

Antiproliferative and Antimicrobial Activities of *Citrus limon* (L.) Burm. f. Stem Bark Extract

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ABSTRACT

Citrus limon is traditionally use for the treatment of several ailments including infectious diseases. This study was designed to evaluate antiproliferative and antimicrobial activities of *Citrus limon* stem bark extract. The plant material was collected, authenticated, air-dried and pulverized. Two hundred grams of powdered plant material was extracted into distilled methanol by cold maceration and the extract was concentration *in a vacuo*. The extract was subjected to *in vitro* phytochemical screening and bioassays including; antiproliferative assay (*Sorghum bicolor* radicle and *Allium cepa* root growth inhibitory assays) and antimicrobial susceptibility against *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter ferundii* and *Candida albicans*. Phytochemical evaluation detected the presence of alkaloids, flavonoids and cardiac glycoside in the extract. The extract displayed concentration-dependent antiproliferative activity with an IC₅₀ of 1.10±0.07 and 0.62±0.04 mg/mL compared to cyclophosphamide (IC₅₀ of 0.17±0.02 and 0.83±0.08 mg/mL) for *Sorghum bicolor* radical growth and *Allium cepa* root growth inhibitory assays, respectively. The extract displayed antimicrobial activity with the highest activity against *Escherichia coli* and *Citrobacter ferundii* with activity indices of 0.68 and 0.59, respectively compared to gentamicin. *Citrus limon* stem bark extract displayed antiproliferative and antimicrobial activities.

Keywords: *Citrus limon*, Antiproliferative, Antimicrobial, *Escherichia coli*, *Citrobacter ferundii*

INTRODUCTION

Plants are important sources of medicines from prehistoric times (Rather *et al.*, 2020) and are valuable in the discovery of about 70% of all chemotherapeutic agents (antimicrobial and anticancer agents) currently in used (Davison and Brimble, 2019). Infectious diseases are the primary cause of public health concern and they account for over 17 million deaths globally each year (WHO, 2019). In many developing countries, medicinal plants have been helpful for the management of infectious diseases. However, the recent emergence of resistance to commonly used antibiotics has necessitated the need to identify natural products that may serve as a lead compound in the discovery of new antimicrobial chemotherapeutic agents.

Citrus limon (L.) Burm. f. belongs to rutaceae family and it is commonly known for its edible fruit that is consumed as food and medicine. Several parts of the plant have been reported to display different activities such as anti-inflammatory,

antioxidant and antiparasitic activities (Klimek-Szczykutowicz *et al.*, 2020). Hence, this study was carried out to examine antiproliferative and antimicrobial activities of *C. limon* stem bark as a means to identify if the plant material can be further evaluated for the discovery of antiproliferative and antimicrobial chemotherapeutic agents.

MATERIALS AND METHODS

Plant Collection and Preparation

The stem bark, leave and floral parts of *C. limon* (L.) Burm was collected from Fiditi forest in Afijio Local Government Area of Oyo State, South-West, Nigeria and authenticated at the Forest Herbarium in Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher number (FHI: 110008) was issued. The plant's stem bark was air-dried and pulverized. Two hundred grams (200 g) of the powdered plant material was extracted into distilled methanol by cold maceration over 72 h, filtered and

concentrated *in vacuo*. The dried extract was refrigerated at 4°C until needed for further studies.

Qualitative Phytochemical Analysis

The extract was evaluated for the presence of saponins, tannins, alkaloids, anthraquinones, cardiac glycosides, flavonoids and terpenoids using phytochemical screening protocol described by Evans (2009).

Antiproliferative Assays

Sorghum bicolor Radical Growth Inhibitory Assay

Viable seeds of *Sorghum bicolor* were used in this assay. Two hundred (200) mg of extract was dissolved in 20 mL of 5% DMSO (Sigma-Aldrich, Germany) to achieve 20 mL of 10 mg/mL stock solution. Thereafter, 10 mL of 2.50, 0.63, 0.16 and 0.04 mg/mL were prepared by serial 1 in 2 dilutions of 10 mL of stock solution. The same concentration as the extract was prepared for cyclophosphamide (positive control). The different concentrations of the extract (10 mL) were added into different petri-dish already lined with cotton wool and filter paper. Ten seeds were spread on the filter paper in each petri-dish, thereafter the plates were incubated in a dark cupboard at room temperature and the lengths of the radicle emerging from the seeds were measured after 96 h incubation. The negative control test group contained seeds treated with 10 mL of 5% DMSO in distilled water (Ayinde and Agbakwuru, 2010). The experiment was repeated in three replicates for all concentrations and controls. The radicle lengths were measured and recorded in millimetres. The percentage of radical growth inhibition was calculated using the formula;

$$\% \text{ growth inhibition} = \frac{(A-B)}{A} \times 100$$

Where: A = the mean length of untreated.

B = the mean length of treated with plant extract.

Allium cepa Root Growth Inhibitory Assay

Allium cepa root growth inhibition was determined using a method described by Akinboro and Bakare (2007). Clean *A. cepa* bulbs (50 ± 10 g) were incubated at room temperature in the dark for 24-36 h until the roots have grown to approximately 2-3 cm length. Two hundred (200) mg of extract was dissolved in 20 mL of 5% DMSO (Sigma-Aldrich, Germany) to achieve 20 mL of 10 mg/mL stock solution. Thereafter, 10 mL of 2.50, 0.63, 0.16 and 0.04 mg/mL were prepared by serial 1 in 2 dilutions of 10 mL of stock solution. The same concentration as the extract was prepared for cyclophosphamide (positive control), different concentrations were poured into different petri-dish and the base of each of three *A. cepa* bulbs were placed on a Petri-dishes containing each extract (0.04 - 10.00 mg/mL). The same concentrations were prepared for cyclophosphamide (positive control), while the negative control bulbs were treated with 20 mL of 5% DMSO in distilled water. The root lengths were measured at 0 and 96 h for each concentration of extract and control. The percentage root growth inhibition after treating with extract/cyclophosphamide at 96 h was determined (Akinboro and Bakare, 2007) using the formula;

$$\% \text{ growth inhibition} = \frac{(A-B)}{A} \times 100$$

Where: A = the mean length of untreated.

B = the mean length of treated with plant extract.

Determination of Antimicrobial Activity

Test Organisms

Clinical isolates of bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Citrobacter ferundii*) and fungi (*Candida albicans*) were used as test organisms to determine the antimicrobial potential of *C. limon* stem bark extract. Discrete colonies from overnight culture were picked and emulsified in 5 mL sterile normal saline, vortexed and visually compared to 0.5 McFarland turbidity scale to yield an approximate final concentration of 1.5 × 10⁸ and 2.0 × 10⁶ colony forming units

(CFU/mL) for bacteria (Baker *et al.*, 1983) and fungus (Ado *et al.*, 2013), respectively. This turbidity scale was prepared by adding 9.6 mL of 1% aqueous solution of barium chloride in 0.4 mL of 1% sulphuric acid giving the approximate microbial density (Baker *et al.*, 1983).

Antimicrobial Susceptibility Assay

Antimicrobial assay was performed using the disc diffusion technique earlier described by Usman *et al.*, (2007). A stock solution (50 mg/mL) of the test extract was prepared by dissolving 500 mg of extract in 10 mL of sterile distilled water. The bacterial test strains were spread over the nutrient agar plates by using separate sterile cotton buds. The fungal strain was spread over the potato dextrose agar plates. Four concentrations (50.00, 25.00, 12.50 and 6.25 mg/mL) of the extract were prepared by serial dilution. Thereafter, 5 mm diameter discs was impregnated with 100 µL of 50.00, 25.00, 12.50 and 6.25 mg/mL of extract to achieve a 5, 2.5, 1.25 and 0.63 mg/disc. The discs were mounted on inoculated agar and incubated. All bacterial plates were incubated at 37°C for 24 h and fungal plates at 25°C for 72h. Moreover, filter paper discs (5 mm diameter) containing standards antibiotics; gentamicin (0.04 mg/disc) and fluconazole (0.05 mg/disc) were used as positive controls. The zones of inhibition were recorded in millimetres (mm) as the diameter of growth free zones around discs. The extract and standard antibiotics were independently tested in three replicates and the results were presented as mean±SEM. The extract is considered active against the organism upon which it showed a zone of inhibition greater than ≥10 mm (Usman *et al.*, 2007). An estimated zone of inhibition at a concentration of control (E-ZIC) was derived from a linear regression curve using GraphPad Prism. Activity index (AI) which compares the antimicrobial activity of the extract to the positive control was calculated using the method described by (Gopalakrishnan *et al.*, 2012). The formula below was used in calculating AI;

$$AI = \frac{E-ZIC}{ZOI}$$

AI = Activity Index

E-ZIC= Estimated Zone of Inhibition at Concentration of Control

ZOI = Zone of Inhibition of Positive Control

Data Analysis

Data obtained were analyzed using the GraphPad prism computer program. The concentration with 50% growth inhibition (IC₅₀) in *Sorghum bicolor* radical growth (**SBRG**) and *Allium cepa* root growth (**ACRG**) inhibitory assays were estimated from a concentration-response inhibition curve using a non-linear regression curve data analysis. The results are displayed as the mean ± SEM of three replicates.

RESULTS

Qualitative Phytochemical Screening

Phytochemical evaluation of the crude extract of *C. limon* stem bark led to the identification of alkaloids, flavonoids and cardiac glycoside in the extract as shown in Table 1.

Table 1: Phytochemical analysis of *Citrus limon* stem bark extracts

BIOACTIVE CONSTITUENT	CHEMICAL TEST	EXTRACT
Alkaloid	Wagner's	+
	Meyer's	+
Flavonoids	Lead acetate	+
	Tannins	+
Saponins	FeCl ₃	+
	Frothing	+
Anthraquinone	Emulsifying	+
	Combined	-
Cardiac glycoside	Free	-
	Kedde	+

Keys: - = Not detected; + = Detected

Antiproliferative Effect of Extract on the Growth *Sorghum bicolor* Radicle and *Allium cepa* Root

The extract displayed concentration dependent antiproliferative activity with 100 % SBRG inhibition at 10 mg/mL and at 0.63 mg/mL the extract displayed less than 50 % inhibition compared to the standard drug that displayed up to 87 % at 0.63 mg/mL. Hence, the calculated IC₅₀ value for the extract was 1.1 ±0.07 mg/mL while the standard drug has an IC₅₀ of 0.17±0.02 mg/mL. The extract displayed only 1/6th of the activity of cyclophosphamide in SBRG inhibitory assay as shown in Table 2. Similarly, in ACRG

inhibitory assay, the extract displayed concentration dependent inhibitory effects with 85.07 % inhibition at 10 mg/mL and 51.54±2.7 % inhibition at the lowest concentration. The positive control displayed 100 % inhibition at 50 mg/mL and 46.15 % inhibition at 0.63 mg/mL. However, the calculated IC₅₀ of the extract was 0.62±0.04 mg/mL compared to the standard drug with IC₅₀ of 0.83±0.08 mg/mL. The extract displayed some ACRG inhibitory effect better than the standard drug as shown in Table 2.

Table 2: Percentage inhibition and IC₅₀ of *C. limon* stem extract on *sorghum bicolor* radicle and *Allium cepa* root growth

CONC. (MG/ML)	SBRG INHIBITORY ASSAY		ACRG INHIBITORY ASSAY	
	CLS	CTZ	CLS	CTZ
10.00	100.00±0.00	100.00±0.00	85.07±1.43	100.00±0.00
5.00	99.32±0.53	100.00±0.00	71.64±2.37	100.00±0.00
2.50	83.11±1.33	97.97±0.57	62.69±2.16	97.01±1.01
1.25	62.16±1.53	95.27±1.73	59.70±1.57	50.75±2.57
0.63	41.59±2.45	87.02±1.58	51.54±2.37	46.15±1.43
IC ₅₀ (mg/mL)	1.10±0.07	0.17±0.02	0.62±0.04	0.83±0.08

Key: CLS= *Citrus limon* stem bark; CTZ = Cyclophosphamide/Positive control; SBRG = *Sorghum bicolor* radicle growth; ACRG = *Allium cepa* root growth

Antimicrobial Effects of the Extract on Clinical Strains of Microorganisms

The extract displayed concentration-dependent antimicrobial activities as shown in Table 3 against *E. coli*, *S. aureus* and *C. ferundii*. The extract displayed 15±1.33, 12±1.53 and 15±1.48 mm zones of inhibition at 5 mg/disc and 5±0.58, 0±0.00 and 9±0.56 mm at 0.63 mg/disc, respectively. Gentamicin used as the positive control displayed 20±3.33, 30±1.67 and 24±1.33 mm zone of inhibition against *E. coli*, *S. aureus* and *C. ferundii*, respectively at 40 µg/disc. The

extract displayed anti-fungi activity with 13±1.25 mm zone of inhibition at 5µg/mL and 5±0.46 zone of inhibition at 0.63 mg/disc against *C. albicans*. Fluconazole used as the positive control displayed 22±0.00 mm zone of inhibition against *C. albicans* at 0.50 mg/disc. The extract's estimated activities at the concentration of the standard drug suggest that the extract displayed its highest activity against *E. coli* and *C. ferundii* with an AI of 0.68 and 0.59, respectively. However, the extract displayed weak anti-fungi activity with an AI of 0.22 as shown in Table 3.

Table 3: Mean zones of Inhibition (mm) of different concentration of *C. limon* stem bark extract against different microorganisms

CONC. (mg/disc)	ZONE OF INHIBITION (mm)			
	<i>E. coli</i>	<i>S. aureus</i>	<i>C. ferundii</i>	<i>C. albicans</i>
5.00	15±1.33	12±1.53	15±1.48	13±1.25
2.50	12±0.46	10±0.76	14±0.77	10±0.88
1.25	8±0.73	8±1.24	12±0.93	7±0.57
0.63	5±0.58	0±0.00	9±0.56	5±0.46
E-ZIC	13.61	10.39	14.18	4.91
Positive Control	20±3.33 ⁺	30±1.67 ⁺	24±1.33 ⁺	22±0.00 ⁺⁺
Activity Index (AI)	0.68	0.35	0.59	0.22

Keys: E-ZIC= Estimated Zone of Inhibition at Concentration of Control; ⁺ = Zone of Inhibition of gentamicin at 0.04 mg/disc; ⁺⁺ = Zone of Inhibition of fluconazole at 0.005 mg/disc

DISCUSSION

The extract of *Citrus limon* stem bark tested positive for the presence of alkaloids, flavonoids and cardiac glycoside similar to constituents present in leave extract *Citrus limon* (Ewansiha *et al.*, 2016). The extract displayed growth inhibitory effect on *Sorghum bicolor* radical (1.1±0.07 mg/mL) and *Allium cepa* root (0.62±0.04 mg/mL) however, lesser compared to cyclophosphamide. This is the first report of *bicolor* radicle and *Allium cepa* root growth inhibitory activity of *C. limon* stem bark extract. The positive growth inhibitory effect of the extract suggests that the plant may inhibit tumour growth thereby complementing earlier report of the cytotoxic effects of *C. limon* stem bark against cancer cell line (Ajaiyeoba *et al.*, 2016). Several morphological parts of *C. limon* have been reported to demonstrate antimicrobial activities including essential oil obtained from fruit rind that was reported to showed considerable antioxidant and antimicrobial properties (Ifesan *et al.*, 2013). In another earlier report, the stem back of *C. limon* was tested against and *S. aureus*, *C. albicans* and *B. subtilis* the extract only showed activity against *Staphylococcus aureus* (Musa *et al.*, 2017). In this current study, the extract displayed highest antimicrobial activity against *E. coli* and *C. ferundii* and lesser activity against *S. aureus* and *C. albicans*. This work corroborates earlier reports of anti-*S. aureus* activity of the extract and further observed that the extract is capable of

higher antimicrobial activities against *E. coli* and *C. ferundii*.

CONCLUSION

Methanol extract of *Citrus limon* stem bark contains alkaloids, flavonoids and cardiac glycoside and displayed antiproliferative effects and antibacterial activities against *Escherichia coli* and *Citrobacter ferundii*.

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