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Optimization Of Concentration Of Clove (*Syzygium Aromaticum*) Extract On Reduction Of Histamine Content During Fermentation Of Idli Batter

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Abstract: Fermented foods are rich in nutritional profile and idli batter has its attention as it is a short term stored fermented food and now considered globally. The fermented batter may contain histamine which are produced during fermentation. The clove extract was known for its antimicrobial activity along with lots of medicinal value. Hence an attempt has been made to optimize the concentration of clove extract for addition in idli batter during fermentation to reduce the production of histamine. The effect of clove on the physiochemical parameters such as pH, color, titratable acidity was analyzed during storage period (0,2,4,6 hours) and it was found that the physiochemical parameters were not affected during the storage hours. The titratable acidity of the idli batter and clove added idli batter was found to be in the range of 0.16-0.18 and 0.10 - 0.28 % during the storage period. The Microbial load were also observed by analyzing the total plate count during storage hours and it reduced the microbial count from 9.05 CFU/g to 8.60 CFU/g during the storage period of 6 h in the clove extract added idli batter. Histamine producers were isolated using Nivens agar media. The histamine content in the clove extract added idli batter was found to be low at 6th hr (0.06 mg/kg.) The GC-MS analysis on phytocompounds in clove revealed that eugenol is a major compound present in clove which may be responsible for antibacterial activity and reduce the histamine producing organism. The histamine content in the control and clove extract idli batter was analyzed using modified colorimetric method and revealed that the addition of clove extract reduced the histamine content during storage period that can help in increased shelf life of idli batter.

Keywords: Clove extract, idli batter, colorimetric assay, phytocompounds, GC-MS, eugenol

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1. INTRODUCTION

Biogenic amines (BAs) are poisonous chemicals produced by microbial action in foods during fermentation process and storage.[1]The biogenic amines such as histamine, tyramine, putrescine, cadaverine are reported as most frequently occurring amines.[2] Histamine is a most active biogenic amine found in meals and soft drinks. It arises in food because of the removal of the alpha carbonyl group by microbial action from the amino acid histidine during the decarboxylation reaction.[3] The high levels of histamine were reported in various foods like fish and fish products, dairy-based products, fermented vegetables and meat-based products.[4] The FSSAI recommends a maximum of 200 mg/kg of histamine in fermented fish products. The limit has been fixed less than 20 mg/kg for alcoholic and nonalcoholic refreshments, aged vegetables and soya items, and up to 700 mg/kg for certain sausages.[5,6] The health issues like headaches, flushing, itching, tachycardia, urticarial,pruritus, hypotension, nasal discharge, bronchospasm and asthma can also be caused by eating foods high in histamine.[7]Idli batter is a fermented cooked food that has a limited shelf life and is commonly used in global cuisine.[8] The idli batter has a one day shelf life at room temperature and its shelf life can be increased up to five days when stored in refrigerated condition. Rice (*Oryza sativa* L.) and split black gram dal (*Phaseolusmungo*) are commonly used in different proportions of 2:1, 3:1, and 4:1 (w/w) to prepare idli batter.[9,10] The lactic acid bacteria and yeasts are found to be the most common microbes involved in fermentation, and lactic acid bacteria is responsible to produce biogenic amines like histamine in idli batter.[11,12] The scientific understanding of the microbial population and physicochemical characteristics is required for safe idli batter storage and its use.[13] The aim of this study is to optimize the addition of clove extract concentration for histamine reduction during fermentation of idli batter. The studies were conducted on inclusion of numerous bioactive components in sausage and found that the spice extracts could reduce biogenic amine.[14] Various thermal treatments are also used in extending the shelf life of idli batter.[15] The various spice extracts have been shown to suppress bacterial growth which results in the reduction in production of histamine. The clove has an antibacterial effect against microorganisms that produce histamine. The clove has been shown to delay the synthesis of biogenic amines by blocking particular bacteria .[16] The objective of the study was to reduce the histamine content in idli batter during fermentation by adding clove extract to increase the shelf life of the batter without affecting pH, color and titratable acidity.

2. MATERIALS AND METHODS

2.1. *Preparation of Idli batter*

The idly batter was prepared with parboiled rice and black gram dhal in the ratio 4:1. The rice and black gram dhal were washed and soaked for 5h and wet ground separately.[17] Then both the batter was mixed, made into consistency and required amount of salt was added. It was allowed for fermentation at ambient temperature for 12 h.

2.2. Preparation of clove extract

The ethanolic extract of clove was prepared according to existing method.[18] The clove was coarsely powdered and soaked in 100 ml of 95% food grade ethanol at ambient temperature for 12 h. It was mixed well and filtered through the Whatman No.1 filter paper to obtain the extracts of clove.

2.3. Addition of clove extract with idli batter

The idli batter of 100 ml was taken in three different containers and the clove extract at 0.5, 1 and 1.5% was added to the batter after 2 h of preparation.

2.4. Physiochemical properties of Idli batter

The batter samples were drawn from clove extract and added idli batter at 2 h interval up to 6 h and analyzed the various physiochemical parameters using standard procedures.

2.4.1 pH

The idli batter (10 g) was mixed with 100 ml of distilled water and mixed thoroughly using a vortex for 2 min. The pH was determined using digital pH meter (Horiba scientific 1) as described earlier.[19]

2.4.2 Titratable acidity

The idli batter (10 g) was mixed with 20 ml of distilled water. The mixer was titrated against 0.1 N Sodium hydroxide using phenolphthalein as indicator to determine titratable acidity in terms of lactic acid produced.[11]

2.4.3 Color

The Hunterlab Hue Flex EZ (Make: Virginia, USA; Model: CFEZ0925) was used to determine the colour of the clove extract added idli batter. The L* indicates the whiteness level. The a* scale will show colour ranges from green (negative) to red (positive), while the b* scale is a yellow-blue scale with yellow being positive.

2.5. *Microbial flora of idli batter*

The microbial flora in the idli batter was investigated to find out the total plate count and histamine producing organisms.

2.5.1. *Total plate count*

The idli batter sample of 10 g was diluted in 90 ml of 0.85% saline solution. After that, it was serially diluted before being distributed on plate count agar.[20] The colonies were enumerated and expressed as CFUg-1 of the batter after 48 hrs of incubation at 37°C.

2.5.2. *Histamine producers*

The idli batter sample of 10 g was diluted in 90 ml of 0.85% saline solution. To discover the organism that produces histamine, it was serially diluted and distributed on Niven's agar.[21] Single colonies that grew in the media were isolated and kept in tryptic soy agar for further investigation.

2.6. *Antibacterial activity*

The antibacterial activity of clove extract was tested against histamine-producing bacteria using the agar well diffusion

method.[22] The isolated histamine producing bacterium was inoculated into the nutrient agar medium using the spread plate method. The wells were created in the inoculated agar media using a punch borer (4 mm), and the extracts of 50 and 100 µL were poured into the wells individually. The antibacterial activity was measured by measuring the diameter of the zone of inhibition after 24 hrs of incubation at 37 °C.

2.7. Phytochemical analysis

The screening of the clove extract for the various compounds responsible for the antibacterial activity was done using GC-MS (Make: Bruker; Model: Scion 436-GC). The prepared clove ethanolic extract was vortexed, centrifuged, and the supernatant liquid was injected into the GC-MS. The GC-MS spectral output provides information on the compounds present in the extract and were compared to those in the NIST library.[23]

2.8. Analysis of histamine content (Colorimetric assay)

The clove extract added idli batter was analyzed for histamine content at 0, 2, 4, 6 h in triplicates using colorimetric assay.[24] The schematic representation of histamine determination in idli batter is shown in Fig.1.

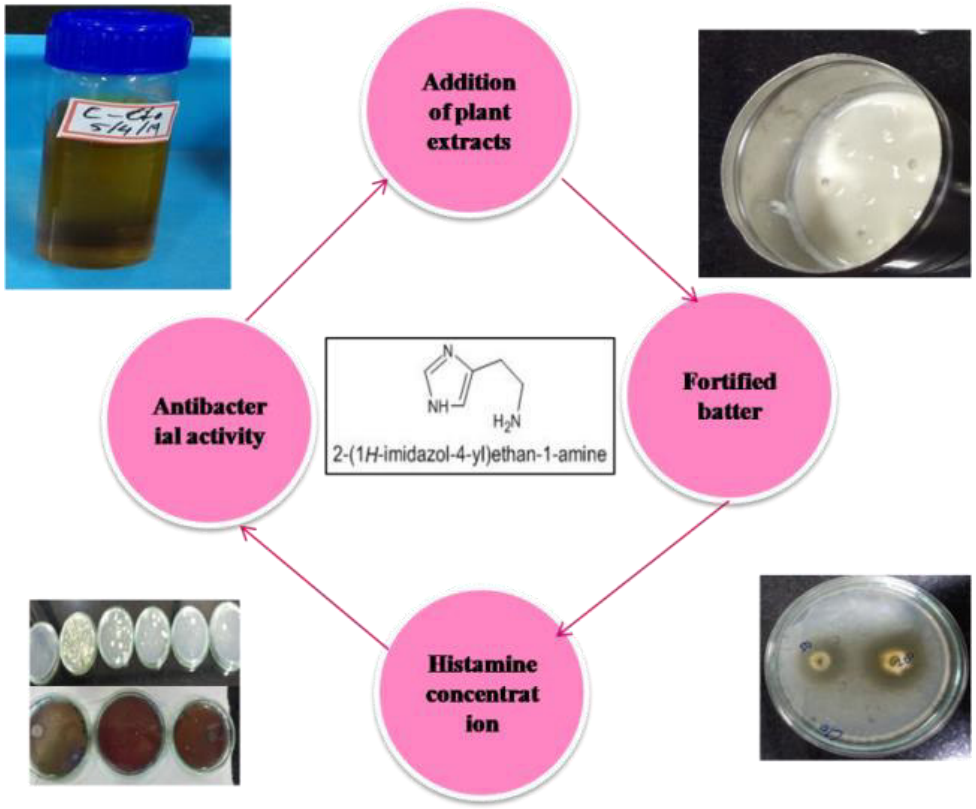

Fig 1 Pictorial representation of optimization of clove concentration

The p-phenyldiazoniumsulfonate reagent was prepared according to modified method.[24] In a 50 ml standard flask, 4 % HCl 1.5 ml of iced 0.9% sulphanilic acid and 5% of sodium nitrite each were added, mixed and kept in an ice bath for 5 mins. After 5 mins, 6 ml of 5 % sodium nitrite was added and made up to 50 ml with chilled distilled water. Further it was stored in an ice bath for 15 mins prior to usage and stable for 12 h. In a centrifuge tube, 5 g of idli batter was taken and diluted with 20 ml of 5% TCA, centrifuged at 5000 rpm for 10 mins and filtered using Whatman No.1 filter paper. The filtrate was then made up to 50 ml with 5% TCA. From the filtrate,1 ml of sample was drawn in centrifuge tubes separately, 2 ml of 5% TCA solution and 0.5 g of mixed salt (6.25 g of anhydrous sodium sulphate& 1 g of trisodium phosphate monohydrate) were added. It was mixed thoroughly; 2 ml of methanol was added to each of the tubes and shaken to shatter the protein gel. The tubes were centrifuged at 3000 rpm for 15 mins. After centrifugation, 1 ml of the top layer was transferred to the clean test tube and evaporated. The residue was collected in a test tube and added 1 ml of distilled water. In a clean empty tube, 5 ml of 1.1% sodium carbonate and 2 ml of *p*-phenyldiazoniumsulfonate reagent were mixed. This mixture was added to the tube containing the residue along with 1ml distilled water and made to react with the *p*-phenyldiazoniumsulfonate reagent. After 5 minutes of reaction, the absorbance was noted at 496 nm using UV visible spectrophotometer (Make: Shimadzu, Japan; Model: UV Vis 1800). The different concentration (20, 40, 60, 80 and 100 µg) of standard histamine (1ml each) was added in distilled water and analyzed the histamine content following the colorimetric assay. The standard curve was plotted against the concentration of histamine using a UV-visible spectrophotometer (Make :Shimadzu, Japan; Model: UV-Vis 1800).

2.9. Experimental design

The level of independent variables were decided through preliminary experiments. The extract concentrations (0.5, 1 and 1.5ml) and storage time (0, 2,4 and 6 h) were used as

factors while histamine content, color, microbial load and physiochemical properties were defined as the response.

3. STATISTICAL ANALYSIS

All the experiments were carried out in triplicates and expressed in mean ± SD. Minitab 19 statistical software was used to analyze the data.

4. RESULT & DISCUSSION

4.1 *Physiochemical properties of Idli batter*

pH and titratable acidity are the key attributes that help in quality evaluation of idli batter. The pH of the idli batter and clove added idli batter was in the range of 5.43 - 5.12 and 4.70 - 4.20 respectively (Fig.2 & 3) during the storage period. The fermenting lactic acid bacteria involved in pH maintenance and responsible for lowering of the pH and carbon dioxide by leavening the batter.[20] The titratable acidity of the idli batter and clove added idli batter was found to be in the range of 0.16-0.18 and 0.1 - 0.28 % during the storage period. The total acidity percentage increases during the fermentation time due to the air pockets and leavening action of batter.[25]The results revealed that clove extract added idli batter was not affected in terms of pH, titratable acidity and color. There was no significant color difference between control idli batter and clove extract idli batter. The L* the value which denotes the whiteness index of the idli batter was 89.1 for control idli batter and 86.06 upto 6th hour of storage period (Fig 4(a,b,c)). It was reported that the addition of mustard oil as preservative affected the colour value of the idli.[11]

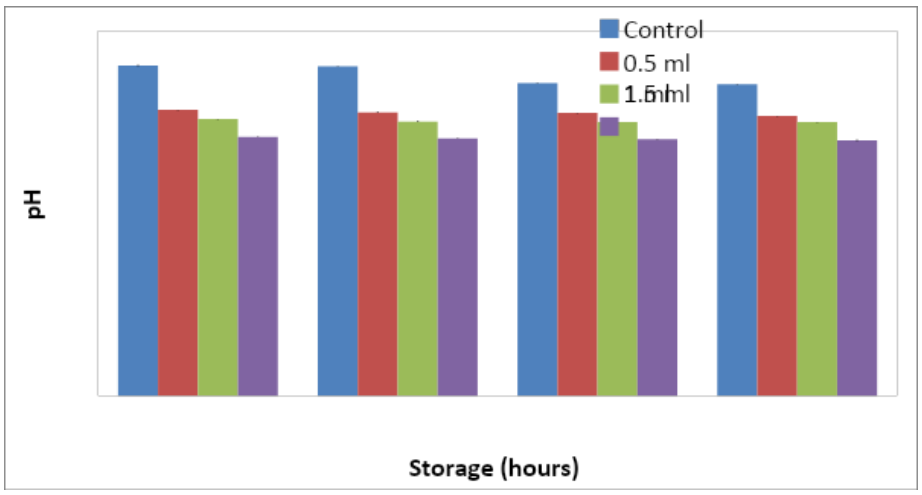

Fig 2. Variation of pH of idli batter with clove extract during the storage hours.

All values as been expressed as mean ± SD were p<0.05

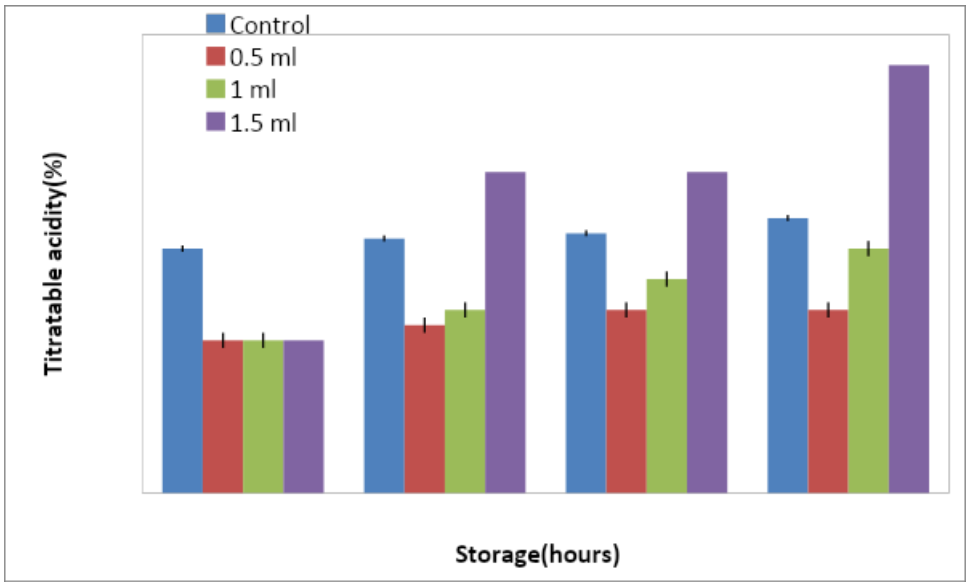

Fig 3. Variation in the titratable acidity of idli batter with clove extract during the storage hours.

All values as been expressed as mean ± SD were p<0.05.

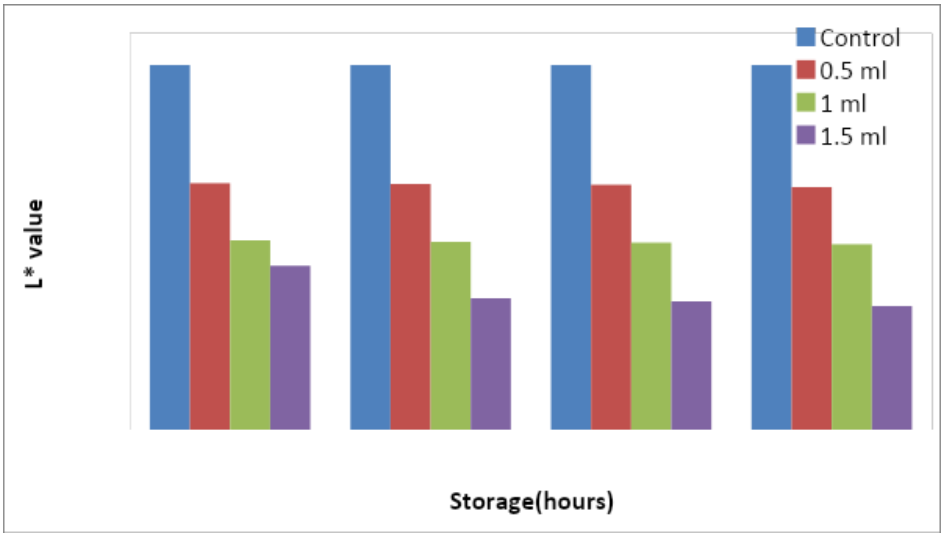

Fig 4 (a) represents the L* value

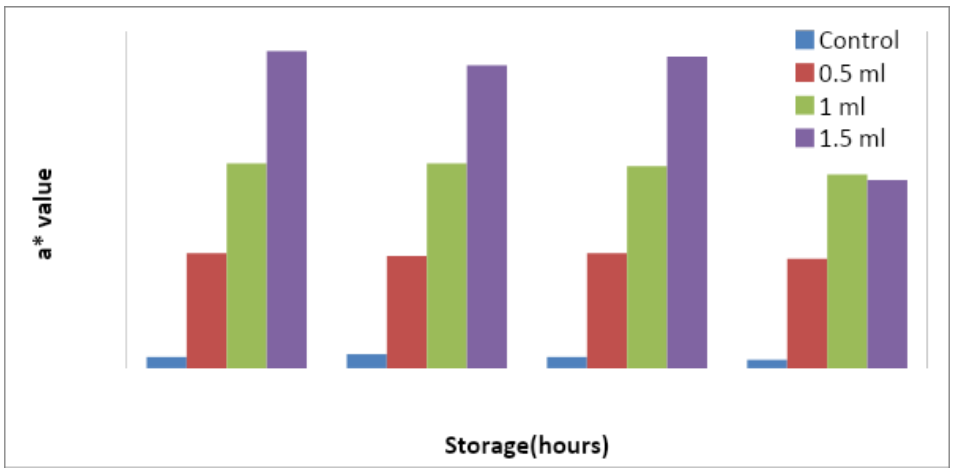

Fig 4 (b) represents the a* value

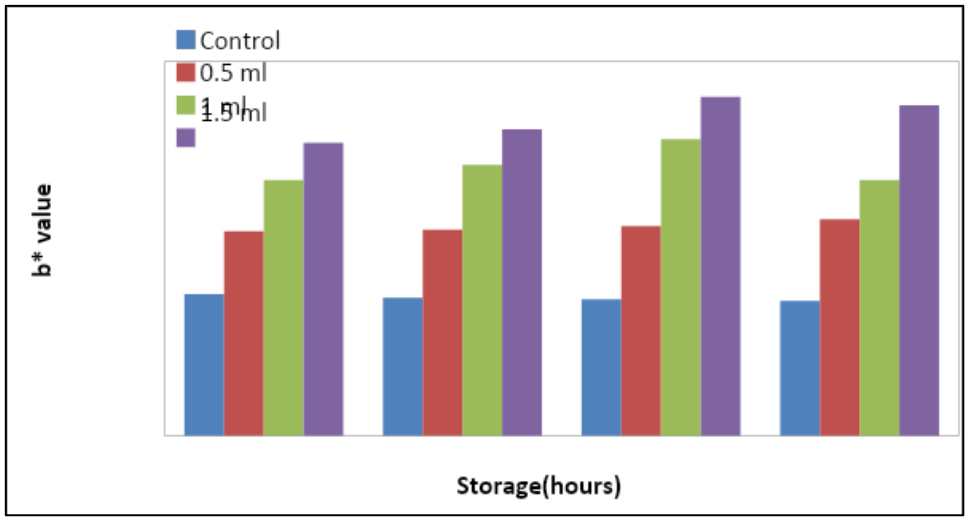

Fig 4(c) represents the b* value

Fig 4. Variation in the colour values of idli batter with clove extract during the storage hours.

4.2 Microbial flora of idli batter

The total plate count of idli batter during the storage period has been shown in the Fig 5. The log_{10} reduction of microbial count from 9.05 CFU/g to 8.60CFU/g during the storage period of 6 h in the clove extract added idli batter was observed. These results showed that the overall reduction capability of clove extract added idli batter towards the microbial population. Earlier studies on mustard oil on batter reduced the overall count of bacteria.[11]It was also observed that the increase in concentration of clove extract leads to reduction of total plate count.

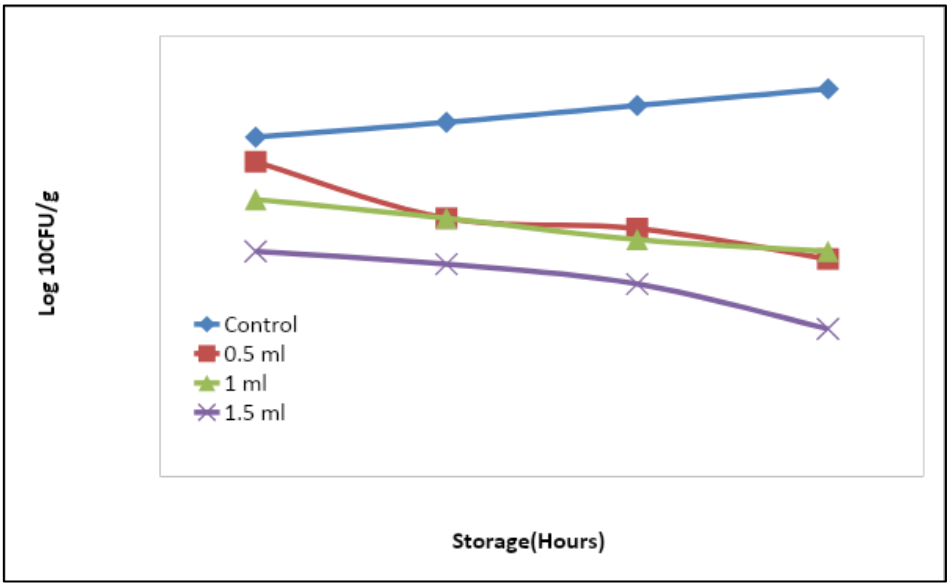

Fig 5. Total plate count during storage period

4.3 *Histamine producers*

During natural fermentation of idli batter, various groups of microorganisms formed depending on pH. The various *spp*. of histamine producing organisms are also formed along fermenting bacteria upon prolonged storage. In the present study, histamine producing bacteria has been isolated using Niven's agar media and it was found to be *Staphylococcus warneri*. The log_{10} reduction was found in the range of 8.74-8.77 CFU/g for idli batter while for clove extract added idli batter it was 8.82-7.30 CFU/g. The histamine forming bacteria was found to be less in clove extract added idli batter when compared to the control idli batter(Fig 5).

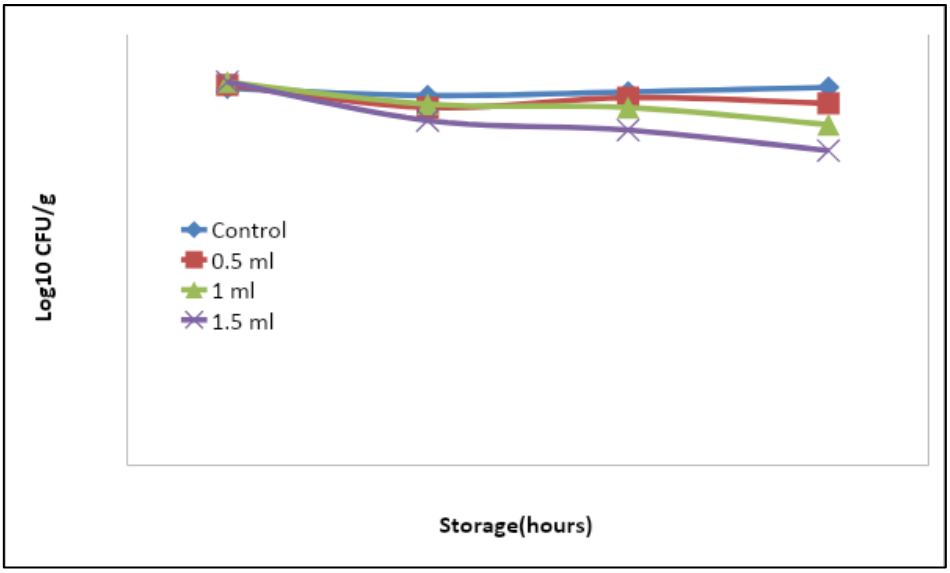

Fig 5. Histamine producers during storage period

4.4 Phytochemicals in clove extract

The GC-MS analyses showed the major phytochemicals present in clove extract (Table 1) and the chromatogram was shown in Fig 6. Among the phytochemicals present in the clove, eugenol was found in higher quantity with peak area of 65.97% and it was found to be responsible for antibacterial activity against histamine producer.[26]Phytocompounds such as vanillin, caryophyllene, copaene, alpha - farnesene, caryophyllene oxide may also be responsible for antibacterial activity against histamine forming bacteria as reported earlier.[27]

Table 1: Phytochemicals present in ethanolic extract of clove

Compound	Retention time (min)	Peak area (%)
Vanillin	10.908	0.15
Caryophyllene	11.308	9.41
Copaene	10.764	0.34
Alpha.-farnesene	12.053	0.28
Alpha.-cubebene	12.453	0.07
Caryophyllene oxide	13.019	0.41
Eugenol	10.520	65.97
Alpha.-Cubebene	10.764	0.34

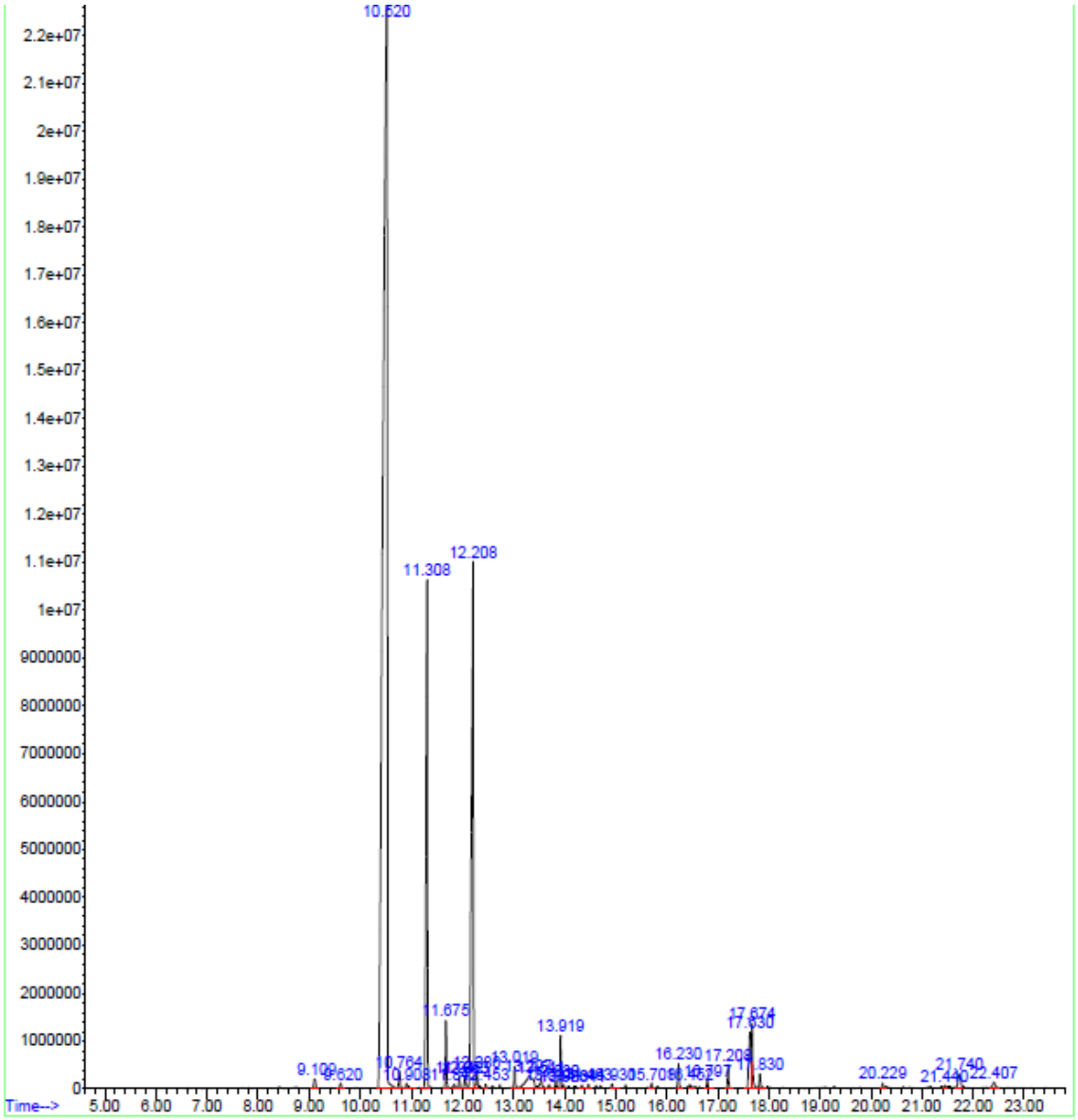

Fig 6. GC-MS chromatogram of clove extract

4.5 *Antibacterial activity of clove extract*

The antimicrobial study showed that the zone of inhibition was 12 mm for 50 µl and 14 mm for 100 µl of clove extract against histamine producing organism, *S. warneri*. Similar reports also revealed that the clove extract exhibited antibacterial activity against histamine forming bacteria.[28] The GC-MS analysis on phytocompounds revealed that eugenol is a major compound present in clove which may be responsible for antibacterial activity. Similarly it is evident that eugenol is an interesting bioactive compound with antimicrobial activity against some food-decaying microorganisms and also human pathogens.[29,30] Hence, addition of clove extract not only helping in reducing the histamine producers but also act as antimicrobial compounds. Earlier studies shown the antimicrobial activity of the extracts of *Cymbopogoncitratus* and *Adiatumcapillus-veneris* against four bacteria due to presence of phytochemicals and used in medicine.[31]

4.6 *Effect of addition of clove extract on histamine content in idli batter*

The histamine content in fermented idli batter has been analyzed at 2 hrs interval for 6 hrs using colorimetric assay during the storage period (Table 2). The histamine content in the control idli batter was high at 6thhrs (1.20 mg/kg) compared to the clove idli batter on 6th hr of storage with various concentrations of clove extract. There was a gradual decrease in the histamine content in all the concentration of clove extract. The histamine content in the idli batter was in considerable limit which is less than the toxic levels. These results revealed that the extract was solely responsible for the reduction of fermenting and histamine producing bacteria by controlling in their metabolic activity.[11] It was also observed that the reduction of histamine content when the concentration of clove extract increases.

Table 2 Effect of clove extract on histamine content in idli batter during storage

Storage duration (hrs)	Histamine content (mg/kg)			
	Control	0.5 ml	1 ml	1.5 ml
0	1.35^{b}	1.25^{a}	0.95^{a}	0.28^{b}
2	1.95^{a}	0.95^{c}	0.51^{c}	0.28^{b}
4	1.17^{c}	1.17^{b}	0.73^{b}	0.51^{a}
6	1.20^{c}	0.06^{d}	0.28^{c}	0.06^{c}

Different letters in the superscript indicate a significant difference ($p < 0.05$) within the same column.

5. CONCLUSION

There are possibilities of contamination of idli batter during fermentation with histamine and also aflatoxins due to microbial action and also contamination.[2,32] The results of the present study revealed that the addition of clove extract is able to control the production of histamine in the idli batter. The GC-MS analysis on phytocompounds in cloves revealed that eugenol is a major compound present in clove which may be responsible for antibacterial activity. It is one of the major constituents of clove (*S. aromaticum* (L.) Merr.)oil and is used in foods as a flavoring agent. Recent scientific evidence supports the beneficial effects of eugenol on human health. The eugenol has also shown excellent antimicrobial activity in earlier studies against fungi and a wide range of bacteria. The addition of clove extract did not affect the physical parameters such as pH, titratable acidity and color of the idli batter upto 6 hrs of storage study. Hence it is concluded that the addition of clove extract in idli batter helps in reduction of histamine and can be used as potential biogenic amine reducers.

6. ACKNOWLEDGEMENTS

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7. AUTHORS CONTRIBUTION

Lakshmi Praba K: Investigation and writing original draft, carrying out experiment Jagan Mohan R: Reviewing and editing, Loganathan M: Conceptualization, Experimental planning, and Supervision

8. CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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