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## Research Article

# In-Vitro Anti-Inflammatory and Anti-Obesity Activity of Crude Extracts from Silkworms Supplemented with Royal Jelly and Selenium

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## ABSTRACT

Obesity is a global health concern associated with chronic inflammation and metabolic dysregulation. Natural sources with anti-inflammatory and anti-obesity properties are being explored for potential therapeutic interventions. This study investigated the in-vitro anti-inflammatory and anti-obesity effects of crude extracts from silkworms supplemented with royal jelly and selenium. The aim of this study was to assess the potential anti-inflammatory and anti-obesity activities of crude extracts derived from silkworms supplemented with royal jelly and selenium. Silkworm extracts were supplemented with royal jelly and selenium, and their anti-inflammatory and anti-obesity effects were evaluated using assays targeting cyclooxygenase (COX), lipoxygenase (LOX), and lipase inhibitors. Flavonoids and polyphenols present in the extracts were analyzed to determine their potential contribution to the observed activities. The supplemented silkworm extracts demonstrated promising anti-inflammatory and anti-obesity effects against COX, LOX, and lipase inhibitors. Flavonoids and polyphenols present in the extracts were implicated in mediating these activities. The study highlighted the potential of royal jelly and selenium supplementation in enhancing the anti-inflammatory and anti-obesity properties of silkworm extracts. Silkworm extracts supplemented with royal jelly and selenium exhibit significant anti-inflammatory and anti-obesity effects in-vitro. These findings support the potential use of royal jelly and selenium as natural sources of anti-inflammatory and anti-obesity agents. Further research is warranted to identify the active components in the silkworm extracts and elucidate their mechanisms of action for future therapeutic applications.

## INTRODUCTION

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Inflammation is a defense mechanism that protects against infections, burns, toxic chemicals, allergens, and other harmful stimuli to living tissue. It is a complex biological response of vascular tissues to harmful stimuli (1). Pain, redness, heat, and swelling are the primary indicators of inflammation. When there is an injury to the human body, blood circulation increases, arterioles dilate, and redness occurs in that area (2). Arachidonic acid plays a crucial role in the mechanisms of inflammation, involving a series of events metabolized by Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX). These enzymes act as chemical mediators in various inflammatory events. COX leads to the production of prostaglandins and thromboxane A<sub>2</sub>, while 5-LOX leads to the production of eicosanoids and leukotrienes (LTs) (3). Current anti-inflammatory drugs inhibit both enzyme activities to relieve symptoms, but they can have serious side effects (4). Therefore, it is important to administer anti-inflammatory drugs with fewer side effects.

Excess body weight with an abnormal proportion of body fat is defined as obesity. It is a chronic metabolic disorder caused by an imbalance between energy intake and energy expenditure that poses risks to health and reduces life expectancy (5). Obesity is a global health issue that is increasingly prevalent in both developing and developed countries. Referred to as the "new world syndrome," obesity is associated with numerous non-communicable diseases such as diabetes, metabolic syndrome, cardiovascular diseases, musculoskeletal disorders, and cancer, disrupting the body's homeostasis (6). Pharmacotherapy can help control obesity by reducing fat absorption or increasing metabolism. However, the use of drugs is linked to various side effects, ranging from tolerable to intolerable, including hypertension, heart-related complications, psychiatric problems (mental illness), and constipation (7). Therefore, there is a need to develop safer alternative drugs

that are effective and associated with minimal side effects.

The silkworm (*Bombyx mori*) is a lepidopteran insect of significant agricultural and economic importance. It is valued not only as a producer of silk but also as a potential model organism in life science research, thanks to its short generation time, clear genetic background, abundant genetic resources, and a substantial number of genes that are homologous to those found in humans (8). The utilization of silkworms has expanded significantly, with applications in drug screening and discovery (9), environmental safety assessments, screening of anti-pathogenic drugs, research on antiviral agents (10, 11), and toxicological studies (12).

Royal jelly (RJ) is a yellowish-white creamy substance secreted by the hypopharyngeal and mandibular glands of worker bees. It serves as the primary nourishment for developing larvae and queen bees throughout their lives (13). Due to its exceptional nutritional value and numerous health benefits, it has earned the reputation of a "superfood". Royal jelly contains a diverse array of essential components, such as proteins, lipids, carbohydrates, minerals, amino acids, vitamins, enzymes, and hormones (14). RJ is commonly used as a dietary supplement due to its anti-inflammatory, anticancer, antioxidant, hypotensive, anti-aging, and antimicrobial properties (14, 15).

Selenium (Se) is an essential trace element known to influence physiological processes in humans and animals (16). Serving as a micronutrient, Se plays a crucial role in various biological functions, including regulating vitamin absorption, metabolism, anti-tumor and anti-aging effects, enhancing the immune system, and preventing diseases (17). The effectiveness of Se is supported by studies on important molecular mechanisms involved in obesity-related processes such as lipogenesis, lipolysis, oxidative stress, and



inflammation within adipose tissue. Both Se deficiency and excess can lead to dysfunction in adipose tissue and metabolic changes (18). Research by (19) demonstrated that selenite improved insulin resistance induced by palmitate in 3T3-L1 adipocytes. In a study, crude extracts from silkworms supplemented with royal jelly and selenium were screened as potential anti-inflammatory and anti-obesity agents by assessing their lipoxygenase (LOX), cyclooxygenase (COX), and anti-lipase activities.

## MATERIALS AND METHODS

### Preparation of extracts

In the study described, 3rd instar (df1s) of bivoltine FC1×FC2 silkworms were raised under standard recommended conditions at a temperature of  $26 \pm 20^\circ\text{C}$ , relative humidity of  $75 \pm 5\%$ , and a 12:12 (light: dark) photoperiod. The larvae were randomly assigned to four groups, each containing 50 worms. The groups were as follows: Group I - Control, Group II - Royal Jelly supplemented (RJ), Group III - Selenium supplemented (Se), and Group IV - Royal Jelly + Selenium Supplemented (RJ+Se). The MR2 variety mulberry leaves (*Morus alba* L.) were sprayed with the different supplementations, with the control batch sprayed with distilled water and dried. These leaves were fed to the silkworms three times a day from Day 1 of the third instar larva until spinning. The dried silkworms were then ground into a fine powder, which was used to prepare an extract with ethanol using a Soxhlet apparatus (20).

### In vitro anti-inflammatory activity

#### COX inhibition assay

The assay was conducted using the Colorimetric COX (human ovine) Inhibitor Screening assay kit (21). In summary, the reaction mixture consisted of 150  $\mu\text{l}$  of assay buffer, 100  $\mu\text{l}$  of heme, 100  $\mu\text{l}$  of enzyme (either COX-1 or COX-2), and silkworm extract samples (1 mg/ml) at various concentrations. The assay relies on the peroxidase component of the COX catalytic

domain. Peroxidase activity was assessed calorimetrically by measuring the production of oxidized N, N, N, N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm. Aspirin (acetylsalicylic acid, 1 mM) was used as the standard drug in the assay. The percent COX inhibition was calculated using following equation:  $\text{COX inhibition activity (\%)} = 1 - T/C \times 100$

#### Assay of 5-Lipoxygenase

In a procedure, 4 ml of nonoxygenated water was used to dissolve linoleic acid (70 mg) and an equal weight of interpolation, followed by pipetting of sodium hydroxide (0.5 N) and the addition of nonoxygenated water (25 mL). The resulting solution was then divided into small portions of 0.5 ml each, rinsed with nitrogen, and frozen. A reaction was conducted in a quartz cuvette at  $25^\circ\text{C}$  with an optical path of 1 cm. The absorbance was measured at 234 nm using a mixture of tris buffer (2.75 ml, pH 7.4), sodium linoleate (0.2 ml), and enzyme (50 ml) (22). The following formula was served to determine percent inhibition:

$\text{LOX inhibition activity (\%)} = 1 - TC \times 100$

### In vitro Anti-obesity Activity

#### Lipase Inhibitory Activity

The lipase inhibitory activity of the prepared sample was assessed using a method outlined by (23). The evaluation involved measuring the rate of oleic acid release from triolein to determine the lipase inhibitory effect. A suspension was created containing 1% (v/v) triolein and 1% (v/v) Tween 40 in 0.1 M phosphate buffer (pH 8) and emulsified. Porcine pancreatic lipase (0.5 gm) was dissolved in 15 mL of 0.1 M phosphate buffer (pH 8). Subsequently, 800  $\mu\text{L}$  of the triolein emulsion was combined with 200  $\mu\text{L}$  of porcine pancreatic lipase, and various concentrations of fractions of methanolic extracts (50, 100, 150, and 200  $\mu\text{g/mL}$ ) were added. Orlistat, a potent pancreatic lipase inhibitor, was used as the reference standard drug. Immediately



after mixing the contents, the absorbance was measured at 450 nm

### Statistical analysis

The IC<sub>50</sub> value, which represents the concentration of a sample that reduces cell viability by 50%, was determined by extrapolating from the dose-response graph. Data points were plotted over a concentration range, and the IC<sub>50</sub> values were calculated using linear regression analysis in the PRISM program. The dose-response curve analysis was carried out using the software GraphPad Prism 5.0.

## RESULTS AND DISCUSSION

### Anti-inflammatory activity

The results of COX and LOX inhibition using methanol extracts of the four groups of supplemented silkworms are summarized in Table 1 and Figures 1 & 2. The inhibitory activities of all fractions were calculated as a percentage of inhibition at five different concentrations (50, 100, 150, and 200 µg/mL), with IC<sub>50</sub> values calculated through statistical analysis using GraphPad Prism software.

The anti-inflammatory effects of Group I, Group II, Group III, and Group IV were evaluated based on their inhibitory activities against COX-1 and 5-LOX enzymes at an initial concentration of 50 µg/mL. During the initial screening, Group IV exhibited a superior inhibitor profile against COX-1 and 5-LOX enzymes. IC<sub>50</sub> values were calculated by plotting concentration against percentage enzyme inhibition, revealing that the methanolic extract of Group IV displayed significant inhibition of COX with an IC<sub>50</sub> value of 115.28 µg/mL compared to the other groups. Group II also demonstrated notable COX inhibitory potential with an IC<sub>50</sub> value of 157.56 µg/mL compared to the control group. Group I and Group III extracts of silkworms showed the lowest COX inhibition at 231.80 µg/mL and 215.35 µg/mL, respectively.

An overview of the COX inhibition profiles by the four groups of silkworms indicates that the methanol extract of Royal jelly and Selenium-supplemented Group IV exhibited more effective COX inhibitory properties compared to Groups II, III, and I, which showed moderate or no inhibition. Group IV was identified as a significant inhibitor of LOX with an IC<sub>50</sub> value of 110.91 µg/mL compared to the other groups. Group II also displayed considerable LOX inhibitory potential with an IC<sub>50</sub> value of 149.48 µg/mL. The lowest LOX inhibition was observed with the extracts of Group I and Group III silkworms at 226.50 µg/mL. The initial response of an organism to injury or infection is represented by inflammation, a complex process that triggers various physiological and immunological pathways. However, when inflammation is dysregulated due to certain factors, it can damage adjacent tissue and lead to a variety of pathologies (24, 25). In general, inflammatory activity is caused by elevated levels of prostaglandins and leukotrienes in the body. The enzymes COX-1 and 5-LOX are responsible for the production of prostaglandins and leukotrienes (24, 26). Therefore, the inhibition of COX-1 by anti-inflammatory drugs such as NSAIDs decreases prostaglandin production, ultimately resulting in the reduction of inflammation and pain (27). However, the inhibition of prostaglandins alone can activate the alternative 5-LOX pathway, leading to increased production of pro-inflammatory and gastro-toxic leukotrienes. Hence, drugs that inhibit both COX and 5-LOX (dual inhibitors) reduce leukotriene and prostaglandin production, effectively inhibiting inflammation completely (28). RJ administration has been shown to successfully inhibit the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α in a dose-dependent manner. The presence of 10-HDA in RJ indicates a stronger anti-inflammatory effect (29). Selenium, which plays a crucial role in regulating various inflammatory



processes in the organism, also demonstrates significant importance (30).

Adequate selenium supplements are crucial for the immune system. Supplementing diets with sufficient levels of selenium can impact immune cell function, influencing aspects of inflammation and immunity (31). Piglets that were fed organic selenium showed reduced levels of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-1 $\beta$ , TLR4, and nuclear factor- $\kappa$ B (NF- $\kappa$ B) in the liver and thymus when exposed to oxidative stress (32). Recent research has highlighted the synergistic effect between RJ and selenium, demonstrating significant anti-inflammatory activity in mice and promoting intestinal health by enhancing the gut microbiota (33). The findings of the current study indicate that royal jelly and selenium function as anti-inflammatory agents, effectively inhibiting both COX and 5-LOX enzymes.

#### **Anti-obesity activity**

Methanolic extracts of dried powder from four groups of supplemented silkworms were evaluated for porcine pancreatic lipase inhibitory activity, and the results were compiled in **Table 1**. The inhibitory effects were calculated as a percentage at various concentrations (50, 100, 150, and 200  $\mu$ g/mL). Orlistat, a hydrogenated derivative of lipstatin, is currently the only pancreatic lipase inhibitor approved for long-term obesity treatment. The crude extracts from Group I (control), Group II (royal jelly), Group III (selenium), and Group IV (royal jelly and selenium) silkworms were further analyzed for their porcine pancreatic lipase (PPL) inhibitory effects at different concentrations, and a dose-response curve was generated (refer to **Figure 3**).

In comparing the inhibitory properties of the extracts using Orlistat as the reference standard, the Group IV extract demonstrated the highest inhibitory activity. Among the four groups tested for anti-obesity inhibition, the methanolic extract

from Group IV silkworms exhibited strong inhibition with an IC<sub>50</sub> value of 134.53  $\mu$ g/mL, while the control group (Group I) had an IC<sub>50</sub> value of 244.86  $\mu$ g/mL. Group II and Group III inhibited the PL enzyme with IC<sub>50</sub> values of 178.44  $\mu$ g/mL and 232.48  $\mu$ g/mL, respectively.

Obesity is a complex disease involving various factors, with excessive accumulation and storage of white adipose tissue being a key pathway of adipogenesis, often linked to energy imbalance (34). Pancreatic lipase plays a crucial role in triglyceride absorption in enterocytes and is significant in obesity management (35). Orlistat is the standard medication for obesity treatment, but it can lead to side effects such as flatulence, diarrhea, oily spotting, incontinence, abdominal cramping, among others (36). Therefore, the search for new inhibitors from natural sources without these adverse effects is essential. In this study, different extracts from supplemented silkworms were examined for their pancreatic lipase inhibitory effects compared to Orlistat. Group IV (Royal jelly and Selenium) silkworms exhibited the highest inhibitory activity. Recent research has indicated that selenium treatment demonstrates anti-obesity effects by inhibiting PRMT5 and HAT activity at a concentration of 10  $\mu$ M (37). Daily supplementation of Royal Jelly significantly enhanced insulin sensitivity and led to a decrease in body fat in overweight individuals (38). The bioactive compounds present in Royal Jelly may contribute to its anti-obesity effects.

Major royal jelly proteins have been found to enhance insulin sensitivity and reduce inflammation associated with obesity, indicating their potential role in weight management (39). RJ also contains unique lipids, such as conjugated linoleic acid (CLA), which has been linked to decreased fat accumulation and improved body composition by inhibiting adipogenesis and promoting lipolysis (40, 41). The flavonoids found in RJ, such as quercetin and kaempferol, impact

adipocyte differentiation and fat metabolism through their anti-inflammatory and antioxidant properties, potentially lowering the risk of obesity (42-44). Furthermore, the polyphenols in RJ, including various phenolic acids and flavonoids, regulate lipid metabolism and boost energy expenditure, contributing to the prevention of obesity (45). Wang et al. reported that rats supplemented with high doses of selenium (200 µg/kg/day) experienced a significant decrease in body weight and adipose tissue ratio, attributed to a lipolytic effect in adipose tissue alongside hepatic storage of free fatty acids (46). Our study findings indicate that the combined treatment of royal jelly and selenium effectively enhances anti-obesity activity.

## CONCLUSION

In conclusion, the current study demonstrated that the extract from silkworms supplemented with royal jelly and selenium exhibits significant anti-inflammatory and anti-obesity effects against COX, LOX, and lipase inhibitors. The presence of flavonoids and related polyphenols in the extract may be responsible for these activities. This study aimed to explore the anti-inflammatory and anti-obesity potential of natural sources with higher potency and fewer side effects. Therefore, Royal Jelly and Selenium could serve as natural sources of anti-inflammatory and anti-obesity agents. Further research is needed to identify the active components of the silkworm extract and understand their mechanisms of action.

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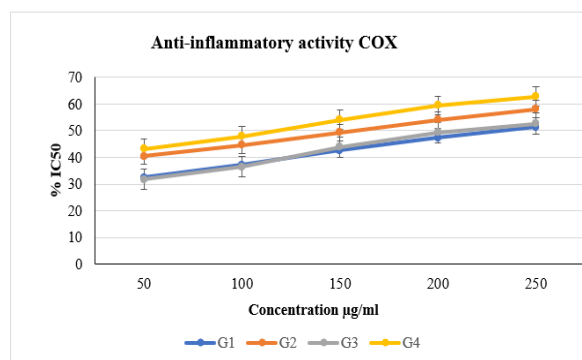


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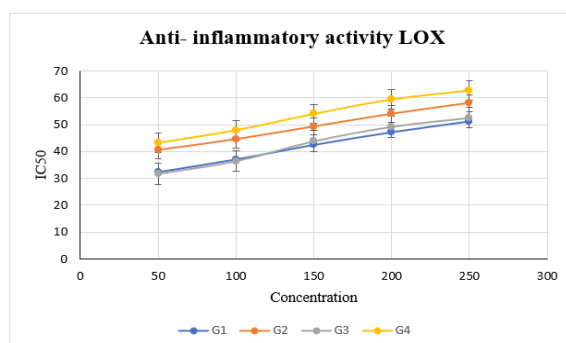
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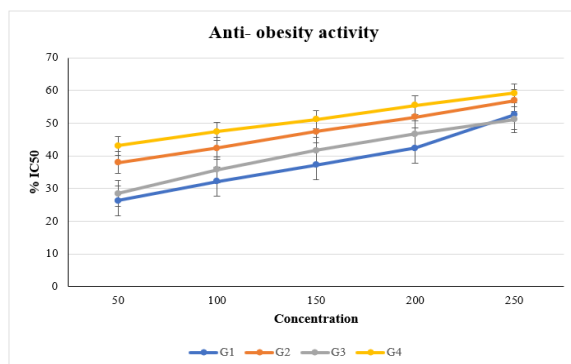
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**Figure 1. The in vitro COX activity four groups of supplemented silkworms**



**Figure 2: The in vitro LOX activity of four groups of supplemented silkworms**



**Figure 3:** The in vitro anti-obesity activity of four groups of supplemented silkworms

**Table 1.** The in vitro anti-inflammatory and anti-obesity activity of four groups supplemented silkworms

Groups	Anti-inflammatory activity		Anti-obesity activity (IC50)
	COX (IC50)	LOX (IC50)	
I	231.80	226.50	244.86
II	157.56	149.48	178.44
III	215.35	209.44	232.48
IV	115.28	110.91	134.53