Chapter 5

Anti-atherogenic potential of Flavonoid rich Eugenia jambolana seed extract and Anthocyanin rich Brassica oleracea leaf extract.
Anti-atherogenic potential of Flavonoid rich *Eugenia jambolana* seed extract and *Brassica oleracea* or anthocyanin rich red cabbage extract.

**INTRODUCTION**

Cardiovascular disease (mainly atherosclerosis) accounts for substantial number of deaths annually in both developed as well as developing countries. High plasma total cholesterol and LDL level are the major contributors that lead to the development of atherosclerosis and cardiovascular diseases (Carmena et al., 2004). Oxidation of LDL (ox-LDL) plays a crucial role in induction and progression of atherosclerosis. Ox-LDL is known to initiate a series of physiological events such as accumulation of plasma lipoproteins in the intima, endothelial cell damage and subsequent macrophage mediated uptake of ox-LDL via scavenger receptors. These events transform the macrophages into foam cells and account for formation of a fatty streak. Increased expression of adhesion molecules, migration of monocytes into the intima and platelet aggregation are key events during onset and progression of atherosclerosis (Stocker and Keaney, 2004).

Flavonoids are polyphenolic compounds are ubiquitous in fruits, vegetables, tea, wine, nuts, seeds, herbs and spices (Mink et al., 2007) as evidenced from the available literature. The therapeutic effects of flavonoids in prevention of cardiovascular ailments are clearly established. Anti-atherogenic property of flavonoids is attributed to its ability to prevent LDL oxidation, a key initiator of onset and progression of atherosclerosis (Maron, 2004). Also, studies have correlated cardioprotective effects of flavonoids with
its potent anti-inflammatory action, ability to improve endothelial function and inhibit platelet aggregation (Patel, 2008).

Among the various classes of flavonoids; anthocyanins are the largest group of water-soluble pigments. Chemically, they are polyhydroxylated or polymethoxylated glycosides or acylglycosides of anthocyanidins which are oxygenated derivatives of 2-phenylbenzopyrylium or flavylum salts (Mazza and Miniati, 1993). Role of anthocyanins in prevention of cardiovascular diseases, cancer, obesity and diabetes (Giuseppe, 2007) has been established and World Health Organization has also endorsed the multiple health benefits of daily consumption of anthocyanin rich diet.

Flavonoids rich fraction of *Eugenia jambolana* seed extract (EJSE) has been shown to possess hypoglycemic, hypolipidemic and anti-diabetic properties (Sharma et al., 2008a). These properties have been attributed to its ability to modulate carbohydrate and lipid metabolizing enzymes in diabetic rats and attenuate *in vitro* adipocyte differentiation (Sharma et al., 2008b). We have previously observed potent hypolipidemic/hypocholesterolemic potential of Anthocyanin rich red cabbage extract (ARCE) and EJSE (Chapters 3 & 4). In continuation, the present inventory focuses on investigation of the protective role of ARCE and EJSE against *atherogenic diet induced* experimental atherosclerosis in rats.

**MATERIALS AND METHODS**

*Preparation of ARCE and EJSE*

As mentioned in chapter 3 and 4
**HPLC fingerprinting ARCE and EJSE**

As mentioned in chapter 3 and 4

**Experimental animals**

Male Sprague Dawley rats weighing 300 ± 20 (Obtained from Sun pharmaceutical advanced research centre, Vadodara, India and were maintained in clean polypropylene cages and fed with laboratory chow (M/S Pranav agro, Ltd Baroda, India) and water ad libitum. The experimental protocol was executed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India and approved by the animal ethical committee of the Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

**Induction of atherosclerosis in rats**

A total of twenty four rats were divided into three groups of 8 animals each. Group I served as control (CON) and was fed with standard laboratory chow (Pranav Agro Ltd, Baroda, India) and orally administered with 0.5 % CMC for 8 weeks. Group II (ATH) and III (ATH+EJSE) were given single dose of vitamin D3 (600,000 IU/kg, i.p.) and later fed with a ATH diet (3% cholesterol, 0.5% cholic acid, 0.2% 6-propyl 2-thiouracil, 5% sucrose, 10% lard, and 81.3% powdered laboratory chow) for 8 weeks (Cai et al., 2005). Group III (ATH+ARCE) and IV (ATH+EJSE) was orally administered with 100 mg/kg ARCE or EJSE (Chapter 3 & 4) while, group II received equal volume of vehicle (0.5% carboxy methyl cellulose).
At the end of the experimental period, rats were fasted overnight and blood was collected via retro-orbital sinus puncture. The blood samples were cold centrifuged (at 4°C, 3000 rpm for 10 min) to obtain serum. Later, animals were sacrificed by cervical dislocation under mild ether anesthesia and, liver, heart, thoracic aorta and kidney were excised and, stored at -80°C (Cryo Scientific Ltd, India) for further biochemical analysis.

*Fecal cholic acid and deoxycholic acid*

Fecal samples from each experiment group were collected on every 3rd day during 5th and 7th week of the study. Fecal samples were dried, eluted with absolute alcohol, filtered and processed for estimation of cholic acid (CA) and deoxycholic acid contents (DCA) (Mosback et al., 1954).

*Estimation of Cholic Acid and Deoxycholic Acid from feces.*

500 mg faces (dry)

\[
\text{Extracted with absolute alcohol (2 ml) (keep for 1 hour)}
\]

Filter

\[
\text{Filtrate dried}
\]

Residue

\[
\text{Hydrolysed with 2 ml 5\% NaoH at 15 LBS presence in autoclave for 1.5 hours.}
\]
Neutralize with ether and evaporate to dryness (2 ml).

Residue dissolved in 1 ml Acetone.

Centrifuged, Supernatant dried to which 1.5 ml 65% H2SO4 is added.

Heat at 60°C for 15 min.

Cool at room temperature.

Read O.D. at 320 nm for cholic acid 385 for deoxycholic acid.

**Hepatic HMG CoA reductase activity (Rao and Ramakrishnan, 1975)**

**Principle:** 3-Hydroxy-3-methylglutaryl-CoA and mevalonate concentrations in the tissue homogenate are estimated in terms of absorbance and the ratio between the two is taken as an index of activity of the enzyme, which catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate.

**Reagents:**
- **Saline arsenate solution:** 1 g of sodium arsenate per liter of physiological saline.
- **Dilute perchloric acid:** 50 ml filter.
- **Hydroxylamine hydrochloride reagent for mevalonate:** Mix equal volumes of hydroxylamine hydrochloride reagent and water freshly before use.
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- Hydroxylamine reagent for HMG-CoA: Mix equal volumes of hydroxylamine hydrochloride reagent and sodium hydroxide solution (4.5 mol/liter) freshly before use.

- Ferric chloride reagent: Dissolve 5.2 g of trichloroacetic acid and 10 g of ferric chloride in 50 ml of 0.65 mol/liter hydrochloric acid and dilute to 100 ml with the latter.

Procedure: - Mix equal volumes of the fresh 10% tissue homogenate and diluted perchloric acid. Allow to stand for 5 mm and centrifuge (2000 rpm, 10 mm). Treat 1.0 ml of filtrate with 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA), mix, and after 5 mm add 1.5 ml of ferric chloride reagent to the same tube and shake well. Take readings after 10 mm at 540 nm vs. a similarly treated saline/arsenate blank.

Von Kossa Staining for Calcium deposition (Sheehan and Hrapchak, 1979)

Fixation: Formalin fixed, paraffin embedded tissue section or alcohol fixed, frozen sections.

Solutions & reagents:

1. Aqueous Silver Nitrate Solution: 1ml Silver Nitrate in 100ml D/W.

2. 5% Sodium Thiosulfate: 5gm Sodium Thiosulfate in 100ml D/W.

3. 1% Nuclear Fast Red Solution: 0.1gm Nuclear Fast Red + 100ml D/W + 5gm Aluminium Sulfate. {Dissolve aluminium sulphate in water. Add nuclear fast red & slowly heat to boil & then cool, Filter & add a grain of Thymol as a preservative.}
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Procedure:

1. **Deparaffinize the paraffin sections & rehydrate to water.**
   - Keep the slide in xylene for 10-15 min
   - Keep the slide in 100% alcohol for 1 min
   - Keep the slide in 70% alcohol for 1 min
   - Keep the slide in 50% alcohol for 1 min
2. Rinse in several change of D/W.
3. Incubate sections with 1% silver nitrate solution in a clear glass coplin jar placed under UV light for 25-30 minutes (or in front of a 60-100 watt light bulb for 1 hr or longer).
4. Rinse in several change of D/W.
5. Remove un-reacted silver with 5% sodium thiosulfate for 5 min.
6. Rinse in D/W.
7. Counter stain with Eosin for 7-10 mins.
8. Rinse in D/W.
   
   **GRADED ALCOHOL**
   - 50% alcohol for 1 time only.
   - 70% alcohol for 1 time only.
   - 100% alcohol for 1 time only.
The stained sections were visualized under a Leica DMRB microscope were photographed.

**Staining of thoracic aorta for Elastin (Alcântara dos Santos et al., 2004)**

**Reagents:**

1. **Van Gieson:**
   - Acid fusc in: 250mg
   - Nitric acid: 0.5mg
   - Glycerine: 10ml
   - Picric acid: up to saturation
   - D/W: 90ml

2. **Acidified KMnO₄**
   - KMnO₄: 0.5% in 950ml
   - H₂SO₄: 3% in 50ml
   - TOTAL: 1000ml

3. **Resorcinol fusion:**
   - Basic fusc in: 2gm
   - Resorcinol: 4gm
   - D/W: 200ml

   • Boil & add 25ml 29% FeCl₃ , Continue boiling for 2-5 mins, Cool and filter it. Discard the filtrate, Dry precipitates, Dissolve 200ml 95% ethanol. Heat till dissolve, After dissolving add 4ml concentrated HCl.

**Procedure:**

1. Deparaffinize & dehydrate to water.
2. Treat with acidified KMnO₄ for 2mins.
3. Wash in water.
4. 1% oxalic acid for 1min
5. Wash in water or 70% alcohol (one deep only).
6. Stain with weigert resorcinol fussin for 45mins.
7. Wash in water 10 times.
8. Differentiate in acid alcohol.
9. Wash in water.
10. Stain in haematoxylin for 5min.
11. Wash in water.
12. Dehydrate through graded alcohol & clean in xylene.
13. Coverslip using permanent mounting medium.

The stained sections were visualized under a Leica DMRB microscope were photographed.

**Preparation of thoracic aorta for gross microscopic evaluation**

Aorta of control and experimental rats fixed in 4% buffered paraformaldehyde, was dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor. Sections of five-μm thickness cut on a microtome were stained with haematoxylin and eosin for microscopic observation. The sections were observed under Leica DMRB microscope and photographed.

**Statistical analysis**

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni’s multiple comparison test. The results were expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.
RESULTS

Serum lipid profile
In the present study, ATH diet fed rats recorded elevation in serum TC (70.40%), TG (49.29%), LDL (80.43%), VLDL (49.28%) and along with decrement in serum HDL (42.10%) while, ARCE and EJSE treatments were successfully able to prevent ATH diet induced increment serum TC (58.13 & 49.77%), TG (20.72 & 37.98%), LDL (63.57 & 55.30%), VLDL (25.44 & 37.96%) along with decrement in serum HDL (37.47 & 34.86%) (Table 1 and Figure 1).

Fecal lipid profile
There were non-significant (p>0.05) alterations in the fecal TC and TG however, ATH+ARCE and ATH+EJSE rats recorded significant increment (p<0.05) in contents of fecal TC (38.98 & 45.36 %) and TG (36.00 & 45.81 %) compared to the CON rats respectively (Table 2 and Figure 2).

Fecal cholic acid and deoxycholic acid
ATH diet fed rats did not show any significant changes in fecal CA and DCA contents (Table 3 and Figure 3) compared to CON rats. However, ARCE treatment to ATH diet fed rats recorded significant increment in fecal CA (37.38 & 10.35 %) and DCA (46.96 & 48.70 %) contents compared to ATH diet fed rats (Table 3 and Figure 3).
Hepatic HMG CoA reductase activity

Hepatic HMG CoA reductase activity registered a non significant alteration in ATH diet fed rats compared to CON rats (Table 4 and Figure 4). However, ATH+ARCE rats recorded significant decrement in the activity levels of HMG CoA reductase activity compared to ATH and CON rats (Table 4 and Figure 4).

Histopathological observations of thoracic aorta

Photomicrographs of thoracic aorta section (HXE stained) of ATH rats revealed formation of a necrotic core due to accumulation of foam cells, deposition of lipids and loosening of smooth muscle cells of tunica media (Figure 5). Calcium deposition in tunica media and intima (von kossa staining) and derangement/defragmentation of elastin layer (weigert’s staining) was also evident (Figure 6 & 7). However, ATH+ARCE and ATH+EJSE rats showed no evidence for atheromatous plaque formation and depicted moderate vascular injuries, calcium deposition and minimal elastin derangement (Figure 5, 6 & 7).
Table 1 Effect of ARCE and EJSE on serum lipid profile in atherogenic diet (ATH) fed rats.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>ATH</th>
<th>ATH+ARCE</th>
<th>ATH+EJSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>65.23±4.43</td>
<td>220.43±14.00</td>
<td>92.17±9.95*</td>
<td>110.7±12.64***</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>44.56±3.33</td>
<td>87.88±7.89##</td>
<td>69.67±4.55*</td>
<td>54.50±3.11***</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>31.09±1.11</td>
<td>18.00±1.32***</td>
<td>28.79±2.17***</td>
<td>27.63±3.00***</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>25.23±2.79</td>
<td>184.86±13.11###</td>
<td>67.33±9.77***</td>
<td>72.17±9.01***</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>8.91±0.67</td>
<td>17.57±0.88***</td>
<td>13.10±0.70**</td>
<td>10.90±0.62***</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M, for n=6. Where, ###P<0.001 compared with CON (rats fed with laboratory chow), **P<0.01 and ***P<0.001 compared with ATH (rats fed with high cholesterol diet).
Table 1 Effect of ARCE and EJSE extract feeding on serum lipid profile in atherogenic diet (ATH) fed rats.

Results are expressed as Mean±S.E.M, for n=8. Where, ###P<0.001 compared with CON (rats fed with laboratory chow), **P<0.01 and ***P<0.001 compared with ATH (rats fed with high cholesterol diet).

Results are expressed as Mean±S.E.M, for n=8. Where, ###P<0.001 compared with CON (rats fed with laboratory chow), **P<0.01 and ***P<0.001 compared with ATH (rats fed with high cholesterol diet).
Table 2 and Figure 2: Effect of ARCE and EJSE on fecal lipid profile in atherogenic diet (ATH) fed rats.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>ATH</th>
<th>ATH+ARCE</th>
<th>ATH+EJSE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol (mg/g)</strong></td>
<td>5.54±0.08</td>
<td>6.57±0.09*</td>
<td>9.08±0.08**</td>
<td>10.14±1.00***</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/g)</strong></td>
<td>7.89±1.00</td>
<td>7.33±1.23*</td>
<td>12.33±1.00***</td>
<td>14.56±1.89***</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, *** p<0.001
Table 3 and Figure 3: Effect of ARCE and EJSE on fecal bile acid contents in atherogenic diet (ATH) fed rats.

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>CON (μg/g feces)</th>
<th>ATH (μg/g feces)</th>
<th>ATH+ARCE (μg/g feces)</th>
<th>ATH+EJSE (μg/g feces)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholic acid</td>
<td>26.98±1.09</td>
<td>30.98±2.34</td>
<td>43.09±2.98</td>
<td>34.56±1.34</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>20.01±1.11</td>
<td>23.45±1.45</td>
<td>37.65±1.78</td>
<td>39.01±2.11</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± S.E.M, for n=8. Where, * P < 0.05, **P<0.01, ***P<0.001 and ns non significant compared with CON (rats fed with laboratory chow), * P < 0.05, **P<0.01 and ***P<0.001 compared with ATH (rats fed with atherogenic diet).
Table 4 and Figure 4: Effect of ARCE and EJSE on activity levels of hepatic HMG CoA reductase activity.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>ATH</th>
<th>ATH+ARCE</th>
<th>ATH+EJSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG CoA reductase</td>
<td>2.32±0.45</td>
<td>2.88±0.51</td>
<td>4.56±0.39</td>
<td>4.80±0.47</td>
</tr>
<tr>
<td>(HMG CoA / mevalonate ratio)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as Mean±S.E.M, for n=8. Where, *P < 0.05, **P<0.01, ***P<0.001 and ns non significant compared with CON (rats fed with laboratory chow), *P < 0.05, **P<0.01 and ***P<0.001 compared with ATH (rats fed with atherogenic diet).
Figure 5: Photomicrographs of thoracic aorta of control, ATH, ATH+ARCE and ATH+EJSE rats stained with hematoxylin and eosin (100X).
Figure 6: Photomicrographs of thoracic aorta of control, ATH, ATH+ARCE and ATH+EJSE rats stained with von kossa (100X).
Figure 7: Photomicrographs of thoracic aorta of control, ATH, ATH+ARCE and ATH+EJSE rats stained with Weigert’s stain (200X).
DISCUSSION

ARCE and EJSE supplementation were capable of preventing ATH diet induced dyslipidemia and improved serum HDL level. Presently recorded increment in the HDL level in EJSE fed rats could be considered to be of great significance as synthetic hypocholesterolemic drugs are not able to elevate HDL level and, presence of high amount of flavonoids in the EJSE and anthocyanins in ARCE could be responsible for the said effect. These observations are in keeping with reported therapeutic potentials of herbals rich in anthocyanins (Jadeja et al., 2010).

Previous studies have reported that, herbal extracts are capable of increasing elimination of dietary TC and TG through feces primarily by inhibiting its intestinal absorption (Jadeja et al., 2010). Hence, it can be presumed that hypolipidemic and hypocholesterolemic properties of ARCE and EJSE is possibly due to its property of preventing absorption of TC and TG in intestine and the same needs further scrutiny. It has also been reported that hypocholesterolemic property of certain herbal extracts is mediated via catabolism of cholesterol into bile acids that are subsequently eliminated through feces (Khanna et al., 2004). Presently recorded higher elimination of CA and DCA through feces of ATH+ARCE and ATH+EJSE rats are possibly due to the modulatory effect of ARCE and EJSE on bile acid metabolism. Further, hepatic HMG CoA reductase is a rate limiting enzyme in the de novo cholesterol biosynthesis thus; HMG CoA reductase inhibitor drugs are the widely used treatment for hypercholesterolemia (Vasu et al., 2004; Thounaojam et al., 2009). In the present study, ARCE and EJSE induced inhibition of HMG CoA reductase activity is in accordance with previous reports (Vasu et al., 2004; Thounaojam et al., 2009). Thus, it can be
assumed that, hypocholesterolemic property of ARCE and EJSE is mediated via inhibition of \textit{de novo} cholesterol biosynthesis and their subsequent catabolism into bile acids.

The \textit{in vivo} investigation of atherosclerosis involves a profound structural analysis of histoarchitectural changes of thoracic aorta. The extent of atherosclerosis in the thoracic aorta of rat was analyzed by haematoxyline and eosin staining. Also, weigert’s stain for elastin derangement and von kossa for calcium localization were performed (Pang et al., 2010). Sections from thoracic aorta of ATH rats were characterized by massive foam cell accumulation, pronounced vascular calcification and elastin derangement. These results are in accordance with previous reports on ATH diet induced histopathological alterations in thoracic aorta of rats (Pang et al., 2010). Studies have established a close link between atherosclerotic lesion and vascular calcification that is thought to be mediated by atherogenic lipids (Parhami et al., 2002). Also, \textit{in vitro} studies have reported that ox-LDL could promote vascular calcification (Tang et al., 2006) but the underlying mechanism still remains unclear. In the present study, EJSE supplementation to ATH diet fed rats significantly minimized plaque formation, vascular calcification and elastin derangement. Presently observed favorable changes in the histoarchitecture of thoracic aorta of ATH+EJSE rats could be linked with its ability to prevent LDL oxidation and thus minimize structural aberrations in thoracic aorta.
Summary

Present study evaluates anti-atherogenic potential of anthocyanin rich *B. oleraceae* leaf and flavonoid rich *E. Jambolana* seed extract using atherogenic diet fed rats as an experimental model. Effect of ARCE and EJSE administration on serum and fecal lipid profile, fecal bile acid, hepatic HMG CoA reductase activity and histopathological evaluation of thoracic aorta was evaluated. Feeding of ARCE and EJSE prevented ATH diet induced dyslipidemia. Further, significant elimination of fecal lipids and bile acids were observed in ATH+ARCE and ATH+EJSE groups. Atherogenic diet feeding resulted in atheromatous plaque formation along with significant calcium deposition and elastin derangements in thoracic aorta of rats. However, ARCE or EJSE supplementation resulted in significant prevention of atheromatous plaque formation, calcium deposition and elastin derangements. Thus, it can be concluded that ARCE and EJSE possesses anti-atherogenic potential and elimination of fecal lipids and catabolism of cholesterol into bile acids could be the possible mechanism.