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Antimicrobial, Cytotoxicity, Acute Oral Toxicity, and Qualitative Phytochemical Screening of the Aqueous and Methanolic Stem-Bark Extracts of *Croton megalocarpus* Hutch. (Euphorbiaceae)

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ABSTRACT

Microbial infections are feared to cause over 10 million deaths by the year 2050, whereby 50% of the global burden squarely lies in less developed countries of Africa and Asian continents. The current drugs have suffered resistance by previously susceptible strains, are associated with severe side effects, among other therapeutic and economic drawbacks, hence the need for alternatives. Despite the widespread usage of medicinal plants by over 80% of the global population to treat common ailments, including microbial infections, only a few have been empirically validated. *Croton megalocarpus* is used to treat microbial-associated infections like pneumonia and typhoid among the Agikuyu community of Kenya. However, its healing claims and safety have not been evaluated empirically to date, hence this study. We investigated the antimicrobial, cytotoxicity, acute oral toxicity, and qualitative phytochemical composition of the aqueous and methanolic stem bark extracts of *C. megalocarpus*. The disk diffusion and broth microdilution techniques described by the Clinical Laboratory Standards Institute (CLSI) were adopted for antimicrobial assays. The acute oral toxicity effects of the studied plant extracts were evaluated according to the Organisation of Economic Co-operation and Development (OECD) guideline document number 425. The brine shrimp lethality assay technique was used to appraise the cytotoxic effects of the studied plant extracts. Qualitative phytochemical screening was performed following standard procedures. The results revealed that all the studied plant extracts had varied antimicrobial effects on selected microbial strains and showed MIC values of <1000 µg/ml indicating their antimicrobial potential. Moreover, the studied plant extracts had LC_{50} values of >100 µg/ml and >2000 mg/Kg bw in the brine shrimp lethality and acute oral toxicity assays, respectively, demonstrating their safety. Antimicrobial- associated phytocompounds were detected in the studied plant extracts suggesting they were responsible for the reported bioactivity. Further studies to establish the specific mode(s) of antimicrobial action, toxicological, and safety should be performed. Furthermore, antimicrobial investigations of the studied plant extracts on other clinically significant microbial strains and the isolation, characterization, and optimization of antimicrobials from the studied plant extracts should be done.

Keywords: Antimicrobial activity, *Croton megalocarpus*, Disk diffusion, Broth microdilution, Brine shrimp lethality assay, Acute oral toxicity.

INTRODUCTION

The rapid increase in antibiotic resistance by various pathogenic microbes, such as the multidrug-resistant *Staphylococcus aureus*, Enterobacteriaceae, and pseudomonads, is a major global health challenge [1, 3]. Research has indicated that resistant bacterial strains arise from inappropriate use of antibiotics, patient non-compliance to antimicrobial therapy, widespread usage of antibiotics in animal feeds as growth promoters, the transboundary transmission of resistant strains, among other factors [2]. This has complicated the treatment of various pathogenic infections with the current antimicrobial agents. Furthermore, the scarcity of novel, safe, affordable, and accessible antimicrobials, especially in the developing countries of the African and Asian continents, which account for over 50 % of all global infectious disease-associated deaths, warrants urgent intervention [4, 5]. Moreover, the presently used antimicrobials exhibit undesirable effects in patients, such as nephrotoxicity, hepatoxicity, gastrointestinal complications, among others [6].

Medicinal plants play an integral role in promoting the health and wellbeing of humans and animals [7, 9]. The World Health Organization (WHO) report indicates that over 80% of the global population, especially from the developing countries in the African and Asian continent, largely depend on traditional medicine to treat and manage common ailments [10, 11]. The reliance on traditional

medicine, mostly medicinal plants, is attributable to their wide cultural acceptability, presumed safety, accessibility, affordability, and potency [10].

Research has shown that medicinal plants synthesize diverse pharmacologically active phytochemical compounds, some of which have been isolated and characterized [12, 14]. These phytochemical compounds primarily protect plants against biotic and abiotic stresses, and when consumed, they confer both dietary and pharmacological benefits to the body [15, 16]. The presence of antimicrobial compounds in various medicinal plants presents a promising source of natural products that can serve as viable alternatives to synthetic antibiotics [17, 18].

Despite the longstanding usage of medicinal plants to treat various ailments in traditional medicine, there are scanty empirical studies to validate their pharmacologic efficacies [19]. Moreover, various concerns regarding medicinal plants' safety have been raised due to the lack of standardized methods and regulations for the preparation, packaging, storage, dosage regimes, and administration [20, 21]. Besides, there is insufficient empirical data on herb- herb and herb-conventional drug interactions and associated toxicity profiles of medicinal plants [22]. Consequently, it is imperative to scientifically investigate medicinal plants' efficacy and safety to validate their usage and offer empirical data to guide the isolation, characterization, and development of safe, potent, accessible, and affordable alternative medicines.

Therefore, this study was designed to investigate the antimicrobial, cytotoxicity, acute oral toxicity effects, and qualitative phytochemical screening of the aqueous and methanolic extracts of *Croton megalocarpus* Hutch. (Euphorbiaceae). *Croton megalocarpus* is a deciduous tree belonging to the Euphorbiaceae family, which grows up to 35 m above the ground [23]. Its cylindrical bole measures up to 120cm wide and is unbranched up to 20 m high. This plant offers timber, firewood, and medicine to local communities, which has led to its wide cultivation. It is locally known as 'mukinduri' by the Agikuya community of Murang' a County, Kenya [24].

Various parts of *C. megalocarpus* are ethnomedically used to manage various ailments ranging from coughs, typhoid, pneumonia, wounds, joint pains, and helminthic infections, among other maladies [23, 25]. Previous studies have revealed the presence of alkaloids, flavonoids, flavones, saponins, glycosides, terpenoids, sterols, and tannins [23]. Moreover, clerodane diterpenoids, including epoxy-chiromodine and chiromodine have been isolated from the stem bark of *C. megalocarpus*. Furthermore, lupeol, β-sitisterol, 3-β-O-acetoacetyllupeol, E-feruric, botulin among other compounds have been isolated [23]. Various bioactivities of *C. megalocarpus* including, antinociceptive, molluscicidal, anti-inflammatory, antioxidant, among others have been investigated [23, 26, 27]. However, this plant has not been empirically validated, hence the present study.

MATERIALS AND METHODS

Plant collection, identification, and processing

In the present study, fresh stembarks of *C. megalocarpus* were collected, with the help of an acknowledged herbalist from Murang' a County, Kenya, based on its ethnomedical information on antimicrobial therapy [24]. A taxonomist later authenticated the plant at the East Africa Herbaria housed at the National Museums of Kenya under voucher specimen number: NMK/02/2019. The collected plant material was spread evenly to air-dry for two weeks at the Department of Public Health, Pharmacology, and Toxicology laboratories, of the College of Agriculture and Veterinary Sciences, Kabete Campus, University of Nairobi, Kenya. After drying, the stem barks were ground into a powder using an electric plant mill and stored in a labelled plastic container on a laboratory shelf awaiting extraction.

Extraction procedures

The methanolic and aqueous stem bark extracts of *C. megalocarpus* were prepared according to the procedures described by Harborne [28]. The methanolic extract was briefly obtained by cold maceration method, whereby 250 g of the powdered material was soaked in a litre of analytical-grade methanol in a 2-liter conical flask and covered with an aluminium foil paper. The merc-menstruum mixture was shaken once daily for two days, then decanted and filtered through Whatman filter paper (No.1). The filtrate was concentrated *in vacuo* using a rotary evaporator. The resultant extract was transferred into a glass bottle and further dried in a hot- air oven at 35 ºC for five days.

On the other hand, the aqueous extract was prepared by macerating 50 g of *C. megalocarpus* powder in 500 ml of distilled water and then heating for ten minutes at 58 ºC. The mixture could cool to room temperature, after which it was filtered through a Whatman filter paper. After that, 200 ml portions were transferred into freeze-drying flasks, fitted into a freeze-dryer, and lyophilized for 48 hrs. The dried extracts were weighed, and their respective percentage yields were determined. All the extracts were stored in a refrigerator (4 ºC) and only retrieved during experimentation.

Investigation of the antimicrobial Activities

In this study, *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), and *Candida albicans* (ATCC 10231) were selected based on their clinical significance and availability at the Department of Public Health, Pharmacology and Toxicology of the College of Agriculture and Veterinary Sciences, University of Nairobi, Kabete Campus. The disc diffusion and broth microdilution techniques, described by the Clinical and Laboratory Standards Institute, were followed [29].

Preparation and standardization of microbial inocula

The bacterial strains (*E. coli*, *S. typhimurium*, and *S. aureus*) were cultured in Mueller-Hinton agar as per the CLSI guidelines [29] for 24 hrs. The inocula were then standardized to a turbidity equivalent to a 0.5 McFarland scale of approximately 1-2×10^8cfu/ml [29]. The fungal strain (*C. albicans*) was grown in Sabouraud dextrose agar (SDA; Oxoid) for 24 hrs according to the directions of the M100-S23 document of the CLSI [29]. After that, sterile normal saline was used to standardize the inoculum to achieve a 0.5 McFarland standard at 530 nm using a UV-Vis spectrophotometer. The OD_{530} values ranges of between 0.11 and 0.14, representing 1-5×10^6cfu/ml, were obtained.

Disc diffusion assay for antimicrobial susceptibility

In this assay, the aqueous and methanolic stem bark extracts of *C. megalocarpus* were weighed appropriately taken and dissolved in 10 ml of 1.4 % DMSO (in sterile water) in a 15 ml centrifuge tube and thoroughly vortexed to make stock solutions containing 100 µg/ml. The stocks were then serially diluted two-fold to obtain sic concentrations (100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, and 3.125 µg/ml, respectively). After that, 20 µl aliquots were

aspirated and carefully impregnated on sterile Whatman paper discs with a 6 mm diameter. The discs were gently pressed onto the media containing 1 ml of either bacterial or fungal inocula to allow for proper drug-microbe contact. The assays were performed in triplicate with DMSO as the negative control and streptomycin/ciprofloxacin/ amphotericin B as positive controls. All the plates were incubated for 24 hrs at 37ºC, after which the diameters of zones of inhibition of microbial growth were measured in millimetres using a digital zone reader.

Determination of the minimum inhibitory concentration (MIC)

The modified CLSI microdilution technique described by Golus *et al*. [30] was adopted to determine the MICs of the studied plant extracts on the studied microbial strains. Briefly, respective microbial cultures were prepared and adjusted in Mueller-Hinton Broth media to a 0.5 McFarland equivalent turbidity. Then, 10 µl of previously prepared test extracts at a 10- fold concentration was mixed with 90 µl of molten Mueller-Hinton agar in Eppendorf tubes in triplicate and gently vortexed. Two-fold microdilution was done in volumes of 100 µl in sterile 96-U-shaped multiwell plates. In each of the micro-titer plates, the growth, sterility, and negative (1.4 % DMSO) controls were included for each of the tested microbial strains. All the multiwell plates could settle at room temperature for the agar to solidify. Afterward, 2 µl of freshly prepared inocula at concentrations of 10^4 cfu/spot were dispensed into the wells using a multichannel micropipette and allowed to interact at room temperature. Sterile water was added to the side wells were added, and the plates were carefully covered in zip-lock plastic bags and incubated at 35ºC for 18 hrs. The lowest concentrations of the studied extracts, which could completely inhibit microbial growth, were considered the MICs as per the CLSI guidelines [29].

Brine shrimp lethality assay

The brine shrimp lethality assay method described by Meyer *et al*. [31] was used to determine the cytotoxic effects of the studied plant extracts in *Artemia salina* nauplii. Briefly, approximately 0.5 g of *Artemia salina* cysts (Sanders Great SaltLake, Brine Shrimp Company LC., USA.) were incubated for 48 hrs in 500 ml of brine water (artificial sea) under continuous normal bulb illumination (25 Watt) at 25 – 29 ^{0}C temperature and ample aeration to hatch into nauplii. After that, ten nauplii were transferred using Pasteur pipettes into three sets of sample vials containing either the studied plant extracts at concentrations of 0, 10, 100, and 1000 µg/ml or podophyllotoxin in 5 ml brine solutions in triplicate. The nauplii were further incubated for 24 hours after which the number of survivors in each test vial were counted and documented. The percentage lethality was determined as a ratio of surviving nauplii in the test groups to those in the control (vehicle treated) group. The Median Lethal Concentration (LC50) values were derived from the line of best fit in a plot of percentage survival against concentration.

Acute Oral Toxicity study

In this study, the Organization for Economic Co-operation and Development (OECD) protocol document number 425 was adopted [32]. Briefly, female Winstar Rats weighing 150± 20g were obtained from the Department of Public Health, Pharmacology, and Toxicology animal breeding unit and acclimatized for 72 hrs before experimentation. The studied plant extracts were reconstituted in normal saline to achieve the appropriate dose for administration. On the experimentation day, the animals were fasted for 4 hrs and randomly assorted into groups of three rats. The experiment was initiated by administering a single dose of 175 mg/kg bw of the studied plant extracts orally to the first group and normal saline (10 ml/Kg bw) to the control group.

Observations of wellness parameters (skin fur, eye colour, mucus membrane, salivation, lethargy, sleep, coma, convulsions, tremors, and diarrhoea) were recorded at intervals of 30 min, 4 hrs, 24 hrs, 48 hrs, 7 days, and 14 days, respectively, for each individual rat. In the absence of observable signs of toxicity or mortality during the 14-day experimentation period, the next subsequent higher doses of 550 mg/kg and 2000 mg/kg, respectively, were administered into new groups of rats [32]. At the end of the experiments, the experimental rats were euthanized and disposed of according to the University of Nairobi Ethical Review guidelines.

Qualitative phytochemical screening

In this study, the standard protocols for detecting alkaloids, flavonoids, tannins, saponins, anthraquinones, and phenols described by Harborne [29] were followed.

Data management and statistical analysis

Quantitative data from antimicrobial and brine shrimp lethality experiments were tabulated on an Excel spreadsheet (Microsoft 365) and exported to Minitab version 19.2 statistical software (State College, Pennsylvania) for analysis. The data were subjected to descriptive statistics, and resultant values were presented as $\bar{x}$±SEM. One-Way Analysis of Variance (ANOVA) was performed to determine significant differences among means followed by Tukey's *post hoc* test for pairwise comparisons and separations of means at α=0.05. Acute Oral Toxicity results were treated according to the OECD guidelines [32]. Qualitative data on wellness parameters in the acute oral toxicity and qualitative phytochemical screening studies were only tabulated. The obtained findings were presented in tables.

RESULTS

Antimicrobial activities of the studied plant extracts

Antimicrobial effects of the aqueous bark extract of C. megalocarpus on selected microbial strains

The effects of the aqueous bark extract of *C. megalocarpus* on selected microbial strains were investigated. The results revealed no significant difference in zones of inhibition observed in *E. coli* at all the studied extract concentrations except at 3.125 µg/ml ($p>0.05$; Table 1). Similarly, the zone of inhibition observed at a concentration of 3.125 µg/ml of the studied extract was significantly like that of the negative control in *E. coli* ($p>0.05$). However, the positive control produced the largest zone of inhibition compared to the zones of inhibition observed at all the extract concentrations ($p<0.05$; Table 1).

The zones of inhibition produced by the aqueous bark extract of *C. megalocarpus* in *S. typhimurium* at concentrations of 3.125 µg/ml and 6.26 µg/ml, and at concentrations of 25 µg/ml and 50 µg/ml were not significantly different ($p>0.05$; Table 1). However, the standard drug produced a significantly larger inhibition zone in *S. typhimurium* than the zones of inhibition observed in all the extract concentrations and the negative control ($p<0.05$; Table 1).

Also, in this study, the antibacterial effects of the aqueous bark extract of *C. megalocarpus* on *S. aureus* were determined. The results indicated that the zones of inhibition observed at extract concentrations of 3.125 µg/ml and 6.25 µg/ml were not significantly different from that of the negative control in *S. aureus* bacteria (p>0.05; Table 1). The zones of inhibition produced by the aqueous extract of *C. megalocarpus* at concentrations of between 12.5 µg/ml and 100 µg/ml in *S. aureus* were not significantly different (p>0.05; Table 1). In this strain, the reference antibiotic showed a significantly larger zone of inhibition than the zones recorded in all the other setups of *S. aureus* strain (p<0.05; Table 1).

Furthermore, when the effects of the aqueous bark extract of *C. megalocarpus* on *C. albicans* were evaluated, the results indicated no significant differences in zones of inhibition observed at concentrations of 3.125 µg/ml and 6.35 µg/ml, 12.5 µg/ml to 50 µg/ml, and, between the control setups (p>0.05; Table 1). However, *C. albicans* was more susceptible to 100 µg/ml of the aqueous bark extract of *C. megalocarpus* with a significantly larger zone than the zones observed in all the other setups (p<0.05; Table 1).

Table 1: Antimicrobial effects of the aqueous bark extract of *Croton megalocarpus* on selected microbial strains

Concentration (µg/ml)	Zone of inhibition (mm)			
	E. coli	***S. typhimurium***	***S. aureus***	***C. albicans***
3.125	6.33±0.33^{c}	11.00±0.00^{d}	6.17±0.17^{c}	9.00±0.00^{d}
6.25	7.00±1.00^{bc}	11.00±0.00^{d}	6.50±0.29^{c}	9.67±0.33^{cd}
12.5	8.67±1.67^{bc}	11.33± 0.33^{cd}	7.16±0.44^{bc}	10.33±0.33^{bc}
25	8.83±1.30^{bc}	11.83± 0.17^{c}	7.33±0.33^{bc}	10.33±0.60^{bc}
50	9.00±1.00^{bc}	12.00±0.00^{c}	8.33±0.88^{bc}	10.66±0.44^{b}
100	10.33±2.03^{b}	13.00± 0.58^{b}	9.00±1.00^{b}	12.17±0.17^{a}
-ve control	6.00±0.00^{c}	6.00±0.00^{e}	6.00±0.00^{c}	6.00±0.00^{e}
+ve control	28.00±1.15^{a}	27.00±0.00^{a}	22.33±1.76^{a}	6.00±0.00^{e}

Values are expressed as x̄±SEM; means which do not share a lowercase alphabet superscript within the same column are significantly different (One-Way ANOVA followed by Tukey's test (p<0.05); +ve: Positive control: For *E. coli* and *S. typhimurium* it was Ciprofloxacin (10 µg); For *S. aureus* it was Streptomycin (µg), and for *C. albicans* it was Amphotericin B (µg); -ve: Negative control: DMSO (1.4 %).

Antimicrobial effects of the methanolic bark extract of C. megalocarpus on selected microbial strains

The results showed significant differences in zones of inhibition of *E. coli* by the methanolic bark extract of *C. megalocarpus*, which ranged from 7.00±0.58 mm at a concentration of 3.125 µg/ml to 15.00±1.00 mm at 100 µg/ml concentration (p<0.05; Table 2). At the lowest tested concentration of this extract (3.125 µg/ml), the zone of inhibition produced was like that in the negative control (p>0.05). The positive control drug exhibited a significantly larger inhibition zone than the zones of inhibition produced by the studied plant extract, at all concentrations, in *E. coli* (p<0.05; Table 2).

The effects of the methanolic bark extract of *C. megalocarpus* on *S. typhimurium* bacterial strain were determined in this study. The results indicated no significant differences in zones of inhibition of *S. typhimurium* by the methanolic bark extract of *C. megalocarpus* at concentrations of 3.125 µg/ml and 6.25 µg/ml (p>0.05; Table 2). Similarly, no significant differences in zones of inhibition were observed between plates treated with this extract at concentrations of 50 µg/ml and 100 µg/ml, between 25 µg/ml and 50 µg/ml, and, between 6.25 µg/ml and 12.5 µg/ml(p>0.05; Table 2). However, the standard antibiotic recorded a significantly larger inhibition zone of *S. typhimurium* compared with the zones of inhibition observed in all the extract concentrations (p<0.05).

Upon subjecting *S. aureus* bacterial strain to the methanolic bark extracts of *C. megalocarpus* at various concentrations, the results revealed no significant differences in zones of inhibition among concentrations of 12.5 µg/ml, 25 µg/ml and 50 µg/ml (p>0.05; Table 2). Generally, in this bacterial strain, a dose-dependent increase in inhibition zones was observed with significantly small zone at 3.125 µg/ml and significantly larger zone at 100 µg/ml (p<0.05; Table 2). However, the reference drug produced a significantly larger inhibition zone than the zones produced in all the other setups (p<0.05; Table 2). The susceptibility of *C. albicans* to the methanolic bark extract of *C. megalocarpus* was also investigated in this study (Table 2). The results indicated that at concentrations of

3.125 µg/ml, 6.25 µg/ml, and 12.5 µg/ml of the methanolic bark extract of *C. megalocarpus*, the zones of inhibition produced were not significantly different (p>0.05; Table 2). Similarly, the zones of inhibition observed at concentrations of 25 µg/ml, 50 µg/ml, and 100 µg/ml of the extract were significantly similar (p>0.05; Table 2). It was also noted that *C. albicans* was not susceptible to the positive control antibiotic; hence, the resultant zone of inhibition was like that of the negative control (p>0.05; Table 2).

Table 2: Antimicrobial effects of the methanolic bark extract of *Croton megalocarpus* on selected microbial strains

Concentration (µg/ml)	Zone of inhibition (mm)			
	E. coli	***S. typhimurium***	***S. aureus***	***C. albicans***
3.125	7.00 ± 0.58^{fg}	7.00 ± 0.58^{ef}	11.00 ± 1.53^{d}	9.33 ± 0.33^{b}
6.25	9.00 ± 1.00^{ef}	7.67 ± 0.88^{de}	13.67 ± 1.86^{cd}	9.00 ± 0.00^{b}
12.5	10.83 ± 1.17^{de}	9.00 ± 0.58^{cd}	15.00 ± 0.00^{bc}	9.67 ± 0.33^{b}
25	$11.6670.33^{cd}$	9.33 ± 0.33^{c}	15.67 ± 0.33^{bc}	11.00 ± 0.50^{a}
50	14.00 ± 0.58^{bc}	10.17 ± 0.44^{bc}	16.33 ± 0.67^{bc}	11.33 ± 0.33^{a}
100	15.00 ± 1.00^{b}	11.00 ± 0.58^{b}	17.67 ± 0.88^{b}	12.00 ± 0.58^{a}
-ve control	6.00 ± 0.00^{g}	6.00 ± 0.00^{f}	6.00 ± 0.00^{e}	6.00 ± 0.00^{c}
+ve control	27.67 ± 0.88^{a}	27.00 ± 0.00^{a}	24.00 ± 1.00^{a}	6.00 ± 0.00^{c}

Values are expressed as x̄±SEM; means which do not share a lowercase alphabet superscript within the same column are significantly different (One-Way ANOVA followed by Tukey's test ($p<0.05$); Positive control: For *E. coli* and *S. typhimurium* it was Ciprofloxacin (10 µg); For *S. aureus* it was Streptomycin (µg), and for *C. albicans* it was Amphotericin B (µg); Negative control: DMSO (1.4 %)

Minimum inhibitory concentrations (MICs) of the aqueous and methanolic extracts of C. megalocarpus on selected microbial strains

The MICs of the aqueous extract of *C. megalocarpus* were 100 µg/ml for both the *E. coli* and *S. aureus* bacterial strains and 25µg/ml on *S. typhimurium* and *C. albicans* microbial cultures (Table 3). On the other hand, the MICs of the methanolic bark extract of *C. megalocarpus* were 50 µg/ml for both *E. coli* and *S. aureus* and 100 µg/ml for both the *C. albicans* and *S. typhimurium* strains (Table 3).

Additionally, Ciprofloxacin (reference antibiotic) exhibited the lowest MICs on of 0.30µg/ml on *E. coli* and *S. typhimurium* bacterial cultures, while Streptomycin had a MIC of 0.62µg/ml on *S. aureus* bacterial strain. Amphotericin B showed a MIC of > 100µg/ml on *C. albicans* fungus (Table 3).

Table 3: Minimum inhibitory concentrations of the aqueous and methanolic stem bark extracts *Croton megalocarpus* on selected microbial strains

Microbial strain	Minimum inhibitory concentration(µg/ml)		
	Aqueous stem bark extract of *C. megalocarpus*	**Methanolic stem bark extract of *C. megalocarpus***	**Positive control drug**
E. coli	100	50	0.30
S. typhimurium	25	100	0.30
S. aureus	100	50	0.62
C. albicans	25	100	>100

Positive control: For *E. coli* and *S. typhimurium* it was Ciprofloxacin (10 µg); For *S. aureus* it was Streptomycin (µg), and for *C. albicans* it was Amphotericin B (µg).

Cytotoxic effects of the studied plant extracts in brine shrimp nauplii

The concentrations of the aqueous and methanolic stem bark extracts of *C. megalocarpus* that could kill 50 % of the exposed brine shrimp nauplii were determined and considered as mean lethal concentration (LC50), which indicates cytotoxic potential (Table 4). The LC50 values obtained for both extracts of *C. megalocarpus* were significantly higher than that of the positive control drug ($p<0.05$; Table 4).

Table 4: Effects of the studied aqueous and methanolic plant extracts *Croton megalocarpus* in brine shrimp nauplii

Plant extract	LC_{50} (µg/ml)
The aqueous stem bark extract of *C. megalocarpus*	486.67 ± 3.15^{a}
The methanolic stem bark extract of *C. megalocarpus*	458.33 ± 2.87^{b}
+ve control	10.00 ± 1.31^{c}

Values are presented as x̄±SEM; means with different lowercase alphabet superscript within the same column are significantly different by One-Way ANOVA followed by Tukey's test ($p<0.05$);aq: aqueous extract; met: Methanolic extract; +ve: Positive control; cyclophosphamide

Acute oral toxicity effects of the aqueous and methanolic extracts of *C. megalocarpus* on a rat model

The results showed that, at all the orally administered doses (175 mg/Kg bw, 550 mg/Kg bw, and 2000 mg/Kg bw) of the aqueous and methanolic bark extracts of *C. megalocarpus*, there were no observable signs of toxicity recorded in all the experimental rats (Table 5). Since the wellness parameters were not altered by the studied plant extracts, at all dose levels up to the limit dose of 2000 mg/Kg bw, the LD50 values of all the studied plant extracts were considered to be >2000 mg/Kg bw according to the OECD/OCDE document No. 425 guidelines.

Table 5: Acute Oral Toxicity effects of the aqueous and methanolic bark extracts of the studied plants of *C. megalocarpus* in experimental rats

Wellness parameter	Observation											
	30 minutes		4 hours		24 hours		48 hours		7 days		14 days	
	EGR	CGR	EGR	CGR	EGR	CGR	EGR	CGR	EGR	CGR	EGR	CGR
Skin and Fur appearance	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fecal matter consistency	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Urination and urine appearance	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Mucous membrane appearance	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Itching	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Salivation	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Convulsions and tremors	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Breathing	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Coma	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Aggression	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Grooming	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Teeth	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Mortality/Death	None	None	None	None	None	None	None	None	None	None	None	None

EGR: Experimental group Rats (Administered with the studied plant extracts); CGR: Control group Rats (Administered with Normal saline only)

Qualitative phytochemical composition of aqueous and methanolic bark extracts of *C. megalocarpus*

The results showed the presence of alkaloids, saponins, tannins, glycosides, flavonoids, and phenols in the aqueous and methanolic extracts of *C. megalocarpus* (Table 6). However, anthraquinones were absent in the extracts (Table 6).

Table 6: Qualitative phytochemical composition of the aqueous and methanolic bark extracts of *C. megalocarpus*

Phytochemical	Stem bark extracts of *C. megalocarpus*	
	Aqueous	Methanolic
Alkaloids	+	+
Saponins	+	+
Tannins	+	+
Glycosides	+	+
Flavonoids	+	+
Anthraquinones	-	-
Phenols	+	+

+: Present; -: Absent

DISCUSSION

Globally, over 2 million individuals are diagnosed with deadly infections, with over 20,000 deaths due to the failure of antimicrobial therapy every year. The burden of microbial infections is worsened by the rapidly increasing antimicrobial-resistant strains, which are feared to cause over 10 million deaths by the year 2050 if urgent measures are not undertaken [33]. Antimicrobial resistance is complex and has rendered the current antibiotics useless because they are incapable of modifying the course of disease [34, 35]. Therefore, there is an urgent need to find efficacious, safe, affordable, and accessible antimicrobials, especially from medicinal plants, which have a higher propensity to offer potent molecules to avert pathogenic infections [36, 37].

Medicinal plants have for long promoted health, especially in the less developed regions of the world [7, 9]. Despite the longstanding usage of herbal remedies to treat microbial infectious microbial infections, especially among the Kenyan communities, only a handful of the traditionally important medicinal plants have been empirically validated. In light of this, we investigated the antimicrobial, cytotoxicity, acute oral toxicity, and qualitative phytochemical composition of the aqueous and methanolic stem bark extracts of *C. megalocarpus* in the quest for better, efficacious antimicrobials. This plant is traditionally used to treat to treat microbial infections and related diseases among the Agikuyu community of Kenya [23, 25]; however, this plant's therapeutic potential and safety have not been investigated to date. Therefore, scientific exploration of this plant provides important data for validating its use and discovering novel antimicrobial compounds against pathogenic infections [24, 38].

This study adopted the disk diffusion and broth microdilution techniques described by the CLSI [29] to investigate the studied plant

extracts' *in vitro* antimicrobial effects. According to these methods, the sizes of inhibition zones and the minimum inhibitory concentrations (MICs) are indicators of *in vitro* antimicrobial susceptibility.

Research has indicated that in the disk diffusion assay, plant extracts that exhibit zones of inhibition of microbial growth of >6 mm have antimicrobial activity [39, 42]. In particular, zones of inhibition of between 6 mm and 9 mm indicate weak antimicrobial activity. Besides, zones of inhibition of between 9 mm and 12 mm show moderate antimicrobial activity. On the other hand, zones inhibition measuring 13-16 mm indicate high antimicrobial activity, while inhibition zones ranging from 16-19 mm have very high antimicrobial activity. Additionally, zones of inhibition with diameters ≥20 mm indicate remarkable antibiotic efficacy [40, 42, 43]. Based on these criteria, the aqueous stem bark extract of *C. megalocarpus* demonstrated weak to moderate antimicrobial activities against *E. coli* and *S. aureus* bacterial strains and moderate antimicrobial activity against *S. typhimurium* and *C. albicans* microbial strains *in vitro*. Notably, the aqueous stem bark extract of *C. megalocarpus* exhibited moderate to high antimicrobial effects against *S. typhimurium*, indicating its antimicrobial potential in treating this bacterial strain. These results corroborate those of Kariuki *et al.* [44] and Matu and Staden [45].

Furthermore, the antimicrobial activities of the methanolic stem extract of *C. megalocarpus* were graded as slight to moderate on *S. typhimurium* and *C. albicans* strains, slight to high activities for *E. coli* strain and slight to very high for the *S. aureus* strain, respectively. These results are consistent with an earlier report involving the ethyl acetate and isobutanol extracts of this plant on selected microbial strains of human pathogenic bacteria [46]. Moreover, research has shown that plant extracts with MIC values that are less than 1mg/ml (1000 µg/ml) are noteworthy and may potentially offer potent antimicrobial agents [46]. In this study, the studied plant extracts exhibited low MIC values on selected microbes. Our study demonstrates that the studied plant extracts have great potential to offer novel strong antibiotics to treat infectious diseases.

Research has shown that secondary metabolites of plants, the phytochemicals, including tannins, phenols, flavonoids, terpenoids, which are antioxidants, have antimicrobial activity, among other pharmacologic effects [13, 28, 49-51]. Consequently, the antimicrobial effects of the studied plant extracts are attributable to these phytoactive compounds. Moreover, the varying degrees of antimicrobial effects reported herein suggest that the antimicrobial-associated phytocompounds were variedly distributed in the studied plan extracts and targeted different strains better than others [13, 50]. Thus, it is suggestive that some of the antimicrobial-associated phytochemicals were extracted better with water than methanol, or otherwise, increasing their concentration and stability, hence the differences in the pharmacologic effects [52–54].

Despite the demonstrable efficacies of medicinal plants, their safety has been questioned because there is a lack of standardized guidelines for preparation, dosing, packaging, labelling, and storage of herbal preparations [21, 55]. Additionally, there are no documented dosage guidelines, contraindications, herb-herb, conventional drug- herbal drug interactions, and toxicity profiles of medicinal plants [20, 56]. Therefore, improper usage of herbal preparations could potentially result in life-threatening effects. Accordingly, we investigated the cytotoxicity and acute oral toxicity effects of the aqueous and methanolic extracts *C. megalocarpus* to provide scientific data to appraise their safety and guide future studies geared towards its utilization as a potential source of potent antibiotics.

The brine shrimp lethality assay technique is a rapid and inexpensive method widely used to screen for cytotoxic effects of plant extracts and chemicals thought of therapeutic value [31]. We adopted this method to assess the safety of the aqueous and methanolic stem bark extracts of *C. megalocarpus* in exposed brine shrimp nauplii. The plant extracts' concentration that could cause a 50 % mortality of the nauplii was considered the LC50. Previous reports indicate that a plant extract or chemical with an LC50 of < 30µg/ml is very cytotoxic, those with LC50 values of 30-100 µg/ml are toxic, and those having LC50 values of >100µg/ml have a low toxicity profile hence safe [57, 58]. Considering these criteria [57, 58], both the aqueous and methanolic extracts of *C. megalocarpus* proved to be non-toxic to brine shrimp nauplii, and thus safe as their LC50 values were >100 µg/ml.

We also evaluated the studied plant extracts' acute oral toxicity effects using the top-down procedure described by the OECD [32]. The results showed that all the studied plant extracts were non-toxic at oral doses and, therefore, safe. Therefore, the studied plant extracts may be safe for use in traditional medicine. However, extensive toxicity studies using other advanced models and in clinical settings to fully establish the safe doses and toxicity profiles.

The secondary metabolites synthesized by medicinal plants as a retort to biotic and abiotic stresses are responsible for a broad spectrum of pharmacologic properties in biological systems [15, 59, 60].

Overwhelming evidence shows that antioxidant phytocompounds, such as flavonoids, tannins, and phenols, have antimicrobial effects [61]; whereas alkaloids and anthraquinones have cytotoxic effects. Therefore, our findings suggest that the antioxidant and other antimicrobial-associated phytochemicals conferred the studied plant extracts' antimicrobial effects. Besides, their safety in brine shrimp nauplii and experimental rats is attributable to the low concentration or absence of toxic phytocompounds [62, 63]. Therefore, our study suggests that the aqueous and methanolic stem bark extracts of *C. megalocarpus* are potential alternative sources of efficacious, cheap, accessible, and safe antimicrobials.

CONCLUSIONS AND RECOMMENDATIONS

Based on the study's findings, the aqueous and methanolic stem bark extracts of *C. megalocarpus* have varied antimicrobial effects against the studied microbial strains, and they do not cause acute oral toxicity effects in Winstar rats and cytotoxicity in brine shrimp *nauplii*. Furthermore, the studied plant extracts contain antimicrobial-associated phytochemicals. Therefore, the studied plant extracts can be potential sources of alternative antimicrobial molecules. Further studies aimed at establishing the specific mechanism(s) by which the studied plant extracts exert their pharmacologic effects are encouraged. We also recommend quantitative phytochemical analysis, isolation, characterization, and optimization of antimicrobial compounds from the studied plant extracts. Moreover, antimicrobial efficacy evaluation of the studied plant extracts on other microbial strains of clinical significance should be done. Extensive and focused toxicological and safety studies of the studied plant extracts on other advanced experimental models and in clinical setups should be undertaken to establish their safety.

Data availability

All data in this study are included within the manuscript; however, any additional information is available from authors upon request.

Conflict of interest

The authors declare that there is no conflict of interest whatsoever regarding this study.

Author Contributions

Joseph Kathare conceived the research idea and performed the experiments under the close supervision of James Mbaria and Joseph Nguta. Gervason Moriasi designed, guided the experiments, and assisted with data analysis and interpretation. All authors reviewed and approved the final manuscript for publication.

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