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Phytochemical Investigation and Antimicrobial Activity of *Cyperus kyllingia* Root Extracts against *Staphylococcus aureus* and *Escherichia coli*

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ABSTRACT

Antibiotic resistance has been a global concern which prompted the need to look for effective and safer natural alternatives as a potential source of novel drugs to treat infections. The main goal of this study was to evaluate the antimicrobial activity of *Cyperus kyllingia* aqueous (CkA), chloroform (CkC), and methanol (CkM) root extracts. Phytochemical screening was done to assess for secondary metabolites present while disk diffusion technique was followed to evaluate the extracts' antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. Results showed that alkaloids and phlobatannins were present in CkA, CkC, and CkM extracts. Terpenoids were detected in CkA and CkC, while tannins, steroids, flavonoids, and volatile oils were present in CkC and CkM extracts. *S. aureus* was found to be more susceptible to CkM at 100 mg ml^{-1} concentration with a mean inhibition zone of 18.33±1.53. However, CkA (14.67±4.93) and CkC (14.67±2.89) showed slight inhibitory activity against the bacteria at the same concentration. The little inhibitory effect was also observed for CkC (14.33±2.08), CkM (14.33±4.04), and CkA (14.33±4.93) extracts against *E. coli*. The overall result of this study suggests that the root extracts of *C. kyllingia* have the potential in suppressing bacterial growth recommending that further studies be done on the plant's bioactive constituents that may have the potential in combating drug-resistant bacteria.

Keywords: *Antimicrobials, Cyperus kyllingia, drug resistance, human pathogens, secondary metabolites*

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1 Introduction

Antimicrobial resistance continues to be a threat in the prevention and treatment of infections caused by pathogens. More alarming is the spread of multi-and pan-resistant bacteria known as "superbugs," which cause infections not treatable with the existing antibiotics (WHO, 2015). This prompts the search for new and effective anti-infective agents.

Plants have been widely ventured in searching for bioactive compounds with antimicrobial properties. The literature is rich with published research evaluating different plant extracts' potential antimicrobial activities. The invasive weed *Cyperus kyllingia* (Hutch & Dalz) (Family: Cyperaceae), locally known as busikad, is distributed all over pan-tropical countries worldwide (Majunder, 2013). Synonyms include *Cyperus kyllingia* Endl, *Kyllingia monocephala* Rottb, and *Kyllingia cephalotes* (Jacq.). Common names include whitehead spike sedge, white kyllingia, whitewater sedge, white-flowered kyllingia, and poverty grass (Raju et al., 2011).

Ethnomedicinally, the leaves were used as anti-venom and relief from malarial chills and allegedly reported to cure pruritus of the skin and thirst attributable to both fever and diabetes (Sindhu et al., 2014). On the other hand, the rhizomes are fragrant, sweet, refrigerant, antidiarrheal, diuretic, stomachic, and anthelmintic (Majunder, 2013). The roots' paste is treated to sprains

Volume 3 | Issue 1 | December 2021
PRINT ISSN: 2408-3623 | ONLINE ISSN: 2408 3631

and contusions (Karithkeyan et al., 2009). It has also been observed that the rhizome paste mixed with milk is efficient in curing ailments such as worm infection, fever, hepatopathy, diabetes, and tumors (Majunder, 2013; Sindhu et al., 2014). The root of *C. kyllingia* can become an essential source of novel drugs to treat certain human diseases (Khamsan et al., 2011;Quanico et al., 2008). Its roots had been used as a diuretic, demulcent, refrigerant, and antipyretic, prescribed for fistula, pustules, tumors, measles, diarrhea, and other intestinal infections (Raju et al., 2011). However, root extracts of the plant have not been assessed for their antimicrobial potential against common human pathogens. Thus, this study aimed to examine the phytochemical constituents and evaluate the antimicrobial activity of the different root extracts of *C. kyllingia* against selected human pathogens—*Staphylococcus aureus* and *Escherichia coli*.

2 Materials and Methods

Plant Sample

Plant samples of *C. kyllingia* were collected in the middle grounds of the New Science Building of Caraga State University, Ampayon, Butuan City (8° 57' 18.746" North by 125° 35' 49.286" East) where vegetation mainly consists of grasses and a few trees. Taxonomic identification of the plant was made by Ms. Rowena Japitana-Ligalig, a Botanist from the Department of Biology, Caraga State University.

Enough samples of the plant roots were cut and washed with running water to remove dirt, and unwanted material, then rinsed with distilled water. The roots were further cut into smaller pieces to approximately 3 cm and air-dried for two weeks. Mortar and pestle were used to produce ground samples of the air-dried roots. The ground plant material was then stored in an airtight container until use (Gupta et al., 2015).

Preparation of the Plant Extracts

The plant root extraction procedure was adapted from Vinoth et al. (2012) with slight modifications. One thousand (1000) grams of air-dried roots were soaked with 1 L of chloroform for 24 hours. The same weight of samples was soaked with 1 L of methanol. The resulting extract was filtered separately with Whatman filter paper No. 1 and concentrated in vacuo at 40°C using a rotary evaporator (Yamato/ Cole Palmer A-286) to afford methanol (CkM) and chloroform (CkC) extracts. On the other hand, *C. kyllingia* aqueous extract (CkA) was prepared via the decoction process. A 1000 g of dried root samples were added with 1 L of distilled water and boiled for 1 hour. The resulting aqueous extract was filtered and subjected to freeze-drying at the Chemistry Department, Mindanao State University-Iligan Institute of Technology, Iligan City, Philippines. The different concentrations of extracts (50 mg ml^{-1}, 75 mg ml^{-1}, and 100 mg ml^{-1}) were then loaded into sterilized tubes and subjected to qualitative phytochemical screening.

Phytochemical Screening

The qualitative test was adopted from the standard phytochemical methods of Vinoth et al. (2012). Extracts of different solvents were analyzed for the presence of terpenoids, alkaloids, saponins, reducing sugar, flavonoids, steroids, tannins, glycosides, phlobatannin, and volatile oils. The test was repeated in triplicates.

Test for Alkaloids

A 0.2 mg of each plant extract was placed in a test tube mixed with 2% of sulphuric acid and was warmed for two minutes. The solution was filtered in separate test tubes, and a few drops of Dragendorff's reagent was added. The appearance of orange-red precipitates indicated the presence of alkaloids.

Test for Terpenoids

A 0.5 mg of each plant extract was placed in separate test tubes containing 2 ml of chloroform. A concentrated sulphuric acid was carefully added to form a layer. The presence of a red-brown color interface validated the presence of terpenoids.

Test for Reducing Sugar

Three (3) test tubes with 2 ml of each plant extract were added with 5 ml of distilled water and added five to eight drops of Fehling's solution. It was heated over a water bath. Redbrick precipitates indicated the presence of reducing sugars.

Test for Saponins

Saponins were determined using the froth test. Each test tube was loaded with 1 g of plant

Volume 3 | Issue 1 | December 2021
PRINT ISSN: 2408-3623 | ONLINE ISSN: 2408 3631

material with 10 ml of distilled water, boiled for 5 minutes, and filtered. The mixture was filtered, and 2.5 ml was added to a test tube with 10 ml of distilled water. The mixture was shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. Honeycomb froth indicated the presence of saponins.

Test for Tannins

A small quantity of each plant extract was mixed with distilled water and heated on a water bath. The mixtures were filtered, and ferric chloride was added. The occurrence of a dark green color validated the presence of tannins.

Test for Steroids

Each plant extract was added with 2 ml of acetic acid and 2 ml of sulphuric acid. The change of color from violet to blue or green indicated the presence of steroids.

Test for Phlobatannin

A 0.5 mg of plant extracts in each test tube was dissolved in distilled water and filtered. The filtrate was boiled with 2% of HCl solution. The formation of red precipitates was an indication of the presence of phlobatanin.

Test for Glycosides

A 25 ml of diluted sulphuric acid was added to 5 ml of plant extracts in different test tubes and boiled for 15 minutes. The mixture was left to cool down and were neutralized by adding 10% of NaOH. Five (5) ml of Fehling's solution was also added, and the formation of red brick precipitates indicated the presence of glycosides.

Test for Flavonoids and Flavones

A 4 ml of extracts were added with 1.5 ml of 50% methanol solution, warmed, and mixed with metal magnesium. Five to 6 drops of concentrated HCl were added to the settlement. Manifestation of a red color indicated the presence of flavonoids and an orange color for flavones.

Test for Volatile Oils

A 2 ml of plant extracts were loaded on separate test tubes shaken with 0.1 ml of diluted NaOH and a small quantity of diluted HCl. The presence of white precipitates confirms the presence of volatile oils.

Antibacterial Assay

Source of Microorganisms

Pure strains of gram-positive *Staphylococcus aureus* (BIOTECH 1582) and gram-negative Escherichia coli (BIOTECH 1634) were obtained from the University of the Philippines, Manila, Philippines. Before use, it was maintained and preserved on nutrient agar slants at 4°C.

Antimicrobial Assay

The paper disk diffusion technique described by Tendencia (2004) was adopted with slight modifications. Nutrient agar was used as a medium.

Preparation of Nutrient Agar (NA)

NA was prepared as per the standard protocol described in the work of Hudzicki (2009). The medium was heated with frequent agitation and boiled to dissolve completely. It was sterilized for 15 minutes at 121°C in the autoclave. The agar medium was cooled to 40-50°C, poured into sterile glass petri dishes, and incubated with lids partly ajar at 37°C for 30 minutes.

Preparation of Inoculum

Five colonies from pure bacterial cultures of *S. aureus* and *E. coli* were taken using a wire loop and were transferred to separate sterile test tubes containing 5 ml of nutrient broth. The broth was incubated at 30°C until it reached the turbidity of 0.5 McFarland standard (Clinical and Laboratory Standards Institute, 2014).

Inoculation of Plate

A sterile cotton swab was dipped into the standardized bacterial suspension. The excess inoculum was reduced by lightly pressing the cotton swab against the wall of the tube above the liquid. The cotton swab containing the inoculum was streaked on the agar plate. The plate was rotated at 60 degrees to rub the inoculum evenly on the plate. This procedure was repeated twice to ensure an even distribution of inoculum to the agar medium. The surface of the agar medium was dried for three to five minutes to allow absorption of excess moisture.

Preparation of Disk

Approximately 7 mm diameter disks of

Volume 3 | Issue 1 | December 2021
PRINT ISSN: 2408-3623 | ONLINE ISSN: 2408 3631

Whatman filter paper (No. 1) were sterilized and loaded with 30 mg ml^{-1} of Amikacin (positive control), negative control (distilled water, chloroform, and methanol), and the root extracts.

Application of Disk to Inoculated Agar Plates: Antimicrobial Disks

The prepared disks were dispensed on the surface of the inoculated agar plate. Each disk was pressed firmly to ensure complete contact of the disk with the agar surface. A maximum of five disks were placed in the petri dish. The disk was placed on the medium approximately 24 mm apart, incubated at 5°C for an hour to permit diffusion, and transferred to the incubator for 24 hours at 37°C, and the zone of inhibition was observed.

Evaluation of Antimicrobial Activity

The Kirby-Bauer test, also known as the disk diffusion method, is a standardized procedure for antimicrobial disk susceptibility testing where bacterial growth inhibition (zone of inhibition) is measured to determine the susceptibility of an organism to a particular antibiotic. Interpretative guidelines or criteria for zone sizes set by the Clinical and Laboratory Standards Institute (CLSI) were used as the basis for the interpretation of the results of the antimicrobial assay. Based on the criteria, the organism can be classified as being Resistant, Intermediate, or Susceptible (Hudzicki, 2009).

Statistical Analysis

The triplicate data reading values of inhibition zones in diameter were based on CLSI standard. Each experimental value was expressed in terms of mean ± standard deviation (SD).

3 Results and Discussion

Qualitative phytochemical analysis of C. kyllingia root extracts

Qualitative phytochemical screening demonstrates the presence and absence of secondary metabolites in plant extracts. Plant drugs are distinct because they contain end-products of long biosynthetic pathways – like secondary metabolites such as alkaloids, phenolics, flavonoids, essential oils, and organic constituents. All plants contain these active compounds, which is a potential explanation of the success of natural products as a drug (Parekh et al., 2005; Nagaraja & Biradar, 2014).

The result of the qualitative phytochemical investigation of the root extracts of *C. kyllingia* (Table 1) indicated the presence of alkaloids and phlobatannins in aqueous, methanol, and chloroform extracts. Volatile oils, tannins, steroids, and flavonoids were present in methanol and chloroform extracts, while glycosides were present only in aqueous extracts. Reducing sugar and saponins were absent in all extracts.

Antimicrobial Analysis

The Kirby-Bauer disk diffusion susceptibility test was utilized to evaluate the antimicrobial activity of the root extracts. Standard zones of inhibition and corresponding inferences for the susceptibility test results of the extracts in this study were based on the work of Hudzicki (2009). Aqueous (CkA), methanol (CkM), and chloroform (CkC) root extracts of *C. kyllingia* with 50 mg ml^{-1}, 75 mg ml^{-1}, and 100 mg ml^{-1} concentrations were

Table 1. Qualitative Phytochemical Screening of *C. kyllingia* Plant Extracts

	Aqueous	Chloroform	Methanol
Alkaloids	+	+	+
Terpenoids	+	+	-
Reducing sugar	-	-	-
Saponin	-	-	-
Tannins	-	+	+
Steroids	-	+	+
Phlobatannin	+	+	+
Glycosides	+	-	-
Flavones	-	-	+
Flavonoids	-	+	+
Volatile Oils	-	+	+

Legend: (-) Absent (+) Present

Volume 3 | Issue 1 | December 2021
PRINT ISSN: 2408-3623 | ONLINE ISSN: 2408 3631

Table 1. Qualitative Phytochemical Screening of *C. kyllingia* Plant Extracts

Plant Extract	Concentration	*Staphylococcus aureus*	*Escherichia coli*
Chloroform	50 mg ml^{-1}	9.33±0.58	7.33±0.58
	75 mg ml^{-1}	9.33±0.58	10±3.61
	100 mg ml^{-1}	14.67±2.89	14.33±2.08
Methanol	50 mg ml^{-1}	8±1.73	8±1
	75 mg ml^{-1}	11.33±4.51	8±1.73
	100 mg ml^{-1}	18.33±1.53	14.33±4.04
Aqueous	50 mg ml^{-1}	9.33±4.04	8±1
	75 mg ml^{-1}	11.33±4.51	12.67±4.04
	100 mg ml^{-1}	14.67±4.93	14.33±4.93
Negative Control			
Chloroform		7.33±0.58	10.33±4.04
Methanol		9.33±2.08	8.33±1.53
Aqueous		7	7
Amikacin		30	30

Data are means of three replicates (n = 3) ±SD
Criteria for *S. aureus* and *E. coli*: Resistant (≤14) Intermediate (15-17) Susceptible (≥18)

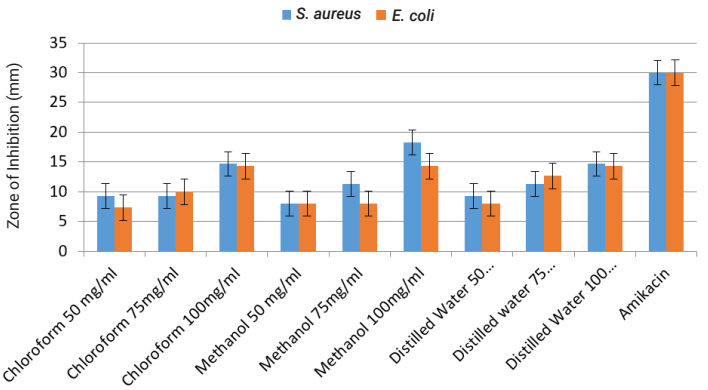

Figure 1. Zone of Inhibition (ZoI): Concentrations Against *S. aureus* and *E. coli*
Error bars represent the standard deviation of the data

tested against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria. Results were recorded in Table 2 and illustrated in Figure 1. Data showed that all plant extracts were potentially effective in restraining the growth of the two pathogens with variable potency. Table 2 revealed that *S. aureus* was more susceptible to CkM at 100 mg ml^{-1} concentration (18.33±1.53). CkA (14.67±4.93) and CkC (14.67±2.89) showed slight inhibitory activity against the bacteria at the same concentration. Against *E. coli*, CkC, CkM, and CkA at 100 mg ml^{-1} concentration (Table 2) exhibited lesser microbial inhibition with ZoI of 14.33±2.08, 14.33±4.04, and 14.33±4.93, respectively.

Despite its little inhibitory effect against E. *coli*, the results still suggest that the root extracts of *C. kyllingia* have promising antimicrobial activity, especially against the *S. aureus* bacteria. The slight differences in bacterial susceptibility can be attributed to their morphological differences, as gram-positive bacteria have a lesser outer peptidoglycan layer that is not an effective permeability barrier making them more vulnerable, while gram-negative bacteria have complicated cell walls with an outer phospholipid membrane that carries a structural lipopolysaccharide component, making the cell wall less impermeable to lipophilic solutes (Vital & Rivera, 2009).

Volume 3 | Issue 1 | December 2021
PRINT ISSN: 2408-3623 | ONLINE ISSN: 2408 3631

Moreover, the slight differences in the efficacies of the extracts can be due to the degree of solubility of active constituents in the three solvents used (Doughari, 2006). Compounds of natural origin have been proven to exhibit antimicrobial properties (Ncube et al, 2008). The consistent presence of alkaloids in all three extracts could have contributed to their antimicrobial activity. Alkaloids have been used as a component in developing antibiotics and as antibiotic-enhancer such as metronidazole, quinolones, and other antibiotics (Cushnie et al., 2014). By forming irreversible complexes with proline-rich proteins, tannins, on the other hand, inhibit cell wall synthesis of bacteria (Mamtha et al., 2004). Terpenoids cause cell wall dissolution by weakening the membranous tissue (Hernández et al., 2000). Flavonoids can form a complex with bacterial cell walls and are effective antibacterial substances against diverse microorganisms in vitro (Mujeeb et al., 2014). Furthermore, steroids exhibit bacterial growth inhibition specifically associated with membrane lipids and cause liposome leakage (Epand, 2007). Lastly, phenolics are said to control the growth and multiplication of bacteria by disturbing the function of the bacterial cell membrane (Simpson & Inglis, 2001). The presence of these compounds in *C. kyllingia* possibly explains its effectiveness in suppressing the growth of *S. aureus* and *E. coli* bacteria.

4 Conclusions and Recommendations

Phytochemical screening of the root extracts of *C. kyllingia* revealed the presence of secondary metabolites. Alkaloids and phlobatannins were present in chloroform, methanol, and aqueous extracts. Terpenoids were only detected in aqueous and chloroform extracts, while tannins, steroids, flavonoids, and volatile oils were present in chloroform and methanol extracts. Among the secondary metabolites that were tested, reducing sugar and saponins were the only ones absent. Evaluation of the antimicrobial activity of the three extracts showed their potential as antimicrobials when tested against the *Staphylococcus aureus* and *Escherichia coli* bacteria. Among the three extracts tested, CkM at 100 mg ml^{-1} concentration was revealed to have been most effective in suppressing microbial growth of the two bacteria compared to CkC and CkA. The results of this study suggest that *C. kyllingia* can be a source of potential antimicrobials which can be attributed to the secondary metabolites present in the extracts.

Lastly, between the two bacteria, *S. aureus* was more susceptible to the treatments than *E. coli*. This can be attributed to their differences as gram-positive and gram-negative bacteria. The results of this study may then be used as a foreground to validate its folkloric uses as a diuretic, hypoglycemic, antioxidant, anthelmintic, and antipyretic. Moreover, the output of this study can significantly aid further research in the development of novel drugs to treat infections, especially against drug-resistant bacteria. It is recommended that further studies be done on the plant's bioactive constituents that may have the potential in combating drug-resistant bacteria.

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Volume 3 | Issue 1 | December 2021
PRINT ISSN: 2408-3623 | ONLINE ISSN: 2408 3631

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