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Antimicrobial and In Vivo Antifungal Potentials of Crude and Degummed *Citrullus lanatus* Seed Oil †

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Abstract: Background: *Citrullus lanatus* (watermelon) seed oil is one of the neglected and underutilized seed oils in Nigeria. This study was aimed at evaluating the therapeutic efficacy of crude and degummed *Citrullus lanatus* seed oil (CLSO) in the treatment of oral candidiasis. **Methods:** In Vivo antifungal activity of the extracts was analyzed by microbiological and histopathological analyses, along with renal function tests at days 7 and 14 post infection treatments. The observations in the test animal groups were compared with that of control group treated with fluconazole. **Results:** Treatment with CLSO resulted in significant body weight loss. Also, there was a significant reduction in the kidney fungal burden (cfu/mL/g) of rats treated with CLSO after fourteen days post infection treatment, compared to group 3 (untreated control) rats. Histologically, group 3 (untreated) showed multifocal aggregation and widespread distribution of fungal blastospores appearing singly or in small clusters within the renal parenchyma when compared with CLSO-treated groups, which had minimal fungal blastospores that appeared singly in the renal tissues. Thus, histological data were corroborated with microbiological tests for crude and degummed CLSO, but not for the fluconazole-treated group which showed multifocal and widespread distribution of blastospores at day 14. Furthermore, within the CLSO treatment group, a significant increase in the (serum) levels of creatinine was observed, while no significant difference in blood urea values was recorded after day 14 post infection study. Histopathological alterations were mitigated to confirm the biochemical indices. **Conclusion:** CLSO could be considered as a potential antifungal agent especially the degummed CLSO, which also, improves the antifungal efficacy of the crude seed oil.

Keywords: blastospores; candidiasis; degummed oil; fungal burden and renal function

1. Introduction

Fungal infections are becoming increasingly prevalent in the human population, and contribute to morbidity and mortality in healthy and immunocompromised individuals respectively [1]. *C. albicans* causes opportunistic infections such as oral candidiasis, oesophageal candidiasis and vaginal candidiasis. Oropharyngeal candidiasis is the most common opportunistic infection associated with oral injuries, tongue pain and taste disturbance [2]. The expression of *Candida albicans* virulence in the oral cavity is strongly correlated with impairment of the immune system, particularly in patients with human immunodeficiency virus infection [3]. Studies have indicated that *C. albicans* are resistant to azoles or polyenes, particularly amphotericin B [4, 5]. *Citrullus lanatus* seed oil is rich in essential fatty acid (18:2*n*-6). The seed of watermelon which belongs to a class of curcubit seeds is under-exploited and utilized in Nigeria despite its abundant nutritive values. Adewuyi *et al*. [6] reported

the antibacterial activity of biosurfactant of *Citrullus lanatus* seed oil. Similarly, Olubunmi *et al* [7], had also reported the antioxidant and cytotoxicity of the seed oil. Since the watermelon seed contains rich essential fatty acids, this study was therefore undertaken to study antifungal properties of crude and degummed *Citrullus lanatus* seed oil.

2. Materials and Method

2.1. Plant Materials

The plant material used in this study was the fresh *Citrullus lanatus* seeds. The seed of *C. lanatus* (water melon) was procured from the dealers within Zuba in Bwari area council of FCT Abuja, Nigeria. The identity of the plant was confirmed, while the Voucher specimens UNN/PSB/Consult/2017/2721-03 were deposited at the herbarium at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

2.2. Fungal and Bacteria Strain

Candida albicans strain (SC5314) and bacteria strain used in this study was obtained from Department of Microbiology University of Nigeria, Nsukka.

2.3. Collection and Processing of Citrullus lanatus

The fruits were washed, cut and the seeds selected manually from the pulp. Tap water was used to wash off pulp on the seeds before sun-drying for 72 h. Samples of watermelon seeds were crushed using a commercial blender (TSK-949, WestPoint, France), preserved in air tight container, and stored in a desiccator ready for analyses.

2.4. Oil Extraction

The extraction was carried out according to the method of Zufarov [8]. The concentrated oil (642 ml) sample was then stored in a bottle in a cool dry place until degumming.

2.5. Degumming of Citrullus lanatus Seed Oil

The oil was degummed according to the modified method of Zufarov [8].

2.6. Susceptibility Assay (In Vitro)

Mueller-Hinton agar (Oxoid) plates supplemented with chloramphenicol were prepared for the antifungal activity assay, according to the CLSI agar well diffusion test [9].

2.7. Result and Interpretations

At the end of incubation, antimicrobial activity of both crude and degummed oil at a particular concentration was indicated by inhibition of growth around each well. The Zone of inhibition was measured with a metre rule in mm and recorded as inhibition zone diameter (IZD) of the oil.

2.7.1. Interpretation

The IZD, was interpreted according to the method of Irobi *et al*. [10].

2.7.2. Animal Grouping for In Vivo Antifungal Activities

This was according to method of Apeh et al. [11]. The rats were sacrificed on the 14th day, blood samples were collected through ocular puncture for biochemical and haematological analyses.

2.7.3. Ethical Statement

The animals were handled according to the guidelines of the Ethical Committee on the use and care of experimental animals of the Department of Biochemistry, University of Nigeria, Nsukka (UNN) and approved by the Departmental Animal Ethics Committee (DAEC), UNN (Approval No. UNN/DAEC/2017/B79).

2.7.4. Preparation of *Candida albicans* for Animal Infection

Stock cultures of *C. albicans* (SC5413) cells were kept at 4 °C and passaged once a month to maintain viability. Before each experiment, *C. albicans* (SC5413) cells were grown in Sabouraud's dextrose agar at 37 °C for 48 h. Cells were harvested by centrifugation at 3500× *g* for 10 min. Cells were washed three times with 50 mL of sterile non pyrogenic phosphate buffered-saline (PBS), counted with a haemacytometer, and resuspended in PBS to the required concentrations (1×10^7).

2.7.5. Induction of Oropharyngeal Infection with *Candida albicans* in Rat

This was described by Apeh et al. [11].

2.7.6. Determination of Tissue *Candida albicans* Burden

At 7 and 14 days treatment, rats were euthanised using diethyl ether, and target organ (kidney) were excised, homogenized, and serially-diluted (10 fold dilutions) in sterile phosphate-buffered saline (PBS). The tissue dilutions (0.1 mL) were then inoculated on SDA plates and incubated for 48 h at 37°C. The resulting *C. albicans* colonies were enumerated and expressed as mean $\log_{10}$ CFU/g tissue.

2.7.7. Histological Examination

Tissue Handling

The kidneys from the rats were excised and immersed (fixed) in Bouin's solution for 24 h and transferred to neutral buffered formalin for routine processing. Fixed tissues were sectioned, embedded in paraffin, and stained with Perodic acid-Schiff stain (PAS).

Light Microscopy

C. albicans infection was assessed by evidence of lesions and by hyphal/blastospore colonisation on the kidney [12] with a digital imaging system. Photomicrographs were taken using Motic™ 5 megapixels microscope camera at magnifications x100, x200 and x400.

Renal function test was determined by the method of Bartels and Bohmer [13].

Statistical Analysis

The results were analyzed using a statistical software package – SPSS Version 21. Data were expressed as mean ± standard deviation (mean ± SD). Where the variables to be compared are three or more, one-way analysis of variance (ANOVA) was used, Duncan test was used for post-hoc. $p < 0.05$ was considered statistically significant.

3. Results

3.1. In Vitro Antimicrobial Test

The crude and degummed oil (CO and DO respectively) of watermelon seed oil exhibited inhibition at different concentrations (Table 1). There was varied zone of inhibition at various concentrations of both the crude and degummed oil. The antimicrobial activity of the oil was higher in 125.0 mg/ml concentration compared to other concenrations while *Pseudomonas aeruginosa* resisted the antimicrobial activity of the oil at all the concentrations. The highest inhibition (16 mm) was recorded against *Candida albicans* by dCLSO while the standard drug (Fluconazole 5 μg/ml) resisted

the growth with a diameter of 11 mm. Free fatty acids are potent inhibitors of enzymes, unsaturated FFAs can also inhibit bacterial activity [14]. This could cause altered and inappropriate cell membrane fluidity and permeability, leading to the membrane destabilization and can allow internal contents to leak from the cell, which can cause growth inhibition and eventual death. Zheng *et al*. [15] observed the importance of –OH group in the antimicrobial activity of free fatty acid.

Table 1.

Test Organisms	Antimicrobials	Inhibition Zones Diameter (mm)				
		250.0 mg/mL	125.0 mg/mL	62.5 mg/mL	31.3 mg/mL	5 µg/mL
E. coli	cCLSO	10	6	6	5	3
	dCLSO	11	14	12	0	0
	Ciprofloxacin					13
Staphylococcus aureus	cCLSO	0	12	8	0	0
	dCLSO	12	13	3	0	0
	Ciprofloxacin					14
Pseudomonas aeruginosa	cCLSO	0	0	0	0	0
	dCLSO	0	0	0	0	0
	Ciprofloxacin					10
Candida albicans	cCLSO	13	10	10	0	0
	dCLSO	10	16	8	0	2
	Fluconazole					11

cCLSO = Crude *Citrullus lanatus* seed oil. dCLSO = Degummed *Citrullus lanatus* seed oil.

3.2. Effect of the Crude and Degummed Oil on Candida albicans Colonization of Kidney

The result of colony forming unit (CFU) (689.67 ± 4.93) {at 1 in 100 dilutions} counted in group 3 {suppressed, inoculated but not treated} showed a significant increase ($p < 0.05$) when compared with groups 4 (326.67 ± 5.13), 5 (477.67 ± 3.51), 6 (278.33 ± 6.03), 7 (336.00 ± 4.00), 8 (273.33 ± 3.06) and 9 (186.00 ± 4.00). There was also a significant increase ($p < 0.05$) CFU counted in group 5 when compared to group 4 (treated with 500 mg/kg bw and 300 mg/kg bw of degummed oil respectively). In the same vein, there was a significant increase ($p < 0.05$) in CFU counted in group 7 when compared with group 6 (treated with 500 mg/kg bw and 300 mg/kg bw of crude oil extract Groups 6 and 7 (crude) had a lower ($p > 0.05$) count than groups 4 and 5 (degummed). When group 9 (treated with 5 mg/kg bw of Fluconazole) was compared with other treatment groups, there was marked decrease ($p < 0.05$) in CFU in group 9 when compared with other treatment groups.

At the 14th day, there was a significant increase ($p<0.05$) {at 1 in100 dilutions} in CFU counted in group 3 (882.33±8.50) when compared with groups 4 (145.33±5.86), 5 (62.00±18.52), 6 (165.67±2.08), 7 (174.00±2.65), 8 (226.33±7.51) and 9 (210.33±8.50). While there was a reduction ($p<0.05$) in CFU count in the groups treated with oil extracts, there was no significant reduction ($p>0.05$) in CFU count in group 8 when compared with the 7th day CFU counts. When group 9 of day-14 was compared with group 9 of day-7, there was a slight increase ($p>0.05$) in day -14 CFU count. The oil's antibacterial activity was also attributed to the presence of the saponin bioactive component which antibacterial activity have been previously reported by Soetan et al. [16]. It is conceivable that immunosuppression facilitated fungal colonisation and evasion in group 3 rats. The suppression of immune status of the animals could have created a suitable environment for severe epithelial degeneration and clusters of blastospores within the renal parenchyma; by encouraging fungal penetration of keratin epithelial linings and facilitated adherence and multiplication of the organism for proper colonisation and evasion. Mucosal manifestation may be associated with primary immunodeficiency that are characterised by an inability to clear fungal infection [17].

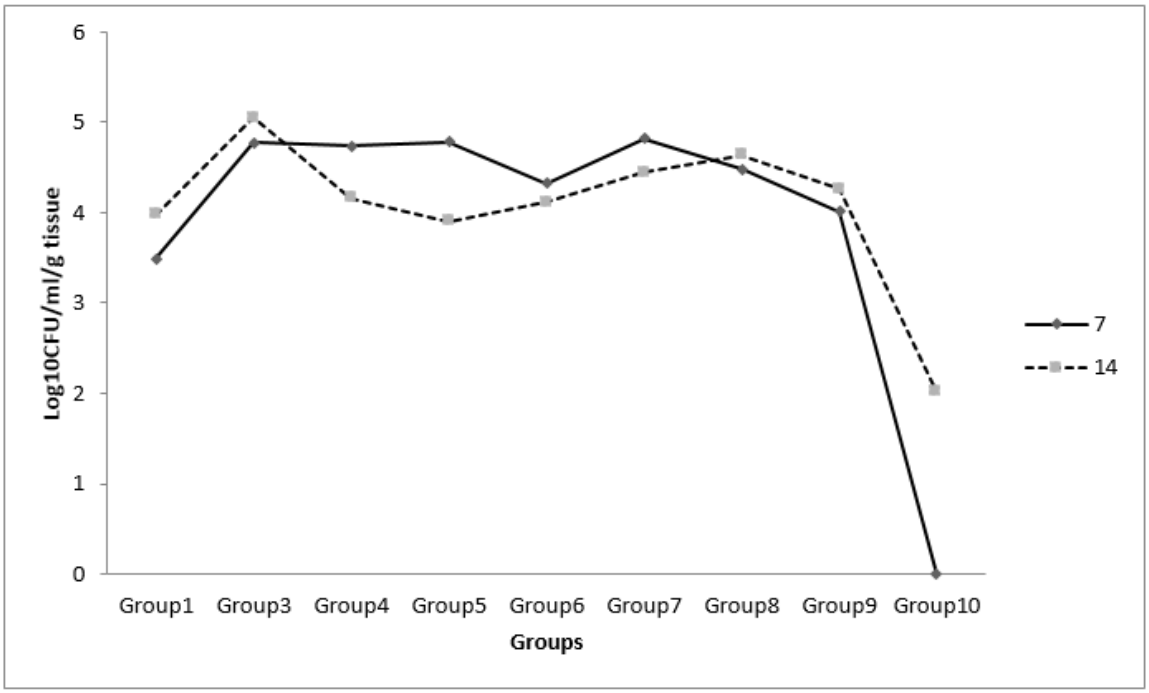

Figure 1. Fungal Burden of Kidney Colonised with *C. albicans* (Colony Forming Unit of 10^{-2} dilutions of tissue homogenate on SDA). Group 1 = inoculated without immunosuppression; Group 3 = inoculated + suppresses without treatment; Group 4 = inoculated + suppressed + treated with 300 mg/kg body weight of DO; Group 5 = inoculated + suppressed + treated with 500 mg/kg bw DO; Group 6 = inoculated + suppressed + treated with 300 mg/kg bw CO; Group 7 = inoculated + suppressed + treated with 500 mg/kg bw CO; Group 8 = inoculated + suppressed + treated with 25 mg/kg bw levamisol; Group 9 = inoculated + suppressed + treated with 5 mg/kg bw Fluconazole; Group 10 = Control.

3.3. Effect of Candida albican on the Urea and Creatinine Function

The urea value of all the groups in the first seven day revealed that there was no significant difference in urea value in all the treated groups when compared with the controls. But the treated groups had a lower ($p > 0.05$) urea value when compared with group 10 {7.33 ± 2.58} (normal control). However, the trend slightly changed at 14th day which had a significant increase ($p < 0.05$) in the treated groups. There was also an elevation of urea value in group 3 (immunosuppressed, inoculated but not treated) at day 14.

After 7 days of treatment, there was a significant ($p<0.05$) increase in creatinine value ($p<0.05$) in all treated groups except group 5 (64.67±16.33) when compared with groups 1 (42.43±4.92) and 10 (48.91±12.07). While at 14th day, there was a significant increase ($p<0.05$) in all treated groups when compared with group 1 (52.95±12.06). But group 3 (117.73±6.42) showed a significant increase ($p<0.05$) when compared with the treated groups. This might be as a result of compromised renal function due to immunosuppression and *C. albicans* infection; this could alter glomerular filtration rate (GFR). Kidney disease is usually associated with reduced urea excretion and consequent rise in blood concentration. This result suggests that CLSO treatments ameliorated the effect of *Candida albicans* on renal function when compared with group 3.

Table 3. Effect of *Candida albicans* on Urea and Creatinine.

Groups	Urea (mMol/L) Day 7	Urea (µMol/L) Day 14	Creatinine (µMol/L) Day 7	Creatinine (µMol/L) Day 14
Group1	9.52 ± 0.94 ^{b}	24.47 ± 19.38 ^{a}	42.43 ± 4.92 ^{a}	98.42 ± 8.22 ^{b}
Group2	5.34 ± 0.91 ^{a}	14.08 ± 1.51 ^{b}	78.67 ± 3.19 ^{b}	89.87 ± 11.54 ^{b}
Group3	6.55 ± 0.55 ^{a}	18.27 ± 2.02 ^{ab}	82.82 ± 3.34 ^{b}	117.73 ± 6.42 ^{c}
Group4	4.63 ± 0.83 ^{a}	10.11 ± 0.72 ^{b}	79.56 ± 4.02 ^{b}	99.23 ± 3.34 ^{b}
Group5	5.40 ± 1.20 ^{a}	10.37 ± 0.97 ^{b}	64.67 ± 16.33 ^{ab}	99.67 ± 7.23 ^{b}
Group6	5.13 ± 1.24 ^{a}	11.14 ± 3.33 ^{b}	84.86 ± 6.21 ^{b}	99.23 ± 6.55 ^{b}
Group7	5.47 ± 1.14 ^{a}	12.12 ± 3.30 ^{b}	85.76 ± 5.71 ^{b}	99.45 ± 6.55 ^{b}
Group8	7.50 ± 3.43 ^{ab}	9.13 ± 1.36 ^{b}	76.97 ± 8.63 ^{b}	100.48 ± 5.33 ^{b}
Group9	6.38 ± 1.79 ^{a}	9.21 ± 3.46 ^{b}	79.26 ± 2.84 ^{b}	100.48 ± 9.93 ^{b}
Group10	7.33 ± 2.58 ^{ab}	7.66 ± 0.70 ^{b}	48.91 ± 12.07 ^{a}	52.95 ± 12.06 ^{a}

3.4. Histopathologic Findings

Histologic results revealed several features of the degenerative characteristics of *C. albicans*. Results demonstrated that the severity of tissue pathology is related to the integrity of the immune system and the concentration of *C. albicans* inoculated (result of pilot study not shown here).

This therefore has shown an increased level of colonisation in immunosuppressed groups through extensive hyphae penetration of epithelial lining, compared to *Candida* control (group 1); suggesting that increased risk of mucosal infections with *Candida albicans* was ameliorated by *Citrullus lanatus* seed oil as treatment progressed. The level at which Candida hyphae were seen to penetrate appeared to coincide with the level of immunosuppression. Systemic/physiologic factors antagonistic to *Candida* organisms could constitute a barriers that inhibited fungal penetration apart from treatment with the oil; such factors might be absent in immunosuppressed rats and this allowed the fungus to superficially colonise the organs.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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