ejpmr, 2018,5(12), 589-598

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Review Article

ISSN 2394-3211

EJPMR

ISOLATION AND CHARACTERIZATION OF WATER SOLUBLE ANTIOXIDANTS COMPONENTS FROM BLACK CUMIN (*Nigella sativa*) AND SWALLOW ROOT (*Decalepis hamiltonii*)

Vulli Venkata Rao[1]*, Vempati Poornodai[2], Nancy Bonareri Mitaki[3] and Suberu Safiu Adewale[4]

[1]Sunrise University, Rajasthan, India.
[2]Biomedical Department, College of Medicine and Health Sciences, Dilla University, Ethiopia.
[3]Department of Biochemistry, Kampala International University in tanzania (KIUT), Daressalaam, Tanzania.
[4]Department of Biochemistry, Kampala International University- Health Sciences, Uganda.

***Corresponding Author: Vulli Venkata Rao**

Sunrise University, Rajasthan, India.

Article Received on 19/10/2018 **Article Revised on 08/11/2018** **Article Accepted on 29/11/2018**

ABSTRACT

Spices are known for good antioxidant potency and they have been implicated against varieties of biological disorders. However majority of the studies have reported the presence of lipid soluble antioxidant. In human diseases major diseases including cancer are initiated by oxidative stress and oxidative stress mediated membrane damage, cellular/DNA damage, tissue damage etc. For efficient quenching of these oxidative stress induced damage in cells and molecules, both water soluble and lipid soluble antioxidants are essential. Current study thus focuses on the identification of any newer water soluble antioxidants and their potency in selected spices-black cumin (*Nigella sativa*) and swallow root (*Decalepis hamiltonii*). Results from this study shows that aqueous extracts of Black cumin – BCAE and Swallow root – SRAE showed multi-potent antioxidant properties including free radical scavenging, reducing power, inhibition of lipid peroxidation and cyto/DNA protectivity. BCAE and SRAE showed 1.9-2.5 fold increase in antioxidant potency as evaluated by free radical scavenging effect than their respective solvent (methanol) extracts – BCME and SRME.

INTRODUCTION

Plant and plant products are being recognized in recent days as safer and potential health promoting (Khan and Mukhtar, 2007), disease curing (Rathore *et al.*, 2005), immune system boosting (Bin-Hafeez *et al.*, 2003), detoxifying (Kaviarasan and Anuradha, 2007) and nutritive (Bovell-Benjamin, 2007) sources. Phenolic compounds constitute a group of substances that are widely distributed in the plant kingdom with different structures and biological activities. Among the phytochemicals, phenolic acids especially hydroxy cinnamates and hydroxy benzoates, the secondary metabolites, are commonly found in fruits, vegetables, seeds and other plant derived food sources (Herrmann, 1989). They have been reported to possess pharmacological properties such as antioxidant, antibacterial, and anticancer (Breinholt, 1999; Shahidi and Naczk, 1995).

The role of natural products are gaining more popularity in both developed and developing countries and much appreciated towards their applications as "alternatives" against chronic diseases such as diabetes, ulcer, cancer etc; particularly those diseases that require a long-term treatment, mainly due to complexities in their disease pathogenicity pattern. Although synthetic drugs are required for immediate relief, long term use of these drugs not only cause side effects such as nausea, allergy, immnosuppression etc., but leading themselves to be a causative factor for several disorders.

Black cumin (*Nigella sativa*) is an annual herb belonging to the family Ranunculaceae. Its seeds have been used since antiquity by Asian herbalist and pharmacist for its therapeutic effects against oxidative stress induced diseases such as cancer (Medenica *et al.*, 1997), immune disorders, as analgesic and antipyretic activity (Al-Ghamdi, 2001). Recent studies have indicated that a number of plant products (Oldham & Bowen, 1998), medicinal herbs (Andrea, 1997; Craig, 1999) and spices (Shobana and Naidu, 2000; Reddy and Lokesh, 1994) exhibit potent antioxidant activity. Black cumin has been reported to possess various biological activities (Morsi, 2000; El-Dakhakhny, Barakat, El-Halim and Aly SM, 2000; Zaoui, 2000; Burits and Bucar, 2000) mainly in its seed oil.

Swallow root (*Decalepis hamiltonii*), belongs to the Asclepiadaceae family. It is one of the most potent sources with varieties of bioactivities (Georgea *et al*., 1999; Anup and Shivanandappa, 2006; Naik *et al*., 2007), and the roots are being used in Ayurveda, the

ancient Indian system of medicine to stimulate appetite, relieve flatulence and as a general tonic. The roots are also used as a substitute for *Hemidesmus indicus* in Ayurvedic preparations (Nayar *et al*., 1978). Various laboratories have also put sustainable efforts in exploring the biochemical constituents present in swallow root and to understand their role as health beneficial sources.

Even though the presence of various phytochemicals have been noted in black cumin (Duke, 2005), they were all lipid soluble components associated with the oil fraction. There have been no reports on the role of water soluble components towards biological activities including antioxidant activity in black cumin. Current study thus focuses on the identification of any newer antioxidants and their potency in selected spices-black cumin (*Nigella sativa*) and swallow root (*Decalepis hamiltonii*). Studies also aimed at aqueous extracts since these extracts were found effective in Ayurveda and traditional medicinal formulations

MATERIALS AND METHOD

Plant material

Black cumin (BC) seeds and fresh swallow roots (SR) were purchased from three different vendors (n = 3) from a local market (Devaraja market, Mysore, Karnataka, India), sun dried for 3 days, and powdered in a mixer (Gopi, C. Lal Electrical and Mechanicals Co. Ambala, India) and preserved in dry condition at 4^{o}C until further extraction.

Preparation of water extract

One gram of defatted (refluxed with hexane) powdered sample was mixed with 10 mL of distilled water and boiled for 5 min, cooled and centrifuged at 5000 *g* for 10 min. The supernatant was collected, stored at 4 ºC till the completion of the experiment and referred as aqueous extract.

Black Cumin decoction was prepared by boiling 1 g of sample (black cumin seeds) in 100 mL of water for 20 min, cooled, filtered and concentrated to 10 mL, stored at 4 ºC till the completion of the experiment. The extract was referred as black cumin decoction (BCD).

Preparation of methanol extract

One gram of powdered sample was mixed with 10 mL of methanol and stirred for 20 min and centrifuged at 5000 *g* for 10 min. The supernatant was collected, stored at 4 ºC till the completion of the experiment and referred as methanol extract.

Determination of total phenol content

The total phenolic content of the samples were determined colorimetrically using the Folin-Ciocalteau method (Singleton and Rossi, 1965).

Determination of antioxidant activity

Antioxidant activity on human Low-Density lipoprotein (LDL) oxidation

Oxidative modification of low-density lipoprotein (LDL) in the arterial wall plays a key role in the pathogenesis of atherosclerosis. Hence, LDL is a good substrate for oxidation and to study the antioxidant activity of phytochemicals from spice sources (Aviram *et al.*, 2005). Plasma was collected from blood of human volunteers and stored at 4^{o}C. The LDL was prepared from the plasma using a differential ultracentrifugation method (Havel *et al*., 1995)**.** The amount of protein was estimated by using Folin-phenol method (Lowry et al., 1951) and the results were expressed as amount of malondialdehyde (MDA) formed in nmoles/mg LDL protein in presence and absence of different concentrations of samples.

Measurement of LDL protection in gel shift assay

Electrophoretic mobility of LDL before and after oxidation and in presence and absence of samples (BC) were examined by agarose gel electrophoresis according to the method of Nobel (1968).

Inhibition of lipoxygenase dependent lipid peroxidation

Lipoxygenases are iron-containing enzymes that catalyse the dioxygenation (Oxidation) of polyunsaturated fatty acids (PUFA).

Fatty acid (PUFA) + O2 → Fatty acid hydroperoxide

To study the inhibition of enzymatic lipid peroxidation by spice antioxidants, lipoxygenases are the best enzyme system. Lipoxygenases are found in plants, animals and fungi. In this study lipoxygenase from soybean has been employed. Enzymatic lipid peroxidation was measured spectrophotometrically by following an increase in absorbance due to the formation of lipid hydroperoxides according to the method of Narayan *et al.* 1999.

Scavenging effect of extracts on DPPH radical

1,1-Diphenyl-2-picrylhydrazyl is a stable free radical that accepts an electron or hydrogen to become a stable 1,1, diphenyl-2-picrylhydrazine molecule. The reduction in DPPH was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH is generally employed as a substrate to evaluate antioxidant activity of plant extracts (Lai *et al*., 2001).

The absorbance was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\text{Scavenging effect (\%)} = \frac{\text{(Absorbance of control - Absorbance of sample)}}{\text{Absorbance of control}} \times 100$$

Measurement of reducing power

The presence of reductants (i.e. antioxidants) in the sample causes the reduction of the Fe3+/ferricyanide complex to the ferrous form. Therefore, the Fe2+ can be monitored by measuring the formation of the Perl's Prussian blue colour at 700 nm. The FeCl3/K3Fe(CN)6 system offers a sensitive method for the semi-quanitative determination of dilute concentrations of antioxidants, which participate in the redox reaction. The reducing

power of samples and standard antioxidants were determined according to the method of Yen and Chen (1995).

Cytoprotective assay of BC & SR extract on cultured NIH 3T3 fibroblast cells exposed to tert – butyl hydroperoxide.

A cytoprotective test on NIH 3T3 cells was conducted as previously reported (Nardini *et al*., 1998).

DNA protection assay

DNA protection activity was performed using phage DNA according to the method of Henry and Steven (1998) with little modification. Briefly, phage DNA (0.5 g) was added to Fenton's reagent (0.3 mM H_2O_2, 0.5 M ascorbic acid and 0.8 M FeCl3) containing 0.16 – 0.64 g GAE phenol of BC and 0.6 µg each of SR phenolic acid extracts per reaction mixture. The final volume of the mixture was brought up to 20 L and then incubated for 30 min at 37°C and the DNA was analyzed on a 1 % agarose gel followed by ethidium bromide staining

Identification of phenolic acids by HPLC

The potential active components in sample (BC & SR) extracts were characterized by HPLC (model LC-10A. Shimadzu Corporation, Tokyo, Japan) analysis on a reverse phase Shimpak C_{18} column (4.6 x 250 mm) using a diode array UV-detector (operating at 280 nm).

Spectroscopic measurements of antioxidant molecules

The purified phytochemicals from the samples were characterized by UV absorption, infrared, mass and NMR studies. Mass were obtained with Finnigan MAT 95 mass spectrometer by injecting the sample dissolved in millipore water. Infrared spectroscopy of bioactive antioxidant isolated from the samples (~1.0 mg) were made by blending the sample thoroughly with potassium bromide and made into a thin disc. Its spectra was obtained with Perkin-Elmer (2000 system GC-IR) operating at 4 cm-1 resolution. 13C and 1H NMR spectra were recorded on a Bruker 500 MHz spectrometer. The samples (50 mg) were dissolved in DMSO (1 mL) for recording the spectra. The spectral data are reported as ppm downfield from tetramethylsilane (TMS) (=0).

Statistical Analysis

All the experiments were carried out in triplicates and the results were expressed as mean standard deviation (n=3). The significance of difference was calculated by Student's *t* test, and values *p < 0.05 and **p < 0.01 were considered to be significant. One way ANOVA followed by Duncan's multiple range test was also used to determine the difference in mean values between sample groups.

RESULTS

Total Phenolic content in Black Cumin and Swallow root extracts

The total phenolic contents of various extracts of black cumin as estimated by the Folin-Ciocalteau method are shown in Table 1. Our results indicated 3 - 4 fold higher phenolic content in BCAE (3.2 mg/g) compared to BCD (1.02 mg/g) and BCME (0.82 mg/g).

Table 1: DPPH radical scavenging activity, total antioxidant activity, reducing power and phenolic content, of black cumin extracts and known standard antioxidants.

Sample / Standard Antioxidants	DPPH radical Scavenging activity (IC_{50} in µg/mL)	Total reducing power in Units*	Total phenol (in mg/g GAE phenol)	Total AOA in Units** (% contribution)
Ascorbic acid	$1.30 \pm 0.02_b$	$8.56 \pm 0.11_f$	Standard	-
Tannic acid	$1.15 \pm 0.03_a$	$4.56 \pm 0.12_d$	$1.49 \pm 0.02_c$	1296_a (77%)
Protocatechuic acid	$1.35 \pm 0.08_b$	$3.88 \pm 0.15_c$	$0.51 \pm 0.01_a$	378_b (23%)
Gallic acid	$1.1 \pm 0.04_a$	$6.41 \pm 0.17_e$	Standard	-
Ferulic acid	$2.55 \pm 0.05_c$	$2.8 \pm 0.07_b$	Standard	-
Vanillic acid	$40.00 \pm 0.7_f$	$0.089 \pm 0.02_a$	Standard	-
BHT	$8.5 \pm 0.08_e$	$0.85 \pm 0.05_a$	Standard	-
BHA	$3.9 \pm 0.05_d$	$1.36 \pm 0.05_a$	Standard	-
BCAE	$1.36 \pm 0.06_b$	$4.16 \pm 0.05_{cd}$	$3.18 \pm 0.16_d$	-
BCME	$2.55 \pm 0.09_c$	$1.06 \pm 0.05_a$	$0.82 \pm 0.08_{ab}$	-
BCD	$1.77 \pm 0.04_b$	$2.89 \pm 0.09_b$	$1.02 \pm 0.06_b$	-

* 1 Unit = 0.1 OD at 700 nm; ** 1 Unit = IC_{50}. Values are expressed as mean ± SD (n=3). Mean value followed by different letters (a, b, c, d, e, f, g, h) in the same column are significantly different ($p \leq 0.05$).

Swallow root was fractionated into polysaccharide (SRAE-PS), protein (SRAE-protein), phenolic (SRAE-phenolic) fractions as shown in table 2. The total of ~ 98 mg/g of phenolics in SRAE, 75.7 mg/g has been recovered in SRAE-phenolic fraction. Remaining ~ 4 % has been found associated with polysaccharide and negligible amount (~1 %) in protein fraction of swallow root. In fact total yield of protein in itself is less ~ 3 % in case of aqueous extract which is ~ 7-fold lesser than carbohydrates. SRME – SR methanolic extract indicated 83.6 mg/g of total phenolic content.

Table 2: Total phenol, protein and carbohydrate content of various fractions of swallow root.

Swallow root fraction	Total phenol (in mg/g GAE phenol)	Total protein (in mg/g)	Total Carbohydrate (in mg/g)
SRAE	98.1 ± 1.01$_h$	3.76 ± 0.08$_c$	31.68 ± 1.34$_c$
SRME	83.6 ± 1.53$_g$	--ND--	--ND--
SRAE-PS	3.9 ± 0.09$_b$	0.063 ± 0.002$_a$	12.79 ± 0.19$_b$
SRAE-Protein	0.94 ± 0.02$_a$	2.9 ± 0.06$_b$	0.53 ± 0.02$_a$
SRAE-Phenolic	75.7 ± 3.59$_f$	0.06 ± 0.001$_a$	12.7 ± 0.21$_b$
SRFP	20.72 ± 0.77$_e$	--ND--	--ND--
SRHP	7.97 ± 0.50$_c$	--ND--	--ND--
SRBP	11.52 ± 0.54$_d$	--ND--	--ND--

Values are mean ± SD (n = 3). Mean value followed by different letters (a, b, c, d, e, f, g, h) in the same column are significantly different ($p \leq 0.05$); ND – Not determined.

SRAE – swallow root aqueous extract; SRME – swallow root methanol extract; SRAE-PS – polysaccharide fraction of swallow root aqueous extract; SRAE-Protein - protein fraction of swallow root aqueous extract; SRAE-Phenolic – Phenolic fraction of swallow root aqueous extract; SRFP, SRHP and SRBP are swallow root free , hydrolyzed and bound phenolic acid fractions, respectively.

Effect of Black Cumin extracts in inhibiting LDL oxidation

Figure 1 shows the antioxidant activity of various fractions of black cumin. It is evident from the results that BCAE had a higher LDL protective ability than that of BCD and BCME. Even though all the extracts showed activity with a significance level of $p < 0.05$, 3 and 4 fold higher activity was observed in BCAE compared to BCD and BCME at equal concentration of 7.5□g GAE phenol/mL respectively.

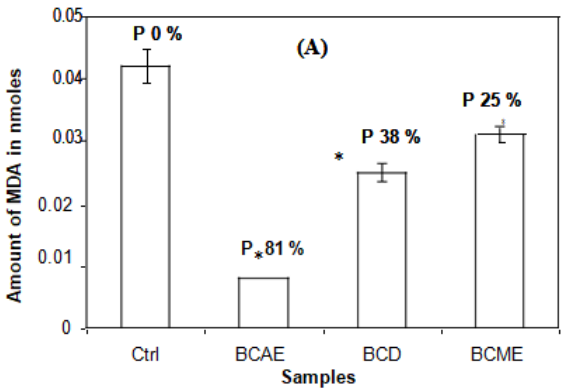

Fig. 1: Effects of BCAE, BCME and BCD on Cu++ induced LDL oxidation. Equivalent amounts of GAE phenol from each extract were used for inhibition. Results are expressed as mean □ SD (n = 3). * Represent $p < 0.05$ compared with control. % protection is indicated on top of the respective bar.

Figure 2 illustrates the protective effect of BCAE, BCD and BCME on LDL oxidation as evidenced by agarose gel shift assay also. Oxidized LDL (lane 2) showed an increased anodic mobility relative to that of unoxidized native LDL (lane 1). At equal concentrations (2 □g GAE phenol/mL), BCME and BCD showed lesser protective effect while BCAE had a higher LDL protective ability, which was nearly equal to that of the standard antioxidant ascorbic acid, tested at 2 □g/mL concentration.

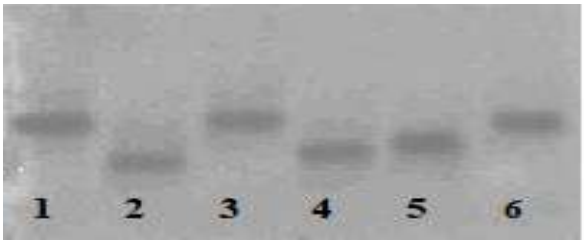

Fig. 2; Electrophoretic analysis of LDL oxidation induced by Cu++ and its protection by BC extracts and ascorbic acid. Native LDL - lane 1; oxidized LDL - lane 2. Antioxidant and black cumin extract treated - ascorbic acid 2 □g - lane 3; 2 □g GAE phenol of BCME – lane 4; 2 □g GAE phenol of BCD – lane 5; 2 □g GAE phenol of BCAE – lane 6.

Effect of Black Cumin extracts on soybean lipoxygenase induced linoleic acid peroxidation

Soybean lipoxygenase enzyme inhibitory activity of BCME increased gradually in a stoichiometric manner (Figure 3). In the case of BCAE there is a higher inhibitory activity at similar concentrations compared to BCME and gradually reached a saturation point showing 100 % inhibition at 120 □g GAE phenol/mL concentration. An IC50 of 60 □g GAE phenol/mL was therefore observed for BCAE extract compared to BCME, which showed an IC_{50} of 316 □g GAE phenol/mL and BCD which showed an IC_{50} of µg GAE phenol/mL.

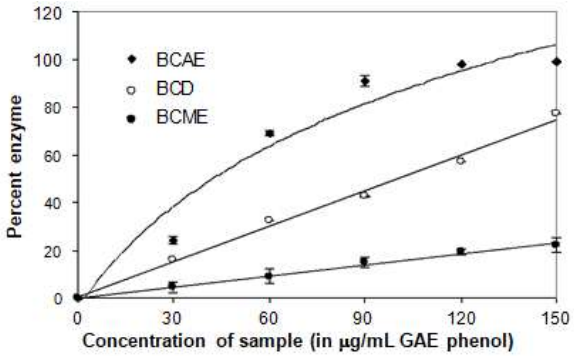

Fig. 3: Effect of BCAE, BCD and BCME on soybean lipoxygenase induced linoleic acid peroxidation. Results are expressed as mean □ SD (n =3).

Reducing power ability

The reducing power ability of BCAE was compared with BCME, BCD and standard antioxidants. Reducing power of the phenolic extracts of swallow root (Figure 4) indicated a dose dependent increase in activity of phenolic acid fractions of swallow root. The increased absorbance at 700 nm due to the reduction of potassium ferricyanide/ferric chloride complex indicates the presence of reducing power in all the phenolic acid extracts tested including the standard antioxidant BHA. The reducing power of BCAE indicated 1.4 and 3.9-fold higher activity compared to BCD and BCME, respectively (Figure 4).

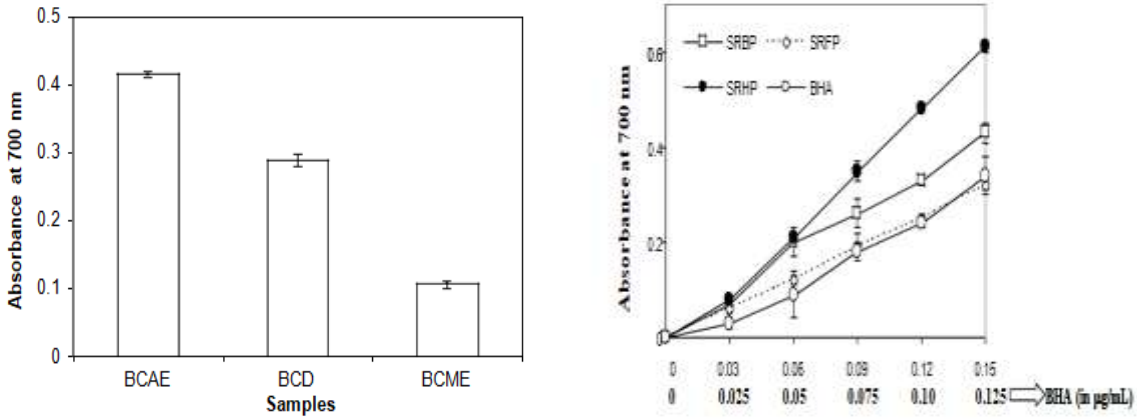

Fig 4: Reducing power of black cumin and swallow root extracts. BCAE-black cumin aqueous extract; BCD-black cumin decoction and BCME-black cumin methanol extract. SRFP-swallow root free phenolic acids; SRHP-swallow root hydrolyzed phenolic acids; SRBP-swallow root bound phenolic acids and BHA-butylated hydroxyl anisole.

Values are expressed as mean ± SD

Cytoprotective effect of BCAE and SR-Fractions

The cytoprotective effect of BCAE at various concentrations on the NIH 3T3 fibroblast cells treated with tert-butylhydroperoxide is shown in Figure 5. The results indicated an increased cell protection by BCAE and it followed a dose dependent response. The maximum cell protection of 65 % against oxidative stress was observed at a dosage of 32 □g GAE phenol/mL extract indicating the presence of antioxidant components in the water-soluble extract of black cumin.

It is also evident that phenolic acid extracts of SR showed dose dependent protection from 0.03 to 0.15 □g/mL concentrations. Among the phenolic acid extracts tested, at equal concentration of 0.12□g/mL, SRHP extract showed highest cytoprotectivity with ~ 87 % protection. SRFP and SRBP extract protected cells up to 47 and 65 %, respectively. Standard antioxidant BHA could show ~ 67 % cytoprotectivity at 0.125 µg/mL concentration.

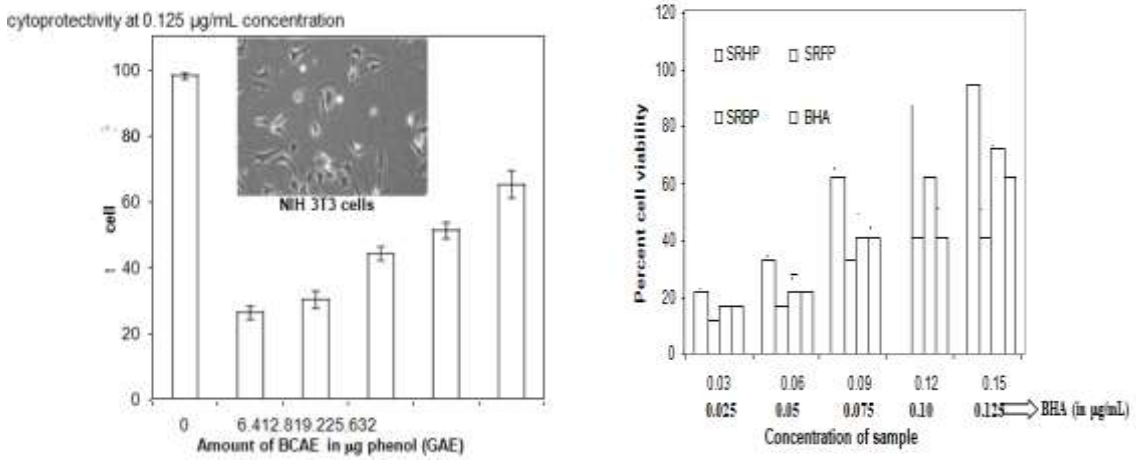

Fig. 5: Cytoprotective effect of black cumin and swallow root extracts on tert-butyl hydroperoxide induced cell damage of NIH 3T3 fibroblast cells.
Results are mean □ SD (n = 3). ** Represent $p < 0.01$ compared with control.

DNA Protective effect

To examine the DNA protective effect of BC extracts, □ phage DNA was subjected to oxidation using Fenton's reagent (hydrogen peroxide, ferric chloride and ascorbic acid). Figure 6 shows the extent of DNA damage induced by Fenton's reagent and the protection offered by BC extracts as revealed by the relative electrophoretic mobility of the oxidized and extracts treated DNA compared to that of undamaged DNA (lane 1). Addition of Fenton's reagent caused the fragmentation of DNA (lane 2) and hence increased the electrophoretic mobility. However, BCAE showed an increased DNA protection (lane 3-6, Figure 6A) with an increase in the dose (0.16 - 0.64 □g GAE phenol) of the extract. A maximum DNA protection was observed at 0.64 □g GAE phenol of the extract (lane 6 of 6A).

Figure 6B shows the DNA protective activity of SRFP, SRHP and SRBP extracts including BHA. Results indicated higher protection (82 %) in BHA treated (1 □g) while 80, 67 and 42 % protection were observed for SRHP, SRBP and SRFP extracts at each 0.6 □g GAE phenol concentration, respectively.

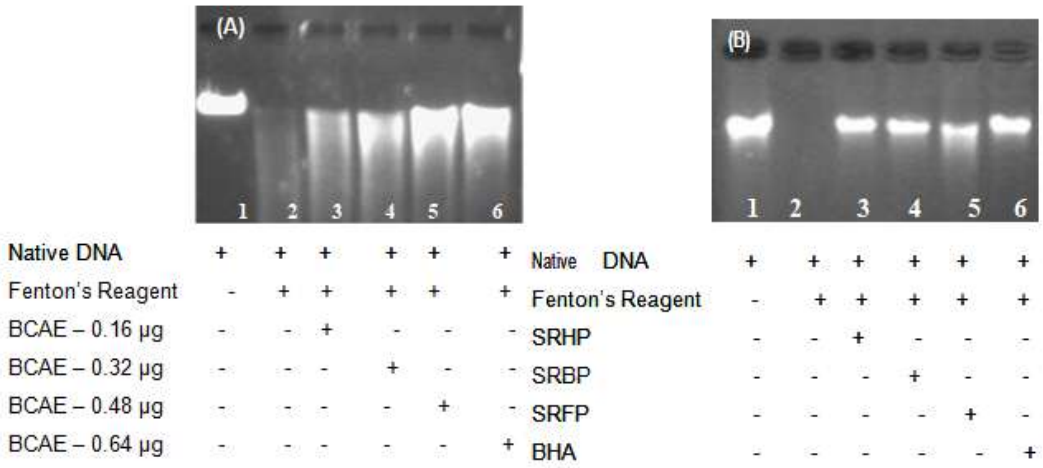

Fig. 6: Electrophoretic analysis of DNA damage and its protection by BC (A) and SR (B) extracts. (A) Lane 1 – Native DNA; Lane 2 – Oxidized DNA; Lane 3 – 6, BCAE treated (0.16-0.64 □g GAE phenol). (B) Electrophoretic analysis of DNA protection by free (SRFP), bound (SRBP) and hydrolyzed phenolic acid (SRHP) extracts (0.6 □g GAE each) in addition to standard antioxidant BHA (1 □g). Lane 1- native DNA; lane 2-oxidised DNA; lane 3-SRHP treated DNA; lane 4-SRBP treated DNA; lane 5-SRFP treated DNA and lane 6-BHA treated DNA.

Identification of phenolic acids in BC and SR extracts

BCAE profile in Figure 7 indicated a major component with retention time of 6.33 min similar to tannic acid and 8.63 min peak of protocatechuic acid was identified. Although other peaks with retention time of 5.88, 10.15 and 11.75 min were observed, only 6.33 min fraction exhibited activity as evaluated by the isolation of each fraction and determining antioxidant activity in them. In order to confirm that there are no overlapping peaks, different conditions of HPLC were performed and

compared the peaks with standard tannic acid. A peak coinciding with retention time equivalent to that of tannic acid was observed (in BCD) indicating that tannic acid is a major antioxidant.

In phenolic acids extracts, a total of 12 phenolic compounds were detected of which five were hydroxybenzoate derivatives and four were cinnamate derivatives. Also, two hydroxybenzaldehyde derivatives were identified. In total 12, 9, and 9 phenolic compounds were identified in SRFP, SRHP and SRBP extracts of swallow root, respectively. In SRFP extract, gentisic acid, 2-hydroxy-4-methoxybenzaldehyde (HMBA), vanillin, vanillic acid and *p*-hydroxybenzoic acid were the major phenolic compounds as evaluated by their retention time with standards. In SRHP extract, gallic acid and gentisic acid were the abundant phenolic acids, while the SRBP extract was constituted by HMBA, vanillic acid and *p*-hydroxybenzoic acid contributing to more than 70 % of phenolic acid content.

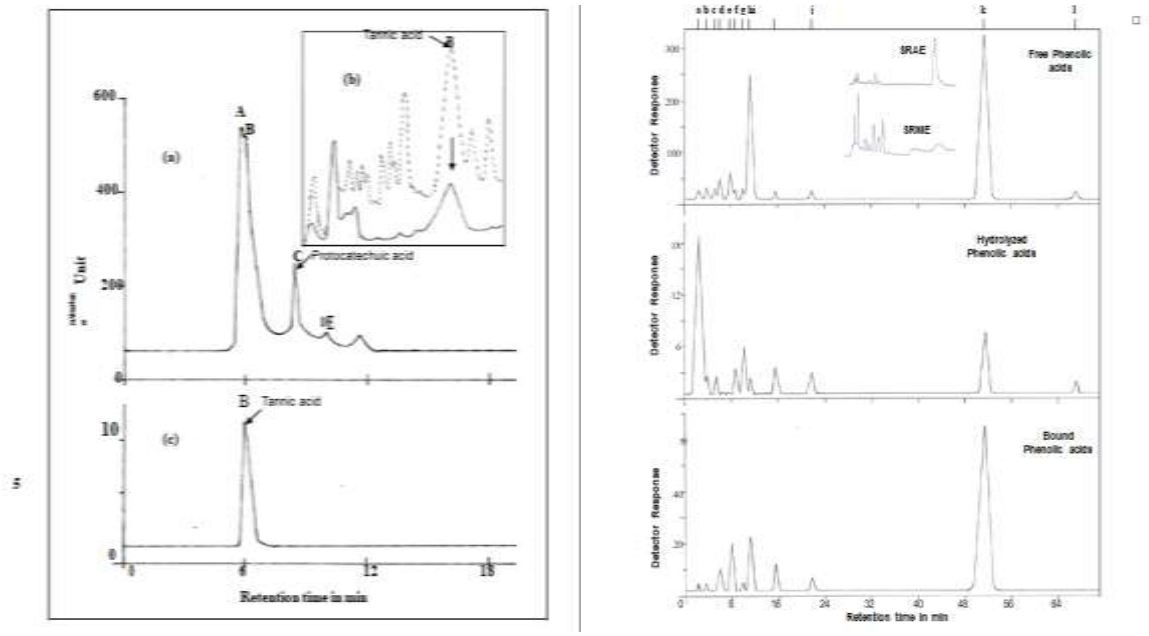

Fig. 7: HPLC profile of black cumin water extract, decoction and standard tannic acid on C-18 column. (a) A – 5.88 min; B – 6.33 min (active); C – 8.63 min; D – 10.15 min; E – 11.75 min. (b) HPLC profile of BCD and standard tannic acid (STA) on an amino column (inlaid). (c) HPLC profile of standard tannic acid on C18 column with RT – 6.33.

DISCUSSION

Lipid peroxidation caused by oxidative stress is detrimental to the cell both at membrane and genetic level, and has been attributed in the diseases such as cancer, cardiovascular diseases and diabetic complications (Cai and Harrison, 2000; Ames, 1987; Perry *et al*., 2000; Phull *et al.*, 1995; Hannon-Fletcher *et al.*, 1999). The hypothesis that the oxidative modification of low-density lipoprotein (LDL) plays a pivotal role in the progression of atherosclerosis has been widely accepted (Niki, 2004). This prevalent oxidation hypothesis implies that the antioxidants, which inhibit oxidation of LDL should be effective for suppressing atherosclerosis. It is evident from the results that BCAE had a higher LDL protective ability than that of BCD and BCME. The antioxidant activity in black cumin decoction may be due to the leaching of water soluble components during boiling. In addition, the ability of BCAE, BCME and BCD to protect LDL against Cu++ induced oxidative damage was also studied and the results showed higher activity in BCAE compared to BCME and BCD. These results indicated that lipoprotein peroxidation could be inhibited effectively by BCAE.

The oxidation of low density lipoprotein (LDL) by lipoxygenase has been implicated in the pathogenesis of atherosclerosis. It has been known that lipoxygenase mediated lipid peroxidation proceeds in general via regio, stereo and enantiospecific mechanisms, but that it is sometimes accompanied by a share of random hydroperoxides as side reaction products (Kuhn *et al*., 1994). Results indicated higher inhibitory activity in BCAE at similar concentrations compared to BCME. These results clearly showed that the water extract of black cumin was a potent inhibitor of lipoxygenase mediated lipid peroxidation, at least 10 fold better than BCME in *in vitro* inhibition of LDL oxidation.

DPPH is a stable free radical that accepts electron or hydrogen to become a stable diamagnetic molecule. BCAE exhibited an IC50 of 1.36 mg/mL, which was nearly equal to the IC50's of tannic and ascorbic acid. Also, 2-fold reduction in scavenging activity was observed in BCME in comparison with BCAE. In swallow root extract, the IC50's of SRFP, SRHP and SRBP for DPPH radical scavenging activity were in the range 0.04-0.13 µg GAE phenol/mL, which are nearly 34-98 folds more active than black cumin extract. These

results indicate the presence of antioxidant components which acts as free radical inhibitors both in BC and SR, but the activity is substantially low in BC compared to SR. The higher antioxidant activity of SR extracts over BC extracts is evident in all the antioxidant assays. The antioxidant components present in these extracts have effective activities as hydrogen donors and hence may stabilize the free radicals avoiding its reaction with cellular components.

The reducing power of a compound is related to its electron transfer ability and may, therefore, serve as an indicator of its potential antioxidant activity (Yildirim *et al*., 2003). Moreover, extracts with phenolic substance-mediated antioxidant activity were shown to be concomitant with the development of reducing power (Siddhuraju and Becker, 2003) thus, BC and SR extracts can act as electron donors and can react with free radicals and convert them to more stable products and terminate radical chain reaction (Chung *et al*., 2002).

The maximum cell protection (65 %) against oxidative stress was observed at a concentration of 32 □g GAE phenol/mL for BCAE and an equal percent protection was offered by SR phenolic acid extracts in the concentration range of 0.12-0.15 µg GAE phenol/mL. These results indicate the presence of antioxidant components in the water-soluble extract of BC and the phenolic acid fractions SR. Therefore it is possible that the intracellular reactive oxygen species in NIH 3T3 cells may also be reduced after treating cells with BC and SR extracts.

In biochemical systems the superoxide radical is converted by superoxide dismutase to hydrogen peroxide, which can subsequently generate extremely reactive hydroxyl radicals in the presence of transition metal ions especially iron (Halliwell & Gutteridge, 1981). Hydroxyl radicals can damage DNA. Addition of Fenton's reagent caused the fragmentation of DNA (lane 2) and hence increased the electrophoretic mobility. However, BCAE showed an increased DNA protection (lane 3-6) with an increase in the dose (0.16 - 0.64 µg GAE phenol) of the extract. A maximum DNA protection was observed at 0.64 µg GAE phenol of the extract (lane 6). In SR extracts treated study, SRHP showed higher DNA protection followed by SRBP and SRFP at 0.6 µg dosage. These results indicate that BC and SR extracts protects DNA from damage by quenching the hydroxyl radicals generated by the Fenton's reagent.

To assess the active molecule (s) exhibiting antioxidant activity in BCAE, the extract was fractionated by HPLC. A major component with retention time of 6.33 min constituting 1.49 mg/g and a minor component with a retention time of 8.63 min constituting 0.51 mg/g were isolated. The major component bound effectively to a tannin specific affinity matrix, and was confirmed as tannic acid by 2D NMR analysis. The other component matched with that of protocatechuic acid. Of the total activity in BCAE 77 % contribution is from tannic acid and the remaining is from protocatechuic acid (23%).

In swallow root, the study addresses whether the bioactivity is attributed to HMBA, a predominant component as reported earlier (Nagarajan *et al*., 2001) or to some other component. We determined the bioactive capacities employing various antioxidant assays along with standards and calculated the precise contribution of these components responsible for antioxidant activities of various fractions of swallow root. However, there are no reports available on the presence of phenolic acids and their contribution towards cytoprotective and antioxidant activity in swallow root. SRFP, SRHP and SRBP were isolated from swallow root by differential extraction procedure. Since phenolic acids were known to possess biological activity (Hsu *et al.*, 2006), the isolated phenolic acid extracts were evaluated for cytoprotective effect, antioxidant activity and identification of their constituent phenolic acids. The demonstrated cytoprotective and antioxidant ability may be involved in the health benefits that are attributed to swallow root phenolics and may work synergistically with other cytoprotective (Srivastava *et al*., 2007) and bioactive molecules reported earlier (Harish *et al*., 2005; Srivastava *et al*., 2006). A correlation coefficient was established between the phenolic content and their activity in each assay. Results indicated no significant correlation co-efficient suggesting that not only phenolic content but different phenolic constituent present in each fraction may be responsible for the activity. Even though the total phenolic content is higher in SRFP extract, at equal GAE phenolic concentration SRHP (1.2-fold) and SRBP (1.3-fold) showed higher antioxidant and cytoprotective activity than SRFP probably because of the presence of gallic acid, a very good antioxidant molecule up to 75 and 58 %, respectively. SRFP and SRBP indicated the presence of abundant poor antioxidant molecules like vanillin, HMBA, and *p*-hydroxy benzoic acid compared to SRHP. The differences in antioxidant activity may be attributed to the presence of different phenolic acids with different antioxidative potential and their synergistic effects. These results may strengthen the view of use of swallow root for their medicinal properties in Ayurveda and folklore medicine.

The presence of phenolic acids in bound form particularly in association with polysaccharides/lignin has been reported earlier (Iiyama *et al.*, 1990; Lapierre *et al*., 2001). Cinnamic acid derivatives are usually seen bound to polysaccharides (Shyama Prasad Rao and Muralikrishna, 2004). In swallow root however, the presence of derivatives of hydroxybenzoate (gallic aicd) and hydroxybenzaldehyde (vanillin and HMBA) were found in the bound form. Vanillin, HMBA and *p*-hydroxybenzoic acid being poor antioxidant molecules, the presence of these compounds had little contribution towards total antioxidant activity evidently as in the case

of SRFP and SRBP. Hence, the cytoprotective and antioxidant properties can be attributed to the phenolic acids like gallic, protocatechuic, gentisic, and vanillic acid in SRFP; gallic and gentisic in SRHP and gallic and *p*-coumaric acid in SRBP extracts. These phenolic acids were reported to possess good antioxidant activities (Miller & Rice-Evans, 1997). The presence of phenolic acids both in free and bound form attached to various polysaccharides is of significant interest in preventing oxidative stress induced diseases. The free phenolic acids are easily absorbed into the circulation while the phenolic acids bound to the polysaccharides are released by the intestinal enzymes as well as by the colonic microflora and can be absorbed into the circulatory system (Andreasen *et al*., 2001). This may have a significant role in the reduction of oxidative stress in lower alimentary canal also.

In conclusion, Black cumin and swallow root phenolic acid extracts showed antioxidant activity including cytoprotectivity, reducing power, radical scavenging ability and protection to DNA damage induced by hydroxyl radical. Black cumin aqueous extract had significant activity than their methanolic extact and decoction. While, in swallow root, hydrolyzed phenolic acid extract (SRHP) showed good antioxidant activity followed by bound (SRBP) and free (SRFP) phenolic acid extracts.

REFERENCE

1. Al-Ghamdi, M. S. *Journal of Ethnopharmacology*, 2001; 76: 45-48.
2. Ames, B. N. *Annals of Internal Medicine*, 1987; 107: 526-545.
3. Andrea, T. B., Robert, M. H., Carl, L. K., Judith, S. S and Gershwin, M. E. *American Journal of Clinical Nutrition*, 1997; 66: 1303-1312.
4. Andreasen, M. F., Kroon, P. A., Williamson, G and Garcia-Conesa, M. T. Free Radical Biology and Medicine, 2001; 31: 304-314.
5. Anup, S and Shivanandappa, T. Hepatology Research, 2006; 35: 267–275
6. Bin-Hafeez, B., Haque, R., Parvez, S., Pandey, S., Sayeed, I and Raisuddin, S. *International Immunopharmacology*, 2003; 3: 257-265.
7. Bovell-Benjamin, A. C. Advances in Food and Nutrition Research, 2007; 52: 1-59.
8. Breinholt, V. Desirable versus harmful levels of intake of flavonoids and phenolic acids, In *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*; Kumpulainen, J., Salonen, J., Eds.; The Royal Society of Chemistry: Cambride, UK, 1999.
9. Burits, M and Bucar, F. *Phytotherapy Research*, 2000; 14: 323-328.
10. Cai, H. and Harrison, D. G. *Circulation research*, 2000; 87: 840-844.
11. Craig, W. J. *American Journal of Clinical Nutrition*, 1999; 70: 491S-499S.
12. Chung, Y., Chang, C., Chao, W., Lin, C and Chou, S. *Journal of Agricultural and Food Chemistry*, 2002; 50: 2454-2458.
13. El-Dakhakhny, M., Barakat, M., El-Halim, M. A and Aly, S. M. *Journal of Ethnopharmacology*, 2000; 72: 299-304.
14. Georgea, J., Ravishankar, G. A., Keshava, U. N and Udayasankar, K. Fitoterapia, 1999; 70: 172-174.
15. Halliwell, B and Gutteridge, M. C. FEBS Letters, 1981; 128: 347-352.
16. Hannon-Fletcher, M., Hughes, C., O'Kane, M. J., Moles, K. W., Barnett, C. R and Barnett, Y. A. An investigation on *in vivo* antioxidant status and DNA damage in patients with IDDM, In. *Antioxidants in human health and disease*, Ed. Basu, T. K., Temple, N. J & Garg, M. L. CAB International, Wallingford, Oxon, UK, 1999; 259-269.
17. Harish, R., Divakar, S., Srivastava, A and Shivanandappa, T. *Journal of Agricultural and Food Chemistry*, 2005; 53: 7709-7714.
18. Havel, R. J., Eder, H. A and Bragdon, J. H. *Journal of Clinical Investigation*, 1995; 34: 1345-1352.
19. Henry, R and Steven, A. A. Free Radical Research, 1998; 29: 499-510.
20. Herrmann, K. *Critical Reviews in Food Science and Nutrition*, 1989; *28:* 315-347.
21. Hsu, C. L., Huang, S. L and Yen, G. C. Journal of Agricultural and Food Chemistry, 2006; 54: 4191-4197.
22. Iiyama, K., Tuyet Lam, T. B and Stone, B. A. Phytochemistry, 1990; [29: 733-737.
23. Kaviarasan, S and Anuradha, C. V. Pharmazie, 2007; 62: 299-304
24. Khan, N and Mukhtar, H. Life Sciences, 2007; 81: 519-533.
25. Kuhn, H., Belkner, J., Suzuki, H and Yamamoto, S. *Journal of Lipid Research*, 1994; 35: 1749-1759.
26. Lai, L. S., Chou, S. T. and Chao, W. W. *Journal of Agricultural and Food Chemistry*, 2001; 49: 963-968.
27. Lapierre, C., Pollet, B., Ralet, M. C and Saulnier, L. Phytochemistry, 2001; 57: 765-772.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L and Randall, R. J. *Journal of Biological Chemistry*, 1951; 193: 265-276.
29. Medenica, R., Janssens, J., Tarasenko, A., Lazovic, G., Corbitt, W., Powell, D., Jocic, D and Mujovic, V. *Proceedings/Annual Meeting of the American Association for Cancer Research*, 1997; 38: A1377.
30. Miller, N. J and Rice-Evans, C. A. *British Food Journal*, 1997; 99: 57-62.
31. Morsi, N. M. *Acta Microbiologica Polonica*, 2000; 49: 63-74.
32. Nagarajan, S., Rao, L and Gurudutt, K. N. *Flavor and Fragrance Journal*, 2001; 16: 27-29.
33. Naik, Y., Jayaram, S., Harish Nayaka, M. A., Lakshman and Dharmesh, S. M. *Journal of Ethnopharmacology*, 2007; 112: 173–179.
34. Narayan, M. S., Akhilender Naidu, K., Ravishankar, G. A., Srinivas, L and Venkataraman, L. V.

Prostaglandins, Leukotrienes, and Essential Fatty Acids, 1999; 60: 1-4.
35. Nardini, M., Pisu, P., Gentili, V., Naterlla, F., Felice, M. D., Piccolella, E and Scaccini, C. Free Radical Biology and Medicine, 1998; 25: 1098-1105.
36. Nayar, R. C., Shetty, J. K. P., Mary, Z and Yoganarasimhan, S.N., *Proceedings of the Indian Academy of Science*, 1978; 87B: 37–48.
37. Niki, E. *Biochemical Society Transactions*, 2004; 32: 152-159.
38. Noble, R. P. *Journal of Lipid Research*, 1968; 9: 693-700.
39. Oldham, K. M and Bowen, P. E. *Journal of the American Dietetic Association*, 1998; 98: 1001-1008
40. Perry, G., Raina, A. K. L., Nonomura, A., Wataya, T., Sayre, L. M and Smith, M. A. Free Radical Biology and Medicine, 2000; 28: 831-834.
41. Phull, P. S., Green, C. J and Iacyna, M. R. *American Journal of Gastroenterology and Hepatology*, 1995; 7: 265-274.
42. Rathore, D., McCutchan, T. f., Sullivan, M and Kumar, S. Expert Opinion and Investigation on Drugs, 2005; 14: 871-883.
43. Reddy, A. C and Lokesh, B. R. *Annals of Nutrition and Metabolism*, 1994; 38: 349-358.
44. Shahidi, F. and Naczk, M. 1995. *Food Phenolics*. Technomic publishing Co, Lancaster, PA/Basel, Switzerland.
45. Shobana, S and Naidu, K. A. Prostaglandins, Leukotrienes, and Essential Fatty, 2000.
46. Shyama Prasad Rao, R and Muralikrishna, G. Food Chemistry, 2004; 84: 527-531.
47. Siddhuraju, P and Becker, K. *Journal of the Science of Food and Agriculture*, 2003; 83: 1517-1524.
48. Singleton, V. L and Rossi, J. A. *American Journal of Enology and Viticulture*, 1965; 16: 144-158.
49. Srivastava, A., Harish, R and Shivanandappa, T. *Journal of Agricultural and Food Chemistry*, 2006; 54: 790-795.
50. Srivastava, A., Rao, L. J and Shivanandappa, T. *Food Chemistry*, 2007; 103: 224-233.
51. Yen, G. C and Chen, H. Y. *Journal of Agricultural and Food Chemistry*, 1995; 43: 27-32.
52. Yildirim, A., Mavi, A and Kara, A. A. *Journal of the Science of Food and Agriculture*, 2003; 83: 64-69.
53. Zaoui, A., Cherrah, T., Lacaille-Dubois, M. A., Settaf, A., Amarouch, H and Hassar, M. Therapie, 2000; 55: 379-382.