Sodium alginate-based nanoformulation: optimistic approach for enhancing the anti-obesogenic effect of rutin

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Background

Obesity is a global epidemic that affects all age groups and social classes. Prevention and treatment strategies against obesity have gained little long-term success. Obesity has been shown to be increased rapidly in the last few decades. It is anticipated that by 2030, up to half of the human population will be classified as obese or overweight.

Objective

This study aimed to develop a polymeric nanoformulation for the oral delivery of rutin in an obese rat model and to explore the anti-obesogenic impact of this nanosized rutin versus free rutin to provide insightful information for understanding the mechanism of action.

Materials and methods

Sodium alginate-based nanorutin was prepared and characterized by transmission electron microscope and Zetasizer. The obtained rutin nanoformulation and its free form were applied for obesity management in rats fed a high-fat diet.

Results and conclusion

The data demonstrated that rutin nanoparticles are made up of single spherical units with a diameter that ranged between 96.1 and 157 nm and exhibited negative zeta potential at −16.6 mV. Treatment of obese rats with chitocal, free rutin, or rutin nanoformulation provoked significant reduction in the body weight, thoracic circumference, BMI, and Lee index. Also, they elicited a significant drop in serum cholesterol, triglycerides, low-density lipoprotein, glucose, insulin, insulin resistance, toll-like receptor 4, nuclear factor kappa B, and leptin levels associated with a significant rise in serum adiponectin and spexin levels. Furthermore, treatment of obese rats with chitocal or rutin nanoformulation elicited significant reduction in body length and abdominal circumference along with significant enhancement in serum high-density lipoprotein level. In conclusion, this approach provides a sparkling proof for the anti-obesogenic potential of rutin nanoformulation through its hypolipidemic, hypoglycemic, and anti-inflammatory effects as well as its inhibitory action on hyperleptinemia and adipogenesis. The superior antiobesity impact of the formulated nanorutin than its free form may be attributed to the enhancement of the solubility and bioavailability by nanoencapsulation.

Keywords:
chitocal, nanoformulation, obesity, rats, rutin, sodium alginate

Abbreviations:
AC, abdominal circumference, ADP, adiponectin, HDL, high density lipoprotein, LDL, low density lipoprotein, NF κB, nuclear factor kappa B, SPX, spexin, TC, thoracic circumference, TG, triglycerides, TLR4, toll like receptor 4

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Introduction

Obesity is a common public health issue as the rates of obesity have been shown to be increased rapidly in the last few decades [1]. It is anticipated that by 2030, up to half of the human population will be classified as obese or overweight [2]. Obesity is often accompanied by an enhanced risk for different metabolic, cardiovascular, skeletal comorbidities, and cancer [3], besides a crucial influence on psychosocial health [4]. Moreover, obesity is associated with increased mortality as the latest data of the Global Burden of Disease study in 2017 showed that a BMI more than or equal to 25 kg/m^2 is linked to 2.4 and 2.3 million deaths in females and males, respectively [5]. Obesity is a multifactorial disease, with various factors contributing to the increased weight, involving dietary mode, physical activity, sleep modalities, medications, besides genetic, epigenetic, and

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environmental factors. As societies consume processed foods high in saturated fats, sugars, and low in essential nutrients, it results in cardiovascular diseases, obesity, and metabolic disorders [6].

The presence of low-grade chronic inflammation, both systemically and in adipose tissue, is a major factor in the development of chronic diseases linked to obesity. The mechanisms behind obesity-related inflammation differ from those in traditional infection-induced inflammation and involve various signaling pathways. Dysfunctional adipocytes produce inflammatory cytokines and chemokines, which attract immune cells to adipose tissue, thereby intensifying the inflammatory response [7].

Oxidative stress plays a fundamental role in obesity. The mechanisms of ROS generation, including superoxide production from oxidative phosphorylation, NADPH oxidases, protein kinase C activation, glyceraldehyde auto-oxidation, and the polyol as well as hexosamine pathways, contribute to cellular and systemic oxidative stress associated with obesity [8].

Treatment guidelines for obesity emphasize that the suitable strategy for weight management must be multidisciplinary, comprising lifestyle alterations, behavioral medication, pharmacotherapy, and/or bariatric surgery. Besides that, the high cost of obesity medication needs to assessed from an economic perspective. The reduced availability of antiobesity medications and increased withdrawals may be attributed to the rising prices over the past two decades [9,10]. Antiobesity drugs are recommended for patients with a BMI more than or equal to 30 kg/m^2 or if more than or equal to 27 kg/m^2 in the existence of one or more comorbidities. Nevertheless, the history of antiobesity drugs was characterized by the impairment of numerous ones after their extensive use in the market, consequential to critical side events, particularly cardiovascular effects, suicidality, risk for abuse, and dependence [11] and recently cancer [12]. Thus, the European Medicines Agency (EMA) and the Food Drug Administration (FDA) upgraded the guidelines of antiobesity drugs approval, indicating the significance of cardiovascular and central nervous system safety [13]. While essentially dubbed as an adjustable as well as forbidding health issue, inhibition and management approaches against obesity have gained little long-term success [14].

Flavonoids in plant foods have generated attention owing to increasing poof of their favorable impact on human health. Dietary plants are the primary sources of flavones [15], and the antiobesity capability of flavonoids like ruin and quercetin is actually applicable and their modulatory influence is recorded on diminishing food intake, decreasing the absorption of nutrients, ameliorating adipogenesis and the life cycle of adipocytes, promotion of thermogenesis and energy exhaustion, and regulating intestinal microbiota and exhibiting antimicrobial activity [16]. Rutin is an important flavonoid merging one molecule of quercetin and rutinose each. It is well recognized to have numerous profitable health effects, involving anti-allergic, antiviral, anticancer, anti-inflammatory, and antioxidant activity [17]. The antiobesity effect of rutin stems from its ability to increase the mitochondrial number and the consumption of oxygen during brown adipogenesis *in vitro* [18]. It has been found that rutin activated brown adipose tissue (BAT) and white fat browning [17]. The functional BAT plays fundamental roles in the metabolism of systemic energy. So, the advancement of energy expenditure by increasing the mass and activity of BAT represents a promising approach in obesity treatment [19] as the elevation of the function of mitochondria and energy expenditure may synergistically participate to proper metabolic profiles versus obesity and metabolic disorders [17].

Despite numerous activity of rutin as an antiobesity agent, it is known as a fat-soluble molecule that is dissolved in organic solvents such as pyridine, methanol, and ethanol [20]. Likewise, it possesses imperfect durability as well as bioavailability and such rutin physicochemical characteristics are particularly attributed to its scare water dissolution [21]. To solve poor solubility of drugs, a new approach like the loading of the drug into polymeric nanosystems or hybrid polymeric systems has been emerged [22]. Also, nanotechnology-based drug nanoformulation provides the usefulness of the augmented bioavailability of active ingredients [23]. Thus, the rutin nanoformularization for the different therapeutic purposes indicated the elevated aqueous solubility and enhanced efficiency in comparison with traditional delivery of rutin [24].

This research work was constructed to develop sodium alginate-based nanorutin formulation for enhancing its therapeutic impact. This study was focused on exploring the anti-obesogenic potential of this nanosized rutin compared with free rutin in an obese rat model and tracking the associated mode of action.

Materials and methods

Preparation and characterization of a polymeric nanoformulated rutin

Chemicals

Rutin, sodium alginate, and Tween 80 (T80) were provided by Sigma-Aldrich Co. (St Louis, Missouri, USA). Miglyol 812 N, a medium chain triacylglyceride mixture, was purchased from Sassol Germany GmbH (Brunsbüttel, Germany). All other chemicals used were of analytical classification and also acquired by Sigma-Aldrich Co.

Nanoformulation

In the current investigation, the nanoformulated rutin was prepared using a modified procedure based on Terjung *et al.* [25]. This involved homogenization with a homogenizer PRO400 PC (SN: 04-01198, USA) in a matrix containing sodium alginate and Tween 80 (T20). Specifically, 1 g of rutin was put in 100 ml solution comprising an oil phase mass fraction of 10 wt% and an aqueous phase mass fraction of 90 wt %, with the water phase containing 1 wt% Tween 80 and sodium alginate (3 g). The emulsion was formed by blending the solution in a high-pressure homogenizer for 30 min at 18 000 rpm, with the process conducted in an ice bath to keep the temperature at 35°C. Then, the rutin nanoformulation was stored at 4°C until used.

Transmission electron microscopy

A drop of the nanoformulated rutin was placed into a carbon-coated copper grid and allowed to adhere to the carbon substrate for 10 min. Excess fluid was eliminated using filter paper. Subsequently, a drop of phosphotungstic acid solution (2%) was applied, and the excess liquid was again deparated using the tip of the filter paper. Samples were observed by operating at 160 kV [26] under transmission electron microscopy (Jeol JEM-2100, Tokyo, Japan).

Zeta potential determination

Zeta potential was analyzed by the light scattering technique [27] using a Zetasizer (Nano ZS, Malvern Instrument, Worcestershire, UK). For zeta potential measurements, the sample was diluted with bidistilled water (1 : 100 v/v) [28]. Following dilution, the sample was transferred to a 1 ml quartz cuvette and measured at room temperature. Three cycles were taken for the sample.

Biological experimentation

Animal caring and housing conditions

The rats were housed in facilities that were authorized and complied with established regulations on ordering, equipment, and maintenance of the experimental biological clinics. Fifty adult female Wistar strain albino rats, weighing 130±10 g at 90-day old, were enclosed in this work. These rats were provided by the Animal Care Unit of the National Research Centre, Giza, Egypt. They were maintained in polypropylene cages under standard environmental conditions, including a temperature of 24±1°C, a cycle of 12-h light/12-h dark, and humidity of 60±5%, and provided with *ad libitum* approach to tap water as well as standard rodent diet. The rats were permitted to acclimatize to these circumstances for 2 weeks before the experiment initiation.

Ethical statement

The research protocol adhered to the standards and universal ethical concepts of animal experiments. This research was performed following the principles and guidelines established by the Ethics Committee for Medical Research of the National Research Centre, Egypt (approval no: 18/175).

Research protocol

Following the adaptation period, 10 rats were fed a standard rodent chow with carbohydrate (40%), fat (3.8%), protein (26.5%), and crude fiber (4.5%) in 100 g of chow, as described by Buettner *et al.* [29] for 18 weeks and assigned as the control group. The remaining 40 rats were fed with a high-fat diet (HFD) comprising carbohydrate (45.7%), fat (38.8%), and protein (15.5%) by calories for 12 weeks, according to Soliman *et al.* [30]. The diet was prepared as recorded by Levin and Dunn-Meynell [31] with slight modification as follows: 72% powdered rat feed, 18% milk powder, 5% corn oil, and 5% ghee. All ingredients of the HFD were thoroughly mixed and baked in an oven at 65°C overnight.

The obese rats were classified to four groups (10 rats/group). Group 1: untreated obese group; group 2: obese rats treated orally with 50 mg/kg body weight of chitocal (Chitocal capsules, which contain 500 mg chitosan, 100 mg ascorbic acid, and 50 mg *Gymnema sylvestre* leaves powder extract, dBK Pharma S.A.E.) [32], administered daily for 6 weeks; group 3: obese rats treated orally with of 20 mg/kg body weight free rutin suspended in 1 ml distilled water/rat [33], administered daily for 6 weeks; group 4: obese rats treated orally with 20 mg/kg body weight rutin nanoformulation dissolved in 1 ml distilled water/rat, administered daily for 6 weeks.

Anthropometric parameters

At the end of the experimental period (18 weeks), the abdominal circumference (AC), measured immediately

anterior to the forefoot and the thoracic circumference (TC), measured immediately behind the foreleg, along with the body length (nose–anus length or nose-to-anus), were estimated in rats under anesthesia. Body weight and body length measurements were recorded for the calculation of BMI [34]:

BMI (g/cm^2)=(body weight (g)/length^2 (cm^2))

Also, Lee index was estimated according to the following equation:

Lee index=cube root of body weight (g)/nose-to-anus length (cm).

Both BMI and Lee index values are indicators for the existence of obesity.

Blood sampling

Following completion of the animal treatment regimen, the rats were fasted overnight (12–14 h), and blood specimens were withdrawn from the tail vein into clean, dry centrifuge tubes. These tubes were then let to coagulate at room temperature for 45 min to get sera. Serum specimens were collected by centrifugation at 4° for 15 min at 1800*g* using a cooling centrifuge. Collected serum was then cryopreserved at −20°C till further biochemical analyses. Subsequently, after blood collection, rats were killed by cervical dislocation under full anesthesia induced by intraperitoneal injection with 90 mg/kg of ketamine and 5 mg/kg xylazine [35].

Biochemical analyses

Total cholesterol (Chol), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and glucose concentration were determined in serum by colorimetric methods with kits procured by Reactivos GPL (Barcelona, Spain). The methods used were based on Meiattini *et al.* [36] for Chol; Bucolo and David [37] for TG; Naito [38] for HDL and LDL as well as Young and McEneny [39] for glucose.

Quantitative estimation of serum insulin concentration was carried out by an ELIZA kit obtained from Immunoscopec Co. CA (the Netherlands), following the method outlined by the National Committee for Clinical Laboratory Standards procedures for the collection of diagnostic blood specimens by venipuncture [40]. The homeostasis model assessment of basal insulin resistance was calculated to estimate the index from the product of the fasting concentration of serum glucose (mmol/l) and serum insulin (mU/ml) divided by 22.5, as described by Duncan *et al.* [41]. The quantitative evaluation of serum toll-like receptor 4 (TLR4) level was performed according to manufacturer's manual using an ELIZA kit purchased from Sunlong Biotech. Co. Ltd (Hangzhou, China). Serum nuclear factor kappa B (NF-κB) levels were estimated using an ELIZA kit purchased from SinoGeneClon Biotech. Co. Ltd (Hangzhou, China) according to manufacturer's protocol. Furthermore, quantitative assessment of serum leptin, adiponectin (ADP), and spexin (SPX) levels were carried out using ELIZA kits provided by SunRed Biotechnology Co. Ltd (Shanghai, China), following the operating instructions.

Statistical procedure

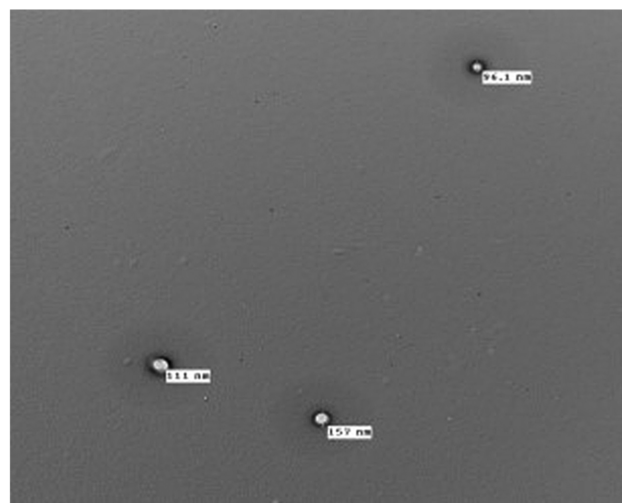
The obtained data were presented as arithmetic means with their standard errors (mean±SE). Statistical analysis was performed using one-way analysis of variance with the Statistical Package for the Social Sciences program, version 20 (Chicago, USA). Post-hoc analysis was conducted using the least significant difference method to compare significance between groups [42]. A significance level of *P* value less than 0.05 was estimated statistically significant.

Results and discussion

Transmission electron microscopy observations

The shape of the formulated rutin nanoparticles is illustrated in Fig. 1. The observed structures consist of single spherical units with diameters ranging between 96.1 and 157 nm, indicating a nanoscale size. The external surface exhibited a smooth and regular appearance, suggesting that sodium alginate gel polymer creates a continuous film around rutin.

Figure 1



Transmission electron microscopy (TEM) morphology and size of the formulated rutin nanoparticles in corporates in sodium alginate gel.

Zeta potential findings

The rutin nanoformulation zeta potential is recorded in Fig. 2, which showed a negative zeta potential at −16.6 mV.

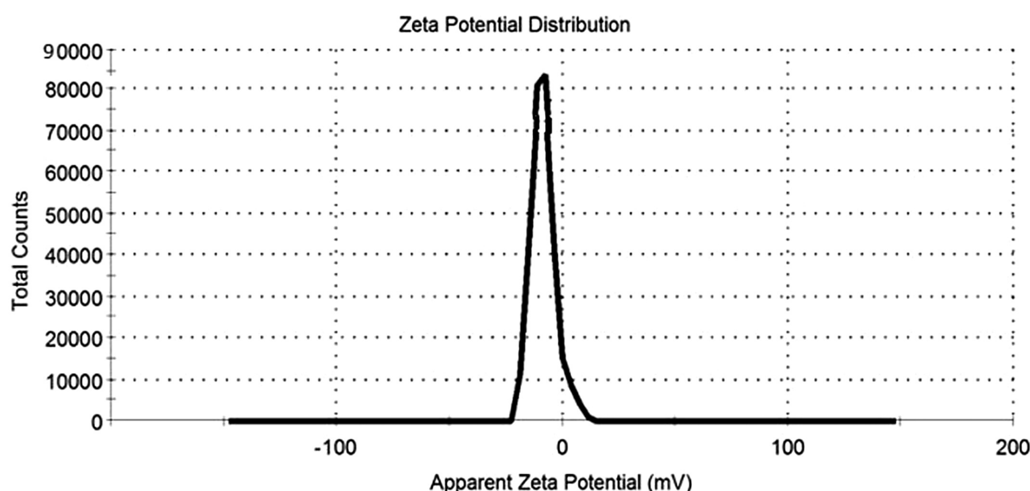
Rutin, a bioflavonoid commonly found in citrus fruits and leafy vegetables, possesses a vast range of biological practices. Some studies have highlighted its pharmacological advantages for the treatment of different chronic diseases such as cancer, diabetes, hypertension, and hyperlipidemia [43]. The common obstacle accompanied with rutin is its poor water solubility [44], which is responsible for its poor bioavailability. Improving its bioavailability will introduce this optimistic botanical product to the primacy of medication for the treatment of different chronic diseases. For poorly water-soluble agents, particle size often performs an essential impact in accomplishing sufficient oral bioavailability. Reduction of particles of poorly water-soluble drugs to the nanosize range is a beneficial strategy to enhance saturation solubility, dissolution rate, and consequently to improve oral bioavailability. During the last decades, nanoparticle-based formulations have protruded as a solution for improving the bioavailability of inherent therapeutic candidates [45].

Sodium alginate, a naturally occurring polyanionic polysaccharide derived from marine algae, is soluble in an aqueous medium. It consists of two β-D mannuronic acid (M), hexuronic acid residues, and α-L-guluronic acid (G), linked together by 1,4-glycosidic bonds. Sodium alginate possesses degradation properties in vivo. Its common properties, including controlled minimum toxicity, biocompatibility, gelation, and relatively low cost, reserve it for use in drug delivery systems and various biological applications [46]. In the current investigation, rutin was incorporated in sodium alginate gel to form rutin nanoformulation and the obtained data clarified that the developed rutin nanoparticles were made up of single spherical units with a diameter that ranged between 96.1 and 157 nm and exhibited negative zeta potential at −16.6 mV. These results fit with those of Gagliardi *et al.* [47], who recorded that poly(lactide-coglycolide) nanoparticles containing rutin are characterized by a smooth spherical shape and negative zeta potential. Furthermore, Ali *et al.* [45] registered that the designed chitosan–alginate nanoparticles of rutin exhibited an average particle size of 141.29±5.11 nm. Electron microscopy images showed all nanoparticles with a spherical shape and appearance as a nonaggregated smooth.

Effectiveness of chitocal, free rutin, and nanorutin administration on anthropometric parameters of obese rats

Table 1 presents the influence of treatment with chitocal, free rutin, and rutin nanoformulation on anthropometric measurements of obese rats. Prolonged feeding of rats with HFD motivated significant ($P<0.05$) rise in body weight, body length, TC, AC, BMI, and Lee index compared with the control group. However, treatment of obese rats with the proposed treatments provoked significant ($P<0.05$) drop in body weight, TC, BMI, and Lee index compared with the untreated obese group. Furthermore, treatment of obese rats with chitocal or rutin nanoformulation elicited a significant ($P<0.05$) decline in body length and AC when compared with those left without treatment.

Figure 2



Zeta potential of rutin nanoformulation.

Table 1 Effectiveness of chitocal, free rutin, and nanorutin administration on anthropometric parameters of obese rats

Parameters groups	Body weight (g)	Body length (cm)	TC (cm)	AC (cm)	BMI (g/cm^2)	Lee Index
Control group	120.1±3.1	20.1±0.3	11.1±0.2	12.4±0.2	0.29±0.01	0.246±0.004
Obese group	283.8±3.3[a]	21.1±0.1[a]	13.2±0.4[a]	14.9±0.4[a]	0.72±0.02[a]	0.341±0.01[a]
Ob+chitocal	175.5±3.4[b]	20.2±0.2[b]	11.3±0.2[b]	13.6±0.4[b]	0.43±0.01[b]	0.275±0.004[b]
Ob+free rutin	181.5±4.1[b]	20.5±0.2	11.7±0.4[b]	14.2±0.4	0.45±0.01[b]	0.290±0.004[b]
Ob+nanorutin	180.5±1.9[b]	20.3±0.3[b]	11.4±0.2[b]	13.9±0.3[b]	0.44±0.01[b]	0.287±0.004[b]

Data were expressed as mean±SE of 10 rats/group. AC, abdominal circumference; TC, thoracic circumference. [a]Significant change at *P* value less than 0.05 as compared to the control group. [b]Significant change at *P* value less than 0.05 as compared to the obese group.

However, treatment of obese rats with free rutin or rutin nanoformulation caused insignificant (P>0.05) increase in the measured anthropometric parameters in comparison with obese rats treated with chitocal. Also, obese rats treated with rutin nanoformulation showed an insignificant (P>0.05) decrease in these parameters relative to those treated with free rutin.

The data of the current study documented successful induction of obesity in experimental rats after being fed on HFD for 12 weeks, which is indicated by the increase in body weight, body length, TC, AC, BMI, and Lee index. These findings are in harmony with those of Pérez-Corredor *et al.* [48] and could be explained by the high deposition of fat due to the HFD regime [49].

Chitocal is a mixture of chitosan, ascorbic acid, and *G. sylvestre*. It consists of high-density chitosan, which is a natural polysaccharide containing copolymers of glucosamine and N-acetyl glucosamine, obtained through fractional deacetylation of chitin, from crustacean shells [50]. The present data revealed that treatment of obese rats with chitocal reduces body weight, length, TC, AC, BMI, and Lee index. These findings are in line with other reports published by Ahmed *et al.* [51]. Also, Bukhari *et al.* [52] found that rats treated with chitocal display a highly significant reduction in body weight gain versus the control positive counterparts. Furthermore, Safavi *et al.* [53] recorded that using antiobesity medication, such as chitocal, resulted in significantly higher mean percentage decrease in baseline weight than nonusers.

Reduction in measured parameters of anthropometry is demonstrated among the treated experimental groups with free rutin or rutin nanoformulation contrary to the untreated obese group indicating the antiobesity impact of the proposed medications. This could be referred to their higher content of the antiobesity major metabolites. Anti-inflammatory, antioxidant, antiplatelet, and antihypertensive characteristics of rutin have been established *in vitro* and *in vivo* [54,55]. By mitigating inflammation, lipogenesis, oxidative stress, and regulating glucose as well as lipid metabolism in both liver and adipose tissue [56–59] rutin could refine obesity and its attributes. It has been reported that feeding of rats with rutin shows a significant decline in body weight without altering food intake [58,60]. Moreover, rutin consumption reduced adipose size, adipogenic gene expression of sterol regulatory element-binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor gamma (PPAR-γ), AP2 adaptor complex, and AMP-activated protein kinase activity of epididymal adipose tissue, evidencing the rutin antiobesity characteristics [61]. Moreover, it has been reported that rutin administration reduces HFD-induced weight gain and adipose tissue mass, associated with enhanced mitochondrial DNA and genes expression contributed in muscular mitochondrial biogenesis and function. These data suggest that the antiobesity properties of rutin may be linked to its ability to activate muscle mitochondria [62].

Effectiveness of chitocal, free rutin, and nanorutin administration on serum lipid profile of obese rats

The data illustrated in Table 2 shows the impact of treatment with chitocal, free rutin, and rutin nanoformulation on serum lipid profile of obese rats. Prolonged feeding of rats with HFD triggered significant (P<0.05) elevation in serum Chol, TG, and LDL levels, accompanied with significant (P<0.05) depletion in serum HDL level compared with control rats. However, treatment of obese rats with chitocal, free rutin, or rutin nanoformulation caused a significant (P<0.05) drop in serum Chol, TG, and LDL levels when compared with untreated obese rats. Moreover, treatment of obese rats with chitocal or rutin nanoformulation emerged significant (P<0.05) enhancement in serum HDL level versus those left without treatment. However, in comparison with obese rats treated with chitocal, the rats treated with free rutin or rutin nanoformulation showed an insignificant (P>0.05) increase in serum Chol, TG, and LDL levels along with an insignificant (P>0.05) decrease in serum HDL level. Furthermore,

Table 2 Effectiveness of chitocal, free rutin, and nanorutin administration on serum lipid profile of obese rats

Parameters groups	Chol (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control group	72.8±4.5	75.4±2.3	38.6±1.3	26.3±2.4
Obese group	135.9±3.3[a]	122.4±4.5[a]	27.7±0.6[a]	83.7±2.6[a]
Ob + chitocal	79.8±3.1[b]	86.2±4.3[b]	35.5±1.4[b]	27.9±2.2[b]
Ob + free rutin	93.9±6.7[b]	92.9±2.9[b]	31.8±2.3	29.2±2.2[b]
Ob+ nanorutin	88.2±6.6[b]	89.7±4.5[b]	32.4±1.6[b]	28.1±1.6[b]

Data were expressed as mean±SE of 10 rats/group. HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides. [a]Significant change at *P* value less than 0.05 as compared to the control group. [b]Significant change at *P* value less than 0.05 as compared to the obese group.

the group of obese rats treated with rutin nanoformulation triggered insignificant ($P>0.05$) decline in serum Chol, TG, and LDL levels and an insignificant ($P>0.05$) rise in serum HDL level in comparison with that treated with free rutin.

Table 3 demonstrates the influence of treatment with chitocal, free rutin, and rutin nanoformulation on serum glucose, insulin, and insulin resistance values of obese rats. Supplementation with HFD brought about a significant ($P<0.05$) rise in serum glucose, insulin levels, and insulin resistance values relative to control rats. However, treatment of obese rats with the offered treatments created a significant ($P<0.05$) decline in serum glucose, insulin, and insulin resistance values compared with untreated obese rats. Also, treatment of obese rats with free rutin or rutin nanoformulation elicited an insignificant ($P>0.05$) increase in serum glucose, insulin, and insulin resistance values when compared with obese rats treated with chitocal. However, treatment of obese rats with rutin nanoformulation caused an insignificant ($P>0.05$) drop in serum glucose, insulin, and insulin resistance values in comparison with those treated with free rutin.

In this work, rats that administered an HFD manifested high serum Chol, TG, and LDL levels parallel low serum HDL level. Also, extended administration of HFD to rats triggered augmentation of serum glucose and insulin levels as well as insulin resistance value. These findings are consistent with Sowmya and Ananthi [63], Godea Lupei *et al.* [64], and Faraji *et al.* [65]. The deleterious effects of HFD on the lipid serum profile could be attributed to the induced impairment in lipid metabolism that is indicated by an increment of cholesterols, TGs, LDL, and very low density lipoprotein (VLDL) levels [63]. Rezvani-Kamran *et al.* [66] registered that the levels of Chol and LDL are increased following the suppression of receptor-dependent LDL transport in the liver. Furthermore, Videla *et al.* [67] cited that lipid accumulation initially produces insulin resistance and visceral obesity, which trigger the synthesis of fatty acids from glucose and suppress their β-oxidation resulting in overflowing of fatty acids leading to an increase in TG synthesis. Also, the dysregulation of adipokines by obesity and inflammation caused systemic insulin resistance [68].

Supplementation of HFD-fed rats with chitocal generated a reduction of serum Chol, TG, and LDL levels paralleled by an elevation of serum HDL level. Moreover, treatment of HFD-fed rats with chitocal motivated a decline in serum glucose and insulin levels as well as insulin resistance value. These findings are greatly supported by those of Bukhari *et al.* [52]. It has been reported that chitocal triggered hypolipidemic effect by the suppression of fatty acid absorption in rats [69]. Furthermore, the strong positive charge carried by the chitocal molecule (amino groups)

Table 3 Effectiveness of chitocal, free rutin, and nanorutin administration on serum glucose, insulin, and insulin resistance values of obese rats

Parameters groups	Glucose (mg/dl)	INS (mIU/ml)	Insulin resistance value
Control group	64.9±2.1	11.2±0.1	1.8±0.1
Obese group	166.3±3.8[a]	25.6±1.4[a]	10.1±0.2[a]
Ob+chitocal	66.8±4.0[b]	14.4±1.0[b]	2.3±0.2[b]
Ob+free rutin	75.2±3.8[b]	15.4±0.6[b]	2.8±0.2[b]
Ob+nanorutin	69.4±2.9[b]	14.7±0.9[b]	2.6±0.2[b]

Data were represented as mean±SE of 10 rats/group. [a]Significant change at *P* value less than 0.05 as compared to the control group. [b]Significant change at *P* value less than 0.05 as compared to the obese group.

enables it to bind with the negatively charged compounds such as lipids [70]. The possible interpretation for enhancing lipid metabolism by chitocal is related to the ability of chitocal to promote the peripheral tissue Chol transport to the liver and thereby reduce Chol in the blood [71]. Moreover, the composition of chitocal regarding the polysaccharide contents is believed to inhibit fat and Chol absorption [72]. Meanwhile, the possible underlying mechanism for increasing serum HDL by chitocal may be owing to the increase in lecithin activity [71]. However, the mechanism behind the reduction of LDL-Chol by chitocal may be related to the oxidation of LDL [69]. However, the *G. sylvestre* (present in chitocal) has been found to decrease glucose and some nutrient absorption in the gastrointestinal tract [73]. Gymnemic acid (the active compound of *G. sylvestre*) has similar configuration of glucose molecules but with a larger molecular weight giving it a high affinity to bind with glucose receptors and competitively inhibits the binding of glucose to its receptor in the small intestine [74]. Moreover, it has been cited that chitosan (present in chitocal) elicited an improvement of insulin resistance and impaired glucose tolerance in high-fructose-diet-fed rats [75]. Muanprasat and Chatsudthipong [76] registered that chitosan treatment upregulates GLUT4 gene expression in the soleus muscle and adipose tissue in type 2 diabetes mellitus-bearing rats leading to the enhancement of muscle glucose uptake. Furthermore, Bai *et al.* [77] stated that chitosan improves the impaired glucolipid metabolism in HFD-fed mice through suppressing the upregulated pro-inflammatory cytokines.

The data from the current study showed that supplementation of HFD-fed rats with free rutin or rutin nanoformulation induces a depletion of serum Chol, TG, and LDL levels paralleled by the promotion of serum HDL level. These outcomes are consistent with those of Livingston Raja *et al.* [78], who observed that rutin shows significant lipid-lowering effectiveness on Triton WR-1339-induced hyperlipidemia in rats. The lipid-lowering effects of polyphenols are attributed to multiple mechanisms including the suppression of lipid synthesis and absorption, inhibition of hepatic production of apolipoprotein B and foam cell formation, transcription factor PPARγ stimulation, LDL receptor upregulation, enhancement of HDL, and stimulation of fatty acids metabolizing enzymes such as lipoprotein lipase (LPL), 3-hydroxy-3-methylglutaryl coenzyme A, cholesterol-7α-hydroxylase, and hepatic lipase. It has been found that rutin increases plasma HDL Chol and decreases LDL and VLDL Chol. This could be explained by the enhancement of plasma lecithin cholesterol acyltransferase enzyme and LPL enzyme, which is an essential enzyme for upgrading lipoprotein metabolism and accelerating the maturation of HDL elements [79,80]. The lecithin cholesterol acyltransferase enzyme mode of action is related to the conversion of free Chol to cholesteryl ester, which is further sequestered into the core of lipoprotein particle, facilitating the formation of new HDL. Subsequently, HDL can undergo transformed back into the liver as intermediate density lipoprotein and VLDL [81]. Furthermore, the decreased TG levels due to rutin or rutin nanoformulation administration could be attributed to the intensified impact of the endothelium LPL enzyme. The LPL functions by hydrolyzing TG into fatty acids and inhibiting the reformation of fatty acids into TG [82].

Prior researches indicated that rutin has a significant impact on reducing glucose in the blood [83,84]. In line with these studies, our findings showed that treatment of HFD-fed rats with free rutin or rutin nanoformulation caused a detraction of serum glucose and insulin levels and insulin resistance value. Huang *et al.* [85] registered that rutin reduces blood glucose level and generation of advanced glycation end products that aggravate insulin resistance and oxidative stress response. When insulin binds to cell membrane insulin receptor (IR), it triggers the phosphorylation of the receptor itself and the phosphorylation of the tyrosine site of IRS-2 protein. Activation of IRS-2 by phosphorylation leads to the subsequent phosphorylation of downstream signaling factors, including Akt and glycogen synthase kinase-3 beta (GSK-3β), and phosphoinositide 3-kinase. GSK-3β phosphorylates glycogen synthase (GS) to suppress its activity in a non-phosphorylated state. However, phosphorylation of GSK-3β at Ser9 reduces GS phosphorylation, thereby increasing its activity and promoting glycogen synthesis, ultimately leading to depleted blood glucose levels [86,87]. Also, Liang *et al.* [88] elucidated that rutin has a favorable function in insulin signal transduction pathway, efficiently mitigating insulin resistance, encouraging uptake and utilization of glucose.

Effectiveness of chitocal, free rutin, and nanorutin administration on inflammatory markers of obese rats

The results in Table 4 describe the impact of treatment with chitocal, free rutin, and nanorutin on serum TLR4 and NF-κB levels of obese rats. Extended

feeding of rats with HFD elicited significant ($P<0.05$) augmentation in serum TLR4 as well as NF-κB levels contrary to control rats. On the opposite side, treatment of obese rats with chitocal, free rutin, and nanorutin triggered significant ($P<0.05$) depletion in serum TLR4 and NF-κB levels versus the untreated obese rats. Obese rats treated with free or nanorutin showed insignificant ($P>0.05$) elevation in serum TLR4 and NF-κB levels relative to those treated with chitocal. Moreover, obese rats treated with nanorutin exhibited an insignificant ($P>0.05$) drop in serum TLR4 and NF-κB levels in comparison with those treated with free rutin.

The data of the current study revealed that feeding rats with HFD for a long duration displayed an elevation in serum TLR4 and NF-κB levels. TLRs, a family of transmembrane proteins, played a fundamental role in the innate immune system [89]. They exhibit differential expression across various cell compartments. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are predominantly expressed on the cellular membrane, whereas TLR3, TLR7, TLR8, and TLR9 are primarily found in intracellular compartments like endosomes and the endoplasmic reticulum [90]. Specifically, TLR4 is expressed in innate immune cells such as monocytes, macrophages, dendritic cells, and in other cell types including enterocytes, adipocytes, and muscle cells [91]. Notably, the direct release of free fatty acids (FFA) and adipokines from visceral adipose tissue to the liver can trigger immune responses, leading to the secretion of inflammatory compounds [92]. The possible mechanism through which FFAs, and particularly dietary saturated fatty acids, can intercede adipose tissue dysfunctions participating to the onset of inflammation induces the TLR4 [93]. Indeed, the adipocyte secretion profile alterations, induce the recruitment and activation of immune cells [94]. Particularly, in obese individuals, adipose tissue macrophages relocate from the anti-inflammatory profile (like that observed in normal weight individuals) toward a pro-inflammatory phenotype [95], producing a change in the output/stimulation of key factors that aggravate local and systemic inflammation [96], such as TNFα, IL-6, IL-1β, TLR4, and NF-κB, that may intensify the inflammatory status [97]. It has been supposed that FFAs can join and activate TLR4; thus, the increased plasma level of FFAs demonstrated in obesity could stimulate TLR4. A recent study of McKernan *et al.* [98] denoted that stimulation of TLR4 can also induce inflammatory responses by impairing adipogenesis, which subsequently leads to dysfunctions in adipocytes and resident immune cells.

Table 4 Effectiveness of chitocal, free rutin, and nanorutin administration on serum toll-like receptor 4 and nuclear factor kappa B levels of obese rats

Parameters groups	TLR4 (pg/ml)	NF-κB (ng/l)
Control group	1631.5±134.9	314.9±10.4
Obese group	3593.5±223.8[a]	596.6±15.4[a]
Ob+chitocal	1857.0±84.0[b]	330.6±19.9[b]
Ob+free rutin	2204.8±141.1[b]	364.9±22.9[b]
Ob+nanorutin	2133.0±86.2[b]	341.9±13.9[b]

Data were expressed as mean±SE of 10 rats/group. NF-κB, nuclear factor kappa B; TLR4, toll-like receptor 4. [a]Significant change at P value less than 0.05 as compared to the control group. [b]Significant change at P value less than 0.05 as compared to the obese group.

Obesity-associated inflammation arises from changes in the communication between adipocytes and macrophages as a result of increased infiltration of macrophages into adipose tissue, activation of pro-inflammatory pathways, alterations in adipokine secretion, and upregulation of expression and release of various inflammatory cytokines [7]. The TLR4 pathway upregulates the pro-inflammatory cytokine expression, like TNF-α, IL-1, and IL-6, by stimulating the transcription factors NF-κB and activator protein-1 [99]. Therefore, the expression of pro-inflammatory cytokines is regulated by the stimulation of the transcription factor NF-κB. In its inactive form, NF-κB binds the NF-κB light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) in the cytoplasm. The inhibitor of κB kinase (IKK) complex, composed of subunits IKKα and IKKβ, regulates the activity of IκBα [100]. FFAs can stimulate the IKK kinase complex, leading to the proteasomal degradation of IκBα and subsequent translocation of NF-κB into the nucleus, where it induces the expression of various inflammatory mediator genes. Obese individuals often exhibit elevated activation of the NF-κB pathway, likely contributing to the increased release of pro-inflammatory cytokines [101].

Treatment of obese rats with chitocal instigated decline in both serum TLR4 and NF-κB levels. Chitosan has been demonstrated to have specific immunomodulatory impact. It can polarize the balance of cytokines toward Th1 cytokines, decreases the generation of the pre-inflammatory cytokines IL-6 and TNF-α, downregulates the expression of CD44 and TLR4 receptor, and inhibits the proliferation of T cells [102]. The study of Xiao *et al.* [103] indicated that chitosan supplementation decreases the expression of TLR4 mRNA and Xiao *et al.* [104] extended these

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findings by demonstrating that chitosan supplementation decreases TLR4 protein expression in weaned pigs challenged with entrotoxigenic *Escherichia coli*.

Chitosan or its derivatives have been found to play an important role in mitigating oxidative stress inflammatory reaction by the suppression of NF-κB signaling pathways under an inflammatory stimulus [105]. The more recent study of Mohyuddin *et al.* [106] reported the inhibitory effect of chitosan on mRNA expression level of NF-κB in rats under heat stress. Furthermore, TLR4 stimulates NF-κB through mobilization of the adaptor proteins toll/interleukin 1 receptor domain-containing adaptor protein inducing IFN-β (TRIF) and myeloid differentiation primary response gene 88 (MyD88), which results in the successive induction of NF-κB signaling genes, like TNF-α, IL-6 and IL-1 [107]. Thus, one can postulate that the inhibitory effect of chitosan on TLR4 as shown in the current approach could result in the reduction of NF-κB level.

Treatment of obese rats with free rutin or rutin nanoformulation lessened serum TLR4 and NF-κB levels. It has been cited that rutin acts as a TLR4 inhibitor in the activated hepatic stellate cells (HSCs) [108]. The anti-inflammatory mechanisms of rutin were further confirmed through its effects on TLR4, TNF receptor-associated factor 6 (TRAF6), MYD88, and NF-κB signaling pathways. The regulatory impact of rutin on TLR4, MyD88, and TRAF6 protein expression was similar to the trend observed in gene expression levels. Rutin has been found to exhibit anti-inflammatory influence by suppressing MyD88, TRAF6, and TLR4 genes as well as protein expression in LPS-induced RAW 264.7 cells [109].

It has been demonstrated that the suppression of TLR4 pathway activation leads to the inhibition of NF-κB activation [110]. Moreover, rutin can inhibit the generation of TNF-α and activation of NF-κB induced by LPS, playing a crucial role in the treatment of vascular inflammatory diseases [111]. Rutin displayed significant attenuating effect in cyclophosphamide-induced inflammation by downregulating the levels of inflammatory mediators, like IL-6 and TNF-α, and the expression levels of NF-κB, p38-mitogen-activated protein kinase (MAPK), COX-2, and iNOS [112]. Furthermore, rutin could ameliorate the inflammatory reaction by repressing the phosphorylation of IκB and p65 in LPS-induced RAW 264.7 cells [109].

Effectiveness of chitocal, free rutin, and nanorutin administration on serum leptin, adiponectin, and spexin levels of obese rats

The data presented in Table 5 elucidate the impact of treatment with chitocal, free rutin, and nanorutin on serum leptin, ADP, and SPX levels of obese rats. Feeding of rats with HFD for a long period of time promoted significant ($P<0.05$) increase in serum leptin along with significant ($P<0.05$) reduction in ADP and SPX serum levels compared with control rats. However, the recommended treatments brought about a significant ($P<0.05$) decline in leptin serum level along with a significant ($P<0.05$) rise in ADP and SPX serum levels of obese rats relative to their untreated counterparts. Meanwhile, treatment of obese rats with free or nanorutin triggered insignificant ($P>0.05$) increase in serum leptin levels associated with an insignificant ($P>0.05$) drop in serum ADP and SPX levels when compared with obese rats treated with chitocal. However, obese rats treated with nanorutin exhibited an insignificant ($P>0.05$) decline in serum leptin level and an insignificant ($P>0.05$) increase in serum ADP and SPX levels in comparison with those treated with free rutin.

Feeding rats with HFD for an extensive time elevated serum leptin levels and reduced serum ADP and SPX levels significantly. Leptin is a hormone generated by the adipose tissue, mainly by the white adipose tissue; it consists of 167 amino acids. The level of circulating leptin in the body is proportional to the amount of fat of the same body [113]. This means that leptin level is positively correlated with fat mass, being elevated in obesity [114].

ADP is synthesized as a 28–30 kD monomer, which assembles into homooligomers of varying molecular weights: low molecular weight trimeric form, medium molecular weight hexameric form, and high molecular weight oligomers [115]. Both total ADP and high molecular weight ADP oligomers are negatively

Table 5 Effectiveness of chitocal, free rutin, and nanorutin on serum leptin, adiponectin and spexin levels of obese rats

Parameters groups	Leptin (pg/ml)	ADP (mg/l)	SPX (pg/ml)
Control group	130.0±9.8	7.5±0.1	208.2±2.1
Obese group	214.7±14.7^{a}	3.7±0.3^{a}	136.7±6.5^{a}
Ob+chitocal	158.8±9.4^{b}	6.6±0.3^{b}	179.7±3.5^{b}
Ob+free rutin	176.4±6.9^{b}	6.1±0.4^{b}	167.7±4.2^{b}
Ob+nanorutin	165.0±9.8^{b}	6.3±0.1^{b}	176.4±6.5^{b}

Data were represented as mean±SE of 10 rats/group. ADP, adiponectin; SPX, spexin. ^{a}Significant change at *P* value less than 0.05 as compared to the control group. ^{b}Significant change at *P* value less than 0.05 as compared to the obese group.

correlated with BMI, glucose, insulin, and TG levels, as well as the degree of insulin resistance and visceral fat deposition [116]. The process by which adipose tissue expands through increases in cell size (hypertrophy) and/or cell count (hyperplasia) regulates the synthesis and production of ADP. Drolet *et al.* [117] observed a negative correlation between the mean diameter of adipocytes and ADP production. It has been found that adipoR1 and adipoR2 expression is significantly decreased in type 2 diabetes mellitus and obesity. In type 2 diabetes mellitus and obesity, the changes in ADP expression and its adipoRs diminish the sensitivity of ADP resulting in insulin resistance, which in turn exacerbates hyperinsulinemia [116].

SPX, also known as neuropeptide Q, is an endogenous neuropeptide. The SPX gene and protein are excessively expressed in the CNS and peripheral tissues in humans, rodents, goldfish, etc. [118]. The prepropeptide SPX consisting of 116 amino acid residues was encoded by the *C12ORF39* gene [119]. The amino acid sequence of SPX is highly preserved across different species, suggesting that this peptide has a fundamental role through biological development and may be actively contributed in the regulation of different pathological and physiological functions [120]. In the diet-induced obesity (DIO) in rats, chronic subcutaneous injection with SPX decreased the food intake [121]. These findings denote a scientific evidence for the inhibitory influence of exogenous SPX on food intake and indicate a close correlation between SPX and food control. It is well known that the uptake and storage of long-chain fatty acid are the key players for controlling body weight [122]. Treatment with SPX induced an inhibitory influence on long-chain fatty acid uptake into adipocytes isolated from untreated DIO in mice. Chronic intraperitoneal injection of SPX decreased the respiratory exchange ratio at night and elevated locomotor activity in DIO in mice [121]. Indeed, the treatment with the synthetic SPX in DIO in mice has been found to decrease body weight probably by its inhibitory effect on fatty acid uptake into hepatocytes [123,124]. In human adipocytes and murine 3 T3-L1 cells, SPX displayed a prompting impact on lipolysis and a suppressing impact on lipogenesis [125]. A study on a healthy adult woman shows that serum SPX levels are inversely correlated with age, BMI, fasting glucose, and TG. This suggests that SPX could independently predict the risk of high BMI and elevated fasting glucose [126]. Moreover, in obese women and children serum SPX levels are considerably decreased compared with the lean ones, indicating the relation between SPX and body weight regulation [125]. Also, SPX has a possible relation with glucose homeostasis; the reduction of fasting glucose also modifies the levels of SPX. The significant decrease in fasting glucose levels correlated with a significant elevation in circulating SPX levels [127]. Another study advocates the SPX role in fat tissue metabolism and lipid homeostasis [125]. In addition, a clinical study on obese individuals reveals that the SPX gene and protein are downregulated in omental and subcutaneous fat [121]. Moreover, in obese individuals the level of serum SPX was lesser than that of the healthy counterparts [128]. Furthermore, the serum SPX is negatively correlated with leptin in obese patients [121,128].

Treatment of obese rats with chitocal reduced serum leptin and elevated serum ADP and SPX levels significantly. The suppression of leptin is essential because leptin is turned on by epigenetic modulation when preadipocytes are activated to initiate the process of adipogenesis [129]. Chitosan supplementation has been found to decrease leptin level in obese adolescents. Apart from the anorexigenic (appetite suppressant) function of leptin, experimental findings offer a theoretical basis for the systemic impact of chitosan treatment in enhancing the expression of liver leptin receptor b (LepRb) and promoting signal transducer and activator of transcription 3 (STAT3) and the phosphorylation of janus kinase 2 (JAK2) [130]. Thus by activation of the JAK2-STAT3 signaling pathway chitosan could reduce leptin resistance while partially inhibiting adipogenesis.

Regarding the stimulatory action of chitocal on ADP level in obese rats in the current investigation, Fatahi *et al.* [131] supported our finding in obese adolescents. Chitosan supplementation has antiobesity and antidiabetic impacts in ob/ob mice, in addition to its role in improving ADP plasma concentrations along with its ability to increase the expression of PPAR-gamma in the adipose tissue, a key regulator of ADP production [132].

The increase in serum SPX level in obese rats administered with chitocal in the current study is linked to the negative correlation between SPX level and adiposity markers (BMI and waist and lip circumferences) as well as lipid profile markers (LDL, TG, and Chol) [133]. Moreover, a negative correlation has been found between SPX and leptin based on the serum data obtained from obese versus nonobese individuals [134]. Therefore, the inhibitory influence of chitosan on adiposity and lipid profile markers as well as leptin level in obese rats in the

present work may be the cause of elevated levels of SPX in this group.

Treatment of obese rats with free rutin or rutin nanoformulation lessened serum leptin level and enhanced serum ADP and SPX levels significantly. It has been demonstrated that rutin reduces hyperleptinemia and inhibits leptin secretion from adipose tissue as rutin improved hyperinsulinemia and dyslipidemia, and in turn regulated leptin dysfunction in fructose-fed rats [135]. Moreover, Ganjayi *et al.* [136] recorded that the treatment of 3T3-L1 adipocytes with rutin leads to reduced levels of leptin and increased levels of ADP, which might be due to increased lipolysis and fatty acid oxidation through AMP-activated protein kinase activation. In addition, rutin substantially inhibited the activity of lipase and glucosidase, enhanced glucose uptake, and decreased lipid content and adipogenesis in 3T3-L1 cells by acting on various targets [136]. Rutin could increase ADP mRNA together with promoting ADP secretion from differentiating 3T3-L1 cells. This indicates that the motivating influence of rutin on adipocyte differentiation probably takes place occurs through the upregulation of adipogenic transcription factors and downregulation of adipocyte-specific gene expression [137].

It has been demonstrated that the decreased expression of SPX gene due to elevated serum leptin in obese individuals is accompanied by impaired intake of food and energy metabolism [121]. Therefore, elevated serum leptin levels can decrease SPX expression in obese individuals. Rutin administration in fructose-fed rats could reduce hyperleptinemia and inhibit leptin secretion from the adipose tissue [135]. This finding together with our results regarding the inhibitory effect of rutin on serum leptin level in obese rats suggest that rutin can upregulate SPX gene expression by decreasing serum leptin levels and consequently enhance serum SPX level in obese rats in our study.

Conclusion

In conclusion, the results of the present attempt contribute to developing a new strategy using natural products as a therapeutic agent combined with nanobiotechnology to control obesity. The sodium alginate-based nanoformulation of rutin exhibited prominent anti-obesogenic potential through its hypolipidemic, hypoglycemic, and anti-inflammatory actions as well as its inhibitory effect on hyperleptinemia and adipogenesis. Noteworthy, the formulated nanorutin has promising antiobesity action than its free form, which may be due to the enhancement of the solubility and bioavailability by the development of rutin nanoplatform.

Limitation of the study

Although the fabricated sodium alginate-based nanoformulation of rutin proved its usefulness in the management of obesity in the present investigation, it would be better to design other similar formulations with different ratios for comparison to establish the most promising one in the treatment of obesity. Moreover, the potential toxicity or side effects of the designed nanoformulation should be performed to find out the optimal therapeutic dosage and long-term safety profile. Furthermore, the duration time for the treatment of obese rats with the nanorutin in the current study could be insufficient to achieve significant effects; and so a longer treatment duration could be more effective.

Future directions

The biomarkers assessed in the current study could be determined at the adipose and liver tissues at the protein and gene levels. Furthermore, it is important to develop functional ligand-targeted nanocarriers as antiobesity strategies that target the white adipose tissues and its vasculature for the reversal of obesity.

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Authors' contributions: S.E.K.: performed the *in vivo* antiobesity experiment on the rat model, conducted biochemical analyses, and handled statistical analysis and data interpretation. H.H.A.: proposed the idea, designed the study, and contributed to paper preparation and final revision. O.A.M.M.: performed the *in vivo* antiobesity experiment on the rat model and contributed to the assessment of biochemical results. K.F.M.: responsible for nanoformulations and preparation. H.A.A.: proposed the idea, contributed to the study design, and participated in paper preparation and revision.

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Conflicts of interest

There are no conflicts of interest.

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