Toxicological evaluation and therapeutic potential of *Brassica oleracea* and *Eugenia jambolana*

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CERTIFICATE

This is to certify that the thesis entitled "**Toxicological evaluation and therapeutic potential of *Brassica oleracea* and *Eugenia jambolana***" incorporates results of investigation carried out by the candidate himself in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara.

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Jayanta M Sankhari

Table of contents

Chapters

Introduction

Cardiovascular diseases (CVDs) are a group of disorders of heart and blood vessels that includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. Coronary artery disease is associated with the blood vessels supplying the heart muscle whereas, cerebrovascular diseases are concerned with blood supply to the brain. Peripheral arterial disease causes lack of blood supply to arms and legs and rheumatic heart disease causes to damage heart muscle and heart valves due to rheumatic fever, caused by streptococcal bacteria. Other forms of diseases include congenital heart disease, concerned with malformations of heart structure existing at birth and deep vein thrombosis and pulmonary embolism that causes blood clots in the leg veins often getting dislodged into heart and lungs.

Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or brain. The most common reason for this is a build-up of fatty deposits on the inner walls of the blood vessels that supply blood to the heart or brain. Stroke can also be caused due to internal bleeding from a blood vessel in the brain (WHO 2007).

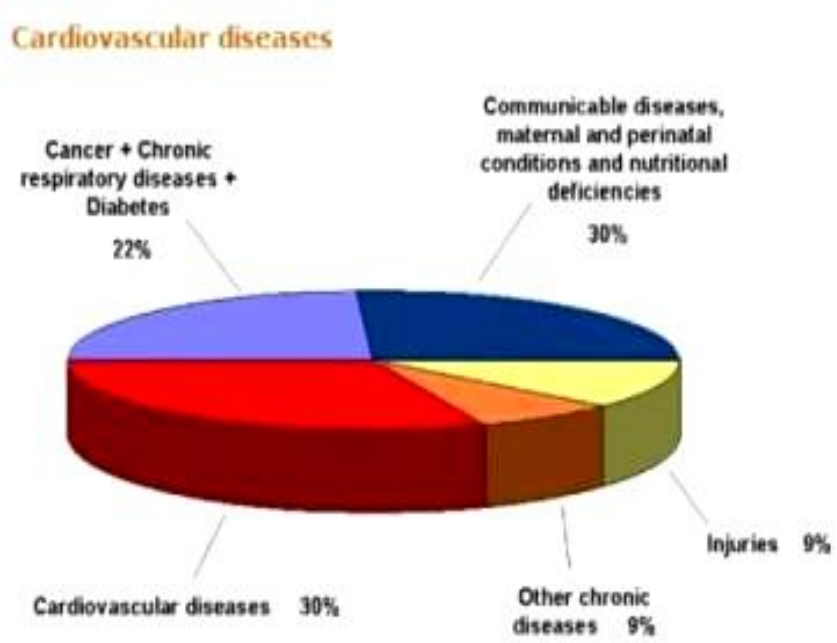


Figure 1 Prevalence of cardiovascular diseases

(From: -http://www.chemicalshealthmonitor.org/spip.php?rubrique10).

Data collected from internet sources reveals that CVDs are also dominantly prevalent (30%) in patients suffering from communicable diseases, nutritional deficiencies and other physiological ailments. CVDs are the number one cause of death globally as more people die annually from CVDs than from any other causes. An estimated 17.3 million people died due to CVDs in 2008, representing 30% of all global deaths. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. Low- and middle-income countries are disproportionally affected: over 80% of CVD deaths take place in low- and middle-income countries and occur almost equally in men and women. By 2030, it is assumed that almost 23.6 million people may die due to CVDs, such as heart disease and stroke.

Cardiovascular disease is a leading cause of global mortality, accounting for almost 17 million deaths annually (Smith et al., 2004; Martinello et al., 2006); atherosclerosis, in particular, is the main contributor for the pathogenesis of myocardial and cerebral infarction. Elevated levels of plasma low-density lipoprotein cholesterol (LDL) and triglycerides, accompanied by reduced high-density lipoprotein (HDL)levels, is often associated with an increased risk of coronary heart disease (Smith et al., 2004; Martinello et al., 2006).High cholesterol diet is regarded as an important factor in the development of hyperlipidemia, atherosclerosis and ischemic heart disease. Cardiovascular disease is the primary cause of mortality in the United States, Europe and most parts of Asia (Braunwald, 1997; Ross, 1999; Ling et al., 2001). In hypercholesterolemia, the cholesterol content of erythrocytes, platelets, polymorph nuclear leucocytes and endothelial cells increases. This increase is reported to activate

these cells and cause the enhanced production of oxygen free radicals (Kok et al., 1991; Prasad and Kalra, 1989; Sudhahar et al., 2006). Erythrocytes, because of their intrinsic potential for free radical generation, might be a very suitable environment for cholesterol to exert its prooxidant action (Kay, 1991). The heart of hyperlipidemic patients adapts poorly to oxidative stress, suggesting that the endogenous adaptive mechanisms against myocardial stress are impaired (Roberts, 1995). Intracellular lipids accumulate in cardiomyocytes and cause several alterations in the structure and functional properties of the myocardium (Hexeberg et al. 1993). Effective cholesterol-lowering drug therapy delays the development or progression of coronary heart disease (Blankenhornet al., 1987; Sudhahar et al., 2006).

Hypercholesterolemia (HC) is a major risk factor for atherosclerosis and coronary heart disease. It is characterized by coronary endothelial dysfunction that is characterized by altered vasodilatation of endothelial dependent vasodilators (Heistad et al., 1991; Luscher et al., 1992). It may also promote ischemic tissue damage by enhancing the vulnerability of the microcirculation to the deleterious effects of ischemia and other inflammatory stimuli, thus increasing the incidence of myocardial ischemia and cardiac events (Stokes et al., 2002). Hexeberg et al. (1993) have also observed in experimental rats that there is intracellular lipid accumulation in cardiomyocytes that alters its structure and property. Hypercholesterolemia is one of the most important risk factors for atherosclerosis and subsequent cardiovascular disease (Steinberg, 2002). Feeding animals with cholesterol has often been used to elevate serum or tissue cholesterol levels to study the etiology of hypercholesterolemia-related metabolic disorders (Bocan, 1998). Exogenous hypercholesterolemia causes fat deposition in the liver and depletion of the

hepatocyte population. This in turn, may result in malfunctioning of the liver, which apparently follows microvesicular stenosis due to the intracellular accumulation of lipids (Gupta et al., 1976; Assy et al., 2000). Also, feeding cholesterol-rich diets induces free radical production, followed by oxidative stress and hypercholesterolemia (Stehbens, 1986; Bulur et al., 1995).Oxidative stress results from impairment of the equilibrium between production of free radicals and antioxidant defense systems. It is one of the factors that link hypercholesterolemia with atherogenesis (Halliwell, 1996). Thus, there is evidence that oxidative stress contributes to the development of atherosclerosis in the vascular wall through the formation of reactive oxygen species (ROS) (Shi et al., 2000). In order to protect the tissues from damage caused by ROS, organisms possess enzymatic and non-enzymatic antioxidant systems (Parthasarathy et al., 2000). Protection against ROS and the breakdown products of peroxidized lipids and oxidized proteins is provided by enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (Gpx) and glutathione-S-transferase (GST). Non-enzymatic antioxidants such as reduced glutathione (GSH), vitamins C and E, play a vital role in protecting cells from oxidative stress by participating in various biochemical pathways (Ramesh et al., 2009).

History of herbal medicine

Herbs have played a major role and have always been an integral part of the development of modern civilization. Much of the medicinal use of plants has developed through observations of wild animals and by trial and error. With passage of time, each tribe added the medicinal power of herbs in their area to its traditional knowledge base. They methodically collected information on herbs were later translated into well-defined herbal

pharmacopoeias. By the end of 20th century, much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native populace. Many drugs commonly used today are of herbal origin with about 25% of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material (Charles and Ramani, 2011; Srichaikul et al., 2012). Some are made from plant extract whereas, others are synthesized to mimic a natural plant compound. Many drugs listed as conventional medications were originally derived from plants. Salicylic acid, a precursor of aspirin, was originally derived from white willow bark and the meadowsweet plant. Cinchona bark is the source of malaria-fighting quinine. Vincristine, used to treat certain types of cancer is derived from periwinkle. The opium poppy yields morphine, codeine and paregoric that is used in the treatment of diarrhea. Laudanum, a tincture of the opium poppy, was a favored tranquilizer in Victorian times. Even today, morphine-the most important alkaloid of the opium poppy-remains the standard against which new synthetic pain relievers is compared.

The World Health Organization (WHO) estimates that 4 billion people, 80% of the world population, presently use herbal medicine for some aspect of primary health care (WHO, 2002). Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental and Native American Indian medicine. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures (WHO, 1993). Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential

medicinal value. It is important to note that each chemical compounds or drugs also manifest extensive contraindications and possible side effects. Rather than using a whole plant, pharmacologists identify, isolate, extract, and synthesize individual components, thus capturing the active properties.

Substances derived from the plants for commercial medications used today are treatment of heart disease, high blood pressure, pain, asthma and other problems. Active ingredients, plants contain minerals, vitamins, volatile oils, glycosides, alkaloids, bioflavonoid and other substances along with isolated or synthesized active compounds can become toxic in relatively small doses. Ephedra is an herb used in Traditional Chinese Medicine for more than two thousand years to treat asthma and other respiratory problems. Ephedrine, the active ingredient present in ephedra, is used in the commercial pharmaceutical preparations for the relief of asthma symptoms and other respiratory problems as it helps the patient to breathe more easily (Srichaikul et al., 2012). Modern pharmacology looks for one active ingredient and seeks to isolate it to the exclusion of all the others. Most of the research that is done on plants continues to focus on identifying and isolating active ingredients, rather than studying the medicinal properties of whole plants. Plants used as medicines offer synergistic interactions between ingredients both known and unknown. The efficacy of many medicinal plants has been validated by scientists abroad, from Europe to the Oriental regions (Srichaikul et al., 2012).

Herbal drugs and safety concern

Herbal products are not completely free from side effects. Well-controlled randomized clinical trials have revealed that undesirable side effects are manifested in

patients who have used herbal drugs on a consistent basis. Cardiovascular problems with use of ephedra, hepatotoxicity by kava-kava consumption, anti-cholinergic effects leading to reduced visceral activity associated with asthma medicine containing Datura metel, water retention by liquorice are reported examples of side effects due to herbal drugs (Elvin-Lewis et al., 2001; Cuzzolin et al., 2006). Due to increased reports on adverse effects regulatory/monitoring agencies in many countries have brought out alerts on herbal drugs. In 1993, the American Herbal Products Association (AHPA) issued an alert to restrict the use of comfrey (herbal medicine that contains pyrrolizidine alkaloids; PAs) for external applications. Cardiovascular ailments were reported with excessive use of a Chinese herb containing ephedra (used to promote weight loss) in the US led to its ban by USFDA in 2004 (Sahoo et al., 2010). The use of three herbal medicines that contain aristolochic acids (AAs), namely Radix Aristolochiae Fangchi (Guangfangji), Caulis Aristolochiae Manshuriensis (Guanmutong) and Radix Aristolochiae (Qingmuxiang), were banned in China due to the potential risk of nephrotoxicity (Li et al., 2008). The most commonly reported adverse reactions are hypertension, hepatitis, face edema, angiodema, convulsions, thrombocytopenia, dermatitis and death (Uppsala Monitoring Centre, 2009; Sahoo et al., 2010).

The safety problems emerging with herbal medicinal products are due to a largely unregulated growing market wherein, there is a lack of effective quality control. Lack of strict guidelines on the assessment of safety and efficacy, quality control; safety monitoring and knowledge on traditional medicine/complementary and alternative medicine(TM/CAM) are the main aspects which are found in various regulatory systems. Under some regulatory systems, plant may be defined as a food, a functional food, a

dietary supplement or a herbal medicine (Sahoo et al., 2010). As per WHO, herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain as active ingredients parts of plants, or other plant materials, or combinations. Unprocessed exudates are also considered as herbal drugs (Sahoo et al., 2010). When herbal drugs are subjected to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation, they are known as herbal drug preparations. This includes powdered herbal drugs, tinctures, extracts, essential oils, expressed juice or process exudates. A botanical product which is derived from one or more plants, algae, or macroscopic fungi and prepared from botanical raw materials by one or more of the processes such as pulverization, decoction, expression, aqueous extraction, ethanolic extraction, or other similar process, intended for use as a drug is known as Botanical Drug Product (http://www.nistads.res.in/indiasnt2010-11/T3Industry/Indian%20Herbal%20 Drug%20Industry.pdf).

Phytochemicals and human health

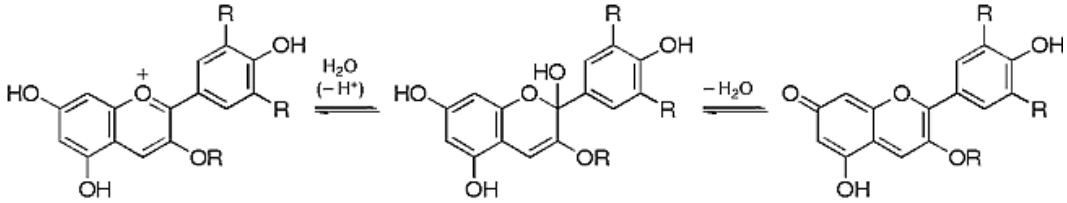
Phytochemicals are substances that plants naturally produce to protect themselves various exogenous against such as bacteria, viruses, and fungi. There has been a lot of interest in phytochemicals recently because many of them can help to slow the aging process and reduce the risk for cancer, heart disease and other chronic health conditions (Kennedy and Wightman, 2011). Till date, more than 900 types of phytochemicals have been reported from various herbs and many are yet to be discovered. Fruits, vegetables, whole grains, soy and nuts are rich sources of polyphenols. Phytochemicals are usually related to plant pigments, so fruits and vegetables with bright colors (yellow, orange, red, blue,

purple, green) are rich sources of these disease fighting substances and the same have been recommended to be consumed daily (http://www.carrotmuseum .co.uk/phyto.html).

Phytochemicals, in combination with vitamins, minerals and fiber can provide immunity against diseases. Much of the protective effect of fruits and vegetables has been attributed to phytochemicals, which are the non-nutrient plant compounds such as the carotenoids, flavonoids, isoflavonoids, and phenolic acids (Boyer and Liu, 2004). Phytochemicals may inhibit induction and proliferation of cancer, regulate inflammatory and immune response and protect against oxidative modification of lipids (Hollman and Katan, 1997; Liu, 2003). We live in a highly oxidative environment, and many processes involved in metabolism may result in the production of more oxidants (Boyer and Liu, 2004). Both cardiovascular disease and cancer are thought to be resulting due to oxidative stress, leading to damage of the larger biomolecules, such as DNA, lipids, and proteins. It has been estimated that there are 10,000 oxidative hits to DNA per cell per day in humans (Ames et al., 1993). Hence, a major role of the phytochemicals is in providing much needed protection against oxidation of biomolecules.

Importance of Anthocyanins

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom (Mazza et al., 1993). Chemically, they are polyhydroxylated or polymethoxylated glycosidesor acylglycosides of anthocyanidins which are oxygenated derivatives of 2-phenylbenzopyryliumor flavylium salts.



flavylium (red) carbinol (colourless) quinone (blue)

Figure 2: Major classes of anthocyanins.

They belong to the family of compounds known as flavonoids and they are distinguished from other flavonoids as a separate class by their ability to form flavylium cations. Anthocyanins are responsible for the red, blue and purple colors of fruits, vegetables, flowers and other plant tissues or products. They are particularly abundant in berries and other fruits with red, blue, or purple color and in red wines (Brouillard et al., 1982, Brouillard et al.,1989, Mazza et al., 1993, Mazza et al., 2004). Till date, about 400 individual anthocyanins have been identified. The six anthocyanidins commonly found in plants are classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus and are named pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin. The most commonly occurring anthocyanidin in nature is cyanidin. The antioxidant activity (scavenging free radicals, metal chelation, protein binding) of anthocyanins including the protection of LDL against oxidation, has been demonstrated in a number of different *in vitro* systems (Wrolstad et al., 2000, Mateus et al., 2001, Mateus et al., 2003)

Flavonoids and their importance

Flavonoids are a group of phenolic compounds widely distributed in plant kingdom and they are important components of various foods of plant origin. Over 4000 structurally unique flavonoids have been identified in plant sources (Patel, 2008). These are recognized as the pigments responsible for the colors of leaves. They are rich in seeds, citrus fruits, olive oil, tea, and red wine. They are low molecular weight compounds composed of a three-ring structure with various substitutions. This basic structure is shared by tocopherols (vitamin E). Flavonoids can be subdivided according to the presence of an oxy group at position 4, a double bond between carbon atoms 2 and 3, or hydroxyl group in position 3 of the C (middle) ring (Middleton et al., 2000).

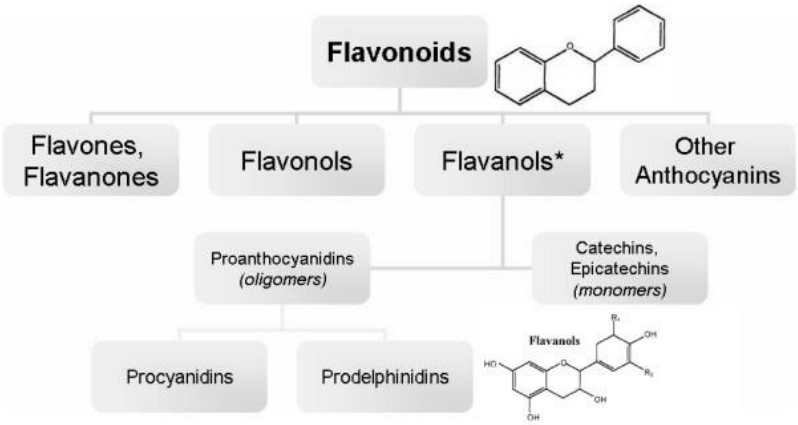


Figure 3: Classification of flavonoids.

Dietary flavanoids have received considerable attention since epidemiological studies suggesting that regular consumption of flavonoid-rich foods or beverages is associated with a decreased risk of cardiovascular mortality (Hertog et al., 1993; Knekt et al., 1996). For instance, the Mediterranean diet, which is rich in these bioactive

compounds, has been shown to protect against chronic diseases, including coronary heart diseases. Flavonoids have been shown to display a wide range of biological effects although their health benefits have been attributed primarily to their antioxidant properties (Patel, 2008). Certain plants and spices containing flavonoids have been used for thousands of years in traditional Eastern medicine. Flavonoids have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances and pigments. It has been shown to possess several biological properties such as hepato-protective, anti-thrambotic, anti-inflammatory, and antiviral activities primarily due to their antioxidant and free-radical-scavenging ability (Patel, 2008). The antiradical property of flavonoids is directed mostly toward HO; and 02 - as well as peroxyl and alkoxyl radicals. Furthermore, as these compounds present a strong affinity for iron ions (which are known to catalyze many processes leading to the appearance of free radicals), their antiperoxidative activity could also be ascribed to a concomitant capability of chelating iron. Several flavonoid compounds have been shown to have antioxidant properties *in vitro*, inhibiting the oxidation of low density lipoproteins and reducing thrombotic tendencies by inhibiting platelet aggregation (Cook and Samman, 1996).

Red cabbage (Brassica oleracea* var. capitata f. rubra*)

The red cabbage (RC; *Brassica oleracea* var. capitata f. rubra) is a variety of cabbage (family *Cruciferae*) with dark red/purple colored leaves that is grown in Northern Europe, America and China (Singh et al., 2006). The plant changes its color according to the pH value of the soil and amount of flavins (a type of anthocyanins). Wherever the soil

is acidic, the leaves of RC grow more reddish while in alkaline soil they are greenish-yellow in color. This explains the reason for their color variation and hence the same plant is known by different names based on color and geo-climatic variations. The juice of RC can act as a pH indicator as it turns red in acidic and blue in basic solutions. (http://en.wikipedia.org/wiki/Red_cabbage).

Figure 4: Classification of *B.Oleraceae* (Red cabbage).

RC needs well fertilized soil and sufficient humidity to grow. It is a seasonal plant which is seeded in spring and harvested in late fall. (http://en.wikipedia.org/wiki/Red_cabbage).

Recently, RC has attracted much attention because of its physiological functions and applications. The anthocyanins from RC are stable under the acidic gastric digestion conditions, although all of the anthocyanins are reduced after pancreatic digestion but acylated forms were notably more stable than non-acylated forms (McDougall et al.,

2007). Thus, RC dye has been used as a pH indicator in pharmaceutical formulations (Chigurupati et al., 2002) and as a colorant in food systems (Giusti and Wrolstad, 2003).

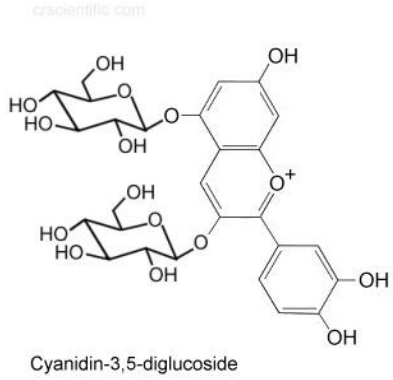
Phytochemistry of red cabbage

A total of 23 anthocyanins have been detected in red cabbage leaves, the major acylated anthocyanins in this vegetable included cyanidin-3-diglucoside-5-glucoside derivatives with various acylated groups connected to the diglucoside, mostly sinapoyl esters (Wu and Prior 2005; Scalzo et al. 2008). It was shown that *Brassica oleracea* rubrum extract was the source of cyanidin-3,5-diglucoside and cyaniding 3-sophoroside-5-glycoside acylated with sinapic, ferulic, caffeic, coumaric or malonic acids (Stintzing et al. 2002). Red cabbage (*B. oleracea*) leaves contain cyanidin 3,5-diglucoside, cyanidin3-sophoroside-5-glucoside, cyanidin 3-sophoroside-5- glucoside and cyanidin-3-sophoroside-5-glucoside acylated with 1 and 2 mol of sinapic acid (Tanchev & Timberlake, 1969). Also, presence of natural antioxidants such as, ascorbic acid, α-tocopherol and β-carotene, lutein etc. has been reported in the RC extract (Singh et al., 2006).

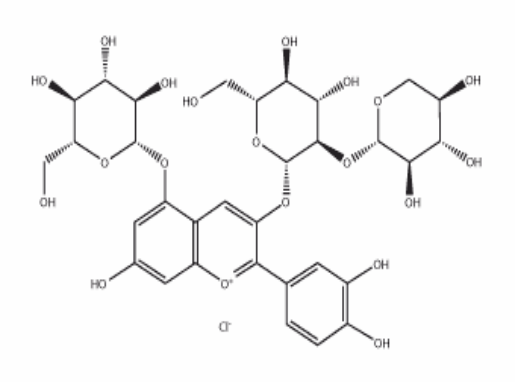
Pharmacology of Red cabbage

Published reports on RC extract have established its hypocholesterolemic (Komatsu et al., 1998), hepatoprotective (Igarashi et al., 2000; Singab et al., 2009), neuroprotective (Lee et al., 2002; Heo and Lee, 2006), nephroprotective in diabetic rats (Kataya and Hamza, 2008), anti-inflammatory (Lin et al., 2008) and anti-cancer (Fowke et al., 2003; Morsy et al., 2010) properties. Further, it has been shown to limits copper stress injury in

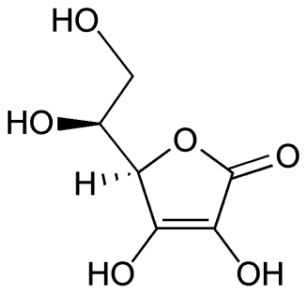
meristematic cells of *Vicia faba* (Posmyk et al., 2008) and protects blood plasma proteins and lipids from hydrogen peroxide (Kolodziejczyk et al., 2011).



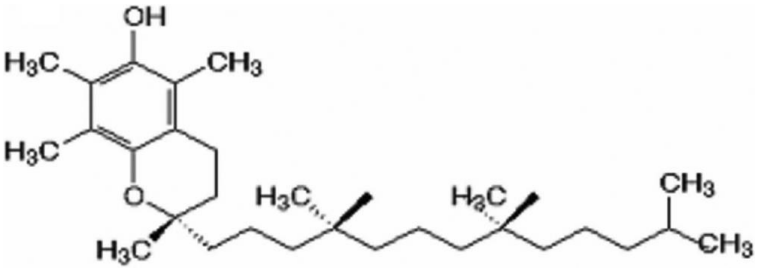
Cyanidin 3,5-diglucoside



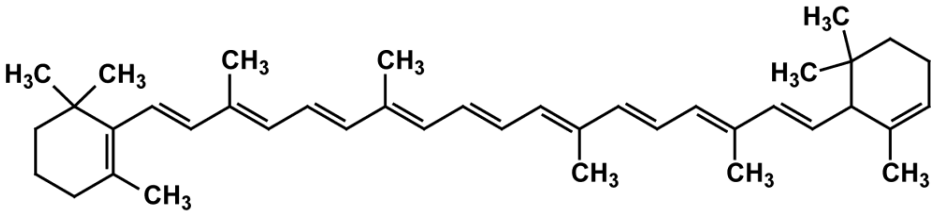
Cyanidin 3-sophoroside-5-glucoside



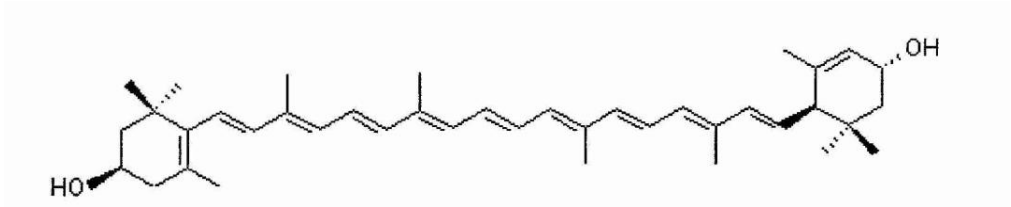
Ascorbic acid,



α-tocopherol



β-carotene



Lutein

Figure 6 Phytochemical constituents of red cabbage.

Eugenia jambolana

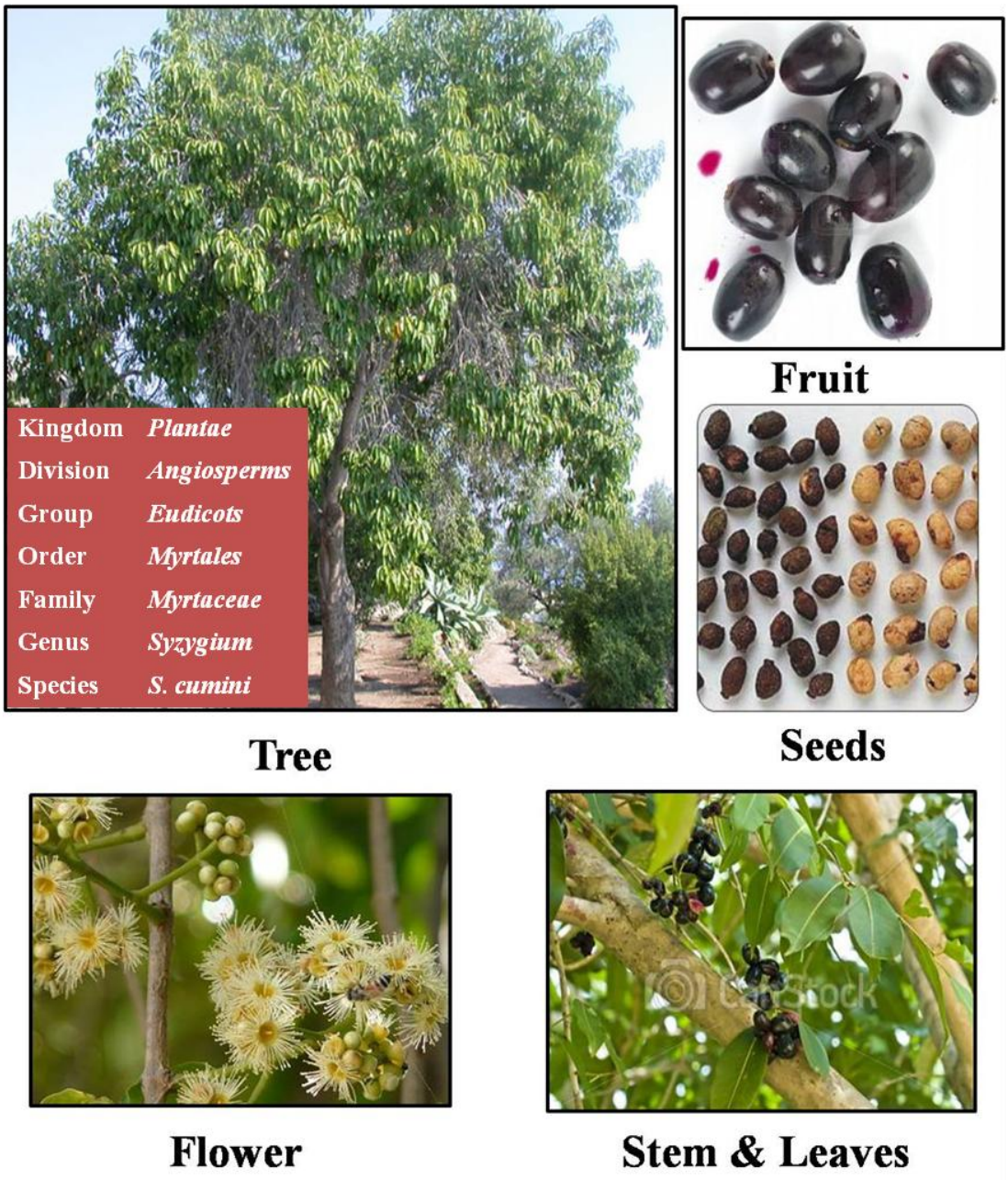


Figure 7: Aerial parts and classification of *Syzygium cumini* (*Eugenia jambolana* Lam).

Eugenia jambolana Lam. (Syn. *Syzygium cumini* Skeels or *Syzygium jambolana* Dc or *Eugenia cuminii* Druce; family Myrtaceae) is a large evergreen tree indigenous to the Indian subcontinent. However today these trees are found growing throughout the

Asian subcontinent, Eastern Africa, South America, Madagascar and have also naturalized to the armer regions of the United States of America (in Florida and Hawaii) (Li et al. 2009; Warrier et al., 1996). The trees are famous for their fruits and their colloquial names, which include Java plum, Portuguese plum, Malabar plum, black plum, Indian blackberry, jaman, jambu, jambul and jambool are attributed to the fruits (Warrier et al. 1996). Based on the morphological and organoleptic features, there are two main morphotypes of Jamun - the Kaatha jamun (which are small and acidic to taste) and the Ras Jaman (oblong, dark-purple or bluish, with pink, sweet fleshy pulp and small seeds) found in the Indian subcontinent (Jabbar and Jazuddin 1994; Morton, 1987).

Traditional uses

Jamun is used extensively in the various traditional systems of medicine like in the Ayurveda, Unani, Siddha, in the Sri Lankan, in the Tibetan and in the Homeopathy systems of alternative and complementary medicine (Bhandary et al., 1999; Warrier et al., 1996). Prior to the discovery of insulin in Europe, Jamun was used for treating diabetes either alone, or in combination with other hypoglycemic plants (Helmstädter, 2007). According to Ayurveda, their barks are acrid, digestive and astringent and useful for treating sore throat, bronchitis, asthma, thirst, biliousness, dysentery and ulcers (Warrier et al. 1996). The ash of the leaves is used as a dentrificant and is effective at strengthening the teeth and the gums. The bark is also known to possess wound healing

Table 1: Scientific and vernacular names of *Eugenia jambolana* (Baliga et al., 2011).

Language	Names
Scientific name	*Syzygium jambolanum, Eugenia cumini, Syzygium cumini, Eugenia jambolana*
English	Jaman, black plum, damson plum, duhat plum, Indian blackberry, jambolan, jambolan plum, Java plum, Malabar plum, Portuguese plum, black plum, black plum tree, Indian blackberry, jambolan, jambolan-plum, Java plum, malabar plum, Portuguese plum
Indian languages	
Assamese	Jamu, kala jamu
Bengali	Kala jam
Guajarati	Jambu, jaambu
Hindi	Jamun, duhat, jam, jaman
Kannada	Nerale hannu
Konkani	Jambul
Malayalam	Kaattucaampa, njaaval, njaara, perinjaara
Manipuri	Gulamchat, jam
Marathi	Jambool
Mizo	Hmuipui
Nepalese	Jamunu, phanrir
Nepalese	Jamunu, phanrir
Pali	Jambu
Prakrit	Jambulo, jammulo
Punjabi	Jaman
Sanskrit	Jambu, jambulah, meghamodini
Tamil	Kottai-nakam, naval
Telugu	Neredu
Urdu	Jaman

properties. In the Siddha system of medicine, Jamun is considered to be a haematinic, semen promoting and to decrease excessive heat of the body (Warrier et al. 1996) whereas, in the Unani system of medicine, it has been reported as a liver tonic, to purify blood, strengthen teeth and gums. The decoction is supposed to be a good lotion for removing ringworm infection of the head (Warrier et al. 1996).

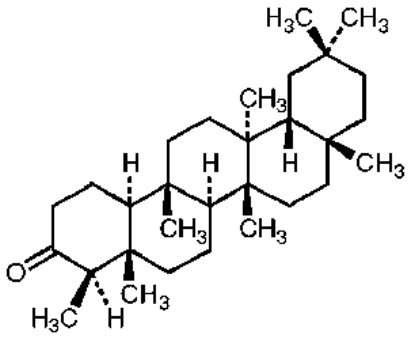
Phytochemistry of *Eugenia jambolana* (EJ)

The leaves of Jamun plant are known to contain β-sitosterol, betulinic acid, mycaminose, crategolic (maslinic) acid, n-hepatcosane, n-nonacosane, n-hentriacontane, noctacosanol, n-triacontanol, n-dotricontanol, quercetin, myricetin, myricitrin and flavonol glycosides myricetin 3-O-(4″-acetyl)-α-L-rhamnopyranosides, acylated flavonol glycosides (Mahmoud et al., 2001; Sagrawat et al., 2006). The essential oil from the leaves is shown to contain the phytochemicals pinocarveol, α-terpeneol, myrtenol, eucarvone,muurolol, α-myrtenal, cineole, geranyl acetone, α-cadinol andpinocarvone (Shafi et al., 2002). The stem bark is reported to possess friedelin, friedelan-3-α-ol, betulinic acid, β-sitosterol, kaempferol, β-sitosterol-D-glucoside, gallic acid, ellagic acid, gallotannin and ellagitannin and myricetine (Rastogi and Mehrotra, 1990; Sagrawat et al. 2006).The flowers are observed to contain oleanolic acid, ellagic acids, isoquercetin, quercetin, kampferol and myricetin (Sagrawat et al. 2006). Studies conducted by various research groups have shown that the pulp of Jamun contains theanthocyanins, delphinidin, petunidin, malvidin-diglucosides, andthese compounds are responsible for their bright purple color (Li and Seeram 2009; Sagrawat et al. 2006; Veigas et al. 2007; Sharma et al., 2008a; Sharma et al., 2008b). Seeds of EJ are the most studied and reported to contain jambosine, gallic acid, ellagic acid, corilagin,3,6-hexahydroxy diphenoylglucose, 4,6-hexahydroxydiphenoylglucose,1-galloylglucose, 3-galloylglucose, quercetin, β-sitoterol (Rastogi and Mehrotra 1990; Sagrawat et al. 2006). Details shown in Figure 7.

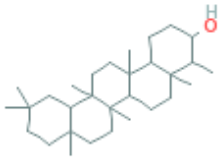
Table 2: Phytochemical constituents of *Eugenia jambolana* (Baliga et al., 2011).

Plant part	Phytochemicals	References

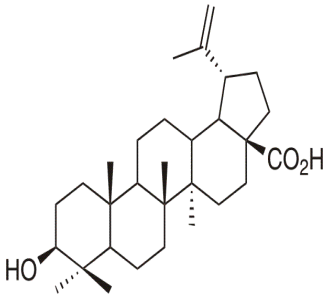
Stem bark	Friedelin, friedelan-3-α-ol, betulinic acid, β-sitosterol, kaempferol, gallic acid, ellagic acid, gallotannin and ellagitannin and myricetin	(Rastogi and Mehrotra, 1990; Sagrawat et al., 2006)
Leaves	β-sitosterol, betulinic acid, mycaminose, crategolic (maslinic) acid, n-heptacosane, n-nonacosane, n-hentriacontane, n-octacosanol, n-triacontanol, n-dotriacontan, quercetin, myricetin, and the flavonol glycosides	(Rastogi and Mehrotra, 1990; Sagrawat et al., 2006).
Flowers	Oleanolic acid, ellagic acids, isoquercetin, quercetin, kampferol and myricetin	(Sagrawat et al., 2006).
Fruit pulp	Anthocyanins, delphinidin, petunidin, malvidin-diglucosides	(Li et al., 2009a; Sagrawat et al., 2006; Veigas et al., 2007)
Seed	Jambosine, gallic acid, ellagic acid, corilagin, 3, 6-hexahydroxy diphenoylglucose, 1-galloylglucose, 3-galloylglucose, quercetin, β-sitoterol,4,6-hexahydroxydiphenoylglucose	(Rastogi and Mehrotra, 1990; Sagrawat et al., 2006).
Essential oils	α-terpeneol, myrtenol, eucarvone, muurolol, α-myrtenal, 1, 8-cineole, geranyl acetone, α-cadinol and pinocarvone	(Shafi et al., 2002).



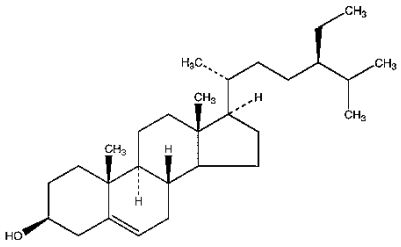
Friedelin



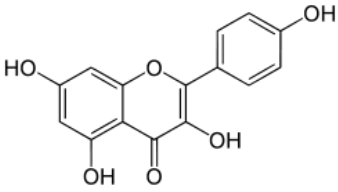
Friedelan-3-α-ol



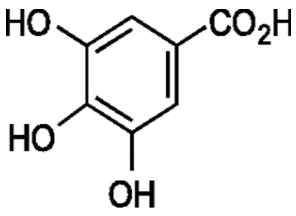
betulinic acid



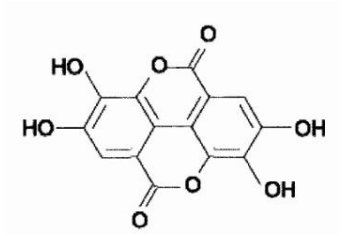
β-sitosterol

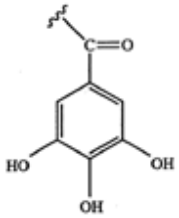


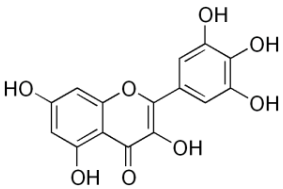
Kaempferol



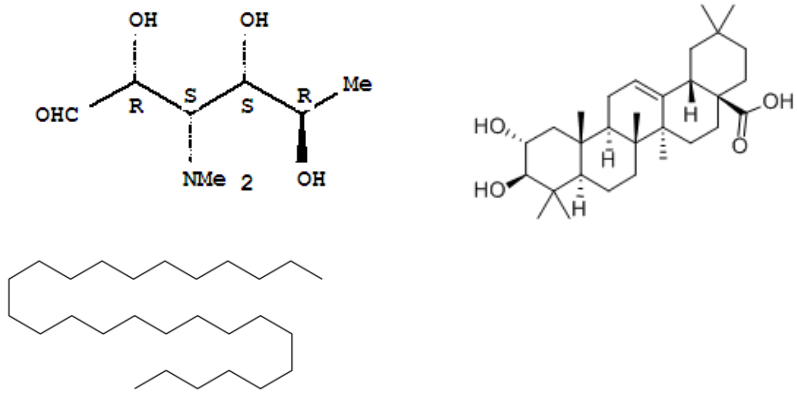
Gallic acid





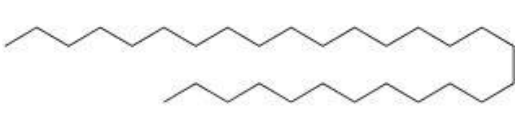


Ellagic acid **Gallotannin** **Myricetin**

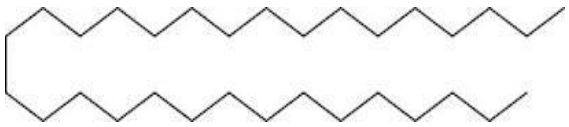


Mycaminose **Maslinic acid** **n-heptacosane**

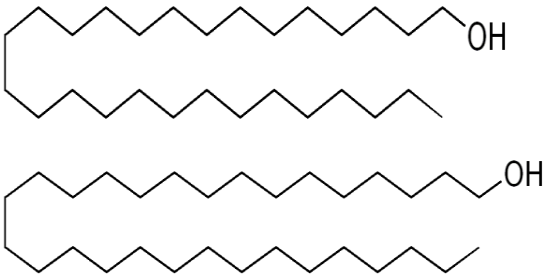
Figure 8: Phytochemical constituents of *Eugenia jambolana*.



n-nonacosane

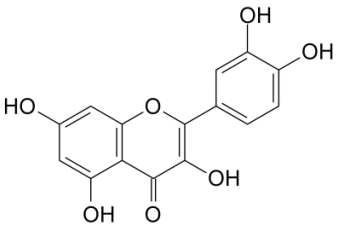


n-hentriacontane

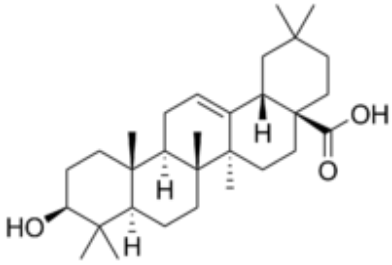


n-octacosanol

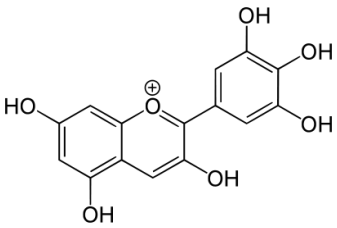
n-triacontanol



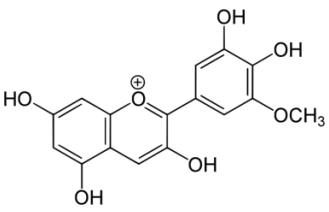
Quercetin



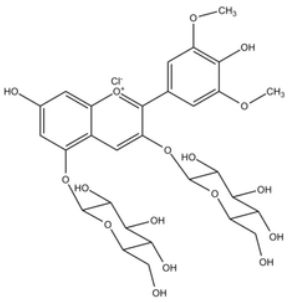
Oleanolic acid



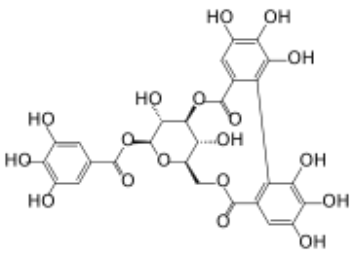
Delphinidin



Petunidin

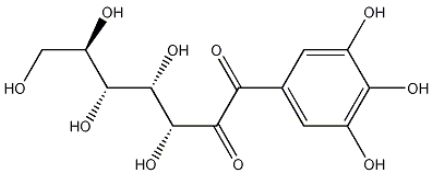


Malvidin-diglucosides

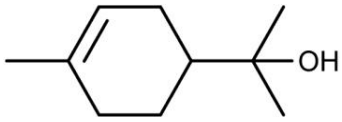


Corilagin

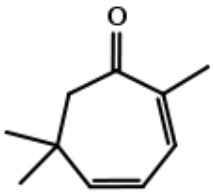
Figure 9: Phytochemical constituents of *Eugenia jambolana*.



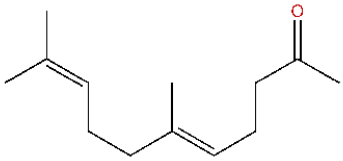
1-galloylglucose



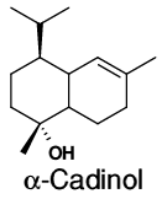
α-terpeneol



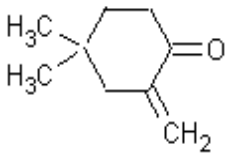
Eucarvone



Geranyl acetone



α-cadinol



Pinocarvone

Figure 10: Phytochemical constituents of *Eugenia jambolana*.

Pharmacology of Eugenia jambolana

Jamun has been thoroughly investigated for its antidiabetic effects and the seed, pulp and bark have been found to have effective antidiabetic action (Sharma et al., 2006; Gohil et al., 2010; Saravanan and Leelavinothan, 2006; Sharma et al., 2006; Pepato et al., 2005), while the leaf was ineffective (Pepato et al., 2001). The seed is the most studied and the effective in causing anti-hyperglycemic effects in different experimental models of study (Achrekar et al., 1991; Panda et al., 2009; Rathi et al., 2002; Ravi et al., 2005, 2004a, 2004b, 2004c, 2004d; Sharma et al., 2008a, b; Sharma et al., 2003; Sridhar et al., 2005). Jamun seeds prevent the diabetes-induced secondary complications like nephropathy (Grover et al., 2002), neuropathy (Grover et al., 2002), gastropathy (Grover et al., 2002), diabetic cataract (Rathi et al., 2002) and also decreased peptic ulceration (Chaturvedi et al., 2007). Human studies have shown that Jamun possess promising anti-hyperglycemic effects (Kohli and Singh 1993; Sahana et al., 2010). Other reported pharmacological activities include, Antibacterial (Shafi et al., 2002), Antifungal (Jabeen and Javaid, 2010), Antiviral (Bhanuprakash et al., 2008),Free radical scavenging (Nahar et al., 2009),Anti-inflammatory (Muruganandan et al., 2001), Gastroprotective (Chaturvedi et al., 2007, 2009a,b), Hepatoprotective (Sisodia and Bhatnagar, 2009),Hypolipidemic (Sharma et al., 2008a,b),Cardioprotective (Mastan et al., 2009),Anti-diarrheal (Mukherjee et al., 1998),Antifertility (Rajasekaran et al., 1988), Anti-allergic (Brito et al., 2007), Antipyretic (Chaudhuri et al., 1990), Chemopreventive (Goyal et al., 2010).

Objectives and Work envisaged in a nutshell

1. Acute and subchronic toxicity evaluations of *Eugenia jambolana* seeds (EJSE) and *Brassica oleracea* leaf extracts or anthocyanin rich red cabbage extract (ARCE).

Preparation of Anthocyanin rich extracts of the above mentioned herbals.

To assess acute toxicity of these herbal extracts in mice model.

To assess subchronic toxicity of these herbal extracts in rat model.

2. Assessment of therapeutic potentials of *Eugenia jambolana* seeds (EJSE) and *Brassica oleracea* (ARCE) leaf extracts against experimentally induced cardiac and hepatic oxidative stress.

Hypothesis to be tested: Can anthocyanin rich extracts of these herbals successfully mitigate isoproterenol induced myocardial necrosis or CCl_4 induced hepatotoxicity in rats.

3. Assessment of *Eugenia jambolana* seeds (EJSE) and *Brassica oleracea* (ARCE) leaf extracts in mitigating experimentally induced atherosclerosis.

Hypothesis to be tested: Can these herbals successfully mitigate PTU + vitamin D3 + high fat diet induced atherosclerosis in Sprague dawley rats.

Chapter 1

Acute and sub-chronic toxicity evaluation of *Eugenia jambolana* seed extract.

INTRODUCTION

India is considered to be the largest producer of medicinal herbs and is rightly called the botanical garden of the world (Dubey et al., 2004). Use of herbal medicines is an age old practice in India and recent studies have documented a rise in usage of alternative therapies not only in the developing countries like India but also in other developed countries worldwide (Singh and Prakash, 2008). In USA, 12-19 % of the total use of complementary and alternative medicines comprises of herbal drugs (Tindle et al.2005). However, there are questions raised on the efficacy and safety of these drugs as majority of the traditional herbal preparations lack of scientific data pertaining to its toxic dose and clinical use. A prevailing myth associated with drugs of herbal origin is that they are always 'safe' with no 'side effects' but, studies have reported hepatotoxicity (Larrey and Pageaux, 1995), nephrotoxicity (Saxena and Panhotra, 2003; Singh and Prakash, 2008) and associated side effects. According to United States Food and Drug Administration act (USFDA), herbal drugs do not fall in the category of medicine and hence there are no rigorous safety evaluations. This necessitates determinations of toxicity dosage of any herbal preparation through preclinical acute and sub chronic toxicity evaluations (Jadeja et al., 2011).

There is no dearth of literature available on beneficial and medicinal uses of EJSE but, the available data lacks a systematic safety/toxicity evaluation of the same. Hence, it

was thought pertinent to evaluate the same in acute and chronic toxicity model as per the Organisation for Economic Co-operation and Development (OECD) guidelines.

MATERIALS AND METHODS

Preparation of extract

Fruits of *Eugenia jambolana* were handpicked from fruit shop. The pulp was removed and the seeds were washed several times with Milli-Q water and shade dried at room temperature. Kernel of the seeds was separated from the seed coat, powdered in an electrical grinder. Hundred gm of kernel powder was suspended in 1000 ml of Milli-Q water overnight and then sieved through several layers of sterile muslin cloth. The resultant residual paste was extracted with 70% ethanol at room temperature and later, was evaporated in a rotatory evaporator at 40–50 ºC (Ravi et al., 2003. 2004, 2005; Sharma et al., 2003). The yield of ethanolic extract was 5 g/100 g of dried seed powder.

Experimental animals

Swiss albino mice of either sex (Obtained from Zydus research centre, Ahmedabad, India) were housed and 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 carried out 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).

Acute oral toxicity in mice

The acute oral toxicity study was conducted using the limit test procedure as per OECD test guidelines on acute oral toxicity test 401 (OECD, 2001). Thirty two *Swiss* albino mice of either sex were divided into four groups (n=8) and were orally administered with a single dose of 1000, 2000, 3000, 4000 or 5000 mg/kg BW of EJSE. Animals were observed for possible behavioural changes such as tremors, convulsions, sleep, altered feeding, salivation, altered somato-motor activities and diarrhoea till 72 hr post treatment.

Sub chronic oral toxicity in mice

The sub chronic oral toxicity study was conducted according to OECD Test 407 (OECD, 1995). Thirty two *Swiss* albino mice of either sex were divided into four groups (n=8) and maintained for 28 days for this experiment. Group I was orally fed with carboxy methyl cellulose (CMC; 0.5 %) that served as control whereas, Groups 2, 3 and 4 were orally administered with 1000, 2000 or 3000 mg/kg of EJSE respectively. After 28 days of treatment, blood was collected from overnight fasted mice via retro-orbital sinus under mild anaesthesia and plasma was separated for further biochemical analysis. Thereafter, the animals were sacrificed by cervical dislocation under mild ether anaesthesia, and brain, heart, lungs, liver, spleen, kidney and adrenal were excised and weighed.

Cage side observations

After treatment with EJSE, the mice were observed daily for possible changes in appearance of skin, fur and eyes. Also, the animals were closely observed for

somatomotor activity, respiratory and behaviour changes, tremors, convulsions, salivation, diarrhoea etc.

Body Weight and Food and Water Intake

Body weight of each experimental animal was recorded using a weighing balance (CITIZEN Model 1621K, Taiwan) at the end of every week during the entire study. Food and water intake in all the experimental groups were monitored daily at 09:00 hrs. Known quantity of food was given to the respective experimental groups and the leftover food was weighed and the quantity was subtracted from the total to record food intake per mice. Also, mice were provided with known volume of reverse osmosis grade water in a 500 ml measuring cylinder and leftover volume of water was measured every 24 hr interval to calculate water intake.

Plasma biochemical analysis

Plasma sodium, potassium (Tulip Diagnostics, Pvt. Ltd), calcium (Lab Care Diagnostics India Pvt. Ltd), Creatine Kinase-MB (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, cholesterol, HDL(Reckon diagnostics, Vadodara, India), triglyceride (Beckon Diagnostics, Vadodara, India) and fasting blood glucose (Bayer diagnostics, India Ltd) were assayed using commercially available kits as per the instruction of the manufacturer.

Estimation of Plasma Sodium

Principle: Sodium is precipitated as a triple salt with magnesium and uranyl acetate. The excess of the uranyl ion are reacted with ferrocyanide in an acidic medium to develop a brownish colour. The intensity of the colour produced is inversely proportional to the concentration of sodium in the sample.

Uranyl ion + Mg ions + Na+ = Uranyl Mg-Na precipitate

Free Uranyl ions + K4Fe(CN) = Brown colour complex

Procedure: Precipitating regent 1ml L1 is mixed well with 20µl of serum and kept at room temperature for five minutes and then centrifuged. Clear supernatant is then separated out and it is then mixed with Acid reagent L2 (20 µl) against blank (distilled water) within 15 minutes.

Readings is taken at 530nm/Green filter.

Estimation of Plasma Potassium

Principle: Potassium reacts with sodium tetraphenyl boron in a specially prepared buffer to form a colloidal suspension. The amount of the turbidity produced is directly proportional to the concentration of potassium in the sample.

Tetraphenyl Boron + K+ - - - - → White turbidity

Procedure: Pipette into clean dry test tubes labelled as Blank , standard and test. Potassium reagent 1ml is mixed with 20 microns of serum, and in standard 20 micron of K+ standard is taken. Asorbance are taken against blank with plain K+ reagent (L1)

Readings is taken at 630nm/Red filter.

Estimation of Plasma Calcium

Principle: At a neutral pH, the Ca2+ forms with arsenazo III a complex, the colour intensity of which is directly proportional to the concentration of calcium in the sample.

Procedure: Serum (25 micron) is incubated at room temperature for 5 minutes. The final absorbance of the sample and standard against the blank is read at red filter.

Readings is taken at 650nm filter

Estimation of Creatine Kinase-MB

Principle: Enzopak CK-MB is based on the principle of specific immunoinhibition by a blend of monoclonal antibody which completely inhibit CK-MM activity and 50% of CK-MB activity. While not affecting the B subunit activity of CK-MB and CK-BB, the CK-BB activity is measured. The CK-MB activity is obtained by multiplying the CK-BB activity by two increased levels may be found due to severe exercise and by large multiple intramuscular injections. While other symptoms and suggestive history, serum CK estimation is an important parameter of choice for myocardial infraction and follows up.

Creatine Phosphate + ADP → Creatine + ADP

ATP + Glucose → G-6 PO_4 + ADP

G-6 PO_4 + NADP → 6-Phosphogluconate + NADPH + H

Procedure: Working reagent 1ml is incubated at 37 degree for 5 minutes and then mixed with 50 microns of serum. The first absorbance of the test is taken at 300 seconds and

thereafter at 30, 60, 90, and 120 seconds at 340 nm. Determining the mean change in absorbance per min.

The reaction is monitored by measuring the increase in absorbance at 340 nm.

Estimation of Lactate dehydrogenase

Principle: Potassium reacts with sodium tetraphenyl boron in a specially prepared buffer to form a colloidal suspension. The amount of the turbidity produced is directly proportional to the concentration of potassium in the sample.

Tetraphenyl Boron + K+ → White turbidity

Procedure: Pipette into clean dry test tubes labelled as Blank, standard and test. Potassium reagent 1ml is mixed with 20 microns of serum, and in standard 20 micron of K+ standard is taken. Absorbance are taken against blank with plain K+ reagent (L1)

Readings is taken at 630nm/Red filter.

Estimation of Alanine aminotransferase

Principle: L-Alanine and alpha-ketoglutarate reacts in the presence of GPT in the sample to yield pyruvate and L-glutamate.

L-Alanine + alpha ketoglutarate → Pyruvate + L-Glutamate

Pyruvate + NADH → Lactate + NAD

Procedure: Type of reaction is kinetic and the factor taken is 3376. 1ml reagent is taken and 50micons of serum is added. The absorbance is measured at an interval of 30 seconds for 2 minutes at 340nm.

Estimation of Alkaline phosphatase

Principle: Alkaline Phosphatase in a sample hydrolyses parnitrophenyl phosphate into paranitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405nm due to liberation of paranitophenol is proportional to the alkaline phosphatase activity.

p-Nitrophenyl Phosphate + H_2O $\rightarrow$ p-Nitrophenol + Phosphate

Procedure: Type of reaction is kinetic and the factor taken is 2713. Four readings are taken at an interval of 30 seconds. Buffersubstrate reagent 1ml is mixed with 20 microns of serum sample. The readings of the test are taken immediately as it is a kinetic reaction. The reaction is read at 30, 60, 90, and 120 seconds at 405 nm. The mean change in absorbance per minute are calculated as test result.

Estimation of Urea

Principle: Urease breaks down urea into ammonia and carondioxide. In alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicaroxyindophenol, a coloured compound. The reaction is catalysed by sodium nitroprusside. The intensity of colour produced is measured photometrically at 570nm.

Urea + H_2O $\rightarrow$ Ammonia + CO_2

Ammonia + Salicylate → 2-2 Dicarboxy Indophenol

Procedure: Type of reaction is End Point reaction. 5 microns of sample serum is incubated at room temperature with working enzyme reagent at 37 degrees for five minutes. After that 1ml working colour reagent (c) is mixed well and incubated for ten minutes at room temperature. The absorbance of test and standard against reagent lank is read at 570nm with red filter.

Estimation of Creatinine

Principle: Creatinine present in the serum reacts with alkaline picrate to form a coloured complex. The rate of formation of coloured complex is directly proportional to creatinine concentration. This rate of reaction (intensity of colour produced) is measured photometrically at 510nm and is compared with that of the standard.

Creatinine + Alkaline Pictrate → Creatinine Picrate Complex.

Procedure: Type of reaction is fixed time and readings are taken at 510nm. Flow cell temperature is maintained at 30 degree throughout the test procedure. 1ml of reagent is mixed with 100 microns of sample serum. Mix and aspirated. Absorbance is recorded at 20 second and again at 80 second against distilled water. The absorance of test and standard against reagent blank is read at 510nm.

Estimation of Cholesterol

Principle: The cholesterol esters are hydrolysed to free cholesterol esterase. The free cholesterol is then oxidised by cholesterol oxidase to cholesten4en30ne with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a coloured complex which is read at 505 nm.

Cholesterol ester → Cholesterol + Fattyacid

Cholesterol + O_2 → Cholesterol-4en-3one + H_2O_2

Procedure: It is a end point reaction. Flow cell temperature is maintained at 30 degree. 1ml cholesterol reagent is mixed with 10 microns of sample serum. It is incubated at 37 degrees for 10 minutes and the absorbance is read. The absorbance of test and standard against reagent blank is read at 505nm.

Estimation of Triglyceride

Principle: Triglycerides incubated with lipoprotein lipase are hydrolysed to free fatty acid and glycerol. Glycerol kinase catalyzes the conversion of glycerol and ATP to glycerol-3-phosphate and ADP. The glycerol-3-phosphate gets oxidised to dihydroxy acetone phosphate by glycerol phosphate oxidase. Hydrogen peroxide formed in this reaction with the help of peroxidase, reacts with chromogens. 4-aminoantipyrine/ ESPT to gives a purple coloured complex which is read at 546nm filter.

Tryglyceride → Glycerol + Free Fatty Acids

Glycerol + ATP → Glycerol-3-p + ADP

Glycerol-3-p + O_2 → DHAP + H_2O_2

H_2O_2 + 4-aminoantipyrine + ESPT → Purple Quinonimine

Procedure: Enzyme reagent 1ml and 10microns serum is mixed well and incubated for 10 minutes at 37 degree. After 10 minutes the absorbance of standard and sample against blank is taken. The absorbance of test and standard against reagent blank is read at 546nm green filter.

Estimation of HDL

Principle: High density lipoproteins(HDL) are separated from other lipoprotein fraction by treating serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipitated; cholesterol content of which is estimated by enzymatic method.

Serum + PTA Reagent → Supernatant(HDL) + Precipitates

Procedure: Fresh clear serum under fasting condition with no haemolysis is the specimen of choice. Sample serum 20microns is added to 20 micron of 3-HDL-cholesterol and it is mixed well. After 5 minutes it is centrifuged at 3500 rpm for 10 minutes. This supernatant 50 microns is incubated with 1ml cholesterol reagent for 10 minutes. The reading is taken in green filter.

Estimation of Blood glucose

Principle: Glucose oxidase oxidises the specific substrate, beta-D-glucose, to gluconic acid and hydrogen peroxide is generated. Hydrogen peroxide thus produced is acted upon by peroxidase and oxygen is liberated. The liberated oxygen is transferred to chromogen system consisting of 4-aminoantipyrine and phenolic compound to produce red quinoneimine dye. The intensity of colour is directly proportional to the concentration of glucose and is measured photometrically at 505 nm green filter.

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{Phenolic Compound} \rightarrow \text{Coloured Complex} + H_2O_2$$

Procedure: Fresh clear serum under fasting condition with no hemolysis is the specimen of choice. The test is performed as early as possible to prevent glycolysis. Sample serum 10microns is added to 1m of working reagent. It is mixed well and incubated for 5 min at 37^{o}C. The absorbance is then measured with Green filter.

Hematological analysis

At the end of experimental protocol blood was collected in K_3-EDTA coated tubes and haemoglobin content, total red and white blood cell counts (RBC and WBC), hematocrit (HCT) value**,** mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution of width-coefficient of variance (RD-WCV), red cell distribution width standard deviation (RD-WSD),platelets count (PLT), mean platelet volume (MPV), red cell distribution width (RDW) and platelet crit (PCT) of blood samples were measured using BC-2300 Haematology Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd).

Histopathological Examination

At the end of experimental period, all animals were subjected to a detailed gross necropsy viz. examination of external surface of the body, all orifices and, the content in cranial, thoracic and abdominal cavities. Autopsy of brain, heart, lungs, liver, kidney and spleen was done and their wet weights were recorded immediately.

Heart, liver and kidney of experimental mice were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor and 5µm thick sections were cut and stained with hematoxyline and eosin and examined under Leica DMRB microscope. Photographs were taken with Canon power shot S70 digital Camera at 100 X magnification.

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

Acute oral toxicity

There were no noticeable behavioural changes recorded in rats subjected to acute toxicity. There was no mortality recorded and hence, LD_{50} could not be determined. As per an arithmetic calculation, it can be concluded that LD_{50} of the EJSE is > 5000mg/kg bodyweight (Table.1).

Sub-chronic oral toxicity

Plasma metabolites and electrolytes

Effect of EJSE administration on plasma metabolites and electrolytes were assessed. EJSE caused significant decrement in plasma TC, TG and LDL levels without any alteration in plasma HDL and VLDL levels. The plasma Na^{+}, K^{+} and Ca^{+2} levels recorded non significant alterations in the experimental groups (Table 2, Figure, 1 & 2).

Plasma markers of cardiac, hepatic and renal damage

EJSE administration did not alter plasma markers of cardiac damage (LDH and CK-MB). However, plasma urea, creatinine and ALP levels were significantly increased after administration of high dose of EJSE (3000mg/kg BW) while, plasma AST, ALT, and billirubin levels were unaltered (Table.3 and Figure 3 & 4).

Hematological analysis

Haematological parameters such as haemoglobin, WBC, RBC, haematocrite, MCV, MCH, MCHC and platelets were assessed in control and treated animals. Sub chronic administration of EJSE did not alter any of the haematological parameters (Table 4, Figure 5 & 6).

Body weight gain, food and fluid intake and, organ weight

A dose dependent decrement in the body weight and food intake however, non significant alteration recorded in water intake of experimental groups (Table 5 and Figure 7). Organ

weights and their necropsy analysis revealed no major changes in experimental groups administered three doses of EJSE (Table.6, Figure 8).

Histopathological evaluations

There were no gross aberrations in the structural integrity of heart, liver and kidney after administration of EJSE (1000, 2000 or 3000mg/kg) (Figure 9). The cells appeared normal with intact nuclei. There was no evidence of cellular condensation observed in any of the tissues. Also, there were no visual evidences of apoptotic or necrotic changes.

Table 1: Arithmetic calculation of 50 % lethal dose (LD_{50}) after acute administration of EJSE to *Swiss* albino mice.

Experimental groups	Dose (mg/kg)	Dose difference (DD)	No of mice (N)	No of Dead	Death (%)	Mean dead (Md)	DD x Md
Control	00	00	8	0	0	0	0
EJSE1000	1000	1000	8	0	0	0	0
EJSE2000	2000	1000	8	0	0	0	0
EJSE3000	3000	1000	8	0	0	0	0
EJSE4000	4000	1000	8	0	0	0	0
EJSE5000	5000	1000	8	0	0	0	0
Total		5000					0

LD_{50} was calculated from the above table as follows:-

$LD_{50} = LD_{100}$ - DD X MD

Where, LD_{50}= 50% lethal dose, LD_{100} = 100% lethal, N = No. of animals/group, MD = Mean death for a group, DD = Dose deference (between two consecutive doses).

Therefore, LD_{50} of EJSE = (> 5000) - 1000 X 0

> 5000 - 0

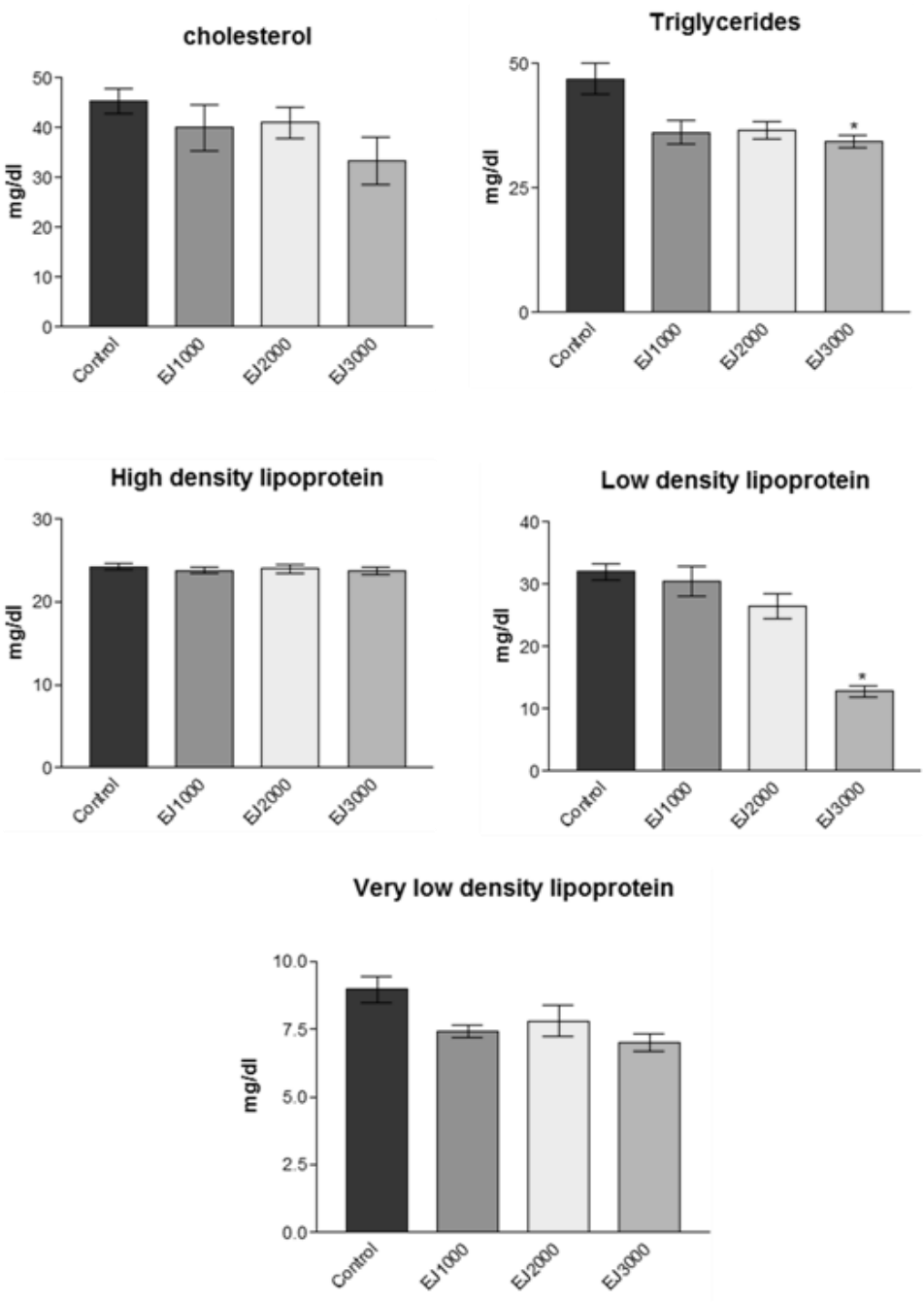
> 5000 mg/kg bodyweight

Tables 2: Effect of chronic (28 days) administration of EJSE on plasma metabolic indices in *Swiss* albino mice.

	CON	EJSE1000	EJSE2000	EJSE3000
Cholesterol (mg/dl)	45.33±2.55	40.00±4.51	41.00±3.05	33.33±4.80
Triglycerides (mg/dl)	46.95±3.05	36.22±2.42	36.59±1.79	34.43±1.29*
HDL (mg/dl)	24.30±0.35	23.83±0.36	24.00±0.56	23.78±0.46
LDL (mg/dl)	32.00±1.33	30.50±2.36	26.56±2.04	12.81±0.93*
VLDL (mg/dl)	8.98±0.48	7.415±0.23	7.812±0.56	7.02±0.32
Glucose (mg/dl)	93.83±7.52	86.28±1.92	76.76±4.69	73.50±3.03
Sodium (mmol/l)	135.6±1.47	135.4±1.63	137.4±1.50	137.2±2.71
Potassium(mmol/l)	4.22±0.06	4.26±0.07	4.440±0.09	4.440±0.11
Calcium (mmol/l)	9.38±0.18	9.73±0.08	9.33±0.12	9.511±0.14

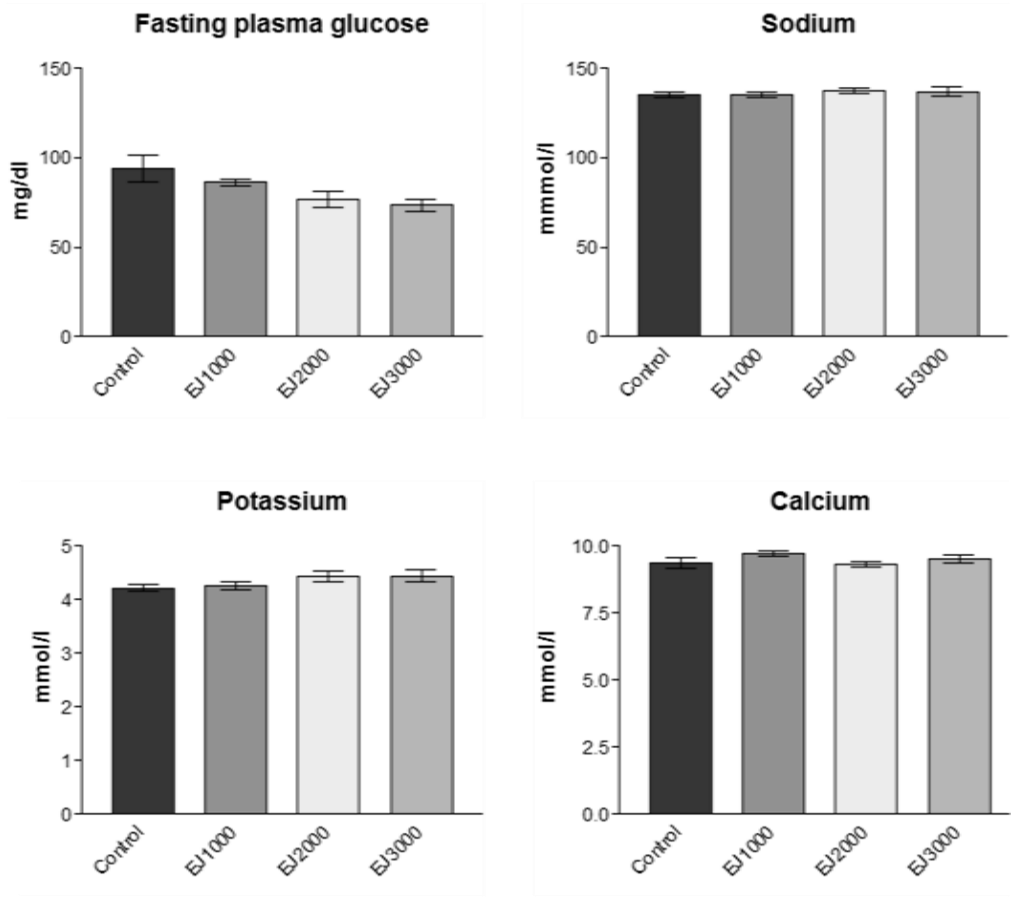
Results are expressed as Mean ± S.E.M for n=8. *$p<0.05$ compared to CON.

Figure 1: Effect of chronic (28 days) administration of EJSE on plasma metabolic indices in *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 2: Effect of chronic (28 days) administration of EJSE on plasma metabolic indices in *Swiss* albino mice.



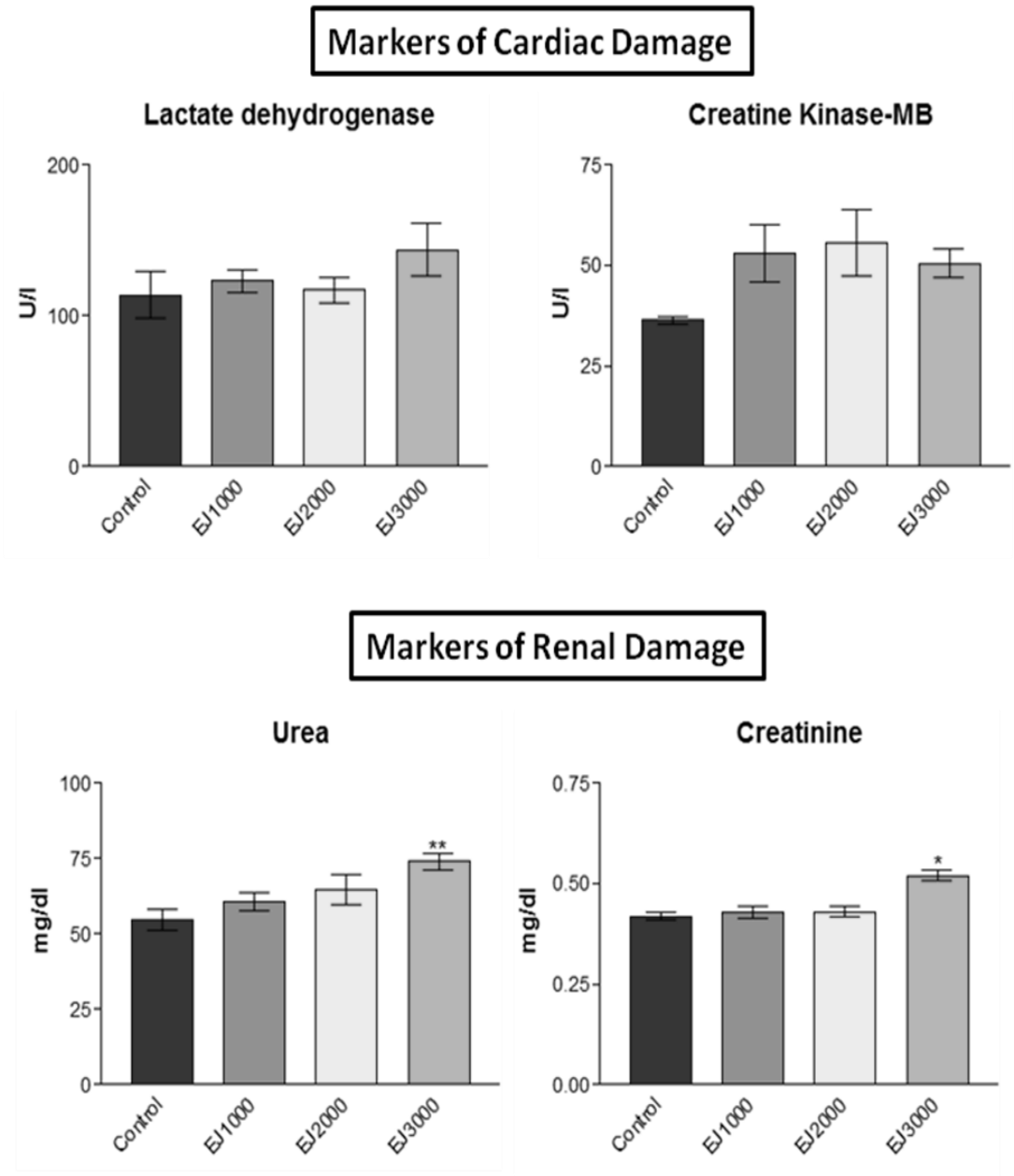
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Tables 3: Effect of chronic (28 days) administration of EJSE on plasma markers of cardiac, hepatic and renal function in *Swiss* albino mice.

	CON	EJSE1000	EJSE2000	EJSE3000
Cardiac injury markers				
LDH (U/l)	113.6±15.41	123.2±7.44	117.0±8.86	143.9±17.45
CK-MB (U/l)	36.40±1.03	53.00±7.21	55.60±8.30	50.60±3.58
Hepatic injury markers				
AST (U/l)	35.87± 1.16	34.00±3.31	40.60±3.47	43.00±1.51
ALT (U/l)	32.50±2.99	24.83±2.40	27.00±1.53	26.67±1.66
ALP (U/l)	10.23±1.09	11.34±1.21	14.53±1.67	15.32±1.99
Billirubin (mg/dl)	0.33±0.021	0.40±0.027	0.47±0.046	0.49±0.011*
Renal injury markers				
Urea (mg/dl)	54.72±3.28	60.66±2.87	64.72±4.97	74.13±2.78**
Creatinine (mg/dl)	0.42±0.011	0.43±0.015	0.43±0.013	0.52±0.013*

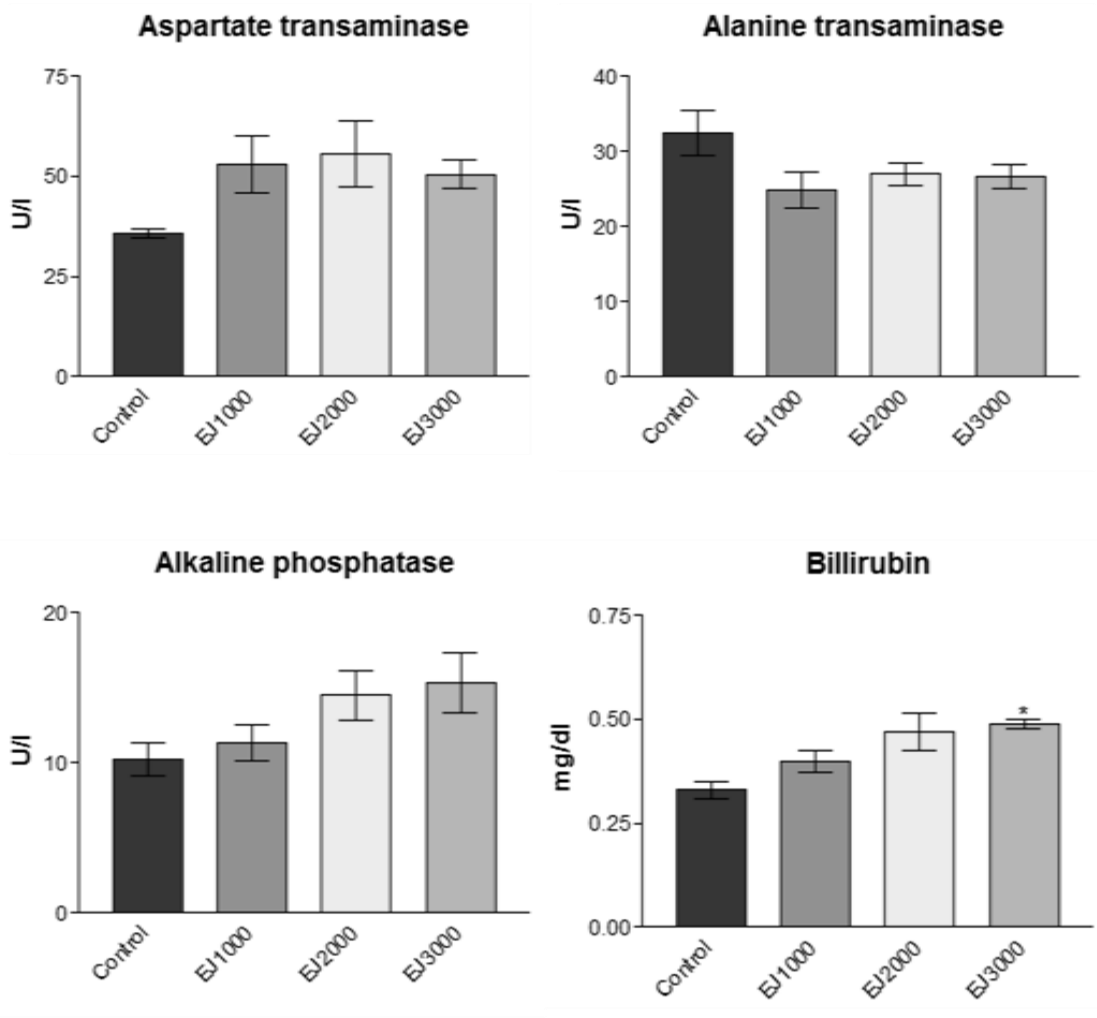
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 and **p<0.05 compared to CON

Figure 3: Effect of chronic (28 days) administration of EJSE on plasma markers of cardiac and renal function in *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 and **p<0.05 compared to CON

Figure 4: Effect of chronic (28 days) administration of EJSE on plasma markers of cardiac, hepatic and renal function in *Swiss* albino mice.



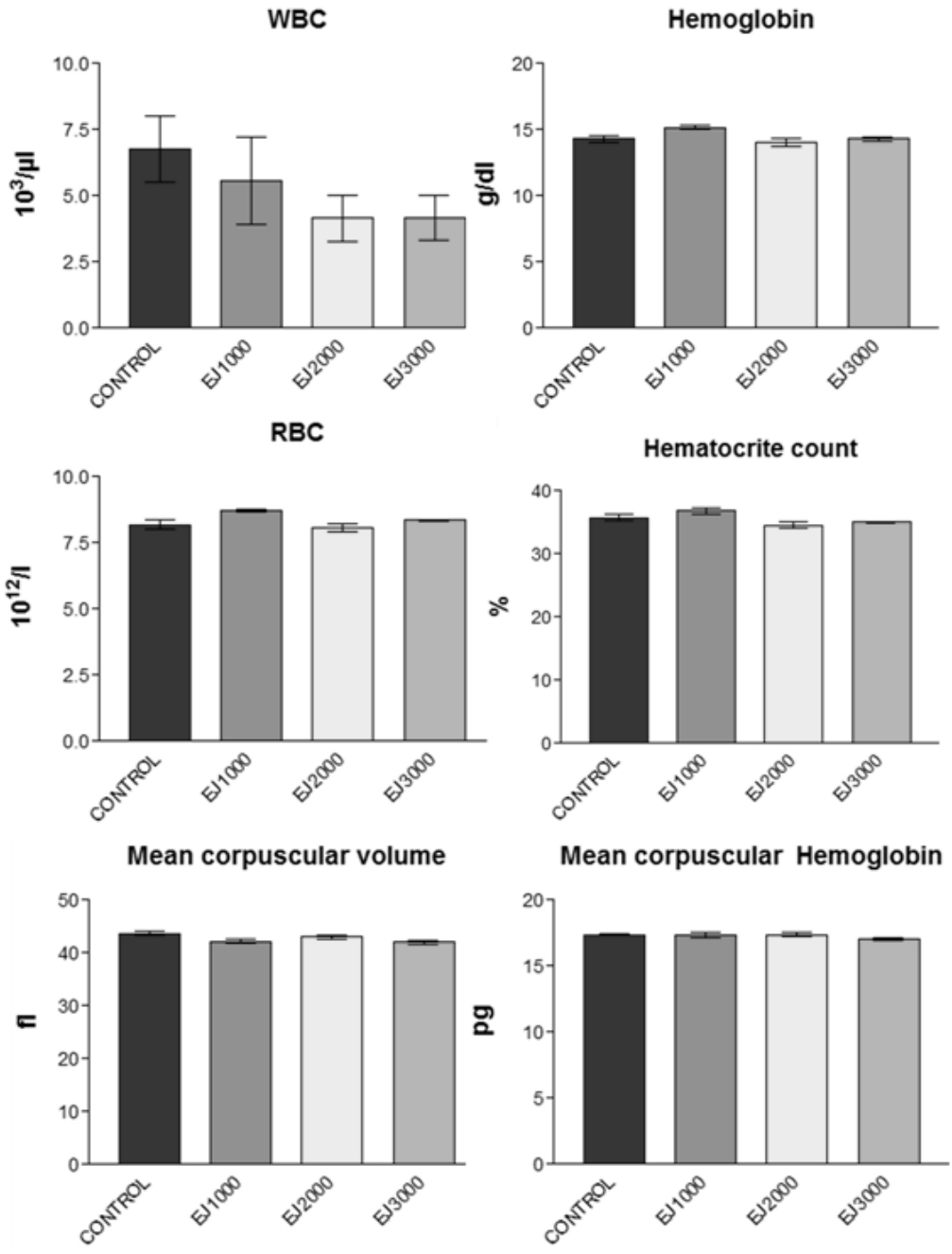
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 and **p<0.05 compared to CON

Tables 4: Effect of chronic (28 days) administration of EJSE haematological parameters of *Swiss* albino mice.

	CON	EJSE1000	EJSE2000	EJSE3000
WBC (10^3/µl)	6.76±1.26	5.57±1.67	4.16±0.88	4.20±0.85
Hemoglobin (g/dl)	14.30±0.25	15.17±0.16	14.07±0.29	14.30±0.11
RBC (10^{12}/l)	8.20±0.17	8.73±0.03	8.06±0.16	8.35±0.04
Hematocrite (%)	35.80±0.49	36.83± 0.46	34.60±0.60	35.03±0.1
MCV (fl)	43.70±0.37	42.20±0.45	43.00±0.49	42.03±0.40
MCH (pg)	17.40±0.05	17.34±0.20	17.40±0.10	17.07±0.12
MCHC (g/dl)	39.90±0.26	41.13±0.20	40.60±0.25	40.77±0.38
RCD-WCV (%)	16.10±1.66	16.60±1.84	16.60±1.72	16.60±1.72
RCD-WSD (fl)	19.87±0.23	19.40±0.09	19.87±0.46	19.17±0.23
Platelets (10^3/µl)	5.88±0.09	5.22±0.26	5.26±0.38	5.44±0.12
MPV (fl)	10.17±0.03	9.70±0.05	9.80±0.15	9.90±0.05
RCDW	14.80±0.05	14.50±0.11	14.63±0.06	14.60±0.05
Plateletcrit (%)	0.59±0.008	0.50±0.02	0.51±0.04	0.53±0.01

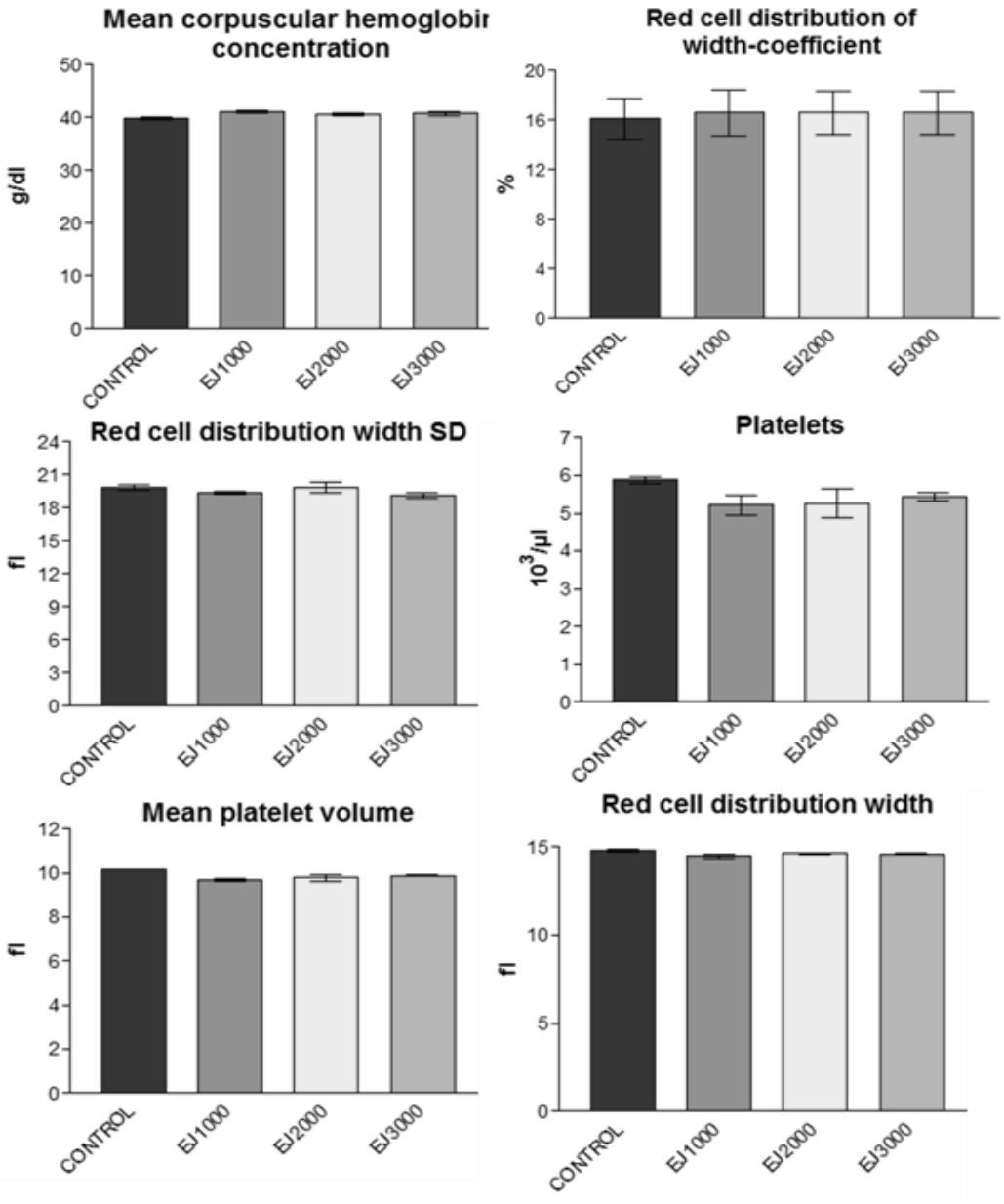
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON

Figure 5: Effect of chronic (28 days) administration of EJSE haematological parameters of *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 6: Effect of chronic (28 days) administration of EJSE haematological parameters of *Swiss* albino mice.



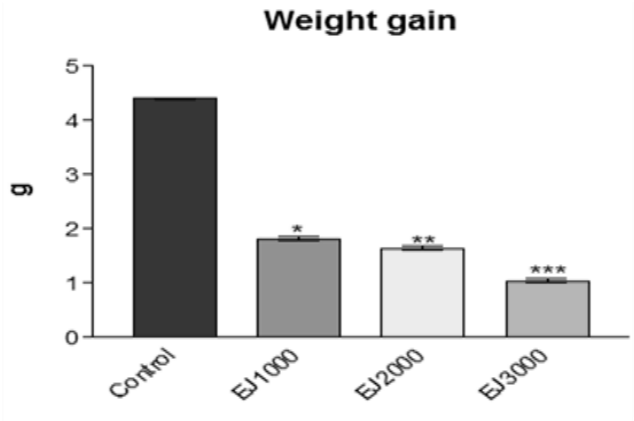
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

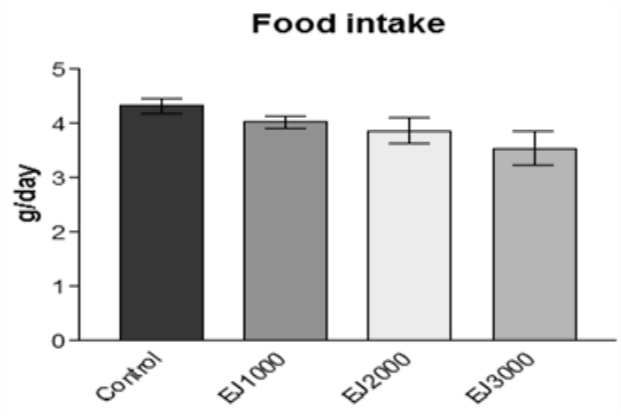
Tables 5: Effect of chronic (28 days) administration of EJSE on bodyweight, food and fluid intake of *Swiss* albino mice.

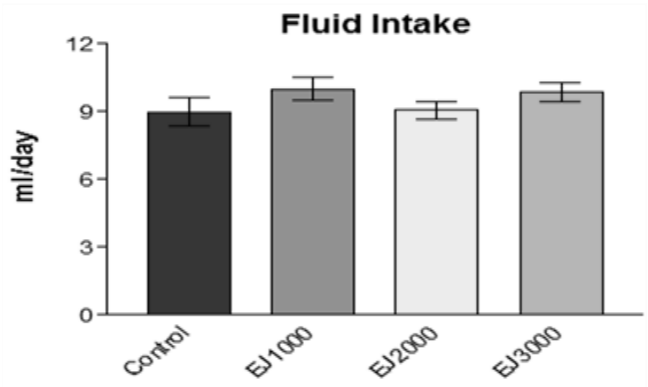
Body weight[#]	CON	EJSE1000	EJSE2000	EJSE3000
Initial (g)	22.45±1.33	23.57±1.12	24.02±0.51	23.50±0.34
Final (g)	26.85±0.59	25.40±0.41	25.67±0.66	24.55±0.72
Weight gain	4.40±0.02	1.82±0.04*	1.65±0.05**	1.05±0.03***
Food intake (g/day)	4.33±0.13	4.03±0.11	3.87±0.24	3.55±0.31
Fluid intake (ml/day)	8.99±0.63	10.01±0.50	9.08±0.39	9.88±0.41

Results are expressed as Mean ± S.E.M for n=8. *p<0.05, **p<0.01 and ***p<0.001 compared to CON.

Figure 7: Effect of chronic (28 days) administration of EJSE on bodyweight, food and fluid intake of *Swiss* albino mice.







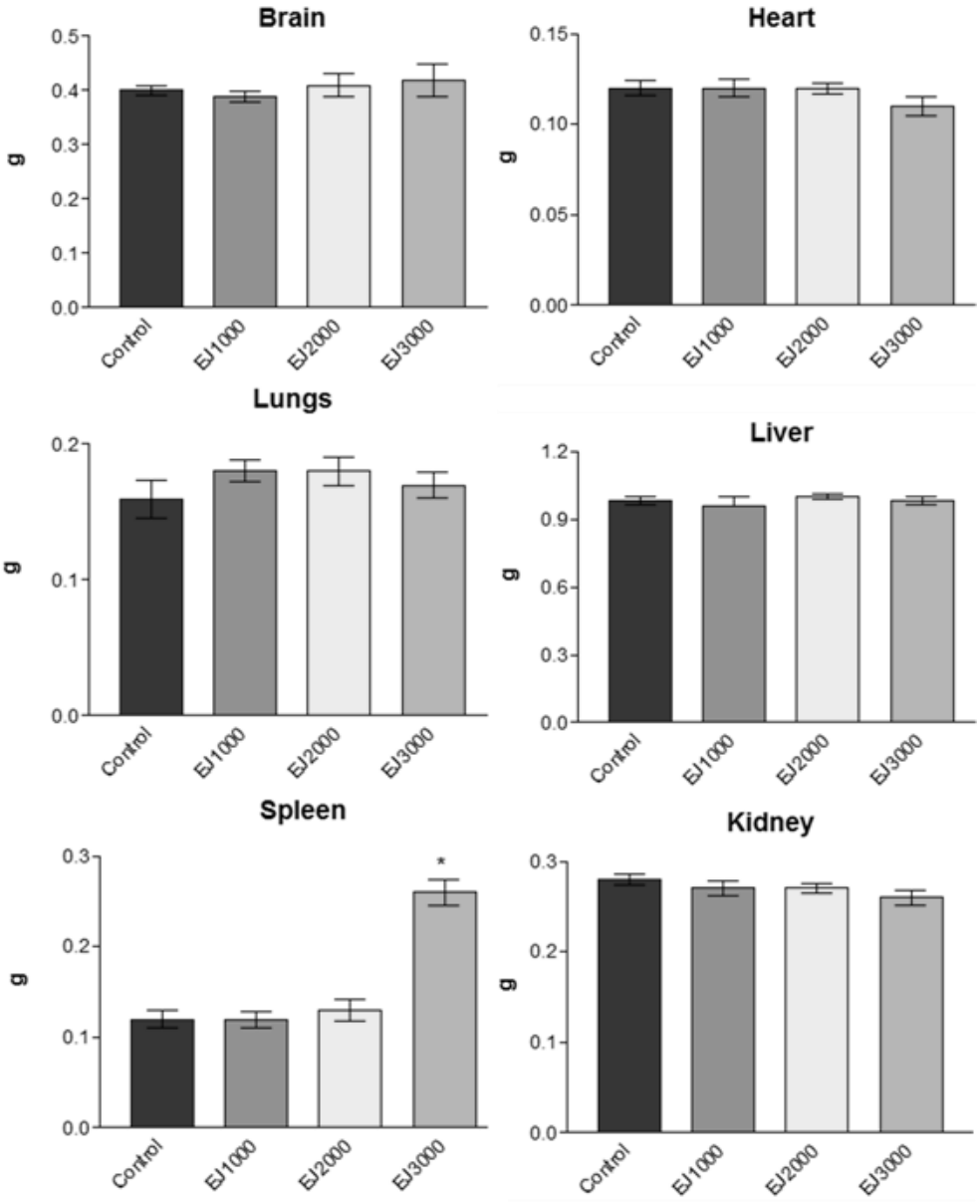
Results are expressed as Mean ± S.E.M for n=8. *p<0.05, **p<0.01 and ***p<0.001 compared to CON.

Tables 6: Effect of chronic (28 days) administration of EJSE organ weights of *Swiss* albino mice.

	CON	EJSE1000	EJSE2000	EJSE3000
Brain (g)	0.40±0.01	0.39±0.01	0.41±0.02	0.42±0.03
Heart (g)	0.12±0.004	0.12±0.005	0.12±0.003	0.11±0.005
Lungs (g)	0.16±0.014	0.18±0.008	0.18±0.01	0.17±0.009
Liver (g)	0.98±0.02	0.96±0.04	1.00±0.02	0.98±0.02
Spleen (g)	0.12±0.010	0.12±0.009	0.13±0.012	0.26±0.014
Adrenal (g)	0.03±0.001	0.03±0.001	0.04±0.002	0.037±0.002
Kidney (g)	0.28±0.006	0.27±0.008	0.27±0.005	0.26±0.008

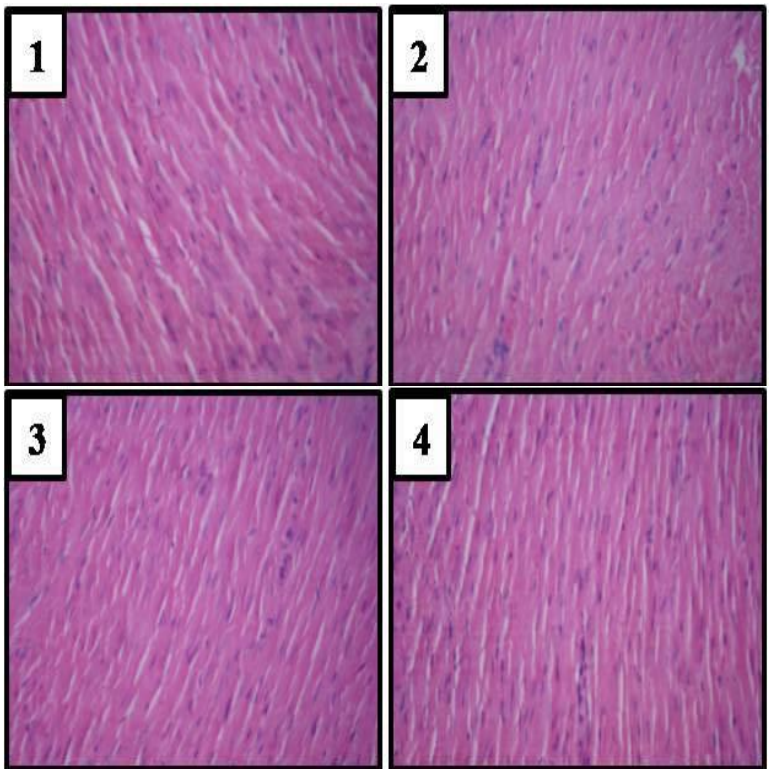
Results are expressed as Mean ± S.E.M for n=8. *$p<0.05$ compared to CON

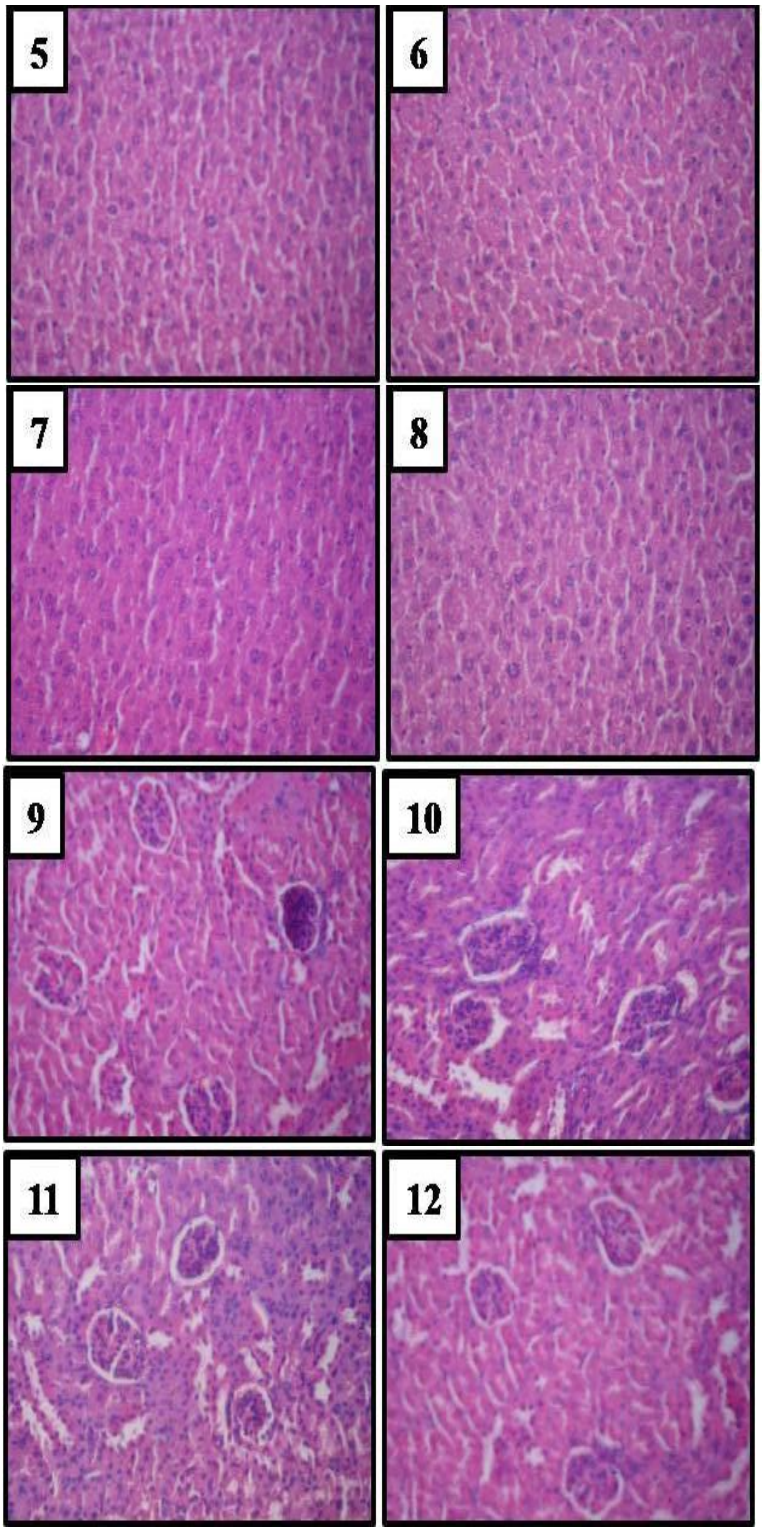
Figure 8: Effect of chronic (28 days) administration of EJSE organ weights of *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON

Figure 9: Photomicrographs of Heart of control (1) and 1000, 2000 and 3000mg/kg EJSE treated mice (2, 3, 4), Liver of control (5) and 1000, 2000 and 3000mg/kg EJSE treated mice (6, 7, 8) and kidney of control (9) and 1000, 2000 and 3000mg/kg EJSE treated mice (10, 11, 12) stained with hematoxyline and eosin (100X).

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DISCUSSION

No mortality was recorded following a single acute dose of EJSE (5000 mg/kg bw), LD_{50} was arithmetically calculated to be > 5000 mg/kg bw (Dede and Dogara, 2004; Saidu et al., 2007). There were no observable symptoms of any behavioural alterations post 72 hours, indicating that EJSE has no major side effects even at high dose (5000 mg/kg bw).

Alterations in plasma lipid content and fluctuations in electrolytes following administration of an herbal extract are imperative to be assessed. It is also mandatory to monitor the same following low as well as higher doses of EJSE. Observed decrement in plasma TC, TG, LDL and VLDL and unchanged HDL levels are in accordance with one observed hypolipidemic potential of EJSE (Chapter 3). However, non significant alterations were recorded following administration of low dose of EJSE (1000 or 2000 mg/kg BW) suggesting that, hypolipidemic effect of EJSE in normolipidemic mice manifests only at a higher dose. No significant alterations were observed in plasma glucose and electrolyte levels suggesting that EJSE does not manifest any major changes in the glycaemic status or electrolyte balance even at higher doses (1000, 2000 or 3000 mg/kg BW).

Plasma levels of Ck-MB and LDH have been used as a marker of cardiac damage under various toxic manifestations (Jadeja et al., 2010; Thounaojam et al., 2010). Our study recorded non significant alterations in the level of plasma CK-MB and LDH after sub chronic administration of EJSE indicating no damage to cardiomyocytes. These observations get further substantiated with the microscopic evaluation of cardiac tissue wherein, no observable alterations in their cellular integrity could be noted after administration of different doses of EJSE.

Some herbal extracts can be heptotoxic and their oral administration leads to elevated levels of plasma AST and ALT (Mukinda et al., 2010; Udem et al., 2010). Oral administration of EJSE has been reported to reduce streptozotocin and carbon tetrachloride induced elevation in plasma AST and ALT at a dose range of 100 to 500 mg/kg bw (Jasmine and Daisy, 2007; Sisodia and Bhatnagar, 2009; Sundaram et al., 2009). In our study the plasma AST and ALT levels in EJSE administered mice were comparable with those of control animals and the same was further corroborated with comparable histoarchitectural details of liver in control and EJSE administered groups. It is inferable from these observations that, EJSE is non toxic to hepatic tissue in the dosage employed herein. EJSE can therefore be considered as safe and non toxic to liver thus, adding further validity to its already reported hepatoprotective potential.

Studies have shown that, use of traditional herbal medicines in treating renal diseases is limited by their adverse effects on renal functions (Saxena and Panhotra, 2003; Singh and Prakash, 2008). No significant alterations in plasma markers of renal damage (urea and creatinine) could be after sub chronic administration of EJSE in low doses (1000 and 2000 mg/ kg bw). However, significant elevation in plasma levels of these markers could be were observed after administration of high dose of EJSE (3000 mg/kg bw). However, no histoarchitecture destruction was observable in the renal tissue of mice administered lower as well as higher doses of EJSE. It is inferable from these observations that, EJSE may manifest moderate levels of renal damage at very high dose (3000 mg/kg bw) but, is never the less safe at therapeutic dosage (100 - 500 mg/kg bw).

A haemogram reflects upon the haemolytic property of any herbal extract in question (Mukinda et al., 2007, 2010) and the same was assessed in our study. No significant alterations were seen in the haematological profile of any of the experimental groups studied herein. Also, food and water intake and absolute weight of vital organs were comparable in control and EJSE treated groups. However, a dose dependent decrement in body weight gain was recorded at the end of 28 days. Significant decrement in plasma lipid profile and body weight gain observed after EJSE administration is attributable to reduced fat absorption through intestine and effective elimination of lipids through faeces as observed in EJSE treated atherogenic rats (Chapter 3).

It can be concluded from the study that, EJSE is non toxic to cardiac and hepatic tissue and moderately nephrotoxic at high dose (3000 mg/kg BW). Hence, the Lowest Observable Adverse Effect Level (LOAEL) for EJSE is 3000 mg/kg BW while, the No Observable Adverse Effect Level (NOAEL) is up to 2000 mg/kg BW (Rhiouani et al., 2008). According to World Health Organization toxicity guidelines for herbal extracts, it can be concluded that the calculated Acceptable Daily Intake (ADI) is equal to NOAEL (2000mg/kg BW) ÷ 100; wherein, 100 is safety factor (Rosidaha et al., 2009). Thus, ADI for EJSE for mice is 20 mg/kg BW. An extrapolation of these results to an average adult human (70kg) would be 1.4 g of EJSE or 28g of dried EJ powder (based on final percentage yield (5% w/w) after extraction).

Summary

The aim of the present study was to investigate safety evaluation of ethanolic seed extract of *Eugenia jambolana* (EJSE) using acute and sub-chronic toxicity assays in Swiss albino mice as per OECD guidelines. Mice administered a single dose (1000, 2000, 3000, 4000 or 5000mg/kg BW) of EJSE and changes in patterns of behavior and mortality were observed. Also, plasma levels of metabolites, hepatic, cardiac and renal function markers, electrolytes, blood count and histopathology of major organs were monitored in mice chronically treated with EJSE (1000, 2000 or 3000 mg/kg BW) for 28 days. Since no mortality was recorded in the acute toxicity evaluation up to a dose of 5000mg/kg bodyweight of EJSE, LD50 was assumed to be >5000mg/kg BW. In the sub-chronic toxicity evaluation, no adverse observations were recorded in mice administered with 2000mg/kg EJSE; however at 3000 mg/kg dose, moderate increase in the plasma levels of urea and creatinine was observed. Hence, LOAEL for EJSE was found to be 3000 mg/kg BW and NOAEL was adjudged as 2000 mg/kg BW.

Chapter 2

Acute and sub-chronic toxicity evaluation of *Brassica oleracea* leaf extract

INTRODUCTION

In recent times the field of "green medicine" has witnessed a renaissance of interest as drugs of herbal origin are relatively inexpensive, accessible and considered to be safe compared to synthetic counter parts (Parek and Chanda, 2006; Venkatesh and Krishnakumari, 2006). Drugs of herbal origin therefore, remain the main source of active drugs from a natural source and are still indispensable in the traditional field of alternative medicine for treating a number of diseases. According to the World Health Organization (WHO), about 80% of the world's population depends on alternative medicines (WHO, 1996). Various herbal drugs/ food supplements, dietary supplements such as garlic (Nakagawa et al., 1984), turmeric (Deshpande et al., 1998) and coriander leaf (Reyes et al., 2010) have been reported to be non toxic at lower doses though associated with minor side effects at higher doses. Although, herbal medicines/dietary supplements are not covered under US-FDA drug regulatory criteria due to the consideration of their being safe, their safety profile may not have been adequately documented. Hence, preclinical acute and sub chronic toxicological evaluations using Organisation for Economic Co-operation and Development (OECD) guidelines need to be undertaken to establish safety profiles of drugs of herbal origin (Jadeja et al., 2010).

Chapter 2

Red cabbage is a popularly consumed food stuff that has been ubiquitously reported to exert medicinal properties. It is mandatory to understand the highest permissible consumption limit of any food supplement to avoid toxicity. The present study therefore aims at investigating the acute and sub-chronic oral toxicity of ARCE extract applying the recommended OECD guidelines for safety or dose dependent toxicity in mice.

MATERIALS AND METHODS

Preparation of extract

Red cabbage was purchased from the local market; leaves were separated and slice into small pieces and dried in the hot air oven at 50°C and powdered using a mechanical grinder.Hundred grams of dried powder was extracted in 1000 ml of 70% aqueous ethanol using ultrasonic treatment at an intensity of 70 W/cm^2 and oscillation frequency at 20 kHz for 5 min (Enertech electronics pvt. Ltd. Mumbai, India). After overnight maceration, the extract was filtered through filter paper and ethanol was evaporated under reduced pressure at 50°C by using a rotary evaporator. The remaining water extract was dried using a freeze dry system under reduced pressure (Kataya and Hamza, 2008). The resultant yield was 12%w/w. The dried extract was dissolved in 0.5% carboxy methyl cellulose (CMC) to obtain different doses of anthocyanin rich red cabbage extract (ARCE).

Experimental animals

Swiss albino mice of either sex (Obtained from Zydus research centre, Ahmedabad, India) were housed and 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 carried out 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 Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Acute oral toxicity in mice

The acute oral toxicity study was conducted using the limit test procedure as per the OECD test guidelines on acute oral toxicity test 401 (OECD, 2001). Thirty two *Swiss* albino mice of either sex were divided into four groups (n=8) and were orally administered with a single dose of 1000, 2000, 3000, 4000 or 5000 mg/kg body weight of ARCE extract. Animals were observed for possible behavioral changes such as tremors, convulsions, sleep, altered feeding, salivation, altered somatomotor activities and diarrhoea till 72 hr post treatment.

Sub chronic oral toxicity in mice

The sub acute oral toxicity study was conducted according to OECD Test 407 (OECD, 1995). Thirty two *Swiss* albino mice of either sex were divided into four groups (n=8) and maintained for 28 days for this experiment. Group I was orally fed with carboxy methyl cellulose (CMC; 0.5 %) that served as control whereas, Groups 2, 3 and 4 were orally administered with 1000, 2000 and 3000 mg/kg of ARCE extract respectively. Food and water intake in all the experimental groups were monitored daily at 09:00 hrs. After 28 days of treatment, blood was collected from overnight fasted mice via retro-orbital

sinus under mild anaesthesia and plasma was separated for further biochemical analysis. Thereafter, the animals were sacrificed by cervical dislocation under mild ether anaesthesia and brain, heart, lungs, liver, spleen, kidney and adrenal were excised and weighed.

Cage side observations

As mentioned in chapter 1.

Body Weight and, Food and Water Intake

As mentioned in chapter 1.

Plasma biochemical analysis

As mentioned in chapter 1.

Haematological analysis

As mentioned in chapter 1.

Pathological Examination

As mentioned in chapter 1.

Extrapolation of no-observable adverse effect level from mice data

For extrapolation of this data to human, the NOAEL for human was calculated base on the equivalent body surface area of both the species (Li et al., 2010) as follows:-

Human NOAEL = mouse NOAEL X human surface area

Mouse surface area

Where, human surface area = 1.71 m^2 for an average 65 kg human and

Mouse surface area = 0.007m^2 for an average 20g mice.

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

Acute toxicity studies

No mortality was recorded in acute toxicity study with ARCE extract up to a dose of 5000mg/kg body weight till 72 h. No abnormalities in behaviour could be monitored on extract administration suggesting, no adverse effect on the motor activity of experimental animals. Further validation of sub chronic toxicity studies by oral administration of 1000, 2000 and 3000mg/kg of ARCE extract for 28 days showed neither mortality nor any behavioural abnormality in gross motor activities in any of the doses employed.

Sub-chronic oral toxicity

Plasma metabolites and electrolytes

Biochemical evaluation of plasma glucose and lipid profile showed ARCE induced hypoglycaemic but no alteration in lipid profile (Table 1 and Figure 1). The evaluation suggests no effect whatsoever any of the doses of ARCE extract on Na^{+}, K^{+} and Ca^{++} (Table 2 and Figure 2).

Plasma markers of cardiac, hepatic and renal damage

Except for a significant elevation in bilirubin no significant alteration could be recorded for AST, ALT or ALP, suggesting no inflammatory or necrotic damage of liver due to ARCE extracts (Table 2 and Figure 3 & 4). Results show no significant alteration in the creatinine level due to ARCE treatment but a significant increase in urea level in mice supplemented with 3000mg/kg of ARCE extract (Table 2 and Figure 3 & 4).

Hematological analysis

All hematological parameters of ARCE extract supplemented mice were within the reference range and were comparable to that of control mice. However, there was a moderate decrease in RBC count of mice that were administered with 3000mg/kg of ARCE extract (Table 4 and Figure 5 & 6).

Body weight gain, food and fluid intake and, organ weight

There was dose dependent (1000 and 2000 mg/kg) decrease in mean body weight gain in ARCE treated mice compare to control group, with negligible weight gain in 3000mg/kg

dose (Table 5 and Figure 7). Food intake was decreased in dose dependent manner, while fluid intake registered non significant changes (Table 5 and Figure 7). Further, non significant differences in mean organ weight were recorded between the control and treated animals at the end of 28 days (Table 6 and Figure 8).

Pathological evaluations

Histological observation of sections of liver of ARCE treated mice attests to the fact that, there is no hepatic damage, as marked by the normal looking integrity of hepatocytes (Figure 9). Histological observation however showed no major toxic manifestation in the cortical or medullary areas of kidney. It is assumed herein that elevated levels of urea can be due to a possible initiation of renal damage that is not fully structurally manifested. The histoarchitecture of glomeruli and tubules in control and ARCE extract treated mice being comparable, the non-toxic nature of ARCE extract stands validated (Figure 9).

LD_{50} of ARCE extract

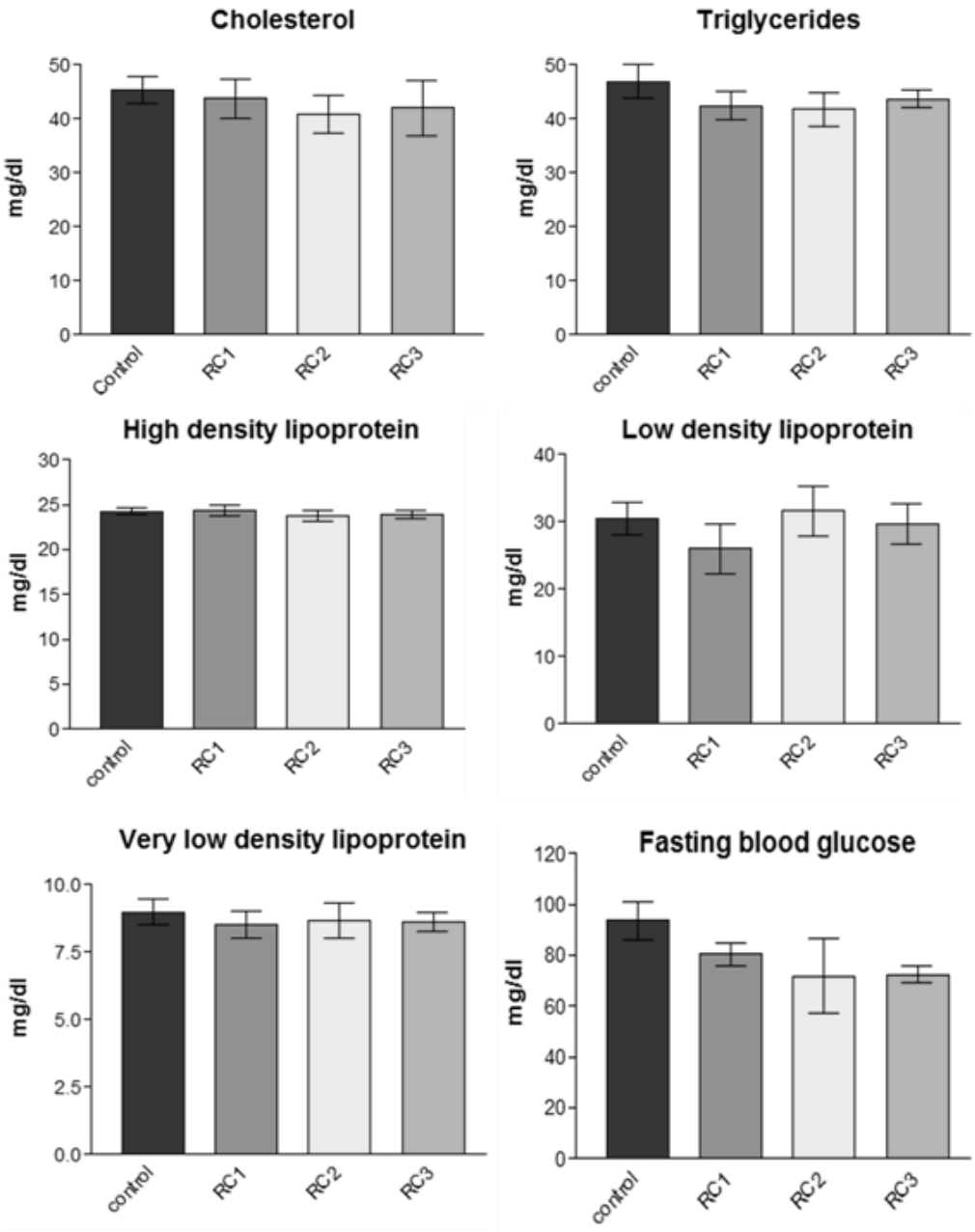
Since no mortality was recorded even after administration of 5000mg/kg of ARCE extract the LD_{50} of ARCE extract could not be established. It is surmisable from the present observations that, the LD_{50} of ARCE extract is likely to be higher than 5000mg/kg BW. Since the doses tested herein fall within the dose range recommended by OECD. ARCE extract qualifies itself to be nontoxic.

Tables 1: Effect of chronic administration of ARCE extract on plasma metabolic indices in *Swiss* albino mice.

	CON	ARCE1	ARCE2	ARCE3
Cholesterol (mg/dl)	45.33±2.55	43.83±3.65	40.83±3.44	42.00±5.09
Triglycerides (mg/dl)	46.95±3.05	42.46±2.59	41.80±3.13	43.67±1.60
HDL (mg/dl)	24.30±0.34	24.35±0.60	23.77±0.53	23.92±0.50
LDL (mg/dl)	30.50±2.37	26.04±3.74	31.66±3.71	29.67±3.04
VLDL (mg/dl)	8.99±0.48	8.51±0.51	8.67±0.67	8.64±0.35
Glucose (mg/dl)	93.83±7.51	80.49±4.66	71.74±5.20	72.56±3.48

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 1: Effect of chronic administration of ARCE extract on plasma metabolic indices in *Swiss* albino mice.

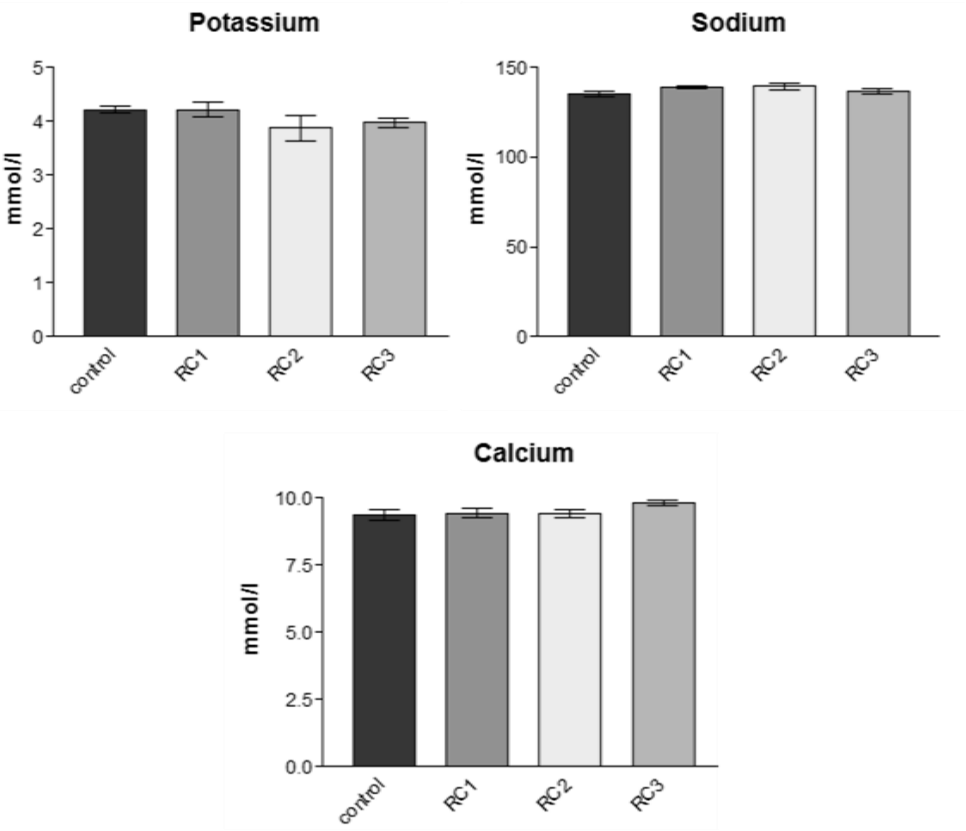


Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Tables 2: Effect of chronic administration of ARCE on plasma sodium potassium and calcium levels in Swiss albino mice.

	CON	ARCE 1	ARCE 2	ARCE 3
Sodium (mmol/l)	135.6±1.47	139.0±0.83	139.6±1.86	137.2±1.46
Potassium(mmol/l)	4.22±0.06	4.22±0.14	3.88±0.24	3.98±0.09
Calcium (mmol/l)	9.38±0.18	9.44±0.19	9.423±0.16	9.81±0.10

Figure 2: Effect of chronic administration of ARCE on plasma sodium potassium and calcium levels in Swiss albino mice.



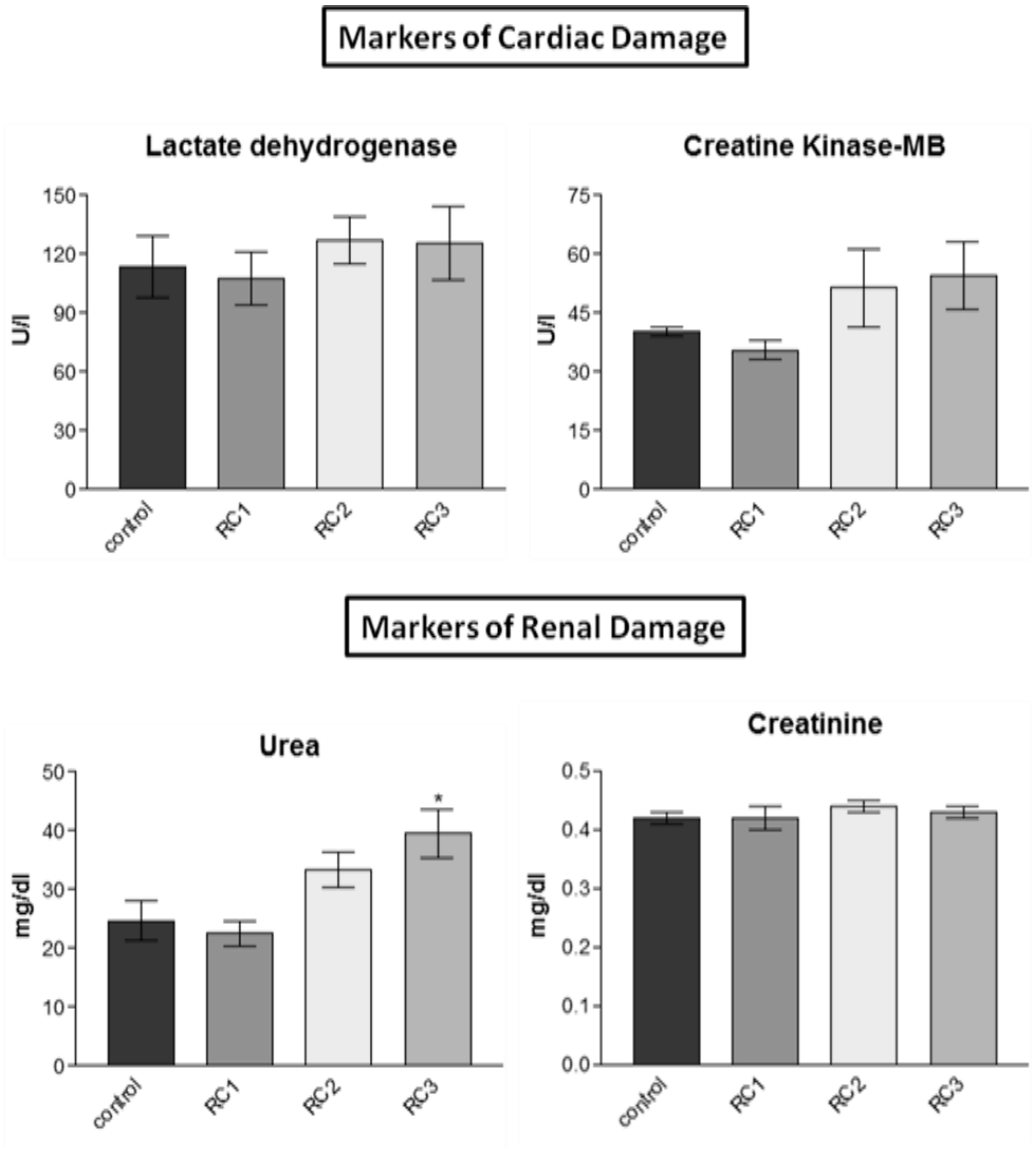
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON

Tables 3: Effect of chronic administration of ARCE on plasma indices of cardiac, hepatic and renal function in *Swiss* albino mice.

	CON	ARCE **1**	ARCE **2**	**ARCE 3**
Markers of Cardiac function				
LDH (U/l)	113.6±15.41	107.8±13.43	127.2±11.83	125.8±18.70
CK-MB (U/l)	40.40±1.03	35.60±2.34	51.40±10.00	54.60± 8.58
Markers of Hepatic function				
AST (U/l)	29.40± 1.16	35.20±2.41	39.80±1.24	33.20±3.51
ALT(U/l)	32.50±2.98	27.17±2.51	32.67± 3.66	29.67±4.16
ALP (U/l)	10.09±1.01	12.43±0.98	11.98±1.00	12.12±1.01
Billirubin (g/l)	0.39±0.02	0.30±0.04	0.39±0.04	0.49±0.04*
Markers of Renal function				
Urea (mg/dl)	24.72±3.28	22.52±2.21	33.40±2.95	39.55±4.07*
Creatinine (mg/dl)	0.42±0.01	0.42±0.02	0.44±0.01	0.43±0.01

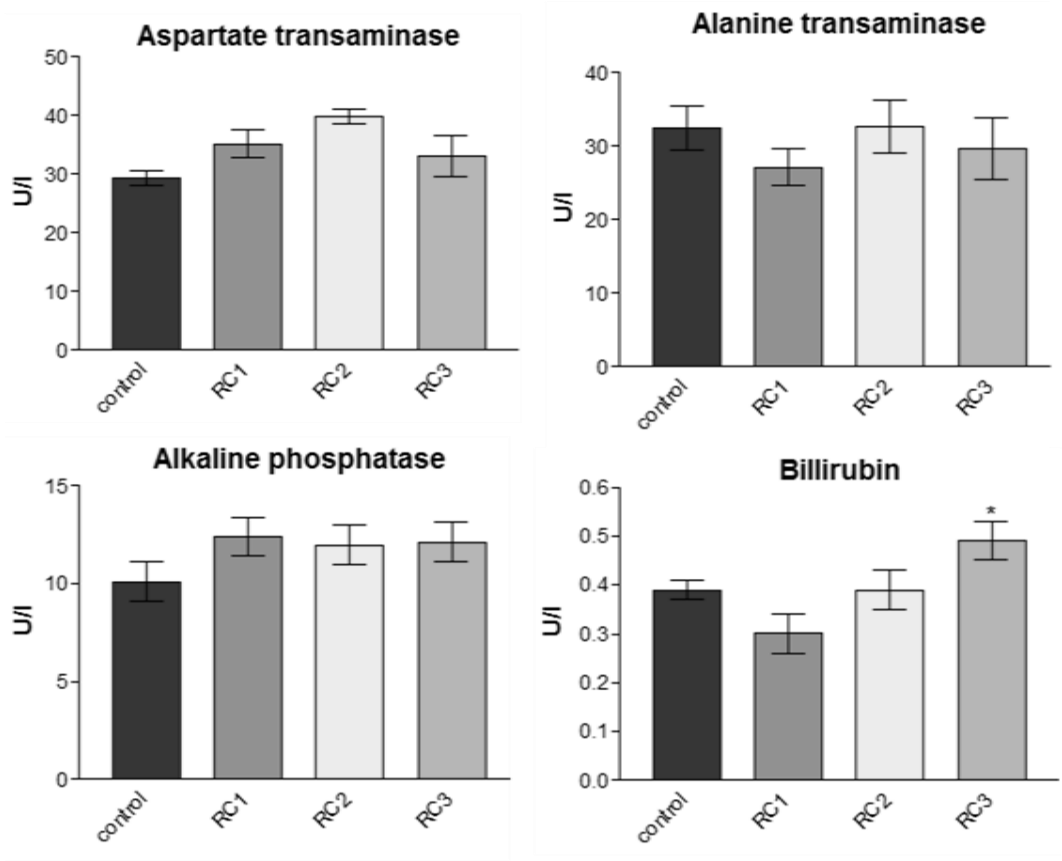
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 3: Effect of chronic administration of ARCE on plasma indices of cardiac and renal function in *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 4: Effect of chronic administration of ARCE on plasma indices of hepatic function in *Swiss* albino mice.



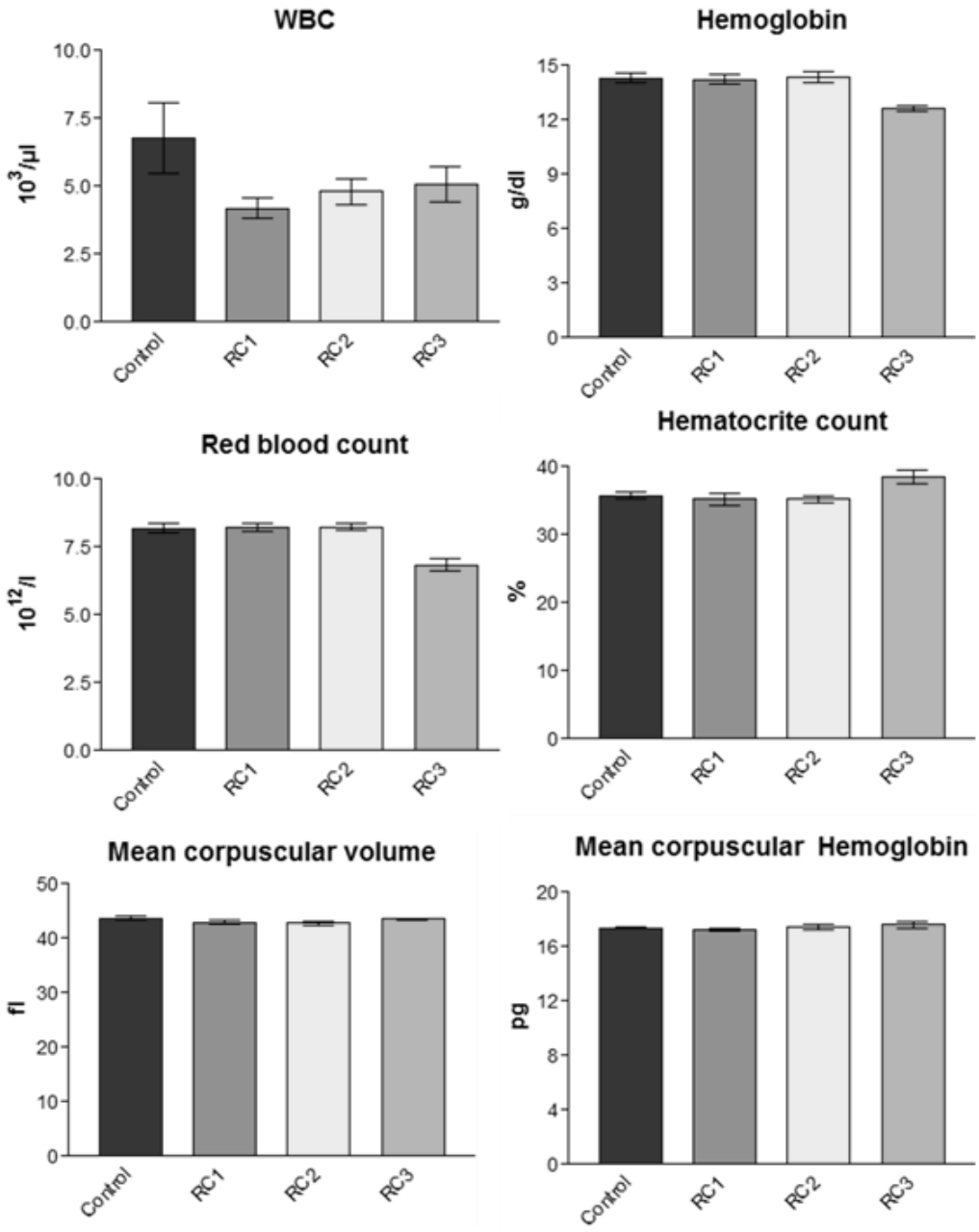
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Tables 4: Effect of chronic administration of ARCE leaf extract on hematological parameters of *Swiss* albino mice.

	CON	ARCE **1**	ARCE **2**	ARCE **3**
WBC(10^3/µl)	6.77±1.28	4.20±0.38	4.80±0.46	5.07±0.64
Hemoglobin (g/dl)	14.30±0.25	14.23±0.26	14.40±0.30	12.63±0.13
RBC(10^{12}/l)	8.20±0.17	8.21±0.15	8.24±0.129	6.85±0.21
Hematocrite (%)	35.80±0.49	35.20±0.90	35.20± 0.45	38.43±1.03
MCV(fl)	43.70±0.38	42.90±0.40	42.77±0.38	43.50±0.17
MCH(pg)	17.40±0.06	17.30±0.10	17.43±0.22	17.60±0.30
	39.90±0.26	40.40±0.29	40.87±0.32	40.67±0.87
RCD-WCV (%)	16.10±1.60	17.20±1.66	17.80±1.72	17.20±1.61
RCD-WSD (fl)	19.87±0.24	20.11±0.40	20.17±0.87	19.87±0.23
Platelets (10^3/µl)	5.89±0.10	6.03±0.73	5.63±0.36	5.50±0.32
MPV (fl)	10.17±0.03	9.73±0.23	9.90±0. 15	10.13±0.07
RCDW	14.80±0.06	14.43±0.20	14.50±0.21	14.70±0.06
Plateletcrit (%)	0.59±0.03	0.51±0.02	0.55±0.04	0.55±0.03

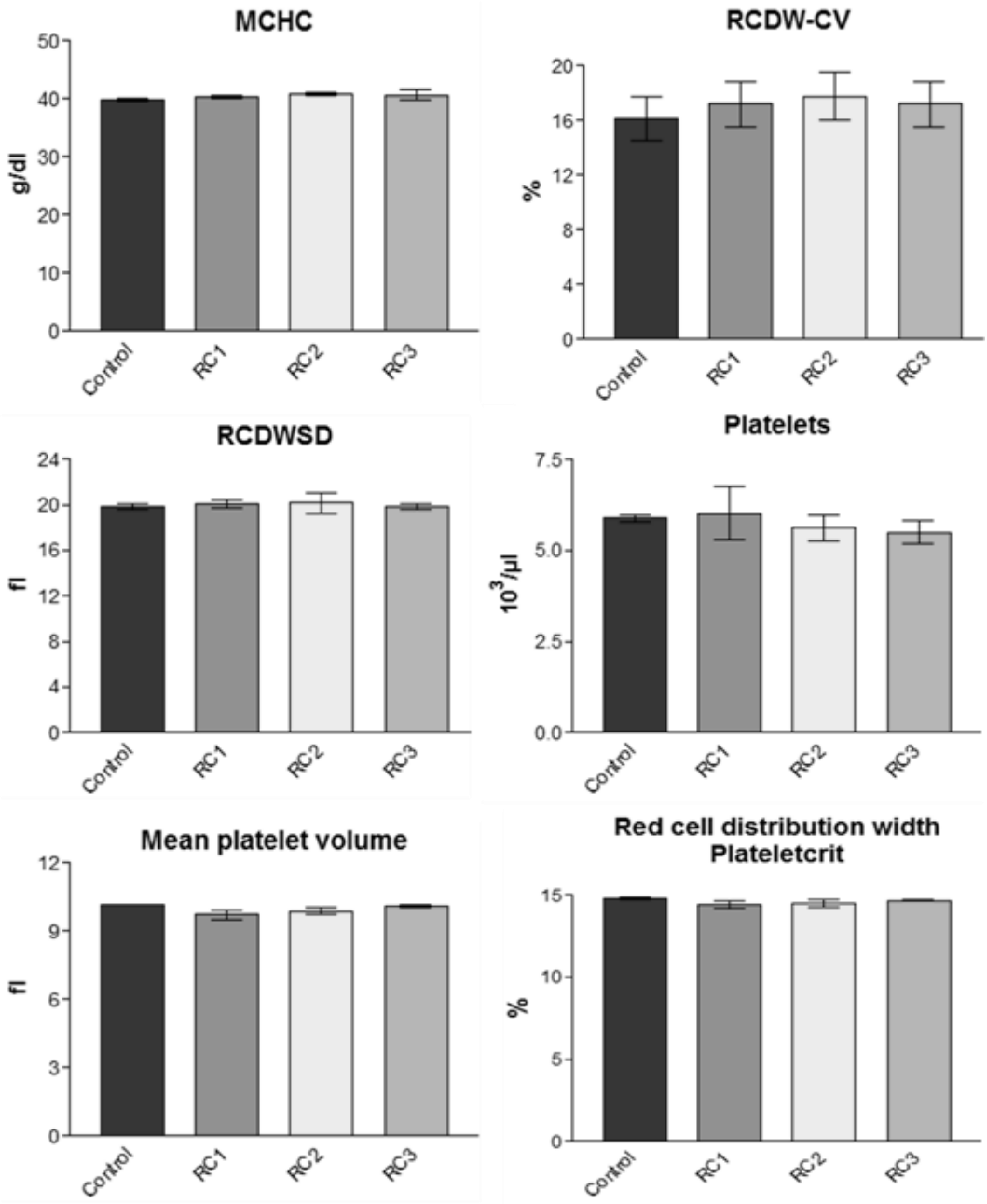
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 5: Effect of chronic administration of ARCE leaf extract on hematological parameters of *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 6: Effect of chronic administration of ARCE leaf extract on hematological parameters of *Swiss* albino mice.



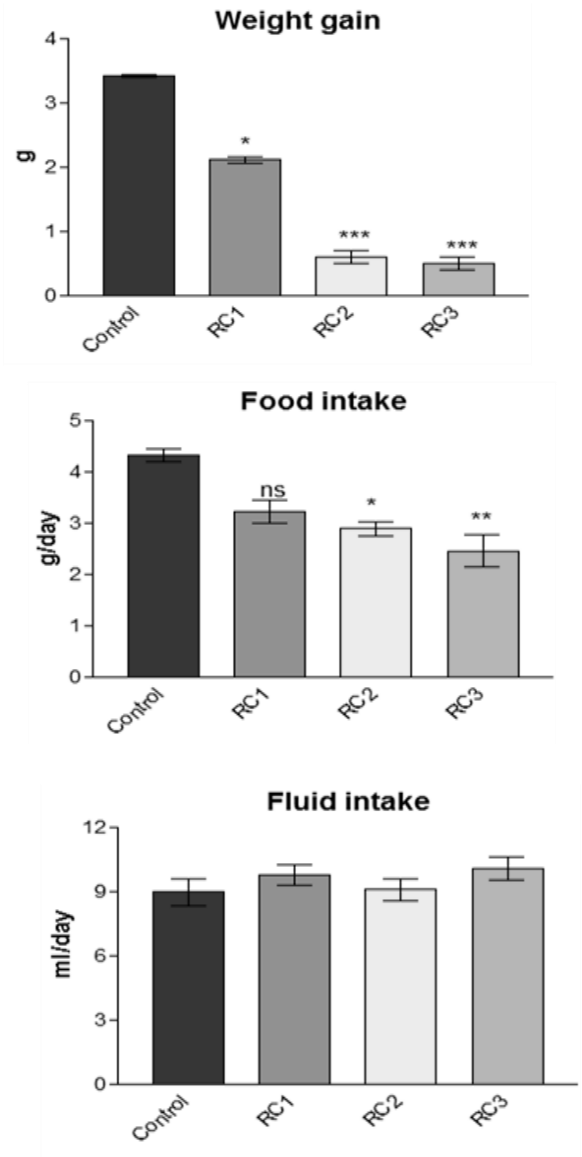
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Tables 5: Effect of chronic administration of ARCE extract on bodyweight, food and fluid intake of *Swiss* albino mice.

	CON	ARCE **1**	ARCE **2**	ARCE **3**
Body weight				
Initial (g)	22.45±1.33	25.83±0.60	24.62±0.69	25.65±0.66
Final (g)	25.85±0.60	27.95±0.45	25.22±0.79	25.70±0.62
Weight gain (g)	3.42±0.02	2.12±0.05*	0.60±0.09***	0.50±0.101***
Food intake (g/day)	4.33±0.13	3.22 ± 0.22^{ns}	2.89±0.14*	2.46±0.31**
Fluid intake (ml/day)	8.99±0.63	9.80±0.47	9.11±0.50	10.08±0.51

Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 7: Effect of chronic administration of ARCE extract on bodyweight, food and fluid intake of *Swiss* albino mice.



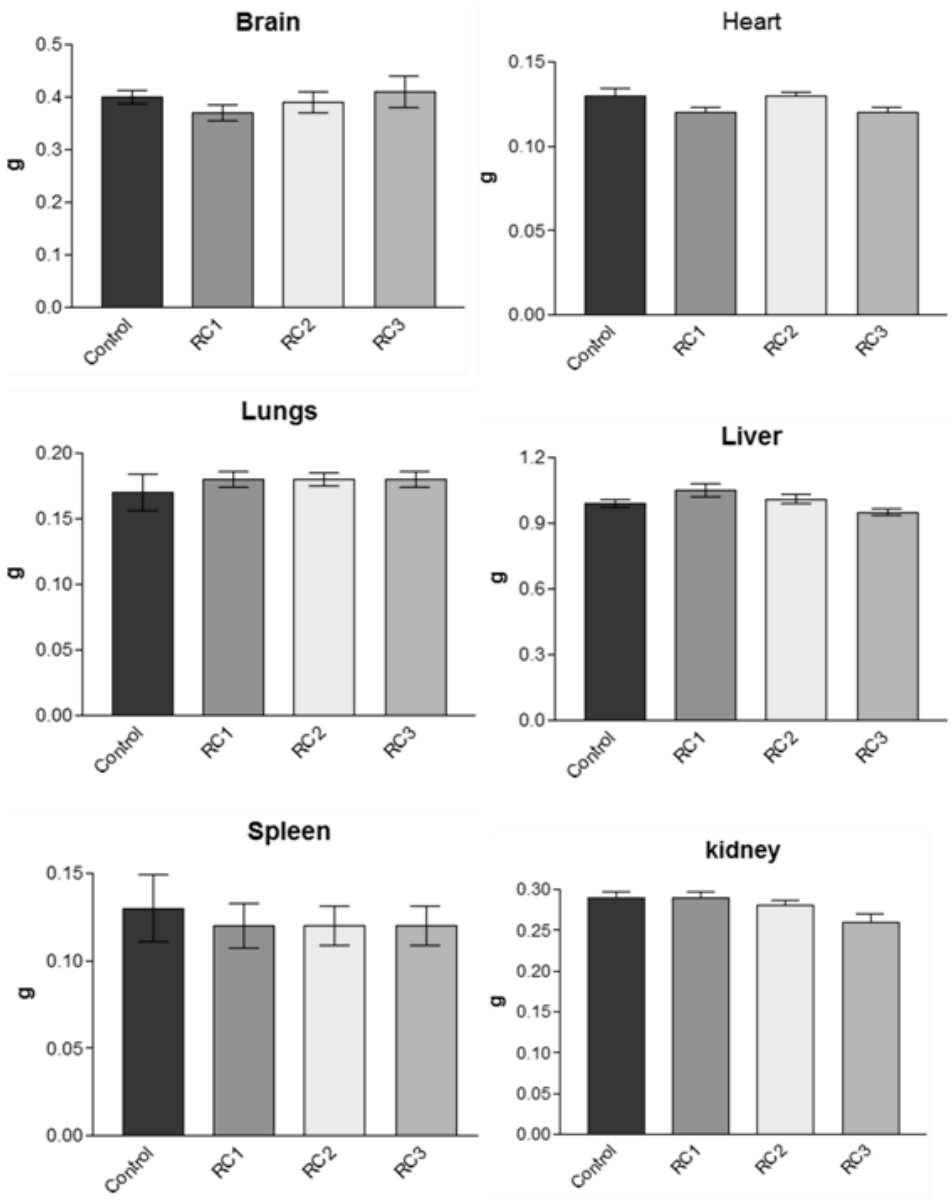
Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Tables 6: Effect of chronic administration of ARCE extract on organ weight of *Swiss* albino mice.

	CON	ARCE 1	ARCE 2	ARCE 3
Brain (g)	0.40±0.012	0.37±0.014	0.39±0.020	0.41±0.031
Heart (g)	0.13±0.004	0.12±0.003	0.13±0.002	0.12±0.003
Lungs (g)	0.17±0.014	0.18±0.006	0.18±0.005	0.18±0.006
Liver (g)	0.99±0.020	1.05±0.030	1.01±0.021	0.95±0.016
Spleen (g)	0.13±0.019	0.12±0.013	0.12±0.011	0.12±0.011
Adrenal (g)	0.03±0.001	0.03±0.001	0.04±0.002	0.04±0.002
Kidney (g)	0.29±0.007	0.29±0.007	0.28±0.006	0.26±0.010

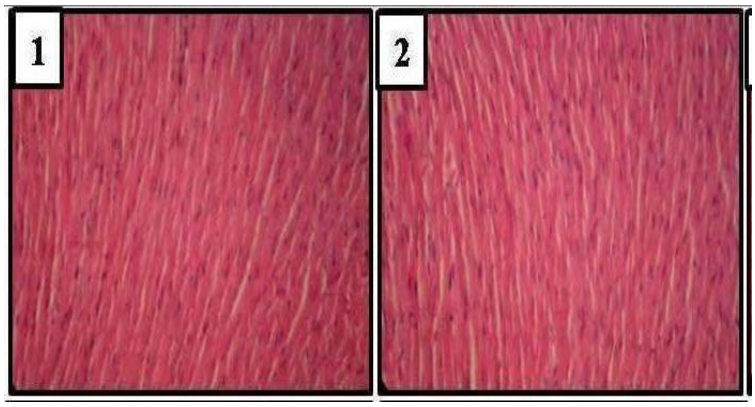
Results are expressed as Mean ± S.E.M for n=8. *$p<0.05$ compared to CON.

Figure 8: Effect of chronic administration of ARCE extract on organ weight of *Swiss* albino mice.



Results are expressed as Mean ± S.E.M for n=8. *p<0.05 compared to CON.

Figure 9: Photomicrographs of Heart of control (1) and 1000, 2000 and 3000mg/kg ARCE treated mice (2, 3, 4), Liver of control (5) and 1000, 2000 and 3000mg/kg ARCE treated mice (6, 7, 8) and kidney of control (9) and 1000, 2000 and 3000mg/kg ARCE treated mice (10, 11, 12) stained with hematoxyline and eosin (100X).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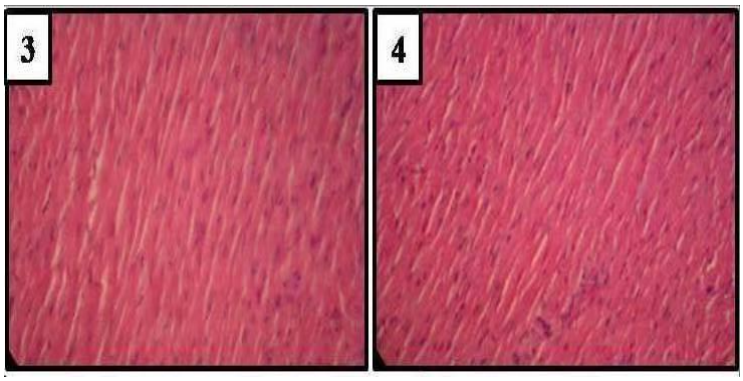
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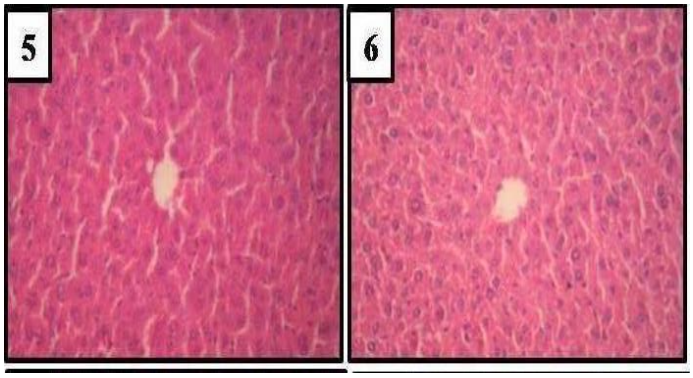
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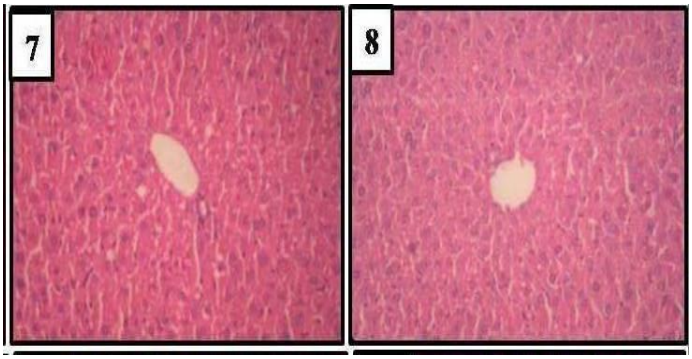
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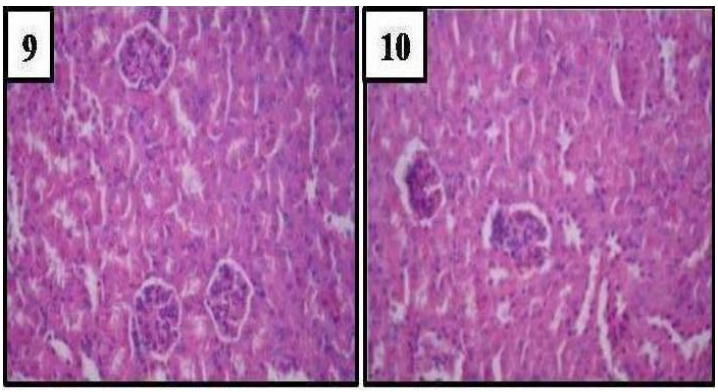
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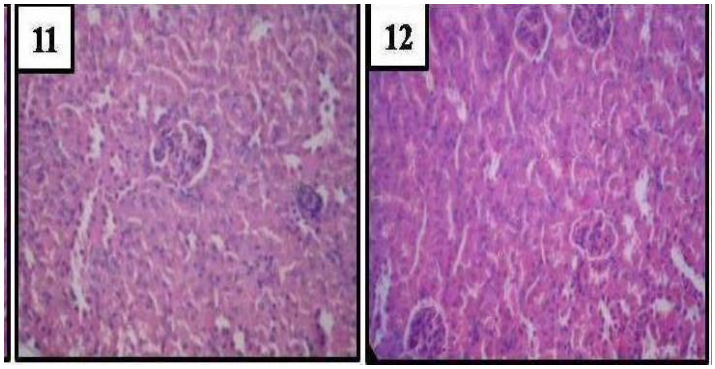
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DISCUSSION

No abnormalities in behaviour could be monitored on extract administration suggesting, no adverse effect on the motor activity of experimental animals. No mortality was recorded in acute toxicity study with ARCE extract up to a dose of 5000mg/kg. Further validation of sub chronic toxicity studies by oral administration of 1000, 2000 and 3000mg/kg of ARCE extract for 28 days showed neither mortality nor any behavioural abnormality in gross motor activities in any of the doses employed. Since no mortality was recorded even after administration of 5000mg/kg of ARCE extract the LD_{50} of ARCE extract could not be established and the LD_{50} of ARCE extract is likely to be higher than 5000mg/kg BW.

Sub chronic administration of ARCE extract did not alter plasma lipid profile but significantly induced hypoglycaemia in a dose dependent manner. The observed mild hypoglycaemia property of ARCE extract is in accordance with previous reports (Kataya and Hamza 2008) and justifies its usage for the treatment of diabetes. The homeostatic balance of plasma electrolytes such as Na^+ and K^+ is regulated by fine mechanism of ionic changes and osmotic balance (Tilkian et al., 1979). Sodium is the major cation of the extracellular fluid that regulates acid-base equilibrium and protects the body against excessive fluid loss. Potassium is the major intracellular cation with similar role to that of Na. Also, Plasma calcium level is considered as a marker of bone metabolism in humans and laboratory animals (Houillier et al., 2006). Since alterations in plasma ionic balance are likely to cause pathological disturbances, it becomes necessary to evaluate the

possible effect of ARCE extract on plasma electrolyte balance. ARCE extract did not alter plasma levels of electrolytes after sub chronic administration.

Evaluation of cardiac, hepatic and renal functions is of prime importance to assess the inherent toxic property, if any, of the phytochemicals in the extract. Possible toxicity of phytochemicals is liable to cause myocardial damage by altering the membrane permeability and/or by disturbing the integrity to myocytes, resulting in leakage of cardiac enzymes (CK-MB and LDH) into blood. Some phytochemicals found in medicinal plants is known to cause hepatotoxicity, marked by elevated levels of marker enzymes of hepatic function in the blood (Geidam et al., 2004). In this light, plasma markers of hepatic damage such as AST, ALT, ALP and billirubin were assayed to investigate the possible hepatotoxicity of ARCE extract. We could not observe alteration in the plasma markers of cardiac and hepatic damage in ARCE administered mice indicating at no adverse effect on these vital organs.

In the present study we observed evaluation in the indices of billirubin with sub chronic ARCE administration. Evidence for elevated levels of plasma billirubin as an indicator of hemolytic anemia in humans is available (Tripathi, 2003). The observed hyperbilirubinemia may be attributed to haemolytic anemia caused due to the phyto-ingredients like, S-methyl-l-cysteine sulfoxide and isothiocyanates (glucosinolate) in ARCE extract (Repetto and Llesuy, 2002; Jagdish Singh et al., 2006) as these compounds have been reported to be associated with haemolytic anemia (Benevenga et al., 1989).

Kidney is a sensitive organ, whose function is known to be affected by a number of factors such as drugs including phytochemicals of plant origin that ultimately lead to

renal failure (Saidu et al., 2007). Assessment of possible renal damage due to ARCE extract was made by assaying plasma urea and creatinine levels. It was found that ARCE extract did not have any adverse effect on kidney functions.

For extrapolation of this data to humans, the calculated NOAEL for human will be 485g of ARCE extract or 4041g of raw ARCE (Li et al., 2010). According to WHO toxicity guidelines 2 weeks to 1 month administration to rodents can be extrapolated as a single administration or repeated administration for less than 1 week in humans (Zhang, 2000) hence, it can be interpreted that 4041g of raw ARCE consumption by a 65kg human for less than a week is considered to devoid of side effect and toxicity.

It can be concluded from this inventory that, ARCE extract is non-toxic and its NOAEL in rodents is 2000mg/kg bw. Extrapolation of these results into human subjects and a realistic comparison is possible. However, haemolytic anaemia and hyperbilirubenemia are likely at higher doses of ARCE extract. Overall, it can be concluded that, ARCE extract falls within the recommended quantity/ dose as per WHO toxicity guidelines and hence can be considered as a non-toxic food supplement.

Summary

The present study has carried out safety evaluations on an ethanolic extract of red cabbage leaves (ARCE) in terms of acute and sub-chronic oral toxicity tests as per Organisation for Economic Co-operation and Development (OECD) guidelines in *Swiss* albino mice. Single dose administration of ARCE extract (1000, 2000, 3000, 4000 or 5000 mg/kg bodyweight) to *Swiss* albino mice did not manifest toxicity or any significant adverse behavioral alterations. Chronic administration of ARCE extract (1000, 2000 and 3000 mg/kg bodyweight) for 28 days also did not register any significant alterations in fluid intake, organ weights, plasma lipid profile, plasma CK-MB, Lactate dehydrogenase, aspartate transaminase, alanine transaminase, creatinine, electrolytes and calcium levels and the total blood count showed a non significant change. However, significant reduction in bodyweight gain, food intake, red blood cell count and hemoglobin content along with higher alkaline phosphatase, billirubin and urea levels were observed in mice treated with 3000 mg/kg bodyweight for 28 days. Since, there was no mortality up to a dose of 5000 mg/kg bodyweight, 50% lethal dose (LD_{50}) could not be determined and hence, it can be assumed that, LD_{50} of ARCE extract is > 5000mg/kg. No observable adverse effect level (NOAEL) dose of the ARCE extract was found to be 2000mg/kg BW. Hence, consumption of ARCE extract for various medicinal purposes is safe. These results can be of relevance for the scientific fraternity as well as laymen who consume this vegetable or its phytochemical preparation.

Flavonoid rich *Eugenia jambolana* seed extract attenuates cardiac and hepatic oxidative stress in high cholesterol diet fed rats.

INTRODUCTION

Hypercholesterolemia is one of the most important risk factors for atherosclerosis and subsequent cardiovascular diseases (Steinberg, 2002). Although, the focus of research so far has been mainly on the vascular effects of hyperlipidemia, it is now quite evident that hyperlipidemia exerts direct effects on the myocardium and hepatocytes via oxidative tissue damage resulting in gross functional and pathological manifestations in the said tissues (Ferdinandy et al., 1998; Ferdinandy, 2003). Although, recent studies have proved that cardiovascular diseases are a leading cause of global mortality (Smith et al., 2004). recent studies have reported beneficial role of dietary supplementation with natural antioxidant to attenuate oxidative stress-induced pathogenesis of diseases (Ramesh et al., 2009). In this context, dietary supplements such as, *Ocimum sanctum* and *Cynara scolymus* have been reported to ameliorate high cholesterol diet (HCD) induced cardiac and hepatic oxidative stress in experimental models (Küçükgergin et al., 2010; Suanarunsawat et al., 2010).

Till date, there are no reports on protective role of EJSE against HCD induced oxidative stress and tissue damage. The present study evaluates protective effect of EJSE against HCD induced cardiac and hepatic oxidative stress in rats.

MATERIALS AND METHODS

Preparation of extract

Fruits of *Eugenia jambolana* were handpicked from the local fruit market. The pulp was removed and the seeds were washed several times with milli-Q distilled water and shade dried at room temperature. Kernel of the seeds was separated from the seed coat manually and the seeds were powdered in an electric grinder. The resultant powder was soaked in 70% ethanol for 24 h with continuous stirring and the mixture was filtered with Whatman's No. 1 filter paper. The resultant filtrate obtained was centrifuged at 10,000 rpm at room temperature (25 °C) and the pellet was discarded. The supernatant was concentrated *in vacuo* by means of rotary evaporator. The concentrated extract was then suspended in water and washed thrice with chloroform. The residual layer was extracted three times with ethyl acetate. All the extracts were finally pooled and concentrated using a rotator evaporator [10, 11]. The total yield at the final step was 5.66 % (w/w).

HPLC fingerprinting of the extract

HPLC fingerprinting of EJSE was developed as described earlier [10, 11]. The separation was carried out on XTerra TM RP C18 column, 4.6×250mm, 5 mm particle size and the temperature was maintained at 30 °C. 25 µl of sample was injected into the column and eluted with a constant rate of 1.0 ml/min. HPLC grade methanol and milli Q distilled water (pH 3.0; adjusted with HPLC grade phosphoric acid) were used as mobile phase in 1:1 ratio. The dual λ absorbance detector (Waters 2487, USA) was operated at 265 nm. Mixtures of authentic standards of quercetin, rutin and kaempferol were dissolved in HPLC grade methanol and injected under the same conditions as for the sample.

Experimental animals

Male *Charles foster* rats (Obtained from Animal resource facility, Department of Biochemistry, The M.S. University of Baroda, Gujarat, India) were housed and 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 carried out 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 Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Experimental design

A total of Twenty four rats were divided into three groups of 8 animals each.

Group I: Control (CON); fed with standard laboratory chow (Pranav Agro Ltd, Baroda, India) and orally administered with 0.5 % carboxy methyl cellulose (CMC) for 8 weeks.

Group II: High calorie diet fed animals (HCD). They were administered single dose of Vitamin D3 (600,000 unit/kg, *i.p.*) and later fed with a HCD (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: High calorie diet and EJSE fed animals (HCD+EJSE). They were orally administered with 100mg/kg EJSE (Ravi et al., 2003, 2004, 2005) along with HCD as mentioned above and groups II and III received equal volume of vehicle (0.5% CMC).

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, 1500 rpm for 10 min) to obtained serum. Later, animals were sacrificed by cervical dislocation under mild ether anaesthesia and, liver, heart, thoracic aorta and kidney were excised and, stored at -80°C (Cryo Scientific Ltd, India) for further biochemical analysis.

Serum markers of cardiac and hepatic damage

Serum based markers such as creatine phospokinase-MB (CK-MB), lactate dehydrogenase (LDH), alanine amino transaminase (ALT) and aspartate amino transaminase (AST) were analyzed using commercially available kits (Reckon diagnostics) and read on semi-autoanalyzer (Mecrk, Germany).

Tissue lipids

Cardiac and hepatic lipids were extracted in chloroform: methanol (2:1) mixture and dried (Folch et al., 1957). The tissue was homogenized with chloroform:methanol (2:1) to a final volume 20 times the volume of the tissue sample (1 g in 20 ml of solvent mixture). After dispersion, the whole mixture was agitated for 15-20 min at room temperature. The solvent was washed with 0.2 volume (4 ml for 20 ml) of 0.9% NaCl solution, vortexed and centrifuged at 2000 rpm to separate the two phases. Upper phase was removed by siphoning the upper phase; lower chloroform phase containing lipids was evaporated under to dryness. Dried lipid extract was dissolved in 1% triton X 100 (Thounaojam et al., 2009) and TC and TG were analyzed using commercially available kits.

Cardiac and hepatic antioxidants and lipid peroxidation

Cardiac and liver tissue pieces from control and treated groups were excised, weighed, homogenized (10% w/v) in chilled tris buffer (10 mM, pH 7.4) and centrifuged at 10,000×g for 20 min in high speed cooling centrifuge (0°C). Clear supernatant was used for assaying lipid peroxidation (LPO; Buege and Aust, 1978), superoxide dismutase (SOD; Kakkar et al., 1984), catalase (CAT; Aebi, 1984), reduced glutathione (GSH;

Beutler et al., 1963) and protein (Lowry et al., 1951) whereas, ascorbic acid (AA; Roe and Küether, 1943) were assayed in tissue sediments.

❖ **ASSAY OF SUPEROXIDE DISMUTASE (SOD)**

Principle: - The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measured at 560 nm.

Reagents:- Sodium pyrophosphate buffer (0.025M, pH 8.3), Phenazine methosulphate (PMS) (186µM), Nitroblue tetrazolium (NBT) (300µM), NADH (780µM), Glacial acetic acid, n-butanol, Potassium phosphate buffer (50mM, pH 6.4)

Procedure: - The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of the enzyme preparation and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer (Genesys 10-S, USA).

One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute and expressed as U/min/mg protein.

❖ **ESTIMATION OF ASCORBIC ACID**

Principle: - Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2, 4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 540 nm.

Reagents: - TCA (4%), 2, 4-dinitrophenyl hydrazine reagent (2%) in 9N H2SO4, Thiourea (10%), Sulphuric acid (85%), Standard ascorbic acid solution: 100µg / ml in 4% TCA

Procedure: - Ascorbate was extracted from 1g of the plant sample using 4% TCA and the volume was made up to 10ml with the same. The supernatant obtained after centrifugation at 2000 rpm for 10 min was treated with a pinch of activated charcoal, shaken vigorously using a cyclomixer and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation.

Standard ascorbate ranging between 0.2-1.0ml and 0.5ml and 1.0ml of the supernatant were taken. The volume was made up to 2.0ml with 4% TCA. DNPH reagent (0.5ml) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5ml of 85% sulphuric acid, in cold. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. The tubes were cooled in ice and the absorbance was read at 540nm in a spectrophotometer.

A standard graph was constructed using an electronic calculator set to the linear regression mode. The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

❖ **ESTIMATION OF REDUCED GLUTATHIONE**

Principle: - Reduced glutathione on reaction with DTNB (5, 5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412 nm.

Reagents: - TCA (5%), Phosphate buffer (0.2M, pH 8.0), DTNB (0.6mM in 0.2M phosphate buffer), Standard GSH (10nmoles/ml of 5% TCA)

Procedure: - Tissue samples for GSH assay were homogenized on ice with 20 volumes of precipitating solution (1.5 mL 100 mmol/L Na-phosphate/5 mmol/L EDTA buffer, pH 8.0 and 0.4 mL 25% metaphosphoric acid) and then centrifuged at 10,000 g for 30 min at 4 °C. The reaction mixture contained 0.5 mL of supernatant, 0.75 mL of Na-phosphate puffer (0.2 M, pH 7.4), 0.1 mL DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) and 0.04 mL NaOH. The solution was kept at room temperature for 15 min and then read at 412 nm on a spectrophotometer.

The values were expressed mg/g sample.

❖ **ASSAY OF CATALASE (CAT)**

Principle: - The UV absorption of hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

Reagents: - 0.067 M Phosphate buffer (pH 7.0), Hydrogen peroxide (2mM) in phosphate buffer

Procedure: - H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of homogenate and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a spectrophotometer. The enzyme solution containing H2O2-free phosphate buffer served as control.

One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units

❖ **DETERMINATION OF TISSUE LIPID PEROXIDATION**

Principle: - Lipid peroxidation, an indicator of tissue injury induced by reactive oxygen species will be measured as thiobarbituric acid reactive substance (TBARS).

Reagents: - TBA reagent containing 0.375% TBA, 15% trichloroacetic acid and 0.25N HCl, 1,3,3,3 tetra-ethoxypropane.

Procedure: - Briefly, 0.5 ml of tissue homogenates was reacted with 2 ml of TBA reagent containing 0.375% TBA, 15% trichloroacetic acid and 0.25N HCl. Samples will be boiled for 15 min, cooled and centrifuged. Absorbance of the supernatants will be spectrophotometrically measured at 532 nm. TBARS concentrations will be calculated by the use of 1,3,3,3 tetra-ethoxypropane as a standard.

The results were expressed as nmol of MDA formed/min/mg protein. Commercially available 1, 1, 3, 3-tetraethoxypropane (Sigma-Aldrich Ltd) was used as a standard for calculation of MDA content.

Microscopic and morphometric evaluation of cardiac and hepatic tissue

Cardiac and hepatic tissue samples of control and experimental rats were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax using automated tissue processor. Five µm sections were cut and stained with haematoxylin and eosin and examined under Leica microscope. Photographs were taken with canon power shot S70 digital camera at 100 X magnification.

For cardiac histopathological scoring, lesions were graded as (0) nil, (1) minimum (focal myocytes damage), (2) mild (small multifocal degeneration with slight degree of inflammatory process), (3) moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process) and (4) severe (necrosis with diffuse inflammatory process) as per Joukar et al. 2010.

The degree of hepatic steatosis and inflammation in each specimen was determined as per Kleiner et al. (2005), as follows:

0 = none, 1 = mild (< 30%), 2 = moderate (30-60%) and 3 = marked (> 60%)

Inflammation was graded by the presence or absence of inflammatory cells, as follows: 0 = absent, 1 = minimal or focal occasional single clusters of inflammatory cells present in a few microscopic fields, 2 = mild inflammation, 3 = moderate inflammation and 4 = marked inflammation.

Statistical analysis

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's multiple comparison tests. 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. Histo-morphometric data was analysed using chi-square method.

RESULTS

HPLC fingerprinting of EJSE

The HPLC chromatogram of EJSE is shown in Figure. 1. The overlap chromatogram of extract and standards confirmed the presence of quercetin (Rt. 2.51), rutin (Rt.4.87) and kaempferol (Rt.15.17) in EJSE used in this study.

Cardiac and hepatic lipids

The HCD diet fed rats recorded elevation cardiac and hepatic TC (52.15 and 64.07%) and TG (54.01 and 54.23%) compared to CON rats (Table.1 & Figure 2). However, EJSE treatment was successfully able to prevent HCD diet induced increment in cardiac and hepatic TC (48.67 and 40.50%) and TG (45.99 and 45.70%) compared to HCD rats (Table.1 & Figure 2).

Serum markers of cardiac and hepatic damage

Effect of EJSE treatment on serum markers of cardiac (CK, CK-MB and LDH) and hepatic (AST and ALT) damage was assessed herein. HCD rats recorded significant increment in the serum levels of CK (58.17 %), CK-MB (55.79 %), LDH (40.21 %), AST

(39.70%) and ALT (33.45%) compared to CON rats (Tables 2 & 3 and Figures 3 & 4). HCD diet induced elevation in the serum marker enzymes of cardiac and hepatic damage were significantly attenuated by EJSE supplementation (Tables 2 & 3 and Figures 3 & 4).

Cardiac and hepatic antioxidants and lipid peroxidation

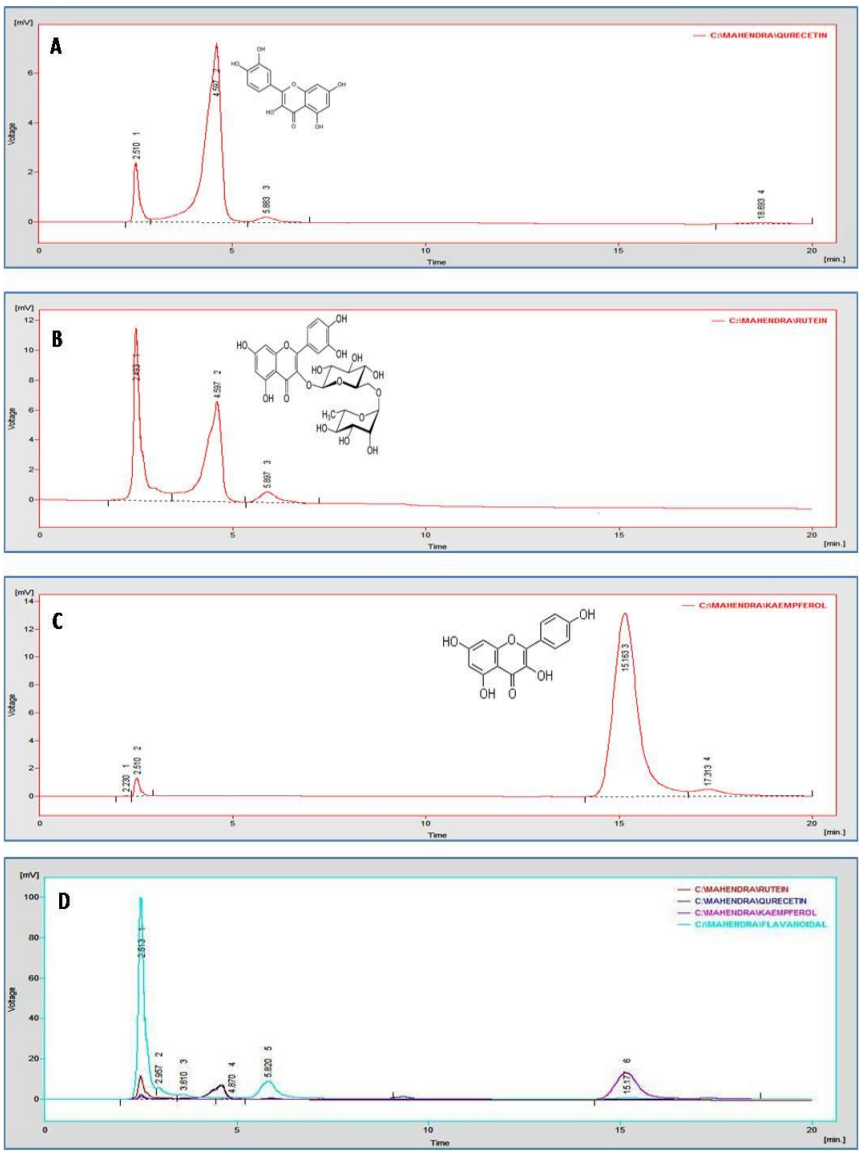
High cholesterol diet fed rats recorded higher cardiac and hepatic oxidative stress as indicated by significant decrement ($p<0.05$) in the activity levels of SOD (47.07 and 49.83 %) and CAT (59.12 and 51.41 %) and, contents of GSH (55.94 and 51.81 %) and AA (56.00 and 59.16 %) along with significant increment in LPO (62.40 and 58.96 %) level compared to CON rats (Tables 4 & 5 and Figures 5 & 6). However, supplementation of EJSE to HCD diet fed rats was capable of minimizing decrement ($p<0.05$) in the activity levels of SOD (31.96 and 43.58 %) and CAT (56.98 and 31.89 %) and, contents of GSH (51.43 and 45.98 %) and AA (50.28 and 52.04%) along with significant increment in LPO (41.86 and 48.06 %) level compared to HCD rats (Tables 4 & 5 and Figures 5 & 6)

Microscopic evaluation of cardiac and hepatic tissue

In the present study, rats fed with HCD diet showed marked structural alterations in cardiomyocytes such as membrane damage, focal myonecrosis, fibroblastic proliferation, infiltration of inflammatory cells (Fig.7B and Table 6), microvesicular hepatocytes lipid accumulation, hepatocyte ballooning and mallory's hyalinization (Fig.8B and Table 7). However, EJSE supplementation to HCD diet fed rats showed optimal cellular integrity

in tissue sections of heart (Fig.7 C and Table 6) and liver (Fig.8C and Table 7) respectively and the same were comparable to that of control rats (Fig.7A & D).

Figure 1: HPLC Chromatogram of EJSE showing overlap with rutin (peak 1, Rt. 2.51), quercetin (peak 4, Rt. 4.87) and kaempferol (peak 6, Rt. 15.17).



The separation was carried out on a 150 mm × 4.6 mm, i.d., 5 µm particle, nova pack column (Waters, USA) at 30 °C with a constant rate of 1.0 ml/min using methanol and milli Q water (pH 3.0 adjusted with HPLC grade phosphoric acid) as mobile phase in 1:1 ratio. The detection was carried out at 265 nm.

Table 1: Effect of EJSE seed extract feeding on tissue (heart and liver) lipid profile in high cholesterol diet (HCD) fed rats.

	CON	HCD	HCD+EJSE
Heart			
Cholesterol (mg/g)	2.88±0.04	6.02±0.07$^{###}$	3.09±0.09**
Triglycerides (mg/g)	1.89±0.02	4.11±0.06$^{###}$	2.22±0.08***
Liver			
Cholesterol (mg/g)	10.09±0.98	28.09±1.89$^{###}$	16.70±1.99***
Triglycerides (mg/g)	7.68±1.00	16.78±1.55$^{###}$	9.11±1.78***

Results are expressed as Mean±S.E.M, for n=8. Where, $^{###}$P<0.001 and ^{ns} non significant compared with CON (rats fed with laboratory chow), **P<0.01 and ***P<0.001 compared with HCD (rats fed with high cholesterol diet).

Figure 2: Effect of EJSE seed extract feeding on tissue (heart and liver) lipid profile in high cholesterol diet (HCD) fed rats.

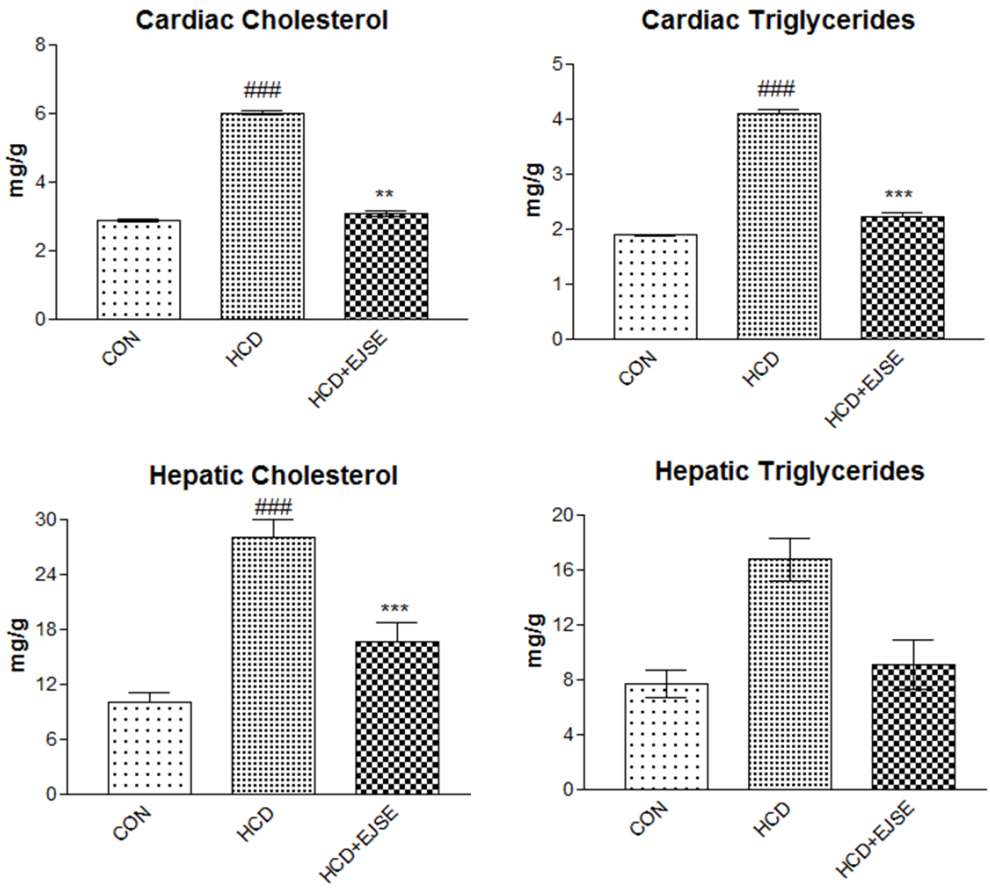
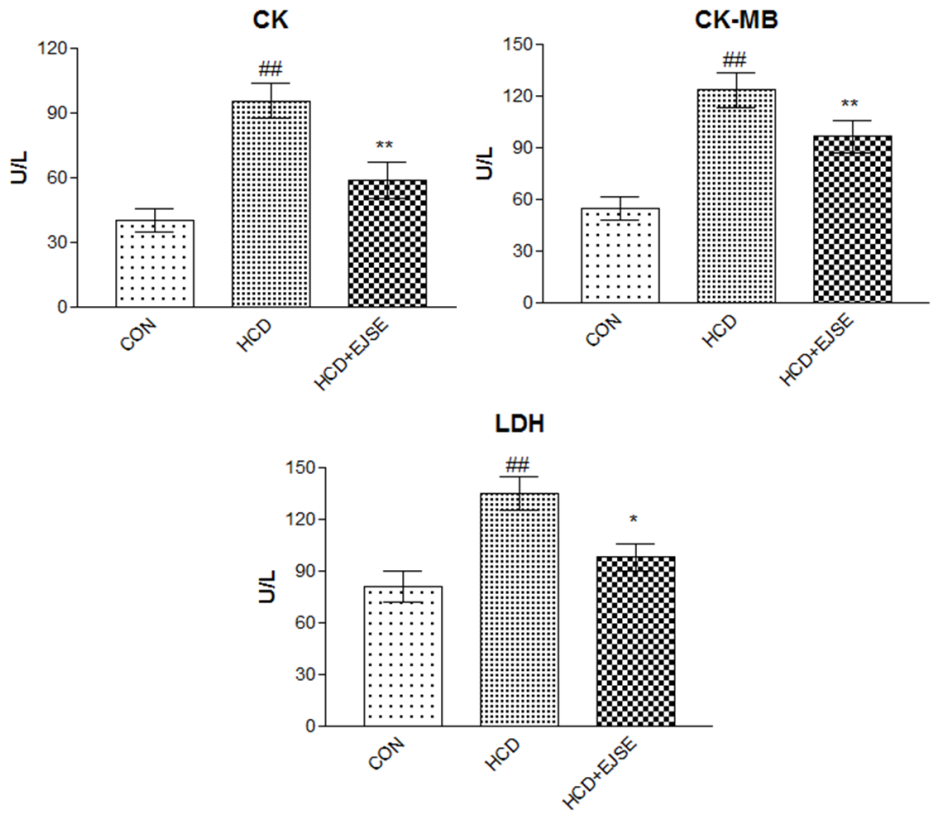


Table 2 & Figure 3: Effect of EJSE seed extract feeding on serum markers of cardiac damage in high cholesterol diet (HCD) fed rats.

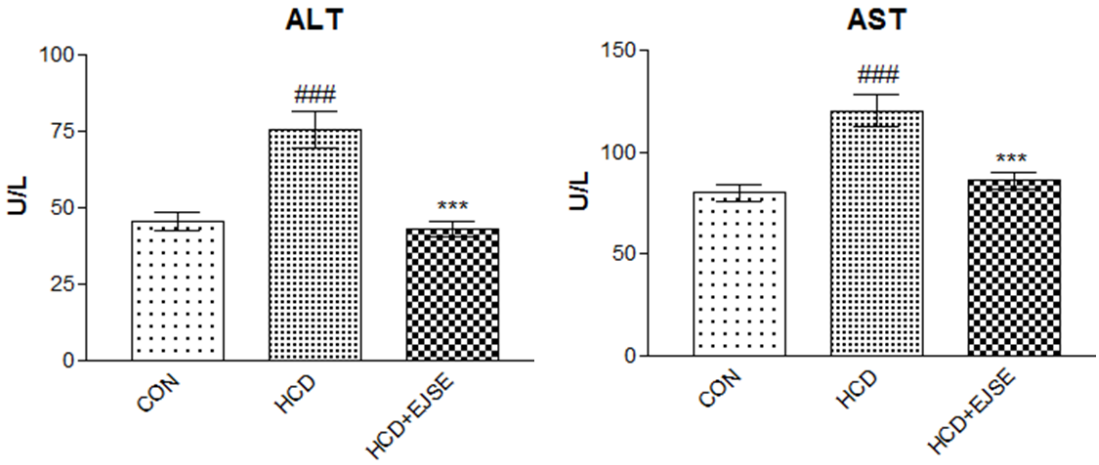
	CON	HCD	HCD+EJSE
CK (U/L)	40.00±5.46	95.64±7.89$^{\#\#}$	58.83±8.14**
CK-MB (U/L)	54.56±6.78	123.43±10.00$^{\#\#}$	96.50±9.28**
LDH (U/L)	80.77±8.99	135±10.00$^{\#\#}$	98.09±8.00*



Results are expressed as Mean±S.E.M, for n=8. Where, $^{\#\#}$P<0.01and $^{\#\#\#}$P<0.001 compared with CON (rats fed with laboratory chow), *P<0.05, **P<0.01 and ***P<0.001 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Table 3 & Figure 4: Effect of EJSE seed extract feeding on serum markers of hepatic damage in high cholesterol diet (HCD) fed rats.

	CON	HCD	HCD+EJSE
ALT (U/L)	45.55±3.00	75.55±6.00$^{###}$	43.00±2.32***
AST (U/L)	80.00±4.30	120.22±7.90$^{###}$	86.00±4.15***



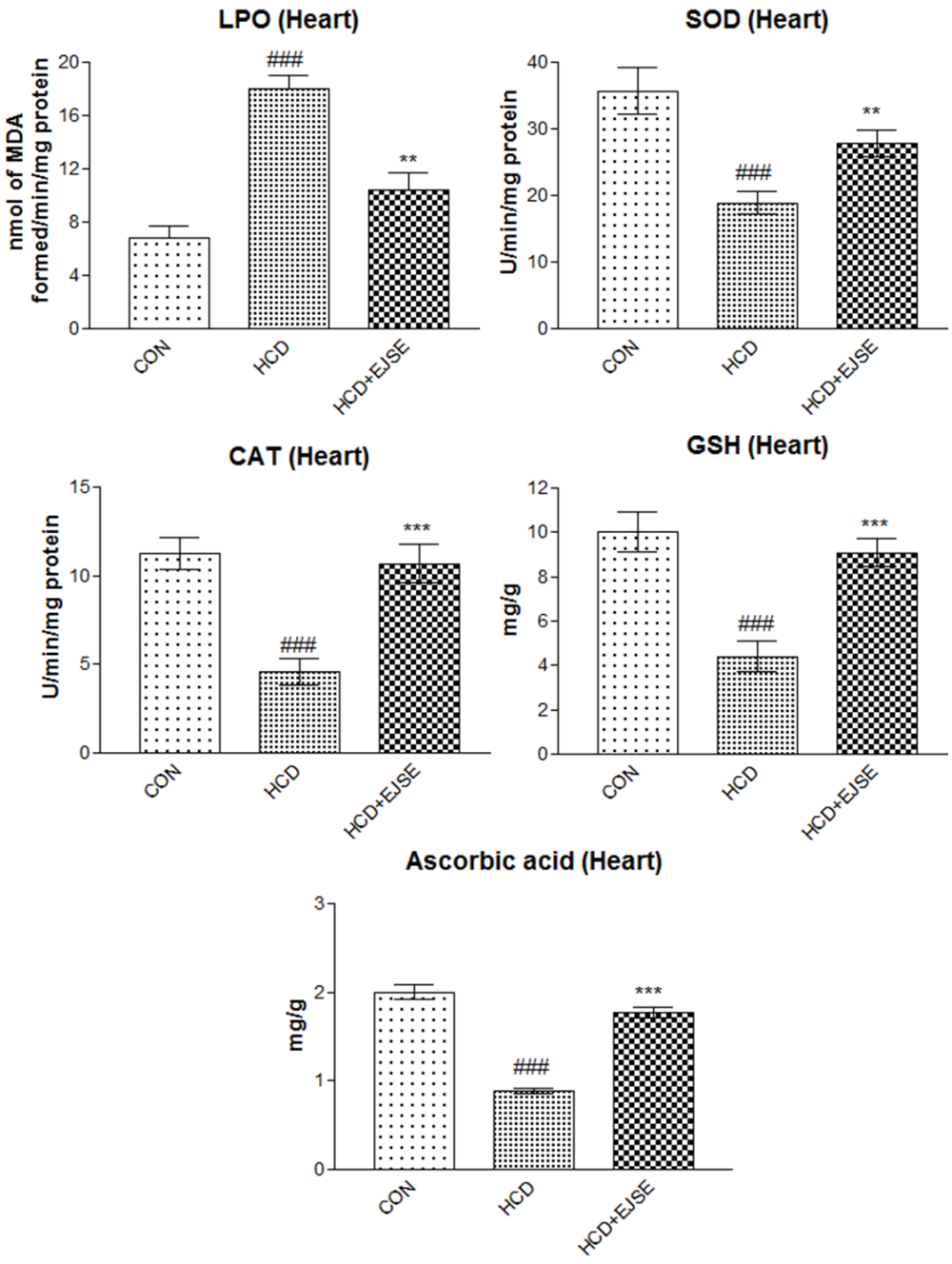
Results are expressed as Mean ± S.E.M, for n=8. Where, $^{##}$P<0.01and $^{###}$P<0.001 compared with CON (rats fed with laboratory chow), *P<0.05, **P<0.01 and ***P<0.001 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Table 4: Effect of EJSE seed extract feeding on cardiac lipid peroxidation and antioxidants in high cholesterol diet (HCD) fed rats.

	CON	HCD	HCD+EJSE
LPO (nmol of MDA formed/min/mg protein)	6.76±0.98	17.98±1.00$^{###}$	10.45±1.23**
SOD (U/min/mg protein)	35.67±3.45	18.89±1.67$^{###}$	27.77±2.00**
CAT (U/min/mg protein)	11.23±0.89	4.59±0.77$^{###}$	10.67±1.09***
GSH (mg/g)	10.01±0.88	4.41±0.67$^{###}$	9.08±0.65***
AA (mg/g)	2.00±0.08	0.88±0.03$^{###}$	1.77±0.06***

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 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Figure 5: Effect of EJSE seed extract feeding on cardiac lipid peroxidation and antioxidants in high cholesterol diet (HCD) fed rats.



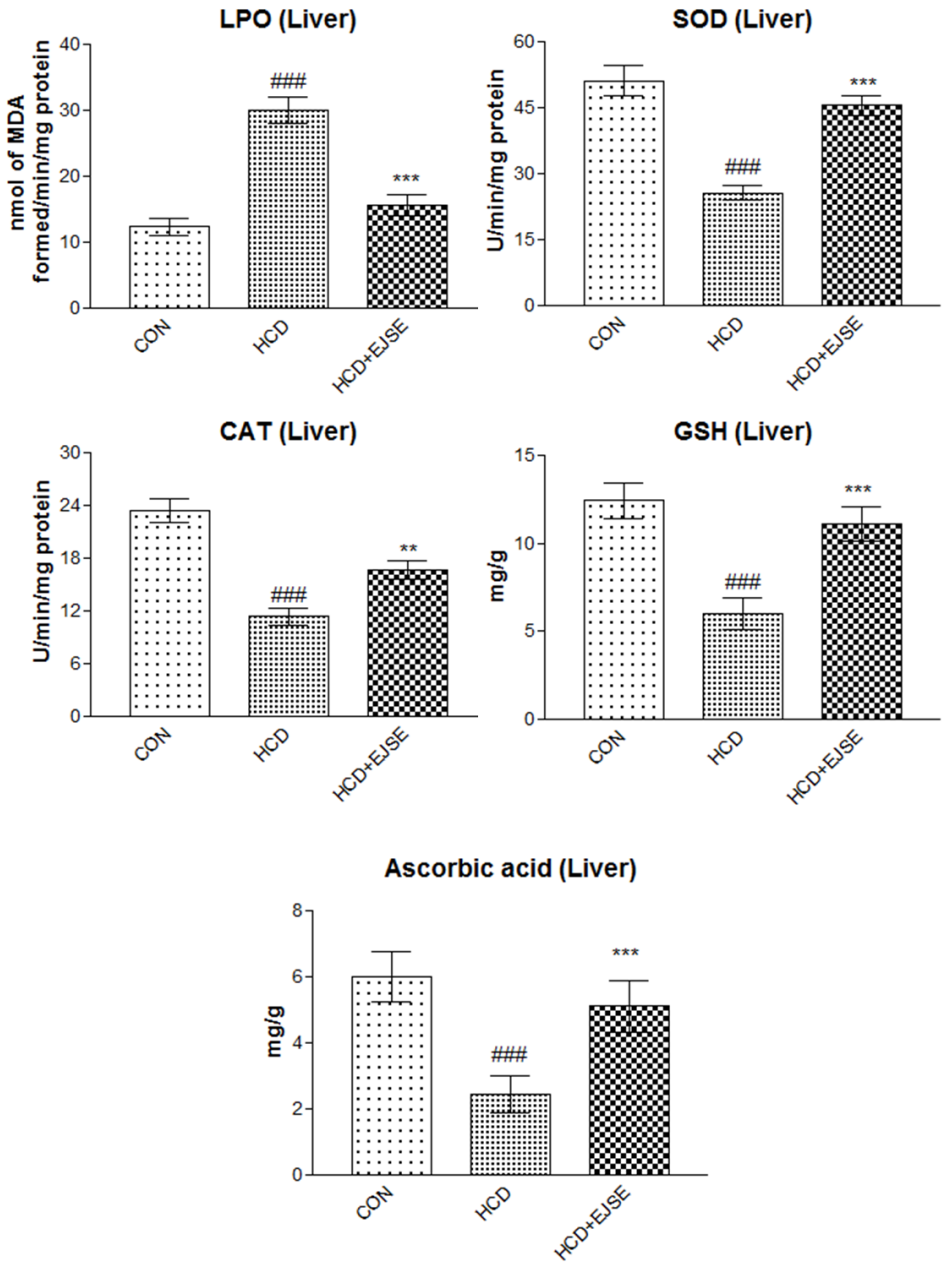
Results are expressed as Mean±S.E.M, for n=8. Where, $^{###}$P<0.001 compared with CON (rats fed with laboratory chow), **P<0.01 and ***P<0.001 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Table 5: Effect of EJSE seed extract feeding on hepatic lipid peroxidation and antioxidants in high cholesterol diet (HCD) fed rats.

	CON	HCD	HCD+EJSE
LPO (nmol of MDA formed/min/mg protein)	12.32±1.22	30.02±1.98$^{###}$	15.59±1.55***
SOD (U/min/mg protein)	51.11±3.42	25.64±1.56$^{###}$	45.45±2.30***
CAT (U/min/mg protein)	23.34±1.34	11.34±0.98$^{###}$	16.65±1.00**
GSH (mg/g)	12.43±1.00	5.99±0.88$^{###}$	11.09±1.00***
AA (mg/g)	6.00±0.77	2.45±0.56$^{###}$	5.11±0.78***

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 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Figure 6: Effect of EJSE seed extract feeding on hepatic lipid peroxidation and antioxidants in high cholesterol diet (HCD) 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 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Figure 7 Morphological and hematoxyline and eosin stained photomicrographs (100x) of rat heart of control (A) showing normal histoarchitecture of hepatocytes (black arrow), HCD (B) showing parenchymatous fat accumulation and ballooning hepatocytes (red arrow), infiltration of inflammatory cells (blue arrow) and formation of Mallory's hyline (green arrow) and HCD+EJSE (C) showing scattered cells with parenchymatous lipid accumulation along with majority of normal hepatocytes (black arrow).

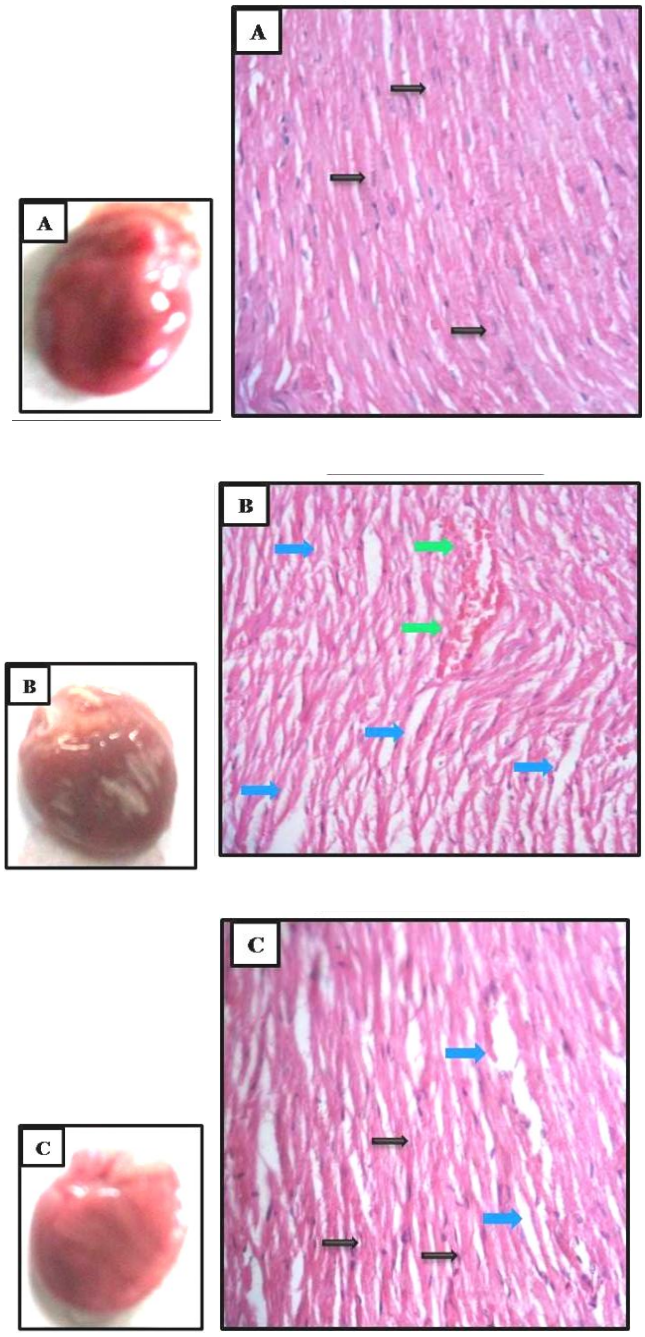


Table 6: Effect of *E.jambolana* seed extract feeding on scoring of cardiac inflammatory changes in experimental groups*

Pathology score		Experimental groups		
		CON	HCD	HCD+EJSE
0	Count	16	0	5
	% within group	100	0	31.25
1	Count	0	0	7
	% within group	0	0	43.75
2	Count	0	4	3
	% within group	0	25	18.75
3	Count	0	4	1
	% within group	0	25	6.25
4	Count	0	8	0
	% within group	0	50	0
Total	**Count**	**16**	**16**	**16**

*Pathology scores: 0 nil, 1 minimum (focal myocytes damage), 2 mild (small multifocal degeneration with slight degree of inflammatory process), 3 moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process), 4 severe (necrosis with diffuse inflammatory process).

Figure 8 Morphological and hematoxyline and eosin stained photomicrographs (100x) of rat liver of control (A) showing normal arrangement of cardiac myofibrils (black arrow), HCD (B) showing loosening of cardiac myofibrils due to lipid accumulation (blue arrow) and infiltration of inflammatory cells (green arrow) and HCD+EJSE (C) showing normal (black arrow) and loosened cardiac myofibrils (blue arrow).

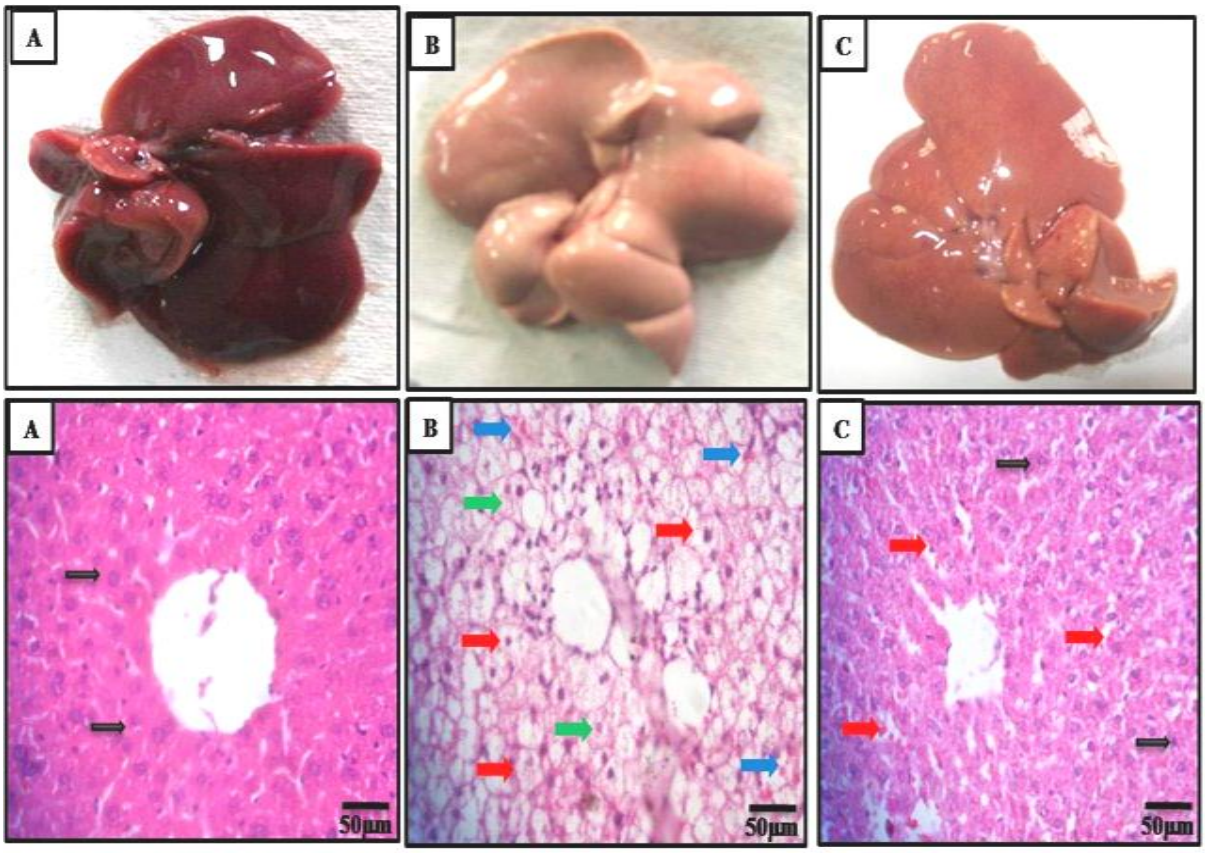


Table 7: Effect of *E.jambolana* seed extract feeding on liver histopathology scoring for steatosis and inflammation

	N	Steatosis[a]				Inflammation[b]			
		0	**1**	**2**	**3**	**0**	**1**	**2**	**3**
CON	8	8	0	0	0	8	0	0	0
HCD	8	0	1	4	3	0	0	3	5
HCD+EJSE	8	4	3	1	0	3	4	1	0
Chi-square		P<0.05				P<0.05			

[a]Steatosis: grade 0 absent; grade <33%; grade 2: 33–66%; grade 3:>66%.

[b]Inflammation: 0 normal; 1 mild; 2 moderate;3 severe.

Discussion

High cholesterol diet induced cardiac and hepatic damage in rats is an accepted experimental model that is popularly used to assess therapeutic role of a drug or herbal principle in question (Deepa and Varlakshmi, 2004; Parmar and Kar, 2007). High cholesterol diet containing 5% cholesterol, 1% cholic acid and 0.5% 2-thiouracil has been widely reported to induce marked hypercholesterolemia and related cardiac and hepatic oxidative stress (Deepa and Varalaxmi, 2003, 2006). In the present study, 8 weeks of HCD feeding to rats resulted in significant increment in cardiac and hepatic lipid profiles. These results are in agreement with the previous reports (Parmar and Kar, 2007; Suanarunsawat et al., 2010). However, EJSE supplementation could successfully minimize these set of changes. Separation techniques such as HPLC, HPTLC of GC-MS etc provide a cutting edge for development of rapid and cost effective methods for standardization and characterization of herbal drugs and to unravel known/novel phytoconstituents (Jain et al., 2010). Hence, in the present study, EJSE was standardized using quercetin, rutin and kaempferol by HPLC analysis.

Experimental rats fed chronically with HCD are known to develop intracellular lipid accumulation in myocardium that is further known to distort its structure and function (Hexeberg, 1993). Also, HCD induced hypercholesterolemia is known to cause malfunctioning of liver through microvesicular steatosis, intracellular lipid accumulation and subsequent oxidative damage to hepatocytes (Küçükgergin et al., 2010).Various pre-clinical and clinical studies have shown that HFD is associated with an increased formation of free radicals (FR) along with an imbalance in antioxidant status which leads to increase in oxidative damage of cellular components (Hopps et al., 2010; Nepal et al.,

2011) and hence, tissue enzymatic (CAT and SOD) and non-enzymatic antioxidants (GSH and AA) are the key for investigating FR induced cellular damage. Presently recorded significant decrement in the enzymatic and non-enzymatic antioxidants along with elevated indices of LPO are in accordance with reports from related labs (Ratheesh et al., 2011) wherein, HCD induced compromised antioxidant status has been pinned as the key cause for oxidative stress induced cardiac and hepatic damage. EJSE supplemented HCD fed rats were able to resist the depletion of endogenous antioxidants and the same was evident in form of near normal levels of enzymatic and non-enzymatic antioxidant levels. Also, the previously reported *in vitro* free radical scavenging property of EJSE is probably instrumental in preventing HCD induced tissue oxidative load (Ahmed et al., 2010) that salves the antioxidant machinery. These observations are attributable to rich content of flavonoids present in the EJSE (Vinson, 1998).

Cardiac and hepatic histopathological alterations were examined in order to comprehensively evaluate the HCD induced damage and subsequent protective role of EJSE. In the present study, HCD fed rats showed structural alterations in cardiomyocytes and hepatocytes in accordance with previous reports (Parmar and Kar, 2007; Suanarunsawat et al., 2010). Cardiac tissue of HCD treated rats showed plenty of evidence of infiltration of inflammatory cells and gross necrotic changes. Liver also showed infiltration of inflammatory cells in the portal area along with lipid laiden and ballooning hepatocytes, formation of Mallory hyaline and nuclear condensation. However, EJSE supplementation to HCD fed rats showed optimal cellular integrity in tissue sections of heart and liver, as the cellular characteristics were comparable to that of

control rats. These results further corroborate the serum and tissue lipid profiles and enzymatic and non-enzymatic antioxidant status of HCD+EJSE rats recorded herein.

Assessment of serum marker enzymes of cardiac (CK-MB, LDH and CK) and hepatic (AST and ALT) damage are confirmatory tests for myocardial and hepatic injuries respectively and their elevated titers in serum is an indicator of extent of tissue damage (Ratheesh et al., 2011). In this context, significantly elevated levels of these marker enzymes in HCD fed rats is in accordance with previous reports (Parmar and Kar, 2007; Suanarunsawat et al., 2010) and further justifies elevated lipid profile, compromised anti-oxidant machinery and histopathological damage recorded herein. However, EJSE could successfully ameliorate elevation of myocardial and hepatic injury markers thus justifying our claim of its cardioprotective and hepatoprotective potentials.

In this study, protective role of EJSE in mitigating HCD induced hyperlipidmia and subsequent oxidative stress induced cardiac and hepatic tissue damage could be established in a relevant experimental model. These attributes add value to the already established therapeutic potentials of EJSE and hence, it can be inferred from the present study that EJSE supplementation has multiple therapeutic potentials in attenuating complex physiological ailments.

Summary

Protective role of *Eugenia jambolana* seed extract (EJSE) on high cholesterol diet (HCD) induced hyperlipidemia/hypercholesterolemia amounting to cardiac and hepatic oxidative stress are assessed in this study. Serum markers of cardiac and hepatic damage, total lipid profile, lipid peroxidation, antioxidant status and histopathological changes in cardiac and hepatic tissue have been assessed in control, HCD fed and HCD+EJSE treated (100mg/kg BW, *p.o.*) rats. It was observed that co-supplementation of EJSE to HCD fed rats significantly ($p<0.05$) minimized elevation in the serum and tissue lipid profiles decrement in the HDL levels. Also, EJSE successfully ameliorated HCD induced cardiac and hepatic oxidative stress ($p<0.05$) and histopathological damage. It can be concluded that, EJSE has the potential of preventing HCD induced experimental hypercholesterolemia and related cardiac and hepatic oxidative stress. It was inferred from this study that EJSE is successfully able to alleviate HCD induced hypercholesterolemia and oxidative damage to cardiac and hepatic tissues of experimental rats.

Anthocyanin rich red cabbage extract (ARCE) ameliorates cholesterol rich diet induced cardiac and hepatic oxidative stress.

INTRODUCTION

The number of overweight individuals is increasing worldwide and has led to an explosion of obesity-related health problems associated with increased morbidity and mortality. The association of higher circulating levels of cholesterol and subsequent development of obesity is well correlated with increased risk of cardiovascular, hepatic, renal and other disease. The risks of other age-related diseases, such as cancer and inflammatory disorders are also often a consequence of the preceding high calorie intake (Park et al, 2010). Oxidative stress has been associated with diverse pathophysiological events, including cancer, renal disease and neurodegeneration. More recently, it has become apparent that reactive oxygen species (ROS) also play a role in the development of vasculopathies, including those that define atherosclerosis, hypertension, and restenosis after angioplasty. Reactive oxygen species (ROS) may lead to the oxidative damage of virtually any biomolecule. Mitochondria are particularly susceptible to damage induced by ROS, which are generated continuously by the mitochondrial respiratory chain. Mitochondria are also a major site for the accumulation of low molecular weight Fe2+ complexes, which promote the oxidative damage of membrane lipids. Recently, a large number of studies have associated mitochondrial dysfunction caused by ROS to both accidental cell death (necrosis) and programmed cell death (Thounaojam et al 2012).

Driven by the need for potent and safe options to treat obesity, diabetes, and the metabolic syndrome, numerous efforts are currently underway to achieve a better understanding of the molecular networks controlling cellular glucose, lipid, and energy metabolism. Medicinal plants with antioxidant and hypolipidemic properties have a potential in protecting against coronary heart disease (Jain et al., 2007). Oxidative stress induced by reactive oxygen species plays a key role in the etiology of several cardiovascular diseases including atherosclerosis. Oral administration/feeding of cholesterol rich diet to experimental animals induce cardiac and hepatic microvascular alterations and oxidative stress. During the onset of atherosclerosis, this is an inflammatory response that appears long before the development of fatty streak lesions in large arteries (Deepa and Varalakshmi, 2004).

Anthocyanins are a group of coloured water-soluble pigments that are attributed to the beneficial effects of many fruits, vegetables, and red wine. There are about 500 types of anthocyanin reported till date from various sources such as fruits, berries and functional foods. Extracts rich in anthocyanins have been reported for their antioxidant, cardioprotective, hepatoprotective properties (Onyesom et al., 2008; Toufektsian et al., 2008). Since, anthocyanins have been reported to be main active ingredient of ARCE in the present study, we evaluated its possible cardioprotective and hepatoprotective roles in high cholesterol diet fed rats.

MATERIALS AND METHODS

Experimental animals

Male *Charles foster* rats were obtained from Animal resource facility, Department of Biochemistry, The M.S. University of Baroda, Vadodara, Gujarat, India and were housed in clean polypropylene cages. The rats were 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 Department of Zoology, The M.S. University of Baroda, Vadodara (Approval No.827/ac/04/CPCSEA).

Preparation of extract and determination of total anthocyanins

Red cabbage (*Brassica oleracea* var. *capitata rubra*) was purchased from the local market, leaves were separated and slice into small pieces and extracted with a mixture of MeOH/H_2O/0.01%HCl, 50/50/1, v/v/w) as described earlier (Glińska and Gabara, 2011). Anthocyanin rich red cabbage extract (ARCE) was evaporated to dryness in a vacuum rotary evaporator in water bath at 40 °C. The resultant yield was 12% w/w. and then the residue was diluted in distilled water of pH 5.6 and total content of anthocyanin in the red cabbage extract was measured spectrophotometrically using molar extinction coefficient of cyanidin-3, 5-diglucoside (26,300 M^{-1} cm^{-1}) (Glińska and Gabara, 2011). The dried extract was dissolved in 0.5% carboxy methyl cellulose (CMC) to obtain different doses of ARCE.

HPLC profile of ARCE

HPLC profiles of ARCE was determined using HPLC (Shimadzu, Kyoto, Japan), column (Hypersil C18 column, particle size 5 mm; 250 x 4.6 mm id; thermo quest, Cheshire, UK). The binary mobile phase consisted of water/formic acid (90:10, v/v) (solvent A) and water/acetonitrile/formic acid (40:50:10, v/v/v) (solvent B). The flow rate was 1 ml/min and a total run time was 40 min. The system was run with a gradient program: 0 min (88% A + 12% B), 26 min (70% A + 30% B), 26-40 min (0% A + 100% B). The anthocyanins were detected at 520 nm as cyanidin 3-glucoside equivalents as per the earlier report (Glińska and Gabara, 2011).

Experimental paradigms

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 and III were given single dose of Vitamin D3 (600,000 unit/kg, *i.p.*) and later fed with a HCD 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 (HCD+ARCE) was give oral gavage treatment with 100 mg/kg ARCE while, group II (HCD) received equal volume of vehicle (0.5% CMC).

At the end of the experimental period, rats were fasted overnight and blood was collected via retro-orbital sinus puncture and, serum was obtained by cold centrifugation (4°C) of the vials at 1500 rpm for 10 min. Later, animals were sacrificed by cervical

dislocation under mild ether anaesthesia and, liver, heart, thoracic aorta and kidney were excised and, stored at -80°C (Cryo Scientific Ltd, India) for further biochemical analysis.

Serum markers of cardiac and hepatic damage

As mentioned in Chapter 3.

Tissue lipids

As mentioned in Chapter 3.

Cardiac and hepatic antioxidants and lipid peroxidation

As mentioned in Chapter 3.

Microscopic evaluation of cardiac and hepatic tissue

As mentioned in Chapter 3.

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

Anthocyanin content in Brassica oleracea L extract

Anthocyanin content in the ARCE was found to be 50.21±3.45 mg/100 g of leaf extract. Total 9 anthocyanin derivatives were detected at 520 nm using HPLC assay with the retention times of 9.47, 14.01, 14.62, 18.03, 23.56, 27.56, 28.34, 33.44 and 34.43 respectively (Figure 1). Further, quantitative spectrophotometric assay recorded 52.17±3.21 mg of anthocyanins in 100g of fresh leaf.

Cardiac and hepatic lipids

The heart and liver tissues were collected from rats of various experimental groups and assessed for the changes in total lipids. Significant increment in the cardiac and hepatic TC and TG was recorded HCD diet fed rats compared to CON rats (Table 1 & Figure 2). However, ARCE treated HCD diet fed rats recorded significant decrement in cardiac and hepatic TC and TG (Table 1 & Figure 2).

Serum markers of cardiac and hepatic damage

HCD diet feeding to rat resulted in elevation in serum markers of cardiac [CK, CK-MB, LDH) and hepatic (AST, ALT) damage compared to CON rats. However, ARCE supplementation to HCD diet fed rats resulted in significant decrement markers of cardiac and hepatic damage compared to HCD diet fed rats (Tables 2 & 3 and Figure 3 & 4).

Cardiac and hepatic antioxidants and, lipid peroxidation

The antioxidant parameters were also assessed in rats of various experimental groups. It was observed that HCD fed rats recorded significant decrement in the activity levels of cardiac and hepatic SOD and CAT and contents of GSH and AA along with significant increment ($p<0.05$) in hepatic and cardiac levels of LPO compared to CON rats indicating at induction of HCD diet induced cardio and hepatotoxicity in these animals (Tables 3 & 4 and Figure 5 & 6). However, ARCE extract supplementation to HCD diet fed rats significantly prevented HCD diet induced alterations in hepatic and cardiac enzymatic and non-enzymatic antioxidants and LPO (Tables 3 & 4 and Figure 5 & 6).

Microscopic evaluation of heart and liver

Histoarchitecture of cardiac tissue of HCD fed rats showed extensive myocyte membrane damage. Also, extensive focal myonecrosis, fibroblastic proliferation and infiltration of inflammatory cells were observed in these tissue sections. However, treatment with ARCE appeared to normalize the myocyte damage. This was evidenced in form of minimal necrotic damage to the myocardium (Figures 7 & 8). Also, microscopic evaluations of hepatic tissue showed lipid accumulation in hepatocytes of HCD rats due to fat overload. Also, hepatocyte ballooning and mallory's hyaline formation was observed in hepatocytes of HCD rats indicating at extensive cellular damage (Figures 7 & 8).

Figure 1: HPLC fingerprinting of ARCE.

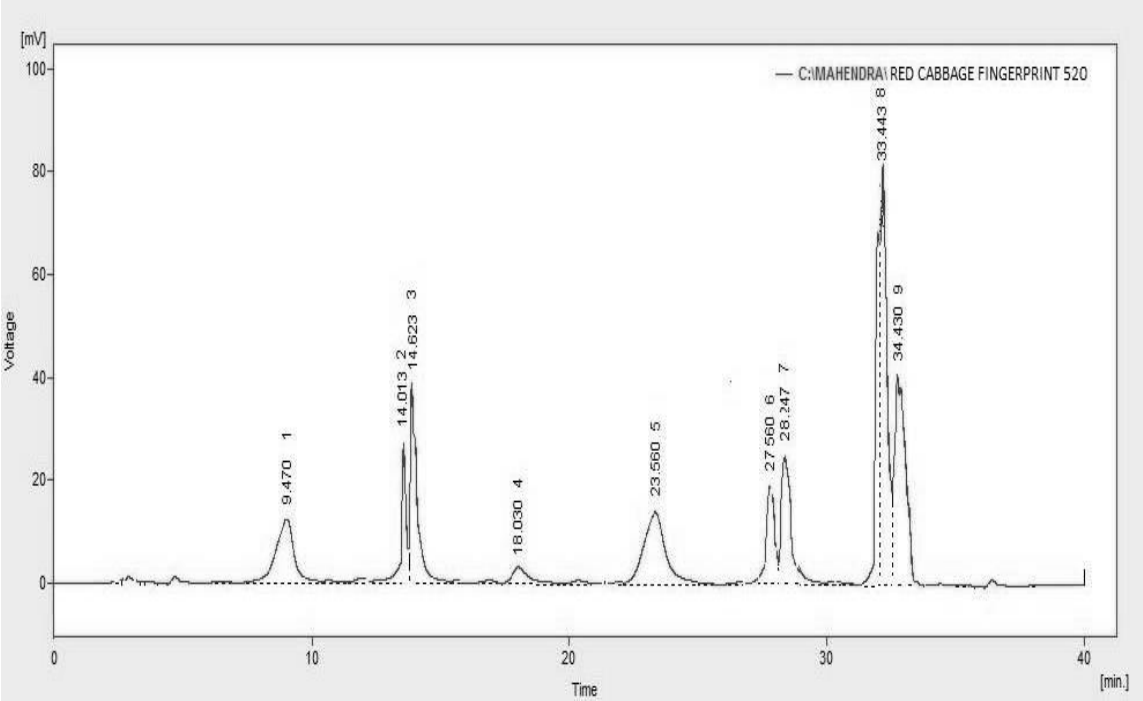
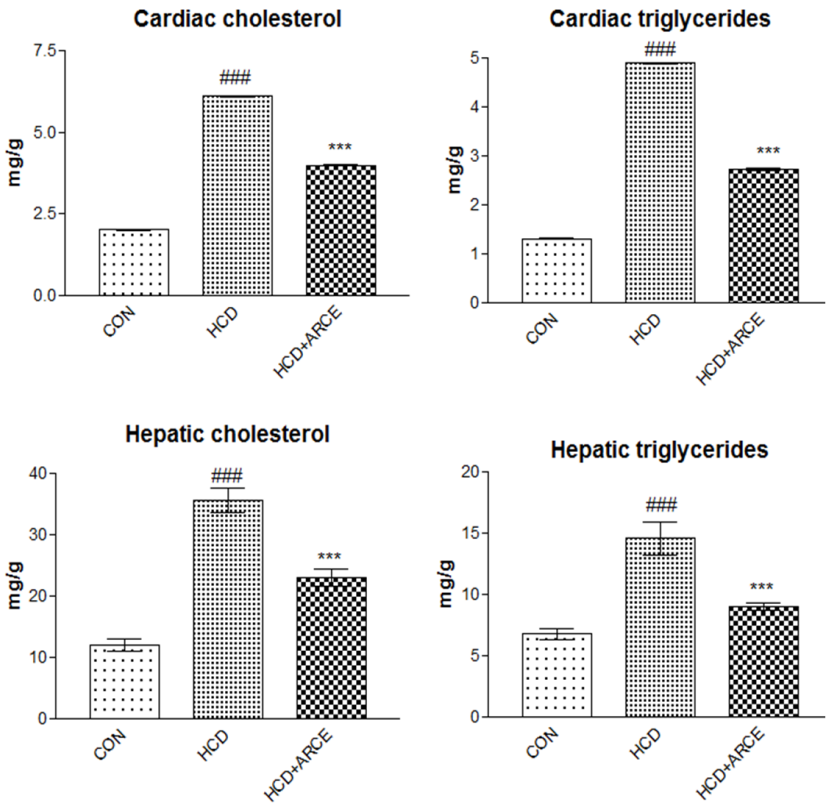


Table 1 and Figure 2: Effect of ARCE on cardiac and hepatic lipid profile.

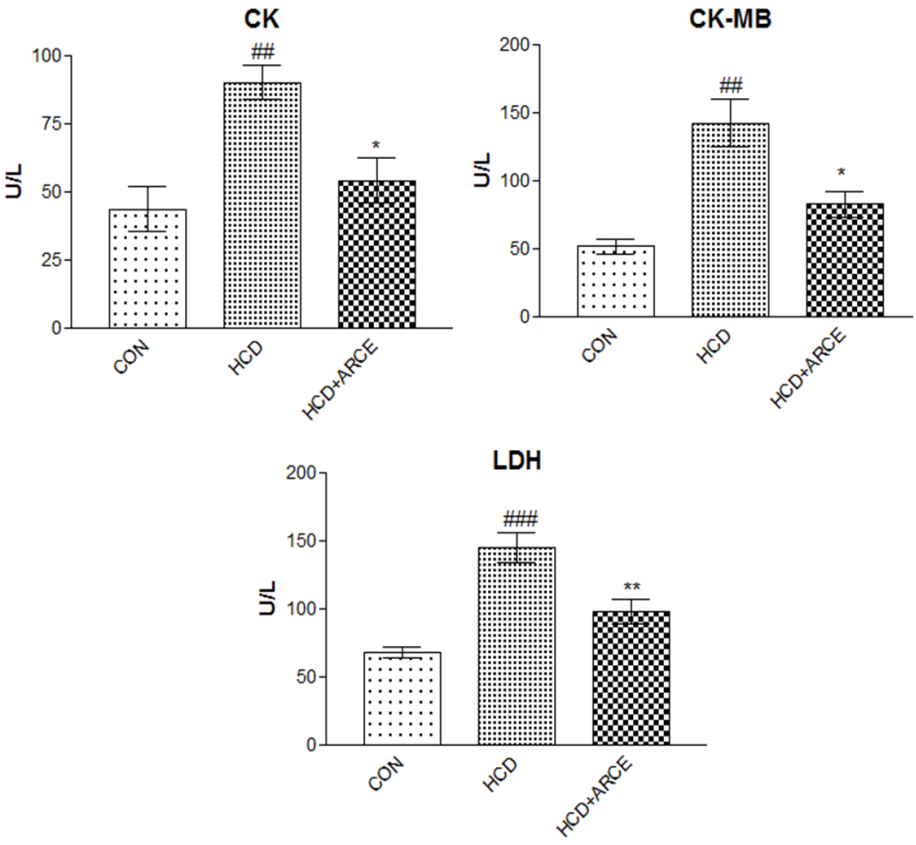
	CON	HCD	HCD+ARCE
Heart			
TC (mg/g)	2.01±0.01	6.11±0.02$^{###}$	3.99±0.02***
TG (mg/g)	1.31±0.01	4.89±0.02$^{###}$	2.72±0.02***
Liver			
TC (mg/g)	12.01±1.00	35.67±2.00$^{###}$	23.01±1.32***
TG (mg/g)	6.75±0.43	14.55±0.39$^{###}$	8.99±0.33***



Results are expressed as Mean±S.E.M, for n=8. Where, $^{#}$ P < 0.05, $^{##}$P<0.01 and $^{###}$P<0.001 compared with CON (rats fed with laboratory chow), *P < 0.05, **P<0.01, ***P<0.001 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Table 2 and Figure 3: Effect of anthocyanin rich red cabbage extract (ARCE) on markers of cardiac damage.

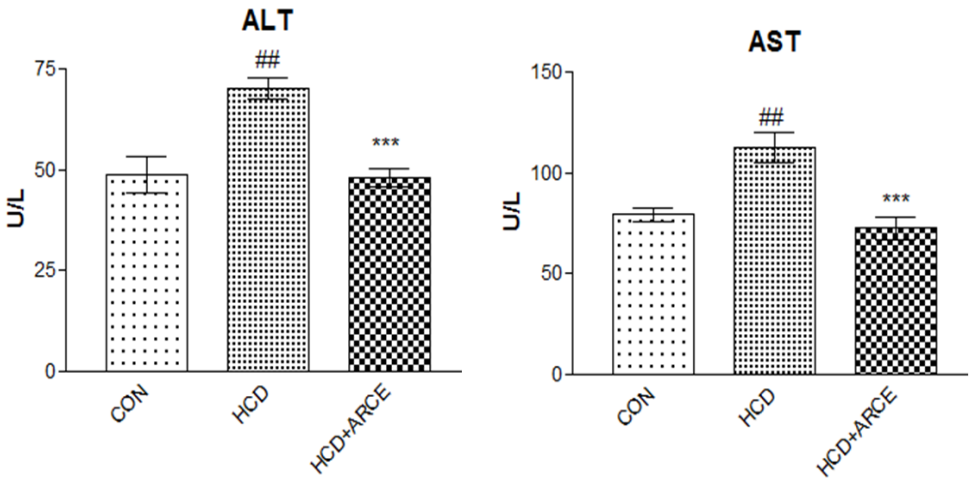
	CON	HCD	HCD+ARCE
CK (U/L)	43.67±8.14	90.17±6.24$^{\#\#}$	54.17±8.09*****
CK-MB (U/L)	51.50±5.83	142.3±17.26$^{\#\#}$	82.50±9.28*****
LDH (U/L)	68.05±4.32	145.21±11.00$^{\#\#\#}$	98.10±9.31******



Results are expressed as Mean±S.E.M, for n=8. Where, $^{\#}P < 0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with CON (rats fed with laboratory chow), $^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Table 3 and Figure 4: Effect of ARCE on markers of hepatic damage.

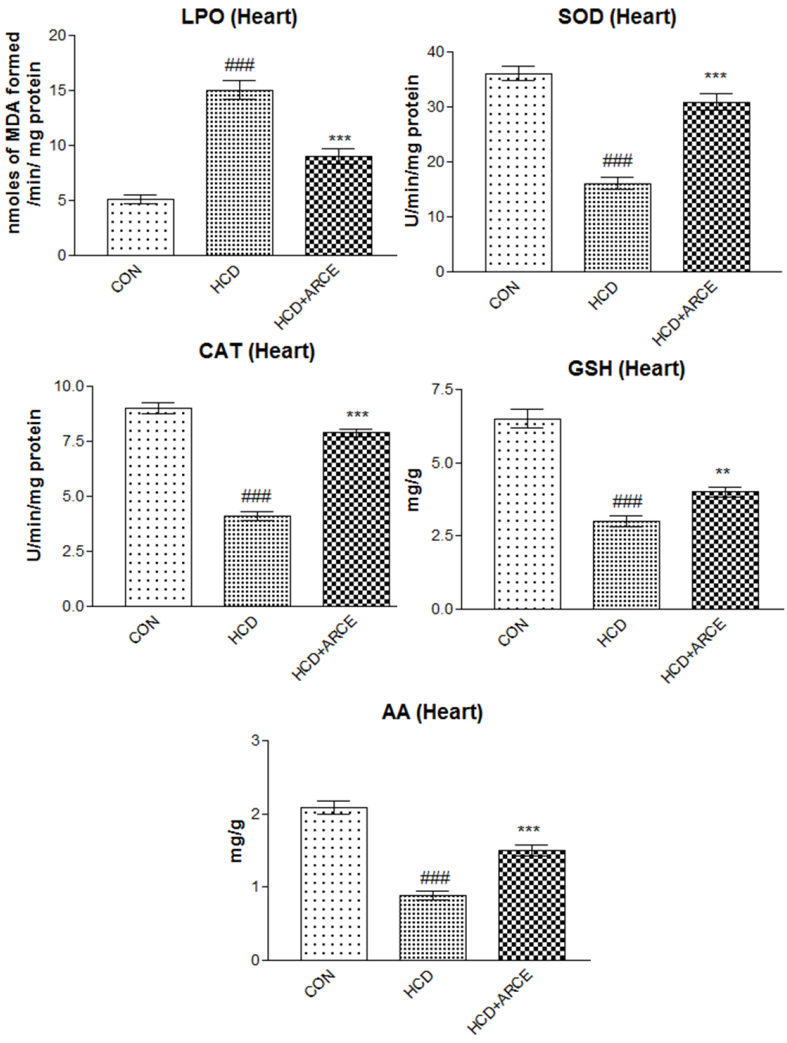
	CON	HCD	HCD+ARCE
ALT (U L^{-1})	48.67±4.485	70.17±2.738$^{\#\#}$	48.00±2.236***
AST (U L^{-1})	79.17±3.582	112.2±7.458$^{\#\#}$	72.50±5.731***



Results are expressed as Mean±S.E.M, for n=8. Where, $^{\#}P < 0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with CON (rats fed with laboratory chow), $^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Table 4 and Figure 5: Effect of ARCE on cardiac lipid peroxidation and antioxidants.

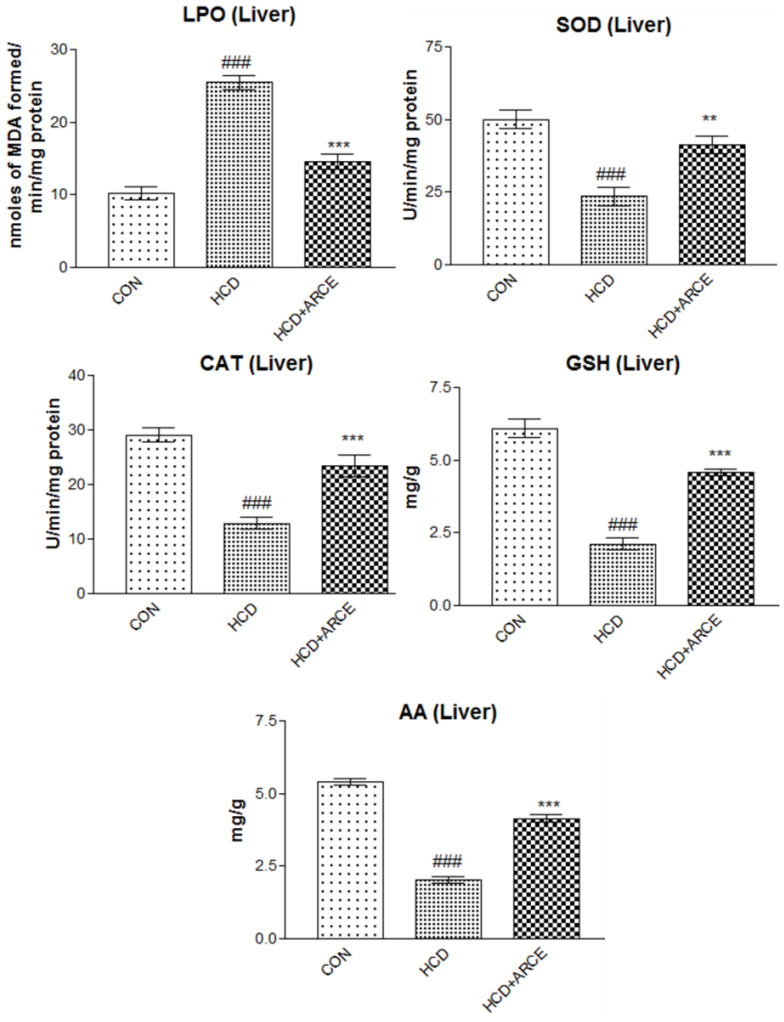
	CON	HCD	HCD+ARCE
LPO (nmoles of MDA formed /min/ mg protein)	5.09±0.44	15.04±0.88$^{###}$	9.00±0.66***
SOD (U/min/mg protein)	36.09±1.23	16.09±1.11$^{###}$	30.89±1.45***
CAT (U/min/mg protein)	9.00±0.24	4.11±0.21$^{###}$	7.88±0.19***
GSH (mg/g)	6.50±0.32	2.99±0.18$^{###}$	4.00±0.17**
AA (mg/g)	2.09±0.09	0.89±0.06$^{###}$	1.50±0.07***



Results are expressed as Mean±S.E.M, for n=8. Where, $^{#}$ P < 0.05, $^{##}$P<0.01 and $^{###}$P<0.001 compared with CON (rats fed with laboratory chow), *P < 0.05, **P<0.01, ***P<0.001 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

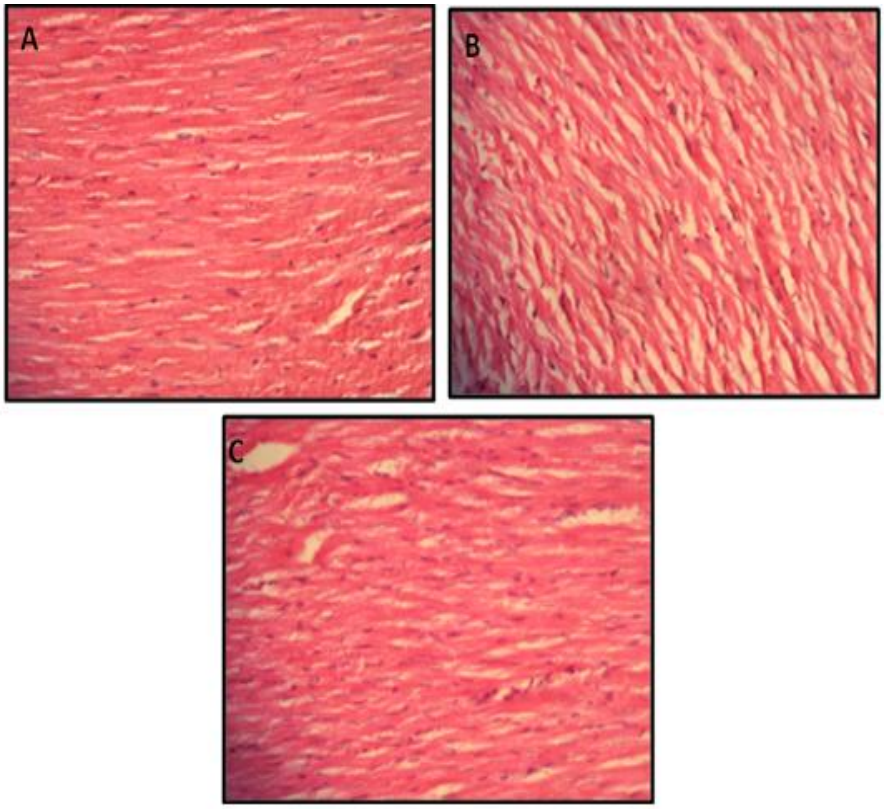
Table 5 and Figure 6: Effect of ARCE on hepatic lipid peroxidation and antioxidants.

	CON	HCD	HCD+ARCE
LPO (nmoles of MDA formed/ min/mg protein)	10.21±0.89	25.46±0.99$^{###}$	14.56±1.00***
SOD (U/min/mg protein)	50.00±3.22	23.45±3.12$^{###}$	41.11±2.98**
CAT (U/min/mg protein)	29.09±1.23	12.90±1.16$^{###}$	23.45±1.98***
GSH (mg/g)	6.09±0.32	2.11±0.21$^{###}$	4.56±0.11***
AA (mg/g)	5.40±0.12	2.01±0.11$^{###}$	4.13±0.13***



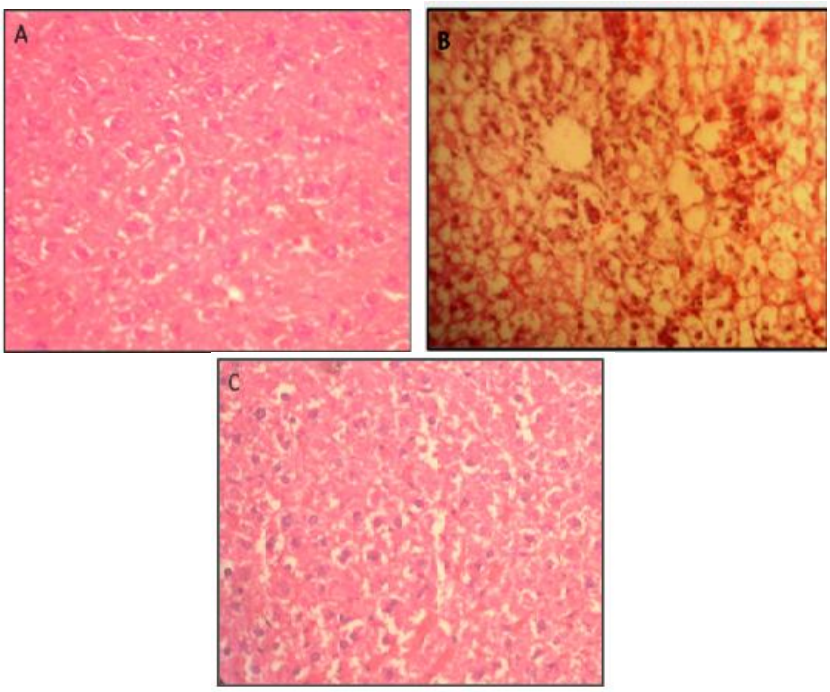
Results are expressed as Mean±S.E.M, for n=8. Where, $^{\#}$ P < 0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with CON (rats fed with laboratory chow), *P < 0.05, **P<0.01, ***P<0.001 and ^{ns} non significant compared with HCD (rats fed with high cholesterol diet).

Figure 7: Photomicrographs of heart of control (A), HCD (B) and HCD+ARCE (C) rats stained with hematoxyline and eosin (100X).



B indicates derangement of cardiac fibers with more spaces. Histoarchitecture of C is comparable to that of A with intact cardiac fibers and minimal spaces.

Figure 7: Photomicrographs of liver of control (A), HCD (B) and HCD+ARCE (C) rats stained with hematoxyline and eosin (100X).



Note the fatty depositions in pic B with fatty degeneration and nuclear condensation. Pic C looks similar to A with intact hepatocytes and no fatty deposits.

DISCUSSION

Supplementation of HCD to rats has been shown to develop hypercholesterolemia and hypelipidemia. Presence of cholic acid in the HCD increases lipid and cholesterol absorption from the intestine and prevents catabolism of cholesterol into bile acids (Wang et al., 1999), whereas, dietary propyl thio uracil decreases the hepatic LDL receptor expression and subsequent clearance of circulating lipoproteins (Hudig et al., 1994). These physiological changes leads to significant elevation in serum lipids and lipoproteins in the HCD diet fed rats. Other research groups have also reported on similar

results after feeding of HCD. In agreement with these reports, we recorded significant elevation in the serum and tissue lipids along with decrement in serum HDL level in the HCD diet fed rats (Parmar and Kar, 2007). However, these changes were significantly minimized by ARCE treatment. Studies have reported hypolipidemic and hypocholesterolemic potential of dietary anthocyanins (Park et al., 2010) and same can be held responsible for beneficial effects of ARCE against HCD induced hyperlipidemia and hypercholesterolemia.

Also, HCD diet induced hypercholesterolemia is known to significantly increase lipid load in cardiomyocytes and induce oxidative damage to hepatocytes amounting to cardiotoxicity and hepatotoxicity respectively (Küçükgergin et al., 2010). Previous studies have shown significant increment in the cardiac and hepatic lipids in the HCD diet fed rats (Suanarunsawat et al., 2010). In the present study, rats that were orally administered ARCE and fed HCD, recorded significant decrement in the tissue lipid load in cardiac and hepatic tissue. This was coupled with observed higher elimination of TC and TG through feces.

Other research group had reported that HCD increases the intracellular lipid load in cardiomyocytes and hepatocytes. This is the prime factor that further induces oxidative stress in cardiomyocytes or hepatocytes (Küçükgergin et al., 2010). Augmented mitochondrial fatty acid oxidation and lipid peroxidation results in imbalance between enzymatic as well as non-enzymatic antioxidant defence systems (Thounaojam et al., 2012). This leads to generation of mitochondrial ROS production and related cellular damages. Depletion of cellular antioxidants leads to situation of compromised endogenous antioxidant status. In the present study, ARCE supplementation significantly

prevented elevation in lipid peroxidation along with decrement in the activity levels of SOD and CAT and contents of GSH and AA in both liver and heart tissues. These observations can be attributed to a high content of anthocyanins in ARCE which has been reported as a potent free-radical scavenger and an antioxidant agent (Wu and Prior, 2005). Therefore, these observations suggest that ARCE extract is capable of restoring the antioxidant status of HCD fed rats indicating its potent cardioprotective and hepatoprotective properties.

Histopathological evaluation of cardiac tissue showed extensive myocyte membrane damage and myonecrosis. Also, histopathological evaluation of hepatic tissue showed intracellular lipid accumulation, and inflammatory changes in the HCD diet fed rats. However, these sets of changes were significantly minimized with co-supplementation of ARCE to HCD fed rats. These results further provide ample testimony to the results of serum and tissue lipid profile and enzymatic (SOD and CAT) and non-enzymatic (GSH and AA) antioxidants recorded herein.

Present study is the first scientific report on hypolipidemic/hypocholesterolemic potential of ARCE extract in HCD fed rats. Also, hepatoprotective and cardio protective potential of ARCE extract against HCD induced oxidative stress and tissue injury observed herein, further indicates at protective role of ARCE against tissue injury associated with hypercholesterolemia. It can be concluded from the present study that hypocholesterolemic, cardioprotective and hepatoprotective properties of ARCE extract observed herein can be attributed to high content of anthocyanins present in the extract.

Summary

This part of the study evaluates protective role of Anthocyanin rich red cabbage extract (ARCE) against high cholesterol diet (HCD) induced hypercholesterolemia and related cardiac and, hepatic oxidative stress in rats. ARCE (100mg/kg BW) treatment to HCD fed rats significantly prevented elevation of total lipids, circulating levels of cardiac and hepatic damage markers. Also, ARCE was able to prevent HCD induced depletion in the cardiac and hepatic antioxidants. Also, peroxidation of membrane lipids and, histopathological changes in cardiac and hepatic tissue were alleviated in ARCE supplemented rats. These results provide first scientific evidence for protective role of ARCE against HCD induced hypercholesterolemia and cardiac and hepatic oxidative stress.

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 flavylium 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 obtained 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)

↓

Extracted with absolute alcohol (2 ml) (keep for 1 hour)

Filter

↓

Filtrate dried

↓

Residue

↓

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 60ºC 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-C0A to mevalonate.

Reagents: -

- Saline arsenate solution: 1 g of sodium arsenate per liter of physiological saline.
- Dilute perchioric acid, 50 ml fliter.
- Hydroxylamine hydrochloride reagent for mevalonate: Mix equal volumes of hydroxylamine hydrochloride reagent and water freshly before use.

- 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% tissuehomogenate 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.}

Procedure:

1. **Deparrafinize the paraffin sections & rehydrate to water.**
    - Keep the slide in xylene for 10-15 min
    - Keep the slide in 100% alcohol for 1min
    - Keep the slide in 70% alcohol for 1min
    - Keep the slide in 50% alcohol for 1min
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 1hr 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.
9. Dehydrate through graded alcohol & clean in xylene.

    GRADED ALCOHOL
    - 50% alcohol for 1 time only.
    - 70% alcohol for 1 time only.
    - 100% alcohol for 1 time only.
10. Coverslip using permanent mounting medium.

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 fuscin	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 fuscin	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 Co A 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 Co A reductase activity compared to ATH and CON rats (Table 4 and Figure 4).

Histopathological observations of thoracic aorta

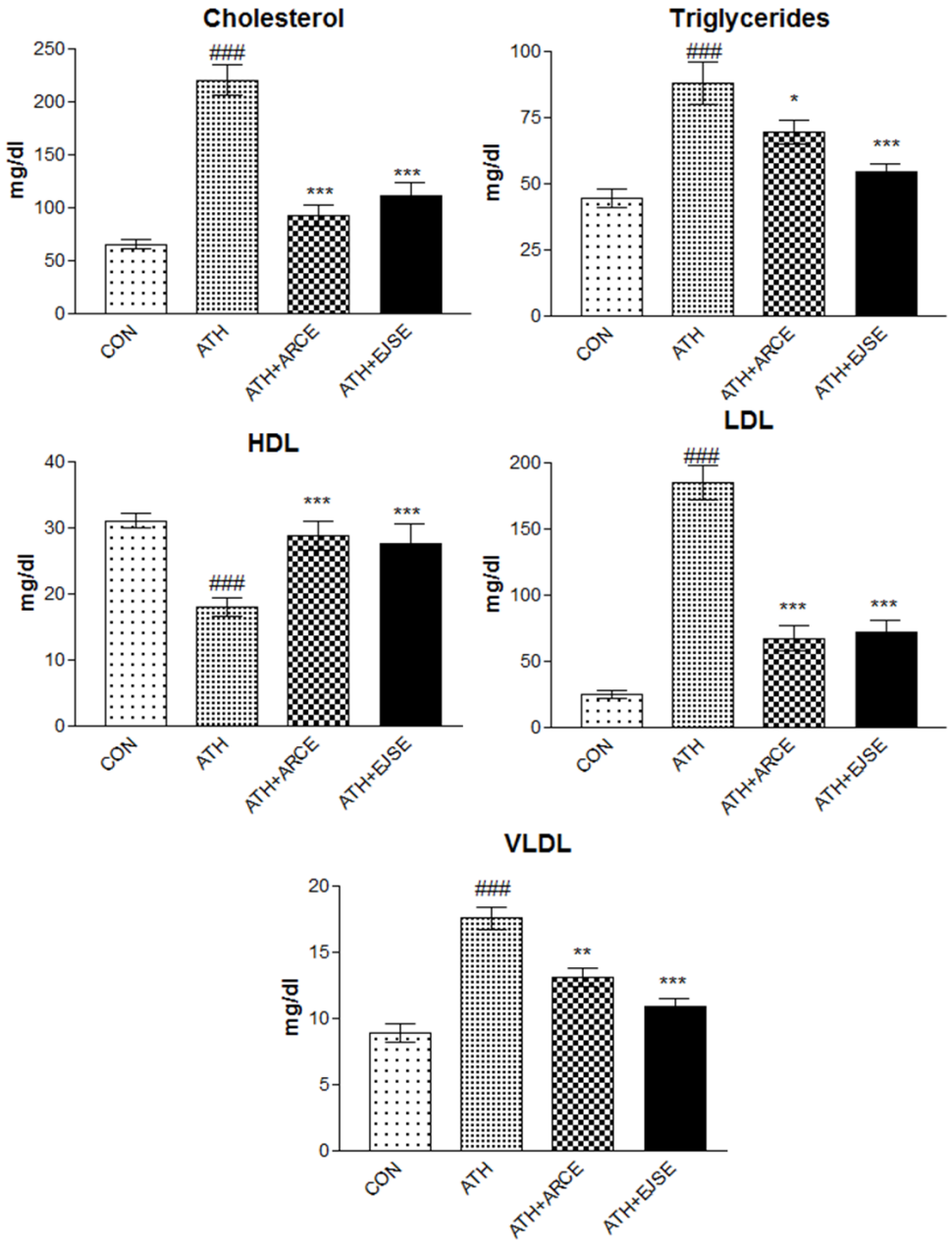
Photomicrographs of thoracic aorta section (HXE stained) of ATH rats revealed formation of a necrotic core due to accumulation of foam cells, deposition of lipids and loosening of smooth muscle cells of tunica media (Figure 5). Calcium deposition in tunica media and intima (von kossa staining) and derangement/defragmentation 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.

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

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<0.001compared 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<0.001compared 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.

	CON	ATH	ATH+ARCE	ATH+EJSE
Cholesterol (mg/g)	5.54±0.08	6.57±0.09^{ns}	9.08±0.08**	10.14±1.00***
Triglycerides (mg/g)	7.89±1.00	7.33±1.23^{ns}	12.33±1.00***	14.56±1.89***

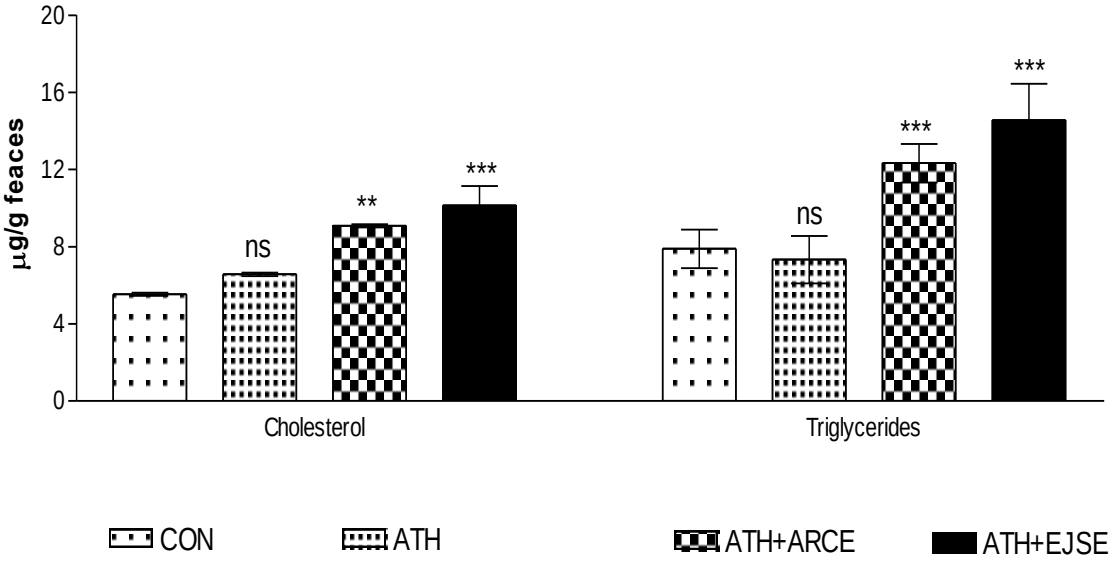
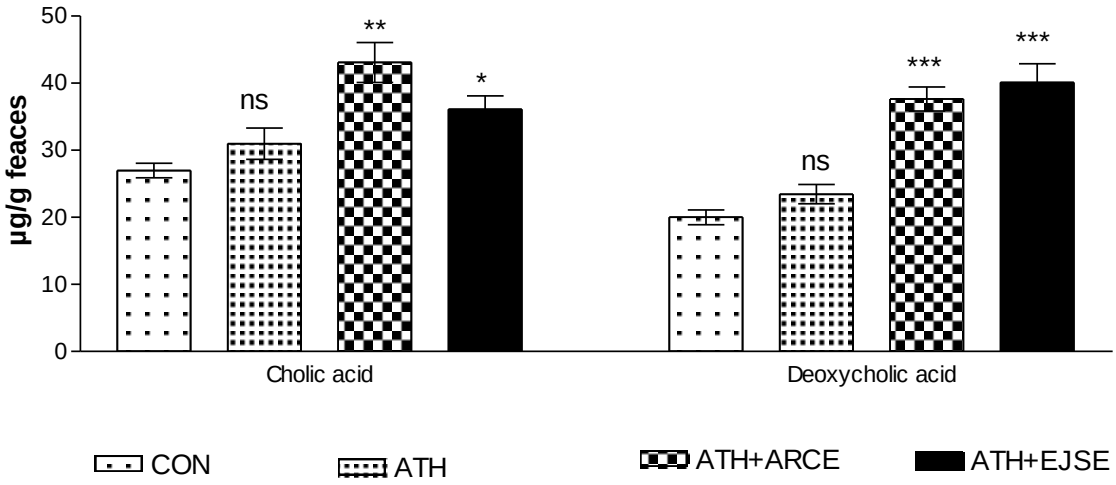


Table 3 and Figure 3: Effect of ARCE and EJSE on fecal bile acid contents in atherogenic diet (ATH) fed rats.

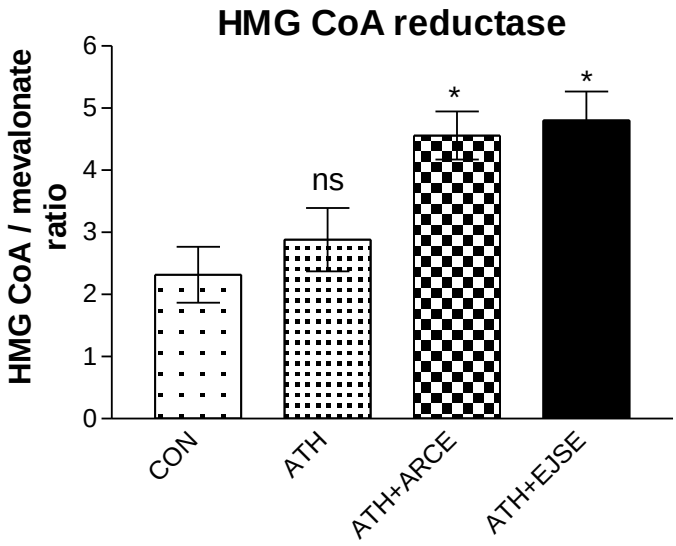
	CON	ATH	ATH+ARCE	ATH+EJSE
Cholic acid (µg/g feces)	26.98±1.09	30.98±2.34^{ns}	43.09±2.98**	34.56±1.34*
Deoxycholic acid (µg/g feces)	20.01±1.11	23.45±1.45 ^{ns}	37.65±1.78***	39.01±2.11***



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 Co A reductase activity.

	CON	ATH	ATH+ARCE	ATH+EJSE
HMG CoA reductase (HMG CoA / mevalonate ratio)	2.32±0.45	2.88±0.51	4.56±0.39	4.80±0.47



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 hematoxyline and eosin (100X).

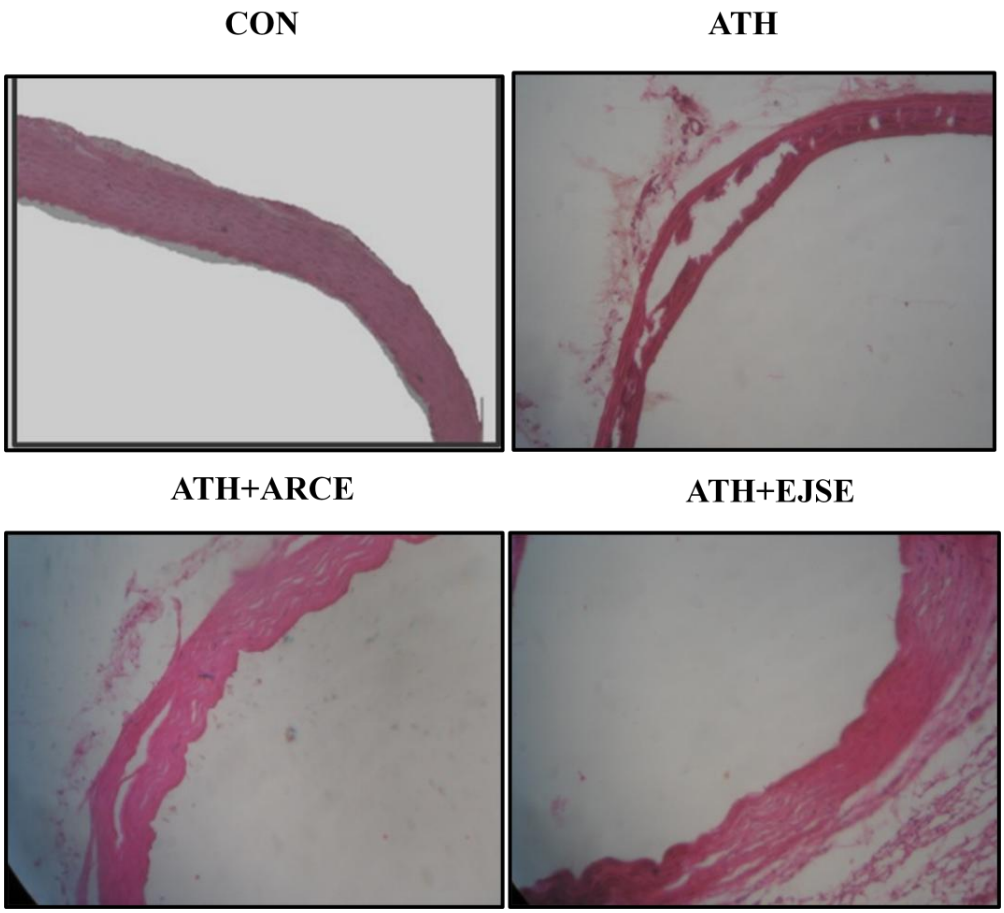


Figure 6: Photomicrographs of thoracic aorta of control, ATH, ATH+ARCE and ATH+EJSE rats stained with von kossa (100X).

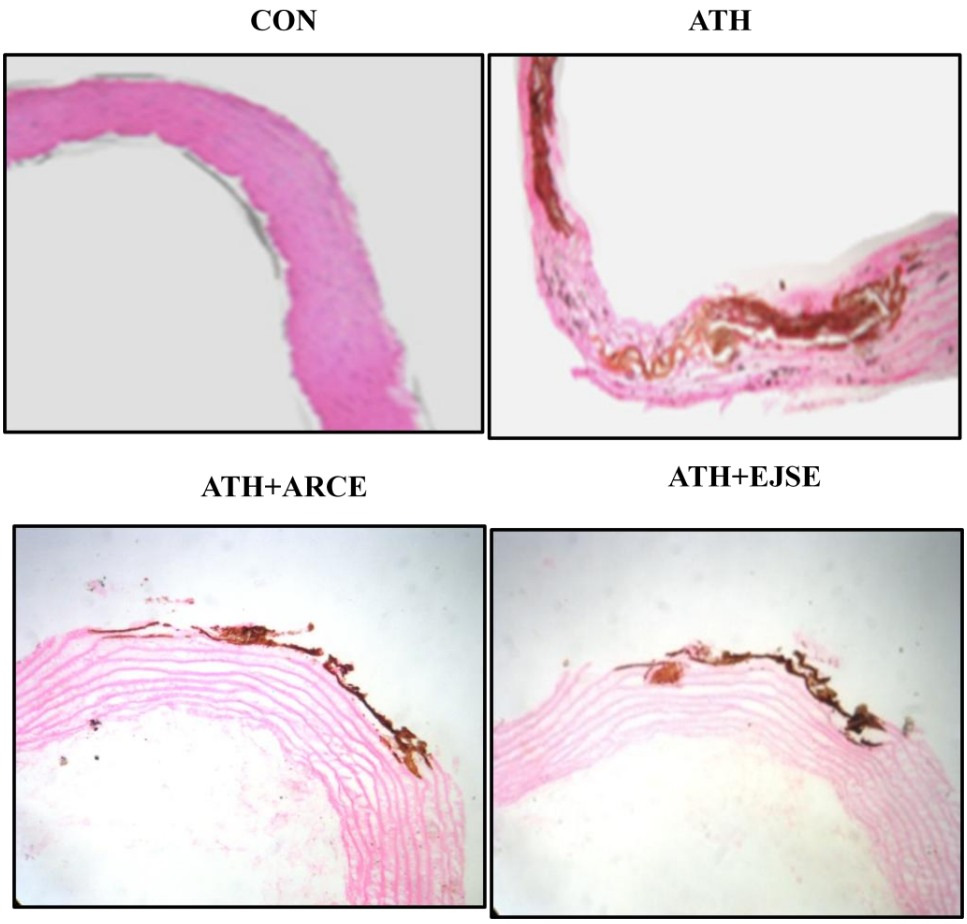
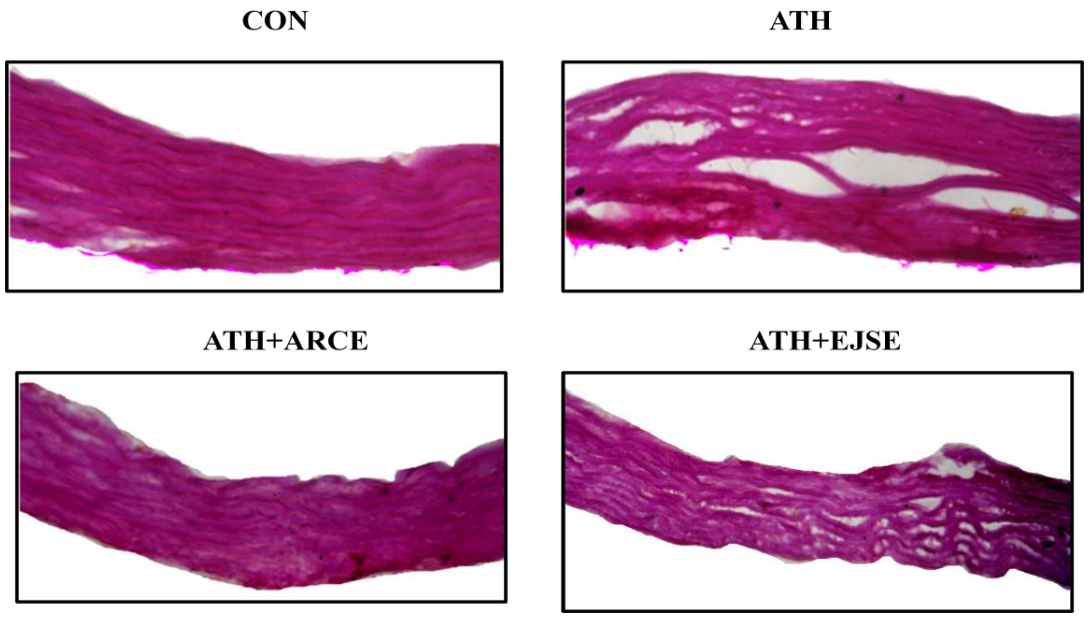


Figure 7: Photomicrographs of thoracic aorta of control, ATH, ATH+ARCE and ATH+EJSE rats stained with Weigert's stain (200X).



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 *de novo* cholesterol biosynthesis and their subsequent catabolism into bile acids.

The *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, *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.

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CONCISE SUMMARY

of the thesis entitled

Toxicological evaluation and therapeutic potential of *Brassica oleracea* and *Eugenia jambolana*

submitted to

The M. S. University of Baroda, Vadodara-390 002, Gujarat, INDIA

For the degree of

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In

Zoology

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CONCISE SUMMARY

Phytochemicals are substances that plants naturally produce to protect themselves various exogenous against such as bacteria, viruses, and fungi. There has been a lot of interest in phytochemicals recently because many of them can help to slow the aging process and reduce the risk for cancer, heart disease and other chronic health conditions. Phytochemicals may inhibit induction and proliferation of cancer, regulate inflammatory and immune response and protect against oxidative modification of lipids Hence, a major role of the phytochemicals is in providing much needed protection against oxidation of biomolecules. Recently, red cabbage (ARCE) has attracted much attention because of its physiological functions and applications. Also, Jamun (*Eugenia jambolana*; EJSE) is used extensively in the various traditional systems of medicine like in the Ayurveda, Unani, Siddha, in the Sri Lankan, in the Tibetan and in the Homeopathy systems of alternative and complementary medicine.

Objectives and Work envisaged in a nutshell

1. Acute and subchronic toxicity evaluations of *Eugenia jambolana* seeds (EJSE) and *Brassica oleracea* (ARCE) leaf extracts.

Preparation of Anthocyanin rich extracts of the above mentioned herbals.

To assess acute toxicity of these herbal extracts in mice model.

To assess subchronic toxicity of these herbal extracts in rat model.

2. Assessment of therapeutic potentials of *Eugenia jambolana* seeds (EJSE) and *Brassica oleracea* (ARCE) leaf extracts against experimentally induced cardiac and hepatic oxidative stress.

Hypothesis to be tested: Can anthocyanin rich extracts of these herbals successfully mitigate isoproterenol induced myocardial necrosis or CCl_4 induced hepatotoxicity in rats.

3. Assessment of *Eugenia jambolana* seeds (EJSE) and *Brassica oleracea* (ARCE) leaf extracts in mitigating experimentally induced atherosclerosis.

Hypothesis to be tested: Can these herbals successfully mitigate PTU + vitamin D3 + high fat diet induced atherosclerosis in Sprague dawley rats.

Salient findings

The aim safety evaluation of ethanolic seed extract of EJSE and ARCE using acute and sub-chronic toxicity assays in Swiss albino mice as per OECD guidelines. Mice administered a single dose (1000, 2000, 3000, 4000 or 5000mg/kg BW) of EJSE/ARCE and changes in patterns of behavior and mortality were observed. Also, plasma levels of metabolites, hepatic, cardiac and renal function markers, electrolytes, blood count and histopathology of major organs were monitored in mice chronically treated with EJSE/ARCE (1000, 2000 or 3000 mg/kg BW) for 28 days. Since no mortality was recorded in the acute toxicity evaluation up to a dose of 5000mg/kg bodyweight of EJSE/ARCE, LD50 was assumed to be >5000mg/kg BW. In the sub-chronic toxicity evaluation, no adverse observations were recorded in mice administered with 2000mg/kg EJSE; however at 3000 mg/kg dose, moderate increase in the plasma

levels of urea and creatinine was observed. Hence, LOAEL for EJSE/ARCE was found to be 3000 mg/kg BW and NOAEL was adjudged as 2000 mg/kg BW. Chronic administration of ARCE extract (1000, 2000 and 3000 mg/kg bodyweight) for 28 days also did not register any significant alterations in fluid intake, organ weights, plasma lipid profile, plasma CK-MB, Lactate dehydrogenase, aspartate transaminase, alanine transaminase, creatinine, electrolytes and calcium levels and the total blood count showed a non significant change. However, significant reduction in bodyweight gain, food intake, red blood cell count and hemoglobin content along with higher alkaline phosphatase, billirubin and urea levels were observed in mice treated with 3000 mg/kg of ARCE for 28 days. Since, there was no mortality up to a dose of 5000 mg/kg bodyweight, 50% lethal dose (LD_{50}) could not be determined and hence, it can be assumed that, LD_{50} of ARCE extract is > 5000mg/kg. No observable adverse effect level (NOAEL) dose of the ARCE extract was found to be 2000mg/kg BW. Hence, consumption of ARCE extract for various medicinal purposes is safe. These results can be of relevance for the scientific fraternity as well as laymen who consume this vegetable or its phytochemical preparation.

In this part of the study, protective role of Anthocyanin rich red cabbage extract (ARCE) against high cholesterol diet (HCD) induced hypercholesterolemia and related cardiac and, hepatic oxidative stress in rats. ARCE (100mg/kg BW) treatment to HCD fed rats significantly prevented elevation of total lipids, circulating levels of cardiac and hepatic damage markers. Also, ARCE was able to prevent HCD induced depletion in the cardiac and hepatic antioxidants. Also, peroxidation of membrane lipids and, histopathological changes in cardiac and hepatic tissue were alleviated in ARCE

supplemented rats. These results provide first scientific evidence for protective role of ARCE against HCD induced hypercholesterolemia and cardiac and hepatic oxidative stress. In another study, protective role of *Eugenia jambolana* seed extract (EJSE) on high cholesterol diet (HCD) induced hyperlipidemia/hypercholesterolemia amounting to cardiac and hepatic oxidative stress are assessed in this study. Serum markers of cardiac and hepatic damage, total lipid profile, lipid peroxidation, antioxidant status and histopathological changes in cardiac and hepatic tissue have been assessed in control, HCD fed and HCD+EJSE treated (100mg/kg BW, *p.o.*) rats. It was observed that co-supplementation of EJSE to HCD fed rats significantly ($p<0.05$) minimized elevation in the serum and tissue lipid profiles decrement in the HDL levels. Also, EJSE successfully ameliorated HCD induced cardiac and hepatic oxidative stress ($p<0.05$) and histopathological damage. It can be concluded that, EJSE has the potential of preventing HCD induced experimental hypercholesterolemia and related cardiac and hepatic oxidative stress. It was inferred from this study that EJSE is successfully able to alleviate HCD induced hypercholesterolemia and oxidative damage to cardiac and hepatic tissues of experimental rats.

In final phase of the study, anti-atherogenic potential of anthocyanin rich ARCE and EJSE were assessed 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.

Conclusion

It can be concluded from the present study that pretreatment of extracts of ARCE / EJSE are potent in ameliorating myocardial and hepatic injuries and prevent onset and progression of atherosclerosis in relevant experimental models. This can be attributed to their already established anti-oxidant and free radical scavenging potentials that minimize oxidative damage and related physiological changes. Thus further justifies our claim of their cardioprotective and hepatoprotective potentials. These attributes add value to the already established therapeutic potentials and hence, it can be inferred from the present study that their supplementation has multiple therapeutic potentials in attenuating complex physiological ailments.

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