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Methanol extract of clove (*Syzygium aromaticum Linn.*) damages cells and inhibits growth of enteropathogens

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Abstract: Damage to cell membrane and inhibition of growth of pathogenic bacteria *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus pumilus* by the clove extract in 80% methanol is examined. The antimicrobial activity was examined measuring cell inhibition zone and minimum inhibitory concentration for the pathogens was determined. The growth inhibition zones observed by agar well diffusion method were 24-30 mm in diameter in presence of clove extract. Minimum inhibitory concentration of clove was 3.9 mg/ml for *E. coli,* 1.95 mg/ml for *P. aeruginosa* and 0.98 mg/ml for *S. aureus,* and *B. pumilus*. Increased release of intracellular nucleotides and proteinaceous materials from the bacterial cells in the presence of methanol clove extract containing polyphenols suggests that the primary mechanism of action of clove extract is membrane damage, which leads to cell death. The results obtained herein further encourage the use of clove extract in antibacterial formulations.

Keywords: Clove, Antimicrobial, Cell damage, Growth inhibition, MIC, Polyphenols; Enteropathogens

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An uprising trend of using processed food, increasing demand for ready to use food products and changes in the dietary habits is followed by increasing reports of food borne pathogenic micro-organisms. Cold distribution of perishable food can help but cannot guarantee the overall safety and quality of products. Maintaining safety and quality of food has become a challenge since there is considerable unease regarding the use of chemical and synthetic antimicrobial compounds to prevent and inactivate growth of pathogenic bacteria. Consumer awareness saying no to synthetic antimicrobial agents has diverted the attention of researchers towards natural antimicrobial components to reduce the need of antibiotics, control microbial contamination, improve shelf life of food and decrease the development of antibiotic resistance by pathogenic micro-organisms

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(Abou-taleb and Kawai, 2008; Sharma et al. 2014; Tajkarimi et al. 2010). The polyphenolics have evolved in plants as antioxidant and antimicrobial agents against environmental stress due to a variety of oxidizing and potentially harmful free radicals. Various herbs and spices, traditionally used in Indian cuisins have good antimicrobial properties, in addition to imparting taste and flavour to food (Fabio et al. 2003; Vaishnavi et al. 2007).

Cloves are the aromatic dried buds of a tree *Syzygium aromaticum (Linn.)* used as spice all over the world. Clove is known for its health benefits over the centuries. The major part of world`s consumption is in food preparations. It is also used as home remedy in curing several ailments. Clove oil has antioxidant, antiviral, antifungal, antidiabetic, anti-inflammatory, antithrombotic, anesthetic, pain relieving and insect repelling effect (Milind and Khanna, 2011). Antimicrobial activity of clove against enteropathogens *Salmonella* species, *Shigella* species and *E. coli,* has been reported by Vashnavi et al. 2007. Aqueous clove extract is found effective against both *Steptococcus* mutants and *Candida albicans* (Pachori et al. 2012). Fabio et al, (2003) have shown inhibitory effect of clove

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extract on the growth of *Aeromonas hydrophila*, *Listeria monocytogenes* and *Yorsinia enterococli* but the mechanism of antimicrobial activity and active components in clove are yet to be explored. An understanding of how the natural substances work to prevent or inhibit the growth of pathogenic bacteria can help to develop new effective methods that rely primarily on their use to enhance food safety (Patrignani et al. 2008). Extracts of plants generally are mixtures of several components such as polyphenols, terpenoids, alkaloids etc and have both antioxidant and antimicrobial characteristics. The present study was undertaken to determine the antimicrobial activity of methanol clove extract against food borne pathogenic bacteria and quantitatively analyse the polyphenolic contents of the methanolic clove extract.

MATERIALS AND METHODS

The Clove (*Syzygium aromaticum)* buds were procured from the local market, identified and authenticated at Department of Botany, Kurukshetra University, Kurukshetra, India.

Extraction

Cloves were dried at 50°C in hot air oven till constant weight was attained. Finely ground clove powder was extracted with 80% methanol (1g/10ml) in a shaker at room temperature for 4 h. Residue was extracted again with 80% methanol for 2 h. Collected extract was filtered through double layered muslin followed by centrifugation at 5000g for 5min to get clear supernatant. Extract was concentrated in a vacuum evaporator and stored at -20°C for further use. The extract was diluted appropriately for different experiments.

Test Organisms

Four enteropathogenic and food-spoiler bacterial strains [two gram negative bacteria i.e. *Escherichia coli* (MTCC 119), *Pseudomonas aeruginosa* (MTCC 741) and two gram positive bacteria i.e. *Staphylococcus aureus* (MTCC 96) and *Bacillus pumilus* (MTCC 7411)] were obtained from MTCC, IMTECH, Chandigarh, India. All bacterial cultures were maintained and subcultured regularly on Nutrient agar media (NAM) containing peptone 5g; beef extract 3g; sodium chloride 5g and agar 2% in a final volume of 1.0L.

The size of inoculum was adjusted to approximately 10^8 colony-forming units per ml by suspending the culture in sterile distilled water. Petridishes containing nearly 25 ml of nutrient agar medium were seeded with 100 µl culture of the respective bacterial strains and kept for 15 min for the absorption of culture.

Bacterial Cell Damage

Bacterial cell cultures incubated in presence and absence of clove extract for 30min at 37°C were analyzed spectrophotometrically to estimate cell damage as described by Wong and Kitts (2006). The culture was grown in nutrient broth up to the log phase of the culture. The cultured broth was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was suspended in 20 mM phosphate buffer of pH 7.0. Culture was washed again and turbidity of each suspension was adjusted to 0.5 McFarland units by suspending the cultures in sterile phosphate buffer. To check the amount of cell damage due to clove extract, 100 µl of bacterial suspension was incubated with 100 µl of clove extract (equivalent to 1.25mg dry weight per ml extract) at 37°C for 30 min in water bath. The spectra were observed at 230 – 350 nm before and after incubation for 30 min against methanol blank.

Well Diffusion Assay

Using a sterile cork borer, nearly 8mm diameter wells were bored in the seeded agar plates and a 100 µl volume of clove extract (equivalent to 25 mg dry weight) diluted in 10 % methanol was added into the wells. All the plates were incubated at 37°C for 24 h. Antibacterial activity was determined by measuring the zone of inhibition around the well using agar well diffusion assay technique (Andrew, 2001). The antimicrobial activities of the clove extract were compared against the standard drugs, ampicillin and chloramphenicol (25 µg/ml; negative control) and 10% methanol (positive control). These tests were performed in triplicate and the mean of inhibition diameter was taken.

Determination of Minimum Inhibitory Concentration

MIC of clove was determined by the agar well diffusion method. Petri dishes containing 25 ml nutrient agar medium were swabbed with the 100 µl culture of inoculum containing approximately 10^8 colony-forming units per ml. Twofold serial dilutions ranging from 125 – 0.243 mg/ml concentrations of clove extract were made in 10% methanol and used to determine antimicrobial activity.

Estimation of polyphenol content

The total content of phenolic compounds in clove extract was determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977). Gallic acid was used as the standard compound. Standard/ Extract (100 µl) was added to 2% Na_2CO_3 (2.0 ml). After 2 min, 50% Folin-Ciocalteu reagent (100 µl) was added to the mixture. Absorbance at 750 nm was measured after 30 min.

RESULTS AND DISCUSSION

Synthetic chemicals are often used as preservatives in food processing and storage to inhibit food-borne pathogens and to extend shelf life. Antibiotics are generally an efficient means of treating bacterial infections. Use of synthetic antimicrobial agents and treatment with antibiotics is not only expensive but the risk of bacterial resistance to antimicrobial agents is also involved. The intake of the antibiotics causes side effects such acidity, burning sensation and damage

to natural fauna of intestine. EOs derived from spices and plants have antimicrobial activity against various pathogens (Burt, 2004; Tajkarimi et al. 2010). Aromatic and volatile oily liquids from flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots of plants have antimicrobial activities. Essential oils from plants such as oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, sage and vanillin have antimicrobial properties (Gutierrez et al. 2009). In the present study, the antimicrobial potential of the methanolic extract of clove against pathogenic bacteria *E. coli, P. aeruginosa* causing gastroenteritis or urinary tract infections, *B. pumilus* causing stomach cramps, food poisoning and *S. aureus* causing pneumonia, food poisoning and toxic shock syndrome (TSS) is examined. Methanol extract of clove was examined for inhibition of bacterial growth by well diffusion method (Fig 1, Table 1). The growth inhibition zone in presence of extract equivalent to 25mg clove buds was 24-30mm for the bacteria tested. These values are higher than 18-26mm for chloramphenicol (25µg/ml), but less than 36-44mm for ampicillin (25µg/ml) standard used indicating that clove extract is an efficient antibacterial agent. Aqueous extract of clove prepared by soaking or boiling are reported to have antimicrobial activity with inhibitory zone of 12.33mm for *Streptococcus* mutants and 14.66 mm for *Candiala albicans* (Pachori et al. 2012). Clove extracts inhibited the growth of *B. cereus, E. coli* and *S. aureus* with inhibition zone measuring 14-25mm (Tajkarimi et al. 2010). Metahnol extract of clove used in present study has inhibited the bacterial growth more effectively. Clove extract has exhibited antimicrobial activity against all the four bacteria tested. Vashnavi et al, (2007) have also reported antimicrobial activity of aqueous clove extract against *Salmonella* sps., *Shigella* sps., and *E. coli*. The Plant substances affect microbial cells by various antimicrobial mechanisms, including attacking the phospholipid bilayer of cell membrane,

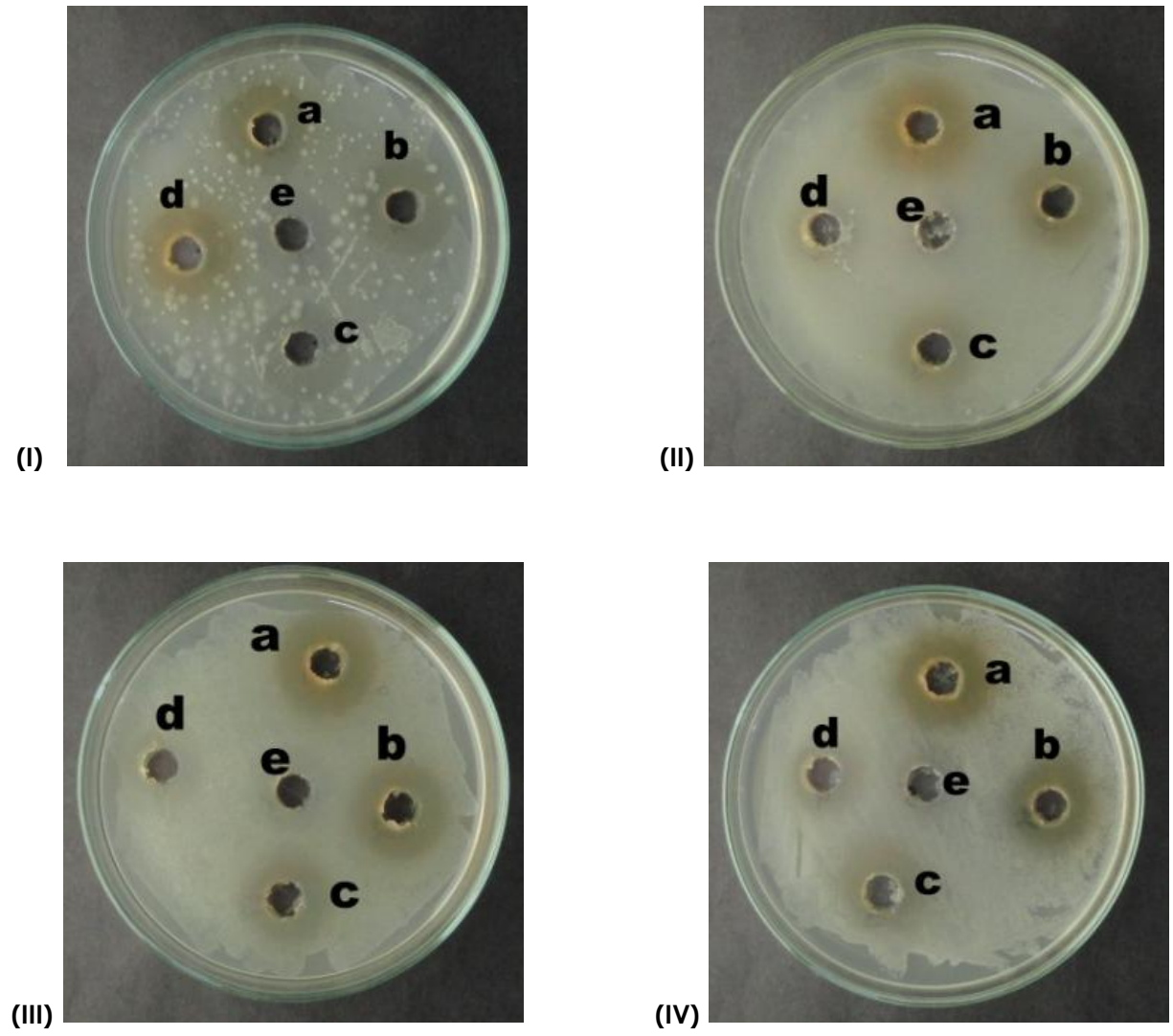

Figure 1 Growth inhibition in presence of different concentrations of clove extract; **(I)** *E. coli*; **(II)** *P. aeroginosa*; **(III)** *S. aureus*; **(IV)** *B. pumilus*. **a-e** concentrations are 12.5, 6.25, 3.125, 1.56, 0.78 mg/ml respectively

Table 1 Diameter of Growth inhibition zone (mm) by clove extract and standard antibiotics

Antibacterial principle	*Escherichia coli*	*Pseudomonas aerogenosa*	*Staphylococus aureus*	*Bacillus pumilus*
Clove extract (25mg/ml)	24	30	28	28
Chloramphanicol (25µg/ml)	18	24	24	26
Ampicillin (25µg/ml)	36	44	41	39
Methanol (10%)	0	0	0	0

disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids (Burt, 2004; Tajkarimi et al. 2010). Damaging impact of clove extract on the cell membrane integrity was observed as increase in absorbance at 260 and 280nm after incubation of the cells with extract. Results in figure 2 show that all the bacterial strains are sensitive to the presence of clove extract in the incubation mixture. Increased absorbance between 260nm to 280nm indicates leakage of intracellular nucleotides and proteinaceous materials into the growth medium and damage to the cell membranes (Wong and Kitts, 2006). Clove extract caused maximum increase in absorbance at 260nm, from 0.25 to 0.45 with *S. aureus* cells. *B. pumilus* culture

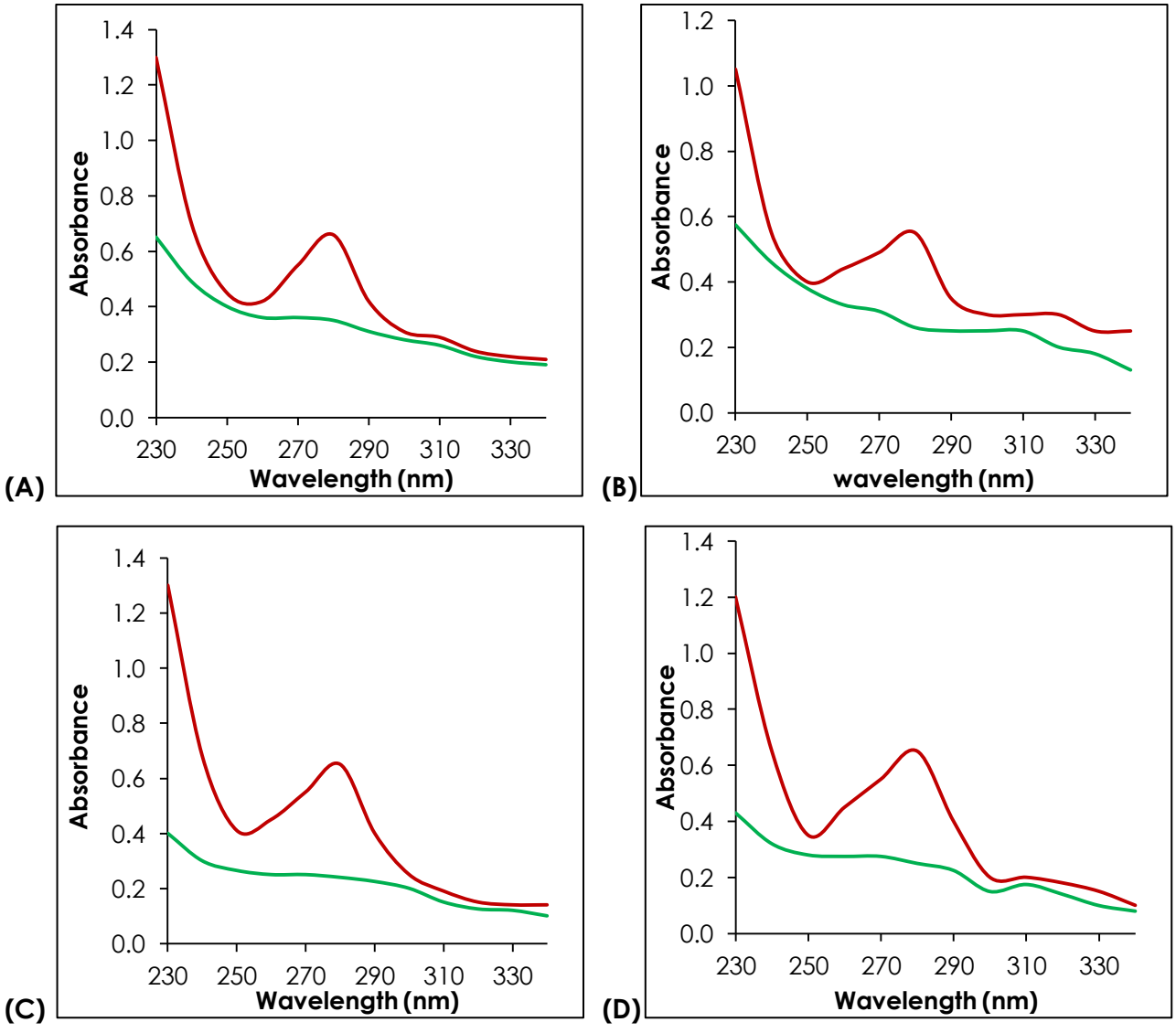

Figure 2 Bacterial cell damage in presence of clove extract (1.25mg/ml); (A) *E. coli*; (B) *P. aeruginosa*; (C) *S. aureus*; (D) *B. pumilus*. The lower (Green) and upper curve (red) indicate the absorbance of control sample and in the presence of extract respectively

Table 1 Minimum inhibitory concentration of clove extract and standard antibiotics

Antibacterial principle	*Escherichia coli*	*Pseudomonas aerogenosa*	*Staphylococus aureus*	*Bacillus pumilus*
Clove extract (25mg/ml)	3.90	1.95	0.98	0.98
Chloramphanicol (25µg/ml)	0.2	0.4	0.44	0.4
Ampicillin (25µg/ml)	0.5	0.5	0.8	0.5

has shown maximum increase in absorbance from 0.25 to 0.65 at 280nm on incubation with clove extract, indicating the highest damage to the membranes of these bacteria (Fig. 2). Incubation of *P. aeruginosa* and *E. coli* cells with clove extract resulted in an increase of absorbance by 0.06 - 0.11 at 260 nm and up to 0.29 at 280 nm. The results showed that clove extract is effectively inhibiting the growth of both gram negative as well as gram positive bacteria. Damage to cell membranes of *B. subtilis* and *E. coli* by parsley and cilantro leaves and stem extracts has been reported (Wong and Kitts, 2006). Methanol extracts of cumin (Dua et al. 2013) and coriander (Dua et al. 2014) are also found to damage the cell membranes of both gram positive and gram negative bacteria.

Minimum concentration of the clove extract, which can inhibit the growth of microbes i.e. MIC for *E. coli* determined by well diffusion method is 3.90 mg/ml, whereas inhibition of growth of *P. aeruginosa* is achieved in presence of extract containing 1.95 mg dry weight/ml. Growth of *B. pumilus* and *S. aureus* was inhibited at a lower concentration of 0.98 mg/ml (Table 2). The antimicrobial properties of aqueous extracts of various medicinal plant species against *E. coli* with an MIC value ranging from 0.09-6.25 mg/ml has been reported (Voravuthikunchai et al. 2004). MIC of 1.5-2.0 mg/ml for *Streptococcus* mutants and *Candiala albicans* is reported when cloves are extracted with distilled water (Pachori et al. 2012). MIC of clove observed here is within the reported extremities. Different antimicrobial activity of herbs against bacterial strains may be due to different bio-reactive compounds in the extracts prepared by different methods.

Growth of bacteria is sensitive to the redox potential of the media. Moderately reducing environment of the growth medium can contribute in part to the growth inhibition of various bacteria (Wong and Kitts, 2006). Extracts of various spices including clove are reported to have various polyphenolic compounds and antioxidant activities (Shan et al. 2005; Milind and Khanna, 2011). Clove extract prepared in 80% methanol for these studies had 11.25 mgGAE/g dry weights. Essential oils extracted from clove are reported to contain 74-82% eugnol (Milind and Khanna, 2011). Polyphenol content of extracts of various spices and herbs is correlated to high antioxidant and reducing properties (Shan et al. 2005). Clove extracts are reported to exhibit considerably high free radical scavenging and peroxide inhibition activity (Ramadan et al. 2013) indicating its reducing character, which may in part explain the inhibition of bacterial growth. Phenolics, in the extracts of the spices, with more than one hydroxyl groups have metal ion chelating property which may also be contributing to the antimicrobial properties by leading to the deficiency of essential metal ions in the growth medium.

Conclusion

Clove extract in 80% methanol has considerable amount of polyphenols, which are known for both reducing and metal chelating properties. Methanol extract of clove damages the cells and inhibits the growth of both gram positive and gram negative bacteria tested here. Clove is commonly used as spice in food preparations and is known for medicinal values in home remedies. The knowledge about the cell damage and inhibition of growth of various pathogens by clove extract adds to its application in the field of pharmacology, phytochemistry and food chemistry for the development of better medicinal or preservative preparations.

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