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ANTIMICROBIAL ACTIVITY OF THE METHANOLIC EXTRACT, FRACTIONS AND ISOLATED COMPOUNDS FROM *CITRULLUS COLOCYNTHIS* (L.) SCHRAD.

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ABSTRACT

The methanolic crude extract prepared from the fruits of *Citrullus colocynthis* (MCCF), its fractions and two isolated compounds namely Ursolic Acid, (compound1) and Cucurbitacin E 2-0-β-D-glucopyranoside (compound 2) were evaluated for their antibacterial activity. Broth microdilution and agar disc diffusion methods were used for the investigations. The antimicrobial assays showed that the crude extract, fractions FC I, FC II, compound 1 and compound 2 were capable of preventing the growth of the bacterial strains (standard strains and their clinical isolates). The lowest minimal inhibitory concentration (MIC) of 62.5µg/ml was recorded for the crude extract and the corresponding values for both the fractions and compounds were 25µg/ml each. The overall results provided promising baseline information for the potential use of the methanolic crude extract of *C. colocynthis*, its fractions and the tested compounds for the treatment of bacterial and fungal infections.

KEYWORDS: **Antimicrobial activity; *Citrullus colocynthis*; Cucurbitaceae; fractions; Ursolic Acid; Glucopyranoside.**

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INTRODUCTION

The search for biologically active compounds from plants has always been of great value towards the new sources of useful drugs against infectious diseases. Plant secondary metabolites such as tannins, alkaloids and flavonoids, are known to have antimicrobial properties[1]and search for other medicinally important compounds in many more plants goes on. *Citrullus colocynthis* (L.) Schrad. (Cucurbitaceae) grows abundantly in the tropical regions of the world and is well recognized in the traditional system of medicine for treatment of constipation, oedema, bacterial infections, cancer and diabetes[2]. The methanolic extract of *C. colocynthis* fruits and seeds has shown high antibacterial activity against Gram positive and Gram negative bacteria including *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Vibrio parahaemolyticus,Vibrio alginolyticus*, *Enterococcusfaecalis, Staphylococcus aureus, Staphylococcus epidermidis,Listeria monocytognes* and *Micrococcus luteus*[3]. Gurudeeban *et al.* investigated *C. colocynthis* for its broad spectrum antimicrobial activity against sixteen clinical microorganisms isolated from HIV positive patients[4]. Qualitative phytochemical screening of active extract of *C. colocynthis* revealed the presence of tannins, saponins, alkaloids and flavonoids. The significant antimicrobial activities of active extracts were compared with standard antibiotic, novobiocin [5]. The aim of the present study was to investigate the antibacterial and antifungal activities of methanolic extract of *C. colocynthis* fruits *as* well as the fractions and compounds obtained following a bio-guided fractionation, for development of medicine against the drug resistant clinical isolates.

MATERIALS AND METHODS

Plants growing in their natural habitats at Dausa, Rajasthan were collected and identified according to 'Flora of the Indian desert'[6]. Voucher specimens were deposited in the herbarium of Department of Botany, University of Rajasthan Jaipur, India. All the analytical grade chemicals and culture media were purchased from Himedia, India. Nutrient Agar (NA), Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were used for bacterial culture, sensitivity testing determined Minimum Inhibitory Concentration (MIC). Gentamycin for GNB (Gram Negative Bacteria) and Vancomycin for GPC (GramPositive Cocci (Himedia™ Laboratories Pvt Ltd, India) were used as reference antibiotics (RA).

(i) Extraction and bio-assay guided purification

The plant material was initially rinsed in distilled water and air-dried in shade. Deseeded fruits were ground into fine powder using an electric blender (Toshiba, Japan). 50 g dried powdered material was extracted with 500 ml methanol by Soxhlet for 72 h at a temperature of 60-80°C[7,8]. The process was repeated till the color disappeared from the solvent and all the extracts were pooled together to get the final volume. The filtrate was then concentrated with the help of the rotary evaporator (Buchi, India) to give dark crude extract. 27g extract was subjected to a silica gel-60 column chromatography and eluted with petroleum ether (100%), petroleum ether: chloroform (80:20, 60:40, 40:60, 20:80), chloroform (100%), chloroform: ethyl acetate (80:20, 60:40, 40:60, 20:80), ethyl acetate (100%), ethyl acetate: acetone (80:20, 60:40 ,40:60, 20:80), acetone (100%), acetone: methanol (80:20, 60:40, 40:60, 20:80) and methanol (100%) respectively. These fractions were then pooled, together on the basis, of their similar TLC patterns. Total nine fractions FCI (2.2 g), FC II (2.9g),FC III(3.0g), FCIV (1.5 g),FC V(3.0 g), FC VI (1.8g),FC VII(1.5g), FC VIII(1.8 g) and FCIX (1.2) were finally obtained. On the basis of antimicrobial activity FC III and FC IX were selected and subjected to TLC (petroleum ether: acetone, 8:2) for further separation.

(ii) Characterization of compounds

Small amount of the eluted compounds were dissolved in $CDCl_3$. Compounds were first subjected to ^{1}H NMR and then tube ^{13}C NMR spectra were recorded. ^{1}H NMR spectra were recorded on JEOL AL-30 at 300.13 MHz and

on Bruker DRX-400 ultra-shield spectrometer at 400 MHz, using TMS as internal standard in $CDCl_3$. The chemical shifts were reported in δ ppm. Elemental analysis was carried out using Perkin-Elmer CHNS/O Analyser 2400. The absorption bands were recorded and reported in cm^{-1}. Mass spectra were recorded on API QSTAR pulsar mass spectrometer. The structures of the compounds were confirmed by comparing with reference data from available literature.

(iii) Microbial strains

Total Fifteen bacterial strains were collected as test microorganisms for antimicrobial activity of the plant extracts, including five standard strains: Ciprofloxacin-resistant *E. coli* (ATCC-25922), Piperacillin-resistant *Pseudomonas aeruginosa (*ATCC-27853), Amikacin-resistant *Proteus mirabilis* (ATCC-25933), Ciprofloxacin-resistant *Klebsiella pneumoniae (*ATCC-13883)and Amoxyclav-resistant *Staphylococcus aureus (*ATCC-29213) with two clinical isolates C1 and C2 of each bacterium, where C1 is resistant and C2 is sensitive to specific antibiotics as mentioned in Table 1, All the experiments were repeated thrice. The microbes were obtained and tested at the Department of Microbiology and Immunology SMS Medical College, Jaipur, Rajasthan and their identity was confirmed by using standard key[9].

Table 1
Testorganismsand their antibiotic sensitivity

Escherichia coli	ATCC25922	Ciprofloxacin- Sensitive
	C1	Ciprofloxacin-Resistant
	C2	Ciprofloxacin-Sensitive
Pseudomonas aeruginosa	ATCC27853	Piperacillin- Sensitive
	C1	Piperacillin-Resistant
	C2	Piperacillin-Sensitive
Proteus mirabilis	ATCC25933	Amikacin- Sensitive
	C1	Amikacin-Resistant
	C2	Amikacin-Sensitive
Klebsiella pneumoniae	ATCC13883	Ciprofloxacin- Sensitive
	C1	Ciprofloxacin-Resistant
	C2	Ciprofloxacin-Sensitive
Staphylococcus aureus	ATCC 29213	Amoxyclav- Sensitive
	C1	Amoxyclav- Resistant
	C2	Amoxyclav- Sensitive

(iv) Preparation of discs and disc dilution test

Whatmann filter paper (No. 1) discs of 6 mm diameter with concentration of 125µg/disc of crude extract and 50µg/disc of fractions and isolated compounds were prepared using dimethylsulfoxide (DMSO) as a solvent. The discs were kept at 37°C for 24 h. The reference antibiotic (RA) and specific antibiotics (SA) discs were prepared as described above to obtain the final concentration of 50µg/disc and 100µg/disc. The disc diffusion test was carried out as per Clinical and Laboratory Standards Institute (CLSI) guideline to check the antimicrobial effect of the plant products and reference antibiotics. Discs prepared with the corresponding volume of DMSO without any drug were used as negative controls. The plates with the test organisms and impregnated discs were incubated at37°C for 24h. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone around the disc. The assay was repeated thrice and results were recorded as mean ± SD.

(v) Determination of MIC

MICs of the crude extract, fractions, C1 and C2 and Reference Antibiotic (Gentamycin for Gram negative bacteria and Vancomycin for Gram positive bacteria) were determined as follows; the test samples of plants extract were

initially dissolved in DMSO. The solution obtained was then added to MHB to obtain a final concentration of 100µg/ml. This solution was further diluted serially to obtain the concentration range of 12.5-50µg/ml and the tubes were agitated. Bacteria were inoculated using a cell suspension of about $1.5x10^6$ CFU/ml obtained from a McFarland turbidity standard No. 0.5 and incubated at 37°C for 24h. The assay was repeated thrice. MIC was defined as the lowest sample concentration that exhibited complete inhibition of bacterial growth.

RESULTS AND DISCUSSION

The bio-assay guided fractionation of crude extract (MCCF) led to the isolation of two main compounds, Ursolic Acid (compound 1) and Cucurbitacin E 2-0-β-D-glucopyranoside (compound 2). The spectral data of both the compounds were found consistent with the earlier reported spectra of the respective compounds [10-12]. Compound 1 was eluted from FC III to yield an off white colored product and identified as Ursolic acid [10] (Fig 1). Compound 2 was eluted from FC IX (chloroform: methanol: ethyl acetate, 3:3:4) to yield yellow colored crystals and identified as Cucurbitacin E 2-0-β-D-glucopyranoside[11,12](Fig 2).

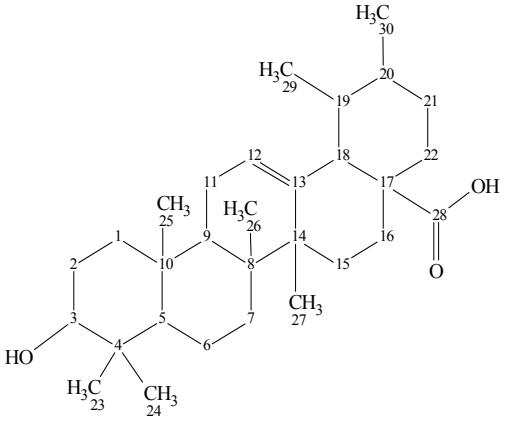

Figure 1
Ursolic acid

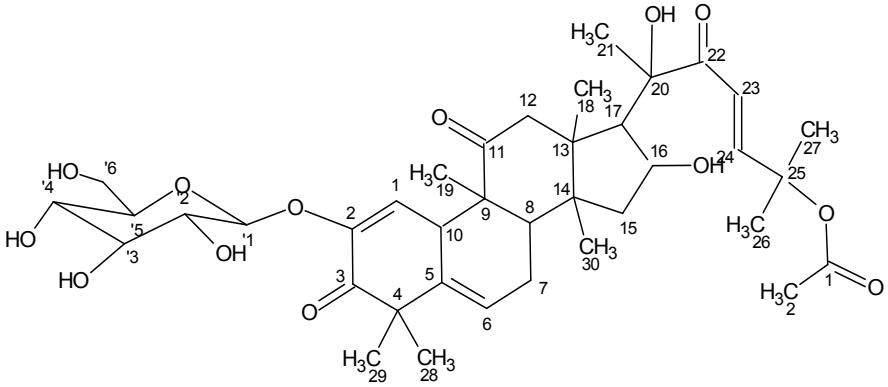

Figure 2
Cucurbitacin E 2-0-β-D-glucopyranoside

Cucurbitacin E 2-0-β-D-glucopyranoside was previously isolated by Torkey *et al*[13] from the plant *C. colocynthis.* The results of antimicrobial activity of these compounds have been presented in Tables 2 and Table 3.

Table 2
Antimicrobial activity of the crude extracts, fractions, compounds isolated from C. colocynthis and reference antibiotics determined by the disc diffusion test.

Tested organism		Tested samples (Zone of Inhibition; mm)						
		Crude extract MCCF	Fraction		Compounds		Antibiotics	
			FC I	FC II	C 1	C 2	1 RA	2 SA
Escherichia coli	ATCC	21.8±0.41	11.8±0.26	12.4±0.45	18.9±0.45	12.1±0.23	23.4±0.25	12.4±0.13
	C1	12.9±0.36	10.6±0.30	12.3±0.35	14.1±0.32	13.1±0.32	20.5±0.15	R
	C2	18.1±0.47	11.0±0.50	12.9±0.36	13.4±0.36	11.1±0.28	21.2±0.17	12.3±0.00
Pseudomonas aeruginosa	ATCC	22.0±0.47	16.7±0.43	14.3±0.47	17.3±0.20	14.1±0.28	20.2±0.05	10.5±0.15
	C1	18.9±0.36	13.1±0.28	12.4±0.45	12.3±0.35	12.5±0.50	19.3±0.15	11.1±0.11
	C2	14.1±0.36	12.9±0.36	10.3±0.32	10.1±0.23	11.1±0.28	20.4±0.03	9.9±0.50
Proteus mirabilis	ATCC	16.3±0.26	10.4±0.60	10.6±0.35	-	-	22.8±0.22	12.1±0.02
	C1	10.1±0.36	10.2±0.25	10.3±0.30	-	-	20.7±0.07	11.9±0.00
	C2	11.4±0.45	9.4±0.36	10.3±0.35	-	-	21.9±0.14	11.0±0.18
Klebsiella pneumoniae	ATCC	13.1±0.51	14.2±0.25	10.6±0.35	15.2±0.25	15.0±0.55	14.5±0.09	8.9±0.14
	C1	18.0±0.45	10.4±0.60	10.0±0.20	12.1±0.28	10.8±0.34	13.9±0.13	7.5±0.21
	C2	18.0±0.40	15.0±0.30	15.1±0.51	13.3±0.30	13.2±0.25	13.5±0.00	7.9±0.16
Staphylococcus aureus	ATCC	23.1±0.47	13.3±0.41	12.9±0.36	21.5±0.50	15.9±0.26	22.5±0.20	13.4±0.00
	C1	22.2±0.58	14.9±0.30	12.5±0.50	13.0±0.11	11.4±0.52	21.6±0.00	12.6±0.13
	C2	23.3±0.47	13.0±0.45	15.1±0.45	12.8±0.32	10.0±0.11	22.3±0.0.01	12.7±0.14

Antimicrobial activity: crude extract was tested at 125µg/disc concentration while fractions, compounds and RA at 50µg/disc concentration. The Tested samples were crude extract from the fruit of *C. colocynthis* (MCCF), Fractions 1 and 2(FC III and FC V), Compound 1: Ursolic acid, Compound 2:Cucurbitacin E 2-0-β-D-glucopyranoside. RA Gentamycin and Vancomycin used for *Staphylococcus aureus*,SA: Methicillin for *Staphylococcus aureus*, Amoxicillin for *Escherichia coli*, Ampicillin for *Klebsiella pneumoniae*, Carbenicillin for *Pseudomonas aeruginosa*, Chloramphenicol for*Proteus mirabilis*. Results of Table 2 demonstrated that the crude extract from *C. colocynthis* (MCCF), fractions FC III and FC IX and the two tested compounds exhibited microbial growth inhibition for all the tested organisms at the tested concentrations, except *P. mirabilis* which was inhibited by the crude extract and Fractions III and IX when tested separately but did not show any inhibition against the compound 1 and compound 2. It was also observed that the zone of inhibition observed with the extract and fractions were smaller for *P. mirabilis* than other microorganisms. *Pseudomonas aeruginosa* which is considered to be a notorious microorganism with respect to drug resistance with alarmingly high antibiotics resistance rates[14], has shown sensitivity to the MCCF, FC III and FC IX and towards both the compounds C1 and C2.

The zone of inhibition (IZ) against all the standard and clinical isolates obtained, ranged from 10.1 to 23.3 mm with MCCF which is in comparison with the reference antibiotic tested. The least IZ was seen for *P. mirabilis and* maximum for *S. aureus,* the only tested GPC (Gram positive cocci). IZ of 9.4 –16.7 mm and 10.3–15.1 mm respectively were observed for FC III and FC IX. Compound 1 and Compound 2 showed IZ ranging from 10.1-21.5 mm and 10.0 – 15.9 mm respectively. The wide range observed in the size of inhibition zones can be the quantitative indication of their antibacterial potency. Some of the organisms tested in this study were multi drug resistant (Table 3) to the conventional antibiotics but they did show growth inhibition by the plant extract.

Table 3
Resistance profile of multi-drug resistant isolates –antibiogram

S. No.	Test microorganisms		Specimen	Resistance pattern of antibacterial and /antifungal Agents
1.	*Escherichia coli*	ATCC (25922)	SMS Medical college & Hospital Jaipur	All Sensitive
		C1	Urine	Cpm, Pc, G, Cf, T, C
		C2	Stool specimen	Cpm, Pc, G T, C
2.	*Pseudomonas aeruginosa*	ATCC (27853)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Wound Swab	Cpm, Ac, Pc, Ak, Cf, T
		C2	Pus Swab	Cpm, Ak, Ao, Pc, G, Ac, Cf
3.	*Proteus mirabilis*	ATCC (25933)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Urine	Ak,G, Cf , T ,C,Ao,Cpm,Pc
		C2	Ear swab	C, Ak, G, Ac,Cpm, Ao, Pc
4.	*Klebsiella pneumoniae*	ATCC (13883)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Urine	Cpm , Ao , Pc, Ak, G
		C2	Sputum	Cpm, Ac, Ao, Pc, Ak,G, Cf
5.	*Staphylococcus aureus*	ATCC (29213)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Wound swab	Cf, Ao, Pc, T
		C2	Pus swab	Cpm Cf, Ao, Ac, Ak, T, Pc
6.	*Candida albicans*	ATCC (10231)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Urine	Fu, Ns
7.	*Aspergillus fumigatus*	ATCC (90906)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Nail scraping	Fu, Ns,
8.	*Aspergillus niger*	ATCC (16404)	SMS Medical college & Hospital, Jaipur	All Sensitive
		C1	Nail scraping	Fu

Antibacterial agents: Cpm =Cefepime (30µg), 2. Ac =Amoxyclav (30µg), 3. Ao =Aztreonam (30µg), 4. Pc =Piperacillin (100µg), 5. AK=Amikacin (30µg), 6. G =Gentamycin (10µg), 7. Cf =Ciprofloxacin (5µg), 8. T =Tetracycline (30µg), 9. C =Chloramphenicol (30µg), 10. Ro =Roxithromycin (30µg), 11. Cr =Cefuroxime (30µg)
Antifungal Agents:Kt =Ketoconazole (10µg), 2. Fu =Fluconazole (10µg), 3. Ns=Nystatin (100 units)

The larger size of the IZ observed for the crude extract as compared to the fractions and the compounds may be an indication of the synergistic antibacterial activity of the compounds present in the extract. This may also be due to the antibacterial effect of other compounds present in the crude extract. The zone sizes also showed that the compound 1 (Ursolic Acid), is more potent antibacterial than compound 2 (Cucurbitacin E 2-0-β-D-glucopyranoside). The results of MIC determinations (Table 4) indicate values ranging from 12.5 µg/ml to 50µg/ml. The role of MIC determination lies in the fact that lesser the MIC better are the chances of it being useful as potential drug for medicinal purpose. As desirable, if lesser amount of the compound will give the required blood levels with effective activity, it will have fewer side effects and reduced toxicity if any.

Table 4
Minimum inhibitory concentration (µg/ml) of the crude extract, fractions and compounds isolated from C. colocynthis and antibiotics.

Tested organism		Tested samples					
		Crude extract MCCF	Fraction		Compounds		Antibiotics
			FC I	FC II	C 1	C 2	1RA
Escherichia coli	ATCC	25	50	50	25	50	3.1
	C1	50	50	50	25	50	6.2
	C2	25	50	50	50	50	6.2
Pseudomonas aeruginosa	ATCC	12.5	25	50	25	25	3.1
	C1	25	50	50	50	50	3.1
	C2	50	50	50	50	50	6.2
Proteus mirabilis	ATCC	25	50	50	-	-	12.5
	C1	50	50	50	-	-	25
	C2	50	50	50	-	-	25
Klebsiella pneumoniae	ATCC	50	25	50	25	25	3.1
	C1	25	50	50	50	50	6.2
	C2	25	25	50	25	50	3.1
Staphylococcus aureus	ATCC	12.50	25	50	12.5	50	25
	C1	12.5	25	50	12.5	50	50
	C2	12.5	50	50	12.5	50	50

The Tested samples were crude extract from the fruit *C. colocynthis* (MCCF), Fractions 1 and 2 (FC III and FC IX), Compound 1: Ursolic acid, Compound 2:Cucurbitacin E 2-0-β-D-glucopyranoside. RA or Reference antibiotics (Gentamycin and Vancomycin used for *Staphylococcus aureus*), SA: specific antibiotics (Methicillin for *Staphylococcus aureus*, Amoxicillin for *Escherichia coli*, Ampicillin for *Klebsiella pneumoniae*, Carbenicillin for *Pseudomonas aeruginosa*, Chloramphenicol for*Proteus mirabilis*. The results from this study are in accordance with the previous biological reports on *C. colocynthis*. Many workers also reported cucurbitacin compounds including the bitter substances (colocynthin and colocynthetin), cucurbitacins A, B, C, D, and E (α-elaterin)[15,16], Cucurbitacins E, I, J, K, and L[17], cucurbitacin glycosides[12,18], flavonoids and flavone glycosides [12,19,20] from *C. colocynthis*. Gurudeeban *et al.*observed high antibacterial activity of *C. colocynthis* fruit. In the present investigation we could observe high antibacterial activity of the crude methanolic extract of the fruit. Eluted Compound Ursolic acid is a triterpenoids and reported to have moderate antibacterial and antifungal activities[21] and can inhibit many strains of *Staphylococcus* and fungus like *Microsporum lenosum* and *C. albicans* at 250-500µg/ml concentrations [22]. Eluted Compound 2 (Cucurbitacin E 2-0-β-D-glucopyranoside) is a glycosidic derivative of cucurbitacin E. Antifeedant activity of Cucurbitacine E was reported by Nielsen *et al.*[23] and Tannin-Spitz *et al*[24].

CONCLUSION

The antibacterial activity of methenolic extract and compounds from *Citrullus colocynthis* was seen for clinical isolates, showing multi drug resistance, is encouraging and provide promising baseline information for the potential use of the crude extract, fractions III and IX as well as the two isolated compounds from the fruit of *C. colocynthis* tested in the treatment of infectious diseases. Further pharmacological and toxicity studies will be necessary to confirm this hypothesis.

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