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Antimicrobial activity of *Syzygium aromaticum* extracts against food spoilage bacteria

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In this study, the emphasis was on extraction of aqueous and methanolic extract from whole clove (*Syzygium aromaticum*) that can be efficiently used as an antimicrobial agent with an ultimate objective of developing replacements for the synthetic chemical additives in food products. Antimicrobial activity of extract revealed that the solvent extract of clove has a great potential for the inhibition of microbial load. The value of antimicrobial activites of solvent extract ranged from 12 to 17 mm in the disc diffusion method as compared to aqueous extract i.e ranged from 12 to 16 mm. Minimum Inhibitory Concentrations were found from 17 to 23 mm for solvent extract and 13 to 17 mm for aqueous exract. The sensory characteristics of bread showed that the treatments had a highly significant effect on volume, color of crust, symmetry of form, evenness of bake, character of crust, grain, color of crumb, taste and texture and overall acceptability of bread. Significantly lower total scores for the bread were exhibited by the bread supplemented with 2 and 2.5% clove extract. The results revealed that bread containing up to 1% clove extract is acceptable.

Key words: Antimicrobial activity, Clove, *Syzygium aromaticum,* food spoilage bacteria, bread, total plate count.

INTRODUCTION

The distinguished inhibitors of microorganisms are plant essential oils and their extracts. The Spices are reputed to possess several medicinal and pharmacological properties and hence find position in the preparation of a number of medicines. Spices impart aroma, colour and taste to food preparations and sometime mask undesirable odours. Volatile oils give the aroma, and oleoresins impart the taste (Proestos et al., 2008).

Due of their antimicrobial nature, spices are used to improve taste and enhance shelf life. Some of spices are also known to contribute to the self-defence of plants against infectious organisms (Kim et al., 2003). There is considerable potential for utilization of natural antimicrobials in foods, especially in fresh fruits and vegetables. Extract derived from spices and plants have antimicrobial activity against *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli* O157:H7, *Shigella dysenteriae, Bacillus subtilis* and *Staphylococcus aureus* at levels between 0.2 and 10µ ml^{-1} (Burt, 2004).

Presently, the major problem is that we can not use chemical preservatives safely now a day due to carcinogenic nature of these chemicals. Residual toxicity is increased due to these chemicals. Due to these reasons, consumers lean to be doubtful of chemical additives and thus the requirement has been increased for natural and adequate preservatives (Skandamis et al., 2001). As a

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consequence, natural antimicrobials are receiving a good deal of attention for a number of micro-organism-control issues. Reducing the need for antibiotics, controlling microbial contamination, improving shelf-life extension technologies to eliminate undesirable pathogens, decreasing the development of antibiotic resistance by pathogenic micro-organisms or strengthening immune cells in humans are some of the benefits (Fisher and Phillips, 2008).

Clove belongs to a tree Eugenia caryophyllata (*Syzygium aromaticum*), is used as a spice in almost all the world's fare. Bud Oil of Clove has natural behavior and the main properties include antioxidant, insecticidal, antifungal and antibacterial properties. By tradition, it has been used in food preservation as flavoring and antimicrobial substance (Velluti et al*.,* 2003). It has a very major role in spice trade and is highly appreciated for their therapeutic properties. Cloves are an excellent source of manganese. They are also a very good source of dietary fiber, vitamin C, vitamin K, and Ω-3 fatty acids and a good source of magnesium and calcium. Cloves consist of a significant amount of proteins, iron, carbohydrates, calcium, phosphorus, potassium, sodium and hydrochloric acid. They are also rich in vitamins A and C, manganese, and dietary fiber (Kim et al*.,* 1998).

The most important constituent of clove is the phenylpropene eugenol due to which it has strong characteristic aroma. Major parts of clove consist of eugenol comprises 70 to 90 % and remaining 15% consist of dry weight (Shobana and Naidu, 2000). Molds, yeast and bacterial growth could be inhibited by the application of clove essential oil (Burt, 2004). Micro-organisms like *Alternaria* sp*., Aspergillus* sp*., Canninghamella* sp*., Lactobacillus* sp*. Fusarium* sp*., Clostridium* sp*; Mucor* sp*., Salmonella* sp*. Penicillium* sp*. Bacillus* sp*.* could be repressed by using clove essential oil (Soliman and Badeaa, 2002). The cloves are antimutagenic, anti-inflammatory, antioxidant, antiulcerogenic, antithrombotic and antiparasitic. The essential oil extracted from the dried flower buds of clove are used for acne, warts, scars and parasites (Miyazawa and Hisama, 2003; Srivastava and Malhotra, 1991; Chaieb et al*.,* 2007b).

B. subtilis is not a human pathogen but is responsible for causing Ropiness, a sticky and stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough (Priest et al*.,* 1988). *B. subtilis* has been associated with outbreaks of food poisoning but the exact nature of its involvement has not been established. *B. subtilis*, like other closely related species in the genus, *B. licheniformis, B. pumulis,* and *B. megaterium*, have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. However, there has not been any correlation between lecithinase production and human disease in *B. subtilis* (Collins et al., 1991). Considereing the importance clove, the present project has been designed to to analyze the physico-chemical properties of clove extract in order to study the inhibitory effect of clove extract against food spoilage bacteria.

MATERIALS AND METHODS

Sample preparation and extraction

Fresh whloe clove samples were purchased from the local grain market of Faisalabad**,** Punjab, Pakistan. The samples were obtained from retail spice-sellers in the amount of 1 kg. Grinding was done with grinder MJ-176P in the laboratory grinder. The samples were kept in closed containers after being chopped into small pieces (1 mm). Two types of clove extract were prepared for aqueous and methanolic extracts.

For the preparation of aqueous extract, 150 ml of distilled water was added into 25 g of chopped clove and the mixture was left for agitation in shaker incubator for 8 h at 300 rpm at 39°C. Afterwards, it was filtered. The dark colored extract obtained at the end of this process was used for furthure analysis. The sample extract was kept in the refrigerator (4°C) for accomplishment of further analysis (Wilson, 1995).

For the preparation of solvent extract, 150 ml of methanol was added into 25 g of chopped clove and the mixture was left for agitation in shaker incubator for 8 h at 300 rpm and 39°C. Afterwards, it was filtered and the methanol was vaporized in rotary evaporator (60°C). The dark colored oily extract obtained at the end of this process was used form for the analysis. The sample extract was kept in the refrigerator (4°C) until the analysis was accomplished (Wilson, 1995).

Physicochemical analysis of extract

Electronic digital type pH meter of Wellium model- Inolab pH 720, WTW 82362 was used for pH determination. Total Acidity, Referactive index, Specific gravity and Brix value was determined by following the methods described in AOAC (2007).

Isolation of bacteria

Glassware should be autoclave before the start of experiment. Spoiled bread samples were collected from different bakeries located in vicinity of Faisalabad city. The samples were drawn in sterilized screw capped bottles and preserved for further studies. Six sterilized test tubes were taken and labeled as 10^{-1}, 10^{-2}, 10^{-3}… 10^{-6}. Serial dilutions for each sample were made by the method as recommended by Cappuccino and Sherman (1996). Nutrient agar, Mueller Hinton agar (MH agar) and Potato dextrose agar (PDA) were prepared according to the methods of NCCLS (2000). In each Petri plate 10ml of medium and 1ml of respective dilution were added carefully. Six sterilized test tubes were taken and labeled as 10^{-1}, 10^{-2}, 10^{-3}… 10^{-6}. Medium and inoculum were mixed immediately by given to and for shaking and circular movement lasting 5 to 7 s. Medium was allowed to solidify. After solidifying inverted Petri plates were placed in incubator at 37°C for 24 to 48 h for bacterial isolation by following the method given by Cappucino and Sherman (1996).

Morphological examination of bacteria

The representative colonies, showing catalase negative and Gram positive, were randomly picked from higher dilution (10^{-5}) of nutrient agar plates. The culture isolates were morphologically observed under microscope by following the method described by Cappuccino and Sherman (1996). Gram's Staining was done according to the method described by Becker et al*. (*2003).

Purification of isolates

The colonies which appeared after 48 h incubation on nutrient agar

Table 1. Mean values for physiochemical analysis of aqueous and methanolic extracts

Physicochemical	Methanolic	Aqueous
Total acidity (%)	0.45 ± 0.02	0.25 ± 0.25
pH	5.20 ± 0.21	6.70 ± 0.24
Specific gravity	1.025 ± 0.23	1.015 ± 0.26
Refractive index	1.53 ± 0.22	1.52 ± 0.22
Brix	9.80 ± 0.24	9.80 ± 0.23

Values are given as mean ± standard deviation

plates were subjected to morphological examination. Pure growth of *B. subtilis* were then transferred to potato dextrose agar and incubated at 37°C and preserved in refrigerator at 4°C for further use. The results of colony characteristics were recorded by following the method as described by Harrigan and McCance (1990).

Determination of antibacterial activity

Microbiological methods used for the determination of antimicrobial activity of clove extract were disc diffusion method and agar well diffusion method.Treatments T1, T2, T3, T4 and T5 for aqueous extract and methanolic extract were used at concentration 0.5, 1, 1.5, 2 and 2.5% respectively. Antimicrobial activity was determined by standard disc diffusion method as described by saeed et al. (2007). The inoculum suspension of each bacterial strain was swabbed on the entire surface of Mueller-Hinton agar (MHA, pH 7.3 ± 0.1, Difco). Sterile 6 mm filter paper discs (Schleicher & Schuell) immersed with clove extract was aseptically placed on MHA surfaces. The plates were left at ambient temperature for 15 min to allow excess prediffusion of extracts prior to incubation at 37°C for 24 h. Diameter of inhibition zones was measured. Each experiment was done in triplicate.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined by agar well diffusion method as described by Ogata et al. (2000). The plates containing Mueller Hinton agar medium were spread with either 0.1 ml of the bacterial noculum. Wells (8 mm in diameter) were cut from agar plates using a sterilized stainless steel borer and were filled with 0.1 ml of clove extract. The plates were incubated at either 37°C for 24 h and the diameter of resultant zone of inhibition was measured. Each combination antimicrobial agent was repeated three times. Microorganisms showing a clear zone of more than 6mm were considered to be inhibited.

Preparation of bread

The breads were prepared from straight grade flour containing clove methanolic extract from 0.5 to 2.5% by method according to the procedure as described in AACC (2000).

Physical analysis of bread

The color of bread was determined with the help of Color meter as described by Rocha and Morais (2003). The textural study of bread was conducted by using Texture analyzer (Model TA-XT2, Stable Microsystems, Surrey, UK) as described by Piga et al. (2005). The water activity of bread was determined by using Hygropalm water activity meter (Rotronic Hygropalm water activity meter, series number 601089738) as described by Piga et al. (2005). The loaf volume of the bread was determined using a rapeseed displace-ment method by the method of Hussain (2009).

Total plate count of bread

Six sterilized test tubes were taken and labeled as 10^{-1}, 10^{-2}, 10^{-3}… 10^{-6}. Nine milimliters of normal saline was poured into each test tube. One grams of crushed spoiled bread sample was shifted into the first test tube and contents were mixed well by gentle shaking. 1m shifted contents from each dilution on to the surface of separate plate count agar plates. Serial dilutions for each sample were made by the method as recommended by Cappuccino and Sherman (1996). One mililiters shifted contents from each dilution on to the surface of separate plate count agar plates and spread well and incubated at 37°C for 24 h. Average number of colonies was counted from those dilutions that showed the colonies size ranging from 30 to 300 with the help of colony counter. The total plate count was calculated using the following formula

Total Plate Count = Average number of colonies × dilution factor/volume factor

Sensory evaluation

The prepared bread loaves were evaluated by a panel of judges for external characteristics such as volume, crust color, symmetry of form, evenness of bake and internal characteristics like grain, crumb color, aroma, taste and texture by following the method of Land and Shepherd (1988).

Statistical analysis

The data obtained from each parameter was subjected to a statistical analysis using analysis of variance techniques to determine the level of significance in different parameters according to the method described by Steel et al. (1997).

RESULTS AND DISCUSSION

Physicochemical analysis of extracts

The results indicated that acidity of clove extracts differed significantly. The results given in the Table 1 indicated that total acidity was found to be 0.45 and 0.25 for methanolic extract and aqueous extract respectively. With increase in solvent concentration, acidity was increased. The results of present sudy are in agreement with the findings of Burt (2004) who reported that acidity ranged from 0.20 to 0.55%. The results were also in agreement with the findings of Hammer et al. (1999). They studied 13 different spices solvent extract and reported that titrateable acidity ranged from 0.35 to 0.55.

The results for pH of clove extract (Table 1) indicated significant variations for pH of clove extract. The results indicated that pH was ranged from 5.20 to 6.70 for methanolic extract and aqueous extract respectively. The results of present study are in concordance with the findings of Koiche and Bouras (2010) who reported that pH of the clove extract was ranged from 5.0 to 5.50. Chaieb

Table 2. Effect of clove aqueous and methanolic extracts on antibacterial activity.

Treatment	Aqueous extract	Methanolic extract
T_0 = control without methanolic extract	-	-
T_1= 0.5% methanolic extract	12.33 ± 0.22	14.00 ± 0.23
T_2= 1.0% methanolic extract	14.66 ± 0.24	14.66 ± 0.25
T_3= 1.5% methanolic extract	14.85 ± 0.25	15.33 ± 0.28
T_4= 2.0% methanolic extract	15.00 ± 0.25	16.00 ± 0.26
T_5= 2.5% methanolic extract	16.66 ± 0.26	17.66 ± 0.29

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract).

et al. (2007) described their results for pH of clove aqueous extract was ranged from 6.50 to 6.90. The results for specific gravity of clove extracts (Table 1) indicated the significant differences for specific gravity.

The results showed that specific gravity was ranged from 1.025 to 1.015 for methanolic extract and aqueous extract respectively. The results of present study are in agreement with the findings of Kumar et al. (2007a) who described that the specific gravity of methanolic extract was ranged from 1.02 to 1.028. Kim et al. (1995) described that the specific gravity of clove extract was ranged from 1.01 to 1.029.

The results pertaining to referective index of clove extracts (Table 1) indicated significant variations of refracttive index. The results showed that refractive index ranged from 1.533 to 1.523 for methanolic extract and aqueous extract respectively. The results of presented study are in agreement to the findings of Dorman et al., 2000 who reported that refractive index of clove extract was ranged from 1.520 to 1.535. These results were also in agreement with the findigs of Gutierrez et al. (2009) who reported the refrective index of clove extract was ranged from 1.518 to 1.536.

The results for brix of clove extract showed that brix of clove extracts had non significant differences. The results (Table 1) indicated that brix of methanolic and aqueous extracts was 9.80 and 9.80 respectively. The results of present study are in contradiction to the findings of Cowan, 1999 who reported that brix of spices extract are in the range of 8.50 to 9.0. The difference in the results might be due to the chemical reaction of solvents with sugars during the mixing operations.

Microbiological analysis of extract

Determination of antibacterial activity

The results for antibacterial activity of aqueous clove extract have been presented in Table 2. The results indicated that aqueous extract of clove extract showed highly significant variations as compared to methnolic extract for antibacterial activity. The highest zone of inhibition of antibacterial activity for clove aqueous extract was showed by T_5 (16.66 mm) followed by T_4 (15.00 mm). Minimum antibacterial activity was showed by T_1 (12.33 mm).

The results for antibacterial activity of methanolic clove extract (Table 2) indicated that clove methanolic extract showed significant results as compared to acueous extract for antibacterial activity. Methanolic extract have highest antibacterial activity at T_5 (17.66 mm) and minimum zone of inhibition was showed by T_1 (14.00 mm). The present results showed that methanolic extract exhibited the strongest inhibitory activity (14 to 17.66 mm) as compared to results of aqueous extract (12 to 16 mm). It is evident from the results that the diameter of inhibition increased significantly as concentration of extract increased. Diameter is showed in millimeter (mm)

The results of present study are in agreement with the findings of Meena and Sethi (1994) who reported that different extracts of clove showed strong antibacterial activity against *P. vulgaris* and *B. subtilis.* They reported the inhibition diameter in the range of 12 to 25 mm. Their results also in concordance with the findings of (Kumar, 2007a) who stated that *S. cumini* showed good activity against S*. aureus* and *B. subtilis*. Both aqueous and methanol extracts of *S. lineare* and *T. asiatica* showed a strong antimicrobial activity against food spoilsge bacteria.

Similar results were found in the previous study of Mytle and others (2006) that determined the inhibitory effect of clove extract against *B. subtilis* on chicken frankfurters. *B. Subtilis* was inoculated at low (102 to 103 CFU/g) or high cell numbers (104 to 106 CFU/g), and stored at 5°C for 2 wk or at 15°C for 1 wk .All strains of *B. subtilis* and grew on control frankfurters at 5 and15°C, but growth was inhibited under both storage conditions in the presence of either 1 or 2% clove oil.

S. aromaticum, *P. granatum, S. cumini and T. asiatica* produced the largest zones of inhibition against *B. subtilis, S. aureus* and *S. epidermidis*. (Bevilacqua et al., 2010) reported good antibacterial activity in clove methanolic extract against *E. coli* using aqueous and methanol extracts. The ethanolic extracts of clove, cumin, and kaffir lime peels showed the broadest antibacterial activity by inhibiting growth of all bacterial strains tested (the diameter of inhibition zone, 8 to 22 mm), while the extracts of cardamom, cinnamon, and kaffir lime leaves inhibited the growth of almost all strains (7 to 12 mm), except for *S.*

Table 3. Effect of clove aqueous and methanolic extracts on MIC.

Treatment	Aqueous extract	Methanolic extract
T_0	-	-
T_1	14.00 ± 0.22	14.66 ± 0.28
T_2	16.00 ± 0.25	15.66 ± 0.27
T_3	17.00 ±0.23	17.00 ± 0.25
T_4	19.00 ± 0.24	20.00 ± 0.26
T_5	21.00 ± 0.27	23.00 ± 0.27

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

Typhimurium, S. London, and *Serratia marcescens*. Plant-derived antimicrobial compounds have been recognized as a means of inhibiting undesirable bacteria and numerous research articles have described the antimicrobial properties of plant extracts. Clove extract has shown inhibiting activity against bacteria (Kildeaa, 2004).

Minimum inhibitory concentration (MIC)

The results pertaining to MIC of aqueous extract have been given in the Table 3 showed highly significant differences beween aqueous extract and methanolic extract for minimum inhibitory concentration. The results indicated that highest value of MIC with aqueous extract was shown by T_5 (21 mm) and minimum value was shown by T_1 (17 mm).

The results for MIC of methanolic extract (Table 3) showed highly significant variations among aqueous extract and methanolic of MIC. Highest value of MIC methanolic extract was shown by T_5 (23 mm) and minimum value 17 mm was shown by T_1. Results showed that MIC value of methanolic extract is greater as compared to aqueous extract because methanol also acts as a natural antimicrobial agent.

The results of present study are in concordance with the findings of Kim et al. (1994) who showed that the methanolic extract of clove showed inhibitory activity against all the six food associated bacteria in which the diameter of zone of growth inhibition varied between 15 to 25 mm (in clove) and 15 to 20 mm (in garlic). The clove ethnaolic extract showed highest diameter of zone of inhibition of 32 mm against *E. coli* followed by *S. aureus* (21 mm) and B*. subtilis* (23 mm) (Burt, 2004).

The clove ethanolic extract showed similar zone of inhibition of 20 mm diameter against *B. megaterium* and *B.* sp*haericus*. The minimum inhibitory activity was recorded against *B. polymyxa*. Our results substantiate the findings of Soliman and Badeaa (2002) that demonstrated the antibacterial activity of clove ethanolic extract against *E. coli, S. aureus* and *B. subtilis* and found that the highest antibacterial activity was against *B. subtilis*.

The MIC values of the clove methanolic extract tested against *L. monocytogenes* ranged in between 20 to 25 mm for selected ethanol extracts ranged from 0.25 to 11.75 mg/mL. It is apparent from the results that the MIC values are high for lavender and verbena, explaining the extent of resistance offered by *L. monocytogenes*, against these ethanol extracts. This study revealed that clove extract showed maximum activity against *L. monocytogenes* with MIC value 0.25 mg/mL followed by mint timija extract with MIC value of 0.315 mg/mL, indicating that clove and mint timija showed excellent antimicrobial activity against *L. monocytogenes*. *L. monocytogenes* is fairly sensitive to all ethanol extracts except lavender and verbena and was showing moderate MIC values against rosemary, geranium and camomile. The MIC values were used as guide for the treatment and battle against undesirable microorganisms. The results obtained showed that the MIC values varied according to the extracts and indicated that clove exhibited the strongest antibacterial activity, followed by mint timija. Similar result has been reported by Meena and Sethi (1994).

Analysis of bread

Based on the results obtained during study, it is stated that methanolic extracts had great antibacterial activity and zone of inhibition than aqueous extract. So bread was prepared from methanolic extracts**.**

Physical analysis of bread

The results pertaining to color of bread have been given in the Table 4. The results indicated that the color value of bread differed significantly due to increasing concentrations of extract. The results illustrated that color of breads prepared from T_0 (control) had the minimum color value that is, 173.0 and its value increased gradually as concentrations of extract increased. The darker color was due to dark oily color of extract. The bread prepared with T_1 was found as best value of color near to control. The breads prepared from T_5 concentration got the maximum color value that is, 186.67. The results of present study are in concordance to the finding of Holley and Patel (2005) who reported that color value of cereal products increase with increase in the concentration of spices extract.

The results regarding the water activity results indicated highly significant variations for water acticity of bread. The results indicated that water activity of breads ranged from 0.77 to 0.83. The maximum water activity was found in the bread prepared with T_5 while the minimum water activity was found in the bread prepared with T_0. The water activity was increased as the percentage of extract increased (Dragland, 2003). The results pertaining to the texture of bread (Table 4) showed highly significant results for texture. The results indicated that the texture (firmness) of bread ranged from 1721 to 2043. Maximum

Table 4. Effect of clove methanolic extract on physical characteristics of bread.

Treatment	Color	Water activity	Texture	Loaf volume
T_0	173.00 ± 0.25	0.77 ± 0.25	1721.3 ± 0.28	620.0 ± 0.25
T_1	175.67 ± 0.26	0.79 ± 0.24	1814.7 ± 0.21	580.3 ± 0.22
T_2	176.67+1.23	0.80 ± 0.25	1920.7 ± 0.23	550.30 ± 0.24
T_3	180.00+2.09	0.80 ± 0.26	2016.0 ± 0.25	520.30 ± 0.21
T_4	183.00+0.76	0.81 ± 0.27	2029.7 ± 0.26	490.30 ± 0.26
T_5	186.67+0.91	0.83 ± 0.26	2043.0 ± 0.27	440.00 ± 0.28

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

Table 5. Effect of clove methanolic extract on external characteristics of breads.

Treatment	Volume	Crust color	Symmetry of form	Evenness of bake	Crust character
T_o	8.80 ± 0.21	8.20 ± 0.26	4.80 ±0.21	2.60 ± 0.25	2.90 ± 0.28
T_1	7.95 ± 0.25	7.60 ± 0.23	4.05 ±0.22	2.60 ± 0.23	2.40 ± 0.21
T_2	7.60 ± 0.22	7.10 ± 0.28	3.80 ±0.23	2.30 ± 0.26	2.30 ±0.27
T_3	6.30 ±0.23	5.70 ± 0.25	3.60 ±0.24	2.20 ± 0.24	2.20 ± 0.22
T_4	5.30 ±0.24	4.30 ±0.24	3.30 ±0.25	2.20 ± 0.22	1.90 ± 0.23
T_5	4.30 ±0 .26	3.40 ± 0.21	3.30 ±0.26	2.20 ± 0.27	1.80 ± 0.25

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

firmness value of bread was 2043 prepared with T_5 (2.5%) methanolic extract. Minimum firmness value of bread prepared with T_0 (control) extract concentration was 1721. The results of present study are in agreement with the findings of Piga et al. (2005) who reported that firmness value is increased as the percentage of spices essential oil increasd.

The results for loaf volume of breads containing methanolic extract (Table 4) indicated highly significant results for loaf volume of bread. The results revealed that the maximum loaf volume (620 ml) was found by the bread produced from T_0 (control) followed by the T_1 extract (580 ml) while the minimum loaf volume (440 ml) was found in the T_5 extract bread. The results indicated that loaf volume of bread was affected significantly by different concentrations level of extract. It is obvious from results that loaf volume of bread containing methanolic extract was higher than prepared from the bread having no extract.

The decrease in loaf volume of the bread may be attributed due to the reduction in wheat structure forming proteins and low ability of dough to entrap air. The protein quantity, alpha amylase activity, damaged starch and genetic factors might have significant effect on bread volume and baking quality for different composite flours (Burt et al., 2007).

Sensory evaluation of bread

The sensory evaluation of bread for various attributes such as volume, colour, symmetry of form, evenness of bake, character of crust, grain, and colour of crumb, aroma, taste and texture was carried out. The product was evaluated by a panel of judges and the results are described below.

External characteristics of breads

The results pertaining regarding volume of breads have been given in Table 5 revealed that methanolic extract showed highly significant effect on the scores assigned to loaf volume of the breads. The results revealed that the scores assigned to volume of breads were affected significantly by the level of extracts concentrations. The results that the scores assigned to loaf volume of breads ranged from 4.30 to 8.80. The judges assigned maximum scores to the volume of control breads (8.80) followed by the breads prepared from the T_1 (0.5% extract). However, the breads prepared from T_5 (*2.5%*) got minimum scores with respect to volume. Decrease in volume was observed after baking of breads. In the present study the breads prepared from methanolic extract showed variable trends as breads from T_1 and control got statistically closest scores for volume where as breads from other conc. got significantly lower scores for volume of breads.

Crust color is an important sensory parameter concerning the consumer's acceptability of bread. The results (Table 5) indicated that the clove methanolic extract showed significant effect on the scores given by judges to crust color of the breads. The results indicated that there was a significant decrease in scores assigned to crust

Table 6. Effect of Clove methanolic extract on internal characteristics of bread.

Treatment	Grain	Crumb color	Aroma	Taste	Texture
T_0	14.00 ± 0.23	8.40 ±0.25	8.70 ± 0.27	15.30 ± 0.21	13.80 ± 0.24
T_1	12.20 ± 0.22	7.50 ± 0.28	7.10 ± 0.26	14.80 ± 0.22	12.80 ± 0.25
T_2	11.30 ± 0.21	6.50 ± 0.27	6.20 ± 0.23	13.30 ±0.24	11.10 ± 0.26
T_3	9.80 ± 0.25	5.20 ± 0.26	5.20 ± 0.24	12.84 ± 0.25	9.10 ± 0.28
T_4	8.30 ± 0.26	4.20 ± 0.29	4.40 ± 0.25	11.30 ± 0.26	7.80 ± 0.26
T_5	7.20 ± 0.27	3.30 ± 0.22	3.40 ± 0.21	10.20 ± 0.27	6.50 ± 0.23

Values are given as Mean ± Standard Deviation, T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic xtract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

color of breads prepared from different extract concentrations as compared to control bread. The breads prepared from T_0 got significantly the highest scores for crust color followed by the breads produced from T_1. The results also indicated that bread prepared with T_5 has minimum crust color value. Crust colour of the bread was light brown which darkened progressively with the increasing level of extract concentration. The darkened colour of crust may be due to the Maillard reaction taking place during baking of loaves, due to high lysine contents. The results of the present study are comparable with those of Lee and Shibamoto (2000) who studied the behavior of different spices extract during bread making process and found that the addition of extract has a positive effect for the crust color of sourdough breads.

Symmetry of form is an important bread parameter in deciding the characteristics like uneven top, low ends and shrunken sides of the bread. The results pertaining to the effect of different clove methanolic extract concentration indicated that scores assigned to evenness of bake differ significantly due to variation in extract concentration (Table 5). It is evident from the results that breads prepared from T_0 clove extract got significantly the highest scores. The scores for symmetry of form were assigned to be the lowest (3.3) to the breads prepared form T_5. It was also evident from the data that the score for symmetry of form decreased proportionally with increase in the concentration of clove extract.The results of present study are in in agreement with the findings of Chavan et al. (1991) who reported that the score assigning to symmetry of form of bread decreased as the concentration level of methanolic extract increased. Burt (2007) also found that spices extracts significantly reduce the score for symmetry of form of bread.

The evenness of bake reflects that all sides including top and the bottom are uniformly baked and it also reflects the intensity of baking whether the sides having lighter or darker shade. The results pertaining regarding the effect of clove methanolic extract indicated that scores assigned to evenness of bake showed non significant variations (Table 5). The results for the evenness of bake of breads indicated that evenness of bake among breads varied from 2.0 to 2.6 prepared from different cocentration of methanolic extract. The scores assigned to evenness of bake decrease significantly. The loaf should be evenly baked on all sides, including the bottom. Pan breads should be evenly colored with no light or burned spots. The shade of the sides and bottom should conform to that of the crust.

The results regarding scores given by judges to crust character of sourdough breads made from clove methanolic extract at different concentration have been presented in Table 5. The results showed that methanolic extract exhibited highly significant effect on scores given to crust character of the breads. The mean scores assigned by the panelists to the crust character of breads prepared from methanolic extract with the difference in concentration showed that the breads prepared from T_0 and T_1 got the highest scores 2.90 and 1.80 respectively for crust character.

Internal characteristics of bread

The results (Table 6) showed that methanolic exhibited highly significant effect on scores given to grain of the breads. The scores assigned by the panelists to the grain of breads prepared from clove extract with the difference in concentration ranged from 7.20 to 14.00 among the breads. The breads from T_5 (7.20) got significantly lower scores while maximum scores were given to the control breads (14.0). The breads prepared from T_1 and T_2 got almost similar scores for grain.

A soft creamy white crumb color is preferred in white breads. However, in some geographic areas, a bright white color is also preferred for the bread crumb. The results for the effect of clove methanolic extract on crumb color of breads have been given in Table 6 indicated that clove extract showed significant effect on crumb color of the breads. The crumb color got significantly the highest scores for breads prepared from the T_0 (8.40) followed by the T_1 bread (7.50). The scores given to the breads decreased with the increase in extract concentrations. The results for crumb color of bread prepared from different concentration levels of clove extract indicate that clove extract significantly affected the crumb color of breads. The scores assigned by the panelists to the crumb color of breads prepared from different concentration of extract. Results indicated that breads prepared with T_0 got

Table 7. Effect of clove methanolic extract on Total Plate Count of bread.

Treatment	Mean
T_o	6.5×10^2
T_1	5.6×10^2
T_2	5.0×10^2
T_3	4.4×10^2
T_4	3.4×10^2
T_5	3.1×10^2

T_0= Control (without methanolic extract), T_1= 0.5% methanolic extract, T_2= 1.0% methanolic extract, T_3= 1.5% methanolic extract, T_4= 2% methanolic extract, T_5= 2.5% methanolic extract.

significantly the highest scores (8.40) for crumb color while the breads prepared with T_5 were ranked at the bottom (3.30) by the judges. It is also evident from the results that breads prepared from T_1 is close to control value followed by T_2 extract concentration.

The results of aroma (Table 6) showed that aroma of breads prepared from different clove extract concentrations significantly affected the scores given to aroma of breads. The scores assigned to the aroma of different breads indicated that breads prepared from T_0 extract got statistically the highest scores (8.7) for aroma followed by breads from T_1 (7.10) whereas the minimum aroma scores were assigned to the breads prepared from T_5. The results are in concordance to the findings of Meena (1994) who reported that aroma of the food products decreased as the concentration of extract increased.

The results of scores allocated to taste of the bread samples prepared from different clove extract concentrations (Table 6) indicated significant effect of clove extract. The scores assigned to taste of breads prepared from clove extract showed that the breads prepared from control i.e. T_0 extract got the highest scores (15.30) for taste by the panelists. It is obvious from the results that breads prepared from control were graded at the bottom with respect to taste scores. The results in Table 6 further exposed that breads prepared from T_1 (14.80) and T_2 (13.30). There was a decline in assigning the scores to breads by increasing the level of extract concentration.

Texture and appearance are two major sensory characteristics of the cereal products. The textural properties of a food has been described as that group of physical characteristics that are sensed by the feeling of touch, disintegration and flow of the food under the application of a force, time and distance. The resuts for texture of breads prepared from different extract concentrations (Table 6) indicated that scores given to texture of breads differed significantly due to differences in extract concentration level. The scores given to the texture of breads prepared with extract indicated that the breads from T_0 extract got the maximum scores for texture (13.80) and the scores decreased progressively as the level of extract concentration increased. The breads prepared from T_5 got the minimum scores by the judges for texture.

Microbial analysis

Total plate count

The results pertaining to total plate count of bread have been presented in the Table 7. The results indicated the highly significant values for Total plate count of bread prepared with clove methanolic extract.

The results indicated that the total plate count of all the bread samples decreased significantly. Decrease in the total plate count occurred from 6.5×10^2 to 3.1×10^2 for all breads. Bread with clove methanolic extract T_1 (0.5%) had TPC value 5.6×10^2. TPC for bread with 1% extract decreased from 6.5×10^2 to 5.6×10^2 and 5.6×10^2 to 3.1×10^2 from the rest. The best results of TPC were obtained from the bread made with 0.25% methanolic extract. Additions of clove extract which is used as antimicrobial substance, showed decrease in the growth rate of microorganisms.

It is evident from the results that there was decrease in the total plate count occurred as the extract level increased. All the results were in the agreement with the Lewis and ausubel (2006) and Lane et al. (1991) who showed there was significant effects on spices extract addition on the TPC of bread.

Conclusion

The preliminary study of this project revealed that spices can be used as antural antimicrobial in food products due to their less lethal effects as compared to synthetic chemical additives. Consumers are are very conscious about food safety nowadays. The Present study reveals that there is a great potential of using spices extract as natural antimicrobials for controlling food spoilage and pathogenic bacteria. Our results signify the fact that natural products like spices can be seen as alternatives to chemical preservatives used in various food industries so as to minimize their side effects and simultaneously improving the shelf life of the food products. The inhibitory factor responsible for the antimicrobial activity can further be identified and used as an alternative to currently used drugs against the pathogenic microbes. Nowadays microbes are increasingly developing resistance against the drugs in use. To combat against these drug resistant microbes, a large library of novel compounds is required. Natural products from plants may give us a solution to this alarming problem.

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