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Full Length Research Paper

Comparative evaluation of the antimicrobial activity of *Citrullus colocynthis* immature fruit and seed organic extracts

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Gastrointestinal problems, dermatological, gynaecological and pulmonary infections produced by micro-organisms are widespread in the entire globe. The treatment of these infections is mainly based on the use of synthetic drugs which have lost, in recent years, their effectiveness, due to the development of resistant strains and the rise of opportunistic fungal infections. Tunisian traditional medicine is a potential source of new remedies namely, *Citrullus colocynthis* Schrad. (Cucurbitaceae). Lyophilized aqueous and organic extracts from immature fruits and seeds were screened for activity against gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Vibrio parahaemolyticus and Vibrio alginolyticus)* and gram-positive (*Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes* and *Micrococcus luteus*) bacteria and various *Candida* spp. (*Candida glabrata*, *Candida albicans*, *Candida parapsilosis* and *Candida kreusei*). Minimal inhibition concentrations (MICs) and minimal bactericidal/fungicidal concentrations (MBCs/MFCs) were used to investigate the antimicrobial activity. Extracts from the two *C. colocynthis* Schrad. organs, at immature state, inhibited the growth of all the tested strains. The highest antibacterial effects were obtained against *E. coli* (MIC = 0.006 mg/ml) with the fruit methanol and the seed petroleum extracts. Regarding the anticandidal activity assessment, seed extracts showed the lowest results. This study demonstrated the broad spectrum antimicrobial activity of *C. colocynthis* immature fruit and seed extracts.

Key words: *Citrullus colocynthis* Schrad, fruits, seeds, organic extracts, antibacterial, anticandidal.

INTRODUCTION

The increase of the incidence of fungal and bacterial infections is due to the emergence of resistant pathogens and their nosocomial dissemination. Scientific efforts to discover new potential antimicrobial drugs are principally leaned towards synthetic and natural products of plant origin. *Citrullus colocynthis* Schrad., belonging to the family of Cucurbitaceae and popularly known as Handhal, Hdaj or Dellaa El-Wad, is a very widespread medicinal plant (Boukef, 1986; Le Flock, 1983). The reproductive organs are traditionally used in Tunisian folk medicine for treating many diseases such as rheumatism, hypertension and various contagious diseases, including dermatological problems and gynaecological, urinary, gastrointestinal and pulmonary infections. In Tunisia, as in the rest of the Mediteranean (Al-Rawi and Chaakravarty, 1964), the parts of plants most often used for medicinal purposes are fruits and/or seeds. Methods of administration are topical, rectal or vaginal suppositories, enema, cervico-vaginal douche and by ingestion (Boukef, 1986).

Some studies have demonstrated the medicinal effect of *C. colocynthis* Schrad. as anti-tumour (Tannin-Spitz et

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Table 1. Yields (%) of *C. colocynthis* seed and fruit extracts.

Yield	Aq		PE		Chl		EA		A		M	
	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit
Yield (%)	2.79	2.94	8.96	0.54	0.71	8.35	0.08	0.93	4.74	6.55	6.83	18.02

Aq, Aqueous extract; PE; petroleum ether extract; Chl, chloroform extract; EA, ethyl acetate extract; A, acetone extract; M, methanol extract.

al., 2007), immunostimulant (Bendjeddou et al., 2003), anti-inflammatory (Marzouk et al., 2010a), antioxidant (Marzouk et al., 2010b) and it is used against hepatic diseases (Gebhardt, 2003), hyperglycaemia (Al-Ghaithi et al., 2004) and hair loss (Roy et al., 2007).

In this study, the antibacterial and the anticandidal activities of five organic extracts obtained from *C. colocynthis* Schrad. seeds and fruits using the broth serial dilution (microdilution method) was examined. Organic extract results were compared to the aqueous ones.

MATERIALS AND METHODS

Plant material

C. colocynthis Schrad. plants were collected in August near Medenine, Tunisia in the municipality of Sidi Makhlouf (33°33 N, 10°27 W). The identification was performed according to the flora of Tunisia (Pottier-Alapetite, 1981) and a voucher specimen (C.C-01.01) was deposited in the biological laboratory of the Faculty of Pharmacy of Monastir.

Extraction protocol

Lyophilized aqueous extracts

100 g of each fresh organ 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 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.

Soxhlet extracts

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 refluxed over the sample (grape pomace). The resulting extracts were evaporated at reduced pressure to obtain the crude extracts. The organic solvents used were 99% pure.

Antibacterial and anticandidal activities

Micro-organisms

Ten reference strains were chosen for antibacterial investigation: Gram-positive cocci (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* CTP 106510, *Listeria monocytogenes* ATCC 19115 and *Micrococcus luteus* NCIMB 8166) and gram-negative bacilli (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* LT2, *Vibrio parahaemolyticus* ATCC 17802 and *Vibrio alginolyticus* ATCC 33787). The antifungal effect of the various *C. colocynthis* extracts was also tested against four pathogenic reference yeasts (*Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida kreusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019).

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

The use of antifungal susceptibility testing against fungi has increased during the last few years. The reference method developed by the CLSI (Clinical and Laboratory Standards Institute), is time consuming and needs many reagents, which may pose a problem for the laboratories or institutions with a large number of samples to process. This is the main reason for exploring other methodologically easier methods such as the E test, the sensitive yeast test and the microdilution method using 96-well microplates.

The MIC and the MBC/MFC values were determined for all strains used in this study as described by Berche et al. (1991) and Hammer et al. (1996). The inoculums of the strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. *C. colocynthis* seed and fruit extracts dissolved in 10% dimethylsulfoxide (DMSO), were first diluted to the highest concentration (1.6 mg/ml) to be tested, and then serial twofold dilutions were made in a concentration range from 0.003 to 1.6 mg/ml in 5 ml sterile test tubes containing nutrient broth. The 96-well plates were prepared by dispensing into each well 95 µl of nutrient broth and 5 µl of the inoculum.

100 µl aliquot from the stock solutions of each extract was added into the first wells. Then, 100 µl from the serial dilutions were transferred into 11 consecutive wells. The last well containing 195 µl of nutrient broth without tested extracts and 5 µl of the inoculum on each strip was used as the negative control. The final volume in each well was 200 µl. The plates were incubated at 37°C for 18 to 24 h. The tested extracts in this study were screened two times against each organism. The MIC of each extract was defined as the lowest concentration which inhibited either bacterial or candidal growth, after incubation at 37°C between 18 and 24 h.

The MBC and the minimal fungicidal concentration (MFC) were determined by subculture on blood agar at 37°C between 18 and 24 h. All tests were performed in triplicate. Levofloxacin, ciprofloxacin and gentamicin were used as antibacterial positive controls and amphotericin B was used for the anticandidal one.

RESULTS

Extraction yields

The yields of the prepared extracts are given in Table 1.

Table 2. Antibacterial MIC (mg/ml) and MBC (mg/ml) of *C. colocynthis* seed and fruit extracts.

Micro-organism		Seeds						Fruits					
		Aq	PE	Chl	EA	A	M	Aq	PE	Chl	EA	A	M
E. coli	MIC	0.400	0.003	0.006	0.025	0.050	0.050	0.200	0.025	0.025	0.025	0.012	0.003
ATCC 25922	MBC	0.800	0.006	0.012	0.050	0.100	0.100	0.400	0.050	0.050	0.050	0.025	0.006
P. aeruginosa	MIC	0.800	0.006	0.006	0.050	0.050	0.050	0.200	0.012	0.012	0.025	0.025	0.006
ATCC 27853	MBC	1.600	0.012	0.012	0.100	0.100	0.100	0.400	0.025	0.025	0.050	0.050	0.012
Salm. typhimurium	MIC	0.800	0.100	0.200	0.200	0.100	0.100	0.800	0.400	0.200	0.200	0.200	0.100
LT2	MBC	1.600	0.200	0.400	0.400	0.200	0.200	1.600	0.800	0.400	0.400	0.400	0.200
V. parahaemolyticus	MIC	0.800	0.200	0.400	0.400	0.200	0.400	0.400	0.400	0.100	0.100	0.050	0.050
ATCC 17802	MBC	1.600	0.400	0.800	0.800	0.400	0.800	0.800	0.800	0.200	0.200	0.100	0.100
V. alginolyticus ATCC	MIC	0.800	0.200	0.400	0.400	0.200	0.400	0.800	0.800	0.200	0.100	0.050	0.050
33787S	MBC	1.600	0.400	0.400	0.800	0.400	0.800	1.600	1.600	0.200	0.200	0.100	0.100
Staph. aureus	MIC	0.800	0.100	0.400	0.400	0.100	0.025	0.400	0.025	0.100	0.100	0.050	0.006
ATCC 25923	MBC	1.600	0.200	0.800	0.800	0.200	0.050	0.800	0.050	0.200	0.200	0.100	0.012
Staph. epidermidis	MIC	0.400	0.100	0.100	0.200	0.200	0.050	0.200	0.100	0.200	0.200	0.100	0.050
CTP 106510	MBC	0.800	0.200	0.200	0.200	0.400	0.100	0.400	0.200	0.400	0.400	0.200	0.100
L. monocytogenes	MIC	0.400	0.100	0.200	0.200	0.100	0.050	0.400	0.400	0.200	0.200	0.100	0.050
ATCC 19115	MBC	0.800	0.200	0.400	0.400	0.200	0.100	0.800	0.800	0.400	0.400	0.200	0.100
E. faecalis	MIC	0.800	0.025	0.200	0.050	0.050	0.050	0.800	0.200	0.200	0.050	0.050	0.012
ATCC 29212	MBC	1.600	0.012	0.400	0.100	0.100	0.100	1.600	0.400	0.400	0.100	0.100	0.025
M. luteus	MIC	0.400	0.100	0.200	0.200	0.100	0.100	0.200	0.100	0.100	0.100	0.050	0.050
NCIMB 8166	MBC	0.800	0.200	0.400	0.400	0.200	0.200	0.400	0.200	0.200	0.200	0.100	0.100

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); Ciprofloxacin (*V. parahaemolyticus* 0.38 µg/ml, *V. alginolyticus* 0.38 µg/ml); Gentamicin (*Salm. typhimurium* 15.62 µg/ml, *Staph. epidermidis* 31.25 µg/ml, *L. monocytogenes* 1.95 µg/ml, *M. luteus* 3.90 µg/ml).
Aq.: Aqueous extract ; P.E.: Petroleum ether extract ; Chl. : chloroform extract ; E.A. : Ethyl acetate extract ; A. : Acetone extract ; M : Methanol extract; *E. coli: Echerichia coli; P. aeruginosa: Pseudomonas aeruginosa; Salm. typhimurium: Salmonella typhimurium; V. parahaemolyticus: Vibrio parahaemolyticus; V. alginolyticus: Vibrio alginolyticus; Staph. aureus: Staphylococcus aureus; Staph. epidermidis: Staphylococcus epidermidis; L. monocytogenes: Listeria monocytogenes; E. faecalis: Enterococcus faecalis. M. luteus: Micrococcus luteus.*

The aqueous extraction yields were close for both organs. Petroleum ether extraction gave the lowest yield for fruits and had better one for seeds. For the seeds, the lowest yield was obtained for ethyl acetate extraction. The higher yield of all is noted for fruit methanol extract.

Antibacterial activity

All the extracts tested for Tunisian *C. colocynthis* imamture fruits and seeds showed antibacterial activity against all tested strains. MIC and MBC were tested for concentrations that ranged from 0.003 to 1.6 mg/ml (Table 2) and all the extracts exhibited antibacterial activity against all tested strains. The strongest inhibitions obtained was against *Escherichia coli* with polar immature fruit extract (methanol extract) and no polar immature seed extract (petroleum ether extract). For both extracts, the MIC was 0.003 mg/ml and the MBC was 0.006 mg/ml. The *Vibrio* sp. strains demon-

Table 3. Anticandidal MIC (mg/ml) and MFC (mg/ml) of *C. colocynthis* seed and fruit extracts.

Candida spp.		Seed						Fruit					
		Aq	PE	Chl	EA	A	M	Aq	PE	Chl	EA	A	M
C. albicans ATCC 90028	MIC	0.400	0.200	0.200	0.100	0.100	0.100	0.100	0.200	0.100	0.100	0.050	0.025
	MFC	0.800	0.400	0.400	0.200	0.200	0.200	0.200	0.400	0.200	0.200	0.100	0.050
C. glabrata ATCC 90030	MIC	0.800	0.200	0.200	0.100	0.200	0.100	0.100	0.200	0.200	0.100	0.050	0.025
	MFC	1.600	0.400	0.400	0.200	0.200	0.200	0.200	0.400	0.400	0.200	0.100	0.100
C. kreusei ATCC 6258	MIC	0.400	0.200	0.200	0.200	0.100	0.100	0.200	0.100	0.200	0.100	0.050	0.050
	MFC	0.800	0.400	0.400	0.400	0.200	0.200	0.400	0.200	0.400	0.100	0.100	0.100
C. parapsilosis ATCC 22019	MIC	0.400	0.200	0.200	0.100	0.100	0.100	0.200	0.200	0.200	0.200	0.100	0.050
	MFC	0.800	0.400	0.400	0.200	0.200	0.200	0.400	0.400	0.400	0.400	0.200	0.100

Positive control, Amphotericin B (MFC 0.5 µg/ml); Aq, aqueous extract; PE; petroleum ether extract; Chl, chloroform extract; EA, ethyl Acetate extract; A, acetone extract; M, methanol extract; *C. albicans, Candida albicans; C. glabrata, Candida glabrata; C. kreusei, Candida kreusei; C. parapsilosi, Candida parapsilosis*.

strated a close sensibility for all the tested seed and fruit extracts. The lowest activity (against all strains) was observed for aqueous lyophilised extracts (MIC ranging from 0.200 to 1.600 mg/ml).

Anticandidal activity

Anticandidal activity was reported as MIC and MFC (Tables 3). All extracts showed significant antifungal activity against all the tested yeasts. Organic extracts were most active than the aqueous ones, for all *Candida*. The methanol fruit extract demonstrated the best anti-fungal activity against *Candida albicans* (with MIC value equal to 0.025 mg/ml and MBC equal to 0.050 mg/ml). In terms of plant organs, the best activities were found for immature fruits at one or more of their organic extracts. For the immature seeds and fruits, polar extracts had the highest anticandidal effects against all strains. Acetone extracts were approximately as efficient as methanol fractions (for both seeds and fruits).

DISCUSSION

Medicinal plants have been used for ages in the treatment of diseases. In recent years, herbal medicines have increasingly been used to treat infections difficult to manage. Although the antibacterial and the anticandidal activities of all organs of *C. colocynthis* has been investigated (Marzouk et al., 2009, 2010c), the screening of the seed and fruit organic extracts was not elucidated. This investigation has provided multifaceted results as made obvious by the extraction yields, antibacterial and anti-candidal activities of *C. colocynthis* tested parts. The efficiency of each organ depends on the plant extract which is generally a crude mixture of non-active and active compounds. The obtained MICs (as low as 0.10 mg/ml) are within the range of what is considered significant for plants and even purified extracts.

The antibacterial activity depended on the tested strain, the plant organ and the nature of the extract: for a given micro-organism, the most effective plant organ can also change according to the organic extract type (for gram negative bacteria, the best antibacterial organ extracts changed from immature fruits in polar extracts to the stronger activity of seeds in the no polar extracts).

The good MIC values of the seeds and fruits against *P. aeruginosa*, known to be the most frequent causes of nosocomial antibiotic-resistant infections (Carmeli et al., 1999), allow a novel confirmation of antibacterial property of these reproductive organs. Anti-*Vibrio* results also made *C. colocynthis* seeds and fruits to be accepted as a plant that serve several purposes for treating problems associated with the digestive system, caused by this halophilic gram-negative bacterium.

Concerning the anticandidal activity, the study showed MICs and MFCs significant for crude plant extracts against all strains; polar extracts were more efficient. An equal strong activity against all *Candida* sp. was noted. The most striking result of this investigation is the document-tation of a remarkable activity against *C. albicans* (MIC up to 0.100 mg/ml for seed extracts and up to 0.025 mg/ml for fruit extracts) (Table 3); this strain caused more than 45% of clinical fungal infections (Gupta et al., 2004).

So, the results proved the idea that plants are known to produce certain toxic chemicals to micro-organism, and a large body of literature has validated the antimicrobial activity of plant extracts, showing a great potential especially against multidrug resistant strains (Mshvildadze et al., 2000; Abdel-Ghani et al., 2008). This is the first report of the antimicrobial activity of organic extracts from *C. colocynthis* seeds as well as from the fruits, against four *Candida* sp. and ten bacteria, including *Vibrio* sp. The results presented in the study indicate that the natural products analyzed seemed to be a good choice for the development of new strategies to treat infections in der-

matology, gynaecological, gastrointestinal and pulmonary infections. Therefore, the traditional use of this plant as antimicrobial agent is validated by the results obtained in this work. So, it can be used as an alternative to conventional formulations for individuals with an interest in naturally-based products (Mimee et al., 2005). The very promising results we have obtained provide a rationale for assessing the activity of *C. colocynthis* Schrad. seed and fruit extracts on infected wounds and surfaces. Further studies are ongoing to identify and purify the chemical compounds of these antimicrobial extracts.

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