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Full Length Research Paper

In vitro Antimicrobial activities of chloroformic, hexane and ethanolic extracts of *Citrullus lanatus* var. *citroides* (Wild melon)

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To test the antimicrobial activities of crude chloroform, hexane and ethanol extracts of leaves, stem, fruits and seeds from *Citrullus lanatus var. citroides* (CL) against bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Proteus vulgaris*) and fungi (*Aspergillus nigar* and *Candida albican*). Antimicrobial properties of CL were tested using cup-plate diffusion method and disc diffusion. Analysis of the data revealed that, the chloroform extract of the fruit exhibited the highest antibacterial activity. It showed antibacterial activity against *S. aureus*: 36 mm, *B. subtilis*; 38 mm, *E. coli*; 37 mm, *P. valgaris*; 23 mm and *P. aerguinosa*; 19 mm. The ethanolic extract of the fruit pulp and stem showed the highest antifungal activity on *C. albican* (41 mm). A*. nigar* was very sensitive to the chloroform extract of the seed (37 mm) and the ethanolic extract of the leaves (37 mm). Results were compared concurrently to standard drugs; clotrimazole and gentamicin. Based on the current findings, it can be concluded that this plant has antimicrobial activity, which is as potent as standard antimicrobial drugs against certain microorganisms.

Keywords: *Citrullus lanatus* var. *citroides,* antimicrobial, medicinal plants.

INTRODUCTION

Herbal medicines have made large contributions to human healthiness (Iwu et al., 1999) and provided a good source of anti-infective agents; emetine, quinine, and berberine remain highly effective instruments in the fight against microbial infections. Phytoremedies have also shown great promise in the treatment of intractable infectious diseases including opportunistic AIDS infections (Parvez et al., 2005; Al-Momani et al., 2007).

Cucurbitaceae plants are known to contain bioactive compounds such as cucurbitacin, triterpenes, sterols and alkaloids (Yuan et al. 2006). Scientific studies mainly refer to Middle East and Asia where cucurbit plants were used actively as herbal remedies have shown tremendous results regarding the use of this botanical family. *Citrullus lanatus var. citroide* (Wild melon), is used widely in traditional herbal medicine. Whereby, fruits of this plant eaten as a febrifuge when fully ripe or even when almost putrid (Grieve and Leyel, 1984). The fruit is also diuretic, being effective in the treatment of dropsy and renal stones (Chiej, 1984). The rind of the fruit is prescribed in cases of alcoholic poisoning and diabetes (Duke and Ayensu, 1985). The root is purgative and in large dose is said to be emetic (Grieve and Leyel 1984). The seed is demulcent, diuretic, pectoral and tonic (Grieve and Leyel 1984; Duke and Ayensu 1985). It is sometimes used in

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the treatment of the urinary passages (Grieve and Leyel 1984) bed wetting (Moerman, 1998). The seed is also a good vermifuge and has a hypotensive action. Fatty oil in the seed, as well as aqueous or alcoholic extracts, paralyze tapeworms and roundworms (Chopra 1958). In Northern Sudan is often used for burns, swellings, rheumatism, gout and as laxative (Schippers and Budd, 1997). Thus, the aim of this current investigation is to evaluate the chloroform, hexane and ethanol extracts of leaves, stem, fruits and seeds from *Citrullus lanatus var. citroides* against several bacteria and fungi *in vitro*.

MATERIALS AND METHODS

Plant materials

Citrullus latanus var. citriode (CL) was collected from AL-Musawarat, Northern Sudan, on February 2008. The taxonomic identification of CL was carried out at by Dr. Abdalla W.E. at the Medicinal and Aromatic Plants Research Institute (MAPRI), National Center for Research, Khartoum, Sudan. A voucher specimen under the scientific name of the plant was deposited at the herbarium of MAPRI.

Preparation of crude extracts

Leaves, stem, fruits were cut into thin slices and dried at room temperature. Seeds were separated and dried under shade. The dry plant materials were ground into powder. Fifty grams of the powdered dried plant materials were extracted following the method of Ciulie (1981). Extraction was performed sequentially using hexane, chloroform, and ethanol. Plant materials were extracted at the beginning with 150 ml hexane for 24 h in conical flask using cool maceration with occasional shaking. Extracts were filtered using filter paper and the extraction procedure was repeated three times. The filtrates were combined and the plant residue was brought to dryness using rotary evaporator and extracted with 150 ml chloroform following the same methods as done previously for hexane. Remaining plants materials were extracted with 150 ml ethanol (90%) following the same method. The different extracts obtained were evaporated using rotary evaporator under room temperature to yield crude extracts. The crude extracts of leaves, stem, fruit bulb and seeds of CL were subjected to antibacterial, antifungal tests and chemical analysis.

Qualitative analysis of chemical constituents

Chemical analysis for the presence of major classes of secondary metabolites (alkaloids, anthraquinones, flavanoids, saponins, terpenes, steroids, tannins, flavones, aglycone and simple phenols) in the crude extracts was carried out according to the method described by Pearson (1976).

Antimicrobial assay

Microorganisms and medium

The bacteria used were originally from the American type culture collection (ATCC), USA. They were obtained from the stock culture of National Sanitary and Biotechnology laboratory, Department of Chemistry, University of Technology Malaysia, Shah Alam, Malaysia. Strains used were *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, *Bacillus subtilis* NCTC8236 and *Proteus vulgaris* ATCC6380. The fungi species used were *Aspergillus nigar* ATCC9763 and *Candida albican* ATCC7596. The media used for antibacterial tests were nutrient broth and Mueller Hinton agar. Whereby, nutrient broth and Sabourout dextrose agar were used for antifungal tests. These media were prepared according to the standard methods. The concentration of the cultures (150×10^6 CFU ml^{-1}) for disc diffusion method was adjusted by comparison with the McFarland method.

Preparation of tested materials

The dilution used for all extracts was 100 mg ml^{-1}. Various extracts of CL were re-dissolved in different solvents whereby, hexane extracts were re-dissolved in hexane. Chloroform was known to be inhibitory to the growth of bacteria. Therefore, the chloroform extracts was re-dissolved in a mixture of petroleum ether and methanol (8:2 v/v). Also, the ethanolic extracts was re-dissolved or suspended in methanol solvent.

Antibacterial assay

Cup-plate diffusion method

Antibacterial activity of plant extracts was carried using cup-plate agar diffusion method (Murray et al. 2009) with some minor modifications. One ml from each standard bacterial stock suspension was mixed thoroughly with 20 ml of sterile Molten Muellur Hinton agar (45 – 50°C), poured into sterile Petri-dishes and left to solidify. Then, four cup-shape wells (10 mm diameter) were made in each plate using sterile cork-borer (No. 9). The agar disks were removed and four alternate cups were filled with extract using sterile adjustable pipettes. Four Petri-dishes with two alternate cups were used with the respective solvent instead of the extracts as control. The plates were then incubated in upright position for 18 to 24 h at room temperature. Two replicates were carried out for each extract. After incubation period, the inhibition zones diameters were measured.

Disc diffusion method

The antibacterial assay for plant extract was also conducted using disc diffusion method as described by Abdel-Wahab et al. (2009). The nutrient agar solution (17 mL) was poured and kept overnight in a refrigerator. Whatman filter paper discs of 6 mm diameter were impregnated with 10 µL of the solution of crude extract (at 4 mg ml^{-1}) dissolved in dimethyl sulfoxide (DMSO). Standard disc of streptomycin sulphate (10 µg/disc) was used as positive control, while DMSO was used as a negative control. The Petri dishes were inverted and incubated for 24 h at 37°C. Clear inhibition zones around the discs indicated the presence of antimicrobial activity.

Antifungal assay

The cup-plate agar diffusion method was adopted with some minor modifications to assess the antifungal activity of prepared extracts (Murray et al., 2009). From each of the fungal stock suspension, 2 ml was thoroughly mixed with 20 ml of sterile molten Sabourout Dextrose Agar (45 to 50°C), distributed into sterile Petri-dishes and left to solidify. Then, four cup-shaped wells (10 mm diameter) were made in each plate using cork-borer (No.9). The agar disks were removed and alternate cups were filled with each extract using

Table 1. Yield and physical properties of different extracts of *C. lanatus* var. *citroides*

Texture	Color	Yield (%)	Extraction solvent	Organ
Solid	Green	1.89	Hexane	Leaves
Solid	Green	3.45	Chloroform	
Gummy	Yellowish green	19.33	Ethanol	
Solid	Light Brown	0.46	Hexane	Stem
Solid	Brown	1.5	Chloroform	
Gummy	Brown	10.51	Ethanol	
Solid	Yellow	0.5	Hexane	Fruit
Solid	Brown	1.68	Chloroform	
Gummy	Brown	7.25	Ethanol	
Oily	Light yellow	15.33	Hexane	Seeds
Solid	Light brown	0.83	Chloroform	
Waxy	Intense brown	6.44	Ethanol	

Table 2. Secondary metabolites detected in *Citrullus lanatus* var. *citroides*

Seed	Fruit	Stem	Leaves	Organic compounds
-	+++	-	++	Alkaloids
-	-	-	-	Anthraquinones
+++	++	+	+++	Flavanoid
++	+	+++	-	Saponins
+++	++	++	+	Terpenes and Steroids
+	-	++	+++	Tannins
-	++	-	-	Flavones aglycone
+	+++	++	+++	Simple phenols

+++ = High concentration; ++ = Moderate concentration; + = Low concentration, - = Absent

sterile adjustable pipettes. The plates were then incubated in the upright position for 24 to 48 h at a 37°C. Two replicates were carried out for each extract against each of the tested organism. After incubation periods, the inhibition zones diameter were measured and the mean values were tabulated.

RESULTS

Extraction yield and qualitative phytochemical screening

The dried plant materials of CL were subjected to successive extraction with a lipophilic solvent (hexane), then extracted with a semi-polar solvent (chloroform) and finally with a polar one (ethanol). Results shown in Table 1 indicate the yield, color and texture of the different extracts. As depicted in Table 1, the ethanol extracts of the studied plant parts, with exception of the seeds, gave the highest yield in form of gummy or waxy extracts, while hexane extracts recorded the lowest yield except that of the seeds which gave quite high yield in form of oily extract. The chloroform extracts gave relatively moderate yield in form of solid extracts.

Phytochemical screening of chemical constituents of different parts of CL showed that the leaves contained mainly flavonoids, tannins and simple phenols. Stem revealed the highest content in saponins. Fruits exerted the highest contents of alkaloids and simple phenols whereas seeds were rich in terpenes and steroids as well as flavonoids. All organs were devoid of anthraquinones (Table 2).

Antimicrobial screening

The results were summarized in Tables 3 and 4. However, results were interpreted in terms of commonly used terms: sensitive, intermediate and resistant. Findings of cup-plate diffusion method for ethanolic extracts of the fruits and seeds against *Pseudomonas aeruginosa* are shown in Figure 1.

Table 3. Antibacterial activity of *Citrullus lanatus* var. *citroides* extracts

Extraction solvent	Part of plant	Inhibition zone (mm)*				
		P.S.	P.R	E.C.	B.S.	S.A.
Hexane	Leaves	-	12	11	13	12
	Stem	16	16	15	14	18
	Fruit	-	-	-	13	17
	Seeds	-	-	-	19	25
Chloroform	Leaves	17	22	31	37	33
	Stem	20	26	36	36	29
	Fruit	19	23	37	38	36
	Seeds	15	19	28	27	26
Ethanol	Leaves	18	18	19	20	17
	Stem	16	23	22	20	27
	Fruit	18	29	26	24	33
	Seeds	18	18	19	17	21
Gentamicin	40 µ/ml	23	25	32	29	35

S.A.: *Staphylococcus aureus ;* B.S: *Bacillus subtilis ;* E.C: *Escherichia coli;* P.R: *Proteus valgaris ;* P.S: *Pseudomonas aerguinosa*. *n=3.

Table 4. Antifungal activity of *Citrullus lanatus* var. *citroides* extracts.

Extraction Solvents	Organ	Inhibition zone (mm)*	
		Aspargellus nigar	***Candida albican***
Hexane	Leaves	-	-
	Stem	-	-
	Fruit	-	-
	Seeds	-	-
Chloroform	Leaves	18	30
	Stem	17	25
	Fruit	20	30
	Seeds	37	37
Ethanol	Leaves	37	30
	Stem	35	41
	Fruit	31	41
	Seeds	25	27

*n=3

Antibacterial activities of *C. lanatus*

Analysis of the data revealed that among the tested extracts, the chloroform extract of the fruit exhibited the highest rates of antibacterial activity. It showed antibacterial activity against *S. aureus*: 36 mm, *B. subtilis*; 38 mm, *E. coli*; 37 mm, *P. valgaris*; 23 mm and *P. aerguinosa*; 19 mm. *B. subtilis* showed more susceptibility towards chloroform extracts of all studied parts. All the tested bacteria were resistant to hexane extracts of the leaves and fruits, while they were intermediate in resistant to hexane extract of stem. *S. aureus* was susceptible towards all extracts (Table 3). *Proteus valgaris* showed the highest sensitivity (29 mm) to the ethanol extract of the fruit. Moreover this bacterium was almost equally susceptible to ethanol extract of the stem and chloroform extracts of the fruits, stem and leaves with inhibition zones in the range of 22-23 mm. Compared to the other tested bacteria, *P. aeruginosa* was less susceptible to extracts of different parts of CL.

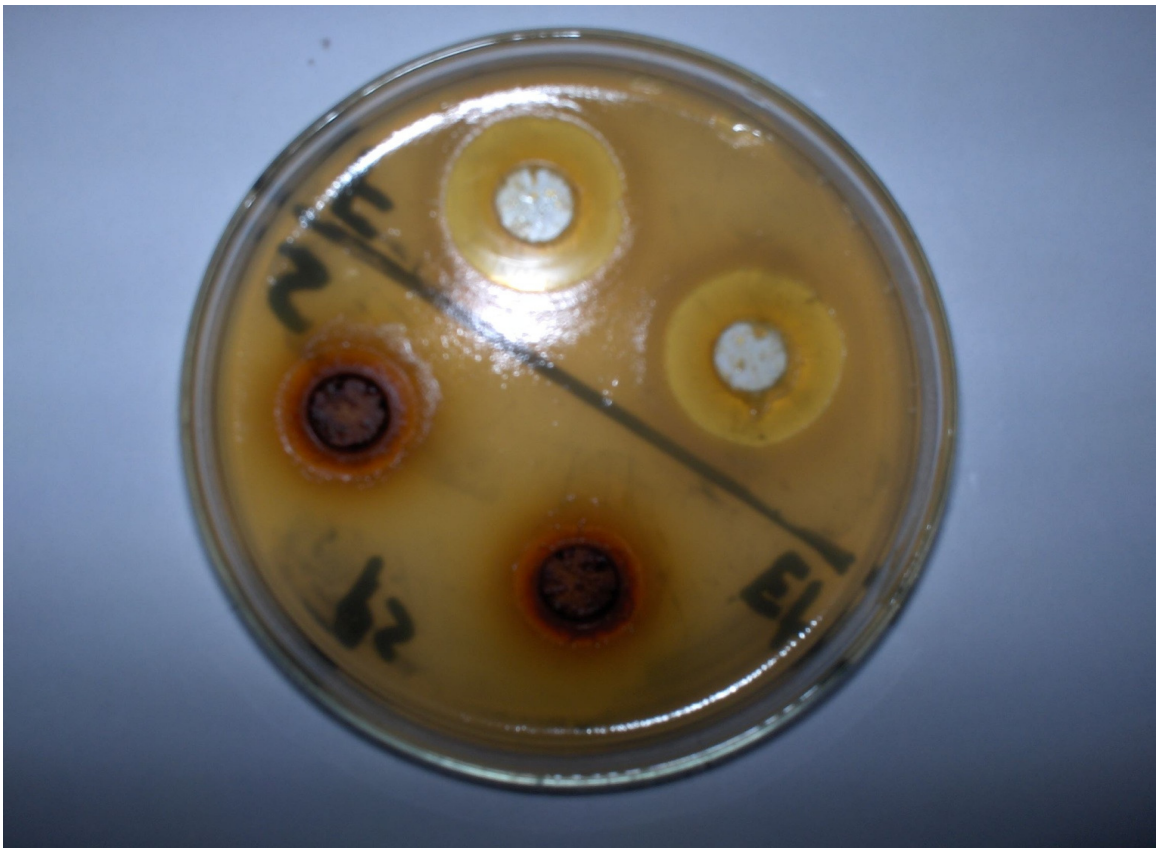

Figure 1. Cup-plate Diffusion Method for ethanolic extracts of the fruits and seeds against *Pseudomonas aeruginosa.*

The chloroform and ethanol extracts exhibited inhibition zones between 15 and 20 mm, and 16 to 18 mm, respectively. Only the hexane extract of stem gave activity with inhibition zone of 16 mm.

Antifungal activities of *C. lanatus*

The antifungal activity of CL against *C. albican* and A. *nigar* is presented in Table 4. The ethanolic extract of the fruit pulp and stem showed the highest antifungal effect compared to the other parts extracts exerting maximum effect (41mm) on *C. albican*. However, chloroform extracts also showed relatively high activity. A. *nigar* was very sensitive to the chloroform extract of the seed (37mm) and the ethanolic extract of the leaves (37mm). The hexane extracts of all studied parts (leaves, stem, fruits and seeds) showed no activity against the two tested fungi species.

DISCUSSION

Traditionally in Sudan, CL is used to treat fungal and bacterial infections. The results from this current study revealed the scientific basis of the traditional usage of CL. The plant under this study was subjected to successive sequential extraction with lipophilic, semi-polar and polar solvents. Phytochemical screening of chemical constituents of different parts of CL showed varied results. Several studies showed that alkaloids (Abdel Gadir et al. 2003) and terpenes (Lavie and Glotter 1971; Ali and Pandey 2007) are widely spread in the genus *Citrullus*, however, in this study alkaloids were detected in fruit and leaves. Terpenes and steroids were found in all plant parts, whereby, seeds exerted the highest contents of terpenes and steroids. Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent (Yagoub 2008).

The screening of bioactive agents from plants is one of the most intensive areas of natural products research today, yet the field is far from exhausted. Sandberg and Bruhn (1979) reported that only around 10% of all plants had been investigated in detail for bioactive agents. For this reason alone it could be argued that further investigation on CL is worthwhile. This report is the first data available on the antimicrobial activity of CL and lends support the traditional use of CL. Therefore, extracts obtained from leaves, stem, fruits and seeds of this plant

were screened for their antimicrobial activity against five standard bacteria namely; *E. coli, S. aureus, P. aeruginosa, B. subtilis* and *P. valgaris* and two fungi species namely; *C. albican* and *A. nigar* at concentration of 100 mg ml^{-1}.

On the basis of the previous reports with standard chemotherapeutic agents against the same standard tested organisms, plant extracts resulting in more than 18 growth inhibition zones were considered to possess relatively high antibacterial activity, and those resulting in 14 to18 mm inhibition are of intermediate, and those resulting in zones below 14 mm are inactive (Sandberg and Bruhn 1979; Kyung et al., 2007). Results obtained for antibacterial activity of the crude extracts of CL are reported in Table 3. Chloroform, hexane, and ethanol used for reconstitution of the extracts showed no activity. The antibacterial activities of different parts were compared with antibiotic gentamicin at a concentration of 40 mg ml^{-1}. Analysis of the data revealed that among the tested extracts, the chloroform extract of the fruit exhibited the highest rates of antibacterial activity. Supporting results are from the work of Cabello et al. (2007) who found that chloroformic extracts are more effective against the same bacterial strains although he used extract of *Piper nigrum*. Our results are also in accordance with the study of Marzouk et al. (2009) on *C. colonthysis* who demonstrated the antibacterial activities of ethanolic extracts of fruits, stem, leaves and roots against Gram positive bacteria *Bacillus pumilus* and *S. aureus*, while fruit and root extracts in double strength gave positive results against Gram positive bacillus (*Bacillus subtilis*). John (2008) also reported that ethanol extract of *C. colocynthis* fruits gave maximum inhibition against *E. coli*, *Pr. vulgaris* and *S. aureus. P. aeruginosa* and *B. subtilis* show moderate activity in chloroform extract. Petroleum ether extract is more active against *Escherichia coli*.

The antifungal activity of CL against *C. albican* and A. *nigar* is presented in Table 4. The ethanolic extract of the fruit pulp and stem showed the highest antifungal on *C. albican*. While, hexane extracts of all studied organs (leaves, stem, fruits and seeds) showed no activity against the two tested fungi species. In this regards, Hadizadeh et al., (2009), have studied the anti-mycotic activity of the ethanol extracts of colocynth (*Citrullus colocynthis* L. Schrad). Floral parts of colocynth were the most effective against *Alternaria alternate* and *Rizoctonia solani.* The inhibition zones displayed by the ethanol extracts of the fruits pulps and stem were comparable to that exhibited by the antibiotic, clotrimazole against *C. albican.* However, ethanol extracts (except that of the seeds) as well as the chloroform of the seeds showed higher activity against *A. nigar* than clotrimazole

Conclusion

The antimicrobial effects of CL extracts against the studied bacteria suggest that, different parts of CL possess remarkable therapeutic action that can support the traditional usage of this plant in the treatment of bacterial and fungal diseases such as gastrointestinal infection, diarrhea, respiratory and skin diseases. These antimicrobial activities are likely due to the presence of secondary metabolites like tannins and flavonoids, alkaloids, saponins, terpenes and steroids in CL. The high potency of CL against these microbes could provide an example of prospecting for new compounds.

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