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Comparative evaluation of the analgesic activity of several populations of Tunisian *Citrullus colocynthis* Schrad. immature fruits

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ABSTRACT

Plant extracts are some of the most attractive sources of new drugs and have shown promising results for the treatment of pains, inflammations and immune-related diseases. *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 level and to evaluate the acute toxicity of different populations of *Citrullus colocynthis* fruit aqueous extracts at immature state and then to screen their analgesic activities. After identification and acute toxicity essay, different populations of *Citrullus colocynthis* Schrad. aqueous fruit extracts were screened for analgesic activity using the acetic acid writhing test in mice. Yields of prepared extracts and alkaloid level are gravimetrically determined. Results show that alkaloid level vary upon the *Citrullus colocynthis* population. All extracts displayed analgesic activity at different doses without inducing acute toxicity. Topic results were obtained with immature fruits from south Tunisia. Experimental results have revealed that *Citrullus colocynthis* Schrad. is a potentially useful drug suitable for further evaluation, and its folk medicinal use, in all Tunisia, as an analgesic agent is validated for all tested Tunisian populations.

INTRODUCTION

The use of plants for medicinal purposes has been practiced for many centuries by a substantial proportion of the population of Tunisia. Interest in ethnopharmacy as a source of pharmacologically active compounds has increased worldwide, particularly in the search for drugs to counter pains. Additionally, in some developing countries, plants are the main medicinal source to treat inflammation diseases due to economic conditions and availability. However, only approximately 20% of the plants found in the world have been submitted to pharmacological or biological testing, despite the substantial number of new remedies derived from natural or semi-synthetic resources being introduced on the market (Mothana and Lindequist, 2005). *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; Marzouk et al., 2009). In Tunisia, as in other Mediteranean countries (Al-Rawi and Chakravarty, 1964), the parts of plants most often used for medicinal purposes are fruits and/or seeds, though other parts of the plants can be used, for example roots to treat urinary infection (Nadhami, 1954) or leaves (Batanouni, 1999).

Traditional healers seem to not pay attention to the plant's degree of maturity. The literature rarely mentions if seeds are present in preparations involving ground fruit/pulp. Modes of preparation and administration vary, even for similar indications. Common preparations use fresh, warmed or dried plant material (often ground), as well as extracts used mostly in a liquid form. Extracts are prepared either in water or in aqueous mixtures containing more lipophilic compounds (hot milk extractions, water/olive oil at various ratios) at a temperature ranged from tepid to boiling. Ground plant material can be mixed with honey for ingestion or topical gynaecological application or with other plants for poultices (for example with *Lawsonia inermis* and *Capparis spinosa*). Methods of administration are topical, rectal or vaginal (fruit), enema, cervico-vaginal douche, and by ingestion (Marzouk et al., 2009; Boukef, 1986).

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Extreme caution should be exercised with ingestion, due to the plant's drastic laxative properties, and with contact with leaves, due to risks of syncope (for all mammals including domestic animals) (Marzouk et al., 2009). Use is contraindicated during pregnancy as the plant is abortifacient (Pottier-Alapetite, 1981; Delazard et al., 2006). Many of today's traditional medicinal uses of the plant are found throughout history (see the Eber's papyrus in ca.1550 BCE Egypt) (Riddle, 1999) over a large geographical zone from Mauritania to India, even extending outside of the plant endemic zone, to Europe (Adams et al., 2009). However plants in the environment are exposed to a range of abiotic stresses like osmotic, salinity, temperature and heavy metal toxicity which effects their growth and other metabolism process such as alkaloid productions. This second metabolite was synthesized by plants to defend themselves against the harmful action of external agents (Heller et al., 1993; Waller and Nowaki, 1978). Thus alkaloid contents depend on the geographical distribution. In a previously published paper immature fruits and seeds are demonstrated the most efficient of all (Marzouk et al., 2009). The current study measured *in vivo* toxicity and analgesic activity of seven populations of *Citrullus colocynthis* Schrad. fruits using mice as models. Preparation and testing were carried out on the reconstituted lyophilized aqueous extracts.

MATERIALS AND METHODS

Plant materials

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

Extraction protocol

The extraction was performed on 100 g of fresh immature fruits. For preparing the aqueous extract, organ was ground with a mixer and added to 500 ml of distilled water. The mixture was allowed to reflux for 30 min, after which the solution was allowed to cool (4 hours 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, on three grams of dried fruits, 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 by sulphuric acid (0.5 N, 3x20 ml). Bases were removed from the extract through precipitation with an excess of ammonium hydroxide (3 ml). The supernatant was then extracted with chloroform (3x10 ml). The precipitate containing alkaloids was separated by means of filtration and treated again by chloroform which was evaporated under low pressure. The presence of alkaloids was verified by Dragendorff's reagent confirmed with Bouchardat's and Meyer's reagents. On the residue, 20 ml of sulphuric acid were added. The excess of sulphuric acid was titrate by sodium hydroxide (0.02 N) using the phenolphthalein indicator. The percentage of total alkaloids was then calculated according to the following formula: Alkaloid level (%) = $((V_{H2SO4}-V_{ex\ H2SO4}) \times 5.788 / W) \times 100$; with V_{H2SO4} = 20 ml, $V_{ex\ H2SO4}$ = volume of sulphuric acid excess, W = 3 g and 1 ml of sulphuric acid correspond to 5.788 mg of alkaloids.

Animals

Swiss albino mice (weighing 18-25 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 the 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)). 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, 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 stoked for next evaluations.

Analgesic activity

Analgesic activity was performed according to the method of Koster et al. (1959) and assessed by the acetic acid abdominal constriction test (writhing test) - a chemical visceral pain model. Swiss albino mice were selected one day prior to each test and were divided into groups of six mice each. One group served as the control and was pretreated under cutaneously with 10 ml/Kg of saline. Another group was pretreated with the reference drug, Acetyl Salicylate of Lysine (ASL), 200 mg/Kg, by the same route. The remaining groups were injected intraperitoneally (i.p.) with 10 ml/kg of 1% acetic acid solution 30 min after the

administration of different seed extracts at the doses of 0.1 and 2 mg/kg. After acetic acid administration, the number of writhes was counted during 30 minutes. Antinociceptive activity was expressed as inhibition percent of the usual number of writhes observed in control animals. The percentages of inhibition were calculated according to the following formula: % inhibition = ((number of writhes) $_{control}$ − (number of writhes) $_{treated\ group}$) x 100 / (number of writhes) $_{control}$

Statistical analysis

Data obtained from animal experiments were expressed as mean ± M.S.E. 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

Experimental results (Table 1, Figure 1) reveal that fruits from Gasrine, Sfax and Mednine have significantly much higher extraction yields than the other populations. Hammamet population shows the lowest yields.

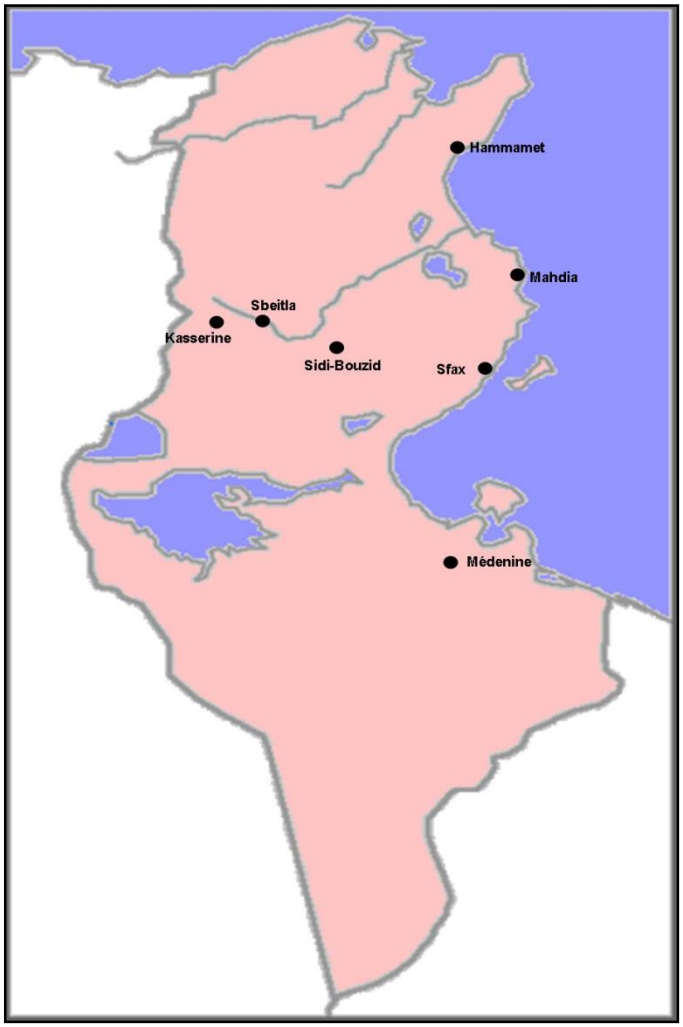

Fig. 1: Geographical map of Tunisia.

Table. 1: Sites and voucher specimens of *Citrullus colocynthis* Schrad. Populations.

Population	Site	Voucher specimens
Hammamet	South Hammamet	C.C-01.07
Mahdia	Chorban	C.C-01.06
Gasrine	Mozgam	C.C-01.05
Sbitla	Sbitla	C.C-01.04
Sidi-Bouzid	Jelma	C.C-01.03
Sfax	Skhira	C.C-01.02
Mednine	Sidi Makhlouf	C.C-01.01

Quantitative alkaloid screening

Alkaloids were found in all *Citrullus colocynthis* organs except the roots (Marzouk et al., 2009) namely immature fruits whose alkaloids are quantified in this study. Results (reported in Table 2) were dependent upon the population. Sfax (2.86 %) and Mednine (2.90 %) populations were the richest in alkaloids containing, but Hammamet organs presented the low amounts of all.

Toxicity studies

Swiss albino mice were observed during 48 h and morbidity and/or mortality were recorded, if happens, for each group at the end of observation period. Due to death index, the LD_{50} of all extracts were determined (Table 1). The seeds LD_{50} were ranged from 385.54 mg/kg (Sfax population) to 799.64 mg/kg (Sbitla population).

Analgesic activity

The inhibition percentages of writhing for all extracts are shown in Table 3. The reference drug inhibited 61.88 % of the number of writhing elicited by acetic acid.

The analgesic effect was tested for concentrations ranging from 0.1 to 2 mg/Kg. The administration of all tested extracts induced a potent dose-dependent antinociceptive activity. The immature fruits from Medenine possess the highest analgesic properties. The lowest activity was observed for Hammamet fruits (90.86 %).

Table. 3: Analgesic activity of different populations of *Citrullus colocynthis* Schrad. aqueous fruit extracts and reference drug (ASL).

Population	Concentration (mg/Kg)	Number of writhes	Inhibition of writhing (%)
Hammamet	0.1	16.17±0.75***	82.08
	2	8.50±1.05***	90.86
Mahdia	0.1	6.16±0.75***	93.37
	2	4.83±0.75***	94.80
Gasrine	0.1	11.00±0.89***	88.17
	2	7.00±0.63***	92.47
Sbitla	0.1	12.5±1.05***	86.56
	2	2.66±0.52***	97.13
Sidi-Bouzid	0.1	16.00±0.89***	82.80
	2	6.50±1.05***	93.01
Sfax	0.1	14.00±0.89***	84.95
	2	2.33±0.52***	97.49
Mednine	0.1	3.33±0.52***	95.52
	2	1.67±0.52***	97.76
Reference drug (ASL, 200 mg/Kg)		28.33±2.06***	61.88

Values are expressed as mean ± M.S.E. (N=6); ***≤0.001 significant from control; ASL: Acetyl Salicylate of Lysine.

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

	Population of *Citrullus colocynthis* Schrad. fruits						
	Hammamet	**Mahdia**	**Gasrine**	**Sbitla**	**Sidi Bouzid**	**Sfax**	**Mednine**
Extraction yields	0.93	1.70	2.94	2.68	2.44	2.81	2.76
Alkaloid levels	1.57	2.08	1.61	1.55	1.71	2.86	2.90
LD_{50}	795.45	749.97	750.03	799.64	795.49	385.54	553.73

DISCUSSION

This is the first study evaluating the in *vivo* acute toxicity and the analgesic activity of different populations of Tunisian *Citrullus colocynthis* immature fruits. The study clarified complexes of ethnopharmacology and biodiversity pictures in terms of plant geographical distribution. Results previously obtained (Marzouk et al., 2009) showed the importance of organs: immature seeds and fruits, the compositions of fruits differed, as made obvious by their extraction yields and analgesic activity. The present investigation showed, in addition, for a given organ, the pharmacological effect can change from a population to another one. The trend over all the populations to have higher activity for immature fruit aqueous extracts points towards various active, potentiate or antagonistic compounds present in various concentrations according to the plant population, especially alkaloids (Moulin and Coquerel, 2002) which possess analgesic property. This phytochemical key family is quantified and results indicated that the geographical distribution of *Citrullus colocynthis* Schrad. influenced second metabolite level and subsequently their toxicity and biological activities. Based on the LD_{50} calculated, the acute administration doses of all population aqueous extracts are estimated (0.01 mg/Kg and 2 mg/Kg for the analgesic activity). In acetic acid-induced writhing in mice, all tested samples extracts reduced significantly the number of writhing which is associated with the release of endogenous substances including serotonin, histamine, prostaglandin and bradykinin (Colier et al., 1986). The results obtained in this test thus suggest that while *Citrullus colocynthis* Schrad. fruis possess peripheral analgesic properties, this particular activity is probably linked to their anti-inflammatory effects. Differences in the LD_{50} and in the analgesic efficacy are related to the plant population compositions. Alkaloids found in *Citrullus colocynthis* fruits were dependent upon the population. At any rate, these results indicate that the analgesic activities could not be imputed to one family of phytochemicals only (or its absence). Like for the antibacterial and the anticandidal activities, analgesic one may be attributed, possibly in combination, to various phytochemicals detected (alkaloids, iridoids, flavonoids, steroids…) (Marzouk et al., 2009). These compounds might be present in various concentrations according to the plant organ (Marzouk et al., 2009). Alkaloids are commonly found to have analgesic property (Moulin and Coquerel, 2002); however, alkaloids cannot be solely responsible for the pharmacological effect. Steroids and iridoids witch are present in this plant may contribute to a better performance. Differences found between the seven populations tested, in the composition levels (alkaloid levels) in the toxicity and in the pharmacological activity, may be attributed to the climatic conditions and soils. 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 to the link between genetics factors and phenotypic variation of qualitative and quantitative composition.

With this analgesic property, *Citrullus colocynthis* Schrad. seeds, whatever the Tunisian population, can be considered an effective nociceptive agent. This immature fruit demonstrated a high activity at very low aqueous extract doses (0.1 mg/Kg and 2 mg/Kg). The study corroborated the analgesic effects of this specie, justified and supported scientifically its ethnopharmacological use to treat pain. Therefore it could account for some of the variations observed in the ethnopharmaceutical preparation methods. From now, the use of this Tunisian plant seeds is validated by the results obtained in this work. Additional studies are ongoing to confirm this *Citrullus colocynthis* Schrad. properties with other models.

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