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Combined Effect of Crude Leaf Extracts of Selected Medicinal Plants against Selected Enteric Bacterial Pathogens and *Candida albicans*

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Abstract

The main aim of the study was to determine the antimicrobial potency of the plant extracts from leaves of *Aloe secundiflora*, *Bulbine frutescens*, *Tagetes minuta* and *Vernonia lasiopus* when used in combinations. The extracts were used against Gram negative bacteria (*Shigella flexineri*, *Salmonella typhi*, *Escherichia coli* and *Enterococcus faecalis*), Gram positive bacteria (*Staphylococcus aureus*) and fungal pathogen Candida albicans by Kirby Bauer method. The combination were determined using the permutation formula of P=N! / (N-R)! Producing six combinations. The combining of the plant extracts from *Bulbine frutescens* and *Vernonia lasiopus* with the others showed improved antimicrobial activity especially against *Escherichia coli*. A decrease in antimicrobial activity was observed when all the plant extracts were used in combinations against *Candida albicans*. The standard antibiotics used were Ciprofloxacin (Gram negative bacteria), Vancomycin (Gram positive bacteria) and fluconazole against *Candida albicans*. The preliminary phytochemical screening of the extracts confirmed the presence of alkaloids, saponins, tannins and flavonoids. Our study revealed that, some of the plant extracts can be used in combination in improving their effectiveness in treating the diseases caused by the bacterial pathogens and fungus *Candida albicans*.

Keywords: Combined effect; Antimicrobial potency; Phytochemical screening; Kirby Bauer; Standard antibiotic

Introduction

Medicinal plants have been identified and used throughout human history [1]. The use of medicinal plants (herbs) to treat diseases is almost universal among non-industrialized societies, and is often more affordable than purchasing expensive conventional drugs [2]. The World Health Organization (WHO) estimates that 80% of the world population especially Asian and African countries use herbal medicine for some aspect of primary health care [3]. Over 120 active compounds currently isolated from the higher plants are widely used in modern medicine and 80% of these show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived [2].

Medicinal plants are used by almost 80% of the world's population for their basic health care because of their low cost and ease in availability [4]. From the dawn of civilization, people have developed great interest in plant based drugs and pharmaceutical products [4]. In the last few decades many bacterial organisms have continued to show increasing resistance against current antimicrobial agents [5]. Herbal drugs made from medicinal plants have been used from ancient times to treat various diseases and their antimicrobial properties make them a rich source of many potent drugs [6]. The use of herbal medicinal plants has always played a positive role in the control or prevention of diseases such as diabetes, heart disorders and various cancers [7]. Some medicinal plants have been used in production of various drugs singly or in combination and even as principal raw material for the production of other conventional medicines [8].

Tagetes minuta L. is also known as Southern Cone Marigold, Stinking Roger or black mint [9]. It is a tall upright plant, with small flowers, native to the southern half of South America [9]. The genus comprises of 56 species which grow either annually or perennially and mostly herbaceous plants [10]. The herbaceous plants were mostly found in North and South America but some species have become naturalized around the world [10]. The total extracts from leaves, flowers, stem and other parts of the plant have shown antibacterial activity against Gram positive and Gram negative bacteria [11]. Extracts from the other common species have also been used as medicine in treating various illnesses such as stomach problems and intestinal disorders [12].

The genus Aloe belongs to Aloeaceae (liliaceae) family which has around 360 to 600 different species [13]. Aloe species have antibacterial, antifungal, anticancer, antiviral and immunomodulatory properties [14]. Aloe secundiflora leaf components have been credited for antibacterial, antifungal and antiviral and antihelmintic medicinal properties [15]. Bulbine is a genus of plants in the family xanthorrhoeaceae and sub family asphodeloideae and its members are well known for their medicinal value [16]. The most common species is *Bulbine frutescens* which is popularly grown in flower gardens [17]. Many species have bulb shaped tuber and they are chiefly found in South Africa with a few species extending to the tropics of Africa and Australia [18]. *Vernonia lasiopus* belongs to the tribe Vernonieae in the family Asteraceae which mostly contains herbaceous plants [19]. Vernonia are shrubs and grow in tropical Africa and have a height of about 2-5 metres, elliptical leaves of up to 20 centimetres and a rough bark [20]. Studies carried out have shown some of the phytochemical components found in their extracts have antimicrobial capability [21]. *Vernonia lasiopus* decoctions from

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the stems and leaves have been traditionally used by herbalists in East Africa to treat, malaria, worms and gastrointestinal problems [22]. The main aim of the study is to provide insight about the combined antimicrobial effects of the leaf extracts from the selected medicinal plants and their use and effectiveness in treatment of bacterial or fungal infections.

Materials and Methods

Plant material collection

The fresh plant material of *Aloe secundiflora*, *Bulbine frutescens*, *Vernonia lasiopus* and *Tagetes minuta* was collected at Kenyatta University Arboretum. Voucher specimen was prepared and deposited in the university herbarium in Plant Sciences Department for future reference. The plants were brought to the laboratory and thoroughly washed in running water to remove debris and dust particles and then rinsed using distilled water and finally air dried.

Preparation of plant extract

The air dried plant materials were tause into powder and soaked in methanol for 72 hours, placed in a Gallenkamp shaker at 65 revolutions per minute. The contents were homogenized and filtered using whatman filter paper no. 1. The filtrate was poured into a round bottom flask and concentrated using a vacuum evaporator and stored in a labelled amber glass bottle at room temperature away from light and heat before being used for antimicrobial efficacy test.

Test bacterial organisms

The microorganisms used were clinical isolates of *Escherichia coli*, *Salmonella typhi*, *Enterococcus faecalis, Staphylococcus aureus*, *Shigella flexineri*, *Enterococcus faecalis* and *Candida albicans* obtained from Kenyatta University Health Centre Laboratory, Nairobi. The microorganisms were tested against methanol extracts of *Tagetes minuta*, *Aloe secundiflora*, *Bulbine frutescens* and *Vernonia lasiopus* impregnated on discs.

Antimicrobial susceptibility testing

The microorganisms used (*Escherichia coli*, *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Shigella flexineri*, *Enterococcus faecalis* and *Candida albicans*) were concentrated by comparing it with a 0.5 McFarland standard. Discs of 6 milliliters were prepared from whatman no.1 filter paper. The discs were sterilized by autoclaving. After sterilization the moisture discs were dried on hot air oven at 50°C [23]. The various plant extracts were halved in every subsequent serial dilution. The discs were impregnated with the extracts from the highest concentration of 1000 mg/ml to the lowest concentration of 1 mg/ml [24].

The antimicrobial efficacy test was carried out using Kirby Bauer method [13]. Hektoen agar was used in the spread plate technique where the clinical isolates were spread using sterilized cotton wool swabs. They were exposed to extracts impregnated discs in milligrams per milliliter from *Aloe secundiflora*, *Tagetes minuta*, *Vernonia lasiopus* and *Bulbine frutescens*.

The discs were placed with equal distance between them on agar plates inoculated with the bacterial pathogens and *Candida albicans*. Positive control discs containing ciprofloxacin was used for the bacteria's *Escherichia coli*, *Salmonella typhi*, *Enterococcus faecalis*, *Shigella flexineri*; vancomycin for *Staphylococcus aureus*, and fluconazole for fungus *Candida albicans*. A negative control of discs impregnated with DMSO was also used. The Petri dishes were incubated at 37°C for 24 hours. Zones of inhibition were measured in millimetres and their average determined. The experiment was carried in duplicates and the diameter of zones of inhibition formed measured.

Minimum inhibitory concentration (MIC) and maximum bactericidal (MBC) test

Minimal inhibitory concentration (MIC) was determine using the broth tube method [25]. 100 µl of 250 mg/ml of methanol extract was added to 100 µl of sterile bacteriological peptone in the first well of the 96 well micro plate and mixed well with a micropipette. 100 µl of this dilution was transferred subsequently to wells two folding each dilution of the original extract. This was done to the extracts of *Aloe secundiflora*, *Bulbine frutescens*, *Vernonia lasiopus*, and *Tagetes minuta*.

An inoculum of 100 µl (0.5 McFarland standard) of overnight clinical cultures of ; *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Shigella flexineri*, *Enterococcus faecalis* and fungus *Candida albicans* were added in each of the wells. Triplicate of each micro plate were made and the procedure repeated for each of the test organisms. The plates were then incubated at 37°C for 24 hours. After incubation 40 µl of 0.2 mg/µl of INT was added in each of the wells and the plates examined after an additional 60 minutes of incubation. Growth was indicated by a red colour (conversion of INT to formazan). The lowest concentration at which the colour was apparently invisible as compared to the next dilution was taken as the minimum inhibitory concentration [26]. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) was determined [26]. 100 µl of suspension was taken from micro plate wells that demonstrated no growth and inoculated on agar plates. The plates were incubated at 37°C for 24 hours. In the case where there was no bacterial growth and also not greater than the minimum inhibitory concentration was used to determine the maximum bacterial concentration and maximum fungicidal concentration.

Combined effect test

The combined effect of the plants extracts was determined by using the minimum inhibitory concentrations of each extract against the microorganisms (Table 1.0). The combination were determined using the permutation formula of P=N! / (N-R)! Producing six combinations. The zones of inhibition formed were measured and compared to the ones when each extract is used separately against the microorganism. The process was carried out in replicates.

Phytochemical analysis

Presence of saponins, tannins, flavonoids and alkaloids in the crude extract were determined [27].

Tannins: Each of the extracts was weighed to 0.5 mg and dissolved in 1 ml of distilled water. Filtration was carried out after 2 ml of $FeCl_3$ was added. If there was presence of a blue or black precipitate then it indicated the presence of tannins.

Flavonoids: Each of the extracts was weighed to 0.5 mg and dissolved in 1 ml of ethanol and filtered. 2 ml of 1% HCl and magnesium ribbon was added to the filtrate. If there was formation of a pink or red colour it indicated the presence flavonoids.

Alkaloids: Each of the extracts was weighed to 0.5 mg and dissolved in 1 ml of methanol and filtered. 1% HCL was added to the filtrate and

the solution heated. Mayor`s reagent was added drop wise and if there was formation of any colored precipitate it indicated the presence of alkaloids.

Saponins: Each of the extracts was weighed to 0.5 mg and dissolved in 1 ml of methanol and filtered. Distilled water was added and shaking done for a few minutes. If there was persistence frothing then it indicated the presence of saponins.

Results

When the plants extracts were used in combination, they showed less antimicrobial activity against *Candida albicans* and *Enterococcus faecalis* as compared to when used singly as compared to the other test microorganisms (Table 1.0). The combination of the plant extracts showed both increased and decreased antimicrobial activity against the other microorganisms as compared to when used singly (Table 1.0).

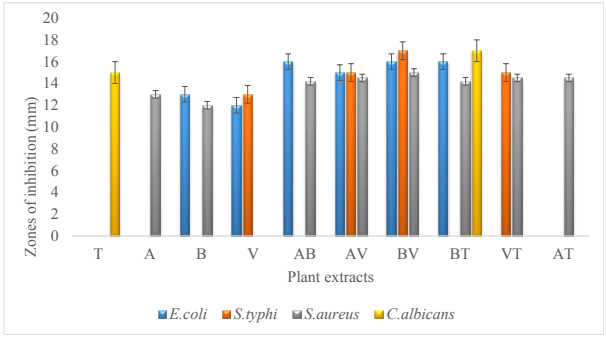
The plant extracts from *Bulbine frutescens* and *Vernonia lasiopus* showed less antimicrobial activity when used singly against *Escherichia coli*. They respectively produced average zone of inhibition of 13 ± 0.97 mm and 12 ± 1.67 mm (Table 1.0). When used in combinations they showed improved antimicrobial activity when used in combinations by producing average zone of inhibition ranging from; AB (16 ± 0.05 mm), AV (15 ± 0.71 mm), BV, BT and VT (16 ± 0.05 mm) (Figure 1.0). *Vernonia lasiopus* extract was less effective against *Salmonella typhi* producing average zone of inhibition of 13 ± 1.68 mm. When used in combination with the other plant extracts, it showed pronounced antimicrobial activity producing larger zones of inhibition with the BV (17 ± 1.06 mm) producing the largest average zone of inhibition whereas AV and VT combinations both produced average zone of inhibition of 15 ± 0.35 mm (Figure 1.0).

Bulbine frutescens and *Aloe secundiflora* extracts were less effective when used singly against *Staphylococcus aureus* producing a zone of inhibition of 12 ± 1.94 mm and 13 ± 0.17 mm respectively. The combining of the extracts showed an improved antimicrobial activity against *Staphylococcus aureus* producing larger average zone of inhibition; AB (14.2 ± 0.18 mm), AV (14.5 ± 0.11 mm), AT and VT (14.5 ± 0.04) both (Figure 1.0). *Tagetes minuta* extract was less effective against *Candida albicans* producing a zone of inhibition of 15 ± 1.06 mm. When used in combination with *Bulbine frutescens*, it showed an increase in antimicrobial activity producing a larger zone of inhibition of 17 ± 2.47 mm (Figure 1.0)

The plant extract from *Vernonia lasiopus* showed high antimicrobial activity at low concentrations against fungus *Candida albicans* (5.5 mg/ml) and *Enterococcus faecalis* (5.0 mg/ml) at low concentrations as compared to the other plant extracts (Table 1.1). The plant extract from *Tagetes minuta* was produced a more effective antimicrobial activity against *Enterococcus faecalis* (6.3 mg/ml) at low concentrations as compared to its activity against *Shigella flexineri* which had an MIC of 12.6 mg/ml. *Aloe secundiflora* extract was more active against *Salmonella typhi* as compared to its activity on *Escherichia coli* (Table 1.1). *Bulbine frutescens* extract was more active against *Shigella flexineri* at low concentrations (6.2 mg/ml) as compared to *Escherichia coli* (Table 1.1)

The plant leaf extracts from *Tagetes minuta*, *Aloe secundiflora*, *Bulbine frutescens* and *Vernonia*

Lasiopus also contained all the phytochemicals namely; saponins, tannins, alkaloids, and flavonoids (Table 1.2). Some of those



Key: T: *Tagetes minuta*, **A**: *Aloe secundiflora*, **B:** *Bulbine frutescens*, **V:** *Vernonia lasiopus*, **AB**: *Aloe* + *Bulbine*, **AV:** *Aloe* + *Vernonia*, **BV**: *Bulbine* + *Vernonia*, **BT**: *Bulbine* + *Tagetes*, **VT**: *Vernonia* + *Tagetes*, **AT**: *Aloe* + *Tagetes*.

Figure 1.0: A comparison of antimicrobial activity of plant extracts against test microorganisms when used singly and in combinations.

phytochemicals present in the plant extracts might be responsible for their antimicrobial activity against fungi, Gram positive bacteria and Gram negative bacteria [11,21,28,29].

Discussion

When plant extracts were used in combinations they showed diverse antimicrobial activity against the tested microorganisms. The study described a first time investigation on combined effect of crude extracts from the medicinal plants used. When the plant extracts were used in combination against *Escherichia coli*, they showed a pronounced antimicrobial activity as compared to when each of them is used separately against it. *Bulbine frutescens* and *Vernonia lasiopus* produced less average zones of inhibition when each of them is used separately against the microorganism (Table 1.0). However, the combinations of the plant extracts of; *Aloe secundiflora* and *Bulbine frutescens*, *Aloe secundiflora* and *Vernonia lasiopus*, *Bulbine frutescens* and *Vernonia lasiopus*, *Bulbine frutescens* and *Tagetes minuta*, showed increased antimicrobial activity against *Escherichia coli* (Figure 1.0). This meant; the plant extracts from *Bulbine frutescens* and *Vernonia lasiopus* when used in combination can enhance antimicrobial activity against *Escherichia coli*.

The use of plant extracts in combinations showed either increased or decreased antimicrobial activity against *Salmonella typhi* as compared to when each of them is used separately against it (Table 1.0). When the extract from *Tagetes minuta* is used in combination with *Vernonia lasiopus*, it showed a decrease in antimicrobial activity against *Salmonella typhi* as compared to when each of them is used separately (Table 1.0). However, other combinations of plant extracts of; *Bulbine frutescens* and *Vernonia lasiopus*, *Aloe secundiflora* and *Vernonia lasiopus*, when used in combination against *Salmonella typhi* showed an enhanced antimicrobial activity; compared to when used separately against the same microorganism (Table 1.0 and Figure 1.0). The findings from the study elucidated that, combining of *Bulbine frutescens* and *Vernonia lasiopus* with the other plant extracts enhanced their antimicrobial activity against *Salmonella typhi*.

The use of plant extracts in combinations against *Staphylococcus aureus* did not significantly increase the antimicrobial activity as compare to when each of them is used separately (Table 1.0). The

Plants Extracts	*E.coli*	*S.typhi*	*S.aureus*	*S.flexineri*	*E.faecalis*	*C.albicans*
AB	16 ± 0.05	15 ± 0.35	14.2 ± 0.18	18.7 ± 0.34	18 ± 0.55	14 ± 0.35
AV	15 ± 0.71	15 ± 0.35	14.3 ± 0.11	18.2 ± 0.01	17.7 ± 0.31	15 ± 1.06
AT	17 ± 071	14 ± 1.06	14.5 ± 0.04	18.7 ± 0.34	17.7 ± 0.31	8 ± 3.89
BV	16 ± 0.05	17 ± 1.06	15 ± 0.39	17.7 ± 0.37	17.5 ± 0.20	13 ± 0.35
BT	16 ± 0.05	17 ± 1.06	14.2 ± 0.18	17.8 ± 0.30	16.3 ± 0.63	17 ± 2.47
VT	16 ± 0.05	15 ± 0.35	14.5 ± 0.04	18.2 ± 0.01	16 ± 0.75	14 ± 0.35
T	16 ± 1.27	17 ± 1.15	17 ± 1.94	19 ± 0.21	19 ± 0.35	15 ± 1.68
A	17 ± 1.38	16 ± 0.68	13 ± 0.17	18 ± 0.38	18 ± 0.35	17 ± 0.38
B	13 ± 0.97	15 ± 0.15	12 ± 1.94	20 ± 0.56	19 ± 0.35	18 ± 0.21
V	12 ± 1.67	13 ± 1.68	14 ± 0.64	18 ± 0.38	18 ± 0.35	20 ± 1.86
+ve control	20 ± 2.48	24 ± 0.35	25 ± 1.10	22 ± 1.10	22 ± 1.10	28 ± 3.19
-ve control	0.00	0.00	0.00	0.00	0.00	0.00
Key: AB: *Aloe* + *Bulbine*; **AV**: *Aloe* + *Vernonia*; **AT**: *Aloe* +*Tagetes*; **BV**: *Bulbine* + *Vernonia*; **BT**: *Bulbine* + *Tagetes*; **VT**: *Vernonia* + *Tagetes*; ± Standard error;:**ve control** (DMSO); **+ve control** (ciprofloxacin, vancomycin and Fluconazole); **T:** *Tagetes minuta*; **A**: *Aloe secundiflora*; **B**: *Bulbine frutescens*; **V**: *Vernonia* lasiopus; 7 mm: 10 mm **Resistant**; 11 mm: 20mm **Intermediate**; 21 mm: 30 mm **Sensitive**						

Table 1.0: Zone of inhibition in millimetres.

Plant extracts	*E. coli*	*S. typhi*	*S. aureus*	*S. flexineri*	*E. faecalis*	*C. albicans*
Tagetes minuta	10.0	8.2	10.0	12.6	6.3	8.7
Aloe secundiflora	10.4	7.3	12.9	8.0	9.7	9.0
Bulbine frutescens	14.0	10.9	13.9	6.2	9.1	8.0
Vernonia lasiopus	11.5	7.5	14.2	7.1	5.0	5.5
$DMSO_4$	-	-	-	-	-	-
Distilled water	-	-	-	-	-	-
Ciprofloxacin	0.005	0.005	-	0.005	0.005	-
Vancomycin	-	-	0.03	-	-	-
Fluconazole	-	-	-	-	-	0.015

Table 1.1: Minimum bactericidal concentration and minimum fungicidal concentration in mg/ml.

Name of test	Plants leaf extracts			
	T. minuta	*A. secundiflora*	*B. frutescens*	*V. lasiopus*
Saponins test	+	+	+	+
Tannins test	+	+	+	+
Alkaloids test	+	+	+	+
Flavonoids test	+	+	+	+
Key: (+) present				

Table 1.2: Phytochemical tests on the plant extracts.

combinations of ; *Bulbine frutescens* and *Vernonia lasiopus*, *Aloe secundiflora* and *Tagetes minuta*, *Bulbine frutescens* and *Tagetes minuta* showed enhanced antimicrobial activity as compared to when; *Bulbine frutescens*, *Aloe secundiflora* and *Vernonia lasiopus* were used singly against *Staphylococcus aureus* (Table 1.0 and Figure 1.0).

Shigella flexineri showed either an increase or a decrease in antimicrobial activity when exposed to the plant extracts combinations. When each of the plant extracts were used separately against *Shigella flexineri* they showed pronounced antimicrobial activity with *Bulbine frutescens* extract being the most active when compared to extracts from other plants (Table 1.0). The combining of *Bulbine frutescens* with; *Tagetes minuta*, *Aloe secundiflora* and *Vernonia lasiopus* each separately against *Shigella flexineri* showed decreased antimicrobial activity as compared to combinations formed using *Tagetes minuta*, *Aloe secundiflora* and *Vernonia lasiopus* (Table 1.0). This meant; combinations of plant extracts from *Tagetes minuta*, *Aloe secundiflora*, *Bulbine frutescens* and *Vernonia lasiopus* had almost similar antimicrobial activity against *Shigella flexineri* as compared to when each of them are used separately against it (Table 1.0).

When the combinations of the plant extracts were used against *Enterococcus faecalis*, they showed a decrease in antimicrobial activity (Table 1.0). From the findings of the study, plant extracts from; *Tagetes minuta*, *Aloe secundiflora*, *Bulbine frutescens* and *Vernonia lasiopus* showed pronounced antimicrobial activity when each of them are used separately against *Enterococcus faecalis* (Table 1.0). Plant extracts combinations from; *Aloe secundiflora* and *Vernonia lasiopus*, *Aloe secundiflora* and *Tagetes minuta*, *Bulbine frutescens* and *Tagetes minuta*, *Vernonia lasiopus* and *Tagetes minuta*, *Bulbine frutescens* and *Vernonia lasiopus*, showed decreased antimicrobial activity as compared to when each of them are used separately against *Enterococcus faecalis* (Table 1.0). This is further supported by the less average zones of inhibition formed when the plant extracts are used in combinations (Table 1.0). This meant, the combination of the plant extracts may be responsible for the decreased antimicrobial activity against *Enterococcus faecalis*.

Candida albicans, showed either an increase or decrease in antimicrobial activity with the latter being more pronounced when the plant extracts were used in combinations. The plant extracts had pronounced antimicrobial activity when each of them was used separately against *Candida albicans* with *Vernonia lasiopus* being the most active as compared to others (Table 1.0). Most of the combined extracts had a decrease in antimicrobial activity with the combination of *Aloe secundiflora* and *Tagetes minuta* showing dismal antimicrobial activity as compared to others (Table 1.0). Plant extracts combination of *Bulbine frutescens* and *Tagetes minuta*, showed enhanced antimicrobial activity against *Candida albicans* as compared to when *Tagetes minuta*

was used separately against it (Table 1.0 and Figure 1.0). This meant, the combination of the extracts mostly reduced the antimicrobial capability of each extracts as compared to when each of them is used separately against *Candida albicans*.

Conclusion

In conclusion, the combination of the plant extracts exhibited diverse antimicrobial activity against the selected bacterial pathogens and fungus *Candida albicans*. When the extracts were used in combination they showed pronounced antimicrobial activity against *Escherichia coli* whereas less antimicrobial activity was observed against *Candida albicans*. This study could provide a new basis on the using of herbal medicinal plants in combination for the effective treatment of the enteric bacterial pathogens and fungus *Candida albicans*.

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