

Rhinacanthins-rich Extract Enhances Glucose Uptake and Inhibits Adipogenesis in 3T3-L1 Adipocytes and L6 Myotubes

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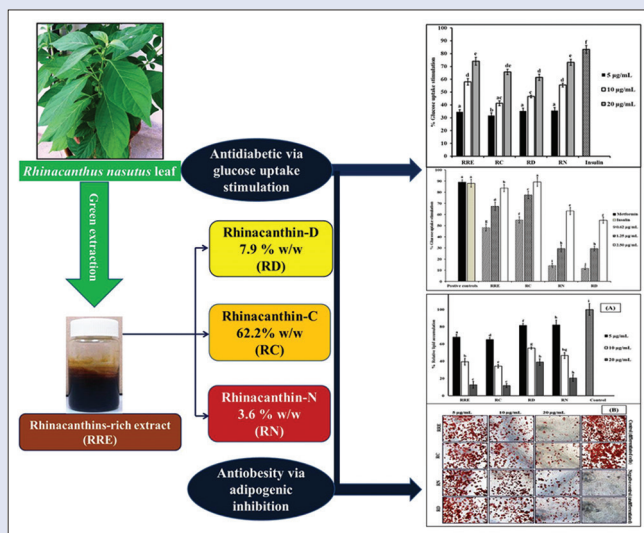
ABSTRACT

Background: Obesity is one of the imperative dynamics in the incidence and intensification of type 2 diabetes mellitus (T2DM). *Rhinacanthus nasutus* leaf extracts are previously reported for their antidiabetic and antiobesity potential. **Objective:** The present study was performed to evaluate glucose uptake stimulatory and antiadipogenic activities of a standardized rhinacanthins-rich extract (RRE) and its marker compounds namely rhinacanthin-C (RC), rhinacanthin-D (RD), and rhinacanthin-N (RN) in 3T3-L1 and L6 cells. **Materials and Methods:** RRE was prepared by a green extraction process, and the marker compounds (RC, RD, and RN) were isolated from the RRE using a silica gel column chromatography. Glucose uptake stimulation in both 3T3-L1 and L6 cells was performed by quantification of residual glucose in the media using glucose oxidase kit. Antiadipogenic activity in 3T3-L1 adipocytes was performed by intracellular lipids quantification using oil red O dye. **Results:** At the highest effective dose, RRE (20 µg/mL) exhibited satisfactory glucose uptake stimulatory effect in 3T3-L1 adipocytes that equivalent to RN (20 µg/mL) and the positive control insulin (0.58 µg/mL) but higher than RC (20 µg/mL) and RD (20 µg/mL). In addition, treatments of L6 myotubes showed that RRE (2.5 µg/mL) exhibited potent and equivalent glucose uptake stimulation (>80%) to RC (2.5 µg/mL) and the standard drugs, insulin (2.90 µg/mL) and metformin (219.5 µg/mL), but higher than RD (2.5 µg/mL) and RN (2.5 µg/mL). Furthermore, RRE (20 µg/mL) exhibited potent antiadipogenic effect in 3T3-L1 adipocytes, which equivalent to RC (20 µg/mL) but higher than RD (20 µg/mL) and RN (20 µg/mL). **Conclusions:** The undertaken study suggests that RRE could be used as an effective remedy in the treatment of obesity-associated T2DM.

Key words: Antidiabetic, antiobesity, rhinacanthin-C, rhinacanthin-D, rhinacanthin-N

SUMMARY

- Rhinacanthins-rich extract and its marker compounds showed potent glucose uptake stimulatory activity in 3T3-L1 adipocytes and L6 myotubes
- Rhinacanthins-rich extract and rhinacanthin-C showed comparable antiadipogenic effect in 3T3-L1 adipocytes
- RRE could be used as an effective remedy in the treatment of obesity-associated T2DM.



Abbreviations used: T2DM: Type-2 diabetes mellitus; RRE: Rhinacanthins-rich extract; RC: Rhinacanthin-C; RD: Rhinacanthin-D; RN: Rhinacanthin-N; α -MEM: α -Minimum essential medium; DMEM: Dulbecco's modified Eagle's medium; HS: Horse serum; FBS: Fetal bovine serum; BSA: Bovine serum albumin; IBMX: 3-isobutyl-1-methylxanthine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GO: Glucose oxidase; NMR: Nuclear magnetic resonance; HPLC: High-performance liquid chromatography.

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INTRODUCTION

According to the World Health Organization, type 2 diabetes mellitus (T2DM) is a major type of diabetes comprising 90% of total diabetic cases.^[1] Hyperglycemia and hyperlipidemia are prime characteristics in the progression of T2DM and chronic cardiovascular disorders.^[2,3] Insulin resistance, the main cause of T2DM, is linked with the release of free fatty acids and proinflammatory cytokines from adipose tissues in obesity or excessive adiposity, which stimulate beta-cells for oversecretion of insulin and its receptors reduction.^[4,5] The global prevalence of obesity and overweight raised to almost double with the

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reported 600 million obese adults and 41 million obese children having high mortality than underweight.^[6] Along with other adverse effects, both insulin and noninsulin therapy in T2DM promote weight gain probably via adipogenesis, the foremost cause of T2DM.^[7,8] The therapeutic molecule that can effectively control hyperglycemia with antiadipogenic potential would be an ideal antidiabetic agent. Therefore, adipogenic inhibition and glucose uptake stimulation in adipose and muscle tissue present the prominent strategies to control obesity-associated T2DM.^[9]

Plant extracts and purified phytochemicals are known as highly valuable sources of novel therapeutic molecules that offer a potential alternative to currently used drugs, which may be associated with side effects. Various plant extracts and phytochemicals have been reported to offer potential as antidiabetic and antiobesity drugs.^[10,11] *Rhinacanthus nasutus* (L.) Kurz (family *Acanthaceae*), a medicinal herb native to Thailand and Southeast Asia, has traditionally been used in the treatment of various disorders including DM.^[12] In China and Taiwan, *R. nasutus* has been consumed as an herbal drink.^[13,14] Methanol extracts of *R. nasutus* leaf have been investigated extensively for antidiabetic and hypolipidemic activity.^[15-19] *R. nasutus* leaf extracts have also been reported for antiobesity effect.^[20,21] Rhinacanthin-C (RC), a major phytochemical of *R. nasutus* leaf, has been recently reported for antidiabetic, hyperlipidemic, and pancreatic protection effects in diabetic rats.^[22] However, the multistage and high-cost purification process of RC hinders drug development. Standardized rhinacanthins-rich extract (RRE) is a semi-purified extract obtained from *R. nasutus* leaf that contains almost 70% w/w rhinacanthins in total, with 60% w/w of RC as the major constituent.^[23] RRE offers significant advantages as an alternative to RC in terms of lower production cost and potentially equivalent or higher bioactivity due to synergism among RRE components.^[23-25] In the present study, RRE was obtained using a simple, environment-friendly, green extraction process to investigate its glucose uptake stimulatory and antiadipogenic effects in 3T3-L1 adipocytes and L6 myotubes.

MATERIALS AND METHODS

Chemicals

α -Minimum essential medium (α -MEM), Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), fetal bovine serum (FBS), and bovine serum albumin (BSA) were obtained from Gibco, Canada. Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), oil red O dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, metformin, insulin, and glucose oxidase (GO) kit were purchased from Sigma-Aldrich, USA. All other chemicals used were of analytical grade.

Cell lines

The 3T3-L1 preadipocytes and L6 myocytes were obtained from the American Type Culture Collection (USA).

Plant material, extraction, and isolation

The fresh leaves of *R. nasutus* were collected from the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus, Thailand, and a voucher specimen (No. 0011814) was kept at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Thailand. The leaves were washed with tap water and dried at 60°C for 24 h in a hot air oven and reduced to powder using a grinder, and the powders were passed through a sieve No. 45.

RRE was prepared using ethanol by previously described method^[23] with some modifications using green extraction process. RC, RD, and RN were purified from the RRE using a silica gel column eluted by hexane and ethyl acetate (99:1, v/v). The structures of all three rhinacanthins [Figure 1] were confirmed by comparing the ¹H and ¹³C-NMR spectral data with those from the literature.^[26,27]

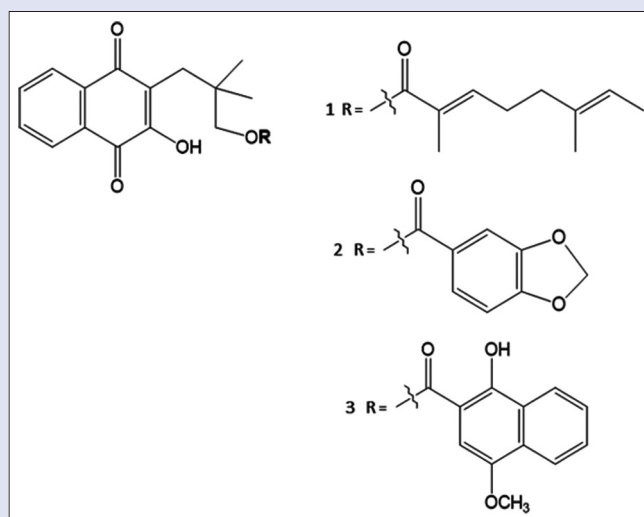


Figure 1: Chemical structures of rhinacanthin-C (1), rhinacanthin-D (2) and rhinacanthin-N (3)

High-performance liquid chromatography analysis of rhinacanthins-rich extract

High-performance liquid chromatography (HPLC) analysis of RRE was performed as previously described method^[23] using a UltraFast Liquid Chromatograph Shimadzu System incorporating a Discovery⁷ C18 (5 μ m, 4.6 mm \times 150 mm) column (Supelco, PA, USA) equipped with a photodiode-array detector and autosampler (Shimadzu Corp., Kyoto, Japan). HPLC analysis showed that RRE contained RC (62.2% w/w) as a major compound, and RD (7.9% w/w) and RN (3.6% w/w) were the minor compounds.

Determination of cell viability

Cell viability of both 3T3-L1 and L6 cells was determined using MTT reduction assay. After treatment with various concentrations of RRE and its marker compounds, the supernatant was removed, and the cells were incubated with 200 μ L MTT solution (0.5 mg/mL) for 4 h at 37°C under 5% CO₂. The supernatant was carefully removed and 200 μ L of dimethyl sulfoxide was added to dissolve the formazan. Absorbance was measured with a microplate reader at 570 nm. Cell viability was expressed as a percentage of control.

Glucose uptake stimulation assay in 3T3-L1 adipocytes

Glucose uptake stimulatory effect of RRE and its marker compounds was evaluated in 3T3-L1 adipocytes by previously described methods.^[28,29] Briefly, the cells were grown in 48-well plates with serum-free DMEM containing 0.2% BSA for 12 h. The cells were washed and incubated with different concentrations of samples in low glucose medium supplemented with 10% FBS for 24 h. Insulin was used as a standard drug. The medium was collected in 96-well plate, and glucose uptake assay was performed by the GO method using commercial GO kit.

Glucose uptake stimulation assay in L6 myotubes

Glucose uptake stimulatory effect of RRE and its marker compounds was determined in L6 myotubes by previously reported method.^[30] Briefly, L6 myocytes were grown in α -MEM with 10% FBS at 37°C under 5% CO₂. Differentiation to L6 myotubes was performed by 2% HS containing medium in 48-well culture plates. The various amounts of

samples were incubated with the cells for 24 h. Insulin and metformin were used as positive controls. After incubation, the media were collected in 96-well plate and the glucose contents were measured by GO method using commercial GO kit.

Antiadipogenic assay in 3T3-L1 adipocytes

Antiadipogenic effect of RRE and its marker compounds was determined by previously described method.^[31] Briefly, the 3T3-L1 preadipocytes were cultured in high-glucose DMEM supplemented with 10% FBS at 37°C under an atmosphere of 95% air and 5% CO₂. Two days postconfluent, the cells were incubated in differentiation medium (1 μM dexamethasone, 10 μg/mL of insulin, and 0.5 mM IBMX in DMEM) along with various concentrations of samples. The level of differentiation or adipogenesis was determined using oil red O staining.^[31]

RESULTS AND DISCUSSION

Glucose uptake stimulatory effect in 3T3-L1 and L6 cells

On the basis of MTT assay, RRE, RC, RD, and RN at various concentrations (0.63–20 μg/mL) showed low cytotoxicity on both 3T3-L1 and L6 cells with cell viability of 80%–100% [Figure 2]. Insulin resistance is the major cause of T2DM, the search of small molecules with insulin-like glucose uptake stimulation potential is an effective approach in diabetic treatment. Based on the previous

glucose uptake report of RC,^[22] RRE and its naphthoquinone constituents, RC, RD, and RN, were evaluated for their glucose uptake stimulation effect in differentiated 3T3-L1 adipocytes by GO method. The results showed that RRE and RN exhibited higher glucose uptake stimulation effect than RC and RD and in a dose-dependent manner (5, 10 and 20 μg/mL). The activity at concentration of 20 μg/mL was almost equivalent to the positive control, insulin (0.58 μg/mL) [Figure 3]. The mechanism of glucose uptake enhancement by 1,4-naphthoquinones of RRE may be via an insulin-independent tyrosine kinase pathway, which is previously reported for shikonin, a 1,4-naphthoquinone of *Lithospermum erythrorhizon*.^[32] This is a preliminary study; however, it provides an interesting research insight to elucidate in-depth and exact glucose enhancement mechanism of rhinacanthins in 3T3-L1 adipocytes. Furthermore, the glucose uptake stimulation along with adipogenic inhibitory potential of RRE provides an interesting strategy to control obesity-associated T2DM and other related complications.

Regarding the body mass, skeletal muscles are the major body part which utilizes 80% of blood glucose, presenting a prominent therapeutic target for diabetic treatment.^[29] Based on the previous reports on muscular glucose uptake stimulatory potential of 1,4 naphthoquinone,^[33,34] RRE and its naphthoquinone compounds (RC, RD, and RN) were determined for their glucose uptake enhancement potential in L6 myotubes. RRE possessed higher glucose uptake-enhancing activity than RD and RN in a dose-dependent manner (0.63, 1.25, and 2.5 μg/mL) [Figure 4]. RRE at a dose of 2.5 μg/mL showed potent glucose uptake stimulating activity (>80%) that equivalent to RC (2.5 μg/mL) and insulin (2.90 μg/mL). The strong glucose uptake stimulatory potential of RRE might be due to the possible synergism among the component rhinacanthins as previously reported in antimicrobial and anti-inflammatory activities.^[24,25] These results provide a strong base for further detail mechanistic study of glucose uptake stimulation by rhinacanthins in L6 myotubes that could be insulin dependent via glucose transporter 4 (GLUT4) or insulin-independent calcium-dependent pathway, as previously reported for other natural 1,4 naphthoquinones.^[33,34]

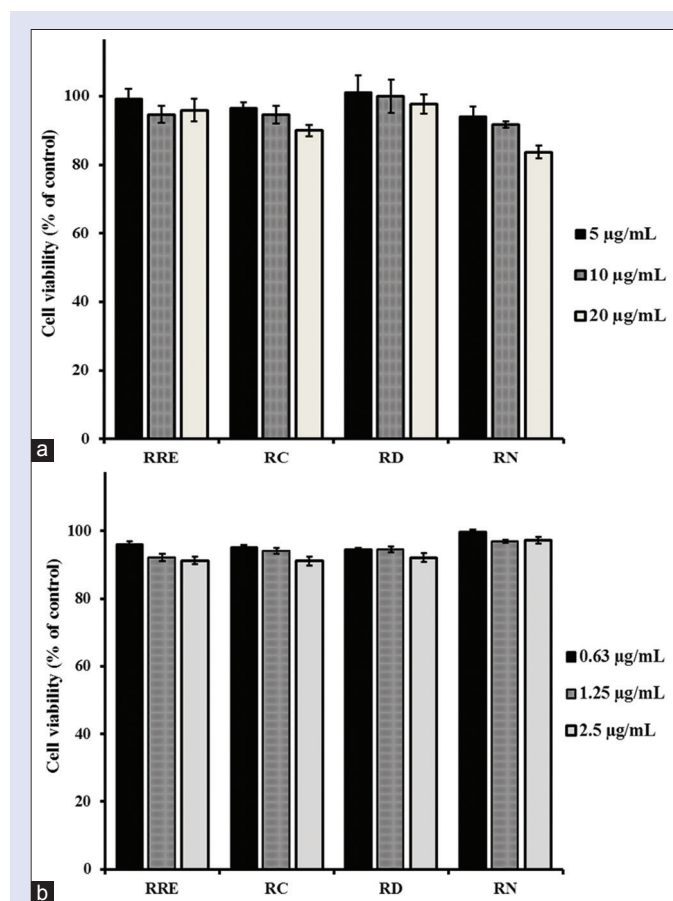


Figure 2: Percentage cell viability of 3T3-L1 (a) and L6 (b) cells after treatment with various concentrations of rhinacanthins-rich extract, rhinacanthin-C, rhinacanthin-D and rhinacanthin-N. Results are expressed as mean ± standard deviation ($n = 3$)

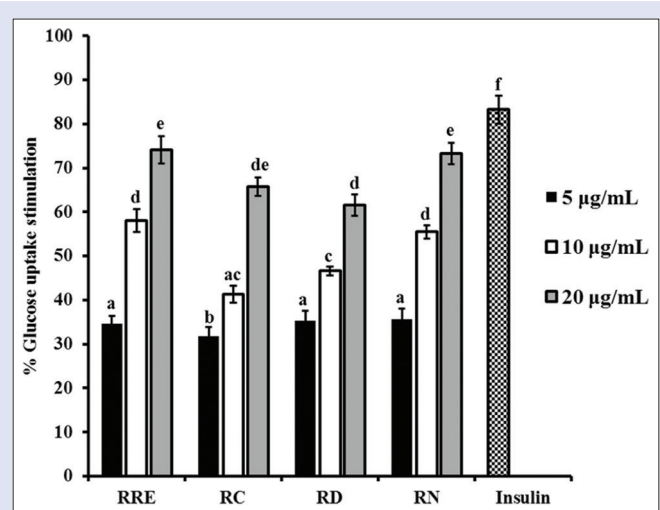


Figure 3: Dose-dependent glucose uptake stimulation in 3T3-L1 adipocytes by rhinacanthins-rich extract, rhinacanthin-C, rhinacanthin-D and rhinacanthin-N in comparison with positive control (insulin = 0.58 μg/mL). Results are expressed as mean ± standard error of the mean ($n = 3$). Mean values followed by different letters are significantly different ($P \leq 0.05$)

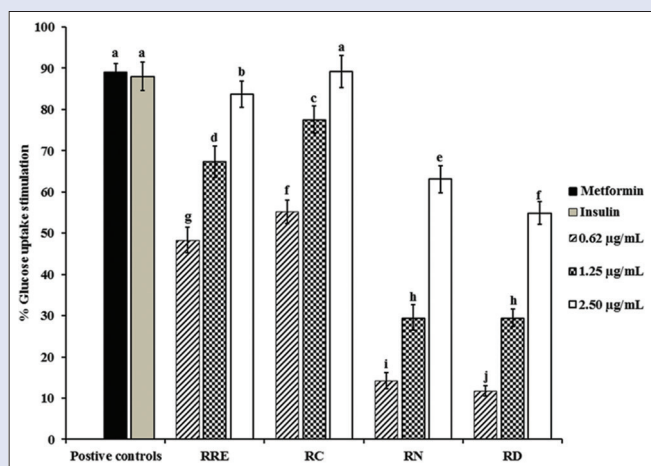


Figure 4: Dose-dependent glucose uptake stimulation in L6 muscle cells by rhinacanthins-rich extract, rhinacanthin-C, rhinacanthin-D and rhinacanthin-N, in comparison with positive controls (metformin = 219.5 µg/mL; insulin = 2.90 µg/mL). Results are expressed as mean \pm standard error of the mean ($n = 3$). Mean values followed by different letters are significantly different ($P \leq 0.05$)

Adipogenic inhibitory effect of rhinacanthins-rich extract in 3T3-L1 adipocytes

Adipogenesis or excess intracellular lipid accumulation is the main factor behind obesity and insulin resistance that leads to T2DM. Adipogenic inhibitory property is therefore an effective strategy to control these pathological disorders.^[35] RRE and its naphthoquinone compounds (RC, RD, and RN) showed potent and comparable dose-dependent adipogenic inhibitory activity in 3T3-L1 adipocytes [Figure 5a]. At the highest effective dose (20 µg/mL), the antiadipogenic activity of RRE (<20% intracellular lipids) was significantly equivalent to RC but higher than RD (20.5% intracellular lipids) and RN (39% intracellular lipids). The microscopic images of stained lipid droplets in various treated cells further confirmed the consistent dose-dependent adipogenic inhibition by RRE and its marker compounds [Figure 5b]. The antiadipogenic potential of RRE correlated with the previous report of shikonin that inhibited adipogenesis via inhibiting FABP4 and LPL genes expression.^[36] 1,4-Naphthoquinones exert their antiadipogenic activity by both upstream (SREBP1C) and downstream (PPAR γ and C/EBP α) regulations.^[36] Rhinacanthins should be therefore subjected to further studies on antiadipogenic molecular mechanism. Apart from diabetes, obesity has been reported to be linked with atheromas, cardiovascular disorders, and malignant tumors.^[37] The epidemiological reports interlinked obesity with metabolic disorders, which is further associated with the increased circulation of inflammatory adipocytokines, such as leptin, interleukin-6, and tumor necrotic factor, which results in malignant growth enhancement.^[38] Adipocytes are supposed to be responsible for the release of tumor-enhancing adipocytokines.^[39] The antiadipogenic effect of rhinacanthins could protect against malignancy via reduction in tumor-enhancing and inflammatory adipocytokines, which can be correlated with the previous anti-inflammatory and anticancer activity of rhinacanthins.^[24]

CONCLUSIONS

This is the first report on the glucose uptake enhancer and antiadipogenic constituents from *R. nasutus* leaf extracts. RRE obtained by green extraction method with 62.2% w/w of RC showed potent glucose

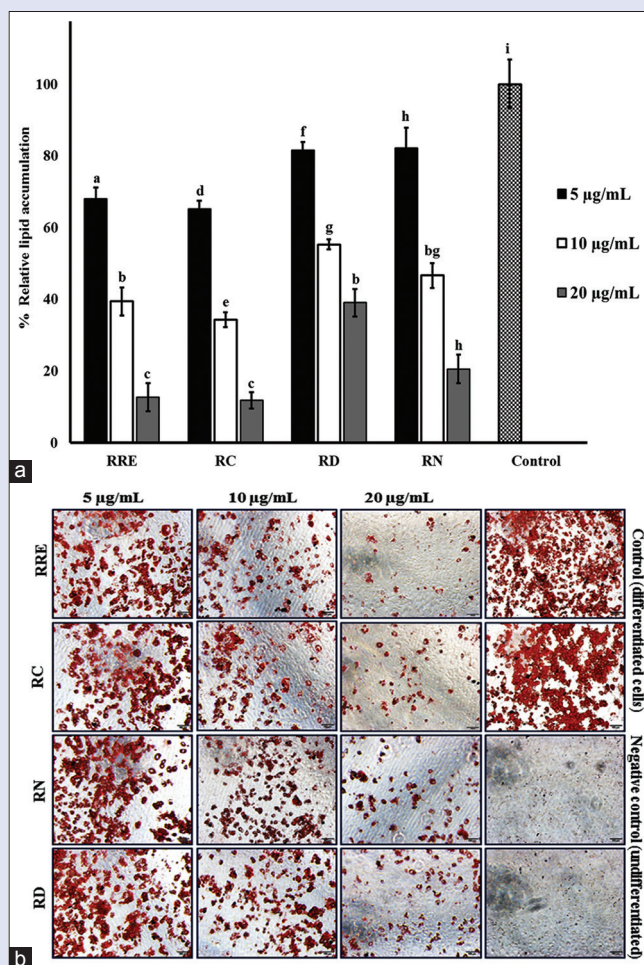


Figure 5: Dose-dependent adipogenic inhibition (a) by rhinacanthins-rich extract, rhinacanthin-C, rhinacanthin-D and rhinacanthin-N in 3T3-L1 adipocytes and microscopic images (b) of treated and untreated cells. Results are expressed as mean \pm standard error of the mean ($n = 3$). Mean values followed by different letters are significantly different ($P \leq 0.05$)

uptake stimulatory and antiadipogenic effects in 3T3-L1 adipocytes and L6 myotubes. RRE may be used as a potential candidate for antidiabetic and antiobesity drug development. Further mechanistic *in vivo* studies of RRE and safety assessment are recommended.

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Conflicts of interest

There are no conflicts of interest.

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