemical analysis

Preliminary phytochemical analysis revealed the presence of tannins, flavonoids and coumarins.Cardiac glycosides, anthracene glycosides and alkaloids were absent in the extract.

Quantitative Phytochemical analysis

The total phenolic content of butanol extract obtained from leaves of *D. gnidium* L. was 152±1.05 45.17 ± 1.70 mg GAE/g, and the total flavonoid content was 250.07±0.66 mg QE/g

Phytochemical *analysis* using HPLC-DAD revealed the presence of daphnetin in the butanol extract at a concentration of 0.814 µg/mg. (Figure 1).

ANTIOXIDANT ACTIVITY

Butanol extract inhibits DPPH and hydroxyl radicals

The radical-scavenging ability of extract is shown in 1.The effect was dose dependent. The highest DDPH radical capacity (78.3%) was found at 200 µl/mL of the extract (Table 1). The IC_{50} value obtained is 12 µg/mL.

A dose dependent effect was also observed for hydroxyl radical scavenging activity. The highest capacity of inhibition (98.32%) was found at 300 µl/mL of the extract (Table 2). The IC_{50} value obtained is 1.75 µg/mL.

Cellular antioxidant activity (CAA)

The results showed a decrease in peroxyl radical produced by splenocytes after incubation with 25 µg/mL of the extract. However, the percent inhibition did not exceed 26.88% when extract was incubated with macrophages (Table 3).

Butanol extract reduces nitric oxide and phosphtase acide produced by macrophges

NO production by macrophages decreased by 58,74 % and 69,45 % when cells were treated for 24 h with 10 µg/mL of butanol extract and 100 µM of daphnetin, respectively (Table 4).

The cellular lysosomal activity in mouse macrophages was assessed. As shown in 5, the butanol extract and its major component (daphnetin) inhibed macrophage lysosomal enzyme activity after incubation for 24 h. This activity reached 98, 49 % and 97, 99 % after treatement with 100 ng/mL and 100 nM of butanol extract and daphnetin, respectively (Table 5).

Anti-inflammatory activity in vivo

The anti-inflammatory activity was found to be dose dependent in xylene-induced ear edema assay. The butanol extract showed significant ($P < 0.05$) inhibition of ear edema, 58.79% and 68.68% on 2 th hour at

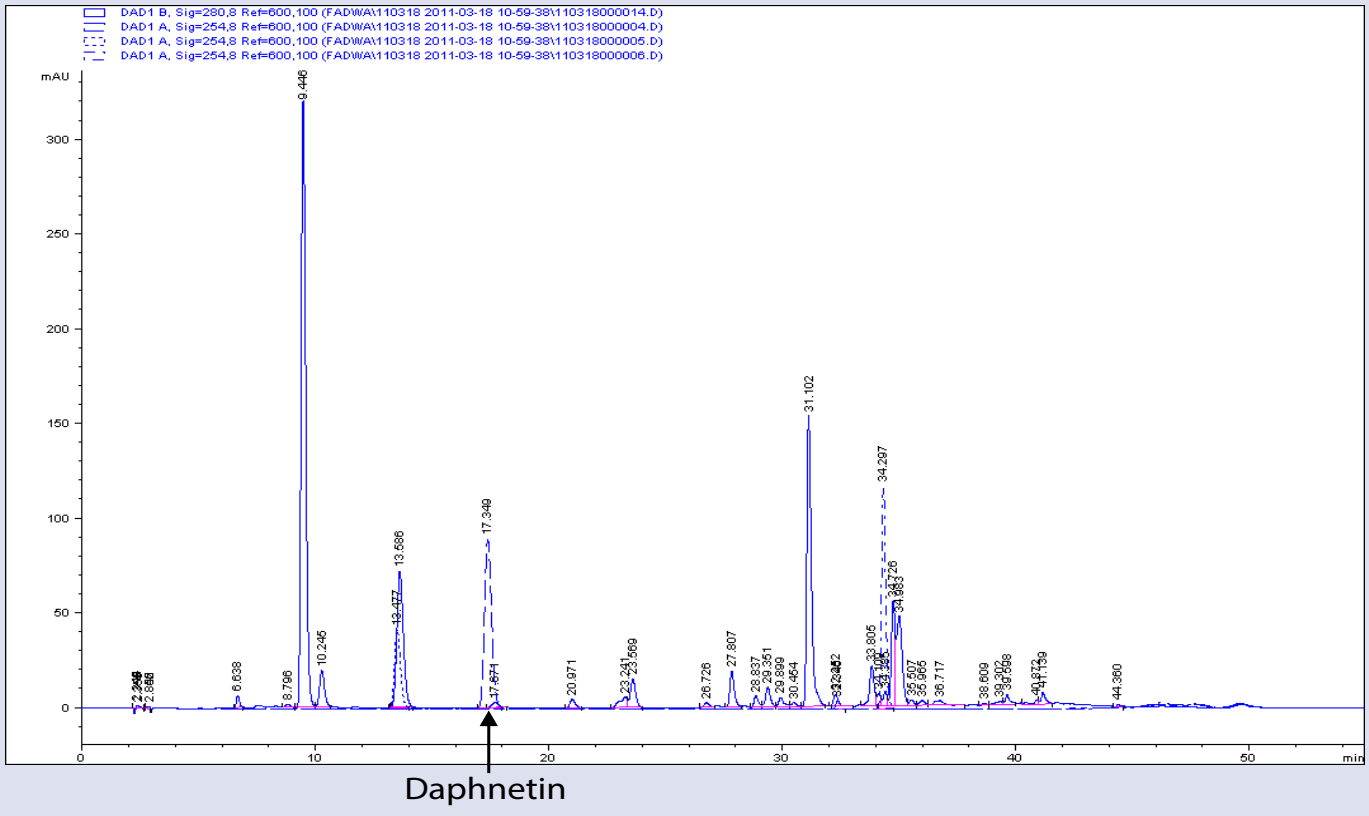

Figure 1: HPLC-DAD chromatogram of butanol extract from leaves of *Daphne gnidium* L.

Table 1: DPPH free-radical scavenging activity of butanol extract from *Daphne gnidium* L.

Concentrations (µg/mL)	Inhibition percentage of DPPH radical (%)
1	27.35
10	47.16
100	72.64
200	78.30

Table 2: Hydroxyl radical scavenging activity of butanol extract from *Daphne gnidium* L. leaves.

Concentrations (µg/mL)	Inhibition percentage of Hydroxyl radical (%)
10	64.31
75	73.42
150	81.59
300	98.32

Table 3: Antioxidant effect of butanol extract from leaves of *Daphne gnidium* L. evaluated by CAA assay in splenocytes and macrophages.

	CAA unit (%)	
Concentrations (µg/mL)	Splenocytes	Macrophages
12.5	92.595±0.073*	4.151±1.256**
25	91.511±0.497**	26.881±8.552**
50	90.781±0.317	28.844±5.455

Table 4: Inhibition of nitric oxide production by butanol extract from *Daphne gnidium* L. Leaves and its major component (daphnetin).

Butanol extract	1 µg/mL	10,18 % ± 0,9*
	10 µg/mL	58,74 % ± 1,37***
Daphnetin	1 µM	10,39 % ± 0,97**
	100 µM	69,45 % ± 0,92***

All values are expressed as mean ± standard deviation.

*Differences in inhibition of NO production were estimated by Student'S t-test (*p<0.05, **p<0.01 and ***p<0.001).*

Table 5: Inhibition of lysosomal activity by butanol extract from *Daphne gnidium* L. Leaves and its major component (daphnetin).

Butanol extract	100 ng/mL	98,49 ± 0,003***
	1 µg/mL	22,34 ± 8,2*
Daphnetin	100 nM	97,99 ± 0,018***
	1 µM	19,15 ± 1,44**

All values are expressed as mean ± standard deviation.

*Differences in inhibition of lysosomal activity were estimated by Student'S t-test (*p<0.05, **p<0.01 and ***p<0.001).*

Table 6: Antitiflammatory activity of butanol extract from leaves of *Daphne gnidium* L. evaluated by xylene-induced ear edema assay.

Treatement	Doses (mg/Kg)	Edema (mm)	Inhibition (%)
Dexamethason	15	0.076* ± 0.05	49.89
Butanol extract	50	0.06 *± 0.03	58.79
	100	0.047** ± 0.01	68.68
Control	-	0.15 ± 0.048	-

All values are expressed as mean ± standard deviation.

*Differences in inhibition of inflammation were estimated by Student'S t-test compared to the control (*p<0.05, **p<0.01 and ***p<0.001).*

Table 7: Analgesic activity of butanol extract from D. *gnidium* L. leaves evaluated by acetic acid-induced writhing assay.

Treatement	Doses (mg/Kg)	Acetic acid writhing contraction	Percentage of inhibition (%)
Aspirin	100	28** ± 3.82	39.78
Butanol extract	50	23.4** ± 7.66	49.67
	100	11.5*** ± 2.14	75.26
Control	-	46.5 ± 7.89	-

*Differences in inhibition of pain were estimated by Student'S t-test compared to the control (*p<0.05, **p<0.01 and ***p<0.001).*

the doses of 50 and 100 mg/kg, respectively. Dexamethasone at a dose 15 mg/kg, i.p. prevented xylen-induced ear edema with a percentage inhibition of 49.89% (Table 6).

Analgesic activity in vivo

The butanol extract at 50 mg/Kg, i.p. and 100 mg/Kg, i.p. produced a percentage inhibition of the acetic acid induced abdominal constriction of 49.67 and 75.26%, respectively. A dose-dependent increase in analgesic activity was found. Aspirin as standard drug reduced the abdominal constriction to 39.78% at a dose 100 mg/kg, i.p.(Table 7).

DISCUSSION

Human cells protect themselves against ''oxidative stress'' by their own weapons. However, these weapons are not always sufficient to protect from damages caused by ROS.[24] These reactive molecules can be stopped by antioxidants administration. [25] Thus, protecting against chronic diseases, such as heart disease and cancer, associated with ''oxidative stress'' can involve natural antioxidants derived from plants.[26]

The butanol extract from leaves of *D. gnidium L* was tested *in vitro* for its antioxidant activity using two different methods. The results showed that extract could act as a radical scavenger in all investigated systems. We also found that it possessed a remarkable scavenging activity on hydroxyl radical, which had high reactivity. These methods *in vitro* might inform about molecular mechanisms such as inhibition radical formation or interaction with organic radicals etc., but did not reflect *in vivo* situation. [27]Therefore, a Cell-based assays, like the cellular antioxidant activity (CAA) assay was used. The organic extract significantly inhibited peroxyl radicals generated from 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP). Daphnetin (7, 8-dihydroxycoumarin), one of coumarin derivatives revelated by HPLC analysis, was showed a free radical scavenging activities. [28]

Our study demonstrated that the extract inhibited NO production. Some flavonoids such as apigenin-7-glucoside, genkwanin, and naringenin are reported to inhibit TNFα production as well as iNOS in LPS-activated macrophages, this effect has been associated with the inhibition of the NF-kB pathway. [21]The decreased release of NO reflected the anti-inflammatory effect.

In present study, we also examined the lysosomal enzyme activity reflected by AP activity in mice macrophages. Butanol extract and daphnetin significantly decreased lysosomal phosphatase activity in treated cells. This enzyme played an important role in killing and digesting pathogens or phagocytosis. In addition, a positive correlation between AP activity and phagocytic stimulation capacity was established. [29]

Previous studies indicated that the presence of hydroxyl groups, which affected the enzymes or electron-transferring system, could modulate phagocytic activity. [21-29] Daphnetin was the major component in the butanol extract. It contained two hydroxyl groups which might explain

the significant decrease of lysosomal activity in cells treated with only 100 nM of molecule.

Moreover, the xylene-induced ear edema in mice was used to evaluate the anti- inflammatory activity *in vivo* of the plant extract. Our study revealed that the administration of the butanol extract reduced the swelling appeared after the application of the irritant agent. In this animal model, edema was due to release of pro-inflammatory components that expanded capillary permeability and inflammatory cells infiltration. [30] The activity of phospholipase A2, PLA2 is also including as established by Ndebia *et al.*[31]

Several mechanisms might explain the anti-inflammatory potential of the extract, including the reduction of vasodilatation improvement of edematous condition.

In acetic acid induced abdominal writhing test, the intraperitoneal of butanol extract significantly reduced the number of abdominal constrictions induced by acetic acid. This animal model allowed detecting peripheral analgesic agents. [32]

As established by Formukong *et al.*[33], analgesic effect might be a consequence of the anti-inflammatory properties. Inhibiting prostaglandin synthesis [34] and other mediators such as histamine and serotonin, which acted, on the peripheral nociceptive neurons would also explain this effect. [35] In addition, flavonoids and polyphenols [36,37,38] might inhibit pain perception [39] and eicosanoid synthesis. [40]

Flavonoids could also inhibit the production of arachidonic acid by stoping neutrophils degranulation [41]and modulate the activity of some enzymes involved in the release of mediator of inflammation as described by Sawadogo *et al.* [39]

CONCLUSION

The present work revealed that the butanol extract from the leaves of *D. gnidium* L. possessed significant anti-oxidant, analgesic and anti-inflammatory potentiel. However, it still necessary to elucidate their mechanisms of action and isolate active constituents responsible for the observed effects in mice.

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CONFLIT OF INTEREST

The authors declare that there are no conflicts of interest

ETHICAL APPROVAL

All experiments were performed in accordance with guidelines for the care and use of laboratory animals as published by the National Institute of Health (USA). All experiments received the explicit approval of the Ethics Animal Committee in Tunisia.

ABBREVIATIONS

NO: Nitric oxide; *D. gnidium: Daphne gnidium*; HPLC-DAD: liquid chromatography with a diode array detector; DPPH: 1,1-diphenyl-2-picrylhydrazyl; i.p.: intraperitoneal;GAE: Gallic acid equivalent; QE: Quercetin equivalent; IC50: Concentration of samples resulting in 50% inhibition.

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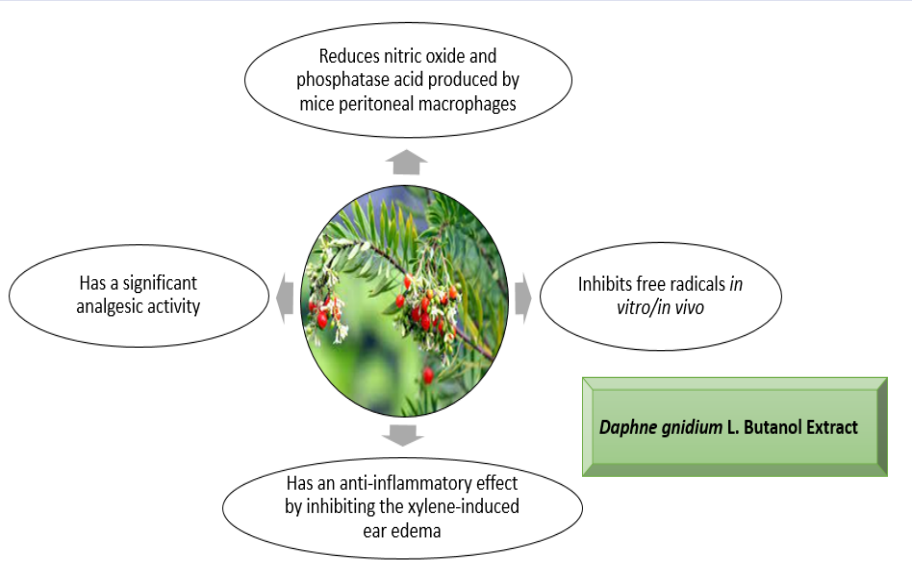
GRAPHICAL ABSTRACT



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