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Phytochemical Composition and Antibacterial Activity of Rosemary (*Salvia rosmarinus*) Leaves Extract on Selected Bacterial Species in Uganda

Okongo Domisiano[1*], Kikweranono Ronald Reagan[2] and Hannington Twinomuhwezi[3]

[1]Kyambogo University, Kampala, Uganda. E-mail: okongodomisia@gmail.com
[2]Mbarara University of Science and Technology, Mbarara, Uganda. E-mail: kirrbadi@gmail.com
[3]Kyambogo University, Kampala, Uganda. E-mail: hantwino@kyu.ac.ug

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Abstract

Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what we would currently characterize as antimicrobial principles, was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies. This study was carried out to determine the phytochemical composition and the antibacterial activity of *Salvia rosmarinus* on *Escherichia coli* and *Staphylococcus aureus*. 100 g of dry powder was macerated by soaking it in 500 ml of 80% methanol. Phytochemical screening was done using documented standard phytochemical screening procedures and the antibacterial Susceptibility Testing was done against *E. coli* and *S. aureus* using the Agar well diffusion method. The results obtained showed that the methanol extract of *Salvia rosmarinus* leaves had antibacterial activity on gram positive *Staphylococcus aureus* and gram negative *Escherichia coli*. The results also showed that the extract was more active on *E. coli* than on *S. aureus*. This was an encouraging result and this indicates that the methanolic extract of *Salvia rosmarinus* leaves might be exploited as a natural antibiotic for the treatment of infections caused by these two pathogenic organisms, namely, *Escherichia coli* and *Staphylococcus aureus*.

Keywords: *Phytochemicals, Rosemary extract, Antibacterial activity, Escherichia coli, Staphylococcuss aureus*

1. Introduction

Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what we would currently characterize as antimicrobial principles, was well

* *Corresponding author: Okongo Domisiano, Kyambogo University, Kampala, Uganda. E-mail: okongodomisia@gmail.com*

accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Rios and Recio, 2005). For example among the local communities of Bwambara sub-county in Rukungiri District, Western Uganda, there are 67 medicinal plant species documented and these are distributed over 27 families and 62 genera. The most commonly reported species belong to Asteraceae family. The most frequently used medicinal species were *Chenopodium opulifolium* (27), *Sesbania sesban* (26), *Thevetia peruviana* (25), *Leonotis nepetifolia* (23), *Momordica foetida* (23), *Euphorbia hirta* (21) and *Cassia mimosoides* (20) (Gumisiriza *et al.*, 2021). Quite a good number of reasons have led to the increasing number study of these plants in hopes of making better the world of medicine. Despite the increasing acceptance of traditional medicine in Africa, this rich indigenous knowledge is not adequately documented. Documentation of plants used as traditional medicines is needed so that the knowledge can be preserved and the utilized plants conserved and used sustainably (Maroyi, 2013).

The healing power of the plants comes from the fact that they contain bioactive nutrient plant chemicals known as phytochemicals. Higher plants represent a rich source of new molecules with pharmacological properties, which are lead compounds for the development of new drugs (Ali *et al.*, 2019). These phytochemicals include tannins, cardiac glycosides, alkaloids, flavonoids, quinones, phenolic acids, anthocyanins, phytosterols, volatile oils, terpenes/terpenoids, and policosanols Western/convectional medicine first gained foot in Africa from 1900 (Roberts, 2017). However with the intense use of the conventional medicine to treat bacterial infections, many bacterial species have developed serious resistance on the convectional antibacterial drugs (Bandow *et al.*, 2003).

The increasingly high numbers of bacteria that are developing resistance to classical antibiotics drive much of the current interest on natural antimicrobial molecules in hope that they may provide useful leads into anti-infective drug candidates (Taylor *et al.*, 2021). On top of that the cost of treatment of some common bacterial infections is too high to be afforded by many people in most developing countries (Okeke *et al.*, 1999).

About 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate directly with their traditional uses. On numerous occasions, the folkloric records of many different cultures have provided information of plants with useful medicinal properties.

The Rosemary plant (*Salvia rosmarinus*) as classified in Table 1, is a luxuriant garden evergreen herb with needlelike leaves, native to the Mediterranean region. It can have white, pink, purple or blue flowers depending on its sub-type. It is beautiful, so it can be used both as an ornamental plant and as a spice. This research therefore seeks to find out the antibacterial activity of Rosemary plant.

Currently, there is a lot of resistance by the bacteria against most common drugs and the resistance continues to grow. Therefore, there is need to come up with alternatively affordable antibiotics from plants

Table 1: Scientific Classification of Rosemary Plant

Kingdom	**Plantae**
Clade:	*Tracheophytes*
Clade:	*Angiosperms*
Clade:	*Eudicots*
Clade:	*Asterids*
Order:	*Lamiales*
Family:	*Lamiaceae*
Genus:	*Salvia*
Species:	*S. rosmarinus*

Note: Binomial name: *Salvia rosmarinus.*

Source: Drew et al. (2017)

which have good antibacterial potential hence the need to study the phytochemical composition and antibacterial activity of plants such as *Salvia rosmarinus*

So the purpose of the study was to determine the antibacterial activity of *Salvia rosmarinus* extract on *Escherichia coli* and *Staphylococcus aureus*. This research will help pharmaceutical industries to determine whether *Salvia rosmarinus* could be a potential source of an alternative antibiotics for Gram negative *Escherichia coli* and gram positive *Staphylococcus aureus*.

2. Materials and Methods

2.1. Research Design

The research used both qualitative quantitative laboratory experiments. The phytochemical analysis strictly followed documented procedures and it was qualitative in nature. The Antibacterial susceptibility testing of *Salvia rosmarinus* leaf extract was determined by agar well diffusion method.

2.2. Sampling, Sample Size and Sample Preparation

Fresh leaves were purchased from Nakawa local market and safely transported to the laboratory for drying and extraction.

2.3. Apparatus and Reagents

- **Apparatus:** Hot plate, cork borers, thermometer, filter funnels, 500 ml beakers and 50 ml measuring cylinders, autoclave, Filter paper, Petri dishes, 100 µl pipette, glass stirring rod and rotary evaporator, oven, sample bottles, grinder.
- **Reagents and Chemicals:** 80% methanol, Mueller Hinton Agar, Tetracycline, DMSO, water, Phytochemical screening reagents.

2.4. Experimental Procedure

- **Preparation of Sample:** Fresh leaves of *Salvia rosmarinus* were dried using an oven at 30 °C for 24 hours. The dried leaves were then grinded to obtain powder. To 100 g of the powder was added 500 ml of 80% methanol, the mixture was shaken 20 minutes and left to stand for 48 hours. This was then followed by filtering through Whatman No. 2 filter paper. The solvent was evaporated using Clevenger apparatus. The resulting sample was divided into two portions. The first portion was used for phytochemical analysis of the crude extract and the second portion was concentrated using a rotary evaporator at 55 °C under reduced pressure. This extract was labelled, stored in tightly, sealed vials and stored in refrigerator at -4 °C.
- **Preparation of Nutrient Agar:** Exactly 12.0 g of Nutrient agar powder was accurately weighed using high precision strain gauge SF-400 electronic balance. It was then dissolved in 500 ml of distilled water in a 500 ml beaker and mixed thoroughly using a sterilized stirrer. It was then further dissolved by heating with frequent agitation and boiled for 1 minute until complete dissolution was achieved. The solution was then dispensed into a 500 ml glass beaker and sterilized in an autoclave at 121 °C (15 psi) for 15 minutes (Tanaka *et al.*, 2014).

2.5. Phytochemical Screening of Salvia rosmarinus Methanolic Crude Extract

Standard phytochemical screening procedures were followed to test for the presence of alkaloids, flavonoids, volatile oils, carotenoids, phenols, saponins, quinones, steroids, tannins and terpenes in the extract of *Salvia rosmarinus* leaves methanolic extract.

2.6. Test for Alkaloids

To 2 ml of the extract, 2 ml of 1% hydrochloric acid was added and steamed. Three (3) drops of Wagner's reagent was then added to the resultant solution. Brown or reddish brown precipitate indicates the presence of alkaloids (Ruskin *et al.*, 2014).

2.7. Test for Carotenoids

To 2 ml of the extract, 85% sulphuric acid was added. A blue colour at the interface indicates the presence of carotenoids (Ajayi *et al.*, 2011).

2.8. Test for Flavonoids

To measured 2 ml of the extract in the test tube was added two drops of sodium hydroxide solution. Appearance of a yellow colour which disappeared or became colorless after addition of two drops of dilute sulphuric acid indicated the presence of flavonoids (Ruskin *et al.*, 2014).

2.9. Test for Phenols

Measured 1 ml of 5% Iron (III) chloride solution was added to 2 ml of the extract in a test tube. A blue, green or dark green colour formation indicates the presence of phenols (Ruskin *et al.*, 2014).

2.10. Test for Quinones

Concentrated sulphuric acid (1 ml) was added to 1 ml of the extract in a test tube. Formation of a red colour showed the presence of quinones (Ruskin *et al.*, 2014).

2.11. Test for Saponins

Distilled water (5 ml) was added to 1 ml of the extract in a test tube. The mixture was then shaken vigorously for 2 minutes. Appearance of a stable foam lasting for 5 minutes indicates the presence of saponins (Ruskin *et al.*, 2014).

2.12. Test for Steroids

Acetic anhydride (2 ml) was added to the extract (1 ml) in a test tube followed by 2 ml of concentrated sulphuric acid. A blue or green reddish black coloration indicated the presence of steroids (Ruskin *et al.*, 2014).

2.13. Test for Tannins

In a test tube containing 2 ml of the extract, 2 ml of distilled water was added followed by three drops of ferric chloride solution. Formation of a blue or green precipitate indicated the presence of hydrolysable and condensed tannins, respectively (Edeoga *et al.*, 2005).

2.14. Test for Terpenes/Terpenoids

To 5 ml of the extract, 2 ml of chloroform and 3 ml of concentrated hydrochloric acid was added. Formation of a reddish brown colour at the interface indicated the presence of terpenes (Balouiri *et al.*, 2016).

2.15. Test for Volatile Oils

To the extract (1 ml), 90% ethanol was added, followed by few drops of ferric chloride. Formation of green colour indicated presence of volatile oils (Ruskin *et al.*, 2014).

2.16. Antibacterial Susceptibility Testing

2.16.1. Preparation of Extract Test Stock Solutions

Four different concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml were prepared in decreasing concentrations using serial dilution. To prepare the initial concentration of 200 mg/ml, 1.2 g of the solid extract was dissolved in 6 ml of DMSO.

To prepare 10 mg/ml of tetracycline solution, 30 mg of tetracycline was dissolved in 3 ml of DMSO.

2.16.2. The Preparation of Mueller Hinton Agar

The broth was prepared by weighing 9.5 g of the powdered media that was dissolved in 250 mls of distilled

water and it was sterilized in the autoclave in the temperature of 121° at 100 kpa (15 psi) above atmospheric pressure for 15 minutes.

The sterilized agar media was mixed in the heat preparation from the Bunsen burner and it was dispensed in the sterilized culture plates where it was left to solidify in the covered plates.

2.16.3. Inoculation

The test microorganism was aseptically inoculated (approx. 1.0 x 10° colony forming units/ml) on sterile Mueller Hinton agar by surface spreading to make uniform microbial inoculums. 100 µl of adjusted bacterial culture of *Escherichia coli* and *Staphylococcus aureus* were surface spread on Mueller Hinton agar in sterile petri plates using sterile glass rod spreader (Okeke *et al.*, 1999).

Using sterile glass cork borers (6 mm in diameter), three agar wells were punched aseptically in a triangular formation in each plate using 6 mm sterile cork borers and marked (Elisha *et al.*, 2017). Wells were carefully made on the agar plate without distorting the media; to contain test extract.

To the first wells 50 µl of extract sample was added. To the second wells 50 µl of standard antibiotic was added. To the third wells 50 µl of the DMSO was added. For each concentration of the methanolic extract the experiment was done in duplicates (Manandhar *et al.*, 2019).

The culture plates were then incubated at 37 °C for 24 hours.

2.16.4. Antibacterial Susceptibility Testing

Escherichia coli and *Staphylococcus aureus* were obtained as working cultures from Microbiology Lab, Department of Biology Kyambogo University.

100 µl of adjusted bacterial culture of *Escherichia coli* and *Staphylococcus aureus* were streaked on Mueller Hinton agar in sterile petri plates. Six agar wells were punched aseptically in a triangular formation in each plate using 6mm sterile cork borers and marked (Elisha *et al.*, 2017).

To the first four wells of the agar plates, 50 µl of extract sample was added, to each a different concentration. To the fifth wells 50 µl of standard antibiotic was added. To the sixth wells 50 µl of the DMSO was added. For each concentration of the methanolic extract the experiment was done in duplicates for both organisms. (Manandhar *et al.*, 2019).

The plates were then inverted and incubated at 37 °C for 24 hours. The inoculated plates were observed for zones of inhibition which were measured in millimeters. The experiment was done in duplicates (Elisha *et al.*, 2017).

Using a metric ruler, the diameter of the zones of inhibition were measured in millimeters (mm) (the diameter of the area of no growth of the microorganism around the wells) was measured for the control in antibiotics and extract (Baris *et al.*, 2006).

2.16.5. Reliability of the Study

The experiment had two controls; positive control that made use of a standard antibiotic, tetracycline and negative control in which DMSO was used.

3. Results and Discussion

3.1. Phytochemical Results of Salvia rosmarinus Leaves

As given in Table 2, screening of the *Salvia rosmarinus* extract of methanol was able to give a number of phytochemicals. A total of ten phytochemicals were tested in the extract, six of ten of the tests were positive and four tests showed negative results. Some phytochemicals were however absent in the methanolic extract such as alkaloids, saponins, carotenoids and phenols. The following phytochemicals were present: flavonoids, tannins, quinones, steroids, terpenes, and volatile oils. Other researchers also managed to obtain results that are in agreement with this (Aziz *et al.*, 2022; Mena *et al.*, 2016).

Table 2: Results for Phytochemical Profile of *Salvia rosmarinus* Leaves

S. No.	Chemical Constituents	Inference
1	Alkaloids	-
2	Flavonoids	++
3	Saponins	-
4	Carotenoids	-
5	Tannins	+++
6	Phenols	-
7	Quinones	++
8	Steroids	+
9	Terpenes/Terpenoids	+
10	Volatile oils	+++

Note: + means present in small quantities, ++ means present in moderate quantities, +++ means present in high quantities and - means absent.

Table 3: Results of Susceptibility Testing on *Escherichia coli* and *Staphyloccocus aureus*

Concentration of the Extract and Tetracycline (mg/ml)	Mean Zone of Inhibition±SEM (mm)	
	E. coli	*S. aureus*
50	15.00±00	12.50±00
100	16.75±00	14.50±00
150	17.75±00	16.50±00
200	18.25±00	17.50±00
+ve (10 mg/ml Tetracycline)	19.75±00	18.50±00
-ve (DMSO)	0.00±00	0.00±00

Standard zones of Inhibition limits: less than 10 mm-inactive, from 10-13 mm-partially active, from 14-19 mm-active and above 19 mm-very active (Gumisiriza *et al.*, 2021).

3.2. Phytochemical Analysis

Analysis of phytochemicals has been always used to show and thus enable isolation of drug lead compound and components from the plant. Phytochemical properties of plants give them unique biological activity. The plant part normally used the most for extraction is leaves.

In this research, *Salvia rosmarinus* extract of methanol was investigated for phytochemical composition and the following phytochemicals were present in the crude extract.

3.3. Flavonoids

Flavonoids consist of a large group of polyphenol compounds having a benzoyl-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by the phenylpropanoid pathway. Available reports tend to show that secondary metabolites of a phenolic nature including flavonoids, are responsible for the variety of pharmacological activities (Mahomoodally *et al.*, 2005). Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection (Dixon *et al.*, 1983).

3.4. Tannins

The term tannin is widely applied to a complex large biomolecule of polyphenol nature having sufficient hydroxyls and other suitable groups such as carboxyl to form strong complexes with various macromolecules. Tannins are generally used in the tanning process and used as healing agents in inflammation, burn, piles, and gonorrhea (Agidew, 2022).

3.5. Steroids

The word steroid is derived from sterol, which is a natural or synthetic chemically active hormone-like element. A steroid is one of a large group of chemical substances classified by a specific carbon structure. Steroids include drugs used to relieve swelling and inflammation, such as prednisone and cortisone; vitamin D; and some sex hormones, such as testosterone and estradiol (Agidew, 2022).

3.6. Terpenoids

Terpenoids are small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids show significant pharmacological activities, such as antiviral, antibacterial, antimalarial, anti-inflammatory, inhibition of cholesterol synthesis, and anti-cancer activities (Agidew, 2022).

3.7. Volatile Oils

Essential oils are found in glandular trichomes, at the bottom of the leaves and within flowering tops. The oil content in leaves ranges between 1.0-2.5%, depending on whether the leaves are young, or fully mature and dry and the composition depends on the region or country where it's grown. The following constituents were identified as shared in common: α-pinene, β-pinene, 1,8-cineole, camphene, borneol, camphor, linalool and β-caryophyllene. Substances found in the majority of oils were: β-myrcene, bornyl acetate, verbenone, limonene and sabinene which are terpene compounds (Pawlowska *et al.*, 2020).

3.8. Antibacterial Susceptibility Testing

From the Figure 1, the sensitivity tests of *Salvia rosmarinus* extract to *E. coli* and *S. aureus* showed that the

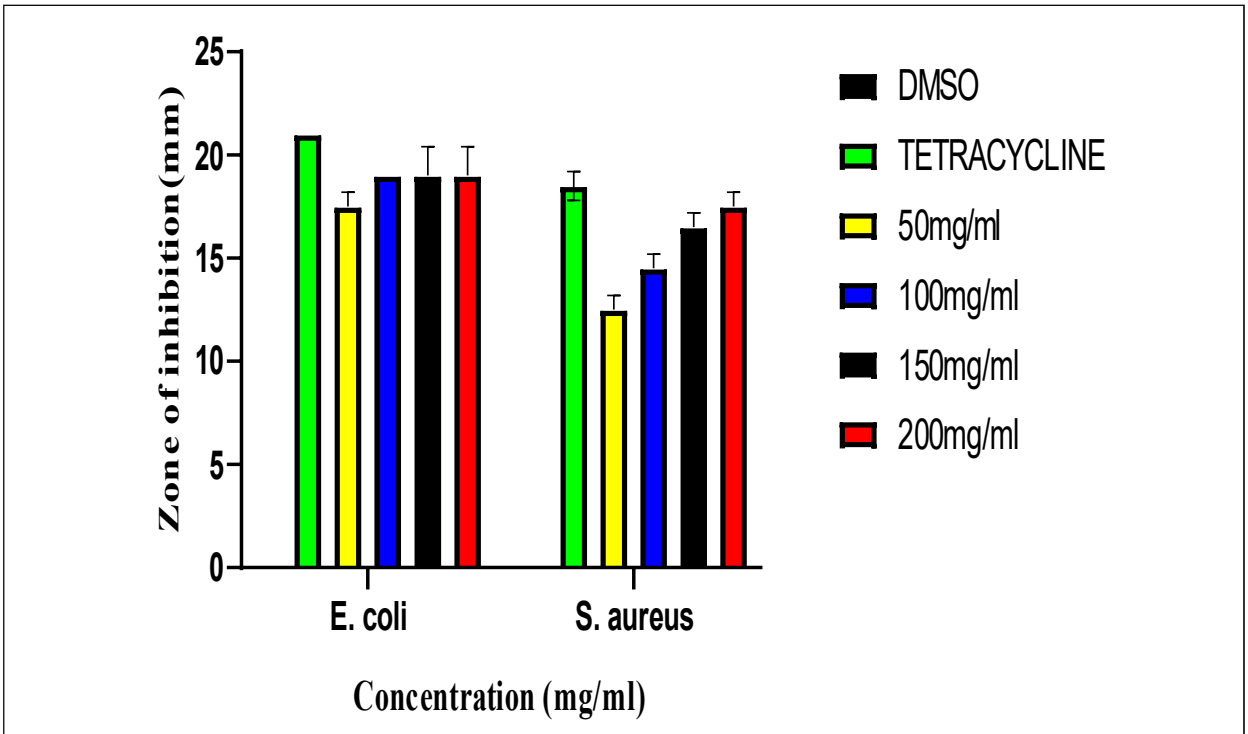

Figure 1: Comparison of Antimicrobial Activity of *Salvia rosmarinus* Extracts on *E. coli* and *S. aureus* with its SEM

methanol extract of the plant material showed antimicrobial activity on the test organisms. The test organisms, *Escherichia coli* and *Staphylococcus aureus* were both more susceptible to the tetracycline as the positive control with zone of inhibition at 18.25±00 mm for *E. coli* and 17.50±00 mm for *S. aureus*. This was the highest zone of inhibition observed for both organisms respectively and at the highest concentration of the extract (Figure 2).

Figure 2: Results for the Zones of Inhibition Shown by the Extracts on *E. coli* and *S. aureus*

With *E. coli* the highest susceptibility was recorded with the methanolic extract at 200 mg/ml with ZOI, zone of inhibition 18.25±00 mm followed by 150 gm/ml at 17.75±00 mm at 100 mg/ml at ZOI of 16.75±00 mm and finally 50 mg/ml with ZOI at 15.00±00 mm.

With *S. aureus* the highest susceptibility was recorded with the methanolic extract at 200 mg/ml with ZOI 17.50±00 mm, followed by 150 mg/ml with ZOI of 16.50±00 mm, followed by 100 mg/ml with ZOI of 14.50±00 mm and finally at 50 mg/ml with ZOI of 12.50±00 mm.

It was also observed that the *Salvia rosmarinus* methanolic extract was more active on gram negative *E. coli* than gram positive *S. aureus* for similar Concentrations. This agrees with Bachir and Benali (2012). This could be due to the difference in their structures. Gram positive *Staphylococcus aureus* has a thicker cell wall but with thin phospholipid bilayer as compared to gram negative *Escherichia coli* with thin cell wall but more phospholipid bilayer.

Escherichia coli was the most sensitive microorganism with the largest inhibition zone of 18.25±00 mm compared to the positive control (19.75±00 mm). *Staphylococcus aureus* was partially active with the plant extract with largest ZOI 17.50±00 mm compared to the positive control (18.50±00 mm). This was in line with the Ethiopian report (Aweke and Yeshanew, 2016).

According to the results above, it was realized that the sensitivity increased with increasing concentration as shown in Table 3, which was in agreement with (Oluduro and Omoboye, 2010). According to Oluduro and Omoboye (2010) the antibacterial activities of most plant extracts are concentration dependent as zone of growth inhibition increased with increasing concentration of the extracts. Ekwenye and Elegalam (2005) also reported that the efficacy of most plant extracts is concentration dependent.

Oluduro and Omoboye (2010) indicated that the presence of phytochemicals in plant extracts is a function of their antimicrobial activities against the test pathogen as they play important roles in bioactivity of medicinal plants. They further explained that phytochemicals exert antimicrobial activity through different mechanisms, Chonoko and Rufai (2011) also indicated that there was a link between the antibactetial activity exhibited by the plant extracts to the presence of steroids, flavonoids, tannins, alkaloids and saponins. Tannins, for example, act by iron deprivation, hydrogen binding or specific interactions with vital proteins such as enzymes in microbial cells (Scalbert, 1991).

Based on the discussion above, the difference between the activity of the extract and the standard antimicrobial drug may be due to the mixtures of bioactive compounds which probably have antagonistic effects against the major bioactive(s) present in the extracts compared to the pure compound contained in the standard antibiotic tetracycline. The standard drug which has the highest zone of inhibition while Dimethyl sulphur oxide (DMSO) had no zone of inhibition it is because DMSO has no active ingredients.

The policy implication of these findings is that more research should be carried out probably using bioassay guided fraction to determine the lead compound(s) in the extract.

4. Conclusion

In conclusion, the study found that the crude plant extract of *Salvia rosmarinus* for 80% methanol extract was active against gram negative Escherichia coli at different concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml and their ZOI were 15.00 mm, 16.75 mm, 17.75 mm, and 18.25 mm respectively and *Staphylococcus aureus* at methanol extraction concentrations of 50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml with the ZOI of 12.50 mm, 14.50 mm, 16.50 mm, and 17.5 m respectively.

Based on the results above it is evident that the methanol extract of *Salvia rosmarinus* contains the following phytochemical compounds: flavonoids, tannins, quinones, steroids, terpenes, and volatile oils and exhibited a significant antibacterial activity hence scientifically validated the ethnobotanical use of Salvia rosmarinus to treat bacterial infections caused by both *Escherichia coli* and *Staphylococcus aureus*.

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