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Staphylococcus aureus photo by Bel Marra Health

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Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Antimicrobial activity and qualitative phytochemical composition of crude extracts from medicinal plants against selected enteric bacterial pathogens, *Candida albicans*

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Abstract *Opinde HR, Nyamache AK, Gatheri GW. 2018. Antimicrobial activity and qualitative phytochemical composition of crude extracts from medicinal plants against selected enteric bacterial pathogens and* Candida albicans*. Bioteknologi 14: 1-12.* Determining the antimicrobial activity and combined effects of the selected plant leaf extracts of *Tagetes minuta*, *Aloe secundiflora*, *Vernonia lasiopus* and *Bulbine frutescens* against selected clinical isolates of *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Shigella flexineri*, *Enterococcus faecalis* and *Candida albicans* were the aims of this study. Moreover, the determination of qualitative analysis of the phytochemicals present in the extracts was also carried out. Kenyatta University arboretum and voucher specimens deposited in the University herbarium, were the source of plant materials. SAS version 9.1 with ANOVA was used to analyze the collected data. Furthermore, the data was subjected to a post hoc test, with $P<0.05$ being considered significant. In the single use or combination of microorganism test, in tested microorganisms the average inhibition zones were found to be significant at $P<0.05$. In the low concentrations on the tested microorganisms, *V. lasiopus* was more active against *S. flexineri* (MIC 3.3 µg/mL, MBC 7.1 µg/mL), *B. frutescens* against *S. flexineri* (MIC 3.2 µg/mL, MBC 6.2 µg/mL), *A. secundiflora* against *S. flexineri* (MIC 3.7 µg/mL, MBC 8.0 µg/mL) and *T. minuta* against *E. faecalis* (MIC 5.1 µg/mL, MBC 6.3 µg/mL)*. The extract combination also showed a significant increased and decreased antimicrobial activity i.e., $P<0.05$. The average inhibition zone formed by the combination of *A. secundiflora* and *T. minuta* plant leaf extracts (8.67±1.86mm) showed a decrease in antimicrobial activity as compared to *T. minuta* (15.17±2.71 mm) and *A. secundiflora* (17.00±2.10mm) respectively in the usage against *C. albicans*. The presence of four phytochemicals; saponins, tannins, alkaloids, and flavonoids is shown in the qualitative phytochemical analysis. An insight into the antimicrobial activities of the plant extracts and their use in the treatment of bacterial or fungal infections is provided by this study. This information might be used in herbal medicine in making concoctions to maximize their effectiveness. To elucidate the actual compounds in the plant leaf extracts responsible for the antimicrobial activity is needed and this elucidation can be used in drug development.

Keywords: Antimicrobial activity, phytochemical, medicinal plants, enteric bacterial pathogens, *Candida albicans*

INTRODUCTION

Almost 80% of the world's population uses medicinal plants for their basic health care. This is often because of their low cost and ease of availability. From the dawn of civilization, people have developed a great interest in plant-based drugs and pharmaceutical products (Shazadi et al. 2010). In recent decades, many bacterial organisms have continued to show increasing resistance against current antimicrobial agents (Nascimento et al. 2000). 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 (Srivastava et al. 2005).

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 (Mohanta et al. 2003). Some medicinal plants have been used in the production of various drugs singly or in combination; even as principal raw material to produce other conventional medicines (Tahir and Khan 2012). *Tagetes minuta* L. is also known as Southern Cone Marigold, Stinking Roger, or black mint. It is a tall upright plant, with small flowers, native to the southern half of South America (Everett 1982). The genus comprises 56 species, of which they grow either annually or perennially, and are mostly herbaceous plants (Soule 1996). Other common species of the family include *Tagetes erecta*, *Tagetes patula* and *Tagetes tenuifolia* (Tereschuk et al. 1997). Most of herbaceous plants are mostly found in North and South America, with some species being naturalized around the world. *T. minuta* extracts have been used as medicinal tea in some areas (Soule 1993). The total extracts from leaves, flowers, stem, and other parts of the plant have shown antibacterial activity against Gram positive and Gram-negative bacteria (Tereschuk et al. 1997). Extracts from the other common species have also been used as medicine in treating various illnesses such as stomach problems and intestinal disorders (Broussalis et al. 1999).

Some of the components contained in *T. minuta* extracts such as flavonoids have been tested and proved to have antimicrobial activity against not only bacteria but also fungi and some nematodes (Cushnie and Lamb 2005). The genus *Aloe* belongs to Aloeaceae (Liliaceae) family which

has around 360 to 400 different species (Newall et al. 1996). Aloe species have antibacterial, antifungal, anticancer, antiviral, and immunomodulatory properties (Holzmuller et al. 2002). *Aloe secundiflora* Engl. is also known as *Aloe floramaculata*, *Aloe engleri* and *Aloe marsabitensis* (Kaingu et al. 2013). Other common species include *Aloe arborescens*, *Aloe chabaudii*, *Aloe turkanensis*, *Aloe greatheadii*, *Aloe cameronii and Aloe excels*. *A. secundiflora* leaf components have been credited for antibacterial, antifungal, and antiviral and anthelmintic medicinal properties (Mwale et al. 2005). *Bulbine* is a genus of plants in the family Xanthorrhoeaceae and subfamily Asphodeloideae and its members are well known for their medicinal value (Acock 1988). They are succulent plants with most species having yellow flowers while others are white, orange, or pink flowers. The most common species is *Bulbine frutescens* which is popularly grown in flower gardens (Van Wyk 2008). 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 (Coopoosamy et al. 2000). *Vernonia lasiopus* O. Hoffman belongs to the tribe Vernonieae in the family Asteraceae which mostly contains herbaceous plants (Keeley 2007). *Vernonia* is a shrub that grows in tropical Africa and has a height of about 2-5m elliptical leaves of up to 20 cm and a rough bark (Ijeh and Ejike 2011).

The plants in this family usually have a bitter taste and in English, they are called bitter leaf (Ijeh and Ejike 2011). Studies carried out have shown some of the phytochemical components found in their extracts have the antimicrobial capability (Koul et al. 2003). *V. lasiopus* decoctions from the stems and leaves have been traditionally used by herbalists in East Africa to treat, malaria, worms, and gastrointestinal problems (Kareru et al. 2008). The study of contemporary medicine has yielded promising and commendable results on the antibacterial activity of medicinal plant extracts as potential drugs that can be added along with contemporary drugs (Coopoosamy et al. 2007).

The aims of this research were (i) To determine the antimicrobial activity of plant extracts from *T. minuta*, *A. secundiflora*, *V. lasiopus* and *B. frutescens* against bacterial pathogens and *Candida albicans*; (ii) To determine the impact of combined plants extracts against the bacterial pathogens and *C. albicans*; (iii) To determine the qualitative phytochemical present in the plant extracts obtained from *T. minuta*, *A. secundiflora*, *V. lasiopus* and *B. frutescens* were the objectives of this research.

MATERIALS & METHODS

Plants sampling

The plants were randomly collected in densely populated areas in the Kenyatta University arboretum and identified by Taxonomist Prof L.E. Newton. The plants were then placed on a table and the leaves were selected from the plants using the following criteria i.e., they have no dead leaves, they have no flowers, and they have the same height. The plant materials were randomly sampled in densely populated areas namely twenty samples of *T. minuta*, ten samples of *V. lasiopus*, five samples of *A. secundiflora* and five samples of *B. frutescens* plants. Voucher specimens were arranged and stored 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, before being rinsed using distilled water and finally air dried.

Preparation of plant extract

The air-dried leaves from the plants were ground into powder and 500 g of them were soaked in 750 mL of Analar grade (AR) methanol in a conical flask for 72 hours. Then they were placed in a Gallenkamp shaker rotating 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 Buchi Rotavapor R-200 yielding 2.8 g of *B. frutescens*, 3.1 g of *A. secundiflora*, 2.6 g of *T. minuta* and 2.1 g of *V. lasiopus*.

The extracts were then stored in labelled amber glass bottles that were slightly opened, where they were further left to dry in the bottle at room temperature away from light and heat in a laminar flow before being used for antimicrobial efficacy test.

Preparation of media

The media were Muller Hinton agar and Potato dextrose agar (Sharau®) which were prepared following commercially given instructions.

Preparation of potato dextrose agar

One L of distilled water was mixed with 39 mg of potato dextrose agar powder in a flat-bottomed conical flask. To dissolve the potato dextrose agar powder, the stirring was done while it was boiling. The flask was then tightly closed using cotton wool and further covered with aluminum foil. The mixture was autoclaved for 15 minutes at 121ºC, after which it was left to refrigerate down to room temperature. 40mg of tetracycline was added to inhibit bacterial growth, and the media was then stirred before dispensing in Petri dishes. The media was poured in the Petri dishes in a laminar flow to give uniform depth of 3-4 mm. The Petri dishes were left to refrigerate and after which they were placed in a sterile plastic bag and stored at a temperature of 2-8ºC before use.

Preparation of Muller-Hinton agar

One liter of distilled water was mixed with 38 mg of Muller-Hinton agar powder in a flat-bottomed conical flask. The mixture was heated with frequent stirring and boiled for one minute to completely dissolve the media. The flask was then tightly closed using cotton wool and further covered with aluminum foil. The mixture was autoclaved for 15 minutes at 121ºC after which it was left to refrigerate to room temperature. The media was poured in the Petri dishes in a laminar flow to give uniform depth of 3-4 mm. The Petri dishes containing the media were

then placed in a sterile plastic bag and stored at a temperature of 2-8ºC before use.

Preparation of susceptibility test discs

Discs of 6 milliliters were prepared from Whatman no.1 filter paper. This was done by punching the filter papers using a paper punch. The discs filled four McCartney bottles. The discs were then sterilized by autoclaving at 121ºC for 15 minutes, after which the autoclave was left to refrigerate before removing the McCartney bottles containing the discs. The discs were dried in a hot air oven at 50ºC to remove moisture (Arunkumar et al. 2009).

The discs were impregnated with formulated stock solution of the plant leaf extracts of 1000 µg/mL of *T. minuta*, *A. secundiflora*, *B. frutescens* and *V. lasiopus* and they were used for the antimicrobial activity. This was done by taking the sterile discs and the stock solutions into the laminar flow. Forceps, which were sterilized on a spirit lamp and refrigerated, were used to pick the discs and place them in the stock solutions of the plants leaf extracts. The forceps were sterilized after every pick. The disc was then left to stay in the plant leaf extracts stock solution for two hours. The discs were then removed and placed in a sterile Petri dish in a laminar flow and left to dry for 30 minutes. The discs impregnated with leaf extracts from each plant were then picked by sterilized forceps and stored in a sterilized McCartney bottle and stored in a refrigerator at a temperature of 4-8ºC before being used for the antimicrobial susceptibility test.

Test bacterial organisms

The tested microorganisms used in the study were clinical isolates of *Escherichia coli*, *Salmonella typhi*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Shigella flexineri*, *Enterococcus faecalis* and *Candida albicans*. The microorganisms were isolated from samples collected from first-time patients at Kenyatta University Health Centre Laboratory, Nairobi. The patients who showed symptoms associated with enteric bacterial infections such as fever, abdominal pain, diarrhea, vomiting and had no known drug resistance were selected.

Isolation, morphological identification, and biochemical test were carried out. The samples for isolating enteric bacteria were from fecal material. Sterile water was used in cleaning infected wounds to get a sample for isolation of *S. aureus* and high vaginal swab for isolation of *C. albicans*. The samples were used in isolation and of characteristic morphological identification of enteric bacteria based on the type of colonies by streaking of the samples on selected media. The morphologically identified microorganisms were then subjected to a biochemical test for identification on biochemical level.

Isolation, morphological identification

The fecal material was first mixed with sterile distilled water. The blend was serially liquefied up to 10^{-6}. A wire loop was sterilized by heating and left to refrigerate. It was then dipped into the serially liquefied sample of 10^{-6} and streaked on selected and differential media in Petri dishes used to support the growth of each of the tested microorganism. The Petri dishes were then tightly closed with a parafilm and were incubated for 24 hours at 37ºC. The plates were then removed, and the bacteria were identified according to their morphological characteristics.

Escherichia coli was isolated by streaking the sample on Eosin methylene blue and MacConkey agar. The Petri dish was tightly closed with parafilm and was incubated at 37ºC for 24 hours. Later, the plate was observed for growth of microorganism. The growth of metallic green colonies on Eosin methylene blue agar, and pink to red brick colonies on MacConkey agar with or without a zone of precipitated bile were a morphological growth characteristic of *E. coli*.

Salmonella typhi was isolated by streaking sample on *Salmonella-Shigella* agar and Hektoen agar. The Petri dish was tightly closed with parafilm and was incubated at 37ºC for 24 hours. Later, the plate was observed for growth of microorganism. The growth of colonies with or without black centers on *Salmonella-Shigella* agar and blue-green colonies with black centers on Hektoen agar was a morphological growth characteristic of *S. typhi*. *S. flexineri* was isolated by streaking sample on *Salmonella-Shigella* agar. The Petri dish was tightly closed with parafilm and incubated at 37ºC for 24 hours. Later, the plate was observed for growth of microorganism. The growth of transparent and translucent colonies on *Salmonella-Shigella* agar was a morphological growth characteristic of *S. flexineri*.

Enterococcus faecalis was isolated by streaking the sample on Columbia agar with 5% sheep blood. The Petri dish was tightly closed with parafilm and was incubated at 37ºC for 24 hours. Later, the plate was observed for growth of microorganism. The growth of diplococcus colonies with gamma hemolysis on Columbia agar with 5% sheep blood was a morphological growth characteristic of *E. faecalis*. *S. aureus* was isolated by streaking the liquefied sample from infected-free wounds on Mannitol agar. The Petri dish was tightly closed with parafilm and was incubated at 37ºC for 24 hours. Later, the plate was observed for growth of microorganism. The growth of yellow colonies with yellow zones on Mannitol salt agar was a morphological growth characteristic of *S. aureus*.

Candida albicans was isolated by streaking the liquefied sample from high vaginal swabs on Sabourauds agar containing tetracycline to prevent bacterial growth. The Petri dish was tightly closed with parafilm and was incubated at 37ºC for 24 hours. Later, the plate was observed for growth. The growth of white to cream colonies, smooth, glabrous, yeast-like colonies on Sabourauds agar was a morphological growth characteristic of *C. albicans*.

Biochemical identification

The biochemical test depended on the ability of the secluded and morphologically recognizable tested microorganisms to cause fermentation of sugars and oxidation. The biochemical tests were: citrate test, urease test, nitrate test, gelatin test, hydrogen sulphide gas test, arabinose test, fructose test, glucose test, inositol test, lactose test, maltose test, mannitol test, mannose test,

raffinose test, sucrose test and sorbitol test. The media used to complete the test were set up as indicated by the manufacturer guidelines and were poured into Nessler tubes.

Durham tubes were inserted into media containing broth and later were observed for gas production. If there were gas production, the result was marked as positive. However, if there were no gas production, then the result was marked as negative. The tubes were then closed and autoclaved at 121ºC for 15 minutes and left to refrigerate before being stored in a refrigerator for utilization. One mL inoculum of the secluded microbes test from a dissolved sample of 10^{-6} was introduced to the media and incubated at 37ºC, and observation was done on it after 24 hours. The tubes containing solid media were observed for color change. If the color change occurred, the results were marked as positive, but if there were no color changes, the results were marked as negative using standards on http://www.microbiologyinfo.com/biochemical test, 15th June 2014.

Table 1. Standard for biochemical tests on isolated microorganisms

Biochemical test	**Microorganisms**					
	E.coli	*S.typhi*	*S.aureus*	*S.flexineri*	*E.faecalis*	*C.albicans*
Citrate	-	-	+	-	-	+
Urease	-	-	+	-	-	-
Nitrate	+	n/a	+	n/a	+	-
Gelatin	-	-	+	n/a	n/a	n/a
H_2S production	-	+	-	-	-	n/a
Arabinose	+	-	n/a	n/a	-	+
Fructose	-	n/a	+	n/a	+	n/a
Glucose	+	+	+	-	+	+
Inositol	-	-	n/a	n/a	n/a	+
Lactose	+	-	+	-	+	-
Maltose	n/a	-	+	n/a	+	+
Mannitol	+	+	+	+	+	+
Mannose	n/a	+	+	n/a	+	n/a
Raffinose	n/a	-	-	n/a	-	-
Sucrose	n/a	-	+	-	+	+
Sorbitol	+	+	n/a	-	+	+

Note: (+)-Positive, (-)-Negative, (n/a)-Not applicable

Maintenance of the bacterial, fungal cultures

The cultures of the clinical isolates of the tested microorganisms were maintained on agar slants. The agar slants were prepared by making 250 mL of nutrient agar and potato Dextrose agar prepared according to r commercial instructions. The solution of the Medias was divided and placed into a lot of bijou bottles of equal amounts of 10 mL. The bijou bottles were then closed and placed in an autoclave and sterilized for about 15 minutes at 121 ºC. The autoclave was left to refrigerate. The bottle was then placed on a wedge in a laminar flow slanting at around 45ºC and left to refrigerate to form agar slants. The bacterial microorganisms were streaked on nutrient agar slant, whereas *C. albicans* was streaked on potato dextrose agar slant. The streaked agar slants were placed in an incubator at 37 ºC and observed for growth every 24 hours. The tested microorganism was subsequently subcultured every 48 hours to maintain their viability.

Antimicrobial susceptibility testing

The microorganisms (*E. coli, S. typhi, E. faecalis, S. aureus, S. flexineri, E. faecalis and C. albicans*) were concentrated by comparing it with a 0.5 McFarland standard. All the plant extracts were dissolved in 4% dimethyl sulphoxide (DMSO) to form a stock solution of 1000 µg/mL.

The stock solution concentration of each extract was formulated using the formula below:

$$C = S \times M/Q$$

Where:
S: Solute weight (mg)
Q: Dilution solvent (mL)
M: Conversion units (µg)
C: Concentration (µg/mL)

The discs were from Whatman filter paper no.1 and contained different plant leaf extracts (*T. minuta, A. secundiflora, V. lasiopus,* and *B. frutescens*) from the highest concentration (1000 µg/mL) to the lowest concentration (1 µg/mL). This was done by serially halving the concentration in subsequent dilutions and then storing in McCartney bottles away from light (Mangoma et al. 2010).

The antimicrobial efficacy test was carried out with the Kirby-Bauer method (Newall et al. 1996). Muller Hinton agar (Sharau®) was used for the tested microorganism bacteria and potato dextrose agar for *C. albicans* in the spread plate technique and the clinical isolates were spread with sterilized cotton wool swabs. They were exposed to extract discs in mg per microliter from *A. secundiflora, T. minuta, V. lasiopus* and *B. frutescens*. The discs were placed with equal distance between them on agar plates inoculated with the pathogen bacteria and *C. albicans*.

Positive control standard discs containing ciprofloxacin (5 µg/mL) was used for *E. coli, S. typhi, E. faecalis, S. flexineri*; vancomycin (3 µg/mL) for *S. aureus,* and fluconazole (15 µg/mL) for *C. albicans*. A negative control of discs containing 4% Dimethyl sulphoxide and 100% Analar (AR) methanol were also used. The Petri dishes were incubated at 37 ºC for 24 hours, then they were removed and observed to see if there was the formation of any inhibition zones by the extracts from the plant against the tested microbes. The experiment was carried out in duplicates, and the diameter of inhibition zones was measured, and their average was determined.

Minimal inhibitory concentration (MIC) was determined with the broth tube method according to Eloff et al. 1998) namely: 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 wells microplate and mixed well with a micropipette. 100 µL of this dilution was transferred subsequently to wells, two foldings for each dilution of the original extract. This was done to the extracts of *A. secundiflora, B. frutescens, V. lasiopus,* and

T. minuta. An inoculum of 100 µL (0.5 McFarland standard) of overnight clinical cultures of; *E. coli*, *S. typhi*, *S. aureus*, *S. flexineri*, *E. faecalis* and fungus *C. albicans* were added in each of the wells. Triplicate of each microplate was made, and the procedure repeated for each of the tested organisms. The plates were then incubated at 37ºC for 24 hours. After incubation, 40µL 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. Red color (conversion of INT to formazan) indicated the growth of organism. The lowest concentration at which the color was apparently invisible, as compared to the next dilution, was taken as the minimum inhibitory concentration (Rabe et al. 2002). Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined according to Rabe et al. 2002 whereby: 100µL of the suspension was taken from microplate wells that demonstrated no growth and inoculated on agar plates. The plates were incubated at 37 ºC for 24 hours. The absence of bacterial growth or the growth, which was not greater than the minimum inhibitory concentration, was used to determine the maximum bacterial concentration and maximum fungicidal concentration.

Combined effect test

The combinations were determined using the permutation formula of P=N!/(N-R)! It produced six combinations namely: *A. secundiflora* and *T. minuta* **(AT)**, *A. secundiflora* and *V. lasiopus* **(AV)**, *B. frutescens* and *T. minuta* **(BT)**, *B. frutescens* and *V. lasiopus* **(BV)**, *A. secundiflora* and *B. frutescens* **(AB)** and *V. lasiopus* and *T. minuta* **(VT)**. The combined effect was determined with ratio of 1:1 using the plants leaf extracts with the concentrations of 1000 µg/mL of each extract. The combined plant leaf extracts were used against the tested microorganisms in replicates and the inhibition zones measured. The average inhibition zones were formed when the extracts which were used in combinations against the tested microbes were compared with the inhibition zones when they are used singly.

Qualitative phytochemical analysis

The method defined by Congesta et al. (2005) was used to determine the presence of saponins, tannins, flavonoids, and alkaloids in the crude extract.

Tannins

Each of the extracts were 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 blue or black precipitate was present, it indicated the presence of tannins.

Flavonoids

Each of the extracts were 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 the formation of a pink or red color, it indicated the presence flavonoids.

Alkaloids

Each of the extracts were 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 per drop, and if there was the formation of any colored precipitate, it indicated the presence of alkaloids.

Saponins

Each of the extracts were weighed to 0.5 mg and dissolved in 1 mL of methanol and filtered. Distilled water was added and was shaken for a few minutes. If there was persistent frothing, it indicated the presence of saponins.

Statistical analysis

The data was exported to Microsoft excel spreadsheet to carry out descriptive statistics. The data was analyzed with SAS version 9.1 whereby; ANOVA (one way) was carried out to show statistical difference with the varying inhibition zones between the test microbes exposed to plant leaf extracts from *T. minuta*, *A. secundiflora*, *V. lasiopus* and *B. frutescens*. Two-way ANOVA was also carried out to determine if any interaction between the plant extracts and tested microorganism with $P \leq 0.05$ was happened and considered significant. The tests were further subjected to a Tukey's post hoc test to find the difference between the means.

RESULTS AND DISCUSSION

All the bacterial pathogens (*E. coli*, *S. typhi*, *S. aureus*, *S. flexineri* and *E. faecalis*) and *C. albicans* were tested against plant extracts with the concentration of 1000 µg/mL found out on discs. The bacterial pathogens and *C. albicans* cultures of 0.5 McFarland standard were used for the efficacy test.

Efficacy test of the plant extracts on the bacterial pathogens, *Candida albicans*

The expose of all bacterial pathogens (*E. coli*, *S. typhi*, *S. aureus*, *S. flexineri* and *E. faecalis*) and fungus *C. albicans* to the plant extract in replicates showed various antimicrobial activity. The largest average inhibition zone (i.e., 16.67±2.58mm) was produced by *A. secundiflora* extract against *E. coli* in comparison to the other plant extracts (Table 2).

The average inhibition zones formed by *T. minuta* and *A. secundiflora* extracts were significantly different to those formed by *V. lasiopus* ($P<0.05$). Moreover, the average inhibition zones formed by ciprofloxacin, methanol and DMSO (negative control) were significantly different to those formed by the plant extract ($P<0.05$). *T. minuta* extract produced the largest average inhibition zone of 17.17±2.48 mm against *S. typhi* when compared to the other plant extracts. The average inhibition zone formed by an extract from *V. lasiopus* was significantly different from those of *T. minuta* ($P<0.05$). Moreover, the average inhibition zones formed by ciprofloxacin, methanol and DMSO (negative control) were significantly different from

those formed by the plant extract (P<0.05). *T. minuta* extract produced the largest average inhibition zone of 16.67±3.44 mm against *S. aureus* when compared to other plant extracts. The average inhibition zones formed by the plant extracts were not significantly different from each other (P>0.05) (Table 2).

However, the average inhibition zone formed by the plant extracts were significantly different from those formed by vancomycin, methanol and DMSO (negative control) (P<0.05). *Bulbine frutescens* extract produced the largest average inhibition zone of 19.50±1.05mm against *S. flexineri* when compared to other plant extracts. The average inhibition zones formed by the plant extracts were not significantly different from each other (P>0.05). However, the average inhibition zones formed by ciprofloxacin, methanol, and DMSO (negative control) were significantly different from those formed by the plant extract (P<0.05). *T. minuta* extract produced the largest average inhibition zones of 18.67±1.03mm against *E. faecalis* when compared to other plant extracts. The average inhibition zones formed by all the plant extracts were not significantly different (P>0.05). However, the average inhibition zones formed by ciprofloxacin (positive control), methanol, and DMSO (negative control) were significantly different from those formed by the plant extract (P<0.05). *V. lasiopus* extract produced the largest inhibition zone of 20.17±2.71mm against *C. albicans* when compared to other plant extracts. The average inhibition zones formed by *T. minuta* and *V. lasiopus* were significantly different from each other (P<0.05). All average inhibition zones formed by the plant extracts were significantly different from those formed by fluconazole, methanol and DMSO (negative control) (P<0.05) (Table 2).

The average inhibition zone formed by the plants extracts against all the test microorganisms were significantly different from those formed by antibiotics, methanol and DMSO (P<0.05). Moreover, zones formed by *T. minuta* extract against all the tested microorganism was significantly different from those formed by *V. lasiopus* and *B. frutescens* (P<0.05). However, it was not significantly different from zones formed by *A. secundiflora* (P>0.05). The average inhibition zones formed by the plant extracts against *E. faecalis*, *C. albicans*, and *S. flexineri* were not significantly different from each other (P>0.05). However, they were significantly different from those formed by *E. coli*, *S. typhi* and *S. aureus* (P<0.05). The interaction between the plant extracts and tested microorganisms were significant (P<0.05) (Table 3).

Table 3 Interactions between the plants extract and tested microorganism on single use.

Extract	**Zone of inhibition±SEM**
Aloe secundiflora	16.69±0.40cd
Bulbine frutescens	16.06±0.56d
Tagetes minuta	17.19±0.42c
Vernonia lasiopus	15.81±0.61d
Controls	
Antibiotics	23.5±0.43b
Methanol	31.17±0.34a
DMSO	0.00±0.00e
Testing microorganisms	
Candida albicans	18.31±1.45a
Escherichia coli	15.26±1.25c
Enterococcus faecalis	18.26±1.41a
Salmonella typhi	16.60±1.37b
Shigella flexineri	18.69±1.45a
Staphylococcus aureus	16.10±1.51bc
***P values* of the main factors, their interactions**	
Extract	<0.001
tested microorganisms	<0.001
Extract*tested microorganisms	<0.001

Note: The value of average inhibition zones ± standard error of mean (SEM) after two-way ANOVA followed by Tukey`s HSD test. A value followed by the same superscript within the same column are not significantly different (P>0.05). Antibiotics: ciprofloxacin, vancomycin, and fluconazole, DMSO: dimethyl sulphoxide.

Table 2. Average inhibition zones in millimeters when plant extracts are on single-use against bacterial pathogens and *Candida albicans.*

Plant extracts	**Tested microorganisms**					
	E. coli	***S. typhi***	***S. aureus***	***S. flexineri***	***E. faecalis***	***C. albicans***
Tagetes minuta	16.50±1.87c	17.17±2.48c	16.67±3.44c	19.00±1.41bc	18.67±1.03c	15.17±2.71d
Aloe secundiflora	16.67±2.58c	16.50±1.87cd	14.17±2.93c	18.17±1.47c	17.67±1.63c	17.00±2.10cd
Bulbine frutescens	13.33±1.75cd	15.33±2.73cd	11.83±2.48c	19.50±1.05bc	18.50±1.05c	17.83±1.72cd
Vernonia lasiopus	12.33±2.58d	13.17±1.84d	13.00±2.61c	18.17±1.47c	18.00±0.89c	20.17±2.71c
Controls						
Antibiotics	21.67±2.42b	25.67±1.63b	24.83±3.54b	21.50±2.07b	21.67±2.66b	27.17±0.98b
Methanol	28.67±2.34a	30.17±2.71a	31.33±2.94a	31.67±2.88a	33.50±2.56a	31.00±3.20a
4%DMSO	0.00±0.00e	0.00±0.00e	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00e
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Note: The value of average inhibition zones ± standard error after one-way ANOVA followed by Tukey`s HSD test. A value followed by the same superscript within the same column are not significantly different (P>0.05). DMSO-Dimethyl sulphoxide, Plant extracts concentration (1000 µg/mL) Antibiotics standard discs of; Ciprofloxacin (5 µg/mL) for Gram-negative bacteria, Vancomycin (3 µg/mL) for Gram-positive bacteria, Fluconazole (15 µg/mL) for *Candida albicans.*

Table 4 Minimum inhibitory concentration in micrograms/milliliter of plant extracts against bacterial pathogens and *Candida albicans.*

Plant extracts	Tested microorganisms					
	E. coli	***S. typhi***	***S. aureus***	***S. flexineri***	***E. faecalis***	***C. albicans***
Tagetes minuta	8.7	6.1	8.9	7.4	5.1	6.2
Aloe secundiflora	9.1	5.5	10.2	3.7	7.0	8.1
Bulbine frutescens	12.5	8.8	10.4	3.2	6.5	6.9
Vernonia lasiopus	10.0	5.6	12.2	3.3	3.9	4.0
Antibiotics	5.0	5.0	3.0	5.0	5.0	15.0

Note: Antibiotics; Ciprofloxacin (5 µg/mL)-Gram-negative bacteria, Vancomycin (3 µg/mL)-Gram-positive bacteria, Fluconazole (15 µg/mL)-*Candida albicans*).

Table 5. Minimum bactericidal concentration and minimum fungicidal concentration in ug/mL of plant extracts against bacterial pathogens and *Candida albicans.*

Plant extracts	Tested microorganisms					
	E. coli	***S. typhi***	***S. aureus***	***S. flexineri***	***E. faecalis***	***C. albicans***
Tagetes minuta	10	8.2	10	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

Tagetes minuta extract was more active against *E. coli* than the other extracts. However, the antibiotic used as a positive control (Ciprofloxacin) was more active against *E. coli* than all other plant extracts. *A. secundiflora* extract had the highest antimicrobial activity against *S. typhi* when compared to other plant extracts, although ciprofloxacin was more active against *S. typhi* than all other extracts (Table 4).

Tagetes minuta extracts were more active against *S. aureus* at low concentration than other plant extracts. The positive control antibiotic (Vancomycin) used against *S. aureus*, showed to be more active in low concentration than all other extracts. *B. frutescens* was more active against *S. flexineri* at low concentration than other plant extracts. Furthermore, the level of concentration of *B. frutescens* extract was lower than standard antibiotic (Ciprofloxacin) against *S. flexineri* (Table 4).

The plant extract from *V. lasiopus* was more active at low concentration against *E. faecalis* than other plant extracts. Its concentration was lower than standard antibiotic (Ciprofloxacin). All plant extracts were more active against *C. albicans* than standard antibiotic (Fluconazole). However, *V. lasiopus* was the most active against *C. albicans* in low concentration among the used plant extracts (Table 4).

Tagetes minuta extract had low concentration bactericidal against *E. coli* when it was compared to the other extracts. The plant extract from *A. secundiflora* was bactericidal against *S. typhi* at low concentration when it was compared to other plant extracts. On the use against *S. aureus*, the extract from *T. minuta* was bactericidal at low concentration when it was compared to the other plant extracts. *B. frutescens* plant extract was bactericidal against *S. flexineri* at low concentration when it was compared to the other plant extracts. On the use against *E. faecalis* and *C. albicans*, *V. lasiopus* was bactericidal and fungicidal respectively at low concentrations on the comparison to the other plant extracts (Table 5).

Combined effect of the plant extracts on the bacterial pathogens, *Candida albicans*

The plant extracts showed to be both more effective and less effective in some instances against all the tested microorganisms in the combination use. *V. lasiopus* and *T. minuta* plant extract combinations formed the largest average inhibition zone of 16.67±1.37mm against *E. coli* when it was compared to the other plant extract combinations. The average inhibition zones formed by the plant extracts combinations were not significantly different ($P>0.05$). However, they were significantly different from those formed by ciprofloxacin, methanol and DMSO (negative control) ($P<0.05$) (Table 6).

Bulbine frutescens and *V. lasiopus*; *B. frutescens* and *T. minuta* plant extract combinations formed the largest average inhibition zones of 16.67±2.58mm and 16.67±2.26mm against *S. typhi* when it was compared to the other plant extract combinations (Table 6). The average inhibition zones formed by all the plant extracts combinations were not significantly different ($P>0.05$). However, the average inhibition zones formed by ciprofloxacin, methanol and DMSO (negative control) were significantly different from those formed by the plant extract ($P<0.05$). *B. frutescens* and *V. lasiopus* plant extract combination formed the largest average inhibition zone of 15.00±2.28mm against *S. aureus* when compared to other plant extract combinations (Table 6). The average inhibition zone formed by all the plant extracts combinations were not significantly different ($P>0.05$). However, the average inhibition zones formed by vancomycin, methanol and DMSO (negative control) were significantly different from those formed by the plant extract ($P<0.05$). *A. secundiflora* and *B. frutescens*; *A. secundiflora* and *T. minuta* plant extract combinations formed the largest average inhibition zones of 18.67±1.03mm and 18.67±0.24mm against *S. flexineri* when compared to other plant extracts combinations (Table 6). The average inhibition zone formed by the plant extract combinations were not significantly different from those formed by ciprofloxacin (positive control) ($P>0.05$) (Table 6).

Table 6. Average inhibition zone in millimeters when extracts are in combinations against the bacterial pathogens and *Candida albicans*

Plant extracts	Tested microorganisms					
	E. coli	***S. typhi***	***S. aureus***	***S. flexineri***	***E. faecalis***	***C. albicans***
AB	16.33±1.97c	14.83±2.14c	14.17±2.32c	18.67±1.03bc	18.00±1.80bc	14.17±2.14b
AV	15.33±2.16c	15.00±3.03c	14.33±1.86c	18.16±1.47c	17.67±1.75c	14.67±2.42b
AT	17.33±1.86c	14.50±2.43c	14.50±1.04c	18.67±0.24bc	17.67±1.51c	8.67±1.86c
BV	16.33±1.75c	16.67±2.58c	15.00±2.28c	17.67±1.63c	17.50±1.64c	13.50±1.52b
BT	15.83±2.32c	16.67±2.26c	14.17±2.17c	17.83±1.47c	16.83±1.47c	16.83±1.47b
VT	16.67±1.37c	14.83±2.64c	14.50±2.17c	18.16±1.47c	16.17±3.19c	14.17±2.99b
Antibiotics	21.67±2.42b	25.67±1.63b	24.83±3.54b	21.50±2.07b	21.67±2.66b	27.17±0.98a
Methanol	28.67±2.34a	30.17±2.71a	31.67±2.88a	31.67±2.88a	33.50±2.26a	30.33±3.20a
4%DMSO	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00e
P values	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Note: The value of average inhibition zones ± standard deviation after one-way ANOVA followed by Tukey`s HSD test. A value followed by the same superscript within the same column are not significantly different (P>0.05). **AB**-*Aloe* + *Bulbine*; **AV**-*Aloe* + *Vernonia*; **AT**-*Aloe* +*Tagetes*; **BV**-*Bulbine* + *Vernonia*; **BT**-*Bulbine* + *Tagetes*; **VT**-*Vernonia* + *Tagetes*; ± Standard error; DMSO – Dimethyl sulphoxide; Antibiotics standard discs of; Ciprofloxacin (5 µg/mL), Vancomycin (3 µg/mL) and Fluconazole (15 µg/mL); 7mm-10mm Resistant; 11mm-20mm Intermediate; 21mm-30mm Sensitive.

However, methanol and DMSO (negative control) were significantly different from those formed by the plant extract and ciprofloxacin (P<0.05). *A. secundiflora* and *B. frutescens* plant extract combination produced the largest average inhibition zone of 18.00±1.80mm against *E. faecalis* when it was compared to the other plant extracts (Table 6). The zones formed by the plant extracts were not significantly different (P>0.05). Moreover, *A. secundiflora* and *B. frutescens* plant extract combination formed zones that were not significantly different from those formed by ciprofloxacin (positive control) (P>0.05). However, the inhibition zones formed by methanol and DMSO (negative control) were significantly different from those formed by the plant extract combinations and ciprofloxacin (P<0.05). *B. frutescens* and *T. minuta* plant extract combination formed the largest average inhibition zone of 16.83±1.47mm against *C. albicans* when it was compared to the other plant extract combinations (Table 6). The average inhibition zones formed by *A. secundiflora* and *T. minuta* extract were significantly different when it was compared to the other plant extract combinations; (P<0.05). Moreover, it was also significantly different from fluconazole (positive control), methanol and DMSO (negative control) (P<0.05) (Table 6).

The average inhibition zones formed by the plant extracts combinations when they were used against the tested microorganisms were not significantly different (P>0.05). However, they were significantly different from those formed by antibiotics, methanol and DMSO (P<0.05). The average inhibition zones formed by the tested microorganisms; *E. faecalis* and *S. flexineri* were not significantly different (P>0.05). However, they were significantly different from those formed by the other test microorganism (P<0.05) (Table 7).

Table 7. Interactions between the plants extract and tested microorganism in combination uses.

Extract	**Zone of inhibition±SEM**
AB	16.03±0.43c
AT	15.22±0.63c
AV	15.86±0.42c
BT	16.28±0.37c
BV	16.11±0.39c
VT	15.75±0.44c
Controls	
Antibiotics	23.50±0.43b
Methanol	30.83±0.34a
DMSO	0.00±0.00e
Tested microorganisms	
Candida albicans	15.56±1.20b
Escherichia coli	16.20±0.96b
Enterococcus faecalis	17.59±1.11a
Salmonella typhi	16.28±1.08b
Shigella flexineri	18.35±1.13a
Staphylococcus aureus	15.74±1.10b
***P values* of the main factors, their interactions**	
Extract	<0.001
tested microorganisms	<0.001
Extract*tested microorganisms	<0.001

Note: The value of average inhibition zones ± standard error of mean (SEM) after two-way ANOVA followed by Tukey`s HSD test. A value followed by the same superscript within the same column are not significantly different (P>0.05). Antibiotics: ciprofloxacin, vancomycin, and fluconazole, DMSO: dimethyl sulphoxide.

Qualitative phytochemical analysis

The qualitative test was carried out on plant leaf extracts from *T. minuta*, *A. secundiflora*, *B. frutescens,* and *V. lasiopus* to find out for the presence of phytochemicals. All the plant extracts were found to contain saponins, tannins, alkaloids, and flavonoids (Table 8).

Table 8 Qualitative phytochemical tests on the plant extracts.

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

Note: (+) present

Discussion

The increase of antimicrobial resistance to many available antimicrobial agents has led to the need for the invention of new drugs. The use of plant extracts to test antimicrobial activity has been brought forward as one of the ways of achieving this goal. The plants used in the study have been said to be of medicinal value. This study evaluated the use of the plants in treating selected bacterial pathogens (*E. coli*, *S. typhi*, *S. aureus*, *S. flexineri*, and *E. faecalis*) and fungus *C. albicans* and to test whether they were effective or not when they were used singly and in combinations. Furthermore, qualitative analysis to test the presence of the phytochemicals was also done.

This is because phytochemicals have been said to be responsible for some of the antimicrobial activity by extracts from plants with medicinal value. From the results, the extracts from the plants were found to have antimicrobial activity in the single-use or combination. However, some of the extracts showed activity when they were used singly and in combinations or otherwise. The extracts had shown both increased and decreased antimicrobial activity when they were used in combinations.

Antimicrobial activity

Medicinal plant extracts from various studies have shown that plants from the similar genera with *A. secundiflora* have shown to have antimicrobial activity. In this study, the antimicrobial activity of methanol extracts from medicinal plants was tested against tested bacterial pathogens and fungus *C. albicans*. It was interesting to note that the plant extracts from *A. secundiflora* showed antimicrobial activity against all the tested microorganisms. The findings from this study were the same as Agarry et al. (2005) about activities against medicinal plants. The plant extract from *A. secundiflora* had antimicrobial activity against tested bacterial pathogens; *E. coli*, *S. typhi*, *S. aureus*, *S. flexineri* and *E. faecalis* and fungus *C. albicans*.

The findings were same with those obtained in a study carried out on Gram-negative and Gram-positive bacteria by Robson (1982) in England; on *E. coli* and Agarry et al. (2005) in Nigeria; on *S. aureus* and *C. albicans* who found out that, extracts from *A. secundiflora* had antimicrobial activity against the tested microorganisms. *A. secundiflora* also showed antimicrobial activity which was significant at $P<0.05$ and the inhibition zones formed was ≥ 12.00mm. These findings were also similar to those obtained in a study carried out in Kenya (Mariita et al. 2011), which resulted the methanol extracts of *A. secundiflora* along the lake region in Kenya to be effective against bacterial pathogens such as *E. coli*, *S. typhi*, and *S. aureus* among others. The study also showed that methanol extracts of *A. secundiflora* indicated a significant antimicrobial activity at $P<0.05$ producing inhibition zones of ≥9.00mm against bacterial pathogens including *S. aureus*, *E. coli*, *S. typhi* and a fungus *C. albicans*. This could probably be due to, the use of same part of the plant (leaves) and most likely at the same age. Msoffe and Mbilu (2009) in Tanzania, also found out that extracts from *A. secundiflora* had antimicrobial activity against *C. albicans*. This may be attributed to the use of the same part of the plant.

The plant extracts from *T. minuta* showed antimicrobial activity against the testing microorganism; *E. coli*, *S. typhi*, *S. aureus*, *S. flexineri*, *E. faecalis* and *C. albicans*. Against *E. faecalis*, the antimicrobial activity was significantly higher than *S. aureus*. From the study, the plant extract from *T. minuta* formed an inhibition zone ≥17.00mm against all test microorganisms. The findings like those obtained from a previous study by Panwar and Bhatt (2014), who found out that extracts from *T. minuta* produced inhibition zone of ≥17.00mm against *S. aureus* which is comparable to the one obtained in this study. This may be due to the same method of extraction and testing. Moreover, the plant used could be of the same age. The findings in this study showed that the minimum inhibitory concentration against these tested microorganisms ranged from 3 to 13 µg/mL. The average inhibition zones were ≥ 17.00mm and the standard antibiotic (Ciprofloxacin) produced inhibition zones of ≥ 20.00mm. The findings were the same as Tahir and Khan (2012) who found out that extracts from *T. minuta* had antimicrobial activity against *S. typhi*, *E. coli*, and *S. aureus*. Furthermore, the minimum inhibitory concentration against these tested microorganisms ranged from 4 to 100 µg/mL, the average inhibition zones were ≥ 17.00mm, and the standard antibiotic (Ciprofloxacin) produced inhibition zones of ≥ 20.00mm. This means that if the concentration of *T. minuta* could be standardized, it could be used as an alternative therapy for Ciprofloxacin, against the tested organisms.

Bulbine frutescens extract showed antimicrobial activity against all the tested microorganisms. The extract antimicrobial activity was significantly higher than that on *E. coli* against *S. flexineri*. From the results of study, the minimum inhibitory concentration of *B. frutescens* against *S. aureus* was 10.4 µg/mL. This finding was in contrary to the one in South Africa by Coopoosamy (2011) who found out that the minimum inhibitory concentration against *S. aureus* was 2.0 µg/mL which was low when compared to the one obtained in the study. Furthermore, Coopoosamy (2011) found out that, extract from *B. frutescens* had no antimicrobial activity against *E. coli*. These differences could be due to different geographical and environmental conditions during the growth of the plant, the method of extraction and the age of the plant. In the study, *B. frutescens* showed antifungal activity against *C. albicans* producing an inhibition zone of ≥18.00mm. The findings were the same as Fennel et al. (2004) and Stafford et al. (2005) who found out that extracts from *B. frutescens* were active against *Candida* spp and *C. albicans* producing an *inhibition zone* of ≥10.00mm. Furthermore, they indicated inhibition between very high (41-50mm), high (31-40mm), medium (21-30mm), low (11-20mm) in fungal species of *Candida* sp, including *C. albicans*. The similarity in the activity could be associated with plant's age and the methods of extraction and testing.

The extract from *V. lasiopus* showed antimicrobial activity against tested microorganisms. The extract had a significantly higher antimicrobial activity against *C. albicans* than *E. coli*. In this study, when the plant extract

from *V. lasiopus* was used against *E. coli* and *S. aureus*, the average inhibition zone were ≥ 12.00mm and ≥ 13.00mm respectively. These findings concurred with those of a previous study by Kareru et al. (2008), who found out that leaf extracts from *V. lasiopus* had antimicrobial activity against *E. coli*. However, the findings were in contrary to a study by Kareru et al. (2008) which showed that *E. coli* and *S. aureus* were resistant (≤6.5mm). This could be due to the difference in the age of the plant, the environmental condition and the aqueous method of extraction used in the study.

Combined effect of the plant extracts

When plant extracts were used in combinations, they showed various antimicrobial activity against the tested microorganisms. When the plant extracts were used in combination against *E. coli*, they showed a pronounced antimicrobial activity as compared to when each of them is used separately against it. *B. frutescens* and *V. lasiopus* produced small average inhibition zones when each of them was used separately against the microorganism.

However, the combinations of the plant extracts of *A. secundiflora* with *B. frutescens*, *A. secundiflora* with *V. lasiopus*, *B. frutescens* with *V. lasiopus*, and *B. frutescens* with *T. minuta* produced large average inhibition zones against *E. coli*. These studies concurred with Nascimento et al. (2000) and Alzoreky and Nakahara (2003), which resulted that when the plant extracts were used in combination against *E. coli*, there was an increase in their antimicrobial activity. This could be due to the same method of extraction and testing.

Against *S. typhi*, the combination use of plant extracts of *B. frutescens* with *V. lasiopus*, and *A. secundiflora* with *V. lasiopus* produced larger average inhibition zones than on the single-use against the same microorganism. The findings showed that combining of *B. frutescens*, *A. secundiflora* and *V. lasiopus* with other plant extracts enhanced their antimicrobial activity against *S. typhi*. The findings were similar to studies Cutter (2000) and by Anjeza and Mandal (2012), which showed that combining of the plant extracts increased their antimicrobial activity against *S. typhi*. This could be due to the use of the same part of the plant or the same age of the plant.

The use of plant extracts in combinations against *S. aureus* significantly increased the antimicrobial activity as compared to the use of the extracts singly. The combinations of *B. frutescens* with *V. lasiopus*, *A. secundiflora* with *T. minuta*, and *B. frutescens* with *T. minuta* showed enhanced antimicrobial activity as by producing larger average inhibition zones than when they were used singly against *S. aureus*. The findings were in agreement with Alzoreky and Nakahara (2003) and Adwan and Mhanna (2008), which showed that combining the plant extracts had increased their antimicrobial activity against *S. aureus.* Furthermore, Adwan and Mhanna (2008) found out that their combination with conventionally available antibiotics also increased their antimicrobial activity. This means that if the concentration of the combined extracts can be standardized, it can be used in combination therapy with antibiotics against *S. aureus.*

Shigella flexineri showed either an increase or a decrease in antimicrobial activity when it was exposed to the plant extracts combinations. If each of the plant extracts was used separately against *S. flexineri*, it showed obvious antimicrobial activity with *B. frutescens* extract producing the largest average inhibition zone when it was compared to extracts from other plants. However, the combining of *B. frutescens* with; *T. minuta*, *A. secundiflora*, and *V. lasiopus* each separately against *S. flexineri* showed decreased antimicrobial activity by producing small average inhibition zones as compared to combinations formed using *T. minuta*, *A. secundiflora*, and *V. lasiopus*. The findings concurred with previous studies by Chanda and Rakholiya (2011) and Barkarnga et al. (2015), which showed that combining the plant extracts resulted both an increase and decrease in their antimicrobial activity against *S. flexineri*. This similarity in activity could be associated with the method of extraction and testing, the plant's age and the part of the plant used.

When the combinations of the plant extracts were used against *E. faecalis*, they showed a decrease in antimicrobial activity as compared to when they were used separately against it. From the results of the study, plant extracts of *T. minuta*, *A. secundiflora*, *B. frutescens* and *V. lasiopus* showed obvious antimicrobial activity by producing a large average of inhibition zones when each of them is used separately against *E. faecalis.* Against *E. faecalis*, plant extracts combinations of *A. secundiflora* with *V. lasiopus*, *A. secundiflora* with *T. minuta*, *B. frutescens* with *T. minuta*, *V. lasiopus* with *T. minuta*, and *B. frutescens* with *V. lasiopus*, showed decreased antimicrobial activity as compared to that when each of them was used separately. The findings were similar to previous studies in India by Chanda and Rakholiya (2011) and in South Africa by Olajuyigbe and Afolayan (2012) which showed that combining of the plant extracts resulted in decreased antimicrobial activity against *E. faecalis*. This could be due to the use of the same part of the plant (leaves) and, most likely, the same age of the plants.

Candida albicans showed either an increase or decrease in antimicrobial activity with the latter being more obvious when the plant extracts were used in combinations. The plant extracts had obvious antimicrobial activity when each of them was used separately against *C. albicans* with *V. lasiopus* being the most active by producing the largest average inhibition zone as compared to others. Plant extracts combination of *B. frutescens* with *T. minuta* showed increased antimicrobial activity by producing a larger average inhibition zone against *C. albicans* as compared to when *T. minuta* was used separately against it. The findings were same as those of Bakarnga et al. (2015), which resulted that combining of the plant extracts showed increased antimicrobial activity against *C. albicans*. However, the findings in the study also showed that the combined extracts had a decrease in antimicrobial activity with the combination of *A. secundiflora* with *T. minuta* and it was indicated by the production of smallest average inhibition zone when compared to others. The findings concurred with a previous study carried out by Adwan et al. (2011), which stated that combining of the plant extracts

showed decreased antimicrobial activity against *C. albicans*. This similarity in the activity could be due to the plant's age and the methods of extraction and testing.

Phytochemicals

The extract from *A. secundiflora* showed that the plant contained pharmacologically active components. The extract contained flavonoids, saponins, alkaloids and tannins which may be responsible for the antimicrobial activity. Similar studies carried out have shown that some of the pharmacologically active components have antimicrobial activity. These findings concurred with those obtained in a previous study by Mariita et al. (2011), which after qualitative analysis of phytochemical components of *A. secundiflora* extract used against *E. coli*, *S. typhi*, *S. aureus* and *C. albicans* from plant collected along the Kenya lake region found out that it contained tannins, saponins, flavonoids, and alkaloids. Furthermore, similar findings were obtained in a study carried out in India by Arunkumar and Methuselvan (2009) which confirmed the presence of flavonoids, saponins, tannins, and alkaloids in *Aloe* extract when it was used against *S. aureus* and *E. coli*. Qualitative analysis for the presence of phytochemicals in *T. minuta* extract showed the presence of flavonoids, saponins, alkaloids, and tannins. This active component might be responsible for antimicrobial activity of *T. minuta* extract against the tested microorganisms.

The study results were like those obtained in studies carried out in Pakistan by Tahir and Khan (2012) and in Argentina by Tereschuk (1997) which also confirmed the presence of phytochemicals flavonoids, saponins, tannins and alkaloids in *T. minuta* extract used against Gram-positive and Gram-negative bacteria. The extract from *B. frutescens* contained pharmacologically active compounds namely saponins, tannins, alkaloids, and saponins which could be responsible for antimicrobial activity. This study concurred with that of a previous study by Coopoosamy (2011), which figured out that when the plant extract from *B. frutescens* was used against *S. aureus* and *E. coli*, the qualitative analysis of phytochemicals showed the presence of alkaloids, saponins, tannins, and flavonoids. However, the findings were in contrary to Van Staden et al. (1994), which resulted that the extract from *B. frutescens* did not contain any of the four phytochemicals. This could be due to diverse plant metabolites associated with a geographical and ecological difference and the age of the plant. The extracts of *V. lasiopus* had active pharmacological compound, i.e., flavonoids, saponins, tannins and alkaloids which could be responsible for the antimicrobial activity.

These findings were like those of a previous study by Kareru et al. (2008), which showed that extract from *V. lasiopus* contained alkaloids, flavonoids, saponins, and tannins when it was used against Gram positive and Gram-negative bacteria (*E. coli* and *S. aureus*). Ayoola et al. (2008) in Nigeria also found out that extract from the plant from the family *Vernonia* contained flavonoids, alkaloids, tannins, and saponins. However, the findings were in contrary to those obtained in a study carried out in Southwestern region in Nigeria by Ibrahim et al. (2012) which showed the extract from *V. lasiopus* did not contain alkaloids. This difference could be associated with the geographical and environmental factors of the area from which the plant was collected.

In conclusion, the plant extracts showed antimicrobial activity when they were used against the bacterial pathogens and fungus *C. albicans*. The increase and the decrease in antimicrobial activity were also observed when the plant extracts were combined and used against the tested microorganism. There was the presence of phytochemicals in the plant extracts, i.e., saponins alkaloids, tannins, and flavonoids.

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Phenotypic and molecular characterization of Methicillin Resistant *Staphylococcus aureus* from surgical patients and normal dogs

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Abstract. *Njoroge C, Mande JD, Mitema SE, Kitaa JMA. 2018. Phenotypic and molecular characterization of Methicillin Resistant Staphylococcus aureus from surgical patients and normal dogs. Bioteknologi 15: 13-25.* The objectives of this study were to determine bacterial ecology and their antimicrobial susceptibilities from a wound and ear swabs with emphasis on *Staphylococcus aureus* and to determine the prevalence of MRSA/MRSP in normal dogs and surgical patients using phenotypic and genotypic assays. The findings revealed that the most prevalent microbial isolates recovered from dogs diagnosed with wounds, surgical site infections, and otitis externa, were *S. aureus* 50% (133/267) and *Proteus* spp. 14% (38/267). Other frequently recovered isolates included *Pseudomonas* spp. 10% (28/267), other *Staphylococcus* spp. 8.2% (22/267), *Streptococcus* spp. 6.7% (18/267), and *E. coli* 5.6% (15/267). Resistance to antimicrobial drugs was observed in the majority of the isolates in the retrospective study, with 97% (262/267) of the isolates demonstrating antimicrobial resistance to at least one drug. Resistance to sulphonamides (96%), potentiated sulphonamides (89%), ampicillin (68%), amoxicillin (62%) and tetracycline (56%) was relatively high for all bacterial species examined. *S. aureus* isolates showed 95% resistance to sulfamethoxazole, 55% to ampicillin, 52% to tetracycline and 52% to amoxicillin/clavulanic acid. *Pseudomonas* spp. showed the highest multidrug resistance with all (100%) isolates showing resistance to amoxicillin, amoxicillin/clavulanic acid and sulfamethoxazole, the isolates also showed high resistance to cotrimoxazole (93%), ampicillin (93%) and tetracyclines (80%). Low resistance to gentamicin (9%), norfloxacin (24%) and chloramphenicol (33%) was observed in all bacterial isolates. Data from the prospective study revealed that presumptive *Staphylococcus* spp. were isolated from 34% (65/191) of the samples. Coagulase-positive *Staphylococcus* spp. (COPS) accounted for 43% (28/65) of the *Staphylococcus* spp. isolated. Phenotypic resistance to oxacillin was detected in 53.6% (15/28) of COPS. Further analysis of the resistant determinants by BLAST revealed that all the resistant *Staphylococcus* strains were *S. aureus* strains. This study confirms *S. aureus* as the most prevalent bacterial isolate from wounds, surgical site infections, and otitis externa. *Proteus* spp., *Pseudomonas* spp., other *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli*, in descending order, were also frequently isolated. Gentamicin, norfloxacin, and chloramphenicol, in that order, were the most effective antimicrobial agents in the management of wounds, surgical site infections and otitis externa in the retrospective study. The study reports the first case of MRSA strains in dogs in Kenya, which were associated with mobile genetic elements (*SCCmec*) and had the potential to be transferred from dogs to humans. The MRSA resistant determinants observed are like some human-like isolates reported in several countries.

Keywords: Dog, methicillin resistant, *Staphylococcus*, surgical patient

INTRODUCTION

Staphylococcal species are commensal bacteria and leading causes of community and hospital-associated disease in humans and animals worldwide (Vengust et al. 2006). The most clinically relevant staphylococci in veterinary medicine are the coagulase positive *Staphylococcus aureus* and members of the *Staphylococcus intermedius* group, particularly *Staphylococcus pseudintermedius* (Weese and Duijkeren 2009). Although *S. aureus* can colonize and infect companion animal species, the most common commensal staphylococci of canines is *S. pseudintermedius* (formerly *S. intermedius)* with isolation rates of between 46-92% of healthy dogs compared to 10% *S. aureus* (Hanselman et al. 2009; Rubin and Chirino-trejo 2011; Paul et al. 2011). *S. pseudintermedius* can be isolated from the nares, mouth, pharynx, forehead, groin, and anus of healthy dogs and cats. It is an opportunistic pathogen and a leading cause of skin and ear infections, infections of other body tissues and cavities, and post-operative wound infections in dogs and cats (Guardabassi et al. 2004; van Duijkeren et al. 2011).

Staphylococcal infections are frequently treated with antibiotics and, consequently, antibiotic resistance and⁄or acquired resistance have developed (Normand et al. 2000). The increasing prevalence of antimicrobial resistance has made staphylococcal infections more dangerous and costly to treat. Of considerable concern are Methicillin-resistant *S. aureus* (MRSA) and the emergence of Methicillin-resistant *S. pseudintermedius* (MRSP) in dogs and cats. These resistant strains of bacteria pose a new threat to animal health due to the limitations of their management (EMA 2010).

MRSA was first identified in the United Kingdom and was then recognized as a nosocomial pathogen worldwide (HA-MRSA) (Petinaki and Spiliopoulou 2012). Subsequently, there have been reports of MRSA infections occurring in people with no exposure to a healthcare setting; these have been designated community-acquired (CA-MRSA). There are differences in the epidemiology of

HA-MRSA and CA-MRSA including resistance determinants, *SCCmec* types and clonal complexes. HA-MRSA has also been found to be resistant to more antimicrobials than CA-MRSA and to be responsible for more invasive infections (Cohn and Middleton 2010). In animals, methicillin resistance was first documented in the early 1970's, after isolation of MRSA from a dairy cow with mastitis. The emergence of MRSA in livestock and people in contact with the animal, have introduced a new epidemiological dimension to MRSA infections. These strains are designated LA-MRSA and are phenotypically and genotypically distinct from the HA-MRSA and CA-MRSA genotypes (Petinaki and Spiliopoulou 2012). Although pet animals, especially dogs and cats, may become contaminated, colonized, or infected with *S. aureus*, including MRSA, these species are not believed to be natural reservoir hosts for *S. aureus*. The MRSA strains found in companion animals are frequently identical to human epidemic strains of MRSA, making it more likely that MRSA originates from a person than a pet (Cohn and Middleton 2010). Majority of MRSA infections in dogs and cats appear to be in high-risk patients and are acquired by direct contact with human carriers (Duquette and Nutall 2004).

The MRSA isolates in dogs have been associated with clinical samples from surgical site infections, wound infections (Baptiste et al. 2005; Vincze et al. 2014), catheter site infections, urinary tract infections, pneumonia, and skin infections (Vengust et al. 2006). These observations demonstrate the clinical importance and therapeutic challenge of MRSA in the management of conditions of dogs and cats.

Methicillin-resistant *S. pseudintermedius* (MRSP) has recently emerged in small animals worldwide and represents a significant challenge for small animal practitioners due to its characteristic multidrug resistance phenotype (Paul et al. 2011) and its characteristics of a nosocomial pathogen (Frank and Loeffler 2012). It has been isolated from various conditions, including wound infections, otitis externa and canine pyoderma (Beck et al. 2012). An essential aspect of MRSA and MRSP control is the identification of potential sources of exposure.

There are limited reports of MRSA in humans in Kenya; Maina et al. (2013) found MRSA prevalence of 84.1% amongst *S. aureus* isolated from patients with skin and soft tissue conditions. A different study by Aiken et al. (2014) reported low carriage rate of *S. aureus* (85/950) in hospitalized patients, with only 7.0% of these isolates being MRSA. There is limited data in the literature on the prevalence, as well as phenotypic and molecular characteristics of microbial isolates from normal and surgical conditions in dogs in Kenya. Mande and Kitaa (2005) found *S. aureus* as the most common isolate from ear swabs of dogs suffering from otitis externa and reported multidrug resistance among bacterial strains. Available records in the University of Nariobi (UoN), Small Animal Clinic laboratory indicated frequent isolation of bacteria from dogs and cats. However, no systematic data or meta-analysis was available describing the full extent of the phenotypic and molecular characteristics of the different types of microbial isolates in dogs in Kenya.

This study was therefore designed with the aim of addressing the identified gap in the knowledge and skills to improve the therapeutic and clinical management of dogs undergoing surgical or medical procedures in Kenya.

The specific objectives of the study were: (i) To determine retrospectively the prevalence of common bacterial flora in isolates from wounds surgical site infections and otitis externa with emphasis on *Staphylococcus aureus*. (ii) To determine the antimicrobial susceptibility profiles of the various bacterial isolates to antibiotics used in the antimicrobial susceptibility tests. (iii) To assess the prevalence of MRSA/MRSP in normal dogs and surgical patients using phenotypic and genotypic tools. (iv) To sequence resistant PCR amplicons and validate the sequences.

MATERIALS AND METHODS

Study site

The study was undertaken at the UoN Small Animal Clinic, Upper Kabete. This facility receives patients mostly from the suburbs of Nairobi region and its environs. It also serves as a referral center for cases from other small animal clinics in Kenya.

Study design

This study involved both retrospective and prospective components. The retrospective study component involved a review of microbial isolates and antibiogram data from the bacteriology laboratory of samples submitted from surgical patients and dogs with otitis externa at the UoN Small Animal Clinic. The prospective component was a cross-sectional study that involved sampling of surgical patients and normal dogs presented at the UoN Small Animal Clinic and a Community veterinary clinic located in Nairobi County.

Retrospective study: Survey of common bacterial isolates from wounds and otitis externa and their respective antimicrobial susceptibility profiles

Animal patient biodata

The bacteriology laboratory records of clinical samples submitted between January 2004 and December 2013 were investigated. All the samples were from animals presented to the UoN's Small Animal Clinic during the study period. The records were examined to retrieve data on culture samples of dogs and cats presented with otitis externa and wounds. Animal biodata extracted from these records included: date of submission, sex, and the site where the sample was collected from (wound or ear swab).

Bacterial profile

For each clinical sample submitted, the number of microbial isolates and microorganisms isolated from either wounds or ear swab were recorded. The total number of various bacterial flora isolated were calculated and expressed as percentages. Bacteria of the Genus

Staphylococcus were recorded as *S. aureus* or broadly classified as other *Staphylococcus* spp. (for those that did not fit the characteristics of *S. aureus* in biochemical tests).

Antimicrobial susceptibility testing (AST)

Routine disk diffusion procedures were employed in AST by the laboratory. The bacterial isolates were tested against a panel of 8 antimicrobial agents namely, ampicillin (2µg), gentamicin (10µg), cotrimoxazole (25µg), chloramphenicol (10µg), tetracycline (10µg), potentiated amoxicillin (amoxicillin-clavulanic acid) (30µg), norfloxacin and sulfamethoxazole (25µg). Various bacteria in the AST were scored by the laboratory as either being susceptible or resistant to the respective antibiotic. If the zone of inhibition around the disk was found to be ≤14mm, the organism was scored as being resistant to that drug.

Wound characteristics

Patient case records, from which wound and abscess swabs were collected, were retrieved for further review. Information recorded for analysis included the cause and location (body region) of the wound or abscess swab.

Data analysis

All data were entered into a spreadsheet (Microsoft Excel 2010) and a pivot table generated. The frequency of the various parameters (species, breed, sex) over the study period was calculated and expressed as percentages. The total number of bacterial flora isolated was estimated and expressed as percentages. Antimicrobial susceptibility was shown as either susceptible or resistant. Overall resistance for each antimicrobial agent was calculated. Percentage resistance for each bacterium was calculated for each antimicrobial agent.

Prospective study: Prevalence of MRSA/MRSP in dogs

Study population

The following formula was used to calculate an appropriate sample size for the study

$$n = \frac{1.96^2\, p(1-p)}{d^2}$$

Where:
p = Estimate of the expected proportion (15%)
d = Desired level of absolute precision (0.05)

An estimated MRSA prevalence of 15% (Bond and Loeffler 2012) in the population was used at 95% confidence interval. From the formula, we estimated our sample size to be 196 samples.

A total of 191 dogs were enrolled in this cross-sectional study, which entailed convenience sampling at the UoN Small Animal Clinic and a Community Owned Clinic. Criteria for inclusion necessitated: dogs of any age, sex, breed and obtaining written consent from the owner or attending veterinarian to collect samples; preference was given to dogs presented for surgery, those with wounds and otitis externa. A brief questionnaire was filled by the owner or attending veterinarian to obtain information on the patient including biodata like breed, sex, age, presenting complaint, history of the condition (first time/recurrent) and prior treatment administered (antibiotic use) in the past three months preceding the study.

Sample collection

Sampling was carried out between March 2014 and June 2015. Samples were collected from four sites on the affected surgical patients and normal dogs, specifically, anterior nares, buccal mucosa, perianal area, a wound swab if the patient presented with a wound, and an ear swab in patients presenting with otitis externa. A sterile cotton-tipped swab moistened with sterile normal saline was used to collect samples by swabbing the sites above. A separate swab was used for each anatomic location and swabs from each dog were pooled in a bijou bottle containing 3 ml of transport medium (Stuart's medium) and transported to the laboratory where they were stored in a refrigerator at 4^{o}C awaiting processing.

Bacteriological examination

Recovery of isolates. Samples were removed from the refrigerator and kept at room temperature for 4 hours before being cultured onto a nutritive medium, tryptone soya broth supplemented with 6.5% NaCl for selective enrichment of *Staphylococcus.* After incubation at 37^{o}C for 24 hrs, a loopful of broth was taken and cultured to Mannitol Salt Agar (MSA), a selective medium and incubated at 35^{o}C for 24-48 hrs. The growth of yellow colonies on this medium and color change of the media to yellow was taken as positive fermentation of mannitol and presumptive *S. aureus* (Kateete et al. 2010). Pink colonies on mannitol salt agar were also sub-cultured and designated as presumptive *S. pseudintermedius.*

The presumptive *S. aureus* or *S. pseudintermedius* colonies were sub-cultured on 5% sheep blood agar (SBA) and incubated at 37^{o}C for 24 hours to isolate a pure culture. Those SBA plates that did not show any growth after 24 hours were incubated for a further 24 hours. Final identification of the presumptive coagulase positive *Staphylococcus* spp. characteristic colonies were by colonial morphology, gram stain reaction, and positive catalase and coagulase tests. The presumed staphylococcus colonies were subjected to a Gram stain and the slide examined under a light microscope to check for gram reaction, size, and shape of the colonies. Gram-positive cocci, that appeared as grapelike clusters in pairs and singles, were presumed to be *Staphylococcus* spp.

Biochemical tests for confirmation

Catalase test. A sterile loop was used to pick organisms from the plate and place them on a slide. A drop of 3% Hydrogen peroxide was added to the slide and mixed with the organisms. Visualization of bubbles was regarded as a positive reaction.

Tube coagulase test. This test was performed by transferring a single colony of inoculum to 1 ml of reconstituted rabbit plasma. The two were mixed by gently rotating the tubes. The tubes were then incubated at 37^{o}C

and evaluated after 24 hrs. Formation of a clot in the tube was taken as a positive reaction. Presumptive coagulase positive Staphylococcus colonies were sub-cultured on Tryptic soy agar, awaiting susceptibility testing.

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Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed according to the Kirby-Bauer disc diffusion method. A sterile loop was used to pick organisms from the tryptone soy agar plate.

The organisms were added to a tube containing 4.5 ml of sterile physiological saline. The mixture was vortexed to create a smooth suspension. The turbidity of the suspension was adjusted to 0.5 McFarland standard. A sterile swab was dipped into the inoculum suspension. The Mueller Hinton (MH) plate was then inoculated by streaking across the agar surface ensuring that the entire plate was covered. The lid of the plate was left slightly open for 3-5 minutes for the agar surface to dry up.

Oxacillin was used as the surrogate antibiotic to methicillin (CLSI 2008). Oxacillin (1 µg) discs (HiMedia Laboratories Pvt. Ltd, Mumbai, India) were peeled from the cartridge using forceps. The lid of the MH agar was lifted to allow placement of the discs on the agar surface. Once the disc was placed, it was gently pressed with forceps to ensure total contact with the agar surface. Plates were incubated at 35-37°C for 24 hrs. The zone diameters of complete inhibition, including that of the discs, were measured to the nearest whole millimeter using a ruler. To measure the zones of inhibition, the ruler was held on the back of an inverted petri dish while keeping it a few inches from a black non-reflecting background illuminated with reflected light.

For each isolate, antimicrobial susceptibility testing was done in duplicate and the mean zone diameter of inhibition calculated. The resistance zone diameter of <17mm around a one µg oxacillin disc was used as an indicator for methicillin resistance as recommended by Bemis et al. (2009) and approved by the Clinical and Laboratory Standards Institute (CLSI) subcommittee on Veterinary Antimicrobial Susceptibility Testing (CLSI 2013).

Molecular identification and PCR detection of *mecA*

Isolates found to be resistant were amplified by polymerase chain reaction (PCR). *S. aureus* ATCC 25923 served as the reference quality control strain. Primer pairs, sequences and amplicon size of primers used in the PCR reactions are shown in Table 2.

DNA extraction

Extraction of DNA was performed as described by Diaz-Campos (2012). Two or three colonies were obtained from 18 -48 hours cultures inoculated on tryptic soy agar (4.1%) and suspended in 400 µL of sterile distilled water. The bacterial suspension was boiled at 95°C for 7 minutes and then centrifuged at 15,000 *g* for 1 min and the supernatant collected. The DNA supernatant extracts were stored at -20°C until used as a template for the PCR reactions.

Validation of isolates

Amplification of 16S rRNA gene of all strains was performed at first to confirm that they were *Staphylococcus* strains. This was accomplished in a protocol adapted from Kondo et al. (2007). PCR reaction was done in a total volume of 20 µL containing 5 µL of DNA template and 0.25 µL of primers Staph-F and Staph-R. Thermal cycling reactions consisted of initial denaturation at 94°C for 10 min; followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, extension at 72°C for 1 min; and a final elongation at 72°C for 5 min. Amplification products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Gels were visualized under U.V light. Amplification of the 416bp PCR product indicated the strain to belong to the genus *Staphylococcus.*

Identification of coagulase positive staphylococci

Primers for species identification were designed to amplify a portion of the *nuc* gene. The procedure used was adapted from Asfour and Darwish (2014). The reaction was established in 25 µL reaction volume containing 10 µL of DNA as a template. The amplification cycles were carried out in a thermocycler. Reaction conditions were optimized to be 94°C for 5 min, as initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 60 seconds. A final extension step at 72°C for 10 min was followed. DNA isolated from *S. aureus* ATCC 25923 was used as positive control. Amplification of 295 bp and 381 bp indicated the isolate to be *S. aureus* and *S. pseudintermedius* respectively.

Detection of mecA

Detection of the *mec*A gene was performed as previously described by Kondo et al. (2007). PCR reaction was performed in a final reaction volume of 25 µL containing 5 µL of DNA template. Amplification was done in an MJ minicycler (MJ Research Inc., USA) under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 2 minutes, annealing temperature at 57°C for 1 minute, extension temperature at 72°C for 2 minutes, and a final extension step of 72°C for 2 minutes. A 1.5% agarose gel was used for electrophoresis after staining with ethidium bromide. Gels were visualized under ultraviolet illumination. A 100 bp DNA ladder was run simultaneously as a DNA marker. Amplification of the 286 bp band indicated the strains to harbor the *mecA* gene.

Sequencing of resistant genes

The PCR products obtained using gene-specific primers for resistance were purified and submitted for sequencing. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, USA). This was done to remove excess primers, salts and Taq polymerase, which can interfere with the sequencing reaction. The purified products, together with the forward and reverse primers initially used for the PCR detection of resistance, were submitted to International Livestock Research Institute (ILRI), Segolip laboratory for sequencing which was done using the ABI PRISM 3770 genetic analyzer (Applied Biosystems, US).

Basic Local Alignment Sequence Tool (BLAST) analysis

The BLASTN tool of the NCBI Genbank database (http: /blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyze the sequenced DNAs. The nucleotides were first read using GeneRunner software for further analysis. Analysis of the BLAST output was used to determine the *Staphylococcus* spp. harboring the assayed resistance genes, their geographical distribution, and hosts from which these homologs had been previously isolated. The homologs to the sequences including their nucleotide and amino-acid identity were identified using the BLASTN output.

Submission to NCBI GenBank

The sequenced resistance gene, that was longer than 200 bp, was submitted to the NCBI GenBank database for validation and assignment of an accession number.

Ethical issues

Ethical approval for the study was granted by the UoN's Faculty of Veterinary Medicine's Biosecurity, Animal Use and Ethics Committee (Ref No. 15/10). Informed consent was sought from the owners before sample collection. Permission to collect samples from the UoN Small Animal Clinic was requested and granted by the Chairman of the Department of Clinical Studies. The dogs used were the sole responsibility of the owner. Hospitalized dogs were housed in kennels located at the clinic.

RESULTS AND DISCUSSION

Retrospective study: Survey of common bacterial isolates from wounds and otitis externa of dogs and their antimicrobial susceptibility patterns

During the period between January 2004 and December 2013, a total of 291 samples were recorded from 191 individual dogs. The swab samples were obtained from wounds 27% (n=80) and ear infections 73% (n=211) respectively. Of these samples, growth was observed in 267 (92%) of the samples with 24 (8%) of the samples showing no growth after culture.

Animal patient biodata

The samples (n=291) were submitted from 200 dogs; of which of which 145 were sampled once, 34 sampled twice, 15 sampled thrice, 3 sampled four times, 2 sampled 5 times and one animal sampled 12 times over the study period. Adult animals accounted for 89% (178/200) compared to 6% (12/200) young animals. Males accounted for 68% (136/200) compared to 27% (53/200) females, while the sex of 11 animals was not indicated. Of the 200 samples from dogs, 119 (59.5%) were German shepherd dogs, 29 (14.5%) dogs were cross breeds, 8 (4%) Japanese spitz, 7 (3.5%) rottweilers dogs. The rest were breeds with 4 or less dogs in each breed.

Microbial isolates

The predominant isolates were *S. aureus* 50% (133/267) and *Proteus* spp. 14% *(38/267)*. Other frequently isolated bacteria included *Pseudomonas* spp. 10% (28/267), *Staphylococcus* spp. 8.2% (22/267), *Streptococcus* spp. 6.7% (18/267), *Escherichia coli* 5.6% (15/267). The frequency and source of isolation of the different spp. is represented in Table 3**.**

Table 2. Primer pairs and sequences used in the PCR reactions for identification of the Genus *Staphylococcus*, species identification of *Staphylococcus aureus* and detection of *mec*A medicated resistance.

Primer name	Sequence 5'-3'	Gene	Amplicon size
MecA1	F-TGC TAT CCA CCC TCA AAC AGG R-AAC GTT GTA ACC ACC CCA AGA	mecA	286bp
MecA2	F-AGA AAT GAC TGA ACG TCC GAT TT R-CAC CTG TTT GAG GGT GGA TAG	mecA	887bp
Sau	F-CGA AAG GGC AAT ACG CAA AG R-GGA TGC TTT GTT TCA GGT GTA TC	Nuc	295bp
Staph	F-GTA GGT GGC AAG CGTTAT CC R-CGC ACA TCA GCG TCA G	16S rRNA	416bp

Note: F-Forward Primer, R-Reverse Primer, Sau-*Staphylococcus aureus*

Table 3. Prevalence of bacterial isolates from clinical samples of wounds and ear swabs in dogs

Isolate	Ear (Percent)	Wound (Percent)	Total	Percent
Staphylococcus aureus	103 (48.8%)	30 (37.5%)	133	49.8
Proteus spp.	35 (16.5%)	3 (3.75%)	38	14.2
Pseudomonas spp.	26 (12.3%)	2 (2.5%)	28	10
Staphylococcus spp.	19 (9%)	3 (3.75%)	22	8.2
Streptococcus spp.	10 (4.7%)	8 (10%)	18	6.7
Escherichia coli	3 (1%)	12 (16.3%)	15	5.6
Corynebacterium spp.	3 (1.4%)	2 (2.5%)	5	1.9
Actinomyces pyogenes	1 (0.5%)	1 (1.25%)	2	0.75
Diphtheroids	1 (0.5%)	-	1	0.4
Klebsiella spp.	-	1 (1.25%)	1	0.4
Norcardia spp.	-	1 (1.25%)	1	0.4
Pasteurella spp.	-	1 (1.25%)	1	0.4
Total	201	66	267	100

Table 4. Resistance (%) of six bacterial isolates from dogs to various antimicrobial agents (n=262).

Antimicrobial agent	*S. aureus* n=139	Proteus n=40	Pseud n=28	*E. coli* n=15	Staph n=24	Strep n=19	Total n=262
Amoxycillin	69%	100%	100%	N/A	67%	N/A	62%
Amoxicillin/ Clavulanic	51%	58%	87%	100%	75%	46%	58%
Ampicillin	55%	94%	93%	79%	68%	65%	68%
Chloramphenicol	24%	45%	64%	33%	33%	7%	32%
Gentamicin	12%	0%	4%	8%	0%	29%	9%
Norfloxacin	28%	14%	15%	33%	21%	8%	22%
Tetracycline	50%	69%	79%	36%	50%	71%	56%
Cotrimoxazole	87%	97%	93%	93%	89%	67%	89%
Sulfamethoxazole	95%	100%	100%	91%	92%	100%	96%

Note: *S. aureus*-*Staphylococcus aureus*; Proteus-*Proteus* spp.; Pseud-*Pseudomonas* spp.; Staph; Other *Staphylococcus* spp; Strep-*Streptococcus* spp.

Staphylococcus aureus remained the most common isolate, regardless of the source of the sample. *Proteus* spp. were more frequently isolated in ear swabs (16.5%), than from wounds (3.7%). *Pseudomonas* spp. were also recorded as important pathogens in ear infections (12.3%) but were found to be minor pathogens in wound infections with isolation rate of 2.5%. *E. coli* was a common cause of contamination in wounds (16.3%) but did not seem to be an important cause of ear infections (0.95%).

Antibiogram profile

Resistance to antimicrobial drugs was observed in most of the isolates in the study, with 97% (262/267) of the isolates demonstrating antimicrobial resistance to at least one drug. Four isolates were not resistant to any drug and one of the isolates was a fungal, thus antimicrobial susceptibility was not done. Resistance to sulphonamides (96%), potentiated sulphonamides (89%), ampicillin (68%), amoxicillin (62%) and tetracycline (56%) was relatively high for all bacterial species examined (Table 4).

Staphylococcus aureus isolates displayed high multidrug resistance to sulfamethoxazole (95%), cotrimoxazole (87%), ampicillin (55%) and amoxicillin/ clavulanic acid (51%). Resistance to sulfamethoxazole was a common finding, with more than (90%) of the isolates being resistant to this drug. *Proteus* spp. isolates were 100% resistant to amoxicillin and sulfamethoxazole and showed high level resistance to ampicillin (94%), cotrimoxazole (97%) and tetracyclines (69%). All *Pseudomonas* spp. isolates (100%) were resistant to sulfamethoxazole and amoxycillin. High level resistances to ampicillin (93%), amoxicillin/clavulanic acid (87%) tetracyclines (79%) and chloramphenicol (64%) were also observed among the *Pseudomonas* spp. isolates (Table 4).

Low resistance to gentamicin (9%), norfloxacin (22%) was observed in all bacterial isolates. The results of antimicrobial susceptibility testing are presented in Table 4. Multidrug resistance was also observed with most of the isolates displaying resistance to 2 or more drugs (Table 5).

Table 5. Phenotypic multidrug resistance profiles displayed by the bacterial isolates from dogs to various antimicrobial agents

Resistance profile	Number of isolates resistant
COT, SXT	7
AMP**, COT**	5
AMP, COT, TET	7
AMP, COT, AMC, SXT	6
AMP, COT, TET, SXT	5
AMP, TET, AMC, SXT	5
AMP, COT, TET, AMC, SXT	7
AMP, CEF, COT, TET, AMC	6
AMP, COT, CHP, TET, AMC, SXT	6

Note: AMP-Ampicillin; AMC-Amoxicillin/Clavulanic Acid; COT-Cotrimoxazole; TET-Tetracycline; CEF-Cefaclor; CHP-Chloramphenicol; SXT; Sulfamethoxazole.

Wound characteristics

Of the 80 samples collected from wounds in the retrospective study, only 58% (46/80) of records were retrievable from the medical records. Wounds commonly involved the limbs of the affected animals, with hindlimbs (32.6%) more affected than forelimbs (27.8%). The head region was also frequently presented with wounds 8 out of 46 (17.3%), Table 6.

Surgical site infections were a more frequent source of wound swabs than other causes, representing 23.9% of the sources. Bite wounds and traumatic wounds were also frequently sampled for culture and susceptibility testing (Table 7). The cause of 18 wounds tested (33%) was not specified.

Prevalence of MRSA/MRSP from normal dogs and surgical patients

Clinical history and animal biodata

Samples from the Community veterinary clinic accounted for 103 (54%) of the samples, while 88 samples (46%), were collected from the University of Nairobi Small Animal Clinic. It was seen in 72 (37.7%) of the 191 dogs sampled, that wound (s) were present on their body. Males were the predominant dogs sampled accounting for 56% (n=107) of the samples, with 44% (n=84) being females. Majority of the animals (60%) had received antimicrobial treatment in the past three months before sampling.

Prevalence of staphylococci

All the 191 samples successfully formed colonies in the enrichment media (Tryptone soya broth). The selective media, MSA, detected 65 (34%) presumptive staphylococci species, the other samples yielded gram -ve bacteria, which were not considered for further screening. The *Staphylococcus* spp. Were subjected to a tube coagulase test, and only 28 (14.7%) of the isolates tested positive and thus designated coagulase positive staphylococci.

Phenotypic characterization of resistance

Antimicrobial susceptibility testing was done on the 28 coagulase positive isolates of which, 13 isolates (46.4%) were susceptible to oxacillin. Phenotypic resistance to oxacillin was observed in 15 isolates (53.6%).

Validation of isolates

The control PCR was performed to exclude any false positive results. It was done using a control primer pair targeting 416 bp fragment of 16S rRNA gene of genus Staphylococcus. Eleven out of the 15 presumptive coagulase-positive staphylococci were confirmed to be staphylococci (Figure 2).

Identification of coagulase-positive staphylococci (COPS)

This PCR assay was done to differentiate the COPS by amplification of the 295 bp and 381bp specific PCR product for *S. aureus* and *S. pseudintermedius* respectively. Out of the 11 confirmed *Staphylococcus* species, this assay identified 7 (63.6%) *S. aureus* strains. No *S. pseudintermedius* strains were detected in this study.

MecA gene

Two *mecA*-positive MRSA strains were isolated from two dogs (Figure 3). One of the strains was from the wound of a dog with a post-operative infection that resulted after inguinal herniorrhaphy, while the other was from a normal healthy puppy presented for vaccination.

BLAST analysis

Identification of DNA sequences. Analysis of the sequenced resistant determinants from the two samples revealed the genes were harbored by *Staphylococcus* spp. strains. The nucleotide sequence of isolate 1 (Lab ID: CS 100), was 97% identical to GenBank accession number AB547235.1, which is a *Staphylococcus sciuri mecA* gene and 96% identical to GenBank accession number KF058902.1 which is a *S. aureus mecA* gene.

The nucleotide sequence of isolate 2 (Lab ID: CS 148) revealed 99% nucleotide identity to sequences in the NCBI databases belonging to different *Staphylococcus* spp. This isolate was 99% identical to GenBank accession numbers KR187111.1, KP265312.1 and HE984157.2 which were *mecA* genes from *S. aureus*, *Staphylococcus epidermidis* and *S. pseudintermedius* respectively (Table 8).

Geographical distribution and host diversity of the homolog genes. The homologs containing the *mecA* gene showed a varied global distribution with isolates from Brazil, Japan, China, Madagascar, Israel, and Ireland. These strains were isolated from diverse sources including human, dogs, rodents, and primates and with different conditions (Table 9).

Accession numbers. The sequenced resistance gene submitted to the GenBank database was validated and subsequently assigned the accession number KX689749.

Table 6. Number of dogs presented with injury to different regions of the body.

Region	Number of dogs	%
Abdomen	4	8.7
Cervical region	4	8.7
Forelimbs	13	28.3
Head	8	17.3
Hindlimb	15	32.6
Pelvic region	1	2.2
Thorax+abdomen	1	2.2
Total	46	100

Table 7. Causes of wounds sampled for culture and sensitivity in dogs presented to the clinic.

Cause	Number of dogs	%
Bite wound	10	21.7
Cellulitis	1	2.2
Fracture	1	2.2
Pododemodicosis	1	2.2
Surgical site infection	11	23.9
Traumatic	7	15.2
Unknown	15	32.6
Total	46	100

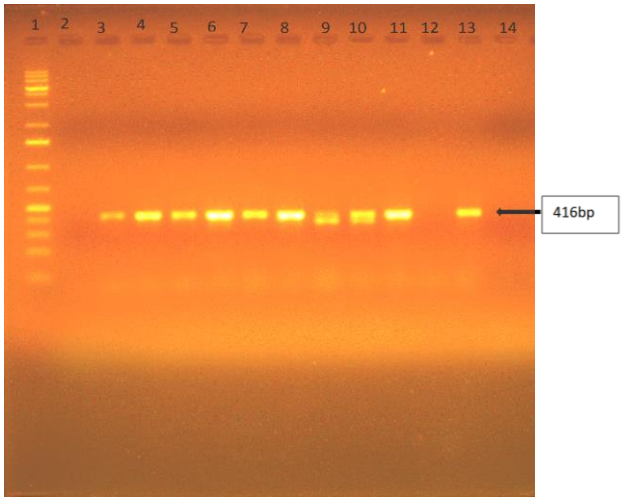

Figure 2. Agarose gel electrophoresis of PCR products of control PCR done for identification of *Staphylococcus* targeting 16S rRNA gene. Lane 1: 100bp ladder DNA marker, Lane 2: Negative Control; Lanes 3-11 Representative *Staphylococcus* (PCR Product 416 bp); Lane 13: *Staphylococcus aureus* ATCC 25923.

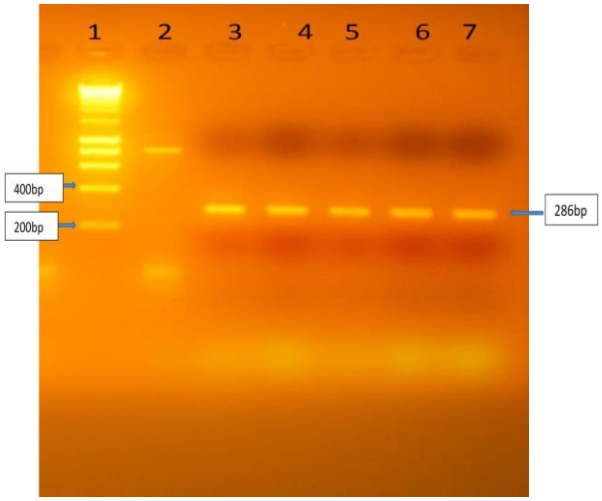

Figure 3. Agarose gel electrophoresis of PCR products of mecA positive strains. Lane 1: 1KB ladder DNA marker, Lane 3-6 mecA positive isolates (Positive PCR product 286 bp), Lane 7 Positive control.

Table 8. Resistant gene nucleotide homologues and their identities in expressed in percentages.

I.D	Homologue	%Identity	Accession number
CS 100	*Staphylococcus sciuri mecA* gene	97%	AB547235
	Staphylococcus sciuri mecA gene	96%	JX094435.1
	Staphylococcus aureus mecA gene	96%	KF058902.1
CS 148	*Staphylococcus aureus mecA* gene	99%	KR187111.1
	Staphylococcus epidermidis mecA gene	99%	KP265312.1
	Staphylococcus aureus mecA gene	99%	KF058908.1
	Staphylococcus pseudintermedius mecA gene	99%	HE984157

Table 9. Diversity of hosts and geographical distribution of resistant gene homologues.

Isolate I.D	Accession number	Host	Country
CS 100	AB547235	Rat	Japan
	JX094435.1	Primate (Sifaka)	Madagascar
	KF058902.1	Bovine (Mastitic milk)	Brazil
CS 148	KR187111.1	Bovine (Mastitic milk)	China
	KP265312.1	Canine (Fracture site)	Ireland
	KF058908.1	Human	Brazil
	HE984157	Canine (Rhinitis)	Israel

Discussion

In this study, *Staphylococcus* spp. were the most common isolates from samples submitted in the laboratory. The findings of the retrospective study confirmed the etiological and clinical importance of *Staphylococcus* organisms as colonizers of skin and important causes of infection in the skin of animals. The high percentage of staphylococci (59.1%) was expected since Staphylococcal species are present on or in clinically normal individuals as commensals (Weese 2010). However, they are opportunistic pathogens including *S. pseudintermedius* as well as *S. aureus* as leading cause of surgical site infections in animals (Vengust et al. 2006; Turk 2015). The observation in this study that *S. aureus* was the most prevalent isolate from wounds is like reports from Bangladesh (Rahman et al. 2003) In contrast, Vincze et al. (2014) recorded a low prevalence of *S. aureus* from wounds with isolation rates of 5.8% and 12.2% for dogs and cats, respectively, in Germany. *S. aureus* has been recognized as an important wound pathogen and a major cause of delayed wound healing and infection. The prevalence of *S. aureus* (37.5%) isolated from wounds of dogs in Kenya has previously not been reported.

The high prevalence of *S. aureus* in this study was surprising. Other authors (Meyers et al. 2007; Urumova et al. 2012; Padhy et al. 2014) have reported *S. intermedius* to be the primary isolate from wounds in dogs. While dogs and cats may become colonized, contaminated, and infected with *S. aureus*, they are not considered reservoir hosts of this organism (Cohn and Middleton 2010). The predominant *Staphylococcus* spp. in dogs has been reported to be *S. pseudintermedius* (Griffeth et al. 2008; Hanselmann et al. 2009). This finding may be because at the laboratory, all coagulase positive Staphylococci were designated as *S. aureus.*

In the present study, *E. coli*, *Streptococcus* spp., other *Staphylococcus* spp., and *Proteus* spp. were other microorganisms isolated from the wound swabs. This finding is like the study by Rahman et al. (2003) in Bangladesh, who isolated *E. coli*, *Klebsiella* spp. and *Proteus* spp. in wound swabs. The results of the present study are also in agreement with Urumova et al. (2012) who also found a high incidence of Enterobacteriaceae in particular *E.coli* in wounds. In this study, the polymicrobial growth was demonstrated, with 24% of the swabs yielding more than one organism was consistent with other reports of similar nature conducted elsewhere (Meyers 2007; Padhy et al. 2014). Colonization in wounds is mostly polymicrobial involving different potentially pathogenic microorganisms (Bowler et al. 2001). The number and diversity of microbes in any wound is influenced by several factors among them are wound type, depth, location, and quality, the level of tissue perfusion, and the antimicrobial efficacy as well as the host immune response.

In vitro antimicrobial agent susceptibility of the isolates showed a high frequency of resistant strains, with 97% of the isolates showing resistance to at least one drug. These observations are the cause for concern, as they are an indication of the existence of multidrug-resistant isolates among dogs that might pose a clinical as well as therapeutical challenges.

In the retrospective study, 58% of bacteria isolated from ear swabs belonged to the Genus *Staphylococcus*; this is comparable to other studies (Lilenbaum et al. 2000; Lyskova et al. 2007; Petrov et al. 2013). Malayeri et al. (2010) reported a high prevalence of 73.8% of *Staphylococcus* spp. in Iran. Other bacteria isolated in the present study included *Proteus* spp. 16.5%, *Pseudomonas* spp. 12.3% and *Streptococcus* spp. 4.7% . This was comparable to a previous study by Mande and Kitaa (2005) where *S. aureus* was found to be the most prevalent isolate (51.2%), and *Streptococcus* spp. (14%), *Pseudomonas* spp. (14%) and *Proteus* spp. (10%). This study demonstrated an increase in staphylococcal isolation from otitis externa to 58% vs. 51.2% compared to a previous Kenyan study. This study also shows that *Proteus* spp. is increasingly becoming an important pathogen with a prevalence of 16.5% up from 10% in the survey by Mande and Kitaa (2005).

Previous studies in dogs, reported the pathogens isolated from wounds to be most sensitive to potentiated sulphonamides and amoxicillin/clavulanic acid preparations (Meyers et al. 2007; Urumova et al. 2012). This observation is not in agreement with findings in this study, where comparatively higher resistance rates were observed to potentiated sulphonamides (89%) and amoxicillin/ clavulanic (58%). Interestingly, Pedersen et al. (2007) found no resistance to amoxicillin/clavulanic acid in their study which involved bacterial isolates from clinical submissions in Denmark. Earlier reports by Authier et al. (2006), suggested amoxicillin/clavulanic acid to be an appropriate antimicrobial for the treatment of skin infections by *Staphylococcus* spp. However, based on the results of this study, the use of these antimicrobials as the first line of treatment for empirical therapy might result in treatment failure if the observation made represents the general population in Kenya. The findings of this study demonstrated that gentamicin and norfloxacin were the most effective antimicrobial agents against many of the isolates. Gentamicin has been indicated for the treatment of Staphylococcal infections (Lilenbaum et al. 2000). However, Authier et al. (2006) suggest that its use should be limited to cases where initial treatment has failed.

In the present study, surgical site infections were found to be a frequent cause for wound swabbing representing 24% of the wound swabs. Seventy five percent of surgical site infections sampled resulted from fracture fixation using an implant. Turk et al. (2015) reported the use of implants increases the risk for surgical site infections. Gallagher et al. (2012) and Turk et al. (2015) further points out that, implants frequently become colonized with bacteria and may also act as substrates for bacterial biofilm formation. Majority of the wounds sampled were located on the extremities with the hindlimbs being more affected than the forelimbs. These results are comparable with the report by Shamir et al. (2002) where the extremities and the head were reported as the most frequent sites of bite wounds in dogs. Similarly, Meyers et al. (2007) also observed most wounds to involve the cranial half of the body, especially the head and thoracic limbs in dogs.

Concerning the susceptibility of coagulase positive *Staphylococcus* spp. to various antimicrobials the present study found 97% of the *Staphylococcus* isolates to be resistant to at least one drug. This finding agrees with a previous report report by Lilenbaum et al. (2000), who reported Staphylococcus isolates to display a high level of resistance in Brazil. They found 90.9% of the isolates in their study to show resistance to at least one drug. However, the findings of this study were in contrast with the findings reported by Junco and Barrasa (2002), who reported only 64.8% of COPS displaying resistance.

In the present study, the least effective antimicrobials against *S. aureus* were sulphonamides (sulfamethoxazole), potentiated sulphonamides (cotrimoxazole), ampicillin and tetracycline. The highest level of resistance noted was for potentiated sulphonamides with a resistance rate of 95%. Lilenbaum et al. (2000) also found many staphylococcal isolates in Brazil resistant to this drug though at a lower rate 72.7%. On the other hand, a study in Denmark by Pedersen et al. (2007) described very low resistance of *S.intermedius* ear isolates to this drug combination. The most effective agent against Staphylococci was gentamicin, chloramphenicol, and norfloxacin. Gentamicin

susceptibility rate was 88% which is like the one reported by Lilenbaum et al. (2000). Most of the isolates were also found to be susceptible to amoxicillin (62%), suggesting that this drug can be used as the first line of treatment before results of antimicrobial susceptibility testing.

Pseudomonas and *Proteus* isolates observed in this study displayed the highest resistance to most antimicrobial agents. *Pseudomonas* spp. are mostly isolated in chronic cases of canine otitis externa (Scott et al. 2001; Greene 2006). This organism has been reputed for its high level of resistance to most antimicrobials. The multidrug resistance was observed to be the case in this study, with 92% of *Pseudomonas* spp. isolates showing resistance to 4 or more drugs. Highest resistance was recorded to amoxicillin and sulfamethoxazole, with all the isolates tested against these drugs showing 100% resistance. These isolates also showed high resistance to ampicillin (93%) and amoxicillin/ clavulanic acid (87%). Significant resistance to chloramphenicol (64%) and tetracycline (79%) was also observed in the *Pseudomonas* spp. isolates in this study. Pedersen et al. (2007) in their study found that all the *Pseudomonas* spp. isolates were resistant to ampicillin, amoxicillin/clavulanic acid, and erythromycin. Malayeri et al. (2010) also concurred with these observations with all *Pseudomonas* spp. isolates in their study showing 100% resistance to ampicillin, amoxicillin/clavulanic, erythromycin, rifampin, and penicillin G. In another study, Hariharan et al. (2006) found that Pseudomonas isolates to be highly resistant to chloramphenicol (99%) and doxycycline (98%). The least antimicrobial resistance in this study was observed against gentamicin (4%) and norfloxacin (15%). This observation agrees with Petersen et al. (2002), who reported most *Pseudomonas aeruginosa* isolates to be 100% susceptible to the two drugs.

In the present study, *Proteus* spp. was the second most frequently isolated microorganism after *S. aureus*, accounting for 14.2% of all isolates; a finding that was like reports by other researchers (Pedersen et al. 2007; Lyskova et al. 2007; Petrov et al. 2013). The present study found all isolates to be resistant to amoxicillin and sulphonamides, but susceptible to gentamicin. High resistance was observed against ampicillin (94%), cotrimoxazole (97%) and moderate resistance to tetracyclines (69%), amoxicillin/clavulanic (58%) and chloramphenicol (45%). Similar results have previously been reported by Petrov et al. (2013), who found all isolates to be susceptible to gentamicin. Also, they observed resistance to tetracycline (81%) and chloramphenicol (74%) though at higher rates, and the isolates in their study were resistant to ampicillin. In contrast to this study, Pedersen et al. (2007), found all *Proteus* spp. isolates in their study to be resistant to tetracyclines and majority of the isolates susceptible to gentamicin and ciprofloxacin, which agrees with the findings in this study.

Prospective screening of dogs in this study showed a carriage rate of 34% (65/191) of *Staphylococcus* spp. This may be due to the prolonged storage time of some samples (up to 8 months for a few samples). In other studies, samples were cultured within 12 hrs (Gingrich et al. 2011) and 24 -36 hours of collection (Bergstrom et al. 2012; Walther et al. 2012). The recent use of antimicrobial agents in most of the study animals before sampling may have led to the suppression of the number of commensal bacteria, especially that resident on the skin.

Detection of the *mecA* gene by PCR revealed 2 out of the 15 (13%) phenotypically resistant isolates to be genotypically resistant to methicillin (oxacillin). These two isolates contained the *mecA* gene that encodes for resistance to β-lactam antibiotics. Ozturk et al. (2010) reported similar results in their study where all 5 *Staphylococcus* spp. isolates that were phenotypically resistant to oxacillin were *mecA* negative on PCR. This discrepancy between phenotypic and genotypic resistance in the isolates has been reported by Schmidt et al. 2014 and Elhassan et al. 2015. This discrepancy could be due to the existence of the so-called borderline (low-level resistant) strains. These *mecA* negative strains are thought to result from overproduction of β-lactamase (Chambers 1997). Other mechanisms associated with borderline resistance include the acquisition of modified PBPs (Elhassan et al. 2015). The existence of these borderline strains emphasize the need to screen *mecA* negative strains for other resistance mechanisms.

Two genes are known to encode for methicillin resistance in *Staphylococcus* spp. namely *mecA* and *mecC*. However, in this study, only *mecA* was investigated for genotypic characterization of methicillin resistance since reports of *mecC* positive MRSA isolates are low with the prevalence of 0-3% reported in European countries (Paterson et al. 2014). Previous studies have also shown *mecA* to be the most common gene encoding for methicillin resistance in *Staphylococcus* spp. (Weese 2010; van Duijkeren et al. 2011).

No MRSPs were observed in this study, which is like reports by Garbacz et al. (2011). Their research involved 39 *S. pseudintermedius* isolates from clinical submissions and found all the isolates to be susceptible to oxacillin. Several studies by different authors have also failed to isolate any MRSP isolates (Murphy et al. 2009; Rubin and Chirino-Trejo 2011; Schmidt et al. 2014). In most of these studies, the investigators collected samples from healthy animals. In the present study, samples were collected both from normal and clinically sick animals, some of which had received antibiotic treatment before sampling. This study found a prevalence rate of 7% (2/28) of MRSA among coagulase positive *Staphylococcus* spp. and an overall prevalence of 1% (2/191). In a Swedish animal hospital, no MRSA was isolated from surgical patients and healthy animals, although the prevalence of MRSA in the environment was found to be 5.3% (Bergstrom et al. 2012). The low prevalence of MRSA in this study is also like reports by Couto et al. (2011), who reported MRSA prevalence of 1% from 287 dogs and cats presented to a veterinary teaching hospital in Portugal and is consistent with findings of Quitoco et al. (2013). A higher prevalence of MRSA of 15.8% was observed in a study on surgical site infections (Turk et al. 2015). A recent report by Aiken et al. (2014), found a similar prevalence (7%) of MRSA among *S. aureus* strains isolated from patients admitted to a hospital in Kiambu County, Kenya. One of the

MRSA isolates in this study was recovered from a patient with a surgical site infection. The patient had been hospitalized for a week before the infection and was under treatment with an antimicrobial agent. This finding is like that by Middleton et al. (2005), whose sole postoperative MRSA isolate was from a canine patient with an orthopedic pin tract infection. MRSA has emerged as an important pathogen in post-operative infections in previous studies (Tomlin et al. 1999; Turk et al. 2015). Antimicrobial drug therapy, hospitalization, and surgery have been cited as factors predisposing to MRSA infection (Loeffler et al. 2005; Faires et al. 2010; Magalhaes et al. 2010; Davis et al. 2013). Multidrug-resistant Staphylococci isolated from dogs with post-operative infections and wounds should raise suspicion of MRSA infection and appropriate care taken in handling such patients.

Analysis of the sequenced resistant determinants showed that the resistant genes were harbored by *Staphylococcus* spp. strains and that the resistant determinant is geographically widespread across various regions of the globe having previously been isolated from countries such as Ireland (McManus et al. 2015), China (Wu et al. 2015), Brazil (Melo et al. 2014), Israel (Perreten et al. 2013). The strains have been isolated from different *Staphylococcus* spp. isolated from bovine (milk), canine (orthopedic implant and rhinitis) and human clinical submissions. The *mecA* genes contained in *SCCmec* have been reported to be almost identical regardless of the *Staphylococcus* species carrying it (Tsubakishita et al. 2010). This observation alludes to the fact that the *mecA* gene is transferable from different *Staphylococcus* spp. The *mecA* gene encodes for penicillin-binding (*PBP2*) protein. PBP 2a is a low-affinity penicillin-binding protein (PBP) that mediates methicillin resistance in *Staphylococcus* spp. (Weese 2010). This modified PBP2 has a low affinity for β -lactams and therefore cell wall construction is not prevented by these antimicrobials (van Duijkeren et al. 2011).

Haphazard use of antimicrobials before testing can lead to the selection of multidrug-resistant strains. This phenomenon may have been the case for one of the *S. aureus* strains isolated that showed resistance to the complete panel of the 8 drugs tested against. This strain was isolated from a dog suffering from recurrent otitis externa. Selective pressure exerted by previous antimicrobial treatment in recurrent cases may lead to the emergence of resistant strains (Guardabassi et al. 2004). The occurrence of antimicrobial resistance in companion animals is of significance to human health (Hawkey 2008). The close contact between household pets and humans offers favorable conditions for the transmission of bacteria by direct contact or indirectly through contamination of the environment. Transfer of mobile resistance determinants between companion animals and humans may also occur (Guardabassi et al. 2004).

In conclusion, the present study confirms that the most prevalent microorganisms associated with wounds and otitis externa were *S. aureus* (50.5%), *Proteus* spp. (14.04%), *Pseudomonas* spp. (9.82%), Other *Staphylococcus* spp. (8.42%), *Streptococcus* spp. (7.67%) and *E. coli* (5.62%). From the results of our study, gentamicin, an aminoglycoside, is the most effective antimicrobial agent against all the isolates from wounds, surgical site infections and otitis externa in dogs. Norfloxacin, a fluoroquinolone, is relatively effective against Gram-negative isolates (*Proteus* spp. and *Pseudomonas* spp.) and *Streptococcus* spp. *Pseudomonas* spp. and *E. coli* from otitis externa and wounds respectively, are the most challenging organisms to treat in dogs. The study findings report the first two cases of MRSA isolated from a healthy dog and a dog with a surgical site infection in Kenya. The study observed MRSA prevalence of 7% among coagulase positive *Staphylococcus* spp. The resistant determinant *mecA* in this study was like some MRSA strains from human patients in other parts of the world and therefore demonstrates the zoonotic importance of these resistant strains.

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Optimization of D-amino acid oxidase production by *Trigonopsis variabilis* using glucose syrup from cassava as carbon source

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Abstract. *Rusli Z, Suryadi H, Wibisana A. 2018. Optimization of D-amino acid oxidase production by Trigonopsis variabilis using glucose syrup from cassava as carbon source. Bioteknologi 15: 26-32.* Glucose syrup from cassava (GSC) contains glucose as the main sugar suitable for an economical and efficient production of D-amino acid oxidase (DAAO) by *Trigonopsis variabilis*. The aim of this study is to optimize the DAAO production by *T. variabilis* using GSC as the carbon source via response surface methodology (RSM) with Face-centered Central Composite Design (FCCD). In the first step, the effects of six factors (concentration of GSC and DL-alanine, pH, temperature, incubation time, and inoculum concentration) were screened to obtain the significant factors on DAAO production by Plackett-Burman design. As a result, the incubation time, the concentration of GSC and DL-alanine showed to be significant factors for DAAO production ($P<0.05$). These factors were then optimized using RSM with FCCD. The optimized RSM results demonstrated that a quadratic polynomial model was found suitable to define the relation between the incubation times, concentration of GSC and DL-alanine parameters. Moreover, the observed high R^2 value (0.9782) confirms a strong evaluation of the experimental data. The optimum condition for DAAO production is as follows: GSC and DL-alanine concentration was 12.3% and 0.3%, respectively, and the culture incubation time was 56.1 hours. Using this condition, we got a DAAO activity of 195.38 U/g dry weight of yeast.

Keywords: D-amino acid oxidase, face-centered central composite design, glucose syrup from cassava, Plackett-Burman design, *Trigonopsis variabilis*

Abbreviations: API: Active pharmaceutical ingredient, DAAO: D-Amino Acid Oxidase, GSC: Glucose syrup from cassava, RSM: Response surface methodology, FCCD: Face-centered central composite design

INTRODUCTION

The production of pure chiral compounds via a cost-effective method has become an important approach to fine chemical, agrochemical, and pharmaceutical industries. The use of biocatalysts to produce pure chiral compounds has been widely applied in large scale and quickly becoming a cost-effective approach. The recent development of biocatalysts processes was designed to be employed on a large-scale and to produce inexpensive enzyme with good properties, *i.e.*, high activity, stability and selectivity (Caligiuri et al. 2006). Over the last few years, researchers have focused on several enzymes (proteases, oxidases, and hydrolases), investigating and improving the property of the enzyme's protein scaffold to meet the requirement of different applications and utilized in various fields (Li et al. 2012; Gurung et al. 2013).

Chiral compounds containing enantiomeric amine or amino acid groups can be produced using a variety of enzymes, such as amino acid dehydrogenases (EC 1.4.1.X), amine oxidases (EC 1.4.3.22), amino acid oxidases (EC 1.4.3.X), aminotransferases (EC 2.6.4.X), ammonia lyase (EC 4.3.1.X), and lipases (EC 3.1.1.X) (Molla, Melis, and Pollegioni 2017). Deracemization of a DL-mixture to obtain L-isomer can be done by employing a D-amino acid oxidase (DAAO, EC 1.4.3.3) and non-selective chemical reduction step to obtain enantiopure L-amino acid after numerous cycles (Turner 2004). DAAO catalyzes the oxidative deamination of D-amino acids to produce α-keto and ammonia.

DAAO is used in the first step of bioconversion of cephalosporin C (CPC) to 7-ACA, whereby the 7-ACA is used as a precursor for the synthesis of API (Pollegioni, Rosini, and Molla 2013). This process involves two-step cleavage with D-amino acid oxidase (DAAO) and glutaryl acylase (GAC) (Barber et al. 2004). The recent innovation has used a one-step mono-enzymatic process using cephalosporin C acylase (Gröger et al. 2017). DAAO has also been used in biosensors, in the resolution of natural (*e.g.* methionine and phenylalanine) and synthetic (*e.g.* naphthylalanine) amino acid racemic solutions (Findrik and Vasić-Rački 2007), and in keto acids production (Song et al. 2016). DAAO can be found in mammalian organs, mainly in the kidneys. DAAO can also be produced by fermentation from microorganisms, such as yeasts *Trigonopsis variabilis*, *Rhodotorula gracillis*, *Candida tropicalis*, and *Neurospora crassa* fungi (Pollegioni et al. 2007). *T. variabilis* DAAO has a high catalytic activity for

CPC oxidation to produce intermediate compound glutaryl-7 amino cephalosporanic acid (Isoai et al. 2002).

DAAO can be produced through fermentation using media that contains carbon, nitrogen, mineral sources, etc. For industrial scale, the utilization of raw materials from local resources for the extractive and fermentative process has to be considered in order to benefit economically (MoH 2016). Indonesia has abundant agro-industrial products that can be used as raw materials for different purposes. Agro-industrial products, such as molasses and cassava, have been used as a substrate in the fermentation industry. Based on preliminary studies, it is known that glucose syrup from cassava is better than molasses or fructose syrup from sorghum; thus, it can be used as an alternative carbon source. Glucose syrup from cassava (GSC) contains glucose as the main sugar (Pontoh and Low 1995), and glucose is the best carbon source for DAAO production (Gupta, Gundampati, and Debnath 2012b). GSC also contain a little amount of nitrogen needed during culture cultivation and enzyme biosynthesis; hence making DAAO production economical and efficient. As an agricultural country, Indonesia is among the top 3 cassava producing countries in the world, producing more than 24 million tons and increasing every year (BPS 2016). Thus, this availability of the main raw materials can be guaranteed for the large-scale production of DAAO in Indonesia.

A statistical technique that combines the Plackett-Burman design (PBD) and central composite design (CCD) is a suitable and the most widely used method for optimization of biological processes (Wibisana et al. 2015; Gupta, Gundampati, and Debnath 2012a). PBD was developed by R.L. Plackett and J.P. Burman in 1946. It was designed to improve the quality control process that could be used to study the effects of design parameters on the system state so that intelligent decisions can be made. Plackett and Burman's devised orthogonal arrays are useful for screening, which yield unbiased estimates of all main effects in the smallest design possible (Vanaja and Shobha Rani 2007). CCD is the most popular among many classes of response surface methodology (RSM). It is widely used for estimating second-order response surfaces. The choice of axial distance α is based on the region of interest. Choosing the appropriate values of α specifies the type of CCD. In addition, for biological reasons, one cannot experiment outside the cube, even though experimentation at the extremes in the region is permissible and, in fact, desirable. This scenario, which occurs frequently in many scientific areas, suggests a central composite design in which the eight corners of the cube are centered and $\alpha = 1$, often called the Face-centered central composite design (FCCD) (Myers, Montgomery, and Anderson-Cook 2009). FCCD has been widely used in enzyme production (Salihu, Bala, and Alam 2016; Tari, Gögus, and Tokatli 2007).

The aim of this study is to optimize the production of DAAO by *T. variabilis* using GSC as carbon source. The optimization was done by response surface methodology (RSM) to enhance the production. Important production parameters were screened by Plackett-Burman design and the optimization of significant factors was done using Face-centered Central Composite Design (FCCD).

MATERIALS AND METHODS

Materials

Trigonopsis variabilis was used as a source of the enzyme obtained from Biotech Center-BPPT (Serpong, Tangerang, Indonesia). Glucose syrup from cassava (GSC) was obtained from PT. Rejo Madusari (Pati, Central Java, Indonesia). DL-alanine were purchased from HiMedia (Manufacturer's city, Country). K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $CaCl_2$, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $CuCl_2.3H_2O$, H_3BO_3, $FeCl_3.6H_2O$ were purchased from Merck (Darmstadt, Germany). Other chemicals, such as thiamin, biotin, o-phenylenediamine, horseradish peroxidase were purchased from Sigma-Aldrich Corp. (St. Luois, MO, USA). All experiments were carried out in duplicate.

Procedures

Inoculum preparation

The *T. variabilis* strain was maintained in a yeast malt medium. The cultures were kept at 4°C and subcultured at regular intervals of 30 days. Preculture medium (pH 6) contained: 22 g/L CGS, 4 g/L DL-alanine, 2 g/L K_2HPO_4, 0.21 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L NaCl, 0.1 g/L $CaCl_2$, 0.105 g/L $MnCl_2.4H_2O$, 0.0231 g/L $ZnSO_4.7H_2O$, 0.042 g/L $CuCl_2.3H_2O$, 0.105 g/L H_3BO_3, 0.0714 g/L $FeCl_3.6$ H_2O, 0.24 mg/L thiamine and 0.02 mg/L biotin. GSC and DL-alanine were sterilized using a 0.2 µm filter. Other components were sterilized by autoclaving at 121°C for 15 min. The inoculum was prepared by taking a loop full of fresh culture (72 h) and suspended in 5 mL of sterile saline water to get an appropriate suspension (OD_{600} = 0.1-0.2), then 1 mL of the culture suspension was inoculated to 50 ml pre-culture medium in 250 ml Erlenmeyer flask. The culture was then incubated in an orbital shaker at 30°C and 200 rpm for 24 h for inoculum development.

Production of DAAO

Production medium (pH 6) contained: 32 g/L GSC, 6.2 g/L DL-alanine, 2 g/L K_2HPO_4, 0.21 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L NaCl, 0.1 g/L $CaCl_2$, 0.105 g/L $MnCl_2.4H_2O$, 0.0231 g/L $ZnSO_4.7H_2O$, 0.042 g/L $CuCl_2.3H_2O$, 0.105 g/L H_3BO_3, 0.0714 g/L $FeCl_3.6H_2O$, 0.24 mg/L thiamin and 0.02 mg/L biotin. GSC and DL-alanine was sterilized using a 0.2 µm filter. The other components were sterilized in an autoclave at 121°C for 15 min.

Production of DAAO was carried out by transferring 10% inoculum (OD_{600} = 0.7-0.8) to the production medium and then incubated in an orbital shaker at 30°C and 140 rpm for 72 h.

Permeabilization of cell

Trigonopsis variabilis cells were harvested and neutralized to pH 6.0 using potassium hydroxide, then centrifuged at 10,000 g at 4°C for 10 min to obtain the cell, and then washed with potassium phosphate buffer (pH 8.0) twice. A certain volume of cells was taken and centrifuged, washed with distilled water and dried at 105°C to reach a

constant mass to determine dry cells weight (Kujan et al. 2001). The rest of the washed cells were suspended using the same buffer. The suspension cells were permeabilized using 5% toluene-ethanol (1:1) and held for 1 h at 37°C. The permeabilized cells were used for enzyme assay.

Enzyme assay

DAAO activity was measured by o-phenylenediamine/ horseradish peroxidase coupling assay (Wang, Yu, and Kuan 2008). The reaction mixture contained 30 mM D-alanine, 0.03% o-phenylenediamine, 1700 U horseradish peroxidase and DAAO of interest in 100 mM potassium phosphate buffer (pH 8.0). The reaction was monitored by an increase in absorbance at 450 nm for 3 min at 25°C.

One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 micromole of H_2O_2 per min at 25°C and pH 8.0.

Data analysis

Optimization was done in two stages; the first stage was to identify the most significant factors for DAAO production using Plackett-Burman design and the later stage the optimization of significant factors by using RSM with a face-centered central composite design. The experimental design and statistical analysis were done using Design Expert software (version 7.1).

Plackett-Burman design

The Plackett-Burman design is very useful for screening the most important factors with respect to their main effects. This model does not describe interaction among factors, it is used to screen and evaluate the important factors that influence the response. Six different independent variables, including carbon (glucose syrup from cassava) concentration, DL-alanine concentration, pH, incubation time (period), temperature and inoculum quantity were selected for screening. Each variable was tested at two levels, namely a high level denoted by (+1) and a low level denoted by (-1) as listed in Table 1. Six variables were screened by twelve experiments conducted by Plackett-Burman design (Table 2). All experiments were carried out in duplicate and the average value of DAAO activity was used for statistical analysis.

Table 1. Six factors and their levels used in Plackett-Burman design.

Code	Variables	Low level (-1)	High level (+1)
A	GSC (%)	10	15
B	DL-Alanine (%)	0.3	0.7
C	pH	6	8
D	Temperature (°C)	28	32
E	Incubation time (Hours)	48	72
F	Inoculum (%)	5	10

Face-centered central composite design (FCCD)

This step involved the optimization of the levels and the interaction effects between various significant variables. In this study, the experimental plan consisted of 20 trials and the independent variables were studied at three different levels: low (-1), middle (0), and high (+1) (Oyejola and Nwanya 2015). The center points were repeated six times to evaluate the curvature and the experiment replication facilitated the pure error estimation so that the significant lack of fit of the models could be predicted. All the experiments were done in duplicate and the average of DAAO activity (U/g yeast cell dry weight) obtained was taken as the dependent variable or response (Y).

The levels of factors used for experimental design are given in Table 3. For three factors, a fractional factorial design with six replications of center points consists of 20 runs.

Table 2. Plackett-Burman screening, representing the response of DAAO production as influenced by GSC (A), DL-alanine (B), pH (C), temperature (D), incubation time (E), and inoculum (F).

Run no.	A	B	C	D	E	F	DAAO activity (U/g YCDW)
1	10	0.3	8	28	72	10	87.6509
2	10	0.3	6	28	48	5	85.8477
3	15	0.3	8	32	72	5	43.8850
4	15	0.7	8	28	48	5	66.3733
5	10	0.7	8	32	48	5	68.5606
6	15	0.7	6	28	48	10	66.6269
7	15	0.7	6	32	72	10	32.6922
8	10	0.7	6	32	72	5	41.7822
9	10	0.7	8	28	72	10	50.2795
10	15	0.3	6	28	72	5	52.4000
11	15	0.3	8	32	48	10	79.5999
12	10	0.3	6	32	48	10	87.0552

Table 3. Matrix of Face-centered central composite design.

Run no.	A	B	C
1	1	1	-1
2	-1	0	0
3	-1	1	-1
4	0	0	-1
5	-1	-1	1
6	1	1	1
7	-1	1	1
8	0	0	0
9	1	0	0
10	0	0	0
11	0	0	1
12	0	0	0
13	0	-1	0
14	1	-1	-1
15	0	0	0
16	1	-1	1
17	0	0	0
18	-1	-1	-1
19	0	0	0
20	0	1	0

Note: A: GSC (%), B: DL-alanine (%), and C: incubation time (hours)

RESULTS AND DISCUSSION

Screening by Plackett-Burman design

Plackett-Burman design was used to identify the important parameters for DAAO production. Table 3 represents the results of the screening of significant variables for DAAO production and the corresponding response (Y) using Plackett-Burman design. Statistical analysis of DAAO activity was performed and represented in Table 4. pH and inoculum concentration positively affect DAAO production, whereas the other variables, GSC and DL-alanine concentration, temperature, and incubation time negatively affect DAAO production (Table 3). The T and P values were used to identify the effect of each factor on DAAO production, as shown in Table 4. Incubation time, GSC and DL-alanine concentration had a significant effect on DAAO activity ($P<0.05$). The R^2 values provide a measure of how much variability in the observed response values can be explained by the experimental factors. In this study, the R^2 was 0.9400 which indicates that 94% of the variability in the response were attributed to the given independent variables and only 6% of the total variations are not explained by the independent variables. The Pareto chart illustrates the order of significant variables affecting DAAO production in Plackett-Burman experimental design. We found that incubation period, the concentration of GSC and DL-alanine were the significant parameters in DAAO production (Figure 1).

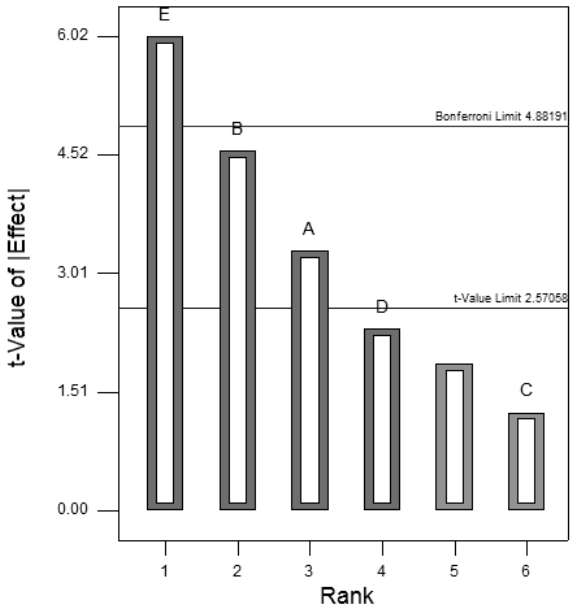

Figure 1. Pareto chart illustrates the order of significance of the variables affecting D-amino acid oxidase production by *Trigonopsis variabilis* (A, B, D, E had a positive effect; C and F negative effects; t-value ranging from 1.24 to 6.02); A. Represent GSC concentration; B. Represent DL-alanine concentration; C. Represent pH; D. Represent incubation time; E Represent temperature; and F. Represent inoculum concentration.

Table 4. Statistical analysis results from Plackett-Burman design showing major effects, coefficient values, t-test (T), P-values for each variable affecting on DAAO production and analysis of variance.

Variables	Main effect	Coef.	T	P value
A-GSC	-13.27	-6.63	-3.30	0.0215
B-DL-alanine	-18.35	-9.18	-4.56	0.0060
C-pH	4.99	2.50	1.24	0.2696
D-Temperature	-55.60	-4.63	-2.30	0.0694
E-Incubation time	-4.04	-12.11	-6.02	0.0018
F-Inoculum	7.51	3.75	1.87	0.1208

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	344.27	6	57.38	12.69	0.0068*
GSC	53.87	1	53.87	11.91	0.0182*
Inducer	87.61	1	87.61	19.37	0.0070*
pH	2.75	1	2.75	0.61	0.4709
Temperature	24.71	1	24.71	5.46	0.0666
Incubation time	161.62	1	161.62	35.73	0.0019*
Inoculum	13.70	1	13.70	3.03	0.1422
Residual	22.62	5	4.52		
Cor Total	366.89	11			

Note: T: Student's test; P: corresponding level of significance; R square 0.9400 and adjusted R square 0.8680

Optimization by face-centered central composite design

The face-centered central composite design was employed to study the optimal levels and the interactions among the selected significant factors. The other factors were maintained at a constant level, which gave maximal yield in the Plackett-Burman experiments. In this study, a total of 20 experiments with different combination of GSC concentration (A), DL-alanine concentration (B), and incubation time (C) were performed and the results of experiments for studying the effect of three independent variables on DAAO activity are presented along with predicted response and residuals (Table 5). The minimum DAAO activity (46,799 U/g YCDW) were observed in run number 6, while the maximum DAAO activity (193.880 U/g YCDW) were achieved in run number 8.

Analysis of variance (ANOVA) was used to analyze the data (Table 6). The goodness of fit of the model was indicated by its coefficient of determination (R^2), which found to be 0.9782. The present R^2-value reflects a very good fit between the observed and predicted responses and implies that the model is reliable for DAAO production. The P-values denote the significance of the coefficients and are also important in understanding the pattern of the mutual interactions between the variables. Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient).

Table 5. The face-centered central composite design represents the response of DAAO production as influenced by GSC (A), DL-alanine (B), and incubation time (C) along with the predicted DAAO production.

Run no.	A	B	C	DAAO activity (U/g yeast cell dry weight)	
				Experimental	Predicted
1	15	0.7	48	115.280	113.984
2	10	0.5	60	155.235	158.368
3	10	0.7	48	132.521	129.619
4	12.5	0.5	48	141.311	147.529
5	10	0.3	72	94.320	93.662
6	15	0.7	72	46.799	44.664
7	10	0.7	72	77.155	77.401
8	12.5	0.5	60	193.880	173.939
9	15	0.5	60	129.079	133.762
10	12.5	0.5	60	176.962	173.939
11	12.5	0.5	72	85.828	87.425
12	12.5	0.5	60	168.451	173.939
13	12.5	0.3	60	189.201	190.930
14	15	0.3	48	130.277	128.077
15	12.5	0.5	60	163.189	173.939
16	15	0.3	72	59.137	60.085
17	12.5	0.5	60	188.640	173.939
18	10	0.3	48	144.371	144.552
19	12.5	0.5	60	168.142	173.939
20	12.5	0.7	60	169.668	175.754

Note: A: GSC (%), B: DL-alanine (%), and C: incubation time (hours)

Table 6. Statistical analysis of face-centered central composite design showing coefficient values, standard error (SE) coefficient, t-test (T), P-values and analysis of variance.

Variables	Coefficient	SE coefficient	T	P
A	114.92	24.589	4.673	0.001
B	-269.97	190.093	-1.420	0.186
C	46.40	5.123	9.057	0.000
A*A	-4.46	0.933	-4.778	0.001
B*B	235.08	145.859	1.612	0.138
C*C	-0.39	0.041	-9.678	0.000
A*B	0.42	6.841	0.061	0.952
A*C	-0.14	0.114	-1.250	0.240
B*C	-0.14	1.425	-0.097	0.925

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	36220.10	9	4024.46	42.99	< 0.0001*
A-GSC	1513.64	1	1513.64	16.17	0.0024*
B-DL-alanine	575.85	1	575.85	6.15	0.0325*
C-Incub. time	9031.39	1	9031.39	96.48	< 0.0001*
AB	0.35	1	0.35	0.00	0.9523
AC	146.24	1	146.24	1.56	0.2398
BC	0.88	1	0.88	0.01	0.9246
A^2	2136.71	1	2136.71	22.83	0.0007*
B^2	243.16	1	243.16	2.60	0.1381
C^2	8766.89	1	8766.89	93.65	< 0.0001*
Residual	936.11	10	93.61		
Lack of Fit	174.63	5	34.93	0.23	0.9341
Pure Error	761.48	5	152.30		
Cor Total	37156.21	19			

Note: A: the coded value of GSC, B: the coded value of DL-alanine, and C: the coded value of incubation time. T: Student's test; P: corresponding level of significance; F: Fisher's function. R square 0.9748 and adjusted R square 0.9521

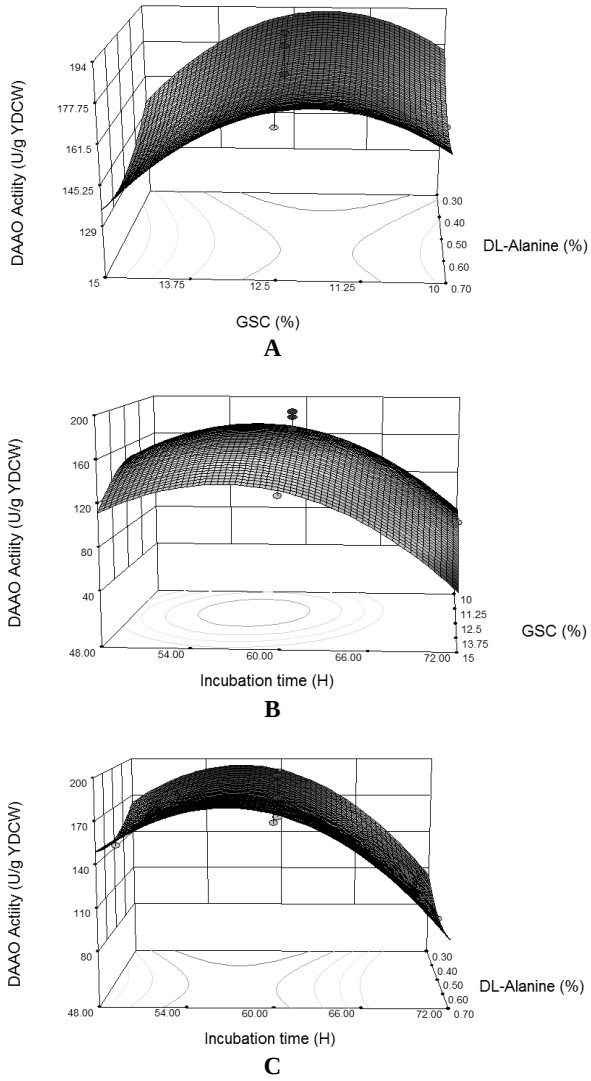

Figure 2. Three-dimensional response surface plots showed the effect of several variables on DAAO production. A. The effect of GSC and DL-alanine concentration with 60 H incubation. B. The effect of GSC concentration and incubation time at constant DL-alanine concentration of 0.5%. C. The effect of DL-alanine concentration and incubation time on DAAO activity at constant cassava glucose concentration of 12.5%.

Table 6 shows that the GSC (A), DL-alanine (B), incubation time (C). The quadratic effect of cassava glucose syrup (A) and incubation time (C) are significant. All the interactions (A*B; A*C; and B*C) are not significant, indicating that there is no significant correlation between each variable pair; thus, they do not have a significant effect in increasing the production of DAAO. By applying the multiple regression analysis of the experimental data, the equation that defines the predicted response (Y) can be shown as follows:

$$Y(\text{DAAO activity}) = 173.94 - 12.30*A - 7.59*B - 30.05*C + 0.21*A*B - 4.28*A*C - 0.33*B*C - 27.87*A^2 + 9.40*B^2 - 56.46*C^2$$

The interaction effects and optimal levels of the variables were determined by plotting the three-dimensional response surface curves (Figures 2.A-C) when one of the variables is fixed at an optimum value and the other two are allowed to vary.

Figure 2.A represents the effect of varying GSC and DL-alanine concentration on DAAO production at a constant incubation time of 60 h. DAAO yield increased with the increase of GSC concentration at DL-alanine concentration from 0.3% to 0.4%. Further increase of DL-alanine concentration would decrease the DAAO yield. According to this interaction effects, the maximum yield of DAAO activity was ≈ 193.88 U/g at GSC ≈ 12.5% and DL-alanine concentration ≈ 0.3%.

Figure 2.B shows the cooperative effect of GSC concentration and incubation time at a constant DL-alanine concentration of 0.5%. As shown in Figure 2.B, at the low and high value of GSC concentration and incubation time, the DAAO activity decreases. The maximum DAAO activity ≈ 193.88 U/g was obtained at GSC concentration ≈ 12.5% and incubation time ≈ 56 h.

Figure 2.C shows the effect of both DL-alanine concentration and incubation time on DAAO activity at a constant cassava glucose concentration of 12.5%. It is obvious that with the increase of DL-alanine concentration above 0.3%, the DAAO activity decreases. The maximum DAAO activity ≈ 193.88 U/g was obtained at the lowest DL-alanine concentration ≈ 0.3% and incubation time ≈ 56 h.

Verification of the model

To determine the accuracy of the model and to verify the result, an experiment under the optimal conditions from RSM was performed and compared with the predicted data. The measured DAAO activity obtained was 195.38 U/g, close to the predicted one 196.38 U/g, indicating a high degree of accuracy. The predicted optimal levels of the process variables for DAAO production by *T. variabilis* were 12.3% GSC, 0.3% DL-alanine, and 56.1 hours incubation time.

In conclusion, Plackett-Burman experimental design is useful to enhance the DAAO production. The design can reduce factors that do not significantly affect the production. Results of Plackett-Burman design showed that the incubation time, concentration of cassava glucose syrup and DL-alanine were significant factors in DAAO production. Optimization of significant factors was done using Face-centered Central Composite Design (FCCD) and the results were evaluated using RSM. The optimum condition for DAAO production as obtained from RSM were as follows: GSC concentration 12.3%, DL-alanine (inducer) concentration 0.3% and 56.1 hours of incubation time. At these optimum levels, DAAO activity reaches 195.38 U/g dry weight of yeast. The results of this study could be used to design a suitable medium using GSC as the carbon source, for an economical and efficient production of DAAO.

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Comparison of physicochemical properties and anti-microbial activities of tea and cocoa-based kombucha

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Abstract. *Adzadogo RS, Gbewoyo WSK. 2018. Comparison of physicochemical properties and anti-microbial activities of tea and cocoa-based kombucha. Bioteknologi 15: 33-37.* Kombucha tea is a slightly sweet, slightly acidic refreshing beverage consumed worldwide. Kombucha is a symbiosis of the Genera *Acetobacter* and *Gluconobacter*, with *Acetobacter xylinum* as a characteristic species, and various yeasts such as genera of *Brettanomyces, Zygosaccharomyces, Saccharomyces* and *Pichia* depending on the source. The tea fungus broth is composed of two portions, a floating cellulosic pellicle layer and a sour liquid broth. Black and green tea are known to be the best substrate for kombucha preparation. Black tea and cocoa powder served as the substrate in this study. The study aimed to compare the physical and chemical as well as the anti-microbial properties of tea and cocoa-based kombucha. The study revealed that tea kombucha is more acidic compared with cocoa kombucha; both tea and cocoa kombucha have similar phytochemicals. Tea and cocoa kombucha were found to inhibit the growth of *Candida albican* and *Shigella* spp., confirming its anti-microbial activity. The presence of phytochemicals and anti-microbial properties support the school of taught that kombucha drink provides a maximum health benefit to humans.

Keywords: Anti-microbe, tea, cocoa, kombucha

INTRODUCTION

Kombucha tea is a fermentation product of microbe symbiosis from the genera *Acetobacter* and *Gluconobacter*, with *Acetobacterxylinum* as a characteristic species, and variety of yeasts such as genera of *Brettanomyces, Saccharomyces, Zygosaccharomyces,* and *Pichia* depending on the source (Mayser et al. 1995). This tea is consumed worldwide is also known as Tea Fungus, Kargasok Tea, Haipao and Manchurian Mushroom. It has a slightly sweet, slightly acidic, and refreshing flavor.

The yeast ferments the sugar to ethanol in the cultivation medium, which is further oxidized to acetic acid by bacteria. The fermentation process results in a reduced pH of the medium. The final product is a sour, acidic beverage, slightly carbonated, comprised of sugars, tea components, organic acids, vitamins, and minerals. Many flavor compounds, including alcohols, ketones, aldehydes, esters, and amino acids have been identified. The tea fungus consists of two portions, a floating cellulosic pellicle layer, and a sour liquid broth.

The primary metabolites found in the fermented beverage are acetic acid, gluconic acid, lactic acid, glucuronic acid and the alcohol and glycerol (Blanc,1996; Chen and Liu 2000; Jayabalan et al. 2007). The changes that occur in the beverage are due to the metabolic activity of the bacterial and yeast ('tea fungus') in the medium. Some of the changes that occur include changes in phenols, minerals, total acidity, and vitamin content (Malbasa et al. 2006; Chen and Liu 2000).

Kombucha tea exhibits anti-microbial activity due to the presence of acetic acid (Steinkraus et al. 1996), as well as some antibacterial agents such as bacteriocins produced during the fermentation. This beverage also contains gluconic acid, which is known to be useful in detoxification of the liver and other antioxidant and anti-inflammatory properties such as gluconates and vitamins (Sreeramulu et al. 2001). Earlier studies on kombucha has shown potential phytochemicals having anti-microbial activity against a spectrum of disease caused by microbes. In agreement, concentrates of unfermented tea components had anti-microbial properties.

In Ghana, kombucha was first introduced in the mid-1980s. Its popularity has increased ever since regarding numerous health benefits associated with this beverage. However, there is lack of scientific evidence on the physiochemical and phytochemical properties of kombucha brewed in Ghana. Furthermore, the current development of antibiotic resistance by human pathogens calls for the search into new anti-microbial substances from various sources. Hence, the objectives of this study are: (i) To determine pH and total acid in tea and cocoa kombucha. (ii) To determine phytochemicals content in tea and cocoa kombucha. (iii) To evaluate the anti-microbial activity of tea and cocoa kombucha.

MATERIALS AND METHODS

Materials

Black tea (Lipton, Ghana), sucrose (St. Louis refined sugar, France), cocoa powder and starter culture were provided by Rev. Dr. W. S. K. Gbewonyo of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, Accra, Ghana. The organisms used, *S. aureus*, *C. albicans*, and *Shigella* spp. were also obtained from the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, Accra, Ghana.

Methods

Preparation of tea kombucha and cocoa kombucha

Sweetened black tea was prepared by infusing one bag of black tea (Lipton) and sucrose (37.7g) into boiled distilled water (1 L) for 3 min, then allowed to cool to room temperature. Previous starter culture kombucha was inoculated to the sweetened black tea. The fermentation took place for 14 days, after which the tea kombucha was harvested and kept in a refrigerator. Cocoa kombucha was prepared according to the method described elsewhere (Gbewonyo 2013).

Freeze-drying of samples

Two hundred milliliters of both tea and cocoa kombucha samples (fermented and unfermented) were freeze-dried to obtain a dried residue of the tea and cocoa samples. The weights of freeze-dried samples were measured. Each sample was reconstituted in distilled water to get a stock solution at a concentration of 1g/ml and was stored in a refrigerator until ready for use. All experiments were performed at the School of Agriculture in the College of Basic and Applied Sciences of the University of Ghana.

Determination of pH and total acid content

The pH was determined from 50 ml of freshly prepared broth of cocoa and tea kombucha. The titratable acid contents of tea and cocoa kombucha samples were determined by the volumetric method. A standardized $NaOH_{(aq)}$ (1.0M) was titrated against 10 ml of each of tea and cocoa kombucha samples. The concentration of total acid present in each sample was then calculated (Greenwalt et al. 1998).

Qualitative analysis of sugars in tea and cocoa kombucha

The stock samples were applied to Thin-layer Chromatography (TLC) silica gel 60 F_{254} plates with fluorescent indicator. A solution of (0.02g/ml) fructose, glucose, and sucrose served as standards. Plates were run using a combination of chloroform, methanol, and acetonitrile (1: 1: 0.5 V/V). The plate was developed using 10% H_2SO_4 and heated at 110 ^{O}C. Retention factor (Rf) was then calculated for both kombucha samples and the standard sugars used.

Phytochemical analysis

Fermented and unfermented samples were screened for saponins, tannins, alkaloids, terpenoids, and flavonoids.

Test for saponins

An equal volume of a test sample and distilled water were shaken vigorously for 2 minutes, then observed for persistent foaming, indicative of the presence of saponins (Kokate, 1999).

Test for tannins

Two drops of $FeCl_3$ (5% W/V) were added into 2ml of the aqueous test samples; formation of green precipitate confirms the presence of tannins (Trease and Evans, 1996).

Test for alkaloids

Two milliliters of each test sample were dried using heat, cooled and 5 ml of 2M HCl was then added to each reaction mixture. Upon complete dissolution of all the residues, three drops of Wagner's reagent was added. The formation of reddish-brown flocculation suggested the presence of alkaloids (Geetha and Geetha 2014).

Test for terpenoids.

One milliliter of chloroform was added to 2 ml of the test samples, diluted 1 in 2 with distilled water. Aliquot of concentrated H2SO4 was added gently to the mixture. If reddish brown precipitate or ring at the junction of the two solutions were observed, this suggests the presence of terpenoids (Rimjhim and Kumari 2014).

Test for flavonoids

Three milliliters of 1M NH_3 was added to an equal volume of the test samples and allowed to stand for 2 minutes after which 2 ml of concentrated H_2SO_4 was added. A yellow precipitate formed indicated the presence of flavonoids (Evans, 1997).

Test for reducing sugar

Three milliliters of Fehling's solution A and B was added to the test samples and heated at 60^{o}C. Formation of a brick-red color suggests the presence of reducing sugar (Geetha and Geetha 2014).

Anti-microbial activity

Media was made by dissolving an appropriate mass of nutrient broth and nutrient agar in a designated volume of distilled water. The media was sterilized by autoclave, pour into sterile petri dishes, and allowed to cool or set. Indicator strains for anti-microbial activity assay of tea and cocoa kombucha were *C. albicans. Shigella* spp., and *S. aureus.* Sterile Whatman filter paper discs (6.0 mm diameter) were impregnated with each of the fermented and unfermented extracts of tea and cocoa. After inoculating the plate with the cultured organisms using cotton swabs, the dried paper disc impregnated with the samples were placed on the plates. Positive control was set up using ceftriaxone and fluconazole; and a negative control using unfermented tea and cocoa. The plates were then incubated at room temperature for 24 hours to allow growth of the microorganisms. The anti-microbial activity of the kombucha samples was evaluated by measuring the diameter of the inhibition zone after 24 hours (Mounyr et al. 2016).

RESULTS AND DISCUSSION

Physiochemical properties and phytochemical screening

We recorded a decreased pH in the fermented tea and cocoa kombucha where tea kombucha was shown to be more acidic (Figure 1). This result indicates that tea kombucha produced more organic acids compared with cocoa kombucha as reported that, the pH value of kombucha beverage decreases due to the production of organic acids during fermentation (Dufresne and Farnworth 2000). Accordingly, the total acid content of tea kombucha is higher compared with cocoa kombucha (Table 1).

The phytochemical analysis results revealed the presence of tannins, terpenes, flavonoids, reducing sugar and saponins in tea and cocoa kombucha. Alkaloids were absent in both tea and cocoa kombucha (Table 2). Glucose and fructose were detected in tea and cocoa kombucha by TLC. Sucrose was detected in unfermented tea; fructose was present in fermented tea and unfermented cocoa; glucose was found to be present in fermented cocoa (Table 3).

Anti-microbial activity of tea and cocoa kombucha

Freeze-dried samples of tea and cocoa kombucha were tested against *C. albicans*, *Shigella* spp and *S. aureus*. Freeze-dried unfermented tea and cocoa served as negative control, whereas the antibiotics fluconazole served as positive control for *C. Albicans*. Ceftriaxone served as positive control for *Shigella* spp and *S. aureus*. Figure 2.A-B shows the anti-microbial activity of cocoa kombucha and tea kombucha towards *C. albicans*. Figure 3.A-B shows the anti-microbial activity of cocoa kombucha and tea kombucha towards *Shigella* spp. Figure 4.A-B shows the anti-microbial activity of cocoa kombucha and tea kombucha towards *S. aureus*. Figure 5 shows the quantification of zone inhibition by the test samples towards the tested pathogenic microorganisms. Although the positive control showed a high level of inhibition against *S. aureus*, neither fermented or unfermented tea and cocoa showed inhibition toward *S. aureus*.

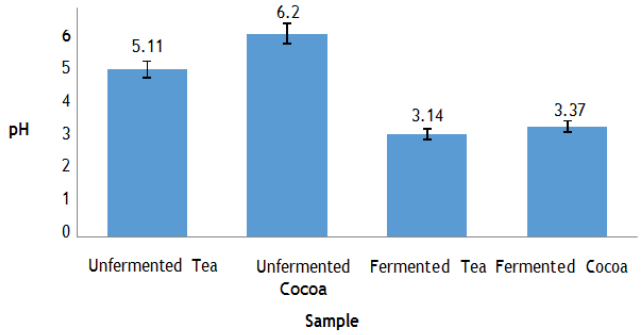


Figure 1. The pH of tea and cocoa kombucha.

Table 1. Total acid content of tea and cocoa kombucha.

Sample	Total acid concentration (g/L)
Tea kombucha	0.126
Cocoa kombucha	0.087

Table 3. TLC analysis of sugars present in tea and cocoa kombucha.

Samples	Retention factor of sugars		
	Sucrose	Fructose	Glucose
Unfermented			
Tea	0.36	-	-
Cocoa	-	0.50	-
Fermented			
Tea	-	0.52	-
Cocoa	-	-	0.58
Standards	0.36	0.50	0.58

Table 2. Qualitative phytochemical analysis of tea and cocoa kombucha.

Test	Observation	Inference			
		Unfermented		Fermented	
		Tea	Cocoa	Tea	Cocoa
Tannins	Formation of a green ppt.	+	+	+	+
Terpenoids	Reddish-brown ppt formed at the interphase	+	+	+	+
Saponins	Forming persist after shaking vigorously.	+	+	+	+
Flavonoids	Yellow solution formed, No ppt	+	+	+	+
Phenol	Red coloration formed	+	+	+	+
Reducing sugars	Brick-red color formed	+	+	+	+
Alkaloids	Brown color of solution persist	-	-	-	-

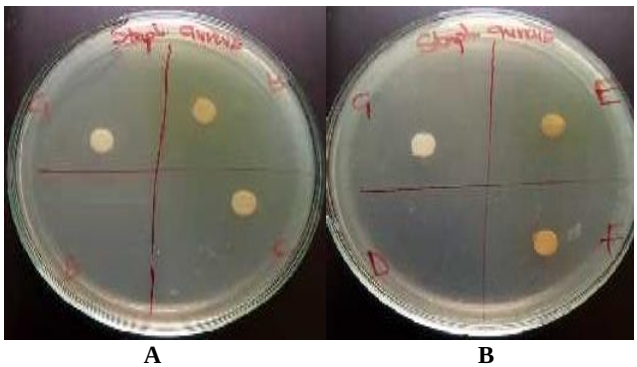
Note: + : Presence, - : Not present, ppt : Precipitate

A B

Figure 2. Anti-microbial activity of cocoa kombucha (A) and tea kombucha (B) towards C. *albicans*. Note: A: fluconazole, B: fermented cocoa, C: unfermented cocoa, D: untreated, E: fermented tea, F: unfermented tea.

A B

Figure 3. Anti-microbial activity of cocoa kombucha (A) and tea kombucha (B) towards *Shigella*. Note: B: fermented cocoa, C: unfermented cocoa, D: untreated, E: fermented tea, F: unfermented tea, G: ceftriaxone.



A B

Figure 4. Anti-microbial activity of cocoa kombucha (A) and tea kombucha (B) towards *S. aureus*. Note: B: fermented cocoa, C: unfermented cocoa,D: untreated, E: fermented tea, F: unfermented tea, G: ceftriaxone.

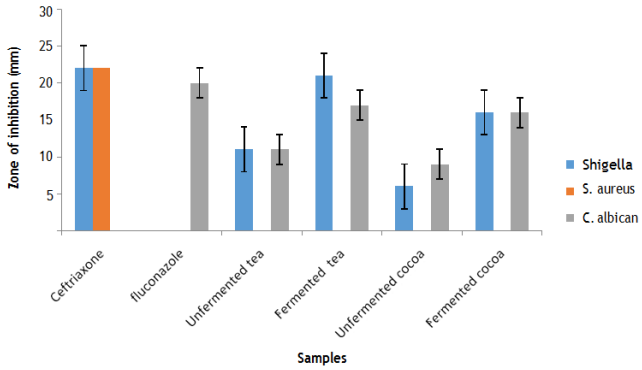


Figure 5. Zone of inhibition of tea and cocoa kombucha against indicator strains.

Discussion

Kombucha is a symbiotic culture of bacteria and yeast. Yeast cells hydrolyze sucrose to glucose and fructose. Meanwhile, acetic acid bacterial convert glucose and fructose into gluconic acid and acetic acid during the fermentation process. These organic acids produced throughout the fermentation of the tea and cocoa kombucha resulted in a corresponding decrease in pH value (Dufresne and Farnworth 2000) that prevents the symbiotic culture from becoming contaminated by undesirable microorganisms not contained in the tea fungus. In our study, a decrease in pH was also recorded in both the fermented tea and cocoa kombucha confirming the result of the previous report (Greenwalt et al. 1998; Dufresne and Farnworth 2000; Jayabalan et al. 2007).

Phytochemicals screening in the tea and cocoa kombucha showed that they contain compounds commonly found in tea and cocoa (Table 2). A study by Subhashini et al. (2010) revealed that cocoa contains tannins, saponins, terpenoids, and flavonoids. Tea is also known for its richness in tannins, phenols, flavonoids, terpenoids, etc. These phytochemicals were detected in both tea and cocoa samples (fermented and unfermented) in this study.

Phytochemicals are known to offer protection against human diseases. The consumption of flavanol-rich cocoa improves cerebral blood flow, which is critical for optimal brain function and reduced dementia. The presence of these phytochemicals in both fermented and unfermented tea and cocoa-based kombucha, indicates that preparation of kombucha does not compromise the potential health benefits derived from its consumption. Thus, the health benefit of kombucha beverage reported by Dufresne and Farnworth (2000) and Teoh et al. (2004) may be attributed to the presence of these phytochemicals.

Glucose and fructose, the hydrolyzed product by yeast, were detected in tea and cocoa kombucha by TLC. Table 3 shows that tea kombucha contains fructose and cocoa kombucha contains glucose. Loncar et al. (2006) described that during the fermentation process yeast cells hydrolyze sucrose into glucose and fructose. Glucose produced during the fermentation is used by the yeast to produce ethanol and carbon dioxide as metabolites. The bacteria then oxidize ethanol in the presence of air to acetaldehyde, then to acetic acid (Reiss 1994; Loncar et al. 2006). The primary

metabolites of ethanol and acetic acid act as catalyzing agents yeasts are stimulated to produce ethanol by acetic acid. Whereas, ethanol stimulates the growth of acetic acid bacteria and their production of acetic acid (Liu et al. 1996). The acetic acid bacteria also utilize some of the glucose to produce gluconic acid. Fructose is used to a lesser extent, and some remain after the fermentation process (Greenwalt et al. 1998). The presence of the unmetabolized glucose, together with the remaining fructose, is responsible for sweetness associated with the beverage.

Kombucha beverages exert anti-microbial properties against a spectrum of known human pathogens (Greenwalt et al. 1998; Sreeramulu et al. 2001) perhaps due to its acetic acid content (Steinkraus et al. 1996). Fermented cocoa kombucha showed anti-microbial activity towards *C. albicans* with a zone of inhibition of 16 mm, higher than that of the unfermented cocoa kombucha. Also, fermented tea showed anti-microbial activity on *C. albicans* with an inhibition zone of 17 mm as compared to its unfermented form which gave an inhibition zone of 13 mm. Kombucha beverages reduced the growth of *C. albicans*, albeit still lower than the positive controls. Based on the size of inhibiting zone, tea has a higher anti-microbial activity than cocoa against *Shigella* (Figure 5). The result might be attributed to lower pH of tea than cocoa kombucha (Figure 1). Furthermore, because anti-microbial activity against pathogenic microorganisms is attributable primarily to acetic acid (Greenwalt et al. 1998), we speculate there might be more acetic acid in tea than cocoa kombucha. Sreeramulu et al. (2000) reported that the anti-microbial activity of kombucha tea is determined by the presence of organic acids, mainly acetic acid, large proteins, and catechins.

Nevertheless, unfermented tea did inhibit the growth of the indicator strains used in this study. Many reports reveal that the polyphenols or tannins extracted from tea inhibit a broad spectrum of Gram-positive and gram-negative bacteria (Zhu et al. 2000). Toda et al. (1991) reported that unfermented tea at high concentrations (using 20% dry tea) inhibit *Staphylococcus epidermidis, Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis, Shigelladysenteriae, Shigellaflexneri, and Vibrio* spp.

Conclusion

The results suggest that tea kombucha is more acidic compared with cocoa kombucha as demonstrated by their pH and total acid content. The presence of phytochemicals in the tea and cocoa kombucha, especially phenols which are known for their anti-microbial activity, might be associated with their significant inhibitory action against the growth of *Shigella* and *C. Albicans*. However, phenol was also detectable in the unfermented tea and cocoa; thus, it could be the reason why the unfermented samples display anti-microbial activity although in a lesser extent as compared to the fermented ones. The study highlights tea and cocoa-based Kombucha beverage as potential anti-microbial agents.

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Public perception and environmental concerns on genetically modified maize production in Kenya

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Abstract. *Mbugua AW, Mwaura F, Thenya T. 2018. Public perception and environmental concerns on genetically modified maize production in Kenya. Bioteknologi 15: 38-50.* The purpose of this study was to establish: (i) the public's perception towards the importance of biotechnology on maize production for food security in Kenya, (ii) the environmental implications of maize-related agro-biotechnology on national biodiversity conservation and biosafety and (iii) the public health concerns on the introduction of maize-related agro-biotechnology in Kenya. The study population included two clusters, namely maize farmers and consumers. The farmers were interviewed from two maize farming zones, namely Githunguri Ward (Kiambu County) as a small-scale production area and Moiben Ward (Uasin Gishu County) as a large-scale production area. The consumers, on the other hand, were interviewed from Umoja 1 Ward (Nairobi County). The number of enumeration areas (EAs) was based on planned 10-household interviews per EA, resulting in a total of 12 EAs; 6 from Umoja 1, 3 from Githunguri and 3 from Moiben with a total of 120 respondents including 60 maize producers and 60 consumers. The data analysis was undertaken using descriptive statistics and inferential statistics, like Man U Whitney test, with the initial phase using frequencies and cross-tabulations. The findings showed that majority of consumers believe that genetically modified (GM) maize will solve the problem of food insecurity in the country because there is a critical need to produce more maize to meet the increasing food demand. Out of the 120 surveyed respondents, 69% agreed that GM maize would play a significant role in solving food insecurity in Kenya. Only 34% of the respondents agreed that current maize production methods are sufficient to meet Kenya's food security needs. The respondents portrayed confidence that GM maize will be beneficial to farmers. 65% of the respondents agreed that GM maize would improve the profitability of the growers due to the increased yields per unit area. 60% of the respondents believed that GM maize would benefit society because it will allow farmers to produce food more efficiently. 51% thought GMO foods would play a significant role in solving malnutrition problem in Kenya. On the flip side, the majority of the respondents were concerned about the likely environmental and health risks. 76% of the respondents were concerned that GM maize will contaminate the conventional crops through cross-pollination. More than 6 in every 10 respondents believed that GM maize would be harmful to non-target insects, while 49 % thought that some of the GM maize would invade the environment and become uncontrollable. With regards to public health and religious concerns on the introduction of GM maize, it was established that 90% of the respondents were in favor of food labeling to show the presence of biotech ingredients. 55% of the respondents believed that GM maize might lead to human sickness and death. The study concluded that Kenyans would like a more integrated approach to address the food security issue without relying on any particular technology.

Keywords: Genetically Modified Organism, GMO, Kenya, maize

INTRODUCTION

Kenya's population is predicted to double by the year 2050; therefore, there is a need to more than double the food production to be a secure food country. Several ways exist in which agricultural output can be improved sustainably. These include, but are not limited to, the reliance on food imports from other countries, expansion of agricultural land including and irrigation in arid areas, use of biological fertilizers, better pest control, soil and water management, and the use of enhanced plant varieties, increased production either by conventional or biotechnological means.

Applications of biotechnology in crop production include tissue culture, genetic engineering, and molecular transformations. This study was primarily focused on genetic engineering, which has, over time, generated concerns. Confirmation from the survey by Kimenju et al. (2005), indicates that consumer groups, conservationists and other non-governmental organizations are apprehensive about genetically modified (GM) foods based on food safety, ethical grounds, religious concerns and the possible side effects on the environment.

If consumer acceptance matters are not sufficiently addressed, then the prospective economic and social profits of modern biotechnology may not be achieved (Stenholm and Waggoner 1992). It is therefore essential to institute the level of consumers' consciousness, perception, and apprehensions about GM foods since the acceptance of GM food is one of the most critical success factors with regards to the future of this technology.

Although numerous studies have analyzed consumer reception of GM foods in the industrialized countries and Asia, there is negligible evaluation work done in Sub-

Saharan Africa, though this expanse could advance significantly from this technology (De Groote et al. 2003). Generally, it is agreed that opinions of risks and benefits are a crucial driver of people's reactions to a specific activity or technology, such as GM food (Slovic 2000).

In Kenya, corn is the staple food crop. The overall consumption is valued at 98-125 kilograms per person per year, which converts to about 2700 thousand metric tons annually (Nyoro et al. 2007). And as such, it plays a significant part in food security; and for any technology to be successfully introduced in maize production, acceptance by the society is vital. Currently, the government of Kenya is investing in research, development, and capacity building on modern biotechnology. There are several on-going biotechnology projects on 5 crops under confined field trials (CFT). The plants include cotton, corn, cassava, sorghum, and sweet potatoes. As this development continues, it calls for more studies to be done to gauge the public's perception of the adoption of genetic engineering as a tool towards sustained food security. Understanding the society's perspective will be instrumental in coming up with a proper strategy as the world moves towards genetic engineering. Scientific studies on this issue are necessary to establish the degree of public willingness to embrace and adopt biotechnology as an acceptable solution to the problem of food insecurity. This kind of research has not been undertaken because most discussions on the matter are usually conducted at high levels by upstream experts, technocrats, and scientists. This study aims to fill this gap.

MATERIALS AND METHODS

Study area

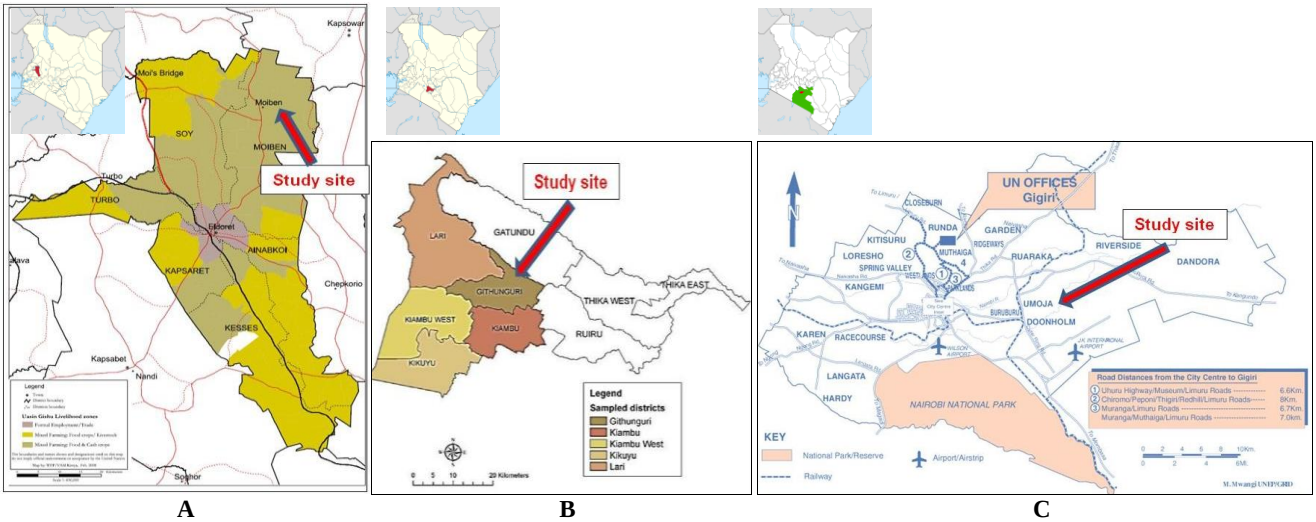
The study was conducted in three areas, including two potential GM maize production areas, namely, Uasin Gishu and Kiambu and one possible GM maize consumption area, namely Nairobi.

Uasin Gishu

Uasin Gishu County is a highland plateau whose altitudes extending from 2,700 m to about 1,500 m above sea level. Uasin Gishu County lies between longitudes 34 degrees 5'' East and 35 degrees 3'' West and latitudes 0 degrees 0'' South and 0 degrees 5'' North. The county shares conventional boundaries with Trans Nzoia County to the North, Elgeyo Marakwet County to the East, Baringo County to the Southeast, Kericho County to the South, Nandi County to the Southwest and Kakamega County to the Northwest. It occupies an overall area of 3,345.2 km^2. The county is one of the vital large-scale maize growing zones in Kenya with typical farm size at 2-10 acres and up to 224,890 acres under maize farming. The study was restricted to the Moiben Sub-County which located in the northern part of the County (Figure 1.A).

Kiambu

Kiambu County is situated in central Kenya. It borders Murang'a County to the North and Northeast, Machakos County to the East, Nairobi and Kajiado counties to the South, Nakuru County to the West, and Nyandarua County to the Northwest (Figure 1.B). The county lies between latitudes 00 25' and 10 20' South of the Equator and Longitude 360 31'and 370 15'East. According to 2009 census, Kiambu County has a population of 1,623,282, with a total area of 2,543 km^2. Up to 60.8% of Kiambu's population lives in urban areas. The study was conducted in the Githunguri Sub-County.



A **B** **C**

Figure 1. Map of study sites in Kenya. A. Uasin Gishu County (Yego 2013), B. Kiambu County (Omwenga et al. 2016), C. Nairobi County (UN-Habitat – www. unhabitat.org)(Wikipedia 2016).

Nairobi

Nairobi County borders Kiambu County to the North and West, Kajiado to the South and Machakos to the East. The county has an overall area of 696.1 km^2 and is situated between longitudes 36o 45‘ East and latitudes 1o 18‘ South (Figure 1.C). It lies at an altitude of 1,798 meters above sea level. Umoja estate is a middle-income residential area. Many of the houses were owner-occupied, but this has now shifted to most of them being let while others demolished the houses and built flats. The average asking rental price is about KES 20, 000 per month for the standalone dwellings. The servant's quarters go for about KES 5, 000. One-bedroom apartments are about KES10,000 and KES15,000 for two-bedroom apartments. About 150,000 people are residing in Umoja I, according to the National Population Census of the year 2009. Maize remains to be the vital staple food in Nairobi, based on the kilograms consumed per adult equivalent. Muyanga et al. (2004) established that up to 97% of the middle-income people in Nairobi used maize related products (maize meal, grain, or green maize) regularly.

Procedure

Research design

A household survey design was used in this study. The respondents (N= 120) were drawn from 3 counties: Githunguri (Kiambu), Moiben (Uasin Gishu), and Umoja 1 (Nairobi). Data on individual perceptions towards GM maize was generated from self-completed questionnaires that were administered.

Study population

To provide a sub-national assessment of public perception about the production and consumption of GM maize, the study population included two clusters: farmers and consumers. The farmers were interviewed from two maize farming zones, Githunguri ward (Kiambu County) and Moiben ward (Uasin Gishu County). The population of Githunguri ward and Moiben ward is 36,378 and 25,774 people respectively (2009 Housing and population census). The former represented small-scale maize growers while the latter represented the large-scale producers. On the other hand, the consumers were interviewed from a stand-alone livelihood cluster zone in Umoja 1 ward (Nairobi County). The population in Umoja 1 ward was 50,739 at the time of the survey (2009 Housing and population census).

Sampling strategy

In an ideal situation, one would like to study the whole population. However, in most cases, it is not possible or not feasible to do this, and therefore, one must take a sample. According to Black and Champion (1976), a sample is a portion of elements taken from a population that is deliberated to be representative of the community. Cochran (1977) tackled this subject of representation by affirming that "One method of defining sample size is to stipulate margins of error for the items that are regarded as most critical to the study. An estimate of the sample size required is first made separately for each of these vital items. When these calculations are finalized, researchers will have a range of n's, typically ranging from smaller n's for scaled, continuous variables, to larger n's for dichotomous or categorical variables. More frequently, there is an adequate variation among the n so that we are hesitant to choose the largest, either from budgetary concerns or because this will give an over-all standard of precision significantly higher than originally intended. In this event, the preferred standard of accuracy may be relaxed for some of the items, to allow the use of a smaller value of n (Cochran 1977).

To determine the correct sample size for continuous data (unknown population) at 95% confidence, with value of the selected α=.025 being 1.96 and margin of error of .03, Cochran’s sample table for population size more than 10,000 yielded a sample size of 119; rounded off to 120 for purposes of convenience in allocation. Since the study involved a household survey, determining the households was informed by the 2009 Population and Housing Census in Kenya (KNBS NASSEP IV Sampling Manual, 2009). In each stratum, representative enumeration areas (EAs) were randomly selected from a list of all EAs using a simple random sampling method. Each enumeration area had 150 households (KNBS NASSEP IV Sampling Manual, 2009). Ten homes were selected for interviews in each EA, to reduce the sample ratio to population. The selection is outstanding because it reduces cluster variability (United Nations Statistics Manual, 2005). According to the KNBS Housing and Population Census (2009), Umoja 1 has approximately 15,000 households, which are about 100 EAs. Githunguri and Moiben each have around 7000 households. Therefore, the sample was distributed according to the ratio of the number of enumeration areas. In Umoja 1, 6 EAs were randomly selected, while in both Githunguri and Moiben, 3 EAs each were selected. The distribution of the sample size is presented in Table 1.

Data collection

Data collection was commenced using a semi-structured questionnaire administered through informed adult consent with the household as the primary sampling unit. The standard questionnaire was structured following the Likert Scale, whereby the respondents were given a choice of five pre-coded answers with the neutral point being neither agree nor disagree (Joshi et al. 2015). The questionnaire was structured to enable the assessment of the level of public acceptance and public views on maize related biotechnology, including GM maize concerning biodiversity conservation and biosafety, public health, and religious ethics.

Table 1. Sample distribution across regions (clusters).

Region	Sample size	Clusters of the respondents
Umoja 1 (Nairobi County)	60	Maize consumers
Moiben (Uasin Gishu County)	30	Maize farmers
Githunguri (Kiambu County)	30	Maize farmers
Total	120	

Interviews

Five field interviewers were selected, and they were trained on the objectives of the project and the methodology of fieldwork. It was a directive that during fieldwork, each interviewer was to identify a landmark from the sampling point and skip 10 households in urban and 5 households in the rural as directed by KNBS NASSEP IV methodology. Since the questionnaire was short, each interviewer was expected to conduct 10 interviews daily. With the help of an additional hand, the supervision was stringent to ensure that the interviewers observed all research ethics, as required. Every evening, the questionnaires were collected from the interviewers and challenges discussed. One of the key challenges that came out was that many of the respondents did not have substantial prior knowledge of GM maize. However, the interviewers made sufficient effort to explain the GM concept before the field questionnaire was administered. However, the state of ignorance resulted in several skip routines in search of relevant respondents. A total of 120 questionnaires were collected from the field which translated to a 100% response rate.

Questionnaire

The first data collection was undertaken using a self-administered questionnaire in accordance with Orodho (2004). The general configuration of the survey included a preliminary section on the respondent's profile. The other three parts focused on assessing the respondents' views and perception on (i) The role/importance of biotechnology on food security, (ii) Environmental implication of biotechnology, (iii) Public health and religious concerns on the introduction of maize related agro biotech in Kenya.

The questionnaire was structured following the Likert Scale, whereby the respondents were given a choice of five pre-coded answers with the neutral point being neither agree nor disagree. The Likert Scale is a psychometric response scale which is predominantly used in questionnaires to acquire participant's inclinations or level of agreement with a statement or set of announcements. Likert scales are a non-comparative scaling technique and are unidimensional (only measure a single trait) in nature. Respondents are asked to specify their degree of agreement with a defined statement by way of an ordinal scale. The Likert Scale was used in the study to allow the individual to demonstrate the level of agreement or disagreement with various statements concerning the adoption of GM maize.

Data analysis

Once the questionnaires were collected from the field, they were cross-checked and screened for missing information, especially on the demographics section. Data coding then followed on individual parts of the demographics (occupation, religion, Home County) and additional comments on food security, environmental implications, and public health. Here, responses were allotted specific numerical codes to be entered into the statistical software and for purposes of uniformity and time saving, during data entry. All data was entered using CSPro software, which is best for data entry because it allows for skips and lock of specific values outside the scope of coding. The data was then exported to SPSS for ease of data cleaning and analysis. Data cleaning was done through checking of missing information, wrongly entered codes, and joining similar responses. The scales that were used for food security, environment, and health were then tested for validity and internal consistency (reliability) using Cronbach's alpha. The Cronbach's alpha (α) generated from IBM SPSS 20 for the overall scale was 0.759 while the Cronbach's alpha for various subscales for the following perceptions was also measured; food security (0.750), environmental concerns & biodiversity (0.731) and public health concerns (0.760). This indicated an excellent internal consistency of the data collection instrument. According to Cronbach (1951), an alpha (α) in the range $0.7 \leq \alpha < 0.9$ indicates right internal consistency of the data collection instrument. The data analysis was undertaken using basic summary statistics means, frequencies, and standard deviation. Descriptive data was analyzed in the initial phase using frequencies and cross-tabulations.

Non-parametric tests-Mann Whitney U test - was used to test the hypotheses. Non-parametric tests assume that data do not follow a normal distribution. The dependent variable was subjected to Shapiro- Wilk tests to check the normality. The results at 95% confidence showed that the data did not follow a normal distribution. Therefore, to compare the differences of any two of the independent variable (consumers, small-scale and large-scale growers), a non-parametric test was most appropriate. A Mann-Whitney U test was employed to compare differences between two independent groups, when the dependent variable is ordinal or continuous but not normally distributed. For this study, the data was constant but not normally distributed. As such, only median could be used as a central tendency measure statistic, which made the Mann-Whitney U test most appropriate for testing the difference in any two of the three independent variables.

RESULTS AND DISCUSSION

Response rate

The study targeted a sample of 120 respondents, and a total of 120 questionnaires were filled, giving a response rate of 100%. This response rate was quite representative since it adapts to Mugenda and Mugenda (1999) view that a response rate of 50% is satisfactory for analysis and reporting; a 60% response is rated as useful, while a response rate of 70% and above is rated as excellent.

Data reliability

Cronbach's alpha was used to measure the internal consistency of the data collected. Since each participant was viewed as independent from all others, Cronbach's alpha was calculated for the overall scale and the subscales. The Cronbach's alpha (α) generated for the whole scale was 0.759 while the Cronbach's alpha for various subscales for the distinct perception was also measured as follows; food security (0.750), environmental concerns & biodiversity (0.731) and public health concerns (0.760). These statistics

indicated an excellent internal consistency of the data collection instrument. According to Cronbach (1951), an alpha (α) in the range $0.7 \leq \alpha < 0.9$ indicates right internal consistency of the data collection instrument.

Respondents demographic profile

The demographic profiles of the respondents were analyzed using descriptive methods, including frequencies and cross-tabulations. The patterns were segmented, as shown in Table 2.

Age and gender

Table 2 shows the breakdown of the key demographic characteristics: age, gender, and the highest level of education. The sample consisted of 59.2% males and 40.8% females. The majority of the respondents consisted of the youth between the ages of 25-34 (29%; 35/120) followed by those between the ages of 35-44 (27%; 32/120). There is a slight similarity in the distribution of respondents' ages across the respondents surveyed in Moiben and Githunguri, perhaps because these regions consisted of many farmers. The respondents surveyed in Umoja 1 however, including of those below 65 years old with an overwhelming majority (45%; 27/60) indicating that they were between 25-34 years old. According to the 2009 housing and census data, about 63% of the population in Umoja 1 were between the ages of 18-64 years, with only 2% having 65 years and above (KNHBS, 2010). This age variation in composition is perhaps due to low income and the compelling high cost of living around the urban area, which only favors the young and energetic population.

Education

The Government of Kenya is devoted to the delivery of quality education and training for all Kenyans in harmony with the constitution and international conventions such as the Education for All (EFA) goal. They are keen to develop policies for moving the country in the direction of the realization of this objective. Approximations from Kenya's 2009 Housing and Population Census, show that over 85% of Kenyans aged over 15 years can read and write with over 90% of men being literate as paralleled to 80% of women. Illiteracy was established to be more common among the underprivileged, predominantly poor women who constitute 61% of the total illiterate population.

The present research findings indicate that only 2% (2/120) of the total respondents had informal education. Table 2 shows that these respondents were interviewed in Moiben ward. The majority of those surveyed in Githunguri, consisted of those with secondary education levels. In Umoja 1, 3 in every 4 respondents surveyed indicated that they had tertiary education. This may be because a more significant portion of the population is engaged in a white-collar job.

Occupational analysis

There is a mix of occupations among the respondents. The majority (53%) of the urban respondents in Umoja I was dominated by respondents engaged in small and medium enterprises In the case of the rural areas, 50% of the respondents associated themselves with agriculture as their primary occupation with 20% indicating that they also engaged in small and medium enterprises (Table 3).

Rural farm size and urban household size

Table 4 shows the findings on rural farm size and urban household size respondent characteristics. In Moiben and Githunguri, since all the respondents were farmers, the key point of interest was to determine the farm size as principal maize production factor whereas, in Umoja 1, the key focus was on the household size as a key maize consumption factor.

Table 2. Breakdown of key demographic characteristics of the respondents.

Sample size (n)		Study area		
		Moiben	Githunguri	Umoja 1
		30	30	60
Age	18-24	0%	7%	20%
	25-34	7%	20%	45%
	35-44	27%	33%	23%
	45-54	30%	20%	8%
	55-64	13%	13%	3%
	65+	23%	7%	%
Gender	Male	60%	70%	53%
	Female	40%	30%	47%
Level of education	Informal	7%	-	-
	Primary	-	20%	-
	Secondary	40%	60%	25%
	Tertiary	53%	20%	75%

Table 3. Respondents' occupational analysis.

Primary occupation	Overall	Farmers (Githunguri & Moiben)	Non farmers (Umoja 1)
Agriculture	**26%**	**50%**	2%
Road transport	1%	2%	-
Administrative services	5%	2%	8%
Auxiliary finance and insurance services	2%	-	3%
Student	3%	2%	5%
Teaching	6%	8%	3%
Medical and health care services	3%	2%	5%
Social assistance services	2%	2%	2%
Artistic activities	1%	-	2%
Defense	2%	3%	-
Public services	3%	3%	3%
Computer system design and related services	1%	-	2%
Engineering (Civic, chemical, and mechanical)	6%	2%	10%
Religious services	2%	3%	-
Sales and marketing	1%	-	2%
Small and medium enterprises	37%	20%	53%
Retiree	1%	2%	-
Sample size (n)	120	60	60

Table 4. Farm size and household size characteristics.

Area	Mean	Std Dev.	Min.	Max.
Moiben Farm size (acres)	211.33	524.76	4.0	2,000.0
Githunguri	1.67	2.07	0.25	8.0
Household size of Umoja 1	3.22	1.52	1	7

The large-scale farmers in Moiben were found to own great tracks of land (Mean=211.33 acres, SD=524.76) compared to Githunguri small-scale farmers (Mean=1.67 acres, SD=2.07). The average household size of occupants of Umoja 1 was approximately three people per household, which is an approximate value of the 2009 housing and population census results. The difference among the maize producers was considered suitable for the perception of comparative analysis.

Public perception of GM maize and food security

The respondents were requested to show the extent to which they agree or disagree with statements of public perception on GM and food security items on a 5-point Likert scale. The overall findings by all the respondents considered GM maize as a possible solution to the food insecurity problems in Kenya. 69% of the respondents agreed that GM maize will play a significant role in solving food insecurity in Kenya (Figure 2). The respondents agreed on the need to produce sufficient maize for the increasingly growing population. These respondents viewed the sole cause of food insecurity in the country as associated with the domination of the country by arid and semi-arid areas; which are unproductive, hence produce insufficient food. However, some of the respondents attributed the food insecurity problem with widespread laziness and believed that GM maize would not be the sole solution. Others thought that the youth's overreliance on white-collar jobs affects innovation in the agricultural sector. The respondents suggested that the government needs to advise farmers on the best modern farming methods, while also searching for other safer ways of curbing the issue of food insecurity without necessarily relying on GM maize. Figure 2 shows that 65% of all the respondents agreed with the Likert Scale statement that GM maize could improve the profitability of the growers. Up to 60% believed that GM maize would benefit society by permitting growers to produce food more efficiently.

The statements met mixed reactions from various stakeholders (consumers, small and large holder farmers) surveyed. For instance, the small-scale maize farmers from Githunguri believed that GM food campaigns only focused on the benefits, without unearthing and disclosing the side effects. At the same time, these farmers are optimistic that GM maize will solve the problem of food insecurity stated by more than 73% of the surveyed farmers from the region. The small-scale farmers also believe that with GM maize, difficulties of malnutrition will be solved citing that farmers will significantly improve profitability from growing GM maize as a state by 60% and 70% of the respondents, respectively. This cluster of farmers is a little hesitant that the current maize production methods are sufficient to meet Kenya's food insecurity needs. However, they warn that if farmers rely on GM maize, and it fails, more hunger is more likely to be experienced.

On the flip side, large-scale maize farmers from Moiben believed that the current maize production approach and technology are sufficient to meet Kenya's food security needs. This was indicated by 60% of the farmers surveyed from the region. Many of them were hesitant that GM maize will solve food insecurity and malnutrition problems in Kenya. However, 43% appreciate the fact that GM maize will benefit the society, by allowing farmers to produce more and to a certain level, solve food insecurity level in the country.

Maize consumers from Umoja 1 showed their optimism and trust in the GM maize. More than 80% indicated that GM maize would help solve food insecurity in Kenya, as shown in Figure 3. Similarly, more than 60% of the consumers surveyed believe that GM maize allows farmers to produce food more efficiently, improve farmers profitability/income, and solve the problem of malnutrition in the country.

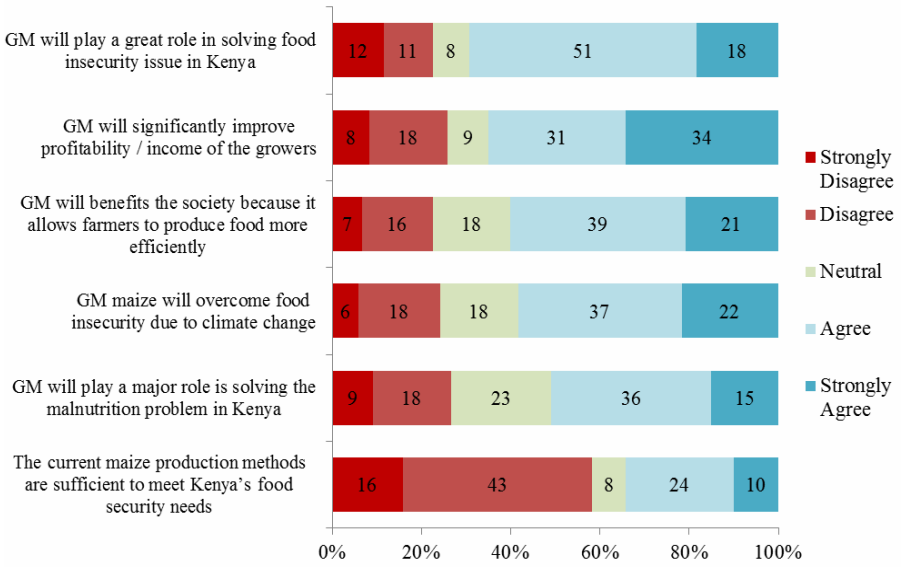


Figure 2. Perception towards the role of biotechnology on food security.

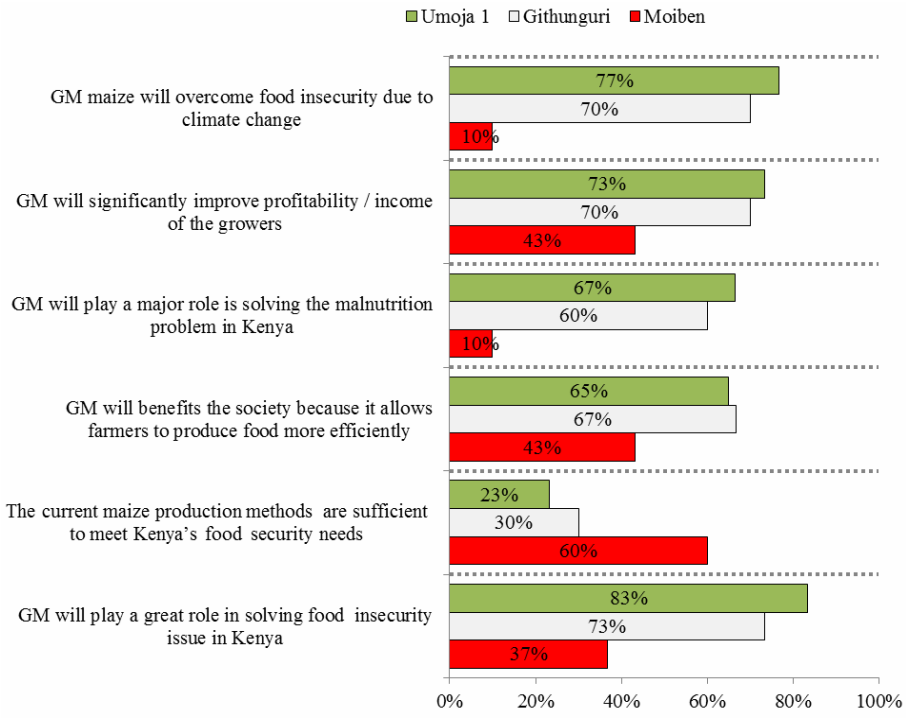


Figure 3. Regional analysis of perception towards the role of biotechnology on food security (Agree + Agree strongly).

Analyzing further comments from the consumers revealed that most of these consumers feel that GM maize is superior and would grow quickly and provide quick earnings, thereby resolving the issue of food insecurity in the long run. Some of them also cited that GM maize would require less fertilizer and biocides, thereby reducing the production costs, which would probably also reduce the cost of maize related products. Some of the consumer respondents, however, had the view that GM maize will create unnecessary competition with the traditional maize.

Environmental implications on national biodiversity conservation and biosafety

The respondents were asked to rank the extent they agree or disagree on whether GM maize affects the environment, especially on national biodiversity conservation and biosafety. The results indicated that 76% of the respondents feared that the introduction of the GM maize would contaminate the conventional crops through uncontrollable cross-pollination. Similarly, more than 50% of the respondents also agree that introduction of GM maize will cause increased pesticide use, which would contaminate the environment (Figure 4).

Small-scale farmers from Githunguri believe that despite the positive attributes expected from the introduction of GM maize, more than 60% of the GM maize would invade the environment and become uncontrollable with the majority (73%) of the surveyed small-scale farmers citing GM maize will contaminate the conventional maize through cross-pollination.

Just as smallholder growers, more than 50% of the large-scale growers from Moiben stated that they agree with the fact that GM maize is likely to affect the environment. They specifically stated that the introduction of GM maize is likely to contaminate the conventional maize through cross-pollination. These farmers added their concerns that harmful herbicides sprayed to control GM related maize weeds would kill beneficial insects like bees and decomposers. As such, large-scale farmers suggest that additional research on the environmental impact of GM maize is required because this is not known in Kenya. These large-scale farmers from Moiben felt that since maize farms will always be fenced, GM maize is likely to have significant effects on wildlife. As most of the farmers demand for a thorough scientific proof to unearth the real side effects of GM maize on wildlife, some claim that research findings found out that GM maize causes animal ulcers (to livestock).

In an equal measure of the magnitude of their optimism with GM maize, consumers were open to the expected adverse effects of GM maize especially on the fact that GM maize would contaminate the conventional maize through pollination, cited by 83% of the surveyed consumers. Similarly, more than 70% of the consumers are deeply concerned that GM maize may be harmful to non-target insects since they believe that GM maize would increase the use of pesticides which will then be detrimental to the environment. The consumers also added other expected effects of GM maize. Some believe that after consuming GM maize, some animals become ill or die, adding that since GM maize is artificially made, it has some genes that affect plants, animals, and even humans. Some of the consumers surveyed from Umoja 1, who claim that GM maize would create unnecessary competition for water and nutrients with other crops, fear that traditional maize varieties may be lost forever.

Additional probing revealed the following public environmental concerns: (i)The GM maize would lead to increased invasion of pests such as weevils. (ii) The GM maize is likely to affect the quality of dairy milk and bee honey. (iii) The GM maize will cause increased use of pesticides, thus contaminating the environment. However, some of the consumers believe that GM maize is pest resistant and will require limited use of pesticides, which is good for the environment. (iv) The GM maize will change the soil structure and mineral composition, thereby affecting the production of traditional indigenous maize. (v) The excessive spraying of the GM maize will contaminate rivers. (vi) The GM maize may lead to desertification. (vii) The GM maize may have adverse effects on wildlife. (viii) The GM maize will invade the environment and become uncontrollable (Figure 5).

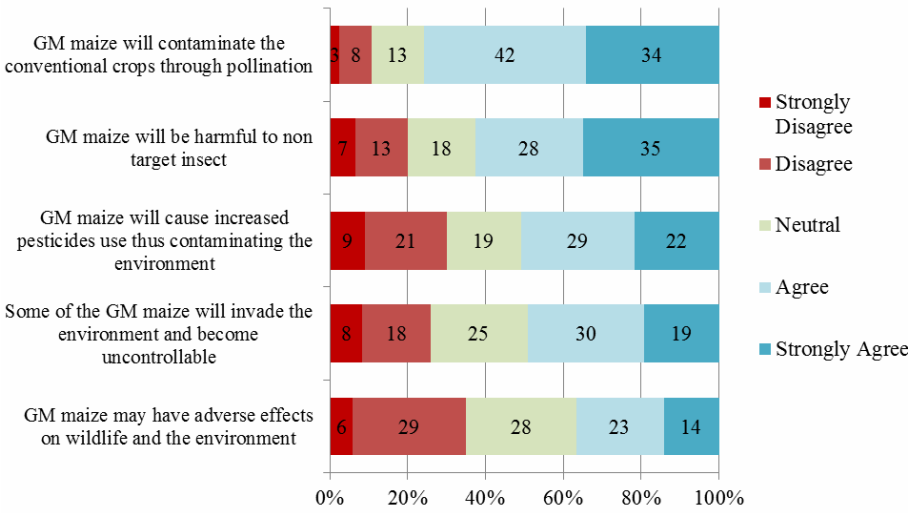


Figure 4. Public perception on the environmental implications of GM maize on national biodiversity conservation and biosafety.

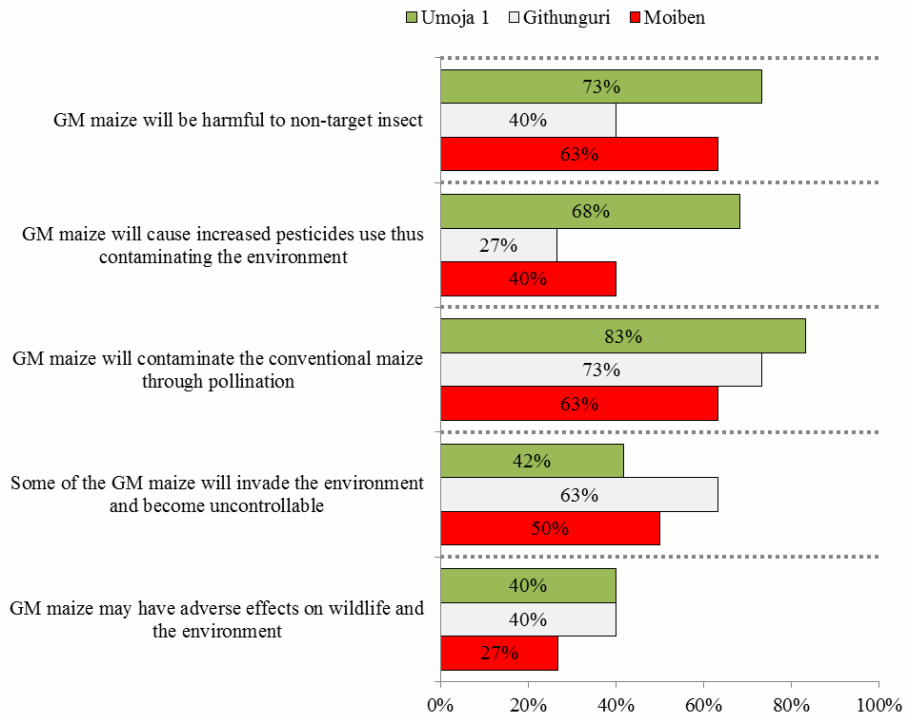


Figure 5. Regional analysis of public perception on the environmental implications of GM maize on national biodiversity conservation and biosafety (Agree + Agree Strongly).

Public health and religious concerns on GM maize

The key message from the survey findings was that GM maize labels need to show the presence of biotech ingredients, because consumers need to understand the health risks associated with this maize. On the contrary, about half of the surveyed respondents feel that GM maize has promising benefits, one of which is its high nutritional value even as others think that GM maize risks undermining God by modifying the conventional crops (Figure 6).

Small-scale farmers from Githunguri showed their support for GM maize, stating that GM maize will benefit the society due to its high nutritional values while also stating that GM maize is reasonably safe for human consumption. However, these small-scale farmers are part of those campaigning for labeling of GM foods to show the presence of biotech ingredients to expose the level of health risks that these foods have on their consumers.

More than 90% of the consumers posed their concerns about labeling every GM food to show the contents. While many are optimistic that GM maize will solve malnutrition problems due to its nutritional value, 60% of the consumers fear that GM maize might be harmful for human consumption. Above all, the consumers fear that GM food might lead interfere with God's ordinary creation of the crops.

Just like the other respondents, large-scale farmers from Moiben expressed their wish for labeling GM foods. Moreover, most of these farmers (50%) feel that GM foods aren't suitable for human consumption. The most outstanding negative perception towards GM maize in the large-scale growers sector is that GM maize is likely to interfere with God's creation of conventional crops cited by 67% of the respondents. Only 23% of large-scale growers believe that GM foods will benefit society due to better nutritional value.

Additional probing revealed the following public environmental concerns: (i) There is a need to increase health awareness campaign on GM maize with some calling for in-depth research on the impact of GM maize on human health. (ii) GM maize has a lot of nutrients that lack in traditional maize. On the flipside, some of the consumers raised deep concerns about GM foods stating that GM food accelerates aging of individuals, it may lead to a physically weak generation and that GM maize may cause allergic reactions to some people. (iii) There were claims that GM maize may make people resistant to drugs with others, suggesting that due to many demerits than merits, we. (iv) While others feel that GM interferes with God's creation, some consumers believe that GM is just human improvements of what God already created and as such does not interfere with God's creation at all. (v) Other respondents believe that GM maize being hybrid maize is not harmful to humans at all with others claiming that no reports of serious effects have been received from other countries that use GM maize. Preferred alternative instead of introduction of GM foods

The respondents were asked to give out a choice to be resorted to instead of introducing GM foods. They were presented with 7 options in the order of inclination with 1 being the most favored and 7 the least preferred. The results of Figure 7 indicate that the option with the highest number of 1 is the most preferred while the one with the highest number of 7 considered as the least favorite. The respondents felt that key focus points should be an expansion of agricultural lands including irrigating arid areas, advocating for soil and water preservation, use of better-quality plant varieties and the use of biological fertilizers as measures replacing GM foods.

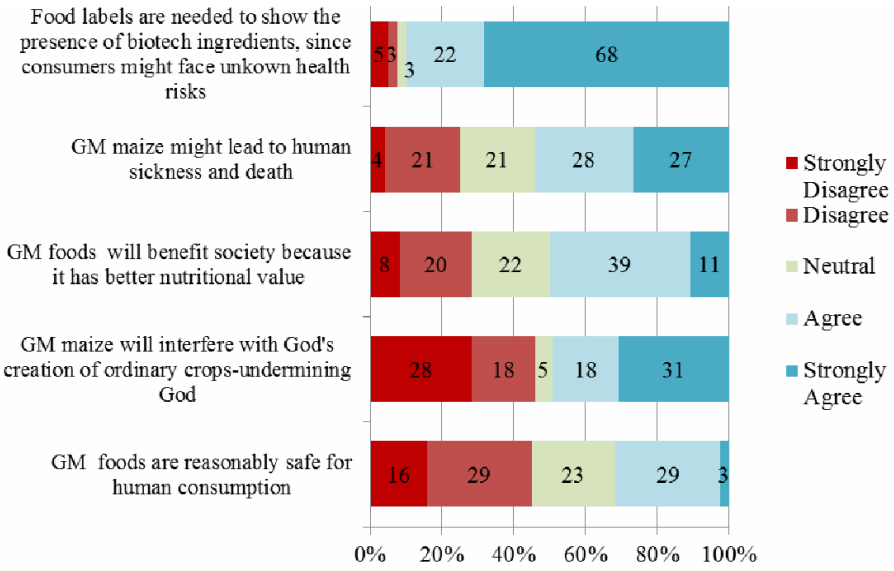


Figure 6. Perceived public health concerns on the introduction of maize related agro biotech.

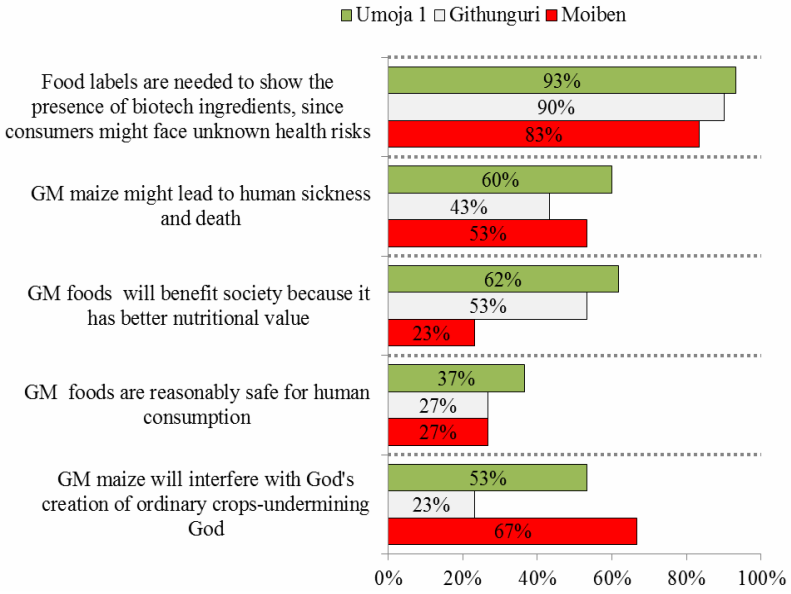


Figure 7. Regional analysis of perceived public health concerns on the introduction of maize related agro biotech (Agree +Agree Strongly).

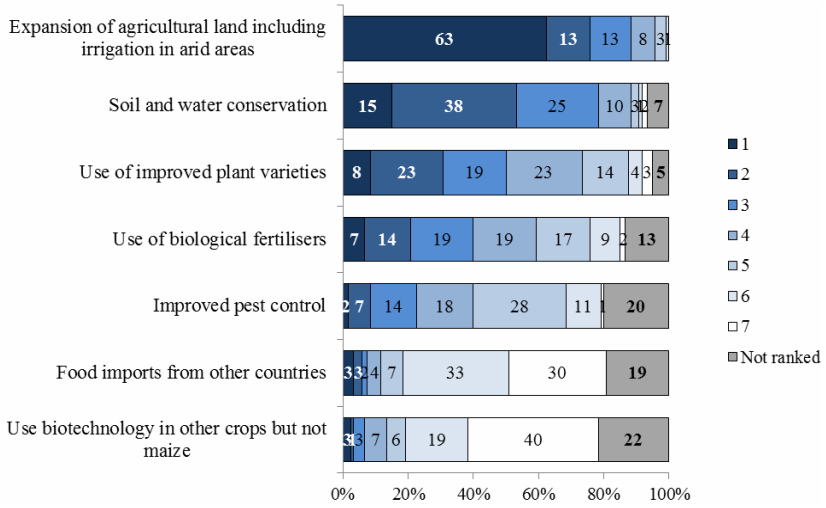


Figure 8. Rank of suggested alternatives to GM foods.

However, due to significant concerns about the side effects of GM foods, most respondents felt that the use of biotechnology on other crops and not maize would solve the problem of replacing GM maize. The other method that respondents felt least comfortable with was the option of importing food from other countries. Lastly, the respondents showed that another option that would be considered is the use of improved pest control methods. However, this is not a method that many would prefer. The ranking of options that would replace GM foods is shown in Figure 8.

Test of hypotheses

The four hypotheses that were to be tested included: (i) There is a public perception that GM maize may not play a significant role in solving the food insecurity issue in Kenya. (ii) There is a public perception that GM maize may not have adverse effects on the environment. (iii) There is a public perception that GM maize may not lead to human sickness and death. (iv) There is a public perception that GM maize may not interfere with God's creation of ordinary crops-undermining God.

Non-parametric test, using a Mann Whitney U test, was used to understand the hypotheses above. This is because the test variables were ordinal.

Hypothesis 1: There is a public perception that GM maize will not play a significant role in solving food insecurity issue in Kenya

The results of Table 5 indicates that there is a statistically significant difference in perception that GM maize will address food insecurity between small-scale farmers and large-scale farmers at 95% confidence

(W=712.5, Z=-3.12, p<.005). Similarly, there was a significant difference in perception of the same between large-scale farmers and consumers (W=939, Z=-3.921, p<.005). Finally, the difference between small-scale farmers and consumers over the fact that GM maize would help in solving food insecurity in the country was not significant at 95% confidence. This implies that while both small-scale farmers and consumers are optimistic that GM maize will solve food insecurity in the country, their counterpart large-scale maize producers warn that introducing GM maize would not address food insecurity in Kenya. Since the 69% agree and 23% disagree that introduction of GM maize into the country will improve food security, the hypothesis that there is a public perception that GM maize will not play a significant role in solving food insecurity issue in Kenya is rejected at 95% confidence. It can, therefore, be concluded that there is sufficient evidence that the introduction of GM maize will help solve food insecurity in the country.

Hypothesis 2: There is a public perception that GM maize may not have adverse effects on the environment

There is no statistically significant difference in perception that GM maize will solve food insecurity between small-scale farmers and large-scale farmers (W=907, Z=-.122, p>.005), large-scale farmers and consumers (W=1304.5, Z=-.538, p>.005) and small-scale farmers and consumers (W=1308.5, Z=-.497, p>.005) at 95% confidence (Table 6). Since only about 37% of the respondents agree while 35% disagree that GM maize may have adverse effects on wildlife and the environment, a difference of 2% which is insignificant (within the margin of error of 3%), the hypothesis that there is a public perception that GM maize may not have severe effects on wildlife and the environment is therefore accepted at 95% confidence. The results indicate that there is not sufficient proof to conclude that there is a public perception that GM maize may have adverse effects on the environment.

Hypothesis 3: There is a public perception that GM maize might not lead to human sickness and death

The results of Table 7 indicates that there is no statistically significant difference in perception that GM maize might lead to human sickness and death between small-scale farmers and large-scale farmers (W=866, Z=-.746, p>.005), large-scale farmers and consumers (W=1235, Z=-1.151, p>.005) and small-scale farmers and consumers (W=1181, Z=-1.624, p>.005) at 95% confidence. Since 56% of the respondents agree, while only 25% disagree that GM maize might not lead to human sickness and death, a difference of 31% which is very significant (far much more than the margin of error of 3%). The hypothesis that GM maize might not lead to human sickness and death is therefore accepted at 95% confidence. The results indicate that there is sufficient proof to conclude that there is a public perception that GM maize might lead to human sickness and death.

The results of Table 8 indicates that there is a statistically significant difference in perception that GM maize will interfere with God's creation of ordinary crops-undermining God between: small-scale farmers and large-scale farmers (W=661, Z=-.3.928, p<.005), large-scale farmers and consumers (W=2527, Z=-1.794, p>.005) and small-scale farmers and consumers (W=1014.5, Z=-3.113, p<.005) at 95% confidence. Due to mixed perceptions among the respondent groups, it is necessary to check the actual descriptive scores. It is shown that 49% agree , while 46% disagree that GM maize will interfere with God's creation of ordinary crops-undermining God, which is a difference of 3%, therefore insignificant. The hypothesis that GM maize will not interfere with God's production of usual crops or undermining God is accepted at 95% confidence. The results, therefore, indicate that there is not sufficient proof to conclude that there is a public perception that GM maize will interfere with God's creation of ordinary crops-undermining God between.

Hypothesis 4: There is a public perception that GM maize may not interfere with God's creation of ordinary crops-undermining God.

Table 5. Mann Whitney U test on perception differences between groups (small-scale farmers, large-scale farmers and consumers) on the public perception that GM maize will solve food insecurity in the country.

Test variable 1	Test variable 2	Mann-Whitney U	Wilcoxon W	Z	p-value
Small-scale farmers	Large-scale farmers	247.5	712.5	-3.12	0.002
Large-scale farmers	Consumers	474	939	-3.921	0.001
Small-scale farmers	Consumers	828	1293	-0.695	0.487

Table 6. Mann Whitney U test on perception differences between groups (small-scale farmers, large-scale farmers and consumers) on the public perception that GM maize may have adverse effects on the environment.

Test variable 1	Test variable 2	Mann-Whitney U	Wilcoxon W	Z	p-value
Small-scale farmers	Large-scale farmers	442	907	-0.122	0.903
Large-scale farmers	Consumers	839.5	1304.5	-0.538	0.591
Small-scale farmers	Consumers	843.5	1308.5	-0.497	0.619

Table 7. Mann Whitney U test on perception differences between groups (small-scale farmers, large-scale farmers and consumers) on the public perception that GM maize might not lead to human sickness and death.

Test variable 1	Test variable 2	Mann-Whitney U	Wilcoxon W	Z	p-value
Small-scale farmers	Large-scale farmers	401	866	-0.746	0.456
Large-scale farmers	Consumers	770	1235	-1.151	0.25
Small-scale farmers	Consumers	716	1181	-1.624	0.104

Table 8. Mann Whitney U test on perception differences between groups (small-scale farmers, large-scale farmers and consumers) on the public perception that GM maize may not interfere with God's creation of ordinary crops-undermining God.

Test variable 1	Test variable 2	Mann-Whitney U	Wilcoxon W	Z	p-value
Large-scale farmers	Consumers	697.5	2527.5	-1.794	0.073
Small-scale farmers	Consumers	549.5	1014.5	-3.113	0.002

Discussion

Public perception on GM maize and food security

The findings in this study showed that the majority (69%) of the respondents considered the introduction of GM maize as a possible solution to the food insecurity problems in Kenya. This finding is like the study conducted in Kenya by Kimenju et al. (2005), in which most people believed that the adoption of the GM technology would have positive impacts, with above than 80% approving that it can offer an answer to the world's food production hitches. Similarly, the study conducted by Anunda (2009) showed that majority (79%) of the respondents from all the four clusters (consumers, farmers, academia, and scientists) believed that the introduction of GM drought-tolerant beans in arid areas of Kenya was desirable.

Anunda (2009) asked the respondents if genetically modified crops (GMCs) will improve yields and offer an answer to Kenya's food issue, 50% of the respondents felt that GMCs could improve yields which portrayed some level of public confidence with GM crops performance. In this study when we asked respondents if they believe growing GM maize will significantly improve profitability/income of growers, 65% of them were positive about it which was quite like the findings by Anunda (2009).

In an opinion survey conducted by Ombewa and Otunge (2012), on awareness and perceptions of agricultural biotechnology by the Seed Traders Association of Kenya (STAK) members, the study sought to know whether the respondents would be willing to produce, package and sell genetically modified crops. 100% of the respondents indicated that they would be ready, implying that they are aware of the benefits of biotechnology crops.

In the 2010, Eurobarometer which is a sequence of public opinion surveys carried out frequently on behalf of the European Commission, 53% of respondents in Europe expected biotechnology and genetic engineering to have a positive effect in twenty years, while 11% expected no impact, and only 20% expected a negative effect. Although this is not directly linked to food security, there is an overall agreement that biotechnology will have a positive effect.

Environmental implications of biotechnology on national biodiversity conservation and biosafety

Findings from this study established there are various public ecological concerns that the introduction of GM maize is likely to contaminate the conventional crops through pollination, as suggested by 76% of the surveyed respondents. The study by Kimenju et al. (2005), established that 51% of the respondents believed the introduction of GM crops would lead to loss of original plant varieties while 40% were of a contrary view.

More than 6 in every 10 respondents in this study believed that the introduction of GM maize would be harmful to non-target insects. The level of public environmental concern in this study was, therefore, is slightly above a survey conducted by Anunda (2009), where respondents were asked if 'Genetically Modified crops that are insect resistant might lead to the death of beneficial insects/non-pests and other non-targeted insects such as bee's or even birds.' The responses showed that a significant number (47%) of respondents disagreed that GMCs that are insect resistant may be detrimental to birds and bees with only 38% agreeing while 15% were undecided.

There was a substantial public environmental concern in this study about the possible contamination of traditional crops through cross-pollination with GM maize. Scientific studies have shown that best way of preventing cross-pollination between adjacent non-GM and GM crop fields is probably by maintaining sufficient distance between the two or engaging in human-pollination of GM crop under controlled conditions which is feasible. The application of GM buffer zones might be difficult in Kenya, where small-scale farmers' seldom have any land to spare. There is a significant likelihood that the risks of non-GM contamination by GM crop will come with numerous controversies and court cases in Kenya and the Biosafety Act does not appear to have adequate legal mechanisms to deal with these issues especially the livelihood damages on the emerging organic farmers whose model will almost be rendered impossible.

Public health and religious concerns on the introduction GM maize

In this study, 32% of the respondents agreed that GM foods are reasonably safe for human consumption. This was like the survey conducted in the USA on public perceptions of labeling genetically modified foods by Hallman et al. (2013), in which 45% of the respondents agreed that GM food was safe for human consumption with 8% strongly agreeing that such food was safe. However, 63% of the respondents in the study indicated that they would be distraught if they were served GM food without disclosure. In addition, 54% of the respondents reported that they would be enthusiastic about paying more for non-GM food.

One of the issues raised by the opponents of the GM technology was that GM maize may cause allergic reactions to some people and that GM maize may make people resistant to medical drugs. These public health concerns are similar with the results of the study conducted by the Centre for Disease Control and Prevention (2001), which suggested that there is potential for the unintentional transfer of allergens to formerly hypoallergenic foods.

Up to 68 % of the respondents in this study strongly agreed that food labels are required to confirm the presence

of biotech ingredients, to avoid the likelihood of consumers facing undisclosed health risks in the future. This finding concurs with the results of the study by Hallman et al. (2013), in which up to 59% of the respondents felt that it was tremendously important to inform the consumers whether food products have GM ingredients on a label. An almost similar percentage indicated that it was necessary to disclose information on whether a food product was cultivated while using hormones (63%), pesticides (62%), or antibiotics (61%).

Finally, the findings in this study established that majority of the respondents believed that the development and introduction of GM crops would interfere with God's creation of conventional plants, thereby undermining God as the Creator of the Universe and all the plants in the world as indicated in the Book of Genesis. Up to 49% of the respondents were concerned, while 46 % were not, and 5% were non-committal on this matter. From a study conducted by Anunda (2006), most of the consumers (64%) disagreed that genetic modification of crop plants can be considered as an act of "Playing God." In the study by Kimenju *et al*. (2005), it was established that 23% of the respondents were concerned that of the adoption of GM foods was tantamount to "playing God." This indicated a significant similarity with this study. Elsewhere in the world, a study was conducted in India by Gene Campaign and the University of Hyderabad to assess the level of public awareness, attitudes, and perceptions on the adoption of GM technology and GMOs among farmers, consumers, and other stakeholders. The findings showed that farmers across all ages and education levels felt that they would never offer 'genetically modified' food in temples or use it in religious ceremonies and festivals and were also unwilling to serve such food in weddings.

In conclusion, there is a robust public willingness on the introduction of GM maize in Kenya to deal with recurrent food insecurity, which might increase under the expected impacts of climate change. Kenya's vulnerability to climate change is predominantly acute due to its geographic exposure, low incomes, and more dependence on climate-sensitive sectors like agriculture, tourism, health, and energy. The agricultural industry in Kenya is already under pressure from climate change. It is anticipated that climate change will lead to a temperature rise of about 4^{o}C and a rainfall variability of up to 20% by the year 2100. This will seriously affect many economic sectors, especially agriculture, where a production decline of between 1- 22% is expected within the humid and dryland zones. Climate change is likely to increase challenges in the economic and social fabric of society. The government should, therefore, explore ways of enhancing the application of environmentally sustainable methods biotechnology for food security in the country as an intervention against increasing food demand, shrinking available arable land, and the looming climate change. There is substantial public fear and concerns on the likely environmental impacts of GM technology in the country. However, much of this is associated with inadequate public awareness and misinformation on biotechnology. This might also stem from insufficient scientific knowledge on the environmental impacts of biotechnology in the world, with most of the studies so far having been conducted in the developed world, which also controls the technology. There was apperception that introduction of GM maize might lead to human sickness and eventual death after consumption, and almost a similar weight was given to GM foods having better nutrition and eventually benefitting the society. This shows that fear and admiration of GM technology adoption are present in almost equal measure. The community is split between the possible benefits as well as the potential downside of GM technology. However, there is no doubt that there is a strong preference to have food labels indicating the presence of biotech ingredients. This is an indication that people still want to have the liberty to make a choice and have free will as far as consumption of bioengineered foods is concerned. There is also a strong indication that the introduction of GM maize in Kenya, like in other parts of the world could result in some resistance from some religious circles.

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