Original Article

Investigating the Preventive Effects of Oral Consumption of Dactylorhiza Maculate (Salep) Hydro-alcoholic Extract on Appetite and Body Weight in Male Rats

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ABSTRACT

Obesity is the result of positive energy balance in which various hormones and neurotransmitters are involved. Using Dietary supplements is a common and popular method to lose weight. Medicinal plants with specific effects on metabolizing enzymes, blocking adipogenesis, and improving energy metabolism can be a suitable alternative to these supplements. In this study, the role of hydro-alcoholic extract of Dactylorhiza maculate (Salep) plant on obesity and its related hormones and antioxidants was investigated. Forty male Wistar rats were divided into five groups: Control, Sham, and Salep extract (three doses): 80, 160, and 320 mg/kg. The extract was fed by gavage for 29 days. After the 29th day, blood and tissue samples were taken. Rats' ELISA kits were used to measure adiponectin, obestatin, resistin, orexin-A, insulin, epinephrine, Agouti Related Neuropeptide (AgRP), omentin, chemerin, amylin, neuropeptide-Y (NPY), and ghrelin. In addition, we measured leptin, cholecystokinin (CCK), antioxidants, and lipid profile factors. Evaluation of weight changes showed that Salep extract helped the animals to lose weight significantly in the 160 and 320 mg/kg Salep groups. Leptin, adiponectin, AgRP, obestatin, CCK, chemerin, adiponectin, and total antioxidants displayed a significant increase compared to the control group. In contrast, ghrelin, omentin, resistin, NPY, amylin, orexin-A, epinephrine, and Malondialdehyde (MDA) decreased in the Salep groups. The lipid profile was also affected by the extract. These findings suggest that the Salep extract prevents appetite, reduces ghrelin, and affects digestive factors; the Salep extract can change the secretory factors of adipose tissue and lipid profile and ultimately help to lose weight.

Keywords: Appetite, Body Weight, Rat, Salep

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1. Introduction

Obesity, as a result of positive energy balance, is a chronic disease in which various hormones and neurotransmitters are involved. Adipose tissue hormones, adipokines, and cytokines (1, 2) such as leptin and adiponectin (3), brain neuropeptides related to energy homeostasis and appetite, such as neuropeptide Y (NPY) (3,9), gastrointestinal hormones such as insulin, ghrelin, cholecystokinin (CCK), and peptide YY (PYY), and finally peripheral signals participate, such as fatty acids are affected by energy imbalance and obesity (4). Dietary changes, exercises, and medications are common treatments for obesity (5, 6). In obese patients, with changes in energy levels, NPY expression increases, and plasma leptin and insulin levels decrease (7). Today, the use of dietary supplements is the most common method for managing weight loss. Herbal medicine can be a suitable alternative to these supplements due to their function in reducing lipid and carbohydrate metabolizing enzymes, blocking adipogenesis, and improving energy metabolism (8). For example, the use of ginseng would prevent food over-intake by affecting leptin resistance, CCK expression, and NPY secretion (6), or the use of a traditional Japanese Kampo medicine, including 12 natural components (11 plants and a fungus), increase intracellular calcium in NPY neurons to treat anorexia (8). Oxidative stress (OS) and increased reactive oxygen species (ROS) can affect obesity. The OS plays a critical role in obesity-related disorders. In this regard, the benefits of antioxidant supplements such as carnitine and vitamins in obesity management were studied and proved (9). Investigating herbal plants with antioxidant activity is vital. Studies have shown that these plants can affect the expression of genes associated with obesity, including inflammation (10, 11). Dactylorhiza maculate (Salep) belongs to the orchid family; it has many species and grows almost all over the world. Despite its proven antioxidant role, this plant contains water-soluble fiber called glucomannan, which has long been known for its role in weight loss, blood sugar control, and cholesterol reduction (12). This component can control body weight by reducing the rate of emptying and absorbing in the digestive system, increasing the secretion of CCK, and regulating the secretion of leptin (13). In this study, we attempted to investigate the role of the hydro-alcoholic extract of Orchis maculate L. roots on obesity and its related hormones in male Wistar rats. The benefit (efficiency) of Salep extract on neuropeptide-Y, agouti protein, insulin, leptin, ghrelin, cholecystokinin, amylin, orexin-A, obestatin, adiponectin, chemerin, omentin-1, epinephrine, and resistin was investigated.

2. Materials and Methods

2.1. Preparation of Salep Extract

Around 100 g Salep roots were mixed with ethyl alcohol (500 cc) and placed in a rotary for 24 h in a cool environment. The filtered solution was mixed by centrifugation (5 min/3,000 rpm). The solution was kept constant, at room temperature, for 48 h to turn into a solid and dry alcohol-free extract. The obtained dry extract was dissolved in distilled water (14).

2.2. Determination of Extract Purification

2.2.1. Phenol and Flavonoid Determination:

Phenol and flavonoid measurement was performed according to the Tali procedure kit. The flavonoid concentration based on the quercetin standard curve was reported (40.96 µg/ml), and phenol concentration based on a gallic acid standard curve in 100mg/ml Salep extract (8.81 mg/ml) was reported.

2.2.2. Total Antioxidant Activity Determination:

The antioxidant activity of the extract (39.7%) was assessed using the ROS inhibitory method called 2,2-Diphenyl-1-picrylhydrazyl DPPH. In this way, first, 1 ml of Salep extract is combined with 3 ml of DPPH methanolic solution. Then, it is stored in a dark and warm place for 30 min. After that, the absorbance of the solution was read in 517 nm wavelength. Methanol was also used as a blank sample (15). Finally, inhibitory activity was calculated based on the following formula:

$$\% \text{ Inhibitory activity} = [(A_{control} - A_{sample})/A_{conntrol}] * 100$$

2.3. Animals of Study

Forty male Wistar rats (180-200 mg/kg, aged 8-9 weeks) were used with a light/dark cycle of 12/12 h, 23 ± 2°C room temperature, 50-55% humidity, and free access to food (Don Pars, Iran), and water.

2.4. Study Design

Animals were divided into six groups: control group (received no substances), sham group (1ml of distilled water and alcohol (50:50)), and four doses of hydroalcoholic extract of Salep, 80, 160, and 380 mg/kg (Sa 80, 160, and 380 mg/kg). Experiments were conducted according to the Ethics Committee of Jahrom University of Medical Sciences guideline (Ethics Code: IR.JUMS.REC.1395.032). The extracts were fed to animals daily by gavage (9-10 a.m.) for 29 days. Animals' glucose level was assessed, using tail blood, on days (zero, seventh, 14th, 21st, and 28th days).

2.5. Sample Collection

After the 29th day, blood samples were collected from the weighted animals; they were anesthetized using Ketamine (100 mg/kg) and Xylazine (20 mg/kg), and the heart, arterial blood, and tissue of sacrificed animals were extracted (16). Then, their serum was collected by the centrifugal device (for 15 min and 3,000 rpm) and kept at

-20°C until the experiment's time. To measure the Gonadotropin hormone-releasing hormone (GnRH), Follicle-stimulating hormone (FSH), Luteinizing hormone (LH) GnRH, FSH, LH, estrogen, and progesterone hormones, ELIZA kits for rats made by Shanghai Crystal Day Biotech Company were used.

2.6. Biochemical Analysis

The samples were used to determine blood insulin (Cat. n:E0707Ra; Assay Range: 0.1-40 mIU/L; Sensivity: 0.05), AgRP (Cat. n:E1274Ra; Assay Range: 5-1500 ng/L; Sensivity: 2.43), adiponectin (Cat. n:E0858Ra; Assay Range: 0.2-60 mg/L; Sensivity: 0.12), leptin (Cat. n:E0561Ra; Assay Range: 0.1-20 ng/ml; Sensivity: 0.05), resistin (Cat. n:E0211Ra; Assay Range: 5-1000 pg/ml; Sensivity: 2.52), obestatin (Cat. n:E0229Ra; Assay Range: 10-3000 ng/L; Sensivity: 5.17), omentin (Cat. n:E0607Ra; Assay Range: 2-600 ng/L; Sensivity: 1.12), cholecystokinin (Cat. n:E0199Ra; Assay Range: 5-2000 ng/L; Sensivity: 2.52), epinephrine (Cat. n:E0545Ra; Assay Range: 0.5-200 ng/ml; Sensivity: 0.26), amylin (Cat. n:E0718Ra; Assay Range: 0.5-200 ng/L; Sensivity: 0.29), Chemerin (Cat. n:E0864Ra; Assay Range: 1-300 ng/L; Sensivity: 0.52), NPY (Cat. n:E0540Ra; Assay Range: 3-900 ng/L; Sensivity: 1.46), ghrelin (Cat. n:E0896Ra; Assay Range: 50-10000 ng/L; Sensivity: 20.59), and orexin-A (Cat. n:E0105Ra; Assay Range: 0.3-1500 ng/L; Sensivity: 0.15), Malondialdehyde (MDA) (Cat. n:E0156Ra; Assay Range: 0.05-10 nmol/ml; Sensivity: 0.024), Glutathione peroxidase (GSHPx) (Cat. n:E0814Ra; Assay Range: 5-1000 U/ml; Sensivity: 2.21), total antioxidant capacity (TAC) (Cat. n:E0871Ra; Assay Range: 0.2-60 U/ml; Sensivity: 0.11), and total oxidant status (TOS) (Cat. n:E1512Ra; Assay Range: 0.2-60 U/ml; Sensivity: 0.13) level were also evaluated by Rat ELISA kits (SHANGHAICRYSTAL DAY BIOTECH COMPANY-CHINA). Lipid profile factors (triglyceride (TG), total cholesterol (TC), LDL (low-density lipoprotein), and HDL (high-density lipoprotein) evaluated by Pishtazteb kits.

2.7. Statistical Analysis

The collected data was analyzed by a one-way ANOVA test using SPSS software (version 21) (SPSS Inc., Chicago, IL, USA). A $P \leq 0.05$ was considered statistically significant. Differences between groups for continuous variables were examined using post hoc Duncan and Tukey tests.

3. Results

Evaluation of weight changes demonstrated that using Salep could help to reduce animals' weight loss. The groups receiving 160 and 320 mg/kg Salep had more weight loss than the control group and the lower doses ($P \leq 0.05$; Figure 1). The evaluation of Agouti Related Neuropeptide (AgRP) and NPY in different doses showed a significant increase in NPY in the higher dose compared to the lower doses and the control group, while AgRP did not show a significant change. Glucose assessment was done over 28 days (days 0, 7, 17, 21, and 28), and data demonstrated that Salep (160 and 320 mg/kg) did not change the glucose level of animals on days 0, 7, and 14, but it was effective on days 21 and 28. The evaluation of these days showed that the use of Salep reduced animals' blood glucose dose-independently, higher doses, lower blood sugar (Figure 1). Leptin showed a significant increase in groups treated with 160 and 320 mg/kg compared to the control group. Higher dosages of Salep were more effective than lower doses. The evaluating ghrelin and insulin indifferent doses showed a significant increase in each dose compared to lower doses and control group (Figure 2). The mean concentrations of cholecystokinin, amylin, omentin, epinephrine, and obestatin in the Salep group showed a significant increase compared to the control group. At the same time, different groups also showed significant differences. Furthermore, the evaluation of adiponectin revealed this effect compared to the control group, although only the highest dose had a significant effect on the concentration of this hormone. Examination of chemerin, orexin-A, and resistin at different concentrations showed that Salep usage (160 and 320 mg/kg) controlled these factors. Examining the effectiveness of Salep showed a significant difference between higher and lower doses of resistin and chemerin. It was also found that higher concentrations greatly reduce orexin-A (Table 1). The lipid profile of different groups is evaluated in Table 2. The HDL increased in Salep groups compared to the control group ($P \leq 0.05$), while LDL, TC, and TG were significantly lower in Salep 160 and 320 mg/kg. Comparing groups showed that 320 mg/kg Salep is more effective in reducing these factors ($P \leq 0.05$; Table 2). Evaluation of TAC, Superoxide dismutase SOD, and GSHPx showed the antioxidant properties of Salep. Three higher doses of Salep (80, 160, and 320 mg/kg) significantly altered TAC and GSHPx levels. At the same time, there were no differences in SOD levels between 160 and 320 mg/kg. Comparing treatment groups showed the role of Salep in MDA level; higher doses were more effective than lower ones (Table 3).

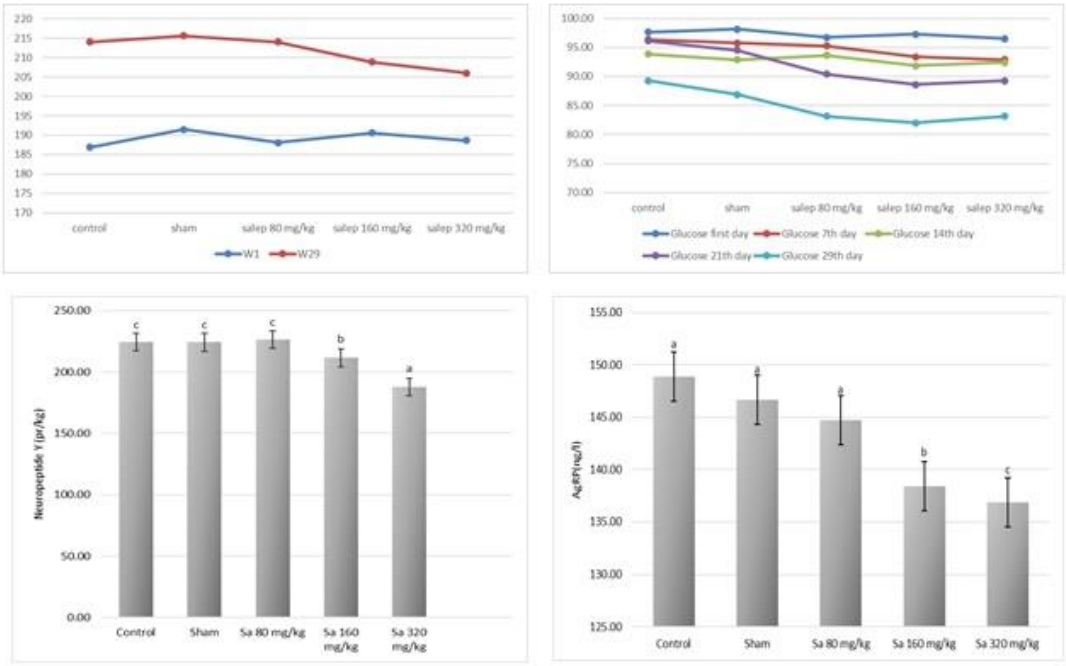

Fig.1. weight loss, AgRP, NPY, and Glucose changes in different doses of Salep extract in comparison with control group.

W1, the first day weight. W29, the 29th day weight.

Each group means with at least one shared letter has no significant difference. P<0.05 is considered statistically significant.

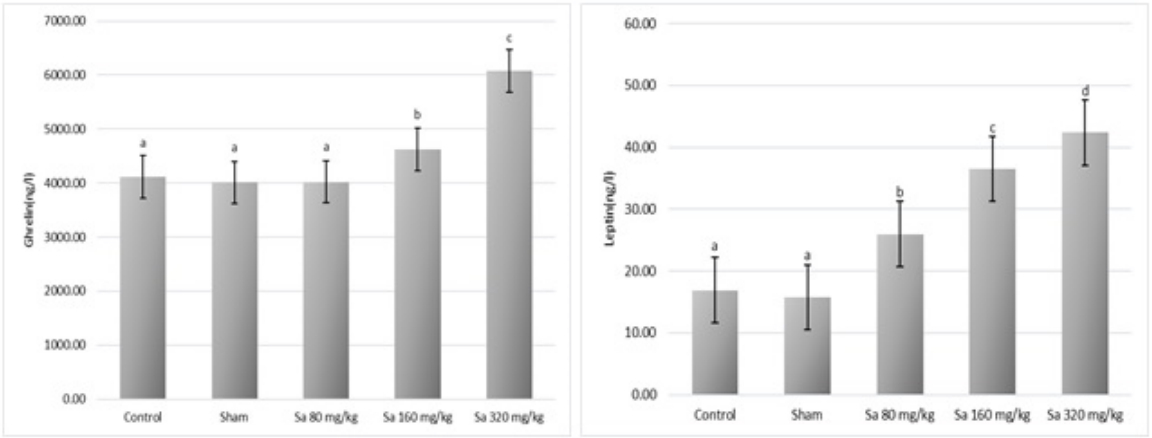

Fig.2. Leptin and ghrelin changes in different doses of Salep extract in comparison with control group.

Each group means with at least one shared letter has no significant difference.

P<0.05 is considered statistically significant.

Table 1. Resistin, obestatin, cholecystokinin, chemerin, orexin-A, omentin-1, amylin, adiponectin, and epinephrine changes in various doses of Salep extract in comparison with the control group

Groups	Control	Sham	Sa 80 mg/kg	Sa 160 mg/kg	Sa 320 mg/kg
Resistin (Mean ± SEM)	175 ± 3.93^{d}	176.58 ± 3.13^{d}	145.55 ± 2.38^{c}	161.64 ± 2.43^{b}	117.63 ± 2.38^{a}
Agouti-related peptid (Mean ± SEM)	148.87 ±2.88^{a}	146.65 ± 2.16^{a}	144.73 ± 3.70^{a}	138.42 ± 4.35^{a}	136.86 ± 7.10^{a}
Obestatin (Mean ± SEM)	105.17 ± 3.08^{a}	103.11 ± 3.12^{a}	125.54 ± 2.64^{b}	137.15 ± 2.65^{c}	149.86 ± 3.33^{d}
Cholecystokinin (Mean ± SEM)	1109.42 ± 12.2^{a}	1091.13 ± 14.1^{a}	1199.98 ± 14.1^{b}	1282.83 ± 12.1^{c}	1370.92 ± 12.7^{d}
Chemerin (Mean ± SEM)	26.80 ± 0.71^{c}	25.99 ± 0.78^{c}	25.54 ± 0.85^{c}	21.98 ± 0.90^{b}	14.69 ± 0.61^{a}
Orexin (Mean ± SEM)	241.96 ± 3.83^{b}	240.08 ± 4.08^{b}	236.59 ± 3.91^{b}	229.40 ± 2.64^{b}	170.80 ± 6.07^{a}
Omentin-1 (Mean ± SEM)	5.39 ± 0.21^{a}	5.46 ± 0.22^{a}	5.41 ± 0.15^{a}	6.66 ± 0.32^{b}	8.49 ± 0.33^{c}
Amylin (Mean ± SEM)	28.71 ± 0.90^{a}	26.65 ± 0.93^{a}	27.59 ± 2.12^{a}	34.05 ± 1.22^{b}	48.40 ± 1.73^{c}
Epinephrine (Mean ± SEM)	47.53 ± 1.67^{a}	49.13 ± 1.48^{a}	49.20 ± 1.77^{a}	55.61 ± 2.29^{b}	72.56 ± 2.46^{c}
Adiponectin (Mean ± SEM)	10.05 ± 0.19^{a}	9.98 ± 0.21^{a}	10.26 ± 0.24^{ab}	11.14 ± 0.47^{b}	13.44 ± 0.37^{c}

Each group means with at least one shared letter has no significant difference. P<0.05 is considered statistically significant.

Table 2. Lipid profile changes in different doses of Salep extract in comparison with control group

Groups	Control	Sham	Sa 80 mg/kg	Sa 160 mg/kg	Sa 320 mg/kg
Total Cholesterol (Mean ± SEM)	53.63 ± 4.54^{c}	50.50 ± 1.66^{c}	45.00 ±3.98^{bc}	41.00 ± 2.56^{ab}	33.38 ±1.22^{a}
Low-density lipoprotein (Mean ± SEM)	111.25 ± 3.67^{c}	113.00 ± 3.23^{c}	91.75 ± 8.39^{b}	80.25 ± 6.98^{b}	32.13 ± 3.08^{a}
High-density lipoprotein (Mean ± SEM)	21.75 ± 0.49^{a}	22.38 ± 0.86^{a}	27.50 ± 1.95^{b}	33.25 ± 1.69^{c}	36.38 ± 1.78^{c}
Triglyceride (Mean ± SEM)	131.13 ± 2.97^{c}	126.63 ± 4.83^{c}	116.00 ± 4.66^{bc}	106.50 ± 5.55^{b}	63.50 ± 7.24^{a}

Each group means with at least one shared letter has no significant difference. P<0.05 is considered statistically significant.

Table 3. Antioxidant changes in different doses of Salep extract in comparison with the control group

Groups	Control	Sham	Sa 80 mg/kg	Sa 160 mg/kg	Sa 320 mg/kg
TAC (Mean ± SEM)	3.42 ± 0.16 [a]	3.57 ± 0.16 [a]	4.24 ± 0.11 [b]	4.59 ± 0.19 [bc]	5.00 ± 0.08 [c]
SOD (Mean ± SEM)	3.37 ± 0.34 [a]	3.55 ± 0.29 [a]	4.01± 0.17 [a]	5.02 ± 0.29 [b]	5.60 ± 0.29 [b]
MDA (Mean ± SEM)	1.66 ± 0.04 [d]	1.57 ± 0.07 [d]	1.41± 0.03 [c]	1.21 ± 0.02 [b]	1.06 ± 0.03 [a]
GSHPx (Mean ± SEM)	60.33 ± 3.08 [a]	62.09 ± 2.73 [a]	67.02 ± 3.31 [a]	81.97 ± 1.83 [b]	97.01± 3.28 [c]
TOS (Mean ± SEM)	1.08 ± 0.02 [d]	1.04 ± 0.02 [d]	0.92 ± 0.02 [c]	0.78 ± 0.02 [b]	0.58 ±0.02 [a]
TOS/TAC (Mean ± SEM)	0.32 ± 0.01 [d]	0.29 ± 0.01 [d]	0.22 ± 0.01 [c]	0.17 ± 0.01 [b]	0.12± 0.00 [a]

Each group means with at least one shared letter has no significant difference. P<0.05 is considered statistically

4. Discussion

The role of plants in weight loss and obesity has been investigated in many studies (8, 17, 18); our study demonstrated the role of Salep on weight loss and obesity-related hormones in animals. Animals treated with Salep had altered appetite hormones and lipid profiles, and this herb had changed the antioxidative system. Salep had increased antioxidants such as SOD, TAC, and GSHPx. On the other hand, treated animals had reduced MDA. Calculation of the TOS/TAC ratio showed that increasing TAC reduced this ratio. The antioxidant potential of this plant was investigated in previous studies (14, 19). These studies believed that Salep flavonoids, polyphenols, and glucomannan are the main factors affecting the antioxidant system (19, 20). Quercetin, a known Salep flavonoid, reduces lipoxygenase and affects gene expression of the oxidative system enzymes (20). Although Salep administration affected appetite-related neuropeptides (e.g., NPY), it had no effect on AgRP. Another member of this family, Ocimum sanctum Linn ethanolic extract (OSE), affected NPY concentration due to its role in hippocampal area neurons (21). Although this study aimed to investigate the effects of OSE on hippocampal neurons in Alzheimer's animal models, a significant increase in NPY expression OSE groups was found. We believe that OSE can stimulate NPY expression. However, previous studies showed that obesity causes long-lasting desensitization of AgRP neurons, and perhaps because of this desensitization, our plant could not affect AgRP in the short term and needs more time (22). The results illustrated that ghrelin decreased and obestatin increased. These hormone balance changes in response to diet or before and after meals (23). In addition, exercise decreases ghrelin or increases obestatin levels (24). Various studies have investigated the role of CCK on meal patterns and caloric intake (25); our results showed an increase in the level of CCK, which is in agreement with the role of some other herbs (26). Data analysis showed that Salep increases the secretion of amylin and weight loss impacted by this plant. The effect of plants on gastrointestinal hormones needs further investigation. Insulin promotes triglyceride storage, fatty acid uptake, and their related gene expression; on the other hand, obesity alters adipocytes and muscle metabolism, so insulin balance is essential in controlling obesity. Salep extract affects animal insulin and glucose and reduces obesity. Animals receiving Salep had reduced resistin and chemerin; it also increased epinephrine and omentin compared to the control group. The role of different plants in disrupting fatty acid-synthesizing enzymes and lipid metabolism was investigated in many studies (27, 28). With the proven role of adipose tissue-related hormones in obesity, the success of Salep in obesity could be through its effect on adipose tissues. The role of leptin and adiponectin in obesity is obvious; therefore, our study illustrated that the use of Salep not only increased leptin but also affected adiponectin in treated animals. Several studies showed this positive relationship between different herbs and serum adiponectin. Still these studies believed the benefits of herbs for leptin reduction, which agreed with our data (29). In conclusion, this study indicated that supplementation with Salep extract improves animal weight loss, especially at higher doses. These findings suggest that Salep reduces NPY and orexin-A, thereby suppressing appetite. This herb also increases obestatin and CCK and reduces ghrelin. Furthermore, Salep has the power to alter secretory factors of adipose tissue to lose weight and reduce lipid profile.

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Authors' Contribution
Acquisition of data: H.H, H.K.J, N.S.J, R.M, S.R, N.S, M.D, and S.D
Analysis and interpretation of data: H.K.J, B.E, and M.M
Drafting of the manuscript: H.H, M.M, B.E, M.D, and H.K.J
Critical revision of the manuscript for important intellectual content: All of the authors
Statistical analysis: H.K.J, H.H and M.M
Administrative, technical, and material support: H.K.J and N.S.J
Study supervision: All of the authors

Ethics
Experiments were conducted according to the Ethics Committee of Jahrom University of Medical Sciences (Ethics Code: IR.JUMS.REC.1395.032).

Conflict of Interest
Authors disclose any conflict of interest in accordance with journal policy.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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