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A STUDY ON ANTIOXIDANT POTENTIAL OF A TRADITIONAL INDIAN SPICE MIX

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ABSTRACT

A popular Indian condiment or spice mix, namely *panchforon* is an equiproportional mixture of fennel, celery, cumin, black-cumin and fenugreek. Antioxidant activity of this condiment extracted in chloroform was evaluated in terms of total phenol content, inhibition to peroxidation in linoleic acid system and free fatty acid content over a span of five weeks. The antioxidation potential of the condiment was further determined and compared with synthetic antioxidant-BHT, post application on homogenized tilapia fish muscle followed by refrigerated preservation (0-4 ^{0}C) for five weeks. Oxidative stability of fish oil, extracted at interval of one week on day 0, 7, 14, 21, 28 and 35 was estimated from peroxide, thiobarbituric acid (TBA) and acid values. Antioxidant activity of the extracted fish oil was also estimated using the mentioned tests on the stated days. It was found that *panchforon* could successfully reduce the amount of malonaldehyde accumulation and free fatty acid formation in fish. Peroxidation was also effectively controlled. Thus *panchforon* showed a comparable efficiency in controlling oxidation whereas *panchforon* enriched fish oil exhibited higher antioxidant activity than the non-spiced control. Increase in antioxidant activity of fish preserved with this spice with time suggests potential health benefits as well.

Keywords: antioxidant, condiment, antioxidant activity, oxidative stability, fish lipid.

INTRODUCTION

Spices, an integral part of cuisine in various parts of the world is considered to be a popular source of natural antioxidants (Rice-Evans, Miller, & Paganga, 1996; Zheng & Wang, 2001). Presently much importance is attached to use of natural antioxidants as substitute of synthetic antioxidants to avoid toxicity **(**Martinez-Tome, et al., 2001**)**. Singular spices and their mixtures in different proportions viz. condiments are used as food seasonings to impart flavour, aroma and taste to food. These spices are potential sources of phenolic compounds, flavones, ascorbic acid, carotenes which exhibits immense biological importance and antimicrobial activity promoting health and even lowering the risk of various diseases (Calucci et al., 2003). Besides all these properties, antioxidant activity of spices has popularized its usage as a food preservative and preparing functional food (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Alvarez JA. 2010).

Panchforon, an equiproportional mixture of five spices (panch = five, foron = spices) is a traditional condiment of Indian origin. The five spices are cumin seed (*Cuminum cyminum L*), fenugreek seeds (*Trigonella foenum-graecum*), celery seed (*Apium graveolens)*, fennel seeds (*Apium graveolens*) and black cumin (*Nigella sativa*) seeds.

The main chemical components of cumin oil are cuminic, cymene, dipentene, limonene, phellandrene and pinene. The major constituent available in ground cumin is cuminaldehedye (Bettaieb 2010). The petroleum ether-soluble fraction of cumin seeds reportedly has antioxidative activity when mixed in lard (Saito & Sakamoto 1976). It has digestive bacteriocidal effect and is used as stimulant and antiseptic (Shetty, Singhal and Kulkarni, 1994).

Seeds of fenugreek contain lysine and L-tryptophan rich proteins, mucilaginous fiber and other rare chemical constituents such as saponins, flavonoids, (including vitexin, vitexin-7-glucoside, orientin arabinoside, homoorientin, saponaretin (isovitexin), vicenin-1, vicenin-2, quercetin, luteolin, and vitexin cinnamate), coumarin, fenugreekine, nicotinic acid, sapogenins (consisting mainly of diosgenin and its isomer yamogenin usually in a 3:2 ratio), phytic acid, scopoletin and trigonelline (Murakami, Kishi, Matsuda &Yoshikawa,2000; Yoshikawa, Murakami, Komatsu, Murakami, Yamahara, & Matsuda. 1997) which are thought to account for many of its presumed therapeutic effects. Saponins and flavonoids has high antioxidant potential. Fenugreek has also been reported to exhibit

pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive activity (Akbari, Rasouli ,& Bahdor 2012). Celery belonging to a part of the Umbelliferae plant family is rich in compounds like coumarins flavonoids, isoimperatorin, isoquercitrin, limonene, linoleic acid and umbelliferone (Zhan Guo Lu, Wei Li & Peng Jun Wang, 2011). The plant accounts for multifold medicinal uses and since its constituents are rich in antioxidants like isoquercetin, flavonoids it has considerable antioxidant potential (Cheng, Lin, Yu, & Peng, 2008). Fennel is a highly aromatic and flavorful herb containing anethole along with α-pinene, myrcene, fenchone, methyl chavicol, limonene, 1,8-cineole and anisic aldehyde (Parejo, Jauregui, Sánchez-Rabaneda, Viladomat, & Bastida, 2004). These have high therapeutic applications and anethole records a high antimicrobial and insecticidal property (Muckenstrum, Foechterlen, Reduron, Danton, & Hildenbrand 1997). Moreover raw fennel bulb contains 14% vitamin C which acts as an effective antioxidant to reduce stress.

Nigella sativa oil from black cumin seeds contains an abundance of conjugated linoleic (18:2) acid, thymoquinone, nigellone, melanthin, nigilline and, tannins. The major fatty acids are linoleic acid (50.2%), oleic acid (19.9%), while palmitic acid (9.4%) is the main saturated one. Antioxidants lignans such as saponin, melanthin (Tulukcu, 2011) are present in considerable proportion which exhibits high antioxidant activity of black cumin. Its hydrating effects, protection of cell tissue property, curative properties and anti-inflammatory action make it an excellent remedy for a large number of ailments. Besides various biological importance and antimicrobial activity presence of thymoquinone, in black cumin seed oil, has actually been shown to help control or modulate the production and release of the immune proteins (Ashraf M. Ahmed, Ebtesam M. Al-Olayan, Mourad A. M. Aboul-Soud and Abdulaziz A. Al-Khedhairy, 2010).

Though much research has been done on these individual spices and their constituents, enough documentation is not available on combination spices. The objective of this study is to determine the total antioxidant activity of *panchforon* since there is large chemical diversity in the constituents and various synergistic interactions occurs among them. The actual potential of delaying oxidation is studied on tilapia fish lipid since the antioxidant activity depends on the constituent of food and medium to which it is added. Variation of this activity has been studied in oil extracted from spiced tilapia fish muscle over a period of five weeks. These values are compared with a synthetic antioxidant BHT.

MATERIAL AND METHODOLOGY

CHEMICALS

2-Thiobarbituric acid was purchased from Loba chemie (India). Ammonium thiocyanate and ferric chloride, choloroform, methanol, isooctane all other solvents and reagents were procured from Merck (India). All chemicals used were of analytical grade.

SPICES

All the spices were bought from local market in south Kolkata and grinded and sieved at125 micron.

PREPARATION OF FISH SAMPLE

Total amount of 2.1 Kg of Tilapia fish (each having an average weight of 300 gram, length 6.5 inches and age of 3 months) was procured from Chowbaga bheri located in east Kolkata wetland. Fish muscles were minced and homogenized in a blender. The mass was further divided in 16 (3x5 + 1) equal portions each weighing 100 gms for mixing *Panchforon* (a mixture of cumin seeds, fenugreek seeds, celery seeds, fennel seeds and black cumin seeds in equal proportion), a synthetic antioxidant BHT, and for using as control. All the spices of *panchforon* were grinded and sieved at 125 micron and then mixed in equal proportion to make the condiment. Both the synthetic and natural antioxidants used were 10% by weight of the sample (ie. 10 gm spice powder was used for each of 100 gm of fish sample) and were put in the press-and-lock polythene freezer bag and stored in the freezer chamber of a refrigerator at 0-4^{o}C. Samples were kept in five batches and taken out for analyses at day 0, 7, 14, 21, 28 and 35. The experiment was repeated thrice.

ESTIMATION OF ANTIOXIDANT ACTIVITY IN SPICES AND FISH LIPID

SAMPLE PREPARATION FOR SPICES

An amount of 5 gm of spice sample was extracted with 50ml chloroform. It was further treated with activated charcoal to decolourize, then centrifuged and filtered with Whatman 1 filter paper. This solution was used for execution of all the antioxidant tests mentioned.

SAMPLE PREPARATION OF FISH LIPID

The fish oil extracted following Bligh Dyer method from various spiced samples and non-spiced control on day 0, 7, 14, 21, 28 and 35 was taken in chloroform and the antioxidant tests were performed following the stated standard protocol.

EXTRACTION OF LIPID

It was done by Bligh and Dyer method (1959). Homogenization of 100gm of blended fishes was done with 1:1 methanolic chloroform and the filtered through Whatman no. 1 filter paper. The organic layer were separated through separatory funnel and dried with anhydrous sodium sulphate. The solvent was removed at low temperature (40^{o}C)

Weight of lipid = (weight of container + extracted lipid) - (weight of container)

Lipid content (%) = amount of lipid extracted (g)/weight of original sample (g) X 100

DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

Total phenolic compound was determined using Folin-Ciocalteu reagent using the modified method of Wolfe et al. (2003). A sample solution of 0.2 ml was pipetted in glass tube and 1 ml of Folin-Ciocalteu reagent, 0.8 ml of sodium carbonate (7.5%) was added to it. The mixture was stored at room temperature for 30 min and absorbance was recorded at 765 nm. Total phenolic compounds were calculated using a standard curve prepared with dilutions of tannic acid. Tannic acid (0 - 800 mg/L) was used to produce standard calibration curve. The total phenolic content was expressed in mg of Tannic acid equivalents (TAE) / g of extract.

ANTIOXIDANT ACTIVITY IN A LINOLEIC ACID SYSTEM

Antioxidant activity was evaluated by the thiocyanate method (Mitsuda, Yasumoto,& Iwami, 1966). Sample was added to 0.5 ml of chloroform, linoleic acid emulsion (2.5 ml 0.02 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0) in a test tube and stored in darkness, at 37^{o}C, to accelerate oxidation. The linoleic acid emulsion was prepared by mixing an equivalent weight of linoleic acid and Tween 20 in phosphate buffer (0.2 M, pH 7.0). The peroxide value was determined by reading the absorption at 500 nm with a spectrophotometer, after colour development with $FeCl_2$ and thiocyanate at various intervals during incubation. The peroxidation of linoleic acid was calculated as peroxidation (%) = (A^1/ A^0) X 100,
Where A^0 = the absorption of the control reaction and
A^1 = the absorption in the presence of sample.

DETERMINATION OF FREE FATTY ACID IN SPICES

Acid value of spices was calculated using IUPAC method 2.201. In a conical flask, 25 ml of diethyl ether was mixed with 25 ml alcohol and 1 ml of phenolphthalein solution. The mixture was carefully neutralized with 0.1N sodium hydroxide. This is called Neutral Solvent. Spice extract of 0.1grams were taken and the sample was dissolved in the neutral solvent and titrated with aqueous 0.1 N sodium hydroxide, shaking vigorously until a pink colour was formed that persisted for 15seconds. Acid Value was calculated as:
Acid value = Volume of sodium hydroxide solution (ml.) X 5.61/ weight of the sample

ESTIMATION OF OXIDATION IN FISH LIPID

ACID VALUE

Acid value was calculated using IUPAC method 2.201. In a conical flask, 25 ml of diethyl ether was mixed with 25 ml alcohol and 1 ml of phenolphthalein solution. The mixture was carefully neutralized with 0.1N sodium hydroxide. This is called Neutral Solvent. 0.1 grams of oil were taken and the sample was dissolved in the neutral solvent and was titrated with aqueous 0.1 N sodium hydroxide, shaking vigorously until a pink colour was formed that persisted for 15 seconds. Acid Value was calculated as:
Acid Value = Volume of sodium hydroxide solution (ml.) X 5.61/ weight of the oil sample

PEROXIDE VALUE

Peroxide value was calculated using IUPAC method 2.501. In a test tube, 0.2 grams of oil sample were taken and saturated solution of potassium iodide was added. A mixture of glacial acetic acid and chloroform (20 ml) was added to the mixture. This was then warmed in water bath for 30 seconds. The content was then poured into a flask containing 20 ml of 5% potassium iodide solution. To it was added 25 ml of distilled water and 1 ml of starch solution. This was then titrated against 0.01N sodium thiosulphate solution. Peroxide Value was calculated as:

$$\text{Peroxide Value} = \frac{\text{Volume (ml) X Strength of sodium thiosulphate}}{\text{Weight of the oil sample}} \times 1000$$

THIOBARBITURIC ACID VALUE

Thiobarbituric acid value was calculated using IUPAC method 2.531. In a test tube, 200 mg of oil sample was taken and 5ml of thiobarbituric acid reagent was added. The mixture was stoppered and warmed in water bath at 95°C for 120minutes. After that the mixture was cooled and the absorbance was measured (A_s) at 530nm in a 10mm cell against water. A reagent blank absorbance (A_b) was also carried out.
Thiobarbituric acid number = 50 X (A_s – A_b)/ weight of the sample

STATISTICAL ANALYSIS

The experiment was performed in triplicate and data from three different experiments were subjected to analysis of variance (ANOVA) ($P<0.05$). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 16.0 for windows, SPSS Inc.).

RESULTS AND DISCUSSION

ESTIMATION OF FATTY ACID AND ANTIOXIDANT ACTIVITY IN SPICES

TOTAL PHENOL CONTENT

The total phenolic content in *panchforon* was determined spectrophotometrically with Folin-Ciocalteu reagent using the modified method of Wolfe et al. (2003). Phenolic compounds are considered to be important plant materials because of their inhibitory effect on autoxidation of oils (Ramarathnam, Osawa, Namiki & Tashiro, 1986) and their radical scavenging ability (Hatano, Edamatsu, Hiramatsu & Mori, 1989). Therefore, it is important to determine the total phenolic compound in the condiment. A gradual and significant ($P< 0.05$) drop in the phenolic content with time was observed in both the additives (Figure -1). The phenolic content of *panchforon* is found to be comparable with the synthetic antioxidant BHT.

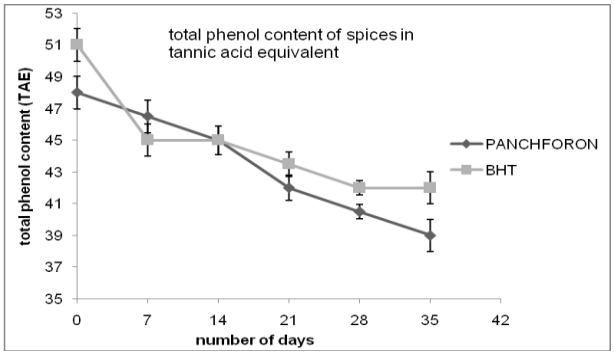

Figure-1: Total phenolic content of spice (additives) is expressed as tannic acid equivalents (TAE; mg tannic acid /g of extract).

ANTIOXIDANT ACTIVITY IN LINOLEIC ACID EMULSION

The values shown in Figure- 2 express the extent of peroxidation in linoleic acid system with addition of spice additives. Comparatively higher value in case of BHT than *panchforon* indicates that higher oxidation is occurring in linoleic acid system when BHT is used. This implies that *panchforon* could control the percentage of peroxidation to a greater extent than BHT. Hence antioxidant activity is higher in *panchforon*. With time the capability of spices to control oxidation increases which is obvious from the significantly ($P<0.05$) decreasing values for both *panchforon* and BHT. The extent of increase in antioxidant potential from initial to final is higher in BHT while *panchforon* comparatively recorded a gradual increase after day 21.

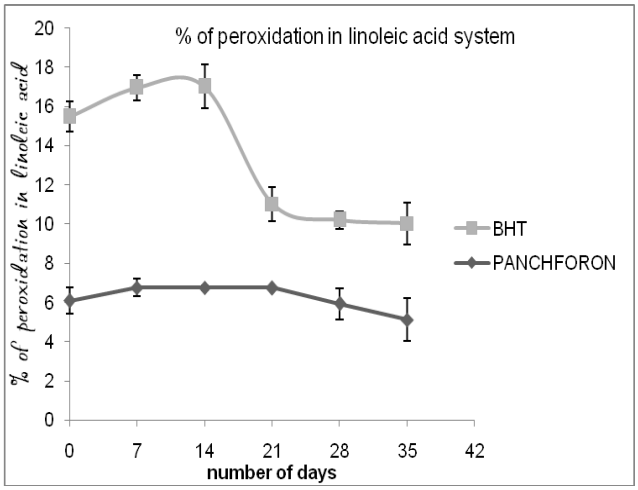

Figure-2: Percentage of peroxidation of spices (additives) in linoleic acid system

ACID VALUE

Spices have appreciable amounts of different fatty acids. The acid value measures the total amount of carboxylic acid groups present in spices. The values recorded for spices (Figure- 3) reveals that the acid content decreased gradually and significantly ($P<0.05$) in *panchforon* throughout the period whereas the acid content steadily ($P<0.05$) increased in BHT.

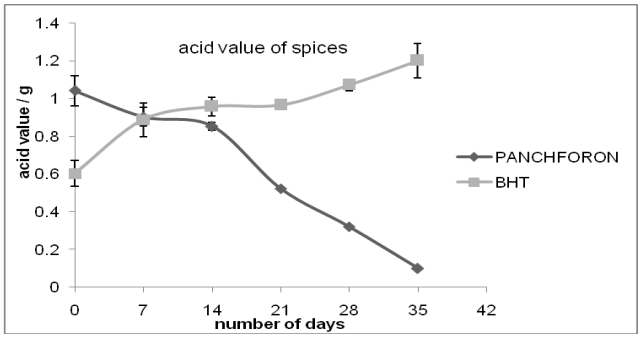

Figure-3: Acid value of spices (additives) per g of sample

TOTAL PHENOL CONTENT

There are several natural compounds that participate in the antioxidative defense mechanism of fish (Hultin 1992). The major phenolic compounds are tocopherol and ubiquinone. The total phenol content in the fish sample was found to increase in all cases with time thereby indicating accumulation of phenolic compounds in the fish oil (Figure-4).Under acidic aqueous conditions a slow hydrolysis of the tocopheryl acetate can be observed which then acts as an antioxidant (Schuler 1990). It is interesting to note that the phenolic concentration in fish oil is higher in *panchforon* and BHT than control. The ability of the spices to increase the phenolic content of fish oil significantly ($P<0.05$) justifies their antioxidant activity. The maximum phenolic content of the *panchforon* spiced fish was noted on the last day which recorded a value even higher than BHT. Total phenol of the fish oil increased significantly with time for each variety.

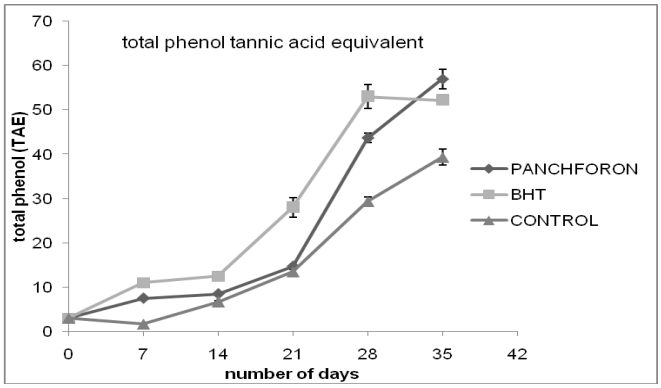

Figure-4: Total phenolic content of extracted spiced and non-spiced fish lipid is expressed as tannic acid equivalents (TAE; mg tannic acid /g of extract)

ANTIOXIDANT ACTIVITY IN LINOLEIC ACID EMULSION

Antioxidant activities of fish lipid extracted from samples mixed with *panchforon* and BHT and non-spiced control were determined according to the thiocyanate method (Mitsuda, Yasumoto,& Iwami, 1966). Figure-5 depicts the percentage of peroxidation with time. The lower peroxidation values in linoleic acid indicate higher inhibition to peroxidation because of these spices. Higher values in control specify lower antioxidant activity

whereas lower values of *panchforon* denote the efficiency of the condiment as antioxidant which has successfully and significantly (P<0.05) arrested the oxidation of the fish lipid in linoleic acid system. Though the lowest peroxidation is observed in case of BHT, *panchforon* values are very much comparable with this synthetic antioxidant. Both BHT and *panchforon* mixed samples have recorded their highest antioxidant activity (lowest peroxidation value) on the 7th day which has decreased the peroxidation remarkably (P<0.05) from day zero. With time the extent of peroxidation in spiced samples has also increased gradually and significantly (P<0.05) which signifies reduction in antioxidant activity with time. In case of control maximum peroxidation is recorded on day 21st which may be due to cumulative effect of peroxidation in fish oil as well as linoleic acid. Extent of peroxidation of spices in linoleic acid system (Figure-2) recorded higher antioxidant potential for *panchforon* whereas the results shown in Figure-5 might be due to more efficient solubilisation of BHT in fish muscle.

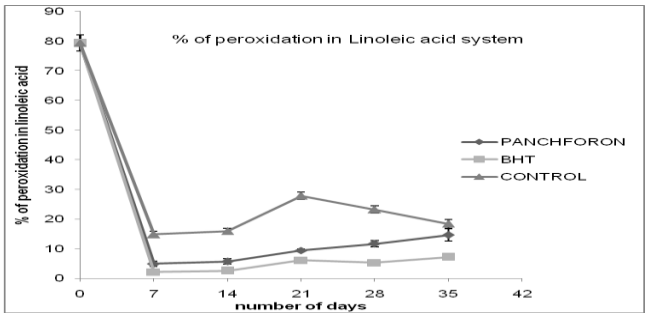

Figure-5: Percentage of peroxidation of extracted spiced fish lipid in linoleic acid system

ACID VALUE

The acid value measures the amount of carboxylic acid groups in free fatty acids generated in the fat due to storage. Increase in this value leads to formation of off-flavour as a result of degradation of fat (Choe &Min 1997). A steady increase in values was observed in case of control as well as BHT, whereas in *panchforon* there was a gradual decrease in the free acid content after day 14th. This decrease in the fatty acid value for *panchforon* in Figure-6 is similar to the trend exhibited in Figure-3.

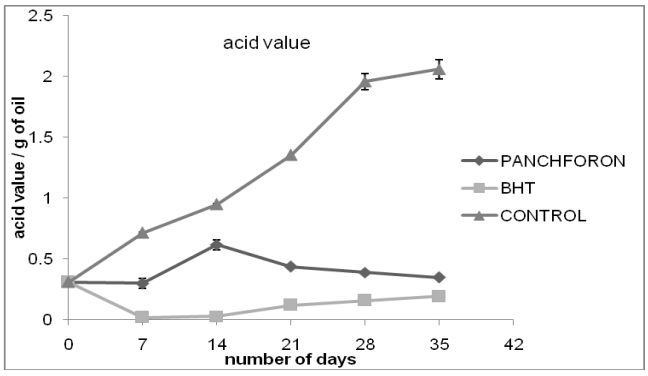

Figure-6: Acid value of extracted spiced fish lipid per g of oil

PEROXIDE VALUE

The significantly increasing peroxide value (P>0.05) in non-spiced control (Figure-7) indicates higher rate of oxidation which leads to greater accumulation of peroxide. In case of *panchforon* added sample the peroxide values although initially increased slightly, underwent a reduction in the subsequent week and then after maintained gradual increase (P<0.05). In BHT mixed fish lipid the peroxide value showed a steady initial increase followed by a gradual decrease. The peroxide values of *panchforon* mixed fish sample though recorded slightly higher than BHT, are of comparable magnitude. The final week recorded no peroxide value. This might be due to decomposition of peroxides to secondary oxidation products on long storage.

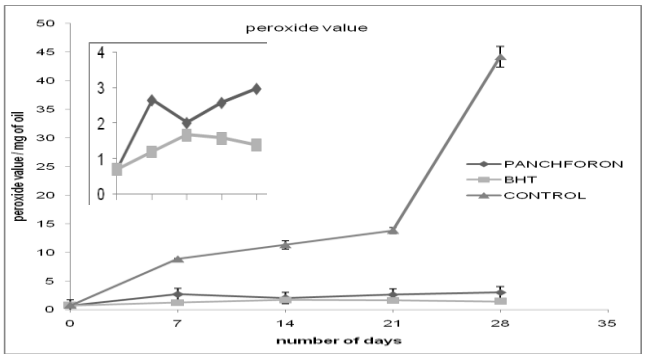

Figure-7: Peroxide value of extracted spiced fish lipid per mg of oil

THIOBARBITURIC ACID VALUE

In Figure-8 the TBA values, an indicator of oxidative rancidity, delineate a gradual increase in malonaldehyde concentration both in case of BHT and control (P>0.05). Malonaldehyde accumulation is found to be much higher in control than BHT and *panchforon*. The most interesting result was observed in *panchforon* where the TBA value gradually decreased thereby arresting the formation of malonaldehyde. Moreover it was noted that much variation in the antioxidant activity of *panchforon* was not found and hence can be concluded to be effective to same extent throughout the storage.

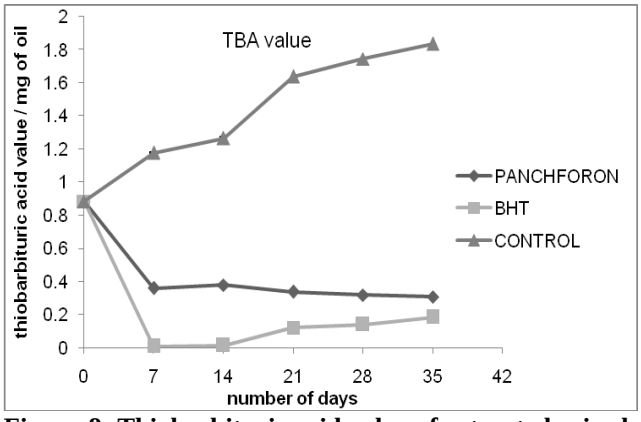

Figure-8: Thiobarbituric acid value of extracted spiced fish lipid measured per mg of oil

CORRELATION STUDY

Correlation between various parameters of lipid oxidation like peroxide value and TBA value were determined. *Panchforon* and BHT added samples were found to record high negative correlation value (-0.9513 and -0.86151 respectively) whereas control showed a high positive correlation of 0.823967. The negative correlation indicates effective control of formation of malonaldehyde. Similarly correlation was found to be over all negative between peroxide value and percentage of peroxidation in linoleic acid for *panchforon*, BHT and control. The correlation for *panchforon* and BHT mixed sample was considerably higher (-0.89722 and -0.8752 respectively) than control (-0.42704). These two correlation values of spices corroborate with each other emphasizing the efficiency of the condiment as an effective antioxidant.

CONCLUSION

This study has provided us with some interesting and important findings. It clearly indicates the high antioxidant activity of the condiment *panchforon* which is comparable with commonly used synthetic antioxidant BHT. Moreover it behaves as an effective antioxidant in storage of fish under domestic refrigerating condition. The interesting finding was that *panchforon* could reduce the malonaldehyde accumulation in fish thereby reducing the potential health risk of this aldehyde. Antioxidant activity of the spices in the fish oil revealed that the fish on long storage with spice becomes enriched with antioxidants which are beneficial to human body.

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