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INCREASE OF OXIDATIVE STABILITY IN FISH OIL USING POTENT NATURAL ANTIOXIDANTS PRESENT IN THREE INDIAN MEDICINAL PLANTS (IMP)

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ABSTRACT

Antioxidants present in Indian Medicinal herbs valued for storing fish oil. The work provides in addition to prevent lipid peroxidation a dosage compensation for illness achieved while using IMP incorporated fish oil. Total phenolic content, Reducing property, β carotene linoleic acid and DPPH assay were performed in extracts of *Aloe barbadensis*, *Oscimum sanctum* and *Boerhavia diffusa.* Storage index analyzed within 10 day intervals for oils added in three increasing concentration percentage of IMP extract. PV and TBA assay compared with a negative control shows a slow degradation in fish oil sample containing IMP extracts. Industries producing fish oil can be seriously pointed the issues concerned with healthy effects of this ingredient.

KEY WORDS

Antioxidant, Fish oil, Auto oxidation and Indian Medicinal Plants.

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INTRODUCTION

Indian Medicinal Plants had a major role in supplying natural antioxidants, which was practiced as Ayurveda medicine since the Vedic period. In Ayurvedic medicines, the rasayanas are prepared from several plant extract which contain strong antioxidants and are used as rejuvenators or nutritional supplements. Among many of the currently available drugs, synthetic drugs do have potential adverse reactions which can be minimized to greater extent through natural compounds Govindarajan *et al* 2005. The selected medicinal plants for the study include *Boerhavia diffusa, Aloe barbadensis* and *Ocimum sanctum* due to its

availability and medicinal importance. *Boerhaviadiffusa* Linn (Family: Nyctaginaceae) is a herbaceous plant, cultivated in fields, widely distributed in the tropical and sub-tropical regions in the world. It contains a number of constituents mainly as alkaloids, others are flavonoids, saponins and steroids HariPratap *et al.* 2012 helping it to be potent natural antioxidant. The juice of this plant's leaves are used against jaundice and the roots are used as a curative for internal inflammation, laxative and urinary diseases Mudgal 1975. *Aloe barbadensis* Mill, (Family: Liliaceae), is a short-stemmed succulent herb widely distributed in Europe, Asia and southern parts of North America. There are more than 300 Aloe species, and among them *A. barbadensis* is officially listed in the Chinese Pharmacopoeia and it has been widely used as ingredients of food products, beverages, cosmetics and pharmaceuticals Chinese Pharmacopoeia 2010; Basmatker *et al.* 2011. The leaves of the plant are formed by a thick epidermis covered with cuticles surrounding the mesophyll, which can be differentiated into chlorenchyma cells and thinner walled cells. A yellow exudate (commonly called aloe) secreted by the inner epidermal cell layers, which has long been recognized as a purgative drug by pharmacopoeias over the world, and also the phenolic compounds abundantly exist in this layer Park and Kwon 2006. Antioxidant activity of *Aloe vera* was studied by Reynolds and Dweck1999 and it showed that three-year-old *Aloe vera* provided a higher antioxidant effect than that two-year-old and four-year-old. The third species *Ocimum sanctum* (Family: Lamiaceae), is an erect hairy annual herb and has been claimed to possess numerous properties. Earlier studies revealed that *O. sanctum* decreased lipid peroxidation and increased the activity of superoxide dismutase Panda and Kar 1998. Abide with the antioxidant property Maulik *et al.* 1997, *O. sanctum* leaves hydroalcoholic and methanolic extracts used in a variety of disorders like bronchitis, rheumatism and pyrexia, anti-inflammatory. Singh *et al.* 1996, immunomodulatory Mediratta *et al.* 2002 andantistress Sen *et al.* 1992, radioprotective and anticarcinogenic Devi 2000.

The use of antioxidants in preventing oxidative deterioration of fish oil commences importance because of the wide area of its application. It has been used in food, pharmacy, cosmetic and paint industries for which the stability and quality have to be maintained. PUFAs in fish oil, however, are vulnerable to oxidation. Fish oil spoilage occurs mainly in two major ways, like oils from animal and vegetable sources: oxidative spoilage and hydrolytic spoilage. It is because of its high content of polyunsaturated fatty acids, including EPA and DHA, fish oil is highly susceptible to oxidative spoilage and the rate of fish oil oxidation is significantly different from that of other oils. The more sensitivity of fish oil to spoil than other oils is due to its high concentration of phospholipids and unsaturated fatty acids. The break in the induction curve is less sharp, and the beginning of the increase in the peroxide value occurs sooner. The course of oxidation is often quite different between extracted fish oils and lipids in fish tissues. Deterioration in quality of fish oil is mainly caused by autoxidation. Non-acceptable flavours and odours develop at very low peroxide values at an early stage of oxidation, even during the induction period. From literatures it was clear that, PUFA auto-oxidation involves free radical generation through a reaction catalyzed by heat, light, trace metals, or enzymes Leclerc *et al.* 2007. This produces various aldehydes and ketones that render unacceptable colours, odours, and flavours to PUFA-containing foods and nutraceutical products Salariya and Rehman 2006. Both synthetic and natural antioxidants are widely used in preventing oxidative deterioration. Antioxidants are added to food to maintain quality and extend shelf life. The use of synthetic antioxidants is restricted in several countries because of possible undesirable effects on human health Hrasia *et al.* 2000. Keen attention has been paid to natural non-toxic antioxidants because lots of synthetic antioxidants such as butylated hydroxyl anisole and butylated hydroxyl toluene that are suspected to have cytotoxicity are used in food.

A comparative, multi-method screening of antioxidant activity for a large number of Indian medicinal plants in relation to their phenolic

compounds is needed to provide a better understanding of their relative importance as natural antioxidants. The natural antioxidants compared to synthetic ones are generally preferred because they are considered safer for human consumption. The antioxidant property of the above medicinal plants can be utilized as fish oil preservative which will supply the possibility of enhanced medicinal effect. Natural antioxidants have recently been extensively used in the food industry. In the present study, the antioxidant properties of three medicinal plants namely *B. diffusa, A. barbadensis* and *O. sanctum* have been determinedby estimating the total phenolic content, DPPH, β carotene and reducing properties, so also to determine the oxidative stability of the fish oil persevered in these medicinal plants extract.

MATERIALS AND METHODS

Herbal Extracts

Leaves of plants for respective antioxidants (*Oscimum sanctum, Boerhaviadiffusa* and *Aloe barbadensis)* were ground to get crude powder. 200 gms of crude powder was shaken with hydro alcoholic for 24 hrs in an orbital shaker at room temperature. Extracts were filtered using Whatman No1 filter paper using vaccum filterate. Filterate was concentrated to dryness under reduced pressure at 40^0C through evaporator. Extract were resuspended in the respective solvent (hexane, ethanol or water).

DPPH radical scavenging activity

The effects of extracts on DPPH radical were estimated according to the procedure described by Moure *et al* 2000. Two millilitres of a 3.6X 10^{-5} M methanolic solution of DPPH (Sigma) were added to 50µl of a methanolic solution of (1mg $ml^{-1)}$ of the antioxidant. The decrease in the absorbance at 515 nm was continuously recorded in a Hitachi U-2000 spectrophotometer for 16 min at room temperature. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH. Radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 min duration as follows:

$IP= (ABS_{t(0\ min)}-ABS_{(t(16min)})/ABS_{t(0\ min)})X100$

β Carotene linoleic acid assay Mokbel and Hashinaga 2006

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated dienehydroperoxide arising from linoleic acid oxidation. A stock solution of β carotene linoleic acid was prepared as follows, first 0.5 mg of β carotene was dissolved in 1ml choloro form, then 25µl of linoleic acid and 200mg of tween40 were added. The chloroform was subsequently evaporated using a vaccum evaporator. Then 100ml of distilled water saturated with oxygen/30 min at 100ml/min was added with vigorous shaking. Aliquote (25µl) of this reaction mixture were transferred to test tubes and 300µl portion of the extracts ($2mgmL^{-1}$ in ethanol) was added before incubating for 48hr at room temperature. The same procedure was separated with BHT at the same concentration and a blank containing only 350µl of ethanol. After the incubation period the absorbance of the mixture was measured at 490nm. Antioxidant capacities of the sample were compared with above BHT and the blank.

Determination of reducing power

The reducing power of extract was determined according to the method of Oyaizu 1986 as described by Yen *et al*. 2000. Lyophilized extract (2.5-15.0mg)in 1ml methanol was mixed with phosphate buffer (5ml, 0-2µ, pH 6-8) and potassium ferricyanide (5 ml, 1%) and the mixture was incubated at 50^0C for 20 min.5 ml of TCA (10%) were added to reaction mixture which was then centrifuged at 3000 * g for 10 min. The upper layer of solution (5ml) was mixed with distilled water (5ml) and ferric chloride (1%) and the absorbance was measured at 700 nm.

Determination of Total Phenolic Content

Total Phenolic content (TPC) of each sample was estimated using the FolinCiocalteu colorimetric method according to Liu et al. 2002 and Cai *et al.* 2004 with minor modification. From the sample of total extract 10, 20, 30, 40, 50µl was taken up to 1 ml using ethanol. 0.1ml of that extract had been added to 5ml distilled water and 0.5 ml of Folin Ciocalteau reagent. After 3 minutes of reaction, 1.5ml of

Na_2CO_3 (20%) and 2.9ml of distilled water was added. After 2 hrs of incubation in dark at room temperature the solution in the test tube were measured in spectrophotometer (765nm).

Peroxide value Lea 1938

3-5 gm of fish oil was taken in an Iodine flask with stopper. 3:2 ratio of glacial acetic acid and chloroform was added to dissolve the fish oil along with 1 ml KI solution. It was kept under dark for 30 minutes. Titrate against .01N sodium thiosulphate using starch as indicator. The test was repeated to obtain a concordant value.

TBA reactivity Assay

The TBA reactivity in fish oils was determined by following the method of Ramachandra Prabhu 2000 with slight modifications. To 0.01 ml of fish oil was added 2 ml of 2-thiobarbituric acid (0.67% W/V) followed by 2 ml of trichloroacetic acid (10% W/V). After mixing, the tubes were covered with marble stones, kept in boiling water for 15 minutes, cooled to room temperature, and centrifuged (2000 g for 10 minutes). The pink TBA chromogen in the aqueous layer was measured at 532 nm against appropriate blanks.

Statitics

SPSS 13.0 for windows was used for statistical analysis. Mean of three determinants were analysed with Pearsons bi variant two tailed correlation coefficient at a level of significance (0.01)

RESULTS AND DISCUSSION

Antioxidant activity of Indian Medicinal Plants (IMP)

Total Phenolic content

Total Phenolic content was expressed in mg/100 gm of Gallic acid. Presence of phenolic contents in the extract determines its antioxidant activity and there is variation for phenolic compounds for each extract. The results of the *invitro* examination of IMP for total phenolic content are expressed as 94, 238, and 256 mg/100 gmgallic acid for *Aloe barbedensis, Boerhavia diffusa* and *Oscimum sanctum* respectively. The presence of phenolic compounds impulse antioxidant activity and the TPC index can be varied according to genetic variation and environment Bravo1998. Phenolic compounds such as flavonoids, phenolic acids and tannins contribute the antioxidant activity of plants.In *O. sanctum* phytochemical investigation on leaf extracts it was found that many of the principle compounds like phenols (eugenol, cirisilineol, isothymonium, apigenin and vosamarinic acid) and flavonoids (orientin and vicennin) and these pharmacophores possess potent antioxidant and anti-inflammatory (cyclooxygenase inhibitory) activity Devi 2000; Kelm 2000.

The leaves of *B.diffussa* are also rich in phenolics than its roots Pratt and Hudson 1990. The leaves of the extract contain camphor, safranal, eugenol, vanillin and flavonoids more than root nodules Pereira 2009. There may be variation in the concentration and principal components of phenolics which changes the total phenolic content of *A. barbadensis, B. diffusa* and *O. sanctum* in experimental trials. In particular flavonoids and catechins are important antioxidants and super oxide scavengers reported in recent studies and their scavenging efficiency depends on the concentration of phenol and the numbers and locations of the hydroxyl groups Benavente-Garia *et al.*1997. Shahidi *et al*. 1992 reported that the phenolic antioxidants function as free radical terminators or metal chelators.

DPPH assay

DPPH is a method based on the scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical, it determines the decrease in sample absorbance at 515 nm. The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple colored methanol solution of DPPH. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. This spectrophotometric assay (Pharmacia, Uppsala, Sweden –LKB-Novaspec) uses stable radical diphenylpicrylhydrazyl (DPPH) as a reagent (Sigma-Aldrich). Experimental results showed *O. sanctum* having more antioxidant potential compared with *B. diffusa* and *A. barbadensis* in DPPH assay. The result percentages of *O. sanctum, B. diffusa* and *A.*

barbadensis were determined as 34.25%±.020, 16.38%±.03 and 7.35%±.055 respectively. The more rapidly the absorbance decreases the more potent the antioxidant activity of the compounds in terms of hydrogen donating ability.

β Carotene assay

The inhibition ratio of the linoleic acid oxidation by methanolic extracts of leaves was determined by β Carotene assay. Plants are the major source of natural antioxidants such as β carotene. β Carotene is a strongly-colored red-orange substance that can be converted into vitamin A in human body. It also acts as an antioxidant, immune system booster and natural pigment. It is a preventive agent for specially skin, liver, and other cancer types. Lack of this vitamin can lead to serious diseases such as night blindness, etc.β Carotene value compared with BHT was 64, 72 and 78 ± 1 for *A. barbadensis, B. diffusa* and *O. sanctum* respectively. The extracts ability to reduce the formation of peroxides in the linoleic acid emulsion where estimated and found not much different in each extracts. But comparing with results *O. sanctum* greatly different from *A. barbadensis* can be due to growing variation of this species. It suggests that *A. barbadensis* of various development stages contains different active components and possesses antioxidant activity to different degrees.

Reducing property

During the reducing power assay, the presence of reductants (antioxidants) in the tested samples would result in reducing $Fe3^{+}$/ferricyanide complex to the ferrous form ($Fe2^{+}$). The $Fe2^{+}$ can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm. In Table No.1 the reducing power of ethanolic extracts of *O. sanctum, A. barbedensis*and *B. diffusa* where expressed in terms of absorbance (ABS). An increase in ABS shows high reducing power. In this assay *O. sanctum* show ABS of 0.653±.001 and 0.389±.001, 0.222±.005 for *B. diffusa* and *A. barbadensis* respectively indicating that *O. sanctum* had more antioxidant activity comparing with others. The antioxidant action of reductones was based on breaking of the radical chain by donation of a hydrogen atom which was reported by Gordon 1990. Duh 1998 and Duh *et al*. 1997 found that the antioxidant properties of mung bean hull and burdock extracts were shown to be concomitant with the development of reducing power.

All the antioxidant assays were put into statistical bi variate correlation found that TPC assay found significance difference comparing with other assay. Antioxidant ability show similar trend in each assay showed typical importance for *O. sanctum*. The others *A. barbadensis* and *B. diffusa* can be comparable but the potent difference in antioxidant capacity was shown by *O. sanctum*. As earlier the phenolic compounds and its concentration found difference and the variation in growing stage and also genetic variation may be the reason. For each species of plant the antioxidant capacity can be differed and more studies needed to explain this phenomenon.

Determination of Peroxide value (PV)

Primary oxidation products (hydroperoxides) were determined by PV measurement. The PV of fish oil stored in *O.sanctum* extract was found to be increase in 20 days with a maximum of 40 meq/O_2. Then the value decreases and increase, but the determinate at .02% concentration of extracts have pattern most adjacent to the positive control (BHT). This shows that the stability of oil preserved in this concentration would be effective. The graph shows, the trend for the concentrations at .05% and 1% had an upper curve when comparing with the concentrations at .02%. The negative control also the curve lies above that of the curve for .02% concentration. So it can be suitable for reducing the oxidative deterioration of fish oil to a lesser end.

TBA assay

The TBA value was determined by reading the optical density (OD) of the sample. It is evident from the data that the sample stored after 40 days cannot be ideal since the OD is beyond 3.0. The fish oil stored in .02% concentration the OD value increases very slowly when compared with. 05% and 1% concentration of extracts. The maximum value obtained at 20^{th} day was 2.348 which was comparable good with the positive control. The optical density of positive control at 20^{th} day was 2.654. This suggests that the concentration for adding *O.sanctum* in reducing the oxidative stress

can be effective at .02% concentration. Since for experimental purposes the refrigerated fish oil had to be thoughed under room temperature before analysis and frequent opening of the bottle can be a reason an early onset of oxidation. But this can be minimized for industrial storage purposes since there is no concurrent opening and thoughing.

Oxidative Stability of Fish Oil in *B.Diffusa* Ethanolic Extract

Determination of Peroxide value

The graph explained that the primary oxidation value is slowly increased to maximum for .05% concentration of *B.diffusa* extracts, with a similar trend observed in the positive control (BHT) also. But in the negative control and other concentration of .02% and 1% of this extract, the pattern is different where there is sudden increase towards the maximum value. From the valuation of PV in a period of 20 days, this effect can be monitored in the graph. An increase of 30meq/kg for .02% and 20 meq/ kg for 1 % was observed but when compared with the result of .05% the value is only 10 meq/kg. These results can be interpreted as the concentration of .05% can be ideal for fish oil preservation while using *B.diffusa* as an extract source.

TBA assay

Secondary metabolic products for the fish oil stored in *B.diffusa* extracts are useful index in determining the condition of spoilage. TBA content measured also for putting out the clarity of the spoilage index. An (optical density) OD below 3.0 was observed only for the concentration of .05% and positive control (BHT) for a period of 40 days indicating that this can be acceptable for minimizing oxidative rancidity. An observed OD of 2.935 and 2.235 for .05% concentration and BHT respectively at 40 days and there is increase in OD above 3.0 was observed at 40 days in concentrations .02% and 1%. The negative control sample without any added antioxidant got an OD beyond 2.0 at 20 days while the others less than that OD indicating that *B.diffusa* has got antioxidant potential beneficial to fish oil storage.

Oxidative Stability of Fish Oil in *A.Barbadensis* Ethanolic Extract

Determination of Peroxide Value

A concentration of .05% found effective for storing fish oil which can be cleared on (the primary oxidation index) PV graph for *A.barbadensis*. This concentration on comparison with that of the negative controls the value after 30 days is less. At this concentration it is below 25 meq/kgbut for negative control it is above 30meq/kg. Other concentrations of .02% and .1% the pattern of graph had high values and the curve is above the positive control. So for *A. barbadensis* a concentration of .05% can be ideal for storage of fish oil.

TBA assay

At 40 days of experiment with the *A.barbadensis* extract, only the OD at .05% concentration and positive control (BHT) had a value below 3.0.The readings at 538nm were shown as 2.845 and 2.345 for .05% and BHT respectively. An OD of below 2.0 was noted at 30 days for the same with 1.794 and 1.704 respectively for .05% and BHT. The other concentrations (.02% and .1%) and negative control got an increased OD and for control it was above 3.0 at 30 days. An OD of 2.094 and 2.734 OD were shown for .02% and 1% concentration respectively which was also less than that of control. Since the secondary metabolite product can be a useful index for storage studies from these observations it can be determined that *A.barbdensis* can be an effective antioxidant for fish oil. The concentration of .05% extract was comparatively good in this study.

Table No.1: DPPH, Reducing property, TPC and β Carotene values were expressed in mean of three determinants of *A. barbadensis, B. diffusa* and *O. sanctum*

S.No	SAMPLE	DPPH	Reducing property	TPC mg/100gm	β Carotene assay
1	Aloe barbadensis	7.35%±.055	0.222±.005	94±1	64%±1
2	Boerhaviadiffusa	16.38%±.03	0.389±.001	238±1	72%±1
3	Ocimum sanctum	34.25%±.020	0.653±.001	256±1	78%±1

Table No.2: TBA reactivity of fish oil stored in ethanolic extract (.02, .05 and 0.1% conc. in sample 1, 2 and 3) of *O. sanctum*, *A. barbadensis* and *B. diffusa* measured in OD at 532 nm per 10µl represented in a, b and c respectively against control and synthetic antioxidant BHT.

a.

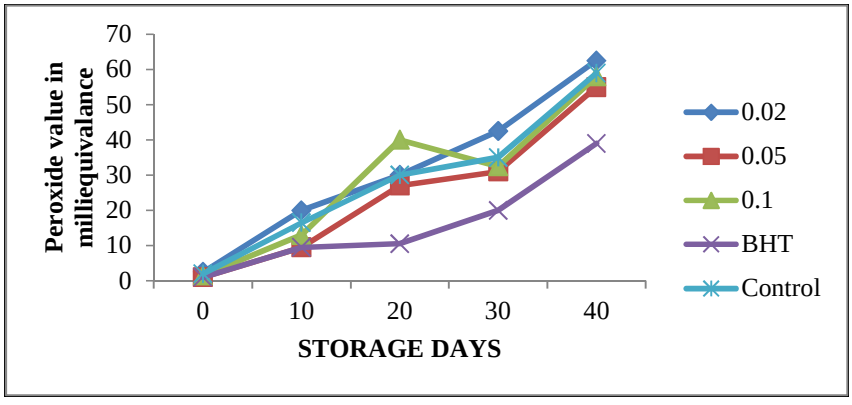
S.No	Storage Days	Sample 1	Sample 2	Sample 3	Control	BHT
1	0	0.288	1.241	1.736	0.530	0.030
2	10	1.353	1.494	1.934	1.745	0.601
3	20	2.348	2.707	2.708	2.158	0.958
4	30	2.708	3.0	3.0	3.0	1.704
5	40	3.0	3.0	3.0	3.0	2.235

b.

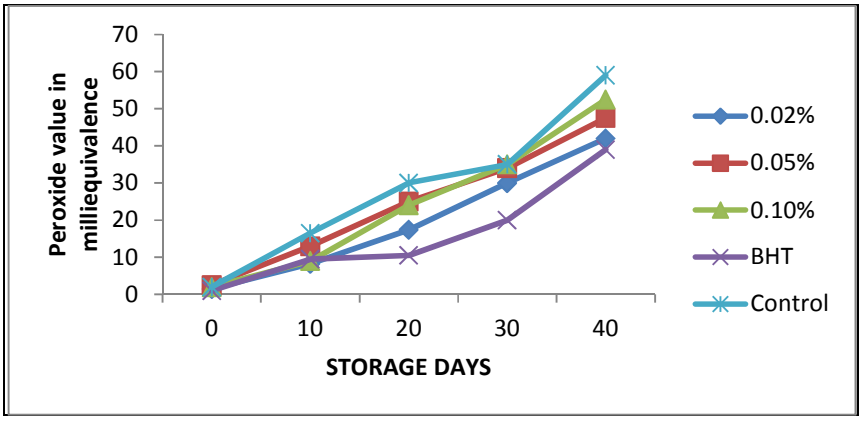
S.No	Storage Days	Sample 1	Sample 2	Sample 3	Control	BHT
1	0	0.861	0.460	0.778	0.530	0.030
2	10	1.399	1.309	1.733	1.745	0.601
3	20	1.812	1.581	2.979	2.158	0.958
4	30	2.094	1.794	2.744	3.0	1.704
5	40	3.0	2.845	3.0	3.0	2.235

c.

S.No	Storage Days	Sample 1	Sample 2	Sample 3	Control	BHT
1	0	0.490	0.358	0.515	0.530	0.030
2	10	1.533	1.445	1.803	1.745	0.601
3	20	1.793	1.528	1.944	2.158	0.958
4	30	3.0	2.443	3.0	3	1.704
5	40	3.0	2.935	3.0	3	2.235



a.



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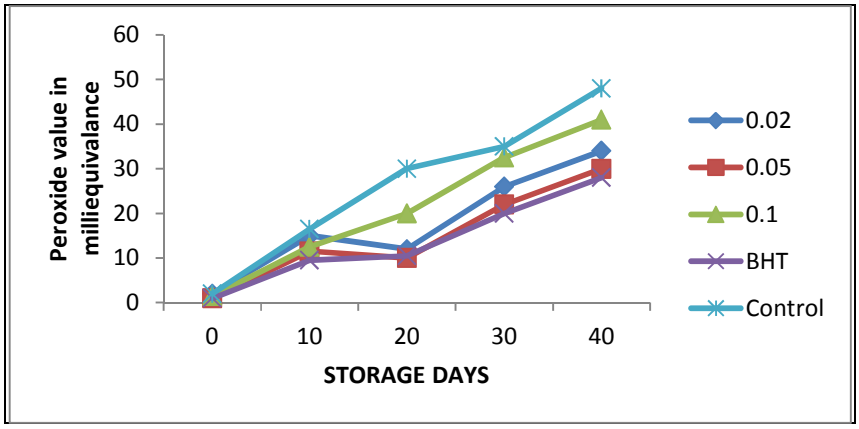

c.

Figure No.1: PV of fish oil stored in ethanolic extracts (.02, .05 and 0.1 % conc.) of *A. barbadensis*, *O. sanctum* and *B. diffusa* (a, b, c resp.) against control and synthetic antioxidant BHT

CONCLUSION

Antioxidant capability of the plant extracts is significantly important which implies its effective role in reducing lipid peroxidation. The adulteration occurred in many products could be prevented by using natural antioxidants. The use of synthetic antioxidants is being strictly banned so an alternative source supplying this property neutralizes the situation. Medicinal plants have other valuable properties which also be contributed in addition to check lipid deterioration. The conditional requirements satisfied well in minimizing oxidative rancidity to be producing best result in fish oil. From the results it can be concluded, that *O.sanctum* showed higher antioxidant properties than *A.barbadensis* and *B.diffusa* extracts, presumably due to differences in structure of the antioxidant components. However, the three medicinal plants ethanolic extracts could be used as natural food antioxidants and a possible substitution of artificial antioxidants. Besides their natural properties, they have the advantages of being easy to obtain, cheap, and effective. Therefore, it would be interesting to do further studies of using these Indian Medicinal Plants extracts as food additives in order to increase the shelf life of foods by preventing lipid peroxidation.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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