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Influence of Environment Conditions on the Chemical Composition and Antioxidant Activity of *Larrea divaricata* Aqueous Extract

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ABSTRACT

Natural antioxidants have gained considerable interest for their role in the prevention of autooxidation of fats and oils. *Larrea divaricata* Cav. is a South American plant widely distributed in Argentina with reported antioxidant, antitumoral and antimicrobial activities. The aqueous extract of the plant exerts antioxidant activity preventing the deleterious effect of temperature and oxygen on the antioxidant activity of vitamin C (Vit C). The aim of this work was: to assess the stability of the aqueous extract of *L. divaricata* (AE) under different environment conditions, such as different temperatures and air oxygen contents. The stability parameters evaluated included the percentage of majority compounds (flavonoid expressed as quercitrin (F) and nordihydroguaiaretic acid (NDGA)) and the antioxidant activity of the extract (AC). Results indicated that both compounds were affected by temperature and air oxygen, with F being more stable to high temperatures than NDGA, finding a decrease of 56% for F and a decrease ≥ 66% for NDGA. Nevertheless, NDGA was more sensitive to air oxygen, as an early decrease of 62% and a maximum decrease of 100% were found. The AC of AE decreased with high temperatures; however, air oxygen seemed to have a more important effect, for a decrease of about 42% was observed after 7 days of incubation. Even though, the environment conditions could affect the chemical composition of the whole extract, it was observed that not all the compounds were equally affected by temperature and air oxygen. Reaching a balance between these two parameters could allow a modulation of the antioxidant activity of this extract.

Keywords: Free radical scavenger activity, flavonoids, NDGA, stability of plant extract

INTRODUCTION

Larrea divaricata Cav. (Zygophyllaceae) is a South American plant widely distributed in Argentina. In popular medicine, it is used for treatment of tumours, inflammatory disease, rheuma and fever. It has been demonstrated that the methanolic extract presents antioxidant activity (Pedernera *et al.* 2006). Moreover, the aqueous extract (AE) exerts antioxidant and antimicrobial activities, preventing the oxidization of vitamin C (Vit C) induced by temperature and air oxygen (Micucci *et al.* 2011). Over the past years, natural antioxidants have gained considerable interest for their role in the prevention of the autooxidation of fats and oils. However, during manufacturing and storage, the active components are exposed to oxidation, hydrolysis, microbial degradation and other environment unfavorable conditions that render the product unstable (Rangari 2008). Therefore, the determination of stability of herbal drugs in formulations is important as the stability ensures that the drug remains within the specifications established in the Pharmacopoeias, regarding its identity, strength, quality and purity.

It is known that environment factors such as temperature, light, air (specifically oxygen, carbon dioxide and water vapors) and humidity can affect stability (Thakur *et al.* 2008).

To date, the stability of the AE of *L. divaricata* under different environment conditions has not been studied. As this extract is considered to be an antioxidant agent that prevents the oxidation of Vit C, the chemical constituents in relation to the antioxidant activity must be addressed.

The presence of nordihidroguaiaretic acid (NDGA) as well as a related compound and a flavonoid expressed as quercitrin have previously been demonstrated in the AE of *L. divaricata* (Davicino *et al.* 2011). It is known that NDGA and the flavonoids possess antioxidant activity (Siddique *et al.* 2006). Flavonoids exert their antioxidant activity by a variety of mechanisms including direct scavenging of reactive oxygen species, inhibition of enzymes responsible for superoxide anion production, chelation of transition metals involved in the generation of free radicals and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals (Peng *et al.* 2003; Veitch and Grayer 2008).

Based on this information, the aims of this work were: to study the stability of an aqueous extract of *L. divaricata* under different temperatures and air oxygen, and to analyze its chemical composition by HPLC, mainly the presence and amount of NDGA and flavonoid, expressed as quercitrin. The antioxidant activity under the same conditions was also evaluated.

This study was performed in view to establish suitable conditions for extract storage and handling, considering its potential use as an antioxidant supplement in food.

MATERIALS AND METHODS

Reagents

Six mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was diluted on 30 ml of absolute ethanol, protected from light and freshly prepared each time. TRISMA (SIGMA) was prepared at 1.2 % w/v in Tris-HCL buffer pH 7.4. Water employed to prepare working solution was of ultrapure quality (MilliQ). Methanol (HPLC grade J.T.

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Baker) and acetic acid (Merck, Argentine) were used. Pure standards were purchased from SIGMA USA: NDGA lot 19C-0504 and Quercitrin lot 14H 0957.

Plant material

Leaves of *Larrea divaricata* Cav., identified by employing morphological, anatomical and histochemical techniques, were used in this study (Anesini et al, 1996). One voucher specimen was deposited at CEFYBO-CONICET and another at the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires. The aqueous extract (AE) was prepared from air-dried leaves. Briefly, 750 mg were infused with 10 ml of sterilized boiling distilled water and heated with stirring for 45 min at 56°C. After maceration at 5°C for 72 h and centrifugation (20 min at 1500 rpm), the supernatant was lyophilized (Anesini *et al*. 1996).

Incubation conditions

The lyophilized AE was dissolved in distilled water to a concentration of 1 mg/ml. The incubations were performed under different temperatures (5, 45 and 100°C) and air oxygen amount to mimic storage conditions. For 5°C samples were placed in glass containers hermetically sealed and kept in a refrigerator. For 45 or 100°C samples were maintained in glass containers hermetically closed and kept in stove set at the selected temperature. When air oxygen was studied, samples were placed into glass containers at room temperature. All the samples were protected from light. Samples were analyzed after 2, 7 and 10 days of incubation (Micucci *et al*. 2011).

Measurement of DPPH free radical-scavenging activity

The antioxidant activity of the samples was determined by analyzing the scavenger activity of the free radical diphenylpicrylhydrazyl (DPPH) (SIGMA, St Louis, Mo, USA) according to Wang *et al*. (2007). The absorbance of DPPH diluted in water was considered as control. The capacity of AE to scavenge the DPPH radical was calculated by the following equation: Scavenging effect (%): [(1- absorbance of sample/ absorbance of control) × 100]. Results were expressed as Mean ± SEM of three experiments carried out in triplicate.

HPLC analysis

The amount of NDGA and flavonoid express as quercitrin present in the AE of *L. divaricata* leaves was determined by HPLC. The HPLC analysis was performed in a Varian Pro Star Instrument equipped with a Rheodyne injection valve (20 µl) and a Photodiode array detector set at 260 nm A Phenomenex – C18 (2) reverse-phase column Luna (250 mm × 4.6 mm and 5 µm) was used. The mobile phase A consisted of water and acetic acid (98: 2), phase B: Methanol and acetic acid (98:2). The elution gradient was from 15% B to 40% B in 30 min, 40% B to 75% B in 10 min; 75% B to 85% B in 5 min and 100% B in 5 min, and 100% B during 10 min and back to initial conditions. The mobile phase was delivered at a flow rate of 1.2 ml/min. The chromatographic procedure was performed at room temperature (18-25°C). Samples were analyzed with a Varian Cromatography deviced equipped with 9012 pumps, 9050 UV detector and 9065 diode array detector. Data were processed with Varian Star 5.5 (USA). Lyophilized AE (1 mg/ml) and pure standards were dissolved in methanol: water (70:30). Retention times for pure NDGA and pure quercitrin were 41.9 and 36.2 min, respectively. Stability was determined measuring the integrated peak area of each compound (Davicino *et al.* 2011).

Statistical analysis

The Student's *t*-test for unpaired samples was used to determine the level of significance. When multiple comparisons were necessary, the Dunnett's test was applied after ANOVA. Differences between means were considered significant when $P < 0.05$.

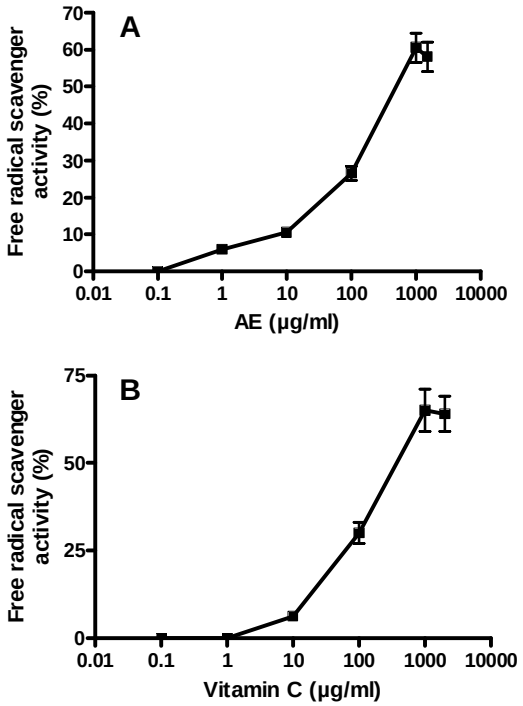


Fig. 1 Antioxidant activity of the aqueous extract (AE) of *L. divaricata* (A) and Vitamin C (Vit C) (B). The AE and Vit C were analyzed at different concentrations (from 0.01 to 1500 µg/ml). Results represent mean ± SEM (n = 3).

RESULTS AND DISCUSSION

In this work the stability of the AE of *L. divaricata* was studied in relation to its chemical composition and its antioxidant activity evaluated under different environment conditions. These conditions were chosen to mimic the storage conditions, mainly 5°C (refrigerator temperature), 45°C (tropical temperature), 100°C was used to mimic food cooking temperature or thermal pasteurization. The direct effect of air oxygen was also studied.

Firstly, the antioxidant activity of different concentrations of the AE was evaluated by the free radical DPPH scavenger test. This was done in order to select the concentration of the AE, with maximum antioxidant activity, to be used in subsequent studies. As it can be seen in **Fig. 1A**, the AE presented free radical scavenging activity that was dose-dependent. It is noteworthy that this activity was comparable to that exerted by Vit C, a positive control for antioxidant activity (**Fig. 1B**). The antioxidant activity of the AE of *L. divaricata* is well known, as it was demonstrated that it possesses a dose-dependent DPPH scavenger activity (Micucci *et al.* 2011). This extract is also capable of inducing the secretion of peroxidase in submandibulary glands of rats (Anesini *et al*. 2004). Moreover, the antioxidant activity of a *L. divaricata* methanolic extract was reported by Pedernera *et al*. (2006), who detected antioxidant activity mainly DPPH scavenging activity.

It has previously been determined that the DPPH scavenger activity exerted by *L. divaricata* AE is partially related to the presence of NDGA (Schreck *et al*. 1992; Turner *et al*. 2011). This extract was also proved to possess antioxidant and inhibitory activity on inflammation mediators (Franchi Micheli *et al.* 1986).

On the other hand, a flavonoid expressed as quercitrin was identified in the AE of the plant (Davicino *et al.* 2011). It is known that flavonoids can act as scavengers of free radicals such as reactive oxygen species (ROS), and also prevent their formation by chelating metals (Santos-Buelga and Scalbert 2000). Moreover, these compounds exert their antioxidant activity by means of enzyme inhibition, and/or induction of the expression of protective enzymes in biological samples (Erlund 2004).

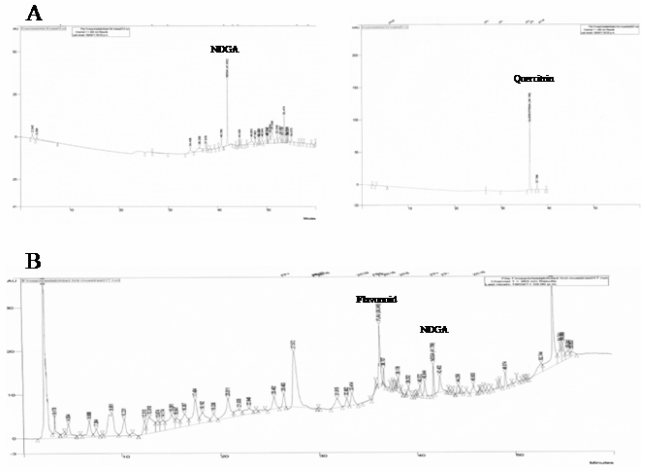
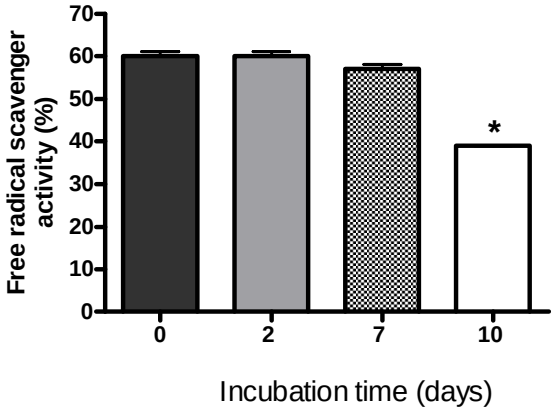


Fig. 2 HPLC analysis of AE. (**A**) HPLC of NDGA and quercitrin standards. (**B**) HPLC of the AE.

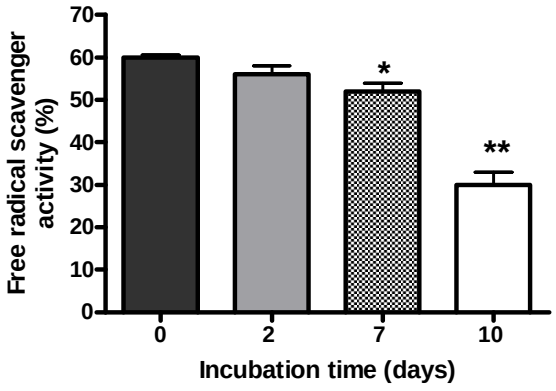


Incubation time (days) at 5 °C	Derivated expressed as quercitrin (%)	NDGA (%)
0	100 ± 8	100 ± 8
2	100 ± 7	100 ±7
7	93 ± 8	90 ± 8
10	85 ± 7	50 ± 4 *

Fig. 3 Effect of low temperature on the AE antioxidant activity (A) and on compounds content (inserted table). The extract was incubated at 5°C during different time periods. Results represent mean ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$ significant differences between time 0 and different incubation times (ANOVA and Dunnet's test).

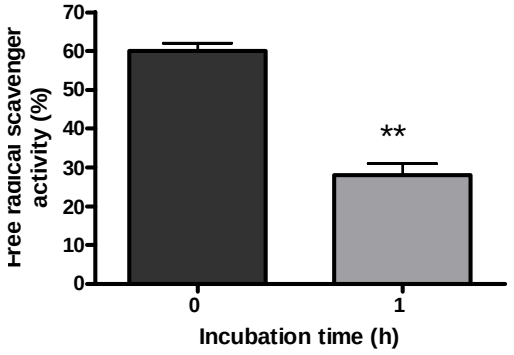
Taking into account these considerations, it was interesting to evaluate the extract at which, the oxidation of NDGA and the flavonoid could influence the overall antioxidant activity of the AE. A HPLC analysis was done in order to identify and quantify NDGA and the flavonoid, expressed as quercitrin, under room temperature and then under different conditions. **Fig. 2B** shows the level of NDGA and the flavonoid expressed as quercitrin in the extract in comparison with NDGA and quercitrin standards (**Fig. 2A**).

The control of storage conditions and the determination of physical and chemical parameters are suitable tools to deal with unstability of natural products. It is known that temperature, moisture and air oxygen are the major factors affecting the quality and stability of naturally occurring antioxidants which could be significantly lost during processing and storage (Jonsson 1991). Among the various processing methods currently used (blanching, slicing sterilization, dehydration, freezing and storage) thermal treatments have been reported to influence the antioxidant activity. Moisture absorbed onto the surface of solid drugs often increases the rate of decomposition of susceptible compounds. **Fig. 3** and the inserted table show that the antioxidant activity decreased significantly after 10 days of incubation at 5°C. The latter effect was observed for the NDGA content, but not for the flavonoid, which presented a slight and not significant decrease. At 45°C, the activity of the AE decreased significantly after 7 and 10 days of incubation, the same was observed with the NDGA content, whereas the flavonoid content only decreased in a significant manner after 10 days of incubation (**Fig. 4** and inserted table). When samples were incubated 1 h at 100°C, the antioxidant activity decreased significantly as well as the level of both compounds (**Fig. 5** and inserted table).



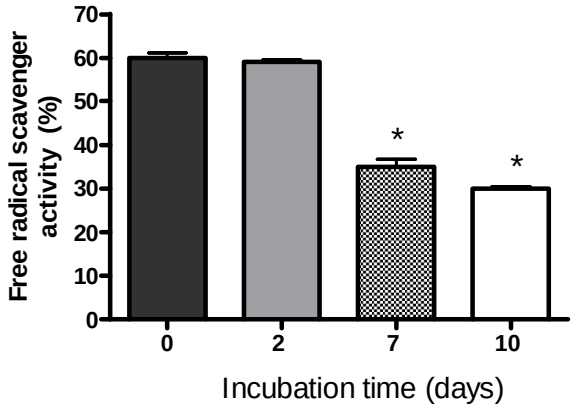
Incubation time (days) at 45 °C	Derivated expressed as quercitrin (%)	NDGA (%)
0	100±8	100±8
2	98±7	85±6
7	100±7	55.35±3**
10	34±2 **	34.05±2**

Fig. 4 Effect of the incubation at 45°C on AE antioxidant activity (A) and on compounds content (inserted table). The extract was incubated at 45°C during different times. Results represent mean ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$ significant differences between time 0 and different incubation times (ANOVA and Dunnet's test).



Incubation time (hour) at 100 °C	Derivated expressed as quercitrin (%)	NDGA (%)
0	100 ± 8	100 ± 8
1	44.03 ± 2 **	Traces

Fig. 5 Effect of the incubation at 100°C on AE antioxidant activity (A) and on compounds content (table inserted). The extract was incubated at 100°C during 1 h. Results represent mean ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$ significant differences between time 0 and different incubation times (ANOVA and Dunnet's test).



Incubation times (days) air oxygen	Derivated expressed as quercitrin (%)	NDGA (%)
0	100 ±8	100 ± 8
2	62 ± 4 *	100 ± 9
7	-	10 ± 0.8 *
10	-	-

Fig. 6 Effect of air oxygen on AE antioxidant activity (A) and on compounds content (inserted table). The extract was exposed to air oxygen during different times. Results represent mean ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$ significant differences between time 0 and different incubation times (ANOVA and Dunnet's test).

Finally, under air oxygen, the antioxidant activity also decreased after 7 and 10 days of incubation, as well as the content of NDGA and the flavonoid which began to decrease significantly on day 2 (**Fig. 6** and inserted table).

From the results it can be concluded that, the antioxidant activity of the AE decreased with high temperatures regardless of the incubation time but the antioxidant activity was more sensitive to air oxygen for an important decrease, about 42% was observed after 7 days of incubation. The fact that the extract decreased its activity under refrigerator temperature could be related to the presence of oxygen in the container.

When the amount of flavonoid and NDGA were determined, it was observed that they were affected not only by high temperature, but also by air oxygen. NDGA appeared to be affected not only by high temperature, as a decrease about 44 to 66% was observed, but also by air oxygen. It is noteworthy that NDGA was more sensitive to air oxygen than to temperature, as a decrease about 90-100% was observed under this condition. The flavonoid was more stable at high temperatures. The latter compound presented a decrease of about 66%, this value was observed after a long incubation time. Moreover, at 100°C the flavonoid exerted a decrease of 56% in comparison with NDGA which exerted a decrease of 100%. Therefore it can be deduced that the flavonoid was also more sensitive to air oxygen than to temperature, for the presence of oxygen, the decrease was observed from day 2 on (about 62%) to reach 100% after ten days of incubation. Besides, the flavonoid appeared to be more sensitive to air oxygen than NDGA for the decrease began early in time.

It is known that, phytoformulations often suffer degradation during storage by different reactions such as oxidation, hydrolysis, crystallization, emulsion breakdown, enzymatic degradation, etc. It has been demonstrated that, pasteurization of grapefruit at 95°C produces a degradation of flavonoids (Igual *et al.* 2011). Boiling, frying, and cooking may also result in the loss of flavonoids in foods (Crozier *et al.* 1997).

In order to correlate the antioxidant activity of the whole AE with the levels of compounds under different conditions, a regression analysis was done between NDGA and flavonoid content and the antioxidant activity of the AE. A good correlation was obtained between NDGA and antioxidant activity (**Fig. 7**). Therefore, the antioxidant activity of the AE can be attributed to the presence of NDGA as a good correlation (r^2 = 0.90) was obtained between antioxidant activity and the NDGA content. On the contrary, no good correlation was found between the antioxidant activity of the whole extract and the flavonoid content (r^2 = 0.65). It could be speculated that, the flavonoid could contribute to the antioxidant activity of the AE, once, the NDGA was completely degraded. At 100°C, the whole AE retained its antioxidant activity, when only traces of NDGA remained, however, a 44% of the flavonoid could be detected. Likewise, at 7 days of incubation at 45°C, the antioxidant activity decreased only 14% whereas the level of NDGA decreased 44%.

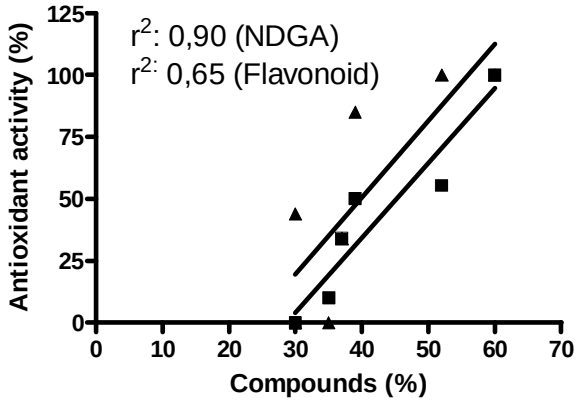


Fig. 7 Correlation between extract antioxidant activity and the compounds content. A correlation study was performed using antioxidant values and compounds content obtained at different conditions. NDGA (■); Flavonoid (▲).

The fact that the extract still maintained its antioxidant activity when NDGA and flavonoid were degraded by air oxygen, could be related to the presence of other antioxidant compounds.

CONCLUSIONS

It is noteworthy that the activity of the compounds evaluated was not equally affected by temperature and air oxygen, reaching a balance between these two parameters could allow the modulation of the antioxidant activity of the whole extract. As demonstrated elsewhere, under the same environment conditions, such as those used in this study, the AE *L. divaricata* was able to prevent Vit C oxidation (Micucci *et al.* 2011).

In conclusion, the NDGA was not the only antioxidant compound present in the extract. The deleterious effect of temperature and air oxygen on the antioxidant activity of these compounds could be overcome by the presence in the extract of others antioxidant (e.g. flavonoids). Finally, as a phytoformulation is a mixture of more than one natural active ingredient, with the potential use as antioxidant supplement, care should be taken to the determination of the stability profile to establish suitable conditions for storage and handling.

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REFERENCES

Anesini C, Genaro A, Cremaschi G, Sterin Borda L, Cazaux C, Borda E (1996) Immunomodulatory action of *Larrea divaricata* Cav. *Fitoterapia* **67**, 329-333

Anesini C, Turner S, Borda E, Ferraro G, Coussio J (2004) Effect of *Larrea divaricata* Cav extract and nordihydroguaiaretic acid upon peroxidase secretion in rat submandibulary glands. *Pharmacology Research* **49**, 441-448

Crozier A, Lean MEJ McDonald MS, Black C (1997) Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. *Journal of Agricultural and Food Chemistry* **45**, 590-595

Davicino R, Alonso R, Anesini C (2011) *In vivo* and *in vitro* activity of *Larrea divaricata* Cav. on EL-4 cells. *Human and Experimental Toxicology* **30**, 965-971

Erlund I (2004) Review of the flavonoids quercetin, hesperedin and naringenin. Dietary sources, bioactivities and epidemiology. *Nutrition Research* **24**, 851-874

Franchi-Micheli S, Luzzi S, Ciufti M, Zilletti L (1986) The effects of lipoxygenase inhibitors and leukotriene antagonist on anaphylaxis. *Agent Actions* **18**, 242-244

Igual M, García-Martínez E, Camacho MM, Martínez-Navarrete N (2011) Changes in flavonoid content of grapefruit juice caused by thermal treatment and storage. *Innovative Food Science and Emerging Technologies* **12**, 153-162

Jonsson I (1991) Thermal degradation of carotenoids and influence of their physiological functions. In: Freidman M (Ed) *Nutrition and Toxicological Consequences of Food Processing*, Plenum Pres, New York, pp 75-82

Micucci P, Alonso MR, Turner S, Davicino R, Anesini C (2011) Antioxidant and antimicrobial activities of *Larrea divaricata* Cav. aqueous extract on vitamin C from natural orange juice. *Food and Nutrition Science* **2**, 35-46

Pedernera AM, Guardia T, Guardia Calderón C, Rotelli AE, de la Rocha NE, Di Genaro S, Pelzer LE (2006) Anti-ulcerogenic and anti-inflammatory activity of the methanolic extract of *Larrea divaricata* Cav. in rat. *Journal of Ethnopharmacology* **105**, 415-420

Peng ZF, Strack D, Baumert A, Subramaniam R, Goh NK, Chia TF, Tan SN, Chia LS (2003) Antioxidant flavonoids from leaves of *Polygonum hydropiper* L. *Phytochemistry* **62**, 219-228

Rangari VD (2008) *A Textbook of Pharmacognosy and Phytochemistry* (Vol 1, 2nd Edn), Career Pub., Nashik, pp 78-100

Schreck R, Albermenn K, Bauerle PA (1992) Nuclear factor KB: An oxidative stress-responsive transcription factor of eukaryotic cells. *Free Radical Research Communications* **17**, 221-237

Santos-Buelga C, Scalbert A (2000) Proanthocyanidins and tannin-like compounds nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture* **80**, 1094-1117

Siddique YH, Beg T, Afzal M (2006) Protective effect of nordihydroguaiaretic acid (NDGA) against norgestrel induced genotoxic damage *Toxicology in Vitro* **20**, 227-233

Thakur AK, Prasad NA, Laddha KS (2008) Stability testing of herbal products. *The Pharma Review* **April-May**, 109

Turner S, Davicino R, Alonso MR, Ferraro G, Filip R, Anesini C (2011) Potential use of low-NDGA *Larrea divaricata* extracts as antioxidant in foods. *Revista Peruana de Bilogía* **18 (2)**, 159-164

Veitch NC, Grayer RJ (2008) Favonoids and their glycosides, including anthocyanins. *Natural Products Reports* **25**, 555-611

Wang KJ, Yang CR, Zhang YJ (2007) Phenolic antioxidants from Chinese toon fresh young leaves and shoots of *Toona sinensis*. *Food Chemistry* **101**, 365-371