Evaluation the hypolipidemic and antioxidant efficacy of diosgenin extracted from fenugreek seeds in experimentally induced oxidative stressed male rats

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Abstract

This study was conducted to assess the antioxidant status and hypolipidemic role of purified diosgenin extracted from fenugreek seeds in hydrogen peroxide (H_2O_2) and atherogenic diet experimentally induced oxidative stress male rats. Hexane and chloroform were used for the extraction of diosgenin from ground fenugreek seeds. Purification of extracted fraction was carried out on Sephadex LH-20 column using ethyl acetate as eluent, homogenous symmetrical peak was obtained, and thin layer chromatography on silica gel confirmed that purified fraction is diosgenin in compression to standard diosgenin and the calculated Rf values. The antioxidant scavenging activity was carried out using 2, 2, 1 diphenyl- 1- picrylhydrazyl (DPPH) assay, the result showed that purified diosgenin has an antioxidant activity 13.68 µg/ml while the corresponding values for vitamin C and butylated hydroxyl toluene were 8.43 and 7.26 µg/ml respectively. Fifteen adult Albino male rats were randomly and equally divided into three groups, and treated for six weeks as follows; G1 group served as control, G2: Animals of this group were received drinking water containing 0.75% H_2O_2 and atherogenic diet, Group 3; Rats of this group were given drinking water containing 0.75% H_2O_2 and atherogenic diet plus 40 mg purified diosgenin/ kg B.W. dissolved in 0.5 ethyl acetate daily by gavages needle. Fasting blood samples were collected by cardiac puncture technique at 0, third and sixth weeks of experiment. The results revealed that rats in G2 exposed to oxidative stress (0.75% H_2O_2 plus atherogenic diet) showed a significant elevation ($P<0.05$) in serum total cholesterol (TC), triacylglycerol (TAG), low density lipoprotein- cholesterol (LDL-C) as well as in malondialdehyde (MDA), and significant suppression in serum high density lipoprotein-cholesterol (HDL-C) and glutathione (GSH) concentrations in comparison to the animals in the first group (G1 group). Intubation of 40 mg/kg B.W. of purified diosgenin was caused a significant alteration in the lipid profile of the treated group (G3), manifested by significant reduction ($P<0.05$) in serum TC, TAG, LDL-C and significant elevation in HDL-C. Antioxidant status also exhibits significant changes manifested by an elevation of serum GSH and reduction in MDA concentration in G3 group animals. This study has shown the significant role of diosgenin as hypolipidemic agent and its role in restores of the oxidative stress.

Keywords: diosgenin, fenugreek seeds, hypolipidemic, antioxidant, oxidative stressed, male rats

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تقييم كفاءة الدايوسجنين المستخلص من بذور الحلبة كمانع للاكسدة وخافض للدهون في ذكور الجرذان المعرضة للإجهاد التأكسدي تجريبيا

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الخلاصة

استهدفت هذه الدراسة تقييم الدور المانع للاكسدة والخافض للدهون للدايوسجنين المستخلص من بذور الحلبة والمنقى بطريقة الكروموتوغرافي في ذكور الجرذان المعرضة للاجهاد التأكسدي بوساطة بيروكسيد الهيدروجين

والغذاء الحاوي على الكولستيرول. استخدم الكلوروفورم والهكسان لاستخلاص الدايوسجنين الخام من بذور الحلبة المطحونة بطريقة السكسوليت، ثم تم تنقيته على عمود من السيفادكس−20 بأستخدام خلات الاثيل كمزيح للنموذج. تم الحصول على دايوسجنين نقي وتم التأكد من ذلك بأستخدام كروموتوكرافي الطبقة الرقيقة وحساب قيمة Rf مقارنة بالقيم القياسية. استخدم اختبار DPPH لتقدير كفاءة الدايوسجنين المنقى كمانع للأكسدة خارج الجسم الحي مقارنة مع فيتامين، C وButylated hydroxyl toluene، وأظهرت النتائج ان الدايوسجنين المنقى لها قدرة مانعة للأكسدة وبمقدار 13.68 مايكروغرام/ مل بينما نتائج فيتامين C و BHT كانت 8.43 و7.26 مايكروغرام/ مل على التوالي. لدراسة دور الدايوسجنين المنقى كخافض للدهون ومانع للأكسدة في الحيوانات التجريبية، تم تقسيم خمسة عشر ذكرا من الجرذان البالغة وبصورة عشوائية إلى ثلاث مجاميع متساوية وعوملت لمدة 6 أسابيع وعلى النحو الاتي: المجموعة الأولى GI واعتمدت كمجموعة سيطرة. المجموعة الثانية GII: جرذان هذه المجموعة اعطيت بيروكسيد الهيدروجين 0.75% في ماء الشرب وعليقة غنية بالكولستيرول (atherogenic diet). اما المجموعة الثالثة GIII فقد اعطيت 0.75% بيروكسيد الهيدروجين في ماء الشرب وعليقة غنية بالكولستيرول (atherogenic diet) وتم تجريع 40 ملغم من الدايوسجنين المنقى والمذاب في نصف مل من خلات الاثيل، علما ان المجموعة الاولى والثانية قد جرعت بنصف مل من خلات الأثيل. تم جمع عينات الدم في اليوم الاول والاسبوع الثالث والسادس من التجربة عن طريق السحب المباشر من القلب لغرض الفحوصات الكيموحيوية بعد 12 ساعة من تجويع الحيوانات في كل مرة وتم فصل المصل لغرض الفحوصات الكيموحيوية. أظهرت النتائج ان جرذان المجموعة الثانية والمعرضة للاجهاد التأكسدي زيادة معنوية ($P<0.05$) في الكولستيرول الكلي والكوليسترول المخنفض الكثافة والكلسريدات الثلاثية الاسيل وكذلك المالونداياالديهايد وانخفاضا معنويا في تركيز كل من الكولستيرول العالي الكثافة والكلوتاثايون المختزل مقارنة بنتائج الحيوانات المجموعة الاولى (مجموعة السيطرة). اظهرت نتائج المجموعة الثالثة والتي تم تجريعها 40 ملغم/ كغم من وزن الجسم من مادة الدايوسجنين تغيرات معنوية بالصورة الدهنية وتجلى ذلك بحصول انخفاض معنوي في الكولستيرول الكلي والكوليسترول المنخفض الكثافة والكليسرول الثلاثي الاسيل وزيادة معنوية في الكوليسترول العالي الكثافة. كما حصل ارتفاع معنوي في الكلوتاثايون وانخفاض في تركيز المالونداياالديهايد في المجموعة الثالثة. تجلت أهمية هذه الدراسة من خلال دور الدايوسجنين كعامل مخفض للدهون واهميته في تقليل الأجهاد التأكسدي.

الكلمات المفتاحية: الدايوسجنين، بذور الحلبة، خافض للدهون، مانع للأكسدة، الإجهاد التأكسدي، ذكور الجرذان

Introduction

Diosgenin, a natural steroidal important metabolite found in various plants predominantly in fenugreek and has diverse biological properties (1, 2, 3). Diosgenin are synthesized from cholesterol in several plants (4) through isoprenoid biosynthetic pathway, and has been shown to have favorable effects, lipid metabolism (5), an anti-inflammatory (6) glucose lowering (7), and antioxidant activates (8, 9). Diosgenin has both antioxidant property, anti-cholesterolomic activity, and as precursor of various synthetic steroidal drugs particulary in metabolic diseases (hypercholesteromia, dyslipidemia, diabetes and obesity) (10). However, cholesterol lowering activity in rats (11), and human trials were attributed to inhibition of the absorption of cholesterol from the small intestine, or the re-absorption of bile acids (12). On the other hand (13) stated that diosgenin enhanced reactive oxygen species (ROS) generation and lipid peroxidation and decreased antioxidant activity in Human epithelial type 2 (Hep2) cells which shows that diosgenin possess prooxidant properties. However, the overall aim of this study was to evaluate the antioxidant and hypolipidemic role of diosgenin extracted from fenugreek in induced oxidative stressed male rats.

Materials and Methods

- **Extraction of diosgenin from Fenugreek seeds:**

- Fenugreek seeds was purchased from local market (Baghdad), and authenticated by the "Iraqi National Herbarium" Botany directorate in Abu-Ghraib.
- Fenugreek seeds was grounded by electrical mill and dried for 2.5 h at 60 ºC. A grounded seed was defatted in a soxhlet apparatus for 48h with hexane. Residual defatted materials (Marc 1) was air dried for 48h (14), hydrolyzed by boiling under reflux with 2N HCl for 2h, cooled and centrifuged at 3000 rpm for 10 minutes. Residual precipitate (Marc 2) was washed with distilled water several times until neutralization (pH= 7) and subjected to centrifugation at 3000 rpm for 10 minutes, the precipitate (Marc 3) was dried at 60 ºC for 2h, re-extracted and refluxed with chloroform, and evaporated to dryness at 45-55 ºC using vacuum rotary evaporator (15, 16).

- **Purification of Diosgenin:** The following procedure was considered for the purification of the crude diosgenin on sephadex LH-20 purification of diosgenin (Marc 3 fraction) was performed on a glass column (38X1.6 cm Id) prepared according to the direction of the supply company, pharmacia- Sweden. Five hundred gm of crude diosgenin was applied on the column and elution was carried out using ethyel acetate as eluent. Three ml fraction collected with a total volume of 120 ml, a volume of 0.1 ml of 70-75% perchloric acid was (color developing reagent) added to each tube and the absorbance of each fraction was measured at 410nm (17). Tubes which showed the highest values were collected and dried under vacuum at 45 ºC. Dried fraction was then subjected to thin layer chromatography analysis.
- **Thin Layer Chromatography:** Thin layer chromatography of purified fraction on Sephadex LH-20 was carried out on a silica gel type G aluminum plate (20X20 cm) at a thickness of 0.25 mm supplied from Fluka Company. Fifty microleter (50 µl) fraction was applied about 1 cm from the front of the plate. The plates were inserted into a saturated TLC chambers containing chloroform: Methanol (94:6) (18), as a mobile phase and rested in stand position, often about 45 minutes the plates were removed, the front end was quickly marked before the solvent evaporated, then left to dry at room temperature. Development of storied compound was conducted by spraying the chromatogram with 50% H_2SO_4 and subsequently drying at 110 ºC in oven for 5 minutes until the development of characteristic color. Rf values were calculated for both the standard and extracted diosgenin.
- **Antioxidant Activity Determination:** The free radical scavenging ability of diosgenin extracted from fenugreek seeds as compared with standard BHT and vitamin C was carried out by spectroscopic method as menthianed by (19).
- **Determination of Serum Reduced glutathione concentration (GSH):** The method of (20) was used for determination of reduced glutathione (mmol/L).
- **Determination of Serum Malondialdehyde (MDA) (mmol):** Thiobarbituric acid was method used and the absorbance was measured at 532nm with an extension coefficient of absorbance $1.53X10^{-1}$ M^{-1} Cm^{-1} (21).
- **Experimental Animals:** Fifteen adult male rats were divided into three equal groups and treated as follows for six weeks.

G1- Animals in this group were received regular standard diet, tap water and 0.5 ml of ethyl acetate daily by gavages needle serving as a control.

G2- Animals in this group were received atherogenic diet and 0.75% H_2O_2 in drinking water, and 0.5 ml ethyl acetate.

G3- Animals in this group were received atherogenic diet and 0.75% H_2O_2 in drinking water plus 40 mg purified diosgenin/ kg B.W. diosgenin dose was considered according to (22). Diosgenin was dissolved in ethyl acetate according to (23). The LD50 of ethyl acetate in rats (ingestion) was found to be 5660 mg/kg B.W. indicting of low toxicity of ethyl acetate (24, 25).

Table (1) Standard pellet diet was prepared as follows

Ingredients	Gm/100 gm
Proteins	16.80
Fats	5.1
Carbohydrates	48.53
Calcium	1.6
Phosphorus	0.77
Crude fiber	6.20
Ash	13.5
Moisture	7.5

Atherogenic diets were prepared by addition of 0.1% cholesterol, 2% casein, 2% cholic acid and 27% sucrose to the standard diet (26). Fasting blood samples were collected at 0 time, 3rd and 6th weeks of the experiment. Blood samples, held for not more than four hours before serum collection by centrifugation (3000 rpm) for 15 minutes and frozen at -20 C until analysis.

- **Analytical Methods:** Serum total cholesterol concentration mmol:

- Serum total cholesterol (TC) was measured according to (27) using Randox assay kit.
- Enzymatic estimation of serum triacyglycerol (TAG) concentration was carried out using Biomerieux kit (28).
- Serum high- density lipoprotein cholesterol HDL-C was measured ezymatically using linear enzymatic kit (Linear chemicals, Barcelona, Spain).
- Serum low density lipoprotein cholesterol LDL-C was calculated according to (28).

Statistical analysis of the data was performed on the basis of two-way analysis of variance (ANOVA) using a significant level of ($P<0.01$ and 0.05). Specific group differences were determined using least significant differences (LSD) as described by (29).

Results and Discussion

- **Extraction and purification of Diosgenin**: Soxhlet de-fating of Fenugreek seeds was carried out with hexane and subsequent acid hydrolysis and refluxing with chloroform. Application of partially purified active fraction on sephadex LH-20 column yield to some extent a homogenous symmetrical peak (Fig. 1). Thin layer chromatography on silica gel (Fig. 2) revealed that the bioactive fraction obtained from sephadex LH-20 column appears as a dark pink spots after development and the calculated Rf was found to be 0.66 which is identified to the Rf value of standard diosgenin obtained from fluka, Germany.

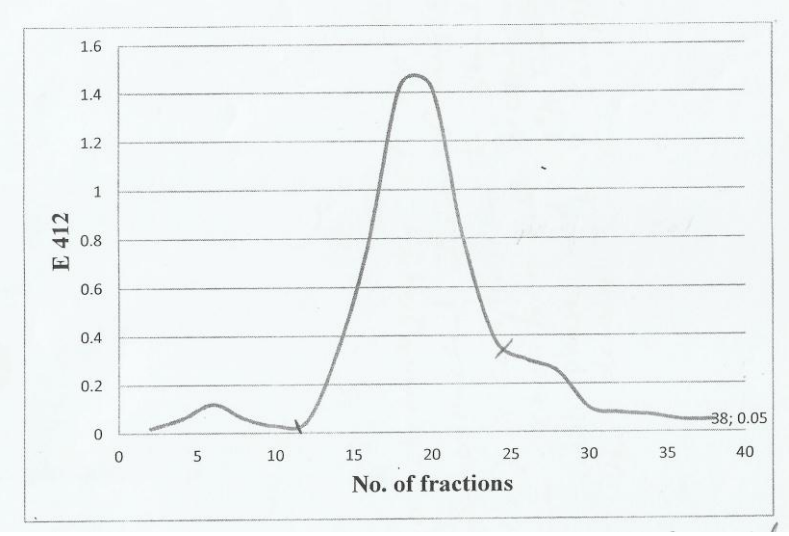

Fig. (1) Purification of de-fated crude extract of powdered fenugreek on sephadex-LH-20 column (38.1.6cm). Ethyl acetate was used as eluent at a flow rate 25ml/hr, fraction size 3 ml, fraction from tubes 11-25 were collected for further confirmation.

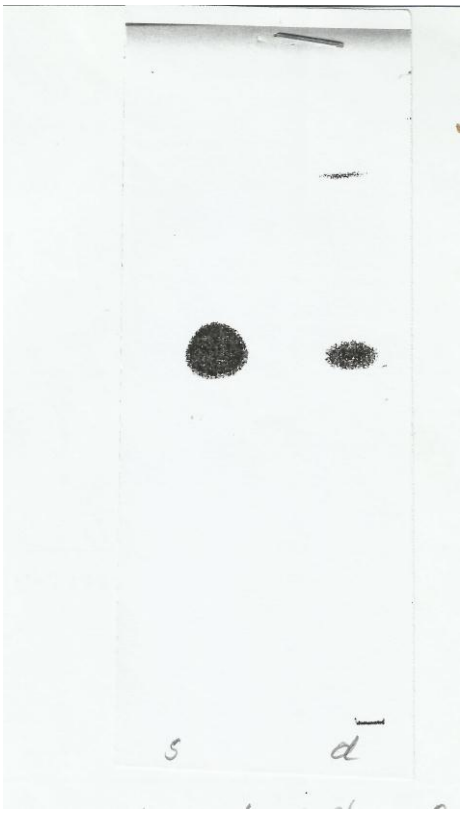

Fig. (2) Thin layer chromatography of purified diosgenin (on silica gel) extracted from fenugreek seeds (d) in compression to standard diosgenin (s). Chloroform and methanol was used as mobile phase and 50% of H_2SO_4 was used for the development of color.

The radical scavenging capacity (EC50) was found to be 13.68 µg/ml (table 2), corresponding values for vitamin C and BHT were 8.43 and 7.26 µg/ml respectively.

Table (2) Antioxidant scavenging activity of 0.02% purified diosgenin of fenugreek seeds as compared to butylated hydroxyl toluene (BHT) and vitamin C by DPPH scavenging assay (19).

Antioxidant	Antioxidant activity mg/ml
Purified saponin	13.68
BHT	7.26
Vitamin C	8.43

The result of this study clarified that purified diosgenin capable for scavenging free radicals by electron or hydrogen donating mechanism and thus should be potent enough to prevent the inhibition of deleterious free radicals mediated chain reactions in vitro (30).

- **Effect of oxidative stress and diosgenin on antioxidative status**

Description of antioxidant status in atherogenic diet plus H_2O_2 treated group (group 2) was manifested from a significant reduction ($P<0.05$) in GSH concentration after three and six weeks of treatments while in a group G3 significant reduction (10.74±0.03) was recorded after three weeks of treatments and no significant changes was observed after six weeks in comparison to control group. Hyperlipidemia and mild oxidative stress should be considered since oxyhemoglobin has the ability to generate a reactive oxidant species, as well as induction of low concentration of H_2O_2 ease with which methaemoglobin release iron in a form that can stimulate lipid peroxidation and OH radical formation (31, 32). Also H_2O_2 may play a potent role in an increasing O_2 production in the stomach followed by a state of tissue hypoxia which in turn leads to excessive formation of free radicals (33, 34, 35).

Table (3) Effect of purified diosgenin on serum rduced glutathione concentration (GSH) (Mmol/l) in male rats fed atherogenic diet plus 0.75% H_2O_2 and control

G / T	G1	G2	G3
Zero	11.38 ± 2.6 A a	11.37 ± 0.19 A a	11.21 ± 0.7 A
3 weeks	11.17 ± 0.8 A a	10.12 ± 0.02 B b	10.74 ± 0.3 B
6 weeks	11.35 ± 1.2 A a	9.33 ± 0.2 C c	10.89 ± 0.15 B

LSD= 0.34
Values are expressed as mean ± SE, n=5 for each group
Capital letters donate differences between groups, P<0.05 Vs control.
Small letters donate differences within group, P<0.05 Vs control.

Table (4) Effect of purified diosgenin extracted from fenugreek on serum malondialdehyde concentration (MDA) (Mmol/l) in male rats fed atherogenic diet plus 0.75% H_2O_2 and control

G / T	G1	G2	G3
Zero	0.449 ± 0.0107 A a	0.48 ± 0.0107 A b	0.460± 0.0214 A b
3 weeks	0.471 ± 0.0085 C a	0.514 ± 0.0054 B a	0.514 ± 0.0032 A a
6 weeks	0.460 ± 0.0107 A b	0.503 ± 0.0086 A a	0.482 ± 0.0021 B b

LSD= 0.0321
Values are expressed as mean ± SE, n=5 for each group
Capital letters donate differences between groups, P<0.05 Vs control.
Small letters donate differences within group, P<0.05 Vs control.

Oxidative glutathione (GSSG) levels can be elevated during oxidative stress mainly through direct interaction of oxidant (H_2O_2) with GSH, causing a depletion of cellular GSH, and differential expression of the GSH- metabolizing enzymes glutathione peroxidase. Glutathione- peroxidase are major cellular antioxidative enzymes that are protected from oxidative damage and cytotoxicity caused by hydroperoxides (36). Moreover, an elevation in free radicals generation caused a gradual cell injury and liberating lipoxygenase enzyme which oxidized long chain unsaturated fatty acids, and a subsequent production of MDA, overwhelming endogenous scavenging system including GSH resulting in oxidative stress (37, 38). Elevation of GSH after six weeks of treatments (10.89 ± 0.15) as compared to third week and reduction of MDA in G3 treated animals after six weeks of tretmets in comparison to third week of treatment (table 3 and 4) may be attributed to saponin compound (diosgenin) which exert their mode of action by suppression the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements (39) as well as can act as hydrogen donors. Beneficial role of diosgenin in oxidative stress induced diabetic rats was also stated by (22). The promising antioxidant activity of diosgenin may be caused by the resonance phenomena of double bonds at carbon atom 5-6 (40). However, diosgenin treatment remarkable down regulated the peroxidation reaction (elevated GSH and reduced MDA) and enhanced the endogenous antioxidant defense system.

- **Lipid profile in Atherogenic Diet plus H_2O_2 and Diosgenin Treated Rats:** Oral administration of H_2O_2 and atherogenic diet to male rats for six weeks caused a case of hyperlipidemia and hypertriglyceredemia manifested by significant (P<0.05) elevation of serum total cholesterol, LDL-C and TAG and significant reduction in HDL-C (table 5, 6, 7 and 8). Serum total cholesterol and LDL-C elevation could reflect the role of H_2O_2 in suppression of lipid metabolism due to changes in lipoprotein metabolism (41) as well as changes in hepatic expression of genes such

as lipoprotein receptors, apolilipoprotein B and the microsomal TAG protein transfer (42, 43). Besides, increment of TAG level in rats received H_2O_2 in combination with atherogenic diet may be due to an increase in serum very low density lipoprotein- cholesterol (VLDL-C) level which acts as a carrier for the TAG in the plasma (44). High sugar content of the atherogenic diet may precipitate in TAG elevation, since TAG leads to a partial deficiency of lipoprotein lipase associated with increased output of lipoprotein from the liver (45, 46).

- **Effect of Oxidative Stress and Diosgenin on Lipid Profile:** It has been hypothesized that cells respond to phytochemicals through direct interactions with receptors or enzymes involved in signal transduction, or through modifying gene expressions that may result in alteration of the redox status of the cell that may trigger a series of redox-dependent reactions (47). Treatment of oxidative stressed rats with dosgenin (40 mg/kg B.W.) restores the lipid profile levels (group 3 tables 5, 6, 7 and 8). The antihyperlipidemic effect of diosgenin may be due to large mixed micelles formation by interaction of diosgenin (saponin) with bile acid account for their increased excretion when saponin rich foods such soybean consumed (48) as well as to upregulation activity of LDL-C receptors (49). The lipid- lowering potential of diosgenin has been demonstrated by (50). Diosgenin decreased the elevated cholesterol in serum LDL-C and HDL-C fractions in cholesterol fed rats because of its capability in suppression of cholesterol absorption and inhibited its uptake in serum and accumulation in the liver. Recently, it was shown that diosgenin (at an oral dose of 0.1% or 0.5% in diet) decreased total cholesterol levels and increased plasma HDL-C levels in both plasma and livers of diet induced hypercholesterolemic rats (8). Several studies review clarify that fenugreek seeds (rich in diosgenin) have an important role in the control of metabolic diseases such as diabetes and obesity (10). Fenugreek decreased the size of adipocytes in diabetic obese KK-AY mice suggesting an increased differentiation of adipocytes leading to decreased adipocytes lipid accumulation because of elevation of mRNA expression levels of differentiation- related genes in adipose tissues (51).

In conclusion diosgenin extracted from fenugreek showed a potent antioxidant and hypolipidemic effect in oxidative stressed rats used in this study restores the antioxidant status and lipid profile to the normal.

Table (5) Effect of purified diosgenin extracted from fenugreek on serum total cholesterol (mmol/l) in male rats fed atherogenic diet plus 0.75% H_2O_2 and control

G / T	G1	G2	G3
Zero	2.663 ± 0.0267 A a	2.643 ± 0.0767 A d	2.615 ± 0.0622 A b
3 weeks	2.652 ± 0.0199 C a	3.449 ± 0.0599 B c	2.827 ± 0.0866 A a
6 weeks	2.554 ± 0.0577 B a	3.842 ± 0.0511 A b	2.696 ± 0.0618 B b

LSD= 0.229

Table (6) Effect of purified diosgenin extracted from fenugreek on serum low density lipoprotein cholesterol (LDL-C) (mmol/l) in male rats fed atherogenic diet plus 0.75% H_2O_2 and control

G / T	G1	G2	G3
Zero	1.127 ± 0.012 A a	1.145 ± 0.055 A d	1.098 ± 0.043 A b
3 weeks	1.147 ± 0.009 C a	1.883 ± 0.021 B c	1.311 ± 0.049 A a
6 weeks	1.035 ± 0.042 B	2.198 ± 0.031 A a	1.080 ± 0.035 B b

LSD= 0.197

Table (7) Effect of purified diosgenin extracted from fenugreek on serum triacylglycerol (TAG) (mmol/l) in male rats fed atherogenic diet plus 0.75% H_2O_2 and control

G / T	G1	G2	G3
Zero	2.399 ± 0.0210 A a	2.409 ± 0.0066 A d	2.419 ± 0.0321 A c
3 weeks	2.282 ± 0.0265 B a	3.002 ± 0.0232 A c	2.011 ± 0.0199 A a
6 weeks	2.304 ± 0.0199 C a	3.584 ± 0.0819 A b	2.652 ± 0.0520 B b

LSD= 0.112

Table (8) Effect of purified diosgenin extracted from fenugreek on serum high density lipoprotein cholesterol (HDL-C) (mmol/l) in male rats fed atherogenic diet plus 0.75% H_2O_2 and control

G / T	G1	G2	G3
Zero	0.906 ± 0.013 A a	0.897 ± 0.017 A a	0.915 ± 0.012 A b
3 weeks	0.907 ± 0.017 A a	0.807 ± 0.021 B b	0.735 ± 0.027 C b
6 weeks	0.942 ± 0.009 A a	0.753 ± 0.006 B c	0.964 ± 0.013 A a

LSD= 0.052

Values are expressed as mean ± SE, n=5 for each group

Capital letters donate differences between groups, P<0.05 Vs control.

Small letters donate differences within group, P<0.05 Vs control.

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