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Research Article

Antimicrobial Activities of Citrus Seed and Leaf Extracts Against Selected Bacterial and Fungal Pathogens Linked to Human Diseases: In vitro Experimental Study in Musanze, Rwanda

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Abstract

Antimicrobial resistance has caused major challenges in both bacterial and fungal infections. Therefore, the current study was designed to evaluate the antimicrobial activity with different crude extracts of leaves and seeds of Citrus limon against Gram-positive (*Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa*) and fungi (*Cryptococcus neoformans* and *Candida albicans*) obtained from different clinical settings. Filtrate extracts after maceration technique and the phytochemical substances detected in plants were flavonoid, saponin, alkaloid, glycoside, and resin under investigation. In vitro, a disc diffusion assay was applied to measure the inhibition zone of the plant effect in millimeters (mm). It was observed that Citrus limon activity showed the highest on ethanolic seed against *Staphylococcus aureus* and the lowest activity plant observed on methanolic leaf extract against *Klebsiella oxytoca*. The mean values of Citrus limon extract convinced there was no statistically significant correlation among extract solvents used in the study ($\rho > 0.05$). Oxacillin was used as the positive control and discs without antibiotic content as the negative control during testing. To conclude, ethanolic seed extract has shown the highest effect against *Staphylococcus aureus* and *Salmonella typhi* from isolate. Indeed, plant extracts that showed potential effectiveness, such as (Citrus leaves and seeds) can be used as natural alternative preventive measures to control pathogenic bacterial diseases and prevent the rise of antibiotic resistance. Furthermore, studies to evaluate the in vivo potential in an animal model are recommended.

Keywords: *Citrus limon,* Crude extract, Bacteria, Fungi, Antimicrobial resistance.

INTRODUCTION

Citrus limon is an important medicinal plant of the family Rutaceae[1]. It is cultivated mainly for its alkaloids, which have many activities including anticancer and anti-bacterial, potential in crude extracts of different parts viz. seeds, leaves, stem, root, and flowers[2]. Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer, and antiviral activities[3] in humans. Flavonoids can function as direct antioxidants and free radical scavengers and can modulate enzymatic activities and inhibit cell proliferation[4]. In plants, they appear to play a defensive role against invading pathogens including bacteria, fungi, and viruses. Plants are a rich source of drugs either direct remedies or production templates for synthetic drugs[5].

The emergence of new drug-resistant strains of pathogenic organisms has made it necessary to investigate plants as sources of novel antimicrobials as they can inhibit drug-resistant bacteria by a mechanism different than that of conventional microbe-derived antibiotics[6] several studies have reported various seed and leaf extracts[7] for their antibacterial activity. Potential pathogens may enter the body by various routes, including the respiratory (K. pneumonia), gastro-intestinal (*E. coli, acinetobacter spp, and S. typhi*), urinary or genital tract (*S. aureus, P. aeroginosa, K. oxytoca, and C. albicans*) and for many gastro-intestinal pathogens such as *Salmonella spp*, the primary source is environmental, and infection follows the ingestion of contaminated food or water [8].

Microbial infections pose a significant health burden, worsened by rising antibiotic resistance over the past forty years, leading to costly and challenging treatment of new and re-emerging diseases; however, plants offer promising antimicrobial alternatives through their diverse bioactive compounds [9]. Many unevaluated medicinal plants may harbor undetected active compounds or pose poisoning risks, yet communities in Africa and Asia have historically used plant extracts to treat microbial infections from bacteria and fungi [10]. Surveillance of AMR in Africa is limited [11], resulting in a lack of reliable data on drug resistance for critical infections; however, medicinal plants [12], which evolved bioactive compounds for self-defense, hold the potential for treatment, as highlighted by initial research on citrus seeds and leaves against *E.coli* in Brazil [13] and Indonesia [14], this study evaluated the antimicrobial activity of *Citrus limon* crude extracts on *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Cryptococcus neoformans* and *Candida albicans* in Rwanda.

METHODOLOGY

Plant materials and extraction

During the study, unripe *Citrus limon* fruits and fresh leaves were collected from local fields and markets in the Musanze District of Northern Province, Rwanda. Raw materials and the whole experiment were performed at the Institute of Higher Education (INES Ruhengeri) Laboratories. The collected samples were washed with tap water. The seeds were then removed from the fruits and allowed to dry at room temperature for a short period. Afterward, they were dried in an oven at 50 º C. This same procedure was followed for the Citrus leaves until a constant weight was achieved. The dried seeds and leaves were ground into a powder using a cutter mill mixer (model/SM-400). Additionally, some plant materials were prepared and air-dried at room temperature. This approach was taken to maximize the presence of natural products in *Citrus limon* without the risk of heat-related degradation of bioactive compounds.

A total of 246 grams of dry powdered plant material was extracted using 200 milliliters of various solvents, including 66% ethanol, 98% methanol, and distilled water. The solid-to-solvent ratio was set at 1:10 (w/v), and different maceration periods ranged from 3 to 5 days. The extraction process occurred at room temperature, with continuous agitation at 200 rpm to enhance extraction efficiency. After the designated maceration periods, the powder-solvent mixtures were filtered through the Whatman No. 1 filter paper. The resulting solutions were then concentrated to 1 milliliter using a rotary evaporator (Heidolph rotary/Laborata 4010 digital) set at 34-78 º C. The concentrated extracts of seeds and leaves were stored at -4 º C until use. The percentage yield of extract was determined by dividing the weight of extracts (a) by the weight of the soaked plant material (b) times one hundred {Yield % = (a/b) *100}.

Phytochemical screening

Detection of groups and active compounds present in *Citrus limon* extracts

Resins: According to the method described by Mason and Wasserman, 5 mL of 95% ethyl alcohol was added to 0.5 g of leaf extracts. The mixture was then heated in a water bath until it boiled for two minutes. Afterward, 10 mL of distilled water acidified with concentrated hydrochloric acid was added to the filtrate, which indicated the presence of resin materials through the formation of turbidity.

Tannins: According to [15], 0.5 g of leaf extracts were boiled in 2.5 milliliters of distilled water and then filtered. The filtrate was divided into two portions; the first portion was treated with 1% ferric chloride solution, which produced a white gelatinous precipitate, indicating the presence of a certain solution, resulting in a bluish-green color, which served as evidence of tannins.

Phenols: The volume 0.1 mL of the leaf's extracts to was added to 0.06 mL of 1% ferric chloride solution, according to phytochemical methods.[16] The appearance of bluish green color indicates the presence of phenols.

Alkaloids: Several drops of Wagner reagent were added to 1.00 mL of an aqueous solution of extract and alcohol to detect the presence of alkaloids.

Glycosides: To detect the glycosides, 10 mL of 50% H2SO4 was added to 1 mL of the leaves extracts and the mixture heated in boiling water for 15 minutes. 10 mL of Fehling's solution was then added and the mixture boiled.

Saponins: Equal volumes of extracts and water were mixed and shaken well to detect saponins presence.

Flavonoids: Few drops of diluted sodium hydroxide were added to 1 mL of crude extract for detection of flavonoids. After the appearance of intense yellow color, few drops of diluted acid were added to confirm the presence of this compound.

Microorganism isolation and identification

In vitro antimicrobial study was carried out on gram positive bacteria (staphylococcus aureus), gram negative bacteria (Escherichia coli, Klebsiella pneumonia, Klebsiella oxytoca, Pseudomonas aeruginosa, Salmonella typhi and Acinetobacter ssp.) and the fungi (candida albicans and Cryptococcus neoformans). From patients' clinical samples collected at the University Teaching Hospital of Butare (CHUB), microorganisms were isolated and identified based on Microbiology Laboratory procedural standards of CHUB and INES – Ruhengeri, Rwanda.

Culture media MC (M081-500G), BA (70133-500G), MSA (M118-500G), XLD (030119093), and SDA (M317C01) agar were prepared by following their respective manufacturer's instructions. Microorganisms were cultured on prepared media; bacterial cultures were incubated at 37 º C for 24 hours whereas yeast cultures were incubated at 30 º C for 72 hours.

Antimicrobial susceptibility testing

The Kirby – Bauer disc diffusion and pour plate methods were used to evaluate three Citrus leaf and seed extract solvents: Methanol, Ethanol, and Distilled water.

Bacterial and yeast cells concentrations were standardized 10^7 to 10^8 cells/mL using a 0.5 McFarland Latex Standard, which corresponds to a bacterial suspension of 10^8 CFU/mL for the disc diffusion method. Suspensions of microbes from stock solutions of both leaves and seeds were prepared for filter paper impregnation. Filter paper discs were soaked in each extraction for one hour to absorb the bioactive compounds from Citrus limon. The discs were then air-dried at room temperature for 10 to 15 minutes before being placed on top of Muller Hinter (MH) agar.

For impregnated discs (**X1**), the sensitivity test was performed according to the standard and then the discs were placed in the petri-plate containing MH agar together with bacterial suspension and incubated over night at 37 ᵒ C for measuring the zone of inhibition to observe Citrus limon antimicrobial effect toward selected microbes.

For discs loaded with extracts (**X2**), sterile 6 mm filter paper discs loaded with 20 µL of plant extract were placed on MH agar plates, with clindamycin discs as positive controls. Plates were refrigerated at 5 ᵒC for 2 hours, then incubated at 35 ᵒ C for 24 hours to allow extract diffusion. Inhibition zones were measured with a ruler to assess antimicrobial activity. The “Pour plate” method involved mixing 20 µL of each extract with bacterial suspension, then pouring it onto petri plates with MH preparation for overnight incubation at 35 ᵒ C. Different solvents (methanol, ethanol, distilled water) served as negative controls, and tests were conducted in duplicate, measuring inhibition zones per CLSI standards.

Statistical analysis

The experimental results were repeated twice, forming duplicates. results were expressed as mean ± SD. When applicable, the data underwent one-way ANOVA. Differences between samples were determined using a two-tailed t-test. Bonferroni error correction was applied to the predictive value. P values greater than 0.05 indicated no statistically significant difference. All analyses were performed using the Microsoft Excel 2010 statistical package.

Ethical approvals

Ethics and research committees at INES–Ruhengeri and University Teaching Hospital of Butare (CHUB) approved the conduct of this study.

RESULTS

Plant extraction yield

Extract yield was found 4.8 g (7.9%) and 15 g (10.2%) from citrus leaves and seeds, respectively. The extraction process involved 188.6 g of dried plant leaves and 47 g of seeds using ethanol. The yield of plant extract and residues varied from 4.8 g to 15 g.

Phytochemical screening

In the chemistry laboratory, a qualitative phytochemical analysis was performed on various extracts to investigate the presence of specific phytochemical compounds. The study focused on commonly found constituents in plants viz. alkaloids, flavonoids, saponins, glycosides, phenols, and resin. Through systematic testing, there was a presence of three compounds, while and other three were not detected as illustrated in (table 1).

Table 1: Phytochemical screening of the extracts

Phytochemical Constituents	*Leaf*	*Seed*
Alkaloids	+	+
Flavonoids	+	+
Saponin	-	-
Glycoside	-	-
Phenols	+	+
Resin	-	-

Note: (+) indicates the presence while (-) indicates the absence of the phytochemical constituent.

Antimicrobial activity analysis

The activity of citrus limon extracts was tested in duplicates and results are shown in (table 2). Results were expressed as mean.

Table 2: Antimicrobial screening test of *C. limon* plant extracts against selected bacterial and fungal strains responsible for human diseases.

	Zone of inhibition (mm)									
	H2O extracts			Methanol extracts			Ethanol extracts (L/S)			
9 microbial species	**X1**	**X2**	**Mean**	**X1**	**X2**	**Mean**	**X1**	**X2**	**Mean**	**SD. drug**
K. oxytoca	7	7	7	7	7	7	7/8	6.5/8	6.7/8	**DA**
P. aeruginosa	8	9	7.5	9	9	9	9/9	9/8	9/8	**OX**
S. typhi	9	10	9.5	11	12	11.5	10/9	11/11	10.5/10	**DA**
S. aureus	8	14	11	11	13	12	11/11	13/15	12/13	**DA**
E. coli	6	6	6	6	6	6	6/6	6/7	7/6	**OX**
K. pneumonia	6	6	6	6	7	6	6/7	7/6	6/6	**OX**
Acinetobacter spp.	6	6	6	6	6	6	6/6	6/6	6/6	**OX**
C. albicans	6	6	6	6	6	6	6/6	6/6	6/6	**DA**
C. neoformans	6	6	6	6	6	6	6/6	6/6	6/6	**DA**

Results of disc impregnation (X1), disc loaded with extract method (X2), L/S: Leave / Seeds

Antimicrobial activity trials

The extracts from dried seeds and leaves of citrus limon exhibited antibacterial activity against *K. oxytoca*, *P. aeruginosa*, *S. typhi*, and *S. aureus* as shown in figure 1.

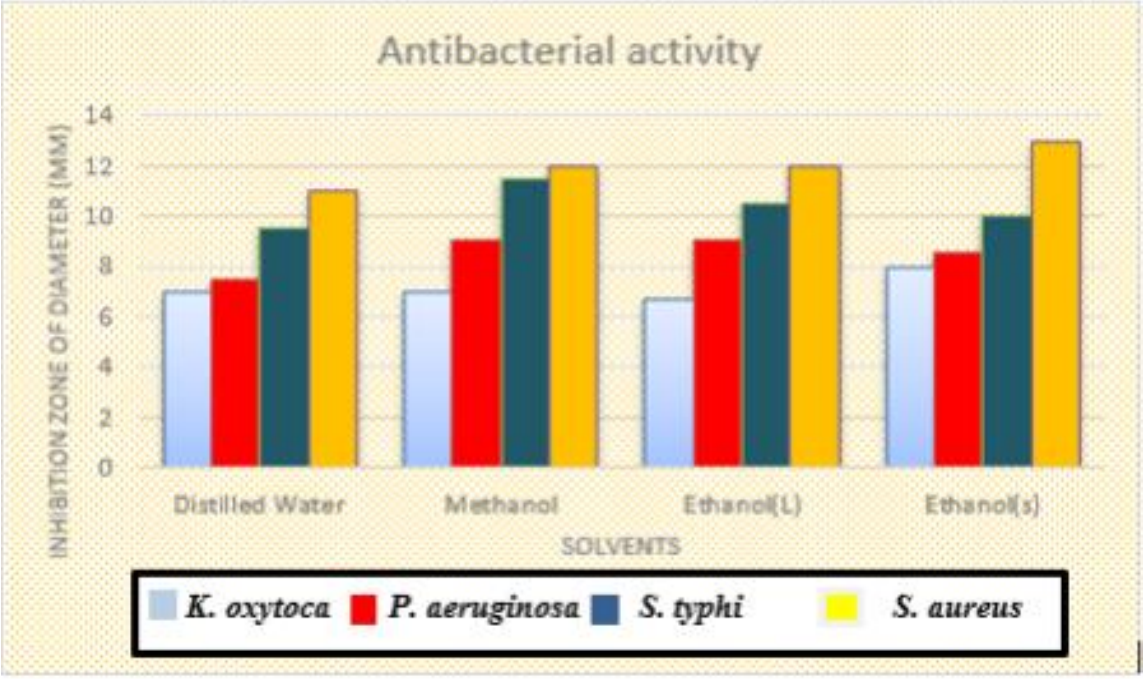

Figure 1: Inhibited zone shown by each fraction of *Citrus limon* extract against microorganisms

DISCUSSION

Phytochemicals

In this study, the phytochemical screening of six import bioactive materials showed the presence of alkaloids, flavonoids and phenol in citrus limon seed and leaf extracts. An article by [17] reported polyphenols in Zest of lemon about 3524 mg EAG/100 g of extracts.

Extracts

The highest yield came from Citrus seeds, which produced 15 g of extracts. In contrast, Citrus leaves yielded the lowest amount at 4.8 g.

Antimicrobial activity of *Citrus limon*

The results of the antimicrobial activity of *Citrus limon* extracts indicated that selected fungi were the most resistant strains. They were followed by *E. coli*, *K. pneumoniae*, and *Acinetobacter* spp. In contrast, *S. aureus*, *S. typhi*, and *P. aeruginosa* were the most susceptible strains to the extracts. Among the extracts, ethanolic extracts of Citrus seeds demonstrated the highest effectiveness. These extracts showed strong antimicrobial activity against pathogenic bacteria associated to human diseases. The active phytochemicals have been reported to have antimicrobial activity against pathogens. The same report showed the contribution of phytochemicals in new antibiotic drug discovery.[18] The evaluation of antimicrobial activity revealed that all extract solvents suppressed growth of pathogenic microorganism. The potent antifungal and antibacterial activities of fractioned crude extract of Zairen plants was only reported in ethyl acetate and aqueous fractions.[19] Another work[20] reported antibacterial and antifungal activities of *Toddalia Asiatica* in hexane, ethyl acetate, methanol, and water extracted fraction. They saw a potential activity in ethyl acetate. Ethanolic extract of lemon seeds was effective retarding microbial growth whereas methanolic and watery extract of citrus leaves was effective.

Methanolic extract of Citrus leaves showed inhibitory effect against two of the pathogenic bacteria (*S. typhi, P. aeruginosa*). Ethanolic extract of citrus seeds was effective against *S. aureus*, *P. aeruginosa* and *S. typhi.* The antibacterial test on β haemolytic *Streptococcus* showed growth inhibition for all extracts of both seeds and leaves. The study of polyphenolic relationship showed that the sensitivity of microorganism to polyphenols depends on itself and the polyphenol.[21,22] However, known mechanisms of bacteria on sensitivity to antibiotics can explain the observed sensitivity.

Results of antimicrobial activity of the citrus limon plant extracts suggest that *Acinetobecter*, *K. pneumonia*, and *E. coli* were resistant strains to plant extracts. The study reported antimicrobial activity of citrus limon peel extracts against *E. coli.*[23] The discordance may be influenced by various factors including geographical locations and climatic conditions for plants used in the extraction could act differently. *S. aureus*, *S. typhi*, and *P. aeruginosa* were susceptible bacteria on Citrus limon plants, in that order.

Lemon juice can inhibit the growth of Enterotoxin *E. coli* (ETEC) that cause diarrhea [24], and moreover, in this study we produced citrus extracts effective in antimicrobial activity against gram positive and negative bacteria.

CONCLUSION

The findings of this study indicate that Citrus limon extracts exhibit varying antimicrobial activities depending on the type of solvent and the pathogen tested. The ethanolic seed extract demonstrated the strongest antimicrobial effect, particularly against *Staphylococcus aureus*, while the methanolic leaf extract showed the least activity against *Klebsiella oxytoca*. Despite these variations, the results revealed no statistically significant correlation between the different extract solvents, suggesting that the solvent type did not significantly influence the antimicrobial efficacy of *Citrus limon* extracts. Furthermore, when compared to oxacillin, the extracts displayed antimicrobial potential, although not as pronounced. This study underscores the potential of *Citrus limon* as a natural antimicrobial agent, with seed extracts showing more promise than leaf extracts. However, further research with a larger range of pathogens and more solvent types is recommended to fully understand the potential applications of *Citrus limon* in combating bacterial and fungal infections.

Limitations of the study: The study was conducted in the mentioned area, Musanze, Rwanda and further works should consider geographical variations for plants to be used in extraction of active compounds.

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Contribution of authors: FU, NGF: Conceptualized and Initialized the study; NGF, HT: Experimental Laboratory, data collection and analysis; JBM: technical customization and original article manuscript writing. HT, NNF: Senior review of the project. All authors have contributed to the review and finalization of the original article manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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