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Formulation of *Daphne gniduim* L. Leaf Extract-based Topical Gel and Evaluation of Anti-inflammatory Activity

AmiraHorchani[1*], Hafedh daly[1], Mohamed Ali Lassoued[3], AimenAbbassi[1], Amine Trabelsi[1], LeilaChekir-Ghedira[1]

[1] Research unit of Natural Bioactive Substances and Biotechnology, Faculty Dental Medicine of Monastir, Avicenne street 5000 Monastir, Tunisia.
[2] Department of surgery A, Faculty of Medicine of Monastir, Avicenne street 5000 Monastir, Tunisia.
[3]Laboratory of GalenicPharmacy, Faculty of Pharmacy Monastir, Avicenne street 5000 Monastir, Tunisia.
[*]Corres.author: amira.horchani@fphm.rnu.tn

Abstract : DaphnegniduimL. has been used in traditional medicine to treat skin diseases, rheumatism and toothache. The objective of the study was to formulate a topical gel from the crudebutanol leafextract of*Daphnegniduim* L. and evaluate itsanti-inflammatory activity in animal model. A preliminary phytochemical screening was carried out for the crude extract. The prepared gel was evaluated for various parameters such as color, appearance, homogeneity, pH, rheological and stability studies. The anti-inflammatory activity was performed in xylene induced mouse ear edema.
The phytochemical screeningrevaled the presence of tannins, flavonoids and coumarines. Organoleptic characteristics and physicochemical properties of gel formulation were found to be satisfactory. The prepared gel showed no irritation on the applied surface up to 24 hrs and possessed a significant reduction of ear edema (76.87 %)compared to marketed gel (standard).
The present study demonstrated that the crudebutanol leafextract of*Daphnegniduim*L has an anti-inflammatory effect when formulated as a gel for topical use. Polyphenolic compound mainly flavonoids may be responsible for this activity. Further preclinical, clinical, and long-term stability on human skin are required.
Keywords : Anti-inflammatory;Crude extract; *Daphne gniduim*L.; Formulation;Topical gel.

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Introduction

The use of medicinal plants for treatment and prevention of certain diseases is considered one of the oldest medical practices in human history[1].Actually, new and approved therapeutic drugs are still be derived from natural sources such as plants[2].There are over 1300 medicinal plants used in Europe [3]**.** In Tunisia, only200 species among 2150 species have been recorded as medicinal plants[4]**.**Thus several efforts are currently undertaken to valorize plants used for traditional medicinal purposes.

*Daphnegniduim*L.an evergreenshrub that grows in the Mediterranean, is used in traditional medicine for the treatment of skin diseases, rheumatism and toothache[5, 6, 7].The ethyl acetate extract of this planthasbeen shown to inhibitpro-inflammatory cytokines (IL-1β and TNF-α) and cyclooxygenase-2-derived prostaglandinE2 (PGE2) productioninmice macrophages[8].Since crude extract cannot easily be administered[9]**,** topicalgel can be used for treatment. Gels are used for their biocompatibility, network structure, and molecular stability of the incorporated bioactive agent[10]**.**

The aim of the present study was to formulate and evaluate a *Daphne gniduim* L. leaf extract based topical gel. Then, the anti-inflammatory activity of this gel was investigated and compared tomarketed gel formulation.

Material and Methods

P**lant material**

The leaves of *Daphnegniduim*L. were collected in the region of Bizerte (Northern of Tunisia) and authenticated by Prof. M. Chaieb (Department of Botany, Faculty of Sciences, University of Sfax, Tunisia), according to the flora of Tunisia[11]**.** A Voucher specimen (D9-11-09) has been preserved in the laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir, Tunisia for future reference.

Extract preparation

The organic extract was obtained by macerating (1Kg) of dried powder of leaves in methanol (1L) during a week with continuous stirring. After evaporation of solvent, the residue is dissolved in water. Several liquid-liquid extractions were realized respectively with chloroform, ethyl acetate and butanol.

Preliminary Phytochemical screening

Phytochemical screening was carried out as per standard methods described by[12]**.**

Chemicals

Carbopol 934 (Sigma Aldrich, Germany), Triethanolamine (Sigma Aldrich, Germany), Propylene glycol-400(LobaChem India), Xylene (Prolabo, France), Ibuphil 5% (Simed, Tunisia), Polyethylene glycol *300* (Prolabo, France), Tween 20 (Prolabo,France), Ethylene diamine tetra acetic acid (Sigma Aldrich, Germany), *Sepicide HB* (Seppic, France),*Sepicide CI* (Seppic, France).

Animals

Male Balb/c mice weighing between 25-30g purchased from the Pasteur Institute (Tunis, Tunisia), were used for the present study. Ethics Committee of the UniversityHospitalFattouma-Bourguiba of Monastir, Tunisia approved the experimental protocol under reference 2019/02/I/CER-SVS/ISBM; 9 January, 2019. The animals were housed at controlled temperature (25±2ºC) with a relative humidity of 30–70% and 12hrs dark-light cycle. They were kept for a seven-day adaptation period prior to treatment and were allowed free access to water and food during the experiment.

Preparation of topical gel containing extract

The gel was formulated by using the dried butanolextract of leaves of *Daphnegniduim*L.In stainless steel beaker, propylene glycol-400 was mixed with distilled water using a magnetic stirrer. Then, a required quantity of extract, dinatrium EDTA and triethanolamine were respectively added under continuous stirring until total dissolution.

Subsequently, the mixture is submitted to a mechanical agitation and polyethyleneglycol (PEG) 300 was progressively added. Further, carbopol 934 was sprinkled into the above mixture with continuous stirring.

The preparation was cooled and preservatives previously dissolved in tween 20 were added. The final gel was stored in the refrigerator for 24 hours. A blank formulation (negative control) was also prepared in the same conditions but without incorporating the extract. Composition of formulated gel was presented in Table 1.

Table 1:Composition of topical gel formulation

Ingredients	**Quantity**
Plant extract (%)	0,8
Carbopol 934 (g)	1
Propylene glycol (g)	4
Polyethylene glycol *300 (g)*	1,5
Sepicide CI (g)	0,1
Sepicide HB (g)	0,1
EDTA (mg)	50
Triethanolamine (g)	1,2
Distilled water up to (g)	100

Evaluation of the formulated gel

Organolepticcharacteristics

The prepared gel was inspected visually for its color, appearance and homogeneity[13, 14]**.**

Measurement of pH

The pH was measured by direct immersion of the glass electrode of the pH meter in the gel system at room temperature. The equipment was calibrated before each use with standard buffer solution at 4.0 and 7.0. The measurements of *p*H were done in triplicate and average values were calculated[15]**.**

Rheological Studies

Viscosity study

The gel viscosity was determined at 25 °C by using a Brookfield viscometer DVII ultra with a spindle **no.7** (Figure 1).The curve giving the variation in viscosity as function of shear rate was obtained by increasing the rotation speed (0.1-100 rpm) in order to have a torque between 10% and 100%. The average of three readings taken in two minutes was noted as the viscosity of gels[16]**.** The rheological behavior was performed with a rheometerContraves AG.

Figure 1:Viscosity measurement using a Brookfield RV DVIII rotating viscometer

Determination of gel consistency

The gel consistency was determined at 25°C by using a penetrometer Prolab. The penetration depth and flow threshold were measured. The measurement of each parameter was carried out in triplicate and the average values were represented[17].

Stability Study

Freeze-Thaw Cycle

The prepared gel was kept in hermetically sealed glass tube and subjected to freeze and thaw cycle. The test was performed for 48 h with five cycles. In each cycle, the gel was kept at a temperature of + 4°C and + 50°C for 30 minutes [18,19].

Long termstability

The gel was kept at room temperature for a period of three months [20].

The appearance, color, pH and viscosity were studied periodically after the 1st, 2nd and 3rd month.

Skin irritation assay

A set of five mice was used in the assay. The gel formulation was applied on the properly shaven skin of mice. Undesirable skin changes, i.e. change in color and changes in skin morphology, were observed for a period of 24 h [21].

In vivo anti-inflammatory activity

The anti-inflammatory activity was assessed in xylene induced mouse ear edema in mice as described by[22]**.**with some modifications. The animals were randomly divided into three groups (n=6). Thirty µL of xylene were applied to the anterior and posterior surfaces of the right ear. After 15 minutes, 25mg of the formulated gel, blank gel and *ibuprofen* 5% gel (standard) were applied on the right ears. The left ear was considered as control. After 3h, the thickness of the ear was measured using a digital caliper and the differences in the thickness were calculated (Figure 2). The degree of ear swelling was expressed as an increase in ear thickness in mm[23].

Figure 2: Measurement of mouse earthicknessusing a difitalcaliper

Statistical analysis

All values were expressed as mean ± SD. The results were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.01$ was considered as significant.

Results and Discussion

Phytochemical screening

Butanol extract obtained from leaves of *Daphne gniduim*L. revealed the presence of tannins, flavonoids and coumarins.

Evaluation of topical gel formulation

The results of physical evaluation of gel formulations are presented in the Table 2 below.

Table 2: Physical evaluation of *Daphnegniduim*L.topical gel formulations at the time of preparation

Formulation	color	Appearance	Homogeneity	pH*
Control	white	clear and transparent	good	6.00±0,02
Formulated gel	brownish	clear and transparent	good	6,37±0,05

*Average of three readings

The formulated gel was brownish in color, clear and transparent in appearance. It was also homogenous by spreading on the skin and the pH was 6,37±0,05which was considered acceptable for topical application[24].The generation of some acid compounds e.g. lactic acid have been proposed for this slight acidity[25] .

Rheological evaluation revealed that the incorporation of natural extract did not significantly change the rheological properties of the prepared gel such as the viscosity(p>0, 05).It also exhibitedpseudoplastic i.e. non Newtonian flow behavior, characterized by a decrease in viscosity with increase in shear rate[26].

This behavior is used in pharmaceutical formulations. Indeed, a topical product should easily flow from the tube when a patient applies force, yet it should return to a sufficient viscosity as to remain on the skin and not flow off after application [27]. Results were as shown in Figures 3 and 4.

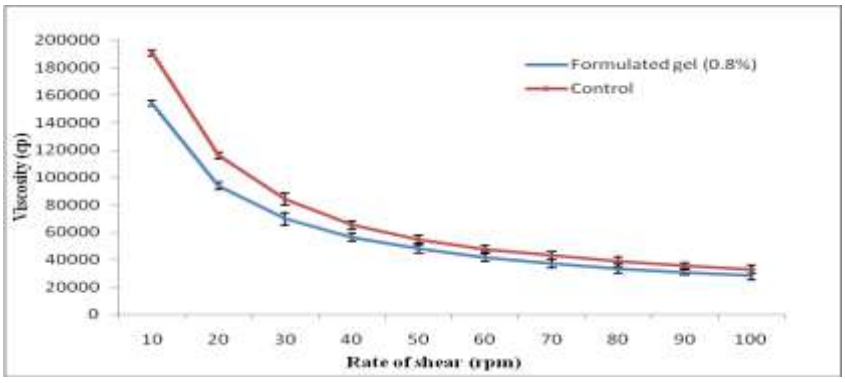

Figure 3: Curve of variation in viscosity as function of shear rate

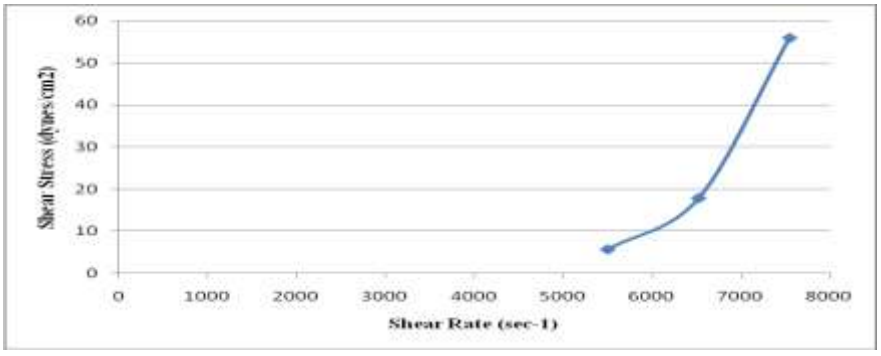

Figure 4:Rheogram (shear stress as a function of shear rate) of formulated topical gel

As for the gel consistency , the penetration depth was 21,50±0,25 mm and the flow threshold was 9,39 ±0,09 g cm-2 . This consistency could be due to the gelling properties of PEG 300[28.]

Stability study

During the stability study, the formulated gel showed no difference in aspect before and after freeze-thaw cycle. There were also no significant changes in color, appearance, pH and viscosity after storing at room temperature for three months (Table 3).Gel formulation provides better application property and stability in comparison to cream and ointments[29.] Furthermore, topical gel showed no irritation on the applied surface up to 24 hrs

Table 3: Stability study at room temperature of topical gel of *Daphnegniduim*L. leaf butanol extract

Parameters	**Initial month**	**2nd month**	**3rd month**
Color	brownish	brownish	brownish
Appearance	clear and transparent	clear andtransparent	clear and transparent
pH*	6,60±0,02	6,53±0,01	6,45±0,01
Viscosity*(cpsx10^3)	476±2	474±1	470±2

*Average of three readings

Anti-inflammatory activity

The anti-inflammatory activity of gel formulation was evaluated and results obtained are shown in Table 4. The reduction of edema produced by topical gel of *Daphnegniduim*L. was 76.87 %while Ibuprofen gel produced 57.8 % reduction of edema.

Table 4: Anti-inflammatory activity of gel formulation

Treatement	**Dose (mg/ear)**	**Edema (×10^{-2}mm± SD)**	**% Reduction of edema**
Blank gel (control)	25	17.3±0.02	-
Ibuprofen *5% gel*(standard)	25	7.3±0.03^c	57.80
Butanol extract 0.8%	25	4±0.05^c	76.87

Values are in Mean ± SEM; a: $p<0.05$, b: $p<0.01$, c: $p<0.001$versus control group.

The incorporation of crude extract in a dosage form such as gel had no effect on the anti-inflammatory activity.Some chemical constituents are considered as secondary metabolites components are bioactive agents[30].

Polyphenolic compounds mainly flavonoids which were found to be the highest content in butanol extract [31]may be responsible for producing anti-inflammatory activity which acts as antioxidant and potential inhibitor of cyclooxygenase, lipooxygenase and nitric oxide synthase[32].

Conclusion

In the present study butanol extract obtained from leaves of *Daphnegniduim*L. was used to formulate a topical gel. The choice of this species was motivated by its use in traditional medicine for antirheumatic and analgesic activities. The prepared gel was found to be satisfactory in physicochemical characteristics such as appearance, homogeneity, pH, viscosity and stability. It also possessed significant topical anti-inflammatory properties and had no skin irritation on applied surface using animal model. Therefore, further preclinical, clinical, and long-term stability as on human skin are required.

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