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Phytochemical, Heavy Metals and Antimicrobial Study of the Leaves of *Calopogonium mucunoides*

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ABSTRACT

In this study, the phytochemical, heavy metal, and antimicrobial characteristics of the leaves of *Calopogonium mucunoides*, a popular plant in Nigeria's folk medicine, were investigated in order to determine its therapeutic potential. The phytochemicals present in the leaves were extracted with n-hexane, methanol, and ethyl acetate, and the extracts were used to investigate the phytochemical constituents and antimicrobial activity. he content of five heavy metals was determined using the methanol extract. Several secondary metabolites were found in varied amounts, according to the results. Lead (0.08 mg/kg) and iron (0.08 mg/kg) were found to be well below the World Health Organization's permissible limits for heavy metals in plants, while cadmium, nickel, and zinc were found to be below detectable levels. Using the disk diffusion method, the extracts were evaluated against thirteen human pathogens (ten bacteria and three fungi). The extracts were shown to have a wide variety of antibacterial properties, with the methanolic extract having the largest zone of inhibition (31 mm) against Bacillus sp., while the n-hexane extract had no antimicrobial action in the entire test species. The results obtained revealed that the leaves of *C. mucunoides* have some therapeutic values and could be exploited in the preparation of herbal drugs for the treatment of various ailments.

1. Introduction

For thousands of years, nature has provided medicinal substances, and an astounding number of modern medications have been identified from natural sources [1]. Medicinal plants are the best natural source of drugs for traditional systems of medicine, modern medicine, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs [2]. According to the World Health Organization, medicinal plants are used by 80% of the world's population to treat ailments, and 25% of medications are made from plants and their derivatives [3]. Herbal formulations have long been utilized in African countries to treat a variety of ailments, and they remain the most economical and accessible healthcare system [4]. Plant-derived medicines are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment, hence the basic reason for the recent increase in the demand for herbal drugs [5]. Secondary plant metabolites, otherwise known as phytochemicals, previously with unknown pharmacological activities, have recently been extensively investigated as a source of medicinal agents [6]. Phytochemicals are non-nutritive chemical compounds that occur naturally in plants and have protective or disease-preventive properties [7-9]. These compounds vary in plants depending on their growing conditions, plant species, location of the plant, extraction methods, soil topography, and age of the plant [8, 10].

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments [11, 12]. In most developing countries, such as Nigeria, where the locals do not have access to quite expensive synthetic drugs, traditional plants become the alternative, and investigations into the phytochemical and antimicrobial activities of these plants have been ongoing [13]. However, Onyema and Ajiwe [14] noted that the illegal use of these plant medications without a prescription coupled with the adaptability of bacteria has resulted in an increase in the number of drug-resistant organisms,

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and there has been growing concern about the era of antimicrobial medications coming to an end. As a result, researchers are increasingly turning their attention to folk medicine in a bid to develop good and efficacious drugs against microbial infections [6].

Despite the economic and nutritional value of medicinal plants, there has been an increasing concern about their safety and toxicity. Studies have shown that heavy metal concentrations in animal sex organs such as testicles and ovaries lead to a loss of sexual drive in the animals [15, 16]. Industrial spills and discharges, indiscriminate dumping of wastes and sewages, agricultural practices and general environmental degradation has resulted to an increase in toxic heavy metals in the soil [17-21]. Plants on the other hand, remain the major link in the transfer of these heavy metals from the contaminated soil to humans [22]

Calopogonium mucunoides Desv (*C. mucunoides*) is a vigorous, hairy annual trailing legume belonging to the family of fabaceae. It is a woody vine listed in the global compendium of weeds as an invasive weed, impacting primarily agricultural and semi-natural ecosystems [23]. Cook et al. [24] reported that *C. mucunoides* is commonly planted as a pioneer species and as a nitrogen-fixing species to reduce erosion and improve soil fertility. Like many other legume forages, the nutritional potential of *C. mucunoides* lies in its protein content [25, 26]. The plant leaf has been used in Nigerian folk medicine for the treatment of ulcers, bacterial infections [27, 28], diarrheal, and scurvy [5]. The presence of various secondary metabolites of pharmacological importance in the plant makes *C. mucunoides* a potential alternative drug for the treatment of such ailments as skin diseases, diarrhoea, and ulcers [29].

Fitriarni and Kasiamdari [29] isolated thirty-seven endophytic fungi from the leaves and stems of *C. mucunoides*, but the study was conducted to determine the diversity of fungi found in the different parts of the plant. The phytochemical, antioxidant, and proximate contents of the plant leaves were determined by Fadeyi et al. [28]. The study assessed the medical efficacy of the herb, among other things, but it lacks complete information to draw a decision on its purported significance. From published literature, the heavy metal content and antimicrobial activity of any part of the plant have not been studied. Bada and Olarinre [30] in their study: characterization of soils and heavy metal content of vegetation in oil spill impacted land in Nigeria, included *C. mucunoides* as part of the vegetation investigated, but the heavy metal composition of the plant has not been properly and exclusively analyzed.

In light of this, the goal of this study was to identify the phytochemical components of *C. mucunoides* leaves, measure the concentration of heavy metals contained in the leaves, and then assess the plant leaves' antimicrobial effectiveness against a variety of human infections.

2. Methodology

2.1. Sample Collection and Extraction

The leaves of *C. mucunoides* were collected from a farmland near the laboratory of Nnamdi Azikiwe University's Department of Pure and Industrial Chemistry in Awka. The plant was identified and authenticated by a taxonomist from the department of Botany of the said institution. They were then air dried for two weeks, after which they were ground in a clean electric motor grinder to get the powered sample which was analysed. A finely ground sample was used to make three different kinds of extracts using methanol, *n-hexane*, and ethyl acetate. In each extract, 10 g of sample was soaked in 100 ml of solvent and allowed to stand for 24 h, after which the solutions were filtered. The filtrates were then used to determine, qualitatively, the presence of the phytochemical components.

2.2. Phytochemical Screening

2.2.1. Qualitative Analysis

Qualitative analysis was carried out on the leaf extracts of *C. mucunoides* for the presence of phytochemicals such as alkaloids, anthocyanins, flavonoids, glycosides, phenols, reducing sugars, saponins, steroids, tannins, and terpenoids, using the methods described by Harbone [7], Trease and Evans [31], and Akpuaka [32].

Test for Alkaloids (Mayer's reagent)

1ml of Mayer's reagent was added to 1ml of each of the extract in a test tube; and a creamy precipitate indicated the presence of alkaloid.

Test for Anthocyanin

2 ml of 2 M HCl and 2 ml of ammonia solution were added to 1 ml of each of the extracts in test tubes. The appearance of pink-red colouration which turns blue-violent indicated a positive test for anthocyanin.

Test for Flavonoids (Sodium hydroxide Test)

Two drops of 10% NaOH solution were added separately to 1 ml of each extract in three different test tubes, and the presence of a yellow precipitate revealed the presence of flavonoids.

Test for Glycosides

To each test tube, 2 ml of each extract were added: 5 ml of distilled water, 5 ml of concentrated H_2SO_4 and boiled in a water bath for 15 min. The test tubes were then allowed to cool and each was neutralized with 20% NaOH, after which 1 ml of Fehling's solution was added and boiled for another 15 min. A brick-red precipitate indicated the presence of glycosides.

Test for Phenols

1 ml of distilled water was added to 1 ml of each of the extracts, followed by the addition of a few drops of

5% NaOH solution. A colour change from yellow to bright orange indicated the presence of phenol.

Test for Reducing Sugars

To the test tubes containing 2 ml of each of the different extracts, were added 5 ml each of Fehling's solution A and B. They were then placed in an 80 °C water bath for 10 min. A brick-red precipitate indicated the presence of reducing sugars.

Test for Saponins (Frothing Test)

8 ml of distilled water was used to dilute 2 ml of each of the extracts, and the content was vigorously shaken for 2 min. Persistent frothing indicated the presence of saponins.

Test for Steroids (Salkowski Test)

In different test tubes, 2 ml of chloroform and 2 ml of concentrated H_2SO_4 were added to 1 ml of the extracts. The chloroform layer appeared red, while the acid layer showed green fluorescence. This indicated the presence of steroids.

Test for Tannins (Potassium hydroxide Test)

0.5ml of 20% freshly prepared KOH was separately added to 1ml of each extract in different test tubes, and the presence of a dirty white precipitate indicated the presence of tannins.

Test for Terpenoids

To 2ml of each of the extracts, 2ml of acetic anhydride and 2ml of concentrated H_2SO_4 were added, and the formation of blue-green rings showed a positive test for terpenoid.

2.2.2. Quantitative Analysis

The quantitative phytochemical analysis of the leaves was determined using the method by Harbone [7].

2.3. Determination of Heavy Metals

The Atomic Absorption Spectrometer (AAS, VGP-210) was used to test for the presence of five heavy metals (cadmium, iron, lead, nickel, and zinc) in the plant using methanol extract. 5 g of the ground sample was soaked in 50 ml of methanol and allowed to stand for 3 h. The solution was filtered, and the residue was left to dry for 72 h at room temperature. After the expiration of the time, 2 g of the dried residue was weighed into a platinum crucible and heated to ash using a Bunsen burner. The ash was then dissolved with deionized water, shaken thoroughly, filtered, and made up to the 100 ml mark with deionized water. This filtrate served as the analyte for the heavy metal determination.

2.4. Antimicrobial Screening

The antimicrobial activities of methanol, n-hexane, and ethyl acetate extracts of the leaves of *C. mucunoides* were tested against human pathogens (bacterial and fungal) using the disk well diffusion control. A total of thirteen organisms were used, ten pathogenic bacteria, namely: *Bacillus sp, Clostridium sp, Escherichia coli, Klebsieela pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella sp, Serratia marcescens, Staphylococcus aureus,* and *Streptococcus sp;* and three pathogenic fungi, *Aspergillus fumigates*, *Candida albicans*, and *Penicillium cyclopium*. These organisms were maintained on nutrient broth for bacteria and Sabouraud Dextrose Broth for fungi.

2.4.1. Antimicrobial Susceptibility Test

This was determined by the modified method described by Agu et al. [33]. Plates that had confluent and/or semi-confluent growth were selected for the antimicrobial susceptibility tests. The disk diffusion method was used to assay the effects of the extracts on the various microorganisms. Mueller Hinton Agar was used for the bacteria and Sabouraud Dextrose Agar (SDA) for the fungus. 24 h broth cultures of the test organisms were serially diluted, then 10^{-1} and 10^{-2} dilutions were used to seed the fungal isolates, while 10^{-2} was used to seed the bacteria plates. Then 0.1 ml of the appropriate dilution of the broth culture of each microorganism was uniformly spread on the surface of the media, and sterile filter papers were soaked in the extracts (*n*-hexane, methanol, and ethyl acetate extracts) and placed on two points on each petri-dish. Clear zones of inhibition around the wells indicated antimicrobial activities of the extracts against the test organisms. The diameter of the zone of inhibition was measured and recorded in millimetres. All experiments were done in duplicates and the mean was taken. Negative controls were set up with sterile water and positive controls were set up using 0.5% Nystatin for fungi and 0.5% Ciprofloxacin for bacteria.

3. Results and Discussion

3.1. Result of the Phytochemical Analysis

Table 1. Result of qualitative phytochemical analysis

Phytochemicals	Ethyl acetate	Methanol	*n*-Hexane
Alkaloids	+	-	++
Anthocyanins	+	+	++
Flavonoids	-	+	++
Glycosides	-	+	++
Phenols	-	-	-
Reducing sugars	-	+	++
Saponins	+	++	+
Steroids	++	+	-
Tannins	++	++	+
Terpenoids	+	+	++

++ = Present; + = Mildly present; - = Below detectable limit

Table 2. Result of quantitative analysis

Phytochemicals	Alkaloids	Flavonoids	Glycosides	Saponins	Steroids	Tannins
Concentration (%)	36.8	1.40	19.6	4.10	19.6	14.2

The qualitative phytochemical screening of the *n*-hexane extract of the leaves of *C. mucunoides* as summarized in Table 1 above revealed the presence of alkaloids, tannins, saponins, flavonoids, glycosides, terpenoids, and reducing sugars, with the exception of phenols, steroids, and anthocyanins. The methanolic extract revealed the presence of all the phytochemicals tested for except for alkaloids and phenols, while ethyl acetate extract showed a positive result for alkaloids, tannins, saponins, steroids, terpenoids, and anthocyanins. The result is in tandem with that obtained by Fadeyi et al. [28], except for the absence of phenols in all the extracts. Because phenol is responsible for the coloration of fruits in plants, its absence is not a problem because *C. mucunoides* has a low palatability.

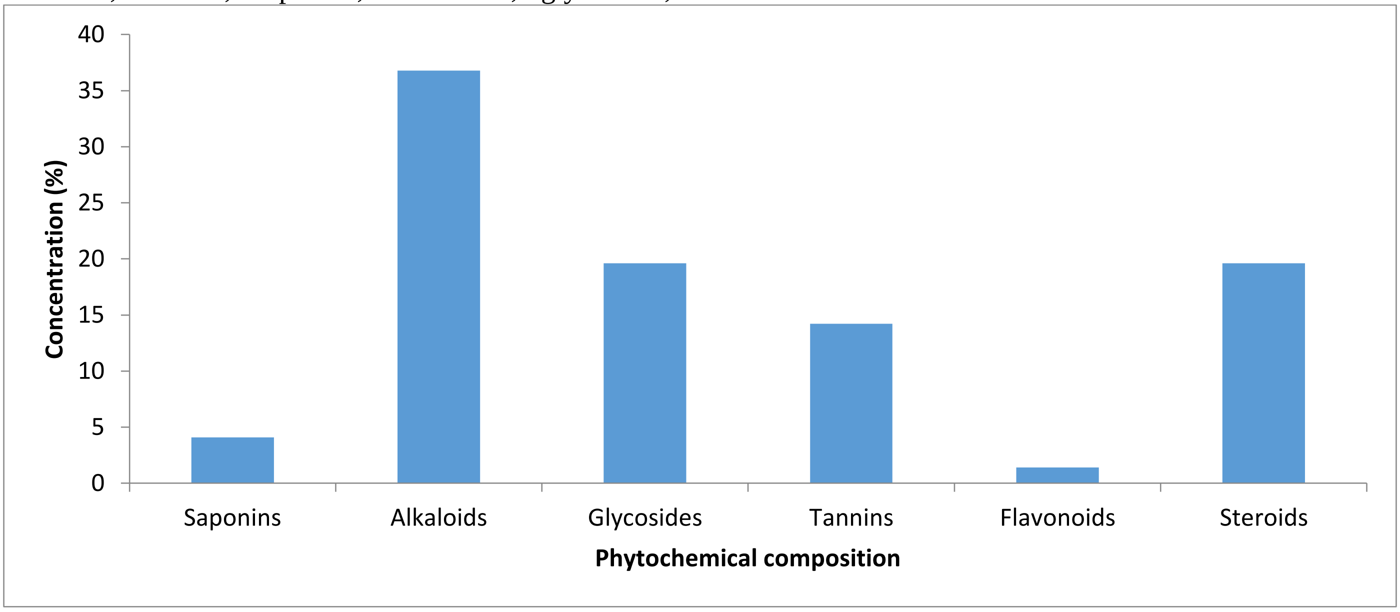

Figure 1. Quantitative phytochemical compositions of *C. mucunoides*

The quantitative values as shown in Table 2 and Figure 1 reveal that alkaloids have the highest concentration (36.80%). Similar results were reported by Enechi et al. [27] and Borokini and Omotayo [5]. They noted that the phytochemical screening of the leaves of *C. mucunoides* revealed a copious presence of alkaloids and flavonoids, among other secondary metabolites. Many plants have alkaloids that confer on them their medicinal efficacy, ranging from the treatment of diarrhoea, reduction and stopping of pains and regurgitation, and many more. Flavonoids have anti-microbial potential and can be used in the treatment of dropsy, hay fever, and ulcers [34], and also reduces the risk of cardiovascular diseases and stroke [31]. Table 2 also showed the presence of glycosides (19.60%), tannins (14.21%), steroids (19.60%), and saponins (4.10%) in relative quantities. Saponins are used in the treatment of fungal and bacterial infections. Since *C. mucunoides* serves as fodder for livestock, the low value obtained for saponins may be ideal. According to Bassey et al. [35], high saponin content in plants may be responsible for the swelling of the rumen in domestic animals. Tannins serve as metal chelators and have anti-inflammatory properties [36]. Its presence in livestock feeds, however, poses nutritional risk such as stunted growth, indigestion, among others [37]. Glycosides are used in the treatment of heart-related diseases such as arrhythmia. Steroids help in the development of male and female sex hormones, and their presence in *C. mucunoides* leaves could be the reason for the morphological deficiencies observed in the testis and ovaries of rabbits after being fed for a long time with the plant leaves [15]. Hence, the need to always ascertain the presence and concentrations of these phytochemicals in forages like *C. nucunoides* before using them. The medicinal values of plants and their derivatives are dependent on the composition of these secondary metabolites [38]. Thus the presence of these phyto-compounds in the leaves of *C. mucunoides* confers on it its medicinal value.

3.2. Result of the Heavy Metal Analysis

Table 3. Concentrations of Heavy Metals

Heavy metals	Cadmium	Iron	Lead	Nickel	Zinc
Concentration (mg/kg)	-0.02	0.08	0.082	-35	-0.6
*Permissible limit (mg/kg)		20			0

*World Health Organization [39]

The result of the heavy metal determination of the methanolic leaf extract of *C. mucunoides* is shown in Table 3**.** Cadmium, nickel, and zinc were found below the detectable limit. This, however, contrasts with the result of Bada and Olarinre [30] for Cd (0.21 mg/kg) and Zn (1.44 mg/kg) and Lead and iron, on the other hand, were present in mild concentrations of 0.08 mg/kg each. This value is well below the permissible limit of 2 mg/kg for Pb and 20 mg/kg for Fe set by WHO [39] for heavy metals in plants. Bada and Olarinre reported a higher value for Pb (0.39 mg/kg) than reported by Bada and Olarinre [30], but their study was centered on plants obtained from oil-contaminated soil, a factor that might have contributed to the high levels of these metals. Pb is a non-essential element that has no known biochemical benefit to both plants and animals [40]. It readily accumulates in different parts of the plant and gets to humans by biomagnifications [41]. Higher Pb concentrations have a number of negative effects on plants, animals, and humans, including chlorosis, blackening of the root system, decreased survival rate, retarded development, impaired mobility, and decreased egg production [41-43]. However, the low concentration of Pb in the leaf extract of *C. mucunoides* implies that the plant is relatively safe, either to be used as fodder for livestock or for the preparation of herbal drugs for the treatment of said ailments. Nevertheless, Pb has been reported to pose adverse health concerns even at levels considered acceptable [44], hence, the need to constantly check the level of Pb concentrations and other heavy metals in plants used for herbal formulations. Fe, on the other hand, is an essential element needed for the production of red blood cells. It aids photosynthesis in plants, helps in the growth of tissues in animals and in the movement of oxygen in the body [45].

3.3. Result of the Antimicrobial Screening

Table 4. Antibacterial Susceptibility Screening

Organisms	Ethyl acetate (mm)	Methanol (mm)	*n*-Hexane (mm)	0.5% Ciprofloxacin(mm)
Bacillus sp	28.5	31	-	37
Clostridium sp	24	28	-	36.5
Escherichia coli	-	12.5	-	37
Klebsiella pneumonia	22	29.5	-	36
Proteus mirabilis	7	8.5	-	34.5
Pseudomonas aeruginosa	-	-	-	34.5
Salmonella sp				
Serratia marcescens	15	19.5	-	37
Staphylococcus aureus	15.5	14	-	38
Streptococcus sp.	22	14.5	-	40.5
	8	16	-	37.5

*0.5% Ciprofloxacin was used as the positive control.

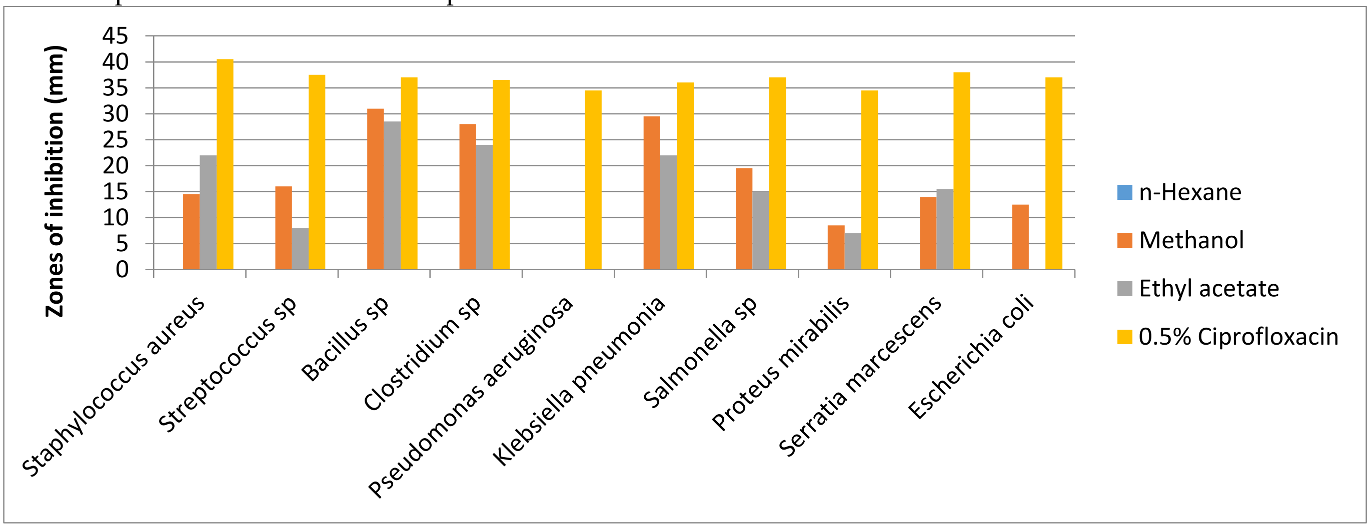

Figure 2. Graphical representation of antibacterial activities of *C. mucunoides* leaf extract against selected pathogenic bacterial

Antimicrobial features of plants are wanted tools in the control of unwanted microorganisms, particularly in the treatment of contagious diseases [3]. The *n*-hexane extract of the leaves of *C. mucunoides* has no antibacterial activity against the organisms tested, as shown in Table 4 and graphically represented in Figure 2**.** The methanolic extract, on the other hand, showed antibacterial activity on the whole organisms except for *Pseudomonas aeruginosa*, with the most effective being against *Bacillus sp*, and the least effective being against *Proteus mirabilis.* Ethyl acetate extract also showed antibacterial activity against the organisms except in Pseudomonas aeruginos and in Escherichia coli. It is most effective against *Bacillus sp.* and least effective against *Proteus mirabilis.* When compared to the positive control, 0.5% Ciprofloxacin, which served as the standard, the methanolic extract appeared to be the most effective, except in *S. aureus* and *Serratia marcescens,* where the ethyl acetate extract was more effective. Another point worth noting from the result is the absence of *Pseudomonas aeruginosa* in the three extracts. This implies that none of the extracts can be used in the treatment of bacterial infections related to *Proteus mirabilis.* According to Onyema and Ajiwe [14], an organism is susceptible to an extract if the inhibitory zone is ≥16 mm in diameter, while 11–15 mm indicates an intermediate effect. Thus, the methanolic and ethyl acetate extracts of the leaves of *C. mucunoides* have some levels of inhibition against most of the organisms tested for and can be used in the treatment of bacterial infections caused by them, except for *Pseudomonas aeruginosa* and *Proteus mirabilis*, while the *n*-hexane extract of the plant leaf should not even be considered at all for any such antibacterial drug because of its inactivity in all the tested organisms.

Table 5. Antifungal Susceptibility Screening

Organism	Ethyl acetate (mm)	Methanol (mm)	*n*-Hexane (mm)	0.5% Nystatin (mm)
Aspergillus fumigates	-	-	-	34.5
Candida albicans				
Penicillium cyclopium	-	-	-	41
	26	26.5	-	36.5

0.5% Nystatin was used as the positive control.

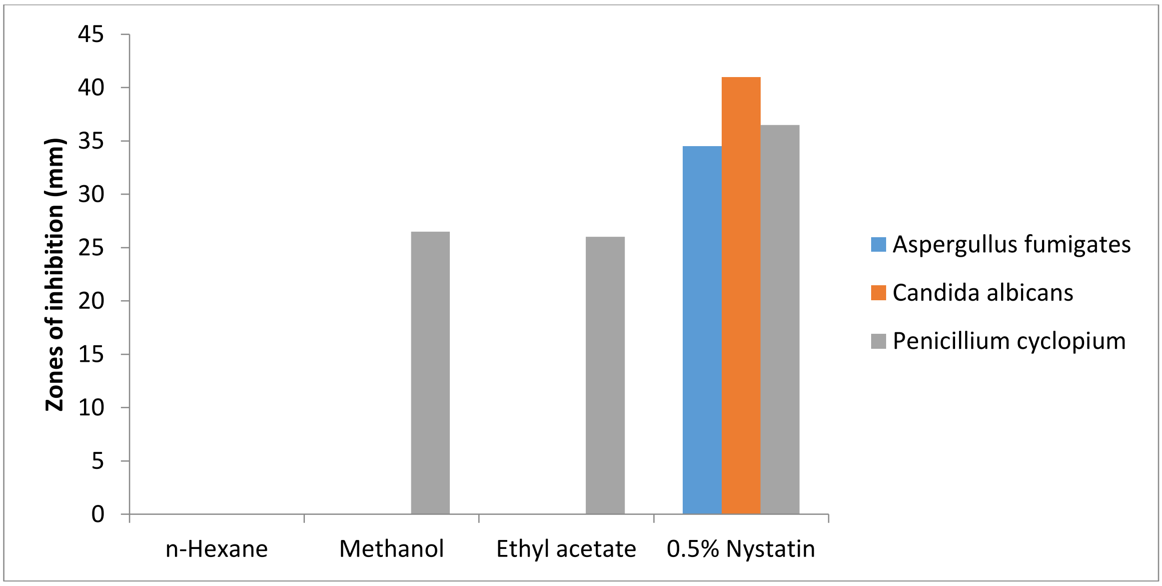

Figure 3. Graphical representation of antifungal activities of *C. mucunoides* leaf extract against selected pathogenic fungal

The results of the three pathogenic fungal tests are reported in Table 5 and Figure 3. It revealed that the three extracts do not show antifungal activity against *Aspergillus fumigatus* and *Candida albicans*. However, while n-hexane extract was also absent in *penicillium cyclopium*, the methanolic and ethyl acetate extracts proved to be highly effective against *Penicillium cyclopium* with high diameter zones of inhibition. This is in agreement with the results obtained in antibacterial screening; thus, *n*-hexane extract of *C. mucunoides* leaves is not a good solvent for antimicrobial activity, while methanolic extract is a better solvent compared to ethyl acetate. While none of the extracts is up to the standards used (0.5% ciprofloxacin for antibacterial and 0.5% nystatin for antifungal), the high zones of inhibition shown is proof that the leaves of *C. mucunoides* are a potential herbal medicine for microbial infections.

4. Conclusion

This work exploited the leaves of *Calopogonium mucunoides* using different solvents in a bid to find the medicinal value of the plant. The findings support the knowledge that *C. mucunoides* appears in the hierarchy of medicinal plants in Nigeria and that there is indeed a very useful potential in the demand for substitute natural medicine for the treatment of various ailments such as ulcers and diarrhoea. This has been proven by the results of the phytochemical screening of the plant leaves that revealed many secondary metabolites of medicinal value. The treatment of bacterial infections using plant extracts is also possible, as seen in the results of the methanolic and ethyl acetate extracts of the plant leaves, which showed high zones of inhibition against most of the pathogenic bacteria tested. The presence of toxic heavy metals such as lead is well below the permissible limit set by WHO. All of this makes the plant's leaves an ideal potential drug source. Consequently, further exploitation of this plant for the probable preparation of cheap and effective drugs is hereby recommended. Further research on other parts of this plant is recommended so as to probe further into the therapeutic potential of the plant.

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Author contributions

Ebuka C. Emenike; Investigation; Data curation; Methodology; Original draft writing - review & editing; Validation
Onyema Chukwuebuka; Conceptualisation, Methodology, Writing - review & editing; Supervision; Validation; Project administration

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