Phytochemical Profile and Antibacterial Activity of Clove (*Syzgium aromaticum*) Methanol Extract Against some Food-borne Pathogens

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Abstract: The Clove (*Syzygium aromaticum)* is an ideal substitute of chemical food preservatives in addition to its pharmacological advantages. The study was aimed to investigate the phytochemical constituents and antibacterial activity of Clove (*S. aromaticum*) methanol extracts against some food-borne pathogens. Methanol extracts from *S. aromaticum* buds were prepared, screened for phytochemical constituents and tested for antibacterial activity against four bacterial foodborne pathogens namely *Enterobacter spp, Salmonella spp, Escherichia coli, and S. aureus*. Phytochemical screening of the methanol extract of *S. aromatic* revealed the presence of Alkaloids, saponins, tannins, Carbohydrates, glycosides, flavonoids, Anthraquinones and terpenoids. The Liquid Chromatography Mass Spectrometer (LCMS) analysis revealed the presence of important metabolites (Neoglucobiase, Chlorogenic acid, Nomilin, Ergosterol and Quassin). Based on the susceptibility of the organisms to the extracts, there was significant difference on the susceptibility of the organisms against the extracts at $p< 0.05$. *Enterobacter spp* was found to be the most susceptible organism with an average zone of 22.50±0.41mm, followed by *staphylococcus aureus* (17.00±0.82 mm), *E. coli* (8.50±0.41mm), while *salmonella spp* (6.00±0.00mm) was resistant. The MIC and MBC values of the extracts ranges from 62.5 to 250 µg/ml. These identified metabolites could be responsible for the antibacterial potential observed. Therefore in addition to its role as food additives, Clove (*S. aromaticum*) can be very useful in drug management against many food-borne pathogens.

Key word: Antibacterial, Clove, Food-borne pathogens, Phytochemicals, Profile

INTRODUCTION

The existence of food-borne illnesses, food spoilage, food wastage, and the resulting negative economic impact of these issues have all pushed the food industries to finding alternative, safe, and natural antimicrobial to use in foods and beverages. The Centers for Disease Control and Prevention (CDC) (2018), estimated that each year in the United States over 48 million people fall ill from food-borne diseases, resulting in approximately 130,000 hospitalizations and 3,000 deaths. In the most recent annual surveillance report published by the CDC (2015), it was stated that the bacterial pathogens responsible for the most outbreak-related illnesses and hospitalizations were *Salmonella* and *Shigella* and toxin producing *Escherichia coli* (Negi, 2012).

Spices have been defined as plant substances from the indigenous or exotic origin, aromatic, with strong taste, used to enhance the taste of foods (Arora *et al*., 1999). Spices possess antimicrobial activities due to the presence of essential oil, alkaloids, glycosides etc. The presence of these bioactive substances is responsible for antimicrobial properties. Some of these spices are also known to contribute to the self defence of plants against infectious organisms (Kim *et al.*, 2003). Clove (*S. aromaticum L.*) is an aromatic spice, belongs to the family *Myrtaceae*. Cloves are used in Ayurveda, Chinese medicine and Western herbalism. It has been shown that some components of clove are useful in bacterial and fungal infections (Zhang *et al*., 1997). The use of spices as colorants and preservatives is becoming fashion in the food industry, and among these spices, *S. aromaticum* is of great importance (Elizabeth *et al.*, 2017). Compared with sodium benzoate, potassium sorbate and other chemical food preservatives, clove oil, the main volatile constituent of *S. aromaticum*, poses various advantages in antimicrobial activity, aroma, and safety, and is an ideal substitute of chemical food preservatives (Elizabeth *et al.*, 2017).

The chemical constituents of clove includes eugenol (Daniel, 2009), acetyl eugenol (5.62%), beta –caryophyllene (1.38%) (Jirovetz, 2006) and vanillin, crategolic acid, tannin such as bicornin (Kamatou *et al*., 2012) gallotannic acid, methyl salicylate (pain killer), the flavonoids eugenin, kaempferol, rhamnetin, and eugentin, triterpenoids such as oleanolic acid, stigmasterol, and campesterol and several sesquiterpenes, rhamnetin and vitamins.

Microorganisms like *Alternaria sp., Aspergillus sp., Canninghamella sp., Lactobacillus sp. Fusarium sp., Clostridium sp; Mucor sp., Salmonella* sp. *Penicillium* sp. *Bacillus* sp. could be retarded by using clove extract (Soliman and Badeaa, 2002). Cloves possess anti-helmenthic, anti- inflammatory ((Kim *et al*., 2003) anti-pyretic, anti-allergic (Feng *et al*., 1987), anti-fungal (Gayoso, 2005**),** anti-carcinogenic, (Abdalrahim *et al*., 2012) anti-allergic (Kim *et al*., 1998), anti-viral (Reichling *et al*., 2009) anti-mutagenic activity (Miyazawa *et al*., 2001), antioxidant insecticidal (Park *et al*., 2000) anti-spamodic, anti-arthritis (Kaur and Sultana, 2012) anti-parasitic properties, to increase hydrochloric acid in the stomach and to improve peristalsis and an anaesthetic. By tradition, it has been used in food preservation as flavoring and antimicrobial substance (Velluti *et al.,* 2003). It has a very major role in spice trade and is highly appreciated for their therapeutic properties.

The prevalence of food borne diseases in Nigeria is alarming despite efforts by government and non-governmental organizations to prevent food borne diseases. The World Health Organization (W.H.O) estimated that more than 200,000 people died of food borne diseases annually in Nigeria, which is as a result of ingestion of food borne pathogens (especially *E.coli* and *Salmonella spp*). Plant extracts and essential oils have the ability to reduce the growth of pathogenic microorganism and extend the shelf life of the food. Therefore, this study is aimed to evaluate the antibacterial activities of clove methanol extract on food borne pathogens and identify the possible compounds responsible for the activity using LCMS.

MATERIALS AND METHODS

Collection and Preparation of Plant Material

The Clove was purchased from Kabuga market, Kano state and was taken to the department of Plant Biology, Bayero University Kano for identification (BUKHAN 342). The clove was sorted and washed with water in order to remove soil debris from it, and then dried under shade at room temperature. After drying, it was pulverized to powder with mortar and pestle, sieved using fine mesh size and very fine powder was obtained (Sukhdev *et al.,* 2008).

Screening of Food borne pathogens

The test isolates including *E. coli, Salmonella spp, Staphylococcus aureus and Enterobacter spp* were obtained from the Department of Microbiology Bayero university kano and were further confirmed using standard biochemical tests (indole, coagulase, oxidase, citrate, triple sugar iron test, urease and vogesproskauer) as described by Cheesbrough (2006).

Extraction of plant materials by maceration method

The method described by Sofowora, (2000) with slight modifications was adopted. One liter of 80 % methanol extraction fluid was mixed with 200 g of powdered clove buds material. The mixture was kept for 2 days in tightly sealed vessels at room temperature and stirred several times daily with a sterile glass rod. This mixture was filtered through muslin cloth. Further extraction of the residue was repeated 3 times until a clear colorless supernatant extraction liquid was obtained indicating that no more extraction from the plant material was possible. The extracted liquid was subjected to water bath at 40 ℃ and the solvent was evaporated. The extract was weighed and portion of it was used for phytochemical screening while the rest was used for the susceptibility test.

Sterility of the extracts

After filtration and evaporation, the extracts were tested for sterility by introducing 1 ml of the extract into 15 ml of sterile nutrient

broth and incubated at 37 ^{0}C for 24 hours.
A sterile extract was indicated by the absence of turbidity or clearance of the broth after the incubation period (Hamilton 2006).

Phytochemical screening of clove extract

The phytochemicals screening was conducted using standard methods:

Test for saponins

Two grams (2 g) of the clove extract was boiled in 20 ml of water in a water bath and filtered. A total of 5 ml of the filtrate was mixed with 3 ml distilled water in a test tube and shaken vigorously. Frothing, which persisted on warming, was considered preliminary evidence for the presence of saponins. A few drops of olive oil were added to the extract and shaken vigorously. The present of saponin was indicated by formation of soluble emulsion (Ngbede *et al.*, 2008).

Test for Carbohydrates (Molisch test)

To a small portion of the extract, distilled water was added and mixed with a few drops of Molisch reagent. 1ml of concentrated sulphuric acid (H_2SO_4) was carefully added down the side of the inclined tube and observed (Parekh and chanda, 2007).

Test for tannins

The clove extract was treated with 15% ferric chloride test solution. A blue color in the mixtures signified the presence of hydrolyzable tannin. For confirmation, 0.5 g of the extract was added to 10 ml of freshly prepared potassium hydroxide (KOH) in a beaker, and shaken to dissolve. The present of tannin was indicated by the appearance of dirty precipitate (Parekh and chanda, 2007).

Test for flavonoids

Three methods were used to determine the presence of flavonoids in the extract sample. A total of 5 ml of 10% ammonia solution was added to a portion of the aqueous filtrate from the extract followed by addition of concentrated H_2S0_4. A yellow coloration observed in each extract was used to validate the presence of flavonoids. A few drops of 1% aluminium solution were added in two ml of each filtrate. A yellow coloration was investigated for the presence of flavonoids. Five milliliters of 20% NaOH was added to an equal volume of the water extract. If a yellow solution was obtained, it was also investigated for the presence of flavonoids (Parekh and chanda, 2007).

Test for alkaloids:

The presence of alkaloids was determined as described by Parekh and chanda, (2007): A total of 0.5 g each of the extract was mixed with methanol containing 1% HCl, and then boiled and filtered. A total of 2 ml of 10% ammonia and 5 ml of chloroform was added to 5 ml of the filtrates and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 2 ml of acetic acid, and Mayer's reagent was added. The formation of cream (with Mayer's reagent) or presence of turbidity was regarded as the presence of alkaloids.

Test for cardiac glycosides (Keller-Killani test)

A total of 2 ml of the extract solution was treated with 2 ml glacial acetic acid containing one drop of ferric chloride solution. This was under laid with 1 ml of concentrated sulfuric acid. The formation of a brown ring of the interface was indicative of the presence of a deoxysugar of cardenolides (Parekh and chanda, 2007).

Test for anthraquinone (Borntrger's test)

About 0.5 g of the clove extract was placed into a dry test tube and 5 ml of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate was shaken with an equal volume of 10% ammonia solution. A pink violet or red color in the ammonia layer indicated the presence of anthraquinones (Parekh and chanda, 2007).

Test for terpenoids (Salkowski test)

A total of 5 ml of each extract solution was mixed in 2 ml of chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to separate the 2 layers distinctly. A reddish brown coloration of the interface was formed to confirm the presence of terpenoids (Parekh and chanda, 2007).

Liquid Chromatography Mass Spectrometer Analysis

The clove extracts were analyzed via liquid chromatography (LC) and mass spectrometer (MS) using LC Waters e2695 separation module with W2998 PDA and couple to ACQ-QDA MS as described by (Piovesana *et al*., 2018) with some modifications. The extracted samples were reconstituted in methanol and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. After filtration, the filtrate (10.0 µl) was injected into the LC system and allowed to separate on Sunfire C18 5.0µm 4.6mm x 150 mm column. The run was carried out at a flow rate of 1.0 mL/min, Sample and Column temperature at 25ºC. The mobile phase consists of 0.1% formic acid in water (solvent A) and 0.1% formic acid in Acetonitrile (solvent B) with a gradient (table 1).

Table 1: Percentage gradient of Solvent used in lcms analysis

Time	%A (0.1% formic acid in water)	%B (0.1% formic acid in Acetonitrile)
0	95	5
1	95	5
13	5	95
15	5	95
17	95	5
19	95	5
20	95	5

From ratio of A/B 95:5 this ratio was maintained for further 1 min, then A/B 5:95 for 13min, to 15min. then A/B 95:5 to 17min, 19min and finally 20min. The PDA detector was set at 210-400nm with resolution of 1.2nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 – 1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8kv (positive) and 0.8kv (negative); probe temperature 600oC; flow rate 10 mL/min; nebulizer gas, 45 psi. MS set in automatic mode applying fragmentation voltage of 125 V. The data was processed with Empower 3. The compounds were identified on the basis of fragmentation pattern, and compared with database as described by Hanafi *et al.* (2018).

Preparation of extract concentration

Stock solution of concentration 2000 µg/ml was prepared by dissolving 2g of the clove extract in 1 ml of dimetyhyl sulphoxide (DMSO) in glass vial bottles. This was then double-diluted to have varied concentrations (2000 µg/ml, 1000 µg/ml, 500 µg/ml and 250 µg/ml) (Kalpana *et al*., 2013).

Preparation of turbidity standard

The turbid solution was prepared as reported by Chessbrough, (2005), and transferred into a test tube as the standard for comparison.

Standardization of Inoculum

Using inoculation loop (wire loop) a loopful of overnight culture of the test organism was transferred into a test tube containing 2 ml of normal saline. This tube containing the suspension for the test organism was compared against another tube containing 0.5 Mcfarland standard. Normal saline was added until the turbidity of the suspension matches that of Mcfarland as described by National Committee for Clinical Laboratory standard (NCCLS, 2008).

Antibacterial assay

The antibacterial activities of clove extract was determined by agar well diffusion method (Okeke *et al*., 2001). Pure culture of each bacterium was first sub-cultured in nutrient broth at 37 ºC for 24h. Standardized inoculum (10^6CFU/ml; 0.5 McFarland) of each test bacterium was spread onto a sterile Muller-Hinton Agar plate (Hi Media) so as to achieve a confluent growth. The plates were allowed to dry and a sterile cork borer of 6.0 mm diameter was used to bore wells in the agar plates. Subsequently, 0.1 ml of the extract was introduced in duplicate wells into Muller-Hinton Agar plate. Sterile DMSO served as negative control. Sodium benzoic acid (standard food preservative) served as a positive control. The plates were allowed to stand for at least 1h for diffusion to take place and then incubated at 37 ºC for 24h. The zone of inhibition was recorded to the nearest size in millimeter.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibited visible growth of microorganisms after overnight incubation (Andrews, 2002). The doubling micro dilution broth method was used to determine the MIC. Two millilitres (2 ml) of the reconstituted crude extract at a concentration of 1000 µg/ml was added to 2ml of sterile Mueller Hinton broth for the bacterial isolates, Two (2ml) millilitres of this extract concentration was transferred serially into test tubes numbered 1-9 until the 10th test-tube was reached, giving extract concentrations ranging from 1000-65.2 µg/ml. 0.1ml of an 18h culture of bacteria previously adjusted to 0.5 McFarland standard was inoculated into each of the test tubes and the contents were thoroughly mixed. A test tube containing the broth and bacterial inoculum was used as negative control. The inoculated culture tubes were incubated at 37 ºC and observed for growth after 24 hours. The lowest concentration of extract showing no visible growth when compared with the control was considered as the MIC as demonstrated by Andrews (2002).

Determination of minimum bactericidal concentration

This is the lowest concentration of antimicrobial agent that prevented the growth of an organism. About 0.1ml aliquot from the tubes that showed no visible bacterial growth from the determination of minimum inhibitory concentration was inoculated on a sterile Mueller Hinton Agar for 24 hours at 37 ºC for the bacterial isolate. The lowest concentration in which no growth occurred was taken as the minimum bactericidal concentration (MBC) (Andrews, 2002).

RESULTS

The phytochemical screening of *S. aromaticum was* presented in table 2. The results showed the presence of alkaloid, carbohydrate, glycoside, steroid, saponin, tannin, terpenoid, flavonoid and Anthraquinone in the methanol extract.

Table 2: Phytochemical constituents of clove methanol extracts

Constituent	Present/absent
Saponin	+
Carbohydrate	+
Tanin	+
Flavonoid	+
Alkaloid	+
Glycoside	+
Anthraquinone	+
Terpenoid	+

Key: + = present, - = absent

Liquid Chromatography mass spectrophotometry (LCMS) profile of methanol clove extract was presented in table 3, it shows that some useful metabolite were detected and their mass to charge rations (M/Z) also reported, however, their tentative compound names were provided, the total ion chromatogram and the molecular fragmentation pattern of the identified compounds also presented in figs 1-5.

Table 3: Liquid chromatography- mass spectrophotometry profile of Methanol Clove Extract

Peak	Retention Time (RT) min	Tentative compound	Molecular mass	MZ(M+H)
1	0.639	Ergosterol	396	397
2	1.981	Chlorogenic acid	354	355
3	7.587	Nomilin (Limonoids)	514	515
4	12.068	Quassin (Quassinoids)	388	389

Key:MZ – Mass to charge ratio, **M-**Molecular mass

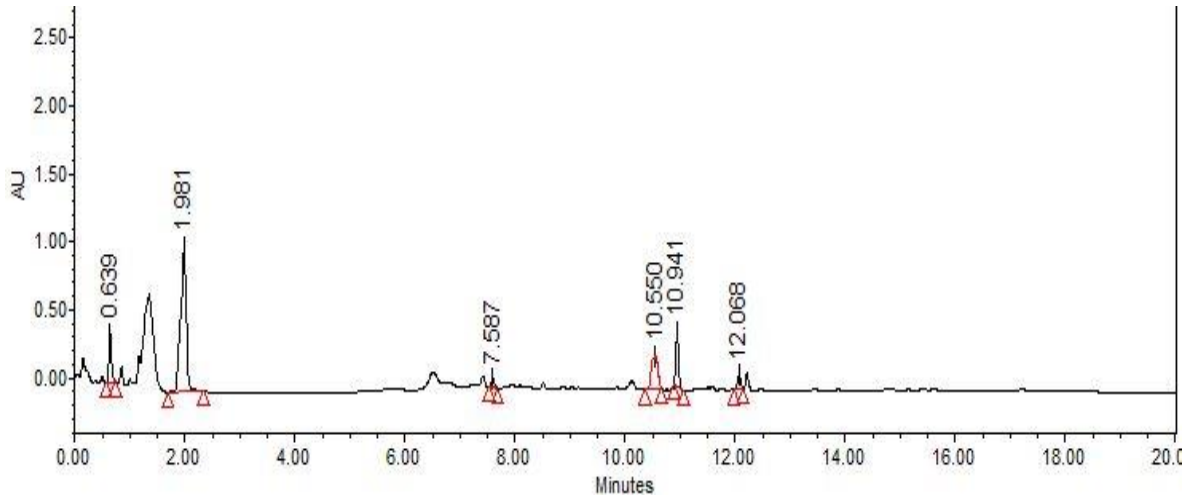

Fig. 1. Total ion chromatogram of the methanol fraction of Clove (*Syzgium aromaticum*)

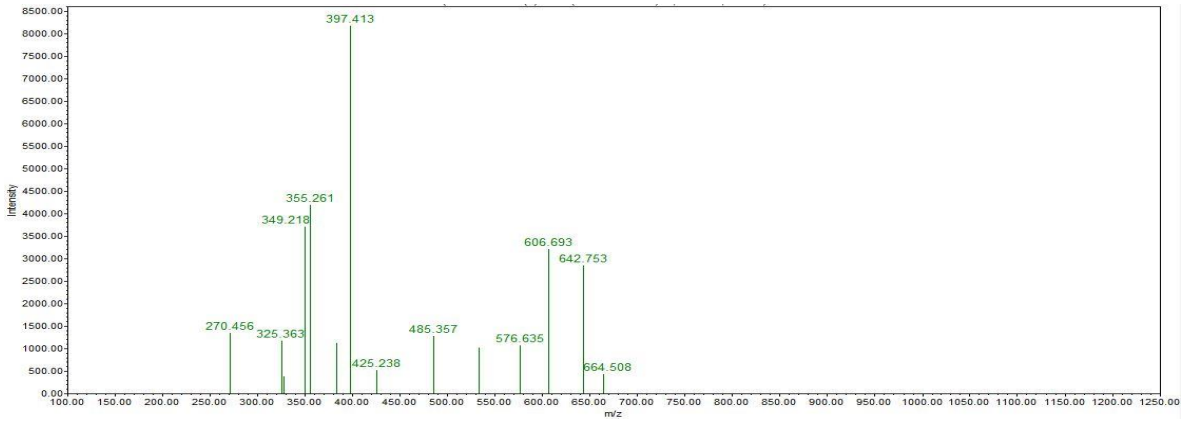

Figure 2: Molecular fragmentation of ergosterol mz 397 (M+H)

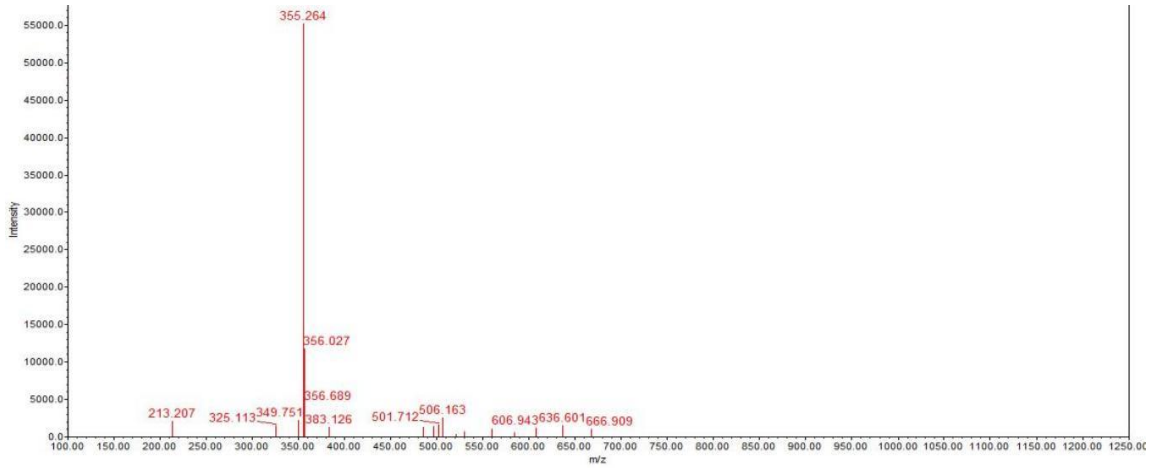

Figure 3: Molecular fragmentation of chlorogenic acid mz 355 (M+H)

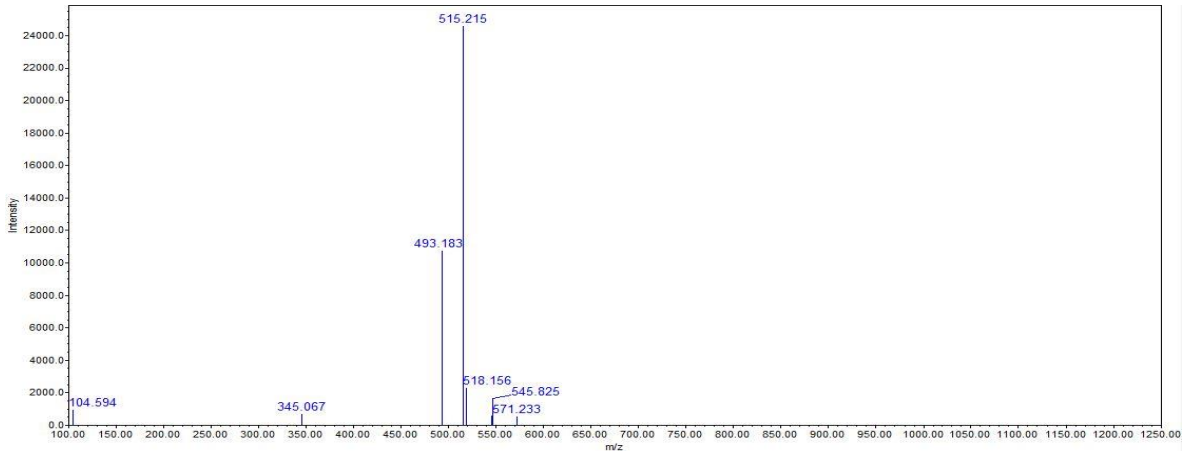

Figure 4: Molecular fragmentation of nomilin (Limonoids) mz 515 (M+H)

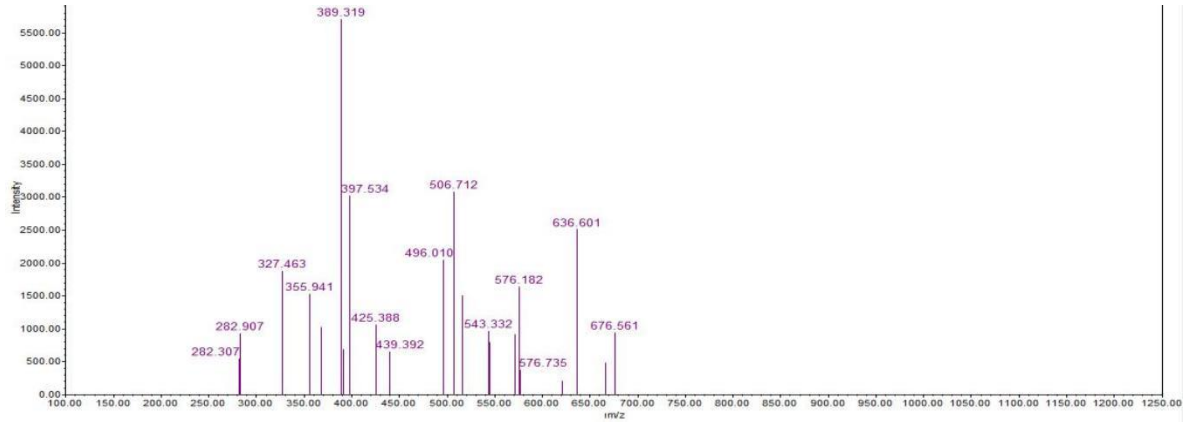

Figure 5: Molecular fragmentation of quassin (Quassinoids) mz 389 (M+H)

Antibacterial activity of clove extract on the food isolates was presented in table 4, where the results revealed highest antibacterial activity on *Enterobacter spp* with a diameter of inhibition zone of 22.5mm±0.41 at 2000µg/ml while the least inhibitory activity was recorded against *E. coli* (8.50±0.41 mm). *Salmonella spp* was resistant to the extract.

Table 4: Antibacterial activity of methanolic clove extract on some food borne pathogens

Food borne pathogens	Diameter of Inhibition Zone(mm) Concentration (µg/ml)				
	2000	**1000**	**500**	**250**	**C 250**
Staphylococus aureus	17.00±0.82	16.0±0.82	14.50±0.41	13.00±0.82	8.50±0.41
Enterobacter spp	22.50±0.41	20.50±0.41	15.50±0.41	13.50±0.41	9.50±0.41
E.coli	8.50±0.41	7.50±0.41	6.00±0.00	6.00±0.00	9.50±0.41
Salmonella spp	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	9.50±0.41

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of clove extract against foodborne pathogens was presented in table 5. The result shows that methanol extract had an MIC value of 125 µg/ml on both *Staphylococcus aureus* and *E.coli*. While the MIC value for *Enterobacter spp* was 250 µg/ml. The MBC value of the extract against *E.coli* was 250 µg/ml while that of both *Staphylococcus aureus and Enterobacter spp* were >2000 µg/ml.

Table 5: Minimum Inhibitory and minimum bactericidal Concentrations (MIC and MBC) of clove extract against some food borne pathogens (µg/ml)

S/N	Pathogens	MIC	MBC
1.	*E.coli*	125	250
2.	*Enterobacter spp*	250	>2000
3.	*Staphylococus aureus*	125	>2000
4.	*Salmonella spp*	>2000	>2000

Key: MIC=Minimum inhibition concentration, MBC=Minimum bactericidal concentration

DISCUSSION

The results of the present study revealed that several phytochemicals are present in *S. aromaticum* methanol extracts. All the phytochemicals (alkaloid, carbohydrate, glycoside, steroid, saponin, tannin, terpenoid, flavonoid and anthraquinone) screened were present, this could be as a results of high polarity of the solvent used compared to non-polar solvents. Phytochemicals give plants their colour, flavour, smell and are part of a plant`s natural defense system protecting them against herbivorous insects, vertebrates, fungi, pathogens, and parasites (Ibrahim *et al.,* 2010). The results are in accordance with the findings of other authors who have studied this plant (Jirovetz *et al.,* 2006). However, many literature had reported the potentiality of alkaloids as cancer chemotherapeutic agents, Central Nervous system stimulants, topical anesthetics in ophthalmology, powerful pain relievers, anti-puretic action, among other uses (Madziga *et al.,* 2010; Neeta *et al.,* 2015), this is majorly due to large group of nitrogenous compounds. Additionally it play some metabolic roles and control development in living system (Edeoga *et al.,* 2006). The presence of flavonoids indicates that *S. aromaticum* will be good for the management of cardiovascular diseases and oxidative stress because flavonoids and phenols are biological antioxidants. Anthraquinone derivatives have been reported to possess antibacterial, antiviral, antifungal and other biological activities (Neeta *et al.,* 2015). In other hand, Aboaba *et al*. (2011) reported that the presence of these phytochemicals which possess antimicrobial potential can be useful in preventing food spoilage and enhance food safety.. The profile of the bioactive compounds of a plant indicates its medicinal value. Antioxidant and antimicrobial properties of various plant extracts is of great interest because of their use as natural additives and replacement of synthetic ones (Neeta *et al.,* 2015).

The presence of phenolic compounds was observed based on the results of lcms profiling of the extract, such as chlorogenic acid these observations could be the reason for antimicrobial activity of this extract. However, it was reported in literature that chlorogenic acid inhibit several food borne pathogens (Li *et al.,* 2014). Similarly in another study conducted by Mengmeng *et al*. (2019), antimicrobial activity of chlorogenic acid against *p. aeruginosa* was reported and it displayed the potential to be developed as a food preservative. This suggests that the *S. aromaticum* extract which have been confirmed to contain several secondary metabolites may also be useful as preservatives/ additives in food.

The results of antibacterial activities of *S. aromaticum* extract against food borne pathogens revealed the effectiveness of the extract against the tested isolates, however, *Enterobacter spp* and *Staphylococcus aureus* were more susceptible to the extracts in comparison to other organisms with inhibition zone diameter of 22.50±0.41 mm and 17±0.82 mm respectively. The result of the antimicrobial activity of *S. aromaticum* extract in this study was in conformity with the study conducted by Burt (2004). However, Ali *et al*. (2018) reported higher inhibitory zone (18.00±1.73 mm) against *E. coli*. The disparities in different studies may be attributed to differences in solvents of extraction and concentrations as well as strain differences. Microbial susceptibility pattern to antimicrobial have been reported to be strain- dependent (Kwon and Lu, 2007). The antibacterial activity could be due to the presence of an array of phytochemicals present in the extract. The antimicrobial activities of some Nigeria spices on some food pathogens have also been reported by Aboaba, *et al*. (2011). The ability of *S. aromaticum* showing sensitivity to three different strains of bacteria (gram positive and gram negative) shows its application as a broad spectrum antimicrobial agent with the largest efficacy being the methanol extract from this study. This justified the use of this plant in traditional medicine practices (Neeta *et al.,* 2015). The Minimum inhibitory concentrations (MICs) was observed at lower concentrations for the three susceptible isolates (125 µg/ml- 250 µg/ml) while the Minimum Bactericidal concentrations (MBC) of the extracts against the tested isolates were at higher concentrations (>2000 µg/ml) except for *E. coli* (250 µg/ml). The findings from this study agreed with the work of Nascimento, *et al*. (2000) who found that the plant extract contained agents (metabolites) that killed *Salmonella*, *C. albicans*, *P. aeruginosa* and *E. coli*.

CONCLUSION

Methanol extract of clove (*Syzygium aromaticum)* contained several secondary metabolites and also exhibited antibacterial activities against some food borne pathogens. Therefore, it can be concluded that *S. aromaticum* could be useful in controlling contaminating food borne pathogens as well as an alternative to synthetic antimicrobial agents used in food industries in addition to its role as food additives.

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