Pak. J. Bot., 50(5): 1999-2004, 2018.

EVALUATING THE POTENTIAL OF BOTANICAL EXTRACTS AND FRACTIONS AS SUBSTITUTES OF CHEMICAL ANTIOXIDANTS IN EDIBLE OILS

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Abstract

In this study, extracts of *Camellia sinensis* (green tea), *Trigonella gracecum foenum* (fenugreek), *Trachyspermum ammi* (ajwain), *Nigella sativa* (black cumin seed), *Viola odorata* (sweet violet) and green tea fractions were evaluated for substitutes of chemical antioxidant in edible oils. All extracts/fractions were tested for free radical scavenging assay by 1, 1-diphenyl-2-picryl hydroxyl (DPPH) in different dilutions. Fenugreek, green tea and ajwain extracts had maximum DPPH values (91.5, 86 and 85.75%) and were significantly better (p<0.05) than others. Each botanical extract/fraction was used (0.1%) in 100 ml samples of soybean, canola, sunflower and fish oil, stored for three months. In canola oil, green tea anthocyanin fraction (GTAF) proved to have least FFA (0.8%) followed by ajwain extracts (1.25%) and both were significantly different (p<0.05) than other antioxidants. FFA values for GTAF were best among extracts used in sunflower oil and soybean oil. After 3 months, canola oil peroxide value (POV) of untreated sample reached to 10.72 meq/kg, which was significantly (p<0.05) reduced by all extracts, especially GTAF (0.85 meq/kg). GTAF was equally leading oxygen scavenger in sunflower oil significantly (p<0.05) reducing POV of un- treated oil from 8.65 to 1.65 meq/kg. Soybean and fish untreated oils POV elevated to 11.53 and14.74 meq/kg respectively, which were reduced down by all antioxidant sources used (p<0.05).

Key words: Edible oils, Plant extracts, Antioxidants, Peroxide value, Fractions.

Introduction

Natural factors like storage time, humidity, light or temperature promote rancidity of edible oils and increase peroxide value (Kaleem *et al*., 2015). Oils cannot be stored for a long time without antioxidants (Anwar *et al.*, 2007). Phenolic chemicals like Butylhydroxyanisole (BHA), but ylhydroxytoluene (BHT) propyl gallate and tertiary butylhydroquinone (TBHQ) are being used to give oxidative stability to edible oils (Aluyor & Ori-Jes, 2008). These toxic chemicals and are carcinogenic to liver, stomach, blood, skin and lungs (Inanc & Maskan, 2012; Khanahmadi *et al.,* 2006). Natural antioxidants like ascorbic acid, tocopherols, carotenoids, chlorogenic acids and polyphenols are available and inhibit oxidation when used in sunflower and soybean oil (Ullah *et al.,* 2003). Botanical sources rich in antioxidant and anti-bacterial potential gain special attention as alternate replacers of chemical antioxidants (Sharma *et al.,* 2013). Aromatic plants like *Violet odorata* (sweet violet) have numerous biological activities derived from polyphenol rich extracts in it (Mittal *et al*., 2015). *Trigonella foenum graecum* (Fenugeek) is an importany spice and exhibits its antioxidant effects by radical scavenging and antimutagenic activities (Bukhari *et al.,* 2008) *Trachyspermum ammi* (Ajwain) is a source of therapeutically active polyphenol structures like Carvacol and thymol which work as strong antioxidants (Bairwa *et al.,* 2012). Fish oil can be stored better by using *Moringa Olieifera* extracts and can supersede synthetic antioxidants (Nascimento *et al.,* 2015). Natural antioxidants proved to be thermally stable in edible oils, which had been a reservation of its use in past (Taghvaei & Jafari, 2015). Olive extracts can be used in foods as natural antioxidants (Fiki *et al.,* 2005). Rosemary extracts, rich in carnosic acid can even prove better substitute of BHA and BHT in sunflower oil (Zang *et al.,* 2010).

Materials and Methods

Plant collection and authentication: *Camellia sinensis* (green tea) leaves, *Trigonella foenum* (fenugreek) seeds, *Trachyspermum ammi* (ajwain) seeds, *Nigella sativa* (black cumin seed), *Viola odorata* (sweet violet) flowers were purchased from local market of Karachi, Pakistan. These leaf, flower or seeds were properly identified by plant taxonomist, Food and Marine Research Center, PCSIR Labs Complex, Karachi.

Extract Preparation from *Trigonella foenum*, *Trachyspermum ammi* and *Nigella sativa* seeds: 200 grams seeds of each *Trigonella foenum*, *Trachyspermum ammi* and *Nigella sativa* were grinded to pass through 30 mesh sieve. These samples were extracted using method of Kim & Lee (2002). The seed powders were soaked in flask with 1000 mL of 80% methanol, sonicated for 24 hrs, filtered with Whatman no. 2 filter paper, took the residue on filter and sonicated for more 24 hours and extracted once more (1000 mL each time). The filtrates were transferred to the round bottom flasks and methanol was evaporated in rotary evaporator under vacuum at 40°C until the volume was reduced to 300 mL. The volume was then made to 400 mL with distilled water to standardize liquid contents.

Extract preparation from *Camellia sinensis* (Green tea): Dried green tea *(Camellia sinensis)* was purchased from local market (200 g), soaked in flask with 1000 mL of 80% methanol, sonicated for 24 hours, filtered with whatman no.2 filter paper, took the residue on filter and sonicated for more 24 hours and extracted once more (1000 mL each time). The filtrates were transferred to the round bottom flasks and methanol was evaporated in rotary evaporator under vacuum at 40°C until the volume was reduced to 300 ml. The volume was then made with

400 ml with water to standardize crude liquid contents. Some of this crude liquid extract of green tea was saved for usage in feed and major portion was further fractioned into acid, neutral, anthocyanin and non-anthocyanin parts.

Fractionation of crude green tea extracts into anthocyanin and non-anthocyanin fractions: Crude green tea extract (GTE) was fractioned into anthocyanins and non-anthocyanin contents using the method of Kim & Lee (2002). Before filtration of green tea crude extract, C18 Sep –pak cartridge was preconditioned by passing 10 mL ethyl acetate, followed by 10 mL methanol and 10 mL of 0.01N HCl. filtered green tea extracts through cartridge and washed with 6 ml of 0.01 N HCl to remove sugars, acid and water soluble compounds. After suction drying, rinsed cartridge with 40 mL ethyl acetate and non-anthocyanin polyphenol contents were collected in a 100mL flask. Anthocyanin portion was washed with 6 ml methanol from C18 cartridge and collected in 100 mL flask. Ethyl acetate was evaporated at room temperature and methanol was evaporated at 40°C using rotary evaporator. In this way, pure anthocyanin and non-anthocyanin fractions of green tea were separated and kept at 4°C till mixing in feed.

Fractionation of crude green tea extracts into acidic and neutral fractions: Crude extract of green tea was fractionated into acidic and neutral polyphenolic part by using the procedure of Oszmianski & Lee (1990). Two polypropylene (82 mm × 20 mm) columns filled with 5 g of Lichroprep RP 18 column SPE (solid phase extraction cartridges), supplied by Merck (25–40 μm) were used for the separation. One column was preconditioned with methanol (10 mL) and then with 10 mL water and subsequently green tea extracts (neutralized to pH 7.0 using 5 N NaOH) were passed through the column to absorb the neutral compounds and was collected as acidic fraction. Now this collected effluent (pH adjusted to 2.0 with 1 N HCl) was passed through another column preconditioned with methanol (10 mL) and 0.01 N HCl (10 mL). Both neutral and acidic green tea fractions were then eluted from their respective columns with 10 mL absolute methanol. Methanol was evaporated and the residues were re-solubilized in water and stored at 4°C till further use.

Extract preparation from sweet violet dried flowers: Green tea dried leaves, sweet violet dried flower and leaves, fenugreek seeds, black cumun seeds, black tea extracts and ajwain seeds were purchased from local market of Karachi, Pakistan. These leaf, flower or seeds were purchased from local market (200 g) and properly identified by plant taxonomist, Food and Marine Research Center, PCSIR Labs Complex, Karachi.

These samples were extracted using method of Kim & Lee (2002). Dried sample was soaked in flask with 15000 mL of 80% methanol, sonicated for 24 hours, filtered with whatman no. 2 filter paper, took the residue on filter and sonicated for more 24 hours and extracted once more (1000 mL this time). The filtrates were transferred to the round bottom flasks and methanol was evaporated in rotary evaporator under vacuum at 40°C until the volume was reduced to 300 ml. The volume was then made with 400 ml with water to standardize crude liquid contents.

Evaluation of antioxidant activity by DPPH radical scavenging method: Free radical scavenging activity of methanolic extracts of fenugreek, black cuminseed, ajwain, sweet violet, green tea and its fractions were measured by1, 1- diphenyl-2-picrylhydrazyl (DPPH) as described by Tailor *et al.*, (2014). In brief, 0.1 mM solution of DPPH in ethanol was prepared. This solution (1 ml) was added to 3 ml. of different extracts at different concentrations (Dilution to 1%, 0.5% and 0.1 %) The mixture was shaken vigorously and allowed to stand at room temp for 30 min., then, absorbance was measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu). Reference standard compound being used was ascorbic acid and experiment was done in triplicate. The IC 50 value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was calculated by using following equation:

$$\text{DPPH scavenging effect (\%) or Percent inhibition} = \frac{A0 - A1}{A0} \times 100$$

where A0 was the Absorbance of control reaction and A1 was the Absorbance in presence of test or standard sample.

Oil storage using botanical extracts and tea fractions: Fresh oil samples of soybean, canola and sunflower were collected from M.A Oils, port qasim Karachi. Fish oil sample was taken from M.A Proteins fish plant Korangi, Karachi. Samples were stored at 4°C in refrigerator before use.168 oil samples were taken in plastic bottles of 100 mL. Samples of soybean were 42 and each treatment applied to 3 samples, so that the average values for 14 treatments be obtained. Similar methods were applied to sunflower, canola and fish oil. Synthetic antioxidants like tertiary butyl hydroquinone (TBHQ), Ethoxyquine and Vitamin E were also applied to all four oils. Extracts of green tea and its acid, neutral, anthocyanin and non-anthocyanin fractions, black cumin seed, sweet violet, fenugreek and cuminseed (ajwain) were used. Ethoxyquine and TBHQ were used at a rate of 300 ppm and vitamin E used at 100 ppm. All extracts and fractions were used at a rate of 0.1% in oil samples. Samples were kept for three months' storage in a laboratory room ranging temperature 20-38°C (march-may).

Evaluating oils for Free fatty acids and peroxide value: After one month storage samples were analyzed for Free fatty acid value according to method described by A.O.A.C (2003). After 3 months, oil samples were analyzed in laboratory for Peroxide value (AOAC, 2003).

Data analysis: Average values on eight treatment diets, fed to three replicates of five birds each, were subjected to One Way Analysis of variance (ANOVA), and significant differences at $p<0.05$ between the mean values were observed using Duncan's multiple range test using SPSS version 17 (SPSS Inc, USA).

Results

DPPH scavenging activity/ Antioxidant activities of different botanical extracts: Antioxidant capability as expressed by DPPH assay of the botanical extracts at 1 % dilution (Table 1), revealed that significantly different values ($p<0.05$) were obtained among them. Fenugreek seeds had maximum value (91.5%), followed by black cuminseed (90.3%), green tea (86%) and ajwain (85.75%), however, all the three extracts were statistically similar ($p>0.05$). Green tea fractions had lower levels of DPPH and non-anthocyanin fraction had least (18%) level.

When these extracts were diluted to 0.5% level (Table 1), higher values were achieved for Ajwain, fenugreek and Green tea extracts (76, 72 and 69 % respectively), and there was non-significant difference in these values ($p<0.05$). There were more declines for black cuminseed and green tea antioxidant potentials, which reached to 62% and 69% DPPH levels. It was noticeable fact that sweet violet extracts lost only 3% scavenging ability.

At 0.1% dilutions, all the extracts declined to minimum levels. Sweet violet and green tea anthocyanin extracts, however lost minimum potencies (6.2% and 4% respectively). Ajwain, fenugreek and green tea had appreciable scavenging capabilities even at 0.1 % dilution and DPPH values were 52, 50 and 42.6% respectively (Table 1). DPPH values of black cuminseed extracts, declined to 18.8% at dilution of 0.1%. Except anthocyanin fraction of green tea, all other fractions had lowest values at 0.1% diluted levels.

Free fatty acid content (%) of different edible oils stored for one month: Canolla oil free fatty acids level was only 0.48% at the start of storage period of one month and it reached to 8.19% within 30 days storage (Table 2). All the synthetic antioxidants like TBHQ, ethoxyquine and vitamin E or natural extracts and their fraction were capable to save oil from oxidative stress and their average FFA content was significantly different among them ($p<0.05$). Green tea anthocyanin fraction proved to be the best agent (Table 2) against oxidation of oil (0.8% FFA) followed by ajwain extracts (1.25% FFA).

Sunflower oil had initially 0.3% FFA before storage and it reached the peak value of 10.5 % after a month. Better FFA values achieved by adding ajwain, green tea and its acid fraction, black cuminseed and vitamin E (Table 2), and the best FFA levels achieved in TBHQ (0.4%), followed by green tea anthocyanin fraction and fenugreek extracts (1.2% and 1.3% respectively). Some elevated levels of FFA were seen in ethoxyquin, sweet violet extracts, Green tea non anthocyanin and green tea neutral fraction (4.55, 4.56, 4.5 and 5.2% respectively). There were significant differences observed among FFA average values ($p<0.05$) of the antioxidant sources used.

Soybean oil had 0.85% FFA and it increased to 13.6% after storage of one month. TBHQ had least FFA value (0.5%). Green tea acid fraction extracts safeguarded the oil at 2.6% FFA and anthocyanin extracts FFA reached 2.5%. These two tea extracts along with sweet violet extracts (2.54%FFA), were statistically same but differed all other antioxidant sources significantly ($p<0.05$%). Green tea non-anthocyanin extracts and ajwain extracts had elevated FFA levels (6.2 and 5.14%, respectively).

Fish oil had 5.9% FFA at the start of the storage time and it reached the level of 18.53% after 30 days' storage. By using all the extracts of botanical origin, FFA was maintained around 8%, and the results were significant for all the applications. Significant differences among antioxidant extracts were observed ($p<0.05$).

Peroxide value (POV) of different edible oils stored for three months: Peroxide values (POV) were significantly different ($p<0.05$) among various antioxidant sources used in Canola oil after three months' storage. Peroxide value of fresh oil increased from 0.86 meq/kg to 10.72 meq/ kg during this time without any antioxidant. Oxidation was partially controlled and oil saved by using all synthetic and natural antioxidant extracts (Table 3). Green tea anthocyanin extracts, ethoxyquin and had better scavenging capability (0.85 and 1.12 meq/kg, respectively).

Sunflower oil, having POV value of 1.25 meq/kg, when stored without antioxidant, got rancid at 8.65 meq/kg level.By using the antioxidant sources, there was significant ($p<0.05$) decrease in peroxide values. Green tea anthocyanins and vitamin E had best potential to combat oxidation of sunflower oil (1.68 and 1.85 POV respectively). Green tea acid and neutral fractions had higher POV values than other applications of extracts (4.53, 4.56 meq/kg respectively), and were significantly different than untreated oil.

Soybean oil was also treated with antioxidant extracts and results among treatment were statistically significant among the treatments ($p<0.05$). Untreated oil POV was elevated to 11.53meq/kg (Table 3), which was just 1.36meq/kg at the start of experiment. However, significant improvement in reduction of oxidation ($p<0.05$) was observed when botanical extracts were used to safeguard oils. TBHQ trated oil had best POV (1.56meq/kg).

Fish oil having POV 0.96 meq/kg, when exposed to oxidative stress for 3 months, elevated its POV to 14.74 meq/kg. There was significant improvement in POV ($p<0.05$), when oil was treated with antioxidant sources. Green tea anthocyanin extract had best recovery of oil being rancid (POV 1.8meq/kg), which almost equalized the potential of Ethoxyquin (POV 1.85meq/kg).

Table 1. DPPH scavenging activities of different botanical extracts (%).

Sr #	Type of methanolic extracts	1.0%	0.5%	0.1%
1.	Green tea leaves dried	86*d*	69*d*	42.6 *d*
2.	Fenugreek grains	91.5*d*	72*d*	50*d*
3.	Sweet violet flowers dried	32*c*	29*b*	22.8*b*
4.	Black cumin seeds	90.3*d*	62*c*	18.8 *a*
5.	Omumseed (ajwain)	85.75*d*	76*d*	52*d*
6.	Green tea non-anthocyanin extract	12*a*	4.2*a*	1.16 *a*
7.	Green tea anthocyanin extract.	36.3*c*	25.2*b*	21*b*
B.	Green tea neutral fraction extract	18*ab*	9.3*a*	3.5 *a*
C.	Green tea acid fraction extract	22*b*	3*a*	0.7 *a*

Means within a column with different letters differ significantly (p <0.05)

Table 2. Free fatty acid content (%) of different edible oils stored for one month.

	Type of extracts	Canola oil	Sunflower oil	Soybean oil	Fish oil
A.	Pure oil fresh (not stored)	0.48*a*	0.3*a*	0.85*a*	5.0*a*
B.	Pure oil / No additive (stored)	8.19*e*	10.5*g*	13.6 *e*	18.53 *d*
C.	*TBHQ 300 ppm*	2.56*c*	0.4*a*	0.5*a*	5.3 *a*
D.	Ethoxyquine 300 ppm	3.88*d*	4.55*e*	3.96*c*	8.93*c*
E.	Vitamin E 100ppm	1.75*b*	3.52*d*	4.3 *c*	6.15*ab*
F.	Crude Omumseed (ajwain) extract. 0.1%	1.25*ab*	2.56*b*	5.14 *c*	6.32*ab*
G.	Crude sweet vilolet extract 0.1%	3.19*d*	4.56*e*	2.54*b*	7.27*b*
H.	Crude green tea extracts 0.1%	2.94*c*	3.25*d*	4.1*c*	7.58*b*
I.	black cuminseed extract 0.1%	1.41*b*	2.1 *b*	4.2*c*	8.54*c*
J.	Crude fenugreek extarcts 0.1%	1.58*b*	1.3*a*	3.8 *bc*	7.28*b*
K.	Green tea non-anthocyanin extract 0.1%	3.1*d*	4.5 *e*	6.2 *a*d	6.4*ab*
L.	Green tea anthocyanin extract 0.1%	0.8*a*	1.2*a*	2.5 *b*	7.3*b*
M.	Green teaneutral fraction extract 0.1%	3.6*d*	5.2 *f*	4.3 *c*	6.57 *ab*
N.	Green tea acid fraction extract 0.1%	2.1*c*	3.6*a*	2.6 *b*	7.3*b*

Means within a column with different letters differ significantly (p <0.05)

Table 3. Peroxide value (POV) of edible oils stored for three months

	TYPE OF FEED	Canola oil	Sunflower oil	Soybean oil	Fish oil
A.	Pure oil fresh (not stored)	0.86*a*	1.25*a*	1.36*a*	1.36*a*
B.	Pure oil / No additive (stored)	10.72*d*	8.65 *f*	11.53 *e*	14.74*e*
C.	*TBHQ 300 ppm*	2.1 *b*	2.06 *b*	1.56 *a*	2.1 *a*
D.	Ethoxyquine 300 ppm	1.12 *a*	3.65 *c*	2.35 *b*	1.85*a*
E.	Vitamin E 100ppm	1.82 *ab*	1.85 *ab*	4.5 *d*	9.98*d*
F.	Crude green tea extracts 0.1%	1.84 *ab*	2.14 *b*	2.14 *b*	5.04*c*
G.	Crude Omumseed (ajwain) extract. 0.1%	1.79 *ab*	4.35 *d*	2.65 *b*	3.92*b*
H.	Crude sweet violet extract 0.1%	2.74 *b*	2.3*b*	3.25 *c*	3.73*b*
I.	Crude fenugreek extracts 0.1%	2.79 *b*	4.32 *d*	3.78 *c*	9.63*d*
J.	black cuminseed extract 0.1%	1.792*ab*	2.13 *b*	1.96 *b*	9.05*d*
K.	Green tea non-anthocyanin extract 0.1%	2.92 *b*	3.15*c*	3.56 *c*	3.99*c*
L.	Green tea anthocyanin extract0. 0.1%	0.85 *a*	1.68 *a*	2.53*b*	2.0 *a*
M.	Green tea neutral fraction extract 0.1%	3.42 *c*	6.56*e*	3.25 *c*	4.65*bc*
N.	Green tea acid fraction extract 0.1%	2.85 *b*	4.53 *d*	2.95 *bc*	3.62*b*

Means within a column with different letters differ significantly (p <0.05)

Discussion

It is very clear that the botanical extracts having higher DPPH values, as in this research, can perform as antioxidants in oils as described by Lee *et al.,* (2016). It has been noticed that extracts and fractions of botanicals like tea can yield equally better DPPH values, like tocopherols, ascorbic acid and citric acid, well known antioxidants in oils (Kim *et al.*, 2007; Carelli *et al.*, 2005). During current study, natural factors like storage time and temperature were considered as stress factors which trigger oxidation of untreated raw oils resulting in increased FFA and POV (Kaleem *et al.,* 2015). During current trial an increase in POV upto 15 meq/kg corresponds to the studies of Anwar *et al.,* (2007), who that documented soybean POV to reached 20 meq/kg level after 180 days' storage. Role of synthetic antioxidants to retard oxidation in oil is well established and the results in current trial by using chemicals like TBHQ, ethoxyquin and Vitamin E confirmed the findings of Ruger *et al.,* (2002) who used TBHQ level up to 1.28% for best results. Performance of botanical extracts against oxidation is very clear due to effective antioxidants in natural forms (*Longato et al*., 2015) when used in sunflower, canola, and soybean or fish oil. Extracts of fenugreek, sweet violet and ajwain gave promising results during our work, having higher DPPH values and effective control against oxidative degeneration equally in different oils, supported findings of Ishtiaque *et al.,* (2013). Similar facts were demonstrated by Krishnaiah *et al.,* (2011) that Plant seed, leaf or flower extracts rich in polyphenols can fight against any oxidative challenge. Natural extracts during our study even proved better than synthetic sources, especially green tea anthocyanin fractions had better FFA and POV than TBHQ, ethoxyquin and vitamin E in different oils. These findings are not different than the previous work of Zhang *et al.*, (2012) who treated sunflower oil with rosemary extracts for long storage. In our recent trial, botanical extracts proved to be the excellent replacers of synthetic antioxidants like ethoxyquin or TBHQ, confirming the POV values betterment as indicated by Kheradmandi *et al.,* (2015) using 800 ppm Mentha pulegium extracts in canola oil. Green tea and its extracts bear extraordinary scavenging capacity due to catechin rich compounds, which had been proven ever better substitutes of rosemary extracts and BHT in canola and chichen oil (Chen *et al.*, 1998). Our trial on different oils using green tea and its fractions proved effective enough to justify antioxidant potentials in reducing peroxide formation.

Conclusion

Synthetic antioxidants are considered to be a matter of concern for human health. Plant derived crude extracts or fractions of extracts supplementation in edible oils justify their antioxidant role and alternates of chemicals like BHT, BHA, ethoxyquin and TBHQ. Exploring natural solutions to replace drugs is recommended. Human health will be more secure by using noval plant resources.

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(Received for publication 27 March 2017)