Research Article

Anti-hyperlipidemic effect of *Vitex agnus castus* Extracts in Mice

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ABSTRACT

Aim of study is to investigate the possible effect of *vitex agnus castus* extracts as anti-hyperlipidemic agent in mice. The fruits of *vitex agnus castus* were dried and grounded into fine powder, and extracted sequentially with Chloroform, methanol and water. Forty eight male albino mice were fed a high cholesterol diet for 28 days to construct hyperlipidemic models. The anti-hyperlipidemic activity of *vitex agnus castus* extracts against hyperlipidemia induced was evaluated in mice. Atorvastatin was used as a standard. Total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels were measured. Free radical scavenging activity has been tested by 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) to discover which extract has the highest free radical scavenging activity and to reveal the possible mechanism of action. Compared with normal mice, hyperlipidemic mice possessed significantly higher lipid and liver enzymes profile outcomes. After treatment with *vitex agnus castus* extracts, TC, TG, LDL and VLDL in hyperlipidemic mice significantly decreased. However, Methanol extract showed the most significant reduction regarding lipid and liver enzyme profile. Moreover, it revealed the most potent free radical scavenging activity in comparison to water extract. The results showed that methanol fruit extract of *Vitex agnus castus* has potential anti-hyperlipidemic activity and this may be due to the high activity in free radical scavenging capability.

Keywords: Anti-hyperlipidemic, *Vitex agnus castus*, free radical scavenging activity.

INTRODUCTION

Hyperlipidemia is caused by excess of lipids or fatty substances in the blood and is an important risk factor in development of atherosclerosis and heart disease. Hyperlipidemia can be designated either primary or secondary, depending on their causes. Alteration in Cholesterol, triglyceride and very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and intermediate density lipoproteins (IDL), which are different forms of lipids, responsible for possible complications in human body such as acute pancreatitis, occlusion of blood vessels and cholesterol Gallstones[1]. Although drugs therapies available for the treatment of hyperlipidemia includes use of drugs like niacin, fibrates, HMG-CoA reductase inhibitors and bile acid binding resins but associated with lots of side effects. Therefore, herbal treatment for hyperlipidemia has been appreciated because of fewer side effects, less cost and easy availability[2]. The fruits of *vitex agnus castus* (chaste berry), belong to *verbenaceae*, use as an herbal medicine for long times. The fruit extract is used as a supplement for estrogen hormone imbalance which can produce menstrual cycle disorders and premenstrual syndrome (PMS) as well as for alleviating menopausal symptoms such as hot flashes[3,4]. The classes of phytochemicals that have been reported in *vitex agnus castus* fruits include fatty acids, flavonoids and terpens[5,6]. The present study was planned to investigate the effect of *vitex agnus castus* fruit extract as anti-hyperlipidemic agent in mice and to determine the free radical scavenging activity of *vitex agnus castus* fruit extracts.

MATERIALS AND METHODS

Vitex agnus castus extraction

The fruits were collected from the plant on December 2014. The dried fruits were separated and then ground into powder. The dried powder fruits (660gm) were extracted sequentially by adding 55 gm in each of the twelve flasks with 200ml of chloroform with continuous shaking by using the water bath for eight hours at 40°C, then filtration done by using filter paper whatman 20cm, the filtrate kept for concentration with rotary evaporator while the residue dried and extracted with methanol and water sequentially. Extraction process repeated three times with each solvent. The extract was then kept in desiccators at room temperature prior to the experiment[7].

Experimental animal

Forty eight apparently healthy, albino male mice 2-3 months age, weight about 20-30g, were obtained from the Higher Institute for Diagnosis of Infertility and Assisted Reproduction Techniques/AL Nahrain University. The animals were acclimatized in standard environmental conditions and fed with food and water *ad libtum* for a week before commencement of the experiment.

Induction of Hyperlipidemia

Hyperlipidemia was induced in mice by addition of High Fat Diet (2% cholesterol and 1% peanut butter) along with the standard for 28 days[8]. Body weights were measured weekly for all groups.

Table 1: Standard and high fat diets composition

Standard diet	High Fat Diet
Seeds (sunflower, groundnut)	Seeds (sunflower, groundnut)
Cereals	Cereals
Fruits (grapes, apple)	Fruits (grapes, apple)
Vegetables	Vegetables
Vitamin A	Vitamin A
Vitamin D_3	Vitamin D_3
Vitamin E	Vitamin E
	Cholesterol powder
	Peanut butter

Experimental design

The mice were divided into 6 groups, 8 mice each group:

Group 1 (normal): standard diet for 28 days.

Group 2 (induced): High Fat Diet (HFD) for 28 days.

Group 3 (treated): HFD for 28 days then atorvastatin 10 mg/kg for further 28 days.

Group 4: HFD for 28 days then Chloroform extract of vitex agnus castus (VAC) at dose of 500 mg/kg for further 28 days.

Group 5: HFD for 28 days then Methanol extract of VAC at dose of 500 mg/kg for further 28 days.

Group 6: HFD for 28 days then Water extract of VAC at dose of 500 mg/kg for further 28 days.

Animals were treated by oral gavage once a day for a period of 28 days.

Blood collection

The animals were fasted for 12 hours prior blood collection. Blood was collected by piercing the facial vein with a lancet. The blood samples were collected in plain glass tubes and allowed to clot for 20 minute at room temperature and centrifuged at 3000 RPM for 20 minute. The serum obtained was kept at 0°C until analyzed. Serum was used for the estimation of the serum lipid profile and liver function test.

Biochemical analysis

Serum lipid total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), very low density lipoprotein (vLDL) , high density lipoprotein (HDL), aspartate aminotransferase (AST), B) Alanine aminotransferase (ALT), Alkaline phosphatase (ALP) levels of mice were detected with a biochemical auto-analyzer (Shimadzu, Japan) and respective commercial test kits (Abbott diagnostic, USA) according to the manual instructions.

Measurement of oxidative stress

The liver was homogenized for malondialdehyde (MDA) investigation. The liver was rinsed in ice-cold PBS (0.02mol/L, pH 7.2-7.4). Remove excess blood thoroughly and weighed before homogenization. The tissues were sliced into small pieces and homogenized them in a certain amount of Phosphate-buffered saline (PBS) (Usually 10mg tissue to 100µl PBS) with a glass homogenizer on ice. The resulting suspension was subjected to two freeze-thaw cycles to further break the cell membranes. After that, centrifugate homogenates for 15 minutes 5000 rpm.

1, 1-diphenyl -2-picrylhydrazyl (DPPH) scavenging activity

The free radical scavenging activity of the active extract was measured by using the DPPH method. 200 µl of 0.1 mM DPPH dissolved in methanol was added to 100 µl of the active extract in the following concentrations (500, 250, 125, 62.5, 31.25, 15.625 and 7.813 µg) and incubated for 30 min. This procedure was executed using 96 well plate and each concentration was tested in triplicate, then the absorbance was measured at 517 nm using an ELISA reader.

Ascorbic acid (Vitamin C) was used as a positive control. Percentage reduction of DPPH was calculated according to the formula below [8]:

$$AA\% = 100 - \left[\frac{(Abs\ \text{sample} - Abs\ \text{blank}) \times 100}{Abs\ control}\right]$$

Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for social Science) version (17), and Microsoft Excel Worksheet 2010. Crude data was analyzed to obtain mean and standard deviation (SD). Student *t- test* was used to compare between two groups. ANOVA test was used to compare between different groups. *P*-value of ≤ 0.05 considered being significant and *P*-value of ≤ 0.001 considered as highly significant.

RESULTS

Serum lipid profile

From the data presented in table 2 it is observed that the administration of high fat diet induced hyperlipidemia in mice (Group 2). Concurrent administration of *vitex agnus castus* at 500mg/kg body weight (Group IV, Group V and Group VI) respectively showed a significant reduction in the levels of serum total cholesterol, LDL, VLDL as well as triglycerides. In vitex agnus castus extracts groups showed profound reduction in lipid profile; however, the methanol extract had the best results among other extracts. In comparison with atorvastatin treated group, group treated with methanolic fruit extract of *vitex agnus castus* showed significant increase in serum TG and statistical significant increase in serum TC, LDL and VLDL.

Liver enzymes activity

In this study, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were significantly high in high-cholesterol fed diet than in normal mice. On the other hands, the methanol extract revealed a significant

reduction in AST level; while the rest of extracts showed insignificant reduction in liver enzymes activity. In comparison with atorvastatin treated group, group treated with methanolic fruit extract of *vitex agnus castus* showed significant increase in serum AST and ALT; table 3.

Antioxidant activities

The MDA were significantly increased in induced (non-treated) group in comparison with healthy group. Meanwhile, the vitex agnus castus fruit extracts had significant reduction in MDA level. However, the glutathione level in all study groups was statistically insignificant; table 2 &3.

DPPH assay

Figure 1 and 2 showed the dose response curve of methanol and water extract on DPPH scavenging activity. The data is represented as mean ± SDV. The IC_{50} of DPPH scavenging activity for ME 80.28µg/ml. water extract scavenging activity was 49954.55 µg/ml. ascorbic acid used (positive control) and it showed scavenging activity through the equation Y= 3.7302ln(x) + 75.075 was 0.00120 µg/ml.

Table 2: Comparison between hyperlipidemic induced (non-treated) group and induced (hyperlipidemic) group *vitex agnus castus* fruit extracts in relation to different parameters.

Group	Induced	Healthy	Chloroform	Methanol	Water
Body weight (g)	35.38±3.05	29.99±2.40 ^{a*}	30.84±2.74 ^{a*}	31.06±2.80 ^{a*}	31.13± 2.83 ^{a*}
TC (mg/dl) ^{I}	381.13± 38.78	117.50±20.06 ^{a**}	296.13±56.98 ^{a**}	125.75± 14.15 ^{a**}	123.5± 16.78 ^{a**}
TG (mg/dl) ^{II}	346.76± 87.91	112.63±32.30 ^{a**}	275.00±52.52 ^{a**}	122±9.01 ^{a**}	145.6± 19.36 ^{a**}
HDL (mg/dl) ^{III}	40.25± 5.92	45.50±7.07 ^{aNS}	39.88±5.64 ^{aNS,}	40.50±6.89 ^{aNS}	40.13± 2.23 ^{aNS}
LDL (mg/dl) ^{IV}	271.53± 53.10	50.85±18.40 ^{a**}	190.00±59.29 ^{a**}	58.65± 14.75 ^{a**}	54.2± 17.36 ^{a**}
vLDL (mg/dl) ^{V}	69.35± 17.58	22.53±6.46 ^{a**}	55.00±10.50 ^{a**}	24.4±1.80 ^{a**}	28.58± 4.59 ^{a**}
AST (U/l) ^{VI}	213.88± 42.19	20.38±7.19 ^{a**}	207.63±27.50 ^{aNS}	174.63± 35.69 ^{a*}	197.5± 54.30 ^{aNS}
ALT (U/l) ^{VII}	194.88± 66.69	26.63±10.32 ^{a**}	200.25±52.84 ^{aNS}	178.68± 50.37 ^{aNS}	186.25± 52.4 ^{aNS}
ALP (U/l) ^{VIII}	226.75± 43.35	77.75±17.02 ^{a**}	220.88±31.96 ^{aNS}	212.88± 27.33 ^{aNS}	219± 40.80 ^{aNS}
MDA (nmol/ml) ^{IX}	4.38±1.36	0.89±0.16 ^{a**}	3.38±1.37 ^{a*}	1.33±0.2 ^{a**}	1.53± 0.24 ^{a**}
GSH (µg/ml) ^{X}	2.49±0.52	2.85±0.45 ^{aNS}	2.25±0.66 ^{aNS}	2.53±0.45 ^{aNS}	2.64± 0.20 ^{aNS}

a: Comparison with induced group, NS: not statistically significant (p>0.05), *: p≤0.05, **: p≤0.001, ^{I} TC: total cholesterol, ^{II} TG: triglycerides, ^{III} HDL: high density lipoprotein, ^{IV} LDL: low density lipoprotein, ^{V} vLDL: very low density lipoprotein, ^{VI} AST: aspartate aminotransferase, ^{VII} ALT: alanine aminotransferase, ^{VIII} ALP: alkaline phosphatase, ^{IX} MDA: Malondialdehyde, ^{X} GSH: glutathione.

Table 3: Comparison of group treated with methanol fruit extract of vitex agnus castus with induced (non-treated) and Atorvastatin treated group in relation to different parameters

Group	Induced	Atorvastatin	Methanol
Body weight (g)	35.38±3.05	33.66±3.36	31.06±2.80 ^{a*,bNS}
TC (mg/dl) ^{I}	381.13± 38.78	101.00±16.86	125.75±14.15 $^{a**, b*}$
TG (mg/dl) ^{II}	346.76± 87.91	87.38±7.58	122±9.01 $^{a**, b**}$
HDL (mg/dl) ^{III}	40.25± 5.92	43.38±4.72	40.5±6.89 $^{aNS, bNS}$
LDL (mg/dl) ^{IV}	271.53± 53.10	34.78±16.08	58.65±14.75 $^{a**, b*}$
vLDL (mg/dl) ^{V}	69.35± 17.58	17.14±2.05	24.40±1.8 $^{a**, b*}$
AST (U/l) ^{VI}	213.88± 42.19	123.63±17.20	174.63±35.69 $^{aNS, b**}$
ALT (U/l) ^{VII}	194.88± 66.69	137.38±47.59	178.63±50.37 $^{aNS, b**}$
ALP (U/l) ^{VIII}	226.75± 43.35	213.00±46.71	212.88±27.33 ^{aNS,bNS}
MDA (nmol/ml) ^{IX}	4.38±1.36	1.15±.056	1.33±0.2 $^{a**, bNS}$
GSH (µg/ml) ^{X}	2.49±0.52	2.55±0.29	2.53±0.45 $^{aNS, bNS}$

a: Comparison with induced group, b: comparison with atorvastatin group, NS: not statistically significant (p>0.05), *: p≤0.05, **: p≤0.001, ^{I} TC: total cholesterol, ^{II} TG: triglycerides, ^{III} HDL: high density lipoprotein, ^{IV} LDL: low density lipoprotein, ^{V} vLDL: very low density lipoprotein, ^{VI} AST: aspartate aminotransferase, ^{VII} ALT: alanine aminotransferase, ^{VIII} ALP: alkaline phosphatase, ^{IX} MDA: Malondialdehyde, ^{X} GSH: glutathione.

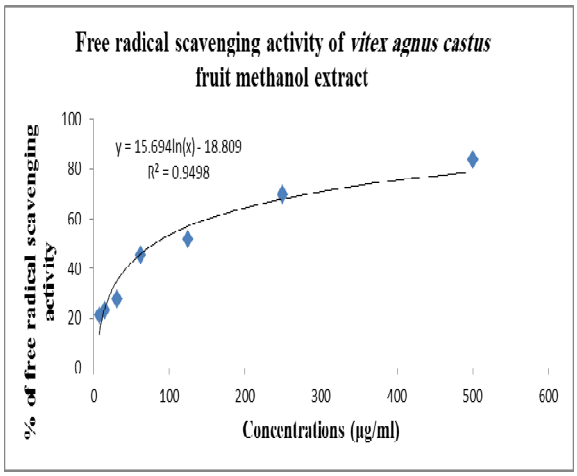

Figure 1: The dose response curve of serial dilutions of Vitex agnus castus fruit methanol extract on DPPH free radical scavenging activity.

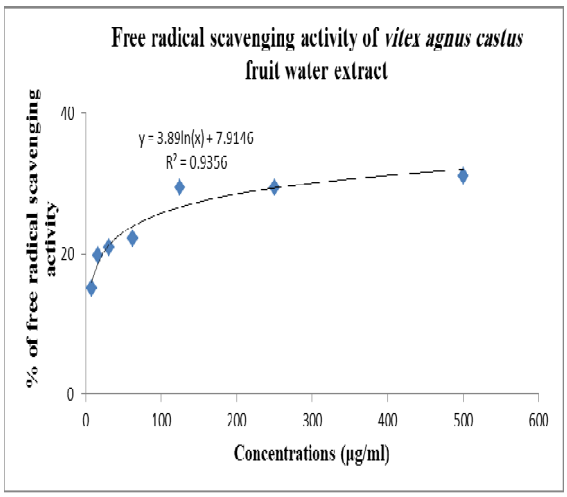

Figure 2: The dose response curve of serial dilutions of Vitex agnus castus fruit water extract on DPPH free radical scavenging activity

DISCUSSION

Different parts of Vitex agnus castus show different pharmacological activity[9].*Vitex agnus castus* fruits have been show to have a variety of biological activities like in the treatment of menstrual disorders resulting from corpus luteum deficiency, including premenstrual symptoms and spasmodic dysmenorrhea, for certain menopausal conditions and for insufficient lactation[10]. Cold method or maceration methods where used in this study so as to avoid any loss or destruction to the compounds inside the fruit from exposure to high temperature[11,12]. In the present study, feeding the mice high fat diet (HFD) for 4 weeks led to highly significant increase in serum total TC, TG, LDL and VLDL in induced (hyperlipidemic) mice as compared to normolipidemic group fed normal standard diet. These changes observed in hyperlipidemic group may be due to that HFD induced hyperlipidemia by demodulating lipid metabolism, mainly by decreasing β-oxidation and increasing cholesterol synthesis and oxidative stress by decreasing free radical scavenger enzyme gene expression[13]. Also, Rui-Li (2006) reported that HFD induced abnormal increases in lipid peroxidation, serum concentrations of total cholesterol, triacylglycerol, and low-density lipoprotein cholesterol in addition to decreased lipoprotein lipase activity, accompanied by a depressed antioxidant defense system[14]. The serum ALT, AST and ALP levels were extensively elevated in high-cholesterol fed diet than in normal mice. This may due to the disturbance of lipid metabolism because of high fat intake, resulting in accumulation of TG in liver and an increased increment of the liver index, and hepatic steatosis occurred[15] since the liver has a crucial role in regulating plasma lipid level all the way through LDL clearance and HDL cholesterol recruitment[16]. Moreover, the elevation in liver enzymes may also due to excess reactive oxygen species (ROS) production in the mitochondria as a result of lipid overload. The surplus ROS generation exhausted the endogenous antioxidants as reported in[17]. The ROS cause hepatic inflammation by activation cytokines[18]. Consequently, the excess lipid infiltration, ROS and inflammatory cytokines elicit a condition of liver toxicity[19]. Hence, the liver function markers (AST, ALT and ALP) showed noteworthy leakage in the serum and indicated the membrane damage of the hepatic cells.

Malondialdehyde (MDA), which is a product of lipid peroxidation or reaction of oxygen with unsaturated lipids[20], was highly significant increased in induced (hyperlipidemic) mice. The elevated levels of MDA in induced (hyperlipidemic) mice suggest increased lipid peroxidation in fat deposits that could be released and have detrimental effects on hepatocytes and other hepatic cells. The serum lipid profile and MDA was found to be declined with extracts of *vitex agnus castus* in comparison with induced (non-treated) group. However, the range of serum TC, TG, LDL and VLDL of chloroform extract still abnormally high despite the reduction. The effect of methanol extract and atorvastatin on serum TC, TG, LDL and VLDL was comparable although atorvastatin seems to be more effective in certain lipid profile parameters. The reason behind the reduction in lipid profile and liver enzymes activity mostly by methanol extract may due to the diversity of phytochemical compounds of methanol extract such as flavonoids, diterpenoids and iridoids which possess a radical scavenging activity and hepatoprotective properties[21]. They protect cells from damage induced by oxidative stress which is generally considered to be a cause of degenerative diseases[22]. Flavonoids may have an additive effect to the endogenous scavenging compounds as they can increase the function of the endogenous antioxidants[23]. In addition, flavonoids and terpenoids may reduce TC, TG, LDL and, VLDL through inhibition of pancreatic lipase which responsible of libration of triglyceride into fatty acids and glycerol[24]. The activity of lipase greatly affects the metabolism of fat and the concentration of triacylglycerols in blood[25]. Glutathione level was not altered in comparison to induced group. Increasing the doses of methanol extract or prolongation

of duration of treatment probably will have impact on the level of glutathione. Free radical scavenging activity of *vitex agnus castus* fruit methanol and water extract was studied and considered important to help understand the mechanism of action of *vitex agnus castus* extract. It may be due to certain chemical constituents such as polyphenols or terpens which possess good oxygen radical scavenging potential[26,27]. The results of current study demonstrate that methanol fruit extract of *vitex agnus castus* had gave the significantly highest anti-oxidant activity in comparison to the water extract, the methanol extract also showed the highest percentage of antihyperlipidemic activity, as shown by biochemical examination. Its potency in inhibiting lipid profile elevation could be contributed to its significant antioxidant activity, as shown in the DPPH scavenging assay. The results of present study showed positive correlation between hypolipidemic effect and free radical scavenging activity in the *vitex agnus castus* methanol fruit extract.

The total phenolic content of fruits of *vitex agnus castus* was determined and found as 114.5±2.704 mg Gallic Acid Equivalent (GAE)/g extract[28]. Plant materials rich in phenolic components exhibit protective role against lipid peroxidation[29].

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