ANISOTES TRISULCUS PREVENTS HYPERCHOLESTEROLEMIA, OXIDATIVE STRESS AND INFLAMMATION AND MODULATES FATTY ACID SYNTHASE IN HIGH CHOLESTEROL DIET-FED RATS

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Abstract

Anisotes trisulcus is a stiff erect shrub with antioxidant and anti-inflammatory activities. This study investigated the protective effect of A. trisulcus extract against oxidative stress, inflammation and hypercholesterolemia in high cholesterol diet (HCD)-fed rats. Rats received HCD and A. trisulcus extract (100 and 200 mg/kg) for 10 weeks and samples were collected for analysis. Serum cholesterol, triglycerides, LDL-cholesterol and vLDL-cholesterol along with cardiovascular risk indices were significantly increased in HCD-fed rats. HDL-cholesterol was decreased, and pro-inflammatory cytokines and ALT, AST and ALP were increased in serum of HCD-fed rats. Treatment with A. trisulcus extract ameliorated these metabolic alterations and decreased hepatic lipid peroxidation levels. In addition, A. trisulcus extract enhanced hepatic GSH and antioxidant enzymes in HCD-fed rats. Furthermore, A. trisulcus extract decreased the expression of hepatic fatty acid synthase (FAS) and stimulated a trend increase in LDL receptor in HCD-fed rats. In conclusion, this study showed the anti-hypercholesterolemic, antioxidant and anti-inflammatory activities of A. trisulcus in HCD-fed rats. A. trisulcus ameliorated serum lipids, cardiovascular risk indices, liver function markers and pro-inflammatory cytokines, and hepatic oxidative stress and FAS expression.

Keywords: Almodh; LDLR; FAS; Cholesterol; Oxidative stress; Inflammation.

Introduction

Hyperlipidemia/dyslipidemia has been demonstrated as a casual risk factor for atherosclerotic cardiovascular disease (Nordestgaard, 2016). It is a disorder of lipid metabolism and manifested as increased levels of low density lipoprotein (LDL) cholesterol and triacylglycerides (Nordestgaard, 2016). Hyperlipidemia may arise through lifestyle factors such as excessive consumption of foods rich in cholesterol and saturated fats, but is also highly heritable (Ma et al., 2012; Yuan et al., 2007). Hypercholesterolemia is a type of hyperlipidemias that elicits atherosclerosis, chronic inflammation and accumulation of hepatic lipids (steatosis) and hence reduce the ability of the liver to lower circulating lipids (Lee et al., 2017). The role of hypercholesterolemia in eliciting oxidative stress has been demonstrated in different studies (Al-Rejaily et al., 2013; Lee et al., 2017; Olorunnisola et al., 2012). The accumulation of cholesterol in different cells, such as hepatocytes and endothelial cells has been associated with diminished antioxidant defenses and excess production of reactive oxygen species (ROS) (Anila & Vijayalakshmi, 2003; Forstermann, 2008). Excess ROS accumulation can induce inflammation, cell death and metabolic alterations (Jones, 2006; Seifried et al., 2007). Hence, agents that can counteract hypercholesterolemia and other hyperlipidemias and exert antioxidant and anti-inflammatory activities can protect against the metabolic alterations induced by high levels of lipids.

Medicinal plants are rich sources of bioactive compounds that can exert anti-hyperlipidemic effects accompanied with attenuation of oxidative stress and inflammation (Aladaileh et al., 2019b). Anisotes trisulcus (family Acanthaceae) is a stiff erect shrub and commonly known as Almodh in Saudi Arabia (El-Shanawany et al., 2011). A. trisulcus has been reported to exert multiple activities, including ant-hypertension, anti-bacterial, local anesthetic and hepatoprotective. In addition, this species is used to limit tobacco consumption, suppress appetite and traditionally in the treatment of jaundice, hepatitis, gallstones and other disorders related to the liver (Al-Rehaili et al., 2011; Al-Rejaily et al., 2002; Ali et al., 2001; El-Shanawany et al., 2011). A. trisulcus has been demonstrated to be a rich source of phenolic compounds, including vanillic acid, veratic acid, β-sitosterol, stigmasterol, α-amyrin and veratic acid, and its methanolic extract exhibited antioxidant and anti-inflammatory activities (El-Shanawany et al., 2014). However, the beneficial effects of A. trisulcus on hypercholesterolemia have not been previously investigated. Therefore, this study evaluated the anti-hypercholesterolemic effect of A. trisulcus and its protective antioxidant and anti-inflammatory activities in high cholesterol diet (HCD)-fed rats. Additionally, the ability of A. trisulcus to modulate hepatic fatty acid synthase (FAS) and LDL receptor (LDLR) expression in HCD-fed rats was studied.

Materials and Methods

A. trisulcus leaf collection and extraction

The leaves of A. trisulcus were collected from the northern are of Saudi Arabis (Sakaka) and were identified and authenticated by an expert taxonomist from the Botany Department, College of Science, Jouf University, KSA. The leaves were washed under running tap water, dried in the shade and then mashed into a powder size and macerated with methanol (80%; v/v) at 4°C for 72 h. The macerated product was filtered, and the obtained supernatant was concentrated using a rotary evaporator to a semi-dry state and then dissolved in distilled water.

Animals and experimental design

Thirty adult male Wistar rats weighing 140-160 g were housed under standard laboratory conditions (12 h light/dark
cycle at 22±25 °C with free access to standard rodent pellet diet and water. All experiments and the study protocols were approved by the Ethics Committee of Jouf University (Ethical approval number: 08/01/41). To investigate the anti-hypercholesterolemic effect of *A. trisulcus* extract, the rats were allocated into 5 groups (n=6) as follows: Group I rats fed a normal diet for 10 weeks and served as control, and groups II-V included rats fed a high cholesterol diet (HCD; normal diet supplemented with 2% cholesterol). Groups II and III received 100 and 200 mg/kg *A. trisulcus* extract, respectively, whereas group V received 10 mg/kg simvastatin for 10 weeks. *A. trisulcus* methanolic extract has shown anti-inflammatory effect in rats at dose of 400 mg/kg as reported previously (El-Shanawany et al., 2014). Therefore, two lower doses (100 and 200 mg/kg body weight) were selected in this study.

At the end of the treatment, all groups were fasted overnight, sacrificed under anesthesia and blood samples were collected and left to coagulate to separate serum by centrifugation. The animals were dissected, and liver was removed and washed and homogenized in cold 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 6000 rpm and the supernatant was collected and stored at -80°C.

**Assay of serum liver function parameters**

Serum liver parameters transaminases [alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP)] were estimated to assess liver function using Randox diagnostic kits (UK).

**Assay of serum lipids and cardiovascular risk indices:**

The levels of serum triglycerides (Fossati & Prencipe, 1982), total cholesterol (Allain et al., 1974) and HDL-cholesterol (Burstein et al., 1970) were assayed using Randox (UK) kits. vLDL- and LDL-cholesterol levels were calculated as follows:

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vLDL-cholesterol = Triglycerides/5
\]

\[
LDL-cholesterol = Total \text{ cholesterol} - (HDL-cholesterol + vLDL-cholesterol)
\]

Cardiovascular risk indices (Ross, 1992) and anti-atherogenic index (AAI) (Guido & Joseph, 1992) in all groups were calculated as follows:

Cardiovascular risk index 1 = Total-Cholesterol/HDL-Cholesterol

Cardiovascular risk index 2 = LDL-Cholesterol/HDL-Cholesterol

AAI = HDL-Cholesterol x 100/Total cholesterol - HDL-Cholesterol.

**Oxidant/antioxidant status analysis**

The assessment of lipid peroxidation (LPO) was based on the amount of formed thiobarbituric acid reactive species (TBARS) according to the described method of Ohkawa et al. (1979). The content of reduced glutathione (GSH) was estimated according to the described method by Ellman (1959). The hepatic activity of superoxide dismutase (SOD) and catalase were assayed based on the method of Nishikimi et al. (1972) and Aebi (1984), respectively.

**Assay of pro-inflammation cytokines**

 Serum levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β were determined using commercial kits (Cusbio, China) according to the manufacturer’s protocols.

**Quantitative Real-Time-PCR (qRT-PCR)**

Hepatic total RNA was isolated using TRIzol reagent (Invitrogen, USA) and its quantity was determined using a nanodrop. Samples with A260/A280 higher than 1.7 were immediately reverse-transcribed into cDNA using RevertAid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Canada) according to the manufacturer’s instructions. For gene expression analysis, qRT-PCR was employed using Quantifast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) and the following primers: fatty acid synthase (FAS): F: 5'-CTGGACCTCGCTCATGGGTG-3' & R: 5'-CATTTCCTGAAGCTCCCGCACG-3', LDL-receptor (LDL-R): F: 5'-CAGCTGTGTGAACCTGGGA-3' & R: 5'-TTCTTCAGGTGGGATCAG-3', and GAPDH: F: 5'-AACCTTGCCATCCTGGAAGG-3' & R: 5'-TACATTGGGGGTAGGAACAC-3'. qRT-PCR reactions were performed using ViiA™ 7 System (Thermo Fisher Scientific, CA, USA) in duplicates. The transcript number was determined using the 2^ΔΔCt method (Livak & Schmittgen, 2001).

**Statistical analysis**

Data were expressed as the mean ± standard error of the mean (SEM) of six rats. Difference between mean values of multiple groups was analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s Post hoc test on Graphpad Prism 7. Statistical significance was considered at P<0.05.

**Results**

**Effect of *A. trisulcus* on serum lipids**

HCD-fed rats showed significantly elevated serum triglycerides (P<0.001), total cholesterol (P<0.001), LDL-Cholesterol (P<0.001) and vLDL-Cholesterol (P<0.001) as shown in Figures 1A-D. HDL-Cholesterol was decreased in HCD-fed rats when compared with the control group (P<0.05) as shown in Figure 1E. Treatment with *A. trisulcus* extract (100 and 200 mg/kg) and simvastatin decreased serum triglycerides, total cholesterol, LDL-Cholesterol and vLDL-Cholesterol (P<0.001) whereas failed to increase HDL-Cholesterol significantly in HCD-fed rats.

**Effect of *A. trisulcus* on cardiovascular risk indices**

HCD-fed rats exhibited significant increase in cardiovascular risk indices 1 (Fig. 2A) and 2 (Fig. 2B) as compared to the control rats (P<0.001). On the other hand, the AAI was decreased significantly in HCD-fed rats (P<0.001; Fig. 2C). Treatment with *A. trisulcus* extract and simvastatin decreased the values of cardiovascular risk indices and increased AAI in HCD-fed rats.
Fig. 1: Effect of *A. trisulcus* on serum lipids in HCD-fed rats. Data are mean ± SEM (n = 6). *P<0.05 and ***P<0.001 compared to Control. ###P<0.001 compared to HCD.

Fig. 2: Effect of *A. trisulcus* on cardiovascular risk indices and anti-atherogenic index in HCD-fed rats. Data are mean ± SEM (n = 6). ***P<0.001 compared to Control. ###P<0.001 compared to HCD.

Effect of *A. trisulcus* on liver function and serum pro-inflammatory cytokines

ALT, AST and ALP were increased significantly in serum of HCD-fed rats (Fig. 3) as compared to the control group (P<0.001). Both doses of *A. trisulcus* as well as simvastatin decreased serum ALT, AST and ALP significantly in HCD-fed rats.

Similarly, serum TNF-α, IL-6 and IL-1β levels were elevated in HCD-fed rats as compared to the control as represented in Figures 4A-C. In contrast, treatment with *A. trisulcus* as well as simvastatin ameliorated the levels of these inflammatory mediators.

Effect of *A. trisulcus* on liver lipid peroxidation and antioxidants

Hepatic TBARS levels were increased significantly (P<0.001) in HCD-fed rats as shown in Figure 5A. On the other hand, hepatic GSH (Fig. 5B), SOD (Fig. 5C) and catalase (Fig. 5D) were decreased in HCD-fed rats. Oral supplementation of *A. trisulcus* (100 and 200 mg/kg) or simvastatin decreased TBARS and increased GSH, SOD and catalase in the liver of HCD-fed rats.

Effect of *A. trisulcus* on LDLR and FAS gene expression

Supplementation of the HCD for 10 weeks exerted non-significant effect on the gene expression levels of LDLR (Fig. 6A) whereas increased hepatic FAS gene expression (Fig. 6B) significantly. Treatment of the HCD-fed rats with *A. trisulcus* (100 and 200 mg/kg) or simvastatin showed a trend increase in LDLR; however non-significant, and decreased FAS gene expression significantly.
Fig. 3: Effect of *A. trisulcus* on liver function markers in HCD-fed rats. Data are mean ± SEM (n = 6).

***P<0.001 compared to Control. ###P<0.001 compared to HCD.

Anisotes trisulcus prevents hypercholesterolemia, oxidative stress and inflammation and modulates fatty acid synthase in high cholesterol diet-fed rats.

Fig. 4: Effect of *A. trisulcus* on serum pro-inflammatory cytokines in HCD-fed rats. Data are mean ± SEM (n = 6).

*P<0.05 and ***P<0.001 compared to Control. ##P<0.01 and ###P<0.001 compared to HCD.
Fig. 5: Effect of *A. trisulcus* on hepatic lipid peroxidation and antioxidants in HCD-fed rats. Data are mean ± SEM (n = 6). **P<0.01 and ***P<0.001 compared to Control. #P<0.05, ##P<0.01 and ###P<0.001 compared to HCD.

**Fig. 6:** Effect of *A. trisulcus* on hepatic LDLR and FAS expression in HCD-fed rats. Data are mean ± SEM (n = 6). ***P<0.001 compared to Control. #P<0.05 and ##P<0.01 compared to HCD.

**Discussion**

This investigation explored the effects of *A. trisulcus* extract on hypercholesterolemia and its associated oxidative stress and inflammation, and gene expression levels of LDLR and FAS in the liver of rats. Hyperlipidemia is a disorder of lipid metabolism associated with the pathogenesis of atherosclerosis, hypertension, metabolic syndrome, and cardiovascular disease (Al-Rasheed et al., 2018; Al-Rasheed et al., 2017; Attia et al., 2002; Mahmoud et al., 2012; Mahmoud et al., 2017c). In this study, HCD-fed rats exhibited hypercholesterolemia and hypertriglyceridemia manifested by increased serum triglycerides, and total, LDL- and vLDL-cholesterols. Accordingly, previous studies have demonstrated dyslipidemia and hypercholesterolemia in HCD-fed rats (Al-Rejaie et al., 2013; Bin-Jumah, 2018; Lee et al., 2017; Olorunnisola et al., 2012). In addition, feeding a HCD resulted in decreased serum HDL levels and increased the values of cardiovascular risk indices, characteristic features of hypercholesterolemia and cardiovascular diseases (Abd El-Twab et al., 2016; Al-Rasheed et al., 2016; Al-Rasheed et al., 2017; Lee et al., 2017). Interestingly, treatment of the HCD-fed rats with *A. trisulcus* extract resulted in significant amelioration of serum lipids and cardiovascular diseases, demonstrating its potent anti-hyperlipidemic, anti-hypercholesterolemic and cardioprotective effects.

The anti-hyperlipidemic effect of *A. trisulcus* extract could be attributed to its ability to modulate hepatic LDLR and FAS expression. Although it didn’t show a significant effect, *A. trisulcus* extract showed a trend increase in LDLR expression in the liver of HCD-fed rats. LDLR is primarily responsible for the absorption of cholesterol by the liver through its mediated endocytosis. Following its absorption, cholesterol is metabolized within the liver and this results in
decreased plasma cholesterol levels (Ma et al., 1986; Yasunobu et al., 1997). Additionally, Anisotes trisulcus extract decreased the expression of hepatic FAS significantly in rats fed HCD. Increased expression of hepatic FAS has been previously reported in animal models of rodents fed high fat diet and HCD (Bin-Jumah, 2018; Inoue et al., 2005; Lee et al., 2017). FAS is one of the fatty acid synthesizing enzymes and its suppression is therefore responsible for decreased lipid synthesis.

HCD feeding was also associated with hepatic injury as shown by increased serum levels of ALT, AST and ALP. Hepatic injury occurs as a result of increased hepatic accumulation as a consequence of dyslipidemia (Lee et al., 2017). Accordingly, liver dysfunction has been reported in rats received HCD for 10 weeks (Bin-Jumah, 2018). HCD-induced rats treated with Anisotes trisulcus extract showed an improvement in serum ALT, AST and ALP levels, demonstrating its hepatoprotective effect. The ameliorative effect of Anisotes trisulcus extract on liver function is a direct consequence of its anti-hypercholesterolemic effect. In addition, the beneficial effect of Anisotes trisulcus extract could be attributed to its antioxidant and anti-inflammatory activities. Hypercholesterolemia is implicated in lipid peroxidation and oxidative modification of LDL (Yang et al., 2008). Oxidative injury has been suggested as the mechanism by which hypercholesterolemia provokes cellular death (Ma et al., 1986; Ma et al., 2012). Accordingly, HCD-fed rats exhibited significant increase in hepatic lipid peroxidation and decreased GSH, SOD and catalase as previously demonstrated (Al-Rejaie et al., 2013; Bin-Jumah, 2018; Lee et al., 2017; Olorunnisola et al., 2012). In the same context, hyperlipidemia has been shown to a risk factor for eliciting oxidative stress, cell death and tissue injury (Huisamen et al., 2012; Vincent et al., 2001). Anisotes trisulcus extract prevented oxidative stress as shown by decreased TBARS and increased antioxidants. These findings pointed to the antioxidant activity of Anisotes trisulcus. By using in vitro DPPH-radical scavenging assay, the antioxidant activity of Anisotes trisulcus has been previously shown (El-Shanawany et al., 2014).

Besides its antioxidant effect, Anisotes trisulcus has demonstrated potent anti-inflammatory effects in carrageenan-induced rat hind paw edema (El-Shanawany et al., 2014). In this investigation, Anisotes trisulcus ameliorated serum levels of the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, in HCD-fed rats. The induction of hypercholesterolemia in rats has been associated with inflammatory responses marked by increased serum cytokines and c-reactive protein (Bin-Jumah, 2018). This inflammatory response could be explained by the hypercholesterolemia-induced oxidative stress which causes inflammation by activating nuclear factor-kappaB (NF-kB) and increasing the production of proinflammatory cytokines, including TNF-α and IL-6. In addition, oxidative modification of LDL particles can induce the expression of adhesive molecules and lead to secretion of cytokine (Rocha & Libby, 2009).

The antioxidant, anti-inflammatory and anti-hyperlipidemic effects of Anisotes trisulcus are attributed to its rich content of bioactive compounds, particularly phenolics. Phenolic compounds possess multiple effects, including antioxidant, anti-inflammatory, hepatoprotective, anti-hyperlipidemic, and anti-diabetic (Al-Dossari et al., 2019; Aladaileh et al., 2019a; Alhusaini et al., 2019; Althunibat et al., 2019; Kamel et al., 2016; Mahmoud, 2012, 2013; Mahmoud et al., 2017a; Mahmoud et al., 2017b). Phytochemical analysis of the methanolic extract Anisotes trisulcus revealed the presence of vanillic acid, veratric acid, α-amyrin and many other compounds with antioxidant and anti-inflammatory activities (El-Shanawany et al., 2014). For instance, vanillic acid has shown protective effects against high fat diet (HFD)-induced hyperlipidemia and inflammation in rats (Chang et al., 2015), and activates thermogenesis in brown and white adipose tissue of HFD-fed mice (Han et al., 2018). Vanillic acid has also been reported to attenuate obesity via activation of the AMPK pathway as well as thermogenic factors both in vivo and in vitro (Jung et al., 2018).

Conclusion

The findings of this study show for the first time the anti-hypercholesterolemic, antioxidant, anti-inflammatory and hepatoprotective activities of Anisotes trisulcus in HCD-fed rats. Anisotes trisulcus ameliorated serum lipids and cardiovascular risk indices. Treatment of the HCD-fed rats exhibited significant improvement of liver function markers, serum pro-inflammatory cytokines, lipid peroxidation and antioxidant defenses. The lipid-lowering effect of Anisotes trisulcus is mediated, at least in part, by its ability to modulate LDLR and FAS expression. However, investigations are needed to determine the exact mechanisms underlying the lipid-lowering effect of Anisotes trisulcus.

Conflict of interest

No conflict of interest

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