

Antioxidants in Leafy Vegetables and Their Efficacy in Preventing Lipid Peroxidation in Heated and Stored Oils

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ABSTRACT

This study was conducted with the objective of determining the antioxidants in four leafy vegetables (LVs), namely cabbage (*Brassica oleracea* var. *capitata*), coriander leaves (*Coriandrum sativum*), hongone (*Alternanthera sessilis*) and spinach (*Spinacia oleracea*) and study their efficacy in preventing lipid peroxidation in heated and stored oils. The vegetables were analyzed for ascorbic acid, total carotene and β -carotene and antioxidant capacity. The antioxidant efficacy of the LVs was determined by analyzing free fatty acid (FFA) and the peroxide values of heated and stored refined groundnut (GN) and sunflower (SF) oils for four weeks. Results revealed that ascorbic acid, total carotene and β -carotene content ranged between 12.8–49.8, 2.8–141.1 and 1.1–21.1 mg/100g, respectively. The total antioxidant capacity was found to be in the range of 6495.1–19902.0 $\mu\text{mol/g}$ of LV extract. Cabbage powder was effective in inhibiting peroxidation in GN and SF oils up to four weeks of storage whereas coriander leaf powder inhibited peroxidation in SF oil for four weeks. In the case of heated oils, all the four LVs significantly inhibited peroxide formation in the two oils (with the exception of spinach in the heated GN oil). Among the LVs, cabbage showed a comparatively better antioxidant property and was also effective in retarding the formation of FFA in stored oils. Overall, the LVs were more effective in preventing lipid peroxidation in SF oil than in GN oil indicating their substrate specificity. It can be concluded that LVs possess antioxidant properties hence, may serve as substitutes for synthetic antioxidants.

Keywords: antioxidant, cabbage, free fatty acids, groundnut oil, peroxides, sunflower oil

Abbreviations: ANOVA, Analysis of variance; BHA, butylated hydroxy anisole; BHT, butylated hydroxy toluene; FFA, free fatty acid; GLV, green leafy vegetables; GN, groundnut; PV, peroxide value; SF, sunflower; TBHQ, tert-butylatedhydroxyquinoline

INTRODUCTION

Edible fats and oils are predominantly composed of triglycerides. The physical and chemical characteristics of fats and oils are influenced greatly by the type and proportion of fatty acids and the way in which they are positioned on the glycerol moiety. The predominant fats are saturated and unsaturated fats (Handoo and Sharma 2000). Processing and storage of fatty food result in physical and chemical changes. While hydrolytic alterations are caused by moisture leading to the formation of free fatty acids, oxidative alterations caused by oxygen and high temperature lead to the formation of hydroperoxide (Paul and Mittal 1997).

The degradation products of lipids are known to cause off flavors in food and are of primary concern to manufacturers and consumers from a quality standpoint. Many of these degradation products are harmful to human health as they destroy vitamins, inhibit enzymes and potentially cause gastrointestinal irritations and other health hazards such as cancer through generating free radicals (Varela 1988; Paul and Mittal 1997; Sandal and Kalia 2000). Control of autoxidation by the use of synthetic antioxidants using BHA, BHT and TBHQ have been found to be efficient in preventing lipid peroxidation in edible oils and fried products but because of the associated health hazards, the utility of alternate suitable natural sources are being studied extensively. Vegetables such as roots and tubers, green leafy vegetables and other vegetables are known to possess significant antioxidant properties against lipid peroxidation (Plumb *et al.* 1996; Ramarathnam 1997; Gazzani *et al.* 1998).

Leafy vegetables, particularly green leafy vegetables (GLVs), are rich sources of vitamins, pigments and may possess medicinal properties. Hence, this investigation was

undertaken to determine the contents of antioxidant vitamins and antioxidant capacity of selected GLVs and study their efficacy in preventing lipid peroxidation in GN and SF oils.

MATERIALS AND METHODS

Refined groundnut (GN) oil and refined sunflower (SF) oil were procured from a supermarket of Mysore city (a district in southern India). These oils were manufactured a month before analysis. Four GLVs namely, cabbage (*Brassica oleracea* var. *capitata*), coriander leaves (*Coriandrum sativum*), hongone (*Alternanthera sessilis*) and spinach (*Spinacia oleracea*) were obtained from the local market. Synthetic antioxidant, BHA was purchased from Hi-media Laboratories, Mumbai, India. All the other chemicals used for the analysis were of analytical grade.

Analysis of antioxidant vitamins of GLV

Leafy vegetables were derooted, washed thoroughly in tap water to remove adhering mud particles, rinsed in distilled water and drained. They were analyzed for moisture content by the air oven drying method (AOAC 2000) and ascorbic acid by titrating against dichlorophenol indophenol dye using L-ascorbic acid as standard (Ranganna 1986). Total carotene was determined by extracting the samples in acetone and transferring to petroleum ether phase. Total carotene was read colorimetrically at 450 nm against petroleum ether. β -carotene was separated by column chromatography and read colorimetrically at 452 nm (Ranganna 1986).

Preparation of leafy vegetable powder

Cleaned vegetables were dried in a forced air oven at $50 \pm 2^\circ\text{C}$ for 8-10 hrs. The dried LVs were ground in a food processor (Kenstar,

India), passed through a 40 µm sieve and stored in airtight containers until further use.

Preparation of extract of leafy vegetable powder

The LV powder was extracted in 100 ml portions of pure ethanol (95%) in a shaker for 8 h in subdued light at room temperature. The extract was decanted and extraction was repeated with fresh ethanol until the extract was colorless. The extracts were pooled and evaporated in a rotary evaporator below 40°C until they were free of solvent.

Analysis of total antioxidant activity

This assay is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) complex at acidic pH (Prieto *et al.* 1999). An aliquot of 0.1 ml sample was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against a blank. A typical blank contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and was incubated under the same conditions as the rest of the samples. For samples of unknown composition, water-soluble antioxidant capacities were expressed as equivalents of ascorbic acid (µmol/g of extract).

Analysis of antioxidant efficacy of leafy vegetable powder

GN and SF oils, free of additives were used as substrates for oxidation studies. Oil samples with 2 g LV/100ml oil were prepared and stored in airtight brown bottles in the dark at room temperature. Oil samples with BHA at the permitted legal limit of 0.02% were prepared for comparative purposes and stored under identical conditions. Oil without additives served as the control. A similar set of oil samples with LV, BHA and control samples were induced to oxidation by heating to frying temperature (175°C) and placed in an incubator maintained at 180°C for 30 min and stored under identical conditions for 4 weeks.

The efficacy of the LVs was tested by determining the FFA and PV contents of both stored and heated oils on a weekly basis for four weeks. FFA (as % oleic acid) was estimated using an alkali titration method (AOCS 2000). PV (meq kg⁻¹ oil) was measured by titration with 0.1 M sodium thiosulfate solution using starch as an indicator (AOCS 2000). All determinations were carried out in duplicate and the data presented represents the mean of duplicate analysis. Significance of differences in the PV and FFA between antioxidant treated and untreated samples were analyzed using

ANOVA using a statistical package Minitab 11.12.

RESULTS AND DISCUSSION

Antioxidant vitamins and total antioxidant capacity of the LVs

Leafy vegetables are known for their high water content. Spinach and cabbage contained ≈93% moisture and coriander and hongone ≈86%. Ascorbic acid is one of the antioxidant vitamins abundantly present in LVs more than in other vegetables. As can be seen from **Table 1**, ascorbic acid content was lower in spinach, moderate in hongone and cabbage and higher in coriander leaves, among the LVs selected. These values were very much lower than those reported by Gopalan *et al.* (1996) for cabbage (124 mg/100 g), coriander leaves (135 mg/100 g) and spinach (28 mg/100 g) and higher for hongone (20 mg/100 g). These differences arose due to the variety, the environment in which vegetables are grown and the time gap between harvest and utility since ascorbic acid is highly prone to oxidation.

Carotenoids are the yellow-orange pigments present in leafy vegetables. β-carotene, which is a precursor of vitamin A, is known to be a powerful antioxidant. The carotenoid content of selected greens showed a wide variation (**Table 1**). It was very high in hongone and low in cabbage. β-carotene content also followed the same trend. However, when β-carotene was considered as percent of total carotene, a reverse trend was evident wherein cabbage had the highest β-carotene content and hongone the lowest.

The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid (Prieto *et al.* 1999). In GLVs, the total antioxidant capacity was found to be high in spinach (20127.4 µmol of ascorbic acid/g of extract) and low in cabbage (6495.1 µmol of ascorbic acid/g of extract). In coriander and hongone, the total antioxidant capacity was found to be 19902.0 and 15294.1 µmol of ascorbic acid/g of extract respectively. The antioxidant capacities were in the order of spinach > coriander > hongone > cabbage. There are not many studies reported in literature on the antioxidant capacity of vegetables as measured by the phosphomolybdenum method.

Antioxidant efficacy of LVs on storage stability of oils

The effect of addition of LVs on the PV and FFA contents of stored oils is presented in **Figs. 1A** and **1B** and **Table 2**. The PV of fresh GN oil was 0.53 meq/kg oil and showed no in-

Table 1 Antioxidant vitamins and total antioxidant capacity of leafy vegetables.

Leafy vegetables	Moisture (%)	Vitamin C (mg/100 g)	Total Carotene (mg/100 g)	β-Carotene (mg/100 g)	Total antioxidant capacity*
Cabbage	93.5 ± 0.05	19.4 ± 2.54	2.81 ± 0.09	1.11 ± 0.09	6495.1 ± 224.6
Coriander	85.4 ± 1.17	49.8 ± 6.35	40.18 ± 3.29	7.36 ± 1.43	19902.0 ± 331.6
Hongone	87.7 ± 1.43	19.1 ± 5.23	141.13 ± 0.57	21.11 ± 0.41	15294.1 ± 320.5
Spinach	94.0 ± 0.32	12.8 ± 2.45	17.70 ± 1.23	3.19 ± 0.20	20127.4 ± 341.8

Values are means and standard deviations.

* - expressed as µmol of ascorbic acid/g of extract.

Table 2 Free fatty acid of stored oils with leafy vegetable powders (%).

Storage Period	Control	BHA	Coriander	Spinach	Hongone	Cabbage	F-ratio
Groundnut oil – 0.02^a							
I week	0.02	0.03	0.04	0.04	0.07	0.03	8.33***
II week	0.03	0.03	0.04	0.04	0.09	0.03	
III week	0.03	0.03	0.04	0.04	0.14	0.04	
IV week	0.03	0.03	0.04	0.04	0.21	0.04	
Sunflower oil – 0.02^a							
I week	0.02	0.02	0.03	0.03	0.04	0.02	9.94***
II week	0.02	0.02	0.03	0.03	0.07	0.02	
III week	0.02	0.02	0.03	0.03	0.09	0.02	
IV week	0.02	0.02	0.03	0.04	0.13	0.02	

^a Initial value.

*** Differences between columns are extremely significant ($P \leq 0.001$).

crease up to 2 weeks, increased by 0.2 on the 3rd week and remained stable thereafter (Fig. 1A). The BHA-treated sample also showed the same trend. Of all the LVs, only cabbage which is devoid of chlorophyll exhibited a protective effect up to three weeks, while the others containing chlorophyll showed an increase in PV from the 2nd week itself, thereby indicating a pro-oxidative behavior. These results are in line with the observations of Fakourelis *et al.* (1987) who demonstrated that PV of olive oil increased with the concentration of chlorophyll on storage showing that chlorophyll acts as a pro-oxidant.

The PV of fresh SF oil was 4.2 meq/kg. On storage, the PV of the oil (control sample) doubled in first week, peaked by the third week and decreased slightly thereafter (Fig. 1B). Oil with BHA and all the LVs showed smaller increase in PV up to the 2nd week but the trend was similar. This is similar to the observations of several other studies which have shown that BHA prevents oxidative rancidity in oils, fried foods and processed foods (Noor and Augustine 1984; Kathya *et al.* 1994; Wanasundara and Shahidi 1994; Gordon and Kourimska 1995; Rehman *et al.* 2003). Oils with coriander and cabbage showed a lower PV than BHA-treated oil and the control for all four weeks, while spinach inhibited oxidation up to two weeks and hongone up to one week. The results indicate that cabbage and coriander leaves were effective in preventing oxidation for longer periods than other two LVs, indicating better antioxidant activities.

The LVs were effective in preventing lipid peroxidation more in SF than in GN oil, as can be seen in Figs. 1C and 1D, which could be attributed to the differences in the composition of oils. SF oil has a higher unsaturated fatty acid content compared to GN oil; hence it is more prone to oxidation. Among the LVs, cabbage can be considered as a potent antioxidant as it prevented oxidation changes in both oils. Hence it can be said that the antioxidant properties of LV is substrate specific.

The FFA of GN oil treated with BHA was 0.03% and remained unchanged until the 4th week. Oil samples treated with the LVs (except for hongone) had a slightly higher FFA than the control or BHA-treated samples but were stable for the entire storage period (Table 2).

The FFA of fresh SF oil was 0.02%, and remained unchanged throughout the study period in the control, BHA-treated and cabbage-added samples. Coriander- and spinach-added samples had a slightly higher FFA than the control or BHA-added samples but did not show any further increase during storage. Except for hongone, all the other LVs had a protective effect against the formation of FFA in both oils (Table 2).

Antioxidant efficacy of LVs on thermal stability of oils

On heating, the PV of GN oil increased from 0.53 meq/kg to 3.5 meq/kg oil by the 1st week, reached 5.78 meq/kg by 3rd the week and reduced to 4.5 meq/kg by the 4th week. PV is a measure of hydroperoxide formation; hydroperoxides are unstable and transitory components formed during oxidation. Hence PV passes through a maximum value at some point (during excessive oxidation) and will then gradually decline (Handoo and Sharma 2000). The PV of BHA-treated samples were higher than in corresponding controls. Coriander- and hongone-treated GN oil samples exhibited a lower PV than untreated samples up to 3 weeks. It is interesting to see that cabbage treated sample yielded a comparatively low PV throughout the storage period (Fig. 1C).

Of all the LVs, cabbage proved to be a comparatively effective antioxidant, but the protection conferred by coriander and hongone to a certain extent cannot be overlooked. The differences in the antioxidant properties between the oil samples were found to be significant at the 5% level ($F = 4.066^*$).

The PV of heated SF oil tripled from the 1st week to 2nd week, whereas that of SF oil heated with BHA showed less

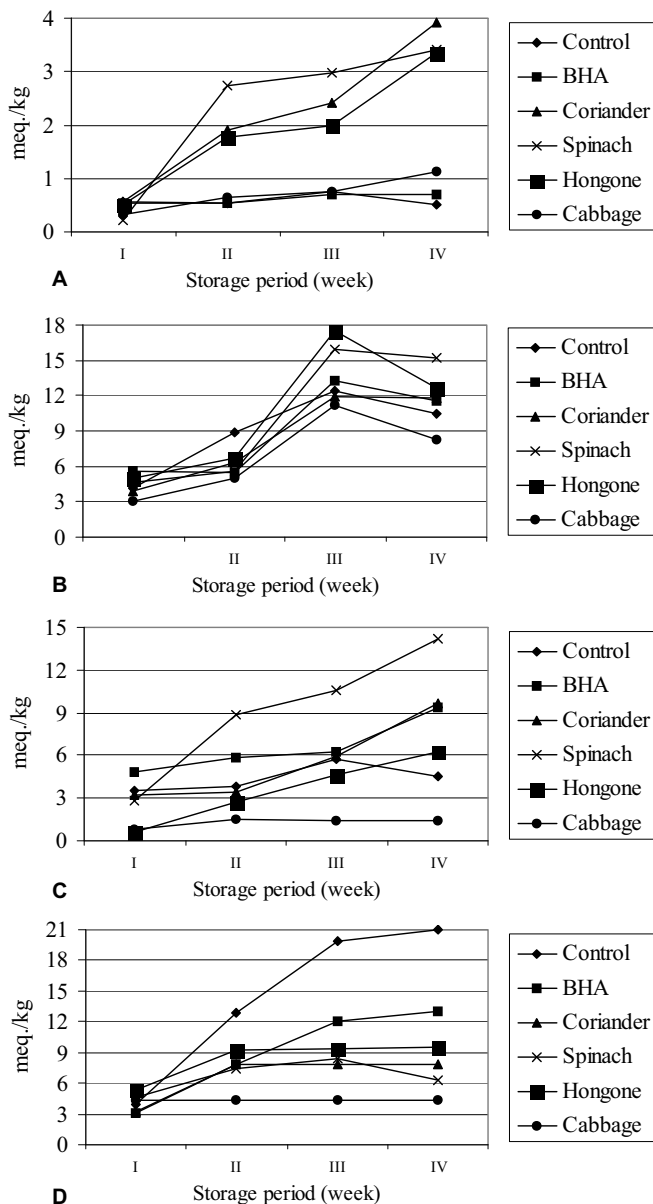


Fig. 1 (A) Peroxide value of groundnut oil stored with leafy vegetable powders. (B) Peroxide value of sunflower oil stored with leafy vegetable powders. (C) Peroxide value of groundnut oil heated with leafy vegetable powders. (D) Peroxide value of sunflower oil heated with leafy vegetable powders.

than a two-fold increase (Fig. 1D). Further increments in PV in both the control and BHA-treated samples were comparatively less than the 1st and 2nd week increment. SF oil heated with LVs showed lower peroxidation than the control and BHA-treated samples. The extent of inhibition of peroxidation varied widely among the LVs tested. SF oil heated with cabbage powder showed a 0.1 meq/kg increase in PV and remained unchanged throughout the storage period indicating excellent antioxidant property, better than the synthetic antioxidants tested. However the other three LVs also contained less peroxides than the control or BHA-treated samples indicating that they possessed better antioxidant activity than BHA. Our results are in line with the observations of Lee and coworkers (2002) who showed that oxidation products reduced in oils with spinach powder as the frying time increased, which was attributed to a greater release of carotene into fried oil that inhibits peroxidation.

ANOVA revealed that the differences that existed in antioxidant activities were significant at the 5% level ($F = 3.0154^*$). The role of LVs as oxidation stabilizing additives was for a shorter period in GN oil when compared to SF oil, which again can be attributed to the fatty acid profile of the oils.

Table 3 Free fatty acid of heated oils with leafy vegetable powders (%).

Storage Period	Control	BHA	Coriander	Spinach	Hongone	Cabbage	F-ratio
Groundnut oil – 0.02^a							
I week	0.03	0.02	0.04	0.04	0.04	0.04	5.76**
II week	0.03	0.02	0.04	0.04	0.04	0.04	
III week	0.03	0.03	0.05	0.04	0.04	0.04	
IV week	0.04	0.03	0.05	0.04	0.07	0.05	
Sunflower oil – 0.02^a							
I week	0.02	0.02	0.04	0.03	0.04	0.04	12.86***
II week	0.02	0.02	0.04	0.04	0.04	0.04	
III week	0.02	0.03	0.04	0.04	0.04	0.04	
IV week	0.03	0.03	0.05	0.04	0.05	0.05	

^a Initial value.** Differences between columns are highly significant ($P \leq 0.01$).*** Differences between columns are extremely significant ($P \leq 0.001$).

Since the formation of peroxides is higher in thermal treatment than in storage, prevention of oxidation by LVs is better reflected in heated samples. Possibly heating would have enabled the dissolution of fat-soluble vitamins and other substances that possess antioxidant activity into the oil and thereby arrested lipid peroxidation and the formation of peroxides.

On heating, the FFA of GN oil increased from 0.02 to 0.04 but remained steady on storage up to four weeks. The FFA of oil with BHA was stable for 2 weeks, increased by 0.01% after the 3rd week and remained stable thereafter. While the FFA of oils with LVs was higher than the control and BHA-treated samples, it remained stable until the end of the study period. ANOVA revealed that an increase in FFA following the addition of LVs was statistically significant ($F = 5.76^{**}$), showing that addition of LVs was not effective in controlling FFA formation or hydrolytic rancidity (Table 3).

The FFA of SF oil remained unchanged on heating up to three weeks of storage, and the BHA-treated sample also showed the same trend. Oils heated with LVs showed a higher FFA than the control with BHA. There were no significant differences in the FFA of oils with different LVs after the 1st week, and no increase was observed on further storage. Comparatively, increase in FFA was slightly more in SF oil treated with LV than in GN oil. On the whole it was evident that LVs were not effective in regulating the FFA content, irrespective of the oil or processing (Table 3).

The data were subjected to analysis of variance to de-

termine the level of significance of variously analyzed parameters. The results are presented in Table 4. As can be seen, SF oil did not exhibit any significant differences in peroxide value on storing and heating but GN oil exhibited marginally significant differences at the 5% level. The effect of LVs as oxidation stabilizing additives was for a shorter period in GN oil than in SF oil, which again can be attributed to the fatty acid profile of the oils. The increase in FFA following the addition of LVs was statistically significant for both the oils either on storage or on heating. This probably could be due to the effect of hongone greens, which did not exhibit protective properties.

CONCLUSIONS

LVs were found effective in preventing peroxidation on thermal processing in both SF and GN oil. Inhibition of lipid peroxidation by LVs was superior to that of BHA. Among the LVs, cabbage proved to be the best. The inhibition of lipid peroxidation was better in SF than in GN oil, which could be due to the differences in their fatty acid profile. LVs were not effective in preventing the formation of peroxides in unheated stored oils, which could be due to poor solubility of the antioxidant components. The significant inhibition of lipid peroxidation on thermal processing also indicates LVs stability at high temperatures. The study results suggest that LVs may serve as substitutes for synthetic antioxidants in food products. In addition, LVs are also a source of micronutrients and are more economical than the synthetic antioxidants.

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Table 4 Analysis of variance for the analyzed data.

Constituent	DF	MS	F-Ratio	P-value
PV of stored SF oil				
Between groups	5	6.6137	0.3456	0.8783 ^{ns}
Within groups	18	19.1350		
PV of stored GN oil				
Between groups	5	2.4936	3.0474	0.0363*
Within groups	18	0.8182		
PV of heated SF oil				
Between groups	5	6.6137	0.3456	0.8783 ^{ns}
Within groups	18	19.1350		
PV of heated GN oil				
Between groups	5	24.8466	4.0667	0.0120*
Within groups	18	7.0932		
FFA of stored SF oil				
Between groups	5	0.0024	9.9414	0.0001***
Within groups	18	0.0002		
FFA of stored GN oil				
Between groups	5	0.0057	8.3303	0.0003***
Within groups	18	0.0006		
FFA of heated SF oil				
Between groups	5	0.0003	12.8630	0.00002***
Within groups	18	0.00002		
FFA of heated GN oil				
Between groups	5	0.0002	5.7564	0.0024**
Within groups	18	0.00004		

DF, degrees of freedom; MS, mean sum of squares; ns, not significant.

Differences between the columns are significant (*, $P \leq 0.05$), highly significant (**, $P \leq 0.01$) or extremely significant (***, $P \leq 0.001$).

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