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Full Length Research Paper

Anti-inflammatory evaluation of immature fruit and seed aqueous extracts from several populations of Tunisian *Citrullus colocynthis* Schrad

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Plant extracts are some of the most attractive sources of new drugs and have shown promising results for the treatment of inflammation and immune-related diseases, including rheumatoid arthritis. *Citrullus colocynthis* Schrad. (Cucurbitaceae) endemic in Tunisia, is widely used in folk medicine to treat many inflammation disorders. The aim of this study is to quantify the alkaloid and the flavonoid levels of different populations of *C. colocynthis* fruit and seed aqueous extracts at immature state. After acute toxicity assay, these extracts were screened for anti-inflammatory activity using the carrageenan-induced paw edema assay in rats. Alkaloid and flavonoid levels vary among the population. The best anti-inflammatory activities were obtained with immature fruits from south Tunisia. Therefore, *C. colocynthis* Schrad. could be a potential useful product suitable for further evaluation for inflammatory diseases.

Key words: *Citrullus colocynthis* Schrad., alkaloids, flavonoids, toxicity, anti-inflammatory, Tunisia.

INTRODUCTION

Since ancient times, several diseases have been treated by administration of plant extracts. Interest in ethno-pharmacy as a source of active compounds has increased worldwide, particularly for the development of anti-inflammatory drugs. Plants with analgesic and anti-inflammatory activities have become more interesting because some of these plants are part of the arsenal of modern medicine and many people are aware of problems associated with the over-prescription and misuse of usual drugs.

Citrullus colocynthis Schrad. (Cucurbitaceae), growing in Tunisia (Pottier-Alapetite, 1981), is widely used in Tunisian folk medicine for treating many diseases such as various contagious diseases, hypertension and rheumatism (Le Flock, 1983; Boukef, 1986). Anti-inflammatory traditional healers seem not to pay attention to the fruit's degree of maturity and literature rarely mention if seeds are present in preparations involving ground fruit/pulp. Common preparations use juice, fresh or dried (often ground) fruit material. Extracts (maceration or boiling) are prepared either in water or in aqueous mixtures (honey, milk and water/olive oil at various ratios). Methods of administration are by ingestion or massage (Le Flock, 1983; Boukef, 1986; Bellakhdar, 1999; El-

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Abbreviations: TOF, Total oligomeric flavonoids; **TLC,** thin layer chromatography; **ASL,** acetyl salicylate of lysine.

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Ghadi and Bshana, 1988).

Some studies have demonstrated the medicinal effect of *C. colocynthis* Schrad. as anti-tumour (Tannin-Spitz et al., 2007), immunostimulant (Bendjeddou et al., 2003), anti-microbial (Marzouk et al., 2009, 2010a) and antioxidant (Marzouk et al., 2010b), and against hepatic diseases (Gebhardt, 2003), hyperglycaemia (Al-Gaithi et al., 2004) and hair loss (Roy et al., 2007).

However, plants in the environment are exposed to a range of abiotic stresses like osmotic, salinity, temperature and heavy metal toxicity which affects their growth and other metabolic processes such as alkaloid and flavonoid productions. These secondary metabolites were synthesized by plants to defend themselves against the harmful action of external agents (Heller et al., 1993; Waller and Nowacki, 1978). Thus, alkaloid and flavonoid contents depend on the geographical distribution.

In a previously published paper, *C. colocynthis* immature fruits and seeds were demonstrated as the most efficient analgesic and anti-inflammatory parts (Marzouk et al., 2010c). The current study measured *in vivo* toxicity and anti-inflammatory activity of seven populations of *C. colocynthis* Schrad. using mice and rat as models. Preparation and tests were carried out on the reconstituted lyophilized aqueous extracts.

MATERIALS AND METHODS

Plant materials

C. colocynthis Schrad. plants were collected in August (2007) from seven stations (Table 1 and Figure 1). The identification was performed according to the flora of Tunisia (Pottier-Alapetite, 1981) and voucher specimens were deposited in the biological laboratory of the Faculty of Pharmacy of Monastir.

Extraction protocol

The extraction was performed on fresh immature fruits or seeds. Each plant part was ground with a mixer and added to distilled water. The mixture was allowed to reflux for 30 min, after which the solution was allowed to cool (4 h at 4°C). The mixture was then filtered using filter paper (Whatman no.1) under the vacuum of a water pump. The filtrate obtained was lyophilized, yielding the lyophilized aqueous extract. Yields are given in Table 2.

Quantitative alkaloid screening

Alkaloids were quantified according to the volumetric procedure of the Official Method of European Pharmacopoeia. Briefly, to 3 g of each dried plant parts, ammonium hydroxide (5 ml), ethanol (10 ml) and diethyl ether (30 ml) were successively added. After 4 h of maceration, the mixture was lixiviated by 150 ml of ether and 50 ml of chloroform. The obtained solution was reduced to 50 ml by a rotavapory (Heidolph) and then treated with sulphuric acid (0.5 N, 3 x 20 ml). Bases were removed from the extract through precipitation with an excess of ammonium hydroxide (3 ml). The supernatant was then extracted with chloroform (3 x 10 ml). The precipitate containing alkaloids was separated by means of filtration and treated again with chloroform which was evaporated under low pressure. The presence of alkaloids was verified by Dragendorff's reagent confirmed with Bouchardat's and Meyer's reagent (Treas and Evans, 1984). To the residue, 20 ml of sulphuric acid was added. The excess sulphuric acid was titrated with sodium hydroxide (0.02 N) using the phenolphthalein indicator. The percentage of total alkaloids was then calculated according to the following formula:

$$\text{Alkaloid level (\%)} = ((V_{H2SO4} - V_{H2SO4ex}) \times 5.788 / W) \times 100$$

Where, V_{H2SO4} = 20 ml, $V_{H2SO4ex}$ = volume of excess sulphuric acid, W = 3 g and 1 ml of sulphuric acid correspond to 5.788 mg of alkaloids.

Quantitative oligomeric flavonoid screening

In order to obtain total oligomeric flavonoids (TOF), the powdered seeds were macerated separately for 24 h in a mixture of acetone/water (2:1). Each extract was then filtered and the acetone was evaporated under low pressure. Tannins were removed from the aqueous phase through precipitation with NaCl for 24 h at 5°C and the supernatant was then extracted with ethyl acetate, concentrated and precipitated with excess chloroform. The precipitate containing TOF was separated by means of filtration (Paris and Moyse, 1976). The presence of flavonoids was verified by the reaction of 'cyanidine' in the presence of hydrochloric acid and magnesium. After release of hydrogen, an orange colour with red purple indicates the presence of the flavonoids. The appearance of the spots corresponding to the flavonoids on the plates of thin layer chromatography (TLC) is done by pulverization using a solution of 2% $AlCl_3$ in methanol. The plates of TLC are then observed under UV with 366 nm; the spots corresponding to the flavonoids have a yellow fluorescence at intense yellow fluorescence (Paris and Moyse, 1976). The percentage of total oligomer flavonoids was then calculated according to the following formula:

Total oligomer flavonoids level (%) = (weight of total oligomer flavonoids / powdered material weight) x 100

Animals

Male adult Wistar rats weighing 160 to 180 g and Swiss albino mice (weighing 18 to 25 g) of both sex were obtained from Pasteur institute (Tunis, Tunisia). They were housed in polypropylene cages and were left for 2 days for acclimatization to animal room maintained under controlled condition (a 12 h light – dark cycle at 22 ± 2°C) on standard pellet diet and water *ad libitum*. Before the day of assay, only the Wistar rats were fasted overnight with free access to water. Housing conditions and *in vivo* experiments approved according to the guidelines established by the European Union on Animal Care (CFE Council (86/609)) were used. The rats were used for the anti-inflammatory evaluation of the aqueous extracts, while the mice were used for the analgesic investigation and for the acute toxicity testing. Animals were divided into drug-treated 'test' and saline-treated 'control' groups of six or eight animals per group.

Acute toxicity

For acute toxicity, mice were divided into groups of eight animals each. One group served as a control and received 0.9% NaCl alone (10 ml/kg) given intraperitoneally (i.p.), while the remaining groups were treated with increasing doses of the aqueous extract; 50, 100,

Table 1. Sites and voucher specimens of *C. colocynthis* Schrad. populations.

Population	Site	Voucher specimens
Hammamet	Hammamet Sud (36°25 N, 10°34 W)	C.C-07.07
Mahdia	Chorban (35°20 N, 10°32 W)	C.C-06.07
Kasserine	Mozgam (35°11 N, 8°46 W)	C.C-05.07
Sbeitla	Sbitla (35°14 N, 9°07 W)	C.C-04.07
Sidi-Bouzid	Jelma (35°16 N, 9°25 W)	C.C-03.07
Sfax	Skhira (34°10 N, 10°04 W)	C.C-02.07
Medenine	Sidi Makhlouf (33°33 N, 10°27 W)	C.C-01.07

N: North; W: west.

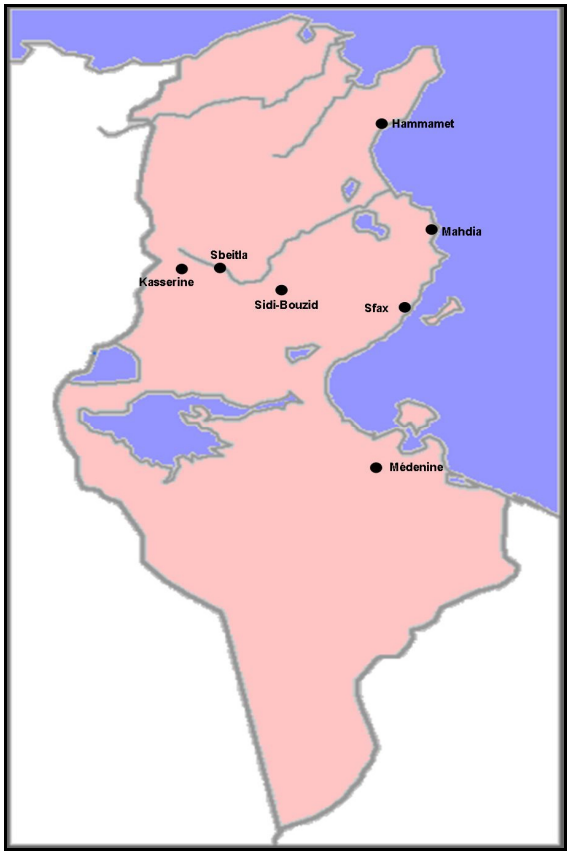

Figure 1. Geographical map of Tunisia.

250, 500, 750, 1000, 1500, 2000, 3000 and 4000 mg/kg (i.p.), respectively.

The mortality rate within a 48 h period was determined and the LD_{50} was estimated according to the method described by Miller and Tainter (1944). According to the results of acute toxicity test, doses were chosen for pharmacological evaluations. After the last observation, the mice were killed and the liver, lungs, heart, spleen and kidneys were withdrawn, weighed and stored for next evaluations.

Anti-inflammatory activity

The anti-inflammatory activity was assessed on the basis of inhibition of paw edema induced by the injection of carrageenan (an edematogenic agent) into the subplantar region of the right hind paw of the rat (Winter et al., 1962). Male Wistar rats were divided into different groups of eight animals. The control group received 2.5 ml/kg of saline, the standard group received the reference drug (acetyl salicylate of lysine (ASL), 300 mg/Kg) and the test groups received different population extracts of *C. colocynthis* at a dose of 1 and 4 mg/kg. Thirteen minutes after intraperitoneal administration of different substances, 0.05 ml of 1% of carrageenan suspension was injected into all animals in the right hind paw.

The paw volume, up to tibiotarsal articulation, was measured using a plethysmometer. The measures were determined at 0 h (V_0: before edematogenic agent injection) and 1, 2, 3, 4, 5, 6 and 24 h intervals later (V_T). The difference between V_T (1, 2, 3, 4, 5, 6 and

Table 2. Extraction yields (w/w %), alkaloid and oligomeric flavonoid levels (%) and LD_{50} (mg/Kg) of different populations of *C. colocynthis* Schrad. seeds and fruits.

Parameter	Population of *Citrullus colocynthis* Schrad.													
	Hammamet		Mahdia		Kasserine		Sbeitla		Sidi-Bouzid		Sfax		Medenine	
	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits	Seeds	Fruits
Extraction yields	1.68	0.93	1.90	1.70	2.88	2.94	1.93	2.68	1.76	2.44	2.15	2.81	2.94	2.76
Alkaloid levels	2.01	1.57	3.10	2.08	2.74	1.61	2.48	1.55	2.90	1.71	3.90	2.86	3.87	2.90
Flavonoid levels	0.24	-	0.18	-	0.18	-	0.20	-	0.17	-	0.13	-	0.10	-
LD_{50}	2043.84	795.45	818.61	749.97	653.51	750.03	1535.21	799.64	2298.48	795.49	438.62	385.54	744.15	553.73

24 h) and V_0 was taken as the edema value. The percentage of inhibition was calculated according to the following formula:

Percentage inhibition = $((V_T-V_0)_{control} - (V_T-V_0)_{treated\ group}) \times 100 / (V_T-V_0)_{control}$

Statistical analysis

Data obtained from animal experiments were expressed as mean ± S.E.M. and as percentage. Results were statistically evaluated by ANOVA and using student's t-test. $P \leq 0.05$ were considered significant.

RESULTS

Extraction yields

After all population (Table 1 and Figure 1) extractions, experimental results (Table 2) reveal that seeds and fruits from Kasserine, Sfax and Medenine have significantly more higher extraction yields than the other population plant parts. Hammamet population showed the lowest yield.

Quantitative alkaloid and oligomeric flavonoid screening

Alkaloid presence was reported in all *C. colocynthis* organs (immature seeds and fruits whose alkaloids are quantified in this study) except the roots (Marzouk et al., 2009). Results (Table 2) were dependent upon the population. Sfax (3.90 and 2.86%, respectively, for seeds and fruits) and Medenine (3.87 and 2.90% correspondingly, for seeds and fruits) populations contained more alkaloids, but Hammamet organs presented the lowest amounts of it. Differences were also noted between plant parts: seeds showed better percentage (from 2.01 to 3.90%) than fruits (from 1.55 to 2.90%). Flavonoids which are only detected in seeds (Marzouk et al., 2009) were present just at low levels (from 0.10 to 0.24%, Table 2). Hammamet was the richest population in these components.

Toxicity studies

Swiss-albino mice were observed for 48 h and morbidity and/or mortality were recorded, for each group at the end of observation period. Due to death index, the LD_{50} of all extracts were determined (Table 2). This value is in relation with plant parts and also with origin states. The LD_{50} of seeds ranged from 438.62 mg/kg (Sfax population) to 2298.48 mg/kg (Sidi-Bouzid population). For the *C. colocynthis* fruits, Sfax population had the lowest LD_{50} (385.54 mg/kg) and that of Sbeitla was evaluated as the less toxic (LD_{50} = 799.64 mg/kg). Except Kasserine population, immature seeds were less toxic than immature fruits. *C. colocynthis* seeds and fruits from Sfax seem to be the most toxic plant parts of all. Regarding the LD_{50}, the quantitative alkaloid and oligomeric flavonoid screening, this toxicity could be, probably, attributed to the alkaloid levels.

Anti-inflammatory effect

In carrageenan-induced rat paw edema, all extracts produced a significant reduction of the edema throughout the entire period of observation (Tables 3 and 4). The intraperitoneal administration of the aqueous extracts of seeds and fruits significantly reduced the paw edema induced by the noxious agent. This inhibition differs with the plant part aqueous extract and its origin state. For this second assessment of the anti-inflammatory activity, the obtained results demonstrated that the reduction of the paw edema vary in a dose dependent fashion with its maximum at 4 mg/kg. On the contrary, at 1 mg/kg, only a weak to moderate activities were noted. In terms of plant parts, immature fruits showed better activity than immature seeds. Experimental results, at 4 mg/kg, reveal that the anti-inflammatory effect seems to remain moderate with Mahdia seeds and Hammamet fruits which showed a very weak property during the first phase and increase

Table 3. Effects of different populations of *C. colocynthis* Schrad. seed aqueous extracts and reference drug on carrageenan-induced paw edema.

Seed population	Dose (mg/Kg)	Mean swelling thickness (10-2) ± S.E.M. (% inhibition)						
		1 h	2 h	3 h	4 h	5 h	6 h	24 h
C1	-	15.00±1.76	34.50±4.33	57.00±6.22	61.50±6.42	67.00±7.37	71.00±6.39	40.50±5.84
C2	-	33.00±3.66	48.00±4.77	61.50±7.20	76.25±7.37	95.00±9.20	106.25±9.85	77.75±6.66
C3	-	15.00±1.76	24.00±2.66	49.00±4.77	57.50±5.56	59.50±5.25	64.50±8.20	32.50±3.51
Hammamet	1 (C1)	14.00±1.41^{ns} (6.67)	30.50±1.91* (11.59)	44.25±2.75*** (22.37)	46.75±3.10*** (23.98)	36.00±2.16*** (46.27)	32.00±2.16*** (54.93)	18.25±1.71*** (54.94)
	4 (C2)	15.75±0.96*** (52.27)	22.50±1.29*** (53.13)	20.50±0.58*** (66.66)	19.00±1.41*** (75.08)	22.75±0.96*** (76.05)	21.75±0.96*** (79.53)	14.00±0.82*** (81.99)
Mahdia	1 (C2)	33.00±0.82 ^{ns} (0.00)	40.25±1.26*** (16.15)	33.00±0.82*** (46.34)	31.00±2.31*** (59.34)	30.50±0.58*** (67.89)	27.00±0.82*** (74.59)	20.50±0.58*** (73.63)
	4 (C2)	27.00±1.15*** (18.18)	35.25±0.96*** (26.56)	32.00±1.41*** (47.97)	26.75±1.26*** (64.92)	29.75±0.96*** (68.68)	25.75±0.96*** (75.76)	17.75±0.96*** (77.17)
Kasserine	1 (C2)	23.75±1.71*** (28.03)	31.00±1.41*** (35.42)	29.00±2.16*** (52.85)	32.50±2.52*** (57.38)	34.50±1.91*** (63.68)	28.25±2.06*** (73.41)	24.50±1.29*** (68.49)
	4 (C2)	12.75±0.96*** (61.36)	18.50±1.29*** (61.45)	23.50±1.29*** (61.79)	24.00±0.82*** (68.52)	29.00±0.82*** (69.47)	19.00±1.41*** (82.12)	13.75±0.50*** (82.31)
Sbeitla	1 (C1)	20.00±1.63*** (39.39)	25.00±1.15*** (47.91)	28.50±1.29*** (53.66)	35..00±0.82*** (54.10)	30.50±1.73*** (67.89)	30.00±1.63*** (71.76)	24.50±1.29*** (68.49)
	4 (C2)	18.00±0.82*** (45.45)	22.00±2.16*** (54.17)	25.75±1.71*** (58.13)	26.75±1.71*** (64.92)	25.75±1.71*** (72.89)	18.25±0.96*** (82.82)	12.75±0.96*** (83.60)
Sidi-Bouzid	1 (C3)	14.00±1.41^{ns} (6.67)	16.75±2.52*** (30.21)	33.25±2.16*** (32.14)	31.75±1.29*** (44.78)	34.50±1.29*** (43.69)	29.50±3.41*** (54.26)	14.50±1.29*** (55.38)
	4 (C2)	14.50±1.29*** (56.06)	20.00±2.16*** (58.33)	24.50±1.29*** (60.16)	28.00±0.82*** (63.28)	24.25±2.06*** (74.47)	23.00±2.16*** (78.35)	16.00±0.82*** (79.42)
Sfax	1 (C1)	15.00±2.45 ^{ns} (0.00)	31.50±2.38^{ns} (8.69)	44.25±2.75*** (22.37)	47.00±3.16*** (23.58)	53.00±1.83*** (20.89)	49.50±1.91*** (25.00)	28.00±0.82*** (30.86)
	4 (C2)	14.75±1.50*** (55.30)	19.75±0.96*** (58.85)	23.50±2.52*** (61.79)	28.00±0.82*** (63.28)	28.75±0.96*** (69.74)	20.50±1.29*** (80.71)	8.75±1.71*** (88.75)

Table 3. Contd.

Seed population	Dose (mg/Kg)	Mean swelling thickness (10-2) ± S.E.M. (% inhibition)						
		1 h	2 h	3 h	4 h	5 h	6 h	24 h
Medenine	1 (C3)	14.50±2.89^{ns} (3.33)	19.25±2.21** (19.79)	33.25±2.21*** (32.14)	32.50±2.38*** (43.48)	39.00±3.91*** (34.45)	29.25±3.86*** (54.65)	14.25±0.96*** (56.15)
	4 (C3)	1.75±0.96*** (88.33)	2.00±0.82*** (91.66)	3.50±0.58*** (92.86)	1.50±1.29*** (97.40)	2.50±1.29*** (95.80)	1.25±0.50*** (98.06)	0.75±0.95*** (97.69)
ASL	300 (C1)	7.25±0.96*** (51.66)	14.25±1.71*** (58.69)	17.50±2.38*** (69.30)	15.75±2.22*** (74.40)	19.50±2.08*** (70.89)	19.50±1.29*** (72.53)	23.75±1.71*** (41.36)

C1,: Control 1; C2, control 2; C3, control 3; 1 (C1) and 4 (C1), in comparison with control 1; 1 (C2) and 4 (C2), in comparison with control 2; 1 (C3) and 4 (C3), in comparison with control 3. Values are expressed as mean ± M.S.E. (n = 8); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 significant from the control; ns, not significant from the control; ASL, acetyl salicylate of lysine.

Table 4. Effects of different populations of *C. colocynthis* Schrad. fruit aqueous extracts and reference drug on carrageenan-induced paw edema.

Fruit population	Dose (mg/kg)	Mean swelling thickness (10-2) ± S.E.M. (% inhibition)						
		1 h	2 h	3 h	4 h	5 h	6 h	24 h
C1	-	15.00±1.76	34.50±4.33	57.00±6.22	61.50±6.42	67.00±7.37	71.00±6.39	40.50±5.84
C2	-	33.00±3.66	48.00±4.77	61.50±7.20	76.25±7.37	95.00±9.20	106.25±9.85	77.75±6.66
C3	-	15.00±1.76	24.00±2.66	49.00±4.77	57.50±5.56	59.50±5.25	64.50±8.20	32.50±3.51
Hammamet	1 (C1)	15.00±2.45^{ns} (0.00)	31.75±1.26* (7.97)	46.50±2.08*** (18.42)	47.00±3.16*** (23.58)	53.00±1.83*** (20.90)	53.25±3.20*** (30.28)	18.00±0.82*** (30.86)
	4 (C2)	24.00±0.82*** (27.27)	27.00±0.82*** (43.75)	31.00±0.58*** (49.59)	23.00±0.82*** (69.84)	23.75±0.96*** (75.00)	15.75±0.96*** (85.18)	11.25±0.96*** (85.83)
Mahdia	1 (C2)	18.75±0.96 *** (43.18)	24.75±1.26*** (48.44)	31.25±0.96*** (49.19)	30.50±1.29*** (60.00)	32.50±2.08*** (65.79)	25.50±2.08*** (76.00)	17.25±0.96*** (77.81)
	4 (C2)	17.00±0.82*** (48.48)	22.75±1.71*** (52.60)	27.5±0.58*** (55.28)	27.75±1.71*** (63.61)	22.50±0.58*** (76.32)	20.50±0.58*** (80.71)	14.25±0.96*** (81.67)
Kasserine	1 (C1)	10.50±1.71*** (30.00)	23.25±1.41*** (32.61)	33.25±2.08*** (41.67)	27.00±0.82*** (56.10)	36.00±2.16*** (46.27)	32.00±2.06*** (54.93)	13.00±1.15*** (67.90)
	4 (C2)	17.75±0.96*** (46.21)	24.25±0.96*** (49.48)	29.75±1.71*** (51.63)	24.00±1.41*** (68.52)	23.00±1.86*** (75.79)	17.50±1.29*** (83.53)	12.75±0.50*** (83.60)
Sbeitla	1 (C2)	23.50±1.73*** (28.79)	26.50±1.29*** (44.79)	33.25±2.21*** (45.93)	32.50±2.38*** (57.38)	30.50±1.73*** (67.89)	30.00±1.63*** (71.76)	24.50±1.29*** (68.49)
	4 (C2)	19.25±0.96*** (41.67)	22.50±1.29*** (53.13)	22.75±2.06*** (63.01)	25.25±1.71*** (66.89)	22.00±2.16*** (76.84)	16.00±0.82*** (84.94)	11.25±0.96*** (85.53)

Table 4. Contd.

Sidi-Bouzid	1 (C3)	14.50±2.89^{ns} (3.34)	21.75±1.50*** (9.38)	42.75±2.16*** (12.76)	37.50±2.38*** (34.78)	39.00±1.29*** (34.45)	29.25±2.08*** (54.65)	17.75±1.71*** (45.38)
	4 (C2)	18.25±1.71*** (44.96)	22.50±1.29*** (53.13)	26.00±0.82*** (57.72)	25.75±1.71*** (66.23)	21.50±2.08*** (77.37)	21.00±2.54*** (80.24)	5.75±0.25*** (92.60)
Sfax	1 (C2)	33.00±2.08 ^{ns} (0.00)	40.25±1.26*** (16.14)	43.75±1.26*** (43.50)	32.50±2.52*** (57.38)	34.50±1.92*** (63.68)	27.00±0.82*** (74.59)	24.50±1.29*** (68.49)
	4 (C2)	14.50±3.11*** (56.06)	16.75±0.96*** (65.10)	20.75±1.71*** (66.26)	15.75±1.50*** (79.34)	17.50±0.58*** (81.58)	14.75±1.26*** (86.12)	5.50±0.50*** (92.93)
Medenine	1 (C3)	13.25±2.22 (11.67)	16.75±3.30** (30.21)	29.00±2.16*** (40.82)	31.75±3.86*** (44.78)	34.50±1.29*** (42.02)	21.75±3.95*** (66.28)	16.75±2.22*** (48.46)
	4 (C3)	1.50±0.57*** (90.00)	1.75±0.96*** (92.71)	2.75±0.50*** (94.39)	1.75±0.96*** (97.65)	2.75±0.96*** (95.38)	0.75±0.96*** (98.84)	0.50±0.58*** (98.46)
ASL	300 (C1)	7.25±0.96*** (51.66)	14.25±1.71*** (58.69)	17.50±2.38*** (69.30)	15.75±2.22*** (74.40)	19.50±2.08*** (70.89)	19.50±1.29*** (72.53)	23.75±1.71*** (41.36)

C1: Control 1; C2: Control 2; C3: Control 3; 1 (C1) and 4 (C1): in comparison to control 1; 1 (C2) and 4 (C2): in comparison to control 2; 1 (C3) and 4 (C3): in comparison to control 3; Values are expressed as mean ± M.S.E. (N=8); *p ≤ 0.05, **≤0.01, ***≤0.001 significant from the control; ns: not significant from the control; ASL: Acetyl Salicylate of Lysine.

significantly at the second one, the other populations throughout the experiment, showed an important effect. This difference may be correlated to the process of synergism or antagonism between all extract components (Marzouk et al., 2009). With all extracts, 3 h after carrageenan injection, the anti-inflammatory activity instigated unambiguous increase to attain the maximum level at 6 and 24 h. The inhibition percentages range at 6 and 24 h respectively, are from 75.76 to 98.06% and from 77.17 to 97.69% for seeds and from 80.24 to 98.84% and 81.67 to 98.46% for fruits. South Tunisia aqueous extracts (at 4 mg/kg) were strongly more active than the other population (1 to 6 h) and seem to have close values after 24 h after the carrageenan injection. The highest activity was found for immature fruit extract from Medenine. Standard drug decreased paw edema by a maximum of 74.39% after 4 h.

DISCUSSION

This is the first study evaluating the *in vivo* acute toxicity and the anti-inflammatory activities of extracts from different population of Tunisian *C. colocynthis* immature fruits and seeds.

The study clarified complexes of biodiversity pictures in terms of plant parts and plant geographical distribution. Results previously obtained (Marzouk et al., 2010c) showed the importance of immature seeds and fruits. Fruits and seeds differed, as made obvious by their extraction yields, analgesic and anti-inflammatory activeties. The present investigation showed, in addition, that for a given plant part, the intensity of the pharmacological activity can change from a population to another. The trend for all the populations to have higher activity for immature fruit aqueous extracts and immature seeds points towards various active, potential or antagonistic compounds present in various concentrations according to the plant

population, especially flavonoids and alkaloids. These two phytochemical key families are quantified and the results indicated that the geographical distribution of *C. colocynthis* Schrad. influenced second metabolite levels and subsequently their toxicity and biological activities.

Based on the LD_{50} calculation, the acute administration doses of all population aqueous extracts were estimated (1 and 4 mg/kg). Carrageenan has been widely used as a noxious agent which is able to induce experimental inflammation for the screening of compounds possessing anti-inflammatory activity. This phlogistic agent, when injected locally into the rat paw, produced a severe inflammatory reaction, which was discernible within 30 min (John and Nodine, 1999). The development of edema induced by carrageenan is a biphasic event: The early phase (90 to 180 min) of the inflammation is due to the release of histamine, serotonin and similar substances. The later phase (270 to 360 min) is associated with the activation of kinin-like substances and the release of prostaglandins, proteases and lysosome (Olajide et al., 1999). All population extracts inhibited hind paw edema and showed a dose-dependent anti-inflammatory activity but the results were different for each population plant parts depending on the early/later phases. All tested extracts inhibited both the phases of the carrageenan-induced edema by reducing the release of histamine and serotonin and also the kinin-like substances and prostaglandins. Pharmacological properties of all *C. colocynthis* Schrad. populations may be attributed to a possible molecular mechanism by effectively decreasing the production of the pro-inflammatory cytokines like IL-6 and IL-1β and the expression of COX-2, and simultaneously elevating the level of anti-inflammatory cytokine IL-4 in the carrageenan-injected rat paw tissues (Moulin and Coquerel, 2002).

Differences in the LD_{50} and the anti-inflammatory efficacy are related to the plant population compositions. Alkaloids and flavonoids found in *C. colocynthis* plant part were dependent upon the population. At any rate, these results indicate that the anti-inflammatory activities could not be imputed to one family of phytochemicals only (or its absence). Alkaloids are commonly found to have anti-inflammatory properties (Moulin and Coquerel, 2002). Flavonoids (detected in all the seed extracts) are known to have the same activities (Borgi et al., 2008). However, alkaloids and flavonoids cannot be only responsible for the pharmacological effect. Steroids and iridoids which are present in this plant (Marzouk et al., 2009) may contribute to better performance as an anti-inflammatory agent (Bames and Adcock, 2009). Differences found between the seven populations tested, in the alkaloid and flavonoid levels, in the toxicity and in the pharmacological activities, may be attributed to the climatic conditions and soils.

But, despite the complexity of the chemistry of the inorganic and organic nature, it is possible to find some general trends like genetics and breeders which are more and more interested in the link between genetic factors and phenotypic variation of qualitative and quantitative composition.

With these anti-inflammatory property, *C. colocynthis* Schrad., the Tunisian population, can be considered an effective agent to treat inflammation diseases. Immature seeds and fruits demonstrated a high activity at very low aqueous extract doses (1 and 4 mg/kg). The study corroborated the pharmacological effects of this species, justified and supported scientifically, the use of *C. colocynthis* as an anti-inflammatory agent to treat pain and rheumatoid arthritis. Additional studies are ongoing to confirm this *C. colocynthis* Schrad. properties with other models. Further, attempts are made to isolate and define the active anti-inflammatory fractions and its components.

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