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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2023 20(2): 557-564. **OPEN ACCESS**

Phytochemical Evaluation and Lipid Profile significance of *Ruta graveolens* through obesity-induced Animal Model

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Anti-obesity drugs are being used by the patient's poses life threatening side effects, therefore, natural remedies could not be overlooked. Ethnobotanically *Ruta graveolens* (*R. graveolens*) is quite common in Pakistan and has been used to cure obesity. To find out anti-obesity action of *R. graveolens* extracts in animal model in comparison with standard drug and to figure out secondary metabolites attributed for it. Phytochemical, fluorescence and proximate analysis were conducted along with ash values, phenolic and flavonoids contents with recommended protocols. Moreover, methanolic (RME) and chloroform extracts (RCE) were screened to determine lipid profile after high-fat diet (HFD) administration in different groups of rabbits via recommended protocol. Both extracts were found rich in alkaloids, carbohydrates, terpenoids, flavonoids, tannins and sterols. However, saponins and glycosides were only reported in RME. Total phenolic contents and flavonoids were found high in methanolic (172.09 mg/g) and ethyl acetate (145.1 mg/g) fractions. Standard group of drug (Orlistat) and methanolic plant extract significantly decreased serum cholesterol, LDL and triglycerides levels in experimental animals ($P < 0.05$). Furthermore, RME showed 65% significantly loss of body weight from 1430 g to 931g, while RCE exhibited 62% fall of rabbits' weight. Anti- obesity of RME owing to the abundance of flavonoids and alkaloids in particular to overcome body fat fluctuations. However, detail study is indispensable to map out the active component(s) from active fraction which might be source of lead compounds in future.

Keywords: *Ruta graveolens*, Anti-obesity, Lipid profile, Orlistat, Terpenoids

INTRODUCTION

Human beings relay on medicinal plants for their basic needs in particular for food, shelter and to mitigate various illnesses. Natural herbs have been reported in literature a backbone to treat different diseases. In developing countries more than 3.3 billion people trust on medicinal plants to cure them (Davidson-Hunt, 2000) Practice of traditional medicine is prevailing all over the world including China, India, Japan, Sri-Lanka, Thailand and Pakistan. Pakistan is a hub of natural herbs especially species of family Rutaceae (150 genera; 150 species) that are quite ubiquitous, including *R. graveolens*. It is an odoriferous herb having unpleasant smell usually saturated with secondary metabolites like isoimperatorin, bergapten, rutin, quercetin, imperatorin, psoralen and xanthotoxin. Among alkaloides rutamarine, rutamine, gravelinine and graveoline, are abundantly found in this plant (Jones, 1995). Seeds of this plant were reported to have nitrogenous substance (21.6 %), fixed oil (36.8 %), linoleic acid (44.5 %), stearic acid (9.1 %), oleic acid (22 %) and palmitic acid (21.8 %) (Parray et al.2012; Saieed et al. 2006). Miscellaneous medicinal uses were reported previously in different traditional system of medicine *viz*., appetizer (mushtahi), carminative (muhallile riyah), to increase eyesight (muqawwie basar), brain tonic (muqawwie dimagh), stomachic (muqawwie meda), abortefacient (musqite janeen) and demulcent (mulattif) (Parray et al. 2012). It is also beneficial for inflammatory, flatulent/colic, sciatica, gout, arthritis and dyspnea (Jarić et al. 2011). Among reported pharmacological activities anti-hyperglycemic, anti-arrhythmic, anti-tumor anti-bacterial and anti-hyperlipidemic are important (Poongothai et al. 2011).

Obesity is the consequence of elevated level of lipids (fats) and usually monitored via lipid profile test. Obesity leads to the cardiac diseases due to increase in LDL level (Buriro et al. 2011). Fenluramine, phentermine and other anti-obesity drugs inhibited fat absorption, suppress

appetite and stimulate energy expenditure. Sibutraminen and some other famous drugs caused reduction in dietary fat absorption by inhibiting pancreatic lipase (Guerciolini, 1997). Some of the anti-obesity drugs were reported in literature with side effect along with therapeutic effects including dyspepsia, dry mouth, headache abdominal pain, insomnia, constipation, and diarrhea (Maahs et al. 2006). Due to all these unfavorable conditions interest was developed in quest of novel natural remedies to reduce the burden of adverse effects and to ameliorate rated disorders.

MATERIALS AND METHODS

Collection of plant material

From Papar Mandi, Lahore (Pakistan), *R. graveolens* fruits were gathered in dried form and deposited at the herbarium of the Botany Department, Government College University Lahore. The fruits of *R. graveolens* were certified under voucher no. GC. Herb. Bot. 2213.

Extraction process

By solvent extraction depending on raising polarity of the solvents dried fruit powder was extracted with methanol and chloroform. In round bottom flasks (5L capacity) 01 kg of powder drug was macerated with 3L of solvent. At different time intervals flasks were agitated periodically and subsequently filtered to obtain specific extract (Fatima et al. 2019).

Solvent-solvent extraction was used to separate crude extracts into different fractions on the basis of principle of solubility of extract in specific solvent. Methanolic extract was mixed with n-hexane and water (1:1) in separating funnel (1 L) and eventually two layer were separated. Hexane layer was concentrated in rotary evaporator until it became colorless to acquire hexane methanolic extract. In separating funnel chloroform was mixed with equal volume of water and concentrated the solvent in rotary evaporator to obtain chloroform methanolic extract. Residual water was dissolved with ethyl acetate fraction and concentrated with the same protocol as described earlier. To obtain butanol and water fraction equal volume of n-butanol was mixed with water and butanolic layer was concentrated, nevertheless, watery layer was dehydrated in freeze dryer. A complete glimpse of flavonoids and phenolic contents found in various extracts were estimated (Fatima et al. 2019).

Phytochemical analysis

Different phytochemical tests to determine secondary metabolites (carbohydrates, glycosides, proteins, fixed oil, saponins, tannins, triterpenoids, alkaloids and flavonoids) were mapped out by adopted standard procedures (Fatima et al. 2019).

Fluorescence analysis

It was done pharmacogenostically to determine contaminants and identification of reliable specimens. Initially crude powder were analyzed under day light subsequently via ultra violet light. Powder (2 g) of plant were treated with 5 ml of sodium hydroxide (5 %), formic acid, ferric chloride (5 %), aniline, sulphuric acid (50 %), barium chloride, ammonium hydroxide, potassium hydroxide (1 M), sulphuric acid (66 %), sodium carbonate, iodine solution, glacial acetic acid, nitric acid (50 %), formaldehyde chloroform and water and left for whole night. Fluorescent properties and extracted materials were analyzed under UV and day light respectively (Xie et al. 2017).

Proximate analysis

Proximate analysis for acid insoluble ash, sulphated ash, total ash, water and alcohol soluble extractive values, moisture contents and water soluble ash were determined by adopted the standard procedures recognized by Indian Pharmacopoeia (Pharmacopoeia, 1996).

Total ash determination

Silica crucible was cleaned, dried and cooled. 2 g of sample was incinerated for 15 min at 450^{o}C in furnace until no carbon analyzed subsequently cooled at room temperature to yield constant weight. Ash percentage was calculated with help of following formula.

Total ash (%) = (weight of ash / weight of powder sample) × 100

Acid insoluble ash

After filtration of insoluble mater (obtained in ash crucible) 25 ml of dilute HCl was added subsequently boiled for 5 min and washed with hot deionized water and filtered. Later on it was shifted to the silica crucible and ignited and cooled to attain constant weight. Percentage of acid insoluble ash was calculated with reference to the dehydrated air specimen (Özgen et al. 2010).

Water soluble ash

After filtration of insoluble mater (obtained in ash crucible) 25 ml of deionized water was added subsequently boiled for 5 min, washed with hot water and collected in sintered glass container. It was stirred at a temperature of 450˚C for 15 min. Difference among the amount of entire ash and insoluble ash was calculated. Percentage of water soluble ash was calculated with reference to the dehydrated air specimen (Özgen et al. 2010).

Sulphated ash

2 g of extracted powder was ignited (to make powder charred in silica crucible), cooled and humidified with concentrated H_2SO_4 until no white fumes were observed. Carbon free ash was obtained after ignition at 800°C ± 25°C and later on it was cooled. Percentage of ash weight

was determined with reference to the dehydrated air specimen (Özgen et al. 2010).

Moisture content and dry matter

Silica pot was placed in an oven for 30 min at 105ºC and was cooled subsequently. 2 g of powder was weighed and left for 30 min at 105°C in the same oven and cooled. Dry matter and water contents were calculated by using the following formulas;

Moisture content (%) = 100 – dry matter (%) and Dry matter (%) = (dried sample weight / sample weight before drying) × 100.

Alcohol soluble extractive value

Alcohol (100 ml, 95 %) and powder (5 g) was mixed for a day. Initially flask was shaken (6 h) and later remained undisturbed for 18 h. To prevent the evaporation of alcohol it was rapidly filtered and 25 ml of extract was collected in petri dish and dried at 105ºC. Percentage of alcohol soluble extracted value was determined with reference to the dehydrated air specimen.

Water soluble extractive value

Chloroform water (100 ml) and powder (5 g) was mixed in a flask. After shaking it was allowed to rest for 24 h while filtrate (25 ml) was collected in tarred petri dish to achieve constant weight at 105º C. Percentage of water soluble extracted value was determined with reference to the dehydrated air specimen.

Entire phenolic content determination by Folin-Ciocalteu's assay

Phenolic contents were determined via spectrophotometric method. Briefly, 01 mg/ml concentration of methanolic extracts and chloroform were prepared with deionized water (9 ml). The extract was mixed with Folin-Ciocalteu's reagent (1 ml) for 5 min followed by addition of diluted 10ml of 7 % sodium carbonate. The blank specimen was prepared with distilled water. Standard solution of Gallic acid were prepared at concentrations of 10, 20, 40, 80, 100, 120 µg / ml, then sample and blank were incubated for 90 min and absorbance was measured at 750 nm. Mean value was calculated by repeating the procedure thrice. From callibration curve total phenolic content was calculated and outcomes were expressed as mg of gallic acid equavalent per g of dry weight or extract (Özgen et al. 2010).

Flavonoid concentrations determination

Flavonoid content were determined via spectroscopic method. In brief specimen extract (200 ul) were prepared with 1 mg/ml of methanol and agitated with potassium acetate 1M (100 µl), distilled water (4.6 ml) and aluminium chloride (100 µl). Further dilutions of standard solution of quercitin (1 mg / ml) were made at concentrations of 10, 20, 40, 80, and 120 µg / ml. Blank sample was prepared with methanol (200 µl). Entire, fractions, extracts, standard and blank were allowed to stand for 45 min and absorbance was determined 415 nm. Mean was calculated by repeating the procedure thrice. Entire phenolic content determined by calibration curve and outcomes were expressed as mg of QE/g of extract (qurecetin equivalent) (Chang et al. 2002).

Animal grouping

The experimental diet comprise of: i- High-fat diet (HFD); desi ghee and butter with ND supplemented; ii- Standard pelleted chow: normal diet (ND). Before the division of rabbits in three of seven groups, they were adapted for 2 weeks and housed at 23 ± 1°C and 55 ± 5 % relative humidity in a room and cage made of standard metallic wire gauge. For 5 weeks three animals were allowed to access fodder. The other rabbits (18 rabbits) were allowed to feed on HFD + ND. They were divided randomly in 04 groups which were as follows: (I). HFD + RME; (II). HFD only; (III). Orlistat+ HFD; (IV). RCE + HFD. In every single week, body weight gain and loss was observed as well as blood sampling was performed. After 12 h fast serum biochemical enzymatic analysis was performed. For serum lipid profile determination the separation of serum was done by centrifugation rate (4,000 rpm), for 10-12 min and at 86°C was stored. The study as validated by Ethical Committee of the University College of Pharmacy and study was carried out according to the standard guideline (ECUCP-R18-I19).

Lipid profile tests

Lipid profile tests (Total Cholesterol, LDL and triglycerides) of the laboratory animals were estimated via commercially available kits as per described (Ting et al. 2018).

Statistical analysis

All numerical values were mentioned as mean ± standard error of mean. By using SPSS version12.0 statistical data was found. Animal group results were mentioned as mean after applying Dunnett test along with variance (ANOVA) analysis) while outcomes were considered significant at $P < 0.05$. Comparison with standard drug was done via student's T test.

RESULTS

The percentage yields of methanolic (RME) and chloroform (RCE) extracts obtained from the crude powder of fruit of *R. graveolens* were 78 g (3.9 %) and 83 g (4.15 %) respectively. The masses of further fractions were attained in Table 1**.** Phytochemical analysis of *R. graveolens* indicates that alkaloids, fixed oils, carbohydrates, flavonoids, triterpenoids and sterols were detected in both RME and RCE. The qualitative properties regarding chemical nature (constituents) of crude extracts and chemical tests given in Table 2. The analysis of ash values exhibited outcomes of 4.5 % (acid insoluble ash),

10.8 % (sulphated ash), 5.25 % (moisture content), 9.7% (extractive water soluble), 9.4 % (total ash) 15.5 % (alcohol soluble extractive) and 7.0 % (water soluble ash). Fluorescence analysis of crude powder of plant and treated with different reagents were analyzed under ordinary light and under 02 different wavelengths of UV light (254 and 365 nm), the color changes were observed in Table 3. From different fractions, phenolic and flavonoids contents were displayed in mg/g and the total contents of phenol and flavonoids were determined in Table 4. Rabbits were given with HFD and weight variation was noticed, the significance rise in weight was seen in all groups after treating with high fat diet from 1017 ± 142 to 1383 ± 156 shown in Figure 1.

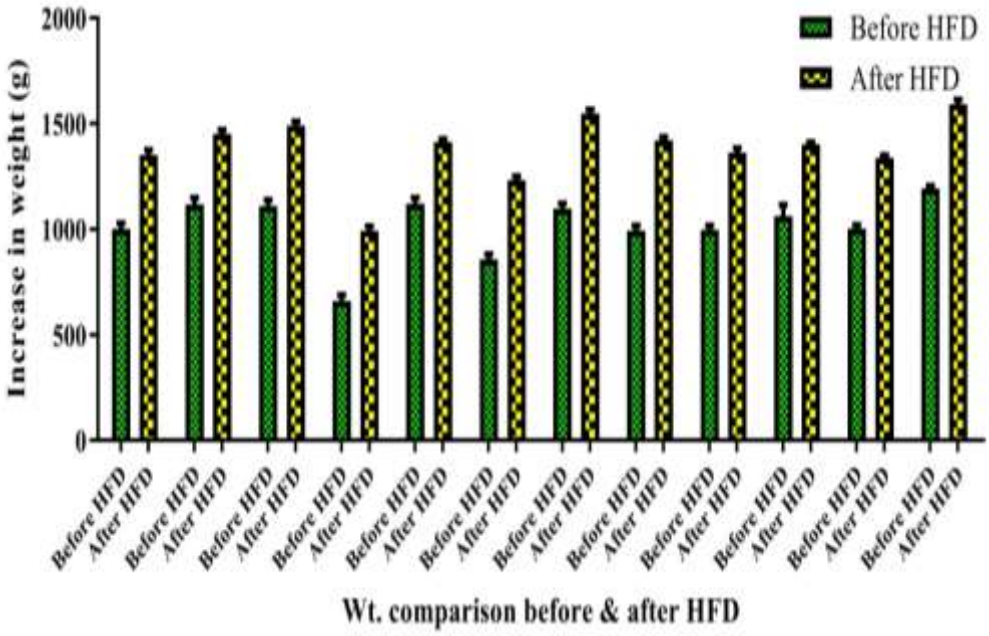

Figure 1: Pair T test was applied before and after high fat diet where values indicated as Mean ± SEM (1017 ± 41.07) and (1383 ± 45.08) respectively, p value <0.0001, df=11.

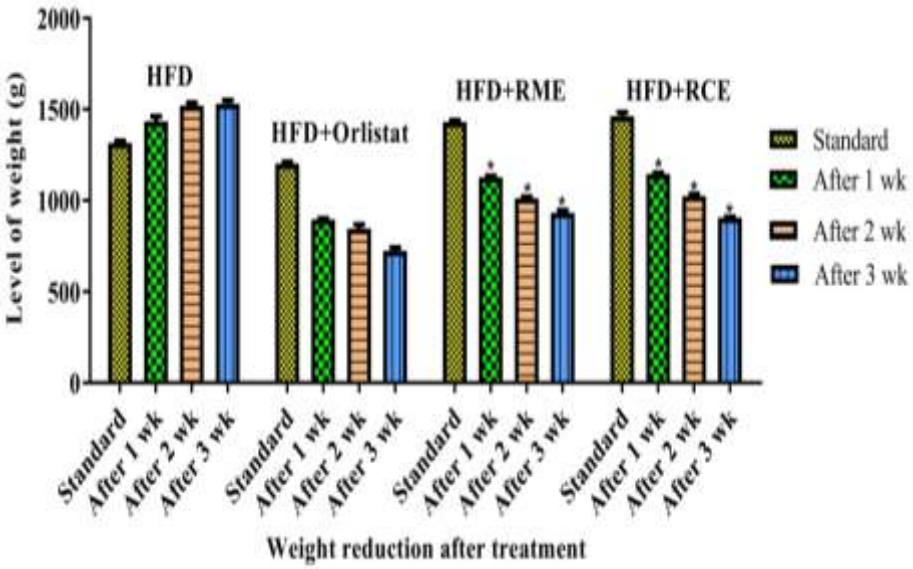

Figure 2: Figures presented with increased fat diet, Mean ± SEM (n=3); One way ANOVA (Dunnett's test); p * P < 0.05 indicates significant decrease of body weight.

Anti-obesity effect of *R. graveolens* were observed after providing HFD, HFD+Orlistat, HFD+RME and HFD+RCE at zero time, after 01, 02 and 03 wk time intervals which showed significant loss of 499g with HFD+RME and 558g with HFD+RCE when compared with standard and reference drug (Orlistat) (480g) shown in Figure 2. Effect of entire serum cholesterol level before and after providing HFD was proved and indicated in various levels from Mean ± S.E.M 67.50 ± 2.786 to Mean ± S.E.M 87.35 ± 3.876 presented in Table 5. The consequence of both plant extracts (RME and RCE) and reference drug were evaluated on the cholesterol level (mg/dl) and found a significant fall with standard drug (Orlistat), RME and RCE when compared with control group. Methanolic extract showed more therapeutically active than chloroform extract with $p < 0.05$ shown in Table 6. Change in serum triglyceride level before and after provision of HFD in rabbits is observed to be raised from mean ± S.E.M 75.37 ± 2.297 to mean ± S.E.M 89.1 ± 2.428 in Figure 3-A. The outcome of *R. graveolens* extracts on the serum triglyceride level (mg/dl) in comparison with standard drug (orlistat) was assessed 59% fall which results in substantial fall on comparison with RME (62%) and RCE (61%) after 3rd week with $p < 0.05$ shown in Figure 3-B.

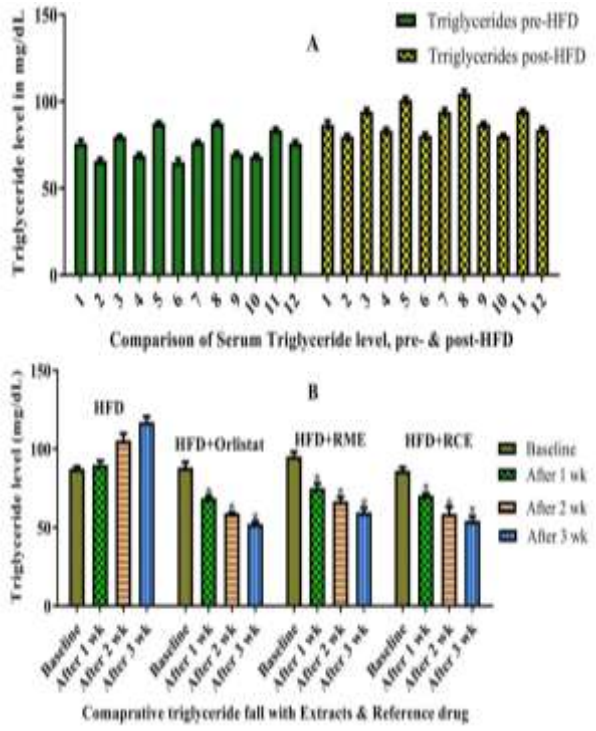

Figure 3: Baseline Post 2 weeks serum triglyceride level (mg/dl) increased after high fat-diet intake; Figures are Mean±SEM (n = 3); One way ANOVA (Dunnett's test); p values are represented by each cell bottom right corner figures after comparing with increased HFD *P < 0.05 indicates significant decrease in body weight.

Change in serum LDL level before and after providing the HFD in rabbits' results in increased in LDL level from Mean ± S.E.M 59.87 ± 2.84 to Mean ± S.E.M 70.36 ± 2.909 in Figure 4-A. The conclusion of our plant's extracts on the serum LDL level (mg/dl) in comparison with Orlistat (36%) indicted the significant decreased of LDL concentration with methanolic extract (38%) and RCE (37%) exhibited a bit better decrease than standard drug shown in Figure 4-B.

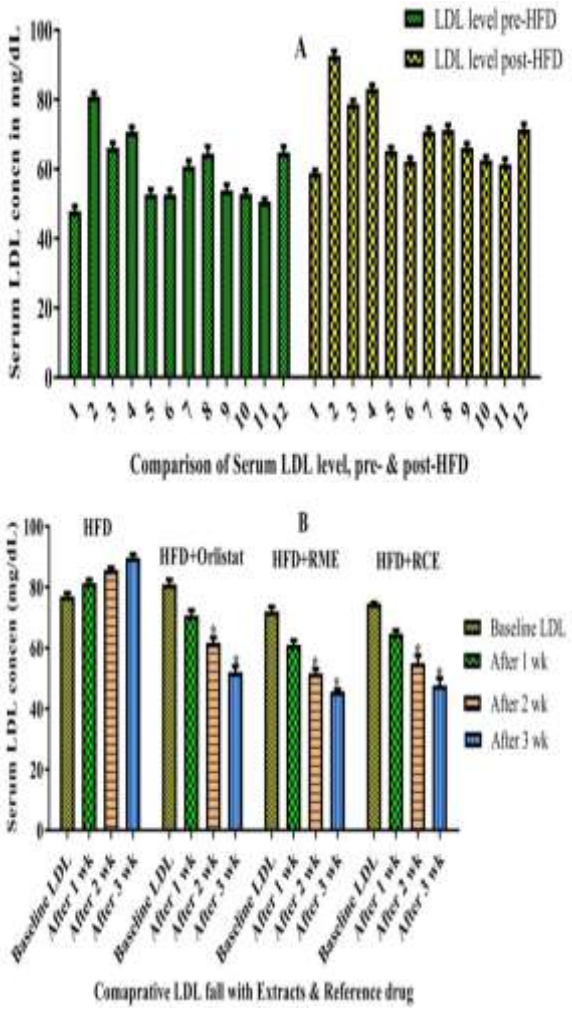

Figure 4: Baseline Post 2 weeks serum LDL level (mg / dl) increased after high fat-diet intake; Figures are Mean±SEM (n = 3); One way (Dunnett's test); p values are represented by each cell bottom right corner figures after comparing with HFD group * P < 0.05 indicates significant decrease in body weight.

Chemical constituents conferred about numerous complications related to the human health like polyphenols and flavonoids for lipid metabolism while saponins attributed to the hypocholesterolemia and anti-obesity (Ono et al. 2006). Phenolic and flavonoids contents were proved antioxidant, carcinogenic risk and to neutralize

mutagenic properties (Borkataky et al. 2014). In this study *R. graveolons* was found abundant in steroids, saponins, flavonoids, alkaloids, cardio glycosides, carbohydrates and tannins (Sivaraj et al. 2011), however, major work was done related to the drug evaluation physicochemical parameters. Earlier, *R. graveolons* was highlighted (proximate analysis) with, acid insoluble ash (4.5 %), total ash (9.4 %), moisture contents (5.25 %) and values for water soluble ash (7 %). In this study entire ash (11.8 %), and moisture contents (7.55 %) were found little higher than previous one, while fluorescence analysis indicated about plant material authentication. Phenolic contents were reported variable in different crude extract fractions (methanolic 23.75 mg/g; chloroform, 172.09; hexane, 22.89 mg/g; aqueous, 12.15 mg/g and for n-butanol portion 18.09 mg/g) as reported previously for other plant extracts in literature (Tiwari et al. 2011). Similarly, flavonoids were observed in different concentrations for each fractions as noticed for phenolic contents, *viz*., for methanolic, 76.6 mg/g; hexane, 92.7 mg/g; aqueous, 36.6 mg/ g and for n-butanol portion 48.5 mg/g. Increased lipid contents led to obesity. Here elevated levels of body weight was significantly reduced ($p < 0.001$) when compared with orlistat anti-obesity drug. Literature review documented earlier about *Cissus quadrangularis* and *Garcina cambosia* were to have anti-obesity and appetite control activities similar to *R. graveolons*, however, exact mechanism is unclear might be due to the secondary metabolites via pancratic lipase inhibition (Han et al. 2005; Kim and Kang, 2005). Previously two studies were conducted on diabetic rats for analyzing antihyperlipidemic activity of *R. graveolons* primarily with aqueous and ethanolic extract resulted in decline of cholesterol and triglyceride levels via reduction in absorption from intestine compared with quercetin (Ahmed et al. 2010; Toserkani et al. 2012). However, chloroform extract resulted in rise in cholesterol molecules ($P<0.425$) and LDL levels ($P> 0.05$). Conclusions were drawn on the facts that flavonoids, alkaloids and cumurin involved in anti-obesity and body fat fluctuations. Present study confirmed *R. graveolons* effectiveness as an anti-obesity drug, however, for the toxicity and other parameters further exploration may also be needed in future study

Table 1: Fractions of crude extract along with their quantity calculated

Scientific Name	Fractions	Wt of Fraction (g)
R. graveolons	Chloroform	3.5
	n-Hexane	4.0
	Ethyl acetate	11.6
	Aqueous	36
	n-Butanol	15.2

Table 2: Phytochemical constituents observed in *R. graveolens* extract

Phytochemicals	*R. graveolens*	
	Chloroform	Methanol
Glycosides	− − −	+ +
Proteins	− − −	− − −
Alkaloids	+ + +	+ + +
Saponins	− − −	++
Fixed oil	+ + +	+ + +
Carbohydrates	+	+ +
Flavonoids	+	+ + +
Triterpenoids	+ + +	+ + +
Tannins	− − −	+ + +
Sterols	+ + +	+ + +

+ indicates presence and – indicates absence

Table 3: Treatment of *R. graveolens* with chemical for fluorescent & behavior analysis

Extract (powder) with chemical reagents	Color obtained (ordinary light)	UV analysis of Fluorescence (254 nm)	UV analysis of fluorescence (365 nm)
Extract (original form)	Brown color	Brown color	Brown color
$BaCl_2$ + powder	Yellow color	Yellow color	Violet color
Chloroform + powder	Light green color	Yellow color	Purple color
5 % $FeCl_2$ solution +Powder	Brown color	Creamy brown color	Brown color
Formaldehyde + powder	Yellow color	Violet color	Blue color
Formic acid + powder	Yellow color	Brown color	Violet color
Glacial acetic acid + powder	Green color	Violet color	Fluorescence of Violet color
Dilute HCl + powder	Yellow color	Orange color	Violet color
Iodine solution + powder	Red color	Red color	Black color
Ammonium hydroxide + powder	Brown shade	Pink color	Blue color
50 % HNO_3 + powder	Yellow shade	Brown color	Green color
Powder + 1 M KOH solution	Shade brownish yellow	Orange color	Green color
Sodium carbonate solution + Powder	Yellow color	Chocolate color	Blue color
5 % NaOH + powder	Carroty color	Orange color	Green color
50 % H_2SO_4 + powder	Brownish green color	Chocolate color	Blue color
66 % H_2SO_4 + present powder	Brownish green color	Chocolate color	Green color
$AgNO_3$ + powder	Yellow color	Yellow color	Blue color
Water + powder	Yellow color	Yellow color	Blue color

Table 4: Total phenolic and flavonoid contents found in *R. graveolens*

Extract/Fraction	Phenolic contents (mg/g)	Flavonoids contents (mg/g)
n-Hexane	22.89	92.7
Aqueous	12.15	36.0
n-Butanol	18.09	48.5
Chloroform	117.02	112.6
Chloroform	23.75	98.6
Ethyl acetate	360.89	145.1
Methanolic	172.09	76.6

Table 5: Effect of entire serum cholesterol level before and after providing HFD

Rabbits involved	Prior HFDCholesterol (mg/dl)	Post HFD Cholesterol (mg/dl)
1	54.55	68.18
2	73.63	101.82
3	88.18	109.09
4	77.27	107.27
5	57.27	75.45
6	60.91	81.82
7	71.82	94.55
8	66.36	70.91
9	63.64	81.82
10	60.91	87.27
11	61.82	84.55
12	73.64	85,45
	Mean±SEM 67.50±2.786	Mean±SEM 87.35±3.876

Table 6: Effect of *R. graveolens* on cholesterol level (mg/dl) in comparison with Orlistat

Food/dose	Standard	After 1 wks	After 2 wks	After 3 wks
HFD	93.03±12.60	100.91±11.17	104.24±10.60	106.36± 9.29
HFD + Orlistat	88.18±9.27	85.15±8.74	79.39±3.20	70.91±2.40*
HFD + RCE	85.76±0.80	96.06±2.98	94.85±4.47	93.94±4.37
HFD + RME	82.43±6.83	84.24±6.06	87.88±7.90	80.30±7.24*

CONCLUSION

Current study proved that chemical constituents in *R. graveolens* showed effective response to reduce body fat (antihyperlipidemic and anti-lipid metabolism). *R. graveolens* could be a beneficial source as an anti-obesity herb as well as in pharmaceutical formulations in future.

CONFLICT OF INTEREST

The authors declare that they have no any conflict of interests to this work and material of manuscript.

ACKNOWLEDGEMENT

We are highly appreciated and grateful to the Principal, College of Pharmacy, University of the Punjab, Lahore, Pakistan, for providing access to laboratories to execute this research work.

AUTHOR CONTRIBUTIONS

We declare that research work and compilation of this article were done by given authors and all responsibilities pertaining to claims relating to the content of this article borne by the writers. IM performed and designed all the experimental work and to write-up. SSH supervised and analyze work at every step as well as provided the technical support throughout the project. MA contributed in compilation and arrangement of material and data into article format. MA, MS, MAG and HA supported in the conception and critical review of the research work.

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