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Influence Of Spices On Bacterial Growth And Histamine Formation

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Abstract

The effect of spices in decreasing histamine forming bacteria and thereby histamine formation is investigated. Quantitative determination of the biogenic amines was conducted using a Waters HPLC system with a Binary pump model M 515 and a C 18 Symmetry column with a flow rate of 1.5 ml/min. Spice treated tuna had significant difference from control sample in their histamine inhibition property ($p<0.05\%$). Fish media inoculated with *S.typhi* and then treated with spices showed l log reduction in viable cell count for all tested spices except clove. *Vibrio cholerae* was susceptible to all treatments and after 24 hrs of spice treatment it was not detectable in the TCBS plates. *S.flexnerii* was also susceptible to the activity of spices. Statistical analysis showed that the control had a significant difference ($p<0.05\%$) from the microbial number in fish media treated with clove. On SEM analysis, the untreated control showed rod shaped bacterial cells of *Salmonella typhi* measuring 1.32-1.65µm long. The growth in nutrient broth with 0.2% spice extract resulted in deformed cells. All the treated samples had pit formation suggesting localized collapse of cell wall. The cells were damaged beyond recognition in cardamom and oregano treated samples. The reduction in biogenic amine was found to be directly proportional to the reduction in histamine forming bacteria in the samples. The inhibitory ability of these spices to arrest toxic amine formation can be used beneficially in food industry.

Keywords: Histamine, Biogenic amines, Spices, Salmonella, Vibrio, SEM analysis

1. Introduction

Biogenic amines such as histamine, cadaverine and putrescine are formed from free amino acids namely histidine, tyrosine, tryptophan, ornithine and lysine respectively. Spermidine and spermine arise from putrescine (Zarei et al., 2011). The amines are important agents of food intoxication and indicators of fish spoilage. Histamine has been implicated in the toxicity of scombroid and even non-scombroid fishes (Taylor, 1986). Formation of histamine in tuna, sardine, horse mackerel and anchovy is reported by many workers (Wendakoon *et al.*, 1990; Mendes, 1999; Yamanaka *et al.*,1986; Marrakchi *et al.*,1990; Okuzumi *et al.*,1990 and Veciana *et al.*, 1990).

Histamine food poisoning (HFP) is common and occurs world wide. Thus, control of the factors that allow seafood to cause HFP deserves to be improved. HFP during 1990s caused 32% of all seafood borne incidents of human disease in England and Wales. The situation is similar in USA where HFP caused 38% of all seafood-borne human disease outbreaks. These outbreaks accounted for 18% of all the people that became ill after consumption of sea food. HFP have been responsible for 8% of all the food-borne disease and 1.2% of all the people that became ill due to food consumption in USA (Mc Lauchlin *et al.*, 2006). Between 1990 and 2003 HFP accounted for 7.5% of all

food borne diseases outbreaks and 38% of all seafood related diseases reported to U.S. Centers for Disease Control and Prevention (Dewaal *et al*., 2006).

Histamine when formed in seafood is relatively stable and not inactivated by freezing or heating such as normal cooking, hot smoking or even canning (Lehane and Olley, 2000; Flick *et al*., 2001; FDA/CFSAN, 2001; Kim *et al*., 2003). Histamine in small amounts is not toxic for humans as it is metabolized prior to reaching the blood circulation. The enzymes histamine-N-methyltransferase (HMT), monoamine oxidase (MAO) and diamine oxidase (DAO or histaminase) transform histamine to less toxic metabolites that are excreted in urine and faeces. The enzymes are found primarily in the small intestine and liver of humans (Taylor, 1986). However if the normal histamine metabolism is reduced or very large amounts of histamine are consumed, then the concentration of histamine in the blood increases. It results in cutaneous, neurological and gastrointestinal symptoms. The symptoms are mainly rashes, utricaria, flushing, headache, diarrhea and vomiting.

Anti microbial agents such as sorbic acid, citric, malic and succinic acids have a diminishing effect on synthesis of biogenic amines (Kang and Park, 1984). In this study, the effect of spices in decreasing histamine forming bacteria and thereby histamine formation is investigated.

2. Materials and Methods

2.1. Raw material

Tuna (*Euthynnus affinis*) were purchased at the day of capture from a local fishing ground located on the Arabian Sea, Vypeen, Cochin. The fish were packed in insulated styrofoam box containing ice and delivered to the laboratory, 6 hour postcapture. The fish was beheaded, gutted and cut into chuncks of 2cmx 1 cm x 1cm (l x b x h). These fish chunks were used for further studies.

2.2. Spice oleoresins

Six different spice oleoresins, i.e., rosemary, garlic, cardamom, turmeric, oregano and clove were obtained from M/s Synthite Industrial Chemicals Ltd. (Synthite Valley, Kolenchery, India).

2.3. Bacterial cultures

Pathogenic bacteria isolated from tuna and type cultures obtained from National Centre for Aquatic Animal Health, Cochin University of Science and Technology were used. The type cultures used were MTCC 3906 *Vibrio cholerae* (0139), MTCC 45 *Escherichia coli*, MTCC 1457 *Shigella flexneri* .

2.4. Bacteriological Media

For microbiological examination, standard culture medias were used (Himedia brand). For preparation of fish media, tuna meat was minced with 5 times its weight of distilled water. One ml each of this meat slurry was transferred to sterilized test-tubes (15cm x 18mm dia) and sterilized by steaming in an autoclave for 30 minutes. The tubes were cooled and used as media for growth studies of pathogenic bacteria in the presence of spice extracts.

2.5. Antimicrobial activity screening

Antimicrobial susceptibility test of the isolated organisms was done by disc diffusion method using the Kirby-Bauer technique (Bauer *et al.*, 1966) and as per modifications of Islam *et al*., (2008). All tests were performed on Mueller-Hinton agar.

2.6. Minimum inhibitory concentration (MIC)

Inoculum preparation and turbidity standard for MIC test were done as per Islam *et al*.(2008). The MICs were determined as the lowest concentration of extract inhibiting the visible growth of each organism on the agar plate.

2.7. Growth of pathogenic bacteria in fish media

Spice extract (0.2%) was added into fish media followed by inoculation of known number of the test culture suspension. 0.2% of the spice extracts were used since that was the MIC produced by the most efficient oleoresin through the *in vitro* experiment. The contents was mixed well and allowed to act for 10 minutes. The control tubes were also kept. At the end of 10 minutes, the contents were transferred to a mortar, diluted with 9 ml of sterile Normal saline. Appropriate dilutions were plated on the respective media and counts were taken.

2.8. Bacterial preparation for SEM

Bactrial culture(10ml) was centrifuged at 8000rpm in a refrigerated centrifuge for 15 minutes. The pellets were washed with sterile seawater of 0.5% salinity and fixed in 2.5% gluteraldehyde prepared in sterile seawater at 4°C overnight. The suspension was washed repeatedly with seawater and dehydrated. Dehydration was done through an acetone series of 70-100%, centrifuged and supernatant removed. After dehydration with 100% acetone, it was kept overnight in a dessicator. The sample was sputter-coated with 25nm platinum. Observations were made on a JEOL model JSM-6390 LV microscope.

2.9. Storage conditions for histamine analysis

The concentration of histamine can vary considerably even between different portions of a single fish (Frank *et al*., 1981). Therefore, in order ascertain uniform histamine level, samples from the dorsal region close to head portion were taken in all cases. This fish chunks were divided in to 8 groups. Each group of fish was subjected to dip treatments of spice extract (0.2%) of clove, cardamom, garlic, oregano, rosemary and turmeric. A commercial antimicrobial, chlorine was also used. Control sample was not subjected to any sort of treatments. The duration of dip treatment was 10 minutes at room temperature. The treated samples of fish were arranged on a plastic tray and stored at 28°C $\pm$ 2°C. Three randomly chosen fish chunks were immediately sampled (day 0), while the rest were kept at ambient temperature for 24 hours. After 1, 4, 9 and 24 hours, three randomly chosen fish samples were removed from the lot and analyzed in triplicate for the presence of histamine forming bacteria and also biogenic amines.

2.10. Biogenic amine analysis

Biogenis amine analysis was done as per Ozogul (2002) using a Waters HPLC system with a Binary pump model M 515, a 600 Gradient mixer solvent delivery system, a dual λ absorbance UV/VIS detector model 2487 and a C 18 Symmetry column (5 µM particle size, 4.6 mm id x 250 mm length column) with a flow rate of 1.5 ml/min. Data analysis was performed using EMPOWER 2 chromatography software.

2.11. Analysis of Histamine Forming Bacteria

Histamine forming bacteria was isolated using Modified Nivens medium (Niven et al., 1981). For enumerating the bacteria, 10g of fish sample was asceptically cut into a sample dish, macerated with 90 ml diluent in a sterile mortar and serially diluted. A 1ml aliquot was taken for enumeration of histamine producing bacteria and mixed with Modified Niven's medium. Allowed the plates to set. Plates were incubated at 25°C for 2 days on the Modified Niven's medium. Purple colonies with or without halos were regarded as positive histamine formers. The test was carried out in triplicates.

2.12. Statistical Analysis

Statistical analysis was done using SPSS version 17.0. Two way ANOVA test was followed by Tukey post-hoc analysis to determine specifically which treatment showed significant difference from control.

3. RESULTS

3.1. Effect of spice oleoresins on bacteria

Table 1 summarizes the antibacterial activity of spice extracts against pathogenic bacteria. Under the test conditions, all bacterial strains showed some degree of susceptibility towards each spice. Table 2 compares the inhibitory zones of spoilage bacteria isolated from tuna. ANOVA between spoilage bacteria and treatments showed that there is significant difference ($p<0.01$) between the inhibition zone produced by the treatments and bacteria used for the study. Inhibitory zone measurements reveal that bacterial strains isolated from tuna was more resistant to the spice extracts compared to the pathogenic strains. The inhibitory zones for pathogens were 6-20 mm and that of bacterial strains from tuna was 7-18mm. Turmeric showed excellent activity towards the spoilage bacteria though the activity was limited for *E.coli*, *V.cholerae* and *S.typhi*.

The post hoc tukey test conducted to analyse the behaviour of different spoilage microorganism against spices revealed that *Lactobacillus* vs *Bacillus* (0.474), *Lactobacilus* vs *Micrococcus* (0.954) and *Bacillus vs Micrococcus* (0.144) had p value greater than 0.05. This explains that there is no significant difference between the susceptibility of these bacteria towards the tested spices. The activity of *Pseudomonas* and *Aeromonas* ranged from 8 to 12mm and 7 to 13 mm, respectively, whereas, those of *Lactobacillus*, *Bacillus* and *Micrococcus* is in a higher range of 10-16mm, 8-18mm, 11-16.5mm, respectively. Since these three bacterial genera (*Lactobacillus*, *Bacillus* and

Micrococcus) belong to the Gram positive group, the difference in degree of sensitivity to the spice extracts may be a reflection of the Gram reaction of the organism.

The MIC of spice extracts obtained by agar dilution method are depicted in Table 3. *V.cholerae* showed the highest sensitivity and *S.typhi* showed highest resistance towards the spices. Two way ANOVA test explained that there was considerable difference between various treatments. Secondary analysis showed that the results also varied significantly between the different pathogens used for the study.

3.2. Effect of spice extracts on growth of pathogenic bacteria in fish media

On exposure to spice extracts, *E.coli* within fish media showed a sharp reduction in viable cell count after 24 hrs when compared to that of control samples (Fig1a). Post hoc analysis reveals that apart from turmeric ($p=0.752$), rosemary ($p=0.980$) and oregano (0.422) all other spice treatments differed significantly from control ($p<0.05$). There was 3 log reduction in *E.coli* count for cardamom treated sample. None of the pathogenic bacteria was detected in 0.2% clove treated tuna sample.

In the inhibition pattern of *S,typhi* (Fig 1b), there was only l log reduction in viable cell count for all spices when compared to control (except clove and cardamom). The tukey test conducted for detailed analysis explains that there isn't significant difference between rosemary, garlic, turmeric and oregano treatment with that of control ($p>0.05$). Complete destruction of the microbe was achieved by clove in fish media inoculated with *S.typhi*. One way ANOVA performed for estimating variance in mean value of viable *Vibrio cholerae* resulted in $p<0.05$. Secondary analysis proves that *V.cholerae* was susceptible to all treatments and after 24 hrs of spice treatment it was not detectable in the TCBS plates except the control sample (Fig 1d). In the case of *S.flexnerii* (Fig 1c), oregano and garlic showed reduced activity. But, all other spice oleoresins exhibited good antimicrobial action against these bacteria. One of the noteworthy fact was that oregano, whose activity was next only to clove, became less effective in inhibiting pathogenic bacteria in the food system.

3.3. Electron microscopic analysis of bacterial cells

Plate 1a shows a cluster of *Salmonella typhi* fixed with gluteraldehyde and observed by SEM. The typical rod shaped bacteria can be seen in this plate. Bacterial cells measures 1.32-1.65μm and is intact. The growth in nutrient broth with 0.2% spice extract resulted in deformed cells. All the treated samples had pit formation suggesting localized collapse of cell wall. Least clumping of cells was showed by garlic treated samples (Plate 1e). The bacterial shape is retained as such by this treatment though the viability of cell is not confirmed. The cells are damaged beyond recognition in oregano

treated samples (Plate 1f). The visible damage caused to the bacterial cells by the spice oleoresins confirms their antimicrobial property.

The mode of action of essential oil in spices is concentration dependent. Low concentration inhibit enzymes associated with energy production while higher amounts may precipitate proteins. 0.2% of spices has resulted in protein coagulation in the tested samples as seen in the SEM images.

3.4. Histidine decarboxylating bacteria (HDB)

The histamine forming bacteria formed in the samples during the 24 hours after treatment with spice extracts is ploted in the Fig2. There is significant difference between treatments ($p \leq 0.05$) and between time intervals ($p \leq 0.05$). The bacterial population in treated and untreated samples showed similar trend till the 4th hour of storage . After eight hours, the number of histamine producing bacteria present in all the samples shot up significantly. The control sample had significantly higher number of HDB than the spice extract treated samples at all stages of the study ($p \leq 0.05$). This difference was evident very clearly after the eight hour of storage at 28±2°C and continued to the 24th hour. This was confirmed by Post hoc tukey test.
Analysis of variance explained that there is significant difference between the treatments too. From the graphical representation it can be seen that after storage for 24 hours, clove treated samples showed the least colony forming units of HDB compared to others. Control sample produced the highest number of HDB followed by chlorine, the common chemical antimicrobial used in seafood industry. The bacterial count in the ascending order is as follows: clove < turmeric < cardamom < garlic < oregano < rosemary < chlorine < control.

3.5. Biogenic amine formation

Biogenic amines produced in tuna samples (without treatment) during the initial stage (0th hour) of the experiment and after 24 hours storage at room temperature is shown in Fig. 3a. The area given by a particular peak is directly proportional to the amine produced. It is evident that there is a drastic increase in the putrescine, cadaverine and histamine during the 24 hour storage period. A comparision between the chromatograms of 24th hour control sample and 24th hour chlorine treated and clove treated samples is shown in Fig 3 b & 3c, respectively. In all the treated samples, the area of the peak is lower than that of control.

Significant difference was observed between the treatment and time period in mean values of histamine produced at $p<0.05$. The amount of histamine produced in control and treated samples during the test period is tabulated in Table 4. The histamine production in the treated and untreated samples stored in boxes at 28 ± 2 °C did not show any significant difference after the first hour of the experiment as seen in Table 4. But, sample analysed after the fourth hour gave a higher value of histamine for the untreated tuna stored at 28 ± 2 °C than spice extract treated tuna. A

strong positive correlation (r = 0.836) was also seen between the amount of histamine produced and the HDB count. This explains that bacterial count is directly propotional to histamine content. For this reason, when the number of viable bacteria increases, amount of histamine also goes up. Therefore, the reduction in amine production in treated samples with various spices can be explained by their strong antimicrobial action.

A mean histamine reduction of 6.5mg/100gm in spice treated samples was observed from the control after 8 hour duration. Except cardamom and turmeric other oleoresin treated samples showed was only negligible increase (average amount =0.416mg/100gm) in histamine content between the 8^{th} and 24^{th} hours storage. The control samples experienced an increase of 6.7 mg/100gm at the above periods. The defect action level was attained by 4 hour of storage in the control. No sample treated with spices exceeded the rejection limit of histamine (50ppm) upto 8^{th} hour. In clove, garlic, oregano and rosemary, the samples remained below the permitted limit of histamine even after 24 hours.

Among the spice treated samples, the tuna chunks dipped in cardamom and turmeric produced a result closer to the control sample. Histamine content in clove, garlic and rosemary treated sample varied significantly from the other treatments with clove giving the lowest value of histamine. The level of histamine was over 18.2 mg in control samples stored for 24 hours at 28 ± 2 °C and 3.91 mg in clove treated samples. Chlorine treated tuna samples were also analysed since, it is used as a commercial antimicrobial in the seafood industry. Though it produced excellent results till the eighth hour after treatment of the samples(Table 4), the build up of histamine in the later hours showed a sharp increase from that of the spice oleoresin treated samples. The volatile nature of chlorine may be the reason for this abrupt increase in histamine content after the 8^{th} hour.

Freshly caught tuna had a cadaverine content of 0.14mg/100gm and putrescine content of 0.047mg/100gm (Table 5 and 6). The concentrations of cadaverine and putrescine increased more rapidly than spice extract treated tuna stored for 24 hours ($p < 0.05$). The concentration of these biogenic amines were typically much lower than the concentration of histamine. Interestingly, the concentration of histamine was propotional to the concentration of cadaverine as well as to the sum of concentrations of histamine, putrescine and cadaverine. Studies have shown that the levels of cadaverine in toxic or decomposed fish are generally several times greater than the levels of putrescine. In this experiment also the cadaverine content formed at the end of 24 hour storage was 9 times greater than that of putrescine content in the control sample.

4. Discussion

Seafood harbours a wide variety of bacterial species which cause spoilage. Their growth and metabolism results in the formation of amines, sulfides, alcohols, aldehydes, ketones and organic acids with unpleasant and unacceptable off-flavours. Microbial

activity is retarded during storage but is not liminated. Hence, the control of microbial growth is the primary step towards ensuring seafood safety.

4.1. Effect of spice oleoresins on bacteria

Deans and Ritchie (1987) had found that clove shows antibacterial properties against 23 genera of bacteria. The lowest MIC (minimum inhibitory concentration) of 0.125% was observed in clove among the spices tested by Yutaka *et al*. (2006). In the present study, the MIC of clove against *V.cholerae*, *S.flexnerii* and *E.coli* was found to be 0.12%, though the MIC of *S.typhi* was set a little higher at 0.20%.

Essential oils of clove, coriander and nutmug were effective in inhibiting the growth of *Aeromonas spp*. A marked reduction of this organism also occurred in inoculated samples of cooked, non cured pork treated with clove (Stecchini *et al*., 1993). 15% of the total bacterial population isolated from tuna was found to consist of *Aeromonas*. Hence, antimicrobial agents which are effective on this species will contribute in decontaminating the fish media more effectively.

E.coli was found to be susceptible to 0.12% of clove extract during the investigation. This is in agreement with the potent antimicrobial activity of clove reported against *Escherichia coli* (De *et al*., 1999). Clove showed the highest inhibitory effect in an investigation carried out by Pokhrel *et al*. (2012) while, coriander, ginger and turmeric showed no inhibitory effect in the case of crude ethanolic extracts.

Oregano generated an MIC of 0.12% towards *E.coli* . The action of oregano is due to damage in membrane integrity, which further affects the pH homeostasis and equilibrium of inorganic ions (Lambert *et a*l, 2001). It is reported that cranberry and oregano, in synergistic combination with lactic acid, can inhibit *V. parahaemolyticus* in seafood systems. Such a strategy can be used for enhancing food safety in food industry.

Activity of garlic was lower than that of clove, cardamom etc. But, still it had reduced 60.56 % of the initial bacterial load in fish media due to the presence of allicin. It is reported by many workers that allicin completely inhibits a variety of Gram-positive and Gram-negative bacteria.

Assays performed by Agaoglu *et al*. (2005) indicate that cardamom seed has inhibitory activity on *Staphylococcus aureus*, *Micrococcus luteus* and many other bacterial strains. In this study, turmeric is seen to have significant effect on spoilage bacteria. The antimicrobial effects of alcoholic extract of turmeric, curcumin and oil from turmeric have been studied by Banerjee and Nigam (1978). Extracts from turmeric as well as active principles from curcuminoids were found to inhibit the growth of numerous Gram positive and Gram negative bacteria, fungi and intestinal parasite, *Entamoeba histolytica*. Curcumin at concentrations of 2.5 -50.0mg/100ml inhibited *in vitro* growth of *Staphylococcus aureus* (Shankar and Srinivasamurthy, 1979).

Antibacterial activity of the essential oil of rosemary against an array of bacterial and fungal species including *Listeria monocytogenes* and *Aspergillus niger* have been reported by Faliero *et al*. (1999) and Baratta *et al*. (1998). Gram positive bacteria such as *Staphylococcus aureus* and *S.epidermid* have been found to be more susceptible to rosemary oil than other Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Pintore *et al*., 2002).

Antimicrobial and synergistic activity of ingredients of betel leaf, betel nut, cardamom, clove and fennel seeds was tested against microbial population of oral cavity and four enteric pathogens namely *Staphylococcus aureus, Salmonella typhi, Escherichia coli and Shigell flexneri*. It was found that the bacteria investigated showed susceptibility against the tested extracts (Ghanwate and Thakare, 2012). In addition to antimicrobial properties some spice extracts like oregano, rosemary and thyme exhibit antioxidant properties also (Kačániová *et al*., 2012) which can be an additional advantage in processed lipid rich foods and fatty fishes like mackerel (Sulochanan, 2008).

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4.2. Activity of spices on food system

There as been relatively few studies of the antimicrobial action of essential oils in model food systems and in real foods. Most of the studies have focused on the activity *in vitro*, and only very few authors have documented their antimicrobial activity on food products (Bajpai *et al*., 2009).

In the present study, tested spices showed marked reduction in total plate count of fish media from that of untreated control. One of the striking factor is that the antimicrobial activity of all the spices is considerably reduced in fish media. The effect is much evident in oregano when compared to its performance in Mueller-Hinton agar. The efficacy of spice extracts *in vitro* is often much greater than *in vivo* or *in situ*, i.e. in foods (Nychas and Tassou, 2000). Growth of *E.coli*, *Salmonella*, *Staphylococcus* was inhibited by oregano extract in agar media and broth cultures. However, the antimicrobial action of this spice in a food system seemed to vary much. It as been reported that the type of oil or fat present in a food can affect the antimicrobial efficacy of spices. Skandamis (2001) has found a reduction in the antimicrobial activity of thyme in full fat cheese. In his experiment, in full fat cheese clove was the only spice which reduced the bacterial count. In marine organisms like tuna, lipid is the second largest biochemical constituent. The interference of lipid molecules can be one of the reason for the reduced activity of oregano and other spices in fish media.

Antimicrobial activity of 14 spice extracts against pathogenic and spoilage bacteria in fresh pork and ham slices showed that individual extracts of clove, rosemary, cassia bark, liquorice was the best inhibitor against the pathogens (Huiyun *et al*., 2009). Sterile reconstituted full-cream milk inoculated with 10^{3} -10^{4} CFU/g of *B.subtilis* and *E.coli*, followed by addition of oregano, marjoram, sage and licorice extracts reduced the bacterial growth by 2.5 log cycle and 0.5 log cycle, respectively after one day storage (Al-Turki *et al*, 2008). Chicken meat patties treated with essential oils of garlic, clove and

cinnamon and inoculated with *Staphylococcus aureus* (MTCC 3103) was studied by Babu *et al*. (2012). The samples were stored at refrigeration temperature and the results revealed that essential oil were effective in reducing the bacterial counts. According to these studies, spices are found to be effective in extending the shelf life of food products.

4.3. Electron microscopic analysis of bacterial cells

Scanning electron microscopic images showed pit formation in spice treated bacterial samples. Exact mechanism of antibacterial action of spices and derivatives is not yet clear (Lanciotti *et al*., 2004). But, pit formation suggests localized collapse of cell wall. Many hypothesis have been given regarding the activity of spices. Hydrogen bonding of phenolic compounds to membrane proteins followed by partition in the lipid bilayer was suggested as the reason for susceptibility of microbes towards the spices by Juven *et al*. (1994). Other hypothesis given by workers involve: i) perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes (Cox *et al*., 2000); ii) membrane disruption (Caccioni *et al*., 2000); iii) destruction of electrons transport systems (Tassou *et al*., 2000) and iv) cell wall perturbation (Odhav *et al*., 2002). Generally, gram-negative bacteria have been reported to be more resistant than Gram-positive to essential oils antimicrobial effect because of their cell wall lipopolyssaccharide (Russel, 1991). This is evident in the increased activity of tested spice extracts towards *Micrococcus*, *Bacillus* and *Lactobacillus* when compared to Gram negative species, *Pseudomonas* and *Aeromonas*. Cell wall lipopolyssacaride may prevent essential oils active compounds reaching the cytoplasmic membrane of Gram-negative bacteria (Chanegriha *et al*., 1994).

Essential oils damage the structural and functional properties of membranes (Ultee *et al*., 1999, 2000, 2002). Carvacol, an active component of many essential oils, has been shown to destabilize the cytoplasmic and outer membranes and act as ‘proton exchanger’, resulting in a reduction of the pH gradient across the cytoplasmic membrane (Lambert *et al*., 2001; Helander *et al*.,1998). The collapse of the proton motive force and depletion of the ATP pool eventually lead to cell death (Ultee *et al*., 2002).

Gill and Holley (2004) had reported that effects on energy generation of bacterial cells play a major role in the activity of eugenol at bactericidal concentrations. The mode of action of essential oil is concentration dependent. At lower concentrations, enzymes associated with energy production is inhibited while higher amounts precipitate proteins. It is uncertain whether membrane damage is quantitatively related to the amount of active antimicrobial compound to which the cell is exposed. It is possible that, once small injuries are caused, the breakdown of the cell follows (Judis, 1963). It has been hypothesized that the inhibition involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability, unavailability of vital intracellular constituents (Juven *et al*., 1994; Kim *et al*., 1995) and/or impairment of bacterial enzymes systems (Wendakoon and Sakaguchi, 1995).

Helander *et al*. (1998) assayed the effect of carvacrol, (+) carvone, thymol and trans-cinnamaldehyde on *E. coli* O157:H7 and *S. typhimurium* and reported that carvacrol and thymol decreased the intracellular ATP content of *E. coli* cells while the extracellular ATP simultaneously increased. This indicated disruptive action of these compounds toward cytoplasmic membrane. Since carvacol and thymol is the major component of oregano, the same reason is responsible for the inhibitory action shown by the spice towards the bacteria.

It is postulated that the antimicrobial activity of garlic is due to the inhibition of succinic dehydrogenase via the inactivation of thiol group. Dellaquis and Mazza (1998) described antimicrobial properties of isothiocyanate derived from onion and garlic. For isothiocyanates, it was hypothesized that they inactivated extracellular enzymes through the oxidative cleavage of disulphide bonds (Brull and Coote, 1999). Dellaquis and Mazza (1998) purposed that the formation of reactive thiocyanate radical could mediate the antimicrobial property.

4.4. Histamine forming bacteria

Pseudomonas, Staphylococcus and bacteria in the family *Micrococcaceae* and *Enterobacteriaceae (Escherichia* and *Salmonella)* were isolated and identified from tuna. These bacteria are known to possess amino acid decarboxylases (Galgano *et al*., 2009;). This enzyme is responsible for producing biogenic amines in food products contaminated with these bacteria. The spice extracts used in the experiment had already shown strong antibacterial activity against these bacteria present on tuna. The decrease in HDB count in the spice treated samples is a result of reduction in the viable count of *Pseudomonas, Staphylococcus* and other bacteria belonging to *Micrococcaceae* and *Enterobacteriaceae.*

The increase in bacterial number over time can proportionately raise the amount of amines formed because amine production has been recognised as a defense mechanism of microorganisms against an acidic environment (Karovičová and Kohajdová, 2005; Suzzi and Gardini, 2003).The fish as a substrate for growth of this bacteria can readily provide an slightly acid environment during its postmortem changes. Tkachenko *et al*. (2001) suggested that some strains, with amino acid decarboxylase activity, could overcome or reduce the effects of temperature, NaCl, and other biological and chemico-physical factors that induce stress responses in the cells, with the production of some biogenic amines.

The importance of using measures focused on the hygienic quality of both raw material and processing units to avoid the development of aminogenic contaminant bacteria and in turn, to reduce biogenic amines content, is well known. However, proper hygiene may not be enough to avoid some biogenic amines formation and other technological measures must be applied (Latorre-Moratalla *et al*., 2010).

The histamine producers include both mesophilic and psychrotolerant species. Strong histamine producers which include mesophiles do not produce toxic concentrations of histamine below 7-10°C. These bacteria include the Gram-negative *Morganella morganii, Hafnia alvei, Raoultella planticola, Raoultella ornithinolytica, Klebsiella oxytoca, Citrobacter braaaki, C.freundii, Enterobacter aerogenes, Proteus vulgaris, Pr.mirabilis, P.damselae subsp damselae, Serratia fonticola* and Gram-positive *Staphylococcus epidermis and Tetragenococcus muriaticus* (Kim *et al*., 2001, 2002; Kanki *et al*., 2002; Takahashi *et al*., 2003). *Photobacterium phosphoreum* and *Morganella psychrotolerance* are also strongly histamine producing psychrotolerant species (Dalgaard *et al*., 2006) which have temperature optimum at 20-30°C (Dalgaard *et al*.,2008).

The present method used for controlling histamine food poisoning mainly target mesophilic species. The control measures used are mostly maintaining a low temperature (0-4°C) with the help of ice cubes and chill rooms at all levels of food processing. But, during frozen storage, the major factor in the histamine production will be psychrotolerant bacteria. *Pseudomonas spp*. are reported as important spoilage organisms in many chilled food products, such as milk (Reddy *et al*.,1969), chicken (Pittard *et al*.,1982) and fish (Miller *et al*.,1973 a,b), in which they become the dominant flora during chilled storage. This dominance is assumed to be attributable exclusively to their rapid growth at chill temperature. *Pseudomonas spp.* and *Bacillus spp.* were found in psychrotrophic conditions in previous studies (Singh and Venkataramana, 1998). It is also reported that these bacteria possess amino acid decarboxylases (Galgano *et al*., 2009). In the present experiment, 18% of the initial bacterial flora on tuna was *Pseudomonas spp.* which implies the risk of low temperature maintenance alone for keeping histamine formation under control. This aspect is not given due care in the present condition. Merely reducing the temperature of the product to be consumed will not help in managing the histamine food poisoning. It seems more promising to reduce growth of the strongly histamine producing bacteria in products of the relevant marine finfish.

In the present study, the spice treatment given to the tuna prior to storage ensured the reduction in the number of histamine forming bacteria. It's use will help in bringing down the risk of histamine food poisoning even under storage conditions by keeping the psychrophilic and mesophilic population in check.

4.5. Biogenic amines formation

The treatment of tuna with spice extracts have resulted in decreased production of histamine with respect to the untreated samples. Studies show that extract of clove flower bud inhibits immediate hypersensitivity in rats by inhibition of histamine release from mast cells in *vivo* and *in vitro* (Kim *et al*., 1998). This property might be responsible for the reduced histamine production in clove treated samples. The inhibitory activity of the essential oil component and other natural antimicrobials on histidine decarboxylase is a possibility that needs further research.

Chlorine is used as an commercial antimicrobial in the seafood industry. Though it produced excellent results till the eighth hour after treatment of the samples, the build up of histamine content in the later hours had a sharp increase from that of the spice oleoresin treated samples. The volatile nature of chlorine may be the reason for this abrupt increase in histamine content after the 8th hour. This information is supported by a study conducted by Baranowski *et al*. (1990). In their study, post-harvest antimicrobial treatments of fish did not show much promise in inhibiting histamine information. Fishes (*Mahi mahi*) were incubated in seawater and in seawater containing 100 ppm of sodium hypochlorite or chlorine dioxide. But, neither histamine formation nor quality loss was inhibited. Moreover, the histamine content in sodium hypochlorite treated samples (2340ppm) were higher than the control (1,230ppm) sample after 18 hours of incubation.

High concentrations of NaCl is found to inhibit the growth of bacteria and thereby histamine formation in seafood (Emborg and Dalgaard, 2006). Histamine formation at toxic level was delayed for one to two days by 3% NaCl and from one to four days by 4% NaCl (Yamanaka *et al*.,1985). But, one drawback is that 3% of NaCl will make the fish too salty for consumption. In the present study it was found that we could reduce the histamine formation by applying 0.2% of spice extracts. Sensory acceptability of spices at this concentration is reported by Dhanya and Mathew (2017).

Histamine alone may not be the culprit in the toxicity of scombroid fishes. There were instances were people became ill due to consumption of seafood with less than 500mg of histamine/kg (Emborg and Dalgaard., 2007). Human subjects given up to 67.5 mg histamine orally did not produce any subjective or objective symptoms of histamine poisoning (Granerus, 1968). Generally, high histamine levels are able to cause a toxic response, but subsequent research has indicated that other factors may also be responsible. A study by Clifford *et al*. (1991) was conducted on mackerel fillets associated with an outbreak of scombrotoxicosis. Statistical analysis failed to detect any differences in amine content between fillets which were shown to be scombrotoxic and those failing to induce symptoms of poisoning. Available data from challege studies with human volunteers suggest that pure histamine cannot always explain the toxicity of histamine-containing seafood. One hypothesis for this discrepensy is that oral toxicity of histamine in seafood can be potentiated by different compounds including other biogenic amines (Taylor,1986). Cadaverine and putrescine have been shown to potentiate the toxicity of histamine (Stratton *et al*., 1991).

When cadaverine was administered simultaneously with histamine, peroral toxicity was observed in the guinea pigs (Bjeldanes *et al*., 1978). Klausen and Lund (1986) reported that at 10°C the high cadaverine contents of mackerel in comparison with herring could be responsible for mackerel often being implicated in scombroid poisoning and not herring, since histamine levels were similar in both. Cadaverine and putrescine, as well as other diamines, have been suggested to facilitate the transport of histamine through the intestinal wall and to increase its toxicity (Fernandez-Salguero and Mackie, 1987).

A variety of microorganisms are able to produce biogenic amines. The production of cadaverine and putrescine by microorganisms is not surprising since the covalent linking of cadaverine and putrescine to the peptidoglycan is necessary for normal microbial growth (Suzuki *et al*., 1988). It may not contribute to significant amounts of putrescine and cadaverine in food samples. Neverthless, a negligible decrease of these amines can occur in the presence of preservatives.

Despite all uncertainties reported, histamine levels above 500 - 1,000 mg / kg (500 - 1,000 ppm) are considered potentially dangerous to human health based on the concentrations found in food products involved in histamine poisoning (Ten *et al*., 1990). The spice oleoresin treatment is found to reduce the production of these biogenic amines along with histamine. Hence, whether or not the toxicity of biogenic amine is proved, use of spices will help in reducing HDB formation and as a result help in reducing the risk of histamine food poisoning.

5. Conclusion

A recap of several notable recent food poisoning outbreaks illustrats the diverse pathways to food borne illness and demonstrated the need for constant vigilance by both individuals and the food industry. A large number of antibiotics have been tried earlier as preservatives to check the growth of psychrophilic flora of fish stored in ice and to extend its shelf life for a reasonably longer period. Addition of antimicrobial chemical preservatives can better protect the meat and fish from microorganisms. Because of the awareness among the consumers, they are preferring the food without any chemical preservatives. This is especially a worrying issue for the seafood exporters. The use of spice oleoresin as an antimicrobial agent in controlling the spoilage and pathogenic bacteria present on fish has been studied.

Bacterial strains showed an MIC ranging from 0.12 % to 0.8%. *Salmonella typhi* was susceptible to clove at a concentration of 0.2%. The spice with the widest spectrum of activity was found to be clove (*Syzygium aromaticum)*. Activity of oregano was comparable to that of clove except in the case of *S.typhi*. Cardamom and Rosemary had almost a similar pattern of inhibition zones and the activity was above 10 mm for all the pathogenic species tested by disk diffusion method.

In model food systems with fish as the growth medium, all spices showed marked reduction in total plate count from that of untreated control. Clove treated samples showed an 88% reduction in the total plate count compared to the untreated control samples. Rest of the spices had a reduction rate above 50%. Fish media inoculated with pathogens also showed a sharp reduction in viable cell count after 24 hrs when compared to that of control samples. None of the pathogenic bacteria was detected in 0.2% clove treated tuna sample.

In case of *S.typhi*, there was only l log reduction in viable cell count for all spices except clove. *Vibrio cholerae* was susceptible to all treatments and after 24 hrs of spice treatment it was not detectable in the TCBS plates except the control sample. *S.flexnerii* was also susceptible to the activity of spices. Thus, all other spice oleoresins exhibited promising antimicrobial action. Statistical analysis showed that the control had a significant difference ($p<0.05\%$) from the microbial number in fish media treated with clove.

On SEM analysis the untreated control showed rod shaped bacterial cells of *Salmonella typhi* measuring 1.32-1.65µm long. The growth in nutrient broth with 0.2% spice extract resulted in deformed cells. All the treated samples had pit formation suggesting localized collapse of cell wall Least clumping of cells was showed by garlic treated samples. In this case, bacterial shape is retained as such. The cells were damaged beyond recognition in Cardamom and Oregano treated samples.

Proper hygiene may not be enough to control biogenic amines formation and other technological measures must be applied.The histamine producers include both mesophilic and psychrotolerant species. The present method used for controlling histamine food poisoning mainly target mesophilic species. But, during frozen storage, the major cause for histamine production will be psychrotolerant bacteria. This aspect is not given due care. Mostly in such cases, merely reducing the temperature of the product to be consumed will not help in managing the histamine food poisoning. At present it seems more promising to reduce growth of the strongly histamine producing bacteria in products of the relevant marine finfish.

In summary, spice oleoresins have potent activity against spoilage and pathogenic bacteria present on tuna. Clove exhibited highest activity at the minimum concentration (0.2%) in *in vitro* studies and in fish media. Use of spices could increase the shelf life and decrease the possibilities of food poisoning and spoilage in processed foods. Treatment with spice oleoresins could delay biogenic amine formation in tuna. Clove, Garlic, Oregano and Rosemary treated tuna had significant difference from control sample in their histamine inhibition property. This reduction in biogenic amine is directly proportional to the reduction in histamine forming bacteria in the spice treated samples. Spices are of great importance to the food industry. The inhibitory ability of these spices to arrest toxic amine formation can be used beneficially and hence, their application on fish can be strongly recommended. Since spices are usually used for flavouring purposes, its consumer acceptability is already proved.

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Treatments	Inhibitory Zone in mm				
	E.coli	***V.cholerae***	***S.typhi***	***S.aureus***	***S.flexnerii***
Garlic	8.0±0.00	9.0±1.00	9.0±1.00	8.0±0.50	10.0±1.2
Clove	20.0±2.00	16.0±2.00	19.0±1.00	15.0±1.40	16.0±1.7
Turmeric	6.0±0.00	6.0±0.00	6.0±0.00	8.0±1.00	14.0±0.5
Cardamom	10.0±1.00	13.0±1.50	11.0±0.00	10.0±1.00	14.0±1.0
Oregano	10.0±0.43	12.0±0.94	17.0±2.00	11.0±0.62	13.0±1.0
Rosemary	10.0±0.62	11.0±0.30	11.0±1.00	10.0±0.46	1.0±1.4

Table 1. Comparision of Inhibitory zones of pathogenic bacteria against spice extracts(5µl)

The given values are expressed as mean±standard deviation, n = 3.

Table 2. Comparision of Inhibitory zones of bacterial strains isolated from tuna against spice extracts(5µl)

Treatments	Inhibitory Zone in mm				
	Micrococcus	***Pseudomonas***	***Bacillus***	***Aeromonas***	***Lactobacillus***

Garlic	16.0±1.00	9.0±0.35	8.0±0.0	8.0±0.60	10.0±1.00
Clove	16.5±1.50	15.0±0.80	18.0±1.4	13.0±1.00	15.0±1.15
Turmeric	15.0±2.00	12.0±1.11	15.0±0.5	7.0±0.00	16.0±2.00
Cardamom	11.0±1.20	8.0±0.13	12.0±1.0	11.0±0.54	13.0±2.00
Oregano	13.0±1.14	12.0±0.56	17.5±1.5	9.0±1.00	14.5±1.04
Rosemary	11.0±0.50	11.0±1.04	12.0±0.42	11.0±1.00	13.0±0.56

The given values are expressed as mean±standard deviation, n = 3.

Table 3. Minimum inhibitory concentration of spice extracts on pathogenic bacteria

Pathogen	**Minimum inhibitory concentration (MIC)**					
	clove	garlic	turmeric	cardamom	rosemary	oregano
S.typhi	0.20%	>0.8%	>0.8%	0.80%	>0.8%	0.40%
E.coli	0.12%	0.40%	0.40%	0.80%	0.80%	0.12%
V.cholerae	0.12%	0.80%	0.24%	0.12%	0.12%	0.12%
S.flexnerii	0.12%	0.12%	0.80%	0.24%	0.12%	0.12%

Table 4. Histamine content in spice extract treated tuna stored at 28 ± 2 °Cfor 24 hours (*mg/100g)

Stor-age hrs	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric

0 1 4 8 24	1.48±0.05 2.34±0.2 8.05±1.1 11.5±1.09 18.2±0.83	1.48±0.05 1.08±0.08 1.59±0.45 1.82±0.39 23.22±1.7	1.48±.05 2.34±0.5 3.07±0.7 4.72±1.3 16.99±1	1.48±0.05 1.45±0.3 1.93±0.58 3.33±0.44 3.91±0.8	1.48±0.05 1.43±0.62 1.63±0.79 3.44±0.34 3.99±0.85	1.48±0.05 1.97±0.61 4.45±0.64 4.47±0.27 4.595±0.73	1.48±.05 0.96±0.8 1.37±0.3 3.24±0.8 4.41±0.5	1.48±.05 3.98±0.6 5.31±0.4 6.09±0.7 16.05±1

* values are given as Mean±SD.

Table 5. Changes in Putrescine content in treated and untreated samples of tuna stored at ambient temperature (*mg/100g)

Storage period (Hours)	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
1	0.047 ±0.01	0.013 ±0.002	0.016 ±0.01	0.022 ±0.005	0.027 ±0.01	0.016 ±0.01	0.024 ±0.01	0.033 ±0.01
4	0.081 ±0.01	0.035 ±0.01	0.046 ±0.01	0.055 ±0.01	0.015 ±0.01	0.022 ±0.01	0.025 ±0.01	0.023 ±0.01
8	0.095 ±0.02	0.064 ±0.01	0.046 ±0.01	0.040 ±0.006	0.032 ±0.01	0.028 ±0.01	0.029 ±0.01	0.028 ±0.01
24	0.115 ±0.013	0.359 ±0.01	0.076 ±0.01	0.052 ±0.01	0.057 ±0.009	0.045 ±0.01	0.050 ±0.011	0.029 ±0.005

*values are given as Mean±SD

Table 6. Change in Cadaverine content in treated and untreated samples of tuna stored at ambient temperature (mg/100g)

Storage period (Hours)	Control	Chlorine	Cardamom	Clove	Garlic	Oregano	Rosemary	Turmeric
1	0.144 ±0.04	0.023 ±0.01	0.022 ±0.01	0.006 ±0.01	0.025 ±0.01	0.005 ±0.01	0.037 ±0.01	0.150 ±0.01

4	0.057 ±0.02	0.030 ±0.01	0.049 ±0.01	0.030 ±0.01	0.011 ±0.01	0.013 ±0.006	0.036 ±0.01	0.154 ±0.01
8	0.206 ±0.01	0.154 ±0.01	0.144 ±0.01	0.076 ±0.013	0.067 ±0.01	0.096 ±0.008	0.079 ±0.003	0.155 ±0.01
24	0.955 ±0.011	0.840 ±0.02	0.294 ±0.01	0.106 ±0.007	0.091 ±0.01	0.183 ±0.01	0.143 ±0.01	0.224 ±0.01

*values are given as Mean±SD

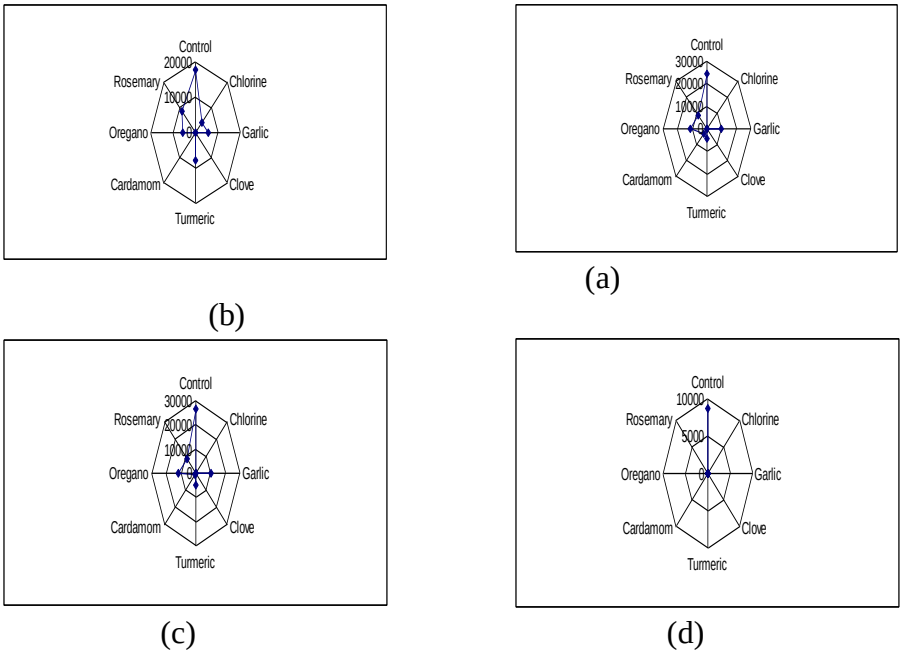


Fig 1. Viable count of a) *S.typhi* b) *E.coli* c) *S.flexnerii* and d) *V.cholerae* in fish media (Radar displays changes in values relative to the central point)

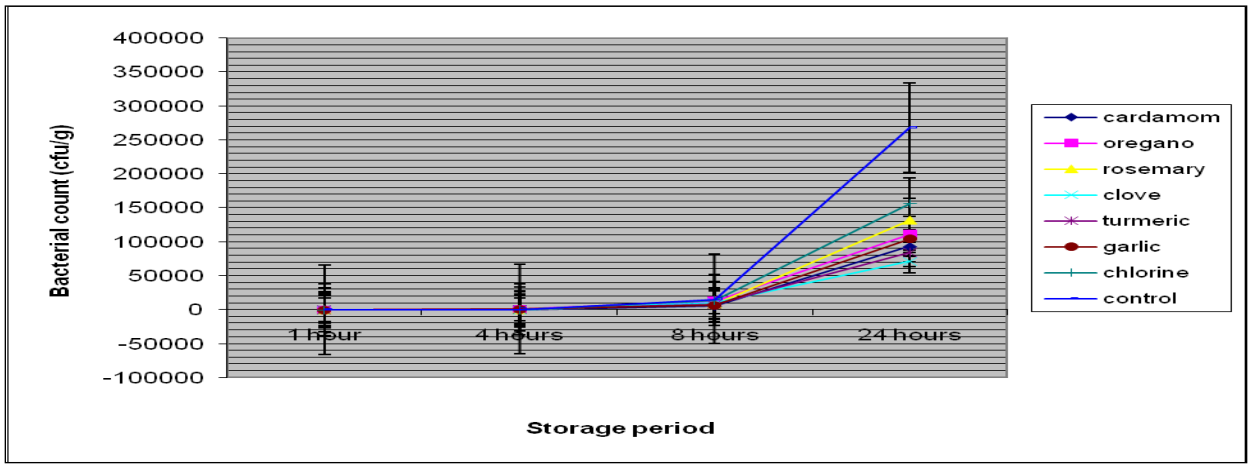
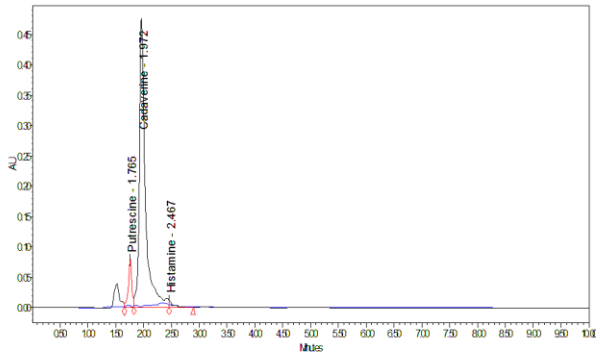
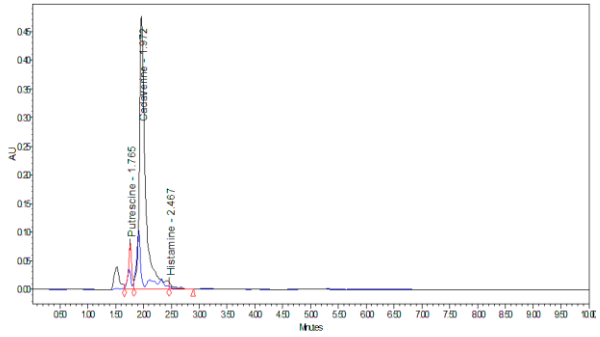


Fig. 2. Histidine decarboxylating bacteria formed in treated tuna samples during storage at ambient temperature.



(a)



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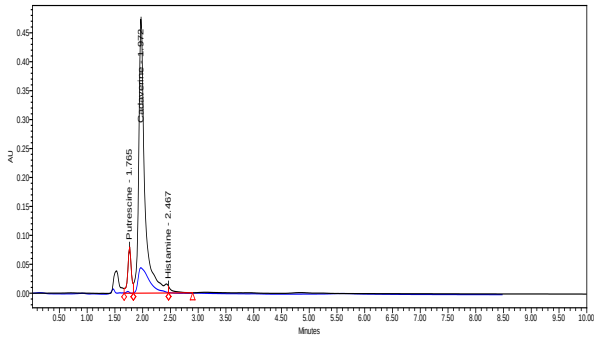

(c)

Fig 3. Comparison of biogenic amines formed during 0hr and 24 hr a) control b) control and chlorine and c) control and clove treated samples of tuna stored at 28±2°C

a) ---- zero hour control, ---- 24th hour control

b) and c) --- 24th hour chlorine/clove treatment, --- 24th hour control