5.1 INTRODUCTION

5.1.1 REGRESSION OF ATHEROSCLEROSIS

The concept that atherosclerosis is a relentlessly progressive disease has been derived from well-documented clinical and pathological observations [Mcgrill 1968]. However studies in animals have demonstrated that atherosclerotic plaques can decrease in size when the atherogenic stimuli are removed, and sequential angiographic observations in humans also suggest that atherosclerotic stenoses can enlarge their lumina under certain circumstances [Malinow 1981]. These processes have been called “atherosclerosis regression” [Armstrong 1976] and the correspondence of regression in humans and animals still needs to be established.

5.1.2 EXPERIMENTAL MODELS

Arterial lesions in many respects resembling human atherosclerosis can be produced in animals by diet. Over the past century, significant advances have been made in the development of animal models of human coronary artery disease. In addition a variety of experimental procedures have been used to investigate the pathophysiology of atherosclerosis. Atherosclerotic lesions have been induced in several animal models including rodents (mice, rabbits, rats, hamsters, guinea pigs) avian (pigeons, chicken, quail), swine, carnivore (dogs, cats) and non-human primates [Mohammed and Moghadasian 2002]. Development of the first line of knock-out animal models, namely apo E - KO mice in the year 1992, is considered an important milestone in experimental atherosclerosis research. This innovation was followed by the production of an ever increasing number of knock out/transgenic animal models. These accomplishments have greatly enhanced our understanding of the disease.

However the numerous animal models used to study the effect of cholesterol and lipoprotein metabolism, differ from humans in the plasma distribution of cholesterol and in the processing of lipoproteins in the plasma compartment. Even data from transgenic or knock out mice are of limited use, because other metabolic pathways and responses to interventions differ from the human condition. The use of appropriate animal models to determine the effects of therapeutic interventions on metabolic processes and gene expression regulating cholesterol and lipoprotein
metabolism is essential to understand the mechanisms underlying their effects on plasma lipids.

Apart from transgenic mice, rabbit, chicken and monkey are more commonly used. Rabbit was the first animal model to be used in atherosclerosis research [Ignatowski 1908]. It shares several aspects with human lipoprotein metabolism except for deficiency in hepatic lipase. Though lesions are readily produced within a few weeks, being a strict vegetarian its serum cholesterol levels are much lower than that of man, and arterial lesions are formed at extremely high plasma cholesterol levels, far in excess of those usually seen in man.

Rat on the other hand is an atherosclerosis resistant species. Unlike humans and similar to mice, rats do not have cholesterol ester transfer protein (CETP) and HDL is the major carrier of plasma cholesterol. Being hypo responsive to dietary cholesterol, hyperlipidaemia and atherogenesis may only be induced in rats by high fat diet containing cholic acid and thiouracil [Joris et al. 1983].

Though spontaneous atherosclerosis was observed in the abdominal aorta of chicken of both sexes over 3 years of age [Weiss 1959], even on cholesterol free diets, cholesterol feeding accelerates the formation of lesions with increasing content of lipids within the lesions. Its non mammalian nature and lesions being less advanced with minimum complications are the drawbacks. Monkey has been another successful model because of the ‘overall’ similarity of lesions in the primate to those of man [Taylor et al. 1954]. Animal size, numbers and difficulty of handling are the disadvantages.

5.1.3 GUINEA PIG MODEL OF ATHEROSCLEROSIS

In this study guinea pig model has been used to investigate the anti atherogenic influence of Ev, Ir(A) and Ir(H) because of its unique inducibility of atherosclerosis. Also it has several striking similarities to humans in terms of hepatic cholesterol and lipoprotein metabolism, such as

- majority of circulating cholesterol is transported in LDL [Fernandez 2001]
- high LDL to HDL ratios [Fernandez et al. 1990]
• the binding domain for the LDL receptor differentiates between normal and familial defective apo B-100

• Apo B mRNA editing in liver is present in negligible amounts (<1%) compared to 18 to 70% in other species [Greeve et al. 1993]

• higher concentrations of free than esterified cholesterol in the liver [Angelin et al. 1992]

• guinea pigs possess plasma CETP (Cholesterol ester transfer protein)[Ha and Barter 1982] - a critical component for human reverse cholesterol transport [Tall 1998], LCAT (Lecithin cholesterol acyl transferase) [Douglas and Pownell 1991] and lipoprotein lipase LPL [Olivecrona and Bengston-Olivecrona 1993] activities for intravascular processing of plasma lipoproteins. This is in contrast to other rodents [Quig and Zilversmitt 1990]

• comparable to humans [Reihner et al. 1991] and in contrast to rats (Swan GPB) guinea pigs exhibit moderate rates of hepatic cholesterol synthesis [Fernandez et al. 1990] and catabolism [Fernandez et al. 1995]

• Guinea pigs lack endogenous ascorbate supply [Sauberlich 1978] and like humans and other primates it is known to have lost the ability to synthesize ascorbate.

• Lipoprotein(a), an extremely atherogenic lipoprotein is found in the plasma and atherosclerotic lesions of guinea pigs, similar to species lacking endogenous ascorbate [Rath and Pauling 1990]

• Females have higher HDL concentrations than males [Roy et al. 2000] and ovariectomized guinea pigs have a plasma lipid profile similar to post menopausal women

• in response to exercise guinea pigs lower plasma TGL and increase plasma HDL-C [Ensign et al. 2002]

• they respond to dietary interventions [Fernandez et al. 1992] and drug treatment [Hokada et al. 1992] by lowering plasma cholesterol
in contrast to hamsters they do not possess a fore stomach which ferments fiber before reaching the small intestine [Fernandez et al. 1999]


they are good models for studying the mechanisms by which statins [Conde et al. 1996], cholestryamine [Fernandez et al. 2000] and apical bile acid transport inhibitors [West et al. 2002] lower plasma cholesterol

they can also be used to study the mechanisms by which certain drugs affect triglyceride metabolism [Aggarwal et al. 2005]

Thus guinea pig was chosen as the model for its striking similarities to human atherosclerosis, making results of the study amenable to extrapolation to human situation

5.2 EVALUATION OF ANTIATHEROGENIC EFFECTS

The modulatory influence of the extracts Ev, Ir(A) and Ir(H) was studied on early atherosclerotic changes following 120 days of high fat diet administration in male Hartley guinea pigs. The parameters studied are

1. Serum lipid estimation for TC, TGL, HDL-C, LDL-C and A.I

2. Liver and heart tissue antioxidant status by the measurement of oxidative stress biomarkers - tissue Thiobarbituric acid reactive substance (TBARS), reduced glutathione (GSH), Super oxide dismutase (SOD) and Glutathione peroxidase (GPX)

3. Histopathological assessment of coronary artery for atherosclerotic changes

4. Enface assessment of aortic lesion areas

5.2.1 MATERIAL AND METHODS

Early atherosclerotic changes were induced in the animals by administration of high fat diet (HFD) containing 0.2%w/w cholesterol for an initial induction period of one month. Animals were grouped and extracts/standard drug atorvastatin calcium
was administered along with a maintenance diet containing 0.15%w/w cholesterol for 90 days. The animals were sacrificed and evaluated for signs of regression of atherosclerosis.

**Chemicals**

Cholesterol, bovine serum albumin, thiobarbituric acid, nitro blue tetrazolium, 5, 5’-dithio bis (2- nitro benzoic acid) and Oil Red O were purchased from Sigma (St.Louis, USA). Atorvastatin calcium was a gift from M/S Ordain Health Care (Pvt) Ltd., Chennai, India. Edible coconut oil was purchased from the local market and guinea pig pellet diet was obtained from Vet Care, Bangalore, India. Commercial enzyme assay kits (Randox Laboratories, Antrim, UK) were used for determination of serum total cholesterol (TC), triglycerides (TGL) and high density lipoprotein (HDL-C). Other chemicals and reagents used in these experiments were of analytical grade and were purchased from reputed commercial sources.

Microscopical examination for histopathological assessment was carried out on a Nikon eclipse TE 2000S microscope. Serum lipid levels were measured using a semi auto analyzer (Star Plus 21) and tissue levels of oxidative stress biomarkers were analysed using a Perkin Elmer lambda 25 UV VIS Spectrophotometer. Gross morphological visualization of lesion areas on Oil Red O stained aortas was enabled by Digital Camera Sony, Cyber shot, Model No: W 305 and total atherosclerotic area was measured using Image Proplus® image analysis software version 6.0.

**Animals**

Male Hartley guinea pigs aged 10 months weighing 700-800 g were purchased from King’s Institute of Preventive Medicine, Chennai-34, India. The animals were housed in specially designed pens group wise at ambient temperature and fed standard Guinea pig pellet diet and water *ad libitum*. They were acclimatized to laboratory conditions for 15 days prior to experimentation. The study was performed in accordance with the protocol approved by the Institutional Animal Ethics Committee (IAEC: Ref: IAEC / SRMC & RI / 41 / 2005).
Experimental Design

Animals (n=36) were fed p.o 0.2%w/w cholesterol dispersed in coconut oil along with normal pellet diet for 30 days while a group of 6 animals received standard pellet diet. 6 animals of the cholesterol fed group were sacrificed and their aorta, coronary artery, heart, liver, gall bladder, lung, kidney and spleen were quickly harvested and preserved in formalin. They were put to paraffin blocks, sectioned, processed, stained with haematoxylin and eosin (H&E) and evaluated microscopically for histopathological changes. The remaining animals of the high fat group were divided into 5 groups of 6 animals each.

The following 60 days they were given pellet diet, water *ad libitum* and 0.15%w/w cholesterol dispersed in coconut oil (atherogenic diet) along with the extracts/ standard drug at the same time between 9.00 AM and 10 AM each day. Weight of the animals was taken at the start of experimentation and thereafter at weekly intervals.

Group design

- **Group I** - Normal control received standard guinea pig pellet diet.
- **Group II** - Atherogenic diet
- **Group III** - Atherogenic diet plus 100 mg/kg bw/ day p.o Ev
- **Group IV** - Atherogenic diet plus 100 mg/kg bw/ day p.o Ir (A).
- **Group V** - Atherogenic diet plus 100 mg/kg bw/ day p.o Ir (H).
- **Group VI** - Atherogenic diet plus atorvastatin calcium 10 mg/kg bw/ day.

Post experimentation

After 90 days the animals were fasted overnight, killed by an overdose of ether anaesthesia and blood collected by cardiac puncture. It was processed for serum lipid estimation. Animals were sacrificed, liver quickly removed, washed and stored under liquid nitrogen. The heart was located and pericardium excised. The left coronary artery was identified and the entire anterior descending left coronary artery was dissected out along with a tiny portion of neighbouring heart tissue and stored in
formalin. Aorta was accessed through the left ventricle with a corneal scissors which was run through to slit the aorta longitudinally. The entire length of the aorta from the base of the aortic arch up to the diaphragmatic hiatus was resected out, trimmed of adventitial fat and stored in formal calcium (10% formalin with 1% CaCl₂). The heart was immediately dissected out and stored in a nitrogen freezer until needed for analysis.

5.2.1.1 Serum Lipid estimation

The blood samples were allowed to clot at room temperature by setting aside for 1 hr and then subjected to centrifugation at 2000 rpm for 15 min. The serum was separated and assayed for TC, TGL and HDL cholesterol on a semi auto analyzer using commercial enzyme assay kits. LDL was calculated by Friedwal’s formula \( \{\text{LDL} = \text{TC} - (1/5 \text{TGL} + \text{HDL})\} \) [Freidwal et al. 1992]. Atherogenic index (A.I) [Yoon et al. 2008] which gives a measure of the risk of atherogenicity was calculated using the formula

\[
\text{A.I} = \frac{\text{TC} - \text{HDL Cholesterol}}{\text{HDL Cholesterol}} \quad \text{Total cholesterol} \quad \text{[Trinder 1969]}
\]

Cholesterol plays an important role in the body as hormone, hormone precursor, an aid in digestion, as energy reserve, metabolic fuel and as a structural and functional component in bio membranes as well as an insulation to allow nerve conduction and prevent heat loss. Strongly associated with lipoprotein metabolism and atherosclerosis, its determination by several methods is of clinical significance. In this study, quantitative \textit{in vitro} determination of serum cholesterol was by enzymatic end point method using reagents provided by the assay kit whose composition was as follows.
<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-aminopyrine</td>
<td>0.30 mmol/L</td>
</tr>
<tr>
<td>Phenol</td>
<td>6 mmol/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 0.5 U/ml</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td>≥ 0.15 U/ml</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>≥ 0.1 U/ml</td>
</tr>
<tr>
<td>Pipes buffer</td>
<td>80 mmol/L; pH 6.8</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td>5.17 mmol/L (200 mg/dl)</td>
</tr>
</tbody>
</table>

**Assay principle**

Serum cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinineimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Fatty acids}
\]

Cholesterol esterase

\[
\text{Cholesterol} + \text{O}_2 \rightarrow \text{Cholestene-2-one} + \text{H}_2\text{O}_2
\]

Cholesterol oxidase

\[
2\text{H}_2\text{O}_2 + \text{phenol} + 4\text{-aminoantipyrine} \rightarrow \text{quinoneimine} + 4\text{H}_2\text{O}
\]

Peroxidase

**Procedure**

10 μl of sample/standard was mixed with 1 ml reagent and incubated at RT for 5 min. 10 μl of distilled water treated similarly served as reagent blank. Cholesterol content was estimated at 500 nm by aspiration into a semi auto analyzer, calibrated with the standard.

**Triglycerides** [Tietz 1990]

TGL measurements are used in the diagnosis and treatment of diseases involving lipid metabolism, various endocrine disorders and liver obstruction.
Quantitative *in vitro* estimation was enabled by the method of enzymatic hydrolysis with lipases using reagents provided whose composition was as follows:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Pipes buffer</td>
<td>40 mmol/L, pH 7.4</td>
</tr>
<tr>
<td>4-chlorophenol</td>
<td>5.4 mmol/L</td>
</tr>
<tr>
<td>Magnesium ions</td>
<td>5.0 mmol/L</td>
</tr>
<tr>
<td>ATP</td>
<td>1.0 mmol/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 0.5 U/ml</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>≥ 0.4 U/ml</td>
</tr>
<tr>
<td>Glycerol-3-phosphate oxidase</td>
<td>≥ 1.5 U/ml</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.05%</td>
</tr>
<tr>
<td><strong>2. Enzyme reagent</strong></td>
<td></td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>0.4 mmol/L</td>
</tr>
<tr>
<td>Lipases</td>
<td>≥ 150 U/ml</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.05%</td>
</tr>
<tr>
<td><strong>3. Standard</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.29 mmol/L(200 mg/dl)</td>
</tr>
</tbody>
</table>

**Assay principle**

Similar to TC the indicator is quinoneimine formed from hydrogen peroxide and 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{Lipases}} \text{Glycerol} + \text{fatty acids (lipases)}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + 4\text{-chlorophenol} \xrightarrow{\text{POD}} \text{quinoneimine} + \text{HCl} + 4\text{H}_2\text{O}
\]
Procedure:

10 µl of sample/standard was mixed with 1 ml reagent and incubated at RT for 5 min. TGL content was estimated against reagent blank at 500 nm by aspiration into a semi auto analyzer upon calibration with the standard.

**HDL-C** [Nauck et al. 1996]

HDL-C is one of the major classes of plasma lipoproteins. They are composed of a number of heterogeneous particles, including cholesterol and vary with respect to size and content of lipid and apolipoprotein. HDL serves to remove cholesterol from the peripheral cells to the liver, where it is converted to bile acids and excreted into the intestine. An inverse relationship between HDL-C levels in serum and the incidence/prevalence of coronary heart disease has been demonstrated in a number of epidemiological studies. The importance of HDL-C as a risk factor for CHD is now recognized. Accurate measurement of HDL-C is therefore of vital importance. In this study serum HDL-C was determined by direct measurement using reagents provided by the assay kit. Direct measurement gives improved accuracy and reproducibility when compared to precipitation methods.

**Principle**

The assay consists of 2 distinct reaction steps

Elimination of chylomicron, VLDL-C and LDL-C by cholesterol esterase followed by oxidation with cholesterol oxidase and catalase.

\[
\begin{align*}
\text{Cholesterol ester} & \xrightarrow{\text{cholesterolesterase}} \text{Cholesterol + Fatty acids} \\
\text{Cholesterol + O}_2 & \xrightarrow{\text{cholesterol oxidase}} \text{Cholestenone + H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 & \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2
\end{align*}
\]
### Reagent composition

<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R1  Enzyme reagent 1</strong></td>
<td></td>
</tr>
<tr>
<td>N,N-Bis(2-hydroxyethyl)-2-aminoethane sulphonic acid</td>
<td>100 mmol/L, pH 6.6</td>
</tr>
<tr>
<td>N-(2-hydroxy-3-Sulfopropyl)-3,5-dimethoxyaniline; sodium Salt (HDOAS)</td>
<td>0.7 mM</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td>≥ 800 U/L</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>≥500 KU/L</td>
</tr>
<tr>
<td>Catalase</td>
<td>≥300 KU/L</td>
</tr>
<tr>
<td>Ascorbate oxidase</td>
<td>≥3000 KU/L</td>
</tr>
<tr>
<td><strong>R2  Enzyme reagent 2</strong></td>
<td></td>
</tr>
<tr>
<td>N,N-Bis(2-hydroxyethyl) 2-aminoethanesulphonic acid</td>
<td>100 mmol/L, pH 7.0</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>4 mM</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥3500 U/L</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.05 %w/v</td>
</tr>
<tr>
<td>Surfactants</td>
<td>1.4%w/v</td>
</tr>
<tr>
<td><strong>HDL Calibrator</strong></td>
<td>1.7 ml (80 mg/dl)</td>
</tr>
</tbody>
</table>

**Procedure**

450 µl of R1 was incubated with 10 µl sample/standard at RT for 5 min. 150 µl R2 was added, mixed quickly and aspirated into the analyzer and absorbance measured at 600 nm. HDL values were read off from the analyser calibrated with the HDL standard.

### 5.2.1.2 Oxidative stress biomarker estimation

Heart and liver tissue was homogenized in ice cold 10% KCl using a polytron homogenizer, centrifuged at 2000 rpm and supernatant used for measurement of Super oxide dismutase (SOD) [Poonam et al. 1984], Glutathione peroxidase (GPX) [Rotruck et al. 1973], Thiobarbituric acid reactive substances (TBARS) [Moran et al. 1979; Okhawa et al. 1979] and reduced glutathione (GSH) [Moran et al. 1979].
Determination of TBARS

TBARS refer to secondary products of lipid peroxidation, derived from lipid peroxides of polyunsaturated fatty acids. Extent of membrane lipid damage was determined by estimating the accumulation of the peroxidative product, thiobarbituric reactive substance, which reacts with thiobarbituric acid (TBA) forming a red pigment under optimum reaction conditions. The absorbance of the formed pigment was measured at 532 nm. BHT was added to prevent initiation of membrane lipid peroxidation during the assay. 1,1,3,3-tetramethoxypropane was used as an internal standard and standard curve plotted. The level of lipid peroxides is expressed in terms of n mol/gm tissue wet weight.

Reagents

Thiobarbituric acid reagent (TBA- 0.8% in 0.5 N HCl)
Butylated hydroxyl toluene (BHT-0.05%)
Saline (0.9%)
1,1,3,3-tetramethoxypropane (TMP)

Procedure

0.2 ml tissue homogenate in 10% KCl was mixed with 0.8 ml saline, 0.5 ml BHT, 3.5 ml TBA reagent and heated for 90 min on a boiling water bath. After cooling, the solution was centrifuged at 2000 rpm for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against blank that contained all reagents minus the biological sample. A standard curve was plotted based on the absorbances of TMP (10-50 n moles in saline) with TBA. Sample TBARS was calculated based on the concentration from the standard curve.

Reduced Glutathione (GSH)

It is the most important of endogenous antioxidants, ubiquitous in living systems. A non enzymatic mode of defense against free radicals, it is a linear tripeptide of l-glutamine, l-cysteine and glycine. The molecule has a sulfhydryl (-SH)
group on the cysteinyl portion, which accounts for its strong electron donating character. Its high negative redox potential combined with high intracellular concentration generates great reducing power. This characteristic underlies its potent antioxidant action and enzyme cofactor properties and supports a complex thiol exchange system, which hierarchically regulates cell activity. Thus GSH is an important constituent of intracellular protective mechanism against a number of noxious stimuli including oxidative stress. Direct attack by free radicals and other oxidative agents depletes GSH. GSH is most concentrated in liver, followed by spleen, kidney, lens, erythrocytes and leukocytes.

Monitoring tissue levels of GSH gives an indication of the extent of oxidative damage.

Principle

Liver and heart tissue GSH content was estimated by measuring the absorbance of the complex formed with DTNB reagent 5, 5'-dithiobis (2-nitrobenzoic acid).

Reagents

1. 5% Trichloroacetic acid (TCA)
2. Phosphate buffer 0.2 M, pH 8.0
3. DTNB (0.6 mM in Phosphate buffer)

Procedure

0.25 ml tissue homogenate was added to equal volume of ice cold TCA. The precipitate was removed by centrifugation at 4000 rpm for 10 min. To the supernatant, 0.25 ml of 0.2 M phosphate buffer, and 0.5 ml DTNB was added and mixed well. The absorbance was read at 412 nm. A standard curve of glutathione was plotted using absorbance values of 8-40 µg of glutathione complex with DTNB. The glutathione content of the samples was taken from the standard curve. The values are expressed in µg/g tissue.
Glutathione peroxidase (GPX)

GPX refers to a family of enzymes with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of GPX is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. There are several isozymes encoded by different genes, which vary in cellular location and substrate specificity. GPX is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. GPX for eg., catalyzes the reduction of H₂O₂ and itself forms glutathione disulphide or oxidized glutathione (GSSG). GSSG is reduced by glutathione reductase to regenerate GSH.

Estimation of tissue levels of GPX indicates extent of tissue oxidative stress.

Principle

GPX in tissues is estimated based on the fact that GSH consumed in a reaction in the presence of H₂O₂ is proportional to the GPX content. Sodium azide is added to inhibit remnant CAT activity.

Reagents

- Sodium azide (10mM)
- GSH (2mM)
- H₂O₂ (1mM)
- 10% TCA
- Potassium EDTA
- Tris HCL Buffer (0.4mM)
- DTNB (0.6mM)

Procedure

200 µl tissue homogenate, 200 µl tris HCl buffer, 0.4 mM Potassium EDTA, 100 µl sodium azide were mixed well. 200 µl of GSH solution was added followed by 0.1 ml H₂O₂. The overall reaction was arrested by adding 0.5 ml 10% TCA. The
precipitate was removed by centrifugation at 4000 rpm for 10 min. To the supernatant 0.5 ml DTNB was added and absorbance measured at 412 nm. The non enzymatic reaction was correspondingly assessed by replacing the enzyme sample by buffer. The results are expressed as n moles of GSH consumed/min/mg protein.

**Super oxide dismutase (SOD)**

This endogenous enzyme antioxidant has been recognized to play an important role in body defense mechanism against the deleterious effects of free radicals in biological systems. Its significance in various types of pathogenic response, especially toxic chemical injury to cells, makes estimation of SOD an important indicator of tissue oxidative status.

**Principle**

SOD activity was measured by nitro blue tetrazolium (NBT) reduction. The superoxide radical reduces NBT to a blue coloured formozan dye that is extracted into butanol layer after inactivation of the reaction with acetic acid. The colour intensity of the chromogen in butanol is measured at 560 nm against butanol blank. SOD activity in Units is calculated based on % absorbance/ min. 50% inhibition/min is taken as 1 unit of enzyme activity.

**Reagents**

1. Sodium pyrophosphate buffer (0.025)
2. Phenazonium metho sulphate (PMS-186 µM)
3. Nitro blue tetrazolium chloride (NBT- 300 µM)
4. NADH (780 µM)

**Procedure**

To 0.05 ml tissue homogenate, 0.3 ml sodium pyrophosphate, 0.025 ml PMS and 0.075 ml NBT were added. The reaction was started by the addition of 0.075 ml of NADH. After incubation at 30° C for 90 sec, the reaction was stopped by the addition of 0.25 ml glacial acetic acid. Reaction mixture was stirred vigorously and
shaken with 2 ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. Its absorbance was measured at 560 nm against butanol as blank. A system without the homogenate, with 0.05 ml PBS served as control.

**Tissue total protein** [Lowry et al. 1951].

Tissue total protein was measured with folin phenol reagent after alkaline copper treatment. Proteins form a colored complex with the reagent in the presence of copper and the intensity of the complex is measured and protein content estimated based on a standard curve of standard protein.

**Reagents**

1. 2% sodium carbonate (Reagent A)
2. 0.5 % Copper sulphate (CuSO₄.5H₂O) in 1% potassium tartrate (Reagent B)
3. Alkaline copper sulphate: Mix 50 ml of Reagent A and 1 ml of Reagent B prior to use.
4. Folin-Ciocalteau reagent (Reagent D)
5. Stock Protein solution: 50 mg of bovine serum albumin dissolved in 50 ml distilled water
6. Working standard: Prepare form the stock, series of 10 dilutions in distilled water ranging from 40-200 µg

**Procedure**

0.1 ml and 0.2 ml of the sample extracts were taken in 2 test tubes and made up to 1 ml with distilled water. 1ml distilled water in another tube served as the blank. 5 ml of reagent was added to each of the test tubes, including blank. They were mixed well and allowed to stand for 10 min. 0.5 ml of reagent D was added, mixed well and incubated at RT in the dark for 30 min. The intensity of the blue color developed is
read at 660 nm. A standard graph of protein was plotted, from which the protein content of the extract was determined.

5.2.1.3 **Histopathological analysis of coronary artery**

Preselected fragments from the formalin fixed coronary artery were put to paraffin blocks, serially sectioned transversely to 5 µm. These were mounted and stained with haematoxylin and eosin and examined microscopically for the presence of lesions.

5.2.1.4 **Assessment of aortic atherosclerotic plaque** [Efendy *et al.* 1997].

*Principle*

The histological mechanism of staining lipids is invariably a function of the physical properties of the dye. It is more soluble in the lipid to be demonstrated than in the vehicular solvent. The poly azo group of dyes includes the Oil Red O series, the Sudan series and the Sudan blacks. All these dyes are interchangeable, and may be used in this method.

**Reagents**

*Oil Red O stock stain*

Oil O red – 0.5 g

Isopropyl alcohol (IPA) – 100 ml

The dye is dissolved in IPA, using very gentle heat of a water bath

*Oil Red O working solution*

30 ml of the stock stain was diluted with 20 ml of distilled water, allowed to stand for 10 min, filtered into a coplin jar and covered immediately. The stain is to be freshly prepared for use.
Glycerin Jelly mounting medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>10 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>60 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>70 ml</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.25 g</td>
</tr>
</tbody>
</table>

Gelatin was dissolved in the distilled water using sufficient heat to melt it, and glycerol and phenol were added. They were mixed well and transferred to a small capped bottle.

Procedure

Briefly formal calcium fixed aorta was rinsed in distilled water and 60% isopropyl alcohol (IPA). The tissue was stained for 40 min by soaking in a saturated solution of Oil Red O in IPA. It was mounted in glycerin jelly to attach the stained tissue to the microscope slide for photography. The % luminal surface area covered by lipid filled Oil Red O stained deposits were measured using Image Pro Plus® Image analysis system.

5.2.1.5 Statistical analysis

Results are expressed as mean ± SEM. The data was analysed using Medcalc software. Comparisons between treatments were made by one-way ANOVA with post-hoc comparisons of the means with that of control being made by Dunnett’s test. A statistical probability of P<0.05 was considered significant.

5.2.2 RESULTS AND DISCUSSION

5.2.2.1 Histopathological changes after one month induction with atherogenic diet

Representative stained sections of left coronary artery and other major organs from the animals, post one month induction with atherogenic diet is presented in Fig 1 (A-F). Spleen (picture not shown) and kidney showed no signs of tissue damage. Lung shows some areas of patchy broncho pneumonitis. Necrotic damage with
Figure 1 One month post-high fat induction changes, (*Haematoxylin & eosin*)
A. intimal changes initiated in the coronary artery, B. Dark stained areas indicating necrotic damage in the myocardial tissue, C. Liver section shows fatty degeneration, D. Fatty changes seen in the villi, E. Patchy areas of inflammation, F. Normal glomeruli of kidney tissue
ballooning degeneration, sinusoidal distension and engorgement can be noted in sections from the liver. These are signs of hepatic dysfunction. Gall bladder shows marked cholesterosis in the villi cells - signs of fatty changes. Myocardial tissue exhibits darker atrophied necrotic areas, probably ischaemia related changes. Coronary artery sections show signs of initiation of intimal disruption. The degenerative histopathological changes observed in the organ systems are all characteristic of lipemic assault. **Thus, these histopathological observations lead us to confirm initiation of early atherogenic changes in the experimental animals as a consequence of administration of atherogenic diet.**

5.2.2.2 General observations

At the time of sacrifice, the animals appeared healthy. Body weight - an indication of general health and adequate food intake - increased gradually during the experimental period in all groups of animals. Fig 2 shows body weight changes of the experimental animals on a 30 day basis during experimentation. Group II has recorded a sharp increase in bodyweight after the 30 day induction period. Subsequent increase however has not been similar possibly due to chronic lipemia associated emaciation. % increase in body weight is least (8 and 7% respectively) in both Group II (Ev) and Group III (Ir(A)) treated groups and they differed significantly (P<0.01) from the positive control. Ir(H) treatment caused a similar increase in body weight as normal control (10.5%). At Ca treated group VI animals showed an increase in body weight by 11.5% Effect of Ev and Ir(A) on body weight is noteworthy in view of the anti obesity claims for the source plants in traditional medicine.

No evidence of disease other than atherosclerosis was found except for occasional focal alopecia in the dorsal thoracolumbar area of group II animals, possibly promoted by prolonged deposition of cholesterol in the skin.
5.2.2.3 Serum lipid levels

Serum lipid profile of the experimental animals is presented in Table 1. High fat diet (HFD) fed positive control-group II recorded a statistically significant rise (P<0.05) in TC, LDL-C compared to normal control group I. There has been a 254% increase in A.I indicating enhanced risk of atherogenicity due to high fat administration. Lipid profile of the treatment groups (III, IV, V & VI) are in comparison with positive control. Ev administration reduced TC, TGL and LDL. A.I decreased by 30%. Ir(A) administration reduced TC, TGL and LDL. HDL-C levels in this group increased by 173%. Ir(H) had no effect on TC and TGL. However HDL-C rose by a remarkable 340% and hence LDL-C decreased by 45%. A.I therefore decreased by 87% compared to NC. Atorvastatin calcium brought about a typical hypolipidaemic response with respect to the tested parameters- TC, TGL, LDL, HDL and A.I. Statistical post testing with Dunnet’s ‘t’ test revealed Ir(A) to be more effective than atorvastatin calcium in reducing TC, while the latter was more effective with respect to other parameters. Ir(H) was most effective in enhancing HDL-C. The improvement in lipoprotein pattern by Ir(A) treatment over PC and more effective reduction of TC than standard drug demonstrates its predominant hypolipidaemic effect.
Table 1 Serum lipid profile of experimental animals

<table>
<thead>
<tr>
<th>Group/Treatment</th>
<th>TC (mg/dl) % Change</th>
<th>TGL (mg/dl) % change</th>
<th>HDL (mg/dl) % change</th>
<th>LDL (mg/dl) % change</th>
<th>AI % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (NC)</td>
<td>27.67±0.79</td>
<td>66.07±4.12</td>
<td>5.32±1.1</td>
<td>7.23±2.67</td>
<td>3.24±1.02</td>
</tr>
<tr>
<td>II (PC)</td>
<td>91.1±10.33</td>
<td>+229.24</td>
<td>75.59±2.28</td>
<td>+14.41</td>
<td>+19.36</td>
</tr>
<tr>
<td>III (Ev-100mg/kg)</td>
<td>60.71±8.06</td>
<td>-33.36</td>
<td>45.95±8.12</td>
<td>-39.21</td>
<td>-7.56</td>
</tr>
<tr>
<td>IV (Ir(A)-100mg/kg)</td>
<td>56.06±6.73</td>
<td>-38.46</td>
<td>42.22±6.73</td>
<td>-44.15</td>
<td>+173.23</td>
</tr>
<tr>
<td>V (Ir(H) 100mg/kg)</td>
<td>82.59±6.05</td>
<td>-9.34</td>
<td>79.15±6.05</td>
<td>+4.71</td>
<td>27.92±0.658</td>
</tr>
<tr>
<td>VI (STD drug-10mg/kg)</td>
<td>65.52±6.4</td>
<td>-28.08</td>
<td>31.28±6.4</td>
<td>-58.62</td>
<td>+330.71</td>
</tr>
</tbody>
</table>

One way F ANOVA df = 5,30
P< 0.001

NC, Vehicle-treated normal control group on normal pellet diet; HFD, High fat diet, Ev- alcohol extract of *Erythrina variegata*, Ir(A)-alcohol extract of *Inula racemosa*, Ir(H)- hexane extract of *Inula racemosa*, STD drug-Atorvastatin calcium. Values are mean±SEM; n=6 animals in each group, P values of groups compared to group II <0.05, ^<0.001, *NS,
Ir(H) specifically enhanced HDL-C without altering TC and TGL. This unique effect does not compare with the action mechanism of any of the known hypolipidaemics and hence exploration of the constituent phyto molecules of the extract and their mechanism of action shall be scientifically worthwhile. Ev on the other hand improved the lipoprotein profile except for modulation of HDL.

5.2.2.4 Liver and heart tissue antioxidant status (Table 2 and Figure 3)

Heart and liver tissue anti oxidant status is reflective of hyperlipidaemia related pro oxidant damage in positive control. In this group there has been a decrease in GSH (P<0.05) and elevation in TBARS (P<0.001) in heart tissue. Endogenous enzyme anti oxidants GPX and SOD decreased in both the tested tissues relative to normal control. Ev treated group III showed an increase in TBARS in heart and a decrease in SOD in liver. Thus no beneficial improvement in the levels of oxidative biomarkers was noted. Relative to positive control SOD, GPX were elevated (P<0.001) in the heart tissue in Ir(A) administered group IV. In this group, tissue TBARS decreased (P<0.001) in liver. Ir(H) administration brought back GPX (P<0.05) to normal control values in heart tissue, while in liver, TBARS (P< 0.001) decreased. Atorvastatin administration caused decrease in TBARS (P<0.001) and increase in GSH (P<0.01) in liver. In the heart tissue SOD (P<0.01) and TBARS increased. Thus Ir(A), Ir(H) and atorvastatin have brought about beneficial alteration of redox status of the tested tissues during HFD administration.

Effect of the extracts on oxidative stress biomarkers almost parallels their hypolipidaemic response. In PC, hyperlipidaemia associated oxidative stress has triggered lipid peroxidation, marked by elevated TBARS, reduced GSH in liver and a decrease in all except TBARS in heart tissue.

Ir(A) elevated SOD, GPX levels in heart without significant improvement of TBARS and GSH. Only TBARS was brought down in liver tissue. This indicates a cardio specific anti oxidant effect of Ir(A). Even Ir(H) beneficially altered SOD, GSH levels in heart, while liver TBARS was reduced. Thus Ir(H) also has ameliorated the HFD induced oxidative damage in the heart tissue. Reduction of liver TBARS by both
Table 2  Liver and heart tissue levels of oxidative stress markers in experimental guinea pigs

| Group/Treatment | Organ weight | SOD  | GPX  | TBARS | GSH  | Organ weight | SOD  | GPX  | TBARS | GSH  |
|----------------|--------------|------|------|-------|------|--------------|------|------|-------|------|------|
| Group I-NC     | 4.16±0.516   | 21.7±1.98 | 33.7±3.13 | 11.39±0.618 | 1.62±0.2317 | 1.33±0.09 | 27.3±1.205 | 57.17±2.42 | 6.17±0.174 | 1.698±0.096 |
| Group II- HFD  | 8.47±1.43    | 18.37±2.65 | 29.22±4.11 | 50.44±7.28 | 0.648±0.01 | 1.82±0.06 | 22.53±1.155 | 35.06±4.74 | 8.06±0.8 | 1.328±0.137 |
| Group III- Ev  | 4.98±0.237   | 10.27±0.571 | 29.87±3.1 | 60.16±3.66 | 0.731±0.018 | 1.61±0.131 | 18.39±1.17 | 48.68±7.33 | 22.98±1.44 | 1.527±0.1 |
| Group IV-Ir(A) | 6.35±0.25    | 16.77±1.41 | 21.6±2.03 | 21.9±2.3 | 0.99±0.1 | 1.62±0.03 | 52.71±3.95 | 144.35±13.44 | 11.95±1.21 | 1.262±0.148 |
| Group V-Ir(H)  | 5.95±0.24    | 10.77±0.571 | 30.48±3.79 | 18.61±3.66 | 0.69±0.1 | 1.16±0.1 | 19.44±1.18 | 59.16±1.7 | 13.62±1.15 | 1.173±0.08 |
| Group VI- Std  | 6.21±0.29    | 14.47±1.07 | 22.76±3.33 | 10.42±0.21 | 1.26±0.166 | 1.46±0.07 | 31.86±2 | 21.22±0.79 | 21.37±4.76 | 1.133±0.102 |

One way ANOVA

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>df</th>
<th>P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>4.98</td>
<td>5.30</td>
<td>0.002</td>
</tr>
<tr>
<td>ANOVA</td>
<td>4.98</td>
<td>5.30</td>
<td>0.001</td>
</tr>
</tbody>
</table>

SOD, superoxide dismutase (units/mg protein); GPX- Glutathione peroxidase (nmol GSH consumed/min/mg protein); TBARS-thiobarbituric acid reactive substances (nmol malondialdehyde/g tissue); GSH-reduced glutathione (μg/g tissue); NC-vehicle treated normal control, HFD- high fat diet treated control. Values are mean ± SEM; n=6 animals in each group, P values of groups compared to group II < 0.05, *NS
Figure 3  Diagrammatic representation of tissue levels of oxidative stress markers in experimental animals
the extracts is possibly the generalized outcome of the improvement in serum lipid profile. Ev showed no significant improvement of tissue antioxidant status over PC. Thus its hypolipidaemic effect has not been augmented by anti oxidant component.

5.2.2.5 Effects on aortic atherosclerosis

Representative photographs of the Oil Red O stained intimal surface of aorta from experimental animals is shown in Fig. 4. The lipid deposits appear brick red with Oil Red O staining. The intimal aortic surface in guinea pigs of group II had variable numbers of flat or raised lipophilic areas. Their long axes are parallel to the longitudinal axes of the aorta. There were no thrombi. In contrast, aortas were grossly normal or showed minimal lesions in NC and in animals treated with the extracts/atorvastain calcium. % of lipid laden areas relative to the total intimal surface is represented in Fig 5. HFD treated group shows visible lesions which measured ~15% of the total intimal surface. The % lesion area is found to be ~ 8, 6.5, 10 and 11 % for Ev, Ir(A), Ir(H) and atorvastatin treated animals respectively. Thus Ir(A) has shown 57% reduction in lesion area, while Ev, Ir(H) and At.Ca reduced the lesion area by 47, 33 and 27% respectively.

Pleotropic effects of statins include stabilization of atherosclerotic plaque by reduction of oxidative stress and vascular inflammation [Crisby et al. 2001]. Greater effective reduction in lesion area by Ir(A) suggests ‘regression’- a progressive decline in ‘size’ or ‘severity’ of lesion. Ev and Ir(H) administration has also minimized the extent of lesions. Their overall hypolipidaemic and anti oxidant effects have possibly counteracted the atherogenicity of dietary cholesterol.

5.2.2.6 Effects on coronary atherosclerosis

The results reported for the gross aortic lesions are complemented by intimal changes in the coronary artery. Representative photomicrographs of stained sections of coronary artery are presented in Fig 6 (A-M). The sections from group I showed almost no atherosclerosis on microscopic observation. In majority of sections intima was not thickened and no foam cells observed in the intima or media. The internal
Figure 4  Representative photographs of intinal surface of aorta from experimental groups showing Oil Red O–stainable lipid deposit. Note marked brick red lipid deposits in positive control
Group I – Normal control

**Figure 6** Representative sections of coronary arteries of experimental animals

**A. (x 10)** normal artery and myocardial tissue

**B. (x 20)** normal coronary vessel-no intimal disruption

**Figure 6** Representative sections of coronary arteries of experimental animals
Group II – Positive control

C. \((x \ 10)\) Coronary artery with damaged intima and luminal fat (black arrows), surrounding myocardial tissue necrotic (white arrow)

D. \((x \ 20)\) large foam cells in the lumen (black arrow)  
E. \((x \ 20)\) discontinuous intima and in the media (white arrow)

Figure 6 Representative sections of coronary arteries of experimental animals
Group III – Ev treated

**F. (x 20)** normal artery with continuous intima despite medial fat deposition

**G. (x 10)** intact ves

*Figure 6* Representative sections of coronary arteries of experimental animals
Group IV – Ir(A) treated

H & I (x10) normal appearance of coronary artery and myocardial tissue

Figure 6 Representative sections of coronary arteries of experimental animals
Group V – Ir(H) treated

**J. (x10)** intact coronary arteries and surrounding tissue, despite some luminal and medial fat

**K. (x 20)** normal artery, no intimal disruption

**Figure 6** Representative sections of coronary arteries of experimental animals
Group VI – Atorvastatin calcium treated

L. (x10) normal coronary artery

M. (x 20) normal arterial laminae, note the fatty infiltration of media

Figure 6 Representative sections of coronary arteries of experimental animals
elastic lamina (IEL) was intact and not reduplicated. Occasional sections had some extracellular lipid. Myocardial tissue appears normal.

The coronary arteries of animals fed high cholesterol diet (group II) showed in almost all animals, accumulation of foam cells in the media and in the much thickened intima. (Fig 6, C, D & E). The intimal disruption is extended to the entire perimeter of the lumen. There are relatively wide acellular spaces in the intima. In the media, extensive aggregates of foam cells, which have sometimes completely replaced the muscular pattern, are often associated with gaps in the IEL. The foam cells have extended occasionally into the adventitia. No thrombi are identified in or on the endothelial lesions. There are areas of necrotic fatty damage in the surrounding cardiac tissue. The appearance of coronary vessel sections is different in Ev and Ir(A) treated animals. They showed evidence of ‘healing’. Foam cells are fewer. Area of the media adjacent to gaps in the IEL are thin and condensed, rather than distended with foam cells. Thus sections from these groups show an almost normal continuous endothelium. Also the cardiac tissue is devoid of fatty degeneration. Sections from Ir(H) and atorvastatin treated animals showed undisturbed intima though there was medial infiltration of fat.
5.3 CHAPTER SUMMARY

*Cavia porcellus* (Guinea pig) is found to be a suitable species for the study of atherosclerosis, because of its amenability to development of early atherosclerotic lesions with high cholesterol supplementation to normal chow diet, within a relatively short period of time (120 days). Literature reveals several similarities between their lesions and those found in man and that certain dietary components or drug treatments can reduce cholesterol concentrations even in the presence of very high dietary cholesterol [Berglund *et al.* 1989]. Thus this study of regression of established atherosclerosis in animals during cholesterol feeding is probably more relevant to conditions likely to be present in man.

It is known that atherosclerosis is an inflammatory disease and atherosclerotic lesions represent different stages in a chronic inflammatory process in the artery [Russel 1999]. Causes of endothelial dysfunction and denudation - the primary event leading to atherosclerosis include among others, elevated and modified LDL and free radicals. Modified LDL trigger and expand the initial inflammatory response, initiating a series of intracellular events leading to formation of lipid peroxides. This facilitates accumulation of cholesterol esters, resulting in the formation of foam cells. Peroxidized lipids unable to maintain cellular integrity damage surrounding membrane proteins resulting in cellular damage as evidenced by coronary intimal and cardiac tissue changes. Pro oxidant and lipid peroxidation associated pro inflammatory changes trigger atherosclerotic changes as evidenced by lipid laden lesion areas in the aorta.

In animals with hypercholesterolemia, antioxidants are known to reduce the size of lesions [Navab *et al.* 1996] and they reduce fatty streaks in non human primates [Chang *et al.* 1995]. The latter observation suggests that antioxidants have anti-inflammatory effect [Fruebis *et al.* 1997]. Antioxidants are known to increase the resistance of human LDL to oxidation ex vivo [Reaven *et al.* 1993] in proportion to antioxidant content of the plasma.

In our study, in the high fat control, hyperlipidaemia associated oxidative stress has triggered lipid per oxidation indicated by altered oxidative stress biomarkers.
Hypolipidaemic effect of Ir(A), accompanied increase in anti oxidant status of heart tissue, akin to atorvastatin calcium, are indicative of improved antioxidant reserve in these tissues. Subsequent possible inhibition of lipid peroxidation, reduced lipid uptake, with the consequent reduction of fatty streak formation, via decreased foam cell formation are presumably a consequence of anti oxidant and/or anti inflammatory and hypolipidaemic changes exerted by Ir(A).

It has also modulated the HFD induced body weight increase, suggestive of an anti obesity effect. Attention is drawn to its phenolic content, as phenols are anti oxidant due to resonance stabilized phenoxy radicals they form with oxidants [Baum and Perun 1962]. By inhibiting LDL oxidation, phenolic antioxidants prevent uptake and degradation of oxidized LDL by macrophages [Mangiapane et al. 1992]

The anti atherogenicity of Ir(H) may be attributed to its phytoconstituent composition. ALT and IALT are the major sesquiterpene lactones reported from hexane fraction. They are reported with antioxidant and anti inflammatory properties. These lactones may have structural similarity to enterolactone and other lignans, which like isoflavones are categorised as ‘phytoestrogens’ on account of their weak estrogenic activity [Setchell et al. 1981].These groups of compounds lower cholesterol and their influence on tyrosine kinases has a role in suppression of cellular processes leading to atherosclerosis [Hodgson et al. 1996].

Ev demonstrated hypolipidaemic, atheroprotective effect, in vitro anti oxidant effect, but little or no anti oxidant effect in vivo. Involvement of mechanisms other than anti oxidant activity may be implicated. Anti inflammatory activity earlier reported for this plant supports this hypothesis.

Isoflavones, a class of ‘phytoestrogens’are reported from EV. Thus 2 different classes of phytoestrogens namely, ‘lignan’ type from IR and isoflavones from EV strongly implicate their profound ‘estrogenic’ ‘anti inflammatory’ and ‘cardioprotective’ activities as the possible mechanism behind the observed atheroprotective effects.
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