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Antibacterial Activity of Syzygium Aromaticum against Food Borne Pathogens and Spoilage Bacteria and Their Antibiotic Sensitivity

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Abstract

Owing to the increasing social concern about chemical preservatives, the need and importance of substituting chemical preservatives with safe natural product is getting more demand. Many researchers have investigated the antimicrobial effect of natural plant products. The study describes the antimicrobial activity of clove (spice), on the fish *Rastrelliger kanagurta* during chilled storage and the direct antibacterial effects of ethanolic and aqueous clove extracts on different strains of bacteria isolated from the stored fish. This work also evaluates the antibiotic sensitivity of the bacterial strains. It indicates that, all strains of isolated bacteria are resistant to penicillin. Different concentrations of spice were added to the fish *Rastrelliger kanagurta* and the fish flesh was stored @ refrigeration temperature 4^0c for12 days. Pour plate technique and well diffusion methods were used to determine the antibacterial effect of clove in 3 days of intervals. For control sample, it exceeds 6 log cfu/gm of total bacteria during the 9 days of storage, but for the samples with addition of clove, it was observed a decrease of log cfu/gm of total mesophilic bacteria during 6 days of storage, there after it shows a slight increase but the growth was generally limited and did not exceed 6 log cfu/gm till the 12^{th} day. Also the isolated strains of bacteria show maximum inhibitory activity towards the aqueous clove extracts as compared to ethanolic clove extracts. Overall the result indicated that clove has very significant antibacterial activity and is increases with increasing the concentration. There for clove is suitable substitute for chemical preservatives and it can effectively used for the preservation techniques.

Key words: antibiotic sensitivity, clove, preservation, *Rastrelliger kanagurta,*.

Introduction

The increasing concern about food safety has recently led to the development and use of natural preservatives to control food borne pathogens and spoilage bacteria. Spices are one of the most commonly used natural preservative and have been used traditionally for preserving foods. Refrigeration at low temperature is usually the most common preservation method for meat and fish products. Today the issue of food preservation has become more serious with increasing concern about the presence of chemical residues in foods so the demand for natural preservatives increasing day by day. The recent negative social perception against synthetic additives shifted the effort towards developing natural preservatives (Shelef, 1984). Antimicrobial properties of herbs and spices have been recognised and used since ancient times for food preservation as well as in medicinal uses (Zaika, 1988).

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Fresh seafood has short shelf life because of being highly perishable (Kykkidou *et al.,* 2009). Refrigeration at low temperature is not enough alone for the preservation of sea food (Gomez-Guillen& Montero, 2007). In growing food industries the increases in processed food items have raised the use of diverse chemical preservatives which delay or prevent the loss of nutrition caused by microbiologic, enzymatic or chemical changes and enhance the shelf life of food items. Unfortunately these chemical preservatives can be dangerous for people health by the reason of accumulating additives in tissue and leading to genotoxicity in case of overdose. However plant extracts and essential oils was an alternative to synthetic chemicals and preservatives (Sultanbawa *et al.,* 2011), especially for providing natural protection without spoilage and extend shelf life in food of animal origin (Holley and Patel, 2005).Therefore enhancing shelf life of sea food with natural preservatives is an important issue to eliminate economic losses and provide safe and good quality food to consumers and reach to distant markets (Kykkidou *et al.*, 2009).

Being plant natural food material, spices appeal to consumers who tend to question the safety of chemical preservatives (Farag *et al.*, 1989). Antimicrobial effects of spices have been recorded in recent years and interest continues to the present (Akgul and Kivanç, 1988; Cosentino *et al.*, 1999; Domans and Deans, 2000; Ristori *et al.,* 2002; Radhakrishnan *et al*, 2003). Still little information is available emphasizing the role of spices as food preservative. (Arora and Kaur, 1999). Spices are recognised to stabilise the foods from the bacterial deterioration. This could be observed when spices show high microbial count initially and as time progresses, the microbial growth become reduced or totally suppressed (Kizil and Sogut, 2003). Antimicrobial activity of spices mainly depends on many factors, which includes; 1) kind of spice, 2) Composition and concentration of spice, 3) Microbial species and its occurrence level, 4) Substrate composition and 5) Processing conditions and storage (Shelef, 1983; Farag *et al.,* 1989).

Cloves (*Syzygium aromaticum)* are the aromatic dried flower buds of the family *Myrtaceae* (Srivastava and Malhotra, 1991; Chaieb *et al.,* 2007). It is a common spice used in local beverages in the middle belt and northern part of the country (SE Atawodi, *et al*, 1970). Syzygium species have been reported to possess antimicrobial and anti-inflammatory activity (Muruganandan S, *et al*, 2011). It was reported that the buds of Syzygium aromaticum were used in folk medicine as diuretic, odontalgic, stomachic, tonicardiac, aromatic condiment properties and condiment with carminative and stimulant activity (Boulos L, 1983). The chief significance of the above study was to carry out the analysis of the antimicrobial activity of clove as an alternative to chemical preservatives for improving the shelf life of food products.

Materials and methods

i. Sample collection

Fresh whole clove buds were purchased from the local market of Trivandrum. The samples were obtained from retail spice sellers. The raw fish mackerel were purchased from the Vizhinjam fishing harbour.

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ii. Preparation of clove powder

Clove powder is prepared by grinding the clove buds using mixer grinder. For this, 50g of clove from 200g of clove buds were taken and after grinding the clove powder were sterilized and kept in air tight containers.

iii. Preparation of clove extracts

Here two types of clove extracts were prepared, Aqueous and ethanolic extracts. For the preparation of aqueous extract, 150ml of distilled water was added into 25g of clove powder and the mixture was left for vaporization in boiled water bath for 3 hours. Afterwards, it was filtered. The dark colored extract obtained at the end of this process was used for the further analysis. The sample extract was kept in the refrigerator at chilled temperature of further analysis. For the preparation of ethanolic extract soxhlet apparatus was used. After 24 hours extract was collected. The dark colored oily extract obtained at the end of the process was used for the analysis. The sample extract kept in the refrigerator at chilled temperature. Here dimethyl sulphoxide(dmso) was taken as the solvent for dissolving the extracts. For dissolving the extracts 1mg of each extract were taken and dissolved in 2ml of DMSO and kept in separate sterile eppendorf tubes.

iv. Preservation of fish

80g of the fresh fish *Rastrelliger kanagurta* were washed, gutted, rewashed, drained and minced. Fish should be planned to store for 12 days from 03/04/2017 to 16/04/2017. The fish flesh was divided into three batches with 24gms each and these each batches again divided into three sets. Each set of samples were mixed with 0.5% and 1.0% of clove powder respectively and one batch left as control. Then all these sets were transferred into separate sterilized containers with 8gms each. Each four sets has 3 containers with 0.5%, 1.0% clove powder and one for control and kept for doing microbial analysis on 3^{rd},6^{th}, 9^{th}, and 12^{th} days of preservation. It is better to avoid contamination by kept in separate containers. The containers with sample were kept in the refrigerator at chilled temperature (4^0c) for 12 days. In addition to this preservation, 8g of fresh fish were taken for the microbiological analysis of the fresh fish sample. All the four sets were taken for the enumeration of bacteria in the corresponding days of observation.

v. Enumeration of bacteria (Standard Plate Count Method)

The bacteria were enumerated by employing serial dilution agar plating method. Serial dilutions of the sample were prepared into 10^{-4} dilution by adding 1g of preserved fish sample to 9ml of sterile physiological saline (0.9%) an amount consisting of 1ml of each dilution was transferred aseptically onto separate petridishes and approximately 15-20ml of molten plate count agar was added. The sample and agar were mixed thoroughly by rotating the plates clockwise and anticlockwise direction in several times. The plates were allowed to set and inverted, and then the plates were incubated at 37°c for 24-48hours. Colony counts were made from plates with counts between 30 and 300 and Colony counts were presented as colony forming units (cfu/ml).

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$$\text{No.of cfu/ml} = \frac{\text{Number of colonies counted X dilution factor}}{\text{Volume of sample taken}}$$

After enumeration, dominant colonies from selected plates were used for doing the species identification.

vi. Identification of SPC bacteria

Selected colonies of all morphological types were picked from agar plate. Isolates were purified by streaking on nutrient agar. Pure cultures were maintained on nutrient agar slants at 5°c. The cultures were identified by using **Bergy's manual of systematic bacteriology**, the main resource for identifying unknown bacteria. According to it, the first approach, identification of bacteria involves preliminary microscopic analysis of the gram-stained preparation for its categorization into gram negative and gram positive groups. After knowing this, the identification was done with the help of various key charts so as to confirm the bacterial identity.

The biochemical tests for the identification of bacterial species are;

1. **Catalase test:** A portion of the colony of the test organism grown in nutrient agar plate was pricked with a sterile glass rod & immersed in 10% h_2o_2 taken in a tube. Noted the effervescence produced.
2. **Oxidase test:** The test disc impregnated with nnnn-tetra methylphenylenediaminedihydrochloride was wetted with one drop of sterile water. A portion of colony of the test organism from nutrient agar plate was pricked with a sterile glass rod & rubbed on the test disc. A purple colour developed within 10s was considered as positive reaction.
3. **Indole test:** The test organism was inoculated in sterile tryptone media. After 24-48hours incubation, kovanc's reagent was added to the culture. The development of red color in the reagent side was considered as positive for indole production.
4. **MR-VP test:** The test organism was inoculated to two tubes containing glucose phosphate peptone water media. After 48 hours of incubation to one tube add five drops of MR reagent, and formation of red color taken as MR positive reaction, and yellow color formation as negative. To the second tube 1ml barritt's reagent a, and 3ml of barritt's reagent b were added and mixed thoroughly. A red color development indicated vp test positive.
5. **Citrate utilization test:** The test organism was inoculated to sterile slants of simmon's citrate agar and incubated for 48 hours. Prussian blue color development was taken as citrate utilization.
6. **Sugar fermentation test:** Peptone water media containing 0.5% test sugar and 0.0025% bromothymol blue was used to test the ability of the organism to ferment various sugars. The test organism was inoculated to the sterile media containing tubes with inverted durham's tubes and incubated for overnight. The colony change from blue to yellow was taken as production and air bubble in durham's tube as gas production due to the sugar fermentation.

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7. **Urease test:** Sterile christensen's urease media slants were inoculated with the test organisms and incubated for 24-48 hours. Development of pink color indicated urease production.
8. **Nitrate reduction test:** To sterile nitrate broth, the test organism was inoculated and incubated for 48 hours. To the culture, nitrate reagent was added and development of red color indicated the reduction of nitrate to nitrite.
9. **Mannitol motility test:** The test organism inoculated by stabbing into mannitol motility test media and incubated for 24 hours. Yellow color developed indicated mannitol fermentation and diffused growth form the stab line indicated motility of the organism.
10. **Use of TSI media:**
11. The ability of utilization of sugars & h_2s production was analyzed by using TSI media. The test organism was inoculated into TSI media first by stabbing into the butt and then streaking on slant portion. The inoculated media were incubated for 24-48 hours and noted the results.

After the identification procedure, the identified species of bacteria were taken for the study of direct antimicrobial effect of aqueous and ethanolic clove extracts.

vii. Evaluation of antibacterial activities of clove extracts.

The clove extracts were screened for its antimicrobial activity against the organism by agar well diffusion method given by Dingle *et al* (1953). Sterile cotton swab was dipped into the prepared culture and used to inoculate the bacteria. With the help of a sterile well cutter, three wells were made in each inoculated plates and labeled properly. 50 micro liters of the aqueous and ethanolic clove extracts were dispensed in the respective wells with the help of the micropipette. The solvent (DMSO) itself was tested for its activity as a control at the same time in the third well. The plates were left for half an hour with the lid closed and then incubated at 37°c for 24 hours. After incubation plates were observed for the zone of inhibition which is suggested by the clear area around the well (WHO, 1991).

viii. Antibiotic sensitivity testing by disc diffusion method

- **Preparation of the culture broth and bacterial inoculation**: Nutrient broth was prepared and inoculated with the test organism. A loop full of microorganisms was taken and inoculated in the nutrient broth and was incubated at 37°c for 24hrs to obtain viscous growth.
- **Preparation of agar plate**: The freshly prepared autoclaved nutrient agar media was poured in the petri plate, after cooling it to 45°c, and was kept to solidify.
- **Inoculation of agar plate**: The inoculum is spread over the entire surface of the petriplate by swabbing in three directions. Inoculated plates were allowed to dry before applying antibiotic discs. Disc should applied to the surface of the agar within 15 minutes of inoculation (BSAC method for antimicrobial susceptibility testing, 2008).
- **Application of discs**: Storage and handling of the discs should be very careful, so that there will be no loss of potency as a result. 4 antibiotic discs were firmly applied to the dry surface of the inoculated petriplate. The contact with the agar should be even.

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- **Incubation**: If plates are left for extended times at room temperature after discs are applied, the antibiotics will diffuse out and the microorganisms starts to grow, results in larger zones of inhibition compared with zones produced when plates are incubated immediately (Andrews, 2004). Plates should therefore be incubated within 15 minutes of disc application. Plates were incubated at 35-37°c for 18-24hrs.
- **Measuring zones**: The diameters of zones of inhibition are measured to the nearest millimeter with a ruler. The zone edge is taken as the point of inhibition as judged by naked eye.

Result

Results of the present investigation indicate that, two different concentrations used for the study such as 0.5% and 1.00% of clove possesses the antibacterial property against bacteria grown in fish during chilled storage. Antibacterial effects were evaluated by pour plate method. The results indicated that different concentrations of clove have broad spectrum antibacterial activity. Among the two different concentrations of clove tested all the two showed antibacterial activity and are different from control. Samples tested with different clove concentrations resulted in decreased bacterial count up to day 9 of storage and thereafter there was a gradual increase in the bacterial counts, but not exceeds over the standard value as log 6. Compared to other concentrations, the inhibitory action of 1.00% clove was found to be most effective. That is the inhibitory effect on the bacterial growth increases with high concentration.

In general, the highest microbial growth was obtained from the control samples, while the lowest microbial development was observed in samples treated with 1.0% clove powder. When the aerobic plate counts reached 6log10cfu/gm, the seafood products were assumed to be at or near spoilage. In this study, microorganisms exceeding 6log10 cfu/gm were observed in the control sample after 9 days of storage while the other samples were under the standard level even after 12 days of preservation.

Table no. 1: Antibacterial effects of different concentrations of clove during 12 days of preservation at 4^0C

Days	Clove Log cfu/gm			
	0.00%	0.5%	1.00%	STD
1	5.30	5.30	4.95	6
3	5.25	5.23	4.74	6
6	5.25	5.13	4.56	6
9	5.36	4.98	4.50	6
12	ND	5.44	5.41	6

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Fig 1: Antibacterial effects of different concentrations of clove during 12 days of preservation at 4^0C

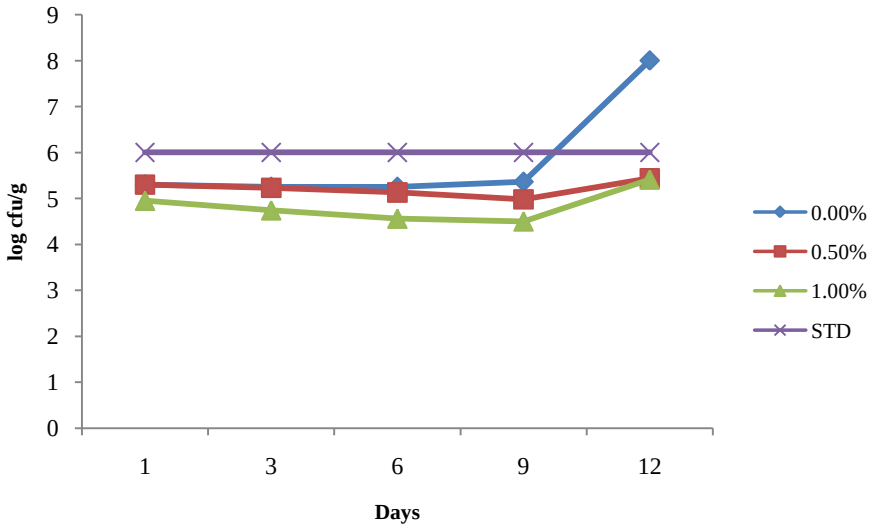
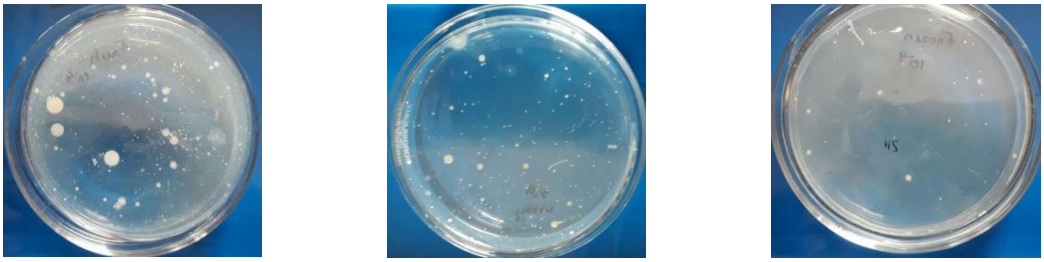


Fig 2:- Total plate count



Control **0.5% clove** **1.0% clove**

Till the 9th day of storage the spice concentrations shows a considerable decrease in the microbial growth. On the 12th day, the microbial count of minced fish without any spice(control) will exceeds the standard limit also there was an increase in counts on two spice concentrations, but they were well within the prescribed standard up to 12 days of storage(table no.1)

The dominated bacterial colonies were isolated from each day of observation is subjected to biochemical tests for the identification. According to the test results, the isolated species of bacteria are *E.coli, Staphylococcus, Bacillus* and *Pseudomonas*.

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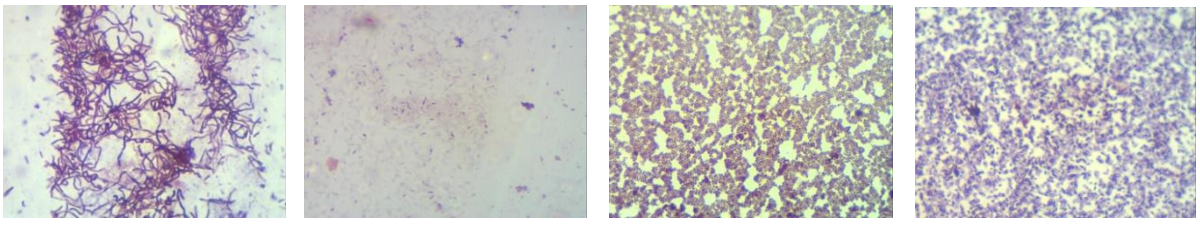
Table no. 2: Biochemical reactions of isolated microorganisms

TESTS	E.coli	Bacillus	Staphylococcus	Pseudomonas
Gram's staining	Gram negative rods	Gram positive bacilli in long chains, with spores	Gram positive cocci	Gram negative bacilli
Growth on MacConkey's agar	Lactose Fermenting	No growth	No growth	Non-lactose fermenting
Glucose fermentation	Fermented With gas	Not utilized	Not utilized	Not utilized
Indole	Positive	Negative	Negative	Negative
Methyl Red	Positive	Negative	Negative	Negative
Voges-Proskauer	Negative	Negative	Negative	Negative
Citrate utilization	Negative	Negative	Negative	Positive
Nitrate	Positive	Negative	Negative	Positive
Urease	Negative	Negative	Negative	Negative
Catalase	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Positive
TSI Media	Acid/Alkaline , Without H_2S	No change	No change	No change/alkaline, No H_2S
Mannitol motility test	Fermented motile	Not fermented, Non-motile	Not fermented, Non-motile	Not fermented motile

Fig 3: Gram staining



Bacillus **E.coli** **Staphylococcus** **Pseudomonas**

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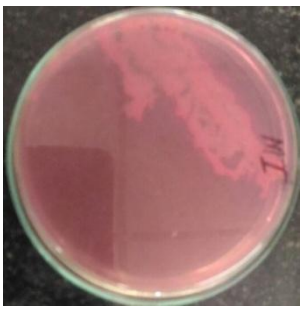
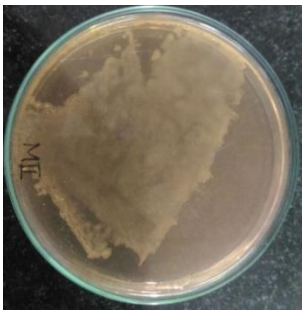
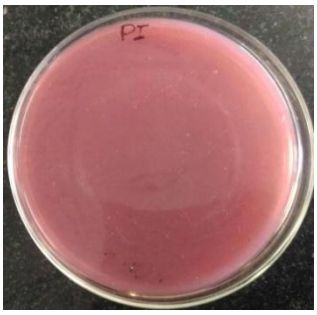
Fig 4: Biochemical results



Lactose fermenting



Non lactose fermenting



Control

Effects of aquas and ethanolic clove extracts

The identified species of bacteria were treated with aqueous and ethanolic clove extracts for analyzing the inhibitory activity of clove extracts.

Table no 3: Zone of inhibition of bacteria against aqueous and ethanolic extracts

Species of bacteria	Zone of inhibition of ethanolic extract (mm)	Zone of inhibition of aqueous extract (mm)	Zone of inhibition in control (mm)
E.coli	2	2. 3	0
Staphylococcus	2	2.5	0
Bacillus	2.2	2.3	0
Pseudomonas	1	3	0

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Fig 5: Zone of inhibition of bacteria against aqueous and ethanolic extracts

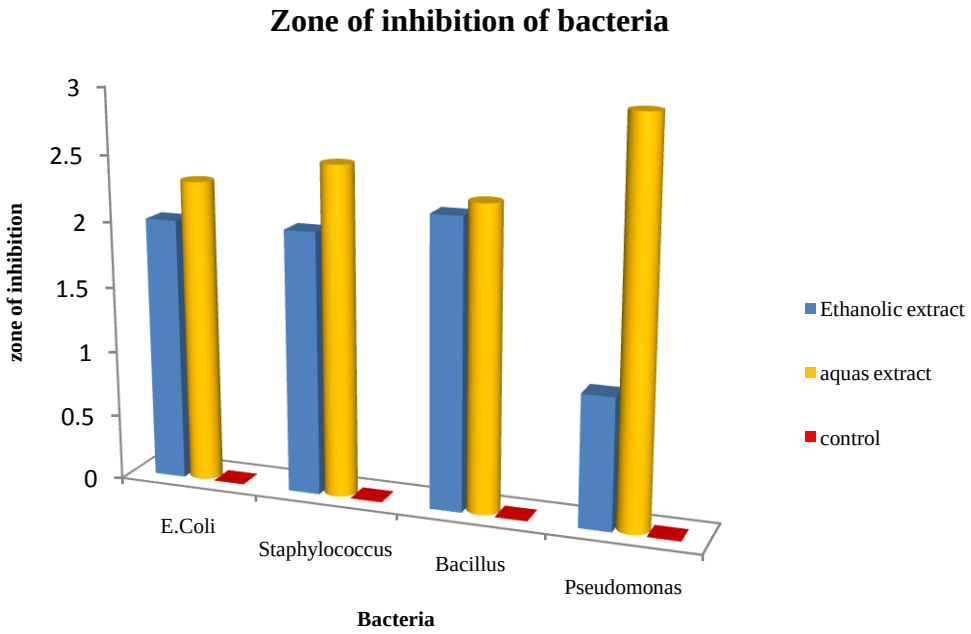

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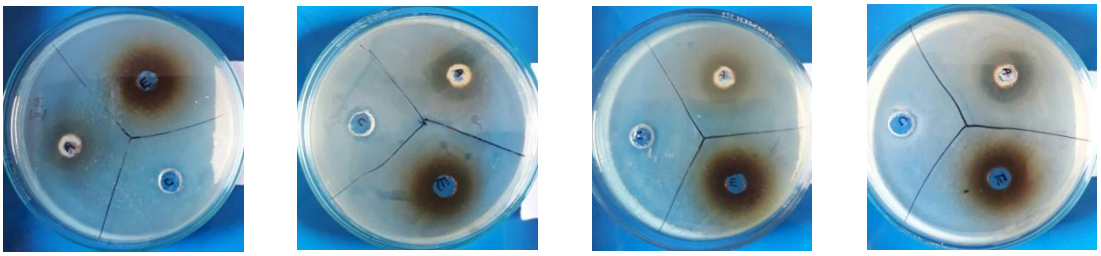
Fig 6: zone of inhibition of aquas and ethanolic extracts



Pseudomonas E. coli Bacillus staphylococcus

The zones of inhibition of four strains of bacteria were measured (Table no.3). The results indicated that antibacterial activity of aqueous extracts is higher than that of ethanolic extracts. The effect of aqueous extract is maximum on gram negative *pseudomonas* species (3.0) and minimum in E.coli and bacillus. The effect of ethanolic extract is maximum on *Bacillus* (2.2) and minimum on *Pseudomonas* species (1.0).

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Antibiotic sensitivity of bacterial isolates.

Table no 4: Antibiotic sensitivity of the bacterial samples

Organism	Zone of inhibition (mm).				
	Ciprofloxacin	Tetracycline	Penicillin	Amikacin	Streptomycin
E.coli	2.3	1.5	0	2.6	2.2
Staphylococcus	2	2.4	0	2.5	3
Bacillus	2.6	1.7	0	2.8	2
Pseudomonas	3.9	2	0	2.1	3.6

Fig 7: Antibiotic sensitivity of the bacterial samples

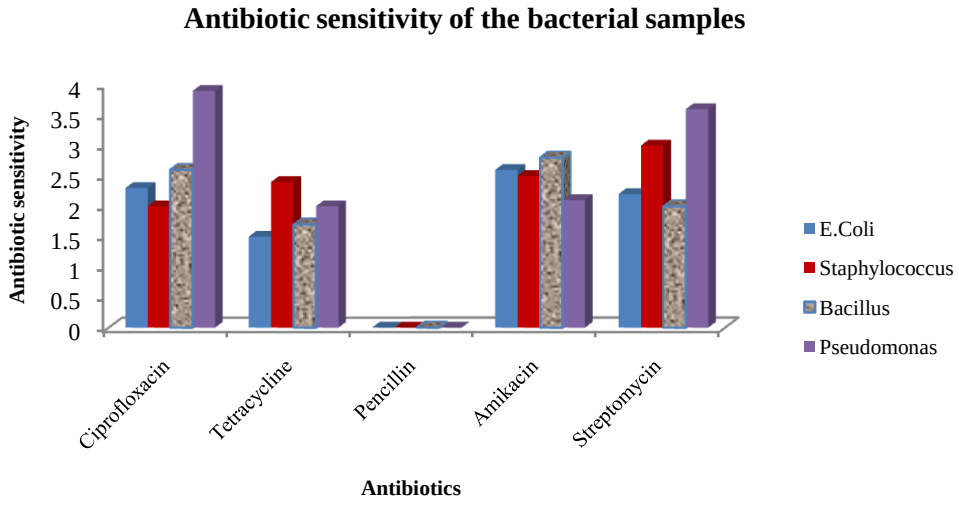

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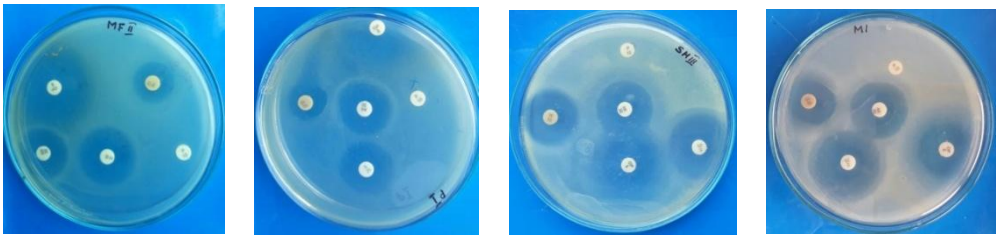
Fig 8: Antibiotic sensitivity



Pseudomonas E.coli Staphylococcus Bacillus

The antibiotic sensitivity of isolated species of bacteria indicated that all the four strains of bacteria were penicillin resistant, inhibition zone is zero. Pseudomonas showed maximum sensitivity toward ciprofloxacin (3.9) and streptomycin (3.6) and less towards tetracycline (2.0). *E.coli* showed maximum sensitivity towards Amikacin (2.6) and minimum in tetracycline (1.5). *Staphylococcus* showed maximum sensitivity towards *streptomycin* (3.0) and minimum towards ciprofloxacin (2.0). *Bacillus* showed high sensitivity towards Amikacin (2.8) and minimum in tetracycline (1.7).

Discussion

The growing concern about food safety has recently led to the development of natural preservatives to control food borne pathogens and spoilage microorganisms (Pundir R K *et al*, 2010). Due to social concerns about the safety of food containing chemicals as preservatives and the increasing antibiotic resistance of bacterial food borne pathogens, there is a growing demand in the use of natural antibacterial compounds such as herbs and spice extracts because they have both characteristic flavor as well as a potential antimicrobial activity (Smid and Gorris, 1999). From this investigation it is found that the addition of clove reduced the growth of bacteria during preservation of fish at chilled temperature. It was also found that the inhibitory efficiency of spices depends on its concentration. Among the two concentrations used in the experiment, 1.00% was found to be most inhibitory to bacterial growth. Although, samples treated with 0.5% also suppressed the growth of bacteria, but not as effectively as 1.00%.

This study also reveal the inhibitory activity of clove extracts against different bacterial species especially E.coli, Bacillus, streptococcus, and pseudomonas. The aqueous clove extract showed the maximum inhibitory effect than the ethanolic extract. According to smith clove is most effective against both gram positive and gram negative bacteria (Smith-Palmer A., Stewart *et al.,* 1998). Similar to the results of above findings by smith, the clove extracts showed good antibacterial activity against the four bacterial strains isolated. Antimicrobial activity of spices has been reported by several researchers. Rahman *et al* investigated the antibacterial activity of some

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spices such as cumin, cinnamon, *S. aromaticum*, fennel, red crushed pepper, mustard, cardamom, *Zingiber officinale*, poppy and anise against some microbes. They tested the diethyl ether treated extracts of spices *in vitro* with the bacteria *Staphylococcus aureus*, *Klebsiella pnumoniae, Pseudomonas* etc. as test strains. The disc diffusion method was used in the trial. Here the result of the disc diffusion test revealed that the ethanolic and aqueous extracts of clove showed different degrees of growth of inhibition depending upon the bacterial strains (table no: 3). Also both extracts of clove showed notable antibacterial activity against gram positive bacteria. But here the antibiotic sensitivity test indicated that both gram positive and gram negative strains are resistant to penicillin. Gram negative bacteria Pseudomonas showed high sensitivity towards Ciprofloxacin and all three gram positive bacteria showed highest sensitivity to Amikacin. The antibacterial activity of clove has been attributed to the presence of some active constituents. Eugenol is the active compound present in clove and the earlier studies suggested that the antibacterial activity of clove was probably due to this compound found in clove and eugenol is also a natural antioxidant. Many of them extracted the clove oil to determine antibacterial activity indirectly by applying on samples or directly by applying on isolated cultures and proved that the antibacterial activity of clove was probably due to the presence of eugenol. The clove oil also showed the inhibitory effect on bacterial growth and fungal growth.

This study also reveals that the refrigeration serves only as a temporary means to retard spoilage and pathogenic bacteria. Therefore, refrigeration cannot be solely relied upon to provide safety of food materials. Nevertheless, refrigeration may be used interactively with food additives, which would provide the desired safety of food materials. Several investigations showed that cinnamon, clove, pimento, thyme, oregano and rosemary had strong and consistent inhibitory effect against several microorganisms and spoilage bacteria. From this present investigation and from previous studies it was found that clove is effective in almost all temperatures and concentrations not only in fish but also in different samples such as meat, sausage, fruits, and vegetables etc. Therefore it can be used as an effective replacement of chemical preservatives.

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