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Antihypercholesterolemic, antioxidative and anti-inflammatory potential of an extract of the plant *Tabernaemontana divaricata* in experimental rats fed an atherogenic diet

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ABSTRACT

The present study highlights the putative anti-atherogenic, antioxidative and anti-inflammatory potential of *Tabernaemontana divaricata* extract (ethanolic) in a Wistar rat model of experimental hypercholesterolemia. The rats were divided into Group I (control), Group II (atherogenic diet-fed, saline-treated) and Group III (atherogenic diet-fed, *T. divaricata* extract-treated [200 mg/kg/b.wt]). The levels of lipid profile parameters (total cholesterol, triglycerides, high-density, low-density and very low-density lipoprotein cholesterol and atherogenic index) and C-reactive protein (CRP) were investigated in serum samples. The levels of enzymatic and non-enzymatic antioxidants, malondialdehyde and nitric oxide (NO), and gene and protein expression levels of CRP, inducible nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNF-α) and gene expression levels of interleukin-1β (IL-1β) were determined in liver tissue samples. Rats of Group II showed significantly heightened mean concentrations of CRP, and lower mean levels of lipid profile parameters, except high-density lipoprotein cholesterol, than control rats; Rats of Group II also exhibited significantly lower mean activities/levels of enzymatic and non-enzymatic antioxidants and heightened mean malondialdehyde and NO content compared to control animals. However, in Group III rats, the values were significantly improved, in contrast to Group II rats. Significantly higher gene expression of IL-1β and significantly higher gene and protein expression of CRP, iNOS and TNF-α occurred in liver samples from hypercholesterolemic rats compared to control rats. In liver samples from Group III rats, gene and protein expression was not markedly elevated. The *T. divaricata* extract thus exhibited anti-atherogenic potential in hypercholesterolemic Wistar rats due to its hypocholesterolemic, antioxidant and anti-inflammatory effects.

1. Introduction

Atherosclerosis, which constitutes a key risk factor for cardiac diseases, is a progressive entity wherein lipids and fibrous elements accumulate in the large arteries. Several steps are discernible in atherogenesis, starting from endothelial cell activation and recruitment of immune cells, leading to differentiation of mononuclear cells and formation of foam cells. These steps are subsequently followed by foam cell death and genesis of fibrotic plaques, with uncontrolled multiplication and spread of smooth muscle cells (SMCs), and, ultimately, rupture of plaques and thrombosis (Libby, 2012). A high blood serum concentration of cholesterol is reportedly associated with evolution of atherosclerotic lesions. Reduced high density lipoprotein (HDL)-cholesterol and increased triglyceride levels are believed to predispose to formation of atherosclerotic lesions, perhaps because of an increase in fat-induced oxidative stress and attenuation of activities of antioxidant enzymes (Furukawa et al., 2004). Reactive oxygen species (ROS) in oxidative stress also are believed to augment evolution of atherosclerotic plaques in blood vessel wall (Shi et al., 2000).

Atherosclerosis is now considered a systemic vascular inflammatory disease, since inflammation occurs throughout the onset of the atherosclerotic lesions. An earlier report suggests that liver-derived inflammatory proteins may directly participate and play key roles in actuating the initiation and progression of the atherogenic process (Kleemann and Kooistra, 2005). Increased inflammatory marker blood serum levels permit a stratification of cardiovascular risk, therein

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helping in assessment of therapeutic response. Low-grade inflammation occurring during atherosclerosis appears to alter the coronary arterial endothelium; this is reflected in enhanced concentrations of inflammatory markers, such as acute-phase proteins and cytokines, in the blood (Madjid and Willerson, 2011). An elevated level of C-reactive protein (CRP) has been reported as a reliable marker of systemic inflammation in cardiovascular disease (Ridker et al., 2002) and an independent predictor of risk of atherosclerosis (Libby and Ridker, 2004). The manifestations of this inflammatory state include nuclear factor kappa B (NF-κB)-dependent overproduction of inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, and augmented expression of inducible nitric oxide synthase (iNOS), that ultimately results in the elevated production of nitric oxide (NO) (Baker et al., 2011). NO reacts slowly with most biomolecules, but is highly reactive with superoxide anion, forming more toxic peroxynitrite and hydroxyl radicals (Beckman et al., 1990), both of which are strong oxidants responsible for cellular oxidative damage. Hence, both oxidative and inflammatory processes apparently predispose to evolution of atherosclerotic lesions.

Increasingly, there are attempts to detect anti-inflammatory molecules in naturally-occurring compounds and/or products by targeting specific inflammatory cascade markers. *Tabernaemontana divaricata (T. divaricata*) is a glabrous, dichotomously-branched tropical shrub, which is a key source of Chinese and Ayurvedic medicines. Constituents with antioxidative activity are noted in abundance in *T. divaricata* leaves (Kalaimagal and Umamaheswari, 2015; Anbukkarasi et al., 2016). *T. divaricata* also reportedly exhibits antioxidant (Mandal and Mukherji, 2001; Nicola et al., 2013), antileukemic, anticoronary and antidiabetic (Daley et al., 2010), anti-obesity (Kanthlal et al., 2012), cytotoxic (in relation to anticancer therapeutics) (Rumzhum et al., 2012), analgesic (Qamruzzamaa et al., 2012) and anti-inflammatory (Jain et al., 2013) properties. A *T. divaricata* leaf extract has been reported to prevent croton-oil induced edema in albino male mice (Jain et al., 2013). However, to our knowledge, the possible anti-atherogenic characteristics of this extract, in terms of its regulatory effects on oxidative and inflammatory mechanisms in atherosclerosis, remain unreported. Hence, the current investigation sought to evaluate the essential parameters that maintain antioxidant status, extent of peroxidation of lipids, the mRNA transcript levels of the genes encoding inflammatory markers CRP, iNOS, TNF-α and IL-1β (by RT-PCR) and the levels of the CRP, iNOS and TNF- α proteins (by immunoblotting).

2. Materials and methods

2.1. Preparation of an ethanolic extract of T. divaricata leaves

The leaves of *T. divaricata* plant, locally known as 'nandiyavattom', were collected from the campus of Bharathidasan University, Tiruchirappalli, India. The plant was identified and authenticated (Authentication No: KV 001) by Dr. S. John Britto, the Director, the Rapinat Herbarium and Centre for Molecular Systematics, St.Joseph's College, Tiruchirappalli, India; the voucher specimen is currently preserved at the Department of Animal Science, Bharathidasan University, Tiruchirappalli, India.

The leaves (fresh and disease-free) of the plant were collected, shade-dried and finely powdered. 30 g of the powdered leaves were extracted with 300 ml of 95% ethanol using a Soxhlet apparatus. The resulting filtrate was finally concentrated to a dry mass (thick paste) by vacuum distillation. This semi-solid ethanolic extract of *T. divaricata* was mixed with a minimal quantity of distilled water and then suspended in saline, the resulting suspension was used for the treatment of experimental hypercholesterolemia.

2.2. Experimental animals

Institutional Animal Ethical Committee (IAEC)-approved protocols (BDU/IAEC/2014/OE/07/Dt.18.03.2014), in addition to national guidelines, were followed for animal maintenance. Albino male rats (Wistar strain; 150–200 g) were used in this study. The animals had unlimited free access to food and water. Control animals (Group I) received a normal diet (Sai Durga Feeds and Foods, Bengaluru, India) while experimental animals (Groups II and III) received an atherogenic diet. This diet was formulated by mixing atherogenic constituents in commercial feed powder. The final composition of this diet was cholesterol (5%), sucrose (20%), sodium cholate (2%), hydrogenated vegetable oil (20%), lactose (20%), choline chloride (0.4%) and thiouracil (0.15%); the remaining 32.45% consisted of the commercial rat feed.

2.3. Experimental design

Experimental rats (150–200 g) underwent random allotment to three groups (Groups I, II and III), each group comprising five animals:

Normal (control) rats (Group I) received an unlimited normal diet daily for 45 days, and saline (200 µl/kg b.wt) by the oral route every day from days 31–45.

Atherogenic diet-fed, saline-treated (Group II) animals received the atherogenic diet in unlimited quantities for 45 days, and saline (200 µl/kg b.wt) by the oral route every day from days 31–45.

Atherogenic diet-fed, *T. divaricata* extract-treated rats in Group III received the atherogenic diet in unlimited quantities for 45 days, and *T. divaricata* extract (200 mg/kg b.wt/day) as an aqueous suspension by the oral route every day from days 31–45.

All animals were sacrificed by cervical decapitation on day 46. Samples of blood were drawn from each rat, and the serum was separated. Hepatic tissue was also excised and the samples were refrigerated (−80 °C) until analysis.

2.4. Processing of hepatic tissue samples prior to analysis

Each sample (100 mg per ml of buffer) was homogenized using phosphate buffer (50 mM, pH 7.2), and centrifuged. The supernatant thus obtained was used for further analysis. The Bradford (1976) method was used to denature the protein in each sample, with crystalline bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO, USA) being a reference standard. Readings were taken at 595 nm using a UV–vis spectrophotometer (Spekol-1300, Analytik Jena, Munich, Germany).

2.5. Biochemical analysis

Enzymatic antioxidants, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), non-enzymatic antioxidants, such as reduced glutathione (GSH), ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E) and the level of lipid peroxidation were determined in hepatic tissue samples of Wistar rats by using a UV–vis spectrophotometer.

2.5.1. Determination of lipid profile levels in serum samples of Wistar rats

By employing a standard cholesterol assay kit (BioSystems, Barcelona, Spain), total cholesterol (TC), triglycerides (TG), and HDL-cholesterol serum concentrations (all in mg/dl) were determined for each sample. Friedewald's formula (Friedewald et al., 1972) was followed to calculate LDL and very low density lipoprotein (VLDL)-cholesterol levels (both in mg/dL) in each sample. The atherogenic index (AI) was also derived per the method of Sivakumar and Sivakumar (2004) as AI = (TC – HDL-cholesterol)/HDL-cholesterol.

2.5.2. Determination of activities of hepatic enzymatic antioxidants

2.5.2.1. CAT. CAT activity (µmol of hydrogen peroxide [H_2O_2] utilised/min/mg protein) was measured by a standard method (Sinha, 1972), wherein dichromatic acetic acid, following heating in presence of H_2O_2, undergoes reduction to chromic acetate, with perchloric acid

(a green-colored unstable intermediate) being formed; this was analysed spectrophotometrically at 590 nm.

2.5.2.2. SOD. SOD activity (units/mg protein) was determined following a well-known method (Marklund and Marklund, 1974), wherein the extent to which hepatic tissue inhibited pyrogallol auto-oxidation was measured. A spectrophotometer (at 470 nm) read variations in absorbance (against blank) every 1 min for 3 min.

2.5.2.3. GPx. GPx activity (μg of reduced glutathione [GSH] utilised/min/mg protein) was assayed as earlier described (Rotruck et al., 1973); the speed at which hepatic tissue Gpx catalyzed oxidation of glutathione by H_2O_2 was determined. The resulting color was read spectrophotometrically at 412 nm.

2.5.2.4. GST. Using a standard procedure (Habig and Jakoby, 1981); GST activity was assayed spectrophotometrically at 340 nm by observing linking of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) substrate; the unit of GST activity was moles of CDNB formed/min/mg of protein.

2.5.3. Determination of activities of hepatic non-enzymatic antioxidants

2.5.3.1. GSH. The content of GSH (μg/mg protein) was assayed by a previously-described procedure (Moron et al., 1979), wherein 10% trichloroacetic acid (0.5 ml) was used to precipitate out protein. To the resulting protein-free supernatant, Na_2HPO_4 (0.3 M, pH 8.0, 4.0 ml) and 5,5′-dithiobis- 2-nitrobenzoic acid (0.04% w/v, 0.5 ml) were added to yield a yellow color that was read spectrophotometrically at 412 nm.

2.5.3.2. Vitamin C. A previously-described procedure (Omaye et al., 1979) was followed to determine hepatic tissue Vitamin C concentration (g/mg protein). Here, ascorbic acid underwent oxidation by copper yielding dehydroascorbic acid, which underwent reaction with 2,4-dinitrophenylhydrazine yielding bis(2,4-dinitrophenylhydrazine) which formed a product of absorption maximum at 520 nm.

2.5.3.3. Vitamin E. The hepatic tissue Vitamin E concentration (μg/mg protein) was determined by a standard procedure (Desai, 1984), wherein tocopherol catalyses the reduction of ferric ions to ferrous ions, yielding a pink color for spectrophotometeric reading at 536 nm.

2.5.4. Measurement of lipid peroxidation in hepatic tissue samples

The MDA content (nmoles of MDA formed/mg protein) was assayed, as described by Ohkawa et al. (1979), as thiobarbituric acid (TBA)-reacting substances. Briefly, MDA (a lipid peroxidation end-product) was allowed to react with TBA, yielding a pink chromogen whose intensity was read spectrophotometrically at 532 nm (tetramethoxypropane was a reference standard).

2.6. Molecular investigations

2.6.1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression of CRP, iNOS, IL-1ß and TNF-α in liver samples of Wistar rats

Trizol (Sigma-Aldrich Chemical Co., St. Louis, USA) reagent (1 ml/100 mg tissue) was used for extracting total RNA from freshly-isolated hepatic tissue. Spectrophotometric determination of total RNA was performed at 260/280 nm to analyze its purity and integrity. RNA integrity was checked via agarose gel electrophoresis by assessing 18S and 28S band intensities.

RT-PCR was run using specific primers for CRP, iNOS, IL-1β and TNF-α genes with a reference, β-actin (ACTB) (Table 1). Using a Qiagen one-step RT-PCR kit (Qiagen, Hilden, Germany) in the prescribed manner, 2 μg of RNA template were reverse-transcribed, and then amplified by PCR using a gradient thermal cycler (Eppendorf, Hamburg, Germany). Reverse transcription was performed at 50 °C for 30 min and initial denaturation of PCR was at 95 °C (15 min). The 3-step PCR cycles included (i) denaturation at 94 °C for 1.5 min, (ii) annealing for 1.5 min at the gene-specific annealing temperature and (iii) extension at 72 °C for 3 min. PCR amplification was performed up to 30 cycles, with a final extension at 72 °C for 10 min to ensure that the products were extended completely. PCR products underwent gel electrophoresis on a 2% agarose gel followed by staining (ethidium bromide) to highlight the amplified products. Markers of molecular weight (100 bp DNA ladder, Genei, Bengaluru, India) were simultaneously run with the RT-PCR products to provide a measure of the molecular size of each amplified product (CRP, iNOS, IL-1β, TNF-α, β-actin). Densitometric scans of (stained) gels allowed normalization of band intensities of the study gene cDNA fragments against the control gene (β-actin) using Quantity One Software (Bio Rad, Hercules, CA, USA). Measurements were performed in triplicate.

2.6.2. Immunoblot analysis of liver tissue CRP, iNOS and TNF-α proteins in Wistar rat samples

Denaturation of samples was achieved using a 100 °C water bath (1 min) with sodium dodecyl sulphate sample buffer, and 60 μg portions of protein were loaded on a 10% polyacrylamide gel with 4% stacking gel, utilizing a buffer system. Following electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes soaked in transfer buffer (25 mM Tris-base, 0.193 M glycine, 20% methanol, pH 8.3) for 60 min at 24 V (Bio-Rad semidry blotting apparatus, California, USA). Transfer conditions were optimized to ensure total protein transfer. Blocking of membranes was achieved using 5% non-fat dry milk in Tris buffered saline-Tween (TBST) [25 mM Tris-base, 150 mM NaCl, pH 7.4; 0.1% (v/v) Tween-20] for 3 h; the membranes were subsequently washed twice for 5 min with Tris buffered-saline (TBS) [25 mM Tris-base, 150 mM NaCl, pH 7.4] followed by incubation with monoclonal antibody (antibodies directed against CRP [1:400 dilution], iNOS [1:1000 dilution] {Sigma-Aldrich, St. Louis, MO, USA} and TNF-a [1:500 dilution] {Santa Cruz Biotechnology, Inc., Santa Cruz, USA}) in TBS containing 5% (w/v) non-fat dry milk for 2 h. The blots were washed 2–3 times with TBST, with a final 5 min wash with TBS, and then incubated (1 h) with secondary antibody (goat anti-rabbit IgG-conjugated with alkaline phosphatase, Genei, Bengaluru, India) diluted (1:2000) in TBS with 5% non-fat dehydrated milk powder. Membranes underwent washing 2–3 times with TBST, using at least 5 min for each wash, with a final 5 min wash with TBS, and kept in a color-developing solution of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Genei, Bengaluru, India). The blots developed finally underwent scanning by gel documentation system (Quantity One Software; Bio-Rad, Hercules, CA, USA) to confirm band intensities.

2.7. Statistical analysis

The values listed represent the mean ± standard deviation of five determinations in each group. One-way analysis of variance (ANOVA), using SPSS software package for Windows (Version 21.0; IBM Corporation, Armonk, NY, USA) was applied to detect the statistical significance of differences between groups. Where ANOVA yielded significant results, post-hoc testing was performed for inter-group comparisons (between two groups) using the least significant difference test. Values were deemed to be statistically significant when $p < 0.05$.

3. Results

3.1. Serum concentrations of lipid parameters in samples from Wistar rats

The serum of hypercholesterolemic rats treated with saline (Group II) exhibited significantly ($p < 0.05$) higher mean concentrations of TC, TG, LDL-cholesterol and VLDL-cholesterol and a higher mean atherogenic index, and a lower mean concentration of HDL-cholesterol,

Table 1
Primer sequences and expected product sizes of genes encoding inflammatory markers used in the current investigation.

Gene studied	Primer	Primer sequence	Amplicon size (bps)
CRP	Forward	5'- AGC CTC TCT CAT GCT TTT GG -3'	280
	Reverse	5'- TGT CTC TTG GTG GCA TACGA -3'	
iNOS	Forward	5'- CCA ACC TGC AGG TCT TCG ATG -3'	258
	Reverse	5'- GTC GAT GCA CAA CTG GGT GAA-3'	
IL-1β	Forward	5'- GCC TCG TGC TGT CTG ACC CA -3'	191
	Reverse	5'- CAG GGT GGG TGT GCC GTC TT -3'	
TNF α	Forward	5'- GGG GGC CAC CAC GCT CTT CT -3'	336
	Reverse	5'- AGG AGC ACG TAG TCG GGG CA -3'	
β-actin	Forward	5'- ATC GCT GAC AGG ATG CAG AAG -3'	108
	Reverse	5'- AGA GCC ACC AAT CCA CAC AGA -3'	

Abbreviations: CRP: C-reactive protein, iNOS: inducible nitric oxide synthase, IL-1β: interleukin-1β, TNF-α: tumor necrosis factor-α.

Table 2
Mean levels of lipid profile parameters in serum samples from Wistar rats.

Parameters tested	Mean activities in Group I rats (received a normal diet for 45 days [days 0–45])	Mean activities in Group II rats (received an atherogenic diet for 45 days and saline for 15 days [days 31–45])	Mean activities in Group III rats (received an atherogenic diet for 45 days and *T. divaricata* extract for 15 days [days 31–45])
Total Cholesterol	58.70 ± 16.77[b,c]	153.55 ± 43.87[a]	72.84 ± 4.67[a,b]
Triglycerides	78.28 ± 8.73[b,c]	147.63 ± 19.71[a]	86.48 ± 11.65[a,b]
HDL Cholesterol	36.95 ± 6.89[b,c]	19.16 ± 10.5[a]	33.81 ± 7.02[a,b]
LDL Cholesterol	14.20 ± 13.78[b,c]	104.86 ± 39.69[a]	21.73 ± 7.12[a,b]
VLDL Cholesterol	15.65 ± 1.74[b,c]	29.52 ± 3.94[a]	17.29 ± 2.33[a,b]
Atherogenic Index	0.64 ± 0.91[b]	8.97 ± 6.71[a]	1.19 ± 0.16[b]

Values represent the mean ± standard deviation for observations made on five rats in each group.
Units: milligrams per deciliter (except for atherogenic index).
Statistical analysis.
[a]Statistically significant difference ($p < 0.05$) when compared with group I values.
[b]Statistically significant difference ($p < 0.05$) when compared with group II values.
[c]Statistically significant difference ($p < 0.05$) when compared with group III values.
Abbreviations: HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein, *T. divaricata*: *Tabernaemontana divaricata.*

when compared to control (Group I) values. However, hypercholesterolemic rats that had been treated with *T. divaricata* extract (Group III) showed significantly lower mean levels of TC, TG, LDL-cholesterol and VLDL-cholesterol and a lower mean atherogenic index, and a significantly higher mean level of HDL-cholesterol, when compared to Group II rats. Although in Group III; TC, TG and LDL-cholesterol values were significantly higher than those in Group I, mean values of HDL-cholesterol, VLDL-cholesterol and AI approximated Group I mean values (Table 2).

3.2. Mean liver tissue enzymatic antioxidant activities

In hypercholesterolemic rats given saline treatment, hepatic tissue SOD, CAT, GPx, and GST mean values were significantly less than those observed in the controls and lower than the mean levels in hypercholesterolemic rats given *T. divaricata* extract treatment (Table 3). Group III sample mean values were significantly lower when compared to control rat mean values (Table 3).

3.3. Mean liver tissue non-enzymatic antioxidant concentrations in samples from Wistar rats

In liver tissue samples from the hypercholesterolemic rats that were provided saline treatment, the mean GSH, vitamin C and vitamin E concentrations were significantly lower when compared to control rat mean concentrations and to mean concentrations in hypercholesterolemic rats that were provided *T. divaricata* extract treatment (Table 3).

3.4. Mean liver tissue MDA content in samples from Wistar rats

The mean MDA concentration was significantly higher in hypercholesterolemic rats that were treated with saline than the mean content in liver samples from control animals and from hypercholesterolemic, *T. divaricata* extract-treated rats; also, the mean liver MDA content in the extract-treated animals significantly exceeded that in control animals (Table 3).

3.5. Hepatic tissue mRNA transcript levels of the CRP, TNF-α, IL-1ß and iNOS genes in samples from Wistar rats

The mRNA transcripts of these genes were generated by RT-PCR. Expression of the *CRP* gene was significantly up-regulated as inferred by a higher mean mRNA transcript level in hypercholesterolemic rats given saline treatment (Group II) in contrast to that observed in control and in hypercholesterolemic, *T. divaricata* extract-treated rats (Fig. 1 A in terms of band intensity; Fig. 1 B in terms of densitometric scan of the same). When the mean mRNA transcript levels of *TNF-α, IL-1ß* and *iNOS* in groups I, II and III were compared, the mean levels in Group II rats all significantly ($p < 0.05$) exceeded those in control and in hypercholesterolemic, *T. divaricata* extract-treated rats (Fig. 1 A and B); expression of these three genes in samples from the extract-treated rats approached the levels of expression noted in control rats.

3.6. Hepatic tissue CRP, iNOS and TNF-α proteins in samples from Wistar rats

When CRP, iNOS and TNF-α protein expression in liver tissue of the three groups of rats was checked by immunoblotting (Fig. 2 A in terms of band intensity; Fig. 2 B in terms of densitometric scan of the same), the mean intensities of the protein bands in hypercholesterolemic rats receiving saline treatment significantly exceeded what was noted in control and in hypercholesterolemic, *T. divaricata* extract-treated rats; the mean protein band intensities in extract-treated rats approached the mean intensities observed in control rats (Fig. 2 A and B).

Table 3
Mean activities of enzymatic antioxidants and mean concentrations of non-enzymatic antioxidants and of malondialdehyde in hepatic tissue samples from Wistar rats.

Parameters tested (units)	Mean activities in Group I rats (received a normal diet for 45 days [days 0–45])	Mean activities in Group II rats (received an atherogenic diet for 45 days and saline for 15 days [days 31–45])	Mean activities in Group III rats (received an atherogenic diet for 45 days and *T. divaricata* extract for 15 days [days 31–45])
SOD (units/mg protein)	8.83 ± 1.79 [b,c]	4.89 ± 1.73[a]	6.50 ± 1.77[a,b]
CAT (μmoles of H_2O_2 utilised/min/mg protein)	73.39 ± 21.54 [b,c]	41.94 ± 5.42[a]	55.98 ± 14.38[a,b]
GPx (μg of reduced glutathione consumed/min/mg protein)	32.24 ± 1.06 [b,c]	19.15 ± 0.80[a]	26.15 ± 1.92[a,b]
GST (μmoles of 1-chloro, 2–4 dinitrobenzene [CDNB] formed/min/mg protein)	1.10 ± 0.14 [b]	0.67 ± 0.06[a]	1.00 ± 0.69[b]
GSH (μg/mg protein)	11.51 ± 0.98 [b,c]	5.19 ± 0.40[a]	7.91 ± 0.83[a,b]
Vit-C (μg/mg protein)	4.47 ± 0.40 [b,c]	1.98 ± 0.96[a]	3.91 ± 0.62[a,b]
Vit-E (μg/mg protein)	2.13 ± 0.49 [b]	1.09 ± 0.43[a]	1.98 ± 0.01[b]
MDA (nmoles of MDA produced/mg protein)	0.97 ± 0.60 [b]	1.92 ± 0.71[a]	1.20 ± 0.30[b]

Values represent the mean ± standard deviation for observations made on five rats in each group.
Statistical analysis.
[a]Statistically significant difference ($p < 0.05$) when compared with group I values.
[b]Statistically significant difference ($p < 0.05$) when compared with group II values.
[c]Statistically significant difference ($p < 0.05$) when compared with group III values.
Abbreviations: SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase, GST: Glutathione S− transferase, GSH: Reduced glutathione, Vit-C: Vitamin C, Vit-E: Vitamin E, MDA: Malondialdehyde.

4. Discussion

While multiple aspects influence the chronic disease process of atherosclerosis, the end-result is elaboration of rupture-prone plaques that may lead to atherothrombotic events. Hypercholesterolemia is considered to be one such major risk factor. When rats consume an atherogenic diet, hypercholesterolemia results, as shown by notable elevations of serum total and LDL-cholesterol levels, with no corresponding significant increase in gross body weight of the animals (Ramesh et al., 2008).

Administration of *T. divaricata* extract to atherogenic-diet fed rats prevented the elevation of serum TC, TG, LDL and VLDL-cholesterol with the significant reduction in mean serum HDL-cholesterol. Earlier literature on hyperlipidemic state revealed the generation of ROS and attenuating potential of antioxidant treatment in animal model (Adigun et al., 2016). The *T. divaricata* extract has been reported to possess scavenging, reducing and metal-chelating activities because of its various antioxidant constituents analysed through gas chromatography and mass spectrometry (GC-MS) (Anbukkarasi et al., 2016). Hence, it is logical to attribute the reduction observed in lipid parameters to the antioxidant components of *T. divaricata* extract. Various antioxidant compounds have also been reported to attenuate elevated serum cholesterol levels, such as EGCG (Ramesh et al., 2008) and eugenol (Venkadeswaran et al., 2014, 2016); a similar effect is seen with *Morus rubra* leaf extract (Sharma et al., 2010) and *Piper betle* leaf extract (Thirumalai et al., 2014; Venkadeswaran et al., 2014), materials that possess antioxidant potential.

The atherogenic index (ratio of LDL-cholesterol to HDL-cholesterol) reportedly predicts incidence of cardiac disease. Therefore, lowering this index is an important correlate of reducing atherosclerosis risk. We noted that in hypercholesterolemic rats being treated with the *T. divaricata* extract, a significantly reduced mean atherogenic index occurred, in contrast to that of atherogenic diet-fed (hypercholesterolemic) rats receiving saline therapy. The data generated by an earlier study confirm a similar pattern of reduction in mean values of this index when hypercholesterolemic rats were given a *Morus rubra* leaf extract, which also serves as a rich source of antioxidants (Sharma et al., 2010).

Oxygen-free radicals are formed in hypercholesterolemic atherogenesis (Vogiatzi et al., 2009). Protection from ROS and the by-products of peroxidised lipids and oxidised proteins is reportedly provided by antioxidants (Shi et al., 2000). We observed significantly abrogated hepatic tissue SOD, CAT, GPx and GST mean activities in hypercholesterolemic rats undergoing saline therapy in contrast to those in controls (Table 3); these lower mean activities may have been because of higher concentrations of oxygen free radicals in hepatic samples from the hypercholesterolemic animals treated with saline. However, hepatic samples of hypercholesterolemic rats undergoing therapy with *T. divaricata* extract showed optimal mean CAT, SOD, GPx, and GST activities (Table 3). Our recent report documents the abundance of antioxidant constituents in an ethanolic extract of *T. divaricata* leaves (Anbukkarasi et al., 2016); this may explain the optimal antioxidant activities noted in hypercholesterolemic rats undergoing therapy with this extract. These results mirror those reported in experimental rat hypercholesterolemia treated using a *Pleurotus ostreatus* extract (Anandhi et al., 2013) or an extract of *Piper betle* (Venkadeswaran et al., 2014).

Non-enzymatic antioxidants play key functions in affording cells protection from oxidative damage. GSH is an essential non-protein thiol in living organisms; thiol groups are observed to confer protection from oxidative damage. α-tocopherol (vitamin E), the principal lipid-soluble antioxidant, is able to quench lipid peroxides, therein protecting biological membranes from oxidative damage. Ascorbic acid (vitamin C), a water-soluble antioxidant, prevents oxidative damage in cellular membranes by reducing and regenerating oxidised α-tocopherol and lipid peroxides (Kojo, 2004; Naziroğlu and Butterworth, 2005). We observed significantly abrogated hepatic GSH, vitamin C and vitamin E mean concentrations in hypercholesterolemic rats receiving saline therapy, compared to controls (Table 3), conceivably because of lipidemic-oxidative stress. This is reflected in the observed elevated level of MDA in the present investigation. However, administration of *T. divaricata* extract orally to hypercholesterolemic rats brought about significant increase in mean hepatic GSH, vitamin C and vitamin E levels and significant reduction in the MDA level, perhaps because of the free-radical scavenging capacity of this extract (Anbukkarasi et al., 2016). These observations mirror what other workers have reported, in particular, hypercholesterolemic rats treated with an antioxidant compound L-carnitine (Adaramoye et al., 2008) and a *Piper betle* extract (Venkadeswaran et al., 2014). Thus, the ability of the *T. divaricata* extract to maintain the antioxidant status and the prevention of lipid peroxidation would have also contributed to its lipid-lowering potential.

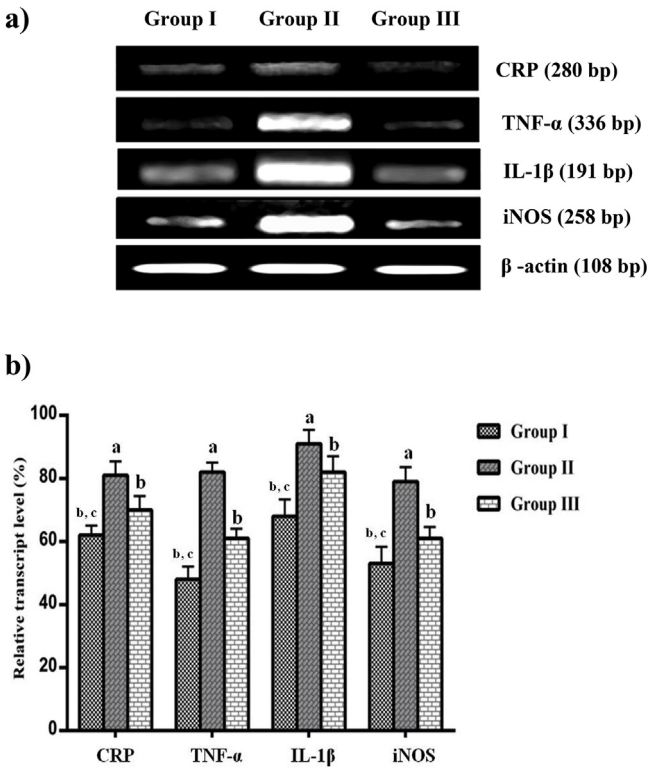

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression of CRP, iNOS, IL-1β and TNF-α in liver samples of Wistar rats. **a.** Differential staining intensity of mRNA transcripts of liver tissue CRP, TNF-α, IL-1β and iNOS visualized on ethidium bromide-stained agarose gels (internal control gene being β-actin). **b.** Bar graph of densitometric scan of the bands represent the mean ± standard deviation values (of readings in triplicate) of liver tissue CRP, TNF-α, IL-1β and iNOS mRNA transcript levels normalized to levels of control gene (β-actin).
Group I - control rats;
Group II - atherogenic diet-fed, saline-treated rats;
Group III - atherogenic diet-fed, *Tabernaemontana divaricata* extract-treated rats.
Statistical analysis
One way analysis of variance [ANOVA] with *post hoc* testing (least significant difference).
[a]Statistically significant difference ($p < 0.05$) when compared with Group I values.
[b]Statistically significant difference ($p < 0.05$) when compared with Group II values.
[c]Statistically significant difference ($p < 0.05$) when compared with Group III values.

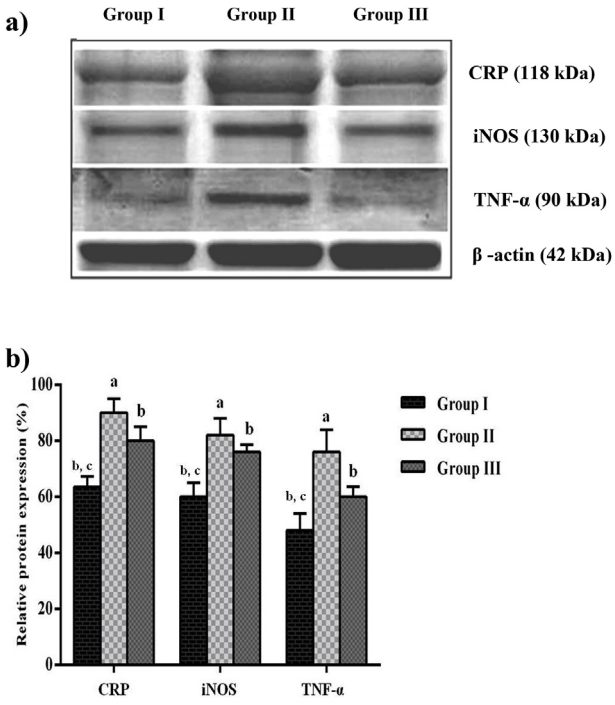

Fig. 2. Immunoblot analysis of liver tissue CRP, iNOS and TNF-α proteins in Wistar rat samples. **a.** Immunoblots showing differential staining intensity of C-reactive protein (CRP), inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF-α) proteins **b.** Bar graph of densitometric scan of the bands represent the mean ± standard deviation values (of readings in triplicate) of band intensity (densitometric) value of each protein.
Group I - control rats;
Group II - atherogenic diet-fed, saline-treated rats;
Group III - atherogenic diet-fed, *Tabernaemontana divaricata* extract-treated rats.
Statistical analysis
One way analysis of variance [ANOVA] with *post hoc* testing (least significant difference).
[a]Statistically significant difference ($p < 0.05$) when compared with Group I values.
[b]Statistically significant difference ($p < 0.05$) when compared with Group II values.
[c]Statistically significant difference ($p < 0.05$) when compared with Group III values.

CRP, a non-specific acute-phase reactant, and a biological marker of inflammation, is documented to strongly predict cardiovascular events (Black et al., 2004). In the saline-treated hypercholesterolemic rats, the serum CRP level was elevated while treatment with *T. divaricata* extract prevented such elevation. Blake and Ridker (2003) have also reported elevated serum CRP levels in cardiovascular disease and acute coronary syndrome. So also in the current investigation, hepatic tissue CRP gene and CRP protein expression were significantly ($p < 0.05$) heightened in hypercholesterolemic animals receiving saline therapy, unlike that occurring in controls. However, in hypercholesterolemic rats undergoing treatment with *T. divaricata* extract, hepatic CRP gene and CRP protein expression were akin to what was seen in controls (Figs. 1 and 2). Thus, treatment using this *T. divaricata* extract may modulate CRP expression at the transcriptional (gene) and translational (protein) levels in hypercholesterolemic rats, therein diminishing severity of hypercholesterolemia-mediated inflammation. These findings are analogous to those of Terra et al. (2009) and Ramesh et al. (2010), who reported lower CRP mRNA transcript and CRP protein levels following administration of procyanidin and EGCG, respectively, to rats ingesting a high fat-diet.

TNF-α, a multifunctional pro-inflammatory cytokine, is documented to be a powerful predisposing factor for cardiovascular diseases (Skoog et al., 2002). TNF-α is conceived to be the decisive mediator of the acute phase response since it modulates production of other inflammatory mediators, including chemokines, with an added crucial function of recruiting white blood corpuscles to the site of inflammation (Bruunsgaard et al., 2000). Interleukins (IL) are also key players in the systemic inflammatory response (Kirii et al., 2003); IL-1β has previously been conceived of as a pro-atherogenic element (Vicenová et al., 2009). Increased levels of IL-1β are related to rupture-prone atherosclerotic lesions (Weber et al., 2010). In clinical studies, high concentrations of IL-1β have been documented within atherosclerotic human coronary arteries (Fearon and Fearon, 2008).

We observed up-regulated expression of TNF-α and IL-1β genes in hypercholesterolemic rats receiving saline treatment, in contrast to that noted in control rats (Fig. 1). In hypercholesterolemic animals undergoing *T. divaricata* extract therapy, however, heightened expression of TNF-α and IL-1β genes was apparently prevented. Similarly, we noted

that the mean liver tissue TNF-α protein concentration was significantly ($p < 0.05$) elevated in hypercholesterolemic rats undergoing saline treatment, contrary to control rat values. Interestingly, in the hypercholesterolemic rats receiving *T. divaricata* extract, such an increase in the TNF-α mean protein level was prevented (Fig. 2). These observations may indicate that the *T. divaricata* leaf (ethanolic) extract can potentially inhibit the production of pro-inflammatory cytokines; thus, the inflammatory response is attenuated. A similar finding was observed when mice that received a cholesterol-rich diet underwent therapy with annexin A5 (a phospholipid binding protein) (Ewing et al., 2011).

iNOS is a key mediator of tissue injury and inflammation. Aberrant functioning of the NOS pathway may precede atherogenesis. An alteration in NO synthesis and/or activity is postulated to modulate the initiation and augment progression of atherosclerosis. NO reacts with superoxide to generate the robust oxidant, peroxynitrite, which sequentially increases lipid peroxidation, protein nitration and LDL-oxidation, therein disrupting multiple pathways of signal transduction (Heeba et al., 2009; Momi et al., 2012). In the present study, in hepatic tissue, there was significant up-regulation of iNOS gene expression and heightened iNOS protein mean concentration in hypercholesterolemic rats receiving saline therapy (Figs. 1 and 2), in contrast to mean levels in control rats. But, hypercholesterolemic rats receiving *T. divaricata* extract displayed neither up-regulation of iNOS gene nor increased iNOS protein concentration in hepatic tissue samples; moreover, hepatic NO level was also reduced (Table 4) (Supplementary material). Thus, treatment with *T. divaricata* extract appears to prevent or attenuate iNOS activation, therein averting inflammation-mediated tissue damage and progression of atherosclerotic complications. These findings are akin to those of Wan et al. (2013), in which berberine (an isoquinoline alkaloid for therapy of diabetes) treatment apparently blocked the production of noxious mediators in genesis of atherosclerotic complications by attenuating iNOS expression at the gene and protein levels, with subsequent attenuated production of NO.

The above observations on the potential of the extract of *T. divaricata* that could prevent the up-regulation of inflammatory markers indicate that its antiatherogenic efficacy is modulated through its anti-inflammatory potential.

5. Conclusion

The findings in this investigation suggest that an ethanolic extract of the *T. divaricata* leaf protects against experimental atherosclerosis in Wistar rats by virtue of its anti-hyperlipidemic and anti-oxidative properties. Moreover, its anti-inflammatory effect appears to arise by blocking the activation of inflammatory markers, namely, CRP, iNOS and pro-inflammatory cytokines, thereby preventing the initiation of atherosclerosis and atherogenic diet-mediated inflammation.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101115.

References

Adaramoye, O.A., Akintayo, O., Achem, J., Fafunso, M.A., 2008. Lipid-lowering effects of methanolic extract of Vernonia amygdalina leaves in rats fed on high cholesterol diet. Vasc. Health Risk Manag. 4, 235.
Adigun, N.S., Oladiji, A.T., Ajiboye, T.O., 2016. Antioxidant and anti-hyperlipidemic activity of hydroethanolic seed extract of Aframomum melegueta K. Schum in Triton X-100 induced hyperlipidemic rats. South Afr. J. Bot. 105, 324–332.
Anandhi, R., Annadurai, T., Anitha, T.S., Muralidharan, A.R., Najmunnisha, K., Nachiappan, V., Thomas, P.A., Geraldine, P., 2013. Antihypercholesterolemic and antioxidative effects of an extract of the oyster mushroom, Pleurotus ostreatus, and its major constituent, chrysin, in Triton WR-1339-induced hypercholesterolemic rats. J. Physiol. Biochem. 69, 313–323.
Anbukkarasi, M., Thomas, P.A., Sundararajan, M., Geraldine, P., 2016. Gas chromatography-mass spectrometry analysis and in vitro antioxidant activity of the ethanolic extract of the leaves of Tabernaemontana divaricata. Phcog. J. 8, 451–458.
Baker, R.G., Hayden, M.S., Ghosh, S., 2011. NF-kappa B, inflammation, and metabolic disease. Cell Metabol. 13, 11–22.
Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U.S.A. 87, 1620–1624.
Black, S., Kushner, I., Samols, D., 2004. C-reactive protein. J. Biol. Chem. 279, 48487–48490.
Blake, G.J., Ridker, P.M., 2003. C-reactive protein and other inflammatory risk markers in acute coronary syndromes. J. Am. Coll. Cardiol. 41, S37–S42.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bruunsgaard, H., Pedersen, A.N., Schroll, M., Skinhøj, P., Pedersen, B.K., 2000. TNF-α, leptin, and lymphocyte function in human aging. Life Sci. 67, 2721–2731.
Daley, C.A., Abbott, A., Doyle, P.S., Nader, G.A., Larson, S., 2010. A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. Nutr. J. 9 10-10.
Desai, I.D., 1984. Vitamin E analysis methods for animal tissues. Methods Enzymol. 105, 138–147.
Ewing, M.M., de Vries, M.R., Nordzell, M., Pettersson, K., de Boer, H.C., van Zonneveld, A.J., Frostegård, J., Jukema, J.W., Quax, P.H.A., 2011. Annexin A5 therapy attenuates vascular inflammation and remodeling and improves endothelial function in mice. Arterioscler. Thromb. Vasc. Biol. 31, 95–101.
Fearon, W.F., Fearon, D.T., 2008. Inflammation and cardiovascular disease. Am. Heart Assoc.
Friedewald, W.T., Levy, R.I., Fredrickson, D.S., 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin. Chem. 18, 499–502.
Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., Shimomura, I., 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. J. Clin. Investig. 114, 1752–1761.
Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Methods Enzymol. 77, 398–405.
Heeba, G., Moselhy, M., Hassan, M., Khalifa, M., Gryglewski, R., Malinski, T., 2009. Anti-atherogenic effect of statins: role of nitric oxide, peroxynitrite and haem oxygenase-1. Br. J. Pharmacol. 156, 1256–1266.
Jain, S., Sharma, P., Ghule, S., Jain, A., Jain, N., 2013. In vivo anti-inflammatory activity of Tabernaemontana divaricata leaf extract on male albino mice. Chin. J. Nat. Med. 11, 472–476.
Kalaimagal, C., Umamaheswari, G., 2015. Bioactive compounds from the leaves of Tabernaemontana divaricata (L.). Int. J. Recent Sci. Res. 6, 3520–3522.
Kanthlal, S.K., Suresh, V., Arunachalam, G., Royal Frank, P., Kameshwaran, S., 2012. Anti-obesity and hypolipidemic activity of methanol extract of Tabernaemontana divaricata on atherogenic diet induced obesity in rats. Int. Res. J. Pharm. 3, 157–161.
Kirii, H., Niwa, T., Yamada, Y., Wada, H., Saito, K., Iwakura, Y., Asano, M., Moriwaki, H., Seishima, M., 2003. Lack of interleukin-1β decreases the severity of atherosclerosis in ApoE-deficient mice. Arterioscler. Thromb. Vasc. Biol. 23, 656–660.
Kleemann, R., Kooistra, T., 2005. HMG-CoA reductase inhibitors: effects on chronic subacute inflammation and onset of atherosclerosis induced by dietary cholesterol. Cardiovasc. Haematol. Disord. - Drug Targets 5, 441–453.
Kojo, S., 2004. Vitamin C: basic metabolism and its function as an index of oxidative stress. Curr. Med. Chem. 11, 1041–1064.
Libby, P., 2012. History of discovery: inflammation in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 32, 2045–2051.
Libby, P., Ridker, P.M., 2004. Inflammation and atherosclerosis: role of C-Reactive protein in risk assessment. Am. J. Med. 116, 9–16.
Madjid, M., Willerson, J.T., 2011. Inflammatory markers in coronary heart disease. Br. Med. Bull. 100, 23–38.
Mandal, M., Mukherji, S., 2001. A study on the activities of a few free radicals scavenging

enzymes present in five roadside plants. J. Environ. Biol. 22, 301–305.
Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469–474.
Momi, S., Monopoli, A., Alberti, P.F., Falcinelli, E., Corazzi, T., Conti, V., Miglietta, D., Ongini, E., Minuz, P., Gresele, P., 2012. Nitric oxide enhances the anti-inflammatory and anti-atherogenic activity of atorvastatin in a mouse model of accelerated atherosclerosis. Cardiovasc. Res. 94, 428–438.
Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim. Biophys. Acta 582, 67–78.
Naziroğlu, M., Butterworth, P.J., 2005. Protective effects of moderate exercise with dietary vitamin C and E on blood antioxidative defense mechanism in rats with streptozotocin-induced diabetes. Can. J. Appl. Physiol. 30, 172–185.
Nicola, C., Salvador, M., Escalona Gower, A., Moura, S., Echeverrigaray, S., 2013. Chemical constituents antioxidant and anticholinesterasic activity of tabernaemontana catharinensis. Sci. World J. 2013, 10.
Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
Omaye, S.T., Turnbull, J.D., Sauberlich, H.E., 1979. Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Methods Enzymol. 62, 3–11.
Qamruzzamaa, A., Ansarib, J.A., Sayyedb, M., 2012. Analgesic and anti-inflammatory effect of ethanolic extract of tabernaemontana divaricata l. flowers in rats. Der Pharm. Lett. 4, 1518–1522.
Ramesh, E., Elanchezhian, R., Sakthivel, M., Jayakumar, T., Senthil Kumar, R., Geraldine, P., Thomas, P., 2008. Epigallocatechin gallate improves serum lipid profile and erythrocyte and cardiac tissue antioxidant parameters in Wistar rats fed an atherogenic diet. Fundam. Clin. Pharmacol. 22, 275–284.
Ramesh, E., Geraldine, P., Thomas, P.A., 2010. Regulatory effect of epigallocatechin gallate on the expression of C-reactive protein and other inflammatory markers in an experimental model of atherosclerosis. Chem. Biol. Interact. 183, 125–132.
Ridker, P.M., Rifai, N., Rose, L., Buring, J.E., Cook, N.R., 2002. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N. Engl. J. Med. 347, 1557–1565.
Rotruck, J., Pope, A., Ganther, H., Swanson, A., Hafeman, D.G., Hoekstra, W., 1973. Selenium: biochemical role as a component of glutathione peroxidase. Science 179, 588–590.
Rumzhum, N.N., Rahman, M., Kazal, K., 2012. Antioxidant and cytotoxic potential of methanol extract of Tabernaemontana divaricata leaves. Int. Curr. Pharm. J. 1, 27–31.
Sharma, S.B., Tanwar, R.S., Rini, A., Singh, U.R., Gupta, S., Shukla, S.K., 2010. Protective effect of Morus rubra L. leaf extract on diet-induced atherosclerosis in diabetic rats. Indian J. Biochem. Biophys. 47, 26–31.
Shi, W., Haberland, M.E., Jien, M.-L., Shih, D.M., Lusis, A.J., 2000. Endothelial responses to oxidized lipoproteins determine genetic susceptibility to atherosclerosis in mice. Circulation 102, 75–81.
Sinha, A.K., 1972. Colorimetric assay of catalase. Anal. Biochem. 47, 389–394.
Sivakumar, V., Sivakumar, S., 2004. Effect of an indigenous herbal compound preparation 'Trikatu' on the lipid profiles of atherogenic diet and standard diet fed Rattus norvegicus. Phytother Res. 18, 976–981.
Skoog, T., Dichtl, W., Boquist, S., Skoglund-Andersson, C., Karpe, F., Tang, R., Bond, M.G., de Faire, U., Nilsson, J., Eriksson, P., Hamsten, A., 2002. Plasma tumour necrosis factor-α and early carotid atherosclerosis in healthy middle-aged men. Eur. Heart J. 23, 376–383.
Terra, X., Montagut, G., Bustos, M., Llopiz, N., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., Salvadó, J., Arola, L., Blay, M., 2009. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. J. Nutr. Biochem. 20, 210–218.
Thirumalai, T., Tamilselvan, N., David, E., 2014. Hypolipidemic activity of Piper betel in high fat diet induced hyperlipidemic rat. J. Acute Dis. 3, 131–135.
Venkadeswaran, K., Muralidharan, A.R., Annadurai, T., Ruban, V.V., Sundararajan, M., Anandhi, R., Thomas, P.A., Geraldine, P., 2014. Antihypercholesterolemic and antioxidative potential of an extract of the plant, piper betle, and its active constituent, eugenol, in Triton WR-1339-induced hypercholesterolemia in experimental rats. Evid. Based Complement. Altern. Med. 2014, 11.
Venkadeswaran, K., Thomas, P.A., Geraldine, P., 2016. An experimental evaluation of the anti-atherogenic potential of the plant, Piper betle, and its active constitutent, eugenol, in rats fed an atherogenic diet. Biomed. Pharmacother. 80, 276–288.
Vicenová, B., Vopálenský, V., Burýsek, L., Pospisek, M., 2009. Emerging role of interleukin-1 in cardiovascular diseases. Physiol. Res. 58, 481.
Vogiatzi, G., Tousoulis, D., Stefanadis, C., 2009. The role of oxidative stress in atherosclerosis. Hellenic J. Cardiol. 50, 402–409.
Wan, X., Chen, X., Liu, L., Zhao, Y., Huang, W.-J., Zhang, Q., Miao, G.-G., Chen, W., Xie, H.-G., Cao, C.-C., 2013. Berberine ameliorates chronic kidney injury caused by atherosclerotic renovascular disease through the suppression of NFκB signaling pathway in rats. PLoS One 8, e59794.
Weber, A., Wasiliew, P., Kracht, M., 2010. Interleukin-1 (IL-1) pathway. Sci. Signal. 3 cm1-cm1.