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Research Article

Centratherum anthelminticum and *Withania coagulans* Improves Lipid Profile and Oxidative Stress in Triton X-100 induced Hyperlipidemic Rabbits

Tooba Lateef, Shamim A Qureshi*

Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan

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ABSTRACT

Hypolipidemic screening of MFEt of *W. coagulans*, ESEt of *C. anthelminticum* and *C. limon* was conducted by using single dose (200 mg/kg) of each for 14 days in normal overnight fasted rabbits, where *W. coagulans* and *C. anthelminticum* gave significant activity. These later subjected to evaluate the hypolipidemic and antioxidant effects in triton X-100 induced hyperlipidemic rabbits. Animals were divided into control and triton-induced hyperlipidemic (TIH) groups. The TIH group was further divided into TIH control, negative control, positive control (simvastatin) and four test groups, treated with MFEt of *W. coagulans* and ESEt of *C. anthelminticum* @ 200 and 600 mg/kg respectively. After 18 hours, rabbits were sacrificed to estimate lipid profile, ALT, CK, CAT, SOD, LPO, HMG-CoA reductase, lipase, atherogenic index (AI) and percent protection. The results demonstrated that both doses of *W. coagulans* and *C. anthelminticum* significantly improved lipid profile, HMG-CoA reductase & lipase activities and antioxidant status of test rabbits. AI was significantly decreased and percent protection was increased by both plant extracts in test groups. It was concluded that *W. coagulans* and *C. anthelminticum* showed good protection against hyperlipidemia and hyperlipidemia-induced oxidative stress by decreasing levels of serum lipids and improving the efficiency of antioxidant enzymes, thereby decreasing the risk of atherogenesis.

Keywords: Triton X-100, atherogenesis, hyperlipidemia, oxidative stress.

INTRODUCTION

Cardiovascular diseases (CVDs) are the major cause of mortality worldwide[1]. Currently, it is predicted that by the year 2020 atherosclerosis (the initiator of CVDs) will be the leading cause of death[2]. The important and initiating factor of atherosclerosis is hyperlipidemia, a disorder of lipid metabolism manifested by elevated levels of triglycerides and bad cholesterols (total cholesterol, low-density and very low-density lipoproteins) and low level of good cholesterol (high-density lipoprotein) in serum[3]. Among the acquired factors, diet having high fat and low fiber contents contributes hyperlipidemia which inturn induce oxidative stress by producing reactive oxygen species (ROS) that accelerates lipid peroxidation (LPO) thereby affecting many body organs particularly heart[4,5]. In order to minimize the risk of life-threatening complications, variety of hypolipidemic medicines are available in commercial market. No doubt these are effective but having few side effects like hyperuricemia, diarrhea, nausea, myositis, gastric irritation, flushing, dry skin and abnormal liver function[6]. On the other hand, herbal medicines are gaining fame in the treatment of different diseases including hyperlipidemia especially in the developing countries of the world[7]. Therefore, researchers are taking keen interest in investigating the new aspects of medicinal plants as they are cheap, easily available and have fewer side effects as compared to conventional medicines[8,9]. *Centratherum anthelminticum* (*Vernonia anthelminticum*; family *Asteraceae*) commonly known as kalijiri, its seeds are well-known for medicinal purpose[10]. Besides containing 18% fixed oil and 0.02% volatile oil[11], different classes of chemical constituents are also reported in seeds of same plant[12] like flavonoids *viz.*,2,3,4,4-tetrahydroxychalcone (Butein), 5,6,7,4-tetrahydroxy flavone, 7,3,4-trihydroxydihydroflavone and sterols *viz.*, sterol-4-alpha-methylvernosterol, vernosterol and avernosterol were isolated from seeds of this plant[13]. Similarly, few steroids including (24a/R)-stigmasta-7-en-3-one, 24(a/R)-stigmasta-7, 9(11)-dien-3-one, 24(a/S)-stigmasta-5 and 22-diene-3β-ol, stigmasta-7 and 22-dien-3β-ol were also reported from the same[14]. Various seed extracts of this plant have been reported for many pharmacological activities like ethanol extract showed antihelminthic, hypotensive, laxative and spermicidal effects[11], ethanol, chloroform and methanol extracts for antifungal, antiviral and larvicidal activities[15-17], petroleum ether and alcohol extracts for antipyretic, analgesic and anti-inflammatory effects[18], ethyl-acetate, acetone, methanol and water extracts showed antifilarial activity[10], polyphenolic extract for antihyperglycemic and antioxidant activities[19], aqueous extract for diuretic[10] and antidiabetic activities[12]. *Citrus limon* (Linn) Burm.f. (family-*Rutaceae*) is commonly known as lemon and ranked third among the other citrus fruits which are

*Author for Correspondence

Table 1: Hypolipidemic screening of *W. coagulans*, *C. anthelminticum*, and *C. limon*

Groups	Group I	Group IIA	Group IIB	Group IIC
TC	166.1±5.45	153.3±1.26*	141.6±6.84*	163.93±2.2
TG	170.56±13.5	129.36±0.77*	152.0±3.46*	160.33±1.52
HDL-c	83.16±1.28	96.33±4.01*	91.8±9.00*	87.46±5.91
LDL-c	48.82±4.17	31.12±3.11*	23.40±6.54*	43.6±4.49
CK	62.47±55.36	37.70±11.76	69.20±21.82	84.48±32.49
ALT	8.73±0.20	8.93±1.27	8.33±1.27	8.86±0.35

*$p<0.05$ when compared with group I.

Table 2: Effect of *C. anthelminticum* and *W. coagulans* on lipase, HMG-CoA /Mevalonate Ratio and AI in TIH rabbits

Groups	Lipase (U/L)	HMGCoA/Mevalonate Ratio	AI
Group I	188.86±18.76	1.17±0.12	1.69±0.22
Group II	114.80±8.69	0.76±0.09	2.99±0.42
Group III	108.6±11.4	0.85±0.12	2.76±0.13
Group IV	124.0±28.75	1.11±0.005	2.22±0.17
Group V	142.13±4.58*	1.12±0.02*	1.70±0.24*
Group VI	153.50±27.71*	1.03±0.01*	1.76±0.30*
Group VII	133.96±9.44*	1.17±0.08*	1.57±0.17*
Group VIII	152.1±8.02*	1.05±0.02*	1.79±0.11*

*$p<0.05$ when compared with respective group II and III.

Table 3: Effect of *W. coagulans* and *C. anthelminticum* on ALT and CK in TIH rabbits

Groups	ALT	CK
Group I	11.36±1.22	32.3±8.09
Group II	15.83±0.46	67.43±9.94
Group III	14.07±1.04	43.17±13.70
Group IV	11.50±1.11^	16.18±8.09^
Group V	13.53±0.36*	35.07±12.36*
Group VI	13.8±0.01*	26.98±9.34^
Group VII	15.03±0.26*	43.15±12.33*
Group VII	14.4±0.52*	37.77±20.36*

^$p<0.05$ when compared with respective group II and III,

*$p<0.05$ when compared with group II.

cultivated all over the world[20]. Lemon fruit is pale yellow, elliptical shaped used for culinary and medicinal purposes[21] whereas its pulp, seeds and peels are considered as wastes but they can be used for the production of many important economical, medicinal and industrial by products[22]. Lemon is considered as good source of potassium, magnesium, calcium, folic acid and vitamin C[23]. Fruit, pulp, peel and seeds of *C. limon* are found to be rich in alkaloids, bitter limonoids, carbohydrates, cardiac glycosides, carotenoids, fixed oils, flavonoids, organic acids, phenols, phytosterols, proteins, saponins, steroids, taninns and volatile compounds[24]. Its limonoids are reported for possessing anti-carcinogenic activity[25]. Ethanolic seed extract of *C. limon* and its essential oil are reported for antioxidant activity[26]. Various leaf extracts of *C. limon* are used in folk medicine for the treatment of obesity, diabetes, hyperlipidemia, cardiovascular diseases, brain disorders and cancer[27] whereas peel and seed extracts have been reported for antifungal and antibacterial activity[23,24]. Its methanolic extract of fruit rind is reported for anti-inflammatory, analgesic, anticonvulsant and insecticidal activities[28]. Flavonoid glycosides (hesperidin and diosmin) are present in all parts of fruit of *C. limon* and reported for reducing hepatotoxicity against carbon tetrachloride while its lipopolysaccharide content reported to decrease blood sugar, oxidative stress induced by nicotine and also showed chemoprotective activity of the bladder[29]. Few coumarin compounds (bergapten and limettin) and flavanones (didymin, erocitrin, naringin and narirutin) are isolated from peels, edible portion, seeds and juice[30]. *Withania coagulans* Dunal (family *Solanaceae*) is commonly known as Indian cheese maker (English) and Punir dodi (Hindi) which is cultivated throughout Central and South Asia[31]. Various organic and aqueous fruit extracts of this plant have been reported for many medicinal properties like antifungal, emetic, antibacterial, antiasthmatic, anti-inflammatory, diuretic, antimicrobial, anti-inflammatory, antitumor, hepatoprotective, antihyperglycemic, antihyperlipidemic, antiatherosclerotic, cardiovascular, immunosuppressive, free radical scavenging and CNS depressant activities[32,33]. Number of withanolides including withaferin A, withanolide A and withanone[34] and coagulin L, coagulanolide, ergostadiene and sitosterol were isolated from this plant[35]. Phytochemical screening of different organic solvent extracted fraction of *W. coagulans* showed the presence of alkaloids, amino acids, carbohydrates, organic acids, phenolic compounds, proteins, saponins, steroids and tannins[36]. Therefore, the present study was designed to compare the hypolipidemic activity of *Centratherum anthelminticum*, *Citrus limon* and *Withania coagulans* in normal and triton X-100 induced hyperlipidemic rabbits.

MATERIALS AND METHODS

Plant Material and Extraction

Plant materials include dried fruits of *W. coagulans* and seeds of *C. anthelminiticum* & *C. limon* were purchased from Hamdard Dawakhana, Sadar, Karachi, identified by expert of Department of Botany, University of Karachi and kept in same department with voucher specimen No.

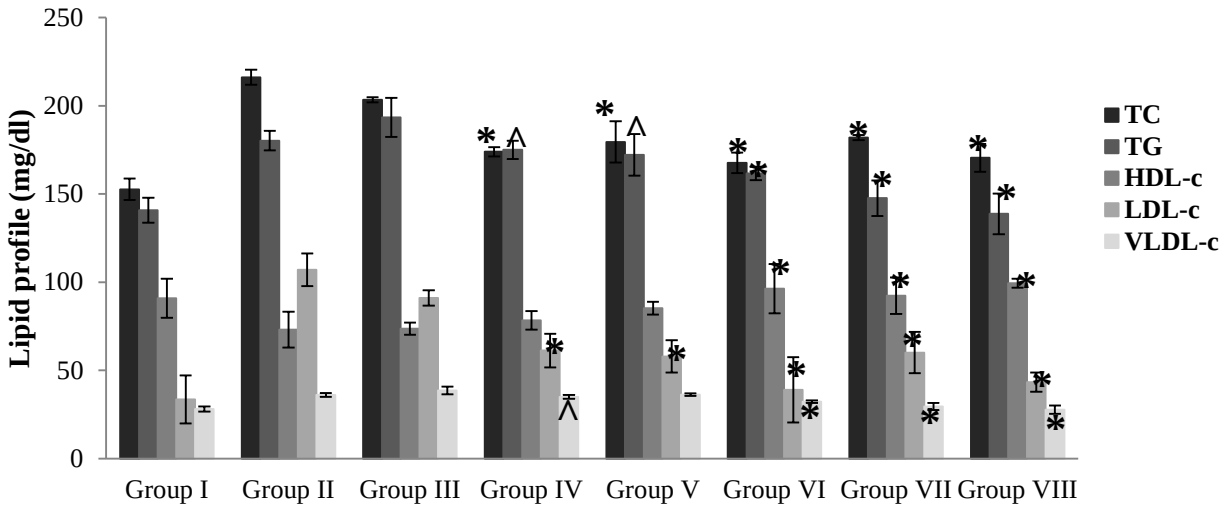

Figure 1: Effect of *C. anthelminticum* and *W. coagulans* on lipid profile in TIH rabbits
*p<0.05 when compared with respective group II and III, ^p<0.05 when compared with group III.

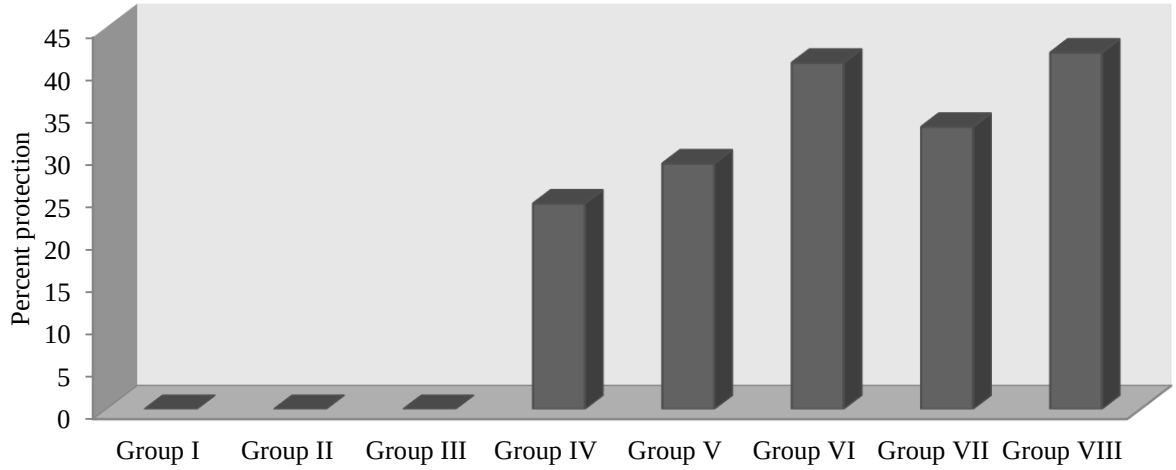

Figure 2: Effect of *C. anthelminticum* and *W. coagulans* on percent protection in TIH rabbits

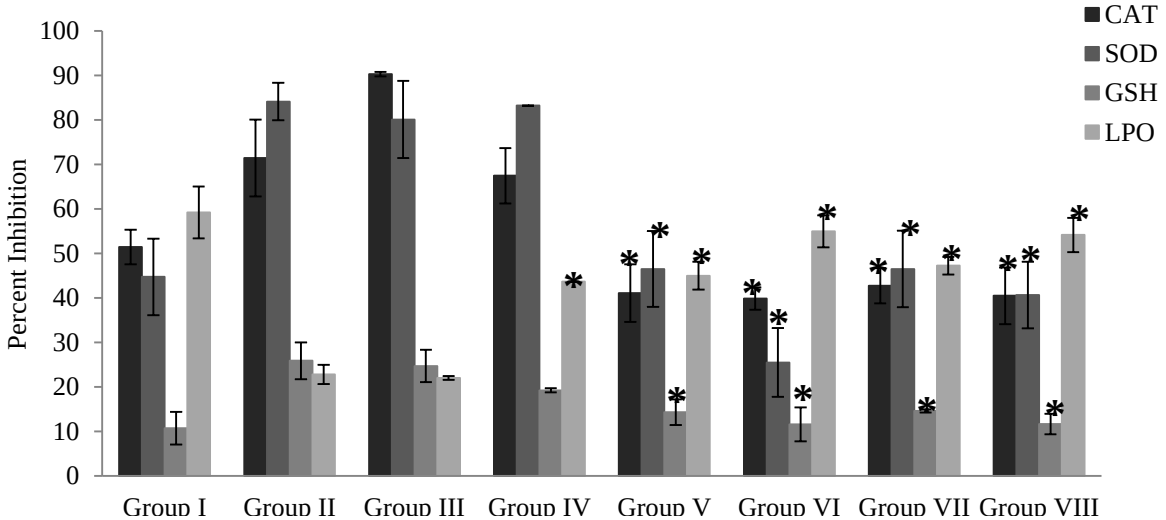

Figure 3: Effect of *C. anthelminticum* and *W. coagulans* on antioxidant enzyme, protein and LPO in TIH rabbits
*p<0.05 when compared with respective group II and III.

KU/BCH/SAQ/06, KU/BCH/SAQ/05 & KU/BCH/SAQ/07 respectively. Three of the plant materials grinded in fine powder separately then 40 g of each of them was soaked in 1 L organic solvent (methanol was used for fruits of *W. coagulans* & ethanol for seeds of *C. anthelminiticum* & *C. limon*) for overnight and filtered

twice through Whatmann No.1 filter paper. Filtrate of each of the plant material was then concentrated till dryness separately in a rotary vacuum evaporator to obtain a dark brown residues that were referred as methanolic fruit extract of *W. coagulans* (MFEt), ethanolic seed extract (ESEt) of *C. anthelminticum* and *C. limon* respectively[37].

Medicine and Vehicle

Hypolipidemic medicine simvastatin (Limitrol) of PharmaEvo (Pvt) Ltd, Pakistan was used as positive control (20 mg/kg) whereas dimethylsulphooxide (DMSO; 0.05%) of Fisher Scientific (UK) used as vehicle for administrating the doses of MFEt and ESEt[38].

Induction of Hyperlipidemia

Hyperlipidemia was induced by giving single intra-peritoneal (i.p) injection of Triton X-100 (BDH Laboratories, England) in a dose of 200 mg/kg in overnight fasted rabbits[39].

Experimental Animals and Protocol

Healthy albino rabbits of both sexes weighing from 1-1.5 kg were purchased from local supplier of University of Karachi, housed individually in separate cages in animal house of same university under standard hygienic condition and provided standard laboratory diet with free access to water *ad libitum* for 1 week before starting the experiment to acclimatize them to the environment. Before commencement of study, the experimental protocol was approved by Board of Advance Study and Research (BASR) of University of Karachi.

Hypolipidemic Screening of C. anthelminticum, C. limon & W. coagulans in normal rabbits

The overnight fasted rabbits were divided into two major groups including control (group I) treated with distilled water (1 ml/kg) and hypolipidemic test (group II) groups that was further divided into three groups *viz.*, group II A, B & C and treated with 200 mg/kg of MFEt of *W. coagulans*, ESEt of *C. anthelminticum* & *C. limon* respectively. Each group contained 6 rabbits and was given its respective treatment orally for 14 days consecutively once in a day. After completing the trial, rabbits were sacrificed to collect blood, serum was separated to analyze biochemical parameters.

Hypolipidemic Activity of C. anthelminticum and W. coagulans in Triton X-100 induced hyperlipidemic rabbits

Plants which showed significant results in hypolipidemic screening were selected to investigate their antihyperlipidemic activity in triton X-100 induced hyperlipidemic model. In this model, the overnight fasted rabbits were divided into two major groups including normal rabbits (group I) which were treated with distilled water (1 ml/kg) and triton X-100 induced hyperlipidemic rabbits (TIH group). This group was sub-divided into different groups and received their treatment immediately after injection (i.p) of triton X-100 (200 mg/kg), as

Group II: TIH control: treated with distilled water (1 ml/kg)

Group III: TIH negative control: treated with 0.05% DMSO (1 ml/kg)

Group IV: TIH positive control: treated with simvastatin (20 mg/kg)

Group V: TIH test group: treated with MFEt of *W. coagulans* (200 mg/kg)

Group VI: TIH test group: treated with MFEt of *W. coagulans* (600 mg/kg)

Group VII: TIH test group: treated with ESEt of *C. anthelminticum* (200 mg/kg)

Group VIII: TIH test group: treated with ESEt of *C. anthelminticum* (600 mg/kg)

Each group contained 6 rabbits and given its respective treatment orally. After 18 hrs of treatment, rabbits were sacrificed to collect blood (serum) and liver to analyze biochemical parameters.

Biochemical Analysis

Serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), liver- & cardiac-specific enzymes including alanine aminotransferase (ALT) & creatine kinase (CK) respectively were estimated by using the commercially available enzymatic kits (Randox, UK). Whereas lipase (lipid hydrolyzing enzyme) was estimated by using commercial kit of Roche, Switzerland. Low-density lipoprotein cholesterol (LDL-c), very low-density lipoprotein cholesterol (VLDL-c), atherogenic index (AI) and percent protection were calculated by using the following formulae,

LDL-c = TC – TG/5 – HDL-c (given in Randox reagent kit)

VLDL-c = TG/5[40]

AI = TC / HDL-c[41]

Percent protection = (AI of control- AI of treated group / AI of control) x 100[42]

The percent inhibition of antioxidant enzymes and protein including catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) were estimated in liver homogenate by manual methods[43-45]. In addition, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity was determined in terms of ratio of HMG-CoA/ mevalonate in liver tissues by method described by Rao and Ramakrishnan[37,46].

Statistical Analysis

Results are presented as mean ± SD (standard deviation) and analyzed by one way ANOVA followed by LSD (least significant difference) test (SPSS, version 17.0). The differences were found significant at $p<0.05$ when compared with respective controls.

RESULTS

Hypolipidemic Screening of C. anthelminticum, C. limon & W. coagulans in Normal Rabbits

A significant decrease ($p<0.05$) was observed in serum TC, TG and LDL-c and increase in HDL-c level by *W. coagulans* and *C. anthelminticum* (200 mg/kg) as compared to control group. Whereas no significant effect was observed in lipid profile of test group subjected with *C. limon*. Whereas no toxicity was observed in liver and heart as the serum ALT and CK levels which were found normal ranging from 8.7 to 8.9 U/L and 37.7 to 84.4 U/L respectively in all groups including control and test groups (Table 1).

Hypolipidemic Activity of C. anthelminticum and W. coagulans in Triton- induced Hyperlipidemic Rabbits

On lipid profile, AI and Percent Protection Test doses (200 & 600 mg/kg) of *C. anthelminticum* and *W. coagulans* were found efficient ($p<0.05$) in decreasing serum TC, TG, LDL-c, VLDL-c and increasing HDL-c levels as compared to TIH control and negative control groups. Simvastatin (20 mg/kg) was also found effective in same aspect in positive control group (Figure 1). Similarly, AI (TC/HDL-c ratio) and percent protection were significantly decreased and increased in all test groups indicating the cardioprotective effect of both plants (Table 2 and Figure 2).

On lipid hydrolyzing enzymes

HMG-CoA reductase activity was determined in terms of HMG-CoA / mevalonate ratio which was significantly improved ($p<0.05$) by both test doses of both plants when compared with TIH control and negative control. A significant increase was also observed in positive control group (Table 2). A significant increase was observed in the level of lipase which is TG hydrolyzing enzyme, by the test groups administered with *C. anthelminticum* and *W. coagulans* (200 and 600 mg/kg) when compared with TIH control groups whereas positive control group showed no significant change (Table 2).

On antioxidant enzymes, protein and LPO

In test rabbits treated with *C. anthelminticum* and *W. coagulans* (200 and 600 mg/kg), a significant ($p<0.05$) decline was observed in percent inhibition of CAT, SOD and GSH when compared with TIH control groups. No significant reduction in same three parameters was observed in positive control group. However, percent inhibition of LPO was found significantly ($p<0.05$) increased in test and positive control groups (Figure 3).

On liver- and cardiac-specific enzymes

The serum ALT was found to be significantly improved ($p<0.05$) in test rabbits treated with *C. anthelminticum* and *W. coagulans* @ 200 and 600 mg/kg and positive control group treated with simvastatin @ 20 mg/kg when compared with TIH control group. Similar improvement was also observed in serum CK levels of test and positive control groups (Table 3).

DISCUSSION

In the present study, hypolipidemic effect of MFEt of *W. coagulans*, ESEt of *C. anthelminticum* and *C. limon* has been observed by using their single dose (200mg/kg) in separate normal overnight fasted rabbits groups for 14 days consecutively. Where, it has been observed that *W. coagulans* and *C. anthelminticum* have more potential in decreasing serum levels of TC, TG, LDL-c, VLDL-c and increasing HDL-c levels in test groups as compared to *C.limon*. However, three of them did not show any hepto- and cardio-toxicities by keeping the serum ALT and CK levels normal. Therefore, *W. coagulans* and *C. anthelminticum* extracts were selected to evaluate antihyperlipidemic and antioxidant activities in triton-induced hyperlipidemic animal model in 2nd phase. Triton-induced hyperlipidemic animal model has been widely used for determining the hypolipidemic activity of natural or synthetic compounds in experimental studies[47]. Triton X-100 is reported to serves as a surfactant and inhibits the lipoproteins uptake in extra-hepatic tissues from circulation, thereby inducing acute hyperlipidemia in many animals including rats, mice, rabbits[7,48]. The same was observed in the present study, where triton X-100 induced hyperlipidemic (TIH) groups showed high levels of TC, TG, LDL-c, VLDL-c and reduced level of HDL-c. However, test groups subjected with oral administration of *W. coagulans* and *C. anthelminticum* after receiving triton injection (i.p) demonstrated improvement in lipid profile like rabbits administered with both doses (200 and 600 mg/kg) of each of *C. anthelminticum* and *W. coagulans* induced significant decrease ($p<0.05$) in TC, TG, LDL-c, VLDL-c and elevate HDL-c level, thus, showed antihyperlipidemic activity. This finding clearly indicates that the both plant extracts have cholesterol reducing ability by inhibiting the activity of HMG-CoA reductase (HMGCR), the rate-limiting enzyme in cholesterol biosynthesis or increasing breakdown of LDL-c *via* its hepatic receptors to get eliminate in the form of bile acids[3]. In order to confirm the first possibility, the concentration of HMGCR was determined in terms of HMG-CoA / mevalonate ratio[40]. All doses of both plants extracts showed improvement in this ratio indicating the inhibition of HMGCR, thereby inhibiting TC biosynthesis. The results also demonstrate decreased levels of TG in test groups of both plant extracts, however the doses of *C. anthelminticum* found better than *W. coagulans* in this respect. The decrease in TG level may be due to increase activity of the endothelium-bound lipoprotein lipase that hydrolyzes TG into fatty acids[3]. Interestingly, in all test groups increase in lipase levels was also observed and showed the lipolytic activity of both plants extracts. The decrease in lipoproteins (LDL-c and VLDL-c) in all test groups is of course the secondary effect of hypocholesterolemic and hypotriglyceridemic impact of ESEt of *C. anthelminticum* and MFEt of *W. coagulans*. This protective role of plant extracts is also confirmed by observing elevated levels of good cholesterol (HDL-c) in serum of test rabbits, thus minimizing the risk of atherogenesis. This antiatherogenic effect of both plant extracts is more fortified by observing a significant decrease in atherogenic index (AI) and increase in percent protection in all test groups clearly indicate that test rabbits treated with plant extracts are less susceptible to atherogenesis. On other hand, simvastatin, a well-known HMGCR inhibitor, used as positive control showed significant decrease ($p<0.05$) in TC, TG, LDL-c and VLDL-c levels but did not show any elevation in HDL-c level. The present results of *C. anthelminticum* and *W.coagulans* are compatible with our pervious hypolipidemic findings of same plant extracts in high fat-induced hyperlipidemic animal model[37,40]. Studies showed that hyperlipidemia-induced oxidative stress is the initiating point of many chronic complications by increasing the formation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen and suppressing the antioxidant enzymes system in body. This stress induced oxidative modification of LDL-c, which plays a key role in the pathogenesis of atherosclerosis[49]. In the present study, ESEt of *C. anthelminticum* and MFEt of *W.*

coagulans showed their potential in reducing the oxidative stress by improving the performance of antioxidant enzymes and protein including CAT, SOD, reduced GSH and inhibiting lipid peroxidation (LPO) in all test groups. An efficient antioxidant defense system is responsible to counteract oxidative damage to macromolecules (membrane lipids, proteins and DNA) by showing free radical scavenging ability[50,51]. Elevated levels of ALT and CK are the reflection of altered cellular integrity of liver and cardiac cells[3]. The same situation was observed in triton-induced hyperlipidemic control rabbits which may be due to microvesicular steatosis and lipid accumulation in these cells[52,53]. On contrary, all test groups treated with *C. anthelminticum* and *W. coagulans* showed significant decrease in levels of both of these liver and cardiac biomarkers, indicating that both plant extracts have hepato- & cardio-protective effects. Therefore, the results conclude that ESEt of *C. anthelminticum* is found effective as same as well-known MFEt of *W.coagulans* in reducing hyperlipidemia and hyperlipidemia-induced oxidative stress in triton-induced hyperlipidemic rabbits.

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