International Journal of Multidisciplinary Research and Growth Evaluation.

Screening of Antimicrobial Activity of Ethanolic Extract of Clove Flower Bud

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Article Info

ISSN (online): 2582-7138
Volume: 06
Issue: 01
January-February 2025
Received: 15-10-2024
Accepted: 11-02-2025
Page No: 1721-1728

Abstract
Several medicinal plant extracts having an antimicrobial property and those extracts are used to treat the infections caused by many microorganisms. This study aimed to assess the antimicrobial activity of *Syzygium aromaticum* (clove) ethanolic extracts. The extracts of clove were prepared in 70% ethanol by cold extraction process. Antimicrobial activity of different concentration of clove extracts was investigated on Bacillus subtillis, Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Klebsiella quasipneumoniae, Pseudomonas aeruginosa, Shigella dysenteriae, Salmonella enterica subsp. enterica serovar Typhi, Staphylococcus aureus, Staphylococcus epidermidis, Candida albicans, Saccharomyces cerevisiae using Modified agar well diffusion method and diameter of ZOI was measured. The broth macro-dilution dilution method was used to determine the MIC and MBC/MFC of clove extracts on these above-mentioned organisms. Klebsiella quasipneumoniae, Pseudomonas aeruginosa and Shigella dysenteriae were found resistant to clove ethanolic extract. The lower concentration of the clove ethanolic extract (6.25mg/ml, 12.50mg/ml, 25 mg/ml and 50 mg/ml) on Enterococcus faecalis, Proteus vulgaris, Salmonella enterica Typhi, Staphylococcus aureus and Staphylococcus epidermidis were found insensitive, however the 100mg/ml concentration of this extract on these bacteria showed the diameter of ZOI 9.65±0.41mm, 10.12±0.70mm, 8.71±0.86mm, 17.52±0.50mm and 17.31±0.68mm respectively. The lower concentration of clove ethanolic extract (6.25mg/ml, 12.5mg/ml and 25 mg/ml) were insensitive to Bacillus subtilis and Escherichia coli however, 50 mg/ml concentration of this extract showed the diameter of ZOI 12.59±1.13mm and 10.32±0.79mm respectively. Similarly, 100 mg/ml concentration of this extract showed the diameter of ZOI 14.75±0.67mm and 13.13±0.41mm on these bacteria respectively. The MBC of this extract for Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Salmonella enterica Typhi, Staphylococcus aureus and Staphylococcus epidermidis were 0.78±0.00 mg/ml, 2.60±0.90 mg/ml, 5.21±1.80 mg/ml, 5.21±1.80 mg/ml, 6.25±0.00 mg/ml, 2.60±0.90 mg/ml, 5.21±1.80 mg/ml respectively. MFC of this extract on the Candida albicans and Saccharomyces cerevisiae were 1.30±0.45 mg/ml and 0.78±0.00 mg/ ml respectively. This finding confirmed that clove ethanolic extract had a potent antibacterial and antifungal activity. Thus, further research is imperative to explore the in-vivo antimicrobial activity and cytotoxicity analysis to suggest the clove ethanolic extract as the alternative treatment in various microbial infections.

DOI: https://doi.org/10.54660/.IJMRGE.2025.6.1-1721-1728

Keywords: *Syzygium aromaticum,* ethanolic extract, antimicrobial activity, ZOI, MBC

Introduction

Drug-resistant pathogenic microbes are spreading throughout the world and causing the significant risk to public health (Mancuso *et al*., 2021) [24]. Emerging trend of antimicrobial resistance in pathogenic microbes causes the need of alternative therapies. Despite the significant advances of the pharmaceutical industries, the antimicrobial agent used for inhibiting or killing the bacteria and fungi are still limited (Arastehfar *et al*., 2020; Lucien *et al*., 2021) [8, 22]. Bacterial and fungal cell resistance to

the old as well as new antimicrobial agents are increasing (Fisher *et al*., 2018; Hasan & Al-Harmoosh, 2020) [16, 19]. Additionally, development of this medications is costly and time-consuming process (Muteeb *et al*., 2023) [27]. Antibiotics and antifungal drugs are often used presently for killing these microbes. However, above mentioned therapies are cost expensive and exhibit the various undesirable adverse effects (Singh *et al*., 2014; Wall & Lopez-Ribot, 2020) [38, 46]. Hence, there is a great need for the development of nontoxic and effective antimicrobials alternatives.

At present, greater than 1,350 plant was reported with antimicrobial activity and more than 30,000 antimicrobial constituents have been isolated from these plants (Omoniyi & Yaqub, 2024) [29]. Many antimicrobial compounds found in secondary metabolites of plants make them effective against Gram +ve and Gram -ve bacteria (Gorlenko *et al*., 2020) [17]. Interestingly, plant extracts exert antimicrobial activity by disrupting the plasma membrane, inhibiting the efflux pump, delocalizing electrons, and conjugating with sugar (Álvarez-Martínez *et al*., 2021) [7]. These extracts are abundantly available, inexpensive, least toxic to host cells (Ahmed *et al*., 2016) [5] and eliminate the infections caused by drug-resistant pathogenic microorganisms (Uzma *et al*., 2018) [44], making them potential alternative therapeutics for microbial infection. So far, several herbal preparations have been devised for their uses as antimicrobial agent (Gyawali & Ibrahim, 2014; Namita & Mukesh, 2012; Parham *et al*., 2020) [18, 28, 31]. The details about the advances in Medicinal Plant antimicrobial activity have been elucidated recently (Vaou *et al*., 2021) [45]. *Syzygium aromaticum* (Family: Myrtaceae). It is widely found in Africa, Asia, Madagascar, and Pacific as well as Oceanic regions (Ullah *et al*., 2023) [43]. It is commercially grown in India, Sri Lanka, Madagascar, China (southern region), and Indonesia (Pandey *et al*., 2024). The Clove flower bud has been traditionally used as a medicinal purpose (Batiha *et al*., 2020) [10]. Batiha *et al*. (2020) [10] have described that this herbal medicine shows the antioxidant, analgesic, anticancer, anti-depressant, antiseptic, antispasmodic, anti-inflammatory, antiviral, antiparasitic activity, hepatoprotective activity and thereby the antibacterial as well as the antifungal properties.

Specifically, Hiwandika *et al*. (2021) [20] intervened that the antibacterial and antifungal activity of *Syzygium aromaticum* occurred due to presence of eugenol and β-caryophyllene. Earlier studies used the various concentration of ethanolic extract of clove flower bud on the *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Salmonella enterica subsp.enterica serovar Typhi, Staphylococcus aureus, Staphylococcus epidermidis, Candida albicans* and *Saccharomyces cerevisiae, Klebsiella quasipneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* and reported the invitro antibacterial activity by determining the zone of inhibition (ZOI) and MIC (Ababutain, 2013; Adhikari *et al*., 2021; Agu & Omebere, 2024; Ajobiewe *et al*., 2022; Dardona & Dardona, 2023; Elisha *et al*., 2022; Hiwandika *et al*., 2021; Liu *et al*., 2021; Omoniyi & Yaqub, 2024; Pundir *et al*., 2010; Saleh *et al*., 2024; Singh, 2018; SKN, 1990; Solanki *et al*., 2022) [1, 2, 4, 14, 20, 29, 33, 46, 39, 40]. However the MBC of clove ethanolic extract on these bacteria wasn't clearly illustrated. So far as no research has investigated the effect of various concentration of ethanolic extract of clove flower bud against the proteus vulgaris. Therefore, the present study was designed to evaluate the antimicrobial activity of ethanolic extract of flower bud of *Syzygium aromaticum* at various concentration by determining the ZOI along with MBC on these above mentioned bacterial and fungal species.

Material and Methods

Collection of plant materials

The dried flower buds of cloves were procured from local retail markets and subsequently transported to the Natural Products Research Laboratory located in Thapathali, Nepal for extraction process.

Preparation of ethanolic extract of flower buds of cloves

The clove flower bud (dried) was crushed using electric blender to make it fine powder. Then the powder was macerated in 100% ethanol for 48 hr with timely shaking. After that, the solution was filtered through the whatman No.1 filter paper. Then, the filtrates were concentrated using a rotary vacuum evaporator under reduced pressure at a temperature of 40–45°C. Then the extracts were dried in low temperature hot air oven to remove excess alcohol and stored in the refrigerator at 4 ºC until further use.

Test microorganisms:

Test organism	strain
Bacillus subtilis	ATCC 6051
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 8739
Klebsiella quasipneumoniae	ATCC 700603
Proteus vulgaris	ATCC 6380
Pseudomonas aeruginosa	ATCC 9027
Staphylococcus aureus	ATCC 6538P
Staphylococcus epidermidis	ATCC 12228
Candida albicans	ATCC 10231
Saccharomyces cerevisiae	ATCC 18824
Salmonella enterica subsp. *enterica* serovar Typhi	Clinical isolates (Tribhuvan University Teaching Hospital, Maharajgunj, Kathmandu, Nepal)
Shigella dysenteriae	Clinical isolates (Tribhuvan University Teaching Hospital, Maharajgunj, Kathmandu, Nepal)

Inoculation suspension:

Colonies from 18–24-hour-old cultures of the test microorganisms were added to normal saline with subsequent vortexing until the turbidity of the suspension matched that of the 0.5 McFarland nephelometer standard. The inoculation suspensions, so prepared, were used within 15 minutes of preparation.

Preparation of test solution:

Dimethyl sulphoxide (DMSO) was added to 1 g of clove extract to make the final volume to 10 mL subsequently vortexing to make the 100 mg/ml concentration of test solution. Similarly, test solutions of concentrations 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml were prepared by two-fold serial dilution of 100 mg/ml in DMSO.

Screening for antimicrobial activity:

Modified agar well diffusion method as described by (Perez, 1990) was followed. Appropriate media, i.e. Muller-Hinton agar (MHA) for bacteria and MHA along with glucose and methylene blue (MHA GMB) for yeasts, were prepared, sterilized and

poured in the sterile 90 mm Petri plates. The inoculum suspensions were applied to the culture medium's dry surface by using a sterile swab. Two such plates were prepared for each microbial species. On one of the two plates, three 6 mm-diameter wells were made, one each at the center of three of the four quadrants of the plate, using sterile cork borer (two wells for 100 mg/ml and 50 mg/ml test solutions and one well for DMSO as a negative control (NC)). On the other inoculated plate, four such wells were made, three at the centers of three quadrants for 6.25 mg/ml, 12.5 mg/ml and 25 mg/ml test solutions, and one at the center of the plate for DMSO as a negative control. 50 µl of the test solutions and DMSO were added to each well using micropipettes. A suitable antimicrobial sensitivity disc (@Ciprofloxacillin for *Bacillus subtillis, Enterococcus faecalis, Escherichia coli, Klebsiella quasipneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enterica subsp.enterica serovar Typhi* and *Shigella dysenteriae* , Amoxicillin for *Staphylococcus aureus* and *Staphylococcus epidermidis* and clotrimazole for *Candida albicans* and *Saccharomyces cerevisiae* , as positive control(PC)), was placed at the center of the fourth quadrant of each of the inoculated petridish. The plates were allowed to remain in upright position for half an hour to allow the test solutions and negative control to diffuse into the media. Then, inoculated plates were incubated in inverted position at suitable temperature for suitable duration (at 35±2˚C for 16-18 hrs for bacteria, at 35±2˚C for 20-24 hrs for *C. albicans* and at 25±2˚C for 24-48 hrs for *S. cerevisiae*). After incubation, zones of inhibition (ZOI) formed around the wells were measured using vernier caliper.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC):

For the microorganisms showing ZOI, broth macrodilution method, as described by (Sykes & Rankin, 2013) and (Balouiri *et al*., 2016), was used to MIC and MBC/MFC of the extract. Two-fold serial dilution of the test solution in Mueller-Hinton broth (MHB) or Sabouraud dextrose broth (SDB) was done in 5 ml screw capped vials so that each vial contained 1 ml of the solutions of extract of concentrations 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.5625 mg/ml, 0.78125 mg/ml and 0.390625 mg/ml. Inoculum suspensions of the test microorganisms of density 5 x 10^5 cfu/ml was prepared in MHB for bacterial species and of density 0.9-4.5 x 10^3 cfu/ml in SDB for fungal species. The suspensions were then added to each vial of the dilution series 1 ml per vial. This resulted in 1:2 dilution of the extract in the vials. Two vials of 1 ml pure MHB or SDB were also maintained. One of them was left uninoculated as negative growth while 1 ml inoculum suspension was added to the other one for positive growth. All the tubes were incubated at suitable temperatures for suitable durations (at 35±2˚C for 16-18 hrs for bacteria, at 35±2˚C for 20-24 hrs for *C. albicans* and at 25±2˚C for 24-48 hrs for *S. cerevisiae*). After incubation the vials were examined for growth as indicated by turbidity in comparison to negative growth vial. The MIC of the extract was determined as the minimum concentration which didn't show any visual growth after incubation. The 0.005 ml of the MIC vial contents were drawn using a calibrated loop and were sub-cultured on suitable fresh media (nutrient agar (NA) for bacteria and Sabouraud dextrose agar (SDA) for fungi) at the same temperature and duration as for MIC vials and observed for the 99.9% killing (Bayoub *et al*., 2010; Maharjan *et al*., 2019). MBC or MFC was interpreted as the minimum concentration for which subculture didn't show any growth.

Statistical analysis

The experiment was done in triplicate. All the data are presented as the Mean ± SD. Statistical analyses were performed using the SPSS information system for windows (SPSS V 27, SPSS Institute Inc. USA). Data of the diameter of ZOI were analyzed using One-Way ANOVA followed by LSD for multiple mean comparisons. The P values less than 0.05 was considered significant.

Results

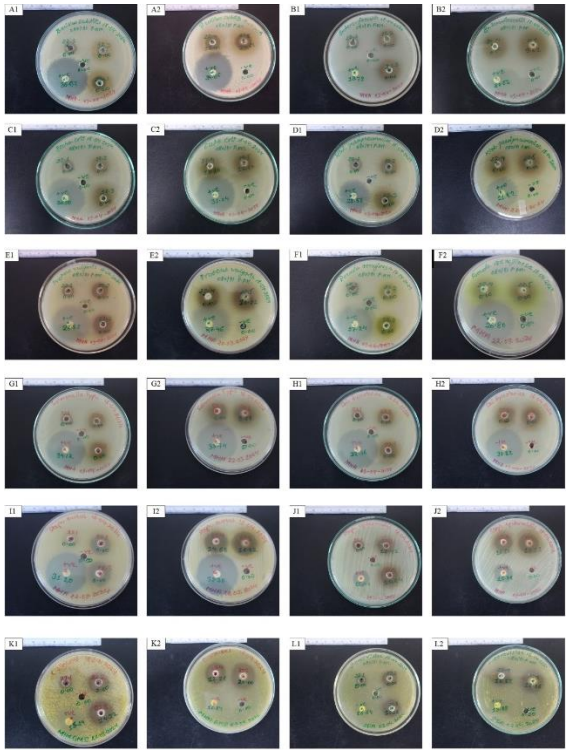

Fig 1: Zone of inhibition (ZOI) determination of clove's ethanolic extract against various bacteria: A1/A2 (*Bacillus subtillis*), B1/B2 (*Enterococcus faecalis*), C1/C2 (*Escherichia coli*), D1/D2 (*Klebsiella quasipneumoniae*), E1/E2 (*Proteus vulgaris*), F1/F2 (*Pseudomonas aeruginosa*), G1/G2 (*Salmonella enterica* subsp. *enterica* serovar Typhi), H1/H2 (*Shigella dysenteriae*), I1/I2 (*Staphylococcus aureus*), J1/J2(*Staphylococcus epidermidis), K1/K2 (Candida albicans),* L1/L2 (*Saccharomyces cerevisiae*)

Table 1: Zone of inhibition (ZOI) of different concentration of clove's ethanolic extract on various bacteria/fungi

Bacteria/Fungi	ZOI (mm)							P-value
	Extract concentration in DMSO (mg/ml)					Control		
	100	50	25	12.5	6.25	NC	PC	
Bacillus subtillis	14.75±0.67	12.59±1.13	0±0	0±0	0±0	0±0	35.47±0.76	0.00
Enterococcus faecalis	9.65±0.41	0±0	0±0	0±0	0±0	0±0	20.59±0.90	0.00
Escherichia coli	13.13±0.41	10.32±0.79	0±0	0±0	0±0	0±0	30.68±0.70	0.00
Klebsiella quasipneumoniae	0±0	0±0	0±0	0±0	0±0	0±0	22.43±0.55	0.00
Proteus vulgaris	10.12±0.70	0±0	0±0	0±0	0±0	0±0	28.60±1.05	0.00
Pseudomonas aeruginosa	0±0	0±0	0±0	0±0	0±0	0±0	27.33±0.57	0.00
Salmonella enterica subsp. enterica serovar Typhi	8.71±0.86	0±0	0±0	0±0	0±0	0±0	33.79±0.30	0.00
Shigella dysenteriae	0±0	0±0	0±0	0±0	0±0	0±0	31.78±1.03	0.00
Staphylococcus aureus	17.52±0.50	14.52±0.77	0±0	0±0	0±0	0±0	32.28±1.07	0.00
Staphylococcus epidermidis	17.31±0.68	16.70±0.46	15.31±0.97	12.40±0.84	0±0	0±0	16.02±0.70	0.00
Candida albicans	20.85±0.65	17.54±0.91	14.81±0.45	0±0	0±0	0±0	16.48±1.10	0.00
Saccharomyces cerevisiae	19.05±0.51	12.75±0.61	11.43±0.89	0±0	0±0	0±0	14.72±0.88	0.00

The different concentration (6.25, 12.5, 25, 50 and 100 mg/ml) of clove ethanolic extract were resistant to *Klebsiella quasipneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae*. The different concentration (6.25, 12.5, 25, 50 and 100 mg/ml) of this extract on *Bacillus subtillis*, *Enterococcus faecalis*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella enterica subsp. enterica serovar Typhi* and *Staphylococcus aureus* were found sensitive and has lower (p=0.00) zone of inhibition as compared to the PC. The 6.25 mg/ml and 12.5 mg/ml concentration of this extract on *Staphylococcus epidermidis* produces the lower (p=0.00) zone of inhibition as compared to the PC. However, 50 mg/ml extract produces diameter of ZOI equally similar(p=0.224) to PC on *Staphylococcus epidermidis*. However, the 100mg/ml clove extract on *Staphylococcus epidermidis* produces the higher (p=0.031) ZOI as compared to the PC. The 6.25 mg/ml, 12.5mg/ml and 25 mg/ml clove extract on *Candida albicans* produces the lower (p=0.00:0.007) diameter of ZOI as compared to the PC. The diameter of ZOI of 50 mg/ml clove extract and PC on *Candida albicans* were equally similar(p=0.062). However, the 100mg/ml clove extract on *Candida albicans* produces the higher (p=0.00) diameter of ZOI as compared to the PC. The 6.25 mg/ml and 12.5 mg/ml and 25 mg/ml clove extract on *Saccharomyces cerevisiae* produces the lower (p=0.00) diameter of ZOI as compared to the positive control. The diameter of ZOI of 50 mg/ml clove extract and PC on *Saccharomyces cerevisiae* were equally similar(p=0.001). However, the 100mg/ml clove extract on *Saccharomyces cerevisiae* produces the higher (p=0.00) diameter of ZOI as compared to the PC.

Minimum inhibitory concentration (MIC)

MIC of clove ethanolic extract against *Klebsiella quasipneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* weren't tested because no ZOI was observed on these bacteria. However, MIC of this extract against *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella enterica subsp. enterica serovar Typhi*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Saccharomyces cerevisiae* couldn't be interpreted due to turbid nature of sample (Bubonja-Šonje *et al*., 2020).

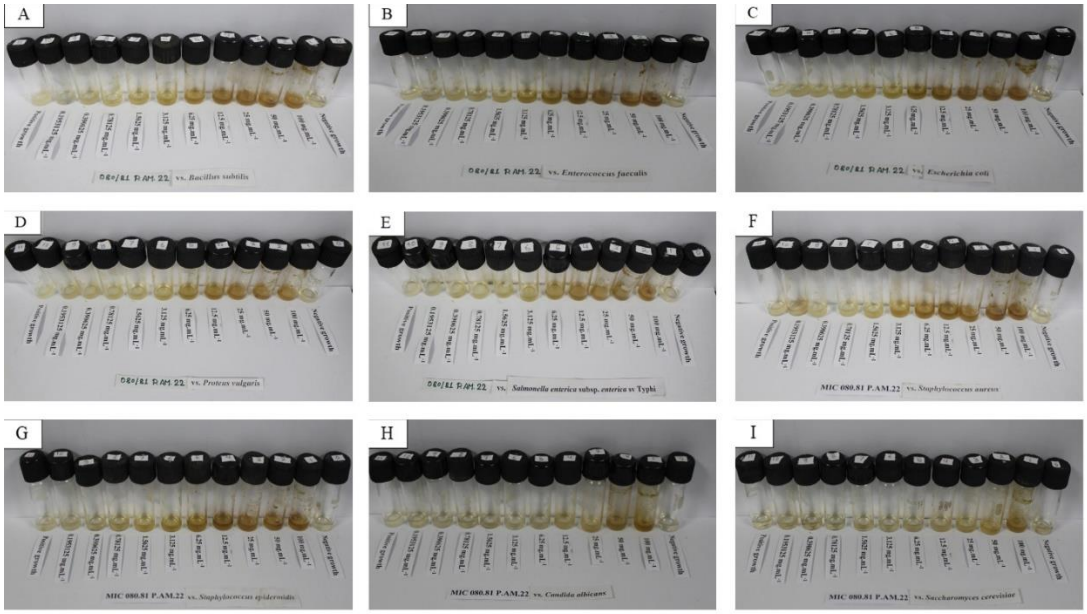

Fig 2: Process of determination of MIC of clove ethanolic extract against various bacteria / fungi: A (*Bacillus subtillis*), B (*Enterococcus faecalis*), C (*Escherichia coli*), D (*Proteus vulgaris*), E (*Salmonella enterica subsp. enterica serovar Typhi*), F (*Staphylococcus aureus*), G (*Staphylococcus epidermidis*), H (*Candida albicans*), I (*Saccharomyces cerevisiae*)

Minimum Bactericidal concentration (MBC)/ Minimum Fungicidal concentration (MFC)

MBC of clove ethanolic extract against *Klebsiella quasipneumoniae*, *Pseudomonas aeruginosa* and *Shigella dysenteriae* weren't tested because no ZOI was observed on these bacteria. However, MBC/MFC of this extract against *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Salmonella enterica subsp. enterica serovar Typhi, Staphylococcus aureus, Staphylococcus epidermidis, Candida albicans* and *Saccharomyces cerevisiae* as presented as below.

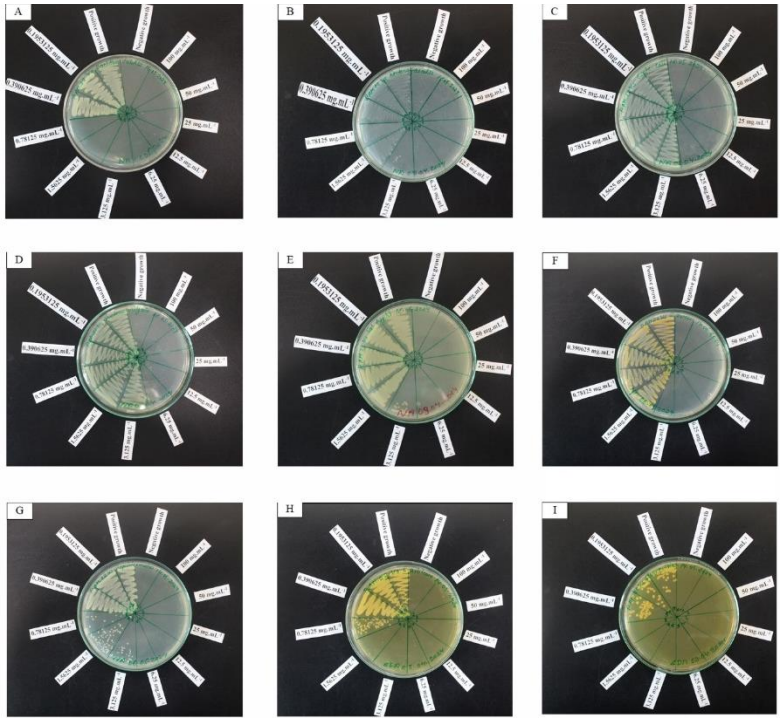

Fig 3: MBC/MFC of clove ethanolic extract against various bacteria / fungi: A (*Bacillus subtillis*), B (*Enterococcus faecalis*), C (*Escherichia coli*), D (*Proteus vulgaris*), E (*Salmonella enterica subsp. enterica serovar Typhi*), F (*Staphylococcus aureus*), G (*Staphylococcus epidermidis*), H (*Candida albicans*), I (*Saccharomyces cerevisiae*)

Table 2: Representation of MBC/ MFC determination of clove ethanolic extract on various microorganisms

Microorganism	Concentration of clove ethanolic extract in mg/ml										
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19	MBC/MFC
Bacillus subtillis	-	-	-	-	-	-	-	-	+	+	0.78mg/ml
Enterococcus faecalis	-	-	-	-	-	-	+	+	+	+	3.125mg/ml
Escherichia coli	-	-	-	-	-	+	+	+	+	+	6.25mg/ml
Proteus vulgaris	-	-	-	-	-	+	+	+	+	+	6.25mg/ml
Salmonella enterica subsp. enterica serovar Typhi	-	-	-	-	-	+	+	+	+	+	6.25mg/ml
Staphylococcus aureus	-	-	-	-	-	-	+	+	+	+	3.12mg/ml
Staphylococcus epidermidis	-	-	-	-	-	+	+	+	+	+	6.25mg/ml
Candida albicans	-	-	-	-	-	-	-	+	+	+	1.56mg/ml
Saccharomyces cerevisiae	-	-	-	-	-	-	-	-	+	+	0.78mg/ml

No growth (-); Presence of growth (+)

Table 3: MBC/MFC value of ethanolic extract of clove against different bacteria/fungi

Bacteria/Fungi	MBC/MFC (mg/ml) Mean ± SD
Bacillus subtilis	0.78±0.00
Enterococcus faecalis	2.60±0.90
Escherichia coli	5.21±1.80
Proteus vulgaris	5.21±1.80
Salmonella enterica Typhi	6.25±0.00
Staphylococcus aureus	2.60±0.90
Staphylococcus epidermidis	5.21±1.80
Candida albicans	1.30±0.45
Saccharomyces cerevisiae	0.78±0.00

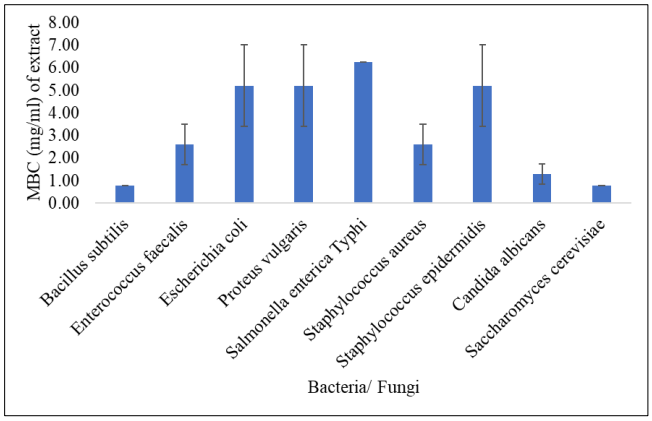

Fig 4: MBC/MFC value of ethanolic extract of clove against different bacteria/fungi

Discussion

The antimicrobial activity of clove ethanolic extract on various bacteria and fungi were investigated on this study. The different concentration (6.25, 12.5, 25, 50 and 100 mg/ml) of clove ethanolic extract were resistant to *Klebsiella quasipneumoniae, Pseudomonas aeruginosa* and *Shigella dysenteriae*. These findings of this research are in contrast with the previous studies (Liu *et al*., 2021; SKN, 1990) , which showed that *Klebsiella quasipneumoniae, Pseudomonas aeruginosa* and *Shigella dysenteriae* were sensitive to clove ethanolic extract. This discrepancy could potentially be attributed to variations in the sample taken (stage of plant) and extraction methods utilized which affect the phytoconstituents of extract. The different concentration (6.25, 12.5, 25, 50 and 100 mg/ml) of clove ethanolic extract are sensitive against *Bacillus subtillis, Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Salmonella enterica subsp. enterica serovar Typhi, and Staphylococcus aureus*. Clove extract has significantly lower diameter of ZOI as compared to the positive control (Ciprofloxacillin and amoxicillin). This bactericidal activity is due to the active compound eugenol (2-methoxy-4-(2-propenyl) phenol), glycosides, flavonoids, saponins and tannins present in the clove ethanolic extract (Faujdar *et al*., 2020) [15]. These compound causes the membrane fatty acid alteration, cell morphology changes and cytoplasmic membrane disruption, effect on ion and ATP transport, generation of intracellular ROS (reactive oxygen species) and inhibit the cellular enzyme, all of which leads to cell death of bacteria (Marchese *et al*., 2017) [25]. These findings are inconsistent with the previous research (Agu & Omebere, 2024; Ajobiewe *et al*., 2022; Elisha *et al*., 2022; Pundir *et al*., 2010; Saleh *et al*., 2024) [4, 6, 14, 33, 35]. The 6.25 mg/ml and 12.5 mg/ml clove extract on *Staphylococcus epidermidis* produces the significantly lower diameter of ZOI as compared to the positive control. The diameter of ZOI of 50 mg/ml clove extract and positive control on *Staphylococcus epidermidis* were equally similar. However, the 100mg/ml clove extract on *Staphylococcus epidermidis* produces the significantly higher diameter of ZOI as compared to the positive control. This concentration dependent bactericidal of this extract is due to eugenol inhibit essential enzyme production of bacteria and cause damage to the cell wall. These findings are inconsistent with the previous research (Singh, 2018) [40]. The 6.25 mg/ml, 12.5mg/ml and 25 mg/ml clove extract on *Candida albicans* produces the significantly lower diameter of ZOI as compared to the positive control. The zone of inhibition of 50 mg/ml clove extract and positive control on *Candida albicans* were almost equally similar. However, the 100mg/ml clove extract on *Candida albicans* produces the significantly higher diameter of ZOI as compared to the positive control. This concentration dependent antifungal activities are due to clove's major bioactive constituents/secondary metabolites: phenols, alkaloids and tannins (Dardona & Dardona, 2023) [13]. The 6.25 mg/ml and 12.5 mg/ml and 25 mg/ml clove extract on *Saccharomyces cerevisiae* produces the significantly lower diameter of ZOI as compared to the PC. The diameter of ZOI of 50 mg/ml clove extract and positive control on *Saccharomyces cerevisiae* were equally similar (p=0.001). However, the 100mg/ml clove extract on *Saccharomyces cerevisiae* produces the higher diameter of ZOI as compared to the positive control. This is due to clove ethanolic extract causes the fungicidal effect on *Saccharomyces cerevisiae* in concentration dependent manner through the granulation of cytoplasm, rupture of cytoplasmic membrane, and intracellular as well as extracellular enzymes inactivation/inhibition. These findings are inconsistent with the previous research (Solanki *et al*., 2022) [40].

The MBC of clove ethanolic extract on *Bacillus subtilis* was 0.78±0.00 mg/ml. This finding of this research diverges from the earlier research (Pundir *et al*., 2010) [33]. Pundir *et al*. (2010) [33] showed the MBC of this extract was 20 mg/ml on *Bacillus subtillis*. The MBC of clove ethanolic extract on *Enterococcus faecalis* was 2.60±0.90 mg/ml. This value was almost similar with the previous research (Saleh *et al*., 2024) [35]. Saleh *et al*. (2024) [35] estimated the MBC of this extract on *Enterococcus faecalis* was 3.125 mg/ml. MBC of clove ethanolic extract on *Escherichia coli* was 5.21±1.80mg/ml. MBC of this extract on *Escherichia coli* wasn't previously determined. Shehu *et al*. (2023) [36] only determined the MIC value of this extract on the *Escherichia coli* which was 6.25mg/ml. MBC of clove ethanolic extract on *Proteus vulgaris* was 5.21±1.80 mg/ml. The antibacterial activity of this extract on *Proteus vulgaris* wasn't previously studied. MBC of clove ethanolic extract *Salmonella enterica Typhi* was 6.25±0.00 mg/ml. This value is higher than the previous findings (Elisha *et al*., 2022) [4]. Elisha *et al*. (2022) [4] revealed the MBC of this extract on *Salmonella enterica Typhi* was 3.9mg/ml. MBC of clove ethanolic extract on *Staphylococcus aureus* was 2.60±0.90 mg/ml. This finding was in contrast with the previous study (Shehu *et al*., 2023) [36] . Shehu *et al*. (2023) [36] showed the MBC value of this extract on *Staphylococcus aureus* was 6.25 mg/ml. MBC of clove ethanolic extract on *Staphylococcus epidermidis* was 5.21±1.80 mg/ml. MBC of this extract on same bacteria wasn't previously determined. However, MBC of clove methanolic extract against *Staphylococcus epididermis* was estimated as 12.5 mg/ml (Mekky *et al*., 2024) [26]. MFC of clove ethanolic extract on *Candida albicans* was 1.30±0.45mg/ml. This finding is in contrast with the result of earlier research (Afanyibo *et al*., 2018) [3]. Afanyibo *et al*. (2018) [3] determined the MFC of this extract on *Candida albicans* was 0.1953mg/ml. MFC of clove ethanolic extract on *Saccharomyces cerevisiae* was 0.78±0.00 mg/ml. This value was lower with the findings of previous study (Wang *et al*., 2017) [47]. Wang *et al*. (2017) [47] showed the MFC of this extract on *Saccharomyces cerevisiae* was 7.16±1.60 mg/ml. The MBC of value of this extract on *Bacillus subtillis, Salmonella enterica Typhi, Staphylococcus aureus, Candida albicans* and *Saccharomyces cerevisiae* obtained in this research varied with the previous studies which might be due to difference in sample obtained from different localities, plant material: solvent ratio and variation of extraction process that leads to different in phytoconstituents of extract

(Stéphane *et al*., 2021) [41].

Conclusion

Klebsiella quasipneumoniae, Pseudomonas aeruginosa and *Shigella dysenteriae* were found resistant to clove ethanolic extract. The lower concentration of the clove ethanolic extract (6.25mg/ml, 12.50mg/ml, 25 mg/ml and 50 mg/ml) on *Enterococcus faecalis, Proteus vulgaris, Salmonella enterica Typhi, Staphylococcus aureus and Staphylococcus epidermidis* were found insensitive, however the 100mg/ml concentration of this extract on these bacteria showed the diameter of ZOI 9.65±0.41mm, 10.12±0.70mm, 8.71±0.86mm, 17.52±0.50mm and 17.31±0.68mm respectively. The lower concentration of clove ethanolic extract (6.25mg/ml, 12.5mg/ml and 25 mg/ml) were insensitive to *Bacillus subtilis* and *Escherichia coli* however, higher concentration (50 mg/ml) of this extract showed the diameter of ZOI 12.59±1.13mm and 10.32±0.79mm respectively. Similarly, higher concentration (100 mg/ml) of this extract showed the diameter of ZOI 14.75±0.67mm and 13.13±0.41mm on these bacteria respectively. The MBC of this extract on *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Salmonella enterica Typhi, Staphylococcus aureus* and *Staphylococcus epidermidis* were 0.78±0.00 mg/ml, 2.60±0.90 mg/ml, 5.21±1.80 mg/ml, 5.21±1.80 mg/ml, 6.25±0.00 mg/ml, 2.60±0.90 mg/ml, 5.21±1.80 mg/ml respectively. MFC value of clove ethanolic extract on the *Candida albicans* and *Saccharomyces cerevisiae* were 1.30±0.45 mg/ml and 0.78±0.00 mg/ ml respectively.

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