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Full Length Research Paper

Antimicrobial and anticoagulant activities of *Citrullus colocynthis* Schrad. leaves from Tunisia (Medenine)

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Traditional medicine is a potential source of new drugs. *Citrullus colocynthis* is a Tunisian plant used in folk medicine against dermatological, gynaecological and pulmonary infections; and against inflammations and cardiovascular and immune-related diseases. The present study was conducted to evaluate the *in vitro* antimicrobial and anticoagulant properties of leaf extracts from an endemic plant, *C. colocynthis* Schrad. The extracts were screened for antimicrobial activity against Gram-negative and Gram-positive bacteria and against four *Candida* spp. using the microdilution method. The anticoagulant property was evaluated using the prothrombin time (PT) and partial thromboplastin time tests. The antimicrobial test results showed that polar extracts using ethyl acetate, acetone and methanol from this species strongly inhibited the growth of microorganisms while petroleum ether and chloroform extracts had moderate antibacterial and anticandidal activities. Investigation of the coagulant activity of different extracts showed that *C. colocynthis* leaves reduced or prolonged the PT and activated or inhibited partial thromboplastin time tested on plasma according to plant extracts and dilution degrees. This indicates that this species possesses both procoagulant and anticoagulant activities. From these results, we confirmed the traditional use of *C. colocynthis*.

Key words: Traditional medicine, *Citrullus colocynthis* Schrad., leaves, antimicrobial, anticoagulant.

INTRODUCTION

Increasingly adverse drug reactions to the synthetic antibiotics and the increasing resistance of some pathogens to synthetic antibiotics, has been another argument against the use of these chemicals as therapeutics (Agnihotri and Vaidya, 1996; Friedman et al., 2002; Dabai et al., 2012).

The biological value of plants has been widely studied and is demanded by consumers, especially for protection against cardiovascular disorder, cancer and other diseases, as well as for general health benefits (Dapkevicius et al., 1998; Campbell and Reece, 2007; Gboko et al., 2012).

Commercial drugs/chemicals used haphazardly in the treatment of many diseases inevitably led multiple drug/chemical resistance in human microorganisms peroxidaion (Service, 1995; Loper et al., 1999). To the best of our knowledge, antimicrobial and anticoagulant properties of *Citrullus colocynthis* Schrad. leaf extracts have not been reported before. *C. colocynthis* Schrad. (Cucurbitaceae) growing in arid areas is endemic in the south of Tunisia (Pottier-Alapetite, 1981). This medicinal plant is widely used in Tunisian folk medicine for treating many diseases such as rheumatism, hypertension and

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various contagious diseases such as dermatological problems and gynaecological or pulmonary infections (Boukef, 1986; Le Flock, 1983).

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).

The objectives of this study were to investigate the antimicrobial and anticoagulant activities of extracts from *C. colocynthis* leaves. The antimicrobial activity was determined by using the microdilution method. Prothrombin time (PT) and activated partial thromboplastin time (APTT) tests on plasma are used to determinate the coagulant-anticoagulant effects.

MATERIALS AND METHODS

Plant material

C. colocynthis Schrad. leaves were collected in August, 2007 from nearby Mednine village, El-Araidha region, Sidi Makhlouf municipality, Tunisia. The taxonomic identification of the plant material was confirmed by a plant taxonomist, Marzouk, Z., in the Biological Laboratory of the Faculty of Pharmacy of Monastir-Tunisia- according to the flora of Tunisia[8]. A voucher specimen (C.C-01.01) has been deposited in this laboratory.

Preparation of extracts

Aqueous extract

One hundred grams (100 g) of fresh leaves were 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 h at 3°C). The mixture was then filtered on filter paper (Whatman No.1) under the vacuum of a water pump. The obtained filtrate was lyophilized, yielding the lyophilized aqueous extract.

Soxhlet extractions

Collected plant materials were dried; the leaves were separated from the stems, and ground in a grinder with a 2 mm in diameter mesh. Different solvents, petroleum ether, chloroform, ethyl acetate, acetone and methanol in ascending polarity, were used for Soxhlet extraction to fractionate the soluble compounds from the grape pomace. The extraction was performed with dried powder placed inside a thimble made by thick filter paper, loaded into the main chamber of the Soxhlet extractor, which consisted of an extracting tube, a glass balloon and a condenser. The total extracting time was 6 h for each solvent continuously refluxing over the sample (grape pomace). The resulting extracts were evaporated at reduced pressure to obtain the crude extracts.

Preliminary phytochemical screening

Aqueous and organic extracts were screened for the presence of key families of phytochemicals (Sakar and Tanker, 1991; Trease and Evans, 1984; Trim and Hill, 1952) using the following reagents and chemicals: alkaloids with Dragendorff's reagent confirmed with Bouchardat's (I_2/MgI_2) and with Meyer's reagents ($KI/MgCl_2$), coumarins with diluted NaOH-UV test, flavonoids with metallic magnesium and hydrochloric acid (HCl), anthraquinones with Borntrager's reagent, cardiac glycosides with Kedde's reagent (and confirmed with Baljet's reagent), iridoids with diluted HCl, saponosids for their ability to produce suds, steroids with acetic anhydride and concentrated sulphuric acid (Liebermann reaction), tannins in general with ferric chloride (confirmed with concentrated HCl, Bath-Smith reaction) and gallic tannins specifically with Stiasny reagent.

Antibacterial and antifungal activities

Organisms

The aqueous and organic extracts from *C. colocynthis* leaves were individually tested against a panel of microorganisms including a total of 8 microbial cultures belonging to 4 bacteria, than 4 *Candida* species. The 4 reference strains were chosen for antibacterial investigation: cocci gram-positive represented by *Enterococcus feacalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 and bacilli gram-negative represented by *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. In order to determine the antifungal effect of these extracts, a range of pathogenic reference *Candida* (*Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida kreusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019) was tested.

Minimal inhibition concentration (MIC) and minimal bactericidal concentration/ minimal fungicidal concentration (MBC/MFC) determinations

The MIC was defined as the lowest concentration that prevents visible growth bacteria. All extracts were dissolved in dimetyle sulfoxyde (DMSO) at 10%. We have applied the dilution method described by Berche et al. (1991). A microdilution technique using 96-well microplates was used to obtain the MIC values of extracts against the tested strains. The concentration for extracts tested was ranged from 6.343 to 3250 µg/ml. The lowest concentration of each extract that inhibited the bacterial growth after incubation, at 37°C between 18 and 24 h, was taken as the MIC.

The MBC and MFC were determined as a concentration where 99.9% or more of the initial inoculum are killed. They were evaluated by subculture in blood agar at 37°C between 18 and 24 h. The levofloxacin was used as antibacterial positive control and Amphotericin B for the anticandidal one.

Assay for prothrombin time (PT) and activated partial thromboplastin time (APTT)

The method described by Brown (1988) was used for the determination of the PT. Plasma was obtained by centrifuging citrated blood for 15 min at 1500 × g. Thromboplastin-calcium reagent (Sigma Diagnostics, St. Louis, MO) was reconstituted with distilled water according to the manufacturer's instructions. It was then prewarmed by placing it in a water bath at 37°C for at least 10 min before commencement of the test. 100 µl of plasma was placed in a test tube and incubated in the water bath for 180 s. For the controls, 100 µl of serum, followed by 200 µl of the prewarmed thromboplastin-calcium reagent was rapidly pipetted into the plasma while simultaneously starting a timer. The test tube was then gently tilted back and forth, until a clot formed, at which time the timer was stopped and the clotting time recorded. For the tests, 100 µl of the prewarmed sample solution was mixed with the

Table 1. Yields (%) of *C. colocynthis* Schrad. leaf extracts.

Chemical group	Aqueous	Petroleum ether	Chloroform	Ethyl acetate	Acetone	Methanol
Yield (%)	11.047	2.046	8.874	1.342	7.376	13.304

Table 2. Preliminary phytochemical screening of *C. colocynthis* Schrad. leaf extracts.

Chemical group	Aqueous	Petroleum ether	Chloroform	Ethyl acetate	Acetone	Methanol
Alkaloïds	+	-	-	+	+	+
Coumarins	+	-	-	+	-	-
Flavonoïds	-	-	-	-	-	-
Anthracenic heterosids	-	-	-	-	-	-
Cardiotonic heterosids	-	-	-	-	-	-
Iridoïds	+	-	-	-	-	+
Saponosids	-	-	-	-	-	-
Steroïds	+	+	-	-	-	-
Gallic tannins	+	-	+	+	-	-

plasma, just before adding the thromboplastin-calcium reagent. All experiments were carried out in at least, triplicates.

The method described by Brown (1988) suitably modified, was used for the partial thromboplastin time tests. Alexin® (Sigma Diagnostics), which is the partial thromboplastin with activator, and calcium chloride (0.02 M) were prewarmed to 37°C separa tely in a water bath. 50 µl of plasma was placed in a test tube. After incubating for 180 s in the water bath, 50 µl of Alexin® was added, and the contents were mixed rapidly. The mixture was then incubated for another 180 s, after which 50 µl of serum, then 50 µl of the prewarmed calcium chloride solution was added while simultaneously starting a timer. The tube was then allowed to remain in the water bath while gently tilting the test tube every 5 s. At the end of 20 s, the test tube was removed from the water, wiped clean with dry gauze, and gently tilted back and forth until a clot was seen and the time recorded. For the test samples, 50 µl of the material was added to the contents of the test tube just prior to the addition of calcium chloride, and readings taken as before. For the two tests, serum and heparin (183 IU/mg) were, respectively, used as negative and positive controls.

RESULTS

Extraction yields and phytohemical screening

Results are shown in Tables 1 and 2. Lyophilised aquous extract and methanol extract showed the better yields. The phytochemical screening showed a significant difference between the tested extracts. We note that the present or the absence of all phytochemical groups is related with the extract polarities and the chemical group types.

Antimicrobial activity

The *in vitro* antimicrobial activity of *C. colocynthis* extracts against the microorganisms employed and their activity potentials were qualitatively and quantitatively assessed by MIC and MBC/MFC values. According to the results shown in Tables 3 and 4, aqueous extract of *C. colocynthis* had great potential of antimicrobial activities against all bacteria and *Candida* species tested. Apolar to polar fractions of the Soxhlet extracts were also found to be effective against all microorganisms examined. MIC and MBC values for bacterial strains, which were sensitive to the aqueous extract and organic ones of *C. colocynthis*, were in the range of 6.343 to 1625 µg/ml and 12.687 to 3250 µg/ml, respectively. The maximum was obtained with the ethyl acetate extract against all bacteria exept *S. aureus*. MIC and MFC values of the *Candida* species sensitive to the tested extracts were < 6.343 to 1625 µg/ml and 6.343 to 3250 µg/ml, respectively. The best results were observed with acetone and methanol extracts against *C. albicans* and also with ethyl acetate extract against *C. Kreusei* (CMI < 6.347 µg/ml and CMF = 6.347 µg/ml).

Based on these results, Soxhlet extracts have a stronger activity and broader spectrum than the aquous one. As emphasized elsewhere, Gram-negative bacteria are more sensitive than Gram-positive ones (Marzouk et al., 2010a). As well, basing on the cell wall differences of bacteria, results show that Soxhlet extracts did possess the same selective antimicrobial activity. By inhibiting the growth of all human *Candida* tested, *C. colocynthis* Schrad. exerted a broad anticandidal spectrum. The antimicrobial nature of this studied plant organ is apparently related to their contents, particularly alkaloids and iridoids, and this finding is in agreement with a previous report (Marzouk et al., 2010a). This claim is further supported by our findings on Soxhlet leaf extrats; indicating, basing on the solvent extraction, high contents of active coumpounds (Table 2) which are response on this antimicrobial effect.

Table 3. Antibacterial MIC (µg/ml) and MBC (µg /ml) of *C. colocynthis* Schrad. leaves.

Strain	Concentration	Aqueous	Petroleum ether	Chloroform	Ethyl acetate	Acetone	Methanol
E. coli	MIC	1625	12.687	12.687	6.343	12.687	12.687
ATCC 25922	MBC	3250	25.375	25.375	12.687	25.375	25.375
P. aeruginosa	MIC	812	12.687	25.375	6.343	12.687	12.687
ATCC 27853	MBC	1625	25.375	50.750	12.687	25.375	25.375
S. aureus	MIC	1625	25.375	25.375	25.375	12.687	12.687
ATCC 25923	MBC	3250	50.750	50.750	101.500	25.375	25.375
E. faecalis	MIC	1625	25.375	25.375	6.343	25.375	25.375
ATCC 29212	MBC	3250	50.750	50.750	12.687	50.750	25.375

MIC positive control, Levofloxacin (*E. coli* 0.61 µg/ml, *P. aeruginosa* 0.3 µg/ml, *S. aureus* 0.3 µg/ml, *E. faecalis* 1.22 µg/ml); *E. coli*, *Echerichia coli; P. aeruginosa*, *Pseudomonas aeruginosa; S. aureus*, *Staphylococcus aureus; E. faecalis*, *Enterococcus faecalis.*

Table 4. Antifungal MIC (µg/ml) and MFC (µg/ml) of *C. colocynthis* Schrad. leaves.

Strain	Concentration	Aqueous	Petroleum ether	Chloroform	Ethyl acetate	Acetone	Methanol
C. albicans	MIC	1625	12.687	12.687	6.347	<6.347	<6.347
ATCC 90028	MFC	3250	25.375	25.375	12.687	6.347	6.347
C. glabrata	MIC	812	12.687	6.347	50.750	12.687	12.687
ATCC 90030	MFC	1625	25.375	12.687	50.750	25.375	25.375
C. kreusei	MIC	1625	25.37	12.687	<6.347	6.347	6.347
ATCC 6258	MFC	3250	50.750	25.375	6.347	12.687	12.687
C. parapsilosis	MIC	812	25.375	12.687	25.375	6.347	6.347
ATCC 22019	MFC	1625	50.750	25.375	50.750	12.687	12.687

Positive control with Amphotericin B (MFC 0.5 µg/ml). *C. albicans*, *Candida albicans; C. glabrata*, *Candida glabrata; C. krusei*, *Candida kreusei; C. parapsilosis*, *Candida parapsilosis*.

Anticoagulant effect

In the PT and APTT tests (Table 5), no extract caused the plasma to coagulate instantly, however, induced the PT and APTT to increase significantly in a concentration-dependent manner. The soxhlet fractions of *C. colocynthis* leaves generally had no significant effect on the PTs and APTTs at low concentrations. On the other hand, further dilutions of the aqueous extract strongly inhibited the plasma coagulation. With the petroleum, chloroform and ethyl acetate extracts, at high concentrations, the plasma coagulated with PTs and APTTs that were slightly higher than negative control values; whereas at the most diluted solutions, we noted a pro-coagulate effect. At high concentrations, acetone and methanol soxhlet fractions were the most active and may be respensable on the aqueous extract anticoagulant effect.

DISCUSSION

C. colocynthis Schrad. leaf activity could be attributed to contents of active components present in the extracts. Moreover, it can be noteworthy to point out that aqueous and organic extracts components are effective on neutralization of microorganisms tested, especially which are responsible for pulmonary infections and on PT and APTT. Also, polar organic extracts exhibited stronger antimicrobial activities than no polar ones, in accordance with PT and APTT results. As a result, organic extracts have excellent capacity in antimicrobial assay than aquous extract, whereas on the PT and APTT tests, organic extracts are less active; this efficacy difference may be attribuated to the phenomena of synergism or antagonism between components in the crude aquous extract tested.

With all these wide spectrum of the antibacterial and antifungal effects, *C. colocynthis* Schrad. leaves can be considered as an effective antimicrobial agent treating infectious diseases. Since, this plant demonstrated activity against some prevalent bacteria and fungi in dermatology, gynaecological and pulmonary infections, namely polar organic extracts, the use of this plant as antimicrobial agent is validated, scientifically supported

Table 5. PT (s) and APTT(s) times of *C. colocynthis* Schrad. leaf extracts.

Extract	Dilution	PT(s)	APTT(s)
	0	31.0 ± 0.2	97.5 ± 10
	½	19.2 ± 0.4	54.1 ± 7.0
	¼	16.0 ± 1.0	49.2 ± 9.0
	1/5	17.0 ± 1.0	41.7 ± 0.1
Aqueous	1/10	15.3 ± 0.2	46.8 ± 1.0
	1/20	15.2 ± 0.3	45.5 ± 2.1
	1/50	15.2 ± 0.2	37.5 ± 0.2
	1/100	15.2 ± 0.2	33.8 ± 0.5
	1/1000	15.0 ± 0.4	33.9 ± 1.0
	0	15.4 ± 0.4	38.5 ± 1.3
	½	15.6 ± 0.5	38.6 ± 1.1
	1/4	15.0 ± 0.8	37.9 ± 0.8
	1/5	14.9 ± 0.5	37.8 ± 0.9
Petroleum ether	1/10	14.5 ± 1.0	37.3 ± 0.8
	1/20	13.8 ± 0.2	37.0 ± 0.5
	1/50	13.5 ± 0.8	37.0 ± 0.5
	1/100	13.5 ± 0.7	36.3 ± 0.7
	1/1000	13.5 ± 0.8	35.9 ± 0.8
	0	14.7 ± 0.2	43.6 ± 1.0
	1/2	14.5 ± 0.2	43.6 ± 1.1
	1/4	14.7 ± 0.3	43.3 ± 1.0
	1/5	14.1 ± 0.5	39.0 ± 0.9
Chloroform	1/10	13.1 ± 0.8	39.2 ± 0.9
	1/20	12.7 ± 0.4	38.0 ± 0.7
	1/50	12.5 ± 0.5	38.0 ± 0.7
	1/100	12.2 ± 0.3	34.8 ± 0.8
	1/1000	12.2 ± 0.2	32.6 ± 0.5
	0	15.1 ± 1.0	41.9 ± 1.9
	1/2	15.0 ± 1.0	40.1 ± 1.4
	1/4	14.2 ± 0.6	40.5 ± 1.4
	1/5	13.1 ± 0.7	38.3 ± 0.9
Ethyl acetate	1/10	12.7 ± 0.6	37.9 ± 0.9
	1/20	12.2 ± 0.5	37.0 ± 0.5
	1/50	12.2 ± 0.4	36.8 ± 0.6
	1/100	12.0 ± 0.2	36.7 ± 0.6
	1/1000	12.0 ± 0.3	35.6 ± 0.5
	0	20.3 ± 1.2	47.2 ± 2.1
	1/2	18.0 ± 1.0	42.1 ± 1.9
	1/4	16.2 ± 1.1	40.7 ± 1.3
	1/5	16.0 ± 1.1	39.7 ± 1.2
Acetone	1/10	15.6 ± 0.9	39.4 ± 1.3
	1/20	15.5 ± 0.7	38.7 ± 0.8
	1/50	15.5± 0.6	38.4 ± 0.7
	1/100	15.1 ± 0.6	37.8 ± 0.5
	1/1000	14.9 ± 0.5	33.7 ± 0.5
	0	15.7 ± 0.8	43.1 ± 1.5
Methanol	1/2	15.0 ± 0.7	40.5 ± 1.0
	1/4	14.7 ± 0.7	40.1 ± 0.9

Table 5. Contd.

	1/5	14.7 ± 0.6	39.1 ± 0.9
	1/10	14.0 ± 0.6	39.6 ± 0.8
	1/20	13.6 ± 0.5	38.6 ± 0.7
	1/50	13.2 ± 0.5	38.1 ± 0.7
	1/100	13.4 ± 0.3	36.8 ± 0.6
	1/1000	13.4 ± 0.3	36.2 ± 0.5
Heparin (31.25 µg/ml)		160 ± 6.0	400 ± 10.0
Serum		13.5 ± 0.3	36.6 ± 0.2

Heparin (183 IU/mg), Positive control; Serum, negative control.

by the results obtained in this work.

Our data suggest that polar Soxhlet extracts from *C. colocynthis* Schrad. leaves have anticoagulant properties. The results obtained on the effect of the whole Soxhlet extracts on the PT and APTT tests further support the conclusion that this species contains both coagulant and anticoagulant activities. The PT and APTT tests are used for distinguishing between the effects of test agents on the extrinsic and intrinsic pathways (Brown, 1988). Substances that affect the PT are thought to act on the extrinsic pathway factors: factors V, VII, X, prothrombin and fibrinogen, while those that affect the APTT act on the components of the intrinsic pathway, that is, all coagulation factors except factors VII and XIII. The results reported here show that the anticoagulant effect examined by the PT test is portrayed by the APTT one.

Antimicrobial and anticoagulant properties of various extracts from many plants are of great interest in both academia and the food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic products by natural ones. In this respect, studying with the endangered species may be of great interest, since their bioactive properties could be lost forever without being tapped. Owing to its strong antimicrobial effect and moderate anticoagulant property, the extracts from the herbal parts of *C. colocynthis* Schrad. could be concluded as a natural source that can be freely used in the pharmaceutical industry as a medicinal herb, but, firstly, immediate and necessary measurements should be taken for the protection of this plant species which is known by its toxicity (leaves LD_{50} = 3903.2 mg/kg) (Marzouk et al., 2010b).

In conclusion, our study can be considered as the first report on the *in vitro* antimicrobial and anticoagulant properties of extracts prepared from *C. colocynthis* Schrad. leaves. From now the use of this plant in traditional medecine is validated by the results obtained in this work. We hope that our results introduce a natural source possessesing strong antimicrobial and moderat anticoagulant coumpounds.

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