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The influence of extraction method on the composition and analgesic activity of *Calligonum comosum* phenolic extracts

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Abstract. The aim of this study was to evaluate the analgesic activity and the effect of extraction methods (ultra-sound: UM and maceration: MM) and solvents (ethanol: EtOH and methanol: MeOH) on the composition of phenolic extracts from *Calligonum comosum*. The results obtained by HPLC analysis demonstrated that the ethanol extracts have shown the highest content of total phenolic and flavonoid compounds. Also, the presence of most known phenolic compounds has been identified in all extracts, especially in the MeOH UM extract. The HPLC analysis showed the presence of ascorbic acid in methanol extracts and caffeic acid in ethanol extracts, and the maceration method shows a high concentration of phenolic compounds, the vanillin was detected in MeOH UM and the appearance of chlorogenic acid in UM extracts, finally the emergence of gallic acid, quercetin and rutin in some extracts. According to the results of the analgesic power, the methanolic extract of the maceration method induces a significant decrease in abdominal cramps compared to the control group and the values obtained are very close from those obtained with the standard anti-inflammatory drug (indomethacin). This result confirmed the beneficial effect of this Saharan plant.

Keywords: methanol extracts, ethanol extracts, maceration, ultrasound extraction, HPLC, analgesic activity.

1. Introduction

The therapeutic use of medicinal plants is very present in certain countries of the world and especially in developing countries, in the absence of a modern medical system [1]. In Africa, herbal medicines are still used by many populations for health care, and their therapeutic power is known empirically [2]. Algeria got a singularly rich and varied flora with approximately 3,000 plant species, 1.5% of which being endemic [3].

In Algerian Sahara, we found *Calligonum comosum* among the medicinal plants used in folk medicine to treat stomach disease [4] and for dental pain [5], as a stimulator and astringent [6]. It appears as a small shrub leafless, growing in sandy deserts of Algeria [7]. Some compounds (anthraquinones) isolated in this plant showed high antimicrobial activity [8], and others, like dehydrodicatechin, showed high cytotoxic and antioxidant activities [9, 10]. They were confirmed against fascioliasis animal disease and used as ethanolic extract of this plant.

The aim of this study was to estimate the effect of the extraction methods (ultrasound or maceration technique), and also the solvents effect by using two solvents, i.e. ethanol (EtOH) and methanol (MeOH). The determination of total and some of the phenolic compounds (ascorbic acid, gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin and vanillin) was performed by High Performance Liquid Chromatography (HPLC). The analgesic activity of methanolic extract of *Calligonum comosum* growing in Oued Souf region (South-East of Algeria) was also studied.

2. Experimental

2.1. Materials

2.1.1. Plant material. The aerial parts of the Larta plant (*Calligonum comosum*) were collected from El Oued region (South-East of Algerian Sahara) in March 2015. The drying took place at room temperature, protected from light and moisture. After drying, the dry plant was crushed and stored in a dry place.

2.1.2. Chemicals. Methanol was purchased from Biochem Chemophara (Montreal, Quebec, Canada). Ethanol and all the other chemicals used in this study, i.e. Folin-Ciocalteu reagent, aluminum chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), gallic acid and quercetin, were purchased from Sigma Aldrich, Chemicals Co (St. Louis, MO, USA).

2.2. Preparation of the extracts

The solvents used in this study are ethanol and methanol in two extraction methods.

2.2.1. Extraction by maceration method (MM). 1 g of dried aerial parts of the plant was introduced in 10 mL of the solvent (MeOH and EtOH) for 24 h. After filtration, these solutions were evaporated by rotary evaporator (type Buchi R-200) at 40 °C for methanol extracts and 45 °C for ethanol extracts [11].

2.2. Extraction by ultrasound method (UM). 1 g of dried aerial parts was added to 10 mL of each solvent then

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takes mixtures to ultra-sound type JP SELECTA (3.1A; 720W) at 30 °C for 30 min. The solvents were then evaporated in a rotary evaporator [12].

2.3. Methods

2.3.1. Determination of total polyphenolic compounds. 0.2 mL of each extract were mixed with 1 mL of Folin-Ciocalteu reagent (10%). Then 0.8 mL of 7.5% Na_2CO_3 solution was added to all blends. The mixtures were incubated at room temperature and protected from the light for 30 minutes. The absorbance was measured at 760 nm. The calibration curve was prepared with gallic acid solutions in the concentration (0.02-0.12 mg/mL). The total polyphenolic content is expressed as mg gallic acid equivalents (GAE)/g extract [13].

2.3.2. Determination of flavonoids. In the quantitative estimation of flavonoids, 0.5 mL of different extracts were added to 0.5 mL of 2% $AlCl_3$ solution. After one hour at room temperature and in the darkness, the absorbance was measured at 420 nm. The calibration curve was prepared with quercetin solutions in the concentration range of 0.03-0.1 mg/mL. Total flavonoids content was calculated as mg quercetin equivalents (QE)/g extract [14].

2.3.3. Instrumentation and chromatographic conditions. A High Performance Liquid Chromatography (HPLC) system we used (Shimadzu LC 20 AL equipped with the universal injector Hamilton 25 µL), the analytical column was a Shim-pack VP-ODS C18 (4.6 mm×250 mm, 5 µm type Shimadzu). UV-VIS detector SPD 20A (Shimadzu) was used. This chromatography is used for identifying the natural compounds non-volatile which are often affected by high heat [15].

2.3.4. Preparation of the mobile phase. The mobile phase was a mixture of acetonitrile and acetic acid 0.1%. The mobile phase was filtered before use through a 0.45 µm membrane filter, sonicated and pumped from the solvent reservoir to the column at a flow rate of 1 mL/min.

The effluent was detected at λ = 300 nm; the volume of injection was 20 µL and the column temperature was maintained at room temperature. Before injection, the column was equilibrated for 40-50 min with the mobile phase.

In this study the quantification of separated peaks was performed by calibration with standards: ascorbic acid, gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin and vanillin.

2.3.5. Evaluation of the analgesic power (writhing test). This study is carried out on male rats weighing between 140 and 160 g, coming from a farm of the central pharmacy of Tunis (SIPHAT). Throughout the experimental period, the animals were treated according to the rules of ethics inherent in animal experimentation. The acetic acid writhing test was used to evaluate the analgesic activity of the methanolic extract of the maceration method (MeOH MM). Four groups of rats (*n* = 6 per group) were injected (i.p.) with 0.1% acetic acid (10 mL/kg body weight), and the intensity of nociception was quantified by counting the total number of writhes over a period of 30 min. The animals received two doses of MeOH MM extract (50 and 100 mg/kg, i.p.) or sterile saline (control group, 0.9%, w/v), 30 min before acetic acid injection. Indomethacin (10 mg/kg) was used as a reference substance (positive control). The numbers of writhes were counted 5 min after acetic acid injection over 30 min [16].

Percentage inhibition of writhing was calculated using the following formula:

$$\% \text{ inhibition} = \frac{MNWc - MNWt}{MNWc} \times 100$$

where: MNWc = mean number of the writhing of the control group; MNWt = mean number of the writhing of the test group.

2.4. Statistical analysis

The obtained results are expressed as an average ± standard deviation. Data analysis was performed by applying Student's T-test, using SPSS and Excel software (Version 2010).

3. Results and discussion

3.1. Total polyphenol and flavonoids content

The polyphenol content was determined from the regression lines (calibration line) of gallic acid. The results showed that the extracts which were prepared in ethanol contained a high level of phenolic content (i.e., 17.53 mg GAE/g extract with ultrasound method, and 12.2 mg GAE/g extract for maceration method), in contrast to a low level of polyphenols registered with methanol extracts (Figure 1).

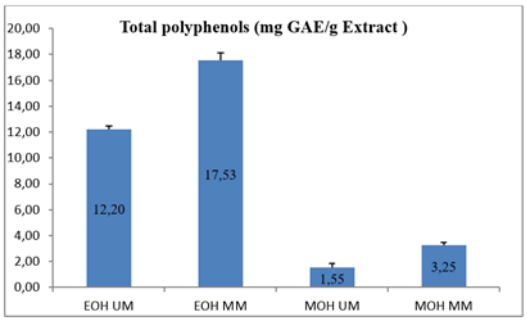

Figure 1. Polyphenols content in different extracts of *C. comosum*.

The flavonoid content was determined from the linear equation of quercetin. Figure 2 shows also that the ethanol extracts of *C. comosum* significantly outperformed the methanol extracts in the content of flavonoids.

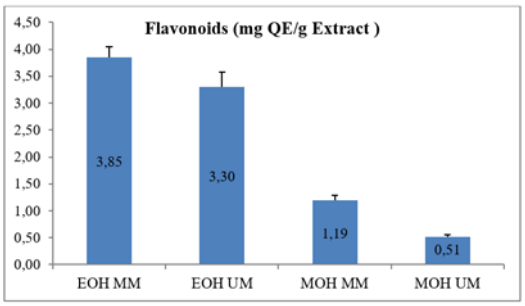

Figure 2. Flavonoids content in different extracts of *C. comosum*.

The variability observed in the values of polyphenol and flavonoid contents through the different extracts of *C. comosum* could be attributed to solvents and the conditions of extractions [17, 18], which affect clearly in the phenolic content of extracts [19].

3.2. HPLC chromatography

According to chromatograms set out in Figures 3-6, we were able to know the number of compounds for each extract (peaks) by comparing their retention times with standard compounds.

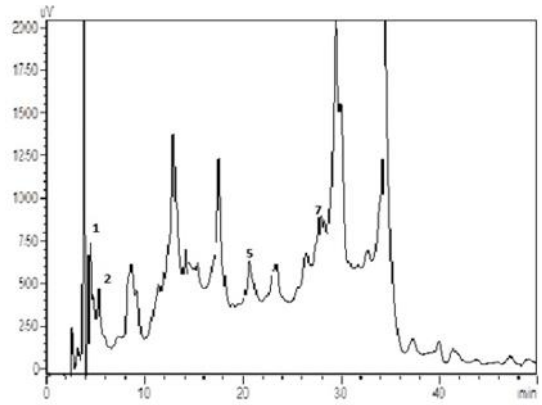

Figure 3. HPLC chromatogram of the extract MeOH MM of *C. comosum* at λ = 300 nm; 1 — Ascorbic acid; 2 — Gallic acid; 5 — Quercetin; 7— Rutin.

In MeOH MM extract were detected 30 phenolic compounds (Figure 3), among them 4 known compounds: ascorbic acid (105.04 µg/mg extract), gallic acid (0.82 µg/mg extract), quercetin (19.78 µg/mg extract) and rutin (0.29 µg/mg extract) (Table 1).

Table 1. The quantitative content of some phenolic acids and flavonoids compounds in the extracts of *C. comosum.*

Extracts of *C. comosum*		**MeOH MM**	**EtOH MM**	**MeOH UM**	**EtOH UM**
Number of peaks		**30**	**12**	**43**	**34**
Phenolic compounds	**Ret. time**	**Quantity (µg/mg extract)**			
Ascorbic acid	4.3	105.04	/	33.55	/
Gallic acid	5.1	0.82	/	0.75	0.53
Chlorogenic acid	13.9	/	/	0.34	0.16
Caffeic acid	16.2	/	0.20	/	0.03
Quercetin	20.5	19.78	/	69.04	9.34
Vanillin	21.2	/	/	0.16	/
Rutin	28.2	0.29	/	1.61	0.61

In the chromatogram of MeOH UM extract we detected a high number of peaks (43 peaks, Figure 4), six of them being known compounds: ascorbic acid, gallic acid, chlorogenic acid, quercetin, vanillin and rutin (Table 1).

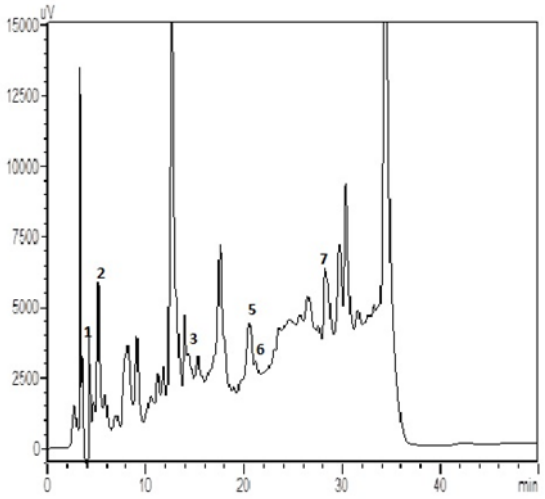

Figure 4. HPLC chromatogram of the extract MeOH UM of *C. comosum* at λ = 300 nm; 1 — Ascorbic acid; 2 — Gallic acid; 3 — Chlorogenic acid; 5 — Quercetin; 6 — Vanillin; 7— Rutin.

The EtOH MM extract does not contain any known phenolic compounds (Figure 5), except for caffeic acid with a low concentration (0.2 µg/mg extract).

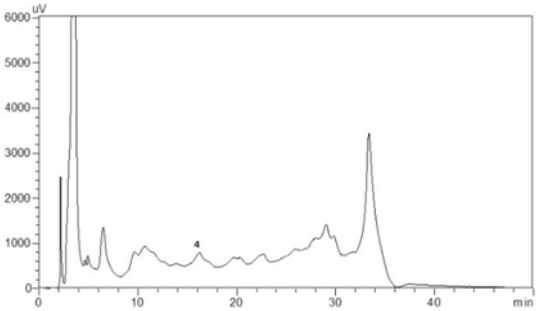

Figure 5. HPLC chromatogram of the EtOH MM extract of *C. comosum* at λ = 300 nm; 4 — Caffeic acid.

In contrast, in the EtOH UM extract were detected all tested phenolic compounds (Table 1) except ascorbic acid and vanillin (Figure 6).

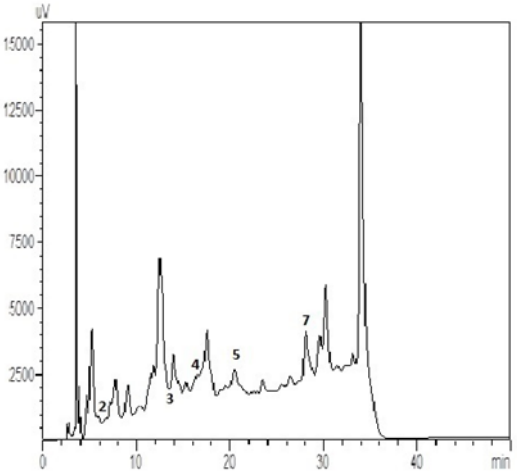

Figure 6. HPLC chromatogram of the EtOH UM extract of *C. comosum* at λ = 300 nm; 2 — Gallic acid; 3 — Chlorogenic acid; 4 — Caffeic acid; 5 — Quercetin; 7— Rutin.

According to these results, the ascorbic acid and caffeic acid may occur in the special solvent (methanol for ascorbic acid and ethanol to caffeic acid), but the maceration method showed a high concentration of these compounds.

The vanillin was observed only in MeOH UM, probably because of solvent properties and the extraction method.

The appearance of chlorogenic acid in both extracts of the UM method could be due to the extraction method.

The emergence of gallic acid, quercetin and rutin in some extracts without the other may be due to the method of extraction and the solvent used.

According to [20, 21], the solvents have a selectivity especially towards phenolic compounds with a low weight molecular.

The methods of extraction greatly influence the quantitative and qualitative content of the phenolic compounds.

3.3. Analgesic activity

In Table 2, we show the dose effects of the MeOH MM extract of *C. comosum* as well as the standard anti-inflammatory drug (Indomethacin) administered in batches of rats given acetic acid. These effects are expressed as a percentage of writhings inhibition.

Table 2. Analgesic activity of the MeOH MM extract of *C. comosum* on acetic acid induced writhes.

Treatment	Dose	Number of writhing responses	Inhibition (%)
Control (acetic acid 0.1%)	10 mL/kg	24 ± 2.0	-
Indomethacin	10 mg/kg	13.33±0.57***	45.83
C1: MOH MM extract	50 mg/kg	16.00±1.0*	33.33
C2: MOH MM extract	100 mg/kg	13.0±1.0 **	45.82

Values are expressed as Mean ± SEM. (n = 6).
*** Significantly different at $p < 0.001$ when compared to control.
** Significantly different at $p < 0.01$ when compared to control.
* Significantly different at $p < 0.05$ when compared to control.

According to these results, the beneficial effect of the methanolic extract of *C. comosum* induces a significant reduction in abdominal cramps compared to the control group. The values are very close to those obtained with the standard anti-inflammatory but at doses five-to-tenfold higher.

The appearance of abdominal torsions under the effect of acetic acid engages the effect of local peritoneal receptors [22] as well as the release of mediators such as prostaglandins and cytokines [23]. The analgesic effect of the extract of *C. comosum* supposes that the biomolecules contained in this plant could inhibit the release of chemical mediators, especially the tannins has been reported as an effective analgesic constituent [24].

4. Conclusions

The evaluation of extraction methods (ultra-sound and maceration) and solvents (ethanol and methanol) effects on the total and some phenolic compounds content (ascorbic acid, gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin and vanillin) by using High Performance Liquid Chromatography (HPLC) revealed that the solvent has an effect on the content of total polyphenols and the appearance of some phenolic compounds, but the method of extraction greatly affects the quantitative and qualitative content of the phenolic compounds.

The methanol extract of *C. comosum* has the best analgesic power compared to the control group and the values are very close to those obtained with the standard used.

Conflict of interest

The authors declare no conflict of interest.

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