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Comparative *In vitro* Antibacterial Properties of Methanol Extracts and Fractions of *Myristica fragrans* Seed and *Thymus vulgaris* Leaf

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Authors' contributions

This work was carried out in collaboration among all authors. Authors RAU and ACE designed the study, performed the experimental procedures, statistical analysis. Author RAU wrote the first draft of the manuscript. Author RAU supervised lab experiments. Authors ACE, IIJ, NAA, AEU and OTU organized data, managed the literature searches, assisted in plant material preparation. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this work was to compare the antibacterial properties of methanol extracts and fractions of *Myristica fragrans* seed and *Thymus vulgaris* leaf on the gram positive and negative bacteria. The *Myristica fragrans* seeds were crushed, defatted and air-dried. The defatted seed and leaf

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powders were separately macerated in absolute methanol for 72 hours. The methanol extracts and fractions were reconstituted at different concentrations of 100mg/mL, 80mg/mL, 60mg/mL, 40mg/mL and 20mg/mL for the antibacterial assay by agar diffusion method with activated cultured *Staphylococcus aureus* and *Escherichia coli* , incubated at 37^{o}C for 24 hours . The results showed that these plants possess antibacterial activity on the basis of their zones of inhibition. Methanol extract of *M*. *fragrans* had a higher activity of 8-19mm on *S*. *aureus* than *E. coli* with 5-14mm range respectively. Ethylacetate fraction had the highest activity with 9-25mm on *S. aureus*, while chloroform fraction had the highest activity on *E. coli* with 8-18mm. For *T. vulgaris*, the methanol extract had a higher activity of 6-18mm on *E. coli* than *S. aureus* of 4-17mm and for the fractions, n-hexane fraction had the highest activity of 7-20mm on *S. aureus ,* while aqueous fraction had the highest activity of 5-18mm on *E. coli*, compared with zones of inhibition of 18mm against *S. aureus* and 28mm against *E. coli* for gentamycin of 2mg/mL which was the reference drug. Methanol extracts and fractions of *M. fragrans* seed and *T. vulgaris* leaf showed excellent activities on the gram positive and gram negative bacteria but the *M. fragrans* had a better activity than *T. vulgaris*.

Keywords: Antibacterials; escherichia coli; inhibition; mcfarland turbidity; myristica fragrans; staphylococcus aureus; thymus vulgaris.

1. INTRODUCTION

Myristica fragrans HOUTT. With the common name “nutmeg” are dried seeds of the plant, from the family Myristicaceae, an evergreen tree about 10 to 20 m high, indigenous to Mollucca Islands. The plant is cultivated in Indonesia, Malaysia (Mollucca Islands, Sumatra, Java and Penang), Ceylon and West Indies(Grenada). It contains both essential and fixed oils. It is used as a psychotropic agent. Pharmacological studies revealed that it has aphrodisiac, antioxidant, anti-inflammatory, anticancer, sedative, antilipemic, anticaries, antidiarrhoeal properties [1]. It has been reported to contain myristicin and elemicin components, the formal relationship of these compounds to Amphetamins, others include terpenes, alcohols and phenols. It has a stimulating, flavouring, and carminative properties [2]. *Thymus vulgaris* Linn. from the family Lamiaceae, is an evergreen herbaceous shrub growing 15 to 30 cm high. It has woody, branched stems and very small, opposite leaves, hairy on the underside linear to oval in shape. Asia is the world producer of thyme. Thyme is commercially cultivated in the Mediterranean region, Southern Europe, North Africa and North America. In India, thyme is grown in the western temperate Himalayas and Nilgiris. The plant has an agreeable aromatic smell and a warm pungent taste. The fragrance of its leaves is due to an essential oil which gives its flavouring value for culinary purposes and is also the source of its medicinal properties. It flowers from May to August [3]. Pharmacological studies also revealed its antispasmodic, anti-inflammatory, antiamoebic and antibacterial properties. Its other important chemical constituents include thymol, carvacrol, camphene and limonene. Thymol oil is used as a flavouring agent, stimulant, antifungal and antibacterial. It is also used topically in lotions, creams and ointments in the concentrations ranging from 0.1 to 1% [4]. The aim of this work was to compare the antibacterial properties of methanol extracts and fractions of *M*. *fragrans* seed and *Thymus Vulgaris* leaf on the gram positive and negative bacteria.

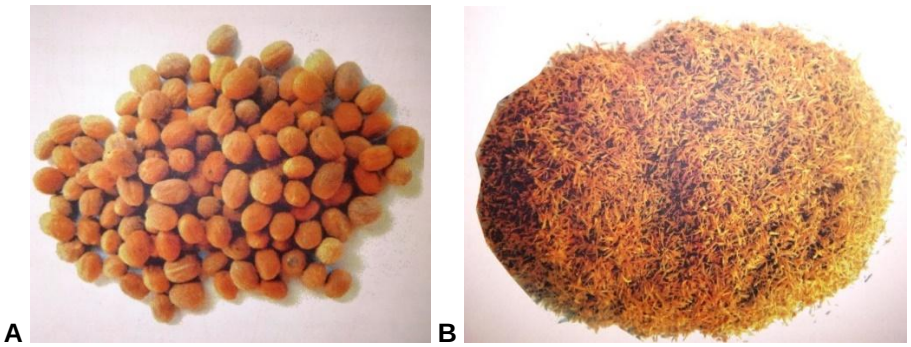

Fig. 1. (A) *M. fragrans* seeds, (B) *T. vulgaris* leaves [5]

2. METHODS

2.1 Collection and Extraction of *Myristica fragrans* Seed and *Thymus vulgaris* Leaf

M. fragrans seeds (Euroma brand) and *T. vulgaris* leaves (Tiger brand) were purchased, crushed into coarse powder and defatted with n-hexane for 24 hours, filtered and air-dried at room temperature. The defatted seed and leaf powders were separately macerated in absolute methanol for 72 hours at room temperature, filtered, concentrated to dryness and the percentage yields were obtained.

2.2 Antibacterial Assay

2.2.1 Source of Test micro-organism

Staphylococcus aureus and *Escherichia coli* were obtained from the Pharmaceutical microbiology Laboratory, Faculty of Pharmacy, University of Uyo, Nigeria.

2.2.2 Activation of micro-organisms

The micro-organisms which were suspended in the agar slants at $4^{O}C$ were incubated at $37^{O}C$ overnight for proper activation of the cultures [6].

2.3 Preparation of Inoculum

After incubation of the micro-organisms at $37^{O}C$ overnight, four to five bacterial colonies were picked and inoculated into a nutrient broth of 20 ml in each bijou bottle and incubated for six hours. The turbidity of the resulting suspension was found to be comparable to 0.5 McFarland turbidity standards. This level of turbidity is equivalent to 10^{8}cfu/mL [7].

2.4 Preparation of Culture Media

The media used for the activation of the micro-organisms was nutrient agar. Nutrient agar of 2.8g was dissolved in 100mL of distilled water in a conical flask. This was sterilized in an autoclave for 15 minutes. After sterilization, the nutrient agar was allowed to cool to $45^{O}C$ then poured into the petri dishes [8].

2.5 Bioassay on Selected Micro-organisms using *M. fragrans* seed and *T. vulgaris* Leaf Methanol Extracts

Agar well diffusion method was employed in this assay [9]. The well-sterilized molten nutrient agar of 20mL and aliquot of 0.1mL of inoculum of each micro-organism were added into each 15cm sterile petri dishes which were well labelled and allowed to set. The solidified agar in each petri dish was bored with cork-borer of 5mm in diameter. The different concentrations of 100, 80, 60, 40 and 20mg/mL of *Myristica fragrans* seed and *Thymus vulgaris* leaf methanol extracts were filled into the wells corresponding to each concentration marked on the petri dishes. Gentamycin of 2mg/mL and sterile normal saline used as positive and negative controls respectively, were introduced into separate agar bored wells. The plates were left on the bench for maximum diffusion to occur and subsequently incubated at $37^{O}C$ for 24 hours. The zones of inhibition were measured and recorded in millimetres (mm).

2.6 Bioassay-guided Fractionation and Testing for the Activity of the Partitioned Fractions

After testing of the methanol extracts for their antibacterial activity using a gram positive and gram negative bacteria, 40mg of both extracts were dissolved in methanol and distilled water in the ratio 3:1 and partitioned by successive extraction with n-hexane, chloroform, ethyl acetate and the residue was considered as aqueous fraction. The liquid fractions were concentrated into thick pastes. The dried fractions were weighed and recorded. The procedures of the bioassay on methanol extracts were repeated on the partitioned fractions and the resulted zones of inhibition were measured and recorded [9].

2.7 Determination of Minimum Inhibitory Concentrations (MICs) of *M. fragrans* seed and *T. vulgaris* Leaf Methanol Extracts and Fractions on *S. aureus* and *E. coli*

From the Cooper and Woodman equation: $X^2 = 4Dt\log \frac{M_0}{M_1}$

Where D = Diffusion coefficient
T = Time to traverse the distance(x)
M_O = Concentration of drug as applied
M_1 = Critical concentration

By varying M_O over a wide range of values and X measured in each case, X^2 was plotted against M_O . This gave a straight line graph, which is the Minimum Inhibitory Concentration (MIC) [10].

2.8 Statisical Analysis

Data are presented as mean ± Standard Error of Mean (SEM).

3. RESULTS

Methanol Myristica fragrans (MMf), Methanol Thymus vulgaria (MTv), n-Hexane Myristica fragrans (n-HexMf), Chloroform Myristica fragrans (chlMf), chloroform Thymus vulgaris (ChlTv), Ethylacetate Myristica fragrans (EthylMf), Ethylacetate Thymus vulgaris (EthylTv), Aqueous Myristica fragrans (AcqMf) and Aqueous Thymus vulgaris (AcqTv).

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Escherichia coli Methanol Crude Myristica fragrans (E.coli MCMf), Staphylococcus Methanol Crude Myristica fragrans (S.aur MCMf).

Escherichia coli n-Hexane Myristica fragrans (E.coliNHMf), Staphylococcus aureus n-Hexane Myristica fragrans (S.aurNHMf).

Escherichia coli Chloroform Myristica fragrans (E.coliCHMf), Staphylococcus aureus Chloroform Myristica fragrans (S.aurCHMf).

Escherichia coli Chloroform Myristica fragrans (E.coliCHMf), Staphylococcus aureus Chloroform Myristica fragrans (S.aurCHMf).

Escherichia coli Ethyl acetate Myristica fragrans (E.coliETAMf), Staphylococcus aureus Ethyl acetate Myristica fragrans (S.aurETAMf).

Escherichia coli Aqueous Myristica fragrans (E.coliAQMf), Staphylococcus aureus Aqueous Myristica fragrans (S.aurAQMf).

Escherichia coli Methanol Crude Thymus vulgaris (E.coliMCTv), Staphylococcus aureus Methanol Crude Thymus vulgaris (S.aurMCTv).

Escherichia coli n-Hexane Thymus vulgaris (E.coliNHTv), Staphylococcus aureus n-Hexane Thymus vulgaris (S.aurNHTv).

Escherichia coli Chloroform Thymus vulgaris (E.coliCHTv), Staphylococcus aureus Chloroform Thymus vulgaris (S.aurCHTv).

Escherichia coli Ethyl acetate Thymus vulgaris (E.coliETATv), Staphylococcus aureus Ethyl acetate Thymus vulgaris (S.aurETATv).

Escherichia coli Aqueous Thymus vulgaris (E.coliAQTv), Staphylococcus aureus Aqueous Thymus vulgaris (S.aurAQTv).

Table 1. Methanol extraction yields

Plant Sample	Weight of Powdered Sample (g) (A)	Weight of Dried Methanol Extract (g) (B)	% Yield B/A × 100
M. fragrans	650	90	13.85
T. vulgaris	640	70	10.94

Table 2. Solvent partition yields

***a) M. fragrans* seed**

Fractions	Weight of Extract (grams) (C)	Weight of fractions (grams) (D)	% Yield D/C × 100
n-hexane	40	3	7.50
Chloroform	40	9.7	24.00
Ethyl acetate	40	4.3	10.75
Methanol	40	10	25.00

***b) T. vulgaris* leaf**

Fractions	Weight of Extract (grams) (E)	Weight of fractions (grams) (F)	% Yield F/E× 100
n-hexane	40	2.5	6.25
Chloroform	40	6.2	15.50
Ethyl acetate	40	4.3	10.75
Methanol	40	13.7	34.25

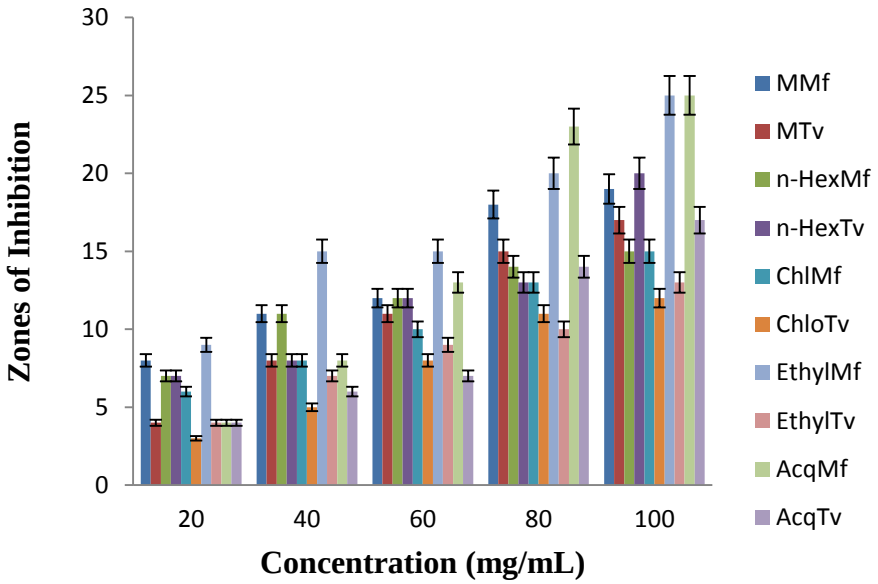

Fig. 2. Zones of inhibition (mm) of *Staphylococcus aureus* for *M. fragrans* seed and *T. vulgaris* leaf

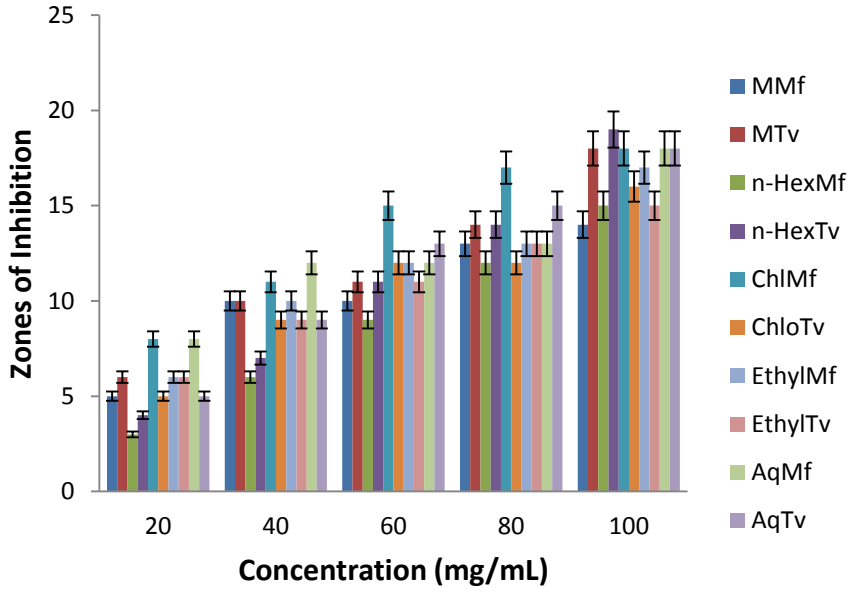

Fig. 3. Zones of inhibition (mm) of *E.Coli* for *M. fragrans* seed and *T. Vulgaris* leaf

Table 3. In vitro antibacterial assay of the methanol extract of *m. fragrans* seed

Concentration (mg/mL)	Log concentration	Zones of inhibition, x (mm)		X^2 ×10	
		E.coli	*S.aureus*	*E.coli*	*S.aureus*
100	2.0000	14	19	19.6	36.1
80	1.9031	13	18	16.9	32.4
60	1.7782	10	12	10.0	14.4
40	1.6021	10	11	10.0	12.1
20	1.3010	5	8	2.5	6.4

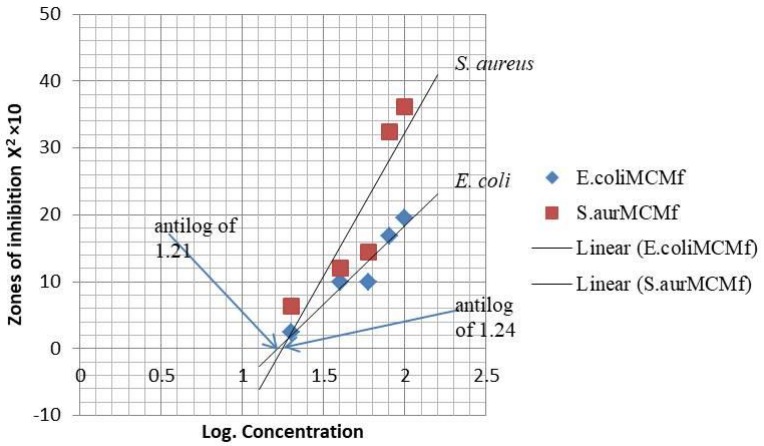

Fig. 4. Graph of MIC of methanol extract of *M. fragrans* seed against *S. aureus* and *E. coli*

Table 4. In vitro antibacterial assay of n-hexane fraction of *M. fragrans* Seeds

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm)		X^2 ×10	
		E.coli	*S.aureus*	*E.coli*	*S.aureus*
100	2.0000	15	15	22.5	22.5
80	1.9031	12	14	12.1	19.6
60	1.7782	9	12	8.1	14.4
40	1.6021	6	11	3.6	12.1
20	1.3010	3	7	0.9	4.9

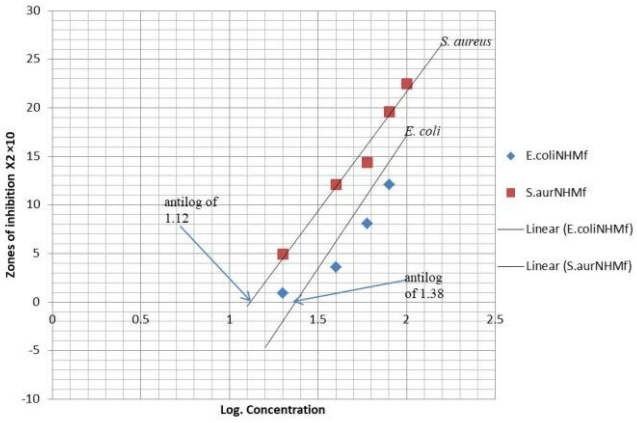

Fig. 5. Graph of MIC of n-hexane fraction of *M. fragrans* seed against *S. aureus* and *E. coli*

Table 5. *In vitro* antibacterial assay of chloroform fraction of *M. fragrans* Seed

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm)		X^2 ×10	
		E.coli	*S.aureus*	*E.coli*	*S.aureus*
100	2.0000	18	15	32.4	22.5
80	1.9031	17	13	28.9	16.9
60	1.7782	15	10	22.5	10.0
40	1.6021	11	8	12.1	6.4
20	1.3010	8	6	6.4	3.6

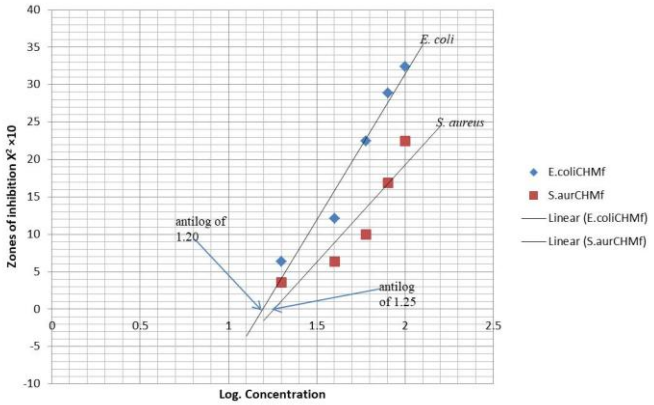

Fig. 6. Graph of MIC of chloroform fraction of *M. fragrans* seed against *S. aureus* and *E. coli*

Table 6. In vitro antibacterial assay of ethyl acetate fraction of *m. fragrans* seed

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm)		X^2 ×10	
		E.coli	*S.aureus*	*E.coli*	*S.aureus*
100	2.0000	17	25	28.9	62.5
80	1.9031	13	20	16.9	40.0
60	1.7782	12	15	14.4	22.5
40	1.6021	10	15	10.0	22.5
20	1.3010	6	9	3.6	8.1

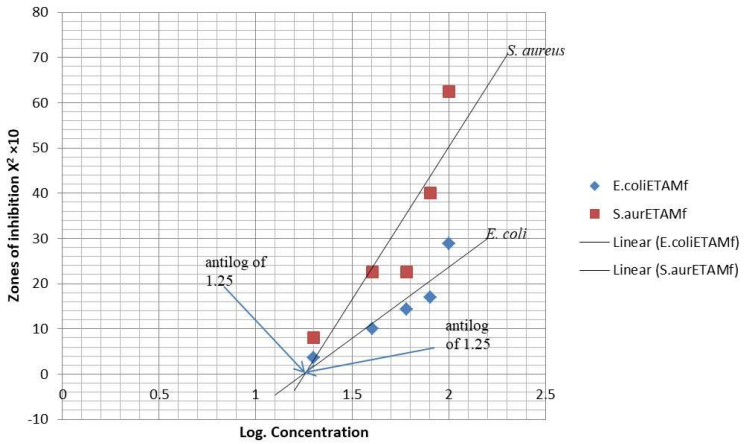

Fig. 7. Graph of MIC of ethyl acetate fraction of *M. fragrans* seed against *S. aureus* and *E. coli*

Table 7. In vitro antibacterial assay of aqueous fraction of *M. fragrans* Seed

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm) *E.coli S.aureus*		X^2 ×10 *E.coli S.aureus*	
100	2.0000	18	25	32.4	62.5
80	1.9031	13	23	16.9	52.9
60	1.7782	12	13	14.4	16.9
40	1.6021	12	8	14.4	6.4
20	1.3010	8	4	6.4	1.6

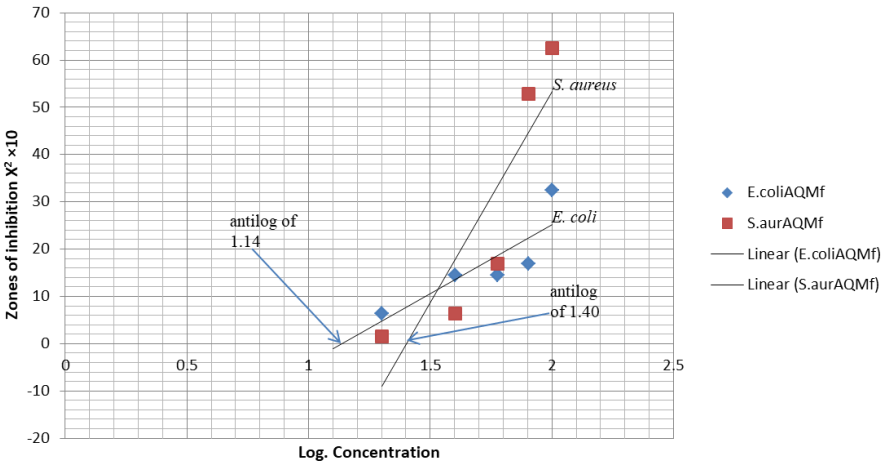

Fig. 8. Graph of MIC of aqueous fraction of *T. vulgaris* leaf against *S. aureus* and *E. coli*

Table 8. In vitro antibacterial assay of methanol extract of *T. vulgaris* leaf

Concentration (mg/mL)	Log concentration	Zones of inhibition, x (mm) *E.coli S.aureus*		X^2 ×10 *E.coli S.aureus*	
100	2.0000	18	17	32.4	28.9
80	1.9031	14	15	19.6	22.5
60	1.7782	11	11	12.1	12.1
40	1.6021	10	8	10.0	6.4
20	1.3010	6	4	3.6	1.6

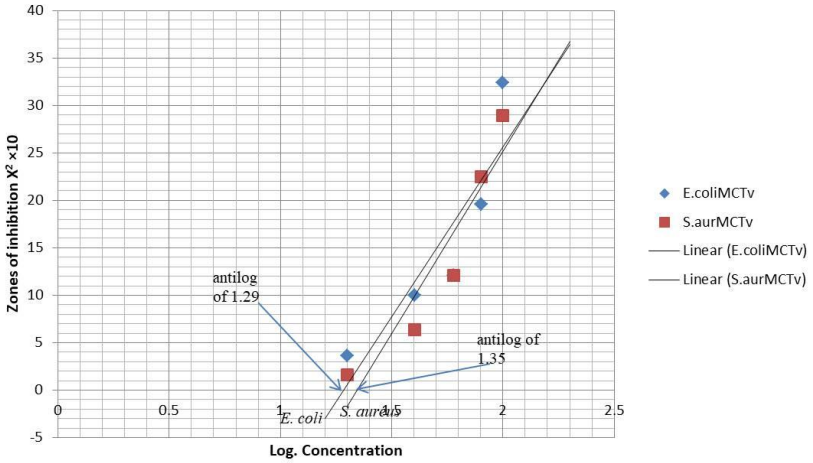

Fig. 9. Graph of MIC of Methanol extract *T. vulgaris* leaf against *S. aureus* and *E. coli*

Table 9. In vitro antibacterial assay of n-hexane fraction of *T. vulgaris* leaf

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm) *E.coli*	*S.aureus*	X^2 ×10 *E.coli*	*S.aureus*
100	2.0000	19	20	36.1	40.0
80	1.9031	14	13	19.6	16.9
60	1.7782	11	12	12.1	14.4
40	1.6021	7	8	4.9	6.4
20	1.3010	4	7	1.6	4.9

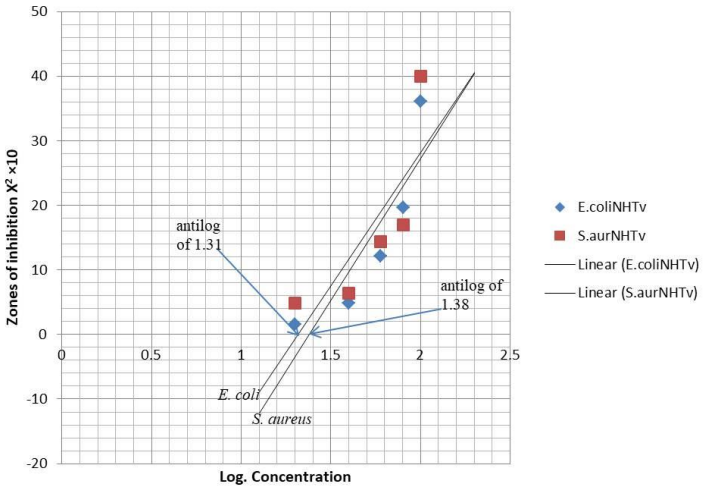

Fig. 10. Graph of MIC of n-hexane fraction of *T. vulgaris* leaf against *S. aureus* and *E. coli*

Table 10: In vitro antibacterial assay of chloroform fraction of *T. vulgaris* leaf

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm) *E.coli S.aureus*		X^2 ×10 *E.coli S.aureus*	
100	2.0000	16	12	25.6	14.4
80	1.9031	12	11	14.4	12.1
60	1.7782	12	8	14.4	6.4
40	1.6021	9	5	8.1	2.5
20	1.3010	5	3	2.5	0.9

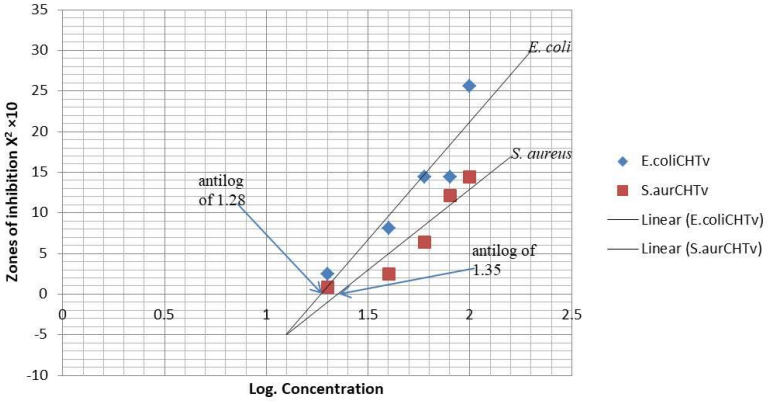

Fig. 11. Graph of MIC of chloroform fraction of *T. vulgaris* leaf against *S. aureus* and *E. coli*

Table 11. In vitro antibacterial assay of ethyl acetate fraction of *T. vulgaris* leaf

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm) *E.coli S.aureus*		X^2 ×10 *E.coli S.aureus*	
100	2.0000	15	13	22.5	16.9
80	1.9031	13	11	16.9	12.1
60	1.7782	11	9	12.1	8.1
40	1.6021	9	7	8.1	4.9
20	1.3010	6	4	3.6	1.6

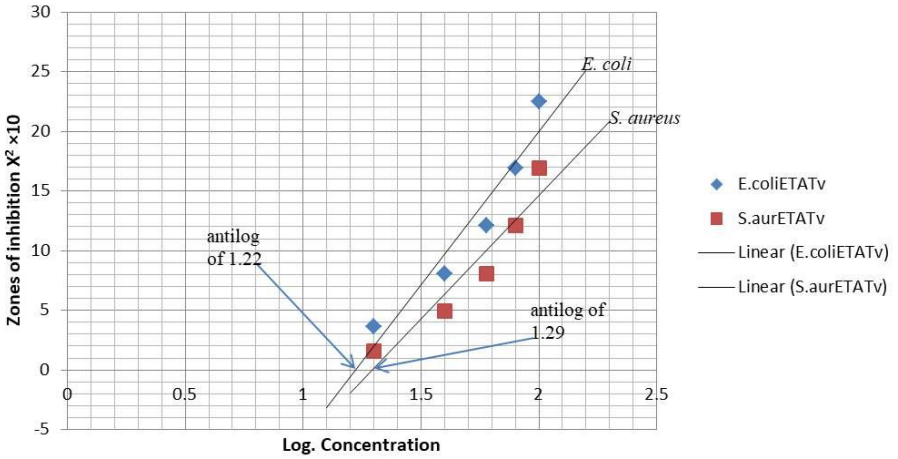

Fig. 12. Graph of MIC of ethyl acetate fraction of *T. vulgaris* leaf against *S. aureus* and *E. coli*

Table 12. In vitro antibacterial assay of aqueous fraction of *T. vulgaris* leaf

Concentration (mg/mL)	Log concentration	Zones of inhibition, x(mm) *E.coli S.aureus*		X^2 ×10 *E.coli S.aureus*	
100	2.0000	18	17	32.4	28.9
80	1.9031	15	15	22.5	22.5
60	1.7782	13	7	16.9	8.1
40	1.6021	9	6	8.1	4.9
20	1.3010	5	4	2.5	0.9

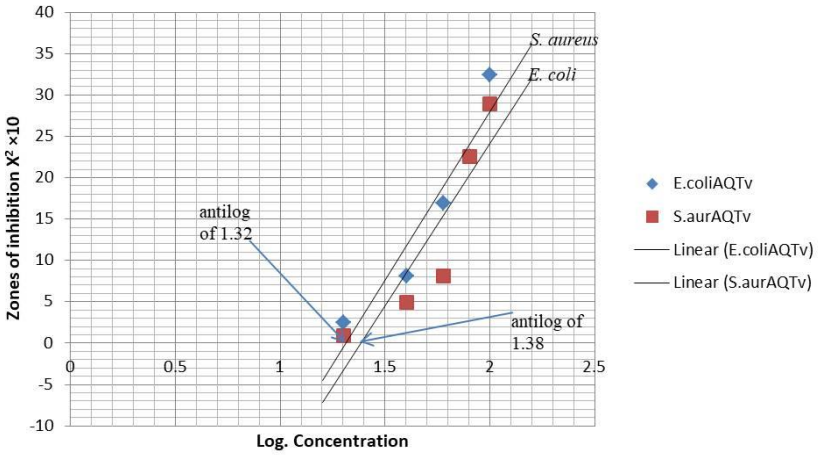

Fig. 13. MIC graph of aqueous fraction of *T. vulgaris* leaf against *S. aureus* and *E. coli*

Table 13. Minimum inhibitory concentrations (MICs) mg/mL

Plants and parts used	Micro organisms	Methanol extract	N-hexane fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
M.fragrans seed	*S.aureus*	17.38	13.18	17.78	17.78	25.12
	E.coli	16.22	23.99	15.85	17.78	13.80
T.vulgaris leaf	*S.aureus*	22.39	24.55	21.88	19.50	19.95
	E.coli	19.50	19.95	18.62	16.60	23.99

4. DISCUSSION

Testing microorganisms for susceptibility to the antimicrobial is a common laboratory procedure that serves as an important tool to chemotherapeutic interventions during cases of infections. The larvicidal property of *Myristica fragrans* seed and *Thymus vulgaris* leaf methanol extracts and fractions was reported by Umoh *et al*. [5] as both plants have potent larvicidal activity, even though that of the *M. fragrans* was more, compared with the nicotine as the positive control and now a comparative antibacterial activity of these plants on gram positive and gram negative bacteria which showed their broad spectrum activities.

The results showed that these plants possess antibacterial activity on the basis of their zones of inhibition. For *M*. *fragrans* on *S. aureus*, methanol extract had a higher activity ranged of 8-19mm with MIC of 13.18mg/mL, while on *E. coli*, it had a lower range of 5-14mm with MIC of 16.22mg/mL. Moreover Ethylacetate fraction had the highest activity on *S. aureus* with the range of 9-25mm and MIC of 17.78mg/mL, followed by Aqueous fraction with the range of 4 -25mm and MIC of 25.12mg/mL and chloroform fraction with the least activity of 6-15mm had MIC of 17.78mg/mL. For *M. fragrans* on *E.coli*, chloroform fraction had the highest activity with the range of 8-18mm and MIC of 15.85mg/mL, followed by aqueous fraction with the range of 8-18mm and MIC of 13.80mg/mL, while n-hexane fraction had the least activity with the range of 3-15mm and MIC of 23.99mg/mL. However, *Thymus vulgaris* methanol extract on *E. coli* had a higher zones of inhibition, ranged from 6-18mm with MIC of 19.50mg/mL compared to that of *S. aureus* of 4-17mm with MIC of 24.39mg/mL. For the fractions, n-hexane fraction had the highest activity on *S. aureus* with the range of 7-20mm and MIC of 24.55mg/mL, while the least was chloroform fraction with 3-12mm and MIC of 21.88. Then on *E. coli*, aqueous fraction showed the highest activity ranging from 5-18mm with MIC of 23.99mg/mL and the least was ethyl acetate fraction with the ranges of 6-15mm and MIC of 16.60mg/mL, using the concentration range of 20-100 mg/mL compared with zones of inhibition of 18mm against *S. aureus* and 28mm against *E. coli* for gentamycin of 2mg/mL which was the reference drug. Sarita *et al*. [11] reported the efficacy of methanol plant extracts on *E. coli* and *S. aureus* using their zones of inhibition. The Minimum Inhibitory Concentrations (MICs) were obtained by extrapolation from the graph using Cooper and Woodman equation [10].

5. CONCLUSION

The results showed potential antibacterial effect of the methanol extracts and fractions of *M. fragrans* seed and *T. vulgaris* leaf against *E. coli* and *S. aureus* but the *M. fragrans* had better activities than *T. vulgaris*. Moreover, same parts of the plants need be studied to evaluate their potential antifungal properties.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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