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Phytochemical screening, antioxidant and antiproliferative activities of successive extracts of *Couroupita guianensis* Aubl. plant

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Couroupita guianensis Aubl. is widely used in Indian traditional medicines to treat stomach aches, cold, skin diseases, microbial and fungal infections. In the present study series of experiments were performed to screen phytochemical constituents, antioxidant and antiproliferative activities of successive extracts of leaves, stem and crude methanolic extract of flowers of *Couroupita guianensis*. The extracts were evaluated for their antioxidant activity by 1,1'-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, nitric oxide free radical scavenging activity, hydrogen peroxide scavenging, lipid peroxide inhibitory activity, hydroxyl radical by deoxyribose method and total antioxidant capacity of extracts and cytotoxic property of extracts as well as total phenolic content. The methanolic leaf extract showed highest total phenol content. Ethyl acetate and methanol extracts were found to possess excellent antioxidant activity in most of the tested methods. In the cytotoxicity study of extracts, the results suggested that ethyl acetate leaf and crude methanolic flower extracts possess better activity.

Keywords: DPPH, Hydroxyl radical, Lipid peroxidation, Nitric oxide free radical, Total antioxidant capacity, Total phenol content.

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Introduction

Couroupita guianensis Aubl. (Family: Lecythidaceae) is widely used in Indian traditional medicines to treat colds, stomach aches and skin diseases, microbial and fungal infections. South America has even used tree parts for treating malaria. The phytoconstituent isatin isolated from flower was reported for antioxidant and anticancer activity[1]. The ethanolic extract of the leaf has shown antinociceptive activity and also responsible for a protective effect against oxygen reactive species[2,3]. Its antimicrobial[4] and antibiofilm properties were also reported[5]. The ethanol extract of leaves of *C. guianensis* was reported for anti-inflammatory and anti-ulcer, anti-oxidant and hepatoprotective properties[6-9]. The oxidation mechanisms and free radical role in living systems have gained increased attention[10]. Oxygen uptake inherent to cell metabolism produces ROS. ROS, which includes free radicals such as superoxide anion radicals (($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and non free-radical species such as hydrogen peroxide and singlet oxygen, are various forms of activated oxygen[11,12]. Antioxidants can protect the human body from free radicals and reactive oxygen species (ROS) effects. These can reduce the progress of many diseases as well as lipid peroxidation[13]. The plant has been reported several times for antioxidant and antimicrobial activities especially on ethanol and methanol leaf extracts. In the present study, the research attempt was made to find successive extracts of *C. guianensis* leaves, stem and flowers for their *in vitro* antioxidant and antiproliferative properties.

Materials and Methods

Plant material

The leaves, flowers and stem of *C. guianensis* were collected from Dasarigatta, Tiptur Taluk, Tumkur District, Karnataka, India, during the month of July 2011. It was authenticated by Mr. Ramu G, Department of Pharmacognosy and Phytochemistry, SAC College of Pharmacy, B.G. Nagara, where a voucher specimen no. 102 has been preserved.

Chemicals

1, 1'-Diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteau reagent, ammonium molybdate, gallic acid, ascorbic acid, sodium nitrite, sodium carbonate,

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petroleum ether, ethyl acetate, chloroform and methanol were purchased from Sigma Aldrich, Bangalore. Potassium dihydrogen phosphate, potassium hydroxide, ferric chloride, ferrous sulphate, potassium ferricyanide, thiobarbituric acid (TBA), butylated hydroxyl anisole (BHA), trichloro acetic acid (TCA), NEDD (naphthyl ethylene diamine dihydrochloride) and aluminium chloride ($AlCl_3$) were purchased from Merck (Mumbai, India). The reagents used were of analytical grade.

Preparation of extracts

The shade dried leaves (310 g) and stem (177 g) were powdered and extracted successively and separately with 1500 mL each of petroleum ether (60-80 °C), chloroform, ethyl acetate and methanol in Soxhlet extractor for 20 hours. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50 ºC) in a rotary evaporator. The extracts were then stored at 4 °C in a refrigerator for further use. PLE- Petroleum ether leaf extract; CLE-Chloroform leaf extract; ELE- Ethyl acetate leaf extract; MLE-Methanol leaf extract; PSE-Petroleum ether stem extract; CSE-Chloroform stem extract; ESE-Ethyl acetate stem extract; MSE-Methanol stem extract.

Crude extraction of *C. guianensis* flowers

The flowers (300 g) were macerated in 80 % methanol (1500 mL) for 7 days with occasional shaking. The macerated solution was concentrated to dryness in a rotary evaporator. The extract was stored in the refrigerator for further use. Crude methanol flower extract (CMF).

Preliminary phytochemical screening

Phytochemical screening of *C. guianensis* extracts was performed according to the method described by Harborne (1973)[14] and Kokate (1997)[15]. This experiment was carried out to detect the presence of carbohydrates, alkaloids, steroids, sterols, amino acids, flavonoids, saponins, terpenoids and glycosides.

Total phenol content of the extracts

Total phenol content of the extracts was determined by using the Folin-Ciocalteu method[16]. To an aliquot of 0.2 mL extract solution (1 mg/mL to 0.1 mg/mL), 1 mL of Folin-Ciocalteu reagent was added. After 4 minutes, 0.8 mL of sodium carbonate solution (4 %) was added to the mixture. After 2 hours, the absorbance was measured at 750 nm by using Shimadzu UV-160 spectrophotometer. Using gallic acid monohydrate, a standard curve was prepared and linearity was obtained in the range of 10-50 µg/mL. The total phenol content of the extract was determined using the standard curve and expressed as gallic acid equivalent in mg/g of the extract. All determinations were carried out in triplicates.

Determination of antioxidant activity

Total antioxidant capacity

The total antioxidant capacity of successive and crude extracts was determined by phosphomolybdenum method[17]. 0.2 mL of the extract solution in DMSO was combined with 2 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in an Eppendorf tube. The tubes were capped and incubated in water bath at 95 °C for 90 minutes. The samples were cooled to room temperature and the absorbance of each solution was measured at 695 nm. The total antioxidant capacity was expressed as mM equivalent of ascorbic acid antioxidant capacity. All the experiments were conducted in triplicates.

DPPH radical scavenging activity

The effects of extracts on DPPH radical were determined by minor modifications. To 2 mL of DPPH solution (100 µM), 0.1 mL each of the extract or standard solution was added separately in test tubes. The test tubes were incubated at 37 ^{o}C for 30 minutes and the absorbance of each solution was measured at 517 nm against blank and converted into percentage radical scavenging activity[18] as per formula.

$$\% \text{ inhibition} = \frac{Zo - Ze}{Zo} \times 100$$

Where; Zo = absorbance of control, Ze = absorbance of extract. The IC_{50} values were calculated by linear regression of plots where x-axis represented the concentration (mg/mL) and y-axis represented the scavenging effect (% inhibition).

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assays were determined by minor modifications. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 mL of the extracts or standards in methanol were added to 2 mL of hydrogen peroxide

solution in PBS (20 mM). The absorbance was measured at 230 nm after 10 min[17].

Nitric oxide radical scavenging activity

The reaction mixture (6 mL) containing sodium nitroprusside (10 mM, 4 mL), phosphate buffer saline (PBS, pH 7.4, 1 mL) and extract or standard (1 mL) in DMSO at various concentrations were incubated at 25 ºC for 150 minutes. After incubation, 0.5 mL of the reaction mixture containing nitrite ion was removed, 1 mL of sulphanilic acid reagent was added to this, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 mL of NEDD was added, mixed and allowed to stand for 30 minutes in diffused light. A pink coloured chromophore was formed. The absorbance was measured at 540 nm[17].

Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory activity of extracts was determined[19]. This was induced by adding ferric chloride 50 µL (400 mM) and L-ascorbic acid 50 µL (400 mM) to a mixture containing egg lecithin (3 mg/mL) in phosphate buffer solution and different concentration of extracts (100 µL). After incubation for 1 hour at 37 ºC, the reaction was stopped by adding 2 mL of 0.25 N hydrochloric acid containing 15 % w/v trichloroacetic acid and 0.375 % w/v thiobarbituric acid, boiled for 15 min, cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm.

Hydroxyl radical scavenging by deoxyribose

To the reaction mixture containing deoxyribose (0.2 mL, 3 mM), ferric chloride (0.2 mL, 0.1 mM), ethylene diamine tetraacetic acid disodium salt (Disodium EDTA, 0.2 mL, 0.1 mM), ascorbic acid (0.2 mL, 0.1 mM) and hydrogen peroxide (0.2 mL, 2 mM) was added 0.2 mL of various concentrations of the extract or standard in DMSO to give a total volume of 1.2 mL. The solutions were then incubated for 30 minutes at 37 °C. After incubation, ice-cold trichloroacetic acid (0.2 mL, 15 % w/v) and thiobarbituric acid (0.2 mL, 1 % w/v) in 0.25 N hydrochloric acid were added. The reaction mixture was kept in a boiling water bath for 30 minutes, cooled and the absorbance was measured at 532 nm[16].

Cytotoxicity studies

Among all the successive extracts of *C. guianensis*, ethylacetate extract of the stem, crude methanolic extracts of the flower has been used for *in vitro* cytotoxicity studies. It is determined by Rita *et al*, the method by slight modification. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/mL using DMEM medium containing 10 % FBS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µL of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37 °C for 3 days in 5 % CO_2 atmosphere and microscopic examination was carried out and observations were noted every 24 hours interval. After 72 hours, the drug solutions in the wells were discarded and 50 µL of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hours at 37 °C in 5 % CO_2 atmosphere. The supernatant was removed and 100 µL of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50 % (CTC_{50}) values is generated from the dose-response curves for each cell line[20].

$$\% \text{ Growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Results

The result of qualitative phytochemical screening has shown that PEL contains carbohydrates and steroids. And CLE, ELE, MLE, CSE, ESE, MSE and CMF contain terpenoids, glycosides, phenols and flavonoids. In the determination of total phenol content, among all the extracts of *C. guianensis,* MLE and CMF were found to have a higher amount of phenol with the value of 96.90 and 92.24 mg GAE/g of extract. The total antioxidant capacity of all successive extracts and crude extract of *C. guianensis* was studied (Table 1). ELE and ESE of *C. guianensis* exhibited highest total antioxidant capacity (0.128 and 0.116 mM). In DPPH radical scavenging assay, ELE and MLE have shown highest antioxidant activity with the lower IC_{50} values of 17.25 and 16.15 µg/mL, respectively. In hydrogen peroxide radical inhibition, a good anti-oxidant activity with the lower IC_{50} value was found in ELE and CMF (55.1±0.04 and

Table 1 — Extracts % yield, total phenol content and *in vitro* antioxidant activity and total antioxidant capacity of *Couroupita guianensis*

Extract/ Standard	% yield of extract	total phenols mg/g [a, b]	IC_{50} values±SD* (µg/mL)					Total antioxidant capacity (mM)[a]
			Nitric oxide	DPPH	H_2O_2	Lipid peroxidation	Hydroxyl radical	
PEL	4.2 %	5.95±0.07	349±0.01	700±0.24	230±0.13	400±0.03	350±0.51	0.065±0.65
CLE	1.42 %	45.18±1.06	450±0.06	465±0.13	290.1±0.29	390±0.11	660±0.11	0.05±0.12
ELE	1.9 %	78.03±0.40	730±0.24	17.25±0.16	55.1±0.04	200.5±0.07	240±0.81	0.128±0.06
MLE	7.5 %	96.90±0.61	560.3±0.04	16.15±1.17	135±0.45	350±0.12	490±0.59	0.096±0.11
CSE	1.08 %	21.79±0.45	270±0.48	80.16±0.14	170.8±0.11	830±0.11	470±0.69	0.01 .16
ESE	0.53 %	35.21±0.29	50.4±0.31	170±0.08	230±0.02	320±0.23	280±0.18	0.116±0.13
MSE	1.2 %	43.31±1.01	750±0.35	115.2±0.21	190±0.21	590.9±0.45	450±0.38	0.01±0.23
CMF	5.67 %	92.24±0.56	750.5±0.31	70.1±0.15	71.6±0.11	440±0.23	460±0.45	0.01±0.08
AA			55.66±0.47	35.5±0.34	57.0±0.25	NA	NA	NA
BHA			NA	NA	NA	85.06±0.28	85.55±0.01	NA

[a]Mean of three replicate determination±SD, standard deviation, [b]gallic acid, equivalent in mg/g of extract. *Values are means of three replicate determination ±SD; NA- not analysed, [a]mM equivalent to ascorbic acid. PEL: Petroleum ether leaf extract, CLE: Chloroform leaf extract, ELE: Ethyl acetate leaf extract, MLE: Methanol leaf extract, CSE: Chloroform stem extract, ESE: Ethyl acetate stem extract, MSE: Methanol stem extract, CMF: Crude methanol flower extract, AA: Ascorbic acid, BHA: Butylated hydroxy anisole.

70.1±0.15 µg/mL). The antioxidant activity of extracts in nitric oxide radical inhibition method, the lower IC_{50} value was found in ESE is 50.4±0.31 µg/mL and followed by CSE, PEL and CLE are 270±0.48, 349±0.01 and 450±0.06 µg/mL, respectively. In the lipid peroxidation assay method, ELE has shown lowest IC_{50} value with highest antioxidant activity (200.5±0.07 µg/mL) and followed by ESE and CLE. The hydroxyl radicals are generated by Fenton reagent and inhibited by ELE with lower IC_{50} value (240±0.81 µg/mL) when compared to other extracts (Table 1). ELE and CMF have been selected based on their *in vitro* antioxidant activity and tested for *in vitro* antiproliferative study. ELE has shown the cytotoxic activity at 300±5.10 µg/mL and CMF has lower CTC_{50} value (220±3.15 µg/mL) and confirms the better cytotoxic activity against HT-29 cell line.

Discussion

Total phenol content was determined by using Folin-Ciocalteu reagent. The test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation, the green-blue complex formed was measured at 750 nm and reported as gallic acid equivalents. In this study MLE, CMF and ELE extracts exhibited high phenol content which is equivalent to gallic acid per gram of extract (Table 1). Total antioxidant capacity was estimated using phosphomolybdenum method based on the reduction of Mo (VI) to Mo (V) by the formation of a green Mo (V) complex at acidic pH. This assay is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The green phosphate molybdenum complex absorbance was measured at 695 nm. ELE, ESE and MLE extracts have shown highest total antioxidant capacity, whereas, remaining extracts showed sign of lower antioxidant capacity.

1,1'-Diphenyl-2-picrylhydrazyl radical scavenging assay is the most widely used method for screening antioxidant activity since it can accommodate many samples in a short period and detective ingredients at low concentration. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow[21]. In this study, ELE and MLE have shown better inhibition of DPPH free radical. H_2O_2 is important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, it is a weak oxidizing agent, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells. It can cross cell membrane rapidly. Once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly with Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. Thus, removal of H_2O_2 is very important for the protection of damage of tissues[22]. Percentage inhibition of hydrogen peroxide by extracts observed highest in CLE, followed by CSE, MLE, MSE and CMF respectively. On the other hand remaining extracts have shown lower percentage inhibition. The IC_{50} value of ELE was lowest (55.1±0.04 µg/mL)

which confirms higher antioxidant activity. The antioxidant activity of extracts by hydrogen peroxide method was higher than the ascorbic acid (57.0±0.2 µg/mL).

Nitric oxide free radicals (NO·) are a well-known inducer of tissue pathogenesis leading to several diseases such as cancer, *Diabetes mellitus* and age-related disorders. The reagent sodium nitroprusside is known to decompose in aqueous solution at physiological pH 7.2 producing NO. Under aerobic conditions, NO· reacts with oxygen to produce stable products (nitrate and nitrite ions)[13,23]. This leads to a reduction of nitrite concentration in the assay media. The percentage inhibition was observed high in CMF and followed by CSE, ESE, CLE and PEL. In lipid peroxidation, lipophilicity and amphiphilic character of compounds play an important role. The antioxidant may present in a compartment where the free radical (lipid peroxide) induces damage. The antioxidants are the phenolic group of their chroman head and this group is may be situated in the lipid membrane near the aqueous phase at a site favourable for scavenging radicals involved in lipid peroxidation[24]. In the lipid peroxide inhibition assay method, ELE has shown good percentage inhibition and other all extracts, CMF, MSE, MLE and ESE have shown concentration-dependent percentage inhibition. The quality of antioxidant potency in the extracts was determined by IC_{50} value whereby, a low IC_{50} indicates strong antioxidant activity. In the hydroxyl radical scavenging by deoxyribose assay method, *C. guainensis* extracts shown dose-dependent percentage inhibition and highest percentage inhibition was observed in the MSE (101.2 %) and followed by MLE (85.93 %), CSE (81.4 %), ELE (80.92 %) and ESE (79.53 %). The molecules which inhibit deoxyribose degradation are those that can chelate the iron ions and thereby prevent them from complexing with deoxyribose and render them as inactive. Overall the scavenging activities of phenolic substances might be due to the active hydrogen donating ability of hydroxyl substitutions. The phenolic compounds present in the extract are good electron donor and they may accelerate the conversion of hydrogen peroxide into water[25]. The IC_{50} value was determined in this method, also found good antioxidant activity in ELE (240 µg/mL). In this assay, the antioxidant activity was lower than the standard BHA.

In the cytotoxicity study, based on *in vitro* antioxidant activity, the potent extracts were chosen for cytotoxicity study on HT-29 cell line. The selected ELE and CMF extracts have shown better cytotoxicity. The CTC_{50} values are 300.0 and 220.0 µg/mL, respectively.

Conclusion

Based on the results, *C. guainensis* has a natural antioxidant capacity as an alternative to synthetic antioxidants. The cytotoxic work on HT-29 cell line was reported first time in this study. Ethyl acetate leaf (ELE) and crude methanol flower (CMF) extracts have shown better antioxidant activity. Further, in future, the plant could be a subject for isolation and other pharmacological studies based on antioxidant activity.

References

1 Premanathan M, Radhakrishnan S, Kulangiappar K, Singaravelu G, Thirumalaiarasu V, *et al*., Antioxidant and anticancer activities of isatin (1H-indole-2,3-dione), isolated from the flowers of *Couroupita guianensis* Aubl., *The Indian J Med Res,* 2012, **136**(5), 822–826.

2 Pinheiro M M G, Bessa S O, Fingolo C E, Kuster R M, Matheus M E, *et al*., Antinociceptive activity of fractions from *Couroupita guianensis* Aubl. Leaves, *J Ethnopharmacol*, 2010, **127**(2), 407–413.

3 Martínez A, Conde E, Moure A, Domínguez H and Estévez R J, Protective effect against oxygen reactive species and skin fibroblast stimulation of *Couroupita guianensis* leaf extracts, *Nat Prod Res,* 2012, **26**(4), 314–322.

4 Manimegalai S, Sridharan T B, Rameshpathy M and Devi Rajeswari V, Antioxidant, phytochemical screening and antimicrobial activity of *Couroupita Guianensis* flower extract, *Der Pharmacia Lettre*, 2014, **6**(6), 251-256.

5 Al-Dhabi N A, Balachandran C, Raj M K, Duraipandiyan V, Muthukumar C, *et al*., Antimicrobial, antimycobacterial and antibiofilm properties of *Couroupita guianensis* Aubl. fruit extract, *BMC Compl Alt Med,* 2012, **12**, 242.

6 Mariana M G, Pinheiro Sidnei B O, Fernandes Catarina E, Fingolo F B and Patricia D F, Anti-inflammatory activity of ethanol extract and fractions from *Couroupita guianensis* Aublet leaves, *J Ethnopharmacol,* 2013, **146**, 324–330.

7 Elumalai A, Naresh V, Eswaraiah M C, Narendar P and Kumar R, Evaluation of antiulcer activity of *Couroupita guianensis* Aubl leaves, *Asian J Pharm Tech*, 2012, **2**(2), 64-66.

8 Elumalai A, Bargavi K, Krishna S and Chinnaeswaraiah M, Evaluation of anti-oxidant and hepatoprotective activity of *Couroupita guianensis* leaves, *J Cell and Tissue Res,* 2013, **13**(2), 3745-3748.

9 Gousia S K, Kumar K A, Kumar T V and Latha J N L, Biological activities and medicinal properties of *Couroupita guianensis, Int J Pharm Pharm Sci Res,* 2013, **3**(4), 140-143.

10 Pryor W Y, The antioxidant nutrient and disease prevention-what do we know and what do we need to find out?, *Am J Clin Nutr*, 1991, **53**, 391.

11 Buyukokuroglu M E, Glucin I, Oktay M and Kufrevioglu O I, *In vitro* antioxidant properties of dantrolene sodium, *J Pharmacol Res*, 2001, **44,** 491-495.

12 Glucin I, Elias R, Gepdiremen A and Boyer L, Antioxidant activity of lignana from fringe tree, *Eur J Food Res Technol,* 2006, **223**, 759-767.
13 Halliwell B and Gutteridge J M C, *Free Radicals in Biology and Medecine*, 2nd edn, Clarendon Press, Oxford, 1989,
14 Harborne J B, Phytochemical methods; Chapman and Hall: London, UK, 1973, 49–188.
15 Kokate C K, Purohit A P and Gokhale S B, *Pharmacognosy*, 7th edn, Nirali Prakashan, Pune, 1997.
16 Meda A, Lamien C E, Romito M, Millogo J and Nacoulma O G, Determination of the total phenolic, flavonoid and praline contents in Burkina Fasan honey, as well as their radical scavenging activity, *Food Chem,* 2005, **91**, 571–577.
17 Jaishree V, Badami S and Suresh B, *In vitro* antioxidant activity of *Enicostemma axillare*, *J Health Sci,* 2008, **54**, 524-528.
18 Subramanion L, Zakaria Z and Sreenivasan S, Phytochemicals screening, DPPH free radical scavenging and xanthine oxidase inhibitiory activities of *Cassia fistula* seeds extract, *J Med Plant Res,* 2011, **5**(10), 1941-1947.
19 Duh P D, Yen G C, Yen W J and Chang L W, Antioxidant effects of water extracts from barley (*Hordeum vulgare* L) prepared under different roasting temperatures, *J Agri Food Chem*, 2008, **49**, 1455-1463.
20 Francis D and Rita L, Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, *J Immunol Methods,* 1986, **89**, 271-277.
21 Sanchez-Moreno C, Larrauri J A and Saura-Calixto F, A procedure to measure the antiradical efficiency of polyphenols, *J Sci Food Agri*, 1998, **76,** 270–276.
22 Sprong R C, Winkelhuyzen-Janssen A M, Aarsman C J, *et al*., Low-dose N-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high-dose increases mortality, *Am J Respiratory Crit Care Med,* 1998, **157,** 1283–1293.
23 Jayaprakasha G K, Jagan Mohan Rao L and Sakariah K K, Volatile constituents from Cinnamomumzeylanicum fruit stalks and their antioxidant activities, *J Agri Food Chem*, 2003, **51**(15), 4344–4348.
24 Afri M, Ehrenberg B, Talmon Y, Schmidt J, Cohen Y, *et al*., Active oxygen chemistry within the liposomal bilayer. Part III: locating Vitamin E, ubiquinol and ubiquinone and their derivatives in the lipid bilayer, *Chem Phys Lip,* 2004, **131,** 107–121.
25 Smith C, Halliwell B and Aruoma O I, Protection by albumin against the pro-oxidant actions of phenolic dietary components, *Food Chem Toxicol,* 1992, **30,** 483–489.