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RESEARCH ARTICLE

PHYTOCHEMICAL VARIABILITY AND ANTIMICROBIAL ACTIVITY OF EXTRACTS OF *Citropsis articulata* LEAVES FROM THREE TROPICAL FORESTS IN UGANDA

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ABSTRACT

Empirical evidence of the presence of bioactive compounds in plants is indispensable, given that medicinal plants play a pivotal role in the quest for new antimicrobials. In this study, leaves of *Citropsis articulata* from three forest reserves (Mabira, Budongo, and Kibale) in Uganda were extracted with distilled water and methanol using infusion and maceration methods, respectively. The total phenolic (TP), total flavonoid (TF), total alkaloid (TA) and total saponin (TS) contents of the extracts were evaluated using UV-Vis spectrophotometric based methods before the fingerprint of each extract was established using high-performance liquid chromatography. *In vitro* antimicrobial activities of the extracts were examined against standard strains of common pathogenic microbes, including *Pseudomonas aeruginosa, Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* using agar well diffusion and microtitre plate-based assays. Results showed that the methanol extract had a significantly higher content of the secondary metabolites compared to the aqueous extract, with samples from Mabira showing the highest level of TP (68.92 mg GAE/g), samples from Budongo had the highest level of TF (11.28 mg QE/g) and TS (101.94 mg DE/g). In comparison, samples from Kibale showed the highest level of TA (66.29 mg AE/g). In the antimicrobial study, methanol extract showed significantly higher potency than the aqueous extract for all samples from the three locations. The plant extracts showed substantially high potency against *Escherichia coli,* with samples from Mabira exhibiting the maximum zone of inhibition (29.5±0.5 mm) at 50% w/v and lowest minimum inhibitory concentration (MIC) of 1.56% w/v. The extracts also demonstrated bactericidal (MBC) and fungicidal (MFC) potency against the test microorganisms in the order; *Escherichia coli* > *Staphylococcus aureus* > *Pseudomonas aeruginosa* > *Candida albicans*. This study demonstrated that *Citropsis articulata* leaves have potentially potent bioactive compounds that could be explored for future antimicrobial drug development.

KEYWORDS

Citropsis articulata, secondary metabolites, antibacterial, antifungal

1. INTRODUCTION

Infectious diseases have plagued humanity throughout history and are among the leading cause of morbidity and mortality globally (Cunha, 2004). Bacteria have often been central in major infections, including respiratory, wound, bloodstream and urinary tract infections (Pallavali et al., 2017). These infections are commonly caused by pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli, Staphylococcus epidermidis*, *Streptococcus pneumoniae, Klebsiella spp, Pseudomonas aeruginosa, Proteus spp, Listeria monocytogenes, Salmonella spp and Enterobacter spp* (Oliveira and Reygaert, 2019; Sizar and Unakal, 2020). Other microorganisms, including fungi have also played a major role as infectious agents; for instance, *Candida albicans* is one of the most prevalent in fungal infections, especially in immuno-compromised individuals (Mayer et al., 2013). Nevertheless, the use of antibiotics and antifungals to treat bacterial and fungal infections coupled with improved hygiene and sanitation, increased knowledge and awareness about microbes have greatly contributed to a reduction in mortality resulting from infectious diseases (WHO, 2001). However, the high genetic variability of microbes coupled with widespread overuse of antibiotics has led to an increase in clinical resistance of microorganisms (Olila and Opuda-Asibo, 2001). This predicament threatens to recreate moments in history when humanity never had effective treatments for several Infectious diseases. Thus, the relentless exploration for new antimicrobial agents remains indispensable.

To date, plants have played a central role in the quest for new antimicrobial agents and other drug developments. Medicinal plants are rich in secondary metabolites with beneficial effects on human health and in the treatment of numerous infections (Ghasemzadeh et al., 2015). These secondary metabolites include tannins, terpenoids, alkaloids, saponins, flavonoids, phenolic compounds, etc. (Das et al., 2010; Shakya, 2016). The herbal medicine sector is reported to support the health care of over 5 billion people around the globe, the highest proportion being in developing countries (WHO, 2011). Medicinal plant products are widely preferred in many parts of the world because they are easily accessible,

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economically viable, and have low toxicity compared to synthetic drugs (Atef et al., 2019). Medicinal plant extracts have also, in the countless past, proved to be potent sources of antimicrobial compounds such as Berberine from *Berberis vulgaris,* Piperine from *Piper nigrum,* and Allicin from *Allium sativum,* among others (Khameneh et al., 2019). The above-mentioned attributes of medicinal plants and their products have caused people to rely on medicinal plants as alternative means of primary health care, including *Citropsis articulata*.

Citropsis articulata Swingle & Kellerm from the family Rutaceae and well-known as the African cherry orange is a tree/shrub usually less than 5 m in height but periodically much taller (Kokwaro, 1982; Swingle et al., 1967). *Citropsis articulata* is native to countries of East, Central and West Africa, including Uganda, Tanzania, Sudan, Congo, Central African Republic, Guinea, Gabon, Nigeria, Ivory Coast, Ghana, Sierra Leone, Togo, Liberia, and Cameroon (Hutchinson and Dalziel, 1958; Kokwaro, 1982). In Uganda, *Citropsis articulata* predominantly occurs in the understory of moist evergreen forests situated primarily in and around the Albertine rift, including Budongo and Kibale forests but is also reported in Mabira forest in central Uganda (Wangalwa et al., 2021). This plant species generally has a low occurrence and has been listed as a vulnerable species in Uganda (MTWA, 2018; Wangalwa et al., 2021). *Citropsis articulata* is reported to have *aphrodisiac and antiplasmodial properties* (Lacroix et al., 2011; Vudriko et al., 2014). The plant contains citropsine A, omubioside and katimborine (Lacroix et al., 2011; Meva'a et al., 2010). However, studies on medicinal plants have indicated that environmental conditions may significantly affect the biosynthesis of secondary metabolites, which may eventually cause variation in the phytochemical content (Liu et al., 2016; Ncube et al., 2012; Yaniv and Dudai, 2014). There is seemingly no empirical evidence of how location influences the phytochemical composition and antimicrobial activity, of Uganda's *Citropsis articulata*. We therefore evaluated the phytochemical composition and investigated the antimicrobial activity of *Citropsis articulata* leaves with respect to different extraction solvents, (methanol and aqueous), preparation and habitat (Kibale, Mabira, and Budongo Forests).

2. Materials and Methods

2.1 Sample Collection

The leaves of *Citropsis articulata* were collected from three forest reserves, namely, Kibale National Park, Budongo and Mabira Central Forest Reserves (CFR). The forest reserves are distantly located in Uganda (Figure 1). Briefly, Mabira Central Forest Reserve is situated in central Uganda, between latitudes 0° 22' and 0° 36' N and longitudes 32° 50' and 33° 07' E with altitudes in the range of 1073 to 1348 m. The forest is also surrounded by three districts, i.e. Mukono, Buikwe, and Kayunga (Wangalwa et al., 2021). Budongo CFR, on the other hand, lies in the western region of Uganda between the latitudes 1° 37' and 2° 4' N and longitudes 31° 20' and 31° 48' E with altitudes ranging from 719 m to 1258 m and is surrounded by Bulisa, Hoima and Masindi districts. Kibale National Park is a tropical moist forest at a medium elevation, ranging from 926 m to 1619 m above sea level. The forest lies between latitudes 0° 13' and 0° 41' N and longitudes 30° 10' and 30° 35' E in Uganda's western region, traversing the districts of Kamwenge, Kyenjojo, and Kabarole (Wangalwa et al., 2021).

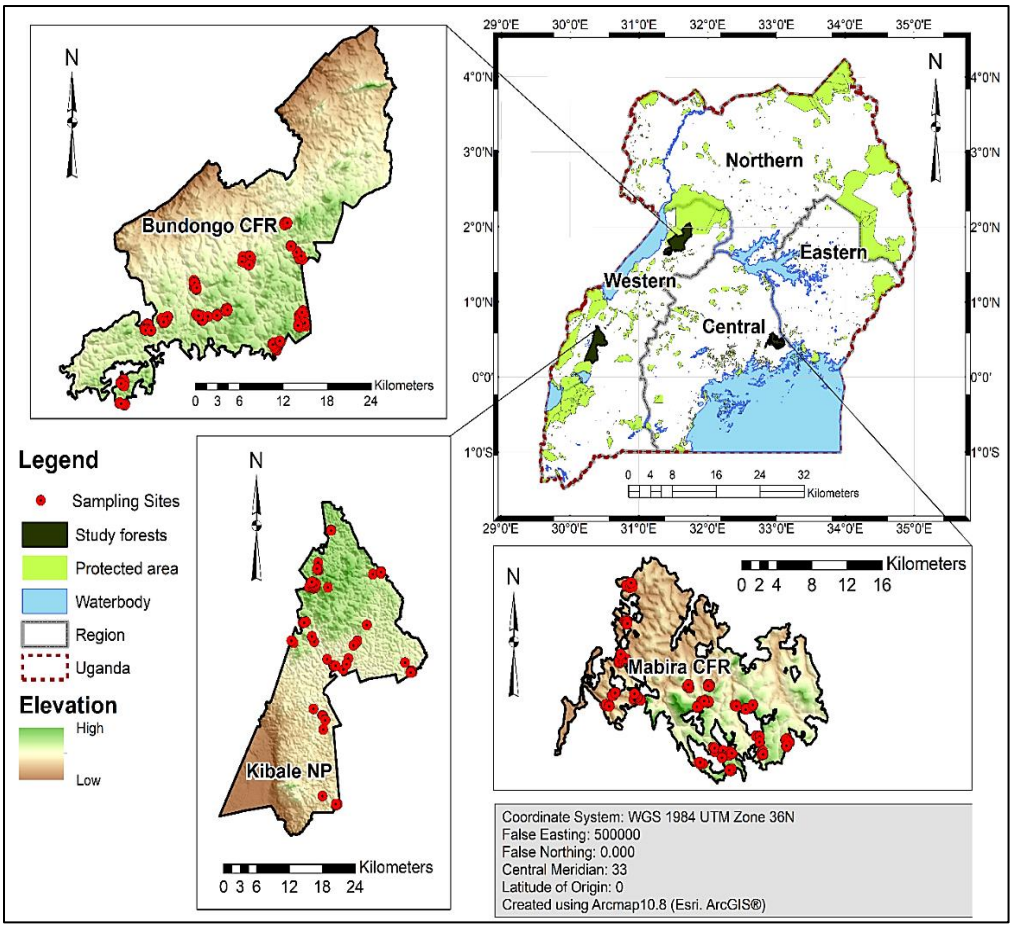

Figure 1: Map showing the study sites: Mabira Central Forest Reserve (CFR), Budongo CFR and Kibale National Park and their respective sampling sites of *C. articulata* in Uganda

2.2 Preparation of Plant Extracts

The leaf samples were washed and air-dried for four weeks in the shade at room temperature. The dried materials were pulverised mechanically (300 mesh size particles) using an electric grinder. The cold maceration method was used for methanol extract in which 100 g of the powdered material was soaked in 600 ml of 70% methanol in amber glass bottles with occasional agitation for 72h (Abubakar and Haque, 2020). The infusion method was used for aqueous extract, and 100g of the powdered material was soaked in 600 ml of boiling water for 30 minutes (Abubakar and Haque, 2020). Each extract was filtered first with muslin cloth before using Whatman No. 1 filter paper (41) to attain a clear filtrate. The filtrates were concentrated *in vacuo* at 40 °C using rotatory evaporator model RV-10 auto V-C (IKA®-Werke GmbH & Co. KG, Germany). The extracts were further lyophilised to powdered form using a freeze dryer (Model 12N, ZZKD®, China). The extract yields were calculated, and the yield percentages were computed using the following formula:

$$\text{PercentageYield (\%)} = \frac{\text{Weight of extract after evaporation of solvent}}{\text{Dry weight of the plant powder before extraction}} x\ 100$$

The extracts were kept at 4 °C in airtight sample bottles for later use.

2.3 Phytochemical Screening

Qualitative phytochemical screening for anthraquinones, quinones, alkaloids, glycosides, phenols, tannins, saponins, flavonoids, steroids, coumarins, anthocyanins, and terpenoids was performed on *Citropsis articulata* extracts using standard methods by (Roghini and Vijayalakshmi, 2018; Sofowora, 1996; Evans, 2002). About 2 g of each plant extract was dissolved in 40 ml of distilled water and filtered. The filtrate was used for phytochemical screening where applicable, as described below.

2.3.1 Test for Phenols

Three millilitres of 10% lead acetate solution were added to four millilitres of extract solution and mixed gently; the formation of a white precipitate, was positive for phenols. In a comparative test, three drops of 10% ferric chloride were added to one millilitre of extract solution; a green colouration revealed the presence of phenols.

2.3.2 Test for Alkaloids

A small portion (0.5 g) of the extract was stirred with five millilitres of 1% aqueous hydrochloric acid in a water bath and then filtered. A few drops of Dragendorff's reagent were added to one millilitre of the filtrate; the formation of an orange precipitate deduced the presence of alkaloids.

2.3.3 Test for Steroids

One millilitre of chloroform was added to one millilitre of extract solution, and a few drops of concentrated sulphuric acid were gently added; the presence of steroids was deduced by the formation of a brown ring at the junction of the two layers.

2.3.4 Test for Anthraquinones

Two drops of concentrated sulphuric acid were added to five millilitres of extract solution, followed by the addition of one millilitre of dilute ammonia solution; the formation of a rose-pink colouration deduced the presence of Anthraquinones.

2.3.5 Test for flavonoids

One millilitre of 10% aqueous sodium hydroxide was added to two millilitres of the extract solution to produce a yellow colouration, followed by the addition of dilute hydrochloric acid; the presence of flavonoids was revealed by a change in the colour of the solution from yellow to colourless. In a comparative test, 0.5 grams of the extract were dissolved in five millilitres of 95% ethanol and then filtered. About 0.5 grams of magnesium tunings were added to the filtrate, followed by a few drops of concentrated hydrochloric acid; the formation of a pink or purple colouration showed the presence of flavonoids.

2.3.6 Test for Tannins

Three drops of 1% ferric chloride solution were added to two millilitres of the extract solution; the presence of tannins was revealed by the formation of a green, blue-green or blue-black precipitate.

2.3.7 Test for Terpenoids

Two millilitres of chloroform were added to one millilitre of the extract solution, followed by the addition of two millilitres of concentrated sulphuric acid. The occurrence of a reddish-brown colouration on the interface was indicative of terpenoids' presence.

2.3.8 Test for Quinones

One millilitre of alcoholic potassium hydroxide was added to one millilitre of the extract solution; the formation of a red colouration was indicative of the presence of quinones.

2.3.9 Test for Saponins

Two millilitres of distilled water were added to two millilitres of extract solution and shaken vigorously for five minutes lengthwise; the formation of a persistent foam that lasted 20 minutes indicated the presence of saponins.

2.3.10 Test for Coumarins

One millilitre of 10% sodium hydroxide was added to one millilitre of the plant extract solution; the formation of a yellow colouration revealed the presence of coumarins.

2.3.11 Test for Anthocyanin

One millilitre of 2N sodium hydroxide was added to one millilitre of the extract solution, and the mixture was heated for five minutes at 100 °C. The formation of a bluish-green colouration confirmed the presence of anthocyanin.

2.3.12 Test for Glycosides

Three millilitres of chloroform were added to two millilitres of extract solution, followed by the addition of one millilitre of 10% ammonia; the formation of pink colouration deduced the presence of glycosides.

2.4 Plant Extract Phytochemical Fingerprinting using RP-HPLC

Ten (10) milligrams of the extract weighed using an ultra-micro balance (Model MYA 21. 4Y. P; RADWAG®, Germany) were dissolved in 10ml of de-ionised water for aqueous extract and methanol for the methanol extract to make a standard concentration of 1 mg/ml. The sample was then filtered through a Whatman cellulose nitrate membrane filter with a pore size of 0.45µm (Ref 7184-004; Whatman® GmbH, Germany). The filtrate was used for the high-performance liquid chromatography (HPLC) analysis. This analysis was conducted using ultrafast liquid chromatography (UFLC; Shimadzu, Japan). The system is composed of SIL-20A HT prominence auto-sampler, CMB-20A communicator, DGU-20A 5R prominence degasser, LC-20AD Pump, CTO-20A column oven, and SPD-20A Prominence UV-VIS Detector. The mobile phase comprised methanol, 0.01% trifluoroacetic acid in water and acetonitrile (60:30:10 respectively) and the separation was conducted using a C_{18} LC column (Luna® 5µm; C18 100Å; 250 x 4.6 mm; Phenomenex®, USA) at 40ºC, a flow rate of 0.6 millilitres per minute and 254nm as the detection wavelength.

2.5 Quantitative Determination of Phytochemical Constituents

2.5.1 Total Phenolic Content

In this study, a modified Folin-Ciocalteau method by Slinkard and Singleton (1977) was used to determine the plant extracts' total phenolic content (TPC). Two millilitres of 10% v/v Folin-Ciocalteu reagent were added to one millilitre of the standard plant extract (1mg/ml) solution, followed by the addition of two millilitres of 7.5% w/v sodium carbonate solution and then incubated at 40ºC for 30 minutes. A Jenway UV-VIS spectrophotometer (Model 6715, Jenway®, UK) was then used to determine the absorbance of the resultant solution at 765 nm. The calibration curve was made using the absorbance values recorded at varying concentrations of the reference standard, i.e. Gallic acid (6.25, 12.5, 25, 50, 100 and 200 µg/ml) at 765 nm. The total phenolic content of the plant extract was measured in milligrams of Gallic acid equivalent (GAE) per gram of extract.

2.5.2 Total Flavonoid Content

This study determined the total flavonoid content (TFC) of plant extracts using the aluminium chloride colourimetric method by Baba and Malik (2015). Four millilitres of distilled water were added to one millilitre of the standard plant extract (1mg/ml) solution, followed by 0.3 millilitres of 5% (w/v) sodium nitrite solution. The resulting solution was incubated for 6 minutes at room temperature. Subsequently, 0.3 millilitres of 10% aluminium chloride solution was added, and the resulting solution was left to stand for another six minutes at room temperature. After that, two millilitres of one molar sodium hydroxide solution were added to the solution and incubated for 10 minutes in the dark at room temperature. A Jenway UV-VIS spectrophotometer (Model 6715, Jenway®, UK) was then used to determine the absorbance of the resultant solution at a wavelength of 420 nm. The calibration curve was made using the absorbance values recorded at varying concentrations of the reference standard, i.e. Quercetin (6.25, 12.5, 25, 50, 100 and 200 µg/ml) at 420 nm. The total flavonoid content of the plant extract was measured in milligrams of Quercetin equivalent (QE) per gram of extract.

2.5.3 Total Alkaloid Content

A method by Ajanal et al. (2012) was used to determine the plant extracts' total alkaloid content (TAC). Ten milligrams (10mg) of the plant extract were dissolved in 10 millilitres of 2N HCl and then filtered using Whatman filter paper. One millilitre of the filtrate was then transferred into a separating funnel and washed with 10 millilitres of chloroform. The solution's pH was adjusted to neutral using 0.1 N NaOH solution. Afterwards, 5 millilitres of Bromocresol green (BCG) and phosphate buffer were added to the resultant solution concurrently. The resulting mixture was vigorously shaken for 3 minutes; the complex formed was extracted with 1, 2, 3 and 4 millilitres chloroform by shaking vigorously for 5 minutes. A 10-millilitre volumetric flask was used to collect the extract, and chloroform was added to a mark. A Jenway UV-VIS spectrophotometer (Model 6715, Jenway®, UK) was then used to determine the absorbance of the resultant solution at a wavelength of 430 nm. The calibration curve was made using the absorbance values recorded at varying concentrations of the reference standard, i.e. Atropine (3.125, 6.25, 12.5, 25, 50 and 100 µg/ml) at 430 nm. The total alkaloid content was measured in milligram Atropine equivalent (AE) per gram of extract.

2.5.4 Total Saponin content

A modified vanillin–sulphuric acid assay by Hiai et al. (1976) was used to determine the total saponin content of the plant extracts. About 0.5 millilitres of 8% (w/v) vanillin (HiMedia™, India) solution was added to one millilitre of the standard plant extract (1mg/ml) solution. Consequently, 5 millilitres of 72% (v/v) sulphuric acid was added to the resultant solution and mixed thoroughly. The mixture was then incubated for 15 minutes at 60°C in a shaking water bath before cooling in ice-cold water for 5 minutes. A Jenway UV-VIS spectrophotometer was used to determine the absorbance of the resultant solution at 550 nm. The calibration curve was prepared from absorbance values recorded at varying concentrations of the reference standard, i.e. Diosgenin (3.125, 6.25, 12.5, 25, 50 and 100 µg/ml) at 550 nm. The total saponin content was measured in milligram Diosgenin equivalent (DE) per gram of extract.

2.6 Preparation of Inoculum

The antibacterial activity of plant extracts was tested against standard strains of known pathogenic bacteria, including *Staphylococcus aureus* ATCC 25923 (gram-positive bacteria) and *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 as the gram-negative bacteria. The antifungal activity of plant extracts was tested against standard strains of a pathogenic fungal species (*Candida albicans* ATCC 10231). The standard stock cultures were obtained from the Microbiology Laboratory, Faculty of Medicine at Mbarara University of Science and Technology. Mueller Hinton Agar (MHA) was used in pre-culturing the bacterial strains at 37 °C for 24 hours. Meanwhile, Sabouraud Dextrose agar was used to culture *Candida albicans* at 37 °C for 48 hours. Fresh colonies of the test microorganisms were harvested and suspended in sterile saline; their concentrations were adjusted to a 0.5 McFarland standard, an equivalent of 1.5×10^8 CFU/ml (Reuk-ngam et al., 2014). The freshly prepared inoculum was used in the subsequent assays within a period not exceeding 30 minutes.

2.7 Antimicrobial Assay of Plant Extracts

The various extracts of *Citropsis articulata* were screened for antimicrobial activity using the agar well diffusion assay described by Morales et al. (2006). Briefly, surfaces of the sterile solidified Mueller Hinton Agar (MHA) [Oxoid, Thermo Scientific™, UK] for bacterial strains and Sabouraud dextrose agar (SDA) [Oxoid, Thermo Scientific™, UK] for *Candida albicans* were inoculated by spreading 1 ml of freshly prepared microbial inoculum. Consequently, a sterile cork borer was used to create 8 mm-diameter wells in agar plates containing inocula. Afterwards, 100 µl of each extract of 50% w/v prepared in Dimethyl sulfoxide (1%) for methanolic extracts and distilled water for aqueous extracts were dispensed into the wells. Two hundred microliters of two conventional antimicrobials, i.e. Ciprofloxacin (1 mg/ml) and Fluconazole (2 mg/ml), were dispensed in free wells to serve as the positive controls for the antibacterial and antifungal activities respectively. Additionally, 200 µl of the solvents used in the experiment, i.e. sterile distilled water and Dimethyl sulfoxide (1%), were dispensed into free wells to serve as the solvent controls. In contrast, an empty well was left to serve as the negative control in each activity assay. The test procedure was performed in triplicate, and the plates were incubated for 24 hours and 72 hours at 37 °C for antibacterial activity assays and antifungal activity assays, respectively. The zone of inhibition revealed on each plate after incubation was measured to establish the antibacterial and antifungal activities of *Citropsis articulata* extracts.

2.8 Determination of Minimum Inhibitory, Bactericidal and Fungicidal Concentrations (MIC, MBC and MFC)

This study also established the minimum inhibitory concentrations (MIC) for each extract. MIC is considered the lowest concentration of an antimicrobial agent that inhibits visible microbial growth after a specific period of incubation (Andrews, 2001). The MICs were determined using procedures described by the Clinical and Laboratory Standards Institute (CLSI) and methods described by Atef et al. (2019). Briefly, 100 µl of Mueller Hinton Broth and Roswell Park Memorial Institute (RPMI) 1640 medium for bacterial strains (*S. aureus, P. aeruginosa,* and *E. coli*) and fungal strain (*Candida albicans*) respectively were dispensed into 96 well microplates. Additionally, 100 µl of serially diluted (from 0.782% w/v to 25% w/v) plants extracts were dispensed to each well, followed by 100 µl of inoculum (1.5×10^6 CFU/ml) of the test organisms prepared in appropriate broth. To ensure that the contents of the wells were properly mixed, the plates were properly sealed with a sterile plate sealer and gently agitated for a few minutes. The plates were then incubated for 24 hours and 48 hours for bacterial and fungal strains respectively at 37°C. This procedure was done in triplicate for each extract. The controls included the following; 1) Growth control (well containing broth with microbial inoculum only); 2) Sterility control (a well containing broth only) and 3) Positive control [a well containing broth with microbial inoculum, and Ciprofloxacin (1 mg/ml) or Fluconazole (2 mg/ml)]. After incubation the MIC of the plant extracts were determined by dispensing 40 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT) [Loba Chemie™, India] in each well and further incubation for 2 hours at 37°C. The MIC was determined as the lowest concentration of extracts in the well that retained the original colour of the indicator (INT). Furthermore, the minimum bactericidal/fungicidal concentration (MBC/MFC) was also established for each extract. MBC or MFC is the least concentration of an antimicrobial agent that kills 99.9% of the test microorganism (Berthel et al., 2019; Misra and Sahoo, 2012). MBC and MFC were determined by subculturing 50 µl of the contents from each well that showed no growth in the MIC determination stage. The subcultures were made on Muller Hinton Agar (MHA) [Oxoid, Thermo Scientific™, UK] for bacteria and Sabouraud dextrose agar (SDA) [Oxoid, Thermo Scientific™, UK] for *Candida albicans* with incubations for 24 hours and 72 hours at 37 °C respectively. MBC and MFC were noted as the least concentrations of the plant extracts that did not exhibit microorganism growth.

2.9 Data Analysis

The quantitative measurements of general phytochemical constituents and antimicrobial activity were presented as mean ± SD where appropriate. Data were subjected to a one-way analysis of variance (ANOVA). Consequently, Tukey's test was used to determine variation in the plant extracts' phytochemical constituents and antimicrobial activity. All statistical analyses were executed at a 5% significance level in GraphPad Prism version 9.0 (GraphPad Software Inc., USA).

3. Results and Discussion

3.1 Phytochemical Composition

The percentage yield of extracts from leaves of *Citropsis articulata* collected from three forest reserves and prepared using two solvents (water and methanol) are illustrated in Figure 2. Findings indicated that the aqueous extracts of leaves collected from Kibale forest presented the highest yield (16.02%) among plant extracts. Similarly, the methanol extract of leaves collected from Kibale forest had the highest yield (10.39%). Results generally indicated that the yield of water extracts for leaves from the three locations was generally higher than methanolic extracts. The high yield observed in the water extract could result from the high polarity of water as a solvent and the high temperature used during extraction (Dhanani et al., 2017; Ghasemzadeh et al., 2015).

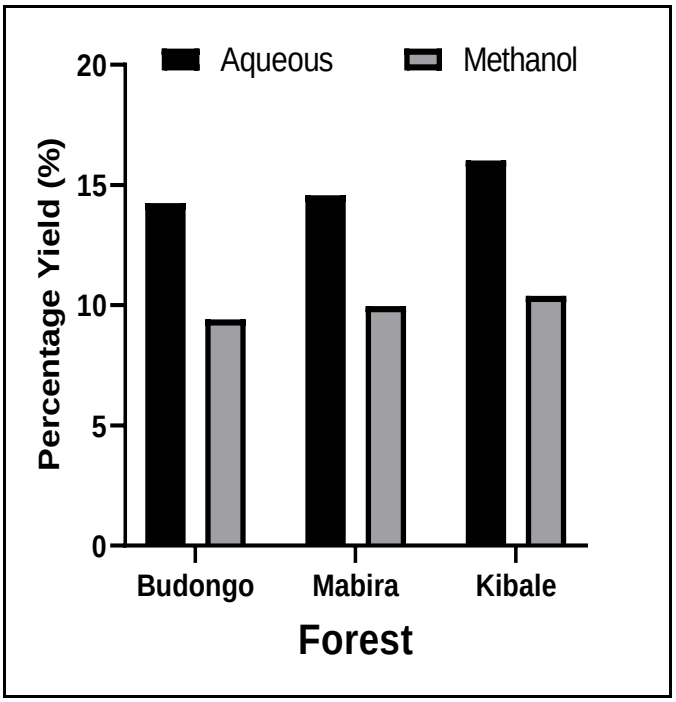

Figure 2: Percentage yield of aqueous and methanol extract of *C. articulata* leaves collected from three tropical forests of Uganda.

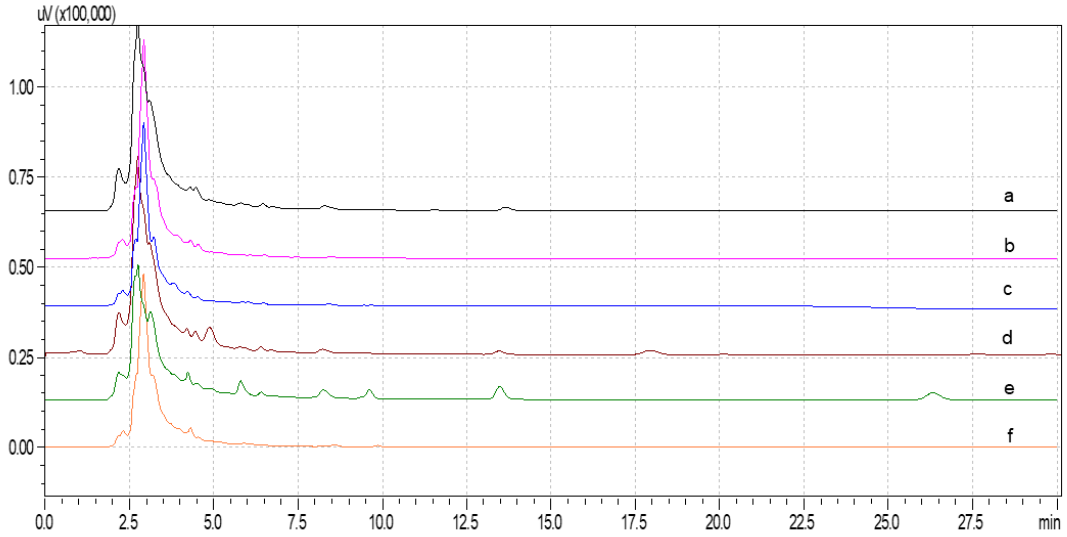

Figure 3: The HPLC chromatogram of *Citropsis articulata* leaf extracts; a) & b) methanol and aqueous extracts of samples from Mabira Central Forest Reserve respectively; c) & d) aqueous and methanol extracts of samples from Kibale national park respectively; e) & f) methanol and aqueous extracts of samples from Budongo Central Forest Reserve respectively

Table 1: Preliminary phytochemical screening of leaf extracts of *C. articulata.*

Phytochemical	Aqueous			Methanol		
	Budongo CFR	Mabira CFR	Kibale NP	Budongo CFR	Mabira CFR	Kibale NP
Anthraquinones	+	+	++	++	++	+
Quinones	-	-	-	-	-	-
Alkaloids	++	+	++	+++	++	++
Glycosides	++	+++	++	++	+++	++
Phenols	+++	+++	+++	+++	+++	+++
Tannins	+	+	+	+	+	+
Flavonoids	++	++	++	+++	+++	+++
Saponins	++	++	++	+++	+++	+++
Steroids	++	+++	++	++	+++	+++
Terpenoids	++	+++	++	+++	+++	++
Anthocyanins	-	-	-	-	-	-
Coumarins	++	++	++	+++	+++	+++

+ Presence of phytochemical constituent: + trace; ++ moderate; +++ adequate

Phytochemical analysis results showed the presence of several phytochemicals, including saponins, alkaloids, glycosides, phenols, tannins, anthraquinones, terpenoids, flavonoids, coumarins and steroids in the aqueous and methanol extracts of leaves collected from the three forest reserves (Table 1). Furthermore, the HPLC fingerprint indicated varying characteristic peaks in the methanol and aqueous extracts (Figure 3) of leaves collected from the three forest reserves. The HPLC chromatograms generally showed more characteristic peaks in the methanol extract than the aqueous extract in all three forest reserves. This variation could be attributed to the affinity of methanol to extract both lipophilic and hydrophilic components. Considering the methanol extract, samples from Mabira forest had the highest number (31) of peaks compared to Kibale national park with 29 peaks and Budongo forest with 26 peaks (Table 2). On the other hand, when considering the water extract, samples from Kibale forest had the highest number (28) of peaks compared to Budongo forest with 27 characteristic peaks and Mabira forest with 24 peaks (Table 2). The variability in the HPLC fingerprint observed in this study as a result of varying geolocations may be attributed to variability in the microclimatic conditions at growth sites of *Citropsis articulata* in the three forests; because these are known to affect the composition of secondary metabolites in plants (Liu et al., 2015). Additionally, the varying abilities of the extraction solvents to extract different phytochemical constituents may also account for the variation in the chromatogram among the solvents. The HPLC chemical fingerprints obtained in this study can play a vital role in representing the chemical integrities, identifying the plant extracts, and monitoring the quality control of herbal medicine products associated with this plant species.

The aqueous and methanol extracts of *Citropsis articulata* leaves collected from three different forests in Uganda were also assessed for variability in phytochemical composition. As indicated in Figure 4a, the concentrations of TPC exhibited significant ($p<0.05$) variability in leaves collected from the different locations as well as the solvent of extraction. The methanol extracts indicated significantly ($p<0.05$) high levels of TPC compared to the aqueous extracts; this could be resulting from the generally high solubility of phenolic compounds in alcohols such as methanol when compared to water as the solvent of extraction (Chew et al., 2011). Methanol extract of the leaves collected from the Mabira forest showed the highest level of TPC (68.92 mg GAE/g), while Budongo forest had the lowest level of TPC (51.65 mg GAE/g). The variability in phenolic content resulting from a change in location of *Citropsis articulata* habitats may be a result of the effect of environmental variability on the accumulation of secondary metabolites in plants (Samaniego et al., 2020). Findings of this study showed that *Citropsis articulata* leaf extracts generally had a higher TPC than that reported previously for other medicinal plants in the same family, such as *Ruta chalepensis* [21.1 mg GAE/g] (Alali et al., 2007; Kolli et al., 2015). Phenolic compounds have numerous chemopreventive properties such as antioxidant, anticarcinogenic, antimutagenic, anti-inflammatory and antimicrobial effects (Huang et al., 2010; Miklasińska-Majdanik et al., 2018). Therefore, reasonably high phenolic content in the *Citropsis articulata* leaf extracts points to this plant species' potentially high medicinal value.

Flavonoids are a significant class of natural products widely found in plants. They are known to have a broad array of health benefits, such as anticarcinogenic, antioxidative, and anti-inflammatory properties (Panche et al., 2016). In this study, total flavonoid content in leaves of *Citropsis articulata* collected from three different forests in Uganda was evaluated. Results indicated that the concentrations of TFC varied significantly ($p<0.05$) in leaves collected from different locations as well as the solvent of extraction (Figure 4b). The methanol extracts exhibited significantly ($p<0.05$) high levels of TFC compared to the aqueous extracts. Methanol

extract of the leaves collected from Bundongo forest showed the highest level of TFC (11.28 mg QE/g), while Mabira forest had the lowest level (with 5.09 mg QE/g). Similarly, samples from Budongo forest showed the highest level of TFC (7.15 mg QE/g), while Mabira forest samples had the lowest level of TFC (3.38 mg GAE/g) in the aqueous extracts. Variation in the TFC with location can be attributed to environmental variability and a possible variation in the genetics of the populations of *Citropsis articulata* from the three forests (Liu et al., 2015; Ghasemzadeh et al., 2015). The results also demonstrated that *Citropsis articulata* leaf extracts generally have a higher TFC than other medicinal plants from the same family such as *Citrus bergamia* [2.11 mg/g] (Yoo et al., 2008). Therefore, *Citropsis articulata* can be a valuable source of flavonoid compounds.

Many plant species contain alkaloids, a group of organic nitrogen-containing compounds. Several alkaloids are valuable medicinal agents that can treat various diseases caused by pathogenic fungi and bacteria (Ain et al., 2016; Marutescu et al., 2017). Total alkaloid content (TAC) in *Citropsis articulata* leaves investigated in this study showed significant ($p<0.05$) variability in leaves collected from different locations as well as the solvent of extraction (Figure 4c). The methanol extracts showed significantly ($p <0.05$) high levels of TAC compared to the aqueous extracts. Methanol extract of the leaves collected from Kibale forest showed the highest level of TAC (66.29 mg AE/g), while those of Budongo forest had the lowest level of TAC (50.55 mg AE/g). Kibale forest also showed the highest level of TAC (28.94 mg AE/g), and Budongo forest had the lowest level of TAC (17.58 mg AE/g) in the aqueous extracts as well. The variation in alkaloid content between solvents may result from the varying polarities of the extraction solvents, resulting in a wide range of variations in the concentration of bioactive compounds of plant extracts (Truong et al., 2019). For instance, alkaloidal compounds generally occur naturally as free bases or their salts, and these typically exhibit substantial variation in their solubility in different solvents. The free bases are generally more soluble in organic solvents, including lower alcohols such as methanol, while sparingly soluble in water, and the reverse is true for most alkaloidal salts (Kukula-Koch and Widelski, 2017; Verpoorte, 2005). The complexity in the chemical nature and composition of alkaloids in the plant extracts could be responsible for the variability in the TAC, as revealed in this study.

Saponins are a class of compounds distinguished by the presence of a steroidal aglycone or triterpene as well as a sugar chain(s) in their structural composition (Güçlü-Üstündağ and Mazza, 2007). Findings on the total saponin content (TSC) in *Citropsis articulata* leaves indicated that both the location and extraction solvent significantly ($p <0.05$) affected TSC (Figure 4d). The methanol extracts exhibited significantly ($p <0.05$) high levels of TSC compared to the aqueous extracts. Methanol extract of the leaves collected from Budongo forest showed the highest level of TSC (101.94 mg DE/g), while Mabira forest had the lowest content of total saponins (94.00 mg DE/g). Budongo forest also showed the highest level of TSC (56.54 mg DE/g), while Mabira forest had the lowest level of TSC (34.06 mg DE/g) in aqueous extracts. It is important to note that saponins have a number of health-promoting effects such as anticarcinogenic, cholesterol decreasing, anti-inflammatory and anti-microbial properties (Schoenlechner et al., 2008). Therefore, high levels of saponins in the leaves of *Citropsis articulata,* which ranged between 34.06 and 101.94 mg DE/g, indicate a valuable role in the medicinal potential of this plant species.

Table 2: HPLC chromatogram attributes of *C. articulata* extracts of leaves from three forest reserves in Uganda

	Methanol							Aqueous					
	Mabira		Kibale		Budongo			Kibale		Budongo		Mabira	
Peak	RT	Area%	RT	Area%	RT	Area%		RT	Area%	RT	Area%	RT	Area%
1	2.179	6.920	1.014	0.561	2.173	5.770		1.656	0.085	2.171	1.496	2.301	5.119
2	2.743	43.026	2.181	6.400	2.747	33.769		2.175	1.629	2.311	2.658	2.697	8.125
3	3.085	30.470	2.741	42.242	3.121	23.202		2.300	2.416	2.916	75.739	2.918	40.304
4	4.292	2.515	3.080	28.830	3.793	3.901		2.685	10.231	3.946	2.963	3.242	26.388
5	4.462	3.525	4.195	2.997	4.223	4.549		2.921	44.329	4.165	1.043	3.995	2.147
6	4.850	1.808	4.445	2.869	4.466	2.746		3.212	19.551	4.307	3.280	4.108	1.898
7	5.151	1.976	4.872	6.861	4.731	1.242		3.800	6.661	4.527	2.110	4.317	4.925
8	5.801	2.195	5.771	1.753	4.897	3.570		4.215	3.249	4.823	1.431	4.818	3.492
9	6.450	1.137	6.393	1.032	5.791	3.876		4.515	2.351	5.127	2.047	5.423	1.272
10	6.749	0.979	6.691	0.849	6.402	1.388		4.923	1.274	5.666	0.506	5.814	0.384
11	7.389	0.756	7.286	0.519	6.763	1.139		5.182	0.859	5.879	0.770	5.961	0.590
12	7.958	0.220	7.899	0.193	7.312	0.756		5.427	1.019	6.075	0.939	6.224	0.306
13	8.281	1.429	8.212	1.113	7.587	0.340		5.843	0.822	6.501	1.332	6.446	0.688
14	9.070	0.228	8.978	0.158	8.241	2.978		6.018	0.883	7.568	0.442	6.976	0.621
15	9.503	0.246	9.356	0.149	8.856	0.374		6.461	0.674	7.807	0.191	7.607	0.888
16	9.657	0.341	9.572	0.161	9.611	2.081		6.752	0.518	8.182	0.297	8.076	0.358
17	10.237	0.282	10.121	0.059	10.104	0.294		7.410	0.347	8.574	0.770	8.569	0.447
18	10.793	0.033	11.114	0.051	10.565	0.183		7.664	0.197	9.030	0.107	9.133	0.301
19	10.900	0.037	11.384	0.070	11.128	0.471		7.959	0.329	9.125	0.194	10.308	0.793
20	11.000	0.047	13.462	0.545	11.400	0.323		8.404	0.588	9.859	0.652	10.867	0.304
21	11.108	0.038	17.938	1.245	12.173	0.871		9.452	0.124	10.520	0.171	11.358	0.293
22	11.242	0.091	20.128	0.198	13.476	3.520		9.668	0.134	11.122	0.051	11.865	0.235
23	11.546	0.223	21.942	0.110	17.696	0.052		13.542	0.089	11.402	0.217	12.308	0.076
24	12.034	0.114	22.033	0.070	19.766	0.048		15.077	0.110	12.174	0.101	12.917	0.046
25	12.330	0.154	22.723	0.177	26.322	2.377		15.268	0.114	13.803	0.175		
26	12.704	0.157	23.646	0.206	28.485	0.181		21.783	0.056	14.900	0.149		
27	13.659	0.833	26.544	0.109				22.042	0.073	15.181	0.170		
28	15.258	0.033	27.671	0.350				22.151	1.290				
29	15.958	0.047	29.806	0.123									
30	26.869	0.108											
31	29.079	0.036											

3.2 Antimicrobial Activity

The aqueous and methanol extracts of *Citropsis articulata* leaf samples collected from different forests at a 50% w/v showed variability in anti-microbial properties against the standard microbial strains. Results showed that both the methanol and aqueous leaf extracts efficiently suppressed the growth of the selected microorganisms, with the methanol extract showing higher potency. As indicated in Table 3, the methanol extract of leaves collected from Mabira forest had the maximum zone of inhibition against *Escherichia coli* (29.5±0.5 mm). In contrast, the aqueous extract of leaves collected from Kibale forest had the lowest zone of inhibition against *Escherichia coli* (19.3±1.0 mm). The methanol extract of leaves collected from Mabira and Budongo forests exhibited the highest zone of inhibition against *Staphylococcus aureus* (23.5±1.0 and 23.5±0.5 mm, respectively). In contrast, the aqueous extract of leaves collected from Kibale forest exhibited the lowest zone of inhibition against *Staphylococcus aureus* (18.5±0.5 mm). The methanol extract of leaves collected from Mabira forest displayed the highest zone of inhibition against *Pseudomonas aeruginosa* (23.0±1.0 mm), while the aqueous extract of leaves collected from Budongo forest had the lowest zone of inhibition against *Pseudomonas aeruginosa* (16.0±1.0 mm). The methanol extracts also yielded valuable results against *Candida albicans,* with leaves from Mabira forest showing the highest zone of inhibition (20.0±1.0 mm) in the antifungal analysis. The antimicrobial activity exhibited by the leaf extracts of *Citropsis articulata* offers support to previous studies that have shown significant antimicrobial activity of plant extracts from family Rutaceae including *Teclea afzelii, Murraya koenigii, Ptaeroxylon obliquum* and *Aegle marmelos* (Meena et al., 2016; Kuete et al., 2008; Vats et al., 2011; Ramadwa et al., 2019). The generally high potency of the methanol extract against the microbial strains may be attributed to the variability in the chemical constituents extracted by the different solvents. This phenomenon is supported by the phytochemical results shown in Figure 4, where the methanol extract generally had more chemical components than the aqueous extract. These findings align with similar studies, e.g. (Al-Hashimi, 2012; Borges et al., 2020; Ghasemzadeh et al., 2015). The variation in the potency of the extracts resulting from varying locations may also be attributed to variation in the phytochemical constituents in the extracts collected from different forest reserves, as exhibited in Figure 4.

Table 3: Diameter of zones of inhibition (mm) of leaf extracts of *C. articulata* against four test microorganisms at 50% w/v concentration

Microbial Test Strains	Zone of inhibition (mm)*					
	Budongo		Mabira		Kibale	
	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol
Escherichia coli	21.5±0.5^{c,d}	23.5±1.5^{b,c}	26.0±1.0^{b}	29.5±0.5^{a}	19.3±1.0^{d}	23.5±0.5^{b,c}
Staphylococcus aureus	20.5±1.5^{b,c}	23.5±1.0^{a,b}	21.5±1.5^{a,b}	23.5±0.5^{a}	18.5±0.5^{c}	20.2±1.0^{b,c}
Pseudomonas aeruginosa	16.0±1.0^{d}	17.5±0.5^{c,d}	19.3±0.5^{b,c}	23.0±1.0^{a}	18.0±1.0^{b,c,d}	20.0±1.0^{b}
Candida albicans	11.3±0.6^{c}	16.7±2.0^{a,b}	14.0±1.0^{b,c}	20.0±1.0^{a}	11.3±1.5^{c}	16.0±1.0^{b}

*Values are means of triplicate measurements ± standard deviations. Means that do not share a letter in the same row are significantly different (P < 0.05) based on Tukey's multiple comparison test.

Results showed that the MIC values recorded in this study ranged from 1.56 to 25% w/v of the extracts on the selected test microorganisms (Table 4). The results also revealed that the MICs varied among plant extracts, with the least MIC recorded in the methanol extracts. The least MIC was exhibited by methanol extract of leaves collected from Mabira forest against *E. coli* (1.56% w/v). On the other hand, the aqueous extract of leaves collected from Budongo and Kibale forests exhibited the highest MIC (25% w/v) against *P. aeruginosa* and *Candida albicans.* On another note, the findings of this study indicated variability in the bactericidal potency of the leaf extracts of *Citropsis articulata* (Table 5). The methanol extract generally exhibited the lowest MBC values than the corresponding aqueous extracts. The lowest MBC value was shown by methanol extract of leaves collected from Mabira forest against *E. coli* (2.08% w/v).

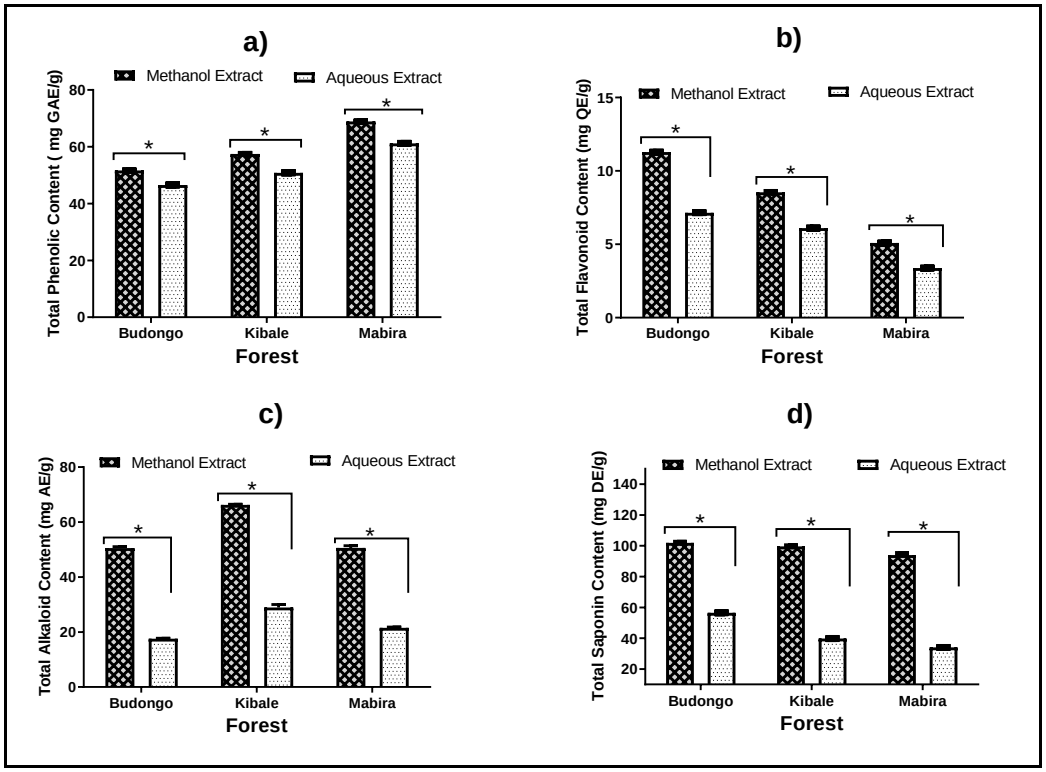

Figure 4: Variation in: a) Total phenolic; b) total flavonoid; c) total alkaloid and d) total saponin content of *C. articulata* leaves extracted with different solvents and collected from three tropical forests of Uganda. All analyses are the mean of triplicate measurements ± standard deviation. On the graph, bars with * were significantly different at p < 0.05. GAE, Galic acid equivalent; QE, Quercetin equivalent; AE, Atropine equivalent; DE, Diosgenin equivalents

Table 4: Minimum Inhibitory Concentration (MIC) of leaf extracts of *C. articulata* against selected test microorganisms

Microbial Test Strains	Minimum Inhibitory Concentration (% w/v)							
	Budongo		Mabira			Kibale		
	Aqueous	Methanol	Aqueous	Methanol		Aqueous	Methanol	
Escherichia coli	12.50	3.13	12.50	1.56		12.50	3.13	
Staphylococcus aureus	12.50	3.13	12.50	3.13		12.50	3.13	
Pseudomonas aeruginosa	25.00	6.25	12.50	3.13		25.00	6.25	
Candida albicans	25.00	6.25	25.00	6.25		25.00	6.25	

Table 5: Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of leaf extracts of *C. articulata* against selected test microorganisms

Microbial Test Strains	Minimum Bactericidal/fungicidal Concentration (% w/v)							
	Budongo		Mabira			Kibale		
	Aqueous	Methanol	Aqueous	Methanol		Aqueous	Methanol	
Escherichia coli	25.0	5.21	25.00	2.08		25.00	6.25	
Staphylococcus aureus	25.0	6.25	25.00	6.25		25.00	6.25	
Pseudomonas aeruginosa	N	12.50	N	6.25		N	12.50	
Candida albicans	N	12.50	N	12.50		N	12.50	

N- No minimum bactericidal/fungicidal concentration found

The aqueous extracts appeared to be generally less active, with bactericidal potency only observed in *E. coli* and *S. aureus.* In contrast, no MBC was recorded in the aqueous extract against *P. aeruginosa*. These findings align with similar studies on medicinal plants (Elisha et al., 2017; Masoko and Makgapeetja, 2015). Regarding antifungal analysis (MFC), the Methanol extracts of leaves collected from the three forest reserves showed a similar MFC value (12.5% w/v) against *Candida albicans*, whereas the aqueous extract did not record MFC against *Candida albicans* for leaves collected from the three forest reserves. Important to note is that presence of phytochemical constituents such as phenolic compounds, alkaloids and saponins (Figure 4) may explain the observed antimicrobial activity of *Citropsis articulata* leaf extracts. These chemical components are known to induce microbial cell death by interacting with proteins of the cell membrane, which leads to its damage and, consequently, leakage of intracellular materials (Burt, 2004; Juven et al., 1994). The differences in the inhibition potency (MIC) among the gram-positive and gram-negative bacteria are explained by the variation in their cell wall and cell membrane compositions (Henley-Smith et al., 2014). Additionally, variability in the potency (MIC and MBC/MFC) of the extracts on the tested microorganisms may be attributed to the variation in the phytochemical constituents of the different extracts based on the solvent and method of extraction (Ghasemzadeh et al., 2015; Mostafa et al., 2018). Since methanol is better at extracting numerous phytochemicals, including phenols, which are known to have antimicrobial activity (Alo et al., 2012), it could explain the high potency of methanol extracts compared to the aqueous extracts. Variability in the antimicrobial potency (MIC and MBC) due to location (different forests) may be explained by the variation in the chemical constituents in the leaves resulting from varying environmental conditions in the habitat of *Citropsis articulata*. Environmental variability in soils, altitude, temperature, illumination, precipitation, and humidity in different production locations or habitats often affects the content of plants' active ingredients (Ncube et al., 2012; Liu et al., 2016; Dong et al., 2011). Therefore variation in the environmental conditions such as altitude and soil factors in the three forest reserves, as reported by Wangalwa et al. (2021), could explain the variability in the phytochemical composition and consequently the inhibition, bactericidal and fungicidal potency of the plant extracts of *Citropsis articulata*. Other factors such as genotype and age of the plant are also known to affect the phytochemical constituents of a plant (Samaniego et al., 2020); therefore, such factors could have contributed to the variability in the inhibition potency of *Citropsis articulata*.

4. Conclusion

The present study showed that methanol, when compared to water, is the best solvent for extracting bioactive compounds from leaves of *Citropsis articulata* since it resulted in the highest content of generic phytochemical constituents. This study also presented a substantial level of variability in the composition and content of secondary metabolites in leaf samples with respect to the location of the habitat of *Citropsis articulata*. Therefore, ex-situ programs such as commercial production/cultivation should pay particular attention to the selection of *Citropsis articulata* accessions with high phytochemical content while also considering the environmental conditions of the habitat. This study has also demonstrated that *Citropsis articulata* leaf extracts have both antibacterial and antifungal potency, with generally high potency recorded against bacteria, thus pointing to the fact that *Citropsis articulata* leaf extracts have potentially potent bioactive compounds that can possibly contribute to the development of antimicrobial agents.

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