ORIGINAL ARTICLE

Effect of Aqueous Extract of Walnut Leaves on Lipid Profile and Atherogenic Ratio in Hypercholesterolemic Rats

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ABSTRACT

Objective: To determine the effect of aqueous extract of walnut leaves on lipid profile i.e. serum total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol triglycerides, LDL/HDL and atherogenic ratio (Total Cholesterol/HDL) of hypercholesterolemic rats.

Study Design: An experimental randomized control study.

Place and Duration of Study: The study was conducted at Islamic International Medical College, Riphah International University, with assistance from National Institute of Health, Riphah Institute of Pharmaceutical Sciences and Citilab, Islamabad, Pakistan. The duration of study was one year from April 2014 to March 2015.

Materials and Methods: A total of 30 male Sprague Dawley rats were included in the study. They were divided into 3 groups i.e. ten rats in each group. Group 1(Control group), Group 2 (Hyper-cholesterolemic control), Group 3 (Aqueous group) treated with aqueous extract of walnut leaves after induction of hypercholesterolemia in a dose of (200mg/kg) through gavage needle once daily, for four weeks. Blood sampling was done at the beginning (baseline), end of week 8, and end of week 12 to perform lipid profile, LDL/HDL and atherogenic ratios TC/HDL-cholesterol. Statistical analysis was applied by using SPSS version 17. All data was shown as mean ±SD and Student t test was applied between groups. p value of < 0.05 was considered as statistically significant.

Results: Hyper-cholesterolemic rats after treatment with aqueous extract (Group 3) had significantly lower levels ($p<0.001$) of serum cholesterol, low density lipoprotein and triglycerides while significantly high ($p<0.001$) levels of HDL-Cholesterol with significantly reduced ($p<0.001$) TC/HDL-Cholesterol and LDL/HDL ratios.

Conclusion: Aqueous extract of walnut leaves has hypo-lipidemic effect on serum total cholesterol, LDL-Cholesterol and triglycerides. It reduces TC/HDL, LDL/HDL ratios whereas it significantly increases the level of HDL-Cholesterol.

Keywords: *Juglans Regia, Atherogenic Ratio, Hypercholesterolemia, Lipoproteins.*

Introduction

Hypercholesterolemia is a condition characterized by elevated serum total cholesterol, triglycerides (Tgs), low-density lipoprotein (LDL-Cholesterol), very low density lipoprotein (VLDL-Cholesterol) and decreased high-density lipoprotein (HDL-Cholesterol) levels.[1] It can develop primarily due to genetic cause or secondary to chronic diseases like hypothyroidism, diabetes mellitus and renal insufficiency.[2] Cholesterol is one of the essential component present in all foods of animal origin and is necessary for synthesis of cell membrane which plays a significant role in the maintenance of cell homeostasis and trans-membrane communication.[3] TGs are mainly synthesized in the liver or present in dietary fat and are carried in the form of chylomicrons and VLDL in capillaries where they are ultimately hydrolyzed by lipoprotein lipase into free fatty acids.[4] HDL plays a significant role in reversing cholesterol transport which is an important step in the eradication of surplus cholesterol from the body.[5] So increased levels of serum HDL prevents development of hypercholesterolemia and cardiovascular disease (CVD).[6] The LDL-Cholesterol are termed as "bad cholesterol", as it carries maximum of cholesterol in circulation which in turn increases risk of CVDs.[7] Studies have shown that hypercholesterolemic diet increase cholesterol, LDL, TG levels and decreases LDL-receptor activities in liver.6 Increased LDL, cholesterol and TGs are considered dangerous and are strongly linked with poor cardiovascular outcomes.[8,9]

TC/HDL and LDL/HDL ratios are two important components and predictors of CVDs. When TC/HDL ratio is more than one to five, the risk of CVDs increases, substantially.[10] The initial step in management of hypercholesterolemia includes diet modification and use of lipid lowering agents.[5] Medicinal plants are being used for the treatment of various diseases as they are considered safer and cost effective as compared to pharmaceutical

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medicines.[11] Walnut (Juglans regia L) is a medicinal plant that belongs to the family juglandaceae and is extensively cultivated in China, Japan, South Asia, South Eastern Europe and United States.[12] Different parts of juglans regia such as kernel, shell, leaves, septum, bark, epicarp have been used in pharmaceutical and cosmetic products.[13]

Previous studies have documented that walnut leaves have been used in folk medicine for the treatment of hypoglycemia, diarrhea, venous insufficiency, hemorrhoids and fungal or microbial infections.[7] Whereas, walnuts leaves extract have antimicrobial, antihyperglycemic, anti-inflammatory and anti proliferative activity.[14-17] Whole walnut improves cholesterol and lipoprotein levels and walnut bark is used for cleaning teeth.[18]

Despite various approaches on walnut, the effect of aqueous extract of walnut leave on lipid profile and LDL/HDL, TC/HDL ratios in hypercholesterolemia have not been explored. So, the purpose of this study is to investigate the effect of aqueous extract of walnut leaves on lipid profile, LDL/HDL and atherogenic ratios (TC/HDL) of hypercholesterolemic rats.

Materials and Methods

This randomized control study was conducted at Islamic International Medical College, Riphah International University, Islamabad with access to Animal Housing Facility for laboratory rats at National Institute of Health (NIH), Islamabad for over a period of 1 year (April, 2014 to March 2015), after taking approval from Ethical Review Committee of Islamic International Medical College. A total of 30 male Sprague Dawley rats, aged 3 months, weighing 250-300 grams were included in the study. Rats were kept for 3 months at NIH Animal Housing Facility in a well ventilated room with 12 hours light and dark cycle, 50-70 humidity % at 24±2 oC room temperature.[19,20] Rats were fed on standard rat diet and availability of water was made ad libitum for a period of one week in order to get rats acclimatized for acclimatization before starting the experiment.

Rats were divided into three groups i.e. 10 in each group. Group 1 (control group) was fed on regular diet till the end of study. Group 2 (hypercholesterolemic group) and 3 (aqueous group) were fed on high fatty diet prepared at NIH comprising 17 % of calories as carbohydrates, 25% as proteins and 58% calories as fat for 8 weeks for induction of hypercholesterolemia.[21] Group 2 was considered as hypercholesterolemic control and then given regular diet till the end of study. Group 3 (aqueous group) which after inducing hypercholesterolemia, was given aqueous extract of walnut leaves (juglans regia) in a dose of (200mg/kg) through gavage needle once daily for four weeks. Aqueous extract was prepared from walnut leaves (juglans regia) collected from Muzaffarabad, Azad Kashmir and were identified and authenticated by Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The leaves collected, were coded and kept under voucher number 57 at the Herbarium, at Quaid-i-Azam University, Islamabad, Pakistan. Walnut leaves were first dried under shade and grounded into a fine powder with the help of electrical grinder. The aqueous extract of walnut leaves was prepared by using one hundred gram of grounded walnut leaves soaked in distilled water for 24 hours. The solution obtained was later filtered using Whatmann Filter paper No.1 and dried in a rotary evaporator at 55°C at research laboratory of Riphah Institute of Pharmaceutical Sciences, Islamabad. The extract obtained was in the form of dark brown semi-solid sticky paste and was stored in air tight glass bottles, protected from light and kept in refrigerator at 2-8 o C to be used throughout the experiment.[22]

Blood samples were collected three times (baseline, at the end of week 8 and week 12). At baseline and at the end of week 8 1.5 ml of blood samples were drawn through tail vein sampling from rats of all three groups while Final 1.5 ml of blood sample was drawn through cardiac puncture at the end of week 12 from rats of all groups.

Blood was then centrifuged at 3000 rev/min for 15min and serum was separated for analysis of lipid profile.[23] Blood samples for serum total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were estimated using Merck (Germany) kits based by enzymatic calorimeter method on automated chemistry analyzer and ratios was calculated as TC/HDL and LDL/HDL.

The data was analyzed using SPSS version 17. All data are shown as mean ±SD and Student t test was applied between group 2 (hyper-cholesterolemic control) and group 3 (aqueous group). p value of <

0.05 was considered as statistically significant.

Results

During the experiment blood was retrieved for lipid profile analysis at various intervals from Sprague Dawley rats i.e. in the beginning of experiment (pre-cholesterol), after 8 weeks (mid-cholesterol) and at the end of experiment at 12 weeks (post-cholesterol) in control, hypercholesterolemic control, and aqueous extract group which are presented in fig 1.

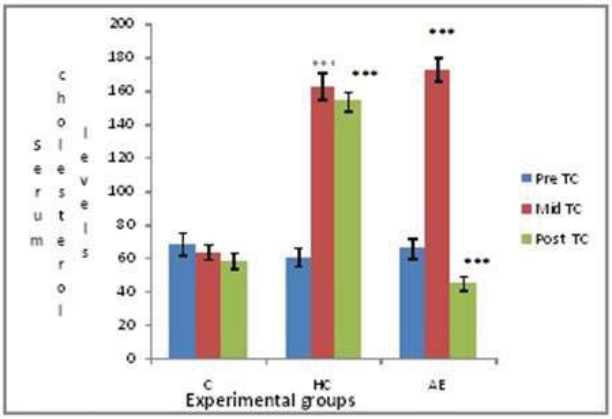

Fig 1: Serum cholesterol levels (mg/dl) in C, HC and AE group at the start of experiment (Pre-Cholesterol), after 08 weeks (Mid-Cholesterol) & after 12 weeks (Post-Cholesterol) in Sprague Dawley rats. C=Control group, HC = Hypercholesterolemic control, AE = Aqueous extract. All Values are expressed as mean +- SD
***** p<0.001 is considered significant on comparison with Hypercholesterolemic control group**

Group 2 and 3 were given hypercholesterolemic diet for 8 weeks, after which mid- cholesterol levels in group 2 (162.5±7.90 mg/dl) and group 3 (172.7±6.95 mg/dl) were significantly raised (p<0.001) as compared to the mid-cholesterol levels of control group 1 (63.5±4.56mg/dl), which confirmed the development of hypercholesterolemia. Group 3 hypercholesterolemic rats treated with aqueous extract for 4 weeks, had significantly reduced (p<0.001) post cholesterol levels (45.0±4.56 mg/dl) when compared with the post cholesterol levels of hypercholesterolemic control group 2 (153.7±5.92 mg/dl).

Analysis of serum TG levels at various intervals during the experiment which are shown in Figure 2. Mid-TG levels after giving hypercholesterolemic diet for 8 weeks in Group 2(153.4.6±3.78 mg/dl) and 3(159.7±2.24mg/dl) were significantly raised

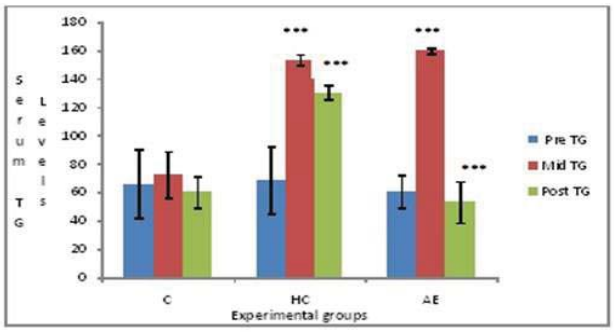

Fig 2: Serum TG levels (mg/dl) in C, HC and AE group at the start of experiment (Pre-TG), after 08 weeks (Mid-TG) & after 12 weeks (Post-TG) in Sprague Dawleyrats C= Control Group, HC = Hypercholesterolemic control, AE = Aqueous extract. All values are expressed as mean +- SD
***** p<0.001 is considered significant on comparison with Hypercholesterolemic control group**

(p<0.001) as compared to mid-TG levels of control group 1 (72.45±16.6mg/dl). Group 3 hypercholesterolemic rats treated with aqueous extract for 4 weeks after which post-TG levels 53.38±14.6 mg/dl were significantly reduced (p<0.001) as compared to post-TG levels of hypercholesterolemic control group 2 (130.5±4.95 mg/dl).

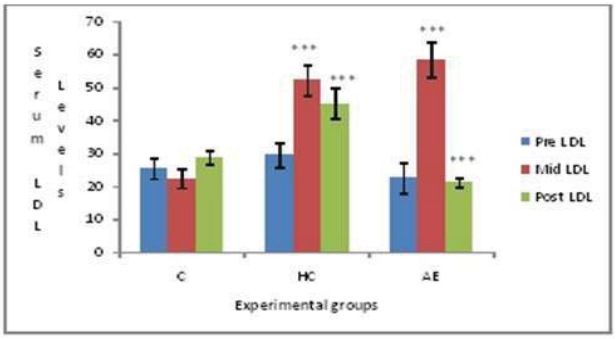

Fig 3: Serum LDL levels (mg/dl) in C, HC and AE Group at the start of experiment (Pre-LDL), after 08 weeks (Mid-LDL) & after 12 weeks (Post-LDL) in Sprague Dawleyrats) C=Control group, HC = Hypercholesterolemic control, AE = Aqueous extract. All Values are expressed as mean +- SD
***** p<0.001 is considered significant on comparison with Hypercholesterolemic control group**

Serum LDL levels analyzed at various intervals during the experiment which are presented in Figure 3.Group 2 and 3 were given hypercholesterolemic diet for 8 weeks after which mid-LDL levels in group 2 (52.25±4.68mg/dl) and group 3 (59.45±5.25mg/dl) were significantly raised (p<0.001) as compared to

mid- LDL levels of control group 1 (22.43±2.89mg/dl). Group 3 hypercholesterolemic

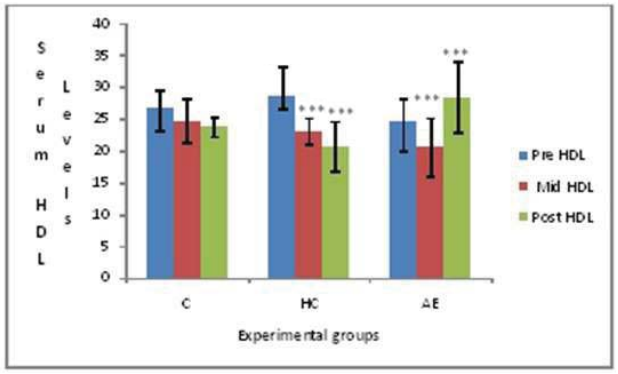

Fig 4: Serum HDL levels (mg/dl) in C, HC and AE Group at the start of experiment (Pre-HDL), after 08 weeks (Mid -HDL) & after 12 weeks (Post-HDL) in Sprague Dawleyrats) C=Control group, HC = Hypercholesterolemic control, AE = Aqueous extract. All Values are expressed as mean +- SD

***** p<0.001 is considered significant on comparison with Hypercholesterolemic control group**

to the mid- TC/HDL and LDL/HDL levels of control group 1. Group 3 hypercholesterolemic rats were treated with aqueous extract for 4 weeks, the post TC/HDL and LDL/HDL ratios were significantly reduced (p<0.05) on comparison with the post TC/HDL and LDL/HDL ratios of hypercholesterolemic control group 2.

Discussion

Hypercholesterolemia is a major risk factor for cardiovascular diseases and diabetes mellitus.

The present study showed that aqueous extract of walnut leaves cause significant reduction in serum cholesterol, TGs, LDL, TC/HDL and LDL/HDL ratio and significant increased in HDL levels of hypercholesterolemic Sprague Dawley rats. This study was in agreement with the work done by Mahmoodi at al., (2011) who studied the hypolipidemic effects of walnut leaf powder on lipid profile in hypercholesterolemic rats. There was significant reduction (p<0.05) in serum total cholesterol, LDL and TGs and increased HDL as compared to hypercholesterolemic control.[6]

Gholamreza (2008) and Divband et al.,(2010)

Table I: Comparison of TC/HDL and LDL/HDL ratio of Sprague Dawley rats at baseline and end of week 8 and week 12 of the experiment

Variables	Time	Group 1 (C) n=10	Group 2 (HC) n=10	Group 3 (AE) n=10
Atherogenic ratio TC/HDL	baseline	2.27 ±0.06	2.23 ±0.06	2.20 ±4.06
	end of 8 week	2.27 ±0.06	7.33 ±0.7**	7.23 ±2.7**
	end of12 week	2.49 ±0.2	6.6 ±0.4	1.84 ±0.1**
LDL/HDL ratio	baseline	0.93 ±0.04	0.96 ±0.34	0.94 ±0.44
	end of 8 week	0.93 ±0.04	2.23 ±0.39**	2.34 ±1.39**
	end of12 week	1.04 ±0.13	2.01 ±0.3	0.85 ±0.06**

TC: Total cholesterol, HDL: High density lipoproteins, LDL: Low density lipoproteins. C=Control group, HC= Hypercholesterolmic control, AE=Aqueous group. Data represents as mean ±SD

* p <0.05 with respect to corresponding control

** p<0.001 with respect to corresponding control

conducted a study in diabetic rats for a period of 4 weeks to study the effect of aqueous extract of walnut leaves on serum lipid profile and blood sugar. They reported that diabetic rats, given aqueous extract of walnut leaves caused a significant decrease in glucose (p=0.009), cholesterol (p=0.045), LDL (p=0.022), TGs (p=0.047) and a significant increase in HDL levels (p=0.045) as compared to diabetic control group. Also TC/HDL (p=0.006) and LDL/HDL ratio (p=0.035) in experimental group were significantly decreased when compared with the control diabetic group.[22,24] This study had showed similar results with the present study but they had used diabetic rats instead of hypercholesterolemic rats. Asgary et al., (2008), studied the effect of administration of ethanolic extract of walnut leaves on biochemical parameters in a dose of 200mg/kg for four weeks in alloxan-induced diabetic rats which showed similar results to our study.[25] Zavvarreza et al (2006), conducted a study and reported that the administration of Iranian walnut oil extract caused dose-dependent decrease in TGs, cholesterol and LDL level in rats that received

hypercholesterolemic diet.[22]

Banel (2009) who conducted a meta-analysis and literature review to investigate the effect of walnuts on blood lipids. When compared with control diets, supplemented diets with walnuts resulted in a significant decrease in total cholesterol, LDL levels and TGs while HDL were not significantly affected by walnut diets.[26] Finding of our study was not in accordance with their study as HDL levels were significantly raised in our study after treatment with aqueous extract.

In present study the HDL levels were significantly raised in aqueous group as compared to hypercholesteremic control group however these findings are not in consistance to work done by Iwamoto et al., (2000) who studied the effect of walnuts consumption on serum lipids in Japanese subjects for a period of 4 week. The study revealed a significant decrease ($p<0.01$) in total cholesterol, LDL levels and LDL/HDL ratio while HDL-Cholesterol were not significantly affected by walnut diets.[27]

The possible mechanism underlying the lipid lowering effect of juglans regia might be due to effects of compounds like phenolic acids and flavonoids which are the major antioxidant present in walnut leaf. Antioxidants like quercitin and cholorogenic acid reduce synthesis of cholesterol in liver through inhibition of HMG COA reductase enzyme and causes increased biliary excretion of cholesterol.[28] Studies have also shown that other components like fiber, micronutrients such as vitamin E and C, folic acid, copper, calcium, potassium, magnesium, plant protein (such as arginine), plant sterols are also present in walnut leaves they distribute lipid properly in physiologically manner to prevent lipid and cholesterol accumulation.[29,30]

Conclusion

The present study concludes that aqueous extract of walnut leaves has remarkable lipid lowering effect in hypercholesterolemic rats that decreases serum cholesterol, triglycerides LDL, TC /HDL and LDL/HDL ratios with concomitant increase in HDL levels. Results suggest that administration of aqueous extract of walnut leave can be another option for treating people with hypercholesterolemia and may have beneficial role in the prevention of cardiovascular disease.

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