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Bioactive components of *Syzygium aromaticum* bud and their effects on selected pathogenic bacteria

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Abstract:

Background: The decline in the effectiveness of common antibiotics is due to microbial resistance and has sparked research interest in discovering new antimicrobial agents from plants. The objective of this study was to evaluate the effectiveness of clove extracts on selected pathogenic bacteria and identify the active antibacterial components.
Methodology: The active components of *Syzygium aromaticum* (clove) buds were extracted using methanol and ethyl acetate and identified by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Antibacterial screenings against selected pathogenic bacteria were conducted using the agar-well diffusion method.
Results: The extracts showed activities against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* at 100 mg/ml, so that the diameters of the methanol extracts were 4.00-25.00 mm, 3.00-26.00 mm and 0.00 -16.00 mm, while the ethyl acetate extracts were 3.00-24.00 mm, 4.00-22.00 mm and 0.00-14.00 mm respectively. The ethyl acetate extract showed higher MIC and MBC at 3.125 and 6.5 mg/mL and 12.5 mg/mL, indicating a more lethal effect than the methanol extract. Nineteen bioactive compounds were identified the extracts.
Conclusion: The study justifies the use of the clove plant by traditional herbalists to treat bacterial infections due to the presence and synergy of the plant's various bioactive components.

Keywords: *Syzygium aromaticum*, Clove extract, Antibacterial activity, bioactive compounds

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Composants bioactifs du bourgeon de *Syzygium aromaticum* et leurs effets sur certaines bactéries pathogènes

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Résumé:

Contexte: Le déclin de l'efficacité des antibiotiques courants est dû à la résistance microbienne et a suscité l'intérêt de la recherche pour la découverte de nouveaux agents antimicrobiens à partir de plantes. L'objectif de cette étude était d'évaluer l'efficacité des extraits de clou de girofle sur des bactéries pathogènes sélectionnées et d'identifier les composants antibactériens actifs.

Méthodologie: Les composants actifs des bourgeons de Syzygium aromaticum (clou de girofle) ont été extraits à l'aide de méthanol et d'acétate d'éthyle et identifiés par analyse par chromatographie en phase gazeuse-spectrométrie de masse (GC-MS). Des criblages antibactériens contre des bactéries pathogènes sélectionnées ont été effectués en utilisant la méthode de diffusion en puits d'agar.
Résultats: Les extraits ont montré des activités contre *Staphylococcus aureus, Escherichia coli* et *Pseudomonas aeruginosa* à 100 mg/ml, de sorte que les diamètres des extraits au méthanol étaient de 4,00-25,00 mm, 3,00-26,00 mm et 0,00-16,00 mm, tandis que les extraits à l'acétate d'éthyle étaient de 3,00-24,00 mm, 4,00- 22,00 mm et 0,00-14,00 mm respectivement. L'extrait à l'acétate d'éthyle a montré des CMI et MBC plus élevés à 3,125 et 6,5 mg/mL et 12,5 mg/mL, indiquant un effet plus létal que l'extrait au méthanol. Dix-neuf composés bioactifs ont été identifiés dans les extraits.
Conclusion: L'étude justifie l'utilisation du giroflier par les herboristes traditionnels pour traiter les infections bactériennes du fait de la présence et de la synergie des différents composants bioactifs de la plante.

Mots-clés: *Syzygium aromaticum*, extrait de clou de girofle, activité antibactérienne, composés bioactifs

Introduction:

Cloves (*Syzygium aromatum* L.) are a tree in the family Myrtaceae, which contains 1200 to 1800 species (1). The plant is grown in Nigeria and other parts of the world and its flower buds produce essential oil used as a spice in cooking and in traditional herbal medicine (2,3). The plant has been found to contain many phyto -chemicals, monoterpenes and eugenol, which have antimicrobial effects that can be studied to combat diseases caused by pathogenic micro-organisms.

Antibiotics have evolved from a purely microbial product to synthetic substances that inhibit pathogenic microorganisms (4). Antibiotics have helped to treat and control the spread of infectious diseases and curbed infections in surgical and pediatric patients, thereby preventing high mortality rates for decades (5). Today, the benefits of antibiotics extend to various areas of human activity, including medical practice and agriculture (4). However, selective pressure from drug abuse has created resistance strains that can evade antibiotic action (6).

The spread of resistant bacteria poses a threat to medicine and is one of the main reasons for the worldwide decline in the effectiveness of antibiotics. In addition, multidrug resistance has increased mortality rates and led to the spread of diseases, particularly in African populations who were unaware of their effects (7). This requires research to focus on the discovery of herbal therapeutics. Therefore, this study was conducted to evaluate the effectiveness of clove extracts on selected pathogenic bacteria and identify the active components.

Material and method:

Study area:

This research was conducted at the Microbiology Laboratory of IBB University in Lapai, Niger State, Nigeria. The clove buds were collected from a community within longitude (9º03 N) and latitude (6º34 E) with an estimated population of 120,000 people, most of whom are farmers and heavy users of herbal medicines (8).

Sample collection and preparation:

Fresh cloves (*Syzygium aromaticum* L.) were purchased in bags and authenticated in the Department of Biological Sciences of the University. They were then washed in distilled water and dried at ambient temperature. The dry flower buds were ground, weighed and stored in a sterile container for experiments. The laboratory repository of the Department of Biochemistry, IBB University in Lapai provided the standard grade methanol and ethyl acetate utilized for the extraction of bioactive components from cloves.

Pure isolates of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from stock cultures at the Microbiology Laboratory of IBB University Lapai Niger State. Isolates were sub-cultured onto nutrient agar plates to assess viability and obtain fresh cultures for the research.

Extraction of cloves flower buds:

The cold maceration method was used to extract the sample ingredients (9). Three hundred grams of the sample powder was transferred to 1000 ml of methanol in a 2-liter conical flask. The same procedure was repeated for ethyl acetate and each flask was sealed with cotton wool, wrapped in aluminum foil and allowed to stand for 48 hours. The flasks were shaken intermittently to achieve maceration and the filtrate was separated through Whatman No. 1 filter paper. The solvents were evaporated on a steam bath leaving only the extract in the container. Then, the yield of the extracts was calculated using the formula; % yield = weight of the plant extract/weight of dry sample (x 100).

Antibacterial activity of plant extracts:

The antibacterial activity of the extracts was determined by the agar diffusion method from zones of inhibition formed around the agar wells due to diffusion against the test organisms (10). Fresh nutrient agar plates were aseptically inoculated with 0.1 ml bacterial culture using the

spread-plate technique before making wells in the agar with a sterile 6 mm cork-borer. One gram of extract was introduced into six separate beakers with different volumes of dimethyl sulfoxide (DMSO) to give 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml of extracts. A positive control with the same concentrations of ciprofloxacin and a negative control with sterile water were setup.

The agar wells were filled with 1 ml of the different concentrations of the extracts and ciprofloxacin. The plates were incubated at 37°C for 24 hours and the diameters of the zone of inhibition around the agar wells were measured with a meter ruler. The experiment was performed in triplicate.

Minimum inhibitory concentration (MIC) by tube dilution method:

The MIC of the extracts was determined according to the tube dilution method of Adegbenu et al., (11). The extracts were serially diluted with DMSO in separate beakers to obtain different concentrations of the extracts (100, 50, 25, 12.5, 6.25 and 3.125 mg/ml). Five milliliters of each concentration were then dispensed into separate test tubes, followed by the addition of 5 ml of Muller-Hilton broth and shaken to mix. The tubes were inoculated with 0.2 ml bacterial solution and examined for the lower concentration of the extracts that showed no turbidity after an incubation period of 24 hours at 37°C

Minimum bactericidal concentration (MBC):

In the MIC study above, test tubes showing no growth were aseptically pour-plated onto fresh nutrient agar. After incubation at the same temperature for 24 hours, the plates were monitored and checked for observable colonies of the test organisms and the lowest concentration that showed no growth was designated MBC.

Identification of compounds by GC-MS analysis:

The oil compounds were identified by GC-MS analysis as described by Ibrahim et al., (12). One ml of the diluted oil was analyzed by GC-MS equipped with Agilent Split/Splitless and a BP5 (30m) equipped with a (0.25m 0.25 m) capillary column (30m 0.25m 0.25m). The carrier gas was nitrogen, and the column was kept at 60°C for 3 minutes before rising to 220°C for 5 minutes and remaining constant at 220°C. The temperature interface was set to 280°C. Mass spectrometric analysis gave 40,800 atomic mass units (AMU) at 2,500°C. Identified compounds have peaks with greater than 90% accuracy com -pared to more than 62,000 spectral samples in the National Institute of Standard and Technology (NIST) library database and Wiley libraries.

Results:

Organoleptic properties of extracts:

The clove extracts were sticky and oily with the ethyl acetate extract being more of a sticky liquid. In addition, methanol extract appears brown while ethyl acetate extracts are light green in color (Table 1). From the result, 40g and 20g of extracts of ethyl acetate and methanol were obtained, corresponding to yields of 13.3% and 6.3%, respectively. In general, it was observed that the extracts produced by the solvents were low.

Table 1: Organoleptic properties and percentage (%) yield of methanol and ethyl acetate extraction

Sample	Extracts	WOP (g)	WOE (g)	POE (%)	COE	TOE
Cloves flower buds	Methanol	300	40	13.3	Brown	Oily
	Ethyl acetate	300	20	6.3	Light green	Oily

WOP: Weight of plant; WOE: Weight of extract; POE: Percentage of extract; COE: Color of extract; TOE: Texture of extract

Chemical compounds composition:

The chromatograms in Fig 1 show the peaks that identified compounds in methanol and ethyl acetate extracts. Nineteen compounds were identified in the extracts with the same retention time, but they had different percentage weights and peak areas. These compounds are listed in Table 2. Of these compounds, -2-methoxy-4-prop-2-enylphenol is the most abundant in the extracts (23.51%-19.03%), followed by -bisabolol (14.81%-12.54%) and acetyl-eugenol (14.11%–9.23%).

A benzene ring that is structurally common could be a relationship between the compounds, as shown in their structures in Fig 2. In addition, several minor compounds were also detected by GC-MS analysis. These compounds include methyl ester, ethyl ester, terpenes, caryophyllene, cedrenol, 1,3-benzodioxol-5-ol, tetradecanoic acid and heneicosanoic acid. They differ greatly in weight from the main components and probably play a role in the effectiveness of clove against pathogenic bacteria.

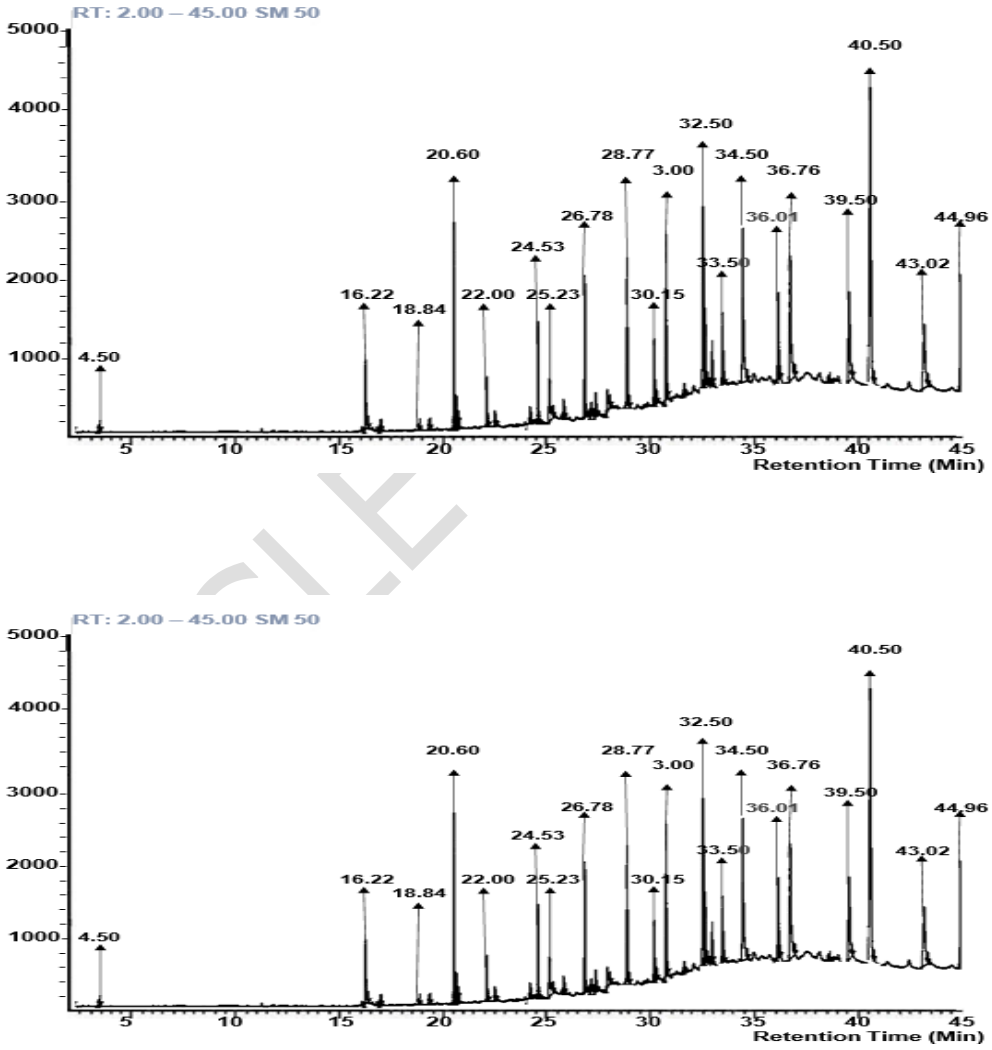
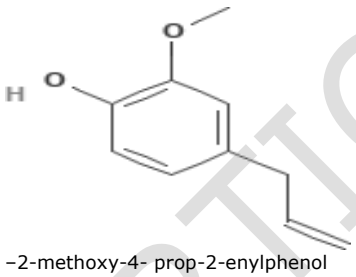


Fig 1: Peaks indicated by GC-MS analysis for identified compounds from the clove extracts

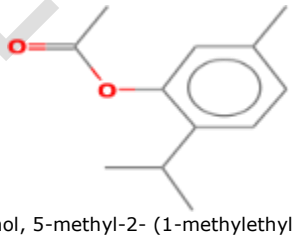
Table 2: Chemical compounds detected in the flower buds of *Syzygium aromaticum*

Retention Time	Compounds	Formula	Molecular Weight	Weight (%)		Peak Area (%)	
				a	b	a	b
4.50	1,3-Benzodioxol-5- ol	$C_7H_6O_3$	138	0.70	1.60	0.59	0.45
16.22	–2-methoxy-4- prop-2-enylphenol	$C_{10}H_{12}O_2$	352	19.03	23.51	3.20	3.11
18.84	Phenol, 5-methyl-2- (1-methylethyl)-, acetate	$C_{12}H_{16}O$	192	11.41	16.12	0.65	0.87
20.60	Acetyleugenol	$C_{12}H_{14}O_3$	206	14.11	9.23	7.93	6.27
22.00	α-Humulene	$C_{15}H_{24}$	204	4.17	2.09	2.56	2.41
24.53	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	1.09	1.24	1.51	2.04
25.23	1,8,11-Heptadecatriene, (Z, Z)-	$C_{17}H_{30}$	234	2.11	2.03	2.28	2.11
26.78	9,12-Octadecadienoic acid (Z, Z)	$C_{38}H_{68}O_8$	242	2.86	3.44	6.85	6.91
28.77	Humulene-1-2- epoxide	$C_5H_{29}O$	220	1.45	1.29	6.71	5.25
30.15	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	336	11.11	7.03	9.78	9.42
32.50	Octadecanoic acid	$C_{18}H_{36}O_2$	284	2.99	5.85	7.31	8.74
33.50	Cholecalciferol	$C_{27}H_{44}O$	384	2.68	3.92	4.15	5.03
34.50	Heneicosanoic acid	$C_{21}H_{42}O_2$	326	5.16	3.01	8.59	10.22
36.01	Caryophyllene	$C_{15}H_{24}$	204	6.07	3.89	6.22	2.60
36.76	Cedrenol	$C_{15}H_{24}O$	220	2.14	2.23	6.54	6.12
39.50	Geramyl-α-Terpinene	$C_{20}H_{32}$	272	2.43	2.78	4.48	4.99
40.50	α-Bisabolol	$C_{15}H_{26}O$	222	0.99	1.52	14.81	12.54
43.02	Hexadecanoic acid, 2-hydroxy-1- (hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330	2.82	2.54	4.79	4.80
44.96	11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324	7.23	6.04	7.68	4.16

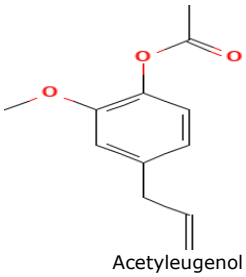
a; Methanol extract, b; ethyl acetate extract



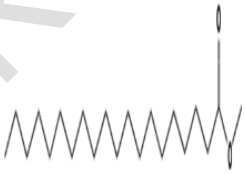
–2-methoxy-4- prop-2-enylphenol



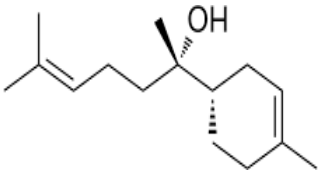
Phenol, 5-methyl-2- (1-methylethyl)-, acetate



Acetyleugenol



Eicosanoic acid, methyl ester



α-Bisabolol

Fig 2: The structure of the main compounds in clove extracts

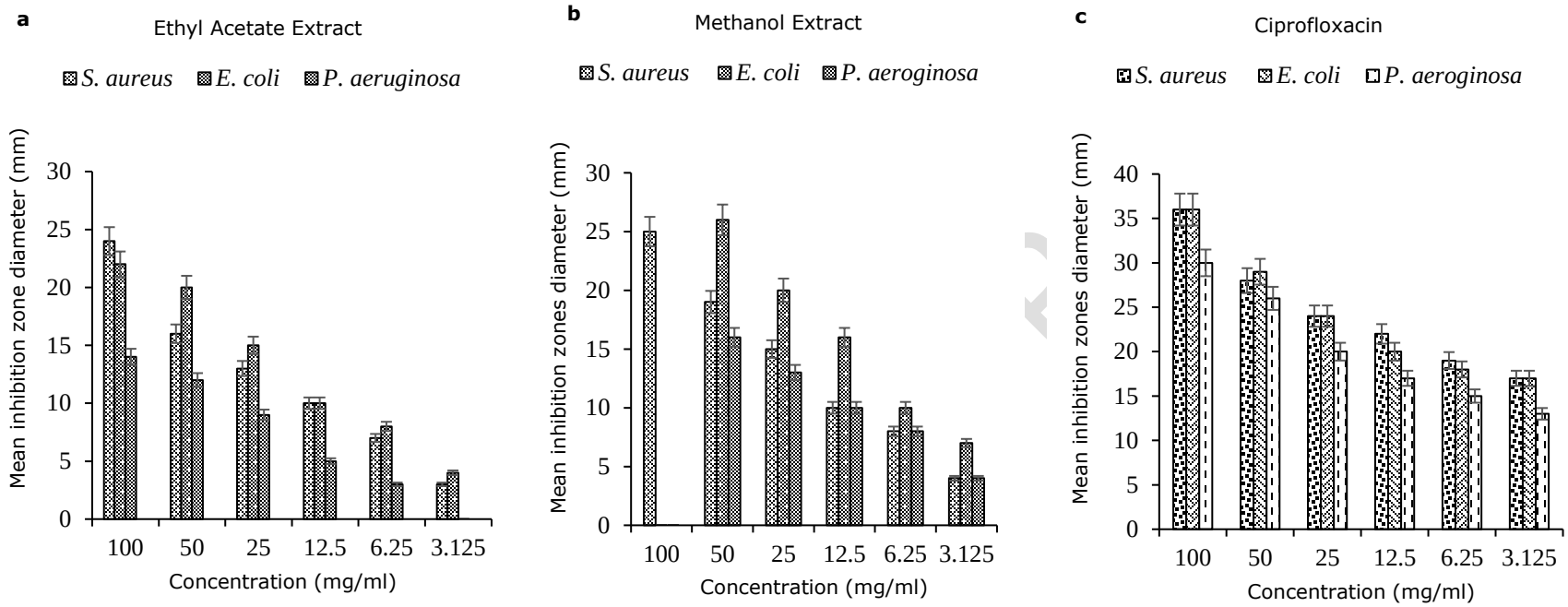


Fig 3: Antibacterial activity (inhibition zone diameter) of (**a**) ethyl acetate and (**b**) methanol extracts compared to that of (**c**) ciprofloxacin control

Results of antibacterial assay of *Syzygium aromaticum* extracts:

The antibacterial screening at 100 mg/ml of ethyl acetate extract produced inhibition zone diameter of 24mm for *S. aureus,* 22mm for *E. coli* and 14mm for *P. aeruginosa*, while methanol extract produced 25mm, 26mm and 16 mm respectively. The antibacterial assay results are presented in Fig 3. From the results, diameters of zones of inhibition increased with increasing concentrations of the extracts which compared with the zones produced by ciprofloxacin control. However, the zone of inhibition was noticeably wider with the extracts, indicating higher activity than the ciprofloxacin control.

Results of MIC and MBC of the extracts:

The MIC results showed the lowest dose that inhibited the three test organisms to be 3.125 mg/ml of ethyl acetate extract, which was below 12.5 mg/ml for the methanol extract, indicating a higher MIC of the methanol extract (Table 3). On the other hand, the test plates for MBC with 3.125 mg/ml of ethyl acetate extract showed no growth of *S. aureus* and *P. aeruginosa* but this occurred for *E. coli* at 6.25 mg/ml, while 6.25 mg/ml methanol extract represented the lowest concentration that suppressed the three test organisms. Ethyl acetate extract was found to have a more suppressive effect on the test organisms at a lower concentration of 3.125 mg/ml than methanol extract, indicating a lethal concentration that is considered the MBC (Table 4).

Discussion:

Herbal products continue to be sources of pharmacoactive properties that correlate well with drug metabolism and drug kinetics (13). Therefore, research still focuses on plants to identify bioactive compounds to treat diseases. In this study, the color differences between ethyl acetate and methanol extracts were consistent with the findings of Leonard (14). Typically, colors represent impurities, and a darker extract has a higher level of foreign matter that differs from the extract's original chemicals (15). In addition, a higher yield for methanol indicates better extraction performance, consistent with the findings of Leonard (14).

This finding was also confirmed by Temesgen et al., (16) who also had higher amount of oil extracted by methanol than by ethyl acetate. However, the observed low extract yield may be due to the non-heat method used in the extraction process. Guan et al., (17) obtained more clove extract with a higher yield than in this study due to the heat applied, confirming the effectiveness of the heating process compared to the cold maceration used in this study. This could be due to the increase in surface area that the heating process allows the solvents to penetrate the sample. This is because previous research has shown higher yields due to the heat used in the extraction process (17).

Table 3: Minimum inhibitory concentration (MIC) results of the extracts

Bacteria	Ethyl acetate extract	Methanol extract
	3.125 mg/ml	12.5 mg/ml
Staphylococcus aureus	++	+
Escherichia coli	++	+
Pseudomonas aeruginosa	++	+

++; lower concentration that showed lethal effect on the test organisms

Table 4: Minimum bactericidal concentration (MBC) results of the extracts

Bacteria	Ethyl acetate extract		Methanol extract
	6.5 mg/ml	3.125 mg/ml	12.5 mg/ml
Staphylococcus aureus		++	+
Escherichia coli	++		+
Pseudomonas aeruginosa		++	+

++; lower concentration that showed lethal effect on the test organisms

The nineteen compounds identified in this study were less than 31 and 40 chemical components identified by (18,19). The number of compounds detected is related to the quality and medicinal value of the clove extract. This investigation found –2-methoxy-4-prop-2-enylphenol as the most abundant compound in the bud of cloves. However, relatively high levels of acetyleugenol in clove buds vary, with most studies reporting high levels of eugenol (20,21). Its occurrence cannot be excluded since the cultivation area, climatic conditions and extraction methods limit the eugenol content in the clove species (22). In addition, the macerate used in this study could not adequately demonstrate the infiltration of the sample to extract eugenol due to the slow reactivity between solvent particles and the sample. This is in contrast to the heating method, which has been reported to extract eugenol from the stem, buds and leaves of cloves (21). Heating causes the movement of solvent particles to penetrate deeper into samples and extracts sensitive compounds.

The chemical components enrich the clove with antibacterial properties of medicinal importance that can be related to the medicinal values reported by traditional healers (23). Despite the complexity of organic compounds in the essential oils, clove oil is very effective and considered safe and non-harmful to health by the United States Food and Drug Administration (USFDA). Therefore, 2.5 mg/kg per day has been recommended for infected patients by the World Health Organization (WHO) due to the healing properties of the oil. Also, recent research has discovered properties in clove oils that inhibit the growth of severe acute respiratory coronavirus 2 (SARS-CoV-2) due to their ability to bind to proteases and inhibit virus replication in the host (24). This underscores the role of clove oil in treatment of diseases caused by the coronavirus, the mechanism for which is not yet fully understood.

The antibacterial activity expresses the effectiveness of the extract in inhibiting microorganisms. It was found that 100 mg/ml extracts inhibited the test organisms, consistent with the findings of Benmakhlouf et al., (20). The increased activity with increasing amount of extract compared to ciprofloxacin indicates strong inhibitory properties. In contrast to that of Moemenbellah-Fard et al., (25), who observed a decrease in activity with increasing concentration. The reason for this behavior could be the diffusion rate of the extracts. Different dilution rates limit the diffusion of the viscous liquid into the agar medium, thus inhibiting the organism.

The MIC and MBC results were inconsistent with those of Fagere and Al Magbou (26), who reported a lower value (1.50 mg/ml) than that reported in our study. As with antibacterial screening, dilution and diffusion rates could be the cause of the differences in MBC and MBC. Lower MIC and MBC values in the ethyl acetate extract indicate higher lethality compared to the methanol extract. This behavior can be related to the combined effect of lipophilic and hydrophilic compounds extracted by ethyl acetate and their synergistic effect on test organisms. Cava-Roda et al., (27) reported that the hydrophobic rings of the major and trace chemicals in cloves interact with cells and disrupt their cytoplasmic membrane.

Conclusion:

This research examined the antibacterial effect of clove buds on pathogenic microorganisms and chemical components responsible for bioactivity. GC-MS analysis revealed 2-methoxy-4-prop-2-enylphenol, phenol, 5-methyl-2-(1-methylethyl), acetate, bisabolol and acetyleugenol as the most abundant compounds in the extracts. At 100 mg/ml, the MIC of ethyl acetate and methanol extracts of the buds were almost identical. However, ethyl acetate extracts had greater bactericidal properties (MBC) and fatal effects on the test bacteria than the methanol extracts. This higher antibacterial activity indicates that ethyl acetate is a better solvent for extracting bioactive compounds from clove buds. The *invitro* efficacy of the clove buds against the test bacteria was demonstrated.

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Contributions of authors:

MA conceived, supervised and wrote the manuscript; HHJ carried out all laboratory works and collation of raw data; BTV performed all statistical analysis of the data collected; MIL analysed and discuss antibacterial results; SM collected literatures related to the plants used in the research; and UB is a chemist that discussed the compounds detected by the GC-MS analysis.

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Conflict of interest:

Authors declared no conflict of interest

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