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Antibacterial potential of polyphenol rich methanol extract of Cardamom (*Amomum subulatum*)

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Abstract: The aim of the present study was to explore the antibacterial potential of cardamom (*Amomum subulatum*) against the enteropathogenic and food-spoiler bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus pumilus*. Bacterial cell membrane integrity was damaged and an increase in the absorbance at 260nm and 280nm was observed after incubation with cardamom extract. Cardamom extract inhibited the growth of all the bacterial strains tested here with MIC 6.24mg/ml for *E.coli* and 4.16mg/ml for other bacteria. The growth inhibition zone observed by well diffusion method in presence of extract equivalent to 33.3mg/ml cardamom was 15-20mm. Cardamom extract had 10.75mg polyphenol/g dry weight. The results indicate good antibacterial activity in polyphenol rich cardamom methanol extract.

Keywords: Antimicrobial, Polyphenol, Methanol, Cardamom, MIC, Inhibition

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Spices are a group of food adjuncts that have been in use for thousands of years not only as flavoring and seasoning agents to enhance the sensory quality of foods but also as folk medicines and food preservatives. These spice ingredients impart characteristic flavor, aroma or piquancy and color to foods to stimulate the appetite. In addition, several spices have long been recognized to possess medicinal properties such as tonic, carminative, stomachic antispasmodic, and antihelminthic (Nadkarni and Nadkarni, 1976). Although these observations are largely empirical, these have earned them pharmacological applications in the indigenous system of medicine. There is an increasing interest both in the industry and in the scientific research of spices and aromatic herbs because of their antimicrobial effects especially against the food borne bacterial pathogens. Extracts from plants such as cinnamon, clove, garlic, mustard, onion and oregano are reported to be effectively controlling the growth of many bacterial pathogens. The antimicrobial compounds in spices and herbs are mostly in the essential oil fraction. Shan et al. (2005) found that more than 50% of 46 spices extract evaluated, exhibited antibacterial activity against pathogens *Staphylococcus aureus* and *Echerichia coli*. Enteropathogens *Shigella* species and *E. coli* are found to be sensitive to the extracts of various kitchen spices (Vaishnavi et al. 2007). Growth of multiple drug resistant pathogenic bacterial strains *Salmonella typhi* (D1 Vi-positive), *Salmonella typhi* (G7Vi-negative), *Salmonella paratyphi* A, *Escherichia coli* (SS1), *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Bacillus licheniformis* is inhibited in the presence of extracts of spices (Naveed et al. 2013). The Gram-positive bacteria were more sensitive to the antimicrobial compounds in spices than Gram-negative bacteria (Agnihotri and Wakode, 2010).

Cardamom (*Amomum subulatum*) also known as Badi ilaychi or brown ilaychi is a terrestrial, rhizomatous herb of Zingiberaceae family, distributed chiefly in Africa, tropical Asia, the eastern Himalayas and cultivated in Nepal, northern West Bengal, Sikkim and Assam hills. The seeds are reported to possess stimulant, stomachic, alexipharmic and astringent properties and are used in folklore medicine for the treatment of indigestion, vomiting, biliousness, abdominal pains and

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rectal diseases (Agnihotri and Wakode, 2010). The seeds are found to promote elimination of bile and are used to treat congestive jaundice. Seeds are also used in gonorrhea, while the pericarp has been reported to be useful in treating headache, stomatitis, skin diseases and fever (Sharma et al. 2002). In the present study, methanol extract of fruits (seeds and rind) of *A. subulatum* was screened for antibacterial potential against foodborne pathogenic bacteria.

MATERIALS AND METHODS

Cardamom (*Amomum subulatum*) fruits procured from the local market were identified and authenticated at Department of Botany, Kurukshetra University, Kurukshetra, India. 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 other chemicals and solvents used were of analytical grade.

Extraction

Cardamom fruits were dried at 60°C in hot air oven till constant weight was attained. Fruits were finely powdered and sieved. Sieved powder was extracted with 80% methanol (1g/10ml) in a shaker at room temperature for 4 hrs. Residue was again extracted with 80% methanol for 2 hrs. Collected extract was filtered through double layered muslin followed by centrifugation at 5000g for 5min in order 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.

Bacterial Culture

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 1L. 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 cardamom extract for 30min at 37°C were analyzed spectrophotometrically to estimate cell damage. The culture was grown in nutrient broth upto 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, 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 bacterial cell damage due to cardamom extract, 100 µl of bacterial suspension was incubated with 100 µl of cardamom extract (equivalent to1.25 mg 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 (Wong and Kitts, 2006).

Bacterial growth inhibition assay

Bacterial growth inhibition was analyzed by well diffusion assay (Andrew, 2001). Twofold serial dilutions of cardamom extract ranging from 100 - 0.097mg/ml concentrations were made in 10% methanol. Using a sterile cork borer, nearly 8mm diameter wells were bored in the seeded agar plates and 100 µl of cardamom extract diluted in 10% methanol was added into the wells. All the plates were incubated at 37°C for 24 hrs. Antibacterial activity was determined by measuring the zone of growth inhibition around the well. The antimicrobial activities of the extract was compared against the standard drugs: ampicillin and chloramphenicol (concentration 25 µg/ml) (negative control) and 10% methanol (positive control). These tests were performed in triplicate and the mean of inhibition diameter was calculated.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of the antibacterial compound that prevents the development of visible growth of micro-organism after incubation. MIC was determined by the agar well diffusion method as described earlier (Dua et al. 2014a,b). Twofold serial dilutions of cardamom extract ranging from 33.3-0.065mg/ml concentrations were made in 10% methanol. Using a sterile cork borer, nearly 8mm diameter wells were bored in the seeded agar plates and a 100 µl volume of different dilutions of extract was added into the wells. These plates were incubated at 37°C for 24 hrs.

Estimation of polyphenol content

The total content of phenolic compounds in cardamom 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, 100 µl of 50% Folin-Ciocalteu reagent was added to the mixture. Absorbance was measured at 750 nm after 30 min.

Statistical analysis

The statistical analyses were performed with the statistical software SPSS/Windows (SPSS 10.0. LNK). The results were expressed as the means ± SEM to show variations in a group. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Food industry is looking for natural preservatives or additives, which are being preferred as these are safer, flavor enhancer and without any side effects as

compared to the synthetic or chemical additives (Brull and Coote, 1999). Resistance of pathogens to different drugs is very common, which is another major concern in treatment of various diseases. Inappropriate use of readily available antibiotics, prolonged hospitalization and poor implementation of infection control measures are the main causes of drug resistance. This has led to the search for new safe and effective antimicrobial agents from alternative natural resources like plant products. Extraction of different bioactive compounds from medicinal plants facilitates pharmacological studies leading to synthesis of a more potent drug with reduced toxicity (Manna and Abalaka, 2000). In the present study, methanol extract of cardamom was examined for inhibition of bacterial growth by well diffusion method (Fig. 1, Table 1). Cardamom extract has exhibited antimicrobial activity against all the four bacteria tested. The growth inhibition zone in presence of extract equivalent to 33.3mg cardamom was 15-20mm for the bacteria tested. These values are comparable to 18-26mm for chloramphenicol (25µg/ml), but less than 36-44mm for ampicillin (25µg/ml) standard used. Growth inhibition studies indicate that cardamom extract is an efficient antibacterial agent. Essential oil extracted from cardamom is reported to have similar antimicrobial activity with inhibitory zone of 20mm for *B. pumilus* and 18.5mm for *E. coli.* Methanol extract of cardamom rind inhibits the growth of *E. coli* more effectively than essential oil or seed extract (Agnihotri and Wakade, 2010). However *E. coli* SS1 and *S. aureus* were resistant to cardamom essential oils (Naveed et al. 2013). Methanol extract of whole cardamom fruit used in present study has inhibited the growth of pathogens tested more effectively. Vaishnavi et al. (2007) have also reported antimicrobial activity of aqueous extracts of various spices including cardamom against *Salmonella* sps, *Shigella* sps, and *E. coli*.

The phytochemicals in plant extracts affect microbial cells by various antimicrobial mechanisms, including attacking the phospholipid bilayer of cell membrane, disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxides caused by oxygenation of unsaturated fatty acids (Burt, 2004; Tajkarimi et al. 2010). Damaging impact of cardamom 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 cardamom 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). Increase in absorbance at 260nm and 280nm is higher for *S. aureus*

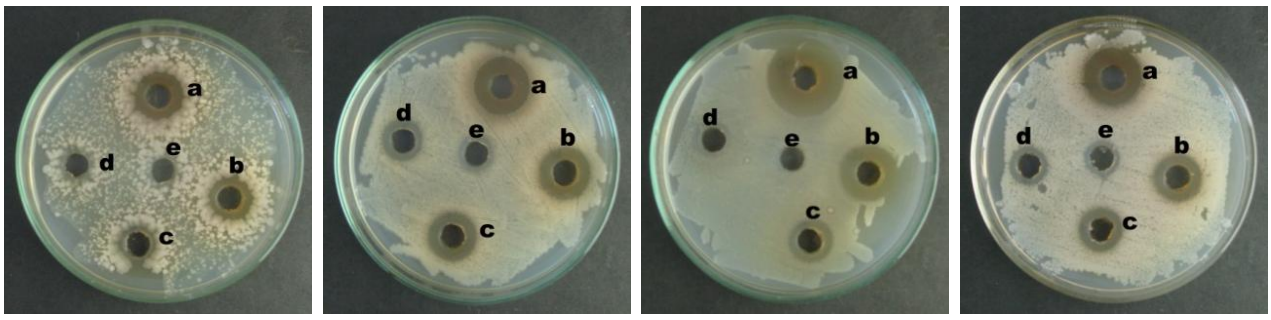

Figure 1 Growth inhibition in presence of different concentrations of Cardamom extract; **(A)** *E. coli*; **(B)** *P. aeroginosa*; **(C)** *S. aureus*; **(D)** *B. pumilus.* **a-e** concentrations are 100, 50, 25, 12.5, 6.25 mg/ml respectively for A; 50, 25, 12.5, 6.25, 3.125 mg/ml respectively for B,C and D.

Table 1 In vitro antibacterial activity of cardamom extract by agar well diffusion method

Compound	Concentration of the compound	Diameter of growth of inhibition zone (mm)			
		E. coli	*P. aeruginosa*	*S. aureus*	*B.pumilus*
Cardamom extract	33.3mg/ml	15.33±0.50	17.20±0.36	20.10±0.47	17.60±0.35
Chloramphenicol	25µg/ml	17.5±0.25	24.7±0.15	23.7±0.16	26.3±0.31
Ampicillin	25µg/ml	35.7±0.21	44.1±0.26	41.4±0.20	38.6±0.31
10% methanol		0	0	0	0

The values represent mean of sample ± SD for n = 3. Diameter of inhibition zone was measured as the clear area centered on the agar well containing the sample. Wells with non-inhibition zone were recorded 0.

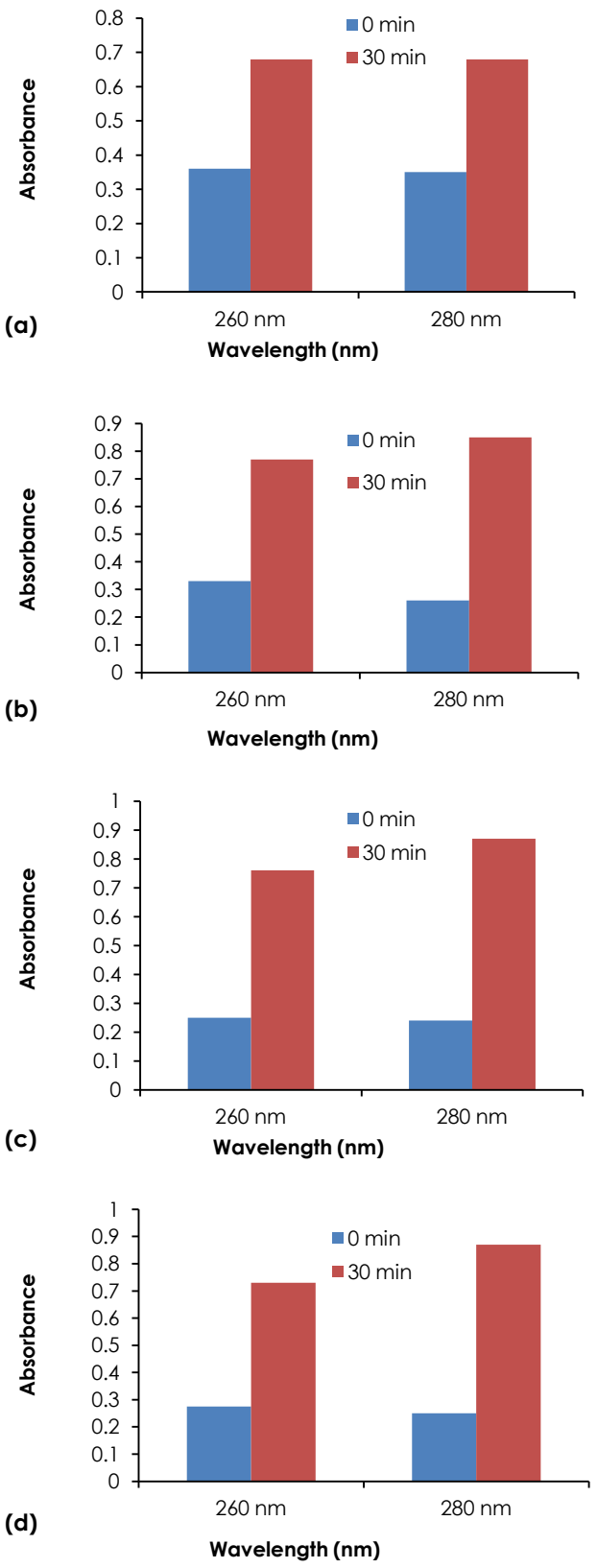

Figure 2 Bacterial cell damage induced by methanol cardamom extract (a) *E. coli;* (b) *P. aeruginosa*; (c) *S. aureus*; (d) *B. pumilus.* Increased absorbance at 260 nm and 280 nm after 30 min indicates leakage of cellular materials (DNA, proteins and enzymes) into growth medium

and *B. pumilus*. *S. aureus* culture has also shown maximum increase in absorbance at 280nm from 0.24 to 0.87 on incubation with the extract, indicating the highest damage to the membranes of this bacterium (Fig. 2). The results showed that cardamom extract causes damage to the cells 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), coriander and clove (Dua et al. 2014a,b) are also found to damage the cell membranes of both gram positive and gram negative bacteria.

Minimum concentration of the cardamom extract, which can inhibit the growth of microbes i.e. MIC for *P. aeruginosa, B. pumilus* and *S. aureus* determined by well diffusion method is 4.16mg/ml, whereas inhibition of growth of *E. coli* is achieved in presence of extract containing 6.24 mg dry weight/ml (Table 2).

Table 2 Minimum inhibitory concentration (MIC) of Cardamom extract

Compound	*E. coli*	*P. aeruginosa*	*S. aureus*	*B. pumilus*
Cardamom extract	6.24	4.16	4.16	4.16
Chloramphenicol	0.2	0.4	0.4	0.4
Ampicillin	0.5	0.5	0.8	0.5

The values for cardamom are in mg dry wt/ml and for standard antibiotics are in µg /ml.

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 cardamom 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). Cardamom extract in methanol has 10.753 $\pm$ 0.35mg polyphenol/g dry weight. Extracts of various spices are reported to have various polyphenolic compounds and antioxidant activities (Shan et al. 2005; Naveed et al. 2013). Polyphenol content of extracts of various spices and herbs is correlated to high antioxidant and reducing properties (Shan et al. 2005), which may in part explain the inhibition of bacterial growth. Certain spices can have a direct effect on the rate of fermentation by stimulating acid production in starter cultures such as grains, seeds, or nutrient liquids that have been well colonized by the microorganisms used for the fermentation. 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. Phenols,

alcohols, aldehydes, ketones, ethers and hydrocarbons have been recognized as major antimicrobial components in spices (Tajkarimi et al. 2010).

CONCLUSION

The results obtained in the present study revealed that methanol extracts of whole fruit including seeds and rind of cardamom possessed broad spectrum antimicrobial activity. Cardamom extract damages the cell membrane integrity and effectively inhibits the growth of both gram positive and gram negative bacteria tested here. The knowledge about the cell damage and inhibition of growth of various pathogens by cardamom extract adds to its application in the field of pharmacology, phytochemistry and food chemistry for the development of better medicinal or preservative preparations. Study of Phytochemicals in the cardamom extract will help to explore its therapeutical potential and use as safe, natural food preservative.

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