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Research Article

Phytochemical analysis and evaluation of anti-hyperlipidaemic effect for ethanolic leaf extract of *Equisetum ramosissimum* L.: in vivo study on rats' models

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Abstract

This study aims to investigate the anti-hyperlipidaemic effect of ethanolic leaf extract of *Equisetum ramosissimum*. 2,2-Diphenyl-1-picrylhydrazyl assay for antioxidants, Folin-Ciocalteu, $AlCl_3$, and UHPLC-MS/MS analysis, focusing on phenols and flavonoid content were performed. Anti-hyperlipidemic effect of the extract on lipid profile and body weight was evaluated alone or in combination with Atorvastatin in rats. The extract was shown to contain phenols (0.032±0.001 µg/g, equivalent to gallic acid), flavonoids (0.044±0.003 mg/g, equivalent to quercetin), and antioxidant IC_{50} value of (1000.00±0.78 µg/mL). UHPLC-MS/MS analysis revealed the presence of 8 different phenols and flavonoids. An in vivo study on healthy standard diet-fed animals and an induced hyperlipidaemic model showed a significant ($P < 0.05$) reducing effect of the extract alone and in combination with Atorvastatin on serum lipid profile. These findings revealed the potential advantages of the extract alone and in combination with statins for preventing or treating hyperlipidaemia, but need to be further explored.

Keywords

Phenols, flavonoids, anti-hyperlipidemic, *Equisetum ramosissimum*, high-fat fed rats

Introduction

Hyperlipidaemia is a condition that describes elevated lipid levels in the blood, which can be caused by a variety of genetic or acquired disorders. In adults, hyperlipidaemia has been shown to be a major risk factor for developing cardiovascular diseases (Stewart et al. 2020). Diabetic dyslipidemia is a typical complication of diabetes. Both type 1 and type 2 diabetes have a correlation between blood cholesterol, triglyceride levels, and atherosclerotic cardiovascular disease. Importantly, there is a piece of solid and clear evidence that people with and without diabetes, who receive cholesterol-lowering treatment have a considerably lower risk of cardiovascular diseases (Schofield et al. 2016).

A recent study performed on diabetic rats utilizing extracts of *E. arvense* L., with a high content of phenolic compounds and flavonoids, which exhibit antioxidant effects, suggested their use as an assistant treatment for diabetes mellitus due to their beneficial impact on insulin resistance and blood glucose level, with a potential effect on reducing diabetic cardiomyopathy, and hence decreasing morbidity risk caused by diabetes (Hegedűs et al. 2020). In another study performed on alloxan-induced diabetic rabbits investigating *E. giganteum* L. extract which contains various flavonoid-derived compounds (kaempferol), results showed significant antidiabetic and antilipidemic effects (Vieira et al. 2020).

According to a recent study on *E. ramosissimum*, the aqueous methanol extracts were shown to have the highest total phenolic content, in addition to containing flavonoids, tannins, alkaloids, and saponins which contribute to the antioxidant, anti-tyrosinase, as well as potent antimicrobial impacts against *Propionibacterium acne* (Savaya et al. 2020). It was also shown to have an effect as haemostatic, diuretics, antifungal, antiviral, antibacterial, antioxidant, and anticancer (Lall 2019; Boeing et al. 2021). One study analyzed the phenolic and antioxidant compounds in the stem extracts of *E. ramosissimum* using GC-MS analysis and reported the presence of several compounds including linoleic acid, palmitic acid, nonacosane, hexahydrofarnesyl acetone, and octacosane as major compounds (Sureshkumar, J., Amalraj, S., Murugan, R. et al. 2021). In addition, the species *E. ramosissimum* has previously shown a very potent anti-diabetic effect (unpublished data), therefore we hypothesize that this species would also exert a hypolipidemic effect if tested on an animal model with induced hyperlipidaemia.

To our knowledge, no previous studies have been designed to link the use of *E. ramosissimum* extract and its impact on lipid profile and on non-high-density lipoprotein (non-HDL). Therefore, the present study aims to screen the phytochemical metabolites present in the alcoholic leaves extract of *E. ramosissimum* and to evaluate the *in vivo* hypolipidemic effect of the extract on hyperlipidaemia-induced rats. To achieve this, ethanolic extract of *E. ramosissimum* leaves was first prepared to explore the phenolic, flavonoid, and antioxidant content. Secondly, an experimental study was designed to evaluate the antihyperlipidaemic effect of the extract using *in vivo* hyperlipidaemia-induced rat models fed with a high-fat diet (HD).

Materials and methods

Standards and reagents

Atorvastatin (S) calcium trihydrate, Vastor 20 mg; Hikma pharmaceuticals company. Ethanol, chloroform, and n-hexane, analytical reagent; LABCHEM Laboratory chemicals, USA. Aluminum chloride 6-hydrate; Laboratory chemicals. Sodium carbonate anhydrous; SD Fine Chem limited (SDFCL), INDIA. 2,2-Di-phenyl-1-picrylhydrazyl, sodium nitrite, sodium hydroxide, gallic acid, ascorbic acid, and quercetin; Sigma – Aldrich, Germany. Folin-Ciocalteu reagent; Sigma-Aldrich, USA. Lipid profile kits including high density lipoproteins, triglycerides, and total cholesterol (HDL, TG, and TC), Glucose– liquicolor, and gel tubes; Human, Germany. $AlCl_3$, NaOH, $NaNO_2$, and Na2CO3; locally laboratory chemicals.

Plant material

The species *E. ramosissimum* used in this study were collected from Mujib reserve in Jordan (84 Km from Amman) and authenticated by Mr. Ibrahem Mahasneh, a professional taxonomist at the Nature Conservation Monitoring Centre, the Royal Society for the Conservation of Nature (RSCN). A voucher specimen is available at the RSCN herbarium (number: E.r-5/7/2017), as it is considered the official authority for plant identification in Jordan. Plant materials were dried under shade and stored at room temperature. When needed, the plant was crushed using a commercial blender to reduce the particle size.

Extract preparation

Briefly, 300 g of plant powder was soaked in 1L of ethanol for 24 hours, with constant agitation at room temperature. The mixture was then filtered, and the solvent was evaporated.

Determination of total phenolic content

Total phenol content was measured using the Folin-Ciocalteu method as described previously (Sadeghi et al. 2015). Briefly, 0.1 g of the plant extract was dissolved in 50 mL of methanol. Then, 2 mL of 10% Na2CO3 solution and 5 mL distilled water with 1 mL of Folin reagent was added to create the reaction's basic medium. The solution was left for 1 hour at room temperature to complete the reaction. The absorbance was recorded at 760 nm by spectrophotometry against the methanol blank solution. Gallic acid (GA) (Sigma Aldrich, Germany) was used as a standard phenolic compound to prepare the calibration curve. The total phenolic content (mg/ mL) was then determined as gallic acid equivalents.

Determination of total flavonoids content

The determination of total flavonoids content was performed using the colorimetric method, based on the formation of a complex flavonoid-aluminum as described previously (Pękal and Pyrzynska 2014). To prepare the reaction mixture, 0.1 g of plant extract was dissolved in 50 mL methanol and added to 1.5 mL of $AlCl_3$, 2 mL of NaOH (4%), 1 mL $NaNO_2$ (10%) and 5 mL distilled water, then completed to a final volume of 10 mL using 80% MeOH (v/v). The absorbance was read at 510 nm. All determinations were calculated using a calibration curve obtained with the quercetin standard (Sigma Aldrich, Germany). The results were expressed as mg/g equivalents to quercetin.

Determination of antioxidant activity using the 2,2-Diphenyl-1- picrylhydrazyl (DPPH) assay

The free radical scavenging activities of the extracts were determined using the 2, 2- Diphenyl- 1-picrylhydrazyl (DPPH) free radical scavenging method as described previously (Paulsamy et al. 2013). For the reaction reagent, 0.4 g of DPPH was dissolved in 100 mL methanol. The reaction was performed by mixing 0.1 g of plant extract with 10 mL methanol. 1 mL of the plant extract solution was mixed with 3 mL of DPPH and completed to a final volume of 10 mL using methanol, then allowed to stand in darkness for 30 minutes. The absorbance was recorded at 517 nm. The calibration curve was done using ascorbic acid (vitamin C) (Sigma Aldrich, Germany) as a standard. The percentage inhibition of DPPH by the plant extract and the IC_{50} were calculated using the following equation:

$$\text{Percentage inhibition}\,(\%) = \frac{A - B}{A} \times 100$$

where A (control) is the absorbance of pure DPPH in oxidized form, and B (sample) is the absorbance of the sample taken after 30 min of reaction with DPPH.

Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS)

The method used in this study was based on previously developed and validated method as described by Bajkacz et al. (2018). Briefly, a Bruker Daltonik Impact II ESI-Q-TOF System with a Bruker Dalotonik Elute UPLC system (Bremen, Germany) was used with a high-resolution Bruker TOF MS and accurate retention times were recorded.

For each analyte following chromatographic separation, including catechol, naringin, chlorogenic acid, gallic acid, caffeine, catechin, caffeic acid, isoferulic acid, kaempferol, kaempferol 3-O-glucoside, and others, identification was based on the exact match for the chromatograms peaks retention time and the mass spectrum and fragmentations fingerprints compared to the authentic samples or to the in house-built library previously established and validated at our laboratory.

The Ion Source Apollo II ion Funnel electrospray source was used to run this device. The dry temperature was 200 °C, the capillary voltage was 2500 V, the nebulizer gas flow was 8 L/min, and the nebulizer gas pressure was 2.0 bar. Elute UHPLC paired to a Bruker Impact II QTOFMS, supplied a TOF repetition rate of up to 20 kHz, a mass accuracy of 1 ppm, and a mass resolution of 50000 FSR (Full Sensitivity Resolution).

Chromatographic separation was performed using Bruker solo 2.0_C-18 UHPLC column (100 mm × 2.1 mm × 2.0 µm) at a flow rate of 0.51 mL/min and a column temperature of 40 °C. Solvents: (A) water with 0.05% formic acid and (B) acetonitrile Gradient: 0 – 27 min linear gradient from 5%–80% B; 27 – 29 min 95% B; 29.1 min 5% B, total analysis time was 35 min on positive and 35 min on negative mode injection volume 3 µl.

Stock solutions of the plant extract were prepared by dissolving the appropriate amount of substance in dimethyl sulfoxide-DMSO (analytical grade), then diluted with acetonitrile and used for identification of exact MS and retention time. All other reagents; acetonitrile, methanol, water, and formic acid used were of HPLC grade.

Study design

Animal groups:

Thirty-six male Wistar rats (185–220 g) were purchased from the animal house laboratories- Applied Science Private University-School of Pharmacy (Amman, Jordan). Rats were placed in metabolic cages, after being acclimated (21–22 °C and 12 h light/dark cycles) for at least one week before starting the experiments. Standard diet and drinking water were equally given for all study animals, and body weight (BW) was recorded weekly. Based on the diet model, animals were randomly divided into 2 divisions as follows:

A. Non-high fat diet (NHD); 2 groups:
   1. NHD: Male Wistar rats supplied with standard diet (30 g/rat/day) for 4 weeks.
   2. NHDP: Male Wistar rats supplied with standard diet (30 g/rat /day) for 2 weeks, followed by further 2 weeks oral treatment with extract of *E. ramosissimum* (200 mg/kg/day).

B. High fat diet-induced hyperlipidemia (HD); 4 groups:
   1. HD: Male Wistar rats supplied with standard diet (20 g/rat /day) + (30% fat lamb fat diet (10 g/rat/day)) for 4 weeks.
   2. HDP: Male Wistar rats supplied with standard diet (20 g/rat /day) + (30% fat lamb fat diet (10 g/rat/day)) for 2 weeks, followed by further 2 weeks oral treatment with extract of *E. ramosissimum* (200 mg/kg/day).
   3. HDS: Male Wistar rats supplied with standard diet (20 g/rat /day) + (30% fat lamb fat diet (10 g/rat/day)) for 2weeks, followed by further 2 weeks oral treatment with daily oral dose of 15 mg/kg of Atorvastatin.
   4. HDPS: Male Wistar rats supplied with standard diet (20 g/rat /day) + (30% fat lamb fat diet (10 g/rat/day)) for 2 weeks, followed by further 2 weeks oral treatment with combination of [*E. ramosissimum* extract (200 mg/kg/day) + 15 mg/kg of Atorvastatin.

Animal dose for Atorvastatin has initially been calculated based on the preliminary experiments, and as described by (Chen Y et al. 2018). Briefly, diluted suspension was prepared to a final concentration of (0.5 mg/ mL) using 20 mg Atorvastatin tablet suspended in 40 mL distilled

water. Atorvastatin suspension was administered by oral gavage at a dose of 15 mg/kg for 2 weeks. A large intermittent single dose (6 ml) by oral gavage was administrated to overcome the weak solubility of statin in distilled water as well as to avoid any possible reflux signs during the oral gavage procedures that were not observed.

Unfortunately, no data have been reported on the toxic effect of *E. ramosissimum*. Nevertheless, previous study by Baracho NC et al. (2009) revealed no significant changes in the hepatic enzymes were observed in Wistar rats who were administrated with the relevant species "*E. arvense*" at a dose of 100 mg/kg for 14 days.

Ethical approval

All procedures were performed in accordance with international regulations for the care and use of laboratory animals. Ethical approval on this study was obtained by the Institutional Review Board at Applied Science Private University, Amman, Jordan. Approval Number: 2021-PHA-40.

Induction of hyperlipidaemia using Male Wistar

The rats were fed lamb fat, which is high in cholesterol, mixed with their daily diet (rat chow) to develop hyperlipidaemia (Irudayaraj et al. 2013). HD-fed animals (4 groups) were subjected to an adjustment phase of 14 days in an extra diet containing 30% (10 g/rat/day) lamb fat. Day 15 was considered day 1 of the treatment, in addition to a high-fat diet. All oral treatments were administered via an intra-gastric tube. At the end of the treatment (day 28), rats were fasted overnight and sacrificed by cervical dislocation.

Treatments preparations

It is well known that statins have very slight solubility in water. Therefore, in accordance with the procedures used by (Balzan et al. 2013; Adejor et al. 2017) with some modifications, Atorvastatin 20 mg were used. One tablet was dissolved in 100 mL of distilled water to achieve a total concentration of 0.2 mg/ml, then administered to each rat orally once daily with a dose of 15 mg/kg rat body weight for a period of 2 weeks.

For the *E. ramosissimum* ethanolic leaves extract, the stock solution was freshly prepared by suspending the dry extract in distilled water. All doses were orally administered via an intra-gastric tube (1 mL/rat) equivalent to a dose of 200 mg dry plant extract/kg rat body weight, based on a previous similar study (Carneiro et al. 2013).

Lipid profile analysis

The serum lipid profile was assayed before the experiment, on day 1, day 15, and at the end (day 28) of the experiment. Blood samples (0.2 mL per animal) were collected into heparinized tubes by puncturing the retro-orbital plexus. The plasma was centrifuged at 2000 × gG, for 10 min. Hyperlipidemia model was confirmed by values of TC greater than (55 mg/dL). Rats that failed to develop the HD-induced hyperlipideamic model were excluded from the study. The TC, TG, and HDL-C levels were quantified using enzymatic kits. The LDL-C and non-HDL levels were calculated using the following equations (Virani 2011; Karkhaneh et al. 2019):

$$\text{LDL-C} = \text{TC}/1.19 + \text{TG}/1.9\text{-HDL}/1.1\text{-}38$$

$$\text{Non-HDL} = \text{TC-HDL}$$

Statistical analysis

The statistical analysis was performed using a Statistical Package for the Social Sciences (SPSS), version 27.0 for Windows (Chicago, IL, USA). The one-way ANOVA test was used to investigate if there are any significant differences in the mean values for each parameter between the different groups of the experiment. *Post hock* (Tukey test) was used to determine the significant differences among groups by analyzing multiple comparisons.

Results

Extraction yield, phenols, and flavonoids content

The extraction yield was calculated at 44.4% (w/w dry weight), for the ethanolic extract of *E. ramosissimum*.

The total phenolic content of *E. ramosissimum* was found to be (0.032± 0.001 mg/mg dry extract equivalent to gallic acid).

The total flavonoid content of *E. ramosissimum* was found to be (0.044±0.003 mg/mg dry extract equivalent to quercetin).

Antioxidant effect (DPPH Assay)

The plant extract DPPH radical scavenging activity was calculated as % inhibition (I%). Equation extrapolated from serial concentrations (µg/mL) verses %inhibition curve: $y = 31.338\ln(x) + 62.9$, $R^2 = 0.9735$ was used to calculate the IC_{50} , and used to determine the antioxidant activity for the ethanolic extract in comparison to ascorbic acid as shown in Table 1.

Table 1. DPPH scavenging effect expressed as IC_{50} (µg/mL) for the *E. ramosissimum* ethanol extract and ascorbic acid.

Radical scavenging agent	IC_{50} (µg/mL) ± SD
Ascorbic acid	14.5 ± 0.7
Ethanolic extract	1.0 ± 0.8

UHPLC – MS/MS

The qualitative analysis of phytocomponents was determined in *E. ramosissimum* for the ethanol extract using UHPLC-MS/MS (Table 2). The total ion chromatograms for the identified compounds are shown below (Figs 1, 2).

The UHPLC-MS/MS for the ethanol extract revealed the presence of 8 compounds, including catechin, caffeic acid, 3-hydroxy-4-methoxycinnamic acid (isoferulic acid), kaempferol-3-O-rutinoside, 2,4-dihydroxyacetophenone, 3-O-neohesperidoside kaempferol, kaempferol-3-O-glucoside and kaempferol. The obtained MS/MS spectra of the identified compounds in the *E. ramosissimum* extract are shown in Figs 3–10.

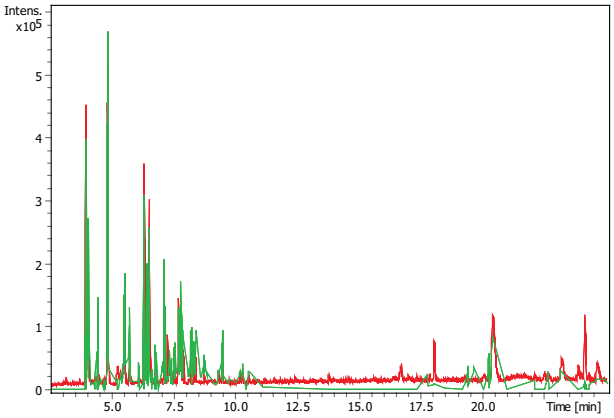
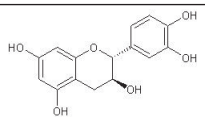
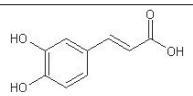
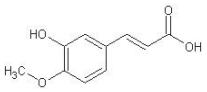
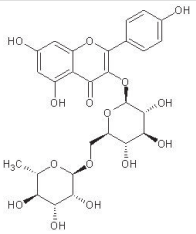
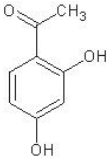
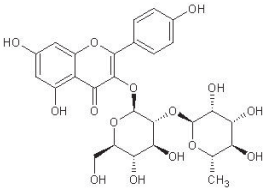
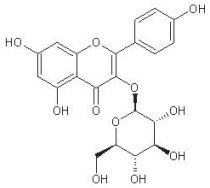
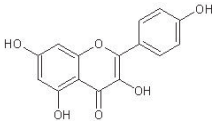


Figure 1. Total ion chromatogram for all compounds detected.

Table 2. Phytochemicals detected in *E. ramosissimum* ethanol extract using UHPLC/ MS-MS.

No.	RT [min]	[M-H]-	Exact mass	Name	Structure	Molecular Formula
1	2.87	289.07179	290.07907	catechin		$C_{15}H_{14}O_6$
2	3.31	179.03493	180.04221	caffeic acid		$C_9H_8O_4$
3	5.55	193.04987	194.05715	3-hydroxy-4-methoxycinnamic acid (isoferulic acid)		$C_{10}H_{10}O_4$
4	5.61	593.15051	594.15778	kaempferol-3-O-rutinoside		$C_{27}H_{30}O_{15}$
5	6.13	151.04018	152.04745	2,4-dihydroxyacetophenone		$C_8H_8O_3$
6	6.4	593.15037	594.15765	kaempferol 3-O-neohesperidoside		$C_{27}H_{30}O_{15}$
7	6.59	447.09298	448.10025	kaempferol-3-O-glucoside		$C_{21}H_{20}O_{11}$
8	10.11	285.03967	286.04695	kaempferol		$C_{15}H_{10}O_6$

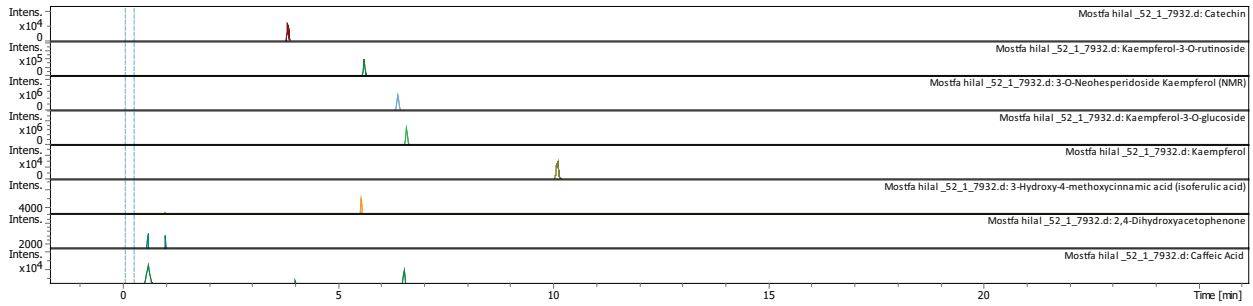


Figure 2. Total ion chromatograms of peaks corresponding to the compounds detected in the *E. ramosissimum* ethanol extract.

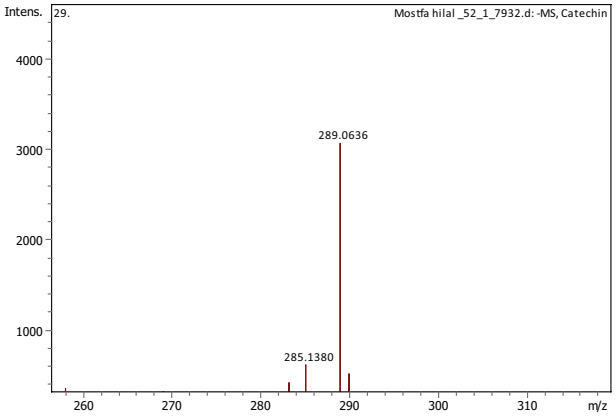


Figure 3. Mass spectrum of catechin.

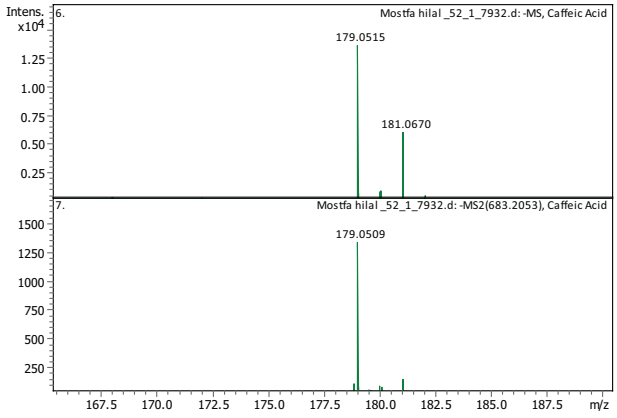


Figure 4. Mass spectrum of caffeic acid.

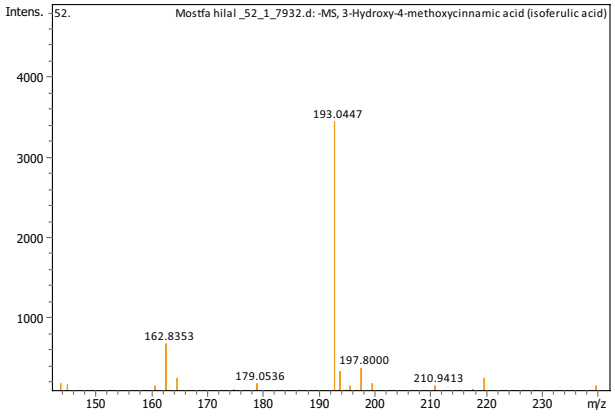


Figure 5. Mass spectrum of 3-Hydroxy-4-methoxycinnamic acid (isoferulic acid).

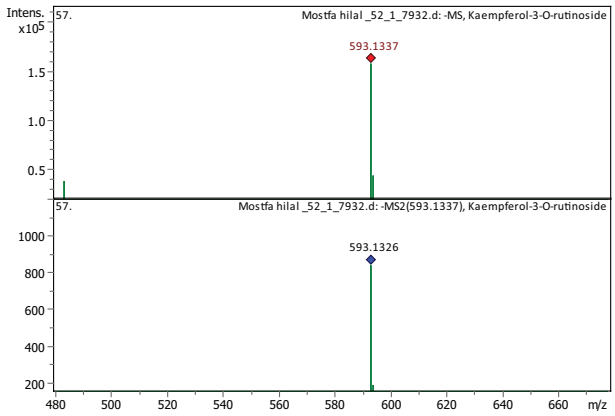


Figure 6. Mass spectrum of kaempferol-3-O-rutinoside.

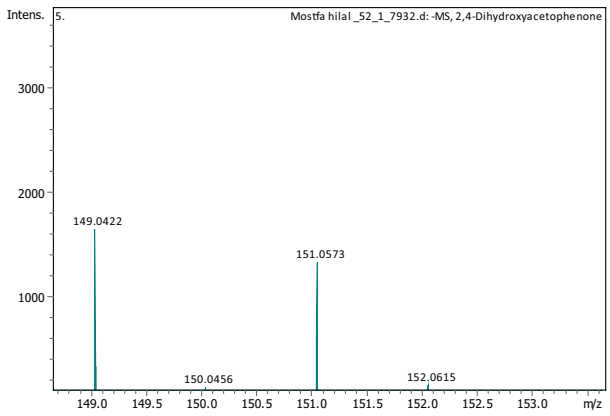


Figure 7. Mass spectrum of 2,4-dihydroxyacetophenone.

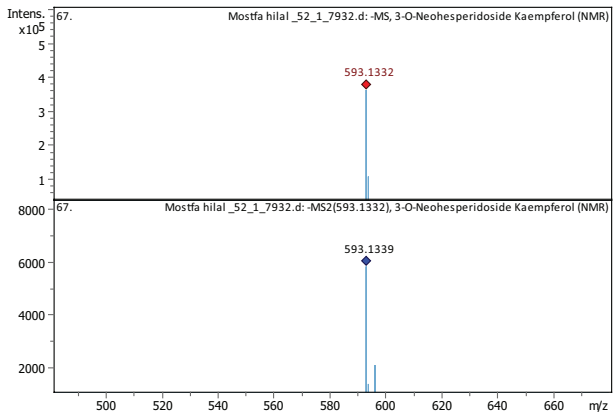


Figure 8. Mass spectrum of 3-O-neohesperidoside kaempferol.

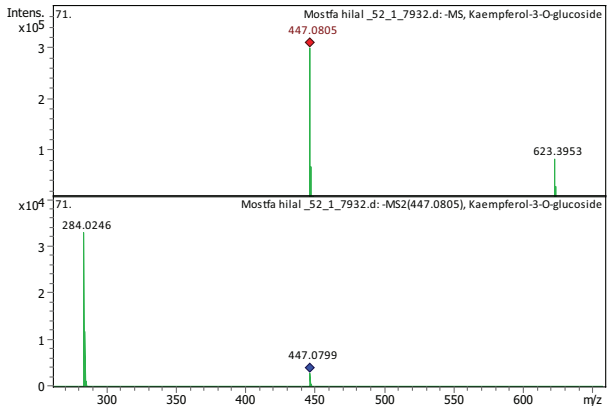


Figure 9. Mass spectrum of kaempferol-3-O-glucoside.

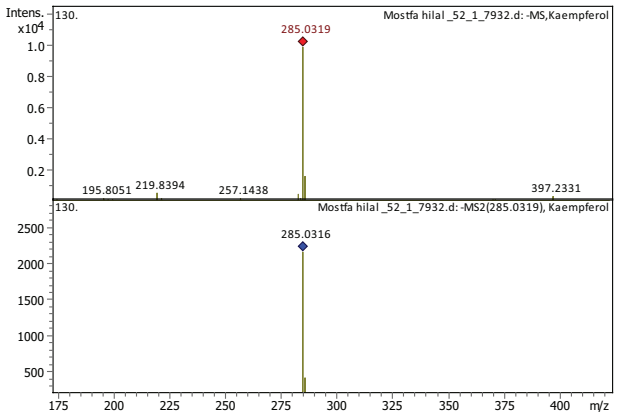


Figure 10. Mass spectrum of kaempferol.

Biological experimental part

Baseline characteristics of the study groups

The baseline characteristics of the HD and NHD received animals at day 14 are shown in Table 3. Generally, BW, levels of TG, HDL, LDL, non-HDL, and TC/HDL ratio in the HD study group were higher than in the NHD group, before starting treatment with plant extract or Atorvastatin.

Table 3. Baseline means and ranges of the BW and lipid profile parameters in NHD and HD study groups (day 14).

Groups	Parameter	Minimum (mg/dl)	Maximum (mg/dl)	Mean (mg/dl)	SD
NHD (n=12)	BW (g)	185.0	210.0	190.8	9.7
	TC	38.0	53.0	46.2	5.1
	TG	64.0	92.0	77.2	9.6
	HDL	20.7	28.3	23.9	2.8
	LDL	16.0	26.6	19.7	4.5
	Non-HDL	17.3	24.9	22.3	2.5
	TC/HDL	1.83	2.01	1.93	0.06
HD (n=24)	BW (g)	185.0	220.0	197.0	14.2
	TC	43.0	56.0	51.2	4.6
	TG	145.0	190.0	163.2	20.1
	HDL	22.5	36.8	28.1	4.9
	LDL	52.6	81.2	65.4	10.9
	Non-HDL	19.2	33.5	25.4	5.7
	TC/HDL	1.68	3.18	2.24	0.54

Abbreviations: NHD= non-high fat diet model in rats (control group); HD = high fat diet induced hyperlipidaemia model in rats. BW: Body weight; TC: total cholesterol; TG: triglycerides; HDL: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; non-HDL: non-high density lipoprotein cholesterol; TC/HDL: total cholesterol-to-high density lipoprotein cholesterol ratio.

Changes in mean BW, and lipid profile among the study groups after treatments (day 28) BW changes

At the termination day (day 28), among the standard diet received groups, a significant difference in the mean BW was observed between plant extract-treated animals (NHDP) and those in control animals (NHD) (228.3 ± 8.2 vs 190.8 ± 9.7, $P = 0.0164$). However, there were no significant differences in the final BWs between the HD groups ($P > 0.005$), with or without treatments (Table 4).

Table 4. The mean BW (g) for the NHD and HD study groups at the end of the experiment (day 28).

Body weight (BW) g					
Group (n=6)	**NHD**		**Group (n=6)**	**HD**	
	Mean	**± SD**		**Mean**	**±SD**
NHD	190.8	9.7	HD	238.33	24.20
NHDP	228.3	8.2	HDP	229.16	20.10
t	2.472		HDS	229.16	42.70
P^a	**0.0164**		HDPS	205.83	10.68
			F	1.5794	
			P^b	0.2256	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment.
Abbreviations: NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

TC changes

At the end of the experiment, there were significant differences in total cholesterol (TC) levels among the four HD groups (F=10.77, $P < 0.05$). Post-hoc multiple comparisons by the Tukey test indicated that the mean TC levels for (HDS) group were significantly different from the other three study groups (Table 5). In the NHD group, mean TC levels were significantly higher than in the NHDP group (F=2.85, $P < 0.05$).

TG changes

As shown in Table 6, there was a significant difference in the mean TG levels between the NHD groups (t=2.46, $P < 0.05$). The mean serum TG level was significantly lower in the NHDP rats than in controls (75.3 ± 7.8 vs 87 ± 7.1 mg/dl). In the HD group, there were significant differences between study groups (F=28.43, $P < 0.001$) at the end of the experiment. The post-hoc multiple comparisons by the Tukey test indicated that the mean TG levels for the control (HD) group were significantly higher than other groups (HDP, HDS, and HDPS). HDP animals showed similar results to their peers (HDS) treated with Atorvastatin (Table 6).

Table 5. The changes in mean TC levels (mg/dl) for NHD and HD study groups at the end of the experiment (day 28).

Total cholesterol (TC) mg/dl					
Group (n=6)	NHD		Group (n=6)	HD	
	Mean	± SD		Mean	± SD
NHD	46.3	18.8	HD	59.3	6.8
NHDP	37.7	5.2	HDP	41.7	4.7
t	2.853		HDS	41.0*1	7.4
P^a	**0.00856**		HDPS	44.3	6.5
			F	10.7716	
			P^b	**0.000199**	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment. *1 Tukey test significant compared to the control group.
Abbreviations: NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

Table 6. The changes in mean TG levels (mg/dl) for NHD and HD study groups at the end of the experiment (day 28).

Triglycerides (TG) mg/dl					
Group (n=6)	NHD		Group (n=6)	HD	
	Mean	± SD		Mean	± SD
NHD	87.0	7.1	HD	190.5*2	48.6
NHDP	75.3	7.8	HDP	81.7*1	8.7
t	2.4625		HDS	81.3*1	5.6
P^a	**0.0167**		HDPS	83.0*1	4.6
			F	28.43	
			P^b	**<0.001**	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment. *1 Tukey test significant compared to the control group. *2 Tukey test significant compared to the other groups.
Abbreviations: NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

Table 7. The changes in mean HDL levels (mg/dl) for NHD and HD study groups at the end of the experiment (day 28).

High Density Lipoprotein (HDL) mg/dl					
Group (n=6)	NHD		Group n=6	HD	
	Mean	± SD		Mean	± SD
NHD	22.9	2.1	HD	29.5*2	3.0
HDNP	27.9	6.4	HDP	22.0*1	4.0
t	–		HDS	23.8*1	4.5
P^a	0.085		HDPS	23.9*1	2.2
			F	4.9117	
			P^b	**0.0102**	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment. *1 Tukey test significant compared to the control group. *2 Tukey test significant compared to the other groups.
Abbreviations: NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

Table 8. The changes in mean LDL levels (mg/dl) for NHD and HD study groups at the end of the experiment (day 28).

Low Density Lipoprotein (LDL) mg/dl					
Group (n=6)	NHD		Group n=6	HD	
	Mean	± SD		Mean	± SD
NHD	25.9	6.2	HD	85.4*2	24.7
HDNP	7.95	2	HDP	19.96*1	7.50
t	8.571		HDS	16.76*1	4.60
P^a	<0.001		HDPS	20.97*1	4.60
			F	37.48	
			P^b	**<0.001**	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment. *1 Tukey test significant compared to the control group. *2 Tukey test significant compared to the other groups.
Abbreviations: NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of E. rasmosissium for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

HDL changes

There was no significant difference in the mean HDL levels between NHD and NHDP groups (t = -1.489, P = 0.0852). The post-hoc multiple comparisons by Tukey test indicated that the mean HDL levels for the control (HD) group were significantly higher than other groups (HDP, HDS, and HDPS) (Table 7).

LDL changes

As shown in Table 8, mean LDL levels were significantly lower in NHDP than in NHD animals (t = 8.571, P < 0.001). There were also significant differences in LDL levels among the four HD groups (F = 37.48, P < 0.001) at the end of the experiment. The mean LDL levels for the control (HD) group were significantly higher than other groups (HDP, HDS, and HDPS). No significant difference between HDP and HDS study groups was observed as presented.

Non-HDL changes

The plant extract-treated rats (NHDP) have shown a significant lowering effect in the mean serum levels of non-HDL compared with the control group (t = 4.641, P < 0.05) at the end of the experiment (Table 9). The mean levels of non-HDL for the HD group were significantly different from the other three groups (F=3.586, P < 0.05), with a higher mean value at the end of the experiment (Table 9).

Table 9. The changes in mean non-HDL levels (mg/dl) for NHD and HD study groups at the end of the experiment (day 28).

Non-High-Density Lipoprotein (non-HDL) mg/dl					
Group (n=6)	NHD		Group (n=6)	HD	
	Mean	± SD		Mean	± SD
NHD	23.4	4.8	HD	26.5*2	3.5
NHDP	11.5	3.1	HDP	19.6*1	7.7
t	4.6418		HDS	17.2*1	3.6
P^a	**0.0004**		HDPS	18.7*1	2.7
			F	3.5866	
			P^b	**0.0342**	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment. *1 Tukey test significant compared to the control group. *2 Tukey test significant compared to the other groups. **Abbreviations:** NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of E. rasmosissium for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

TC/HDL ratio changes

A significant difference in the mean levels of TC/HDL ratio at the end of the experiment between NHD and NHDP groups (t = 3.79, $P < 0.05$) was found. On the other hand, no significant differences in the mean levels of TC/HDL ratio at the end of the experiment were recorded between HD groups (Table 10).

Comparative changes in BW and serum lipid profile among the study groups

Fig. 11 shows comparative changes in BW (g), serum levels of TC, TG, HDL, LDL, non-HDL (mg/dl) and TC/HDL ratio among the study groups. For the normally fed rats, compare to the baseline measurements and control group (NHD), the NHDP group showed lower levels for non-HDL, LDL and TC, with an increase in BW and HDL level.

Table 10. The changes in TC/HDL ratios for NHD and HD study groups at the end of the experiment (day 28).

TC/HDL Ratio					
Group (n=6)	NHD		Group (n=6)	HD	
	Mean	± SD		Mean	± SD
NHD	2.04	0.28	HD	1.89	0.12
NHDP	1.45	0.19	HDP	1.95	0.44
t	3.791		HDS	1.75	0.06
P^a	**0.001**		HDPS	1.79	0.06
			F	0.8419	
			P^b	0.4869	

Notes: t: variation between sample means of NHD and NHDP study groups. P^a: value for independent t test the end of experiment. F: variation between sample means/variation within the samples, P^b: value for ANOVA test at the end of experiment.
Abbreviations: NHD: non-high fat diet model in rats (control group); NHDP: NHD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HD: high fat diet induced hyperlipidaemia model in rats; HDP: HD rats treated with daily oral dose of 200 mg/kg extract of *E. rasmosissium* for 2 weeks; HDS: HD rats treated with daily oral dose of 15 mg/kg of Atorvastatin for 2 weeks; HDPS: HD rats treated with daily oral dose of 200 mg/kg extracts of *E. rasmosissium* and 15 mg/kg of Atorvastatin for 2 weeks.

For the HD-groups, plant extract alone and in combination with Atorvastatin showed to lower TG and LDL levels compared to the baseline measurements and the not treated group (HD). These finding are interesting, particularly for hyperlipidemia prevention and treatment, with special emphasis when TG, LDL, and TC levels are elevated.

Discussion

In this *in vivo* study, statistical analysis for the results showed remarkable benefits on the lipid profile for animals with HD - induced hyperlipidaemia, receiving treatment of the combination of *E. ramosissimum* and Atorvastatin or the herbal medicine alone. These findings support that herbal use would contribute to improving lipid profile when used alone or in combination with statins.

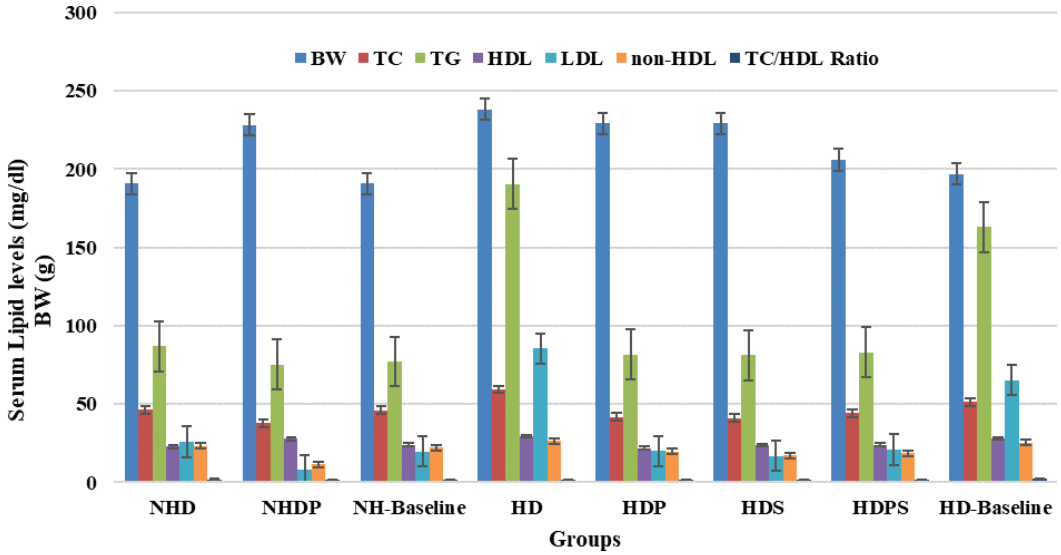


Figure 11. Comparative changes in BW (g), serum levels of TC, TG, HDL, LDL, non-HDL (mg/dl) and TC/HDL ratio among the study groups.

The observed effect would be largely contributed to the plant phenols and flavonoids content, which are compounds of secondary metabolites found in many plants (Mir et al. 2019). These phytochemicals have been widely studied for their biological activities, such as antioxidant, anti-hyperlipidaemic, anti-inflammatory, anti-microbial, anti-cancer, anti-aging, cardioprotective, neuroprotective, immunomodulatory, anti-diabetic, antibacterial, anti-parasitic, anti-viral, and anti-nociceptive properties (Bai et al. 2019; Caro-Ordieres et al. 2020). As well, the observed antioxidant activity of *E. ramosissimum* extract can be linked to its phenolic and flavonoid content.

These findings are similar to a previous study by (Batir-Marin et al. 2021), the anti-oxidative activity was investigated in related species of *Equisetum*, which showed that the contents of total phenolic components were richer in the ethanol extract fractions, with remarkable anti-oxidative activities. The available literature demonstrated that the species *E. ramosissimum* has many bioactive compounds relevant to the treatment of hyperlipidaemia. Of these are polyphenols, which are known to possess hypolipidemic activity (Gandhi et al. 2020; Saadh 2020).

One of the metabolites detected in the studied extract using UHPLC-MS/MS analysis was kaempferol, which has previously shown positive effects on cancer, liver injury, obesity, and diabetes. It can be used to promote glucose metabolism and inhibits gluconeogenesis in the liver. Moreover, it lowers the lipid profile and oxidative stress in rats' post-myocardial injury (Kim and Park 2020). Another metabolite detected in the study extract is catechin. The latter showed to contribute to the lipid-lowering effects, as it has a confirmed cholesterol-lowering and anti-obesity activity in previous animal and clinical studies (Kim and Heo 2022).

As far as we know, neither therapeutic nor protective effects against hyperlipidaemia for *E. ramosissimum* on human or animals model have previously been studied. As expected, the results of the current study are consistent with the results of (Vieira et al. 2020), who studied butanoic and aqueous extracts prepared from Brazilian samples of the relevant species *E. giganteum* on lipid profile in alloxan-induced diabetic rabbits. They concluded that *E. giganteum* can be a therapeutically relevant potential bioactivity in treating hyperlipidaemia, with a powerful reducing effect on blood cholesterol and triacylglycerol.

The presented findings showed the species ethanolic extract to be effective in improving lipid profile in normal rats (NHDP), by significantly lowering TC and TG, but increasing BW and HDL levels. Of special interest is the large significant reduction in LDL, non-HDL, and the ratio of TC/HDL levels in the normal group treated with *E. ramosissimum* extract.

Groups with induced hyperlipidaemia and treated with the plant extract (HDP) showed improvement in their lipid profile, by significantly lowering HDL levels. Of special interest is the large significant reduction in TG, LDL, and non-HDL levels among this group. While the combination between the plant extract and Atorvastatin (HDPS) showed a significant increase in HDL levels, with large and significant reductions in TC, LDL, and non-HDL levels. These data revealed that the prepared extract in combination with the used statin drug was significantly effective, especially in lowering LDL and non-HDL levels in hyperlipidaemic animals. That can be explained by the remarkable reduction found in the TG, LDL, and non-HDL, on the same animal model, when the plant extract was used alone.

Concerning the observed hypolipidemic effect of *E. ramosissimum* in the current experiment, Chang CJ et al. (2011) have previously revealed that kaempferol can regulates the lipid profile in HFD rats, through the lipid metabolism pathway in the liver. Consequently, we hypothesize that gene expression of LDL-receptors might be induced by kaempferol as reported by Ochiai A et al (2016), which increases of LDL uptake and its hepatic metabolism leading to decline in the LDL levels, as was observed in plant extract-treated animal groups in this study.

Therefore, the current findings shed the light on the promising effect of the plant extract alone or in combination with Atorvastatin, as a potential dietary supplement or treatment remedy that can be used for improving hyperlipidaemia. The approach of treatment which stands on the strategy of combining the already used conventional medicines with herbal medicines for the management of chronic diseases is wildly being studied in different complementary and alternative medicine (CAM) systems for its added value and advantages of improving the drug's efficiency (Yeh et al. 2020).

Conclusion

Accordingly, the observed hypolipidemic effect using the ethanolic extract of *E. ramosissimum* at a dose of 200 mg/kg, once daily for 2 weeks, with or without Atorvastatin can be considered as a novel finding. The current study showed that this extract to significantly lowered all lipidaemia linked parameters, especially TG levels in high fat diet fed rats, similar to Atorvastatin. However, the decrease is not so dramatic as with Atorvastatin.

On the other hand, combined treatment for Atorvastatin and the extract improves the blood lipid profile, and the levels of some parameters are similar to the statin-treated rats. Therefore, this extract may be more useful in cases of elevated blood TG.

However, additional research on combining this herbal species with statins medicine for the treatment of hyperlipidaemia, is necessary to explore the combination safety and efficiency. Furthermore, investigation of the combination different patterns at different doses, might be useful in future large-scale research, focusing on potential interaction of this combination.

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