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Research Article

EFFECT OF THYMOQUINONE ON GHRELIN EXPRESSION IN RAT STOMACH

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ABSTRACT. Ghrelin is a polypeptide hormone mainly synthesised from the stomach. Ghrelin also affects carbohydrate metabolism, the gastrointestinal system, the cardiovascular system and cell proliferation. Currently, increasing levels of obesity coupled with sedentary lifestyles have led to increased interest in the study of appetite-controlling factors and herbal agents. One of this plants used for phytotherapy is black cumin (*Nigella sativa, N. sativa*). Thymoquinone is the principal bioactive component of black cumin seed essential oil. The aim of the present study was to evaluate the possible effects of different doses and routes of administration of thymoquinone on ghrelin expression in the stomach. Thirty-five adult female Sprague Dawley rats were divided into five groups as first group (1 mg/kg thymoquinone, ip), second group (2 mg/kg thymoquinone, ip), third group (10 mg/kg thymoquinone, ip), fourth group (20 mg/kg thymoquinone, gavage), and control group (no treatment) and each group consisted of 7 rats. The histological structure was demonstrated with Crossman's trichrome staining. The presence of ghrelin in stomach sections was demonstrated by using the streptavidin-biotin-complex immunohistochemical method. According to our results, the immunohistochemical reaction in epithelial and glandular cells was weak in all experimental groups compared to the control group. Based on our immunohistochemical findings, it is concluded that thymoquinone treatment in both routes of administration decreases the expression of stomach ghrelin. The observation of immunoreactions of different severity in experimental groups indicate that thymoquinone did not inactivate ghrelin in the stomach, but ghrelin expression varied according to the route of administration and dose.

Keywords: *Appetite, ghrelin, stomach, thymoquinone.*

INTRODUCTION

First discovered in 1999, ghrelin also called the appetite hormone, is a peptide hormone [1]. Ghrelin is primarily derived from the precursor preproghrelin produced by gastric oxyntic gland cells [2]. The oxyntic glands are located on the inner surface of the gastric fundus and form the majority of the proximal part of the stomach. Ghrelin-secreting endocrine cells are known to be X/A-like cells. In addition to the stomach, ghrelin expression has been described in other peripheral tissues such as the gastrointestinal tract, pancreas, ovary and testis [3]. Ghrelin hormone, which is known to have growth hormone secretory effects, also has effects on appetite, carbohydrate

metabolism, the gastrointestinal system, the cardiovascular system, and cell proliferation [4]. Ghrelin is first synthesized in the stomach and then reaches the hypothalamic arcuate nucleus (ARC) and other brain parts via the bloodstream. Alternatively, peripherally synthesized ghrelin induces growth hormone-releasing hormone receptor (GHS-R) expression via the vagal nerve and stimulates the hypothalamus via the nucleus solitary [4]. A third view is that ghrelin is released locally from the hypothalamus and increases appetite by directly affecting neuropeptide Y, agouti-related protein, and other cells [5].

Ghrelin secretion is influenced by factors such as food intake, age, gender, insulin level, but the most important factor is nutrition. Plasma ghrelin secretion increases in fasting and decreases following feeding. During fasting, its concentration in saliva and blood increases by 70-80% and decreases to basal level within one hour after feeding [6]. Although the mechanism of post-feeding ghrelin decrease has not been fully elucidated, it has been reported that dietary glucose, lipids, and amino acids inhibit ghrelin levels [7]. A study was pointed that ghrelin administration increased food intake either obese or slim people [8]. Wren et al. showed both intra-cerebrovascular and systemic administration of ghrelin induced adiposity and weight gain by stimulating hyperphagia in rats [9].

Currently, increasing levels of obesity have led to increased interest in factors that control appetite-controlling factors and herbal agents. One of these plants used for phytotherapy is black cumin (*Nigella sativa, N. sativa*) [10]. *N. sativa* seeds in the Ranunculaceae family are used in the treatment of many diseases, such as respiratory system diseases, digestive system problems, nervous system diseases, and diabetes. The plant's most commonly used active ingredient is thymoquinone (thymoquinone). Thymoquinone (2-isopropyl-5-methylbenzo-1, 4-quinone) (18.4-24%) is the main biologically active component of black cumin seed essential oil [11].

There are several studies investigating the possible effects of *N. sativa* on obesity [12-15]. In a study carried out by Safi et al. [16] found that *N. sativa* caused a significant decrease in Body Mass Index (BMI). The same study used a standard questionnaire based on a visual analog scale score containing four questions to evaluate appetite sensation and decreased appetite was determined in the *N. sativa* group [16]. Le et al. [17] concluded that *N. sativa* is a plant with a slight anorexic effect that causes a decrease in food intake and body weight. In another study conducted in 2015 similarly suggests that the commercial oil of *N. sativa* contains appetite-reducing components that induce weight loss [18]. In the literature searches, no research investigating the relationship between thymoquinone, which constitutes approximately half of *N. sativa* oil, and ghrelin, which is known for its orexigenic, in other words, appetite-enhancing activation, has been encountered. In our study, we aimed to evaluate the possible effects of different doses and different routes of administration of thymoquinone on ghrelin expression in the rats' stomachs.

MATERIALS AND METHODS

The study was conducted in the Ondokuz Mayıs University Experimental Animals Application and Research Center and laboratories of Ondokuz Mayıs University Faculty of Veterinary Medicine, Department of Histology and Embryology. Thirty-five adult female Sprague Dawley rats were used as study material. The rats were divided into five groups as first group (1 mg/kg thymoquinone, ip), second group (2 mg/kg thymoquinone, ip), third group (10 mg/kg thymoquinone, gavage), fourth group (20 mg/kg thymoquinone, gavage), and control group (no treatment) and each group consisted of 7

rats. Rats were housed under standard laboratory conditions (temperature: 24 °C; dark/light cycle: 12/12 h; free access to food and water; relative humidity: 60%). Experimental animals were fed *ad libitum* with standard rat diet throughout the study. In the 42-day study, the animals' body weights were measured before the thymoquinone administration, and the amount of thymoquinone to be administered was determined. (All experimental protocols were approved by Ondokuz Mayıs University Animal Experiments Ethics Committee (Experimental Approval No: 2015/51).

At the end of the experiment, rats in all groups were sacrificed after xylazine-ketamine anaesthesia. Then the stomachs of the rats were removed and fixed in a 10% formaldehyde solution and then routine histological procedure was performed. After stomach samples embedded in paraffin, 5 μm thick sections were cut. Crossmon's staining procedure was used to evaluate the histological structures of stomach. [19]. In addition, the presence of ghrelin in stomach sections was demonstrated by the streptavidin-biotin-complex immunohistochemical method [20].

Slides were examined under light microscope (Nikon E-80i Microscope, Tokyo, Japan). Photography was done using Nikon digital sight imaging system (Tokyo, Japan). Immunohistochemical evaluations were performed by assigning values from 0 to 3 according to no staining (-), very weak staining (±), weak staining (+), moderate staining (++) and strong staining (+++) [20].

Immunohistochemical Staining

Five-micrometer sections were stained immunohistochemically by using Rabbit polyclonal Ghrelin antibody (abcam, cat no ab129383) with Streptavidin biotin complex method. Mouse and rabbit specific HRP/DAB (ABC) detection kit (abcam, cat no ab64264) was used as secondary antibody. After deparaffinization, the slides were heated in citrate buffer (pH 6.0) at 700 W for antigen retrieval. In order to block endogenous peroxidase activity, the slides were incubated in 3% hydrogen peroxide solution. After rinsing with phosphate buffer solution (PBS), the blocking serum was dripped to prevent non-specific protein binding in the sections. Then the sections were incubated with primary antibody (1/500 dilutions) at +4 ^{o}C overnight in a humidified chamber. For negative controls only PBS was dripped into the slides. Following the washing step, biotinylated secondary antibody was instilled into sections and incubated at streptavidin-HRP complex after washing step. 3,3'-diaminobenzidine (abcam, cat no ab64264) was used as a chromogen, and the slides were covered with entellan by counterstaining performed with Mayer's hematoxylin [20].

RESULTS AND DISCUSSION

Histological Findings

Crossman trichrome method was employed to determine the standard histological structure. In our study, the fundus (corpus) region of the stomach was examined. In the stomach tissue, which was observed to have a single-layer prismatic epithelium, the presence of cells forming the fundus glands in the area of the propria under the epithelium was detected. When the stomach sections of all groups were examined, the standard histological structure was observed in all of them. There were no differences in the histochemical characteristics of the groups (Fig.1).

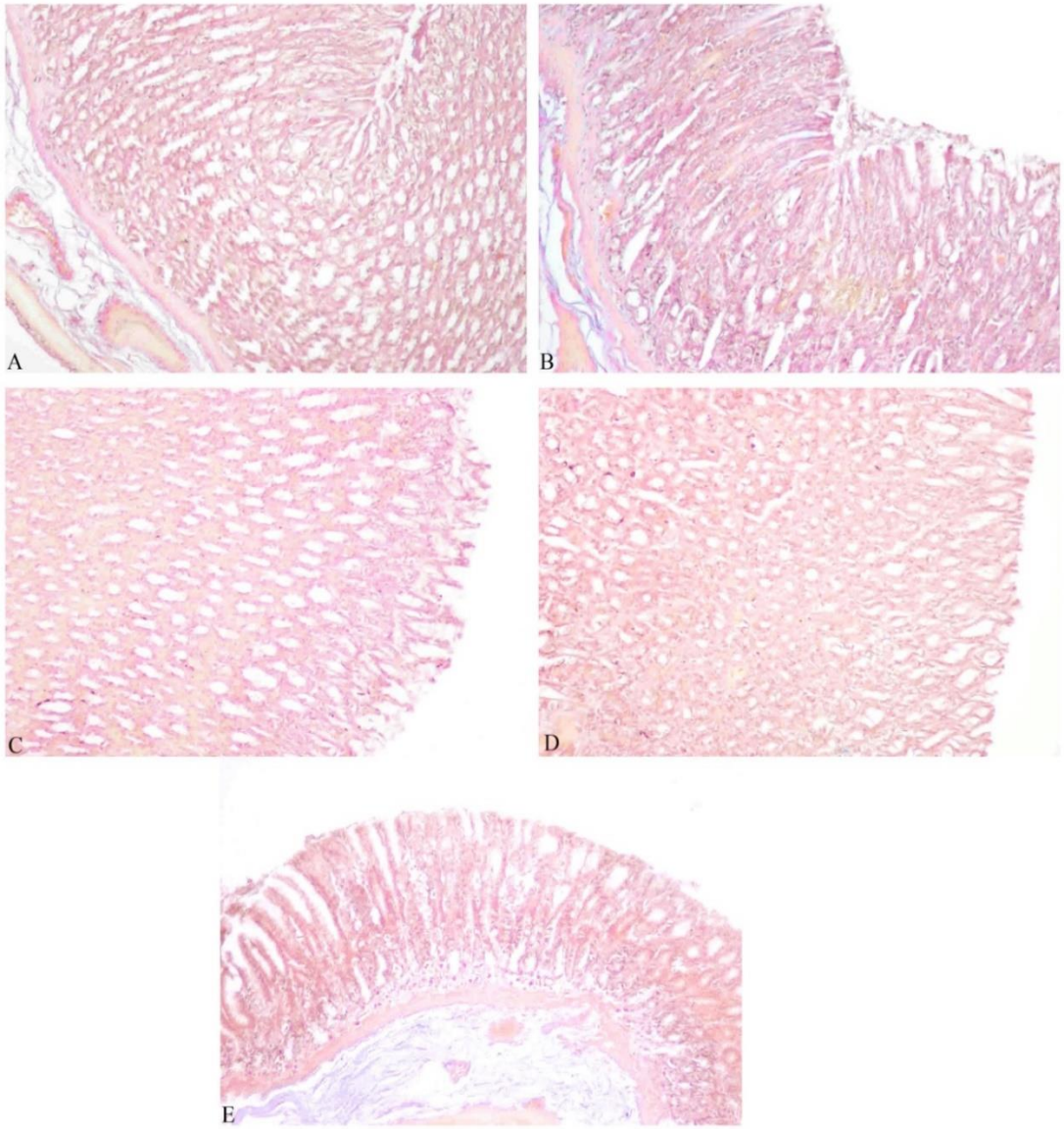

Fig. 1. *Histological structure of fundus in control and experimental groups. A: Control group, B: First group (1mg/kg thymoquinone, ip) C. Second group (2 mg/kg thymoquinone, ip), D. Third group (10 mg/kg thymoquinone, gavage), E. Fourth group (20 mg/kg thymoquinone, gavage), Crossmon's trichrome staining (x20).*

Immunohistochemical Findings

Immunohistochemical stainings were scored in a semi-quantitative manner to determine differences in the distribution patterns of intensity of immunolabelling of surface epithelium cells and glandular epithelium cells in fundus of the control and experimental groups. The intensities of ghrelin immunostainings were shown in Table 1. Differnt severity of Ghrelin expression was detected on the apical side of the fundic mucosal epithelium and different parts of the fundic glands that located in the lamina propria. No reaction was observed in the negative control preparation (Fig. 2A).

Table 1. *Ghrelin immunoreactivity in control and experimental groups.*

	Control group	**First group (1 mg/kg thymoquinone, ip)**	**Second group (2 mg/kg thymoquinone, ip)**	**Third group (10 mg/kg thymoquinone, gavage)**	**Fourth grup (20 mg/kg thymoquinone, gavage)**
Fundus surface epithelium cells	++	+	+	+	+
Glandular epithelium cells in lamina propria	+++	+	++	++	+, ±

No staining (-), very poor staining (±), weak staining (+), modarate staining (++), severe staining (+++).

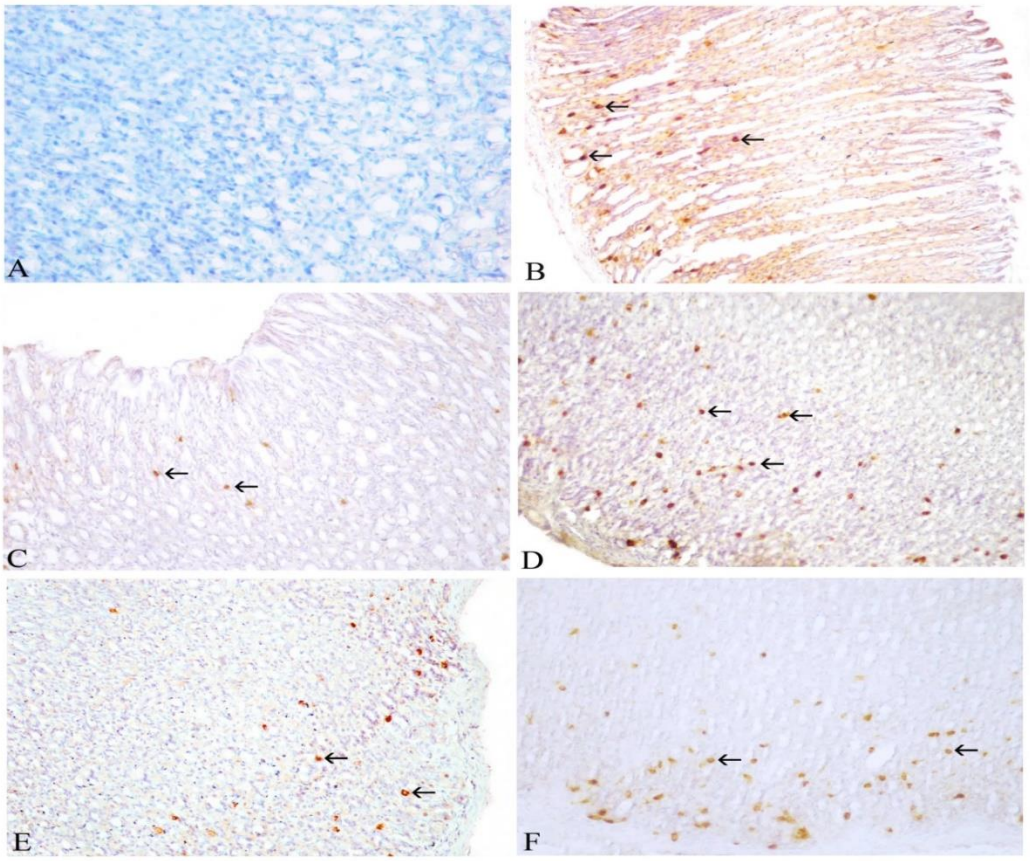

Fig. 2. *Immunohistochemical localization of ghrelin in fundus. A: Negative control x40, B: Control group x20, C: First group (1mg/kg, ip thymoquinone) x20, D: Second group (2 mg/kg, ip thymoquinone) x20, E: Third group (10 mg/kg, gavage thymoquinone) x20, F: Fourth group (20 mg/kg, gavage thymoquinone) x20.*

Ghrelin was mildly expressed in the epithelial cells compared to the lamina propria gland cells in all groups. Ghrelin expression was down-regulated in epithelial cells of all experimental groups compared to the control group. The reaction intensities in the epithelial cells of the experimental groups were weak and there was no significant difference between the groups.Ghrelin was strongly expressed in the glandular epithelium cells found in the lamina propria of stomach tissues in the control group. Moderate and similar intensity of immunoreaction was detected in the second (2 mg/kg thymoquinone, ip) and third (10 mg/kg thymoquinone, gavage) groups. The group receiving 20 mg/kg thymoquinone by gavage decreased the expression of ghrelin when compared with the third group (10 mg/kg thymoquinone, gavage). There was significant reduction in first group compared to all other groups (Table 1, Fig. 2,3).

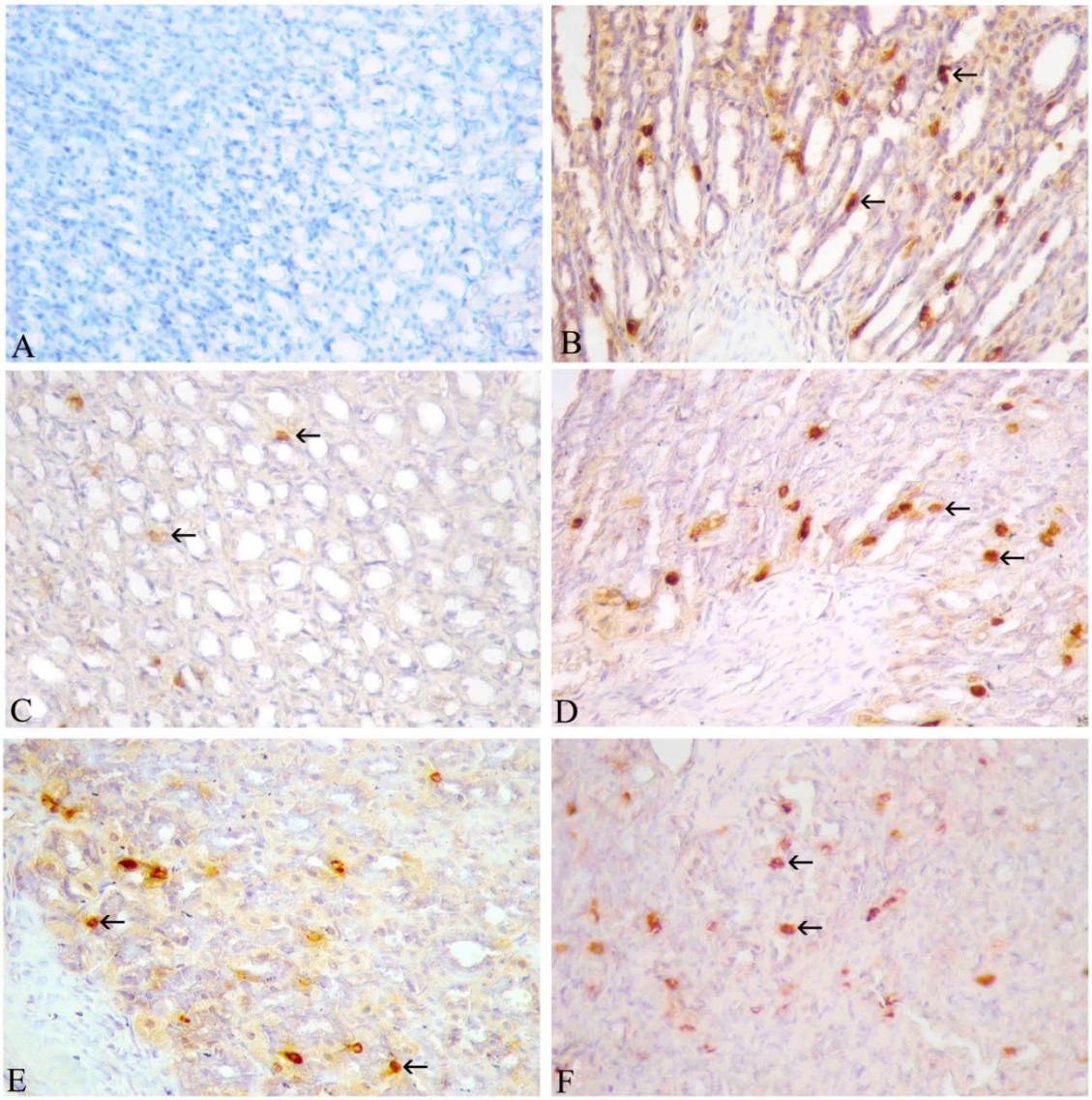

Fig. 3. *Immunohistochemical staining of Ghrelin in fundus. A: Negative control, B: Control group, C: First group (1 mg/kg thymoquinone, ip), D: Second group (2 mg/kg thymoquinone, ip), E: Third group (10 mg/kg thymoquinone, gavage), F: Fourth group (20 mg/kg thymoquinone, gavage), x40*

It is essential to understand the mechanisms of change of ghrelin, an appetite-related polypeptide, in order to prevent putting on weight, which has become an important problem with sedentary lifestyle. Thymoquinone is the active ingredient of black cumin seed, one of the most preferred plants in traditional medicine and is commonly consumed.

The effect of thymoquinone administered at different doses by both gavage and intraperitoneal routes on ghrelin expression in fundus surface epithelium cells and glandular epithelial cells in lamina propria was demonstrated in our study. According to our results, the immunohistochemical reaction in epithelial and glandular epithelium cells was weak in all experimental groups compared to the control group. The immunoreaction severity was mildest in the group receiving 1mg/kg thymoquinone intraperitoneally. The moderate intensity of immunoreaction was determined in the second (2 mg/kg, ip thymoquinone) and third (10 mg/kg, gavage thymoquinone) groups. The group receiving thymoquinone at 20 mg/kg by gavage decreased ghrelin expression compared to the third group receiving thymoquinone at 10 mg/kg by gavage. Based on our immunohistochemical findings, it is concluded that thymoquinone treatment in both routes of administration decreases the expression of stomach ghrelin.

In recent years, several studies have been conducted to investigate the effects of plants and plant extracts on appetite, food intake, energy balance and the level of hormones such as ghrelin [21]. Mazidi et al. [22] evidenced that ghrelin expression were increased adminstarition of Cannabis sativa unlike thymoquinone, which we used in our study. In another study conducted in 2007 [23], it was reported that arabinoxylan obtained from the outer shell of wheat, rye, rice and other cereal grains decreased ghrelin levels, similar to the effect of thymoquinone used in our study. In another study on plants, it was revealed that in addition to regular nutrition, Phaseolus vulgaris supplementation, similar to the effect of thymoquinone which we used in our study, suppressed ghrelin secretion and reduced appetite. Also İlhan et al. [24] demonstrated the effects of capsaicin on ghrelin in their study reported in 2013. As a result of the study, they reported that capsaicin caused decreases in ghrelin levels and expression in the testes. The findings of this study are similar to the effect of thymoquinone which was used in our study in the stomach.

CONCLUSION

In conclusion, our present study comparatively examined the effect of thymoquinone administered at different doses by both gavage and intraperitoneal routes on ghrelin expression in stomach. The localization of ghrelin in the stomach, which has proven effects on appetite, has been shown *in vivo*. Ghrelin expression was detected in cells of the epithelium and connective tissue of the gastric mucosa in all groups. The observation of immunoreactions of different severity in experimental groups indicate that thymoquinone we used in our study did not inactivate ghrelin in the stomach, but ghrelin expression varied according to the route of administration and dose. Our results are encouraging for more detailed studies on the interaction of ghrelin and thymoquinone, which have wide metabolic effects. We believe that future molecular studies will elucidate stomach-ghrelin-thymoquinone interactions, mechanisms of action and pathways.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: S.T., N.D., Design: S.T., N.D., Data Collection or Processing: S.T., N.D., Analysis or Interpretation: S.T., N.D., Literature Search: S.T., N.D., Writing: S.T., N.D.

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