



Phenolic compounds and *in vivo* anti-inflammatory activity of aqueous extract of *Centaurium umbellatum* (Gibb.) Beck. flowers in northern Algeria

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ABSTRACT

This study investigated the high performance liquid chromatography (HPLC-PDA) profiling of phenolic compounds, and evaluated the anti-inflammatory activity of aqueous extract of *Centaurium umbellatum* (Gibb.) Beck. flowers growing in Algeria. The extract was subjected to HPLC for the identification of the major bioactive polyphenols present in it. Anti-inflammatory effect of the aqueous extract in rats for two different test models: Acute-carrageenan induced paw oedema and sub-acute cotton pellet induced granuloma was inspected. Twelve phenolic compounds were identified in HPLC analysis of the etheric extract of *C. umbellatum* flowers. Six of them, quercetin, orcinol, monohydrated gallic acid, β -resorcylic acid, *p*-anisic acid and *o*-coumaric acid, were revealed for the first time in this plant, through HPLC. At a dose of 150 mg/kg body weight, a good acute anti-inflammatory activity was observed in Wistar strain rats with a reduction in the paw oedema of 75.53%, in comparison to indomethacin (62.12%). These results were confirmed by the granuloma test caused by cotton pellet. After 8 days of the treatment with the aqueous extract at 150 mg/kg, the reduction in the paw oedema was 60.86%, compared to Piroxicam, a non-steroidal anti-inflammatory drug (62.17%). In all the two models of anti-inflammatory studies, 100 and 150 mg/kg b.w. doses of the extract showed significant effect ($p < 0.01$). It is concluded that the phenolic compounds are responsible for acute anti-inflammatory activity of *C. umbellatum* and justifies its ethnomedicinal use for fever, eczema, dermatitis and diabetes.

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INTRODUCTION

Centaurium umbellatum (Gibb.) Beck. ssp. *suffruticosum* (Salzm.) Maire, previously known as *Centaurium erythraea* Rafn., belongs to the Gentianaceae family and is usually known as "common centaur" (Rybczyński et al., 2015). In Algeria, it is commonly known as *mraret el*

hanech or *k'lilou*, due to its bitter taste (Trabut, 1935; Quézel and Santa, 1963). This is an erect annual or biennial herb, reaching half a meter in height. *C. umbellatum* inflorescences contain many small bright pink flowers, of about a centimeter across, flat-faced with



Figure 1. Aerial parts of: **a**, specimen of *C. umbellatum* (Gibb.) Beck. ssp. *suffruticosum* (Salzm.) Maire harvested in the region of Medea; **b**, sample of the herbarium of National High School of at El Harrach (Algiers, Algeria).

yellow anthers and usually flowers from June to September. The fruit is a cylindrical capsule.

C. umbellatum is a widespread plant of northern Africa, Europe, western Asia and it has also been carried to Northern America (Newall et al., 1996). It is traditionally used in folk medicine either in mild dyspeptic and/or gastrointestinal disorders (Cunha et al., 2009). In Algeria, *C. umbellatum* is also used, for the treatment of several affections like fever, diabetes, hepatic congestion, painful dyspepsia (flatulence), eczema dermatitis, etc. (Berkan et al., 1991; Hachemi et al., 2013). Besides, in Morocco, *C. umbellatum* is used for kidney disorders treatment (El-Hilaly et al., 2003) and as diuretic (Haloui et al., 2000). *C. umbellatum* utilization is discouraged in young people and should also be avoided by lactating women, due to its bitter constituents (Cunha et al., 2009).

Phytochemical investigations enabled to signal the presence of organic acids or phenols without specifying (Merad, 1973); to highlight the presence of kaempferol (Lebreton and Dangy-Gaye, 1973). Meanwhile, other authors have identified a series of phenolic acids: *para* and *meta*-OH-benzoic, protocatechuic, vanillic, syringic, *p*-coumaric, ferulic, caffeic, and sinapic (Hatjimanoli and Debelmas, 1977). Two phenolic acids were isolated and identified: monohydroxy- and 2,5-dihydroxy terephthalic acids (Hatjimanoli et al., 1988). It should be noted that *C. umbellatum* contains bitter substances

(secoiridoids) and xanthone derivatives (Aberham et al., 2011; Waltenberger et al., 2015).

Today, due to its antioxidant characteristics (Neagu et al., 2016), its gastroprotective effect (Tuluze et al., 2011), its diuretic (Haloui et al., 2000), antispasmodic (Chda et al., 2016), anti-inflammatory and analgesic properties (Berkan et al., 1991; Haloui et al., 2000), it continues to draw the attention of researchers.

The literature survey revealed that there are no research studies carried out related to the flowers of this plant, hence, in the present study, we attempted to investigate the HPLC profiling of bioactive polyphenolic compounds, and evaluate *in vivo* anti-inflammatory activities of aqueous extract of *C. umbellatum* (Gibb.) Beck. flowers, in acute and sub-acute models.

MATERIALS AND METHODS

Collection and identification of plant material

C. umbellatum (Gibb.) Beck. ssp. *suffruticosum* (Salzm.) Maire flowers were collected from Medea, Algeria during the flowering period (June 2012). The samples were identified using the Quézel and Santa flora and confirmed with the herbarium of National High School of at El Harrach (Algiers, Algeria). A voucher specimen (Figure 1, Accession no: CUSF: 2164) has been deposited in the herbarium of Laboratory Medical, Botany and Cryptogamy of Algiers's University. The other samples were shrewdly pulverised.

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Extraction of phenolic compounds

The collected flowers of plant were separated from undesirable materials, plants or plant parts manually and then were properly washed with water, shade dried, and powdered. It was stored in a well-closed container to protect from light and moisture till used.

The extraction of phenolic compounds was realised according to Netien and Lebreton Technic (1964). The powdered plant materials (4 g) were extracted in an orbital shaker with hydrochloric acid 2 N (50 mL) during 40 min, into a water bath at 40°C, to obtain acidified aqueous extract of *C. umbellatum*. The extract was initially filtered in a cotton plug to get rid of the plant debris, and next through Whatman No. 1 filter paper. The resulting filtrate was then extracted with diethyl ether (3 × 50 mL). The combined extracts were dried over anhydrous Na₂SO₄. The solvent was removed in a rotary vacuum evaporator (R-100, Buchi) under reduced pressure. The plant yielded a 5.93% extract of the dried plant material, which was stored in the dark at +4°C for further analysis.

Preparation of aqueous extract

The powdered flowering tops (10 g) was mixed with sterile distilled water (100 mL) in a clean flat-bottomed glass container, and were stirred and heated at 70°C for about 4 h. After cooling, the extract was filtered by using Whatman No. 1 filter paper. The filtrate was collected and frozen in ice cube container. The frozen ice cube was freeze-dried (that is, lyophilisation) to obtain concentrated aqueous extracts in powdered form. Extracts were stored at +4°C in the dark until their use as test drug sample for the animal studies.

Test animals

For the screening of *in vivo* anti-inflammatory activity, 24 inbred male rats Wistar strain weighing 180 to 250 g were acclimated for one week under standard laboratory condition at the Laboratory of Pharmaco-toxicology, Research and Development Center, SAIDAL (Algiers, Algeria), before use. Animals were housed in groups of three or four in solid-bottomed plastic cages with free access to tap water and standard feed: original granulate (National Livestock Feeding Agency, Algiers, Algeria) *ad libitum*. Room temperature was set at 23 ± 1°C and animals were subjected to a 12/12 h light/dark cycle. All experiments were performed in accordance with National Animal Care Guidelines and were in compliance with internationally accepted principles for laboratory animal use and care.

Chemicals and reagents

Unless specified otherwise, all solvents and reagents were

of analytical grade. Kaempferol (KA), quercetin (QU), orcinol (OR), monohydrated gallic acid (GA), β-resorcylic acid (RA), *para*-anisic acid (AA), *ortho*-coumaric acid (CA), syringic acid (SyA), vanillic acid (VA), ferulic acid (FA), sinapic acid (SA), and protocatechuic acid (PCA) were purchased from Fluka Laboratory. Methanol (HPLC), acetonitrile (HPLC), ethyl ether (Merck Laboratory), carrageenan (1% suspension in 0.9% physiological saline, Sigma, USA), indomethacin (Saidal subsidiary pharml, Dar el Beida, Algiers, Algeria), Piroxicam (Andenex-Chemistry), thiopental (Trittau, Germany), hydrochloric acid 2 N (Prolabo Laboratory), sodium chloride 0.35 g/L (conductive solution of the plethysmometer) were of analytical grade.

Identification of polyphenols by HPLC

HPLC was performed using a Discovery® C18 column (25 cm × 4.6 mm, 5 μm.i.d., Supelco) attached to a 2690 HPLC system (Waters) with in-line degasser, autosampler, sample incubator, and column heater. Compound elution was monitored with a Waters 996 UV/Visible photodiode array detector (PDA). Complete baseline separation of all compounds was achieved at a flow rate of 0.8 mL/min with the column incubated at a constant temperature of 40°C. The injection volume was 20 μL.

The mobile phase consisted of water-hydrochloric acid (98:2) (solvent A) and acetonitrile-water- hydrochloric acid (79:19:2) (solvent B). Elution program was adjusted after several trials based on Semmar works (Semmar, 2000). The system was run at a gradient elution program, that is, 0 min at 10% A/90% B, 30 min at 17% A/83% B, 45 min at 17% A/83% B, 100 min at 50% A/50% B, 110 min at 50% A/50% B, 112 min at 70% A/30% B, 118 min at 70% A/30% B, and 120 min at 10% A/90% B. For PDA detection, the wavelength program was set right to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 260 nm (phenols, phenolic acids) and changed to λ 354 nm (flavonols, flavones). The PDA was set at an acquisition range from 190 to 700 nm. The detection and quantification of OR, GA, RA, AA, CA, SyA, VA, FA, SA, and PCA were carried out at 260 nm, of QU and KA at 354 nm, respectively.

Standard and sample preparation

Standard solutions for each of the phenolic compounds were prepared in methanol. All solutions were stored in the dark at +4°C. The calibration curves of the standards were prepared by serial dilution of the standard stock solution (0.2 mg/L) with methanol. The calibration curves were drawn from the chromatograms as peak area versus concentration of standard. A solution of phenolic compounds extracted from *C. umbellatum* (Gibb.) Beck. flowers was prepared at a concentration of 5 mg/mL in methanol. The samples were

stored at low temperature (5°C) in the dark. Spiking of the solution samples was done with phenolic standards to identify the individual polyphenols. Before HPLC analysis was carried out, all solutions were filtered through 0.20 µm PTFE syringe filter and degassed in an ultrasonic bath for 15 min.

Anti-inflammatory activity

Carrageenan-induced oedema test (acute-model)

Carrageenan-induced oedema in hind paw of the rat was used as the animal model to determine acute inflammation, according to the method of Winter et al. (1962). The rats were weighed, identified and was made to fasted for 18 h the day before the experiment.

The rats were divided into four groups (six rats per group). Group I (control) was given distilled water (10 mL/kg), while Group II (positive control) received 10 mg/kg b.w. of indomethacin orally. Group III and IV received 100 and 150 mg/kg b.w. of the *C.umbellatum* (Gibb.) aqueous extract orally, respectively. Acute inflammation was induced in all the four groups by plantar aponeurosis injection of 0.1 mL of 1% carrageenan solution in the right paw of each rat, 1 h after the oral administration of the tested materials. The paw volume was recorded by the Waters plethysmometer 7150 (Apelex), 3 h after the administration of the drug and the extract. The percentage inhibition of inflammatory effect of the aqueous extract, compared to control test, was calculated using the following expression:

$$\% \text{ Inhibition} = [(V_c - V_t) / V_c] \times 100$$

Where v_c is the degree of inflammation by the control group and v_t is the degree of inflammation by the test group.

Cotton pellet induced granuloma (Sub-acute model)

Two autoclaved cotton pellets weighing 20 ± 1 mg were implanted in both sides of the groin region of each rat (D'Arcy et al., 1960). Twenty-four rats were divided into three groups of eight animals each. Group I (control) was supplied with physiological water NaCl 9‰ (10 mL/kg). Group II (positive control) received Piroxicam orally at the dose of 5 mg/kg b.w. Group III was given 150 mg/kg b.w. of *C. umbellatum* (Gibb.) Beck. aqueous extract orally for 8 consecutive days.

Using a strictly aseptic technic, a dorsal incision, after depilation, was made in anesthetized animals, with 15 mg/kg thiopental by intraperitoneal route. Then, the cotton pellets along with the granuloma tissues were dried in an oven at 60°C for 18 h, weighed and resulted weights were compared with the control. The percentage inhibition of granuloma by the test drug was determined.

Statistical analysis

All data were expressed as mean \pm SEM and Student's *t*-test was applied to determine the significance of the difference between the control groups and rat treated with the test compounds.

RESULTS AND DISCUSSION

HPLC assay of bioactive polyphenols in *C. umbellatum* (Gibb.) Beck. flowers

Individual phenolic compounds of *C. umbellatum* (Gibb.) Beck. flowers were identified by HPLC. The chromatographic separations of phenolic compounds in the etheric extract are shown in Figures 2, 3 and 4. It should be noted that the identification of the flavonoglycos, phenols and phenolic acids was realised by their retention time, UV spectrum in methanol and by superposition with standard substances spectrum used. Based on the experimental results, six new phenolic compounds were identified in *C. umbellatum* (Gibb.) Beck. flowers for the first time in this species.

All the revealed compounds represented in Figure 5, are: a flavonol: quercetin (76.13 min), a phenol: orcinol (16.58 min) and four phenolic acids: monohydrated gallic acid (6.96 min, major peak), β -resorcylic acid (31.63 min), *p*-anisic acid (55.11 min) and *o*-coumaric acid (76.34 min). This study also confirmed the presence of a flavonol: kaempferol (61.29 min) and five other phenolic acids: protocatechuic acid (9.83 min), syringic acid (14.12 min), vanillic acid (15.65 min), ferulic acid (32.32 min), and sinapic acid (34.13 min). Other authors had signaled the presence of phenolic acids or phenols without any other precisions (Merad, 1973).

Anti-inflammatory activity

Carrageenan-induced paw oedema

The anti-inflammatory activity of the aqueous extract of *C. umbellatum* (Gibb.) Beck. ssp. *suffruticosum* (Salzm.) Maire flowers' using carrageenan-induced oedema tests is indicated in Table 1. In this test, the positive control (indomethacin 10 mg/kg) decreased significantly ($p < 0.01$) the paw oedema after 3 h of the carrageenan injection with inhibition 62.12%. A maximum oedema paw volume of 0.45 mL was observed in the control rats, 3 h after the carrageenan injection (1%). Rats treated with the extract at 150 mg/kg b.w. significantly ($p < 0.01$) decreased the carrageenan-induced oedema paw volume after 3 h compared to the standard drug indomethacin at a dose of 10 mg/kg b.w. The reduction in the paw volume by the 150 mg/kg b.w. was 75.53%, which was higher than that of the indomethacin (62.12%) at 3 h.

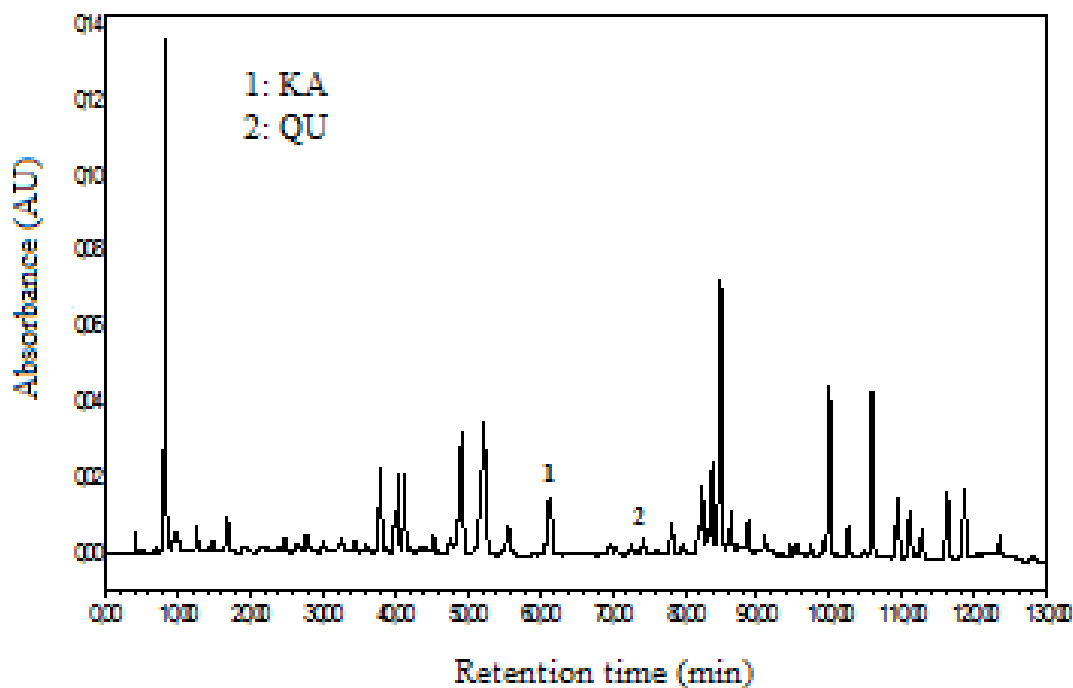


Figure 2. HPLC profile of the flavonoids contained in etheric extract of the flowering tops of *C. umbellatum* (Gibb.) Beck. detected at 354 nm.

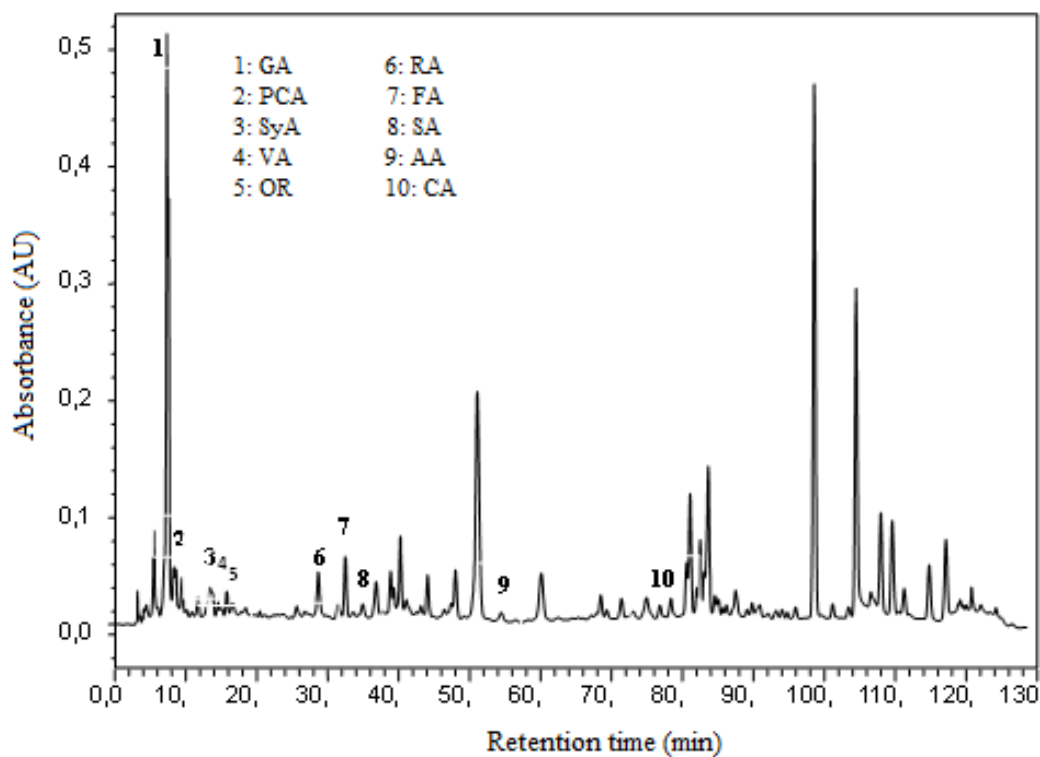


Figure 3. HPLC profile of phenolic acids and phenol contained in etheric extract of the flowering tops of *C.umbellatum* (Gibb.) Beck. detected at 260 nm.

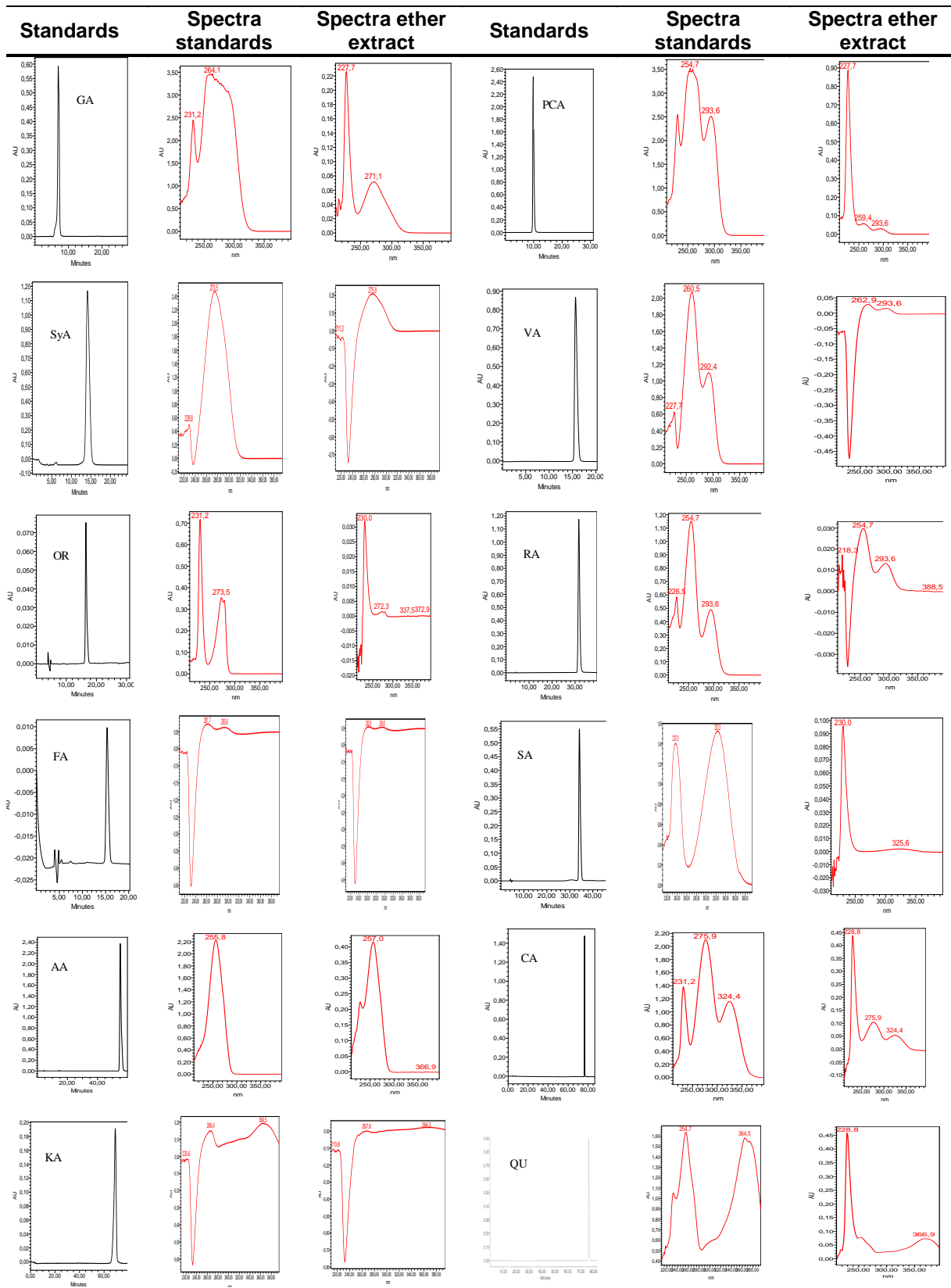


Figure 4. HPLC-UV spectra of phenolic acids, phenol and aglycone detected at 260 and 354 nm in the etheric extract of the *C. umbellatum* (Gibb.) Beck. flowers against corresponding genuine products.

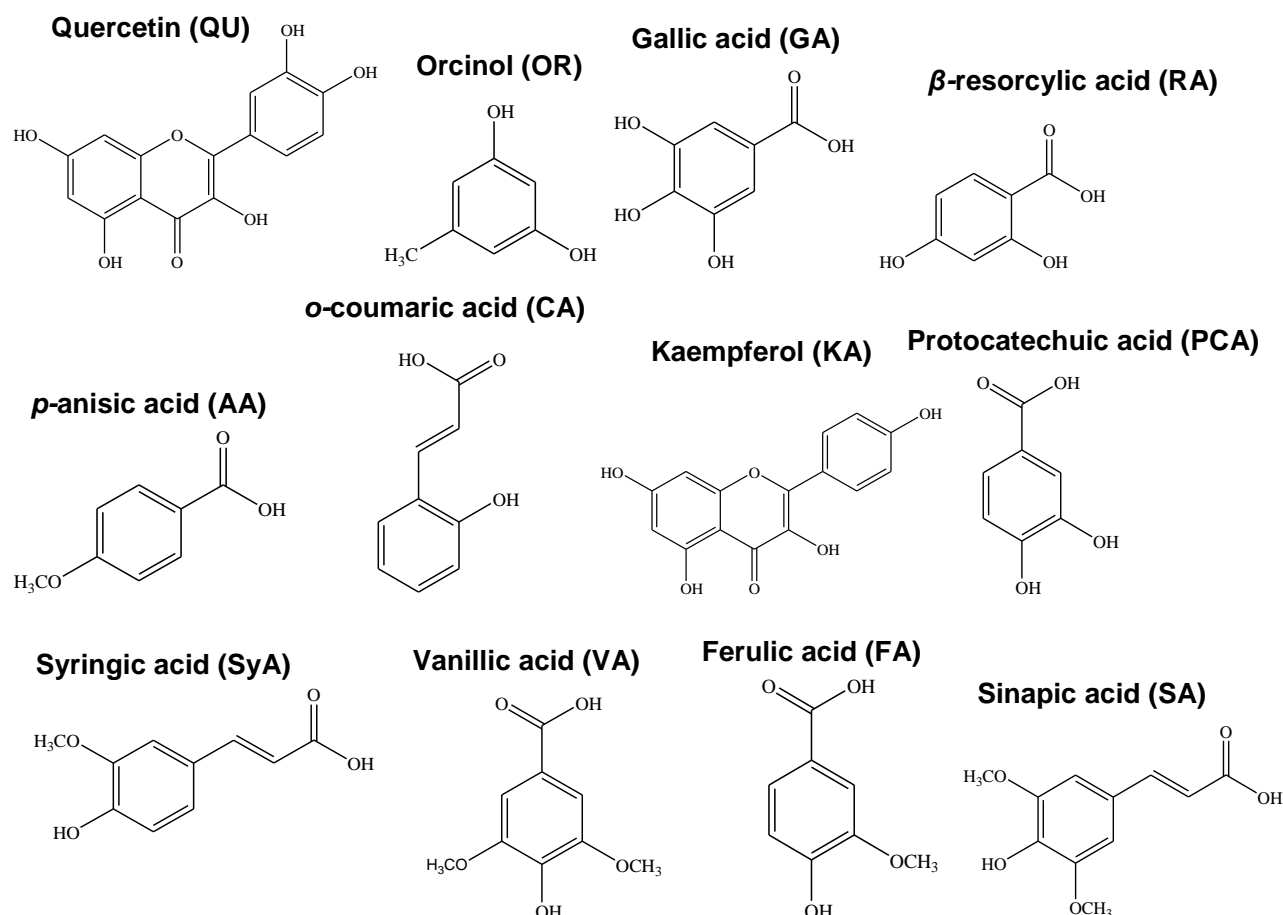


Figure 5. Structures of 12 compounds identified from an etheric extract of *C. umbellatum* (Gibb.) Beck. flowers.

Table 1. Anti-inflammatory activity of aqueous extract of *C. umbellatum* (Gibb.) Beck. on carrageenan-induced paw oedema in rats.

Treatment	Dose (mg/kg b.w.)	W (g)	Paw oedema volume (mL)		Oedema (%)	Oedema reduction (%)
			V _c	V _t		
Control	10 (mL/kg)	172.83	0.270	0.450	66.0	-
Indomethacin	10	175.83	0.268	0.335	25.0	62.12
<i>C. umbellatum</i>	100	174.0	0.250	0.335	34.0	48.48
<i>C. umbellatum</i>	150	172.5	0.258	0.300	16.27	75.53

W, Average weight of rat of each group; V_c, average initial volume of the right leg of each lot before injection of carrageenan solution (1%); V_t, average volume of the right legs of each lot after injection of carrageenan solution (1%).

Cotton pellet induced granuloma (Sub-acute model)

In granuloma induced sub-acute inflammation model, the test of the aqueous extracts of *C. umbellatum* (Gibb.) Beck. flowers at the dose of 150 mg/kg b.w. had significant anti-inflammatory activity ($p < 0.01$) (Table 2). The percentage inhibition of granuloma after drug administration was found to be 60.86% for the aqueous extracts of *C. umbellatum*

(Gibb.) Beck. at the dose of 150 mg/kg b.w. and 62.17% for the standard drug Piroxicam.

Inflammation is the response of the host to infectious or sterile tissue injury and has the physiological purpose of restoring tissue homeostasis (Serhan, 2007; Medzhitov, 2010). However, unresolved inflammation can lead to tissue damage, giving rise to a large group of disorders which underlie a vast variety of human diseases (Nathan

Table 2. Anti-inflammatory activity of aqueous extract of *C. umbellatum* (Gibb.) Beck. on cotton pellet induced granuloma in rats.

Treatment	Dose (mg/kg b.w.)	Mean weight of granuloma (g)	Inhibition of granuloma (%)
Control	10 (mL/kg)	0.230 ± 0.005687	/
Piroxicam	5	0.087 ± 0.030794	62.17
<i>C. umbellatum</i>	150	0.090 ± 0.029063	60.86

Data represent mean ± SEM of 8 animals. $p < 0.01$ compared to control (student's *t*-test).

and Ding, 2010; Ambriz-Pérez et al., 2016). Non-steroidal anti-inflammatory drugs (NSAIDs) have been used to treat inflammation since the nineteenth century and are among the most commonly utilized medications in the world today (Rainsford, 2007). NSAIDs relieve the hyperalgesic symptoms associated with inflammation by inhibiting the COX enzyme and the resultant inhibition of prostaglandins synthesis from arachidonic acid (Vane and Botting, 1996). In this study a positive step was put forward to investigate the anti-inflammatory actions of *C. umbellatum* (Gibb.) Beck. utilized traditionally as an antipyretic especially against fever. The aqueous extract of the flowers of *C. umbellatum* (Gibb.) Beck. was found to have significant ($p < 0.01$) anti-inflammatory property in all the dose level in acute carrageenan induced paw oedema, a test which has significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (Mossa et al., 1995).

In sub-acute study, the inflammatory granuloma is the typical feature (Olajide et al., 2000) which has been reduced significantly ($p < 0.01$) by the aqueous extract of the flowers of *C. umbellatum* (Gibb.) Beck. at the dose level of 150 mg/kg.

The percentage protections of inflammation at the dose level of 150 mg/kg in acute and sub-acute model were 75.53 and 60.86 respectively at 3 h. It provided the feedback that the aqueous extract of the flowers of *C. umbellatum* (Gibb.) Beck. was more effective in acute than sub-acute inflammation.

This study has shown that the *C. umbellatum* (Gibb.) Beck. extract possesses a significant antioedematogenic effect ($p < 0.01$) on paw oedema induced by carrageenan compared favorably with indomethacin in treated rats. Phytochemically, the *C. umbellatum* (Gibb.) Beck. extract was found to be rich in flavonoids, phenols and phenolic acids. The phenolic compounds are naturally occurring compounds, considered to possess anti-inflammatory activities, both *in vitro* and *in vivo* (Ambriz-Pérez et al., 2016). Although the precise mechanisms of this anti-inflammatory activity are not fully elucidated, there is a correlation between the high intake of food rich in these compounds and a down regulation of the inflammatory response (Alarcón de la Lastra and Villegas, 2005).

The acute anti-inflammatory activity of the *C. umbellatum* (Gibb.) Beck. extract might be due to the presence of phyto-constituents such as flavonoids,

phenolic acids etc.

Conclusion

This report contributes to complement the phytochemical analysis of phenolic compounds of the flowering tops of *C. umbellatum* (Gibb.) Beck. The identification of four phenolic acids monohydrated gallic acid, β -resorcylic acid, *p*-anisic acid and *o*-coumaric acid have been reported. This study also allowed the researchers to identify five phenolic acids and one flavonol cited in the literature: Protocatechuic acid, syringic acid, vanillic acid, ferulic acid, and sinapic acid and kaempferol. The richness of *C. umbellatum* (Gibb.) Beck. with phenolic compounds could explain its acute anti-inflammatory activity. The study has rationalized the ethno-medicinal utility of the flowers of *C. umbellatum* (Gibb.) Beck. for various ailments related to inflammatory disorders.

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