Tropical Journal of Pharmaceutical Research July 2017; 16 (7): 1631-1636
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i7.23

Original Research Article

Antimicrobial activity of ethyl acetate extract of Citrullus lanatus seeds

Upe Francisca Babaiwa[1], Osayemwenre Erharuyi[2], Abiodun Falodun[2] and John O Akerele[1]

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City Nigeria, [2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

For correspondence:* ***Email:* *upe.babaiwa@uniben.edu;* ***Tel:*** *+234-8035467818*

Sent for review: 3 August 2015 *Revised accepted: 10 April 2017*

Abstract

Purpose: *To determine the antimicrobial activity and chemical constituents of ethyl acetate extract of Citrullus lanatus seeds.*
Methods: *Antimicrobial activity of the ethyl acetate extract of the seeds of C. lanatus was evaluated against Staphylococus aureus ATCC 25923, Escherichia coli ATCC 25922, Bacillus subtilis NCTC8236, Pseudomonas aeruginosa ATCC 10145 and Candida albicans ATCC 24433 using standard microbiological method. Chemical constituents of the extract were determined with Gas chromatography and mass spectrometry (GC MS) systems.*
Results: *An oily, yellowish orange coloured extract with a yield of 18.7 % was obtained. The extract inhibited the growth of all test microorganisms at minimum inhibitory concentration range of 0.313 -2.5 mg/mL and minimum bactericidal concentration of 0.313-5 mg/mL. GC MS profile of the extract showed the presence of oleic acid (31.22 %), saturated fatty acid (23.85 %) and gamma tocopherol (8.79 %). Other organic compounds accounted for 36.16 % of the extract*
Conclusion: *Ethyl acetate extract of the seeds of Citrullus lanatus has antimicrobial effect against some Gram positive and Gram negative bacteria as well as C. albicans. Twenty-one compounds including fatty acids, hexamethyl-2-ethylacridine, gamma tocopherol and methylphthalate have been identified in the seeds.*

Keywords: *Citrulluslanatus seeds, ethyl acetate extract, antimicrobial activity*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Plant derived substances have become of great interest owing to their versatile application [1].It has been estimated that 14.28 % of higher plant species are used medicinally and that 74 % of pharmacologically active plant derived components were discovered after following up on ethnomedicinal use of the plants [2].

Citrullus lanatus is a plant species that belongs to the family, Cucurbitaceae. It is a vine-like (scrambler and trailer) flowering plant which originated from West Africa and often cultivated for its fruits. This species has two cultivars namely, watermelons (*C. lanatus* (Thunb.) var *lanatus*) and citron melons (*C. lanatus* var *citroides* (LH Bailey) Mansf), In Chinese medicine, the rind *of C. lanatus* fruit is used for the treatment of diabetes, hypertension and acne [3]. Water melon red pulp has been extensively studied for its antioxidant and cancer preventing properties [4]. The seeds are crushed and made into an emulsion with water for

treatment of catarrhal infections, bowel disorders and as worm expeller [5]. Although different solvent extracts of the leaves, roots, flower, fruit pulp and stems of this plant have been evaluated for useful phytochemicals around the world, the few reports that exist on the seeds as a potential new source of antimicrobial agent in Nigeria were preliminary and unstandardized methods for evaluation of antimicrobial susceptibility pattern of crude extracts of other variates of *C. lanatus* seeds were used [6-9].

This study is aimed at determining the antimicrobial property and chemical components of the ethyl acetate extract of *C.*lanatus *var. lanatus* seeds using the standard agar well diffusion and agar dilution methods as well as gas chromatography and mass spectrometry methods.

EXPERIMENTAL

C. lanatus fruits was purchased from a local market (Tamboga market) in Benin City and identified by Prof JO Bamidele of the Department of Plant Biology and Biotechnology, University of Benin, Benin City. A voucher specimen was deposited in the local Departmental herbarium. The seeds were air dried for five days and 1 kg was pulverized and macerated in ethyl acetate for 72 hr. Resultant extract was filtered with Whatmann filter paper no-1 and the filtrate was concentrated, weighed and stored at 4 ^{o}C in an airtight container.

Antimicrobial susceptibility tests

Staphylococus aureus, Escherichia coli, Bacillus stubtilis and Pseudomonas aeruginosa were sub-cultured from stock into sterile nutrient agar plates while or *Candida albicans* was sub-cultured from stock into sterile sabouroud dextrose agar plates. The plates with bacteria were then incubated for 48 hr at 37 ^{o}C while those with fungus were incubated for 48 hr at 28 ^{o}C. Identical colonies from overnight plates were suspended in sterile broth for 12 h and adjusted to 0.5 McFarland standard to give an inoculum size of approximately 10^{8} cfu/mL. Adjusted inocula were diluted 1:100 to give inoculum size of 10^{6}cfu/mL as previously reported [10].

Antimicrobial susceptibility test was carried out using modified agar well diffusion method [11]. Standardized inoculum of each microorganism (200µL) was mixed thoroughly with 30 mL of sterile Mueller Hintons agar (at 45 - 50 ^{o}C) for bacteria or sabouroud dextroxse agar for fungus and poured into sterile plates to set. A cork borer (10 mm) was used to bore six wells in each agar plate and the agar in the well was removed, and each of the sealed wells was filled with 200 µL (containing 100mg) ethyl acetate extract. A similar procedure was carried out with ciprofloxacin (0.5 mg/mL) and ketoconazole (1 mg/mL) standard drugs. Exactly 200 µL of each standard drug was emptied into each well to give 100 µg and 200 µg per well for ciprofloxacin and ketoconazole respectively. Negative controls (without extract and standard drugs) and positive control (viability test for microorganisms used) were carried out for each set of experiment. All seeded plates remained on the bench for 30 min, before incubation in an upright position for 18-24 hr at 37 ^{0}C for bacterial and 25-28 ^{o}C for 48 h in the case of *C. albicans*. Inhibition zone diameters (IZD) were measured in millimetres (mm). All experiments were carried out in triplicates.

Determination of minimum inhibitory concentration (MIC)

Agar dilution method [12] was used for determination of MIC of the extract using ciprofloxacin and ketoconazole on susceptible test microorganisms (*S. aureus* ATCC 25923, *E. coli* ATCC 25922*, B. subtilis* NCTC8236*, P. aeruginosa* ATCC 10145 and *C. albicans* ATCC 24433. Mueller Hintons agar was prepared according to manufacturer's instructions and placed in water bath at 50 ^{o}C. Stock solution of extract was incorporated into the molten agar at different volumes to obtain a concentration range of 3.125 – 400 mg/ mL. Serial dilution of the stock solution of ciprofloxacin (0.5 mg/mL) was also carried out to obtain a concentration range of 0.01562 - 5 µg/mL. Similarly a concentration range of 0.2 – 200 µg/mL was prepared from a stock solution of ketoconazole (1 mg/mL). Agar extract and agar ciprofloxacin/ketoconazole mixtures of different concentrations were poured into separate sterile plates and allowed to set. Each of the test microorganisms was radially streaked onto the prepared plates. Negative and positive controls were set up for each set of experiments. All plates were incubated at 30 ^{o}C for 18-24 h except for *C. albicans* which was incubated at 25 - 28 ^{o}C for 48 h. Experiments were carried out in triplicates and the MICs were defined as the lowest concentrations of extract and standard drugs that inhibited growth of test microorganisms.

Determination of minimum bactericidal (MBC) and minimum fungicidal concentration (MFC)

Plates that showed no visible microbial growth following MIC determination were swabbed with moistened sterile sticks and inoculated onto

sterile Mueller Hinton agar plates containing 5 % Tween 80 [13]. All plates were incubated for 18 - 24 h at 37 ^{o}C except *C. albicans* which was incubated for 48 h at 25 - 28 ^{o}C. Agar plates with least concentration without visible growth were considered as MBC/MFC values.

GC-MS analysis

Determination of the chemical constituents was carried out on Agilent technologies 6890N network GC system and Agilent technology 5973 network mass selective detector coupled with 7683B series injector. Model number of the column and capillary specifications used were; Agilent 122-5533, stationary phase (5% phenylmethylsiloxane) internal diameter (0.25 mm x 30 m), and film thickness (1um). Helium at a flow rate of 1.2 mL/min was used as carrier gas. An initial inlet temperature of 230 ^{o}Cwas programmed at 50 ^{o}C for 5minutes and then increased to 300 ^{o}C at a rate of 10 ^{o}C/min ending with 25 min for a total run time of 45 min. An electron ionization mode of 70 eV was used. Total ion count was used for compound identification and quantification.The peaks were identified using Phero base software which compared the relative peaks with standard data base of known compounds saved in National Institute of Standards and Technologies (NIST02) reference spectra library.

Data analysis

Inhibition zone diameters for antimicrobial assay were expressed as mean ± SEM using Minitab 13. One way ANOVA was used to compare activity of extract with standard drugs.Data analysis and peak area measurementof raw GC-MS data was carried out using Agilent Chemstation and Phero base software.

RESULTS

In this study all the test microorganisms were susceptible to inhibitory effect of the ethyl acetate extract of *C. lanatus* seeds (Table 1). The pattern of MIC values (Table 2) for *E. coli, B. subtilis and C. albicans* were observed to be similar to those of MBC/MFC (Table 3). GC MS chromatogram showed several peaks with their corresponding retention time (Figure 1) and on comparison of this mass spectral with NIST02 reference spectral library, 21 phytocompounds were identified (Table 4). The predominant compounds identified with known antimicrobial properties were fatty acids (Palmitic, Lauroleic and oleic), hexamethyl-2- ethylacridine, dibutyl phthalate, and vitamin E.

DISCUSSION

Seeds of *C. lanatus* var lanatus evaluated in this study were found to possess antibacterial activities against *E. coli, Ps. auregenosa, S. aureus, B, subtilis* and *C. albicans.* The extract had a high concentration of oleic acid (31.22%) and saturated fatty acid (23.85 %), hexamethyl - 2- ethylacridine (17.83 %), in addition to gamma tocopherol (vitamin E, 8.79 %), methylphthalate among the 21 compounds identified.

Table 1: Inhibition zone diameters of extracts against test microorganisms

Test microorganism	Ethyl acetate extract (mm)	Ciprofloxacin (mm)	Ketoconazole (mm)
P. aeruginosa	24 ± 0.74	33 ± 0.33	NA
E.coli	25± 0.78	38 ± 0.27	NA
B.subtilis	26 ± 0.41	35 ± 0.51	NA
S.aureus	24 ± 0.29	30 ± 0.35	NA
C.albicans	25 ± 0.40	ND	32 ± 0.23

NA = Not applicable; Extract has comparative inhibitory activities with standard drugs against all test microorganisms

Table 2: Minimum inhibitory concentrations of extract and standard drugs against the test microorganisms

Test microorganism	Ethyl acetate extract (µg /mL)	Ciprofloxacin (µg/mL)	Ketoconazole (µg/mL)
P. aeruginosa	1500	0.25	NA
E.coli	310	0.0625	NA
B.subtilis	2500	0.125	NA
S.aureus	2500	0.25	NA
C.albicans	1250	ND	10

NA = Not applicable

Table 3: Minimum bactericidal/fungicidal concentration (mbc/mfc) of extract and standard drugs against the test microorganisms

Test microorganism	Ethyl acetate extract (µg /mL)	Ciprofloxacin (µg/mL)	Ketoconazole (µg/mL)
P. aeruginosa	5000	0.25	NA
E.coli	313	0.0625	NA
B.subtilis	2500	0.125	NA
S.aureus	5000	0.25	NA
C.albicans	1250	ND	10

Not = Applicable; High susceptibility of Escherichia coli both to extract and ciprofloxacin has been clearly demonstrated with MBC/MFC data.

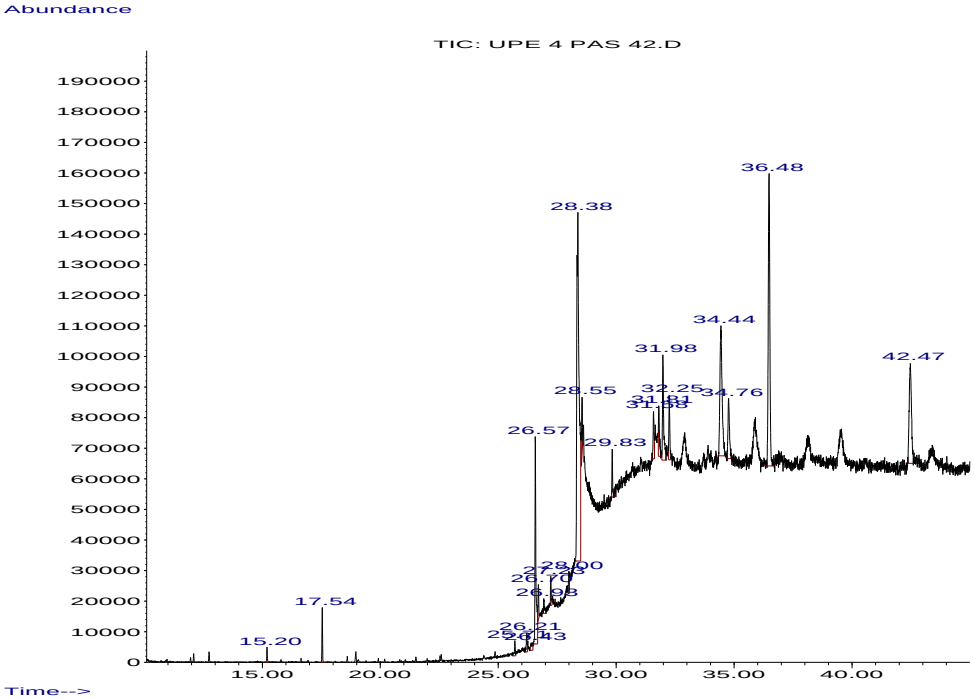

Figure 1: GC MS chromatogram of ethyl acetate fraction of C. lanatus extract

Table 4: Chemical composition of ethyl acetate extract of *Citrullus lanatus* seeds

Peak no.	Retention time (min)	Area (%)	Name of compound
1	15.20	0.33	Ribitol
2	17.54	1.24	1,2,3-Propanetriol monoacetate
3	25.71	0.53	1,2- Benzene dicarboxylic acid
4	26.21	0.61	2- Methyl propyl ester
5	26.43	0.52	Silicic acid
6	26.57	7.77	n- Hexadecanoic acid (Palmitic acid)
7	26.70	1.02	Dibutyl phthalate
8	26.93	0.54	11- Dodecenoic acid (Lauroleic acid)
9	27.23	0.83	3- methylindole-2- carboxylic acid
10	28.00	0.72	Cyclotrisiloxane
11	28.38	31.22	9- Octadecenoic acid (Oleic acid)
12	28.55	1.78	Hexahydropyridine
13	29.83	1.31	Hexamethyltetrasiloxane
14	31.58	1.80	Methyltrisilane
15	31`.81	1.89	Tetrasiloxane
16	31.98	4.72	2,4,6- Cycloheptatrien-1- one
17	32.25	2.27	Silane
18	34.44	10.83	Trimethyl[4- (2-methyl-4-oxo-2-penty) phenoxy] silane
19	34.76	3.45	Diethyl bis(trimethylsilyl) ester
20	36.48	17.83	Hexamethyl-2-ethylacridine
21	42.47	8.79	Gamma-tocopherol (Vitamin E)

The antimicrobial activities of this plant have been reported previously [14]. However, these activities were reported in connection with chloroformic, hexane and ethanolic extracts of *C. lanatus var citroides* against *S. aureus, E. coli, B. subtilis* and *Pseudomonas* species. In some earlier studies, fatty acids and dibutyl phthalate [15] have been associated with both the antibacterial and antifungal activities due to the ability of fatty acids to intercalate into the bacterial cell membrane causing increased fluidity, permeability changes and consequently the lysesof the unstable bacterial cell. Release of lethal secondary degradation products of fatty acid peroxidation like hydrogen peroxide and reactive oxygen species may also be responsible for the observed antibacterial action [16]. Additionally auto oxidation of unsaturated fatty acids create short chain aldehydes which have antibacterial activities [17]. Acridine and it derivatives are known to possess bacteriostatic activity against many Gram positive and Gram negative bacterial [18]. The presence of hexamethyl -2- ethylacridine in this extract may be responsible for the observed bacteriostatic action against *B. subtilis and C. albicans*

Vitamin E is a family of lipophilic antioxidants in cellular membrane that scavenge reactive oxygen species. In aerobic microorganisms, oxygen is the terminal electron acceptor in the generation of energy; thus the non-availability of this oxygen as a result of the antioxidant activity of vitamin E inhibits the generation of energy and consequently reduces metabolic activity of the bacterial cell leading to bacteriostasis. Since growth inhibition cannot continue indefinitely, eventually the growth inhibited bacterium dies (bacteriocidal action).

The presence of oleic acid (31.22 %) as the predominant fatty acid in this study is at variance with previous reports where linoleic acid (62.2 %) was found to be predominant [19]. This variation may be due to difference in agroclimatic conditions.

CONCLUSION

The findings of this study demonstrate the antimicrobial action of the ethyl acetate extract of *C. lanatus* seeds against some bacteria and fungi. Twenty one chemical compounds including fatty acids, hexamethyl-2-ethyl acridine, gamma tocopherol and methyl phthalate have been identified in the seeds. This study offers a scientific proof for the traditional use of *C. lanatus* seed extract in the treatment of infections, having been shown to be active against some bacteria and fungi associated with respiratory, gastrointestinal and urinary tract infections.

DECLARATIONS

Acknowledgement

The corresponding author is grateful for the access to Professor Abiodun's TETFUND and STEP-B laboratory facilities where all the physicochemical studies on the extract were carried out.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

REFERENCES

1. *Baris O, Gulluce M, Sahin F, Ozer H, Kilic H, Ozkan H, Sokmen M, Ozbek T. Biological activities of the essential oil and methanol extract of AchileabiebersteiniiAfan. (Asteraceae) Turkish J Biol2006, 30:65-73.*
2. *Gibbon GW. The diabetic foot amputations and drainage of infections. J Vas Surg 2008; 8(3): 791-793.*
3. *Ellen S (2009). Healing nature of watermelon seed tea. www.areclinic.org/articles.asp? accessed 1 / 12 /2012.*
4. *Silvia O, Daurte A, Cabrita J, Pimentel M, Diniz A, Gomez E. Antimicrobial activity of Guinea Bissau traditional remedier. J Ethnopharmacol1996; 5-590: 53.*
5. *Wallace JR. Antimicrobial properties of plant secondary metabolites. Prod and NutriSoc2004; 63: 621-629.*
6. *ASM. Manual of clinical microbiology, 2007.*
7. *Erhirhie EO and Ekene NE. Medicinal values of Citrullus lanatus (Watermelon): Pharmacological review. J Res. Pharma and Biomed Sci (2014)4:4 1305-1312.*

8. Adewale A, Rotimi AO and Adebobola OA. Antibacterial activity of nonionic and anionic surfactants from Citrullus lanatus seed oil. Jundishapur J. Microbiol. (2013).6;3; 205-208.
9. Braide W, Odiong, IJ and Oranusi SU. Phytochemical and antibacterial properties of seed of watermelon (Citrullus lanatus).Prime J. Microbiol Res.2;3;99-104.
10. Egbuonu E and Anthony C. Comparative investigation of the antibacterial and antifungal potential of extracts of watermelon (Citrullus lanatus) rind and seed. Euro.J Medi. Plants. (2015):9:4: 1-7.
11. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, Eds. Manual of clinical microbiology. 7th ed. Washington DC: ASM (1999).
12. Afolayan AJ, Meyer JM. The antimicrobial activity of 3, 5, 7–trihydroxyflavone isolated from the shoots of Helichrysumaureonitens. J Ethnopharmacol 1997; 57:177-181.
13. Kyung KH, Woo Y, Kim DS, Park HJ, Kim YS. Antimicrobial Activity of an Edible Wild Plant, Apiifolia Virgin's Bower (Clematisapiifolia DC). Food SciBiotech2007;16: 1051-1058.
14. Ehinmidu JO. Antibiotics susceptibility patterns of urine bacterial isolates In Zaria, Nigeria. Trop J Pharm Res 2003;. 2, 223-228.
15. Agoramoorthy G, Chandrasekaran M, Venkatesalu V, and Hsu MJ. Antibacterial and antifungal activities of fatty acid methyl esters of Blind- your eye mangrove from Indian. Brazilian J. Microbiol 2007;38:739-742.
16. Al-Bari MM, Sayeed Ma, Rahman MS and Mossadik MA. Characterization and antimicrobial activities of a phthalic acid derivative produced by Streptomyces bangladeshiensis-A novel species collected in Bangladesh. Res J. Med and Medical Sci, 2006:1 77-81.
17. Wojtezak L and Wieckowski MR. The mechanism of fatty acid induced proton permeability of the inner mitochondrial membrane. J. BioenergBiomembr.1999, 31: 447-455.
18. Adolph S, Bach S, Blondel M, Cueff A, Moreau M, Pohnert G, Poulet SA, Wichard T and Zuccaro A. Cytotoxicity of diatom-derived oxylipins in organisms belonging to different phyla. J Exp. Biol. 2004, 207:2935-2946.
19. Ramesh Kumar, MandeepKaur and MeenaKumari. Acridine: A versatile heterocyclic nucleus. ActaPoloniaePharmaceutica - Drug Res. 2012, 69: 3-9.
20. Braide W, Odiong J, Oranusi S. Phytochemical and antibacterial properties of the seeds of watermelon (Citrullus lanatus). Pri J Microbiol Res 2012; 2 (3) 99-104.