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Studies Related To Antimicrobial And Antioxidative Activity Of Clove (*Syzygium Aromaticum*)

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Abstract: Spics such as garlic black pepper, clove, ginger, cuminod caraway are used extensively in Indian diet (Arora and Kavr, 1999) in spices and herbs used today are valued for their antimicrobial activities and medicinal values in addition to their flavor and fragrance qualities (Shan et al., 2007). These phytochemicals present in the spices are capable to attack beneficial and to repeal harmful organism and they serve as phytoprotectants, moreover numerous class of isoflavones, anthocyanins and flavonoids are also found associated with the spices. Among the difference spices clove extracts have biological activities such as antibacterial antifungal insecticidal and antioxidant properties and are used traditionally as flavoring agent and as a antimicrobial agent in food industry (Huang, Ho, Lee & Yap, 2002; Lee & Shibamoto 2001; Velluti Sanchis Ramos & Marin 2003). Among the five spices extracts (aqueous extract) higher inhibition way observed in clove against five food borne pathogenesis (Staphylococcus aureus, Escherichia coli, Salmonella typhimurium Bacillus cereus and Listeria monocyto genes are selected the clove extract exhibited the highest bactericidal and bacteriostatic activity than other different solvents are used and hot water shows maximum concentration percent than other solvents. By analyzing the total bacterial population of egg, meat and fish against clove extract at difference time intervals, the clove extract highly inhibitory the microbial effect growth at 48h intervals.

Key words:: Staphylococcus aureus, Escherichia coli, Salmonella typhimurium Bacillus cereus and Listeria monocyto

INTRODUCTION:

Spices and herbs used today are valued for their antimicrobial activities and medicinal values in addition to their flavour and fragrance qualities (Shan et al., 2007). These phytochemicals present in the spices are capable to attract beneficial and to repeal harmful organisms and they serve as phytoprotectants. Moreover, numerous class of isoflavones, anthocyanins and flavonoids are also found associated with the spices. Numerous studies have been published on the antimicrobial activities of clove oils against different types of microbes including food borne pathogens (Beuchat, 1994; Smith-Palmer et at, 1998; Hara-Kudo et al, 2004). However, little information is known about the solubility of clove extracts in different solvents and the antimicrobial role in the prevention of food spoilage (Sethi and Meena 1997). In addition to this, the reported data on the relationship between the antimicrobial activity and the antioxidative capacity clove is scarce.

MATERIALS AND METHODS

Spices used for the present study

Ginger (Zingiber officinale), pepper (Piper nigrum), clove (Syzygium aromaticum), nutmeg (Myristica fragrans), cardamom (Elettaria cardamomum), onion (Allium cepa), garlic (Allium sativum) turmeric (Curcuma longa), cinnamon (Cinnamomum zeylanicum) and omum (Trachyspermum amnu) were obtained from the local market in Cuddalore.

Screening of spices extracts

The agar well method was used to screen efficiency of aqueous extracts of five selected spices against five food borne pathogenic bacterial strains viz., E. coli, Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium and Listeria monocytogenes. Clove (Syzygium aromaticum) was selected based on their inhibition zone for further study.

Preparation of spices extracts

The plant samples were collected from local market and washed with distilled water, drained the water, shade dried. Extracts were prepared using hot water, ethanol, acetone, benzene and petroleum ether.

1g of shade dried spices was homogenized with mortar and pestle in 10ml hot water ethanol, acetone, benzene and petroleum ether (1:10 w/v) and filtered through a double layered cheese cloth. Filtrate was collected and stored in refrigerator at 10-14°C.

Method for testing antimicrobial properties of spice extracts

Agar well diffusion method

The selected strains of bacteria were inoculated into 10ml of sterile nutrient broth and incubated at 37°C for 16-18 hours. Using a sterile cotton swab, the nutrient broth cultures were swabbed on the surface of sterile nutrient agar plates. Agar wells were prepared with the help of sterilized cork borer with 6 mm diameter. Using a micropipette, 100 microliters of different concentrations of spices extracts (100%, 75%, 50%, 25% and 10%) were added to different wells in the plate. The plates were incubated in an upright position at 37°C for 24 hours.

Inhibitory studies on the growth of bacterial strains
The method followed for preparing the spices extracts.
Effect of spices extracts on the growth of pathogen in agar well diffusion method

Agar well diffusion method was followed for evaluation of antibacterial activity of known plant extracts viz., garlic, ginger, clove, nutmeg and onion. Nutrient agar medium was prepared and autoclaved at 15 Ib pressure for 20 minutes and cooled to 45°C, poured in sterile petriplates and allowed to solidify. Cell suspension (106 cell mL'1) of the test organisms viz., Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Bacillus cereus and Listeria monocytogenes was prepared separately. "L" rod was used to spread the cell suspension. The stainless steel cork borer used to make the 6 mm diameter well; each well was filled with the ethanol extract, acetone extract, benzene extract, petroleum ether extract and hot water extract of prepared from five selected spices. The plates were incubated at 37°C for 24 hours; observed for the zones of inhibition. Diameter of inhibition zones was measured and expressed in mm.

Bacteriostatic bactericidal concentrations

The bacteriostatic concentration was the lowest concentration at which bacteria failed to grow in TSB, but able to grow when 100 samples were plated on TSA. The bactericidal concentration was the lowest concentration at which bacteria failed to grow in TSB and also after 100 1 samples were plated on TSA. Bacteriostatic and bactericidal concentrations were determined for the five spices extracts selected based on zone of inhibition. The appropriate volume of spices extracts was added to 9.5 ml TSB to give a final concentration of 0.005-1% after the TSB was inoculated with 0.5 ml of an 18 h bacterial culture. (1% was chosen as the maximum concentration because higher levels would probably be unacceptable concentration because higher levels would probably be unacceptable in food). Tubes were inoculated with bacterial cultures, incubated at 35°C with regular shaking. After 24 h, growth was compared with the control both visually and through the measurement of absorbance at 630 nm using a Dynatech MR 500 plate reader (Dynatech laboratories, Billinghurst, UK). 1001 samples from tubes showing no growth were plated onto TSA, incubated for 24 h under the same conditions as used for the TSB.Resazurin mic method Serial twofold dilutions (0.005-1.25% v/v) of the each spices extracts were prepared by vortexing the spices extracts in room-temperature sloppy (of semi-fluid matter) agar. The resazurin assay medium was then inoculated with the test organisms to yield a final cell density 1 log cycle lower than the cell density required to reduce resazurin (usually 5 logio - 6 log 10 cfu ml-7). The inoculum density was confirmed by plate count. A sterile 96 well microtitre tray with lid was setup with each of the test bacteria as follows: column 1-9, 170ml inoculum plus 20ml of an spice extracts dilution; column 10, 170ml inoculum plus 20ml spice extracts dilutent (positive control); column 11 and 12, 170 fal sterile resazurin assay medium plus 200l spice extracts dilutent (negative control and blank, respectively).

Well contents were thoroughly mixed using the micropipettor. Two trays were prepared for each organism and incubated at 37°C for 3.5 h. After incubation, 10 ml of resazurin solution was added to all except column 12, to which 10 ml of distilled water was added. After a second incubation of 2 h at 37°C, three methods were used to Determine the MIC values. First, wells were assessed visually for colour change, with the highest dilution remaining blue indicating the MIC. Absorbance was then read at 570 nm. Immediately after wards, plate counts were carried out on samples from the microwells, to determine whether bacterial numbers correlated with either indicator of MIC.

The cell concentration necessary to cause reduction of resazurin within 2h was determined for each of the test organisms.

Total bacterial population in egg, fish and meat
Total bacterial population in egg, fish and meat

Total bacterial population was determined at different time intervals during storage. The samples (10 g) with the spices extracts was homogenized by using a lab-blender 400 stomacher (laboratory equipment, London, UK) in 90 ml of sterile peptone water. Serial dilutions were prepared and spread-plated on sterile petriplates with plate count agar and plates were incubated at 35 ± 1°C for different time intervals (24, 36 and 48 h). Counts were reported as log 10 cfu/g of egg, fish meat.

Phenolic extraction

Spices were cleaned and carefully washed with tape water, wiped by soft centrifugation and were processed. Ground materials were suspended (1:10 w/v) in 80:20 v/v acetone/perchloric acid 5%, shaken for 30 min at 4°C and then centrifuged for 10 min at 3000g the supernatant was collected and used for the assays. Total phenolic determination

The total phenolic content of spices extracts was determined by the Folin-Ciocalteau method. 0.5 ml diluted extract solution was shaken for 1 min in 6 ml of distilled water added with 100 [al of Folin-Ciocalteau reagent. The mixture was shaken and 2 ml of 15% Na2CO3 was added and shaken once again for 30 seconds. Finally, the solution was brought up to 10 ml by adding distilled water. After 1.5 h, the absorbance at 750 nm was evaluated using a spectrophotometer (Beckman Coulter DU-640, Ca, USA). Total phenol values are expressed in terms of gallic acid equivalent (GAE) (mg g-1 of dry mass), which is a common

reference compound.

Total alkaloid determination

The total alkaloid content of spices extracts was determined by the following method. Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagent is added (Siddiqui and Ali, 1997). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent. The ethanolic spices extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

Total flavonoids determination

Aluminum chloride colorimetric method was used for flavonoids determination as described.

Free radical scavenging activity

The capacity of spices extract to remove l,l-diphenyl-2-picryl-hydrazyl radical (DPPH) was determined by the method described.

Determination of reducing power

Reducing power was measured according to the method described.

RESULTS AND DISCUSSION

Screening and selection of spices extracts against different food borne pathogens Selected five different spice extracts were prepared using cold water as a solvent were examined for antimicrobial activity against S. aureus, E. coli, Salmonella typhimurium, B. cereus and L. monocytogenes. The zone of inhibition values of the spices extracts were given in Table. Among the five spices extracts screened for antimicrobial activity, clove was found to be effective against the test pathogens. Among these extracts, the higher inhibition was noted in clove (1 1.2 mm, 10.5 mm, 9.5 mm, 8.6 mm and 7.5 mm). The zone of inhibition was found to be in the order of S. aureus > E. coli > B. cereus > Salmonella typhimurium > L. monocytogenes.

This antimicrobial nature exhibited by different plant extracts is not surprising as edible plant extracts of crannery, lime, nutmeg, lemon juice turmeric pepper have been documented earlier. The results of different studies provide evidence that some medicinal plant extracts could be potential sources of new bacterial agents. The selected spices extracts were further evaluated for their antimicrobial potential against different food borne pathogenic strains. A closer look at the table reveal that the clover extracts showed the highest antimicrobial activity against S. aureus followed by E. coli.

Estimation of total phenol, flavonoids and alkaloid content of spice extracts

The total phenol, flavonoids and alkaloid content were found to be 65.8, 11.5 and 8.4 mg / 100g respectively against S. aureas, E. coli, S. typhimurium, B. cereus and L. monocytogenes respectively.

Table 1.
Screening of difference spices extracts for antimicrobial activity against certain selected food borne pathogens

S.No	Spices	Zone of inhibition in mm				
		S. aureus	*E.coil*	*S.typhimurium*	*B. cereus*	*L.monocytogenes*
1.	Pepper	10.2 + 0.1	9.4 + 0.20	8.6 + 0.2	8.0 + 0.2	-
2.	Nutmeg	10.2 + 0.2	9.0 + 0.1	8.5 + 0.2	8.0 + 0.2	7.6 + 0.2
3.	Cardamom	9.2 + 0.152	8.6 + 0.1	8.0 + 0.2	7.5 + 0.1	7.0 + 0.1
4.	Turmeric	8.4 + 0.1	7.6 + 0.1	-	6.8 + 0.1	-
5.	Clove	11.2 + 0.15	10.5 + 0.1	9.5 + 0.1	8.6 + 0.2	7.5 + 0.2

Including the size of the disc 6 mm.
Values are given a mean SD of three replications
Aqueous extract of spices
No zone inhibition.

Table 2 .
Estimation of total Phenol, Flavonoid and alkaloid content of clove extract

S. No	Constituents	Content mg /100 g
1.	Phenol	65.8 + 0.2
2.	Flavonoids	11.5 + 0.25
3.	Alkaloid	8.4 + 0.2

Values are referred to mg/ 100 g of spices and are the means with standard deviations of three different determine
Values are given a mean + SD of three replications
Total phenolic content expressed as grams of gallic acid equivalents (GAE) per 100 g dry weight (DW).

Estimation of total radical-scavenging activity and reducing power of different spices extract

The estimation of radical-scavenging activity and reducing power in clove extracts. The radical scavenging activity was found to be 86.0% and reducing power was found to be 86.0% and reducing power was 3.5 (at absorbance of 700nm).

Microbial reduction densities of different ethanolic clove extract against different food borne pathogens

The microbial reduction densities of clove extract against S. aureus, E. coli, S. typhimurium, B. cereus and L. monocytogenes. The clove extract showed a resazurin reduction densities against S. aureus (7.9) log 10 efu m-1, E. coli (7.7), S. typhimurium (7.5), B. cereus (7.3) and L. monocytogenes (7.1) respectively.

Antimicrobial activity of clove extracts against certain food borne pathogens

The antimicrobial effect of hot water, ethanol, acetone, benzene and petroleum benzene and petroleum ether extract of selected five spices extracts viz., garlic, clove, ginger, nutmeg and onion at 10 per cent concentration were calculated against S.aureus, B.cereus, Salmonella typhimurium, E. coli and L. monocytogenes.

Table 3.
Estimation of radical – scavenging activity and reducing power different clove extract

Activity / power	Activity (%) absorbance at 700 nm)
Radical scavenging	86.0 + 0.76[b]
Reducing power	3.5 + 0.2[b]

Inhibitory effect of clove extract against selected food borne pathogens

The result reveals that ethanolic clove extract exhibited the maximum inhibition zone against S. aureus (15.5 mm), followed by E. coli (13.4 mm), S. typhimurium (12.2 mm), B. cereus (11.5 mm) and L.monocytogenes (10.0 mm).

Acetone based clove extract exhibited maximum inhibition zone against S. aureus (14.2 mm) followed by E. coli (12.5 mm), S.typhimurium (11.0 mm) B. cereus (10.6 mm) and L. monocytogenes (9.5mm).

Benzene based clove extract exhibited maximum inhibition zone against S. aureus (12.0 mm), followed by E. coli (11.2 mm), S. typhimurium (10.2 mm), B. cereus (10.0 mm) and L. monocytogenes (9.0mm).

Table 4.
Microbial reduction density and Antimicrobial activity of clove extracts against certain food borne pathogens

S. No.	Text organisms	Intensity of Blue color	Solvent extracts in 10% w/v concentration Zone of inhibition in mm				
			Ethanol	Acetone	Benzene	Petroleum ether	Hot water
1.	S.aureus	7.9	15.5 ±0.2	14.2 + 0.2	12.0 ±0.2	11.5 ±0.2	11.0±0.2
2.	E.coli	7.7	13,4 ±0.2	12.5 ±0.2	11. 2,+ 0.2	10.5 ±0.2	9.8 ±0.305
3.	S. typhimurium	7.5	12.2±0.2	11.0 + 0.2	10.2 ±0.2	9. 5 ±0.152	9.0±0.2
4.	B. cereus	7.3	11. 5 ±0.251	10.6 + 0.2	10.0 + 0.2	9.2 + 0.2	8.5 ±0.2
5.	L.monocytogenes	7.1	10.0 ±0.2	9.5 + 0.152	9.0 ±0.2	8.5 + 0.2	7.5 ±0.2

* Including the size of the disc 6 mm.
* Values are given a mean + SD of three replications.
* log 10 efu ml-1

Petroleum ether based clove extract exhibited maximum inhibition zone against S.aureus (11.5 mm) followed by E.coli (10.5 mm), S.typhimurium (9.5 mm), B. cereus (9,2 mm) and L.monocytogenes (8.5mm).

Hot water based clove extract exhibited maximum inhibition zone against S. aureus (11 mm), followed by E. coli (9.8 mm), S. typhimurium (9.0 mm), B. cereus (8.5 mm) and L. monocytogenes (7.5 mm).

Bacteriostatic Concentration (Percent) Of Different Spice Extracts

The five different spices extracts, against certain food borne pathogens such as Staphylococcus aureus, E. coli, Salmonella typhimurium, Bacillus cereus and Listeria monocytogenes. The clove extract were examined for bacteriostatic concentration in percent among the different solvents. Hot water shows maximum concentration followed by petroleum ether, benzene, acetone and ethanol.

Bacteriocidal Concentration (Percent) Of Different Spice Extracts

Five spices extract were examined in different solvents for their bacteriocidal activity against S. aureus, E. coli, Salmonella typhimurium, B. cereus and L. monocytogenes. Result revealed that the ethanol based clove extract exhibited the maximum inhibition potency and followed by different solvent. The inhibition of growth was decreased using the different solvents extracts were given in the following order.

Ethanol > acetone > benzene > petroleum ether > hot water. The results are our present study is in harmony to those reported has showed that clove oil was found effective against food borne gram positive bacteria (Staphylococcus aureus, Bacillus cereus and L. monocy togenes) and gram negative bacteria E. coli, Salmonella sp. and P. aeruginosa.

The results of the static and cidal concentrations showed that S.aureus and E.coli were more sensitive to spice extracts than S.typhimurium, L.monocytogenes and B.cereus. The difference in sensitivity between the gram-negative and gram-positive bacteria to inhibition by plant extracts is in fine with the earlier researchers including. The reason for the susceptibility of gram negative strain is attributed to the fact that gram-negative bacteria has an outer memberance, which endows the bacterial surface with strong hydrophilicity and acts as a strong permeability barrier.

The results of our present study is in conformity with the earlier reports of Yin and Scheng (2003), reported that garlic extracts showed excellent antibacterial effect on certain food borne pathogens associated with beef. On the other hand, the antibacterial effects of garlic against E. coli, L. monocytogenes, S. aureus, C. jejunii in non-meat foods has been observed (Zhao et al, 2001).

Variations in the yields and phenolic content of various extracts are attributed to polarities of different compounds present in extracts and such difference have been reported earlier in the literature.

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Variations in the yields and phenolic content of various extracts are attributed to polarities of different compounds present in extracts and such difference have been reported earlier in the literature.Influence of Different Clove Extracts on the Total Bacterial Population in Egg, Fish And Meat

Table 5.
Influence of different clove extracts on the total bacterial population of egg, fish and meat

Spice extract	Total bacterial population log_{10} CFU ml/g of sample								
	Egg			Fish			Meat		
	24h	36 h	48 h	24h	36 h	48 h	24h	36 h	48 h
Ethanol	4.2	3.0	2.1	4.7	3.8	2.5	4.1	3.1	2.1
Acetone	4.4	3.2	2.3	4.1	3.1	2.1	4.1	3.1	2.1
Benzene	4.6	3.4	2.5	4.1	3.1	2.1	4.1	3.1	2.1
Petroleum ether	4.7	3.6	2.7	4.1	3.1	2.1	4.1	3.1	2.1
Hot water	4.9	3.8	2.9	4.1	3.1	2.1	4.2	3.1	2.2

*control dipped in sterile distilled water (10 g samples in 100 ml of distilled water).

The total bacterial population as influenced by treatment with different spice extracts in egg, fish and meat at three different time intervals (24, 36 and 48 h). Among the different solvents such as ethanol, acetone, benzene, petroleum ether and hot water the hot water based clove extract exhibited the highest inhibitory effect at 48 h time interval. In general the microbial population on the surface of meat was found to be more susceptible to the clover extracts, followed by fish. The reducing powers of clove extracts using potassium ferricyanide method was studied. The results of our study is confirmly with the earlier reported a mutual correlation between antioxidative activity and reducing effect. Reports regarding the reducing power of ethanol based clove extracts is scarce. However, noted differences in reducing power of various extracts of grape seed.

The clove extracts were found to have potent antimicrobial activity against the test strains. In this regard, the use of clove or other spices in foods is found to be a promising approach in enhancing the keeping qualities of food products.

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