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Antimicrobial activity of crude leaf extracts from medicinal plants against *Enterococcus faecalis*

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ABSTRACT

Enterococcus faecalis is a Gram negative, commensal enteric bacterial pathogen usually located in the intestinal tracts of either animals or human beings. The pathogenic members of enteric bacteria's are usually associated to infections that are characterized by enteric fevers, abdominal pain, and diarrhoea and vomiting. This study aimed at determining the effects of the selected medicinal plants extracts against Clinical isolate of *Enterococcus faecalis* obtained from Kenyatta University health Centre. Kirby Bauer method was used to determine the antimicrobial activity of the plants extracts against *Enterococcus faecalis*. All the plant extracts showed antimicrobial activity against *Enterococcus faecalis* with *Tagetes minuta* producing the largest average zones of inhibition of 18.67 ± 1.03 mm when compared to the other plant extracts. *Vernonia lasioporus* was more active at low concentrations (MIC $3.9 \mu\text{g/ml}$; MBC $5.0 \mu\text{g/ml}$) while *Aloe secundiflora* was less active (MIC $7.0 \mu\text{g/ml}$; $9.7 \mu\text{g/ml}$). Ciprofloxacin ($5 \mu\text{g/ml}$) was used as a positive control producing an average zone of inhibition of 21.67 ± 2.66 mm while Methanol (33.50 ± 2.56 mm) and 4% Dimethyl sulphoxide (0.00 ± 0.00 mm) were used as negative control. Qualitative phytochemical screening showed the presence of four phytochemicals namely; saponins, tannins, alkaloids and flavonoids. The study provides insight about the antimicrobial activity of the plant extracts and if they can be used in treatment of as an antimicrobial agent against infections caused by *Enterococcus faecalis*.

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Keywords

Qualitative phytochemical screening; Antimicrobial agents; *Enterococcus faecalis*; Ciprofloxacin; Clinical isolate.

INTRODUCTION

Medicinal plants since memorial have been used in many communities as a source of medicine [13]. Medicinal plants play an important role in medical system to counter some of the serious diseases in the world. Pharmacological investigations of herbal plants have been carried out overtime to find novel drugs or active components for the development of new therapeutic agents [3]. Aloes are perennial succulent xerophytes which develops water storage tissues in leaves to survive in areas with low or erratic rainfall [28]. *Aloe secundiflora* has been used in treating ailments including; chest problems, polio, malaria and stomach ache by herbalists in the Lake Victoria region [21]. *Aloe secundiflora* leaf components have been credited for antibacterial, antifungal and antiviral and antihelmintic medicinal properties [18]. Aloes contain over 75 nutrients and 200 active compounds including enzymes, vitamins, minerals, lignin, sugars, saponins, anthraquinones amino acids and salicylic acid. Extracts of Aloes especially its leaf gel have shown antibacterial activity by inhibiting the growth of both Gram negative bacteria and Gram positive bacteria [11]. *Bulbine* is a genus of plants in the family xanthorrhoeaceae and sub family asphodeloideae and its members are well known for their medicinal value [1]. They are succulent plants with most of the species having yellow flowers where as some of them have white, orange or pink flowers. *Bulbine frutescens* is mostly grown as ornamental plant in flower garden at homes in South Africa [31]. *Bulbine* plant has been used for medicinal purposes in the early stages of the eighteen century by Dutch and British settlers of South Africa in treating various ailments [31]. The leaves of the plant have been used in the treatment of wound thought to be infected with bacterial pathogens and it has shown antibacterial properties [20]. Some of the species of the plant found in South Africa have been used for blood cleansing, treatment of ringworms and gravel rush by some local communities such as the Xhosa [8]. A decoction of bulbs and roots of some of the species has been used in the treatment of some of the ve-

nereal diseases in women and stomach upsets [30]. *Vernonia* shrubs is a herbaceous grow in tropical Africa and have a height of about 2-5 metres, elliptical leaves of up to 20 centimetres and a rough bark [15]. The plants in this genus usually have bitter taste and in English they are called bitter leaf [15]. Some of the common African names of plants in this genus are Olusia (Luo), Mululuza (Luganda), Onugu (Igbo), Grawa (Amharic) and Chusar-doki (Hausa) [22]. *Vernonia lasiopos* decoctions from the stems and leaves have been traditionally been used by herbalists in East Africa to treat, malaria, worms and gastrointestinal problems [19]. The genus *Tagetes* belongs to the Asteraceae family which presently comprises of 56 species, 27 biennials and 29 perennials. *Tagetes* species are grown all over the world as multipurpose plants [27]. *Tagetes* species and chemo-types from its genus have been largely examined for biological active metabolites that can be used in industry and medicine [12]. Compounds that have antimicrobial activity in the *Tagetes minuta* plant are said to be accumulated in the organs of the plant and their essential oils have not only antimicrobial effect but also insecticidal properties. Plant parts such as flowers and leaves have been known to contain flavonoids that are scavengers for free radicals which enhances the antimicrobial activity of the *Tagetes minuta* extracts [9]. Phytochemicals from the plant such as carotenoids have also been used in pharmacological preparations and they have been found to contain anti-aging and anti-cancer effects [4]. The plant extracts have been used in treating intestinal and stomach problems [5] [29].

MATERIALS AND METHODS

Plant material collection and sampling

The plants were randomly collected in densely populated areas in the Kenyatta University arboretum. The plants were then placed on a table and the leaves selected from the plants using the following criteria: those with no dead leaves, those with no flowers and of the same height. The plant material was randomly sampled in densely populated areas where; twenty samples of *Tagetes minuta*, ten samples of *Vernonia lasiopos*, five samples of *Aloe secundiflora* and five samples of *Bulbine frutescens* plants based on the criteria were collected. Voucher specimens were prepared and deposited in the University herbarium in Plant Sciences Department for future reference. The plants were brought to the laboratory and thoroughly

washed in running water to remove debris and dust particles and then rinsed using distilled water and finally air dried.

Preparation of plant extract

The air dried leaves from the plants were grinded into powder and 500 grams were soaked in 750 millilitres of Analar grade (AR) methanol in a conical flask for 72 hours, 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 1.8 grams of *Bulbine frutescens*, 3.1 grams of *Aloe secundiflora*, 2.6 grams of *Tagetes minuta* and 2.1 gram of *Vernonia lasiopus*. The extracts were then stored in a labelled amber glasses bottle slightly opened where they were further left to dry bottle at room temperature away from light and heat in a laminar flow before being used for antimicrobial efficacy test [24].

Preparation of media

The media used were Muller Hinton agar (Sharau®) was prepared according to commercially given instructions. 38mg of Muller Hinton agar powder was added into one litre of distilled water in a flat bottomed conical flask. The mixture was heat with frequent agitation and boiled for one minute to completely dissolve the media. The flask was then tightly closed using cotton wool and further covered by aluminum foil. The mixture was autoclaved for 15 minutes at 121 degree celsius after which it was left to cool down to room temperature. The media was poured in the petri dishes in a laminar flow to give uniform depth of 3 - 4 millimetres. The petri dishes containing the media were then placed in a sterile plastic bags and stored at a temperature of 2 - 8 degree celsius before use.

Preparation of 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 prepared filled four McCartney bottles. The discs were then sterilized by autoclaving at 121 degree celsius for 15 minutes after which the autoclave was left to cool before removing the McCartney bottles containing the discs. The discs were dried on hot air oven at 50 degrees celsius to remove moisture [2]. The discs used for the antimicrobial activity were impregnated with formulated stock solution of the plant leaf extracts

of 1000µg/ml of *Tagetes minuta*, *Aloe secundiflora*, *Bulbine frutescens* and *Vernonia lasiopus*. A forceps used for picking the discs was first sterilized using a spirit lamp and left to cool. The forceps were then used in picking the sterilized discs and placing them in the stock solutions of the plants leaf extracts. The forceps were sterilized after every pick. The disc were then left to stay in the plant leaf extracts stock solution for two hours. The discs were then removed and placed in sterile petridish 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 - 8 degree celsius before being used for the antimicrobial susceptibility test.

Test bacterial organisms

The test microorganism used in the study was clinical isolate of *Enterococcus faecalis*. The microorganism was isolated from samples collected from first time patients at Kenyatta University Health Centre Laboratory, Nairobi. The samples were collected from patients who showed symptoms associated with enteric bacterial infections such as fever, abdominal pain, diarrhoea, vomiting and have no known drug resistance. Isolation, morphological identification and biochemical test were carried out in Microbiology laboratory, Department of Microbiology. The samples used for isolation of *Enterococcus faecalis* was from faecal material. The collected samples were isolated and identified by streaking of the samples collected on selective media for characteristic morphological identification based on the type of colonies formed. The morphologically identified microorganisms were then subjected to biochemical test for identification up to biochemical level.

Isolation and Morphological identification

The faecal material collected for the isolation and morphological identification of enteric bacteria was first mixed with distilled sterile water. The mixture was serially diluted up to 10^{-6} . A wire loop was sterilized by heating and the left to cool. It was then dipped into the serially diluted sample of 10^{-6} and streaked on selective and differential media in petri dishes known to support the growth of each of the test microorganism used. The petri dishes were then tightly closed using a parafilm and incubated for 24 hours at 37 degrees celsius. The plates were then removed and the bacteria's identified according to their morphological characteristics. *Enterococcus faecalis* was isolat-

ed by streaking the diluted faecal sample of 10^{-6} on Columbia agar with 5% sheep blood. The petridish tightly closed used parafilm and incubated at 37 degrees celsius for 24 hours. The plate was then later observed for growth. Growth of diplococcus colonies with gamma hemolysis on Columbia agar with 5% sheep blood is a morphological growth characteristic of *Enterococcus faecalis*.

Biochemical identification

The biochemical test carried out was based on the capability of the isolated and morphologically identified test microorganisms to cause fermentation of sugars and oxidation. The Medias used to carry out the test were prepared according to the manufacturer commercially used procedure and poured into cork screwed centrifuge tubes. Durham tubes were inserted to media containing broth and later observed for gas production. If there was gas production the result was termed as positive and no gas production as negative. The tubes were then closed and autoclaved at 121 degree celsius for 15 minutes and left to cool before being stored in a refrigerator before use. 1 millilitres inoculum of the isolated test microbes from diluted sample of 10^{-6} were introduced in the media and incubated at 37 degrees celsius and observation done after 24 hours and compared with the standard controls. The tubes containing solid media were observed for colour change. Where colour change occurred the results were termed as positive and no colour change as negative [14].

Antimicrobial susceptibility testing

The discs were impregnated with the extracts from the highest concentration of 1000mg/ml to the lowest concentration of 1mg/ml [17]. The antimicrobial efficacy test was carried out using Kirby Bauer method [25]. Muller Hinton agar was used in the spread plate technique where *Enterococcus faecalis* was spread using sterilized cotton wool swab and exposed to extracts impregnated discs in milligrams per microliter from *Aloe secundiflora*, *Tagetes minuta*, *Vernonia lasiopus* and *Bulbine frutescens*. The discs were placed with equal distance between them on agar plates inoculated with *Enterococcus faecalis*. Positive control discs contained ciprofloxacin while negative control discs were impregnated with dimethyl sulphoxide and distilled water. The Petri dishes were incubated at 37°C for 24 hours. Zones of inhibition formed were measured in millimetres and their average determined. The experiment was carried in duplicates and the diameter of zones of inhibition formed

measured.

Minimum inhibitory concentration and Minimum bactericidal concentration

Minimal inhibitory concentration (MIC) was determine using the broth tube method [10]. 100µl of 250mg/ml of methanol extract was added to 100µl of sterile bacteriological peptone in the first well of the 96 well micro plate and mixed well with a micropipette. 100µl of this dilution was transferred subsequently to wells two folding each dilution of the original extract. This was done to the extracts of *Aloe secundiflora*, *Bulbine frutescens*, *Vernonia lasiopus*, and *Tagetes minuta*. An inoculum of 100µl (0.5 McFarland standard) of overnight clinical culture of *Enterococcus faecalis* was added in each of the wells. Triplicate of each micro plate were made and the procedure repeated. The plates were then incubated at 37°C 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. Growth was indicated by a red colour (conversion of INT to formazan). The lowest concentration at which the colour was apparently invisible as compared to the next dilution was taken as the minimum inhibitory concentration [26]. Minimum bactericidal concentration (MBC) was determined by taking 100µl of suspension from micro plate wells that demonstrated no growth and inoculated on agar plates. The plates were incubated at 37°C for 24 hours. In the case where, there was no bacterial growth and value not greater than the minimum inhibitory concentration the concentration was used as the minimum bacterial concentration.

Qualitative phytochemical analysis

Presence of saponins, tannins, flavonoids and alkaloids in the crude extract were determined [6].

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

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

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

Table 1: Average zones of inhibition in millimeters when plant extracts are used against *Enterococcus faecalis*

Plant extracts	Zone of Inhibition±SD (mm)
Tagetes Minuta	18.67±1.03 ^c
Aloe secundiflora	17.67±1.63 ^c
Bulbine frutescens	18.50±1.05 ^c
Vernonia lasiopous	18.00±0.89 ^c
Antibiotic	21.67±2.42 ^b
Methanol	28.67±2.34 ^a
4%DMSO	0.00±0.00 ^e

The value of average zones of inhibition ± standard deviation (SD) after one-way ANOVA followed by Tukey's test. A value followed by the same superscript within the same column are not significantly different ($P>0.05$). Key: 4% Dimethyl sulphoxide (Negative control); Ciprofloxacin (Positive control), Plant extracts concentration (1000µg/ml).

Table 2: Interactions between the plants extract and *Enterococcus faecalis*

Extract	Zone of inhibition±SEM
<i>Aloe secundiflora</i>	16.69±0.40 ^{cd}
<i>Bulbine frutescens</i>	16.06±0.56 ^d
<i>Tagetes minuta</i>	17.19±0.42 ^c
<i>Vernonia lasiopous</i>	15.81±0.61 ^d
Controls	Zone of inhibition±SEM
Antibiotic	21.67±0.43 ^b
Methanol	31.17±0.34 ^a
DMSO	0.00±0.00 ^e
Test microorganisms	Zone of inhibition±SEM
<i>Enterococcus faecalis</i>	18.26±1.41 ^a
<i>P value</i>	
Extract	<0.001
<i>Enterococcus faecalis</i>	<0.001
Extract*Test microorganisms	<0.001

The value of average zones of inhibition ± standard error of mean (SEM) after two-way ANOVA followed by Tukey's test. A value followed by the same superscript within the same column are not significantly different ($P>0.05$). Key: Antibiotic (ciprofloxacin), DMSO - dimethyl sulphoxide

Table 3: Phytochemical tests on the plant extracts

Plant extracts	Saponins	Tannins	Alkaloids	Flavonoids
Aloe secundiflora	+	+	+	+
Vernonia lasiopous	+	+	+	+
Bulbine frutescens	+	+	+	+
Tagetes minuta	+	+	+	+

Key: (+) present

1% HCL was added to the filtrate and the solution heated. Mayor's reagent was added drop wise and if there was formation of any colored precipitate it indicated the presence of alkaloids.

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

STATISTICAL ANALYSIS

The data collected was exported to Microsoft excel spreadsheet where descriptive statistics were carried out. The data was analyzed using SAS version 9.1 one-way ANOVA (Analysis of variance) was carried out to show statistical difference using the varying zones of inhibition when the plant leaf extracts from *Tagetes minuta*, *Aloe secundiflora*, *Vernonia lasiopos* and *Bulbine frutescens* were used against *Enterococcus faecalis*. Two-way ANOVA was also carried to determine if there was any interaction between the plant extracts and *Enterococcus faecalis*. $P \leq 0.05$ considered significant in both test. Furthermore, a post hoc test was done to find the difference between the means.

RESULTS

All the plant extracts showed antimicrobial activity against *Enterococcus faecalis*. The average of the zones of inhibition formed ranged from 17mm - 19mm. *Tagetes minuta* extract produced the largest zones of inhibition of 18.67 ± 1.03 mm while *Vernonia lasiopos* extract produced the least zones of inhibition of 18.00 ± 0.89 mm (Table 1). The standard antibiotic used as a positive control (Ciprofloxacin) produced the largest zone of inhibition when compared to all the plant extracts 21.67 ± 2.66 mm. This showed that *Tagetes minuta* plant leaf extract was more effective against *Enterococcus faecalis* as compared to the other plant extracts. The average zones of inhibition formed by the extracts when used against *Enterococcus faecalis* were not significantly different; $P > 0.05$ (Table 1). However, the average zones of inhibition formed by antibiotics (positive control), methanol and DMSO (negative control) were significantly different from those formed by the plant extract $P < 0.05$ (Table 4.1).

The average zone of inhibition formed by the plants extracts when used against *Enterococcus faecalis* were significantly different to those formed by antibiotic, methanol and DMSO ($P < 0.05$; Table 2). Moreover, zones formed by *Tagetes minuta* extract against *Enterococcus faecalis* was significantly different to those formed by *Vernonia lasiopos* and *Bulbine frutescens* ($P < 0.05$; Table 2). The interaction between *Enterococcus faecalis* and the plant extracts was significant ($P < 0.05$; Table 2).

When the plant extracts were used in low concentrations against *Enterococcus faecalis*, *Vernonia lasiopos* was the most active with a minimum inhibitory concentration of $3.9 \mu\text{g/ml}$ and maximum bactericidal concentration of $5.0 \mu\text{g/ml}$. *Aloe secundiflora* extract was the less active against *Enterococcus faecalis* with a minimum inhibitory concentration of $7.0 \mu\text{g/ml}$ and a minimum bactericidal concentration of $9.7 \mu\text{g/ml}$ when compared to all the other plant extracts. The other two extracts, *Bulbine frutescens* and *Tagetes minuta* also had a pronounced antimicrobial activity against *Enterococcus faecalis* with minimum inhibitory concentration of $6.5 \mu\text{g/ml}$ and $5.1 \mu\text{g/ml}$; maximum bactericidal concentration of $9.1 \mu\text{g/ml}$ and $6.3 \mu\text{g/ml}$ respectively (Figure 1.0)

The qualitative phytochemicals test on plant extracts from *Tagetes minuta*, *Aloe secundiflora*, *Bulbine frutescens* and *Vernonia lasiopos* showed the presence of phytochemicals. The extracts contained saponins, tannins, alkaloids, and flavonoids (Table 3).

DISCUSSION

The increase of antimicrobial resistance to many available antimicrobial agents has led the need for the invention of new drugs. The use of plant extracts to test for antimicrobial activity has been brought forward as one of the ways of achieving this goals. The plants used in the study have been said to be of medicinal value. This study evaluated the antimicrobial activity of the medicinal plant extracts against *Enterococcus faecalis*. The extracts from the medicinal plants showed antimicrobial activity against *Enterococcus faecalis*. *Vernonia lasiopos* showed the most pronounced antimicrobial activity against *Enterococcus faecalis*. This showed that *Vernonia lasiopos* extract can be used as a more potent source of antimicrobial agent against *Enterococcus faecalis*. Antimicrobial activity in most medicinal plant extracts might be associated with the presence of secondary metabolites

in the plant extracts. From the study, the extract from *Vernonia lasiopos* contained pharmacologically active compounds; flavonoids, saponins, alkaloids and tannins which might be responsible for the antimicrobial activity. Some of the compounds have been associated with antimicrobial activity in medicinal plant extracts against bacterial microorganisms [3] [19]. *Aloe secundiflora* extract showed the less antimicrobial activity among all the extracts used against *Enterococcus faecalis*. This showed that, although the extracts from *Aloe secundiflora* had antimicrobial potential, a lot of it is required to cause antimicrobial effect at low concentrations when compared to other extracts. The extract also had secondary metabolites that might be responsible for its antimicrobial activity against *Enterococcus faecalis*. Similar studies previously carried out have shown that some of the secondary metabolites are pharmacological active components with antimicrobial activity [2] [24]. The extracts from *Bulbine frutescens* and *Tagetes minuta* also showed antimicrobial activity against *Enterococcus faecalis*. *Tagetes minuta* extract was more active against *Enterococcus faecalis* at low concentrations when compared to *Bulbine frutescens*. Preliminary phytochemical screening of *Tagetes minuta* extract showed the presence of secondary metabolites; saponins, flavonoid, alkaloids and tannins. The antimicrobial activity might be associated with the presence of the secondary metabolites that may be pharmacologically active against both Gram positive and Gram negative bacterial microorganisms [16] [23]. The extract from *Bulbine frutescens* also contained the secondary metabolites that might be responsible for its antimicrobial activity against *Enterococcus faecalis*. Similar studies carried out have shown that some of the secondary metabolites are pharmacologically active and can cause antimicrobial activity [7] [32].

CONCLUSION

Plants of medicinal value have been used by traditional healers to treat a lot of infectious diseases. The plant leaf extracts showed antimicrobial activity against *Enterococcus faecalis* with extract from *Vernonia lasiopos* showing a higher activity at low concentrations (MIC) when compared to extracts from the other plants. The present study showed that extracts from the used medicinal plants can be used to treat bacterial infections caused by *Enterococcus faecalis*. Qualitative phytochemical analysis showed that the plant extracts contained saponins, flavo-

noids, tannins and alkaloids. These secondary metabolites found in most medicinal plants are known to be responsible for the antimicrobial activity. However, further biochemical, chemical and pharmacological investigation must be carried to identify purified bioactive components that are responsible for the antimicrobial activity and their mechanism of action.

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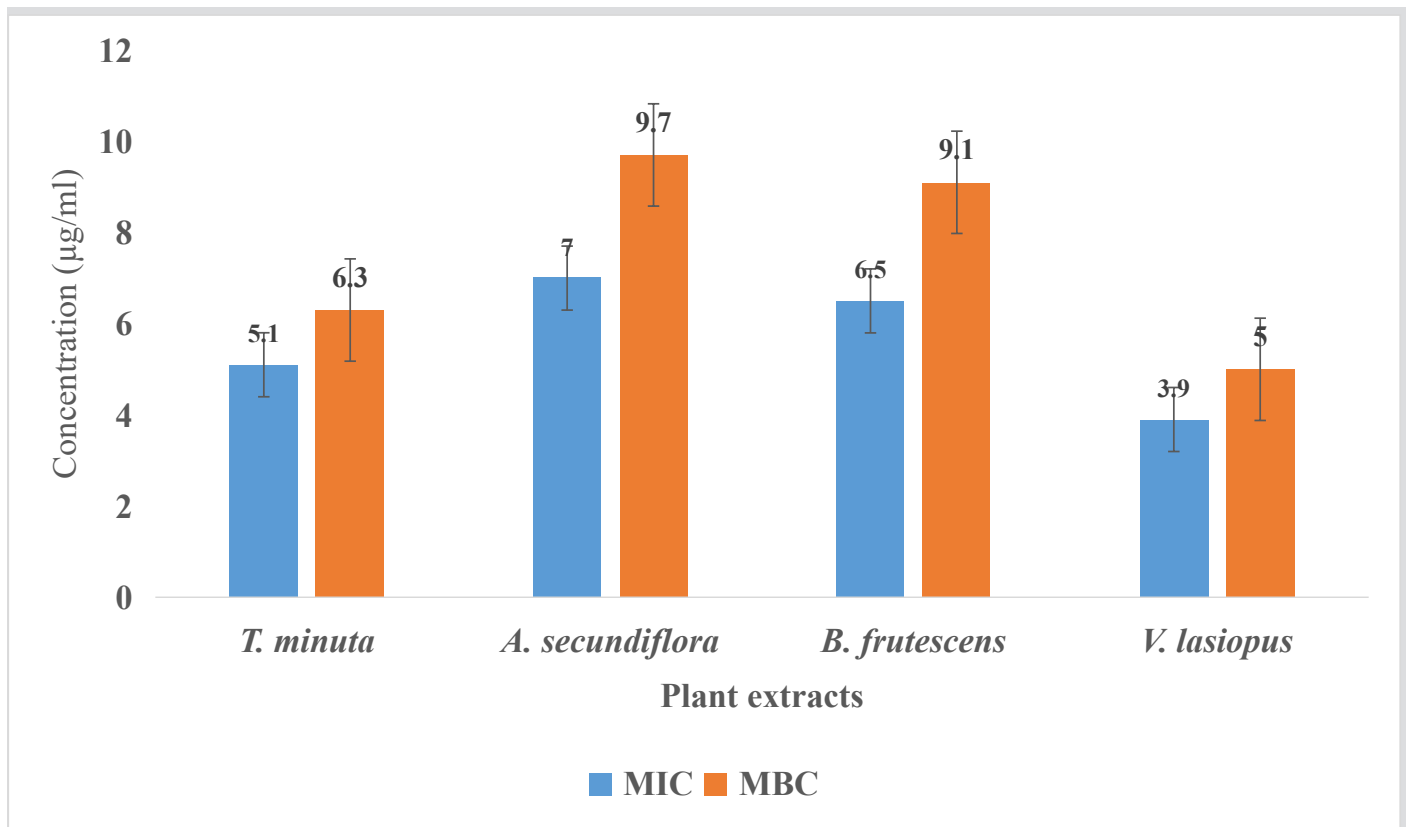


Figure 1. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of plant extracts against *Enterococcus faecalis*. Key: Error bars – represent standard error of mean (SEM).