Oral administration of apigenin 7-*O*-*β*-D glucoside reverses hyperlipidemia and associated vascular dysfunction in rats

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Abstract

Apigenin 7-*O*-*β*-D-glucoside is a flavonoid reported as smooth muscle relaxant, anti-inflammatory, and anti-oxidant, suggesting its role in cardiovascular disease. The present study aimed to investigate the effect of apigenin 7-*O*-*β*-D-glucoside on hyperlipidemia and associated vascular dysfunction in rats. Oral administration of apigenin 7-*O*-*β*-D-glucoside to HFD and tyloxopol-induced hyperlipidemic SD rats for 28 and 10 days that reduced significantly ($p < 0.01$), total cholesterol, LDL, VLDL and triglycerides, and increased HDL levels and also reduced the total body weight and atherogenic index suggesting its antihyperlipidemic effect. This effect was further confirmed when the compound also inhibited the key enzyme HMG-CoA reductase in the biosynthesis of cholesterol. The devastating effects on vascular architecture such as change in the aortic intima, media, adventitia and also the endothelium damage, were reversed in apigenin 7-*O*-*β*-D-glucoside 5 mg/kg/day treated group. In the *in vitro* studies, the compound reversed the endothelial damage demonstrated by significant vasorelaxation in the aortic rings from hyperlipidemic rats treated with apigenin 7-*O*-*β*-D-glucoside 5 mg/kg/day, with EC_{50} value of 0.02

μg/mL (0.01-0.20) compared to hyperlipidemic HFD rats, similar to atorvastatin. These findings indicate that antihyperlipidemic effect of apigenin 7-*O*-*β*-D-glucoside is mediated through decrease in total cholesterol, LDL, VLDL, triglyceride level and increase in level of HDL through inhibition of HMG-CoA inhibition and also improved the associated histopathological changes and endothelium dysfunction.

Keywords: Apigenin 7-*O*-*β*-D-glucoside; HFD-induced hyperlipidemia; Lipid profile; HMG-CoA reductase activity; Histopathological study; Vascular function study;

1. Introduction

Hyperlipidemia or hypercholesterolemia (Durrington, 2003), was identified in the 19th century as cause of atherosclerotic lesion that is also responsible for endothelial dysfunction (Virchow, 1856). Hyperlipidemia is categorized by high serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c) and low high-density lipoprotein cholesterol (HDL-c) levels (Belguith-Hadriche *et al.*, 2016; Hill *et al.*, 2022), All lipoproteins carry cholesterol, however elevated levels of LDL-cholesterol is associated with an increased risk of atherosclerosis due to shear and oxidative stresses (Drexler *et al.*, 1999; Felmeden *et al.*, 2003). One of the main causes of high cholesterol is due to the increasing activity of HMG-CoA reductase (Nakamura *et al.*, 1999). And is target for cholesterol-lowering agents including statins. Different flavonoids, of plant orige n such as astilbin, brutieridin, melitidin also inhibit this enzyme (Zeka *et al.*, 2017).

Research on flavonoids got attention recently, after the confirmation of their benefecial effects on the cardiovascular system such as antioxidant, vasodilators and modulators of vascular endothelial function (Patel *et al.*, 2007; Vargo *et al.*, 2006). Flavonoids like, apigenin controls cholesterol metabolism *in vivo* and help in reducing fats level through cholesterol absorption, and accelerating reverse cholesterol transport. In addition it also resists oxidization and protects blood vessels (Zhang *et al.*, 2017). Apigenin 7-*O*-*β*-D-glucoside is one of the important flavonoids in nature, which is abundantly present in plants, and due to its low toxicity received much attention in recent years (Kumar *et al.*, 2014; Zeka *et al.*, 2017). It is reported as cardio protective, smooth muscle relaxant, anti-inflammatory, and anti-oxidant agent (Ali *et al.*, 2017; Bolzon *et al.*, 2017; Ciumărnean *et al.*, 2020; Guzelmeric *et al.*, 2015; Samet *et al.*, 2015). The cardio protective, potential smooth muscle relaxant, and antioxidant properties of apigenin 7-*O*-*β*-D-glucoside

provide a base for its investigation in hyperlipidemia. Current literature lacks pharmacological investigation of apigenin 7-*O*-*β*-D-glucoside in managing hyperlipidemia.

2. Research methodology

2.1 Pharmacological investigations

2.2 Drugs and standards

The compound apigenin 7-*O*-*β*-D-glucoside was acquired from an authentic source (Sigma Chemical Company, St. Louis, MO). All other chemicals such as acetylcholine chloride, potassium chloride, phenylephrine hydrochloride, dimethyl sulfoxide (DMSO), Tween 80, cholesterol, and cholic acid were purchased from the vendor specified (Sigma Chemical Company, St. Louis, MO). On the day of experiment, stock solutions and dilutions were freshly prepared in the appropriate solvent.

2.3 Experimental animals

The Ethical Committee of the Department of Pharmacy, COMSATS University Islamabad (CUI), Abbottabad Campus, approved the studies following the guidelines and standards of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (Ferdowsian *et al.*, 2011). Sprague-Dawley (SD) rats (200-250 g) were housed and kept at a temperature of 23–25°C, having free access to food and water in the animal house of the Department of Pharmacy, CUI, Abbottabad,

2.4 High-fat diet HFD-induced hyperlipidemic model

HFD consists of butterfat, cholesterol, and cholic acid (5%, 2%, 0.5% w/w) respectively, was added to a normal diet (wheat (5 kg) and oat flour (4 kg), skimmed milk (500 g), fish oil (100 mL), yeast, and multivitamin (100 g) for 10 kg) and considered as HFD (Buettner *et al.*, 2006).

2.5 Protocol

Hyperlipidemia was induced using HFD as described previously (Berrougui et al., 2003), with slight modifications. SD rats 180-225 g were placed in constant hygienic conditions and fed on a HFD diet. In this study 6 different groups were using with 5 rats in each. Group 1: normal control, group 2: HFD (untreated group), group 3: HFD diet + atorvastatin (10 mg/kg), group 4-6: HFD diet + apigenin 7-*O*-*β*-D-glucoside (1, 3, and 5 mg/kg p.o). All rats have free access to water and

food. The consumption of food was observed daily and the increase in weight of the body was notated on weekly base. After 4 weeks of treatment, rats were fasted for 24 hr and were anesthetized with sodium thiopental 60-90 mg/kg, and blood was obtained via cardiac puncture. Blood serum was investigated for lipids.

The rat aorta was also dissected from hyperlipidemic and treated groups for the evaluation of vascular dysfunction *in vitro*.

2.5.1 Tyloxapol-induced model of hyperlipidemia

In this model after administration of normal food for 10 days tylaxapol was administered for once at 500 mg/kg/ip.

1. **Protocol**

SD rats weighing 180-225 g were placed in constant hygienic conditions and fed on a normal diet. In the study 6 different groups were using with, 5 rats in each (Harnafi *et al.*, 2008). Group 1: normal control group, group 2: hyperlipidemic (untreated), group 3: atorvastatin treated (10 mg/kg)**,** group 4, 5, and 6: apigenin 7-*O*-*β*-D-glucoside treated groups (normal diet + apigenin 7-O-β-D-glucoside (1, 3, and 5 mg/kg p.o). After treatment for 10 days, all rats were fasted for 12 hr, group 1 received 10 mL/kg (i.p) saline, and groups 2 to 8 were given 500 mg/kg (i.p) tyloxapol for 24 hr. After cervical dislocation of rats blood was collected via cardiac puncture for analysis of lipids. The liver was also collected for the assay of HMG-CoA reductase enzyme activity.

2.6 Estimation lipid profile

For the calculation of serum TC, , LDL, VLDL, TG and HDL, serum, standard, and blank were taken and85 incubated at 21- 25°C for 10 min. 0.2 mL from each mixture was transferred into 96 well plates after incubation, and the absorbance of each mixture was evaluated against the blank reagent on a microplate reader at 490 nm (Friedewald *et al.*, 1972; Haslam *et al.*, 2020). The atherogenic index was calculated by using the formula described by Friedewald et al. (1972).

Atherogenic index= TC-HDL/HDL

2.7 Assay of HMG-CoA reductase

Preparation of Liver homogenate

Generally, the tissue homogenate was prepared by the isolation of the liver from the rat. The isolated liver was blotted with chilled saline and weighed. For this assay, 1 g of the liver was crushed down into pieces, and added into a 9 mL saline arsenate solution. The preparation was homogenized using a homogenizer and used the assay of HMG-CoA (Rao *et al.*, 1975; Díaz-Zagoya *et al.*, 2021).

Analysis of HMG-CoA reductase activity

The functioning of HMG-CoA reductase was determined through an indirect method, as described by Rao et al. (1975). About 1 g of freshly prepared liver tissue homogenate in 9 mL was taken and mixed with 10 mL of 5% perchloric acid and was kept for (5-7 min) at 25°C. The mixture was centrifuged for 10 min at 2000 rpm. To determine the mevalonate activity, Solution (A) freshly prepared 0.5 mL of 1 M aqueous hydroxylamine hydrochloride was added to 1 mL supernatant, which was obtained during centrifugation. Then for the estimation of the HMG-CoA, Solution (B) alkaline hydroxylamine hydrochloride 0.5 mL was also added in another tube having 1 mL supernatant of the same. The mixture of both tubes was incubated for 5 min at 37°C and then 1.5 mL of 0.06 M ferric chloride reagent was added to each tube and shaken vigorously. After 10 min of incubation, absorbance was read at 450 nm through a kinetic spectrophotometer against a reagent blank and then against each sample.

Enzyme inhibition calculation

Enzyme inhibition was calculated by the following formula.

Solution B (HMG-CoA) ÷ Solution A (mevalonate activity) = Enzyme inhibition (Iqbal, 2015)

2.8 Histopathological examination aorta from of HFD hyperlipidemic SD rats

Thoracic aortae from all groups were studied for histopathological changes. The thoracic aortae were quickly dissected out flushed immediately with saline and freed of all extra vascular tissue. Then clean aortic tube was fixed in 10% formalin. After fixation this tissue was dehydrated with ethanol at different concentration. After dehydration these tissue was dealcoholized with xylene.

Then embedded into paraffin. The tissues were segmented into 5 μm slices via a rotary microtome and stained with hematoxylin and eosin dye at ratio of 1:1. The tissue was examined under a light microscope (Opticam 5.0) for morphology of tunica intima, tunica media, tunica adventitia, macrophages, vacuolation, and lipid deposition (Mohanta *et al.*, 2016).

2.9 *In-vitro* vascular dysfunction

2.9.1 Measurement of endothelium-dependent, and independent effects

Thoracic descending aortae were isolated from HFD hyperlipidemic rats and compound with normal rats were sacrificed by cervical dislocation. The descendant aortae were cut out and shifted instantly into Kreb's–Henseleit solution. The composition of Kreb's solution was (mM) NaCl 118.2, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.3, $C_6H_{12}O_6$ 11.7, $NaHCO_3$ 25.0 and $CaCl_2$ 2.5 (pH 7.4), extra tissues were removed from the aortae, and rings of 1-3 mm were made. Each ring was hung in an organ bath 10 mL, filled with same solution, aerated with 95% O_2 and 5% CO_2 (carbogen), and coupled to a force transducer attached to a PowerLab data acquisition system. Resting tension of 2 gm was applied to each ring and equilibrated for 60-90 min, with a change of Krebs–Henseleit solution every 15-20 min. Phenylephrine (1 μM) was administered to induce contraction and (1 μM) acetylcholine was added to check endothelium integrity by plotting inhibitory concentration-response curves (CRCs) as described previously Shah & Gilani, (2009) and Warnholtz *et al.,* (2011). Under similar conditions, aortic rings from the three different treated groups with apigenin 7-*O*-*β*-D-glucoside, were tested for endothelium integrity. Then CRCs were compared and analyzed.

2.10 Toxicity study

Different groups of mice (25-30 g body weight) with 5 in each group, were treated with different doses (1, 3, 5, 10 and 20 mg/kg/p.o) of apigenin 7-*O*-*β*-D-glucoside, compared to normal saline group. The mice were allowed free access to food, and water for 24 hr, and kept under consistent observation for toxic effects such as anorexia, diarrhoea, gastrointestinal spasms, lethargy, and mortality (Gilani *et al.*, 2005).

3. Results

3.1 Antihyperlipidemic activities

3.1.1 Body weight

HFD fed for 4 weeks caused a significant (p <0.001) increase in body weight in the hyperlipidemic control group compared to normal SD rats. The treatment of hyperlipidemic rats with apigenin-7-*O*-*β*-D glucoside (mg/kg) 3 and 5 caused an approximate 60% and 81% decrease in body weight, respectively, compared to 40% reduction with atorvastatine (Fig. 1).

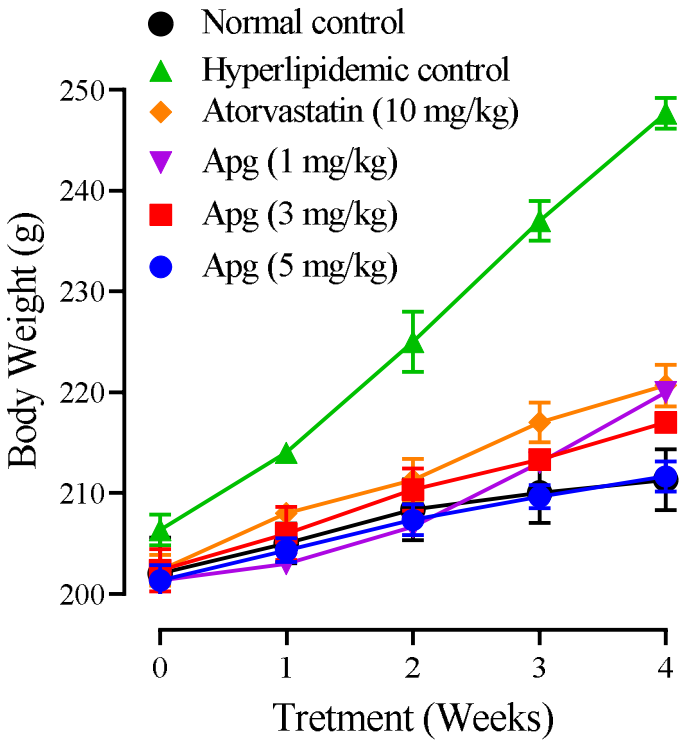
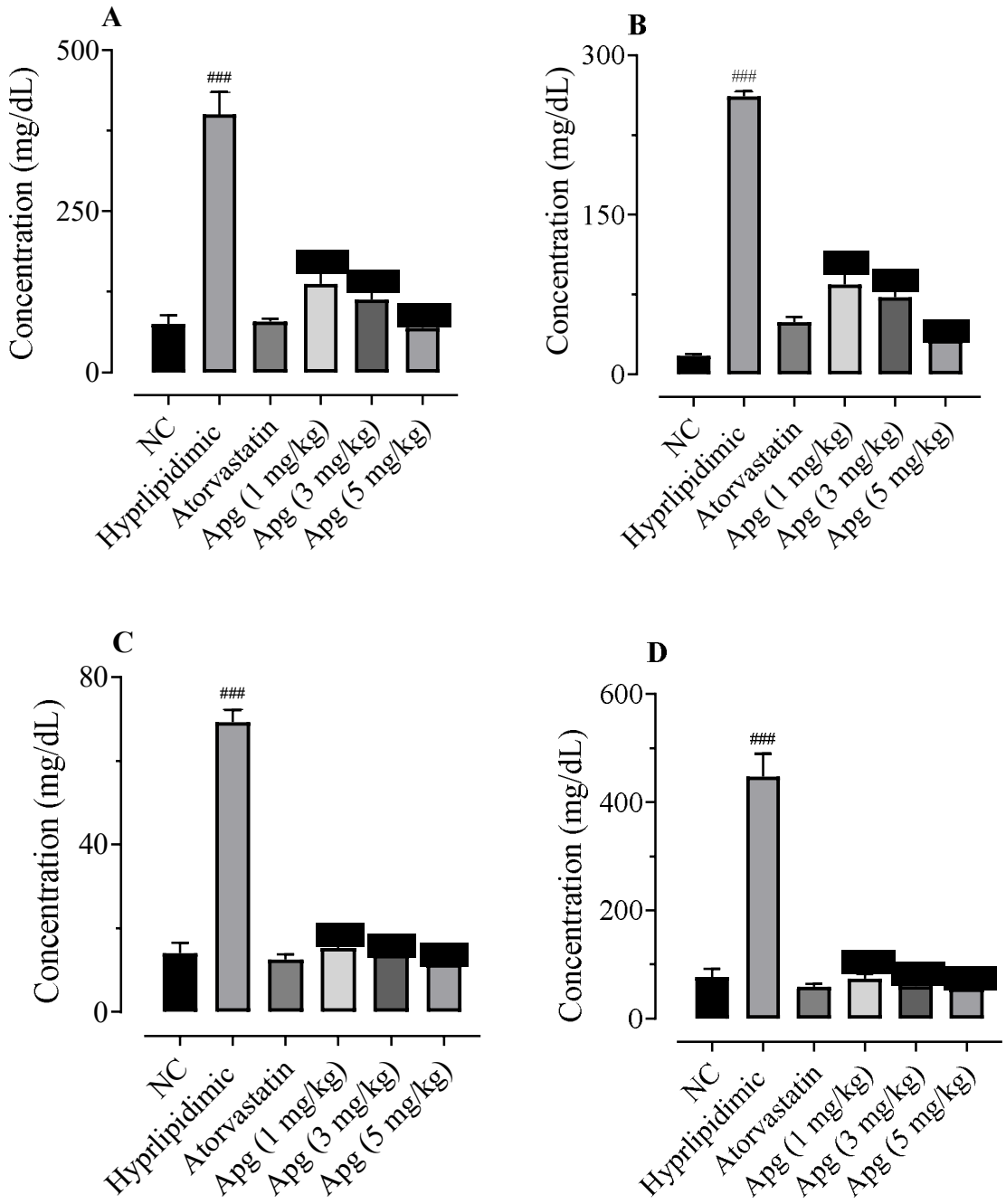


Figure 1: Shows increase in the body weight treated for four weeks with apigenin-7-*O*-*β*-D glucoside (Apg) in HFD hyperlipidemic rats, compared with normal, hyperlipidemic and atorvastatin group. Value shows (mean±SEM, n=3-5).

3.1.2 Serum lipid profile in HFD-induced hyperlipidemic SD rats

Different lipids such as TC, LDL VLDL, TG and HDL were measured in HFD feeding normal control, treated with of apigenin-7-*O*-*β*-D glucoside (1, 3 and 5 mg/kg) and atorvastatin (10

mg/kg) groups. Lipid profile and atherogenic index were calculated, as shown in Fig.2 (A, B, C, D, E and F) and Table.1.



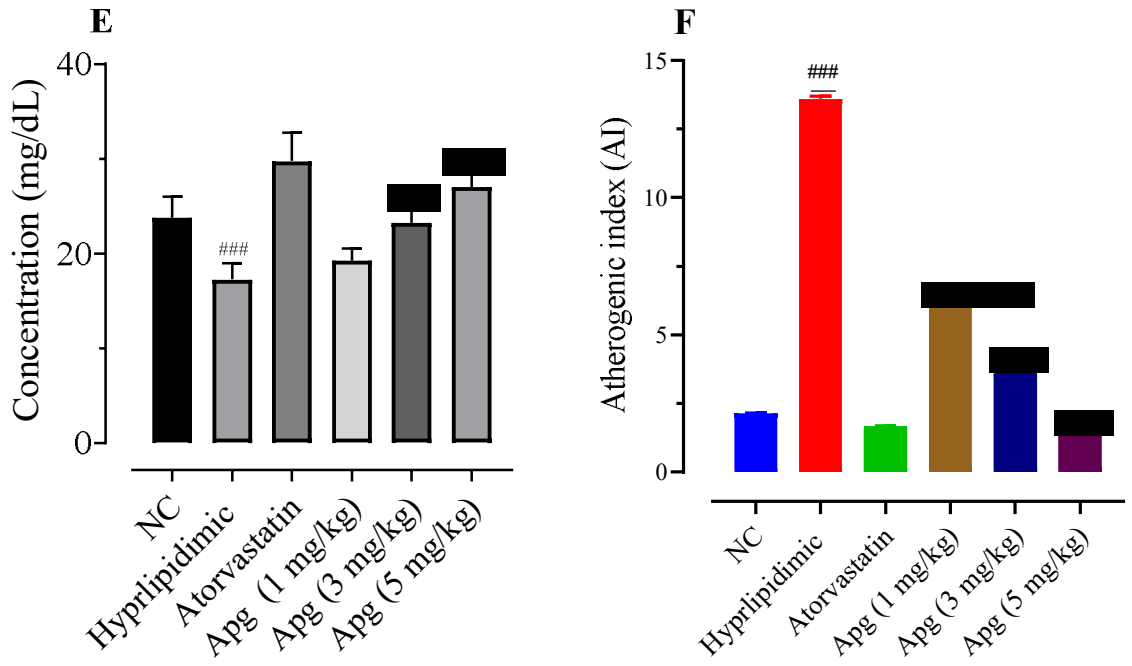


Figure 2: shows inhibitory effect of apigenin-7-*O*-*β*-D glucoside and atorvastatine on (A) TC: Total cholesterol (B) LDL: Low-density lipoprotein (C) VLDL: Very Low-density lipoprotein (D) TG: Triglyceride, (E) (HDL): High-density lipoprotein. (F). AI: Atherogenic index in HFD induced hyperlipidemia in SD rats.Values are expressed as mean±SEM (n=3-5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs hyperlipidemic (HFD-induced) values (One way ANOVA analysis followed by Dunnet's multiple comparisons. NC: Normal control: (Apg) apigenin-7-*O*-*β*-D glucoside. (###) compared with this group.

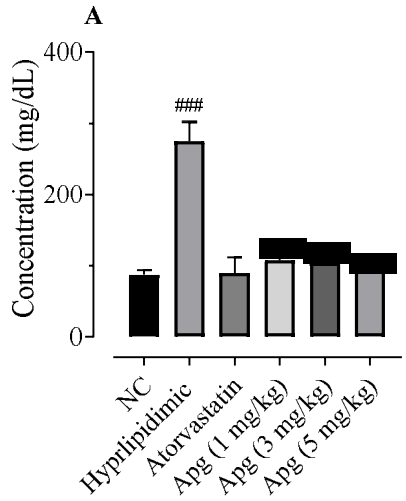
Table 1: Effect of apigenin-7-*O*-*β*-D glucoside at different doses (1, 3 and 5 mg/kg) and atorvastatin (10 mg/kg) on serum lipid profile in HFD-induced hyperlipidemic rats and atherogenic index. Values are expressed as mean±SEM (n=3-5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs HFD-induced hyperlipidemic values

Lipid profile HFD-induced hyperlipidemic(mg/dL) and atherogenic index							
S/N	**Groups**	**TC**	**LDL**	**VLDL**	**TG**	**HDL**	**AI**
1	Normal Ref Range	100.00–150.00	38.00–64.00	19.00–34.00	96.00–174.00	21.00–54.00	1.00
1	Normal Control	74.75±4.24	17.50±1.50	14.00±2.2	76.50±3.50	23.70±1.90	2.15±0.02
2	Hyperlipidemic	399.75±3.80	261.25±4.20	69.20 ± 2.40	447.50±37.20	17.50 ±1.10	13.60±0.30
3	Atorvastatin	79±4.00	48.95±4.50	11.30±1.40	58.50 ± 5.00	29.70±2.50	1.67±0.07
4	Apg (1 mg/kg)	137.20±4.10***	84.50±7.20***	15.27±1.50***	73.75±7.80***	19.25±1.25	6.12±0.12**
5	Apg (3 mg/kg)	113.00±3.87***	72.250±6.10***	13.70±1.00***	57.75±6.70***	23.25±1.70**	3.86±0.04***
6	Apg (5 mg/kg)	69.25±2.50***	32.00 ± 2.40***	11.50±1.33***	55.55±5.50***	27.00±1.82**	1.56±0.03***

S/n= serial no TC; Total cholesterol, TG; Triglyceride, HDL; High-density lipoproteins. LDL; Low-density lipoproteins VLDL; Very low-density lipoproteins AI; Atherogenic index. Normal reference range are according to Ihedioha *et al.*, (2013).

3.1.3 Serum lipid profile in tyloxapol-induced hyperlipidemic SD rat

Different lipids such as TC, LDL VLDL, TG and HDL were measured in HFD feeding normal control, treated with of apigenin-7-*O*-*β*-D glucoside (1, 3 and 5 mg/kg) and atorvastatin (10 mg/kg) groups. Lipid profile were measured, Fig.3 (A, B, C, D and E) and Table.2.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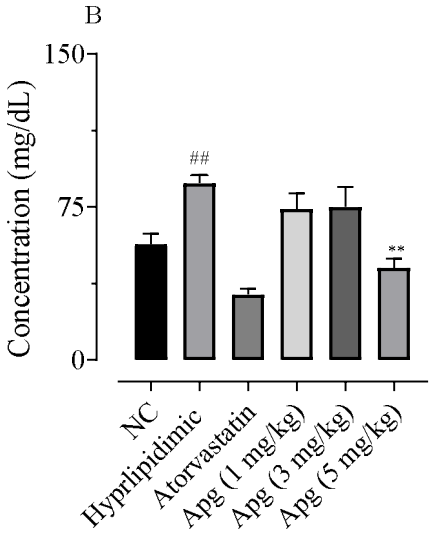
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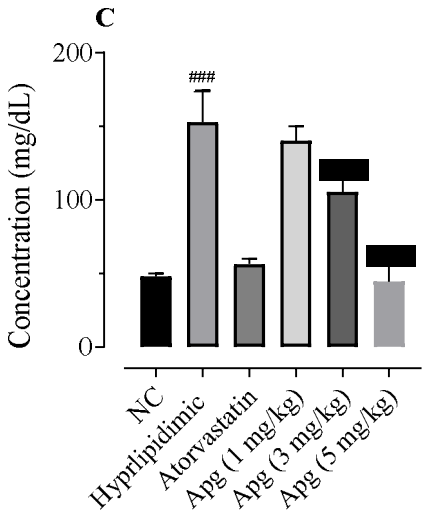
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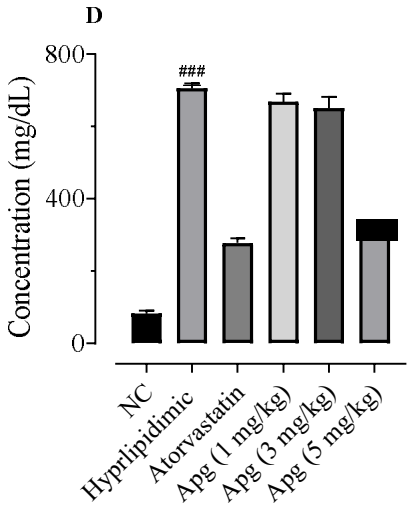
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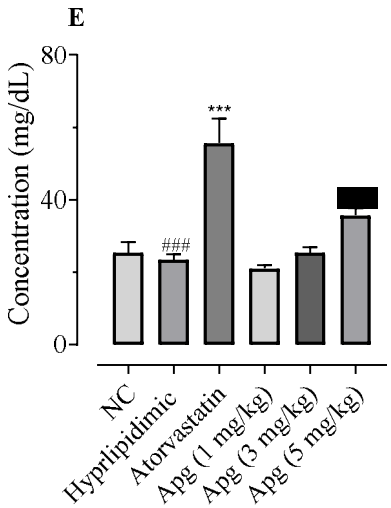
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Apg (3 mg/kg)
Apg (5 mg/kg)

Figure 3: Shows inhibitory effect of apigenin-7-*O*-*β*-D glucoside (Apg) and atorvastatine on Tyloxapol-induced, **(A)** TC: Total cholesterol **(B)** LDL: Low-density lipoprotein **(C)** VLDL Very Low-density lipoprotein **(D)** TG: Triglyceride **(E)** HDL: High-density lipoprotein in tylaoxapol-induced hyperlipidemia . Values are expressed as mean±SEM (n=3-5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs hyperlipidemic (Tylaoxapol - induced) values NC: Normal control and apigenin-7-*O*-*β*-D glucoside (Apg)

Table 2: Effect of apigenin-7-*O*-*β*-D glucoside at different doses (1, 3, and 5 mg/kg) and atorvastatin (10 mg/kg) on serum lipid profile in tyloxapol-induced (TI) hyperlipidemic Sprague-Dawley rats. Values are expressed as mean±SEM (n=3-5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs hyperlipidemic (Tylaxapol - induced) values

Lipid profile Tyloxapol-induced hyperlipidemic model (mg/dL)						
S/No	**Groups**	**TC**	**LDL**	**VLDL**	**TG**	**HDL**
1	Normal Ref Range	100.00–150.00	38.00–64.00	19.00–34.00	96.00–174.00	21.00–54.00
2	Normal control	87±5.00	56.60±4.10	48.00±1.60	82.30±6.60	25.30±2.40
3	Hyprlipidimic	274.30±11.80	86.60±3.30	152.66±7.90	705.60±9.60	27.30±1.60
4	Atorvastatin	89.30±18.00	32.00±2.40	56.30±2.80	114.60±5.70	52.30±1.20
5	Apg (1 mg/kg)	107.33±6.80***	79.60±7.80	131.66±3.05	668.33±11.50	21.00±1.10
6	Apg (3 mg/kg)	103.66±5.13***	71.00±7.50	105.33±3.50***	650.66±9.00	25.30±1.50
7	Apg (5 mg/kg)	94.60±3.60***	45.00±4.50**	44.33 ±7.50***	301.30±6.02***	35.60±2.00**

TC; Total cholesterol, TG; Triglyceride, HDL; High-density lipoproteins. LDL; Low-density lipoproteins VLDL; Very low-density lipoproteins. Normal reference range are according to Ihedioha *et al.*, (2013).

3.2 Effect on HMG-CoA reductase in tyloxapol-induced hyperlipidemic model

An assay of HMG-CoA reductase was performed which shows the absorbance of HMG-CoA/mevalonate as compared with the tyloxapol-induced hyperlipidemic model control group shows that apigenin-7-*O*-*β*-D glucoside 5 mg/kg treated group increase the ratio as compared to normal and atorvastatin 10 mg/kg group (Table.3).

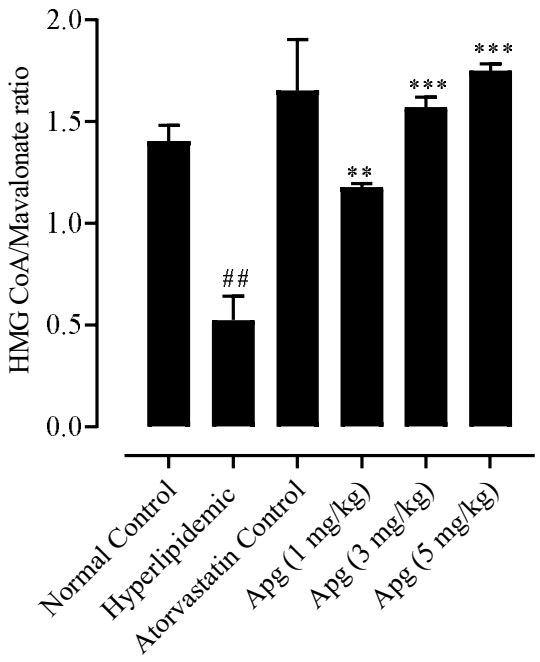

Figure 4: Bar graph shows the effect of the different doses apigenin-7-*O*-*β*-D glucoside in HMG-CoA reductase activity on the tyloxapol-induced hyperlipidemic rats, compared to normal control and atorvastatin (10 mg/kg) treated groups. Significance value represents *p<0.05, **p<0.01 and***p<0.001. (n=3-5)

3.2.1 HMG-CoA reductase activity in High Fat Diet model of hyperlipidemia

An assay of HMG-CoA reductase was performed which shows the absorbance of HMG-CoA/mevalonate as compared with the HFD control group shows that apigenin-7-*O*-*β*-D glucoside 5 mg/kg treated group increase the ratio as compared to normal and atorvastatin 10 mg/kg group (Table.3).

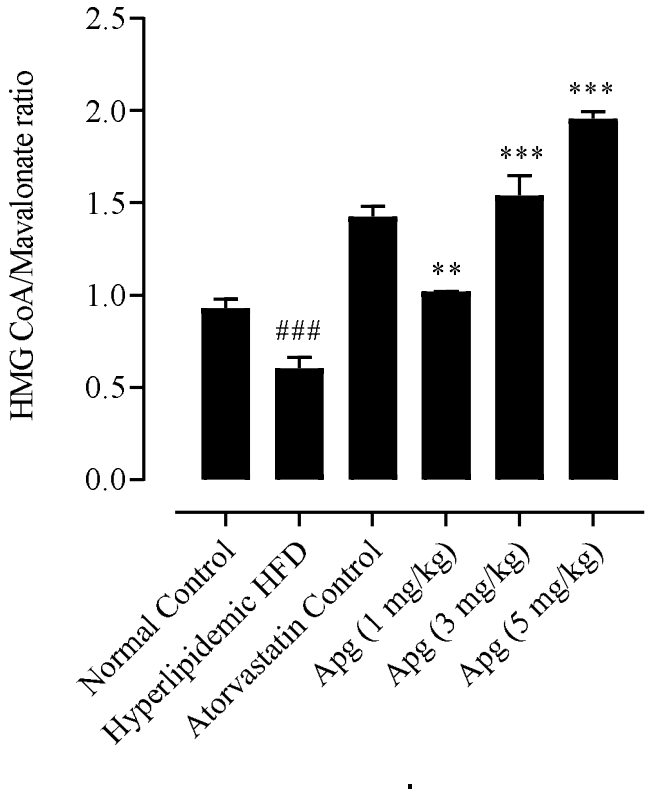

Figure 5: Bar graph shows the effect of the different doses apigenin-7-*O*-*β*-D glucoside in HMG-CoA reductase activity on the HFD-induced hyperlipidemic rats, compared to normal control and atorvastatin (10 mg/kg) treated groups. Significance value represents *p<0.05, **p<0.01 and***p<0.001. (n=3-5)

Table 3: HMG-CoA reductase activity in tyloxapol and HFD-induced hyperlipidemia in rats compared to normal control and atorvastatin (10 mg/kg) treated groups. Data was expressed with standard error of means (mean±SEM) Significance value represents *p<0.05, **p<0.01 and***p<0.001.

Groups	Tyloxapol-induced	HFDs induced
	HMG CoA/Mevalonate ratio	
Normal Control	1.4046±0.08	0.9603±0.008
Hyperlipidemic	0.5242±0.11	0.6312±0.0548
Atorvastatin (10 mg/kg)	1.6535±0.249	1.457912±0.056
Apg (1 mg/kg)	1.17781±0.014 **	1.019143±0.0019 **
Apg (3 mg/kg)	1.57113±0.040 **	1.540772±0.086 ***
Apg (5 mg/kg)	1.7489± 0.0292***	1.95807±0.030***

Apg: Apigenin-7-*O*-*β*-D glucoside

3.3 Effect on histopathological changes in aorta from HFD hyperlipidemic SD rats

Histopathological studies of the aorta of normal control rats showed the normal structure of three tunics of the aorta; tunica intima, tunica media, and tunica adventitia. In the normal control groups, the endothelium was smooth and regular round tunica intima, tunica media with smooth muscle cells and elastic lamina and tunica adventitia regular connective tissues, (Fig. 6A). The aorta from the HFD-induced hyperlipidemic group showed focal discontinuity of tunica intima with infiltration of macrophages, vacuole formation in tunica media associated with a large number of foam cells formation and increase in the matrix (Fig. 6B), while atorvastatin treated rats showed less damage of endothelium and reduced spaces between tunica media and the tunica intima with no macrophages infiltration and reduced lipid deposits (Fig. 6F). Apigenin-7-*O*-*β*-D glucoside (1 mg/kg) orally treated rats showed disruption of the endothelium in the tunica intima and

proliferation of smooth muscle cells in the tunica media while lipid accumulation was reduced (Fig. 6C). Aorta from hyperlipidemic rats treated with apigenin-7-*O*-*β*-D glucoside (3 mg/kg) showed mild disruption of the endothelium in the tunica intima and improvement in a proliferation of smooth muscle cells with reduced lipid accumulation (Fig.6D). Similarly, a photomicrograph of the aorta from hyperlipidemic rats treated with apigenin-7-*O*-*β*-D glucoside (5 mg/kg) showed almost regular morphology of aortic intima, media, and adventitia (Fig. 6E).

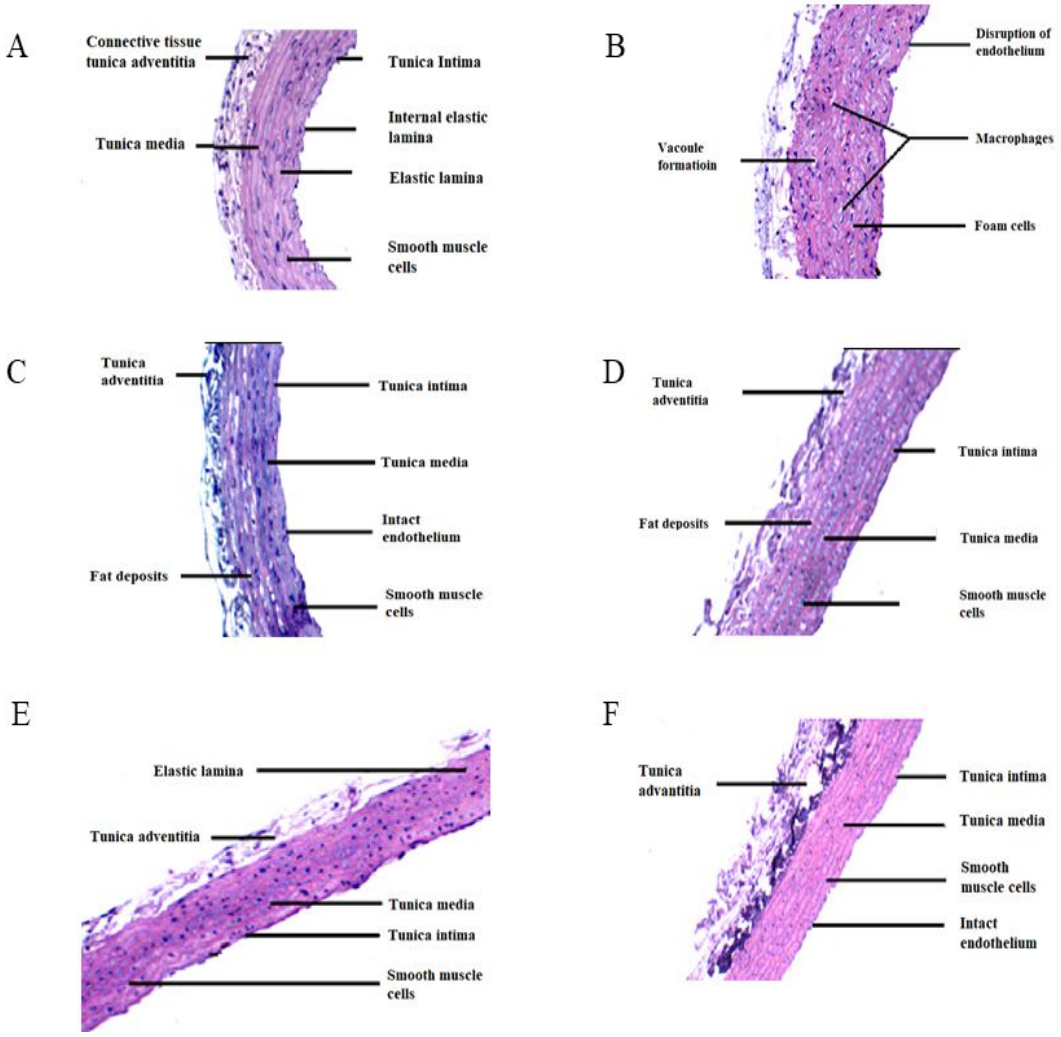

Figure 6: Histopathological slides shows morphology of **(A)** Hyperlipidemic (HFD) **(B)** Normal and effect of **(C)** 1 mg/kg (**D**) 3 mg/kg (**E**) 5 mg/kg apigenin-7-*O*-*β*-D glucoside (**F**) Atorvastatin (10 mg/kg) treated SD rats

3.4 Vascular dysfunction study in HFD hyperlipidemic SD rats

Isolated rat aorta of the normal control group pre-contracted with phenylephrine (1 µM) showed relaxation to acetylcholine with EC_{50} value of 0.08 µg/mL (0.03-0.06). While aortic preparations from HFD hyperlipidemic rats showed < 30% relaxant response to acetylcholine. Atorvastatin-treated rats showed 50% relaxation to acetylcholine with an EC_{50} value of 0.42 µg/mL (0.3-0.5). Aortic ring preparations from apigenin-7-*O*-*β*-D glucoside 1, 3, and 5 mg/kg (p.o.) treated rats showed significant relaxant response (50%, 75 %, and 100 %) with their respective EC_{50} values of 0.40 (0.3-0.5), 0.48 (0.3-0.6), and 0.01 (0.1-0.2) as shown Fig. 7.

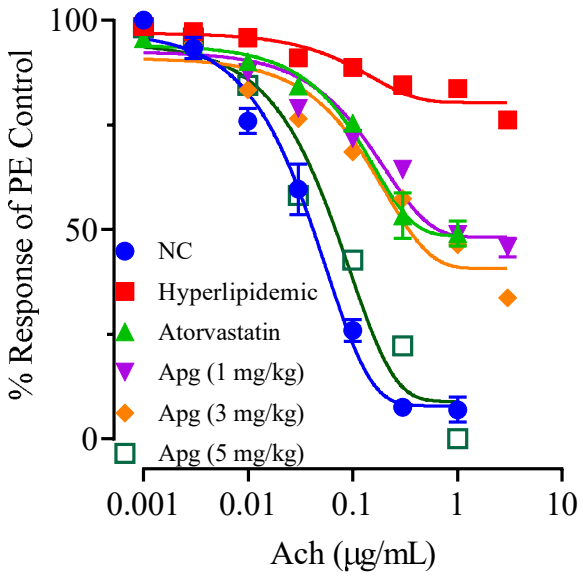

Figure 7: Acetylcholine response on phenylephrine (PE; 1 µM)-induced contraction in isolated rat aortic rings from normal control (NC), HFD hyperlipidemic, atorvastatin 10mg/kg, and apigenin-7-*O*-*β*-D glucoside (Apg) treated groups. Value shown are (mean±SEM, n=3-5).

3.5 Acute oral toxicity study

Overnight fasted, healthy mice in 4 groups (n = 3) were administered orally 1, 5, 10, and 20 mg/kg of the apigenin 7-*O*-*β*-D glucoside and observed continuously for 4 hr and 24 hr for any abnormality and mortality. No visible abnormality and or mortality was found.

4. Discussion

Apigenin 7-*O*-*β*-D-glucoside was orally administered to HFD-induced hyperlipidemic rats for 28. This treatment reduced TC, LDL, VLDL and TG while increased HDL level. HFD causes metabolic problems in rats like human metabolic abnormalities. HFD consists of cholesterol, cholic acid and butter. Cholic acid promotes absorption of cholesterol and butter in the intestine. Literature showed that long-term HFD feeding significantly increase the levels of TC, LDL, VLDL and TG compared to the control group, while HDL decreases (Li *et al.*, 2018). Hyperlipidemia induced by HFD results in oxidative stress which leads to the ultimate production of reactive oxygen species (ROS). literature provides evidence that overproduction of ROS causes cellular damage via oxidation of critical cellular components, such as membrane lipids, and proteins (Jain *et al.*, 2010)

In our study HFD-induced hyperlipidemia, there was significant increase in the levels of serum TC, LDL, VLDL and TG and a decrees in the level of HDL, compared to normal rats. Our results indicated that all doses (1, 3 and 5 mg/kg) of apigenin-7-*O*-*β*-D glucoside have decreased TC, TG, LDL, and VLDL levels, while increase the level of HDL. These finding suggested that improvement in the lipids level might be due to the effect of apigenin-7-*O*-*β*-D glucoside on the key enzyme also involved in the biosynthesis of these lipids. HMG-CoA is the key enzyme for the synthesis of lipids in the body. Therefor a model was required of hyperlipidemia solely related to HMG-CoA reductase. Tyloxapol is nonionic detergent, (an oxyethylated tertiary octyl phenol formaldehyde polymer), is used by several studies to induce hypercholesterolemia in animals (Harnafi *et al.*, 2008). It causes a significant increase in hepatic cholesterol biosynthesis by stimulating the activity of HMG-CoA reductase (Janicki *et al.*, 1962; Bertges *et al.*, 2011). The treated normal SD rats with tyloxapol of (500mg/kg) for one day as a simple prior treated with different doses of apigenin-7-*O*-*β*-D glucoside. Our results indicated that all doses (1, 3 and 5 mg/kg) of apigenin-7-*O*-*β*-D glucoside decreased TC, LDL, VLDL and TG levels, while increased the level of HDL further confirm that the lipid lowering effect of apigenin-7-*O*-*β*-D glucoside is mediated through inhibition of HMG-CoA *in-vivo*. This hypothesis was further confirmed through *in vitro*. Liver from the apigenin-7-*O*-*β*-D glucoside treated groups were investigated and an assay was performed to see the effect on the key enzyme in the biosynthesis of lipids. Ratio increased by apigenin-7-*O*-*β*-D glucoside showing inhibitory effect on lipid biosynthesis. Apigenin-7-*O*-*β*-D

glucoside significant increase in the ratio of HMG-CoA/mevalonate conforming the inhibition of HMG-CoA enzyme activity, at the dose of 5 mg/kg ($p < 0.001$), similar to atorvastatin.

Hyperlipidemia induced changes, the morphology of the blood vessels the aorta of HFD rats showed spaces within the tunica media and tunica intima. These spaces originally contained fat droplets (Fig .7 B) mainly in the endothelial layer. A typical symptom of atherosclerosis and endothelial dysfunction (Dobrian *et al.*, 2000). Additionally, there is evidence to support the idea that increase ROS production which is a significant contributor to hyperlipidemia vascular dysfunction. Increased ROS activity speeds up the inactivation of nitric oxide (NO), which reduces bioactive NO and increases vascular resistance (Shinozaki *et al.*, 1999; Yoshioka *et al.*, 2000). To have insight with the effect apigenin-7-*O*-*β*-D glucoside on the histological changes in blood vessels, aorta from all groups were investigated have found that apigenin-7-*O*-*β*-D glucoside reversed the histopathological changes to rats receiving HFD avoided the buildup of lipids in the aorta. The effect was more prominent at 5mg/kg dose compare to 10mg/kg atorvastatin, showed normal physiology in the aorta. (Fig .7 C, D, and E). However to evaluate effect of apigenin-7-*O*-*β*-D glucoside endothelial dysfunction, acetylcholine (Ach) was used to investigate the effects of HFD on endothelial function in isolated rat aortic rings. Isolated rat aorta of the normal control group, pre-contracted with phenylephrine (1μM), showed (100%) relaxation to Ach. In HFD hyperlipidemic rats, the maximum relaxation induced with 3 μg/mL of acetylcholine was < 30% compared to 100% in the normal control group. Literature shows that about 80% endothelium integrity is required for regulatory function. Apigenin-7-*O*-*β*-D glucoside (5 mg/kg) treated group showed complete (100%) relaxation to Ach. In 3 mg/kg treated rats, aortic rings showed 75% relaxation to Ach.

To investigate the safety of the compound, an acute toxicity study was performed in mice, we observed that the apigenin-7-*O*-*β*-D glucoside was found to be safe up to 20 mg/kg dose. No mortality and morbidity was observed.

Conclusion

Findings on the pharmacological investigation of apigenin-7-*O*-*β*-D glucoside showed anti-hyperlipidemic effect, mediated through inhibition of HMG-CoA reductase and reversed the vascular dysfunction also improved the histopathological changes associated with it. This were very interesting and exiting finding on the reversal of vascular dysfunction of apigenin-7-*O*-*β*-D

glucoside in the hyperlipidemic rats and suggesting apigenin-7-*O*-*β*-D glucoside as a potential and antihyperlipedimic agent.

References

Ali, F., Rahul, Naz, F., Jyoti, S., & Siddique, Y. H. (2017). Health functionality of apigenin: A review. *International Journal of Food Properties, 20*(6), 1197-1238.

Belguith-Hadriche, O., Ammar, S., Contreras, M. d. M., Turki, M., Segura-Carretero, A., El Feki, A.,Bouaziz, M. (2016). Antihyperlipidemic and antioxidant activities of edible Tunisian Ficus carica L. fruits in high fat diet-induced hyperlipidemic rats. *Plant Foods for Human Nutrition, 71*, 183-189.

Bertges, L. C., Mourão Jr, C. A., Souza, J. B., & Cardoso, V. A. C. (2011). Hyperlipidemia induced by Triton WR1339 (Tyloxapol) in Wistar rats. *Braz J Med Sci Health, 1*, 32-34.

Bolzon, L. B., dos Santos, J. S., Silva, D. B., Crevelin, E. J., Moraes, L. A., Lopes, N. P., & Assis, M. D. (2017). Apigenin-7-O-glucoside oxidation catalyzed by P450-bioinspired systems. *J. Inorg. Biochem., 170*, 117-124.

Buettner, R., Parhofer, K., Woenckhaus, M., Wrede, C., Kunz-Schughart, L., Scholmerich, J., & Bollheimer, L. (2006). Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *Journal of Molecular Endocrinology, 36*(3), 485-501.

Ciumărnean, L., Milaciu, M. V., Runcan, O., Vesa, Ș. C., Răchișan, A. L., Negrean, V.,Para, I. (2020). The effects of flavonoids in cardiovascular diseases. *Molecules, 25*(18), 4320.

Díaz-Zagoya, J. C., Marín-Medina, A., Zetina-Esquivel, A. M., Blé-Castillo, J. L., Castell-Rodríguez, A. E., Juárez-Rojop, I. E., & Miranda-Zamora, R. (2021). Effects of high rosuvastatin doses on hepatocyte mitochondria of hypercholesterolemic mice. *Scientific Reports, 11*(1), 1-11.

Dobrian, A. D., Davies, M. J., Prewitt, R. L., & Lauterio, T. J. (2000). Development of hypertension in a rat model of diet-induced obesity. *Hypertension, 35*(4), 1009-1015.

Drexler, H., & Hornig, B. (1999). Endothelial dysfunction in human disease. *Journal of Molecular and Cellular Cardiology, 31*(1), 51-60.

Durrington, P. (2003). Dyslipidaemia. *The Lancet, 362*(9385), 717-731.

Felmeden, D. C., Spencer, C. G., Blann, A. D., Beevers, D. G., & Lip, G. Y. (2003). Low-density lipoprotein subfractions and cardiovascular risk in hypertension: relationship to endothelial dysfunction and effects of treatment. *Hypertension, 41*(3), 528-533.

Ferdowsian, H. R., & Beck, N. (2011). Ethical and scientific considerations regarding animal testing and research. *PloS one, 6*(9), e24059.

Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. J. C. c. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry, 18*(6), 499-502.

Gilani, A. H., Shah, A. J., Ghayur, M. N., & Majeed, K. (2005). Pharmacological basis for the use of turmeric in gastrointestinal and respiratory disorders. *Life sciences, 76*(26), 3089-3105.

Guzelmeric, E., Vovk, I., & Yesilada, E. (2015). Development and validation of an HPTLC method for apigenin 7-O-glucoside in chamomile flowers and its application for fingerprint discrimination of chamomile-like materials. *J. Pharm. Biomed., 107*, 108-118.

Harnafi, H., Caid, H. S., el Houda Bouanani, N., Aziz, M., & Amrani, S. (2008). Hypolipemic activity of polyphenol-rich extracts from Ocimum basilicum in Triton WR-1339-induced hyperlipidemic mice. *Food Chemistry, 108*(1), 205-212.

Haslam, D. E., Peloso, G. M., Herman, M. A., Dupuis, J., Lichtenstein, A. H., Smith, C. E., & McKeown, N. M. (2020). Beverage consumption and longitudinal changes in lipoprotein concentrations and incident dyslipidemia in US adults: the Framingham heart study. *J. Am. Heart Assoc., 9*(5), e014083.

Hill, M. F., & Bordoni, B. (2022). Hyperlipidemia. In *StatPearls [Internet]*: StatPearls Publishing.

Ihedioha, J. I., Noel-Uneke, O. A., & Ihedioha, T. E. (2013). Reference values for the serum lipid profile of albino rats (Rattus norvegicus) of varied ages and sexes. *Comparative Clinical Pathology, 22*, 93-99.

Iqbal, D., Khan, M. S., Khan, M. S., Ahmad, S., Hussain, M. S., & Ali, M. (2015). Bioactivity guided fractionation and hypolipidemic property of a novel HMG-CoA reductase inhibitor from Ficus virens Ait. *Lipids in health and disease, 14*, 1-15.

Jain, P. G., Patil, S. D., Haswani, N. G., Girase, M. V., & Surana, S. J. (2010). Hypolipidemic activity of Moringa oleifera Lam., Moringaceae, on high fat diet induced hyperlipidemia in albino rats. *Revista Brasileira de Farmacognosia, 20*, 969-973.

Janicki, B. W., & Aron, S. A. (1962). Effect of Triton WR-1339 on lipoproteins and lipoprotein lipase of Guinea pig plasma. *Proceedings of the Society for Experimental Biology and Medicine, 109*(3), 507-509.

Kumar, D., & Bhat, Z. A. (2014). Apigenin 7-glucoside from Stachys tibetica Vatke and its anxiolytic effect in rats. *Phytomedicine, 21*(7), 1010-1014.

Li, T.-T., Liu, Y.-Y., Wan, X.-Z., Huang, Z.-R., Liu, B., & Zhao, C. (2018). Regulatory efficacy of the polyunsaturated fatty acids from microalgae Spirulina platensis on lipid metabolism and gut microbiota in high-fat diet rats. *International Journal of Molecular Sciences, 19*(10), 3075.

Mohanta, S., Yin, C., Weber, C., Hu, D., & Habenicht, A. J. (2016). Aorta atherosclerosis lesion analysis in hyperlipidemic mice. *Bio-protocol, 6*(11).

Nakamura, N., Hamazaki, T., Ohta, M., Okuda, K., Urakaze, M., Sawazaki, S., Ishikura, Y. (1999). Joint effects of HMG-CoA reductase inhibitors and eicosapentaenoic acids on serum lipid profile and plasma fatty acid concentrations in patients with hyperlipidemia. *International Journal of Clinical and Laboratory Research,* 29, 22-25.

Patel, D., Shukla, S., & Gupta, S. (2007). Apigenin and cancer chemoprevention: progress, ppotential and promise. *International Journal of Oncology,* 30(1), 233-245.

Rao, A. V., & Ramakrishnan, S. (1975). Indirect assessment of hydroxymethylglutaryl-CoA reductase (NADPH) activity in liver tissue. *Clinical Chemistry, 21*(10), 1523-1525.

Samet, I., Villareal, M. O., Motojima, H., Han, J., Sayadi, S., & Isoda, H. (2015). Olive leaf components apigenin 7-glucoside and luteolin 7-glucoside direct human hematopoietic stem cell differentiation towards erythroid lineage. *Differentiation,* 89(5), 146-155.

Shinozaki, K., Kashiwagi, A., Nishio, Y., Okamura, T., Yoshida, Y., Masada, M., Kikkawa, R. (1999). Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O2-imbalance in insulin-resistant rat aorta. *Diabetes, 48*(12), 2437-2445.

Taylor, L. E., Gillis, E. E., Musall, J. B., Baban, B., & Sullivan, J. C. (2018). High-fat diet-induced hypertension is associated with a proinflammatory T cell profile in male and female Dahl salt-sensitive rats. *American Journal of Physiology-Heart and Circulatory Physiology, 315*(6), H1713-H1723.

Vargo, M. A., Voss, O. H., Poustka, F., Cardounel, A. J., Grotewold, E., & Doseff, A. I. (2006). Apigenin-induced-apoptosis is mediated by the activation of PKCδ and caspases in leukemia cells. *Biochem. Pharmacol., 72*(6), 681-692.

Virchow, R. (1856). Phlogose und thrombose in gefassystem. *Gesammelte Abhandlungen Zur Wissenschaftlichen Medicin*, 458-463.

Warnholtz, A., Mollnau, H., Oelze, M., Wendt, M., & Münzel, T. (2001). Antioxidants and endothelial dysfunction in hyperlipidemia. *Current Hypertension Report*, *3*, 53-60

Yoshioka, S., Uemura, K., Tamaya, N., Tamagawa, T., Miura, H., Iguchi, A., Hotta, N. (2000). Dietary fat-induced increase in blood pressure and insulin resistance in rats. *Journal of hypertension, 18*(12), 1857-1864.

Zeka, K., Ruparelia, K., Arroo, R. R., Budriesi, R., & Micucci, M. (2017). Flavonoids and their metabolites: Prevention in cardiovascular diseases and diabetes. *Diseases, 5*(3), 19.

Zhang, K., Song, W., Li, D., & Jin, X. (2017). Apigenin in the regulation of cholesterol metabolism and protection of blood vessels. *Exp. Ther. Med., 13*(5), 1719-1724.