https://dx.doi.org/10.4314/dujopas.v8i2b.1
ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 8 No. 2b June 2022*

Antibacterial Activity of Sesquiterpenoids and 8-O-methylated Flavonoids from Hexane Fraction of Clove *(Syzgium aromaticun)*

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Abstract
According to World Health Organization reports, there is a major health challenge now and in the future due to increase resistance of bacteria to well-known antibiotics. This study aimed at identifying some vital metabolites from Hexane fraction of clove (Syzygium aromaticum) using Liquid Chromatography Mass Spectrometer (LCMS) and its antibacterial activity against some foodborne pathogens. Syzygium aromaticum buds were extracted with Hexane using maceration method. This was followed by LCMS analysis for secondary metabolites and then antibacterial activity using Agar well diffusion against four foodborne pathogens (Enterobacter spp, Salmonella spp, Escherichia coli, and Staphylococcus aureus). The Minimum inhibitory concentrations (MICs) and Minimum Bactericidal (MBC) of the clove extracts were also determined by the micro-dilution method. The LCMS profile revealed the presence of significant metabolites such as Nevadensin (8-O-methylated flavonoids) and 9-Hydroxymegastigma-4, 6,7- trien-3-one (Sesquiterpenoids) among others belonging to the well-known class of bioactive compounds that shows antioxidant and anti-inflammatory potentials. Based on the results obtained, Enterobacter spp was found to be the most susceptible organism with an average zone of 12.50±1.20 mm, followed by E. coli 10.50±1.20 mm. The MIC and MBC of the extract against the test organisms ranged from 62.5 to 250 µg/ml. Therefore, the antibacterial activities observed might be as a result of these metabolites, and could be recommended for used in drug management or as antibacterial agent.

Keyword: Antibacterial, Clove, Foodborne, Flavonoids, Pathogens.

INTRODUCTION
Clove (*Syzygium aromaticum*) is one of the most important spices that have been used traditionally as food preservative and other therapeutic purposes (Hussain *et al.*, 2017). Clove is cultured in several parts of the world including Nigeria. This plant possessed rich phenolic constituents such as eugenol, and eugenol acetate with great potential for pharmaceutical, cosmetic, food and agricultural applications (Hussain *et al.*, 2017). Cloves contain appreciable

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amounts of volatile oil (used for flavouring foods and pharmaceuticals), which is mainly confined in aerial parts of the plant. The yield and composition of volatile oil are variable and are thought to be linked to growing conditions, genetic factors, different chemotypes, geographic origins, and differences in the nutritional status of plant (Hussain *et al*., 2017). The antioxidant and antimicrobial properties of clove are higher than many fruits, vegetables and other spices (Hussain *et al*., 2017).

The diseases caused by bacteria are due to infection with *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Mycobacterium tuberculosis*, *Bacillus cereus*, *Clostridium perfringens* and others (Chen *et al*., 2017), which are basic foodborne pathogens. The discovery and development of antibiotics helped to cure most of the infectious diseases, and subsequently decreased the mortality rates of humans around the world. Since their developments from the 1940s, antibiotics have become life-saving medicines that are integral to human health (Butler and Paterson, 2020). However, the increasing antibiotic resistance (AR) of pathogenic bacteria was as a result of misuse or overuse of antibiotics, which has become one of the biggest threats to public health (Chandra *et al*., 2017). The AR ability of microorganisms can escape the effects of the drugs designed to kill or inhibit them by different mechanisms, such as neutralizing the antibiotics, flushing them outside of the cell, or changing their outer structure, and subsequent decreasing the effects of drugs to the bacteria (Silhavy *et al*., 2010). Additionally, the use of these antibiotics is becoming increasingly restricted because of the toxicity possessed by themselves as well as their trickish AR. Despite all the efforts that have been devoted to combat AR on the national and international scale, this situation is still on the rise (Hussain *et al*., 2017).

According to the 2019 report from the Centers for Disease Control and Prevention in the USA (Chen *et al*., 2021) more than 2.8 million antibiotic-resistant infections occurred each year, and as a result, more than 35,000 people died. Plant extracts and essential oils can reduce the growth of pathogenic microorganisms and extend the shelf life of the food. Sujana *et al*. (2013) reported higher zone of inhibition by methanol extract of *Mentha paparita* (mint), in a similar study, Bashir *et al*. (2021) reported the potentiality of hexane fraction of the same plant against some food-borne pathogens. Therefore, there is an urgent need to search for safe and effective compounds with antibacterial potentials as alternatives to antibiotics to address these serious problems. This study aimed to investigate the antibacterial potential of clove hexane extract against some food borne pathogens and possibly identify the metabolites responsible for the activity using LCMS techniques.

MATERIALS AND METHODS

Plant Material Preparation

The *S. aromaticun (*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 clean water in order to remove soil debris from it, and then allowed to dry at room temperature under shade. It was pulverized using mortar and pestle and lastly sieved using fine mesh size to remove the fibers in order to obtain very fine powder (Sukhdev *et al.,* 2008).

Extraction of *S. aromaticun*

The extraction was done using method described by Sofowora (2000) with slight modifications. About 200 g of powdered plant material was sucked in one liter of n-Hexane (1L) extraction fluid. The mixtures were kept for 48 hours in tightly sealed vessels at room

temperature and stirred several times daily. The 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 concentrated using water bath at 40°C.
The extracts were tested for sterility as described by Hamilton, (2006). About1 ml of the extract was introduced into 15 ml of sterile nutrient broth and incubated at 37^0C for 24 hours. A sterile extract was indicated by the absence of turbidity or clearance of the broth after the incubation period.

Collection of Food borne pathogens
The test isolate *E. coli, Salmonella spp, Staphylococcus aureus and Enterobacter spp* were obtained from the Department of Microbiology Bayero University Kano and were further confirmed using selected biochemical tests (indole, coagulase, oxidase, citrate, triple sugar iron test, urease and voges proskauer) as described by Cheesbrough (2006).

The LCMS Analysis
The *Syzgium aromaticun (*Clove) hexane extracts was subjected to lcms analysis as reported by Piovesana *et al*. (2018) with some modifications. The extracted samples were reconstituted in hexane and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. 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 were maintained at 25°C. 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: Solvent Gradient

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

Started from ratio of A/B 95:5 which was maintained for further 1 min, then changed to A/B 5:95 for 13 min, to 15 min. then returned to A/B 95:5 to 17 min, 19 min and finally 20 min. The wavelength was set at 210-400nm in the photodiote array (PDA) detector with resolution of 1.2 nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 – 1250, other settings include: ESI source in positive and negative ion modes; capillary voltage 0.8 kv (positive) and 0.8 kv (negative); probe temperature 600 °C; 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 as described by Hanafi *et al.* (2018).

Preparation of extract concentration
The extract was prepared as described by Kalpana *et al*. (2013), starting with stock solution (2000 µg/ml) which was prepared by dissolving 2 g of the plant extracts in 1ml 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).

Preparation of turbidity standard
The turbid solution as the standard for comparison was prepared as reported by Chessbrough, (2005).

Standardization of Inoculum
This was conducted according to National Committee for Clinical Laboratory standard (NCCLS, 2008). Using inoculation loop (wire loop), a loopful of overnight culture of the test organism was introduced into a test tube containing 2 ml of normal saline, then compared against another tube containing 0.5 Mcfarland standard. Normal saline was added until the turbidity of the suspension matched that of Mcfarland.

Antibacterial assay
The antibacterial activities of *Syzgium aromaticun (*Clove) hexane extracts was determined by agar well diffusion method as reported by Okeke *et al*. (2001). Pure isolate of each bacterium was first sub-cultured in nutrient broth at 37^{o}C for 24h. Standardized inoculum (10^{6} CFU/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 6.0mm diameter was used to bore wells in the agar plates. Subsequently, 0.1ml 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 and then incubated at 37^{o}C for 24h. The zone of inhibition was recorded to the nearest size in millimeter.

Determination of Minimum Inhibitory Concentration
The lowest concentration of the antimicrobial agent that retard visible growth of microorganisms after overnight incubation is referred to minimum inhibitory concentration (Andrews, 2002). The doubling micro-dilution broth method was used to determine the MIC. About 2 ml of the reconstituted extract at a concentration of 1000 µg/ml was added to 2ml of sterile Mueller Hinton broth for the bacterial isolates, About 2 ml 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. Then 0.1 ml of 18 h 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 bacteria inoculum was used as negative control. The inoculated culture tubes were incubated at 37 ^{o}C 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 (Andrew, 2002).

Determination of minimum bactericidal concentration:
This was conducted according to the method described by Andrew, (2002). About 0.1 ml 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 ^{o}C for the bacterial isolate. The lowest concentration in which no growth occurred was taken as the minimum bactericidal concentration (MBC).

RESULT
The *Syzygium aromaticum (*clove) hexane extract was obtained and used for Liquid Chromatography Mass Spectrophotometry analysis, the profile (total ion chromatogram) and fragmentation pattern of the identified (Tentative) compounds were presented in table 1 and figures 1-4.

Table 2: Identified compounds from *Syzygium aromaticum* (clove) hexane extract

Peak	Tentative Compound	M	MZ (M+H)
1	Dioctylamine (Dialkylamine)	241	242
2	Nevadensin (8-O-methylated flavonoids)	344	345
3	9-Hydroxymegastigma-4,6,7-trien-3-one (Sesquiterpenoids)	206	207

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

Table 3: Antibacterial activity of hexane clove extract on some food borne pathogen

Food borne pathogen	Diameter of Inhibition Zone (mm)				
	Conc. (µg/ml)				
	2000	1000	500	250	C 250
Staphylococus aureus	10.0±1.00	7.50±0.50	6.50±0.5	6.00±0.00	8.00±1.00
Enterobacter spp	12.5 ±1.20	10.50±1.20	8.0±0.82	7.50±0.41	8.00±1.00
E.coli	10.50±1.20	8.50±1.20	7.50±0.41	6.50±0.40	9.5±1.20
Salmonella spp	7.50±0.41	6.50±0.40	6.00±0.00	6.00±0.00	9.5±0.41

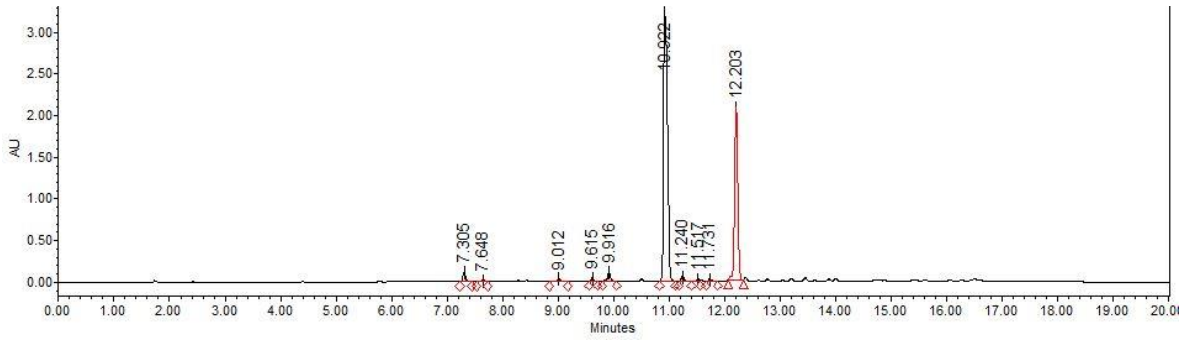

Figure 1: Total Ion Chromatogram of Clove Hexane Extract (*Syzygium aromaticum*)

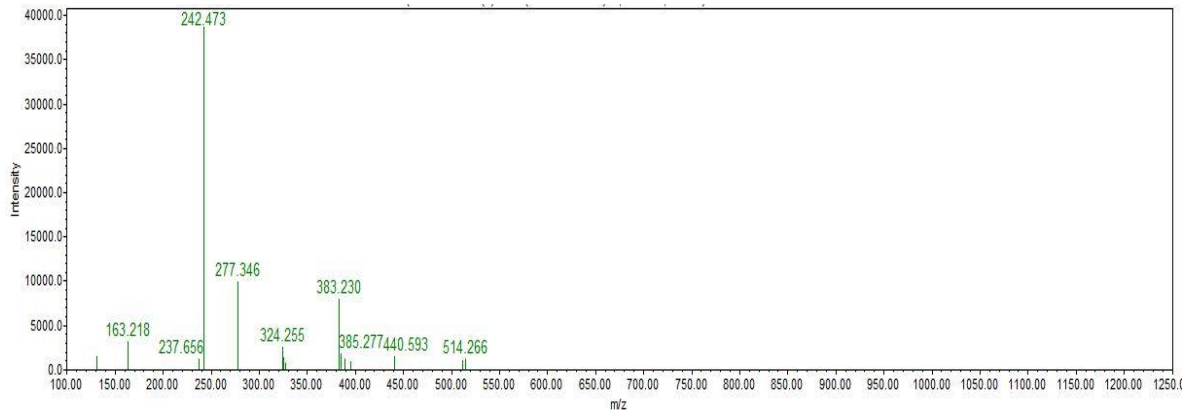

Figure 2: Fragmentation pattern of Dioctylamine (Dialkylamine) m/z 242.473 (M+H)

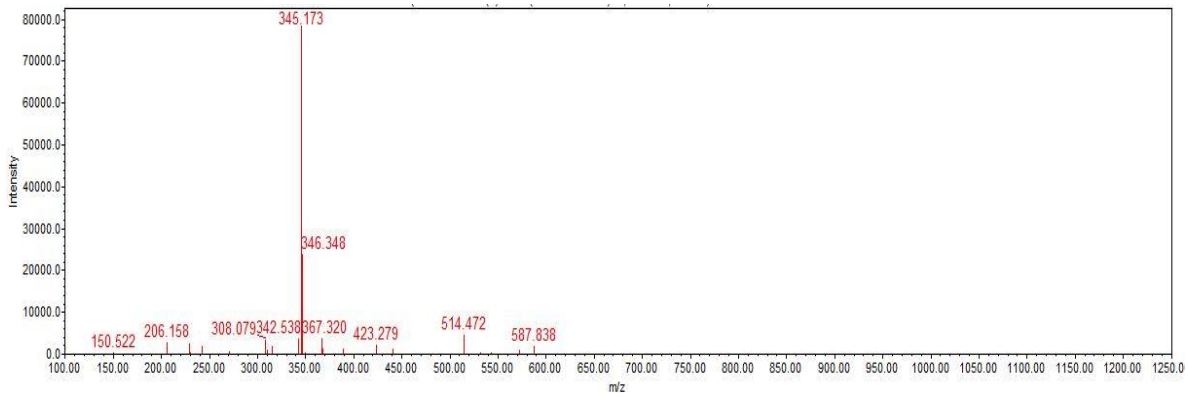

Figure 3: Fragmentation pattern of Nevadensin (8-O-methylated flavonoids) m/z 345.173 (M+H)

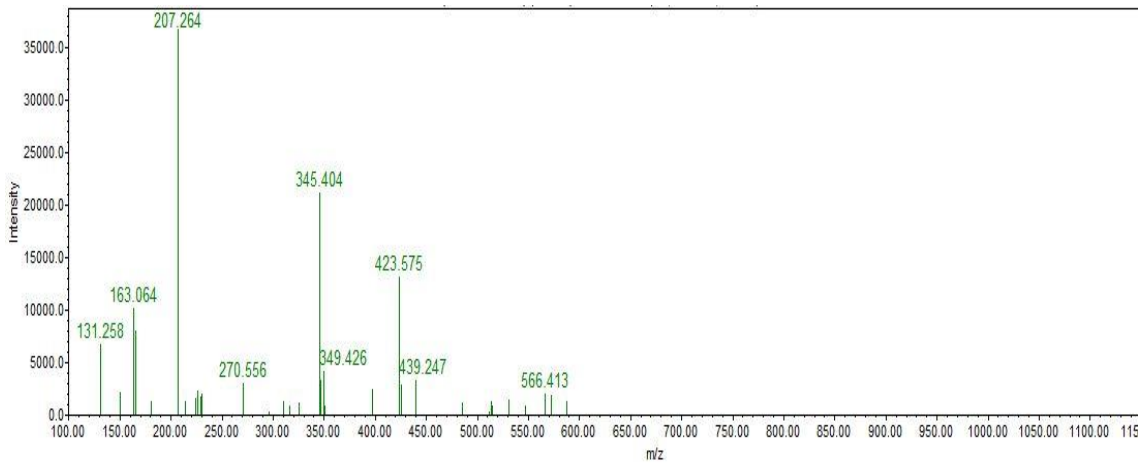

Figure 4: Fragmentation pattern of 9-Hydroxymegastigma-4,6,7-trien-3-one (Sesquiterpenoids) m/z 207.264 (M+H)

The Antibacterial activity of clove hexane extract on the food-borne pathogens was presented in table 2, and the results showed higher antimicrobial activity on *Enterobacter spp* with average zone of inhibition of 12.50±1.20 mm, followed by *E. coli* 10.50±1.20 mm, *staphylococcus aureus* 10.00±1.00 mm and *salmonella spp* 7.50±0.41 mm.

The Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of clove extract against the organisms are presented in table 3. The result shows that Hexane extract was found to have MIC of 62.5 gm/ml on *E.coli, Staphylococcus aureus, Salmonella spp* and 125 µg/ml on *Enterobacter spp*. While the MBC values were 62.5 µg/ml

against *E. coli and* 125 µg/ml against *Staphylococcus aureus*.

Table 4: Minimum Inhibitory and minimum bactericidal Concentrations (MIC and MBC) of clove extract on some food borne isolate

S/N	Isolate	H.C.E (µg/ml) MIC	MBC
1	*E.coli*	62.5	62.5
2	*Enterobacter spp*	125	>2000
3	*Staphylococus aureus*	62.5	125
4	*Salmonella spp*	62.5	>2000

Key: H.C.E= hexane clove extract, MIC= Minimum inhibition concentration, MBC =Minimum bactericidal concentration

DISCUSSION

The LCMS profile of hexane extract of clove revealed some important metabolites such as Dioctylamine (Dialkylamine), Nevadensin (8-O-methylated flavonoids) and 9-Hydroxymegastigma-4, 6, 7- trien-3-one (Sesquiterpenoids). Our finding was slightly in agreement with the work of Lesmana *et al*. (2021), in which they reported higher flavonoid content in clove leaf n-*hexane* fraction (25.6 mgQE ·g−1). Similarly some literature described that the total flavonoid content in the clove bud fraction was found higher by using 80% ethanol solvent and 100% water (higher polarity) than 100% n-hexane and 100% ethyl acetate solvents (Aboelmaati *et al.,* 2012). Such differences on the quality and quantity of flavonoid content on phytoextracts may occur generally due to the biological and environmental background of the plants (Shojaii *et al.,* 2017; Ismail *et al.,* 2017). However, extraction using different techniques, with the same solvent may also affect the flavonoid contents on the phytoextracts (Zhang *et al.,* 2018). On the other hand, previous studies of the ethanol clove fraction were found to have gallic acid (C7H6O5) (*m*/*z* 169.0) and (15Z)-9, 12, 13-Trihydroxy-15-octadecenoic acid at higher concentrations (C18H34O5) (*m*/*z* 329.2) than other compounds, based on LC-MS data.

Many literatures revealed the composition of sesquiterpenes to be predominantly abundant, at 62.26% (sesquiterpenes hydrocarbon 33.88%; oxygenated sesquiterpenes 28.38%), followed by monoterpenes at 35.39% (monoterpenes hydrocarbon 34.88%; oxygenated monoterpenes 0.51%) and others at 2.35%. (Cucho-Medrano *et al*., 2021). Generally, phytochemicals are responsible for plants colour, flavour, smell and are part of a plant`s natural defense system and also protect them against herbivorous insects and vertebrates, fungi, pathogens, and parasites (Ibrahim *et al.,* 2010). The profile of the bioactive compounds of a plant shows its medicinal importance. 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). Nevadensin (8-O-methylated flavonoids) which is flavonoid rich indicated that *S. aromaticum* will be good for the management of cardiovascular diseases and oxidative stress because flavonoids and phenols are biological antioxidants. In many instances, the

aromatic or spices plants contain compounds that possess confirmed strong or potent anti-oxidative properties. Antioxidants are very important to human health, including lowering the risk of cancer. Antioxidant also combats the effects of free radicals. The more the danger of free radicals, the more the plants produces more antioxidant. Antioxidant system prevents these reactive oxygen species from being formed at optimum level (Wankhede, 2015).
The results are in accordance with the findings of other authors who have studied this plant previously (Jirovetz *et al.,* 2006). Aboaba, *et al*., (2011) has reported that the presence of these phytochemicals which possess antimicrobial properties are desirable tools in food spoilage and food safety.

The results of antibacterial activities of clove (*Syzygium aromaticum*) hexane extracts against some food borne pathogens indicated that *Enterobacter spp* was found to be the most susceptible organism with average zone of inhibition of 12.50±1.20 mm, followed by *E. coli* 10.50±1.20 mm, *Staphylococcus aureus* 10.00±1.00 mm and S*almonella spp* 7.50±0.41 mm. The result obtained from this study was in conformity with many studies reported in literature (Burt, 2003). In contrast, Wankhede (2015) and Pande and Singh (2011) reported that extract of petroleum ether showed highest antimicrobial activity in *S. typhimurium* of 17 mm and less in *S. aureus.* Cortés-Rojas (2014), also reported a complete bactericidal effect against all the food-borne pathogens tested *Escherichia coli* (*E. coli*), *Staphylococcus aureus* and *Bacillus cereus* was the aqueous extract of clove at 3%. At the concentration of 1% clove extract also showed good inhibitory action. However, in another study conducted by Ali *et al*. (2018), only *E. coli* was susceptible with inhibition zone diameter (8.00±1.73mm). This could be as a result of polarity of the solvent used. However, higher polarity may extract more content of bioactive compounds.

The Minimum inhibition concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the clove extracts was observed at lower concentrations for the susceptible isolates (*Enterobacter spp*, *Salmonella spp*, *Escherichia coli*, and *Staphylococcus aureus*) which ranged from 62.5 to 125 µg/ml. Akintayo *et al*. (2021) reported moderate antibacterial properties of tested flavonoids and organic acids against clinical strains of Gram-negative pathogens: *E. coli* and *P. aeruginosa* (MIC = 500 µg/mL). Among 19 selected plant substances, only three: kaempferol, quercetin, and chlorogenic acid were inactive against *P. aeruginosa* at all concentrations tested (15.6–1000 µg/mL). However, for up to 10 compounds, no significant activity was found against the Gram-positive bacteria *S. aureus*. Additionally, another microorganism from this group *E. faecalis* showed low sensitivity (MIC = 1000 µg/mL) to most analyzed metabolites (Akintayo *et al*., 2021). Therefore our observations confirmed the result of works which indicated higher activity of natural plant substances, including flavonoids, against some Gram-negative bacteria than Gram-positive ones. The general tendency of bacterial sensitivity to this clove hexane extract was observed as follows: *Enterobacter spp* > *E. coli* > *S. aureus* > *Salmonella spp*. Previous studies revealed greater activity of alkaloids, flavonoids, and phenolic acids especially against *P. aeruginosa*, and also *E. coli* than *S. aureus* (Akintayo *et al*., 2021).
Most sesquiterpenes have not been individually screened for antimicrobial activity, however, three of the major components, β-pinene, linalool, and (*E*)-caryophyllene, were also screened by Akintayo *et al*. (2021) and these compounds showed activities similar to the essential oils themselves.

CONCLUSION

This research revealed the presence of vital metabolites in hexane extract of clove (*Syzygium aromaticum)* which exhibited antimicrobial activity against the food borne pathogens tested. Therefore, the extracts from *Syzygium aromaticum* may act as an alternative to synthetic antimicrobial agent used in food industry, with high degree of safety and less course effective.

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