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Full Length Research Paper

Effect of *Blepharis maderaspatensis* L. Roth. extracts on serum lipids in Triton WR-1339 and high cholesterol diet induced hyperlipidemia in rats

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The present study evaluates anti-hyperlipidaemic and anti-atherogenic activities in Triton WR-1339 and high cholesterol diet induced hyperlipidemia in rat models. Chloroform, ethyl acetate and ethanol extracts of whole plant of *Blepharis maderaspatensis* were evaluated for their anti-hyperlipidaemic and anti-atherogenic activities using Triton WR-1339 induced hyperlipidaemic rats (acute study) and high cholesterol diet induced (chronic study) experimental models. Hyperlipideamia was developed by intraperitonial injection of Triton WR-1339 (200 mg/kg body weight) in acute study and feeding with cholesterol rich diet in chronic study. The animals were divided into various groups and intragastric administration of various extracts of *B. maderaspatensis* (100 mg/kg) body weight was given in both models. After the completion of the treatment, they were evaluated for serum total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), atherogenic index (AI), coronary risk index (CRI) and were compared with the rats treated with simvastatin (20 mg/kg) of the body weight. Pre-treatment of ethanol extracts of *B. maderaspatensis* significantly reduced the serum total cholesterol, triglycerides, LDL, VLDL, AI and CRI as comparable with simvastatin.

Key words: Serum lipids, atherogenic index, cholesterol diet, coronary risk index, Triton WR-1339, *Blepharis maderaspatensis*, high cholesterol diet.

INTRODUCTION

Diseases of the cardiovascular system are the most common cause of death in many developed countries (Epstein, 1992). It is now established that hyperlipidaemia represents a major risk factor for the premature development of atherosclerosis and its cardiovascular complications. Current nutritional, metabolic and toxicological research is extensively involved in elucidating risk factors for hypercholesterolemia and its pathologic consequences which involve the cardiovascular system, the brain and other organs. In experimental studies, hypercholesterolemia is usually produced by dietary and/or genetic manipulations. However, hypercholesterolemia also occurs as a toxic response to certain synthetic detergents. Triton WR-1339 has a direct inhibitor effect on the lipoprotein lipase in muscle and adipose tissue (Kourounakis et al., 2002; Lomnicky et al., 1998). A diet high in cholesterol content is a major environmental contributor to an unbalanced lipoprotein metabolism and is associated with an increased prevalence of atherosclerosis. Epidemiological, clinical, genetic and experimental studies indicate that high serum levels of low density lipoprotein (LDL) cholesterol are associated

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with atherosclerosis and an increased risk of coronary heart disease (Levine et al., 1995; Ballantyne, 1998; Krieger, 1998). The diet-induced hypercholesterolemia animal model has long been used for the assessment of agents with beneficial effects on cholesterol.

Blepharis maderaspatensis L. Roth (Acanthaceae) often used in India by hyperlipidaemic subjects as an alternative therapeutic tool to treat hyperlipidaemia is being used in folk medicine as a diuretic and wound healing agent. It is also used to treat the disorders such as boils, bone fracture, diarrhea and lactation (Ayyanar et al., 2008). However, there are no scientific reports on the anti-hyperlipidaemic activity of these plants and hence the present study is aimed to assess the possible hypolipidaemic activity of various extracts of *B. maderaspatensis* on Triton WR-1339 induced and high cholesterol diet induced hyperlipidaemic rats.

MATERIALS AND METHODS

Plant material

The plant was collected during the month of November, 2009 from the Western Ghats of Srivilliputtur, India, and authenticated by Mr. V. Chelladurai, Research Officer of Botany, CCRAS, Government of India. The voucher specimen (no. CED/Herbarium/B 0008) has been deposited in the herbarium maintained by Centre for Endogenous Development, Department of Pharmacognosy, Arulmigu Kalasalingam College of Pharmacy, India. The plant materials were dried at room temperature under shade for 15 days and then it was ground into coarse powder by electrical grinder. The powdered plant material was stored in a dessicator until extraction.

Plant extraction and fractionation

About 900 g of air dried coarse powder of leaves of *B. maderaspatensis* was soaked in ethanol for 7 days with occasional shaking. The ethanol soluble residue was filtered off and concentrated under vacuum at room temperature using rotary evaporator which yielded a dark greenish semisolid material. The concentrated extract was mixed with small quantity of water and sequentially fractionated with chloroform, ethyl acetate and ethanol and the fractions collected were concentrated separately (El-Mahmood and Doughari, 2008).

Phytochemical Screening

Saponins

The extracts (300 mg) were boiled in 5 mL water for 2 min. Then the mixture was cooled and mixed vigorously, and left to stand for 3 min. The formation of froth indicates the presence of saponins.

Tannins

The extracts (300 mg/mL) were added to 10 mL of distilled water, filtered and mixed with 2 mL 5% $FeCl_3$ solution. Bluish black precipitation indicates the presence of tannins (Igbinosa et al., 2009).

Terpenoids

The extracts (300 mg) were mixed with 5 mL chloroform and warmed at 80°C for 30 min. Few drops of concentrated sulfuric acid was added and mixed well. The appearance of a red color indicates the presence of terpenoids.

Alkaloids

The extracts (300 mg) were digested with 2 M HCl and filtered. Few drops of Mayor's reagents/ Wagner's reagent/Dragondroff's reagent were added to each 1 ml of the filtrate. The appearance of Creamish precipitate/Browinsh-red precipitate/orange precipitate obtained indicates the presence of alkaloids.

Flavonoids

The extracts (300 mg) were dissolved in 10 mL of ethanol and filtered. The filtrate (2 mL) was treated with conc. HCl and magnesium ribbon. Pink-tomato red color indicated the presence of flavonoids (Harborne, 1973).

Experimental animals

Healthy Wistar albino male rats weighing about 150 to 250 g were procured from Perundurai Medical College, Perundurai, Erode district, Tamilnadu, India. They were fed with standard diet and water *ad libitum*. They were housed in polypropylene cages maintained under standard lab conditions (12/12 h light/dark cycle; 25±1°C, 35 to 60% relative humidity). Experiments were conducted in accordance with the internationally accepted principles of laboratory animal use and care. The study protocol was approved by the Institutional Animal Ethics Committee (509/02/C/CPCSEA/2002) for the purpose of control and supervision of animals (CPCSEA, New Delhi.) conducted on 5th May, 2010.

Triton induced model of hyperlipideamia

In this model, overnight fasted male rats were randomly divided into 6 groups containing 6 rats in each group such that the difference in the weights of rats within and between groups does not exceed ±20% of the average weights of the rats. Group 1 rats served as the untreated control and were treated with 10 and 1 ml/kg of 0.5% w/v methylcellulose in distilled water orally and intraperitoneally, respectively. Group 2 (model control) rats were pre-treated orally with 10 ml/kg of 0.5% methylcellulose (w/v) in distilled water for 1 h before receiving 200 mg/kg of Triton WR-1339 (Sigma Chemical Company, St. Louis, U.S.A.) given via the intraperitoneal route. Rats in Groups 3, 4, 5 and 6 were also orally pre-treated with 20 mg/kg of simvastatin, 100 mg/kg of chloroform extract, ethyl acetate and ethanol extract of *B. maderaspatensis* dissolved in 10 ml/kg of distilled water 1 h before intraperitoneal injection of 200 mg/kg of Triton WR-1339 (Khanna et al., 2002; Banerjee et al., 2006; Gajbhiye et al., 2008). Twenty-four hours (24 h) after treatment, rats were sacrificed under diethyl ether anaesthesia and blood samples for serum analysis were obtained directly from the heart chamber (Adejuwon et al., 2010). The rats were also checked for any inflammation and acute phase response at the site of injection of Triton WR-1339.

Hypercholesterol diet induced model of hyperlipideamia

The rats under investigation were housed under standard

Table 1. Effect of 20 mg/kg simvastatin and 100 mg/kg chloroform, ethyl acetate and ethanol extracts of *B. maderaspatensis* on serum total cholesterol, triglyceride, HDL-c, LDL-c and VLDL-c in normal and Triton WR-1339 induced hyperlipidaemic rats.

Group	TC (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal control (Group 1)	110.5 ± 3.5	91.44 ± 2.7	39.8 ± 2.3	51.8 ± 1.8	18.9 ± 1.1
Triton WR-1339 control (Group 2)	192.7 ± 5.4^{b}	188.45 ± 6.2^{b}	41.7 ± 2.3	122.1 ± 4.8^{b}	28.9 ± 1.1^{b}
Simvastatin treated (Group 3)	127.3 ± 3.4^{d}	118.32 ± 4.2^{b}	54.5 ± 2.2^{d}	48.7 ± 1.8^{c}	24.1 ± 1.1^{d}
Chloroform extract treated (Group 4)	149.1 ± 2.2^{b}	176.36 ± 5.6^{b}	43.7 ± 2.1^{d}	78.1 ± 1.9^{d}	27.3 ± 1.7^{b}
Ethyl acetate extract treated (Group 5)	136.8 ± 6.9^{d}	168.88 ± 2.4^{d}	46.2 ± 1.8^{b}	63.9 ± 2.6^{b}	26.7 ± 1.2^{b}
Ethanol extract treated (Group 6)	131.9 ± 4.3^{b}	101.32 ± 5.1^{b}	56.8 ± 2.4^{b}	51.2 ± 1.2	23.9 ± 1.2^{d}

The data were expressed as mean ± S.E.M. a, A significant decrease at $p < 0.001$, when compared to Group 1 value; b, a significant increase at $p < 0.01$, when compared to Group 1 value; c, a significant decrease at $p < 0.01$, when compared to Group 1 value; d, a significant increase at $p < 0.05$, when compared to Group 1 value.

conditions (temperature 25±1°C, humidity 60±10%, light from 6 am to 6 pm) with free access to water. In this model, male rats were randomly divided into 6 groups of 6 rats in each group such that the difference in the weights of rats within and between groups does not exceed ±20% of the average weights of the rats. Group 1 (control) was fed with normal rat chow, which was obtained from Lipton India Ltd [composition: protein (~14%), fat (~10%) and carbohydrate (~76%)]. Other groups of animals were fed with hypercholesterolemic diet (HCD) contained normal rat chow with the supplement of cholic acid (1%), cholesterol (2%) and cooking oil (5.5%) as described elsewhere (Yan et al., 2006; Li et al., 2009). Group 2 (model control) rats were treated orally with 10 ml/kg of 0.5% methylcellulose (w/v) in distilled water. Rats in Groups 3, 4, 5 and 6 were also orally treated with 20 mg/kg of simvastatin, 100 mg/kg of chloroform extract, ethyl acetate and ethanol extract of *B. maderaspatensis* dissolved in 10 ml/kg of distilled water for 4 weeks. At the end of 4 weeks treatment period, rats were fasted overnight, sacrificed by cervical dislocation and tissue samples (blood and aorta) were collected (Kwok et al., 2010).

Blood collection and biochemical assays

At the termination of each experiment, between 07:00 and 09:00 h, overnight fasted rats had their blood samples collected directly from the heart chamber under inhaled diethyl ether anaesthesia. Blood samples were collected into plain sample bottles and allowed to clot at room temperature for 4 h before they were centrifuged using Acmas Technocracy Laboratory Centrifuge (Model AC 4105, India) at 10,000 × g at the same temperature for 20 min to separate the sera. Then they were analyzed for serum triglycerides (TG), total cholesterol (TC),high density lipoprotein (HDL-c) and low density lipoproteins (LDL-c) using Automated Clinical System analyzer (Roche Diagnostic Systems Inc**,** COBAS MIRA Plus, Switzerland).

Determination of atherogenic index (AI) and coronary risk index (CRI)

AI and CRI were calculated as:

AI = (TC-HDL- C)/HDL-C (Makni et al., 2008) and CRI = TC (mg/dl)/HDL-C (mg/dl) (Alladi and Shanmugasundaram, 1989), respectively.

Histopathological studies

Rat aorta from 2 animals from each group selected randomly were excised after sacrificing the animals under ether anaesthesia and fixed in 10% formo-saline. Fixed tissues were completely dehydrated in absolute ethanol and processed routinely for embedding in paraffin wax. From these 5 µm sections were prepared and stained with haematoxylin-eosin dye. Stained slides were photographed using light microscope (Labomed Microscope, India) (Chidambaram et al., 2007; Mahbubeh et al., 2011).

RESULTS

Phytochemical analysis

The phytochemical screening of chloroform, ethyl acetate and ethanol extracts of *B. maderaspatensis* revealed the presence of tannins, saponins, steroids, terpenoids and flavonoids in all the extracts. Alkaloids were absent in all the extracts.

Effect of oral pre-treatment with 100 mg/kg of various extracts of *Blepharis maderaspatensis* on serum lipids, coronary artery risk and atherogenic indices in Triton WR-1339 induced hyperlipidaemic rats

The plasma TC and triglyceride levels of all groups after 24 h are shown in Table 1. In comparison with the normal control group (Group I), Triton WR-1339 is found to have a marked increase of plasma TC and triglyceride levels of Triton WR-1339 control group (Group 2) and 100 mg/kg of chloroform extract, ethyl acetate extract and ethanol extract treated (Groups 3, 4, 5 and 6) rats. In fact, 24 h after Triton administration, the increase of plasma TC concentration were 174% in Group 2, 115% in Group 3, 134% in Group 4, 123% in Group 5 and 119% in Group 6 than that of normal control (Group 1). Triglycerides levels

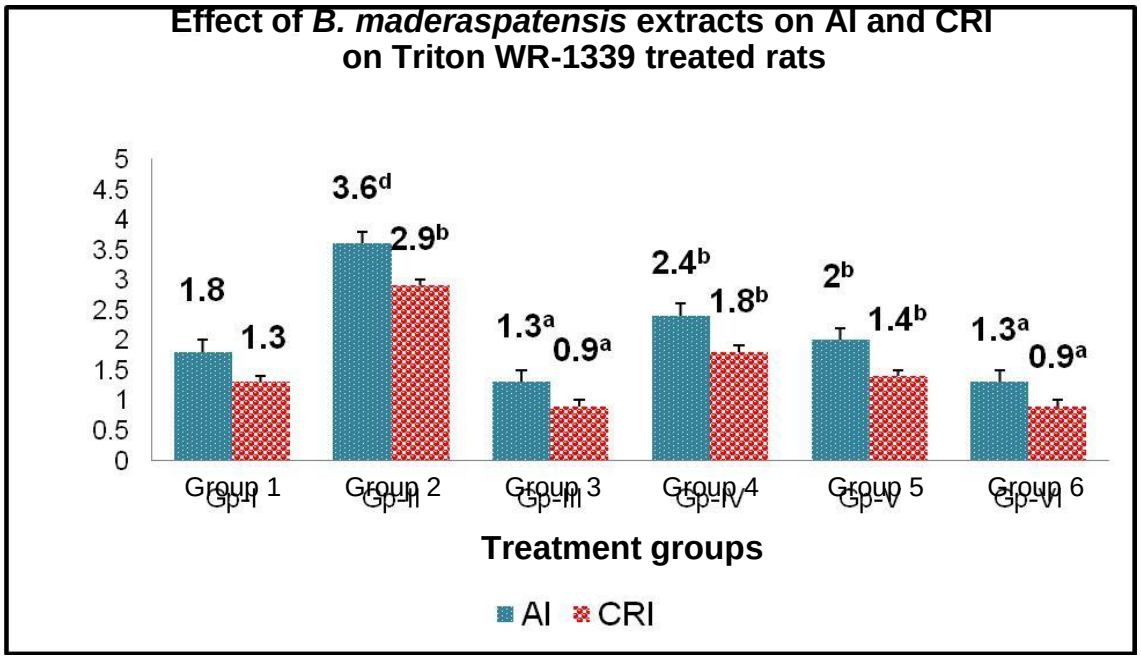

Figure 1. Effect of 20 mg/kg simvastatin and 100 mg/kg chloroform, ethyl acetate and ethanol extracts of *B. maderaspatensis* on AI and CRI in normal and Triton WR-1339 induced hyperlipidaemic rats. a, A significant decrease at $p < 0.001$, when compared to Group 1 value; b, a significant increase at $p < 0.01$, when compared to Group 1 value; c, a significant decrease at $p < 0.01$, when compared to Group 1 value; d, a significant increase at $p < 0.05$, when compared to Group 1 value.

Table 2. Effect of 20 mg/kg simvastatin and 100 mg/kg chloroform, ethyl acetate and ethanol extracts of *B. maderaspatensis* on serum total cholesterol, triglyceride, HDL-c, LDL-c and VLDL- c normal and cholesterol induced hyperlipidaemic rats.

Group	TC (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal control (Group 1)	130.6 ± 7.4	102.3 ± 3.6	45.2 ± 2.2	61.2 ± 3.1	24.2 ± 1.1
Hypercholesterolmic control untreated (Group 2)	223.1 ± 9.1^{a}	267.2 ± 8.7^{a}	24.7 ± 1.5^{e}	155.5 ± 4.6^{a}	43.0 ± 2.4^{b}
Simvastatin treated (Group 3)	153.6 ± 4.2^{d}	141.72 ± 3.4^{c}	40.1 ± 1.1	82.4 ± 3.1^{d}	31.1 ± 1.3^{b}
Chloroform extract treated (Group 4)	210.5 ± 4.8^{c}	195.53 ± 2.7^{c}	25.1 ± 0.8^{e}	145.3 ± 4.5^{c}	40.1 ± 2.3^{b}
Ethyl acetate extract treated (Group 5)	182.6 ± 8.2^{c}	181.18 ± 7.1^{c}	29.1 ± 1.7^{e}	115.4 ± 4.3^{c}	38.1 ± 1.5^{b}
Ethanol extract treated (Group 6)	160.3 ± 6.8^{u}	158.16 ± 4.4^{u}	39.3 ± 2.1	87.6 ± 2.3^{u}	33.4 ± 1.2^{u}

The data were expressed as mean ± S.E.M. a, A significant increase at $p < 0.001$, when compared to Group 1 values; b, a significant increase at $p < 0.05$, when compared to Group 1 values; c, a significant decrease at $p < 0.05$, when compared to Group 2 values; d, a significant decrease at $p < 0.01$, when compared to Group 2 values; e, a significant decrease at $p < 0.05$, when compared to Group1 values.

were also elevated by 206, 129, 192, 184 and 110% in Groups 2, 3, 4, 5 and Group 6, respectively. HDL and LDL-cholesterol concentrations are also shown in Table 1. There was not much significant change in the HDL levels except there was a mild increase in Groups 3 and 6 when compared to Group 1. A significant increase on LDL-cholesterol levels have occurred in 24 h from Triton injection. LDL-cholesterol concentration in Group 2 was 235% higher than those in normal control grouped animals after 24 h of Triton injection. Also, the increase of this parameter was reduced as that of normal control group in Groups 3 and 6, while there was a mild reduction in case of Groups 4 and 5, 24 h after the beginning of the experiment. There was no inflammation and acute phase response produced at the site of Triton WR-1339 injection and the animals did not show any difference in the intraperitoneal site.

Figure 1 shows the changes of AI and CRI of control and treated mice.

Effect of oral treatment with 100 mg/kg of various extracts of *Blepharis maderaspatensis* on serum lipids, coronary artery risk and atherogenic indices in diet-induced hypercholesterolemia rats

The serum TC and triglyceride levels of diet induced hypercholesterolemic rats and normal rats are shown in Table 2. In comparison with the normal control group (Group 1), the diet induced hypercholesterolemic rats

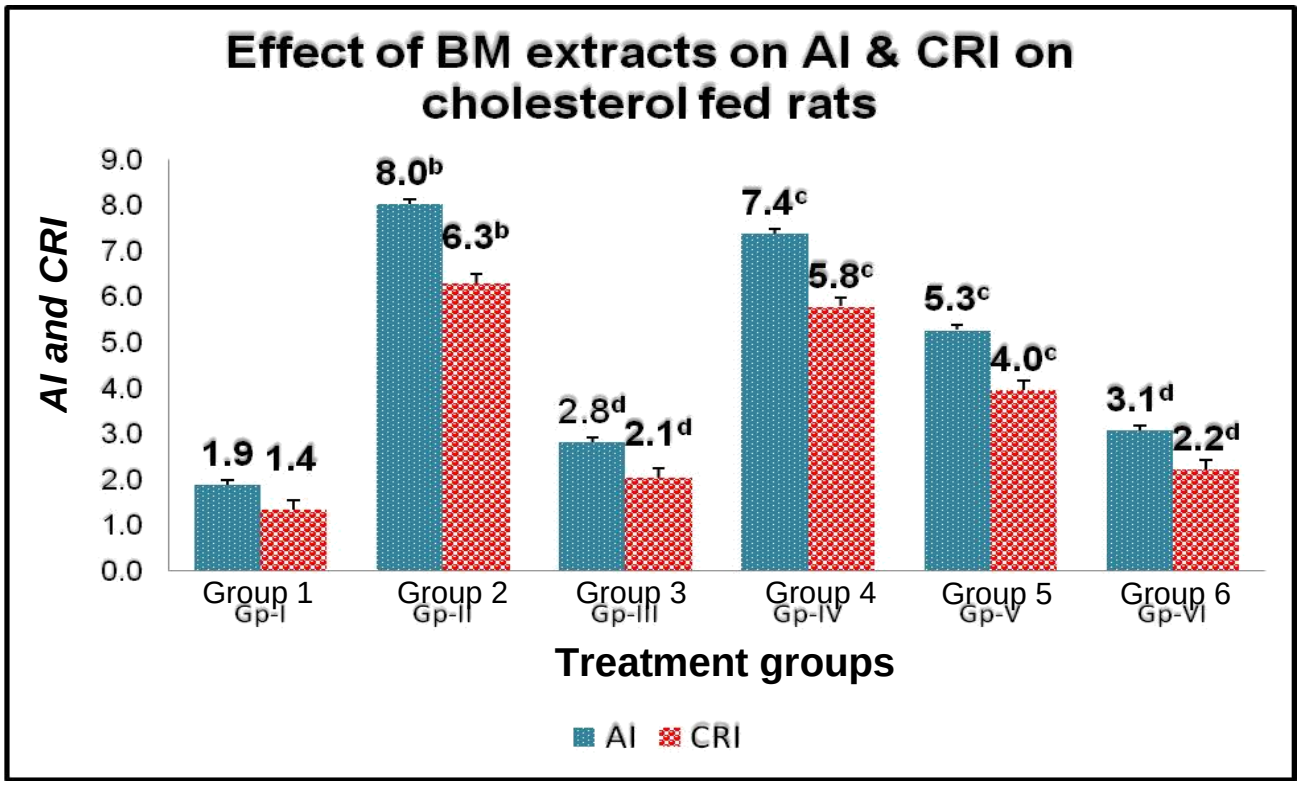

Figure 2. Effect of 20 mg/kg simvastatin and 100 mg/kg chloroform, ethyl acetate and ethanol extracts of BM on AI and CRI in normal and cholesterol induced hyperlipidaemic rats. a, A significant decrease at $p < 0.001$, when compared to Group I value; b, a significant increase at $p < 0.01$, when compared to Group I value; c, a significant decrease at $p < 0.01$, when compared to Group I value; d, a significant increase at $p < 0.05$, when compared to Group I value.

showed a marked alteration of plasma TC and triglyceride levels in Groups 3, 4, 5 and 6 rats. In fact, the increase of plasma cholesterol concentrations were 170% in Group 2, 117% in Group 3,161% in Group 4, 139% in Group 5 and 122% in Group 6 than that of normal control (Group 1). The triglycerides levels were also elevated by 261, 138, 191, 178 and 154% in Group 2, 3, 4, 5 and 6, respectively. HDL and LDL-cholesterol concentrations are also shown in Table 2. There was not much significant change in the HDL levels. While a significant increase on LDL-cholesterol levels have occurred after 4 weeks of high cholesterol diet. LDL-cholesterol concentration in Group 2 was 254% higher than those in normal control group animals after 4 weeks of consuming high cholesterol diet. Also, the increase of this parameter was reduced as that of normal control group in Group 3 and 4, while there was a mild reduction in case of Groups 4 and 5.

Figure 2 shows the changes of AI and CRI in control and treated mice. It is apparent from these results that the rat fed with high cholesterol diet had been significantly altered with the cardiovascular risk markers. The AI was statistically increased in Group 2 (422%) when compared with values of the rats fed with normal diet. The AI values were reduced in Groups 3 and 6, in comparison with the normal diet fed rats. The same type of activity was observed with that of CRI levels also and the Group 2 rats had an increased level of 449%, while the values were found to be lower than the normal control rats in the Groups 3and 6.

Histopathology studies of aorta of normal and hyperlipidaemic diet treated rats

The aorta were stained using haematoxylin–eosin dye and the sections when focused under light microscope showed Lumen, Intima Tunica, Media tunica in Group 1 normal rats. The hyperlipidaemic control rats revealed spaces with the layers of intima and media showing slight thickening of intima tunica and moderate clusters of lipid laden, macrophages and cholesterol clefts. The hyperlipidaemic rats treated with chloroform and ethyl acetate extracts of *B. maderaspatensis* showed comparable improvement in the spaces with the layers of intima and media. While the hyperlipidaemic rats treated with the standard drug simvastatin and ethanol extracts of *B. maderaspatensis* showed normal lumen, intima tunica and media tunica and there was no macrophage infiltration.

DISCUSSION

Hypercholesterolemia, which modifies cholesterol metabolism, is widely recognized as a major risk factor

leading to mortality. Natural compounds have been used in the treatment of various chronic human pathological conditions. Thus, the therapeutic benefits of plant extracts without side effects have been the focus of many extensive studies (Arafa, 2005; Yokozawa et al., 2006; Yokozawa et al., 2003). The non-ionic detergent, Triton WR-1339, has been widely used to block the uptake of triacyl glycerol-rich lipoproteins from plasma by peripheral tissues in order to produce acute hyperlipidaemia in animal models which are often used for a number of objectives, in particular for screening natural or chemical hypolipidaemic drugs (Schurr et al., 1972). With this aim, many medicinal plants, such as *Phylanthus niruri* L. (Phyllanthaceae), have been assessed for their hypolipidaemic activity in a Triton WR-1339 induced hyperlipidaemic model. These investigations reveal that parenteral administration of Triton WR-1339 induced hyperlipidaemia in adult rats. Maximum blood cholesterol and triglyceride levels were reached at 20 h, followed by a decline to normal values. Similar results were reported (Lauk et al., 1989) when investigating (with the same model), the hypolipidaemic activity of *Mucuna pruriens* L. (DC) (Fabaceae). This result establishes the feasibility of using Triton induced hyperlipidaemic mice as an experimental model to investigate the hypolipidaemic effect of *B. maderaspatensis* extracts.

It is well known that diet plays an important role in the control of cholesterol homeostasis. The consumption of cholesterol-enriched diet is regarded as an important factor in the development of cardiovascular diseases as it leads to the development of hyperlipidaemia, atherosclerosis and abnormal lipid oxidation/metabolism (Onody et al., 2003). Thus, natural products with hypocholesterolemic and hypolipidaemic properties may be useful in reducing the risk of cardiovascular disease development.

In this study, an initiative to establish the anti-hyperlipidaemic activity of *B. maderaspatensis* have been carried out on rats using Triton WR-1339 and high-cholesterol diet induced models. From our study, it was found that ethanol extract of *B. maderaspatensis* showed a comparable hyperlipidaemic activity as that of the standard drug simvastatin in both the models. Present results clearly revealed that ethanol extract at a dose of 100 mg/kg significantly lowered both plasma triglycerides and cholesterol levels in both the models as shown in Tables 1 and 2. Large increase in plasma cholesterol and triglycerides due to Triton WR-1339 injection results mostly from an increase of LDL secretion by the liver accompanied by a strong reduction of very low density lipoprotein (VLDL) and LDL catabolism (Otway and Robinson, 1967). Experimental animals that consume high dietary levels of cholesterol develop an increase in LDL cholesterol level and atherosclerosis (Rudel et al., 1986). When there is excess LDL in the blood, it is deposited in the blood vessel wall. Then, it becomes a major component of the atherosclerotic plaque. The LDL-receptor is in-charge of LDL absorption in liver. Changes in hepatic LDL-receptor contribute to the elevation in blood cholesterol levels induced by high-cholesterol diets as well as to the reduction that follows hepatic cholesterol depletion (Brown and Goldstein, 1986). In both models, there were drastic increase in the levels of LDL values and this might have contributed to the abnormalities in TC and triglycerides levels. So the ethanol extract of *B. maderaspatensis*, though not found evidentally, but can be hypothesized that it might have enhanced the catabolism of LDL and VLDL cholesterol and might have led to the anti-hyperlipidaemic activity in both the models.

The histopathology studies of the aorta also revealed a normal aorta in Group 6, which was treated with ethanol extract of *B. maderaspatensis*, while it was not the case with the other extracts. These results also suggest that there might be an increase in the catabolism of LDL and VLDL and hence, there was no cholesterol deposition in the layers. The extract considerably lowered the AI and CRI indicating the decrease of LDL values and TC values and thus, ascertaining the cardio protective and anti-atherogenic effect of ethanol extract of *B. maderaspatensis*.

The results found clearly demonstrate that the bioactive compound(s) contained in this plant have a polar character since the order of anti-atherogenic activity is as follows: chloroform extract <ethyl acetate extract < ethanol extract. In fact, flavonoids a heterogeneous group of ubiquitous plant polyphenols, have exhibited a variety of pharmacological activities, including the anti-atherogenesis effect (Del Bas et al., 2005). Furthermore, tannins contents in plant samples confirmed the results reported by Bruneton (1987). The results strongly suggest that the hypolipidaemic activity of this medicinal plant could be attributed to the presence of the valuable polyphenolic compounds.

In conclusion, our results apparently validate the folk medicinal use of *B. maderaspatensis* ethanol extract to reduce plasma lipid content. This is the first study which investigates the hypolipidaemic activity of ethanol *B. maderaspatensis* extract and the results found are encouraging for further assessment to elucidate both mechanisms, and hypolipidaemic action on chronic hyperlipidaemia models and to identify the bioactive compounds.

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