Discussion
V. DISCUSSION

Consumer demand for fish and fishery products is increasing as a result of awareness of health benefits of fish consumption. With the rising demand for these, it is necessary to develop processes for maintaining good quality fish and its distribution, particularly in the interior distant parts of the country. Preservation of fish with synthetic antioxidant and antimicrobial compounds in ice and frozen storage is still one of the commonest and efficient ways of prevention of spoilage. However use of synthetic compounds has raised questions regarding its safety and toxicity. There is a considerable interest in the natural use of phenolic compounds. These phenolic compounds are commonly found in both edible and non edible plants, and they have been reported to have multiple biological effects, including antioxidant and antimicrobial activity. Crude extracts of fruit seeds, herbs, vegetables, cereals, and other plant materials rich in phenolics are of increased interest in the food industry because they retard oxidative degradation of lipids and improve the microbiological characteristics and nutritional value of food (Loliger, 1991). This chapter deals with the discussion of results obtained from antioxidant and antimicrobial properties of GSE and PSE, storage stability of GSE and PSE in linoleic acid model system with the effect of heat, pH and storage time and effect of GSE and PSE on oxidative, biochemical and microbial spoilage of Indian mackerel during ice and frozen storage conditions.

5.1. Percentage of total extractable matter from grape and papaya seeds

The crude extract of seeds from various sources can be obtained with different extraction methods viz., cold and hot extraction and the percentage of total extractable matter depends on type of extraction method and solvent being used for extraction of natural antioxidant and antimicrobial compounds. Solvent extraction is usually used to
recover a component from either a solid or liquid. The sample is contacted with a solvent that will dissolve the solutes of interest. Solvent extraction is of major commercial importance to the food and biochemical industries, as it is often the most efficient method of separation of valuable products. Some extraction techniques involve partition between two immiscible liquid, others involve either continuous extractions or batch extractions. In the present study, the percentage of total extractable matter from grape and papaya seeds was carried out by cold extraction method. In the present study it was found that percentage of grape seed extractable matter was higher (6.0%) than papaya seeds (2.75%) and is represented in Table 3 and Fig.14. The extractable matter of GSE was found to be 6.0%. Jayaprakasha et al. (2003) reported that, the total extractable matter of GSE in acetone and methanol extraction was found to be 5.60 and 6.10% (w/w) respectively. The results of the present study of percentage of total extractable matter of GSE are in agreement with the findings of Ramchandani et al. (2010). He reported that Bangalore blue and Pandhari sahebi grape seeds possesed 6 and 3% respectively. According to Hesham et al. (2009), total extractable percentage of seeds depends on the percentage and type of solvent used for extraction and found that 50% of ethanol extracted 12.7% of extractable matter from grape seeds. In the present study, the percentage of total extractable matter of grape seeds was 2 folds higher than papaya seeds which is similar to the finding of Zhou et al. (2011). They investigated that, the different fractions of solvent showed different yield percentage as petroleum ether fraction (10.2 g/100 g), ethyl acetate fraction (1.2 g/100 g), n-butanol fraction (1.1 g/100 g), and water fraction (6.4 g/100 g).

5.2. Total phenolic content (TPC) of GSE and PSE

Phenols represents group of compounds that have more than one phenolic hydroxyl group attached to one or more benzene rings. Total phenolic content contributes to overall
antioxidant activity and were determined by colorimetric methods with Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid (H$_3$PW$_{12}$O$_{40}$) and phosphomolybdic acid (H$_3$PMo$_{12}$O$_{40}$) which, after oxidation of the phenols, is reduced to blue oxides of tungsten (W$_8$O$_{23}$) and molybdene (Mo$_8$O$_{23}$), respectively. This reaction, which occurs under alkaline conditions, is carried out with sodium carbonate. Under these conditions, the electron is easily removed from the phenol molecule. The resultant blue coloration has a maximum absorption in the region of 760 nm, and is proportional to the total quantity of phenolic compounds present. The quantification of TPC is usually expressed as gallic acid equivalent per gram of sample.

In the present study, the total phenolic content of GSE and PSE showed significant difference (p < 0.05) as the former contains four times higher phenolic content than the latter as shown in Table 4 and Fig. 15. The GSE in the present investigation possessed higher TPC of 1.23 mg GAE/g DW which is in agreement with the findings of Hogan et al. (2009), who recorded TPC of Norton grapes to be 1.8 mg GAE/g DW. The percentage yield of phenolic content from the plant extracts depends upon several factors and the prime factor being solvent used for the extraction process as the study conducted by Jayaprakasha et al. (2003). They summarized that grape seeds extracted in different solvents viz., acetone and methanol varied in total phenolic content by 46 and 38% respectively. Yi et al. (1997) had documented a slightly higher phenolic content in grape seeds i.e, 1.4 to 3.1 mg GAE/g DW. According to Gibis and Weiss, (2012) TPC of extracts from grape seeds was approximately 26 times higher than rosemary extract. The higher content of phenolics in grapes indicates that it can act as better radical scavenger and prevent lipid oxidation in fish products. PSE possessed total phenolic content of 0.32 mg GAE/g DW which is comparatively much lower than GSE. The lower phenolic content of
PSE indicated that it can’t act as strong radical scavenger and the result are in agreement with the findings of Maisarah et al. (2013), who quantified TPC of tropical American papaya seeds contain 0.32 mg GAE/g DW. The TPC vary with respect to seed varieties, region and climatic conditions as reported by Norshazila et al. (2010). Kwang- Ang et al. (2012) also recorded that TPC of papaya seeds ranged from 0.60 to 6.75 µg GAE/mL extract.

**5.3. Total flavonoid content (TFC) of GSE and PSE**

Flavonoids are most common group of phenolic compounds that are ubiquitous in plants. They exhibit antioxidant activity, which depends upon the number and location of hydroxyl groups. It contributes to overall radical scavenging activity of the crude extracts and is expressed as catechin equivalent per gram of sample. In the present study, the TFC of GSE and PSE showed a significant difference ($p < 0.05$) as the former contain six times more than the latter as shown in Table 4 and Fig. 15. The results of the present study can be compared with the findings of Irondi et al., (2013), who recorded that, papaya seed extract has lower flavonoid content compared to leaves and fruit pulp. The present study revealed that, the TFC of GSE was found to be $1.66 \pm 0.30$ mg CE/g DW which is comparatively higher than the Norton grape seeds (1.19 mg CE/g DW) as reported by Hogan et al. (2009). Cantos et al. (2002) observed that the TFC of seven table grape seeds extract ranged from 0.13 to 0.31 mg CE/g DW. Hogan et al. (2009) investigated that TFC of the cabernet Franc clone 313 contained 0.48 mg CE/g DW which is comparatively lower than the results of present study. The TFC of PSE in the present study was found to be $0.28 \pm 0.30$ mg CE/g DW which is relatively lower than study as reported by Maisarah et al. (2013). They reported that, the TFC of papaya seed extract was 0.59 mg GAE/g DW. The variation in TFC depends upon season, seed cultivar, maturity and condition as
reported by Cantos et al. (2002). The present study revealed that, the GSE possesses higher flavonoid content in comparison to PSE and exhibited superior radical scavenging activity which can prevent lipid oxidation both in model system and fish meat.

5.4. *In vitro* antioxidant activity of GSE and PSE

Grape and papaya seeds were subjected to solvent extraction using petroleum ether and ethanol separately in the ratio of 1:10 (seed: solvent) and extracts were filtered and concentrated using flash evaporator. The residues were kept in frozen storage and freeze dried and the final extracts were stored in refrigerated condition. Various *in vitro* antioxidant properties of the extracts such as DPPH free radical scavenging activity, ABTS radical scavenging activity, metal chelating activity, ferric reducing antioxidant power, linoleic acid peroxidation inhibition and antimicrobial activity of extracts are discussed as follows.

5.4.1. DPPH* radical scavenging activity of GSE and PSE

Antioxidant effectiveness is measured by monitoring the inhibition of oxidation of suitable substrate. In biological systems antioxidant effectiveness is classified into 2 group’s viz., evaluation of lipid peroxidation and measurement of free radical scavenging ability. DPPH* radical scavenging activity is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. The antioxidant activity is then measured by the decrease in absorbance at 515 nm (Shimada et al., 1992). The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. In the present investigation the DPPH scavenging activity of GSE and PSE at different concentration was measured by decrease in absorbance and is
shown in Fig. 16 and Table 5(A), 5(B). The activity of both the extracts increased with increase in concentration (p < 0.05). At the same concentration used, the descending order of DPPH radical scavenging activity of the seed extract tested was as follows: GSE > PSE. The DPPH scavenging radical activity of GSE increased up to 300 ppm and thereafter the activity did not show any further increasing trend (p > 0.05). The result suggests that BHA and BHT showed highest radical scavenging activity of 91.05 and 89.72% compared to GSE and PSE which were 87.02 and 61.43% respectively at 500 mg/L. The high radical scavenging activity of GSE could be due to the presence of flavonoids that can scavenge on free radicals such as superoxide, hydroxyl, 1,1-diphenyl-2-picrylhydrazyl and reduction of hydroperoxide formation and their effects (Jacob et al., 2008). The presence of the functional group “-OH” in the structure and its position on the flavonoids molecule determine the antioxidant capacity (Arora et al., 1998). Addition of -OH group to the flavonoid nucleus will enhance the antioxidant activity, while substitution by -OCH₃ groups diminishes the antioxidant activity. The other reason for higher scavenging activity of GSE could be its high amount of total phenolic content and the presence of monomeric polyphenolic compounds such as (+)-catechin, (−) – epicatechin and epicatechin-3-o-gallate and dimeric and tetrameric procyanidins (Saito et al. 1998). The result of DPPH radical scavenging activity in the present investigation can be compared with the findings of Bozan et al. (2008). They hypothesized that, the higher radical scavenging activity does not depend upon higher polyphenolic content but higher activity might be ascribed to certain constituents which show strong antioxidant activity. The radical scavenging activity of Bangalore blue grape seeds and Pandhari sahebi seeds can be compared with the present investigation as documented by Ramchandani et al. (2010). They found that both the varieties of grape seeds showed concentration dependent scavenging as Bangalore blue
grape seeds showed highest scavenging activity of 81% whereas, Pandhari sahebi grape seeds showed radical scavenging activity of 90% at a concentration of 300 mg/L. The DPPH radical scavenging activity (EC$_{50}$) ranged between 2.71 to 4.62 μg/mL Trolox equivalent/g grape seed as reported by Bozan et al. (2008), which clearly indicated EC$_{50}$ was quite higher in grape seed extracts.

The DPPH radical scavenging activity of PSE showed dose dependent activity and radical scavenging activity increased with the increase in concentration as shown in Table 5(B) and Fig.16. The lowest radical scavenging activity of PSE was found to be 31.90% at 100 mg/L whereas; it showed highest scavenging activity of 61.43% at 500 mg/L. The results of the present study can be compared with the findings of Kwang-Ang et al. (2012), who found that DPPH radical scavenging activity of papaya seed extract ranged from the lowest of 17.59 ± 0.79% to the highest of 57.30 ± 0.41% at a sample concentration of 500 μg/mL. The DPPH radical scavenging activity of PSE as reported by Irdoni et al. (2013) showed lower scavenging activity of 17.85 EC$_{50}$ for papaya seed extract and lower activity of PSE might be due to P-hydrobenzoic acid and vanillic acid which are simple phenolic compounds as documented first time by Zhou et al. (2011). The carboxyl group is the electron withdrawing group which doesn’t benefit the radical scavenging activity of the compound (Thiago et al., 2008). The result indicated that, the GSE is a potential free radical scavanger, which reacts with radicals by donating its hydrogen atom and acts as a primary antioxidant which can be used as a rich source of functional and antiradical compound to prevent lipid oxidation.

5.4.2. ABTS$^{•+}$ radical scavenging activity of GSE and PSE

The ABTS$^{•+}$ assay is based on scavenging ability of antioxidant against long shelf life radical cation ABTS molecule. In this assay ABTS is oxidized by peroxy radical or
other oxidant to its radical cation, ABTS⁺⁺, which is intensely colored and antioxidant capacity is measured by ability of the compound to decrease color reacting directly with ABTS⁺⁺ radical. ABTS⁺⁺ assay measures both the hydrophilic and lipophilic antioxidants since the reagents dissolve well in both aqueous hydrophilic and organic hydrophobic solvent (Kwang-Ang et al., 2012). In the present study, ABTS⁺⁺ scavenging activity of GSE and PSE at different concentrations were measured against Trolox equivalent as shown in Fig 17 and Table 6(A), 6(B). ABTS⁺⁺ radical scavenging activity of both the extracts increased as the concentration increased (p < 0.05). However, the activity varied with the type of extract tested. The dose-dependent scavenging activity is shown in PSE while in case of GSE there was no significant difference at concentration range of 200-600 mg/L (p > 0.05), which showed a similar trend as in the case of DPPH assay.

The ABTS⁺⁺ radical scavenging activity of GSE is in agreement with the findings of Sanchez-Alonso et al. (2008), who found that, ABTS radical scavenging activity of GSE at 100 ppm was found to be 284 ± 24 µmol of TE/g of white grape seed extract. Other researchers also documented that, the radical scavenging activity of GSE shows dose dependent activity as reported by Gibis and Weiss, (2012) who observed ABTS⁺⁺ radical scavenging activity of GSE to be 4.5 µmol/g at 100 mg/L. The extraction of grape seed with different solvents showed varied ABTS⁺⁺ radical scavenging activity and it depends upon polarity of the solvent, the isolation procedure and the purity of the active compounds as well as test system used to evaluate the activity (Meyer et al., 1998). The effectiveness to quench the ABTS radical depends upon the number of aromatic rings in the antioxidant, nature of hydroxyl groups and molecular weight (Hangerman et al., 1998). The Higher TEAC value indicates that the mechanism of antioxidant action of extracts was as a hydrogen donor and it could terminate the oxidation process by converting free radicals to
the stable forms. So it was more likely said that the structure and side group of phenolic compounds had the capacity to scavenge radicals and different compounds have the ability to scavenge differently. As a consequence, different assays should be conducted to verify the antioxidant activity of various compounds, in which mode of action could be different.

The radical scavenging activity of PSE is comparatively lower than GSE and the result of the present work is similar to that of Prasad et al. (2010). They showed that, the lower activity of ABTS$^{•+}$ radical scavenging activity of PSE is due to lower content of phenolic and flavonoid content. The lower scavenging activity of PSE also agreed with the findings of Kwang-Ang et al. (2012). They monitored ABTS radical inhibition activity of PSE at two different concentrations viz., 200 and 500 μg/mL and recorded values were $1.28 \pm 0.24$ and $11.19 \pm 0.62$% respectively. The results strongly suggest that, the GSE is strong ABTS$^{•+}$ radical cation inhibitor as compared to PSE.

5.4.3. Ferric reducing antioxidant power (FRAP) of GSE and PSE

Reduction capabilities of plant extracts can serve as a significant indicator of their potential antioxidant activities. This assay is based on the reaction, which measures reduction of ferric (Fe$^{3+}$) 2,4,6 tripyridyl-5-triazine (TPTZ) to a colored ferrous (Fe$^{2+}$) TPTZ product. The Fe$^{2+}$ formed from the reduction process was then monitored by measuring the formation of Perl’s Prussian blue. Increase in absorbance of the reaction mixture at 700 nm indicates an increase in reducing power, however it is limited to hydrophilic antioxidants. This kind of reduction process is based on the tendency of an antioxidant to donate electron (Medina et al., 2007). The reducing ability of the GSE and PSE were evaluated at different concentrations (100, 200, 300, 400, 500 μg/mL) as shown in Fig. 18 and Table 7(A), 7(B) and were compared to reference, BHA and BHT at 200 mg/L. The BHA and BHT were found to have reducing capacity of 1.27 and 1.01
respectively. All the extracts were capable of reducing Fe$^{3+}$ and did so in a linear dose dependent manner. Among two extracts tested, GSE showed highest ferric reducing activity at all concentrations (p < 0.05) and showed the highest reducing power at 500 µg/ml which is comparable to reducing power of BHA at 200 mg/L, indicating that GSE could easily donate electron to ferric ion (Fe$^{3+}$), thus reducing it to ferrous ion (Fe$^{2+}$). The ability of grape seed to show good reducing power is due to catechin and epicatechin possessing the higher number of higher hydroxyl groups. The results of high reducing power of GSE were in agreement with highest DPPH and ABTS radical scavenging activity. Zhang et al. (2011) also reported that GSE was able to reduce Fe$^{3+}$ to Fe$^{2+}$ and with increase in concentration ferric reducing power increases. The result from the present study of ferric reducing antioxidant power of GSE fall within the usual ranges described for antioxidant obtained from white grape seeds originating from wine production as reported by Saura-Calixto, (1998). In contrary, the papaya seed extract showed a least reducing power and the results were in agreement with the present work on phenolic content, DPPH and ABTS radical scavenging activity. Zhou et al. (2011) found that, the ethyl acetate extract of papaya seed extract possessed the greater reducing power than ethanol extract fraction, n-butanol fraction and water fraction, which is in accordance with the results of the present study. The results suggested that the reducing power of the compound appears to be related to degree of hydroxylation and extent of conjugation in polyphenols, which is seen in GSE as it contains catechin and epicatechin which are highly hydroxylated whereas, in case of PSE it lacked hydroxylation in its structure, which affects ferric reducing ability. This is in accordance with the findings of Kwang Ang et al. (2012), who reported that the papaya peel showed higher reducing power compared to papaya seed
extracts, but he added that both the extracts, showed much lesser reducing power compared to synthetic counterparts like BHA, BHT and tocopherol.

5.4.4. Metal chelating activity of GSE and PSE

An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which catalyze hydroperoxide decomposition in lipid oxidation of fish and fishery products and Fenton-type reaction in which ferrous ions catalyze the conversion of hydrogen peroxide to hydroxyl radical with the production of ferric ion. Metals are essential for life because they are required for transportation, respiration and activity of many enzymes. Redox active metals like iron, copper, chromium, cobalt and other metals undergo redox cycling reaction and possess the ability to produce reactive radicals such as superoxide anion and nitric acid in biological system. Excessive accumulation of metal ion lead to oxidative stress due to increased formation of reactive oxygen species (ROS), which are responsible for DNA damage, lipid peroxidation, protein modification and other effects (Jomova and Valko, 2011). Therefore, it was considered to be important to screen Fe$^{2+}$ chelating ability of extracts. The assay used to determine the chelating activity of Fe$^{2+}$ was based on the chelation of this metal ion with Ferrozine to yield red colored complex. In the presence of chelating agents, the complex formation is disrupted and red color of the complex decreases. Measurement of the rate of color reduction therefore allows estimation of the chelating activity. GSE and PSE extracts were assayed for their metal chelating activity at different concentrations as depicted in Fig. 19 and Table 8(A), 8(B) and this activity was compared with the chelating activity of synthetic metal chelator EDTA at 0.5 mM and 1.0 mM. The metal chelating activity of EDTA at 0.5 mM and 1 mM were found to be 85.31 and 97.22% respectively. GSE chelated more iron than PSE (p < 0.05), although both extracts were less efficient than
commercial chelator EDTA. The higher chelating activity of GSE is due to hydroxylation in its structure as GSE contains catechin and epi-catechin. Metal chelating activity of the compound depends upon number of hydroxyl groups in ortho position. The low chelating activity of PSE was possibly due to the presence of methoxy group, as reported by Zhou et al. (2011). The chemical structures of PSE were identified as p- benzoic acid and vanillic acid and methoxy group of vanillic which could interfere in metal chelating activity of papaya seed extract and that may be the reason for low metal chelating activity than GSE.

5.4.5. Linoleic acid inhibitory activity of GSE and PSE

Fish lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiate lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (Gao et al., 2000). Hydroxyl radicals eliminate hydrogen atom from the membrane lipids, which results in lipid peroxidation.

Lipid oxidation inhibition has been measured by ferric thiocyanate method (FTC). It measures amount of peroxide produced during intial stage of lipid oxidation. The FTC assay consists of ammonium thiocynate and Fe$^{2+}$ in acidic solution. H$_2$O$_2$ induced by lipid oxidation oxidizes Fe$^{2+}$ to Fe$^{3+}$ resulting in the formation of a red colored Fe$^{3+}$-thiocyanate complex. The levels of oxidation are determined by measuring the absorbance at 500 nm (Kosem et al., 2007; Yu et al., 2007). On the other hand, TBA measures the amount of malonaldehyde (MDA) produced during the second stage of the lipid oxidation. This method is based on the MDA reaction with thiobarbituric acid to obtain a red pigment, resulting from the condensation of two molecules of TBA with one molecule of MDA. The
levels of oxidation are determined by measuring the absorbance at 535 nm (Fernández et al., 1997; Zarena et al., 2009b).

The inhibitory capacity of GSE and PSE against the oxidation of linoleic acid model system was tested. As seen in Table 9(A), 9(B) and Fig. 14 the percentage of inhibition of lipid oxidation by GSE and PSE were seen at three different concentrations. At the same concentration, GSE shows higher inhibitory activity than PSE extracts and with the increase in concentration inhibitory activity of both the extracts increases and with maximum of 81.20 and 65.04% respectively for GSE and PSE (p < 0.5). Ramachandani et al. (2010) also reported that the grape seed extract could inhibit lipid peroxidation in the mouse liver microsome model system by 70-80%. In addition, Jayaprakasha et al. (2001) reported that, the ethanolic and water grape seed showed 80% inhibition of the linoleic peroxidation after 100 h. The mechanism involved for interference of grape seed extract inhibition of lipid oxidation is either iron chelating activity or by scavenging of superoxide radicals, which are responsible for reduction of ferric to ferrous, catalyzed by Fenton reaction and the iron chelating activity (Ramchandani et al., 2010). The lipid peroxidation inhibition of PSE were observed at different concentrations and showed 46.43% at 500 mg/L and the present findings are on the similar line as reported by Maisarah et al. (2013) who found that, papaya seed having least tendency to inhibit lipid peroxidation and showed antioxidant capacity of 58.97% at 500 ppm in linoleic acid model system whereas, other parts of fruit showed higher antioxidant capacity compared to seed extracts.

5.5. *In vitro* antimicrobial activity of GSE and PSE

The antimicrobial activity of GSE and PSE is shown in Table 10. Among the two extracts tested against gram +ve and gram –ve bacteria, GSE showed higher antimicrobial activity compared with PSE. GSE were more effective against *Staphylococcus aureus* and
Bacillus subtilis compared to gram –ve bacteria. These results were in agreement with those reported by Jayaprakasha et al., (2003) and Serra et al., (2008). The higher activity against the gram +ve strains may be due to the fact that gram +ve have less stable cell wall, which makes it permeable to some antimicrobial agents, whereas, in gram –ve bacteria the presence of outer cell membrane or cytoplasmic membrane of the bacterium is composed of phospholipid bilayer and proteins, it is difficult for antimicrobial agents to permeate. The antimicrobial properties of GSE could be attributed to the presence of core compounds like 3,4,5-trihydroxyphenyl group found in epigallocatechin, epigallocatechin-3-ogallate and prodelphinidin which might play an important role in their antimicrobial activity (Tagurt et al., 2004). The galloyl groups present in the structure of compounds present in GSE could exhibit antimicrobial activity.

5.6. Changes in antioxidant activity (AOA) of GSE and PSE with the effect of heat, pH and storage stability

The ability to inhibit lipid oxidation differed according to the types of plant extract, oxidizable substrate used, thermal and pH treatment to which the extracts are subjected. It is well known that many factors such as antioxidant concentration, temperature and pH of the media, processing treatment and storage strongly influence the antioxidant activity (Gazzani et al., 1998). In the present study, changes in antioxidant activity in linoleic acid model system with the influence of heat, pH and storage stability were carried out.

5.6.1. Effect of heat treatment on AOA of GSE and PSE in linoleic acid model system

In the present study effect of heat treatment (100 °C, 15 min) on the antioxidant activity of GSE and PSE were determined in linoleic acid model system and the results are presented in Table 11(A), 11(B) and Fig.22. The antioxidant activity of GSE and PSE without any heat treatment was found to be 67.67 and 46.43% respectively at 500 mg/L.
The heat treatment at 100 °C for 15 min decreased the AOA of GSE and PSE and was found to be 52.16 and 46.43% respectively at 500 mg/L. The decrease in AOA of both the extracts might be due to loss of naturally occurring antioxidants present in the extract or formation of novel compounds having proxidant activity upon heating. Thermal processing can induce the formation of compounds with antioxidant properties or improve the AOA of naturally occurring antioxidants (Nicoli et al., 1999). The antioxidant activity of number of vegetable juices is reported to be stabilized by boiling, suggesting that the initial proxidant activity is due to prooxidases which are inactivated at high temperatures (Gazzani et al., 1998). It is reported that crucifer extracts exhibit either a proxidant or an antioxidant activity depending on the thermal processing and variety of vegetable examined (Castenmiller et al., 2002). An increase in AOA of carrot puree due to thermal processing is associated with increased levels of phenolic acids (Talcott et al., 2000).

5.6.2. Effect of pH on AOA of GSE and PSE in linoleic model system

The antioxidant activity of GSE and PSE were seen in a wide range of pH (4, 7, and 9) at concentration of 500 mg /L and the results of the investigation are presented in Table 12(A), 12(B) and Fig.23. The lipid peroxidation inhibition of GSE and PSE at 500 mg/L was recorded to be 67.67 and 46.43% respectively as shown in Table. 9A, 9B and Fig.20. The AOA of GSE was found to be 69.01, 68.11 and 72.18% at pH 4, 7 and 9 respectively, whereas, for PSE lipid peroxidation inhibition of 52.20, 46.02 and 54.06 % at respective pH. The significant difference in AOA of GSE and PSE were found maximum at pH 9 while least at pH 7, which indicates strong dependence of AOA on the pH system. Yen et al. (1993) reported that a methanol extract from peanut hulls had a higher AOA at neutral acid pH. The AOA of different extracts from cocoa by-products was higher at
alkaline pH (Azizah et al., 1999). These differences might be due to different samples used and various compounds being extracted in each case.

5.6.3. Effect of storage time on AOA of GSE and PSE in linoleic acid model system

The effect of storage time on the antioxidant activity of the GSE and PSE at 500 mg/L is shown in Table 13A, 13B and Fig. 24. The GSE and PSE were stored in the dark at 5 and 25 °C and the lipid peroxidation inhibition of GSE and PSE at varied temperature and storage conditions were seen after 15 d. The lipid peroxidation inhibition of GSE showed percentage inhibition of 67.42 and 64.13% at 5 and 25°C respectively while PSE showed lipid peroxidation inhibition of 46.66 and 46.40% at respective temperatures. The results of the study indicated that there was slight change in AOA of GSE with the change in storage time while antioxidant activity of PSE remains unchanged during entire storage period. The results of the present study indicated that, there were no change in AOA during the storage period. Saeedeh et al. (2007) observed that, the storage period did not show any significant difference in AOA of the plant extracts.

5.7. Raw material characteristics of Indian mackerel

5.7.1. Proximate composition

The biochemical composition of fish varies greatly from one species to another depending upon sex, age, environment and season in which fish is being caught. Therefore, a substantial normal variation is observed in the constituents of fish muscle. During starvation period, the fish uses the energy depots in the form of lipids and also may utilize protein, thus depletion of these reserves results in a general diminution of biological condition (Huss, 1995). This condition will influence the quality of different fish products in terms of yield and process efficiency. Therefore the knowledge of biochemical
composition of fish is fundamentally important for the application of different technological processes.

In the present study biochemical composition of fresh Indian mackerel (*Rastrelliger kanagurta*) was analyzed and the results are presented in Table 22. The moisture, crude protein, crude fat and ash contents in fresh mackerel were found to be 71.02, 21.02, 6.96 and 1.20% respectively and the results can be compared with the findings of Sulochanan, (2008), who documented that, the moisture, crude protein, crude fat and ash content of Indian mackerel (*Rastrelliger kanagurta*) from west coast were 71.19, 21.21, 7.51 and 1.33% respectively. The results of the study are in agreement with the findings of Lakshmisha *et al.* (2014) for moisture and lipid content which ranged between 71.31 to 76.63% and 5.90 to 7.25% respectively. Indian mackerel can be classified as a fatty fish based on the percentage of lipid content. According to Ozogul and Ozogul, (2007) fatty fish usually contain a minimum of 5 to 8% fat in edible tissue. Low-fatty fish have higher water content as a result; their flesh is white in colour (Feeley *et al*., 1972). Fatty fish store the fat in muscle tissue resulting in their flesh colour as yellow, grey and pink (Gurr, 1992). The variation in proximate and fatty acid composition of Indian mackerel is due to seasonality and breeding behavior of the fish (Nisa, 2011). According to Mohan *et al.* (2008), the variation in proximate composition of fish not only depends on seasonality and breeding behavior but within the fish, variation in proximate composition is found between dark and white muscle of Indian mackerel. The result of crude protein and ash content of Indian mackerel were in agreement with the values of Mohan *et al.* (2008), Bandara *et al.* (2001), Mehmet, (2008) and Nisa *et al.* (2011).
5.7.2. Biochemical, microbiological and sensory characteristics of fresh mackerel

The freshness of fish meat was assessed by chemical, microbiological and sensory characteristics and it was revealed that, the quality of fish used in the present study was in prime condition. The determination of TMA-N and TVB-N for assessment of freshness of fish is widely used because of its good correlation with sensory quality changes during storage (Nair et al., 1976). In the present study TMA-N and TVB-N content of fresh Indian mackerel were observed to be $3.22 \pm 0.08\, \text{mg}\%$ and $8.45 \pm 0.23\, \text{mg}\%$ respectively which is below the maximum prescribed limit for fresh fish. The limit of TMA-N and TVB-N in the fresh fish is 10-15 mg% and 35-40 mg% respectively as limits prescribed by Gopakumar, (2002) and the values obtained for Indian mackerel meat revealed that, the fish was in fresh condition. The pH of fresh meat of Indian mackerel was found $6.24 \pm 0.06$ and limit of acceptability of marine fish is considered to be 6.5-7.5 as reported by Gopakumar, (2002). The peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) in the fresh mackerel were recorded as $1.62\, \text{mg}\,$ of hydroperoxides/kg of sample and $0.32\, \text{mg}\,$ of malonaldehyde/kg of sample respectively and the results of the present study can be compared with findings of Lakshmisha et al. (2014), who recorded that initial PV and TBA value of Indian mackerel were 2.8 meq O$_2$/kg fat and $0.45\, \text{mg}\,$ of malonaldehyde/kg of flesh respectively. The probability of having rancid odour will be high if PV and TBARS exceed 10 mg of hydroperoxides/kg of meat and 3 mg malonaldehyde/kg of meat respectively as limits prescribed by Connel (1990). The FFA content as % of oleic acid is an index of hydrolytic rancidity, which was observed to be $1.45 \pm 0.14\%$ of oleic acid in fresh mackerel and the results of FFA is in agreement with Lakshmisha et al. (2014). The total plate count (TPC) of fresh mackerel was found to be
4.5 log cfu/g whereas S. aureus and E. coli were not found in fresh mackerel. The sensory score of fresh mackerel was observed to be 8.90 on hedonic scale.

5.8. Effect of GSE and PSE on lipid oxidation products of whole mackerel and steaks during iced storage

5.8.1. Changes in peroxide value (PV) during ice storage

Peroxide value (PV) is a measure of the primary degree of oxidation in the fish muscle (Govindan, 1985; Haard, 2000; Gopakumar, 2002). In the present study changes in PV of mackerel chilled whole control (CWC), mackerel treated with GSE, PSE and BHT were observed during a period of 15 d of ice storage and are presented in Table 15 A and Fig. 25A. On the day of sensory rejection of CWC, the hydroperoxide formation of mackerel treated with BHT, GSE and PSE was found less by 58.82, 56.09 and 16.00% respectively. Hassan et al. (2010) reported that, there was an increase in PV of Indian mackerel throughout the ice storage period. The PV for the present study is in agreement with the above findings. Mackerel contained more dark muscle, and higher quantities of mitochondria, myoglobin, fats, glycogen and cytochromes, than did white fleshed-fish species (Chaijan et al., 2005). The chances of lipid oxidation in mackerel during ice storage will probably be more compared to lean fishes due to the high content of unsaturated fatty acids and prooxidants in the muscle, especially dark muscle (Chaijan et al, 2006).

The changes in PV of steaks treated with GSE and PSE showed lower hydroperoxide formation compared to whole mackerel treated with GSE and PSE during ice storage as shown in Table 15 B and Fig. 25B. This could be attributed to the skin as a barrier for whole fish to the antioxidant extracts in comparison to steaks. The PV of chilled steaks control (CSC) increased significantly throughout the storage period (p < 0.05)
whereas hydroperoxide formation of treated groups was minimum. The initial PV of CSC was found to be 1.60 mg hydroperoxides /kg of sample and reached maximum limit on 9th d of ice storage whereas BHT, GSE and PSE treated steaks were found to be in acceptable condition even at the end of 15 d of ice storage. A reduction in hydroperoxide formation on the day of sensory rejection of chilled steaks control (CSC) was found less by 64.39, 57.75 and 33.44% for BHT, GSE and PSE treated steaks respectively. The results of the present study are in agreement with the findings of Jianyun et al. (2014). They documented that, phenolic compounds from grape seed and clove bud extracts lowers the increase in peroxide value of silver carp fillets during chilled storage which clearly demonstrate the effect on lowering the primary lipid oxidation in silver carp fillets.

The peroxide values of samples treated with GSE and PSE showed relatively lower PV compared to that of CWC and CSC. The lower peroxide value was best seen in GSE treated samples compared to PSE treated samples. This showed that, the GSE could play an active role as a strong natural antioxidant while its inhibitory activity was more pronounced in limiting hydroperoxide formation with respect to PSE. According to Sulochanan (2008), phenolic compounds from natural sources like rosemary and clove extracts lower the peroxide value of Indian mackerel during 10 d of storage in iced condition. The inhibition of peroxides is concentration-dependent which showed a direct relationship between the polyphenolic concentration and the inhibitory efficiency as studied by Bensid et al. (2014). During ice storage, polyphenolic concentration of 100 ppm of GSE inhibited the formation of hydroperoxides about 95% as observed by Iglesias et al. (2012).
5.8.2. Changes in thiobarbituric acid-reactive substances (TBARS) during ice storage

The TBARS value is an index of secondary lipid oxidation measuring malonaldehyde content. Malonaldehyde is a secondary product of oxidation resulting from the degradation of lipid hydroperoxides formed during the oxidation process of polyunsaturated fatty acids (Fernandez et al., 1997). The TBARS test is a sensitive test for the decomposition products of highly unsaturated fatty acids which do not appear in peroxide value determination. The acceptability limit of TBARS value for fish stored in ice was 0-5 mg malonaldehyde/kg flesh (Nunes et al., 1992). In the present study, changes in TBARS content in mackerel during ice storage are represented in Table 16A and Fig. 26A.

The initial TBARS value of CWC, BHT, GSE and PSE samples was 0.32 mg malonaldehyde/kg sample and reached to maximum of 2.25, 1.27, 1.36 and 1.99 mg malonaldehyde/kg sample respectively after 15 d of storage in ice. On the day of sensory rejection of untreated whole mackerel (CWC), the malonaldehyde formation of mackerel treated with BHT, GSE and PSE was recorded as lower by 56.47, 56.47 and 22.35% respectively. This clearly indicated that, treatment with GSE and PSE lowers the formation of malonaldehyde although, the treatment with GSE was more effective compared to PSE treated samples. The TBARS values represent the content of secondary lipid oxidation products, particularly malonaldehyde. The carbonyls are primarily responsible for the production of off-flavors in oxidized meat and meat products. Malonaldehyde is a secondary product of oxidation resulting from the degradation of lipid hydroperoxides formed during the oxidation process of polyunsaturated fatty acids (Fernandez et al., 1997). They react with the TBA reagent to form a pink complex (TBA chromogen) with a maximum absorbance at 532 nm. The intensity of this pink color is directly related to the concentration of the TBA-reactive substances in the original sample. The acceptability
limit of TBARS value for fish stored in ice was 0-5 mg malonaldehyde/kg flesh (Nunes et al., 1992). The results of the TBARS analysis of mackerel for the preliminary study of 15 d in ice storage are presented in Table 16A and Fig.26 A. The overall lipid oxidation increased substantially as the ice storage progressed. On the 9th d of ice storage, TBARS value for the chilled whole control (CWC) was found to be 1.70 mg malonaldehyde/kg meat, GSE and BHT treated mackerel recorded to be 0.74 mg malonaldehyde/kg meat whereas, PSE treated mackerel was observed to be 1.32 mg malonaldehyde/kg meat. Hassan et al. (2010), observed the marked increase in TBARS content of Indian mackerel throughout the chill storage period. The results of the study indicated that, the percentage of reduction in TBARS formation, with the effect of GSE and BHT was recorded to be 56.47% whereas, PSE treated mackerel marked only 22.35% lower in TBARS formation compared to untreated mackerel. These results are in agreement with that of Iglesias et al. (2012), who monitored that grape seed extract at 100 ppm effectively, decreased TBARS formation in fish muscle. They explained that mechanism of the protective effect on lipid oxidation and lowering of TBARS content may be due to the fact that GSE are rich in proanthocyanidins which have multiple mechanisms for its antioxidant activity and ability to sequestrate radicals, chelate metals and synergize with other antioxidants (Lu and Foo, 1999).

The TBARS values of steaks among the treatments at 0th d did not differ significantly (p > 0.05) as shown in Table 16B and Fig.26 B. However, the TBARS values of all treatments were significantly lower than that of the control (CSC) (p < 0.05) during 15 d of ice storage. The percentage inhibition of malonaldehyde formation treated with GSE and PSE was recorded to be 59.66 and 20.99% respectively whereas, BHT showed marginal reduction of 65.57% with respect to untreated samples on the 9th d of ice storage.
The above results clearly indicated that GSE at 500 mg/L can be compared with that of BHT at 200 mg/L whereas, PSE did not show promising effect in limiting the malonaldehyde formation. The above findings of PSE are in agreement with the in vitro antioxidant properties of papaya seed extract. Rababah et al. (2006) and Shirahigue et al. (2010) observed, a marginal reduction in TBARS in chicken meat with the effect of GSE during refrigerated storage. According to Jongberg et al. (2013), GSE at 500 ppm showed strong antioxidant activity in lowering TBARS formation in chill stored beef patties. The results of the present work follow similar trend of lowering TBARS formation as investigated by Brannan and Mah (2007). They monitored the secondary lipid oxidation in chicken samples treated with GSE at 0.1% to 1.0% and concluded that, the higher percentage of phenolic compounds from GSE were more effective in lowering secondary lipid oxidation.

5.9. Effect of GSE and PSE on biochemical changes of whole mackerel and steaks during ice storage

5.9.1. Changes in free fatty acid (FFA) content during ice storage

It is well known that, the free fatty acid (FFA) is a result of enzymatic decomposition of lipids in fish and fishery products (Gopakumar, 2002). Free fatty acids which cause protein denaturation are formed during processing as a result of lipid hydrolysis. FFA may have been involved in reaction with myofibrillar proteins and promote protein aggregation (Pacheco-Aguilar et al., 2000). In the present investigation, effect of GSE and PSE on enzymatic inhibition of mackerel and steaks during ice storage was studied and are represented in Table 17A, 17B and Fig. 27A, 27B. Fatty acid formation of all the treated groups of mackerel and steaks showed significant difference with that of control groups (p < 0.05). On the day of sensory rejection, the free fatty acid
content in whole mackerel with the treatment effect of BHT, GSE and PSE was observed to be lower by 9.36, 12.94 and 6.08% as oleic acid respectively compared with untreated samples. The free fatty acid formation in steaks treated with BHT, GSE and PSE was reduced by 11.59, 8.59 and 2.59% as oleic acid respectively with respect to untreated steaks. The above result clearly indicated that, the effect of treatment had minimum inhibition on lipid hydrolysis and among the two seed extracts tested, inhibition on enzymatic hydrolysis was more pronounced in GSE treated samples compared PSE treated samples. The result of the present study is in agreement with findings of Chaijan et al. (2006). They reported that, the phenolic compounds had less effect in lowering enzymatic hydrolysis of fish muscle. Bensid et al. (2014) reported that, the effect of polyphenols from thyme, oregano and clove extracts leads to lower the formation of free fatty acid. The prime factor of hydrolytic rancidity in fish during ice storage takes place as a result of endogenous enzyme namely, lipases and phospholipases activity and later on, microbial activity should be important, so that FFA formation should mostly be produced as a result of bacterial and enzyme activity. The inhibitory effect of plant polyphenols on endogenous enzyme activity in fish based muscle system occurred in first stage (d 2-6) while antimicrobial effect of polyphenols led in the second stage (d 10–23) to a lower free fatty acid formation.

5.9.2. Changes in trimethylamine nitrogen (TMA-N) content during ice storage

Trimethylamineoxide (TMAO) is the compound which occurs naturally in most of the marine fishes responsible for their characteristic fishy odour and flavor. Trimethylamine is the bacteriologically degraded product from trimethylamineoxide (TMA-O) (Huss, 1995). Trimethylamine (TMA) level in fish is an important factor in the subjective evaluation of fish quality because of its close association with fish spoilage
Fish with a level beyond 10-15 mg TMA-N/100g fish are considered as spoiled (Govindan, 1985). In the present investigation changes in TMA-N content of mackerel and steaks with the effect of GSE and PSE during ice storage were investigated and the results are presented in Table 18A, 18B and Fig. 28A, 28B. GSE and PSE resulted in significant reduction in TMA-N production in whole mackerel and steaks during ice storage period of 15 d (p < 0.05). The percentage of reduction in TMA-N of mackerel with the effect of treatment with BHT, GSE and PSE on the day of sensory rejection with respect to untreated mackerel (CWC) was found to be 36.91, 32.27 and 19.01% respectively whereas, effect of BHT, GSE and PSE on reduction of TMA-N formation with respect to untreated steaks (CSC) was found to be 38.74, 25.39 and 19.63% on the day of sensory rejection respectively. The results of the present study are in agreement with Gao et al. (2014). TMA-N production in fish and fishery products is due to enzymatic and microbial degradation of protein and non-protein nitrogenous compounds. The protein and non-protein nitrogenous content in fish depends on several factors such as age, sex, culture method and locality of fish in which it resides (Delbarre-Ladrat et al., 2006). The increase in TMA-N levels may result from deamination of free amino acid, oxidation of amines and degradation of nucleotides by autolytic enzymes and microbial activity (Ocano-Higuera et al., 2011). The above results clearly indicated that, the polyphenolic compounds from GSE and PSE were effective in controlling TMA-N formation in mackerel and steaks during ice storage. The results of the present study are in agreement with Lopez de Lacey et al. (2014). They monitored crude polyphenols from green tea extract and showed antimicrobial activity which lowered TMA-N formation in hake fillets during 15 d of storage.
5.9.3. Changes in total volatile base-nitrogen (TVB-N) content during ice storage

Total volatile basic nitrogen (TVB-N) levels have been recognized as useful indicators of seafood spoilage; under EU directive 95/149/EEC, the European Commission has specified that, the TVB-N level can be used, if sensory methods raise doubts about the freshness of seafood species (EU, 2005). The limit of acceptability of TVB-N for human consumption is less than 35-40 mg of TVB-N/100g in meat products. It mainly constitutes ammonia in the fresh muscle and is produced by deamination of muscle by adenylic acid which leads to denaturation of muscle proteins. In the present investigation changes in TVB-N content with the effect of GSE and PSE during ice storage of mackerel and steaks are shown in Table 19A, 19B and Fig. 29A, 29B. The significant increase in TVB-N formation was found to be in control samples compared to the treated ones due to the deterioration of fish by bacterial growth and production of vapor gases such as ammonia. The result agreed with the findings of Winarni et al. (2012). They recorded that, the initial TVB-N content of Indian mackerel was 10 mg/100 g and with increase in storage period, TVB-N content reached 39.22 mg/100g after 16 d of storage in ice. The treatment with GSE and PSE contributed to lowering TVB-N formation but GSE seems to be more effective in delaying or reducing the growth of bacteria which are involved in the production of basic compounds (TVB-N) in fish during ice storage. The percentage of reduction in TVB-N with the effect of treatment with BHT, GSE and PSE on the day of sensory rejection with respect to untreated mackerel (CWC) was found to be less by 40.28, 31.85 and 24.70% respectively whereas, the effect of BHT, GSE and PSE on reduction of TVB-N formation with respect to untreated steaks (CSC) was lowered by 29.56, 22.17 and 11.45% respectively on the day of sensory rejection. The present findings of antimicrobial action of polyphenols to lower TVB-N during ice storage are in agreement with the report
published by Unalan et al. (2011) that, the pomegranate and rosemary extract helps to lower TVB-N formation in halibut fillets. Similar trends were reported by Quitral et al. (2009) on lowering TVB-N content of Chilean jack mackerel (Trachurus murphyi) during ice storage with the effect of rosemary and oregano polyphenols.

5.9.4. Changes in pH during ice storage

A change in pH of fish muscle is usually a good index for quality assessment (Gopakumar, 2002). It is important in determination of fish quality as texture of fish and gaping in fish fillets are influenced by pH (Love, 1992). The changes in the pH of iced stored mackerel and steaks are presented in Table 20A, 20B and Fig.30A, 30B. Lactic acid generated in anoxic conditions from glycogen is the principal factor in lowering the post mortem pH in fish muscle. In the present study a marked increase in pH of all treated groups of whole mackerel and steaks during ice storage was observed and were in line with Shinde et al. (2012). According to Ababouch et al. (1996) initially, the muscle pH averaged 6.15 for sardine (Sardina pilchardus) stored at ambient temperature and 6.24 for iced sardines. It increased slightly during storage, reaching a level of rejection (12 d) i.e., 6.85 at ambient storage and 6.55 in ice storage. The increase in pH with storage may be caused by the growth of spoilage bacteria leading to the accumulation of alkaline components (e.g., ammonia and trimethylamine). Similar results were reported by Duan et al., (2010) and Fan et al., (2008). The pH values of all treated samples were consistently lower than that of the control (p < 0.05) during storage, which could be attributed to antimicrobial characteristics of GSE and PSE. The results of the present work are in agreement with the findings of Li et al. (2012) who reported that, the increase in pH value was delayed in Crucian carp treated with tea polyphenols and rosemary extract.
5.10. Effect of GSE and PSE on drip loss (%) of whole mackerel and steaks during ice storage

Changes in drip loss percentage of mackerel treated with GSE and PSE during storage in iced condition for a period of 15 d are depicted in Table 21A and Fig. 31A. On 9th d of ice storage with respect to untreated mackerel, the reduction in drip loss percentage of GSE treated mackerel was found to be 14.51%, 12.05% for PSE treated mackerel and BHT treated mackerel exhibited highest percentage of reduction in drip loss which is 27.55%. The drip loss percentage in steaks are presented in Table 21B and 31B and the percentage increase in drip loss in steaks treated with GSE and PSE showed remarkably lower percentage as compared to whole mackerel. On the 12th d of storage, the percentage reduction in drip loss was found higher in GSE treated sample compared to PSE treated sample. The GSE treated steaks exhibited 27.60% whereas, PSE treated samples showed 15.49% reduction in drip loss compared to untreated steaks. The results of the present study agreed with the findings of Winarni et al. (2012), who reported that, the percentage reduction of drip loss of Indian mackerel treated with crown god fruit extract was 21.36%.

5.11. Effect of GSE and PSE on microbiological characteristics of whole mackerel and steaks during ice storage

It has been estimated that about one third of world’s food production is lost annually on account of microbial spoilage. Microorganism associated with aquatic products usually reflects the microbial population in the environment. Total plate count (TPC) of bacteria present in the food is the most important part of any proposed microbiological standard. A low bacterial count indicates enhanced shelf life. The initial TPC differ from one fish to the other. TPC is not the measure of total bacterial population, only measures fraction of the
microflora that is able to produce colonies under the conditions of growth medium and the incubation (Lyhs, 2009).

In the present study changes in total plate count of whole mackerel and steaks treated with GSE and PSE during ice storage for 15 d are presented in Table 22A, 22B and Fig. 32A, 32B. The results of the study showed that, the microbiological growth was significantly ($p < 0.05$) influenced by the dip treatment of mackerel and steaks with GSE and PSE. The initial TPC of CWC was 4.5 log (cfu/g) and reached the maximum limit of acceptability on the 9th day of ice storage, beyond which it was observed to be spoiled, while GSE and PSE treated mackerel was in acceptable condition on 12th d of ice storage. Similar results were documented by Banon et al. (2007), that, the addition of grape seed and green tea extract to beef patties improved the microbial load and increased the shelf life of patties by 3 d. GSE extracts were found to be more effective in reducing total bacterial load of fish samples. The antibacterial property of GSE was reported to be due to the hydrophobic nature of phenolic compounds (Jayaprakasha et al., 2001). Accumulation and attachments of these phenolics to the bacterial cytoplasmic membrane eventually lead to cell death (Lin et al., 2004). Other researchers also reported antimicrobial effects of GSE in restructured mutton slices (RMS) under aerobic and vacuum packaging conditions during refrigerated storage and result of the investigation revealed that reduction of total psychrophilic counts of GSE in both aerobic and vacuum packaged RMS is probably due to the antimicrobial activity of GSE (Ahn et al., 2004).

The effect of treatment with GSE and PSE was found more effective in steaks as compared to whole mackerel. The initial bacterial load of CSC was 4.6 log (cfu/g) and after 9 d of storage in icing condition, the bacterial load reached 7.3, 5.7, 4.9 and 5.9 log (cfu/g) for CSC, BHT, GSE and PSE respectively. Similar reduction of 2 log cycles was
reported for crown god fruit treated Indian mackerel in ice storage for 15 d as reported by
Winarni et al., (2012). Microbiological quality of Indian mackerel fillets were subjected to
treat with chillies marinades and the results of the investigation showed that, there was a
reduction in 2 log cycles compared to untreated one as reported by Ulfah, (2013).

5.12. Effect of GSE and PSE on organoleptic characteristics of whole mackerel and
steaks during ice storage

The objective of sensory testing is to measure the intrinsic sensory attributes of a
sample through the analytic sensory perceptions of human assessors. The overall quality of
seafood is comprised of both wholesomeness and sensory acceptability by the consumer
(Sikorski and Sunpan, 1994). The wholesomeness is affected by chemical and microbial
factor where as sensory factors are determined by flavor and texture (Sawyer et al., 1988).
Sensory analyses of fish and fishery products have always been a part of the production
process (York and Sereda, 1994).

In the present investigation sensory attributes of mackerel and steaks during ice
storage studies are depicted in Fig.33A, 33B and Table 23A, 23B. The mean panel scores
of all the sensory characteristics were observed to decrease in whole mackerel and steaks
an increase in the storage period. The present study indicated that, using sensory score of 9
as limit of acceptability, the observed shelf life was 9 d in CWC samples, 12 d for PSE
treated samples and 15 d for BHT and GSE treated samples, whereas CSC reached a
maximum limit of 9 d of ice storage and BHT, GSE and PSE treated steaks were in
acceptable condition at the end of 15 d of ice storage. The results of the present study can
be compared with the previous finding of Bennour et al. (1991) and Shinde et al. (2012),
who reported that mackerel were in acceptable condition upto 6-9 d during ice storage. The
sensory attributes for mackerel and steaks treated with BHT, GSE and PSE showed a
declining trend in sensory attributes, however, the deterioration was slow for treated samples compared to untreated control samples. Treated samples had a significant effect on the sensory attributes (p < 0.05) compared to untreated ones. Previous works demonstrated an increased shelf-life and enhancement of sensory quality of Indian mackerel with the treatment of aloevera and god fruit extracts as investigated by Winarni et al. (2012). The increase in sensory attributes of Indian mackerel treated with spice extracts of rosemary, ginger, pepper and clove during chill and frozen storage was reported by Sulochanan, (2008). However results found by Brannan, (2009) were not in agreement with the present results in which the author indicated that there is no effect of GSE on the sensorial attributes of chicken breast but influenced on rancid flavor and off odor.

5.13. Effect of GSE and PSE on lipid oxidation products of whole mackerel and steaks during frozen storage

5.13.1. Changes in peroxide value (PV) during frozen storage

In the present investigation combination of antioxidants with frozen storage of mackerel and steaks were investigated and the results of the study are presented in Table 24A, 24B and Fig.34 A, 34B. The results indicated that the PV of frozen whole control (FWC) and frozen steak control (FSC) increased significantly (p < 0.05) from the initial value of 1.62 up to 14.96 and 12.07 mg of hydroperoxide/kg sample respectively at the end of frozen storage. The peroxide value at the end of 180 d for BHT, GSE and PSE treated mackerel reached maximum of 6.09, 7.12 and 7.28 mg of hydroperoxide/kg sample and for steaks reached maximum of 4.78, 4.93, 7.15 mg of hydroperoxide/kg sample respectively. The PV of steaks treated with GSE and BHT were found to be much lower in hydroperoxide formation compared to mackerel during frozen storage. The decrease in hydroperoxide formation of mackerel treated with BHT, GSE and PSE with respect to
untreated groups on the corresponding day during frozen storage was found to be 55.39, 49.66 and 40.87% respectively whereas, for steaks treated with BHT, GSE and PSE it was found to be 64.55, 61.63 and 32.32% respectively. The above results clearly indicated that, the effect of GSE and PSE in combination with frozen storage inhibited the primary lipid oxidation of whole mackerel and steaks. The results are in agreement with previous findings of Pazos et al. (2005), who studied that, induction periods of formation of peroxides were significantly retarded in samples treated with grape seed extract of frozen muscle of mackerel. The results demonstrated that all flavonoid fractions and propyl gallate were effective for retarding oxidation during frozen storage. The result of present study are in agreement with the findings of Ozogul et al. (2011), who found peroxide value of sardine fillets to be lowered by 3 times with the effect of antioxidant treatment combined with frozen storage along with the findings of Aubourg et al. (2002). They documented that peroxide formation for mackerel fillets were above 10 mg of hydoperoxides/kg of sample within 3-6 months period. It is concluded that, the presence of GSE and PSE in the frozen storage led to a partial inhibition of hydroperoxide formation.

5.13.2. Changes in thiobarbituric acid-reactive substances (TBARS) during frozen storage

The present study was focused on the effect of GSE and PSE on inhibition of secondary lipid oxidation of whole mackerel and steaks during frozen storage for a period of 180 d. Changes in TBARS value of mackerel treated with BHT, GSE, and PSE during 180 d of frozen storage are shown in Table 25A and Fig.35A. The TBARS value of FWC, BHT and PSE showed an increasing trend upto 150 d of frozen storage and thereafter, TBARS value decreased at the end of storage. The decrease in TBARS values may represent the breakdown of the malonaldehyde because of tertiary degradation of lipid
oxidation products (Pezeshk, et al., 2011). The TBARS value of steaks during frozen storage studies are shown in Table 25B and Fig. 35B. The increase in TBARS value of steaks treated with GSE and PSE showed a similar trend as shown in mackerel during frozen storage period. The lower malonaldehyde formation of mackerel treated with BHT, GSE and PSE with respect to untreated groups on the 120th d of frozen storage was found to be 55.71, 50.47 and 30.95% respectively whereas, steaks treated with BHT, GSE and PSE it was found to be 61.29, 45.16 and 33.33% reduction in malonaldehyde formation respectively. The combination of frozen storage and treatment with polyphenolic compounds from GSE and PSE has positive effect on inhibition of TBARS formation and shows significant difference between control and treated samples (p < 0.05). The result of the present study agreed with that of Aubourg et al. (2002). They found that polyphenolic compounds from natural source like Rosmol lowers the TBARS formation of horse mackerel during frozen storage. The GSE shows positive effect in lowering TBARS formation compared to PSE and shows better result in steaks compared to mackerel. The results of the present investigation can be compared with effects of grape seed and green tea extracts on TBARS formation in bonito (Sarda sarda) fillets during frozen storage as reported by Yerlikaya and Gokoglu, (2010) and result can be summarized as grape seed extract showing promising inhibition of secondary lipid oxidation compared to green tea extract. According to Brannan and Mah (2007) GSE had remarkable antioxidant effects in chicken stored in refrigerated and frozen storage. It can be concluded that GSE and PSE had positive effect on inhibiting the quality loss by secondary lipid oxidation products during frozen storage of whole mackerel and steaks.
5.14. Effect of GSE and PSE on biochemical changes of whole mackerel and steaks during frozen storage

5.14.1. Changes in free fatty acid (FFA) content during frozen storage

Lipid hydrolysis can be assessed according to FFA concentrations in fish muscle. Due to the relatively high autolytic activity associated with fish tissue in combination with its high PUFA content, the lipids are very prone to both lipolysis and oxidation during frozen storage (Aryee et al., 2007). A significant increase in FFA (p < 0.05) indicated that hydrolytic changes take place even at low temperatures. The present study investigated the inhibition of free fatty acid formation with the effect of GSE and PSE during frozen storage, which are represented in Table 26A, Table 26B and Fig. 36A, Fig. 36 B. The free fatty acid formation among the different groups showed significant difference (p < 0.05) which clearly indicated the effect of GSE and PSE during frozen storage. On the day of sensory rejection for untreated samples (FWC), the formation of free fatty acid was less by 53.24, 51.46 and 34.56% for BHT, GSE and PSE treated mackerel respectively. The inhibition of lipid hydrolysis was more effective with the effect of treatment and on the day of sensory rejection for FSC, the formation of free fatty acid was less by 74.48, 69.71 and 61.59% for BHT, GSE and PSE respectively. The present results can be compared with the findings of Ozogul et al. (2011). They found that, the combination of freezing with antioxidants from natural sources lowers the free fatty acid formation in sardine fillets. According to Stodolnik et al. (2005) the antioxidant treatment with flaxseed extract lowered the free fatty acid formation in whole mackerel during frozen storage. Hence it can be concluded that the efficacy of GSE was more to inhibit hydrolytic rancidity than PSE.
5.14.2. Changes in trimethylamine nitrogen (TMA-N) content during frozen storage

The present study investigates effect of GSE and PSE in combination with frozen storage condition to lower TMA-N formation in whole mackerel and steaks and the results are presented in Table 27A, 27B and Fig. 37A, 37B. On the day of sensory rejection for untreated samples (FWC and FSC), the percentage of reduction of TMA-N content of mackerel with the effect of treatment with GSE and PSE it was less by 47.92 and 33.22% respectively whereas, for steaks treated with GSE and PSE was less by 44.18 and 35.36% respectively. The present result indicated that, the effect of GSE and PSE in combination with frozen storage lowers the TMA-N content in mackerel and steaks. Erkan and Bilen, (2010) reported that, the effect of GSE prominently lowered the TMA-N content during frozen storage of chub mackerel fillets. According to Yerlikaya et al. (2010), GSE lowers TMA-N content in shrimp coated with grape seed during frozen storage. Similar findings by Amira et al. (2011) also reported that, frozen storage of Nile tilapia in combination with green tea extract lowers the TMA-N content. The lowering in TMA-N could also be attributed to the rapid reduction of bacterial population or decrease in the capacity of bacteria for oxidative deamination of non protein nitrogen compounds due to the effect of phenolic compounds in GSE and PSE. Other researchers also found significant reduction in TMA-N during frozen storage of mullet fillets with the effect of phenolic compounds from natural source of marjoram and thyme as presented by Yasin and Abou-Taleb, (2007).

5.14.3. Changes in total volatile base- nitrogen (TVB-N) content during frozen storage

Changes in TVB-N content of mackerel with the effect of GSE and PSE in combination with frozen storage are presented in Table 28A, 28B and Fig. 38A, 38B. The results of the present study during frozen storage of mackerel and steaks indicated the
effect of GSE and PSE on lowering the TVB-N content and on the day of sensory rejection for untreated samples (FWC and FSC), the percentage of reduction of TVB-N content of mackerel with the effect of treatment with GSE and PSE was less by 42.10 and 25.00% respectively whereas, for steaks treated with GSE and PSE it was less by 53.71 and 32.63% respectively. As the activity of spoilage bacteria increases after the death of a fish, a subsequent increase in the reduction of TMAO to TMA occurs (Ali et al., 2010). According to Rahman (1999), 10–60% of the viable microbial population dies during freezing yet the remaining population gradually increases during frozen storage. Thus, TVB-N production by bacteria could be expected during frozen storage. The present findings coincide with Erkan and Bilen, (2010), who stated that, the effect of GSE prominently lowered the TVB-N content during frozen storage of chub mackerel fillets. Similar findings of GSE lowering TVB-N content in shrimp coated with grape seed during frozen storage has been documented by Yerlikaya et al. (2010). Other researchers also studied the effect of polyphenols from plant extracts during frozen storage and found that they not only lowers the TVB-N content but also reduces the microbial count in fish and fishery products (Ozogul et al, 2011; Amira et al., 2011).

5.14.4. Changes in pH during frozen storage

The changes in pH of whole mackerel and steaks treated with different phenolic compounds during 180 d of frozen storage are presented in Table 29A, 29B and Fig.39A, 39B. A remarkable increase in pH was observed in all the treated groups for a period of 180 d. The frozen storage of steaks follows similar trend of increase in pH with increase in storage period. Bennour et al. (1991) reported that, the pH of fresh mackerel (Scomber scombrus) was 5.69. It varied from 5.95 to 6.02 at different rejection times. At the end of the storage, the pH values were 6.24, 6.29, and 6.52, for the three different ice:fish ratios
like 1:2, 1:3, and 1:4, respectively. This slow rise in pH was observed by three different ice:fish ratios (like 1:2, 1:3, and 1:4, respectively i.e., 1 kg ice: 2 kg fish) as compared to ice:fish (1 kg ice: 1 kg fish). Similarly, Sathivel et al. (2007) reported higher pH for non-glazed and distilled water glazed salmon fillets as compared to that of lactic acid and chitosan glazed samples during frozen storage studies. The increase in pH is related to the accumulation of alkaline compounds, such as ammonia mainly derived from microbial action during spoilage of fish muscle.

5.15. Effect of GSE and PSE on drip loss (%) of whole mackerel and steaks during frozen storage

Drip loss is the exudate tissue fluid that free from muscle during thawing. Drip may vary from 3 to 5% and even up to 20% of the total fish weight following prolonged storage. The dry texture experienced in some thawed fish can be considered to be due to drip loss. Drip formation takes place as result of cell damage and also due to dehydration of protein micelles, which take place as a result of denaturation of proteins. In the present study, drip loss percentage of mackerel and steaks during frozen storage are presented in Table 30A, 30B and Fig. 40A, 40B. The present results revealed an increase in drip loss (%) in all the samples during frozen storage. The reduction in drip loss of mackerel treated with BHT, GSE and PSE with respect to untreated groups on the 120th d of frozen storage was found to be 47.89, 61.16 and 54.41% respectively whereas, for steaks treated with BHT, GSE and PSE there was 64.83, 55.28 and 41.79% reduction in drip loss respectively. The results are similar to earlier observations (Dyer et al. 1964; Reddy et al. 1990; Vishwasrao 2000; Turan et al. 2003; Sathivel et al. 2007).
5.16. Effect of GSE and PSE on microbiological characteristics of whole mackerel and steaks during frozen storage

Freezing and frozen storage lowers the microorganism count and the microorganisms present on the surface and the tissue of fish are generally inactivated. Thus, during frozen storage, microbial changes in fish are minimal. Although some microorganisms survive at low temperature, their activities are suppressed and bacterial numbers may be reduced when recommended temperature are maintained. Microbial activity is controlled by two conditions present in frozen foods; water activity (aw) is limited and the temperature is usually low to prevent microbial growth. In the present study mackerel and steaks treated with GSE and PSE during frozen storage is depicted in Table 31A, 31B and Fig. 41A, 41B. Total plate count determined in mackerel and steaks were initially 4.5 and 4.0 log cfu/g which was higher than those reported for Indian mackerel (Winarni et al., 2012). Total plate count (TPC) of the present study when compared with proposed limit for fresh fish (7 log cfu/g) by ICMSF, (1986) showed that mackerel and steaks were of good quality. During freezing and frozen storage at -18 °C, the bacterial counts reduced in all the samples and thereafter, total plate count slightly increased with the increase in storage days, but growth of microorganisms did not exceed the limit throughout the storage period for all the groups. The GSE and PSE treated samples showed slightly lower microbial count than the control one. The present results can be compared with the findings of Ozogul et al. (2011), who reported that combination of antioxidant and frozen storage resulted in significant reduction of bacterial growth. The treatment of phenolic compounds to fish and fishery products during frozen storage helps to reduce bacterial count and improves shelf life of the product as documented by Amira et al. (2010). Microbiological characteristics of Nile tilapia treated with green tea extract
during frozen storage reduce bacterial count in treated fish samples as studied by Amira et al. (2010).

5.17. Effect of GSE and PSE on organoleptic characteristics of whole mackerel and steaks during frozen storage

Frozen storage prevents food from undesired sensory and chemical changes caused by microorganisms and spoilage reaction, which however, cannot be totally hindered. Reactions especially occur in proteins and lipids which affect sensory properties and cause unpleasant odour, taste and texture changes. Physical and chemical changes in proteins of fish during frozen storage cause texture deterioration. This problem also affects sensory aspects. Formaldehyde formed makes cross-links with protein, lessen protein solubility and decrease water holding capacity (Steen and Lambelet, 1997). In the present investigation sensory scores of mackerel and steaks samples gradually decreased during the frozen storage as can be seen on Table 32A, 32B and Fig. 42A, 42B. The samples treated with BHT were the most preferred samples followed by the GSE and PSE treated mackerel and steaks. According to sensory scores preference of panelists gradually decreased especially in FWC and FSC. Control samples had the equivalent (p < 0.05) sensory scores on the first month, however it did not continue when compared to treatments of GSE and PSE at the end of the storage. Decreasing organoleptic scores during storage were thought to be related to penetration of seed extracts into whole mackerel and steaks. The difference in treatment groups was statistically significant (p < 0.05). These findings were also supported by Yerlikaya and Gokoglu, (2010) and showed that bonito fillets treated with grape seed and green tea extracts during frozen storage were more preferred compared to control fillets. The present findings were in agreement with the reports of Seleni et al. (2011), that there is no significant difference between sensory properties of grape seed
extract and BHT treated chicken meat stored at −18 °C for nine months. The results of the sensory attributes indicated that storage life of mackerel and steaks is affected by treatment with GSE and PSE during ice storage and frozen storage.