

Phytochemical Screening and *in vitro* Bacteriostatic Effects of *Syzygium aromaticum* (Clove) Extracts on Clinical Bacterial Isolates

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ABSTRACT

Medicinal plants have been the richest drug bio-resource for traditional systems of modern medicines, folk medicines, nutraceuticals, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs. Among other spices, clove has been widely used for several centuries as medicinal plant due largely to its antimicrobial and antioxidant activities. Various reports confirmed the antifungal, antibacterial, antiviral and anticarcinogenic effects of spice plants. Precisely, clove has attracted a huge attention as a result of the potent antimicrobial and antioxidant effectiveness standing out among other spice plants. In this study, we report the phytochemical screening and antibacterial effectiveness of *Syzygium aromaticum*, commonly known as clove against clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The plant material was extracted using Soxhlet extraction method with ethanol and methanol as the extracting solvents while the antibacterial effectiveness was tested using agar well diffusion method. The phytochemical analyses revealed the presence of various phytochemicals which include flavonoids, glycosides, phenols, saponins, steroids and tannins. The two extracts were found effective against all the test bacteria with largest and smallest zones of growth inhibition produced against *P. aeruginosa* and *E. coli*, respectively at all the concentrations used. In conclusion, different phytochemicals were detected from the ethanolic and methanolic extracts of clove and high effectiveness of the extracts was observed against all the clinical bacterial isolates tested.

INTRODUCTION

Plant-derived substances are increasingly gaining more recognition due to their versatile applications. Medicinal plants have been the richest drug bio-resource for traditional systems of modern medicines, folk medicines, nutraceuticals, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs [1,2]. Spices such as clove, mint, thyme, oregano and cinnamon have been widely used for several centuries as medicinal plants due largely to their antimicrobial and antioxidant activities. Various reports confirmed the antifungal, antibacterial, antiviral and anticarcinogenic effects of spice plants. Precisely, clove has attracted a huge attention as a result of the potent antimicrobial

and antioxidant effectiveness standing out among other spice plants [3,4].

Clove is scientifically known as *Syzygium aromaticum* (*S. aromaticum*) and, it is a median size tree (8-12 m) from the Mirtaceae family native of Maluku islands in east Indonesia. For several years, the trade and search of clove stimulated the economic advancement of this Asiatic region [5]. Several researchers confirmed the antimicrobial activities of clove on various bacterial and fungal organisms. A report by Sofia *et al.*, 2007 [6] showed that aqueous extract of clove at 3% concentration revealed significant bactericidal effects against different food-borne related bacteria including *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*. The clove extract also showed a good inhibitory property at 1% concentration.

Another study showed that clove oil produced the third widest zones of growth inhibition among three (3) spice plants' oils tested against twenty-five (25) isolates of Gram positive and Gram-negative bacteria [7]. The antifungal activity test of clove oil indicates a higher sensitivity pattern against *Mucor* spp. followed by *Microsporium gypseum*, *Fusarium moniliforme* NCIM 1100, *Trichophyton rubrum*, *Aspergillus* spp. and *Fusarium oxysporum* MTCC 284 [8]. A different study conducted on various dermatophytes, *Trichophyton mentagrophytes* KCTC 6077, *Microsporium canis* KCTC 6591, *Epidermophyton floccosum* KCCM 11667, *Trichophyton rubrum* KCCM 60443 and *Microsporium gypseum* showed an effectiveness of up to 60% at a concentration of 0.2 mg/mL [9]. Although the antimicrobial activity of *S. aromaticum* has been reported by several researchers around the globe, the phytochemical analysis and antibacterial effectiveness of clove extracts derived from ethanol and methanol against the test clinical bacterial isolates is reported from the study area for the first time to the best of our knowledge.

MATERIALS AND METHODS

Collection and identification of plant material

Cloves were purchased from Gombe main market, Gombe State, Nigeria. Identification of the plant was carried out at the herbarium section of Biological Sciences department of Gombe State University and obtained a voucher number, 072.

Preparation of crude plant material

The cloves were dried in shade for about two (2) weeks and then crushed into powder using laboratory mortar and pestle.

Preparation of soxhlet extraction

Ethanol and methanolic extracts of the cloves were prepared by weighing 20 g of the clove powder and partitioned into different places on filter papers. The filter papers were folded and placed into the sample space of the soxhlet extractor. Aliquot of 200 ml each of 70% ethanol and 80% methanol was then put separately into the solvent flask of the soxhlet extractor. The hot plate and condenser attached to the extractor were switched on to begin the extraction. The solvents (ethanol and methanol) were evaporated to recover the extract using a rotary evaporator.

Phytochemical Screening

The plant extracts were evaluated for the presence of various phytochemicals using simple qualitative methods described by Harboune (1973) [10] as demonstrated below:

Test for phenols

Two drops of ferric chloride solution were added to 2 ml of ethanolic or methanolic extract and observed for the presence of phenols.

Test for Tannins

Two drops of ferric chloride were added to 2 ml of ethanolic or methanolic extract and observed for the presence of tannins.

Test for saponins

About 1 g of the extracts was dissolved in distilled water, vigorously shaken and observed for the presence of saponins.

Test for Steroids

About 2-3 drops of chloroform were added into 2 ml of the extracts followed by the addition of concentrated sulphuric acid (H₂SO₄) and observed for the presence of steroids.

Test for glycosides

About 2 ml of the extract was boiled, followed by the addition of a few drops of concentrated hydrochloric acid (HCl). The mixture was boiled again for a few minutes to hydrolyze any glycoside present. The mixture was changed to alkaline by the addition of a few drops of aqueous ammonia solution. Five drops of the mixture were added to 2 ml of Benedict's reagent, boiled and observed for the presence of glycosides.

Test for alkaloids

About 2-4 drops of Dragendroff's reagent were added to 5 ml of ethanolic or methanolic extract. A change of colour confirmed the presence of alkaloids.

Test for flavonoids

A few drops of concentrated sodium hydroxide (NaOH) were added to 2 ml of the extracts, followed by the addition of a few drops of dilute hydrochloric acid and observed for the presence of flavonoids.

Preparation of stock concentrations of extracts

A stock concentration of 200 mg/ml of each extract was prepared by dissolving 0.4 g of the extract in 2 ml of dimethyl sulfoxide (DMSO). Subsequently, 100, 50, 25 and 12.5 mg/ml concentrations were prepared from the stock using 2-fold serial dilution.

Clinical Bacterial Isolates

The clinical bacterial isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were obtained from the culture collection of the Microbiology laboratory, department of Microbiology, Gombe State University. The isolates were originally obtained from the Microbiology Laboratory of Federal Teaching Hospital Gombe, Gombe State Nigeria.

Standardization of Inocula

The inocula were standardized by dissolving 1-2 colonies of the test bacteria using a sterile wire loop from an overnight culture plates and dissolved in 10 ml of normal saline solutions contained in test tubes. The tubes were vortex mixed for homogeneity and compared with 0.5 McFarland standards (approximate cell count density: 1.5×10^8).

Preparation of culture media

All culture media were prepared according to manufacturers' instruction and dispensed into petri dishes before use.

Screening for *in vitro* antibacterial effectiveness of clove

Sterile swab sticks were immersed into the standardized inocula contained in test tubes. Excess fluids were drained by pressing the sticks at the walls of the tubes and swabbed onto prepared Mueller Hinton Agar (MHA) plates. Four wells of approximately 6 mm diameter were cut in each plate using a sterile cork borer. Aliquots of 100 µl of various concentrations of the extracts were added into the wells using a micropipette. The plates were allowed to stand at room temperature for one hour for the extracts to diffuse into the agar and incubated for 24 hours at 37 °C. Antimicrobial activity was determined by measuring the diameter of inhibition zones in millimeter produced against the test bacterial isolates. The experiments were performed in replicates and the mean values were noted.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentrations (MICs) of the extracts were determined from the lowest concentration that

showed activity on the plate. This was performed by preparing four different concentrations, 12.5, 6.25, 3.13, and 1.56 mg/ml of each extract in 2 ml of nutrient broth contained in test tubes. The tubes were inoculated with the bacterial isolates and incubated for 24 hours at 37 °C. In each experiment, two control tubes were prepared alongside by mixing the extracts and growth medium without the standardized inocula in test tubes (positive control) and tube containing the growth medium and the inocula (organism control).

Determination of Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) was determined by sub-culturing all tubes that showed no visible bacterial growth from the MIC on fresh solid media and incubated for 24 hours at 37 °C.

Statistical Analysis

Data analysis was performed using one-way ANOVA of variance [11].

RESULTS (OR RESULTS AND DISCUSSION)

Syzigium aromaticum extraction yields

Table 1 shows the overall physical properties of *Syzigium aromaticum* (Clove) extracts obtained after 20 g powder of the dried plant material has been soaked with 200 ml each of methanol and ethanol, which yielded extract residues of 8 and 3 g, respectively. Both the extracts possessed the same texture (sticky) but different colours as dark brown and golden brown for methanolic extract and ethanolic extract, respectively.

Table 1. Physical properties of the extracts

Type of Extract	Colour	Texture	Weight
methanolic extract	dark brown	sticky	8 G
ethanolic extract	golden brown	sticky	3 G

Qualitative phytochemical analyses of *S. aromaticum* extracts

Phytochemical analyses of *Syzigium aromaticum* extracts revealed the presence of various phytochemicals including tannins, saponins, glycosides, flavonoids, phenols, steroids but there was no evidence of alkaloids detection (**Table 2**). The presence of phenol was confirmed by the production of black coloration after the addition of ferric chloride, whereas the formation of deep green colour after the addition of ferric chloride indicates the presence of tannins. The production of persistent foam after shaking the extract in distilled water confirmed the presence of saponin.

The existence of steroids in the extracts was confirmed by the production of reddish colour at the bottom of a test-tube following the addition of chloroform and concentrated H₂SO₄ while the production of reddish-brown precipitate after heating the extract with the addition of concentrated HCl and benedict's solution shows the presence of glycosides. Flavonoids were detected by the production of intense yellow colour following the addition of concentrated NaOH which disappeared after the addition of dilute HCl. However, there was no evidence of alkaloids detection from both the extracts.

Table 2. Phytochemicals detected from the ethanolic and methanolic extracts.

Phytochemicals	ME	EE
alkaloids	-	-
flavonoids	+	+
Glycosides	+	+
Phenols	+	+
Saponins	+	+
Steroids	+	+
Tannins	+	+

Key: ME: Methanolic extract, EE: Ethanolic extract, +: Present, -: Not detected

Antibacterial effects of *S. aromaticum* extracts

The antibacterial effects of methanolic and ethanolic extracts of clove were investigated against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* using agar well diffusion method. It was observed that, the two extracts showed a remarkable *in vitro* effectiveness against the test bacterial isolates at various concentrations (100, 50, 25 and 12.5 mg/ml) (**Tables 3 and 4**). However, Methanolic extract was found to be more effective compared to the ethanolic extract against *P. aeruginosa*, followed by *K. pneumoniae* and then *E. coli* based on the zones diameters of growth inhibition recorded in Tables 3 and 4 as demonstrated in **Fig. 1**. Moreover, the test bacterial isolates showed significant difference in terms of their sensitivity to both extracts as analysed statistically with P<0.05.

Table 3. Antibacterial effect of *S. aromaticum* ethanolic extract.

Organisms	Concentrations			
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
<i>E. coli</i>	15±0.04	13±0.16	10±0.05	8±0.22
<i>P. aeruginosa</i>	22±0.07	19±0.13	16±0.17	13±0.44
<i>K. pneumoniae</i>	20±0.08	16±0.18	13±0.33	10±0.08

The results were from three different experiments and obtained the means ± S.D. Significant difference was observed among the isolates' sensitivity to the ethanolic extract with P<0.05.

Table 4. Antibacterial effect of *S. aromaticum* methanolic extract

Organisms	Concentrations			
	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
<i>E. coli</i>	21±0.10	17±0.16	13±0.85	11±0.10
<i>P. aeruginosa</i>	23±0.30	20±0.23	18±0.43	17±0.23
<i>K. pneumoniae</i>	22±0.40	19±0.50	14±0.07	12±0.40

The results were from three different experiments and obtained the means ± S.D. Significant difference was observed among the isolates' sensitivity to the ethanolic extract with P<0.05.

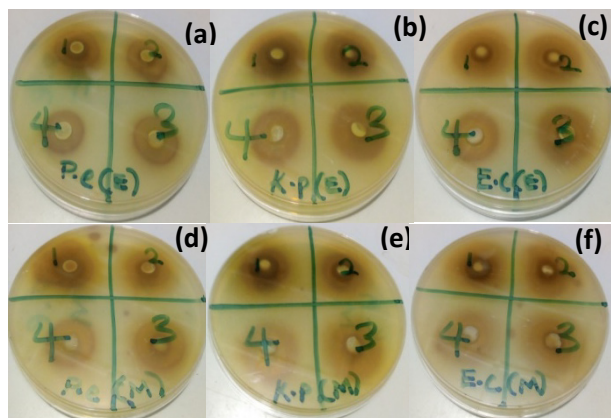


Fig. 1. Bacteriostatic effects of *Syzigium aromaticum* against the test bacteria. The test isolates were swabbed onto Mueller Hinton agar plates and bored 6 mm wells using sterile cork borer. Crude extracts of the plant materials were poured into the respective wells and incubated for 24 hours at 37 °C. a=*P. aeruginosa*, b=*K. pneumoniae*, c=*E. coli* (Tested against ethanolic extract of clove), d= *P. aeruginosa*, e=*K. pneumoniae*, f= *E. coli* (tested against methanolic extract of clove).

Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

Table 5 shows the MIC and MBC values of the extracts determined against the test bacteria using broth dilution method. The MIC was determined as the lowest concentrations of the extracts that inhibited any visible growth when compared with the control tubes. The MIC values of *S. aromaticum* methanolic extract against the test bacteria were recorded as 1.56 mg/ml each for *P. aeruginosa* and *K. pneumoniae* and 3.12 mg/ml for *E. coli* whereas those of ethanolic extract were recorded as 1.56, 3.12 and 6.25 mg/ml for *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively.

The MBC was confirmed by the absence of growth of the test bacteria sub-cultured on agar plates from the tubes not having turbidity corresponding to the suspected MIC. Both the extracts showed potential bactericidal activity against the test bacterial isolates. Methanolic extract had potential MBC value of 3.12 mg/ml for *P. aeruginosa* and *K. pneumoniae*, and 6.25 mg/ml for *E. coli* whereas the ethanolic extract had MBC values of 3.12, 6.25 and 12.6 mg/ml for *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively.

Table 5. MIC and MBC of *S. aromaticum* extracts against the bacteria.

Organisms	Methanolic		Ethanolic extract (mg/ml)	
	Mic	Mbc	Mic	Mbc
<i>P. aeruginosa</i>	3.12	6.25	6.25	12.60
<i>K. Pneumoniae</i>	1.56	3.12	3.12	6.25

Drugs (antibiotics) resistance has significantly increased among pathogens perhaps because of versatile microbial genetic system exposed to the pressure of numerous control agent(s) [12, 13]. The search for novel antimicrobials or new natural alternatives such as plant extracts has received a remarkable interest over the years [14,15]. This has been guided by the success of traditional medicine which is thought to pave ways for new chemotherapeutics to eliminate infections caused by drug-resistant microbes and to alleviate the harmful effects of antibiotics [14].

Clove has proven antimicrobial activities against several organisms such as bacteria and fungi [3]. Many researchers have reported the antimicrobial effectiveness of different spice plants such as cinnamon, mint, mustard, ginger, garlic and clove [3, 6, 16].

According to World Health Organization (WHO), about 80% of the world's population relies on plant extract or their active constituents as folk medicine in traditional therapies [17]. In this study, both ethanol and methanol extracts obtained from cloves were found to exhibit high effectiveness against the three clinical bacterial isolates tested as shown in Tables 3 and 4. Methanolic extract appeared to be more active over the ethanolic extract in virtually all the concentrations used against the test bacteria. The results obtained suggest that *E. coli* and *P. aeruginosa* were the most resistant and sensitive organisms, respectively to the two extracts of cloves, which is comparable to the report of Ashraf *et al.*, (2018) [18]. However, the report of Pundir *et al.*, 2010 [19] confirmed *E. coli* as the most sensitive organism to the ethanolic extract of clove. In general, the results of this work corroborate with the reports of several researchers conducted on similar organisms [17, 20, 21, 22], aqueous infusion, decoction and essential oil of clove [23, 24].

The antibacterial and antifungal properties of cloves could be attributed to its high content of Eugenol [25] and other phytochemicals whose presence was qualitatively detected in this study. The eugenol is described as an antimicrobial compound with a wide range of antimicrobial effects [26, 27]. Phytochemical constituents with phenolic structure like eugenol are highly effective against several tests microorganisms. Additionally, the representatives of this class are recognised as either bacteriostatic or bactericidal agents, subject to the concentration used.

CONCLUSION

Cloves ethanolic and methanolic extracts revealed the presence of different phytochemicals and the extracts showed high effectiveness against *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *E. coli* and *P. aeruginosa* were found to be the most resistant and sensitive bacteria, respectively to the extracts

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