RESEARCH ARTICLE

Screening of Phyllanthus Species for Antimicrobial Properties

Chemical Sciences Journal, Vol. 2012: CSJ-56

Screening of *Phyllanthus* Species for Antimicrobial Properties

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Accepted: July 13, 2012; Published: July 23, 2012

Abstract
The development of resistant pathogenic microorganism against conventional antibiotic drugs has risen to a point of global concern. New antimicrobial compounds with diverse chemical structures and novel mechanisms of action are therefore needed to curb the new and re-emerging infectious diseases. This study has identified two *Phyllanthus* species (*Phyllanthus amarus, Phyllanthus odontadenius)* sampled from Nairobi and Siaya counties in Kenya. *In vitro* activity of extracts of these species and correlated their efficacy was compared with the commercial extracts of *P. niruri* that are in the Kenyan market. Disk diffusion method was employed to screen the antimicrobial activities of both the extracts and two standard antibiotics; 0.32mg mL^{-1} gentamycin and 0.30 mg mL^{-1} Nystatin. The dichloromethane(DCM):methanol (1:1) extracts of *Phyllanthus odontodenius* showed the strongest activity against all the organisms both at 100 mg µL and 50 mg μL^{-1} followed by both the hot water and cold water methanol extracts. The solvents in comparison to antibiotics showed 80% activity for methanol, 48% for DCM:MeOH 1:1, 43% in hot water and 28% for cold water. Thin layer chromatography (TLC) showed that the compounds found in the three species were identical. This study has shown that, the two species possess significant antimicrobial activity and justifies the use of their extracts by herbalists in the treatment of many microbial diseases. Therefore, further bioassay guided fractionation, isolation and characterization studies of compounds from these extracts are needed to confirm the active components and mechanisms of action of these two species.

Keywords: *Phyllanthus amarus; Phyllanthus odontadenius*; *Phyllanthus niruri;* antimicrobial activity; infectious diseases.

1. Introduction

Like in many developing countries, new drugs are often not affordable in Kenya. Approximately, 60–80% of the world's population still relies on traditional medicines as remedies for the treatment of common illnesses [1]. According to World Health Organisation (WHO), medicinal plants are the best source to obtain a variety of drugs to combat serious diseases [2] and it advocates that countries should venture into other aspects of traditional medicine. This should be with a view of identifying safe and effective remedies for ailments of both microbial and non-microbial organisms. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value [3]. In addition, traditional medicine is an important part of African culture [4] and herbs are now very popular in developing countries on account of improved knowledge about their safety, efficacy, and quality assurance of ethnomedicine. In recent years, secondary plant metabolites (phytochemicals) have been extensively investigated as a source of medicinal agents. It is anticipated that phytochemicals with good antibacterial activity will be used for the treatment of bacterial infections, fungi, and viruses [5]. Of the common herb genus that is widely used is the family Phyllanthaceae to which *Phyllanthus (P.) niruri* belongs. This species is indigenous to S. America, India, and China. There are more than 300 species [9] which are widely distributed in many tropical countries where they are considered as weeds. In Kenya, they are commonly found at the Coast, Nairobi, Central, Eastern, Nyanza, and Western provinces. *P. niruri* has been widely used in treating a number of traditional ailments. The genus treats jaundice, gonorrhoea, frequent menstruation, skin ulcers, sores, swelling, flu, dropsy, diabetes, liver disorders, itchiness, gall, bladder calculus, [7], and chronic dysentery/diarrhoea [8]. *P. niruri* primarily contains lignans, alkaloids, and bioflavonoids and it has demonstrated *in vitro* antibacterial actions against *Staphylococcus*, *Micrococcus,* and *Pasteurella* bacteria as well as *in vivo* and *in vitro* anti-malaria properties [10, 11]. Its extracts have been used as antiviral source to treat Hepatitis B [12]. The methanol extracts of *Phyllanthus* species from India were reported to have strong antioxidant activity [13]. The root of *Phyllanthus acuminatus* inhibited the growth of murine P-388 lymphocytic leukemia and B-16 melanoma cell lines [13]. On the basis of results obtained, the ingredients in catliv effectively helped in regeneration of hepatic cells and were an effective liver tonic for calves [14]. In an experiment with cholesterol fed rats, *P. niruri* at

a dose of 100 mg kg lowered the elevated level of low-density lipoprotein lipids in hyperlipidemic and drug fed animals [15].

P. niruri is reported to have inhibitory effect on human immunodeficiency virus [16]. The extracts of five medicinal plants: *Aristolochia indica, Cassia occidentalis, P. niruri*, *Withania somnifera, and Tinospora cordifolia* increased CD4 count in HIV-positive patients [17].

The alkaloidal extract of *P. niruri* is found to exhibit sensitive inhibitory response on cytopathic effects induced by both the strains of human immunodeficiency virus on human MT-4 cells in the tested concentrations. The bioactivity of plants used as herbs appears to be derived from "secondary metabolites," such as the polyphenols [18]. Polyphenols are the most numerous and widely distributed class of phytochemicals. They include classes of chromones, coumarins, lignans, stilbenes, xanthones and the ubiquitous flavonoids [19]. Many polyphenols, particularly the flavonoids, had been found to possess relatively potent antioxidant, antiatherosclerotic, antiinflammatory, antimutagenic, antitumor and antiviral activities [20]. Observational studies [see 19, 21] have repeatedly shown that diets high in plant-based foods and beverages are associated with a lower risk of chronic diseases. Notable are the cardiovascular disease and some forms of cancer. These studies [19, 21] suggest this correlation may be attributable to the phytochemical constituents as well as to the macro and/or micronutrient content of those foods.

Bacteria have evolved numerous defences against antimicrobial agents which have resulted into the rise in drug-resistant pathogens. This resistance is conferred by multidrug resistance pumps (MDRs). MDRs are membrane translocases that extrude structurally unrelated toxins from the cell. These protect microbial cells from both synthetic and natural antimicrobials [22]. The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments and could help curb the problem of these multi-drug-resistant organisms. In a study done with *Pseudomonas aeruginosa*, which is resistant to different antibiotics, its growth was inhibited by extracts from clove, jambolan, pomegranate and thyme [2].

Moreover, the synergistic effects of extracts with antimicrobial activity in association with antibiotics can provide effective therapy against drug-resistant bacteria. These synergistic combinations represent a largely untapped source of new pharmaceutical products with novel and multiple mechanisms of action that can overcome microbial resistance. Recent developments in plant biotechnology have created the tools to produce botanical mixtures at a level comparable to that of pure drug compounds [24]. This therefore limits the depletion of biogenetic resources in forests. The specific objectives of this paper were to: (1) Identify *Phyllanthus* species collected from two ecological regions in Kenya, Nairobi area and Siaya district, Nyanza province; (2) Investigate the *in vitro* activity of extracts of *P. amarus, P. odontadenius, and P. niruri* on *Candida albicans, Bacillus pumilus, Micrococcus luteus, Klebsiella pneumonia, Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli*; (3) Correlate the efficacy of commercial extracts (*P. niruri*) in the Kenyan market vs. the two *Phyllanthus* spp. found in Kenya.

2. Methods

2.1. Sampling

Purposive (Non-probability) sampling was employed. Field collections of *Phyllanthus* spp. were carried out in Langata forest Nairobi area and Ligingo village, Siaya district, Nyanza province. This was conducted in collaboration with the communities in these regions in order to seek traditional uses.

Herbarium specimens were prepared and photographs taken to aid in the confirmation of the identity of the plants. Voucher specimens were deposited in the Herbarium of the S. B. S. where identity of the plants was confirmed by comparison with available voucher specimens. The identification was further confirmed at the National Museums of Kenya Herbarium. The ethno-botanical information of the species in Kenya was collated from literature and local herbalist. This was used to correlate the local herbs' ethno-botany with that of *P. niruri*. Plate 1 shows the *Phyllanthus* herb.

2.2. Experimental design

2.2.1. Processing of the plant materials

Whole plant materials were sorted and chopped into smaller pieces, where necessary, and dried under the shade. The dried plant material was ground to various degrees of fineness depending on their botanical structures using an electric grinder. Plant extracts were prepared by soaking 20g of dried powder in cold water, hot water methanol or dichloromethane:methanol (1:1) according to standard extraction method [see 11]. Antimicrobial screening was then done. This involved different solvent extracts of *P. niruri, P. odontadenius* and *P. amarus* screened against six

bacterial strains and one fungus. The test organisms used were: *Candida albicans, Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, E. coli* and *Klebsiella pneumonia.* These were obtained from the Microbiology laboratory of the S. B. S. However, standardization is required for intra and inter-laboratory reproducibility as results may be significantly influenced by the method used [25].

Plate 1: Identification of a *Phyllanthus* herb.

Bacterial and fungal cultures

Active cultures for experiments were prepared by transferring a loop-full of cells from the stock cultures to test tubes of nutrient agar for bacteria and Sabouraud dextrose broth (SDB) for fungi. These were incubated without agitation for 24 hr at 37°C and 25°C respectively. The cultures were diluted with fresh nutrient agar and SDB to achieve optical densities corresponding to 2.0×10^6 colony forming units (CFU/mL) for bacteria and 2.0×10^5 spore/ml for fungal strains. Antimicrobial susceptibility test was done by Kirby-Bauer disc diffusion and well methods to screen the antimicrobial activity. *In vitro* antimicrobial activity was screened by use of nutrient agar and SDB for fungi. The cultures used are shown in Table 1.

Table 1: The bacterial and fungal cultures.

Item Number	Product Description	Format
NCPF3179	*Candida albicans*	Standard
NC08241	*Bacillus pumilus*	Standard
NC07447	*Staphylococcus aureus*	Standard
NC10400	*Bacillus subtilis*	Standard
NC07743	*Micrococcus luteus*	Standard
ATCC25922	*Escherichia coli*	Standard
***	*Klebsiella pneumoniae*	

*** From School of Pharmacy, University of Nairobi.

2.2.3. The control experiment

A control experiment was set up by using drops of sterile distilled water in place of different solvent systems. A positive control 0.32 mg mL^{-1} gentamycin for bacteria and 0.30 mg mL^{-1} Nystatin for fungi were used. At the end of incubation, the inhibition zones formed around the disc were measured with a Vernier calliper in millimetres. Further tests were carried out using Mueller-Hinton agar (MHA) and Tryptone Soya agar (TSA).

3. Results and Discussion

3.1. Species identification

The *Phyllanthus* spp. collected from Nairobi's Langata forest matched voucher specimen of *P. odontadenius,* Plate 2.

Plate 2: A voucher specimen of *P. odontodenius*.

The herb collected from Ligingo, Siaya district was identified as *Phyllanthus amarus*.

Plate 3: Voucher specimens of *Phyllanthus amarus.*

The ethnomedicinal data of *P. amarus* collected from Siaya, Nyanza province are given in Table 2.

Table 2: Ethnomedicine of *Phyllanthus amarus* as collected from Siaya district.

Vernacular name	Parts used	Preparation method	Application	Condition treated
Anyidhra*	Whole plant	Pounded plant mixed with oil	Massage	Muscle aches and hunch back tendency in children.
		Decoction	Oral	Measles, malaria, stomach ailments, sexually transmitted diseases particularly gonorrhoea.

*Source: [6]

Ethnomedicinal information on *P. amarus* as collated from literature shows that the plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge, and antiseptic. It is useful in curing many ailments as cited earlier [see 7] and diseases of the urino-genital system, scabies ulcers and wounds [27]. However, there was no information on *P. odontadenius* ethnomedicine collated from literature.

The three plant species (*P. odontadenius, P. amarus, and P. niruri)* had at least one extract active against the test microbial organisms. The dichloromethane:methanol extracts were the most active exhibiting high antimicrobial activity followed by methanol extracts and aqueous extracts in that order. Cold water was the least active solvent while all the extracts were less active against *Candida albicans.* All extracts of *P. odontadenius*

showed activity against all the tested bacteria as compared with *P. amarus* where cold water extract did not show any activity. Hence, the *P. odontadenius* extracts had the greatest activity as given in Table 3.

Table 3: Antimicrobial activity of three *Phyllanthus* species extracts of different solvents.

		Microorganism				
		Candida albicans	***Escherichia coli****	***Staphyloccocus aureus****	***Bacillus subtilis****	***Bacillus pumillus****
P. odontadenius	DCM:MeOH	-	+	++	++	++
	MeOH	-	+	++	++	++
	cold water	-	+	++	++	++
	hot water	-	+	++	++	++
P. amarus	DCM:MeOH	-	+	++	++	++
	Meoh	-	+	++	++	++
	cold water	-	-	-	-	-
	hot water	-	+	++	++	++
P. niruri	MeOH	-	+	++	++	++

- No inhibition, + active, ++ very active, ** Gram-positive, * Gram-negative

The aqueous, methanol (MeOH), and dichloromethane(DCM):methanol (1:1) extracts of *Phyllanthus* spp. demonstrated varying levels of antimicrobial activity against the test organisms. The methanol extracts of all plants showed the highest inhibitory activity with effects highest in *Bacillus pumillus.* However, at 2 mg mL^{-1}, there was no activity in *Candida albicans* and there were least activity in *E. coli.* In general, among the tested microbial strains, bacteria were found to be more sensitive to many of the test agents than fungi. These results are in agreement with those in [13], where methanol extracts of five *Phyllanthus* species from India was used. The species reported by the two scholars are those that had strong antioxidant activity. Gram-positive bacteria were more susceptible than the Gram-negative; the susceptibility of the test microbes to the extracts showed high activity with *P. odontadenius* and *P. amarus.* At a concentration of 100 mg μL^{-1}, *P. niruri* matched activity of *P. amarus* at 50 mg μL^{-1} concentration. Besides, at a concentration of above 50 mg μL^{-1}, the activity of the extracts was positive on all the test organisms with *P. odontadenius* extracts showing the greatest activity, Table 4.

Table 4: Antimicrobial activities of methanolic extracts of *Phyllanthus* species at a concentration of 100 mg μL^{-1} and 50 mg μL^{-1}.

	100 mg μL^{-1}			**50 mg μL^{-1}**		
	P. odontadenius	***P. amarus***	***P. niruri***	***P. odontadenius***	***P. amarus***	***P. niruri***
Staphylococcus aureus	+++	++	+	+	+	-
Bacillus subtilis	++++	+++	+	++	+	-
Escherichia coli	++++	++++	+	++	++	+
Bacillus pumilus	+++	++	+	+	+	+
Klebsiella pneumonia	+++	+++	+	++	+	-
Candida albicans	+++	++	+	++	+	+

Inhibition <40% -, Inhibition >40% +, Inhibition >50% ++, Inhibition >60% +++, Inhibition >70% ++++

The antimicrobial effects of the extracts were influenced by the type of media used. MHA showed greater inhibition activity than both nutrient agar and TSA. Susceptibility testing on each of the isolate was first performed on nutrient agar, and then on MHA and TSAs. The solvents in comparison to antibiotics showed 80% activity for methanol, 48% for DCM:MeOH (1:1), 43% in hot water and 28% for cold water as shown in Figure 1.

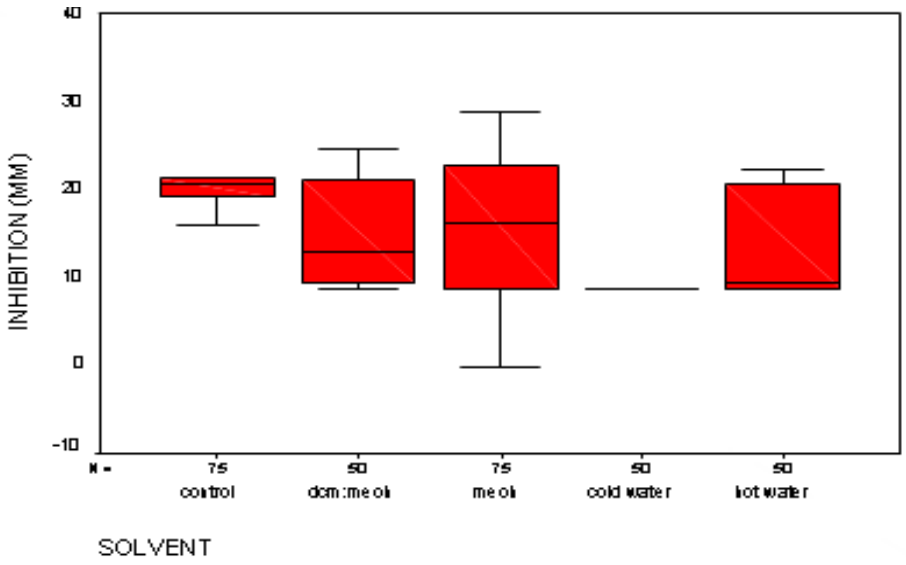

Figure 1: Comparisons between the solvent used.

Figure 2 gives a graphical representation of the antimicrobial activity of methanol extracts at different concentrations. It shows a broad spectrum of activity against all the microorganisms that were employed. The most active solvent was methanol with the effects being highest in *Bacillus pumillus.* At 2 mg mL^{-1} there was no activity in *Candida albicans. Escherichia coli* showed the least activity for bacteria. Fifty (50%) of *B. pumillus* were very close to the median at around 20 mm. Most solvents were active against the bacteria. Susceptibility of the test microbes to the extracts showed high activity with *P. odontadenius* and *P. amarus.* At a concentration of 100 mg μL^{-1} *P. niruri* matched activity of *P. amarus* at 50 mg μL^{-1} concentration. This means that *P. odontadenius* and *P. amarus* contain high concentrations of phytochemicals responsible for antimicrobial activity than *P. niruri* [see 28]. However, our results reveal that the alcoholic extracts were more effective than the aqueous extract in inhibiting the growth of the test microbes. These results reflect those of [29].

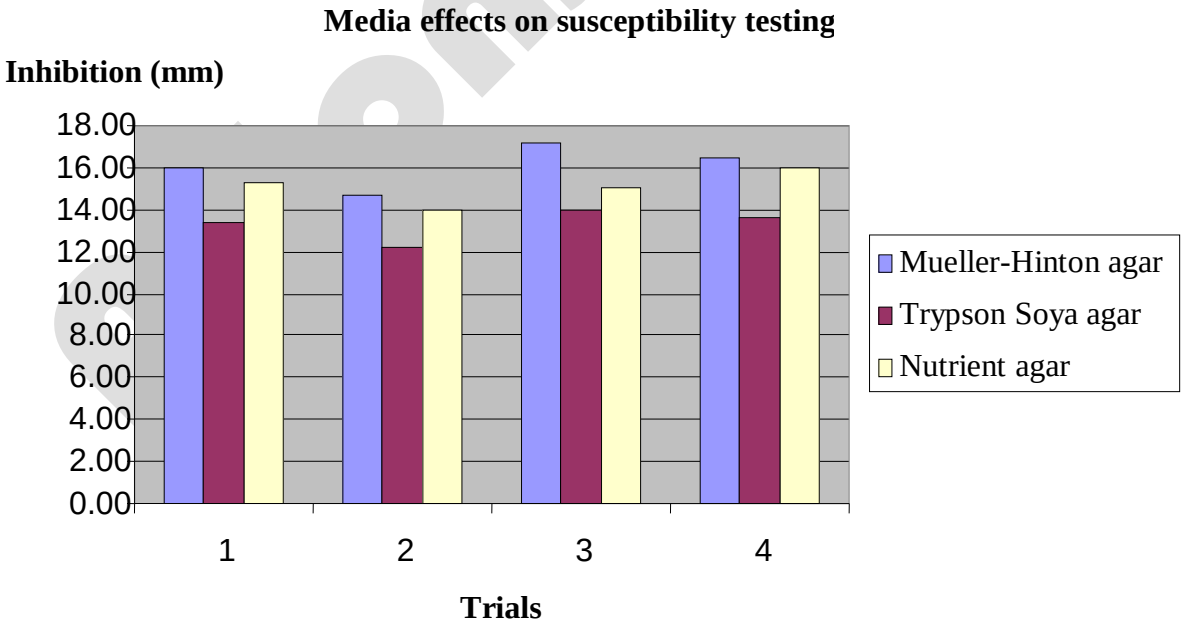

Figure 2: Media influence on susceptibility testing.

The higher activity of the methanol extracts may be due to higher solubility of the active compounds in these solvents. Methanol had a higher power to extract the active antibacterial compounds in the plant which exhibited higher activity with higher zones of inhibition. The aqueous extracts had little activity against the test organisms. Cold water extract showed least activity with minimal activity in *E. coli*. This is supported by [29-31]. These studies showed that water extracts had none or poor antimicrobial activity than those made using organic solvents. This implies that among many reasons, water had some active substances present in it but in concentrations at which bioactivity was no longer detectable or, the active substances were soluble in organic solvents and basically not in water extracts. These findings are in agreement with that of [32].

There was higher activity (large inhibition zones) in *B. pumilus* and *S. aureus* as compared with other bacteria and fungi. It is common observation that Gram-negative bacteria are more resistant to many compounds than Gram-positive ones. This is generally ascribed to the morphological differences between these two microorganisms. It was also observed that, after exposure to a given antibiotic agent, *E. coli* was found to decline in numbers for the first two hours, and then rapidly increased almost at the same rate as the control. This was consistent with the observation that almost all of the extracts did show lesser activity against *E. coli,* than that of *S. aureus*. The Gram-positive bacteria are more susceptible having an outer thin peptidoglycan layer which is not an effective permeability barrier. Plates 4, 5, 6, and 7 show the positive and negative results of the susceptibility tests. The tested plant extracts were most active against Gram-positive microorganisms than Gram-negative microorganisms. This is in agreement with previous studies by several scholars [see 33].

Report that the inactivity of water extracts could be attributed to the extracts not being prepared according to the traditional methods [35] which involved boiling in water for several hours. It is also worth noting that the traditional practitioners use water because that is all they had at their disposal. Nevertheless, their success may be due to administration of the concoctions/decoctions in large quantities, and the treatment in most cases involves the use of the extracts for a long period of time [27, 34]. The aqueous extracts may possibly be active against bacterial strains which were not tested in the current study as reported by [35].

Concentration of 0.32 mg mL^{-1} of gentamycin and 0.30 mg mL^{-1} of Nystatin were used as positive controls for bacteria and fungi respectively. Gentamycin, a standard antibiotic, had a better antibacterial activity than methanol extract of *Phyllanthus* spp. The solvents in comparison to antibiotics showed 80% activity for methanol, 48% for DCM:MeOH (1:1), 43% in hot water and 28% for cold water. The antibacterial activity of various plant species has been compared to that of standard antibiotics [36].

The antimicrobial activity of the extracts of *Phyllanthus* spp. may be due to the presence of lignans; phyllanthin and hypophylanthin, flavonoids, triterpenoids, glycosides, and tannins, in the plant extract [26]. Phytochemical constituents like flavonoids are known to prevent gastric ulcer due to the astringent and antimicrobial properties, which appear to be responsible for gastro-protective activity. P-cymene, a monoterpenoid has also tested for antimicrobial properties using the paper disc diffusion method, in which it revealed a good antimicrobial activity [37]. More importantly, there have been no side-effects or toxicity reports for many years on this plant [38]. Although there has been extensive research on this plant, further research needs to be done especially towards the mechanism of biological activity of phytochemicals from this plant.

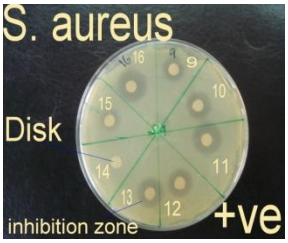

Plate 4: Disk-positive results of *Staphylococcus aureus* showing inhibition zones.

Legend: 9 - *P. amarus* ethanol extract, 10 - *P. amarus* methanol extract, 11 - *P. odontadenius*, 12 - *P. odontadenius* methanol extract, 13 - *P. amarus* MeOH extract, 15 - *P. amarus* root cold water extract, 16 - *P. odontadenius* DCM:MeOH extract.

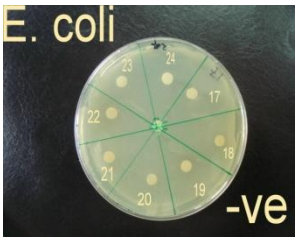

Plate 5: Disk-negative results of *Escherichia coli* showing inhibition zones.
Legend: 18 - *P. amarus* DCM:MeOH extract, 19 - *P. odontadenius* MeOH extract, 21 - *P. odontadenius* hot water extract, 22 - *P. amarus* shoot hot water extract, 23 and 24 blank.

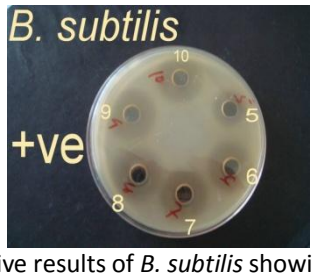

Plate 6: Well-positive results of *B. subtilis* showing inhibition zones.
Legend: 5 - *P. amarus* methanol extract, 6 - *P. odontadenius*, 7 - *P. odontadenius* methanol extract, 8 - *P. amarus* MeOH extract, 9 - *P. amarus* root cold water extract, 10 - *P. odontadenius* DCM:MeOH extract.

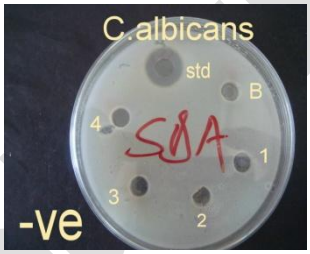

Plate 7: Well-negative results of *C. albicans* showing inhibition zones.
Legend: Std - positive control, B - blank negative control, 1 - *P. odontadenius* cold water extract, 2 - *P. niruri* MeOH extract, 3 - *P. amarus* DCM:MeOH extract, 4 - *P. niruri* cold water extract.

The small inhibition zones (10 mm) of the extract in almost all the strains tested could be associated with diffusion problem of the active constituent(s). If the active constituents were macromolecules, there could be diffusion problem on the agar media as the molecules move slowly on such a matrix system. Even though it was prepared at the same concentration as those of other extracts, it had thicker consistency which might have been responsible for the reduced-size of the colored zone that appeared on the dish containing 15% agar. Moreover, the plant materials were extracted without prior deflating and hence, the high amount of fatty material and pigment might have also inhibited the process of diffusion of the active principle(s). In comparison, the extracts of *P. niruri, P. amarus, and P. odontadenius* did not show much difference in activity, though, *P. amarus* showed slightly higher inhibitory activity.

The outcome of susceptibility testing is known to be influenced by several factors, some of which include the medium used for bacterial culture, type of drug tested, and the type of organism [39]. This study introduced a deviation from the standard MHA results of 6.12% and 17.13% for nutrient agar and trypson agar by using Nutrient and TSA in susceptibility testing respectively. The high discrepancy of susceptibility results observed between MHA and each of Nutrient and TSA for majority of the drugs tested raises doubts about the reliability of the Nutrient and TSA for microbial susceptibility testing. While similarity of susceptibility results have been reported between MHA and certain media including Oxoid sensitivity test medium and Iso-Sensitest agar, high discrepancy have been reported for other media such as Wilkins-Chalgren agar [23]. The suitability of culture media for susceptibility testing is often associated with the composition which could affect growth of the test organism or drug activity in

various ways. For media of poor suitability such as nutrient and TSAs, there is usually the presence of antagonistic substances or unsuitable pH that inhibits drug activity.

4. Conclusion

The results of the antimicrobial screening show that all extracts exhibited appreciable antibacterial properties inhibiting growth of all bacteria. This study therefore, has provided bases to the folkloric use of this plant as a remedy for urinary tract infection, skin disease and other infections caused by the pathogens studied as practiced ethno-medically all over the world. It also justifies the folklore medicinal uses and claims about the therapeutic values of these plants as curative agent. The findings of this study discourage the use of Nutrient and TSAs in the Kirby-Bauer method as practiced by some laboratories in Kenya. This is due to the considerable error margin these media may introduce into microbial susceptibility results. The results yielded by using the box plot method show a mean and confidence interval of 50% of the inhibition in MHA being above 15 mm. Further purification and characterization of the phytochemicals should be done to determine their efficacy as antimicrobial agents. This should be with a view of obtaining useful chemotherapeutic agent. Compounds with high activity should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, specifically, toxicity studies that examine both the effects and benefits on normal microbiota should be undertaken. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search is made more systematic and the interpretation of results facilitated.

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