Journal of Pharmaceutical Research International

33(42B): 316-338, 2021; Article no.JPRI.73114
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Anti-Atherosclerosis and Anti-Hypertensive Effects of Flavonoid Isorhamnetin Isolated from the Bark of Cordia *dichotoma L*

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Authors' contributions

This work was carried out in collaboration among all authors. Author SSA designed conducted the study and wrote the article. Author MA contributed in data analysis. Author NAK supervised the whole study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i42B32450
Editor(s):
(1) Prof. John Yahya I. Elshimali, Charles R. Drew University of Medicine and Science, USA.
Reviewers:
(1) S. Ramalingam, A. V. C. College, India.
(2) T. Ramesh, Annamalai University, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/73114

Original Research Article

Received 22 June 2021
Accepted 29 August 2021
Published 02 September 2021

ABSTRACT

Aims: This study aimed to investigate the anti-atherosclerotic and anti-hypertensive ability of isolated Isorhamnetin from the bark of *Cordia dichotoma L*.
Study design: Experimental study
Place and Duration of Study: The extraction and isolation of bark of *Cordia dichotoma L* bark (ACDL) was done at Biosapience lab, Bhopal, India. Anti-atherosclerotic activity was done at IFTM University, Moradabad, India. Anti-hypertensive activity was done at BilwalMedchem and Research Laboratory, Pvt. Ltd, Jaipur, India. All the studies was conducted from 2015 to 2021 depending upon the availability of resources.
Methodology: Column chromatography was done for Ethyl acetate soluble fraction of successive alcoholic extract of *Cordia dichotoma L* bark (ACDL), followed by successive fractionation using benzene, n-butanol, and acetone. Eluents with similar Rf values were pooled and underwent

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flavonoid test. Only a single eluents that passed the test was underwent purification. The crystallized powder obtained underwent UV, FTIR, Mass, ^{1}H NMR and ^{13}C NMR analysis for structural identification.
The evaluation of anti-atherosclerotic activity of isolated Isorhamnetin from bark extract of plant *C. dichotoma* L was done in high fat induced atherosclerosis rats. The seven groups of Wistar rats were taken and each group containing six rats. To evaluate the anti-hypertensive activity of the plant extract, L-NAME induced hypertension model was used.
Results: The structural identification and confirmation of flavonoid Isorhamnetin was analyzed by characterizations of isolated product. Lipid, lipoprotein profile and body weight were determined in high fat diet induced atherosclerosis rats. Isorhamnetin isolated from bark of *C. dichotoma* L produced a significant and dose-dependent anti-atherosclerotic activity in terms of reduction in low density lipoprotein (LDL), very low density lipoprotein (VLDL), Total cholesterol (TC), and Triglyceride (TG) level; and elevation of high density lipoprotein (HDL). Isorhamnetin isolated from the bark of plant *C. dichotoma* L reduced the elevated arterial pressure of L-NAME induced hypertensive rat significantly to the level of normotensive animal group. The isolated Isorhamnetin have shown its potential as an efficient source for the treatment of hypertension.
Conclusion: The present study has identified the isolation and characterization of isorhamnetin flavonoid from bark of plant *C. dichotoma* L. This study also provides evidences of anti-atherosclerotic and antihypertensive effects of *C. dichotoma* L.

Keywords: Isorhamnetin; flavonoid; C. dichotoma; anti-atherosclerotic; anti- hypertensive.

1. INTRODUCTION

Nowadays, plants are the primary source of drugs of therapeutic property. About half of the total worlds population believes in traditional medicine mainly comprising the use of plant extracts and their active constituents. Plant derivatives are considered to be an initial source as medicines to guard the health and contending several diseases [1,2]. About 72000 plant species were appraised for having remedial properties, out of which 3000 were recognized in India [3]. Foremost indigenous systems enumerated medicinal plants as Ayurveda (700), Unani (700), Siddha (600), Amchi (600) and allopathy with 30 plant species for remedies. Primarily, Ayurveda is the science of life encouraging the thought that good health meant to metabolically proportionate living beings [2]. Amongst such plant species, *Cordia dichotoma* L. Forst., also showed numerable medicinal applications. This is a medium-sized tree that belongs to the family Boraginaceae, grows extensively in the Philippines where it is locally known as anonang. Other common names are lasura, Indian cherry, bhokar, shlesmataka, gonda. Plants yield edible fruits from which a white jelly-like sticky substance could be obtained for sticking or fastening purposes. The screening of bark, fruits and seeds demonstrates the presence of pyrrolizidine alkaloids, flavonoids, coumarins, terpenes, saponins and sterols[4]. Different parts of the plants such as bark, bark, fruit and seeds have been extensively investigated for many medicinal purposes as anti-inflammatory, antiulcer, antidiabetic, antidyspepsia, larvicidal, antidysentery, anthelmintic, analgesic, diuretic, laxative, immunomodulator and hepatoprotective [5-8]. Isolation of three valuable flavonoids quercetin, kaempferol and isorhamnetin from the butanol fraction of *C.dichotoma* Lfruits indicates this plant is rich in pharmacologically active phytoconstituents[9]. Initial researches on the crude extract from the bark of the tree on direct measurement of radical scavenging activity displayed potential antioxidant properties [10]. At a concentration of 100 µg/ml, the isolated taxifolin from the seeds of *C. dichotoma* Lsignposts significant DPPH free radical scavenging activity [11]. It is also reported that crude ethanolic extract from the bark displayed antilarvicidal effectiveness against the brine shrimp *Artemia salina* [12]. Apigenin from *C. dichotoma* L, when screened for ulcerative colitis showed promising reduction and healing in inflammatory enzymes [13]. Another study reported the inhibition of the number of implants in laboratory female rats by the crude methanolic extract of the *C. dichotoma* Lbark [14]. In related research, the anti-implantation activity showed by the extract of leaf from the same tree signifying its possible application as a natural contraceptive drug [15].

Cardiovascular diseases are on the rise which tends to elicit more severe diseases and dwell significant economic drain on countries across

the globe. For example, diseases like atherosclerosis lead to myocardial fibrosis which can cause heart failure. There are about 4000 flavonoids present in plants, many of them are promising pharmacological effects. Several researchers reported that a flavonoid compound, Isorhamnetin has numerous pharmacological effects on cardiovascular and cerebrovascular diseases mainly antithrombosis, anti-hypotension, anti-hypoglycemia, anti-myocardial ischemia and anti-atherosclerosis. Additionally, Isorhamnetin can also protect endothelial cells, improve nerve function, treat neurogenerative disorders and enhance cognition and memory. The anti-oxidation, anti-inflammation and anti-apoptosis properties of flavonoid isorhamnetin are primarily responsible for its therapeutic and preventive elects on the cardiovascular system [16]. As per studies, hypertensive patients have disorders of cellular calcium regulation, and Li et al. investigated on rabbits that isorhamnetin had double inhibitory effects on voltage-dependent (VDC) and receptor-operated calcium channels of vascular smooth muscles [17]. It can also induce a positive inotropic effect in isolated atria of rats [18]. By reducing the intracellular free calcium level, relaxes the blood vessels and lowers blood pressure. The vasodilative effect at low-dose isorhamnetin is through an endothelium-dependent pathway, whereas high-dose isorhamnetin causes vasodilation is independent of the endothelium pathway. The mechanism of vasodilation of isorhamnetin may be related to the endothelial NO/ GC/cGMP pathway and cyclooxygenase pathway. Elevating endothelial NO production and activating cyclooxygenase increase the PGI2 production, thus employing the vasodilator effect which is irrelevant to the ATP-activated potassium channel [19]. For such reasons, the hypotensive mechanism of isorhamnetin is generally endothelium and non-endothelium dependent. On higher concentrations, non-endothelium dependent inhibition of calcium channels reduces intracellular calcium levels, but the low concentration promotes endothelial production of NO and activation of COX which further relaxes blood vessels and resisting hypertension. The stimulation oxidized low-density lipoprotein increase the level of intracellular ROS free radicals, and at 20 mg/kg concentration of isorhamnetin P13 K/AKT pathway gets activated which increases the expression of Nrf2/HO-1, reduces ROS levels and macrophage apoptosis, and inhibited atherosclerotic plaque formation in mice [20]. The subintimal migration, phenotypic transformation, proliferation and collagen synthesis of vascular smooth muscle cells (VSMC) are the basic pathological characteristics of atherosclerosis. Moreover, It was also studied that flavonoid isorhamnetin inhibited VSMC proliferation, collagen and DNA synthesis in a dose-dependent manner [21]. Isorhamnetin is probably useful for clinical prevention and treatment of atherosclerosis. In the present study, we successfully isolated the isorhamnetin from the bark of *C. dichotoma* L and evaluated its antihypertensive and anti-atherosclerosis activity in experimental animal models.

2. MATERIAL AND METHODS

2.1 Materials

Ethyl acetate, n-hexane, methanol, ethanol, chloroform, diethyl ether, hexane were analytical grade and purchased from Himedia Laboratories (Bhopal, India). Reverse osmosis Milli-Q wa¬ter (18 MV) (Millipore, USA) was used for all solutions and dilutions. All other reagents were of the highest commercial grade available.

2.2 Plant Material

Bark of Cordia dichotoma L. were collected from the sides of the railway track, Meerut cantonment, Uttar Pradesh. India. It was collected in August to December 2015. Herbariums were prepared for authentication of plant. One copy of herbarium file was deposited in Department of Pharmacognosy, IFTM University, Uttar Pradesh, India and one copy was deposited for authentication. Authentication of plant was done by National Botanical Research Institute, Lucknow, India. (Voucher no. NBRI/CIF/ 477/2015).

2.3 Drugs and Chemicals

L-N^G– Nitro arginine methyl ester (L-NAME), Isorhamnetin standard, and Hippurylhistadine leucine was purchased from Sigma Aldrich, USA. All the reagents and solvent used for extraction and isolation were analytical grade and were purchased from CDH Fine chemicals, India.

2.4 General Experimental Procedures

UV spectra of the isolated compounds were recorded in methanol over a scanning range of 200-400 nm and λmax of compounds were determined. Spectra were recorded with a Systronics double beam-2203 UV-VIS

spectrophotometer. EIMS (electron impact mass spectrum) in positive mode, were recorded on Brukers aurora M90 (USA) instrument. The isolate was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 375- 7500 cm-1 in FT-IR spectroscopy (Bruker FT-IR Spectrometer, USA). 1H and 13C-NMR spectra were recorded on a Bruker BioSpin Advance III FT-NMR spectrometer, USA, operating at 700 MHz both for proton and carbon using tetramethylsilane (TMS) as internal standard. Chemical shifts were shown in δ values (ppm) with TMS as an internal reference. For column chromatography silica gel 60 (70-230 mesh, Merck, Darmstadt, Germany) was used. Thin-layer chromatography (TLC) was performed using precoated TLC plates (Silica Gel G-60 F254, Merck, Germany).

2.5 Preparation of Extracts and Fractionation

The shade dried bark after pulverization (300g) were extracted exhaustively with petroleum ether (70oC) and then with ethanol (90%) in a Soxhlet apparatus successively. The alcoholic extract obtained was concentrated under vacuum which is further taken for phytochemical screening and isolation of phytoconstituents.

A solvent-solvent extraction technique was used to separate the phytoconstituents. Initially, the semisolid ACDL (5gm) was dissolved in methanol: water (4:1) (200ml) and then subjected to filtration. The filtrate was concentrated up to 1/10th of the volume below 400C. Then acidify the solution to pH 2 by 2M H2SO4. The acidic fraction was then extracted with chloroform thrice the quantity of present volume. The chloroform fraction was then evaporated to dryness. The fraction was then tested for the presence of phenolic phytoconstituents. The fraction was thin layer chromatographed and column chromatographed later on for further purification which was then concluded with spectroscopic studies (ACDL).

2.6 Phytochemical Screening for Flavonoids

Ferric chloride, lead acetate and Shinodas test were performed on all the prepared extracts for the presence of flavonoids with suitable solvents according to the standard procedures [22].

2.7 Chromatographic Characterization

2.7.1 Column chromatography

The fraction of the extract ACDL was subjected to column chromatography. An open glass column (150 by 200 mm) was packed with silica gel (Merck, Darmstadt, Germany, 0.063 to 0.200 mm). The column was eluted with Toluene: Ethyl acetate (93:7). The fractions were collected in 5 to 10 mL portions depending on the visible changes in the colourful bands running out of the column. The eluted fractions (5 mL) were vaporized in the oven at 60°C. Then each fraction again re-dissolved in the corresponding solvent and studied spectro-photometrically.

2.7.2 Thin layer chromatography

Thin-layer chromatography was carried out as per standard procedure. Different extracts of the plant samples were applied on chromatographic plates and a chromatogram was developed in different solvents. Fractions with spots of the same Rf values were combined and re-chromatographed in appropriate solvent systems until TLC pure isolates were obtained.

2.8 Identification and characterization

The melting point was determined using open capillary tubes in a Veego (India) melting point apparatus and is uncorrected. UV-visible spectrum was measured on a double beam UV-Visible spectrophotometer 2204 model Systronics using methanol as solvent. IR spectrum was taken on an FT-IR spectrophotometer (Bruker ALPHA) at room temperature. For the NMR study, a small amount of isolated compound was dissolved in DMSO and 1H and 13C NMR spectra were taken on a Brukers ADVANCE-III 500MHz. The mass spectrum was taken on Bruker microTOF QII high-resolution. TLC study was performed using silica gel-G TLC glass plates of uniform thickness of 0.2 mm. The plate was developed using solvent system toluene-ethyl acetate (93:7) in a chromatography chamber. The detection was carried out under UV at 254 nm and 365 nm. A chemical test (FeCl3 test) was performed for the isolated compound.

HPLC analysis. HPLC analysis was performed with a Water HPLC 450 Controller system consisting of an intelligent pump, a high-pressure mixer, a manual sample injection valve

(Rheodyne 7725i) equipped with a 20-µl loop, and a UV–visible detector. The compound was separated on Thermo Hypersil Keystone C-18 column (250 x 4.6 mm, 5 µm) with 60:40 (% v/v) methanol: water with an isocratic mobile phase at a flow rate of 1.0 ml/min. The injection volume was 20 µl and the detection wavelength was 255 nm. HPLC was performed at ambient temperature.

2.9 Animals

The rats were maintained in an institutional animal house facility with 12 h light and dark cycles. Temperature was maintained at 25±3 °C, relative humidity 60-70% and feeding schedule consisted of rat pellet diet and water adlibitum. The study was duly approved by Institutional Animal Ethical Committee as per CPCSEA guidelines by Animal House facility of IFTM University Moaradabad (Resolution Number: 2015/837ac/Ph.D./09). Some Animal studies were conducted in Bilwal Medchem and Research Laboratory Pvt. Ltd. Jaipur Rajasthan. The study was duly approved by Institutional Animal Ethical Committee as per CPCSEA guidelines (Resolution Number: BMRL/IAEC/ 2021-46).

2.9.1 Acute oral toxicity study

Acute oral toxicity of the extract was performed using OECD guideline 423: Acute oral toxicity – Acute toxic class method. No mortality or morbidity status was observed with single dose of 2000 mg/kg in experimental mice. Hence, two random dose 100 mg/kg BW and 200 mg/kg BW of extract was selected for further pharmacological activity. Dose of isorhamnetin was selected based on previous literature [23].

2.9.2 Anti-atherosclerotic activity

2.9.2.1 Experimental group

Wistar rats were divided into seven group containing 6 rat each.
Group I served as normal control without any treatment.
Group II served as negative control fed with HFD only.
Group III served as positive control-I and received atorvastatin (1.2 mg/kg body weight, p.o).
Group IV served as positive control-II and received standard Isorhamnetin (SIR) (10 mg/kg, B.W, p.o)
Group V served as test group and received isolated Isorhamnetin (IIR) (10 mg/kg, B.W, p.o)
Group VI served as test group and received Low dose test extract (LDTE) (100 mg/kg, B.W, p.o)
Group VII served as test group and received High dose test extract (HDTE) (200 mg/kg, B.W, p.o)

2.9.2.2 Induction of Atherosclerosis

Atherosclerosis was induced by feeding High fat diet to rats of Group II to Group VII for 28 days as per previously reported method[24].High Fat diet consisted of 50% Corn Starch, 11.25% Rice Powder, 01% vegetable oil, 10% egg white, 08% fish meal, 19% Cellulose, 0.125% mineral complex, 0.125% vitamin Complex and 0.50% Salt.

2.9.2.3Biochemical analysis

At the end of the experimental period blood was collected in heparinized tubes from rats via retro orbital plexus under mild ether anesthesia. The tubes were then centrifuged at 3000 rpm for 10 min to give supernatant plasma, which was used of determination of Total cholesterol, HDL, VLDL, LDL, Triglycerides using commercial kit obtained from Span Diagnostic ltd. India by auto analyzer.

2.9.2.4 Body weight

Body weight was determined by digital weighing balance.

2.9.3 Anti-hypertensiveactivity

2.9.3.1 L-NAME induced hypertension

Male wistar were treated with NOS inhibitor, L-NAME (50 mg in 100 mL) in drinking water for 30 days for induction of hypertension as per previously reported method [25].
The standard drug used was Captopril (10 mg/kg, orally).
Male Wistar rats were divided into groups with six animals (n=6).
Normal control rats (Group 1) received distilled water ad libitum only,
negative control rats (Group 2) received L-NAME (50 mg in 100 mL) in drinking water
positive control-I rats (Group 3) received Captopril (10 mg/kg/day) with L-NAME (50 mg in 100 mL) in drinking water
Positive control-II (Group 4) received standard isorhamnetin (SIR) (10mg/kg) L-NAME (50 mg in 100 mL) in drinking water

Test group (Group 5) received isolated isorhamnetin (IIR) (10 mg/kg) with L-NAME (50 mg in 100 mL) in drinking water
Test group (Group 6) received low dose extract (LDTE) (100 mg/kg) with L-NAME (50 mg in 100 mL) in drinking water
Test group (Group 7) received high dose extract (HDTE) (200 mg/kg) with L-NAME (50 mg in 100 mL) in drinking water.

2.9.3.2 Blood pressure measurement using tail-cuff method

Rats were acclimatized to the restrainer and transducer, for about 15min each day for one week before the experiment. Proper breathing was ensured by projecting the nose of the animal through the front nose cone. Body temperature was monitored throughout the experiment. The tails of the rats were exposed to a hot air blower. The rats were allowed to acclimatize inside the cage for 15 min before starting actual blood pressure measurement. Occlusion and sensor cuffs were wrapped around the base of the tail. Afterwards, the cuffs were inflated and deflated a minimum of 6 times to measure tail arterial blood pressure and the data were averaged. This procedure was conducted on Day 0, Day 10, Day 20 and Day 30.

2.9.3.3 Determination of ACE Inhibition Activity

50 µL solution of extract, drug and isolated compound were mixed with 200 µL of phosphate buffer (100 mM, pH 8.3) containing 0.2 M NaCl, and 6.5 mM hippuryl-histidyl-leucine (HHL). 100 µL (0.1 U/ml) of ACE from rat lung prepared in the same buffer was added to start the reaction and the mixture was incubated at 37°C for 30 min. 50 µL of 1 M HCl was added to stop the reaction. The Gly-His bond of HHL was then cleaved and the product was extracted with 1.5 mL ethyl acetate. Ethyl acetate supernatant was taken after centrifugation (1200 × g for 15 min), and ethyl acetate was removed by heat evaporation. The extracted product was dissolved in a 3 mL volume of distilled water and the absorbance was determined at 228 nm using a spectrophotometer (UNICO UV-2102, Shanghai, China). The inhibition activity was calculated using the following equation:

$$\text{Inhibition (\%)} = \left(\frac{Aa-Ab}{Aa-Ac}\right) \times 100$$

Where, Aa is the absorbance with ACE and HHL without the sample (positive control, no inhibition and maximum activity); Ab is the absorbance with ACE, HHL and the sample or standard; and Ac is the absorbance with HHL without ACE and the sample (control) [26].

2.10 Statistical Analysis

Results are expressed as Mean±SEM and were analyzed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Identification and characterization of isolated compound

Isolated fractions were tested for ferric chloride and Shinoda test and give positive results for flavonoids and flavones.

Based on Rf value of TLC spot and wavelength. Fractions having the same Rf value and wavelength were considered as one fraction alike. Total seventeen fractions had been taken out. The fraction was subjected to TLC and spectroscopic studies. All the eluents were dried, TLC chromatographed, purified by solubility analysis and recorded data is presented in Table 1. Further, samples were analyzed with UV spectroscopy and spectra are given in Fig. 1. The eluents were thin layer chromatographed in Benzene:Acetone(9:1), Butanol:Chloroform: Diethylamine (7:2:1) and Toluene :Ethylacetate: Formic acid (10:17:2). Ammonium vanadate solution, iodine chamber and UV chamber are the detection agents. After TLC separation, the eluents displaying similar patterns concerning Rf values were pooled and L4 was taken for further studies. Eluents TE15- TE17 shown a single spot in TLC studies. Further, the re-chromatographed and crystallized fraction of isolated flavonoid was examined for structural analysis. Various analytical techniques and chemical tests were employed for the identification of the isolated compound. The as-obtained compound (78.5 mg) was a buff yellow-coloured amorphous solid with 307-310°C melting point. In this layer chromatography (TLC) analysis, the compound showed a single band on a silica gel plate at Rf value 0.43 insolvent system toluene-ethyl acetate (93:7). The high-performance liquid chromatography (HPLC) profile also suggested a single compound by a single peak. Additionally,

the analysis was carried out under a UV spectrophotometer at 254 nm and 365 nm wavelength. The compound showed a positive ferric chloride test and in UV light at 365 nm after spraying with 1% ethanol solution of aluminium chloride it gives yellow fluorescence, which indicated the presence of flavonoids. The absorption maxima exhibited at 207 nm, 255 nm and 367 nm wavelength in the UV spectrum of isolated compounds in methanol which are characteristics for an isoflavone structure (Fig. 1. (D)).

Table 1. Eluents and their spectral studies for ACDL

S. No.	Eluents	Wavelengths (nm)	Absorbance	Pools	Rf
1.	TE1-TE4	264.8	0.645	L1	0.17,
		351.2	0.882		0.48,
		308.0	0.719		0.69
		384.8	0.764		
		764.0	0.096		
		555.2	0.169		
		677.6	0.087		
2.	TE5-TE10	260.0	0.374	L2	0.28,
		610.4	0.021		0.47
		644.0	0.062		
		773.6	0.017		
		550.4	0.025		
		677.6	0.007		
3.	TE11-TE14	262.4	0.646	L3	0.32,
		346.4	0.752		0.72
		298.4	0.706		
		550.4	0.110		
		764.0	0.058		
		672.8	0.046		
4.	TE15-TE17	207.0	0.413	L4	0.43
		255.0	0.237		
		367.0	0.245		

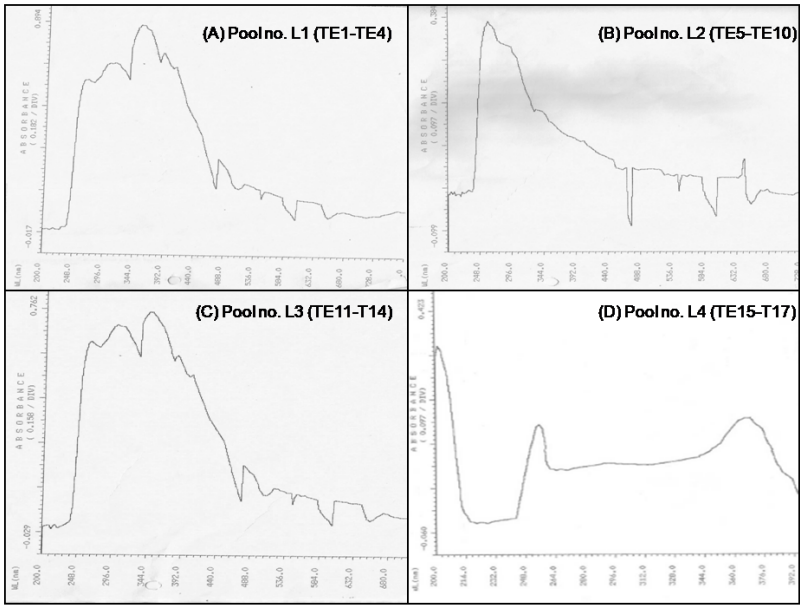

Fig. 1. Primary U.V. Spectroscopy data of eluents

Fig. 2 demonstrates the IR spectrum of isolated compounds which shows prominent absorption bands at 3488-3350 cm-1 (free phenolic OH), 1660 cm-1 (conjugated C=O) and others are tabulated in Table 2. The interpretation of the spectral analysis of the isolated isorhamnetin fraction was done by comparing it with previously reported literature [27-29]. Due to the presence of –OH stretch of phenols or alcohols, C-H stretching vibration of alkenes and alkanes, the absorption bands are present at 3488.47 cm-1, 3021.62 cm-1, 2928.94 cm-1 and 2854.13 cm-1 respectively. The characteristic C=C stretching band at 1498.74 cm-1 confirms the aromaticity of the compound and the strong stretching band at 1660.26 cm-1 indicates the presence of carbonyl group (C=O). Another inference for the confirmation of aromatic structure was interpreted from the bands at 1039.30 cm-1 and 811.67 cm-1 which were represented as C-H deformation in methyl moiety of the aromatic nucleus, C-H deformation in the aromatic ring and for the presence of a di-substituted (meta) benzene ring respectively.

The mass spectra of L4 as shown in figure 3 analyzed by a molecular ion peak at m/z 317.17 [M-1]+ was determined by the molecular formula of the compound as C6H12O7. Another two main fragment ion peaks were shown in mass spectra of L4 at 302 of C15H9O7 and 286 of C15H9O6. The mass fragmentation pattern indicated that one methoxy and hydroxyl group were attached to the ring-A, (Fig.6) while the remaining hydroxyl groups were linked with the ring-B and C at C-3, 5, 7 positions. The C-4 position was blocked by a carbonyl group was also confirmed by the mass fragmentation. As per the mass spectral information, the identification of the compound was done as 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl) chromen-4-one as represented in Fig. 6. By comparing the spectroscopic data with previous reports, the isolated compound was identified as isorhamnetin [31].

Table 2. FTIR Spectral Data of L_4

S No.	Wavenumber in cm^{-1}	Inference
1.	3021cm^{-1}	Presence of aromatic =C-H stretch
2.	3488 cm^{-1} – 3350 cm^{-1}	Presence of OH group
3.	2928 cm^{-1}	Presence of OCH_3 group
4.	1660 cm^{-1}	Presence of C=O group Ɣ pyrone
5.	1620, 1510 cm^{-1}	Aromatic C=C group
6.	1591,1498 cm^{-1}	Presence of aromatic vibrations
7.	811 cm^{-1}	Disubstituted benzene(*o,m,p*)
8.	1039 cm^{-1}	Presence of C-O bond of primary alcohols

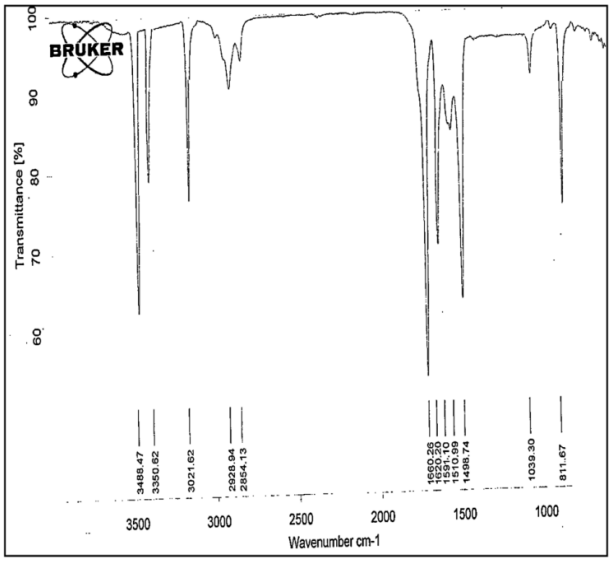

Fig. 2. FTIR Spectra of L_4

Table 3. Interpretation of Mass spectra for L_4

S. no.	Peak/ MW	Formula	Remarks	M^{+1} peak
1.	26	C (2)H(2)		26.0220
2.	28	Carbon monoxide		28.1722
3.	42	CH (2) = C=O		42
4.	273	C (14)H(9)O(6)	A, B, and C excluding methoxy group with carbon at A.	274.2056
5.	274	C(14)H(10)O(6)	A, B and C excluding hydroxyl group with carbon and CH at C	275.2146
6.	285	C(15)H(9)O(6)	At A, B and C excluding methoxy group at C nucleus	286.2187
7.	288	C(15)H(12)O(6)	A, B and C excluding CO group at C	289.2551
8.	290	C(14)H(10)O(7)	At A, B and open C nucleus excluding C(2)H(2)	291.2246
9.	299	C(16)H(11)O(6)	A, B and C excluding para hydroxyl at C nucleus	291.2246
10.	299	C(16)H(11)O(6)	A, B and C excluding hydroxyl at b nucleus	299.1450
11.	299	C(16)H(11)O(6)	At a, B and C without ortho hydroxyl at C nucleus	299.2501
12.	299	C(16)H(11)O(6)	A, B and C excluding hydroxyl at A	302.1915
13.	301	C(15)H(9)O(7)	A, B and C excluding para hydroxyl at C	302.1915
14.	316	C(16)H(12)O(7)	Molecular Ion	317.1761

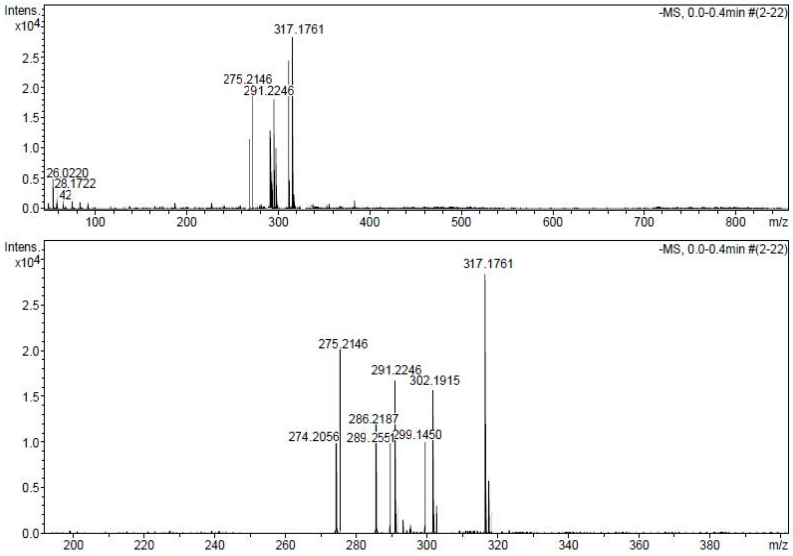

Fig. 3. LCMS Spectra of L_4

NMR spectral studies were performed for the structural confirmation of compound. ^{1}H-NMR and ^{13}C-NMR spectra of isolated compound are shown in Fig. 4 and Fig. 5 respectively. The ^{1}H-NMR spectrum of the compound (Fig. 4) showed a total of 10 signals for 12 hydrogens. The signals observed were allocated as following. The ^{1}H-NMR spectrum shows, the aromatic region exhibited at δ 7.774 (1H, d, J=1.5 Hz, H-2′), 7.680 (1H, dd, J= 1.5 Hz, H-6′) and 6.951 (1H, d, J=8.5 Hz, H-5′) due to a 3, 4 di-substitution of ring B and a typical meta-coupled pattern for H-6 and H-8 protons (δ 6.211 and 6.498, d, J = 2.0 Hz). The presence of the meth-oxy group at 3 was supported by δ 3.184 signals [31]. The ^{13}C-NMR spectrum shows (δ ppm): 148.61 (2-C), 135.62 (3-C), 175.72 (4-C), 156.05 (5-C), 98.11 (6-C), 163.71 (7-C), 93.53 (8-C), 160.32 (9-C), 102.91 (10-C), 121.84 (1-C), 111.56 (2-C), 146.43 (3-C), 147.21 (4-C), 115.33 (5-C), 121.64 (6-C), 55.63 (OCH_3). The chemical properties and spectral data of the isolated compound were identical to those reported in earlier for isorhamnetin [32-35]. The structure of isolated compound is shown in Fig. 6. The data reflects the structure of compound will be 3,5,7,4′-Tetrahydroxy-3′-methoxyflavone which is recognized as Isorhamnetin.

Table 4.^{1}H NMR data of L_4

S.No.	Shift (ppm)	Hs	Type	J (Hz)	Atom	Range (ppm)
1.	12.47	1	s	-	OH	12.46-12.49
2.	10.77	1	s	2.20	OH	10.76-10.79
3.	9.78	1	s	8.78,2.22	OH	9.72-9.78
4.	9.43	1	s	-	OH	9.39-9.41
5.	7.77	1	d	8.75	2H	7.74-7.78
6.	7.68	1	m	-	6H	7.68-7.70
7.	6.95	1	d	8.50	5H	6.94-6.98
8.	6.49	1	d	2.51	6H	6.41-6.50
9.	6.21	1	d	2.49	6H	6.19-6.32
10.	3.18	3	s		OCH_3	3.14-3.16

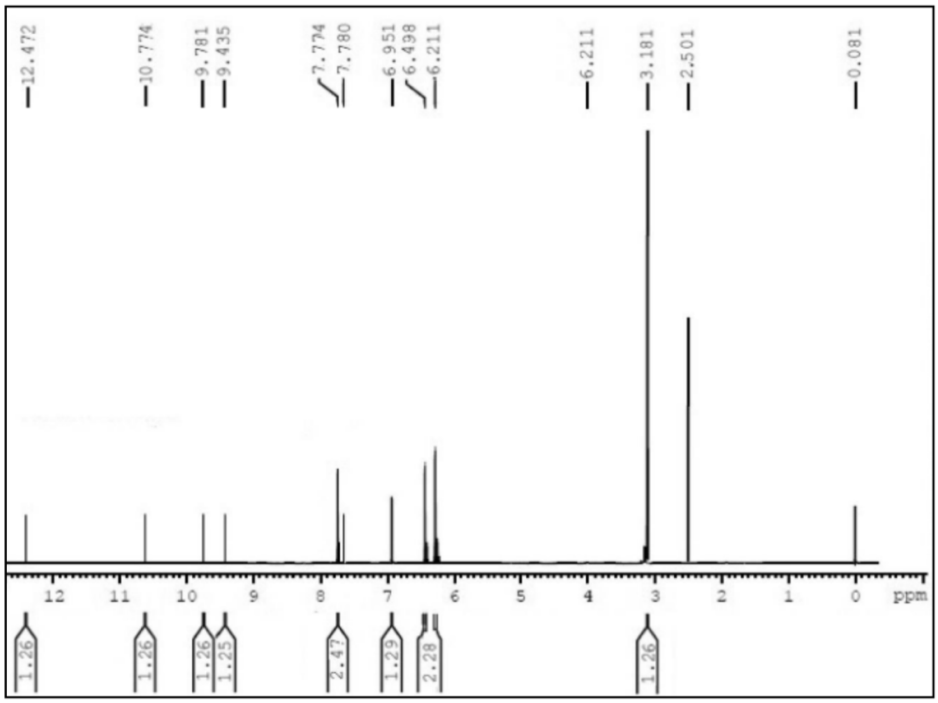

Fig. 4. ^{1}H NMR spectra of L_4

Table 5.^{13}C NMR data of L_4

S. No.	Shift (ppm)	Cs	Atom	Range (ppm)
1.	175.72	4	4-C	175.70-175.74
2.	163.71	7	7-C	163.70-164.13
3.	160.32	9	9-C	160.31-160.33
4.	156.05	5	5-C	156.04-156.06
5.	148.61	2	2-C	148.60-148.63
6.	147.21	4	4-C	147.20-147.22
7.	146.43	3	3-C	146.42-146.44
8.	135.62	3	3-C	135.61-135.64
9.	121.84	1	1-C	121.82-121.85
10.	121.64	6	6-C	121.63-121.65
11.	115.33	5	5-C	115.32-115.34
12.	111.56	2	2-C	111.55-111.57
13.	102.91	10	10-C	102.90-102.92
14.	98.11	6	6-C	98.10-98.12
15.	93.53	8	8-C	93.52-93.54
16.	55.63		OCH_3	55.62-55.64

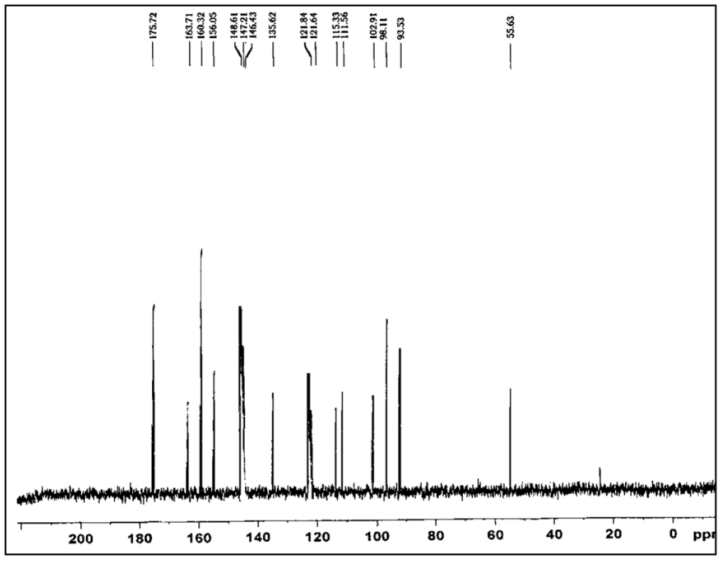

Fig. 5. ^{13}C NMR spectra of L_4

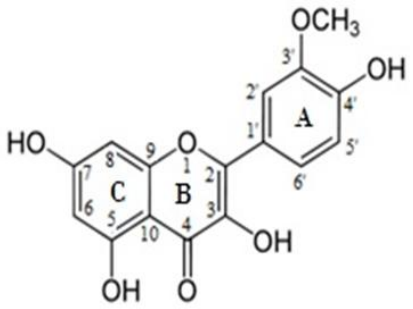

Fig. 6. Structure of Isorhamnetin

3.1.2 Effects of bark extract of *C.dichotoma* L on lipid parameters

Administration of a high-fat diet to various experimental groups induced atherosclerosis as revealed by elevated levels of LDL, VLDL, TC, TG, and lowered levels of HDL compared to experimental rats of normal control groups. Administration of atorvastatin 1.2 mg/kg, B.W lowered LDL compared to negative control group significantly at $p<0.0001$; whereas administration of SIR 10 mg/kg, B.W and IIR 10 mg/kg, BW lowered LDL compared to negative control group significantly at $p<0.001$. Additionally, administration of high dose test extract (200 mg/kg, BW) lowered LDL level compared to the negative control group at $p<0.05$, whereas, administration of low dose test extract does not lower the LDL level compared to the negative control group.

Administration of atorvastatin 1.2 mg/kg, B.W lowered VLDL compared to negative control group significantly at $p<0.05$; whereas administration of SIR 10 mg/kg, B.W, IIR 10 mg/kg, BW, high dose test extract (200 mg.kg, BW), and low dose test extract does not lower the VLDL level compared to the negative control group. Administration of atorvastatin 1.2 mg/kg, B.W elevated HDL compared to negative control group significantly at $p<0.0001$; whereas administration of SIR 10 mg/kg, B.W and IIR 10 mg/kg, BW elevated HDL compared to negative control group significantly at $p<0.001$ and $p<0.0001$ respectively. Additionally, administration of high dose test extract (200 mg/kg, BW) elevated HDL level compared to the negative control group at $p<0.01$, whereas, administration of low dose test extract does not elevate the HDL level compared to the negative control group as shown in table 1.

Tables 2 determine the changes in TC and TG in plasma of various treatment groups. Administration of atorvastatin 1.2 mg/kg, B.W, SIR 10 mg/kg, B.W, IIR 10 mg/kg, BW, low dose test extract (200 mg.kg, BW) and high dose test extract (200 mg.kg, BW) lowered the TC level compared to negative control group significantly at $p<0.0001$. Similarly, the level of TG also lowered with the administration of atorvastatin 1.2 mg/kg, B.W, SIR 10 mg/kg, B.W, IIR 10 mg/kg, BW, low dose test extract (200 mg/kg, BW) and high dose test extract (200 mg/kg, BW) as compared to negative control group significantly at $p<0.0001$.

3.1.3 Body Weight

As depicted from Table 3, there was no significant change seen in body weight of animals on Day 1 of administration of atorvastatin 1.2 mg/kg, SIR 10 mg/kg, B.W, IIR 10 mg/kg, BW, low dose test extract (100 mg/kg, BW) and high dose test extract (200 mg/kg, BW) as compared to the negative control group. On Day 10, animals treated with atorvastatin 1.2 mg/kg experienced a decrease in relative body weight compared to the negative control group significantly at $p<0.01$, whereas no decrease in body weight was observed on administration of SIR 10 mg/kg, B.W, IIR 10 mg/kg, BW, low dose test extract (100 mg/kg, BW) and high dose test extract (200 mg/kg, BW) compared to the negative control group. During the 20 days of induction, administration of atorvastatin (1.2 mg/kg, B.W) decreased the body weight compared to the negative control group significantly at $p<0.01$, whereas administration of SIR (10 mg/kg, B.W) and IIR (10 mg/kg, BW) lowered the body weight compared to negative control group significantly at $p<0.0001$. Additionally, administration of low dose test extract (100 mg/kg BW) and high dose test extract (200 mg/kg BW) does not decrease the body weight compared to the negative control group. On day 30, administration of atorvastatin (1.2 mg/kg, B.W), SIR (10 mg/kg, B.W) and IIR (10 mg/kg, BW) decreased the body weight compared to the negative control group significantly at $p<0.0001$. Moreover, administration of high dose test extract (200 mg/kg, BW) decreased the body weight compared to the negative control group at $p<0.0001$, whereas, administration of low dose test extract does not decrease the body weight compared to the negative control group.

3.1.4 ACE inhibition activity of Bark extract of *C.dichotoma* L

ACE inhibition assay is an important aspect to be determined in antihypertensive studies. ACE is involved in two hypertension cascades i.e. conversion of angiotensin I to angiotensin II and bradykinin degradation [36]. A commercial antihypertensive drug, Captopril, was used as standard. Table 1 demonstrates the results obtained by ACE inhibition assays with different treatment groups. As the ACE inhibition was found to be 86.25±2.88 % , 83.34 ± 1.34, 76.79 ±0.40, 49.34 ±2.60, and 68.66±0.88 in Captopril (1.2 mg/kg, B.W, p.o), SIR (10 mg/kg, B.W, p.o)

and IIR (10 mg/kg, B.W, p.o), Low dose test extract (100 mg/kg), and High dose test extract (200 mg/kg) administered group of experimental animals. Captopril displayed maximum inhibition of ACE followed by SIR, and IIR. While administration of Low dose test extract (100 mg/kg) and High dose test extract (200 mg/kg) were having comparatively lesser capability to inhibit ACE.

3.1.5. Effects on Blood pressure

No significant difference was observed in SBP on Day 1 of treatment in each experimental group. On day 10, blood pressure was significantly higher in L-NAME administered negative control group as compared to the normotensive Wistar group. Administration of Captopril (1.2 mg/kg, B.W, p.o) and, SIR (10mg/kg), Low dose test extract (100 mg/kg BW), and High dose test extract (200 mg/kg, BW) decreased the SBP compared to negative control group significantly at $p<0.0001$, whereas administration of IIR (10 mg/kg BW) significantly decreased SBP at $p<0.001$. At day 20 and 30, administration of Captopril (1.2 mg/kg, B.W, p.o) SIR (10mg/kg, BW), IIR (10 mg/kg, BW), high dose test extract (200 mg/kg BW), and low dose test extract (100 mg/kg BW) decreased the SBP compared to negative control group significantly at $p<0.0001$.

Table 6. Effect of various treatments on plasma VLDL, LDL and HDL level in experimental groups

Treatment group	LDL (mg/L)	VLDL (mg/L)	HDL (mg/L)
Normal control	3.47 ± 0.12****	1.26 ± 0.12**	5.68 ± 0.15****
Negative control	4.74 ± 0.19	2.36 ±0.2	3.37 ± 0.1
Positive control-I, Atorvastation (1.2 mg/kg, B.W, p.o)	3.29 ± 0.22****	1.56 ± 0.23*	5.29 ± 0.12****
Positive control-II, SIR (10 mg/kg, B.W, p.o)	3.48 ±0.2***	1.62 ±0.13^{ns}	5.18 ± 0.16***
Test, IIR (10 mg/kg, B.W, p.o)	3.59 ±0.1***	1.65 ± 0.15^{ns}	5.35 ± 0.41****
Low-dose test extract (100 mg/kg)	4.23 ±0.18^{ns}	1.93 ± 0.25^{ns}	4.29 ± 0.45^{ns}
High-dose test extract (200 mg/kg)	3.9 ±0.22*	1.92 ± 0.25^{ns}	4.91 ± 0.11**

*Results are expressed as Mean±SEM and were analysed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.*

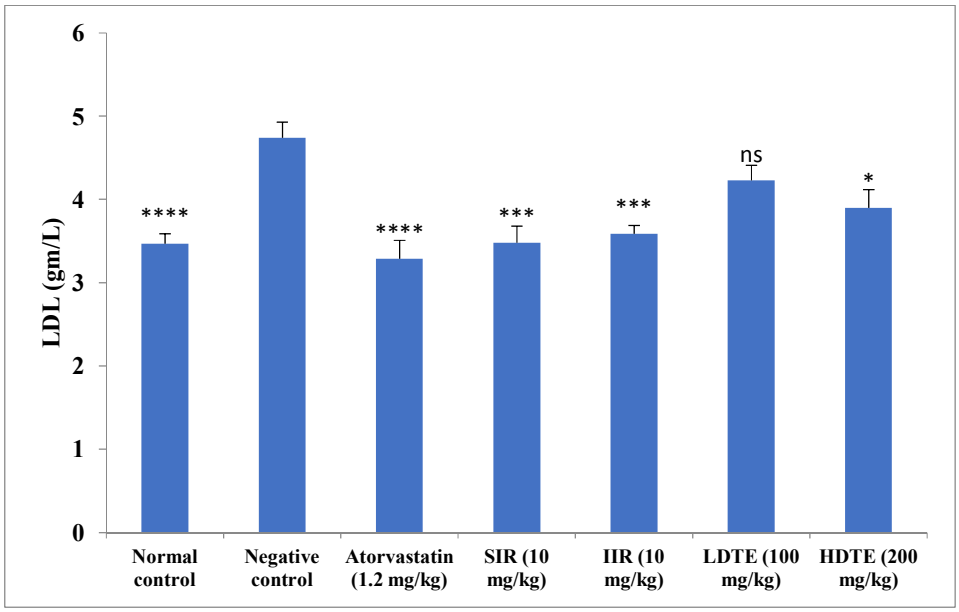

Fig.7. Effects of various treatments on plasma LDL of various experimental groups

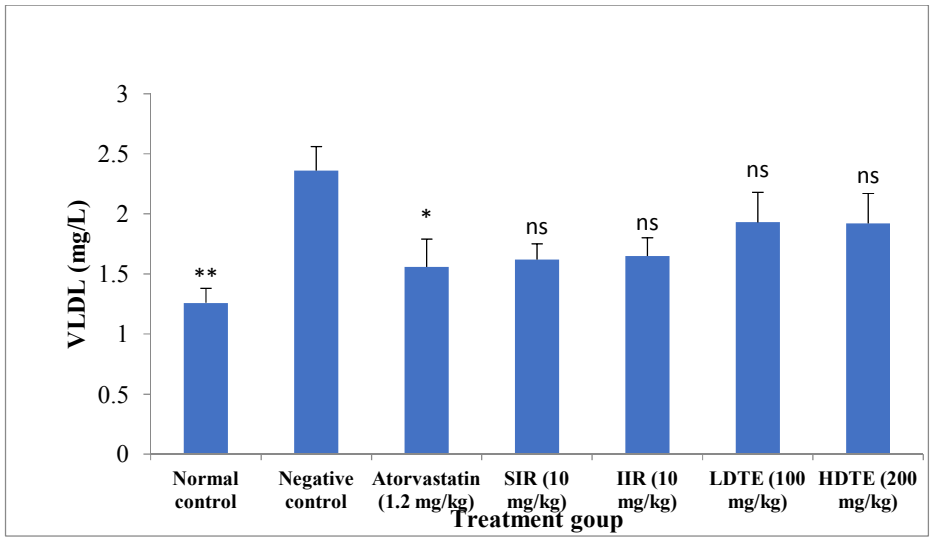

Fig. 8. Effects of various treatments on plasma VLDL level in experimental groups

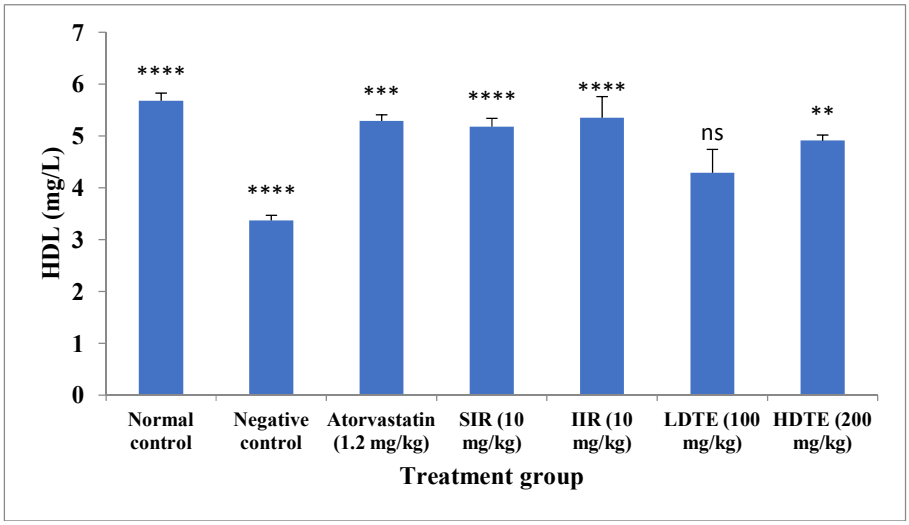

Fig.9. Effect of various treatments on plasma HDL level in experimental groups

Table 7. Effect of various treatments on plasma TC and TG level in experimental groups

Treatment group	TC (mg/L)	TGs (mg/L)
Normal control	8.04 ± 0.44****	6.38 ± 0.16****
Negative control	12.84± 0.32	11.74 ±0.18
Positive control-I, Atorvastation (1.2 mg/kg, B.W, p.o)	8.59 ± 0.43****	6.88 ± 0.22****
Positive control-II, SIR (10 mg/kg, B.W, p.o)	9.00 ±0.22****	7.14 ±0.24****
Test, IIR (10 mg/kg, B.W, p.o)	8.62 ±0.29****	7.59 ± 0.17****
Low-dose test extract (100 mg/kg)	10.86 ±0.16****	9.9 ± 0.14****
High-dose test extract (200 mg/kg)	9.75 ±0.11****	8.83 ± 0.13****

*Results are expressed as Mean±SEM and were analysed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.*

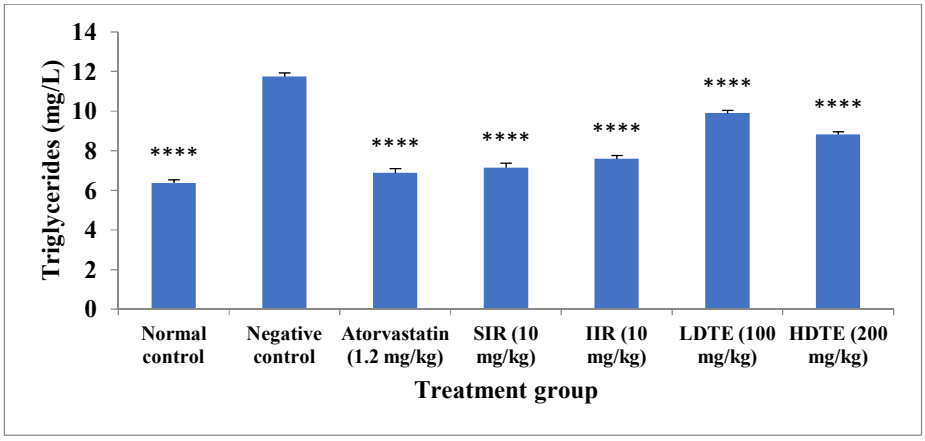

Fig.10. Effect of various treatments on plasma TGs level in experimental groups

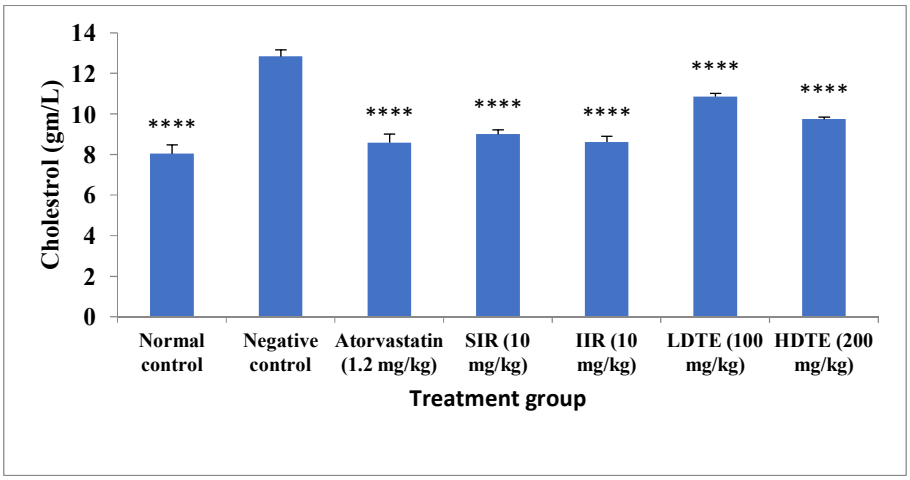

Fig.11. Effect of various treatments on plasma TC level in experimental groups.

Table 8. Effect of various treatments on body weight of various experimental groups

Treatment group	Body weight (gm)			
	Day 0	Day 10	Day 20	Day 30
Normal control	104 ± 1.11^{ns}	109.2 ±1.02****	123.96 ±1.82****	133.81 ±1.22****
Negative control	106.41 ±0.67	117.23 ± 0.6	139.71 ± 0.55	150.96 ± 0.89
Positive control-I, Atorvastation (1.2 mg/kg, B.W, p.o)	105.91 ±0.45^{ns}	113.1 ± 0.84**	133.31±0.83**	141.4 ± 0.59****
Positive control-II, SIR (10 mg/kg, B.W, p.o)	107.25 ± 0.59^{ns}	115.95 ± 0.4^{ns}	131.18± 1.6****	141.46 ± 0.56****
Test , IIR (10 mg/kg, B.W, p.o)	106.08 ± 0.65^{ns}	115.75 ± 0.85^{ns}	130.5 ± 1.4****	141.51 ± 0.64****
LDTE(100 mg/kg)	107.85 ± 1.13^{ns}	118.31 ± 0.6^{ns}	138.2 ± .97^{ns}	148.8 ±0.7^{ns}
HDTE (200 mg/kg)	105.4 ± 0.61^{ns}	117.76 ± 0.74^{ns}	135.7 ± 0.54^{ns}	145.06 ± 0.51****

*Results are expressed as Mean±SEM and were analysed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.*

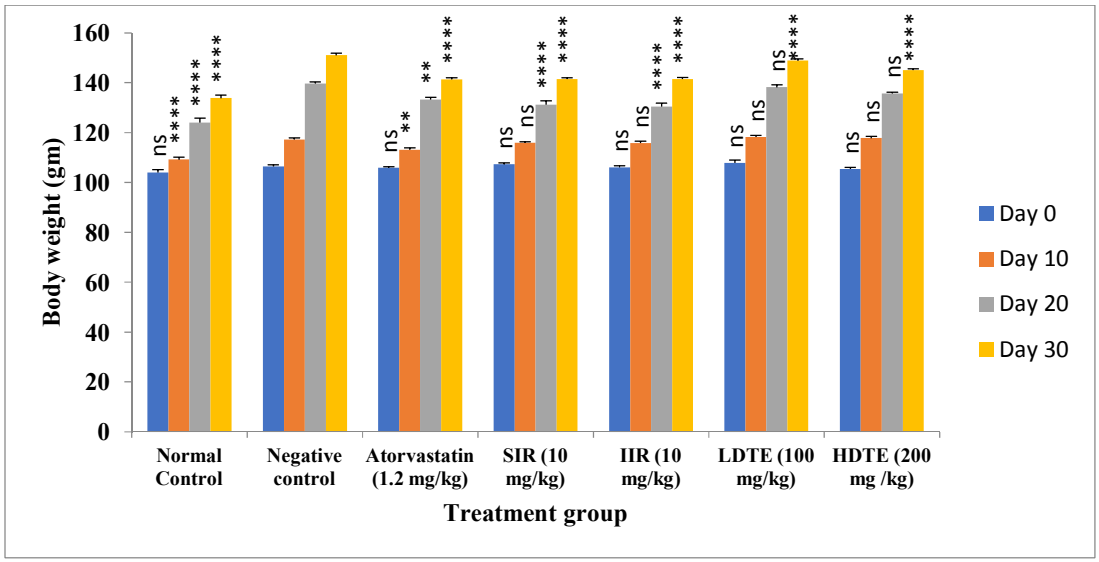

Fig. 12. Effect of various treatments on body weight (gm) in various experimental groups

Table 9. Effect of various treatments percent inhibition of ACE in various experimental groups.

S. No.	Treatment group	% inhibition of ACE
1.	Normal control	-
2.	Negative control	-
3.	Positive control-I, Captopril (1.2 mg/kg, B.W, p.o)	86.25±2.88
4.	Positive control-II, SIR (10 mg/kg, B.W, p.o)	83.34 ± 1.34
5.	Test , IIR (10 mg/kg, B.W, p.o)	76.79 ±0.40
6.	Low dose test extract (100 mg/kg)	49.34 ±2.60
7.	High dose test extract (200 mg/kg)	68.66±0.88

Table 10. Effect of various treatments on SBP in various experimental groups

Treatment group	Day 1	Day 10	Day 20	Day 30
Normal control	118.26 ±1.11^{ns}	118.42± 0.86****	118.76 ±0.72****	119.32 ± 0.54****
Negative control	116.21± 1.14	136.35± 0.55	143.84 ±0.78	161.72 ± 0.92
Positive control-I, Captopril (1.2 mg/kg, B.W, p.o)	118.38 ±0.72^{ns}	118.79 ±1.13****	118.07 ±0.69****	118.07± 0.69****
Positive control-II, SIR (10 mg/kg, B.W, p.o)	116.02 ±0.81^{ns}	128.64 ±0.77****	121.66 ±0.76****	115.84± 0.95****
Test , IIR (10 mg/kg, B.W, p.o)	116.36 ±0.99^{ns}	131.97± 0.47***	125.97 ±0.26****	117.87 ±0.61****
Low dose test extract (100 mg/kg)	117.87± 0.86^{ns}	156.27± 0.48****	149.14 ±0.48****	139.17± 0.344****
High dose test extract (200 mg/kg)	118.47± 0.79^{ns}	148.26± 0.37****	129.24 ±1.15****	121.59± 0.69****

*Results are expressed as Mean±SEM and were analysed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.*

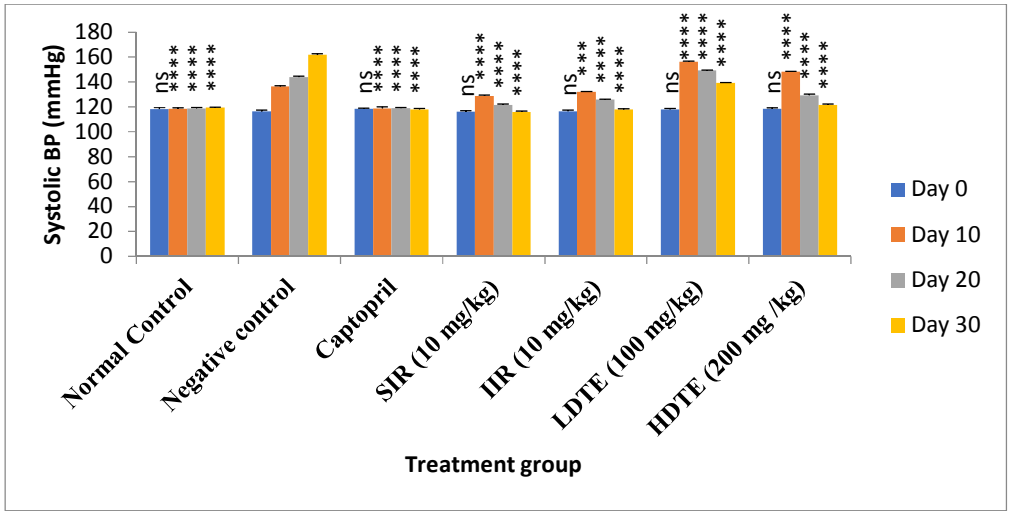

Fig. 13. Effect of various treatments on SBP in experimental groups

Table 11. Effect of various treatments on DBP in various experimental groups

Treatment group	Day 1	Day 10	Day 20	Day 30
Normal control	85.07 ±1.8*	84.14± 1.69****	84.35 ±1.51****	89.72 ± 1.83****
Negative control	89.72± 1.83	108.61± 2.27	123.91 ±2.22	126.49 ± 0.73
Positive control-I, Captopril (1.2 mg/kg, B.W, p.o)	86.57 ±1.02ns	90.48 ±0.68****	89.33 ±0.83****	86.52± 0.66****
Positive control-II, SIR (10 mg/kg, B.W, p.o)	86.44 ±1.19 ns	89.83 ±0.78****	90.52 ±0.51****	89.21± 0.73****
Test , IIR (10 mg/kg, B.W, p.o)	86.4 ±0.35 ns	92.73± 2.19****	89.11 ±0.55****	88.47±1.03****
Low dose test extract (100 mg/kg)	85.76± 0.39 ns	99.84± 0.93***	99.37 ±0.94****	100.70± 0.70****
High dose test extract (200 mg/kg)	85.51± 0.69 ns	98.14± 0.43****	97.99 ±2.02****	95.46± 1.18****

Results are expressed as Mean±SEM and were analysed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.

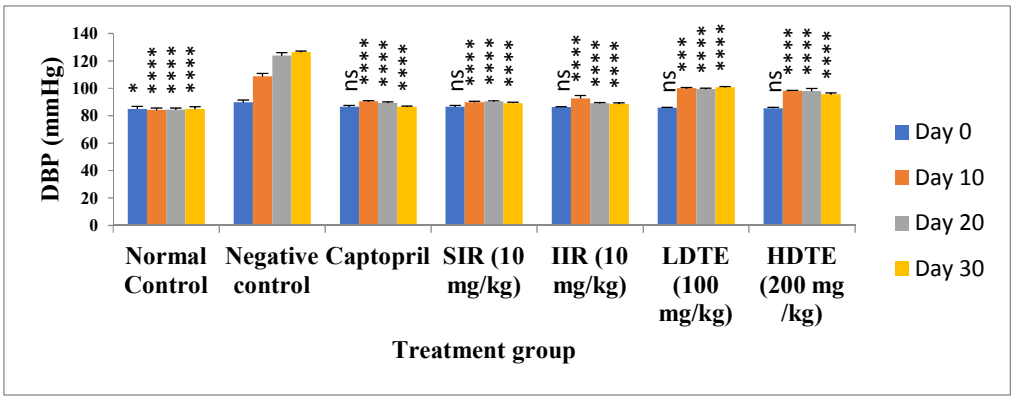

Fig. 14. Effect of various treatments on DBP in experimental groups

Table 12. Effect of various treatments on MBP in various experimental groups

Treatment group	Day 1	Day 10	Day 20	Day 30
Normal control	118.26 ±1.11^{ns}	118.42± 0.86****	118.76 ±0.72****	119.32 ± 0.54****
Negative control	116.21± 1.14	136.35± 0.55	143.84 ±0.78	161.72 ± 0.92
Positive control-I, Captopril (1.2 mg/kg, B.W, p.o)	118.38 ±0.72^{ns}	118.79 ±1.13****	118.62 ±0.62****	118.07± 0.69****
Positive control-II, SIR (10 mg/kg, B.W, p.o)	118.38±0.81^{ns}	128.64 ±0.77****	121.67 ±0.76****	115.84± 0.95****
Test, IIR (10 mg/kg, B.W, p.o)	116.36 ±0.99^{ns}	131.97± 0.47****	125.97 ±0.26****	117.87±0.61****
Low dose test extract (100 mg/kg)	117.87± 0.86^{ns}	156.27± 0.48^{ns}	149.14 ±0.48****	139.17± 0.34****
High dose test extract (200 mg/kg)	118.47± 0.79^{ns}	148.26 ±0.37^{ns}	129.24 ± 1.154****	121.59± 0.69****

*Results are expressed as Mean±SEM and were analysed using one way ANOVA followed by Dunnetts test of multiple comparison. The mean values of treatment groups were compared with the mean values of negative control group. The criteria of statistical significance were ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05.*

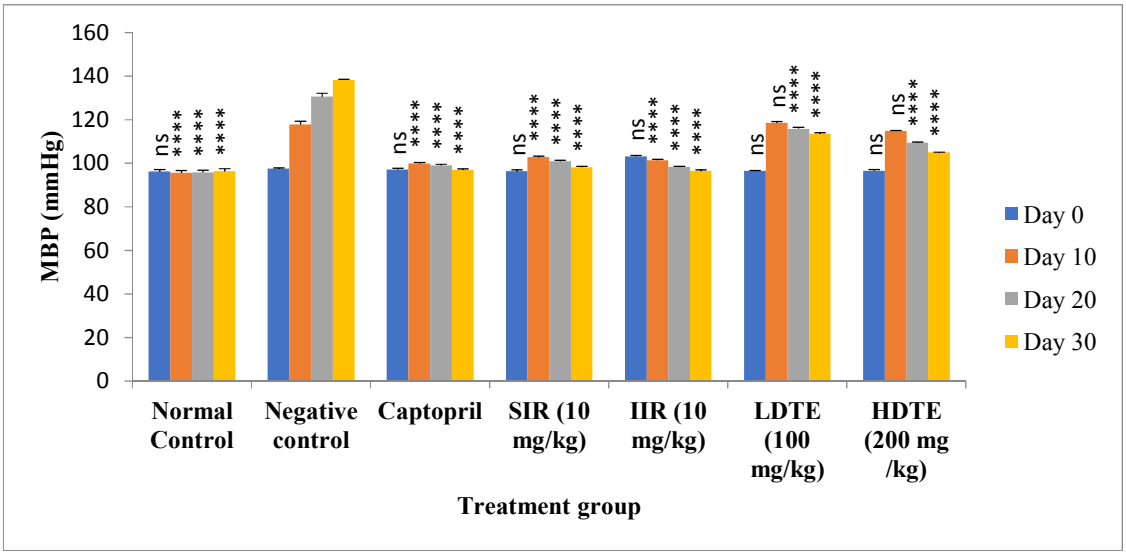

Fig. 15. Effect of various treatments on MBP in experimental groups.

A non-significant difference in DBP was observed among various experimental groups of animals on Day 1 of the treatment. On day 10 of the treatment, a significant decrease in DBP was observed in experimental animals treated with Captopril (1.2 mg/kg, BW), SIR (10 mg/kg, BW), IIR (10 mg/kg BW) and, high dose test extract (200 mg/kg BW) at p<0.0001. While, experimental animals were treated with Low dose test extract (100 mg/kg, BW) significant decrease in DBP was observed at p<0.001. On days 20 and 30 of treatment, a significant decrease in DBP was observed in experimental animals treated with Captopril (1.2 mg/kg, BW), SIR (10 mg/kg, BW), SIR, high dose test extract (200 mg/kg BW), and Low dose test extract (100 mg/kg, BW) compared to negative control group significantly at p<0.0001.

On day 1 of treatment, no significant change was observed in MBP in experimental groups. On day 10 of treatment administration of Captopril (1.2 mg/kg BW), SIR (10 mg/kg, BW), and IIR (10 mg/kg, BW) significant decrease in MBP was observed at p<0.0001 compared to the negative control group. While, in experimental animals treated with Low dose test extract (100 mg/kg, BW), and High dose test extract (200 mg/kg,

BW) the difference in MBP was found to be non-significant compared to negative control groups.

On days 20 and 30 of the treatment, a significant decrease in MBP was observed in Captopril (1.2 mg/kg BW), SIR (10 mg/kg, BW), IIR (10 mg/kg, BW), Low dose test extract (100 mg/kg, BW), and High dose test extract (200 mg/kg, BW) administered experimental animal compared o negative control group at $p<0.0001$.

4. DISCUSSION

Atherosclerosis is a complex, multifactorial inflammatory disease. It is one of the major risk factors for coronary artery disease characterized by the presence of lesions because of the amassing of lipids in the artery walls. Several genetic, environmental and metabolic factors are implicated in the growth of atherosclerosis plaque. In human beings, hypercholesterolemia or total increased cholesterol (TC) and low-density lipoprotein is a well-known risk factor. Saturated fat and cholesterol-rich foods have been associated with it elevations in circulating cholesterol levels. Basically, in murine models of atherosclerosis, Lipid-enriched diets are used to induce or accelerate the rate of the atherosclerotic lesion [37]. Consequently, the use of a high-fat diet for promoting atherosclerosis is an obliging tool for examining the disease and treatment effect. A valuable role is played by the lipoprotein oxidation and oxidative processes in general in the pathogenesis of atherosclerosis [38]. The elevation of plasma lipids and lipoprotein fractions manifested the disorders of lipid metabolism which in turn results in cardiovascular diseases. Obesity is developed in animals fed with a feeding diet rich in cholesterol as their body weights have been seen to be increased significantly. The body weight is increased due to the increase of fat tissue deposit much more on the level of the hip. The changes in body weight must be observed to access the course of disease with the response to therapy and also indicated the adverse effect of doses. As depicted from the results, the bodyweight of rats increased in all six groups from the beginning to the end of the experiment. Cholesterol is an important element of the biological membranes and having its significant importance as a precursor in the synthesis of bile acids, vitamin D and steroidal hormones. The increase in the concentration of serum cholesterol significantly increases the risk of coronary heart diseases. In high-fat diet-induced rats, a considerable increase was seen in the levels of plasma TC, TG. A cholesterol-enriched diet resulted in a significant increase in TC, TG levels accompanied by increase serum LDL and decrease circulating HDL levels and atherosclerosis is linked with the high concentrations of TC, LDL, TG and low concentration of HDL [39-40]. Dyslipidaemia is also amongst the major risk factors for diseases like atherosclerosis which promotes the accretion of oxidized LDL in the arterial wall. An important role is played by oxidized LDL in the initiation and progression of the cardiovascular dysfunction associated with atherosclerosis and hence amelioration of oxidative stress is equally important as controlling dyslipidaemia. The initial step in the conversion of LDL into atherogenic form is considered the oxidative modification of LDL. A significant reduction was seen in serum levels of TC, TG and HDL of rats treated with atorvastatin. HDL is inversely related to total body cholesterol, and a reduction in plasma HDL concentration may accelerate the development of atherosclerosis which leads to ischaemic heart disease, by impairing the cholesterol clearance from the arterial wall [41-44]. HDL is mainly attributed to reserve for the transport of cholesterol, a process by which excess tissue cholesterol is taken up and subsequently processed by HDL particles for further delivery to the liver for metabolism. Thus, an increased level of HDL can contribute towards the lower risk of atherosclerosis. Lipid peroxidation is caused due to the imbalance in lipid metabolism and it is commonly accepted that the elevated levels of plasma LDL and VLDL are risk factors for coronary heart diseases. The direct correlation between LDL level and atherosclerosis as well as the reversibility of related pathological events by lowering the serum LDL has been reported earlier. The high levels of LDL were significantly reduced in the atorvastatin treated group when compared to high-fat diet-fed rats. Several reports confirmed that flavonoids can increase HDL concentration and decrease LDL and VLDL levels in hypercholesterolemic rats [45].

Atherosclerosis is a progressive inflammatory disease caused by multiple factors, one of which is macrophage-induced cell apoptosis. HO-1, an endogenous defence enzyme, prevents oxidative stress injuries and is up-regulated by Nrf2 activation. Studies have shown that the PI3K/AKT pathway is involved in Nrf2 activation in macrophages. The PI3K/AKT pathway is involved in protecting against not only apoptotic insults but also ROS detoxification. It is reported that Isorhamnetin notably increased HO-1

expression, activated AKT phosphorylation, and promoted Nrf2 translocation which indicated that it showed selective effects on AKT from different cells. The addition of the AKT and HO-1 inhibitors may abolish the aforementioned effects of Isorhamnetin. These results suggest that the anti-apoptotic effects of Isorhamnetin were related to the activation of the PI3K/AKT pathway and HO-1 expression. So, the previous studies reported the mechanism of Isorhamnetin on ox-LDL-induced macrophage injuries and anti-atherosclerosis [20]. Thus, it indicates that Isorhamnetin must be a potential candidate in preventing Atherosclerosis.

An antihypertensive effect of bark extract of *C.dichotoma* L which contains isorhamnetin flavonoid was investigated in normotensive and hypertensive rats. The SHR model is used for screening of antihypertensive compounds as it provides a similar pattern of attenuation of blood pressure as in hypertensive humans and to study the mechanism of established hypertension. L-NAME induced hypertension in rats is due to the nitric oxide (NO, endothelium-derived relaxing factor) deficiency-induced endothelial dysfunction and increase in blood pressure [44]. Flavonoids act as ACE inhibitors by chelate complex formation within ACE containing zinc ion and flavonoids, thus inhibiting the ACE efficacy [45]. The polyphenolic compounds, flavonoids act on the RAAS pathway and attenuate the blood pressure, by inhibiting the renin release and ACE. The bark extract of *C.dichotoma* L may be acting on the RAAS pathway and decreasing the blood pressure, as it contains flavonoidIsorhamnetin. It also attenuates the plasma concentration of angiotensin II, indicating that isorhamnetin; flavonoid present in the extract of *C.dichotoma* Linhibits the enzyme renin which converts angiotensinogen into angiotensin I and also inhibiting the enzyme, ACE which converts angiotensin I to angiotensin II. In L-NAME-induced hypertension groups there was a decrease in NO level as compared to their control group respectively. The decrease in NO level produces vasoconstriction of arteries which results in increased cardiac output hence increased blood pressure. Flavonoids produce vasodilation as they have an antioxidant effect, by inhibiting the formation of peroxynitrite, thereby increasing the bioavailability of NO producing vasodilation and lowers BP [46]. Flavonoid also improves endothelial dysfunction and scavenges superoxide anions and therefore may defend NO from superoxide induced inactivation. Flavonoids have an antioxidant effect, vasodilator and cardioprotective effect, thus produce an antihypertensive effect [47]. The SHR rats are genetically hypertensive and increased oxidative stress without any stimulus and increased superoxide anion. There is increased oxidative stress in hypertension. L-NAME inhibits the enzyme NOS which decreases NO formation, thereby causing vasoconstriction and ROS formation, leading to lesion formation and hypertension. Several studies reported that increased cGMP concentration activates PKG1, and mediates a variety of physiological signal mechanisms in VSMC, and decreases in intracellular Ca2+ levels, causing membrane hyperpolarization, and inhibiting myosin light chain phosphorylation, resulting in vasorelaxation thereby decreasing blood pressure [48]. In the present study, the bark extract of *C.dichotoma* L contains isorhamnetin flavonoid which acts as antioxidants. As flavonoids scavenge superoxide anions and therefore may shield NO from superoxide-induced inactivation. As a result, isorhamnetin containing bark extract of *C.dichotoma* Lis rescuing the elevated BP in hypertensive animals.

5. CONCLUSION

The present study has identified the isolation and characterization of isorhamnetin flavonoid from the bark of plant *C.dichotoma*. This study also provides evidence of the anti-atherosclerotic and anti-hypertensive effects of *C.dichotoma* L. This bark extract reduced bad cholesterols, triglycerides and increasing good cholesterols in rats subjected to a feeding regime enriched with cholesterol. Further, the standardized *C.dichotoma* L bark extract significantly reduced SBP, DBP and MBP in L-NAME–induced hypertensive rats. These results support the traditional use of the bark extract of this plant in the treatment of hypertension and atherosclerosis.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. there is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study highlights the efficacy of Siddha, ayurveda which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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