International Food Research Journal 22(1): 289-294 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Selective ABTS and DPPH- radical scavenging activity of peroxide from vegetable oils

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Article history

Received: 28 January 2014
Received in revised form: 25 July 2014
Accepted: 29 July 2014

Keywords

Antioxidants
ABTS
DPPH
Mustard oil
Groundnut oil
Sesame oil

Abstract

Vegetable oils contain natural antioxidants like sterols, phosphatides, tocopherols, tocotrienols etc. In the present study, the efficiency and stability of natural antioxidant in unrefined mustard oil, groundnut oil and sesame oil on heating is studied. The oils undergo five cycles of heating to a frying temperature (210°C) and their antioxidant activity is premeditated using radical scavenging assay. The inhibition concentration of unheated and heated mustard oil, groundnut oil and sesame oil (with solvent benzene) and the stability of natural antioxidant at different concentrations are evaluated using 2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method. The result showed that the antioxidant activity in unheated oils are more compared to heated oils using ABTS radical scavenging activity (IC_{50} 24.31, 25.26 and 24.42 for unheated and IC_{50} 49.7, 54.55 and 46.39 for heated). The scavenging activity is also studied using DPPH analysis (IC_{50} 26.22, 27.37 and 28.25 for unheated and IC_{50} 53.3, 59.49 and 49.18 for heated). The ABTS method is highly correlated with DPPH and the correlation coefficient is computed (Pearson's correlation $r < 0.04$; $p < 0.01$).

Introduction

Oxidation of fats and oils occurs during raw material storage, processing, refining, high temperature exposure and final storage of products. Rancidity in edible oil leads to deterioration producing toxic products like peroxides, dimmers, trimmers etc, which alters taste, aroma, flavors, nutritional quality and safety in foods after cooking and processing (David and Choe, 2003). Existence of antioxidants in oils is technically a simplest way of reducing oxidation and gives health protection. The important role of antioxidants in oil is to delay the oxidation of other molecules by inhibiting the initiation of oxidizing chain reactions by free radicals (Li *et al*., 2009). The products of oxidation (peroxides) may be a significant causative factor in the development of many chronic diseases such as cancer and cardiovascular diseases (Rubalya and Neelamegam, 2012a).

Free radicals and oxidants activate oils and fat to undergo peroxidation, also the oxidation of macromolecules like proteins and DNA causes extensive damage to the body cells (Rubalya *et al*., 2012b). Radicals are chemical species that contains one or more unpaired electrons, and free radicals are a radical that goes out immediately from the molecular surroundings from where they are generated (Byong *et al*., 2006; Duduku *et al*., 2011). There are several endogenous sources of oxidants in the body: that reduces oxygen in mitochondria during cellular respiration which leads to the formation of the radical as by-products of superoxide O_2, hydroxyl HO^+, and hydrogen peroxide H_2O_2; degradation of fatty acids and other molecules in peroxisomes produces H_2O_2 (Davies, 1995; Edwin, 1996; David and Choe, 2003).

Mustard oil consists of 60.4 % of monounsaturated fatty acids, 21.7% of polyunsaturated fatty acids, 12.8% of saturated fatty acids and antioxidant such as phenolic compounds and Vitamin A (Fereidoon, 2005). Groundnut oil includes 46% of monounsaturated fatty acids, 32% of polyunsaturated fatty acids, 17% of saturated fatty acids and antioxidant nutrients such as, vitamin E, polyphenolic, vitamin B. Sesame oil contains 41.5% of monounsaturated fatty acids, 43.5% of polyunsaturated fatty acids, 14.6% of saturated fatty acids, antioxidant like vitamin E, sesame lignans and phyto-estrogen etc. (Mohamed and Awatif, 1998). On degumming, refining, deodorization natural antioxidants are lost hence unrefined oil is taken for study.

Study of antioxidant efficiency in oils using ABTS

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and DPPH radical scavenging assay is an important *in-vitro* analysis with which the total antioxidant stability in oils can be studied for their high-quality reproducibility and simple eminence control (Bakkali *et al*., 2008; Rubalya and Neelamegam, 2012b). Both the methods apply decolorization assays to identify the existence of antioxidant which annul the development of the ABTS radical cation and DPPH radical (Tomaino *et al*., 2005). In most of the assays to determine the antioxidant properties, the ABTS activity was strongly correlated with DPPH because both methods are responsible for the same chemical property of H^- or electron-donation to the antioxidant (Alessandra *et al*., 2003; Amin *et al*., 2004). The radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. The formation of more number of free radicals accelerates the oxidation of oils and decreases its quality. The objective is to study the oxidative stability in unrefined edible oils (mustard, groundnut and sesame oil) and to investigate the antioxidant potential in the oils. The primary product peroxide formed on oxidation is estimated using radical scavenging ABTS and DPPH analysis.

Materials and Methods

The antioxidant stability in unrefined edible oils such as sesame oil, mustard oil and groundnut oil are got from the local oil extraction place at Thanjavur of Tamilnadu, India. Hundred milliliter of the sample oil is heated (Rubalya *et al.*, 2013). The oils are exposed to five cycles of heating (0.5, 1.0, 1.5, 2 and 2.5 hrs) to the temperature at 210°C.

ABTS (2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) radical cation scavenging*

The $ABTS^{*+}$scavenging test is used to determine the antioxidant activity (by estimating peroxide formation) of both hydrophilic and hydrophobic compounds. The assay measures $ABTS^{*+}$ radical cation formation induced by metmyoglobin and hydrogen peroxide. The formation of the colored ABTS radical is suppressed by antioxidants by electron donation radical scavenging and inhibit. The quantity of antioxidant in the test sample is inversely proportional to the ABTS radical development.

$ABTS^{*+}$ is generated by mixing 2.5 ml of 7 mM ABTS with 14.7 mM ammonium per sulphate and stored in the dark at room temperature for 16 hours. The solution is diluted with water to achieve an absorbance of 0.7±0.05 O.D. The radical-scavenging activity is assessed by mixing 2 ml of this $ABTS^{*+}$solution with different concentrations of sample dissolved in benzene (25, 50, 75, 100 µg/ml). After 30 min, the percentage inhibition at 734 nm was calculated for each concentration relative to blank absorbance (Re *et al*., 1999).

$$ABTS + H_2O_2 \longrightarrow ABTS^{*+} + H_2O \text{ ---------- (1)}$$

The reaction between $ABTS^{*+}$and ammonium per sulphate directly generates the blue green $ABTS^{*+}$chromophore, which can be reduced by an antioxidant, thereby resulting in a loss of absorbance at 734 nm. The antioxidant capacity is expressed as percentage inhibition, calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_{\text{Control}} - A_{\text{Sample)}} / A_{\text{Control}}] \times 100 \text{ -------(2)}$$

Where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the sample. The peroxide level is determined by the reading the absorbance using UV- Spectrophotometer. IC_{50} is calculated by plotting percentage inhibition against different concentrations of oil (Rubalya and Neelamegam, 2012b). IC_{50} values denote the concentration of sample required to scavenge 50% of ABTS free radicals. Low IC_{50} values indicate high radical – scavenging activity. The experiment has been performed in triplicate, was recorded as mean ± SD and their variance is analysed using one-way ANOVA procedure as shown in Table 1.

DPPH (2, 2'-diphenyl-1 - picrylhydrazyl) free radical scavenging assay*

$DPPH^*$ method is also used to study the scavenging activity of antioxidants in oils. It is seemed to be endowed with good antioxidant properties. This method is based on the reduction of a methanol solution of $DPPH^*$in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H (Soler *et al*., 2000). The samples are prepared by dissolving oil to benzene and taken (25, 50, 75,100 µg/ml) 2 ml of a methanol added to DPPH• free radical (Brand *et al*., 1995). The reaction mixture is shaken by cyclo-mixer and then kept in the dark for 30 min under ambient conditions.

This transformation results is a change in colour from purple to yellow, which has been measured spectrophotometrically by using UV-Spectrophotometer (Perkin Elmer-Lambda 11). The disappearance of the purple colour change at 517 nm is observed.

$$DPPH^* + AH \longrightarrow DPPH + A^* \text{ ---------- (3)}$$

Table 1. ABTS radical decolourisation of different oils- unheated and heated

Concentration of oil sample (µg/ml)	Mustard oil		Groundnut oil		Sesame oil		P value	F/df
	unheated	Heated	unheated	Heated	unheated	heated		
25	31.8 ± 0.60[b]	44.6 ±0.07[c]	35.0 ±0.16[c]	48.7 ±0.43[c]	13.4 ±1.34[a]	41.5 ±0.17[b]	0.000	34.7/ 11
50	61.5 ±1.10[c]	54.3 ±0.70[c]	57.5 ±1.04[c]	67.7 ±0.36[b]	33.2 ± 0.78 [c]	55.9 ±0.38[c]	0.000	103.6/ 11
75	77.6 ± 1.20 [c]	69.7 ± 1.01[c]	8.9 ± 0.83[c]	71.8 ± 0.7[a]	54.5 ± 0.31 [c]	67.1 ±0.22[c]	0.000	35.1/ 11
100	97.8 ± 0.20 [c]	85.7 ± 0.60[b]	91.7 ±1.41[b]	79.4 ± 0.4[b]	67.2 ± 2.46 [a]	83.1 ±1.13[b]	0.002	138.4/ 11

Mean value ± SD (n=3)
[a] Significant difference between mustard, groundnut and sesame oil (unheated and heated) ($p < 0.05$)
[b] Significant difference between mustard, groundnut and sesame oil (unheated and heated) ($p < 0.01$)
[c] Significant difference between mustard, groundnut and sesame oil (unheated and heated) ($p < 0.001$)

The percentage of inhibition (antioxidant capacity) is computed by measuring the absorbance at 517 nm, using the following formula,

$$\text{Inhibition (\%)} = [(A_{Control} - A_{Sample}) / A_{Control}] \times 100 \text{------- (4)}$$

Where, $A_{Control}$ is the absorbance of the control and A_{Sample} the absorbance of the sample at 517 nm. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH radicals. Antioxidant activity is calculated by plotting percentage inhibition against different concentrations of oil. All observation has been carried out in triplicate, was recorded as mean ± SD and their variance is analysed using one-way ANOVA procedure as represented in Table 2.

Statistical analysis

All data on total antioxidant activity are the average of triplicate. To examine the effect of type of compound and concentration on antioxidant activity, graph pad software version 5.0 is used (r^2 = 0. 0.9949, $p<0.005$, $n>9$). The data were recorded as mean ± SD and analysed by SPSS (version 12). One-way analysis of variance is performed by ANOVA procedures. Significant differences between means are determined by Duncan's multiple range tests, p-Values <0.05 are regarded as significant and p-value<0.001 are very significant. The variance between different groups and two methods (ABTS and DPPH) for mustard, groundnut and sesame oil is also analyzed and tabulated in Table 3.

Results and Discussions

In-vitro *analysis of mustard*

The formation of the ABTS radical cation takes place almost instantaneously after adding ammonium per sulphate to an ABTS solution. The scavenging ability of peroxides against ABTS radicals was

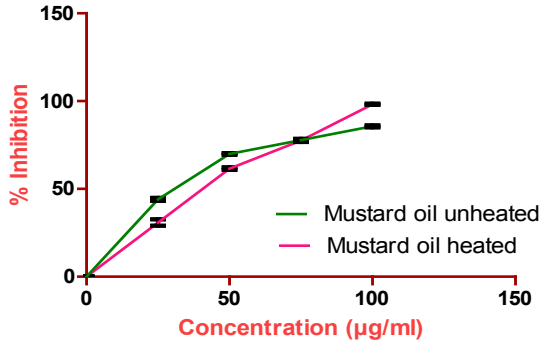

Figure 1. ABTS radical decolourisation of unheated and heated Mustard oil

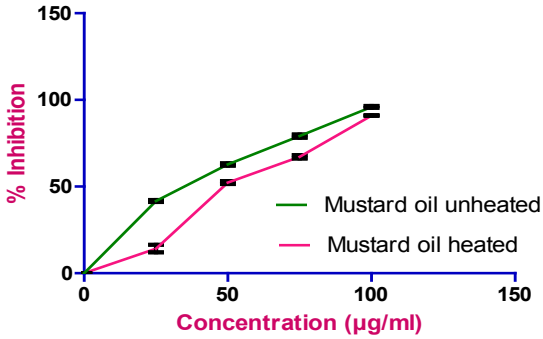

Figure 2. DPPH radical decolourisation of unheated and heated Mustard oil

concentration dependent. A more appropriate format for the assay is decolourisation technique in that the radical is generated directly in stable form prior to reaction with putative antioxidants (Ilhami, 2006). The scavenging activity of unheated and heated mustard oil is shown in figure 1 and 2. During concentration from 25 µg/ml to 50 µg/ml, the gradient of the curves of percentage of inhibition versus concentration for heated is steeper than for unheated mustard oil, indicating that in this concentration the anti-radical activity increased rapidly with concentration.

Table 2. DPPH radical decolourisation of different oils- unheated and heated

Concentration of oil sample (µg/ml)	Mustard oil		Groundnut oil		Sesame oil		p value	F/df
	unheated	Heated	unheated	Heated	unheated	heated		
25	14.3 ± 0.5[b]	41.6 ± 0.2[c]	19.9 ± 0.3[c]	35.8 ± 0.7[c]	14.7 ± 1.4[c]	41.5 ± 0.7[c]	0.000	50.6/ 11
50	52.4 ± 0.7[c]	62.8 ± 1.0[c]	47.8 ± 1.2[a]	59.9 ± 2.1[c]	34.8 ± 0.1[b]	55.9 ± 0.4[c]	0.001	151.5/ 11
75	67.1 ± .3[c]	79.1 ± 1.8[c]	71.4 ± 0.6[c]	75.7 ± 1.4[c]	54.3 ± 1.2[c]	67.1 ± 0.2[c]	0.000	83.1/ 11
100	90.9 ± 0.8[b]	95.9 ± 2.6[c]	95.4 ± 0.9[c]	87.3 ± 0.2[c]	69.2 ± 0.9[c]	83.1 ±1.1[b]	0.011	27.5/ 11

Mean value ± SD (n=3)
[a] Significant difference between mustard, groundnut and sesame oil (unheated and heated) ($p < 0.05$)
[b] Significant difference between mustard, groundnut and sesame oil (unheated and heated) ($p<0.01$)
[c] Significant difference between mustard, groundnut and sesame oil (unheated and heated) ($p <0.001$)

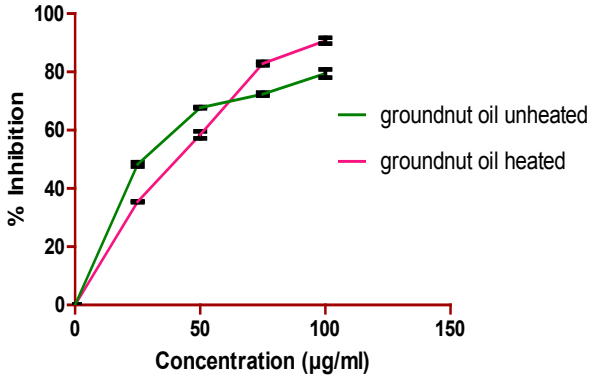

Figure 3. ABTS radical decolourisation of unheated and heated groundnut oil

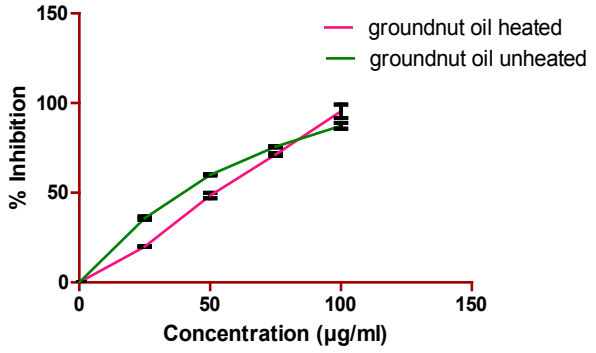

Figure 4. DPPH radical decolourisation of unheated and heated groundnut oil

The heated mustard oil loses its antioxidant activity by 48.9%. The Inhibition concentration (IC_{50}) of unheated is 24.31% and for heated it is 49.70%. Hence unheated has less scavenging activity due to the antioxidant stability than heated oil using ABTS method. In DPPH the heated mustard oil loses its antioxidant activity by 53.9% the Inhibition concentration (IC_{50}) of unheated oil is 28.75% and heated oil is 53.30%. The loss of antioxidant activity in ABTS* and DPPH* radical decolourisation assay is observed lower in heated mustard oil due to loss of antioxidant and increase in saturated compounds. It was observed that mustard oil contain greater amount of erucic acid (42.8%) and low value of polyunsaturated linolenic acid (18.2%); it also has antioxidants like vitamin E and sterols. After heating the content of vitamin E got evaporated and level of sterols get decreased (Md. Abdul *et al*., 2012).

In-vitro *analysis of groundnut oil*

The radical-scavenging activity of unheated and heated groundnut oil is shown in Figure 3 and 4 illustrate the variation of percentage of inhibition with the concentration of oil. In the radical scavenging concentration from 25 µg/ml to 75 µg/ml, the gradient of the curves illustrate the percentage of inhibition versus concentration for unheated groundnut oil is lesser than for heated oil, indicating that in this concentration the anti-radical activity increased rapidly with concentration. The gradients increased slowly and remain constant at higher concentrations of unheated groundnut oil. In these instances, ABTS* may have been largely reduced and the colour is not proportional to the amount of radical scavenger. The Inhibition concentration (IC_{50}) of unheated is 25.26% and heated 54.55%. The IC_{50} value of unheated groundnut oil is found to be lesser than that of heated oil by 46%. In the DPPH method the Inhibition concentration (IC_{50}) of unheated oil is 27.37% and 59.49%. Hence, in heated oil more concentration of antioxidant in the sample is needed to inhibit the peroxide formed in the assay. The free radical scavenging activity of unheated groundnut decreases whereas the heated oil increases along with the concentration. The loss of antioxidant activity in ABTS* and DPPH* radical decolorisation assay is

Table 3. Correlation % of inhibition with ABTS assay and DPPH assay

Name of the oils	ABTS		DPPH	
	R	p	R	P
Groundnut oil	+0.02	<0.001	+0.040	<0.001
Mustard oil	+0.011	<0.001	+0.041	<0.001
Sesame oil	+0.01	<0.001	+0.000	<0.002

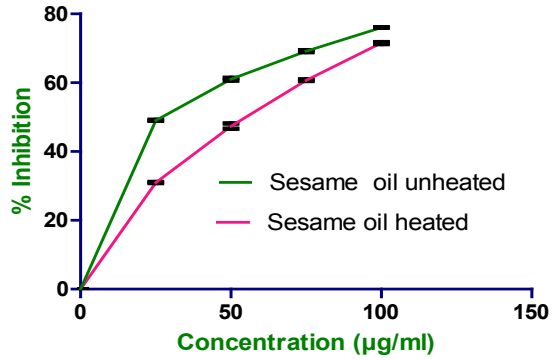

Figure 5. ABTS radical decolourisation of unheated and heated sesame oil

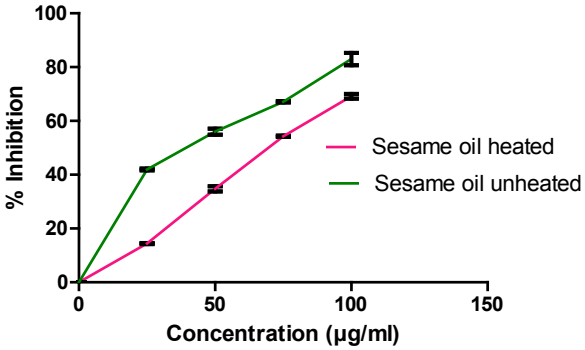

Figure 6. DPPH radical decolourisation of unheated and heated sesame oil

observed lower in heated groundnut oil due to loss of antioxidant in the oil. The result is also supported in the GCMS analysis of groundnut oil antioxidants vitamin E gets totally evaporated and quantification of vitamin B decreases with heating (Rubalya *et al*., 2014).

In-vitro *analysis of sesame oil*

The radical-scavenging activity of unheated and heated sesame oil is shown in Figure 5 and 6 illustrate the variation of percentage of inhibition with the concentration of oil. During concentration from 25 µg/ml to 100 µg/ml, the gradient of the curves of percentage of inhibition versus concentration for heated sesame oil is steeper than for unheated oil, indicating that in this concentration the anti-radical activity increased rapidly with concentration. The gradients increased slowly and remain constant at higher concentrations of unheated sesame oil. In these instances, ABTS* may have been largely reduced and the colour is proportional to the amount of radical scavenger. The IC_{50} value of unheated sesame oil is found to be higher than that of heated oil by 52%. The Inhibition concentration (IC_{50}) of unheated is 24.42% and heated 46.39%. The IC_{50} value of unheated is 26.22% and heated is found to be 49.18% hence the variation is about 53%. Hence, in heated oil more concentration of antioxidant in the sample is needed to inhibit the peroxide formed in the assay. It was observed that quantity of tocopherol in heated and processed sesame oil is 54 mg/100g (Mohamed and Awatif, 1998). It was explained by Edwin (1996) that the ascorbic acid in sesame oil can regenerate tocopherol on heating reacting with inactive chelating agents and increases the antioxidant stability.

At different concentration of unheated and heated sample of mustard oil, groundnut oil and sesame oil the antioxidant activity is found to be better in sesame oil compared to mustard and groundnut oil. Similar results were observed using ABTS analysis the radical scavenging decreases in the order mustard oil, sesame oil and groundnut oil. Using DPPH analysis the radical scavenging decreased in the order sesame oil, mustard oil and groundnut oil (Chandran *et al.*, 2014). The variance between ABTS and DPPH method for the entire sample are statistically significant ($p<0.01$). The correlation coefficient and significance between the two methods for unheated and heated sample is shown in Table 3.

Conclusion

The findings of this study indicate that antioxidant activity in each type of oils has different stability, contributed by the different antioxidant component in them. The efficiency of antioxidants in mustard, groundnut and sesame oil is studied using ABTS and DPPH radical scavenging assay. From the calculated Inhibition concentration for unheated and repeatedly heated oils, the antioxidants (tocopherol, sesame lignans etc.,) efficiency and stability of sesame oils is predicted stable compared to mustard and groundnut oils. The free radical scavenging activity in the sesame oil is comparatively stronger than other oils and it could be recommended for deep frying with less adverse effect.

Acknowledgement

The authors are thankful to the Vice Chancellor,

SASTRA University for allowing us to carry out this work in the University lab and also for his constant support and encouragement.

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