Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Bisflavonoids fraction from *Araucaria bidwilli* Hook., reverses hyperlipidemia induced atherosclerosis in high-fat diet induced hyperlipidemia

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Abstract

Background: Hyperlipidemia is a major cause for atherosclerosis which is a frontline cause for mortality in the world. Bisflavonoids are dimeric flavonoids abundant in few medicinal herbs with various pharmacological effects. However, in vivo anti-hyperlipidemic role of bisflavonoids (BFR) is limited. The present investigation is aimed to study BFR from the leaf extract of *Araucaria bidwillii* Hook. in rat model of hyperlipidemia.

Results: Administration of HFD was significantly ($p < 0.0001$) shown to increase total cholesterol (TC), low-density lipoprotein (LDL), and triglycerides (TG) associated with decrease in HDL. BFR at two doses significantly decreased TC, LDL, and TG in HFD-fed rats. In addition, BFR significantly ($p < 0.0001$) decreased the MDA and significantly ($p < 0.0001$) increased the impaired anti-oxidant enzyme SOD and CAT in heart tissue induced by HFD. Further, 28 days administration of BFR significantly ($p < 0.001$) decreased HFD-induced aortic wall thickness.

Conclusion: It can be concluded that bisflavonoids from *A. bidwillii* Hook. leaf extract administered to high fat-fed rats showed beneficial anti-hyperlipidemic effect by reducing lipid profiles and protecting the heart tissue from oxidative stress.

Keywords: *Araucaria bidwillii*, Lipoproteins, Atherosclerotic plaques, Oxidative stress marker, Anti-oxidant enzymes

Background

Hyperlipidemia is a condition in which there is an elevated level of lipids and lipoproteins in the blood. It is a frontline major cause for atherosclerosis associated with morbidity and mortality in Asian countries [1]. Epidemiological studies have clearly emphasized that the significant role of circulation bad cholesterol including low-density lipoprotein (LDL) is associated with atherosclerosis [2, 3]. Consumption of high-fat diets, living sedentary lifestyle, and lack of exercise are important factors in the pathogenesis and progression of coronary artery disease [4]. Medicinal plant extracts and their bioactive constituents have been shown to possess various biological effects. In particular, polyphenolic phytoconstituents such as flavonoids and flavones have been shown to possess anti-hyperlipidemia potential in in vitro and in vivo experiments [5–7]. In particular, mono-flavonoids from plant extracts have been shown to inhibit oxidation of LDL and reduce triglyceride level in high-fat diet-induced hyperlipidemia [8, 9]. The anti-atherogenic effect of mono-flavonoids such as quercetin, resveratrol, and naringenin has shown the reduction of atherosclerotic plaques in rodent model of hyperlipidemia and atherosclerosis [10–12]. On the contrary, bisflavanoids are dimeric species of flavonoids which occur quite abundantly in some plant species. For example, the genus *Hypericum* contains amentoflavone which is known for various pharmacological properties [13]. The plant genus *Araucaria* belongs to the Araucariaceae

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family and traditionally has been used for amenorrhea [14]. It has a rich source of bisflavanoids which have been isolated and characterized earlier [15, 16]. The leaf extract of *Araucaria bidwillii* Hook. has been shown to possess analgesic and anti-inflammatory effect [17] and cytotoxic effect against various cancer cell lines [18]. Our previous investigation on bisflavonoids separated from the ethyl acetate fraction has shown neuroprotective effect through anti-oxidant mechanism in rat model of oxidative stress [19]. There is no investigation regarding anti-hyperlipidemic effect of bisflavanoids from *Araucaria bidwillii* Hook. leaves which have been reported. Therefore, the evaluation of anti-hyperlipidemic potential of bisflavonoids fraction may have a value-added biological effect and it may have a source for natural anti-hyperlipidemic and nutraceutical. This present investigation was aimed to investigate the anti-hyperlipidemic effect of bisflavonoids separated from the plant *Araucaria bidwillii* Hook., in high-fat diet-induced hyperlipidemia in rats.

Methods

Chemicals

Adrenaline bitartrate, thiobarbituric acid (TBA), and atorvastatin were from Sigma-Aldrich Co., MO, USA. Cholesterol and egg yolk powder were purchased from Hi Media Laboratories, Mumbai. The organic solvents, chemicals, and reagents were purchased from HIME DIA, Mumbai.

Plant collection and authentication

Fresh *Araucaria bidwillii* Hook. leaves were collected and authenticated from Government Botanical Garden, Udhagamandalam, India. It was authenticated by Botanical Survey of India, Coimbatore, Tamil Nadu. A voucher specimen (VCP/PCOL/2008-02) of this plant material has been retained in our school for further reference.

Extraction of *Araucaria bidwillii* leaves

The shed dried leaves weighing around 1 kg were coarsely powdered and macerated with 95% v/v of ethanol. The extract was evaporated by rotary vacuum evaporator and freeze-dried. Percentage yield of the alcoholic extract was found to be 25.5% w/w.

Separation of bisflavanoids' fraction

The separation of bisflavanoids (BFR) was carried out by previously described method [15, 16]. The resultant alcoholic extract was successively reflexing with hexane, dichloromethane, and ethyl acetate until the appearance of colorless solvents. The resultant residue was washed with boiling water, and the final insoluble yellow residue was dissolved in ethanol which showed confirmative test for flavonoids.

Isolation of bisflavonoids by column chromatography

The concentrated bisflavonoids' fraction (1 g) was subjected to column chromatography over silica gel column and eluted with gradients of n-hexane and ethyl EtOAc system with increasing proportion to get 72 fractions. Fraction 26-45 with similar R_f value were labelled as fraction A and its was evoparated under pressure to obtain compound 1. Fractions 50–63 with similar R_f value were labeled as fraction B which was evaporated which yield compound 2 (33 mg). The spectral data of compounds 1 and 2 were compared with published literature [15, 16].

Preparation of high-fat diet and induction of atherosclerosis in rats

The modified high-fat diet was freshly prepared as per the method [20]. The diet contains wheat powder (50 g/100 g), yellow corn (10 g/100 g), barley powder (1 g/100 g), skimmed milk power (20 g/100 g), calcium chloride (4 g/100 g), salt (1 g/100 g), oil (7 g/100 g), egg yolk powder (6 g/100 g), and vitamin B12 (1 g/100 g). Thirty male Sprague-Dawley rats about 150–200 g were divided into five groups. Group I rats were the control group and were fed with normal pellet chow ad libitum. Group II rats were fed with HFD. Group III rats were fed with HFD and BFR 100 mg/kg per oral route. Group IV rats were fed with high-fat diet and BFR 200 mg/kg per oral route. The two doses of BFR (100 and 200 mg/kg) were chosen based on our previous per oral toxicity studies and the effect of *BFR* in rat model of oxidative stress model [19]. Group V rats were fed with high-fat diet and atorvastatin 5 mg/kg per oral route. The high-fat diet and drugs were administered for 28 days ad libitum. Experiments on animals were performed based on animal ethics guidelines of IAEC (290/CPCSEA/12.12.2000/PH07).

Body weight measurement

The body weight of the rats was measured on the 1st, 7th, 14th, 21st, and 28th day using animal weighing scale.

Biochemical estimation

At the end of the experiment, rats were sacrificed by carbon dioxide euthanasia, 2 ml blood was collected from cardiac puncture, and serum was separated and assayed for lipid profile using standard biochemical (Randox Laboratory) assay kit.

Measurement of oxidative stress and anti-oxidant markers

Rat heart was collected for estimation of oxidative stress enzymes and anti-oxidants. Rat heart was homogenized

with RIPA buffer (50 mM pH 7.8). The homogenate was divided into two equal volumes. The first volume of the homogenate was centrifuged at 1000 rpm (REMI CM-12) at 4 °C, and the supernatant was divided into two equal volumes. The first portion of the supernatant was used for malonaldehyde assay. The remaining supernatant was used for anti-oxidant marker assay after centrifuging at 12,000 rpm (REMI CM-12) at 4 °C for 15 min. Protein was assayed by established method [21].

Assay of malonaldehyde

Malonaldehyde (MDA) was measured by already established method [22]. 0.1 ml of tissue homogenate was mixed with 20% acetic acid (1.5 ml), thiobarbituric acid, and sodium dodecyl sulphate (0.2 ml). It was heated at 100 °C for 60 min and cooled. Further, it was mixed with n-butanol-pyridine-water mixture and centrifuged. The organic layer was withdrawn, and absorbance was measured at 532 nm.

Assay of superoxide dismutase

Superoxide dismutase (SOD) was assayed by standard protocol [23]. Sodium carbonate (2.8 ml of 0.05 mM buffer), 100 μl of EDTA, and 20 μl of tissue homogenate were incubated at 30 °C for 45 min. Adrenaline (100 μl) was added, and absorbance was read at 480 nm for 8–12 min.

Assay of catalase

Catalase (CAT) assay, 2.25 ml of phosphate buffer (65 mM), and 100 μl of homogenate were incubated at 25 °C for 30 min. 0.650 ml of hydrogen peroxide was mixed with homogenate, and the absorption was read at 240 nm [24].

Histology of aorta

At the end of the experiment, about 1 cm of rat aorta was transferred into buffer neutral formalin. The tissue was fixed for 24 h and dehydrated with gradient alcohol, made transparent with xylene solution. The tissue was blocked with paraffin, and sections of 5–10 μm thickness were prepared. The sections were then dewaxed with xylene, sequentially exposed to high-to-low concentration of alcohol, washed with distilled water, hematoxylin and eosin stained, dehydrated, made transparent, and fixed. The aortic wall thickness was observed under a microscope [25].

Statistical analysis

Data were expressed as mean ± SD. Data in each group were analyzed by two-way ANOVA (body weight parameter) as well as one-way ANOVA followed by post hoc analysis—Tukey's multiple comparison test using GraphPad Prism statistical software, USA. $p < 0.05$ is considered as statistically significant.

Fig. 1 TLC of crude fraction of BFR

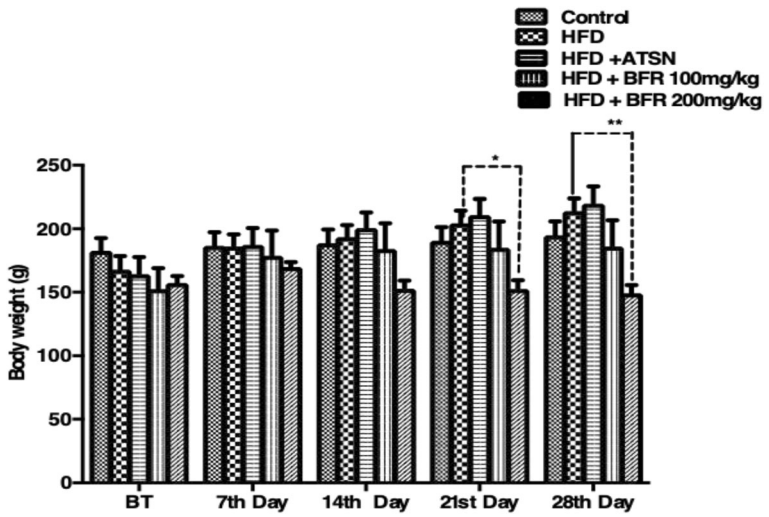

Fig. 2 Effect of BFR and ATSN in body weight of high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at *$p < 0.05$ and **$p < 0.01$

Results

Phytochemical analysis of bisflavonoids

The TLC of the bisflavonoids' crude fraction was carried out using toluene–ethyl acetate–formic acid (4:5:1) (Fig. 1). The TLC plate was developed with natural product-polyethylene glycol reagent. The results from TLC plate for BFR revealed the presence of various spots with different R_f values. The R^f values (0.16 to 0.61) were compared with the earlier literature reported with various bisflavonoids [19]. The spectral data of compound 1 (methylcupressuflavone) is yellow crystalline powder with m/z which was 570.52 (M, 100). IR: 3247.23, 2948.17, 2802.22, 1792.45, 1673.34, 1600.71–1720.31, 1282.63, 1158.62, 917.54, and 724.33. NMR: 13.40–13.24 (2H, s), 9.22–9.51 (H, d), 7.53–7.63 (2H, q), 6.83–6.89 (2H, t), 6.74–6.53 (H, m), 3.33–3.95 (H, m), and 2.15–2.91 (m, benzylic CH). Compound 2 (tetrahydroamentoflavone) is pale yellow amorphous powder with m/z 542.41 (M, 100%). IR: 3275.61, 2902.32, 1880.62, 1603.29–1880.62, 1251.14, 1158.68, 1158.69, and 741.64. NMR: 12.38–12.17 (H, s), 10.80 (H, s), 7.33–7.35 (2H, d), 7.20–7.32 (2H, d), 6.82–6.85 (2H, d), 5.88 (H, s), 3.91-4.06 (H, m), and 3.11–3.36 (m, benzylic CH).

Effect of ATSN and BFR on body weight

Effect of ATSN and BFR on body weight and lipid profile of the normal diet- and high-fat diet-treated rats is shown in Figs. 2 and 3a–d. HFD administration did not affect the body weight significantly. However, reduced body weight was observed in the BFR (200 mg/kg)-treated HFD group at 21st ($p < 0.01$) and 28th ($p < 0.001$) days.

Effect of ATSN and BFR on lipid profile

High-fat diet administration had significantly ($p < 0.01$) increased the total cholesterol (TC) and triglyceride (TG) level indicating hypercholesterolemia and hypertriglyceridemia in rats compared with normal TC and TG level in the control group (Fig. 3a, b). Anti-hyperlipidemic effect was observed in the HFD and BFR treatment group as evidenced by significant decrease ($p < 0.05$) in TC, TG, and low-density lipoprotein (LDL) level compared with the high-fat diet-treated group. The effect of BFR in decreasing elevated lipid profile was dose dependent (Fig. 3a–c).

In addition, there was a significant decrease ($p < 0.05$) in high-density lipoprotein (HDL) in high-fat diet-fed rats than the normal diet-fed control group. BFR administration at the dose of 200 mg/kg significantly ($p < 0.05$) increased the HDL level as compared with HFD-fed rats. ASTN administration also showed a significant ($p < 0.05$) effect on lipid profiles in decreasing TC, TG, and LDL and ($p < 0.01$) increasing HDL of the high-fat diet-given rats compared with the high fat-treated control group (Fig. 3d).

Effect of ATSN and BFR on MDA and anti-oxidant enzymes

Effect of ATSN and BFR on MDA level in the heart of HFD-fed rats is shown in Figs. 4, 5, and 6. High-fat diet administration had significantly ($p < 0.0001$) elevated the rat heart MDA level pointing oxidative stress due hyperlipidemia. BFR significantly reversed ($p < 0.001$) the elevated MDA level in the HFD-treated group compared with the normal diet-treated group. Likewise, ATSN at the dose of 5 mg/kg has shown a significant decrease ($p < 0.0001$) in MDA level as compared with the high fat-treated control group. As shown in Fig. 5, significantly ($p < 0.0001$) decreased SOD level was observed in high-fat diet-administered rats. BFR administration

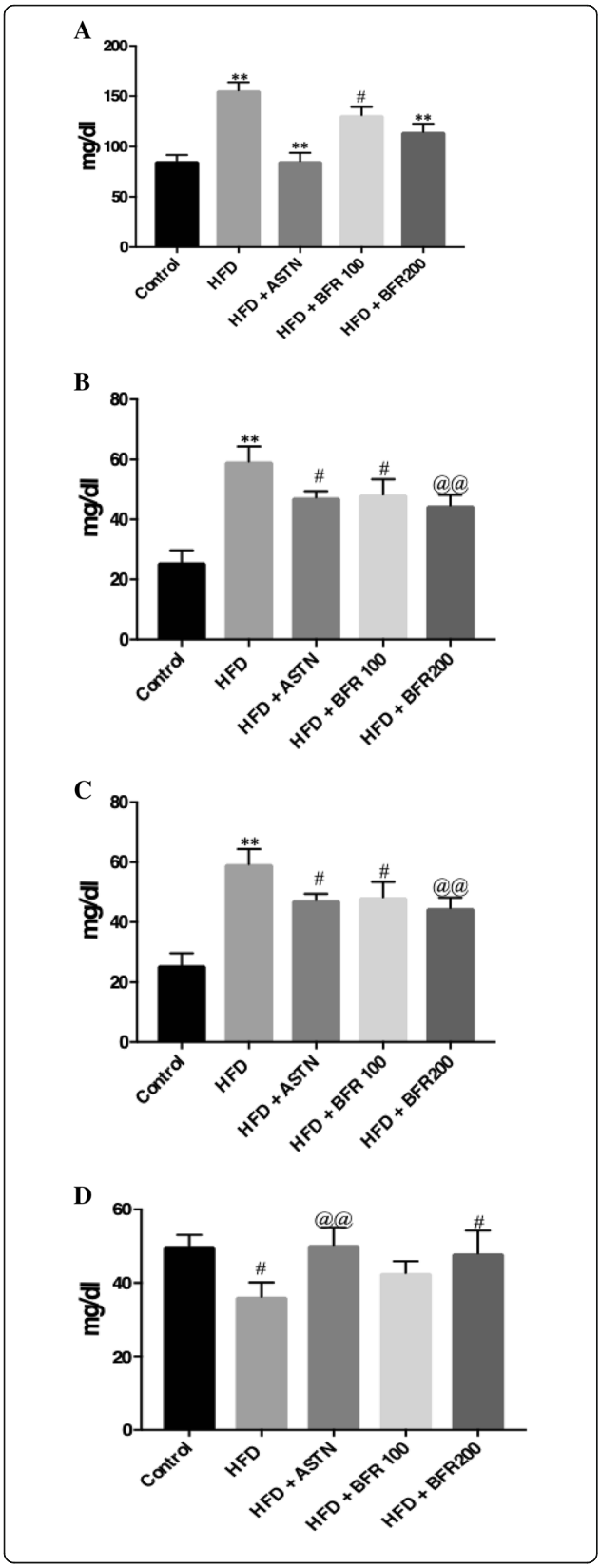

Fig. 3 **a** Effect of BFR and ATSN in total cholesterol level of the high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{\#}p < 0.05$ and $^{**}p < 0.01$. **b** Effect of BFR and ATSN in low-density lipoprotein (LDL) level of the high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{\#}p < 0.05$, $^{@@}p < 0.01$, and $^{**}p < 0.01$. **c** Effect of BFR and ATSN in triglyceride (TG) level of the high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{\#}p < 0.05$, $^{@@}p < 0.01$ and $^{**}p < 0.01$. **d** Effect of BFR and ATSN in high-density lipoprotein (HDL) level of the high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{\#}p < 0.05$ and $^{@@}p < 0.01$

significantly ($p < 0.001$) elevated the anti-oxidant SOD level; the anti-oxidant effect of BFR was dose dependent. In addition, ATSN ($p < 0.001$) for 28 days to HFD-fed rats showed a significant increase in SOD level compared with the high fat-treated control group. Likewise, rats that were ingested with high-fat meal showed a significant decrease ($p < 0.0001$) in catalase compared with the control group treated rats fed with normal diet. Administration of ATSN and BFR significantly ($p < 0.0001$) increased the CAT level compared with high-fat diet rat fed CAT level.

Histopathological examination of rat aorta

Histopathological examination of high fat-fed rat aorta showed the accumulation of atheromatous plaque in subendothelial portion of the aorta as compared to normal diet-fed rat aorta (Fig. 7). There is a significant increase in aortic thickness ($p < 0.0001$) in the HFD-treated group (Fig. 8b) when compared with the normal feed-fed group (Fig. 8a). Administration of BFR at 200 mg (Fig. 8d) significantly ($p < 0.05$) reduced the high-fat diet-induced aortic thickness when compared with the high-fat diet-fed rat group (Fig. 8b).

Discussion

Herbal extracts with various bioactive phytoconstituents have been shown to exert beneficial role in reversing hyperlipidemia-induced oxidative stress [26]. Consumption of high-fat diet can cause hyperlipidemia which is a common cause for atherosclerosis, and prevalence of atherosclerosis in young adults has been increased in developing countries [27]. The *A. bidwilli* Hook. leaf extract has been used traditionally for various illnesses associated with oxidative stress. Our previous work on comparative free radical scavenging ability and in vivo neuroprotective effect of bisflavonoid-rich fraction in rat model of oxidative stress confirmed the in vitro and

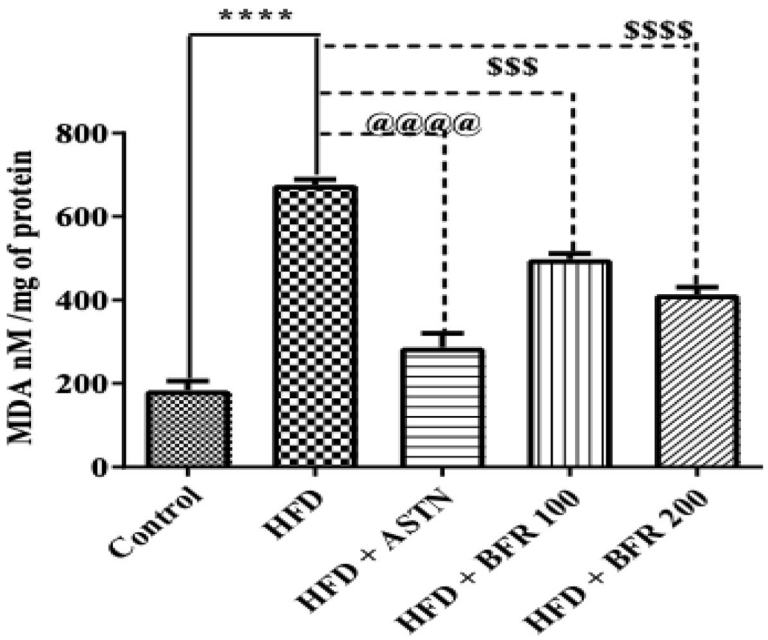

Fig. 4 Effect of BFR and ATSN in rat heart MDA level of high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{****,@@@@}p < 0.0001$ and $^{\$\$\$}p < 0.001$

in vivo bioactivity of the fraction. This prompted us to investigate the effect of crude bisflavonoids' fraction in experimentally induced hyperlipidemia. In this study, rats fed with atherogenic diet for 4 weeks continuously resulted in impaired lipid profile. This is due to altered transport and metabolism of total cholesterol and triglycerides in the rat plasma. It has been shown by various authors that the replacement of normal diet with high-fat diet to rats develops hyperlipidemia [28, 29]. On the other hand, atheromatous plaque deposition in the rat aorta is due to supplementation of atherogenic diet continuously for 4 weeks and altered blood lipoprotein profiles. Previous studies have shown that significant increase in body fat composition in rats was associated with high-fat diet

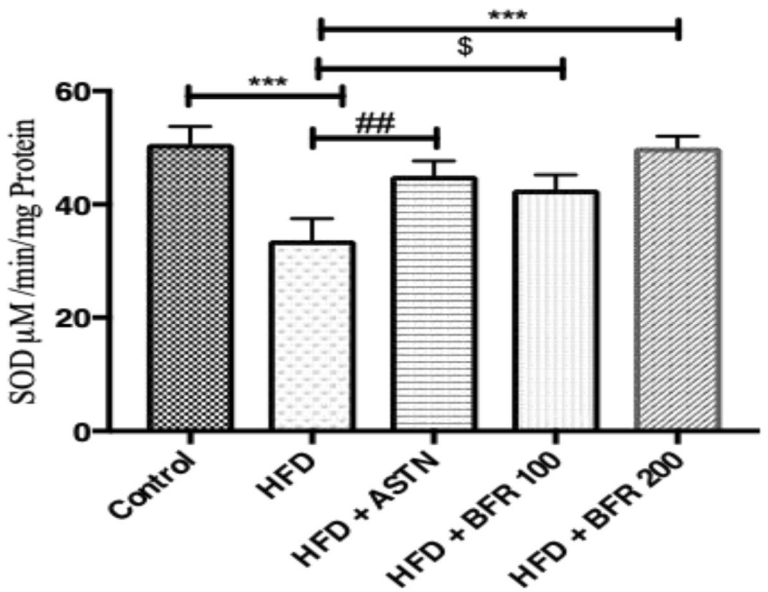

Fig. 5 Effect of BFR and ATSN in heart SOD level of high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{\$}p < 0.05$, $^{\#\#}p < 0.001$, and $^{***}p < 0.001$

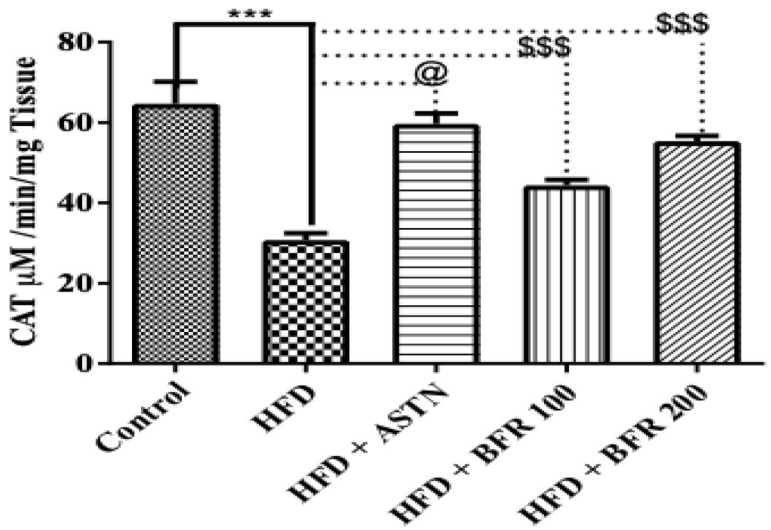

Fig. 6 Effect of BFR and ATSN in heart catalase level of high-fat diet-fed rat. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{@@@,***,\$\$\$}p < 0.001$

consumption in rats [30, 31]. However, our results showed that high fat did not affect the body weight through the entire period of high-fat diet administration. This insignificant body weight change may be due to duration of high-fat diet administration. Interestingly, the BFR-treated group had shown decreased body weight at the 21st and 28th day which suggests the possible interaction of BFR in exogenous absorption, transport, and storage of excess cholesterol and free fatty acids derived from atherogenic diet.

It is well known that plant-derived bioflavonoids with special reference to mono-flavonoids and flavones exhibit anti-hyperlipidemic effect in in vivo and in vitro model, and the beneficial effect of flavonoids on cholesterol transport, LDL, and HDL metabolism has been reviewed recently [32]. The mono-flavones like

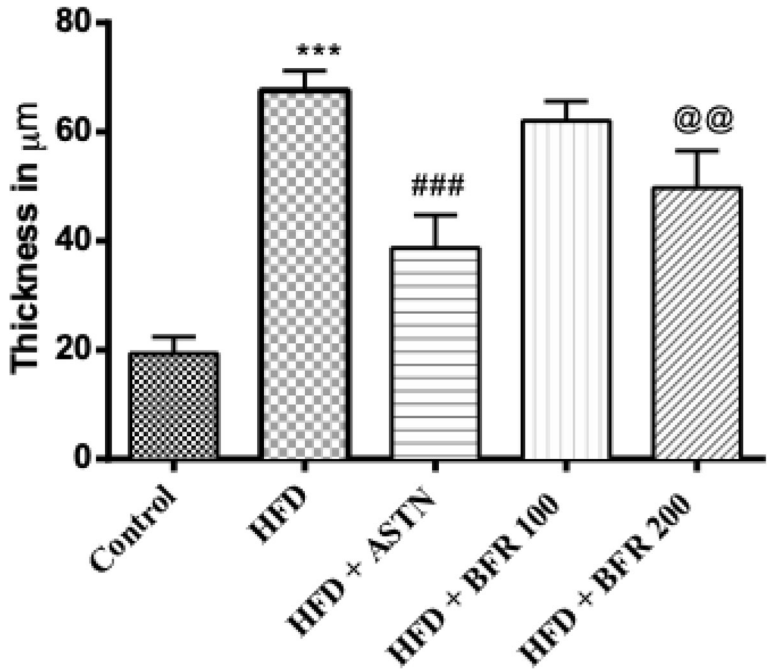

Fig. 7 Effect of BFR and ATSN in aortic wall thickness of high-fat diet-fed rats. Data were expressed as mean ± SD. Comparison made between HFD vs control and HFD + ATSN vs HFD group, HFD + BFR vs HFD. Statistical significance at $^{@@}p < 0.01$ and $^{***,\#\#\#}p < 0.001$

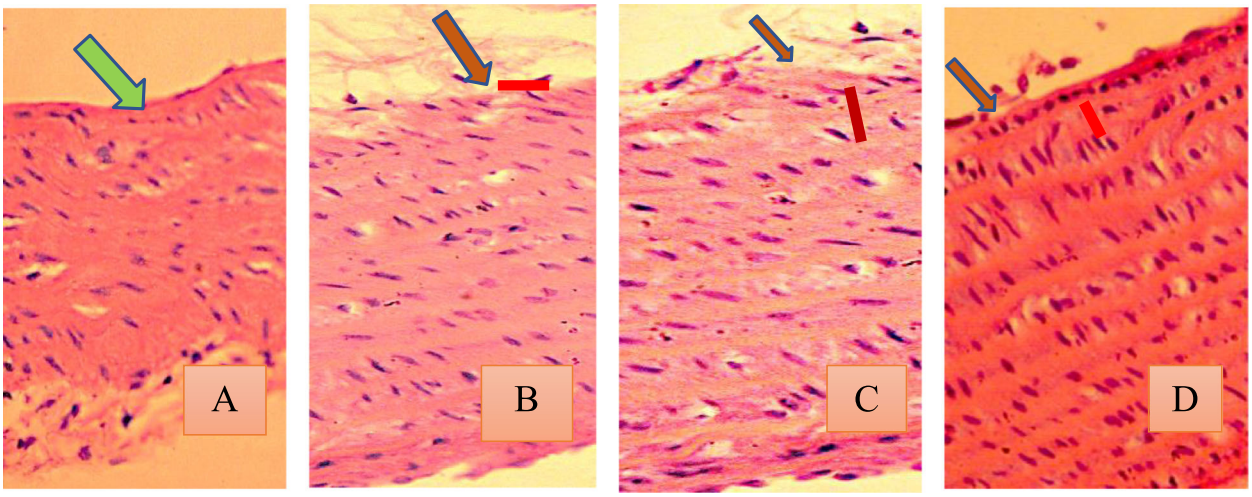

Fig. 8 Histopathology of aortic wall of normal and high-fat diet-fed rats treated with BFR and ATSN. **a** Normal aorta (magnification × 40). **b** High-fat diet fed (magnification × 40). **c** ATSN treated (magnification × 40). **d** BFR 200 mg/kg (magnification × 40). Green arrow and red arrow represent the normal and altered morphology of the aorta layer. Red thick line/bar represents the wall thickness of the aorta layer of normal and BFR, BFR + ATSN BFR treated group

hesperetin and naringenin have been shown to affect cholesterol metabolism by inhibiting endogenous pathway by affecting hepatic production of apo B containing VLDL and LDL in vitro [33–35] and in vivo [36]. In another study, catechins have been shown to facilitate the LDL receptor binding activity in HepG2 cell lines through upregulation of sterol regulated element binding protein (SREBP-1) [37]. It has been reported that monoflavonoids from various medicinal plants affect the lipoprotein lipase (LPL) activity in muscle and adipose tissue of mice and thereby inhibit the conversion and deposition of triglycerides in tissues [38, 39]. The bisflavanoids of *Garcinia kola* seed namely kolavarin A and B showed hypolipidemic effect in the experimental rat model of hyperlipidemia [40, 41]. The observed anti-hyperlipidemic potential of bisflavonoids' (BFR) fraction may be attributed to reduce the lipid absorption from intestine or increase the hepatic metabolism and clearance of lipoproteins.

It is well known that increase in blood cholesterol and triglycerides can generate reactive oxygen species (ROS) [42]. Further, increased LDL-C is associated with oxidative modification of LDL, overproduction of lipid peroxidation, and oxidative stress [43]. Increased MDA and decreased anti-oxidant enzymes in rat heart confirm the oxidative stress, and this impaired anti-oxidant defense was remarkably reversed by BFR which may be attributed to direct scavenging of lipid peroxidation product. The anti-lipid peroxidation mechanism of flavonol through glycoside linkage [44] and interaction of aglycone moiety [45, 46] have been reported. The impaired anti-oxidant enzymes such as SOD and CAT were corrected by BFR suggesting in vivo anti-oxidant enhancing capacity of BFR in high-fat diet-induced oxidative stress. This in vivo anti-lipid peroxidation and anti-oxidant effect was aligned with the neuroprotective potential of BFR in the rat brain following bilateral common carotid artery occlusion-induced oxidative stress. Furthermore, the histopathological investigation of rat aorta showed that attenuation of atheromatous plaque deposition in high-fat diet-fed rats suggests anti-atherogenic effect. Results from our study showed that the *per oral* administration bisflavonoids' fraction from *A. bidwilli* Hook. leaf extract had reversed the atherogenic diet-induced hypercholesterolemia and hypertriglyceridemia in rats. This effect was associated with attenuation of oxidative stress and atherosclerosis by decreased deposition of atheromatous plaque in rat aorta and increased anti-oxidant profiles in the plasma. The anti-hyperlipidemic effect of BFR is may be due to its putative role in exogenous transport of the dietary lipids and inhibition of LDL oxidation and anti-oxidant effect in in vivo.

Conclusion

It can be concluded that bisflavonoids' fraction from *A. bidwillii* Hook. leaf extract administered to high fat-fed rats showed anti-hyperlipidemic effect by reducing blood cholesterol, triglycerides, and LDL level and protecting the heart tissue from oxidative stress.

Abbreviations

BFR: Bisflavonoids; HDF: High-fat diet; TC: Total cholesterol; LDL: Low-density lipoprotein; MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; TBA: Thiobarbituric acid; ATSN: Atorvastatin

Acknowledgements

The authors acknowledge Vels College of Pharmacy for providing necessary laboratory support to carry out this project during his post-graduate study in pharmacy program.

Authors' contributions
HNA and RS designed the idea and prepared the protocol for the work. RS performed the experimental work. IU carried out the histopathological interpretation. RS and HNA are responsible for the data analysis. All authors read and approved the manuscript for submission.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

Availability of data and materials
(a) Data and material are available upon request. (b) The plant was collected from the Government Botanical Garden and authenticated by a field botanist. The plant authentication number was VCP/PCOL/2008-02.

Ethics approval and consent to participate
The preclinical in vivo experimental procedures on Sprague-Dawley rats were carried out after receiving institutional animal ethical committee approval. The approval number is as follows: 290/CPCSEA/12.12.2000/PH07.

Consent for publication
Not applicable

Competing interests
The authors declare that there is no competing interest.

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Received: 5 March 2020 Accepted: 7 September 2020
Published online: 19 November 2020

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