

Amla (*Emblica officinalis* Gaertn.) prevents dyslipidaemia and oxidative stress in the ageing process

Takako Yokozawa^{1*}, Hyun Young Kim¹, Hyun Ju Kim¹, Tsutomu Okubo², Djoing-Chi Chu² and Lekh Raj Juneja²

¹Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

²Bio-nutrition Division, Taiyo Kagaku Co. Ltd, 1–3 Takaramachi, Yokkaichi 510-0844, Japan

(Received 7 October 2006 – Revised 6 December 2006 – Accepted 19 December 2006)

Amla (*Emblica officinalis* Gaertn.) is widely used in Indian medicine for the treatment of various diseases. We have investigated the effects of amla on the lipid metabolism and protein expression involved in oxidative stress during the ageing process. SunAmla or ethyl acetate extract of amla, a polyphenol-rich fraction, was administered at a dose of 40 or 10 mg/kg body weight per d for 100 d to young rats aged 2 months and aged rats aged 10 months. The lipid levels, such as cholesterol and TAG, in serum and liver were markedly elevated in aged control rats, while they were significantly decreased by the administration of amla. The PPAR α is known to regulate the transcription of genes involved in lipid and cholesterol metabolism. The PPAR α protein level in liver was reduced in aged control rats. However, the oral administration of amla significantly increased the hepatic PPAR α protein level. In addition, oral administration of amla significantly inhibited the serum and hepatic mitochondrial thiobarbituric acid-reactive substance levels in aged rats. Moreover, the elevated expression level of bax was significantly decreased after the oral administration of amla, while the level of bcl-2 led to a significant increase. Furthermore, the expressions of hepatic NF- κ B, inducible NO synthase (iNOS), and cyclo-oxygenase-2 (COX-2) protein levels were also increased with ageing. However, amla extract reduced the iNOS and COX-2 expression levels by inhibiting NF- κ B activation in aged rats. These results indicate that amla may prevent age-related hyperlipidaemia through attenuating oxidative stress in the ageing process.

Amla: Ageing: Dyslipidaemia: Oxidative stress

Ageing, defined as a multifactorial phenomenon characterised by a time-dependent decline in physiological function, is related to many metabolic alterations, such as dyslipidaemia, atherosclerosis, obesity, type 2 diabetes, arthritis and neurodegenerative disease (Knight, 2001). In particular, aged mammals, including man, show increasing lipid concentrations in the serum and liver, and develop CVD directly related to lipid metabolism, such as hypertriacylglycerolaemia and hypercholesterolaemia (Ferrari *et al.* 2003). Moreover, the ageing process involves the accumulation of oxidative damage in cells and tissues. Oxidative stress, such as via the increased production of free radicals and other reactive oxygen species and decreased antioxidative defences, is known to occur during ageing (Beckman & Ames, 1998; Wickens, 2001; Yu & Chung, 2006). Numerous studies have shown that oxidative stress causes structural and functional alterations in molecular, cellular, tissue and organ systems (Yu & Chung, 2001; Kim *et al.* 2004; Poon *et al.* 2004). Furthermore, recent molecular studies on oxidative stress have shown altered gene regulation during ageing (Kim *et al.* 2002; Yoon *et al.* 2002). Therefore, functional foods which

possess both a hypolipidaemic effect and antioxidant activity may be effective and useful for reducing the risk of age-associated pathological damage.

Amla (*Emblica officinalis* Gaertn.) is a member of the small genus of *Emblica* (Euphorbiaceae). It grows in tropical and subtropical parts of China, India, Indonesia, and the Malay Peninsula. It is an important dietary source of vitamin C, minerals, and amino acids and also contains phenolic compounds, tannins, phyllembelic acid, phyllembelin, rutin, curcuminoides and emblicol. All parts of the plant are used for medicinal purposes. Especially, the fruit has been used in Ayurveda as a potent rasayana (Thakur, 1985) and traditional medicine for the treatment of diarrhoea, jaundice and inflammation (Deokar, 1998). In addition, the pulp of the fruit is smeared on the head to alleviate headache and dizziness (Perry, 1980). Recently, amla extract has been tested for various pharmacological activities. The fruit extract was reported to have hypolipidaemic (Anila & Vijayalakshmi, 2002), antidiabetic (Sabu & Kuttan, 2002) and anti-inflammatory activities (Asmawi *et al.* 1993) and inhibit retroviruses such as HIV-1 (El-Mekawy *et al.* 1995), tumour development (Jose *et al.*

Abbreviations: COX-2, cyclo-oxygenase-2; EtOAc, ethyl acetate; I κ B- α , inhibitor binding protein κ B- α ; iNOS, inducible NO synthase; PMSF, phenylmethyl sulfonyl fluoride; TBA, 2-thiobarbituric acid.

* **Corresponding author:** Dr Takako Yokozawa, fax +81 76 434 5068, email yokozawa@inm.u-toyama.ac.jp

2001) and gastric ulcer (Bandyopadhyay *et al.* 2000). Moreover, amla extract exhibited antioxidant properties (Bhattacharya *et al.* 1999; Anila & Vijayalakshmi, 2003), and it has been reported that the aqueous extract of amla is a potent inhibitor of lipid peroxide formation and a scavenger of hydroxyl and superoxide radicals *in vitro* (Jose & Kuttan, 1995). In a previous study, we also demonstrated the antioxidative property of amla using Cu²⁺-induced oxidised human LDL (Kim *et al.* 2005). Taken together, this evidence suggests that amla may be an effective modulator against oxidative stress-related alterations under ageing.

In the present study, we investigated the effects of amla on lipid metabolism and oxidative stress in the ageing process and also determined protein expression involved in age-related changes.

Materials and methods

Materials

The following reagents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan): 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid; TBA), bovine serum albumin, 2-amino-2-hydroxymethyl-1,3-propanediol (tri(hydroxymethyl)-aminomethane), Tween 20, phenylmethyl sulfonyl fluoride (PMSF), protease inhibitor cocktail and skimmed milk powder. Precision plus protein standards and the Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories, Japan. Rabbit anti-human PPAR α , rabbit anti-human bax, mouse anti-mouse bcl-2, rabbit anti-human NF- κ B p65 polyclonal antibody, rabbit anti-human inhibitor binding protein κ B- α (I κ B- α) polyclonal antibody, mouse anti-mouse NOS2 monoclonal antibody (primary antibody for inducible NO synthesis (iNOS)), mouse anti-human cyclo-oxygenase-2 (COX-2) monoclonal antibody, goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody, and goat anti-mouse IgG horseradish peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse β -actin antibody was purchased from Sigma-Aldrich (St Louis, MO, USA). ECLTM Western blotting detection reagents were purchased from Amersham Bioscience (Piscataway, NJ, USA).

Preparation of amla extract

Two grades of amla extract have been used. The first one is a commercial product of amla, an enzymic amla fruit juice extract called SunAmla (Taiyo Kagaku Co. Ltd, Yokkaichi, Japan), and the other one is a polyphenol-rich fraction of amla fruits derived from ethyl acetate (EtOAc) extraction. The EtOAc extract was prepared by extracting the air-dried amla fruit pieces in water–EtOAc (1:4) at room temperature for 24 h. The extract was evaporated under reduced pressure followed by lyophilisation. The yield was 12% of the dried fruits. The composition was 81.2% moisture, 14.1% carbohydrate, 3.4% fibre, 0.63% minerals, 0.5% protein, 0.1% fat, 0.05% Ca and 0.02% P.

Total polyphenols and vitamin C contents of amla extracts

Total polyphenol contents of the SunAmla and EtOAc extract of amla were measured by a colorimetric method using gallic acid as a standard. The vitamin C content was measured using HPLC.

Animals and treatments

The 'Guidelines for Animal Experimentation' approved by the University of Toyama were followed during these experiments. Male Wistar strain rats (Japan SLC Inc., Hamamatsu, Japan), aged 2 and 10 months, were used in the experiment. Young rats were 2 months old and weighed 125 (SE 3) g at the beginning of the experiment. Aged rats were 10 months old and weighed 476 (SE 15) g. Rats were kept in a wire-bottomed cage under a conventional light regimen with a dark night at room temperature (about 23°C) and humidity (about 60%). Animals were given laboratory pellet chow (CE-2, comprising 24.0% protein, 3.5% lipid and 60.5% carbohydrate; CLEA Japan Inc., Tokyo, Japan) and water *ad libitum*. Food consumption was measured daily, and body weight was recorded weekly. Twenty-one of the rats were divided into three groups, matched for their serum total cholesterol and body weight. The rats were fed a basal diet (CE-2) and treated with SunAmla extract or EtOAc extract of amla. Amla extract was dissolved in water and given orally to rats at a dose of SunAmla or EtOAc extract of amla of 40 or 10 mg/kg body weight per d for 100 d using a stomach tube. The oral dose was determined by the effective dose in our previous study (Kim *et al.* 2005). Control rats were given access to water alone. At 6 h after the last dose, the rats were decapitated, their blood was drawn, and serum was collected by centrifuging the blood at 1000 g for 15 min at 4°C. Each liver was removed, dried on tissue paper, weighed and stored at –80°C until analysis.

Measurement of serum cholesterol and triacylglycerol levels

Serum total and non-esterified cholesterol levels were determined using commercial kits (Cholesterol E-Test Wako and Free Cholesterol E-Test Wako, respectively; Wako Pure Chemical Industries Ltd, Osaka, Japan). Serum esterified cholesterol levels were calculated by subtracting the non-esterified cholesterol levels from the total cholesterol levels. Serum TAG levels were determined using commercial kits (Triglyceride E-Test Wako; Wako Pure Chemical Industries Ltd).

Measurement of hepatic cholesterol and triacylglycerol amounts

The liver of each rat was homogenised, total lipids of the liver homogenates were extracted with a mixture of chloroform and methanol (2:1, v/v) according to the method of Folch *et al.* (1957), and the amounts of total cholesterol, non-esterified cholesterol and TAG were determined using the Wako kit described earlier. Esterified cholesterol amounts were calculated by subtracting the non-esterified cholesterol amounts from the total cholesterol amounts.

Measurement of serum 2-thiobarbituric acid-reactive substance levels

Serum TBA-reactive substance levels were determined using the method of Naito & Yamanaka (1978).

Isolation of hepatic mitochondria and measurement of 2-thiobarbituric acid-reactive substance levels

Mitochondria were prepared from liver homogenates by differential centrifugation (800 *g* and 12 000 *g*; 4°C; 15 min) according to the methods of Johnson & Lardy (1967) and Jung & Pergande (1985), respectively, with slight modifications. Each pellet was re-suspended in preparation medium and the TBA-reactive substance concentration was determined by the method of Buege & Aust (1978). Briefly, 250 μ l of each re-suspended pellet or working standard was added to 750 μ l TBA–TCA–HCl solution (0.4 % of TBA, 15 % of TCA, 2.5 % HCl) and it was heated at 95–100°C for 20 min and cooled in an ice-bath. Then, samples were centrifuged at 1000 *g* at room temperature for 10 min to transfer supernatant fractions from the denatured protein precipitate. TBA-reactive substance was determined by measuring absorbance at 532 nm. The value of TBA-reactive substance was expressed in nmol malondialdehyde/mg protein by a calibration curve constructed from malondialdehyde (0–25 nmol/ml) in 1,1,3,3-tetramethoxypropane. The protein level was evaluated by the method of Itzhaki & Gill (1964) with bovine serum albumin as the standard.

Homogenisation, isolation of cytosol, and nuclear extracts

Livers were homogenised (Choi *et al.* 1997) at 4°C in homogenisation buffer (25 mM-tri(hydroxymethyl)-aminomethane-Cl (pH 7.5), 250 mM-NaCl, 5 mM-EDTA, 1 mM-PMSF and 1 mM-dithiothreitol), and supplemented with protease inhibitor cocktail that consisted of 100 mM-4-(2-aminoethyl) benzenesulfonyl fluoride, 0.08 mM-aprotinin, 2 mM-leupeptin, 5 mM-bestatin, 1 mM-pepstatin A and 1.5 mM-E-64. Homogenates were incubated for 15 min on ice, added to 10 % Nonidet P-40, and centrifuged at 4000 *g*, at 4°C, for 5 min. For Western blot analysis, each sample (20 μ g protein/lane) was denatured by boiling in Laemmli sample buffer and stored at –80°C until the assay.

Nuclear extracts were isolated using the method of Sakurai *et al.* (1996). Briefly, liver tissue was weighed and homogenised by a Potter Elvehjem homogeniser (Shintokagaku, Tokyo, Japan) in 4 vol. (w/v) of buffer A containing 10 mM-HEPES (pH 7.9), 10 mM-KCl, 0.1 mM-EDTA, 1 mM-dithiothreitol, 0.5 mM-PMSF, and protease inhibitor. Homogenates were incubated for 15 min on ice, added to 10 % Nonidet P-40, and centrifuged at 4000 *g*, at 4°C, for 5 min. Supernatant fractions were discarded and pellets were re-suspended in 2 vol. buffer B containing 20 mM-HEPES (pH 7.9), 0.4 M-NaCl, 1 mM-EDTA, 1 mM-dithiothreitol, 1 mM-PMSF, and protease inhibitors. Homogenates were kept for 15 min at 4°C and then centrifuged at 14000 *g* for 5 min at 4°C. Nuclear extracts were collected in microfuge tubes and stored in samples at –80°C. The protein concentration of the nuclear extracts was determined by the Bio-Rad protein assay.

Western blot analysis

Homogenates (20 μ g for bax, bcl-2, iNOS and COX-2), cytosol extract (I κ B- α) and crude nuclear extract (20 μ g for PPAR α and NF- κ B) from the liver were subjected to SDS-PAGE (10 %, w/v). Proteins were then transferred to nitrocellulose membranes

(Bio-Rad, Hercules, CA, USA) and blocked overnight at 4°C with 5 % non-fat dry milk in 25 mM-tri(hydroxymethyl)-aminomethane-Cl (pH 8.3), 140 mM-NaCl, 2 mM-KCl and 0.1 % Tween 20. Membranes were then incubated for 3 h at 4°C with the primary polyclonal antibody raised against PPAR α (dilution 1:1000), bax (dilution 1:1000), bcl-2 (dilution 1:1000), NF- κ B (dilution 1:1000), I κ B- α (dilution 1:1000), iNOS (dilution 1:1000), COX-2 (dilution 1:1000) and β -actin (1:5000). Western blotting with rabbit polyclonal antibody (dilution 1:1000) or mouse monoclonal antibody (dilution 1:1000) was also performed. Detection was achieved using the ECL chemiluminescence kit for horseradish peroxidase. The density was quantified in a Phosphor Imager (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Results are expressed as mean values with their standard errors. The effect on each parameter was examined using one-way ANOVA. Individual differences between groups were evaluated using the Dunnett's test and those at $P < 0.05$ were considered significant.

Results

Total polyphenol and vitamin C contents of amla extract

The total polyphenol content of SunAmla was about one-third that of the EtOAc extract. However, SunAmla contained vitamin C at 2 %. Vitamin C was not present in the EtOAc extract (Table 1).

Body weight and food intake

Table 2 shows the changes in body weight and food intake during the oral administration of amla to aged rats for 100 d. Body-weight gain was slightly decreased compared with aged control rats after the oral administration of SunAmla or EtOAc extract of amla, but it was not significantly different among the groups. Also, there were no differences in food intake among the groups.

Serum and hepatic cholesterol levels

The serum and hepatic cholesterol profiles of the aged rats fed SunAmla or EtOAc extract of amla are summarised in Table 3. The serum total and non-esterified cholesterol levels in aged

Table 1. Total polyphenol and vitamin C contents of amla extracts

(Mean values with their standard errors for ten replicate samples)

	Total polyphenols (%)		Vitamin C (%)	
	Mean	SE	Mean	SE
SunAmla	29.4	2.2	2.0	0.2
Ethyl acetate extract	80.4	4.3	–	–

Table 2. Body weight and food intake of ageing rats (Mean values with their standard errors)

Group	Dose (mg/kg body weight per d)	Body weight						Food intake (g/d)	
		Initial (g)		Final (g)		Gain (g/100 d)		Mean	SE
		Mean	SE	Mean	SE	Mean	SE		
Control (<i>n</i> 4)	–	476.0	17.6	491.1	19.5	15.1	4.4	19.0	0.4
SunAmla (<i>n</i> 7)	40	476.3	8.9	486.4	9.8	10.1	5.9	20.0	0.4
Ethyl acetate extract (<i>n</i> 7)	10	476.3	14.3	489.8	14.0	10.8	2.5	19.9	0.5

control rats significantly increased compared with young rats, whereas the levels were decreased by the amla extract, especially in the EtOAc extract of amla at 10 mg/kg body weight per d. However, esterified cholesterol levels of the rats were not significantly different among the groups. The hepatic total, non-esterified and esterified cholesterol levels in the aged control rats were significantly increased compared with the young rats ($P < 0.001$). However, the hepatic total cholesterol levels were significantly reduced by 18 or 26 % by the administration of SunAmla extract at 40 or EtOAc extract of amla at 10 mg/kg body weight per d compared with aged control rats, respectively ($P < 0.001$). The hepatic non-esterified cholesterol levels were also significantly decreased by the administration of amla extracts compared with aged control rats ($P < 0.001$). In addition, the esterified cholesterol level in the livers of rats fed SunAmla extract at 40 or EtOAc extract of amla at 10 mg/kg body weight per d was significantly reduced by 19 or 29 % compared with aged control rats, respectively ($P < 0.001$).

Serum and hepatic triacylglycerol levels

Table 4 shows the serum and hepatic TAG levels of aged rats given SunAmla or EtOAc extract of amla. The serum TAG level in aged control rats markedly increased by 2.7-fold compared with young rats ($P < 0.001$). However, the

administration of SunAmla or EtOAc extract of amla significantly reduced the serum TAG level by 30 or 24 %, respectively. Hepatic TAG levels in aged control rats also increased by 2.7-fold compared with young rats ($P < 0.001$). However, the hepatic TAG levels significantly declined from 38.0 to 32.7 or 30.9 mg/g by the administration of SunAmla or EtOAc extract of amla, respectively ($P < 0.001$).

Expression of peroxisome proliferator-activated receptor- α protein

To investigate the effects of amla extract on lipid metabolism in aged rats, hepatic PPAR α protein levels were determined using Western blot analysis (Fig. 1). The PPAR α protein levels in aged control rats were markedly decreased by 60 % compared with young rats ($P < 0.001$), whereas the oral administration of SunAmla or EtOAc extract of amla significantly increased the levels to 48 % compared with aged control rats, respectively ($P < 0.001$).

2-Thiobarbituric acid-reactive substance levels of serum and hepatic mitochondria

Table 5 represents the inhibitory effect of amla extract on lipid peroxidation induced by age-related oxidative stress. The TBA-reactive substance levels of serum and hepatic

Table 3. Serum and hepatic cholesterol profiles (Mean values with their standard errors)

Group	Dose (mg/kg body weight per d)	Total cholesterol		Non-esterified cholesterol		Esterified cholesterol	
		Mean	SE	Mean	SE	Mean	SE
Serum cholesterol (mg/l)							
Young rats (<i>n</i> 4)	–	703	24	492	11	211	14
Ageing rats							
Control (<i>n</i> 7)	–	886***	26	648***	31	238	14
SunAmla (<i>n</i> 7)	40	847**	91	624**	71	223	22
Ethyl acetate extract (<i>n</i> 7)	10	795†	7	583*	17	212	8
Liver cholesterol (mg/g)							
Young rats (<i>n</i> 4)	–	7.33	0.28	2.99	0.06	4.34	0.24
Ageing rats							
Control (<i>n</i> 7)	–	10.63***	0.31	4.29***	0.12	6.34***	0.32
SunAmla (<i>n</i> 7)	40	8.74***‡	0.29	3.58***‡	0.15	5.16*‡	0.16
Ethyl acetate extract (<i>n</i> 7)	10	7.91‡	0.84	3.41*‡	0.26	4.50‡	0.59

Mean value was significantly different from that of the young rats: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Mean value was significantly different from that of the ageing control rats: † $P < 0.05$, ‡ $P < 0.001$.

Table 4. Serum and hepatic triacylglycerols (Mean values with their standard errors)

Group	Dose (mg/kg body weight per d)	Serum (mg/l)		Liver (mg/g)	
		Mean	SE	Mean	SE
Young rats (n 4)	–	394	47	14.1	0.8
Ageing rats					
Control (n 7)	–	1056*	73	38.0*	1.8
SunAmla (n 7)	40	736*†	78	32.7*†	1.6
Ethyl acetate extract (n 7)	10	800*†	156	30.9*†	1.7

*Mean value was significantly different from that of the young rats ($P < 0.001$). Mean value was significantly different from that of the ageing control rats: † $P < 0.01$, ‡ $P < 0.001$.

mitochondria in aged control rats were significantly increased compared with young rats. However, the administration of SunAmla or EtOAc extract of amla significantly reduced the serum TBA-reactive substance levels by 22 or 36 % compared with aged control rats, respectively. Moreover, the TBA-reactive substance levels of hepatic mitochondria were also significantly lower in rats given amla extract than in aged control rats.

Expressions of bax and bcl-2 proteins

We also investigated the effects of amla extract on apoptosis in the liver of aged rats (Fig. 2). As compared with young rats, the bax protein levels in aged control rats were significantly increased by 46 % ($P < 0.001$). However, oral

administration of amla extract significantly decreased the level of bax protein ($P < 0.001$). Bcl-2 protein levels in aged control rats did not show significant differences compared with those of young rats, while those of rats given SunAmla extract were significantly increased ($P < 0.001$).

Expressions of nuclear factor- κ B and inhibitor binding protein κ B- α proteins

As shown in Fig. 3, NF- κ B protein levels in the livers of aged control rats were higher than those of young rats. However, the oral administration of amla extract decreased the NF- κ B protein levels compared with aged control rats, although the I κ B- α protein level did not show any significant difference among all groups.

Expressions of inducible nitric oxide synthase and cyclo-oxygenase-2 proteins

Fig. 4 shows the effects of amla on levels of iNOS and COX-2 protein. The iNOS protein levels in aged control rats markedly increased by 4.9-fold compared with young rats ($P < 0.001$), whereas the oral administration of SunAmla or EtOAc extract of amla led to a significant decrease to 15 or 46 % compared with aged control rats, respectively ($P < 0.001$). In addition, COX-2 protein levels in aged control rats were slightly increased compared with young rats ($P < 0.01$), while those of rats given SunAmla at 40 mg/kg body weight per d or EtOAc extract of amla at 10 mg/kg body weight per d were significantly lower by 25 or 27 % than those of aged control rats, respectively ($P < 0.001$).

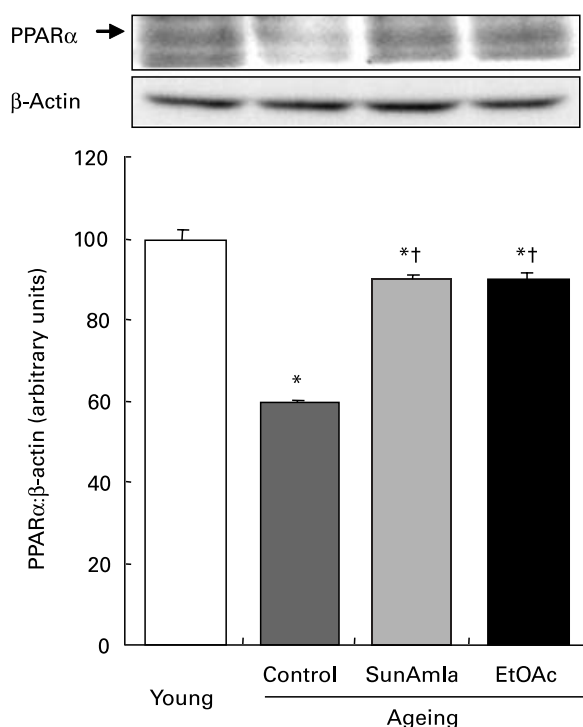


Fig. 1. PPAR α protein in hepatic nucleus. Values are means, with standard errors represented by vertical bars. * Mean value was significantly different from that of the young rats ($P < 0.001$). † Mean value was significantly different from that of the ageing control rats ($P < 0.001$). EtOAc; ethyl acetate.

Discussion

Old age is associated with the appearance of dyslipidaemia, atherosclerosis, obesity and type 2 diabetes mellitus (Knight, 2001). These risk factors steadily increase the incidence of CVD among elder individuals. In addition, aged mammals including man show increasing plasma concentrations of lipids and reduced fatty acid oxidation (Parini *et al.* 1999). Recently, several studies showed that the significant increase of lipid levels in the ageing process is associated with a reduction in the expression and activity of PPAR α , a nuclear transcription factor, in the liver of rats (Lacko & Davis, 1979; Sanguino *et al.* 2004a). Therefore, great effort has been focused on a PPAR α activator for patients with dyslipidaemia requiring lipid-lowering therapy.

In the present study, we investigated the effect of amla extract on lipid profiles and oxidative stress in the ageing process. The present results also showed that the total cholesterol and TAG levels in the serum of aged control rats (13 months) were higher than those of young rats (2 months) and accumulated higher total, non-esterified and esterified cholesterol, and TAG levels in the liver (Tables 3 and 4). However, the oral administration of SunAmla or EtOAc extract of amla for 100 d to aged rats partially prevented age-related increases in cholesterol and TAG levels in the serum and liver. A small fraction of the cholesterol in the liver is incorporated into the membranes of hepatocytes, but most is exported. Esterified cholesterol is one of the exported forms. It is formed in the liver through the action of acyl-CoA-cholesterol

Table 5. 2-Thiobarbituric acid-reactive substance levels of serum and hepatic mitochondria

(Mean values with their standard errors)

Group	Dose (mg/kg body weight per d)	Serum (nmol/ml)		Hepatic mitochondria (nmol/mg protein)	
		Mean	SE	Mean	SE
Young rats (<i>n</i> 4)	–	2.05	0.10	0.217	0.012
Ageing rats					
Control (<i>n</i> 7)	–	2.88*	0.44	0.291**	0.017
SunAmla (<i>n</i> 7)	40	2.24‡	0.22	0.275*	0.025
Ethyl acetate extract (<i>n</i> 7)	10	1.83‡†	0.19	0.243†	0.025

Mean value was significantly different from that of the young rats: * $P < 0.01$, ** $P < 0.001$.

Mean value was significantly different from that of the ageing control rats: † $P < 0.05$, ‡ $P < 0.01$, ‡† $P < 0.001$.

acyl transferase, which catalyses the conversion of cholesterol into a more hydrophobic form that is transported in secreted lipoprotein particles to other tissues that use cholesterol or is stored in the liver. Thus, the reduction of esterified cholesterol level indicates that the cholesterol was used for the synthesis of vital molecules in tissues, including the liver.

PPAR α , a nuclear transcription factor, is known to regulate the transcription of genes involved in lipid, cholesterol, lipoprotein and glucose energy metabolism, and insulin sensitivity (Ricote & Glass, 2001; Barbier *et al.* 2002; Blanquart *et al.* 2003; Berger *et al.* 2005). PPAR α is expressed in tissues such as the liver, muscle, kidney and heart, where it stimulates the β -oxidative degradation of fatty acids. Aged mammals show a decreased capacity for fatty acid oxidation (Toth & Tehernof, 2000) and this may be an underlying cause of

age-related increases in plasma lipid concentrations and dyslipidaemia. In addition, PPAR α deficiency is consistently related to increased plasma levels of TAG and cholesterol (Peters *et al.* 1997). Therefore, we determined the PPAR α expression level in the liver. The present results also demonstrated that the hepatic PPAR α protein level in the liver of aged rats was increased by 48 or 47% with SunAmla at 40 mg/kg body weight per d or EtOAc extract of amla at 10 mg/kg body weight per d, respectively, compared with aged control rats (Fig. 1). Based on these results, we found that serum and hepatic lipids are moderately increased in old animals as a result of the reduction of the hepatic PPAR α protein level, while amla extract decreased the elevated levels of lipids in aged rats by activating the PPAR α level (Tables 3 and 4; Fig. 1). These results suggest that amla may be an effective PPAR α activator and protect against CVD resulting from dyslipidaemia.

It is well known that reactive oxygen species-mediated lipid peroxidation increases with age (Halliwell & Chirico, 1993; Lin & Beal, 2003). In addition, Inoue *et al.* (1997) showed the negative correlation between the liver expression of the PPAR α gene and the plasma TBA-reactive substance level. As shown by the present study, TBA-reactive substance levels of serum and hepatic mitochondria in aged rats were also significantly increased, whereas the administration of SunAmla or EtOAc extract of amla significantly decreased the TBA-reactive substance level compared with aged control rats, respectively (Table 5). It is thought that reduction of the TBA-reactive substance level by amla extract may be due to the decrease of age-related dyslipidaemia through the activation of PPAR α and the antioxidant effect of amla extract which contains polyphenols and vitamin C (Table 1).

Bcl-2 family proteins, anti-apoptotic proteins, protect against cell death by acute oxidative stress (Chan & Yu, 2004). It has been demonstrated that bcl-2 overexpression

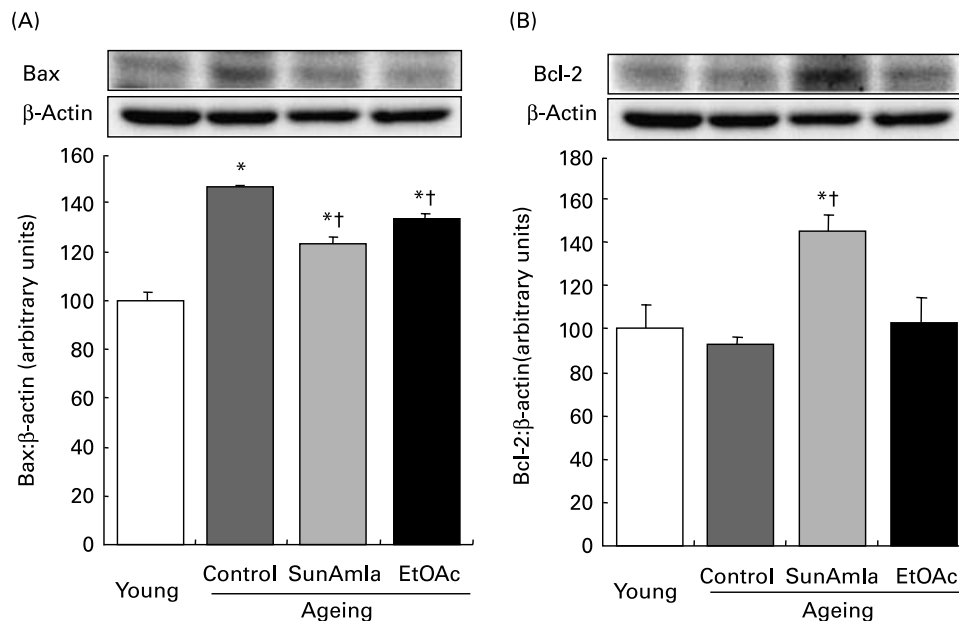


Fig. 2. Bax (A) and bcl-2 (B) proteins in hepatic homogenate. * Mean value was significantly different from that of the young rats ($P < 0.001$). † Mean value was significantly different from that of the ageing control rats ($P < 0.001$). EtOAc; ethyl acetate.

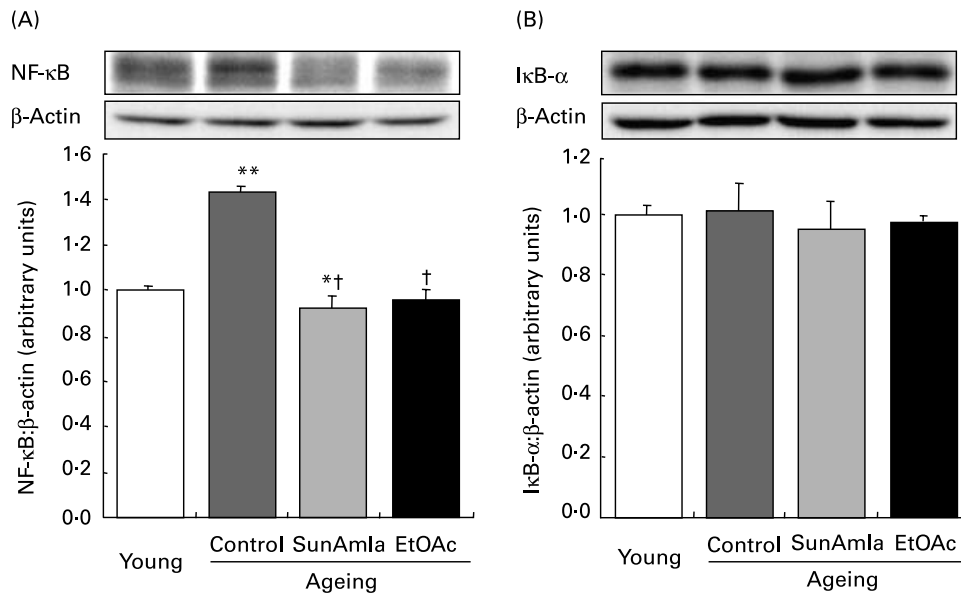


Fig. 3. NF- κ B (A) and inhibitor binding protein κ B- α (I κ B- α) (B) proteins in hepatic nucleus and cytoplasm. Mean value was significantly different from that of the young rats: * $P < 0.05$, ** $P < 0.001$. † Mean value was significantly different from that of the ageing control rats ($P < 0.001$). EtOAc; ethyl acetate.

preserves viability and diminishes lipid peroxidation in cells exposed to oxidative stress (Hockenbery *et al.* 1993). Conversely, some pro-apoptotic proteins such as bax, a mammalian cell-death protein that targets mitochondrial membranes, can induce mitochondrial damage and cell death even when caspases are inactivated (Green & Reed, 1998). However, several workers have reported that antioxidants slow down or block the apoptotic process by stabilising mitochondrial functions (Samhan-Arias *et al.* 2004; Williams *et al.* 2004). In the present results, the bax protein level was significantly enhanced in the aged control rats ($P < 0.001$), while bcl-2 protein was

slightly reduced compared with young rats but not significant between the aged-rat groups. The oral administration of amla extract has a beneficial effect on these proteins (Fig. 2). These results indicate that amla extract has protective effects against cell death by age-related oxidative stress.

Chronic oxidative stress and inflammatory reactions also lead to many age-associated diseases and the ageing process was found to enhance the activation of NF- κ B by down regulating I κ B- α (Spencer *et al.* 1997; Poynter & Daynes, 1998). Under resting conditions, NF- κ B exists in the cytoplasm as a dimer bound to the inhibitory protein I κ B- α . Inducers of

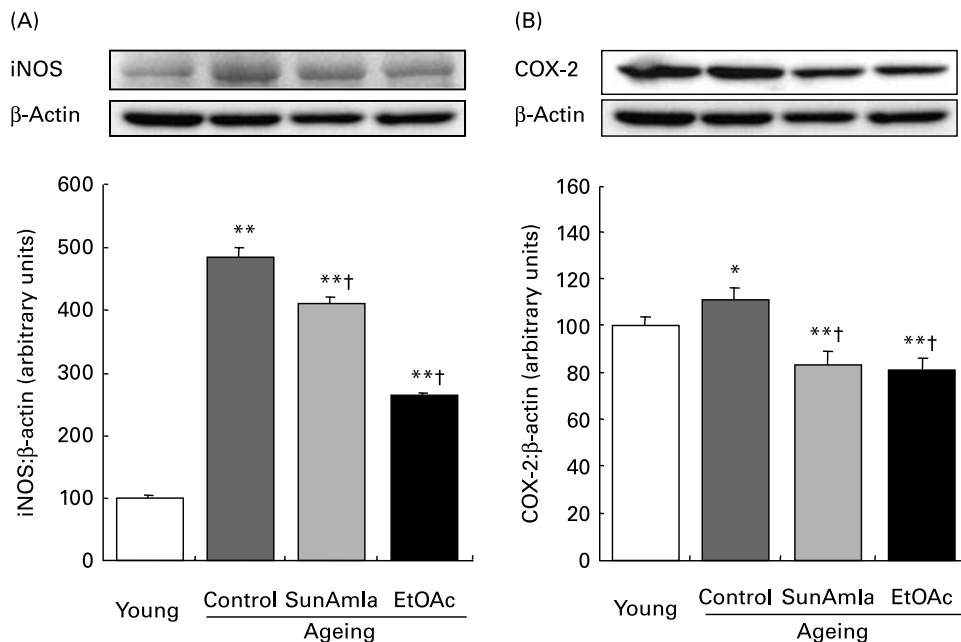


Fig. 4. Inducible NO synthase (iNOS) (A) and cyclo-oxygenase-2 (COX-2) (B) proteins in hepatic homogenate. Mean value was significantly different from that of the young rats: * $P < 0.01$, ** $P < 0.001$. † Mean value was significantly different from that of the ageing control rats ($P < 0.001$). EtOAc; ethyl acetate.

NF- κ B, such as inflammatory cytokines, reactive oxygen species, and viral products, activate a dimeric I κ B- α kinase (IKK) complex, causing the phosphorylation and ubiquitination of I κ B- α and its release from NF- κ B. The free NF- κ B dimer translocates to the nucleus, where it regulates target gene transcription such as iNOS, COX-2, IL-6, IL-12 and TNF α (Baldwin, 2001; Li & Verma, 2002). Transcription factors that are directly influenced by reactive oxygen species and pro-inflammatory cytokines include NF- κ B, AP-1, PPAR, and other members of the nuclear receptor superfamily involved in ageing (Lavrovsky *et al.* 2000; Sanguino *et al.* 2004b). In the present study, we also measured NF- κ B and I κ B- α protein levels during ageing. NF- κ B protein in aged control rats increased compared with young rats, whereas the oral administration of amla extract decreased the elevated NF- κ B protein level compared with aged control rats. Interestingly, the protein level of I κ B- α was not changed in spite of the increased NF- κ B protein level with ageing, indicating that there might be another regulation mechanism of the I κ B- α protein level during ageing (Fig. 3). These results imply that amla protects against oxidative stress by inhibiting NF- κ B activation during ageing.

Recently, PPAR α activation has also been demonstrated to play an important clinical role in the control of the cellular redox balance and inflammatory responses such as iNOS and COX-2 by negatively interfering with NF- κ B transcriptional activity (Spencer *et al.* 1997; Blanquart *et al.* 2003). Modulation of this process may prevent or at least attenuate age-related changes in lipid metabolism and oxidative stress. Many investigators have shown that antioxidants, PPAR α activators and hypolipidaemic drugs slow the ageing process and prevent age-related disease (Poynter & Daynes, 1998; Sanguino *et al.* 2005). Thus, we assessed the contributory roles of iNOS and COX-2 to investigate the effect of amla on oxidative stress in the ageing process. As shown by the present study, old age induced the overexpressions of iNOS and COX-2 proteins, and these age-related changes were inhibited by the oral administration of amla extract (Fig. 4). Moreover, this inhibitory effect was higher in the EtOAc extract of amla, a polyphenol-enriched extract, than SunAmla. Therefore, these findings imply that amla extract can act as an antioxidant to alleviate age-related oxidative stress by the inhibition of both iNOS and COX-2 as a result of the down regulation of NF- κ B activation and up regulation of PPAR α .

Zhang *et al.* (2001) isolated several novel sesquiterpenoids from the roots, new organic acid gallates and polyphenols from the fruit juice, and new ellagitannins and flavonoids from the branches and leaves of *Phyllanthus emblica*. Among them, corilagin (β -1-O-galloyl-3,6-(R)-hexahydroxydiphenyl-*d*-glucose) and its analogue (1,6-di-O-galloyl- β -*d*-glucose) from fresh juice or solvent extracts of amla showed very strong antioxidant effects *in vitro* and *in vivo* (Ghosal *et al.* 1996; Bhattacharya *et al.* 1999; Zhang *et al.* 2001). Moreover, anti-inflammatory and anti-ageing mechanisms of polyphenolic compounds found in various plants involve the down regulation of the inflammatory response through inhibiting factors such as iNOS and COX-2 via its inhibitory effects on NF- κ B or AP-1 (De la Lastra & Villegas, 2005). Hypolipidaemic drugs and PPAR α activators reduce oxidative stress and inhibit NF- κ B activation by activation of PPAR α (Inoue *et al.* 2002; Pahan *et al.* 2002). On the basis of this evidence, the hypolipidaemic

and PPAR α -activating activities of amla extract in the present study can be explained by its antioxidant effect derived from its polyphenolic constituents.

In conclusion, the present results demonstrated that age-related increases of lipids and oxidative stress are mediated by the reduction of hepatic PPAR α expression. However, the administration of amla extract to aged rats attenuates age-related dyslipidaemia and oxidative stress by inhibiting NF- κ B transcriptional pathways through modulating hepatic PPAR α expression, suggesting that amla may be beneficial in preventing age-related atherosclerotic CVD.

References

- Anila L & Vijayalakshmi NR (2002) Flavonoids from *Emblica officinalis* and *Mangifera indica* – effectiveness for dyslipidemia. *J Ethnopharmacol* **79**, 81–87.
- Anila L & Vijayalakshmi NR (2003) Antioxidant action of flavonoids from *Mangifera indica* and *Emblica officinalis* in hypercholesterolemic rats. *Food Chemistry* **83**, 569–574.
- Asmawi MZ, Kankaanranta H, Moilanen E & Vapaatalo H (1993) Anti-inflammatory activities of *Emblica officinalis* Gaertn leaf extracts. *J Pharm Pharmacol* **45**, 581–584.
- Baldwin AS (2001) The transcription factor NF- κ B and human disease. *J Clin Invest* **107**, 3–6.
- Bandyopadhyay SK, Pakrashi SC & Pakrashi A (2000) The role of antioxidant activity of *Phyllanthus emblica* fruits on prevention from indomethacin induced gastric ulcer. *J Ethnopharmacol* **70**, 171–176.
- Barbier O, Torra P, Duguay Y, Blanquart C, Fruchart JC, Glineur C & Staels B (2002) Pleiotropic actions of peroxisome proliferator-activated receptors in lipid metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol* **22**, 717–726.
- Beckman KB & Ames BN (1998) The free radical theory of aging matures. *Physiol Rev* **78**, 547–581.
- Berger JP, Akiyama TE & Meinke PT (2005) PPARs: therapeutic targets for metabolic disease. *Trends Pharmacol Sci* **26**, 244–251.
- Bhattacharya A, Chatterjee A, Ghosal S & Bhattacharya SK (1999) Antioxidant activity of active tannoid principles of *Emblica officinalis* (aml). *Indian J Exp Biol* **37**, 676–680.
- Blanquart C, Barbier O, Fruchart JC, Staels B & Glineur C (2003) Peroxisome proliferator-activated receptors: regulation of transcriptional activities and roles in inflammation. *J Steroid Biochem Mol Biol* **85**, 267–273.
- Buege JA & Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol* **52**, 302–306.
- Chan SL & Yu VC (2004) Proteins of the bcl-2 family in apoptosis signalling: from mechanistic insights to therapeutic opportunities. *Clin Exp Pharmacol Physiol* **31**, 119–128.
- Choi YH, Lee SJ, Nguyen P, Jang JS, Lee J, Wu ML, Takano E, Maki M, Henkard PA & Trepel JB (1997) Regulation of cyclin D1 by calpain protease. *J Biol Chem* **272**, 28479–28484.
- De la Lastra CA & Villegas I (2005) Resveratrol as an anti-inflammatory and anti-ageing agent: mechanisms and clinical implications. *Mol Nutr Food Res* **49**, 405–430.
- Deokar AB (1998) *Medicinal Plants Grown at Rajagaon*, 1st ed., pp. 48–49, Pune: DS Manav Vikas Foundation.
- El-Mekkawy S, Meselhy MR, Kusumoto IT, Kadota S, Hattori M & Namba T (1995) Inhibitory effects of Egyptian folk medicines on human immunodeficiency virus (HIV) reverse transcriptase. *Chem Pharm Bull* **43**, 641–648.
- Ferrari AU, Radaelli A & Centola M (2003) Aging and the cardiovascular system. *J Appl Physiol* **95**, 2591–2597.

- Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**, 497–509.
- Ghosal S, Tripathi VK & Chauhan S (1996) Active constituents of *Emblica officinalis*: Part I – The chemistry and antioxidant effects of two new hydrolysable tannins, emblicanin A and B. *Indian J Chem* **35B**, 941–948.
- Green DR & Reed JC (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1312.
- Halliwell B & Chirico S (1993) Lipid peroxidation: its mechanism measurement and significance. *Am J Clin Nutr* **57**, 715–725.
- Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL & Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241–251.
- Inoue I, Noji S, Shen MZ, Takahashi K & Katayama S (1997) The peroxisome proliferator-activated receptor α (PPAR α) regulates the plasma thiobarbituric acid-reactive substance (TBARS) level. *Biochem Biophys Res Commun* **237**, 606–610.
- Inoue I, Itoh F, Aoyagi S, et al. (2002) Fibrate and statin synergistically increase the transcriptional activities of PPAR α /RXR α and decrease the transactivation of NF κ B. *Biochem Biophys Res Commun* **290**, 131–139.
- Itzhaki RF & Gill DM (1964) A micro-biuret method for estimating proteins. *Anal Biochem* **9**, 401–410.
- Johnson D & Lardy H (1967) Isolation of liver or kidney mitochondria. *Methods Enzymol* **X**, 94–96.
- Jose JK & Kuttan R (1995) Antioxidant activity of *E. officinalis*. *J Clin Biochem Nutr* **19**, 63–70.
- Jose JK, Kuttan G & Kuttan R (2001) Antitumour activity of *Emblica officinalis*. *J Ethnopharmacol* **75**, 65–69.
- Jung K & Pergande M (1985) Influence of cyclosporin A on the respiration of isolated rat kidney mitochondria. *FEBS Lett* **183**, 167–169.
- Kim CH, Park DH, Chung A, Zou Y, Jung KJ, Sung BK, Yu BP & Chung HY (2004) Proteomic analysis of post-mitochondrial fractions of young and old rat kidney. *Exp Gerontol* **39**, 1155–1168.
- Kim HJ, Yokoza T, Kim HY, Tohda C, Rao TP & Juneja LR (2005) Influence of amla (*Emblica officinalis* Gaertn.) on hypercholesterolemia and lipid peroxidation in cholesterol-fed rats. *J Nutr Sci Vitaminol* **51**, 413–418.
- Kim HJ, Yu BP & Chung HY (2002) Molecular exploration of age-related NF- κ B/IKK downregulation by calorie restriction in rat kidney. *Free Radic Biol Med* **32**, 991–1005.
- Knight JA (2001) The biochemistry of aging. *Adv Clin Chem* **35**, 1–62.
- Lacko AG & Davis JL (1979) Age-related changes in rat and primate plasma cholesterol metabolism. *J Am Geriatr Soc* **5**, 212–217.
- Lavrovsky Y, Chatterjee B, Clark RA & Roy AK (2000) Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp Gerontol* **35**, 521–532.
- Li Q & Verma IM (2002) NF- κ B regulation in the immune system. *Nat Immunol* **2**, 725–734.
- Lin MT & Beal MF (2003) The oxidative damage theory of aging. *Clin Neurosci Res* **2**, 305–315.
- Naito C & Yamanaka T (1978) Atherosclerotic disorder and lipid peroxidation. *Jpn J Geriatr* **15**, 187–191.
- Pahan K, Jana M, Liu X, Taylor BS, Wood C & Fischer SM (2002) Gemfibrozil, a lipid-lowering drug, inhibits the induction of nitric oxide synthase in human astrocytes. *J Biol Chem* **277**, 45984–45991.
- Parini P, Angelin B & Rudling M (1999) Cholesterol and lipoprotein metabolism in aging: reversal of hypercholesterolemia by growth hormone treatment in old rats. *Arterioscler Thromb Vasc Biol* **19**, 832–839.
- Perry LM (1980) *Medicinal Plants of East and South East Asia: Attributed Properties and Uses*. Cambridge, MA: MIT Press.
- Peters JM, Hennuyer N, Staels B, Fruchart JC, Fievet C, Gonzalez FJ & Auwerx J (1997) Alteration in lipoprotein metabolism in peroxisome proliferator-activated receptor- α deficient mice. *J Biol Chem* **272**, 28307–28312.
- Poon HF, Calabrese V, Scapagnini G & Butterfield DA (2004) Free radicals and brain aging. *Clin Geriatr Med* **20**, 329–359.
- Poynter ME & Daynes RA (1998) Peroxisome proliferator-activated receptor α activation modulates cellular redox status, represses nuclear factor- κ B signaling, and reduces inflammatory cytokine production in aging. *J Biol Chem* **273**, 32833–32841.
- Ricote M & Glass CK (2001) New roles for PPARs in cholesterol homeostasis. *Trends Pharmacol Sci* **22**, 441–443.
- Sabu MC & Kuttan R (2002) Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *J Ethnopharmacol* **81**, 155–160.
- Sakurai H, Hisada Y, Ueno M, Sugiura M, Kawashima K & Sugita T (1996) Activation of transcription factor NF- κ B in experimental glomerulonephritis in rats. *Biochim Biophys Acta* **1316**, 132–138.
- Samhan-Arias AK, Martín-Romero FJ & Gutiérrez-Merino C (2004) Kaempferol blocks oxidative stress in cerebellar granule cells and reveals a key role for reactive oxygen species production at the plasma membrane in the commitment to apoptosis. *Free Radic Biol Med* **37**, 48–61.
- Sanguino E, Ramón M, Michalik L, Wahli W, Alegret M, Sánchez RM, Vázquez-Carrera M & Laguna JC (2004a) Lack of hypotriglyceridemic effect of gemfibrozil as a consequence of age-related changes in rat liver PPAR α . *Biochem Pharmacol* **67**, 157–166.
- Sanguino E, Roglans N, Alegret M, Sánchez RM, Vázquez-Carrera M & Laguna JC (2004b) Prevention of age-related changes in rat cortex transcription factor activator protein-1 by hypolipidemic drugs. *Biochem Pharmacol* **68**, 1411–1421.
- Sanguino E, Roglans N, Alegret M, Sánchez RM, Vázquez-Carrera M & Laguna JC (2005) Atorvastatin reverses age-related reduction in rat hepatic PPAR α and HNF-4. *Br J Pharmacol* **145**, 853–861.
- Spencer NF, Poynter ME, Im SY & Daynes RA (1997) Constitutive activation of NF- κ B in an animal model of aging. *Int Immunol* **9**, 1581–1588.
- Thakur CP (1985) *Emblica officinalis* reduces serum, aortic and hepatic cholesterol in rabbits. *Experientia* **41**, 423–424.
- Toth MJ & Tehernof A (2000) Lipid metabolism in the elderly. *Eur J Clin Nutr* **54**, S121–S125.
- Wickens AP (2001) Ageing and the free radical theory. *Respir Physiol* **128**, 379–391.
- Williams RJ, Spencer JPE & Rice-Evans C (2004) Flavonoids: antioxidants or signaling molecule? *Free Radic Biol Med* **36**, 838–849.
- Yoon SO, Yun CH & Chung AS (2002) Dose effect of oxidative stress on signal transduction in aging. *Mech Ageing Dev* **123**, 1597–1604.
- Yu BP & Chung HY (2001) Oxidative stress and vascular aging. *Diabetes Res Clin Pract* **54**, S73–S80.
- Yu BP & Chung HY (2006) Adaptive mechanisms to oxidative stress during aging. *Mech Ageing Dev* **127**, 436–443.
- Zhang YJ, Tanaka T, Yang CR & Kouno I (2001) New phenolic constituents from the fruit juice *Phyllanthus emblica*. *Chem Pharm Bull* **49**, 537–540.