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ANALGESIC EFFECT OF QUERCETIN 3,7-O-DIMETHYL ETHER ISOLATED FROM *SALVIA OFFICINALIS*

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Abstract

To evaluate the analgesic effect of flavonoid quercetin 3,7-O-dimethyl ether isolated from ethyl acetate fraction of the methanol extract of *Salvia officinalis.*

The dried leaves of *Salvia officinalis* were subjected to extraction with methanol, and then it was fractioned by water and ethyl acetate solvent. The antinociceptive activity of flavonoid quercetin 3,7-O-dimethyl ether isolated from ethyl acetate fraction was assessed using heat-induced (hot-plate) and chemical-induced (acetic acid and formalin) nociception models in mice and rats. Involvement of opioid system and cyclic guanosine monophosphate (cGMP) pathway were also investigated using naloxone and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), respectively.

Pretreatment with quercetin 3,7-O-dimethyl ether significantly ($p < 0.05$) and dose-dependently increased the hot plate latency time**.** It also reduced the number of abdominal constrictions and paw lickings (in both early and late phases) induced by acetic acid and formalin, respectively**.** Naloxone and ODQ significantly attenuated the antinociceptive activity of quercetin 3,7-O-dimethyl ether in the hot plate and both phases of formalin test.

Antinociceptive action of flavonoid quercetin 3,7-O-dimethyl ether support the traditional use of *Salvia officinalis* in folk medicine**.** This study provide evidence that the antinociceptive effect of quercetin 3,7-O-dimethyl ether is partly mediated by opioid system and cGMP pathway. Finally, the quercetin 3,7-O-dimethyl ether isolated from this plant appears to contribute for the antinociceptive property of different extracts from *Salvia officinalis***.**

Keywords: *Antinociceptive, flavonoid, opioid system, hotplate test, formalin test.*

Introduction

Salvia is the largest genus of Lamiaceae family and includes about 9000 species spread all over the world. One of these species is *Salvia officinalis* L. *Salvia officinalis* L. or Sage is a perennial, evergreen low shrub native to the Mediterranean area but is known throughout the world [1-2]. The plant grows in different places in Jordan and used as spices and flavoring agents by perfumery and in cosmetic industries and folk medicine. In folk medicine the plant is used for the treatment of various ailments, including menopausal complications, inflammation, ulcers, and hyperglycemia [3].

Many studies have been performed to verify the traditional uses of *S. officinalis*. The plant has been reported to possess antiinflammatory, antimicrobial, antinociceptive, antidementia, antioxidant, antimutagenic and hypoglycemic effects [3].

Several bioactive constituents have been isolated and identified from *Salvia officinalis* such as 1,8-cineole, α-pinene, rutin, camphor, α-thujone, β-thujone, borneol, epicatecin, viridiflorol, luteolin-7-glucoside, rosmarinic acid, apianane terpenoids, salvin, carnosic acid, apigenin, luteolin, luteolin glucuronides, vicenin-2, carnosol, and other phenolic glycosides [4-10]. Some of such bioactive constituents are known to have biological activities [3,11-14].

Authors and other studies have reported the anti-inflammatory and antinociceptive properties of *S. officinalis* extracts [15-16]. Since quercetin 3,7-O-dimethyl ether has been isolated as the main constituent of ethyl acetate fraction of *S. officinalis*, it was felt interesting to evaluate this compound for its antinociceptive activity and possible mechanisms of action.

Methods

Plant material

In this study, the plant part utilized was the leaves. Fresh leaves of wild-growing *Salvia officinalis* were obtained during May from Al-Fesaliya (Madaba, Jordan) by Esam Qnais and the sample was authenticated by herbarium of the Hashemite University. A voucher specimen was deposited in the herbarium for future reference.

Isolation and identification of quercetin 3,7-O-dimethyl ether

The leaves of *Salvia officinalis* was air dried and the dried leaves (3500 g) were grounded to fine powder by electric mill. The leaves powder (3400g) was defatted by soaking for 10 days at room temperature in 10000ml of petroleum ether (60–80°C). The plant material was then separated by filtration using cheesecloth. The defatted plant material was extracted twice with methanol (10000ml) for 3 days at room temperature. The crude extract was evaporated under reduce pressure to give 208 g of gummy material. The gammy material was then partitioned between water and ethyl acetate. The ethyl acetate extract was concentrated using rotary evaporator and the main compound isolated by column chromatography on silica gel using a CH_2Cl_2-MeOH gradient system (100:1 → 0:100). At the end quercetin 3,7-O-dimethyl ether (Pale yellowish powder, 85 mg) was obtained and purified by recrystallization using MeOH. Quercetin 3,7-O-dimethyl ether was identified by comparing NMR spectra (^1H, ^{13}C-NMR) and melting point value with that reported [17-18].

Animals

Non-fasting male Swiss albino mice and Wistar rats weighing 24–28g and 170–290g, respectively, were used throughout this study. The animals were housed in standard cages at room temperature (25±3 ◦C) in 12 h dark/12 h light control and with access to food and water *ad libitum*. The studies were carried out in accordance with the Hashemite University animal ethics committee guidelines.

Drugs and chemicals

All chemicals and drugs were supplied by Sigma-Aldrich and were of analytical grade.

Hot plate test

The hot-plate test was assessed using mice. Mice were placed on the hot plate maintained at 55±0.5°C. After 60 min of intra–peritoneal administration of quercetin 3,7-O-dimethyl ether (3, 6, 9 mg/kg, doses were selected based on its solubility) the reaction time (licking paw) was recorded and compared to control (0.9% NaCl) **.** The cut-off time was 60s to avoid any tissue damage to the paws. The percentage of increase in latency (reaction time) was calculated as follows:

% increase in latency= (A-B/B) × 100, where A is the reaction time measured after treatment; B is the reaction time measured before treatment [15].

Acetic-acid-induced writhing test

Acetic-acid-induced writhing test performed using mice. Mice were divided into 5 groups (n=6) consisting of animals pretreated with vehicle (0.9% NaCl, control group), morphine (5mg/kg), or quercetin 3,7-O-dimethyl ether (3, 6, 9 mg/kg) prior intra-peritoneal injection of acetic acid (10ml/kg, 0.9% v/v, 30min after treatment). The number of constrictions of abdominal muscle together with extension of the hind limbs for the 10 min immediately after acetic acid injection in each group was recorded and compared to the response in the control group. The antinociception was expressed as the percent reduction of the abdominal constrictions [15].

Formalin test

Formalin test was assessed using rats. Rats were divided into 5 groups (n=6) and injected s.c with 50µl of 5% of formalin solution (using a microsyringe with a 26-gauge needle) into the plantar surface of the right hind paw to induce pain. The rats were treated with quercetin 3,7-O-dimethyl ether (3, 6, 9 mg/kg), vehicle (0.9% NaCl, control group), or morphine (5mg/kg) 60min before formalin injection. The time spent in licking was measured from 0 to 5 min (early phase) and 15–40 min (late phase). The reduction of the licking time was considered as an antinociceptive response.

The percent inhibition of licking was calculated as follows:

% of inhibition= (A-B/B) × 100 where A is the licking time in treated group; B is the licking time in control group [15].

Evaluation of the mechanism of action

To study the possible involvement of opioid receptor and cGMP pathway in the nociceptive effect of quercetin 3,7-O-dimethyl ether. Animals were pretreated with naloxone, ODQ (10mg/kg i.p., anonspecific inhibitor of guanylyl cyclase), or vehicle 30 min prior quercetin 3,7-O-dimethyl ether injection and subjected to the hot plate and formalin test as described above [15].

Statistical analysis

The values were reported as mean±S.E.M. and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. At the level of $p < 0.05$ the results were considered significant.

Results

Isolation of quercetin 3,7-O-dimethyl ether

One flavonoid that has not been previously isolated from *Salvia officinalis* has been isolated and identified from ethyl acetate fraction (Figure 1). Quercetin 3,7-O-dimethyl ether (Pale yellowish powder, $C_{17}H_{14}O_7$) was identified by NMR spectra (^1H, ^{13}C-NMR) analysis and melting point value. The data are in accordance with that reported in the literature [18].

Hot plate test

Figure 2 shows that quercetin 3,7-O-dimethyl ether given intraperetoneally 60min prior produced significant and dose-related increase in the latency response (expressed as % increase in latency) in mice when assessed using the hot plate test. Morphine (5mg/kg) demonstrated the most effective effect when compared to the quercetin 3,7-O-dimethyl ether at all doses used.

Acetic-acid-induced writhing test

The results in Figure 3 show that when mice were pre-treated with quercetin 3,7-O-dimethyl ether a significant dose dependent inhibition of acetic-acid-induced writhing was observed compared to control group. The inhibition observed was 21.2±3.2, 51.3±6.1, 64.3±5.5% for the doses of 3,6,9 mg/kg respectively. Treatment with morphine (5mg/kg) significantly ($p< 0.05$) resulted in 91.2±5.1% reduction of the number of abdominal constrictions compared to control group.

Formalin test

Quercetin 3,7-O-dimethyl ether showed significant and dose-dependent decrease in licking time in both early and late phases after formalin injection when compared with control rats (Figure 4). The reduction in the early phase and late phase was similar ($p< 0.05$). Treatment with morphine (5mg/kg) significantly ($p< 0.05$) reduced the paw licking response time in early phase (84.2±5.2%) and in late phase (90.5±7.5%) when compared to control group.

Evaluation of the mechanism of action

Tables 1 and 2 show the results of the effect of pretreatment of mice and rats with naloxone and

ODQ, respectively. The antinociception caused by quercetin 3,7-O-dimethyl ether in the hot plate test and formalin test was significantly attenuated by i.p. treatment of animals with the naloxone and ODQ. Naloxone and ODQ had insignificant effect on the control animals (vehicle). It is worth noting that the effect on the hot plate test and formalin test was similar.

Discussion

An earlier studies reported that different extracts of *S. officinalis* have potent antinociceptive activity in animals. In the present study, we for the first time demonstrated that quercetin 3,7-O-dimethyl ether, isolated from ethyl acetate fraction of the methanol extract of *Salvia officinalis,* produced a significant and dose-dependent antinociceptive action in both chemical and thermal-induced nociception models compared to the control and quercetin 3,7-O-dimethyl ether acts both centrally and peripherally. Importantly, the antinociceptive activities of quercetin 3,7-O-dimethyl ether were able to be reversed by pretreatment with naloxone or ODQ, suggesting that both opioid receptors and cGMP pathway contribute to the antinociception of quercetin 3,7-O-dimethyl ether.

Hot plate test on mice is widely used to study centrally acting analgesic drugs which delays the response against heat-induced pain thresholds [19, 20]. More specifically, the hot plate test serves as a popular test to monitor the supraspinal reflex μ1/μ2-opioid receptors [21, 22, 23]. In this study, quercetin 3,7-O-dimethyl ether at 3,6,9mg/kg doses significantly increased the time latency in hot plate test revealing the central antinociceptive activity of quercetin 3,7-O-dimethyl ether.

The acetic acid-induced writhing test is considered a useful tool for the evaluation of peripheral antinociceptive action [24]. In this test acetic acid induced the writhing response by liberating proinflammatory mediators cyclooxygenase (COX), lipoxygenase (LOX), prostaglandins (PGs), and others in the peripheral tissue fluid, which then excite the primary afferent nociceptors entering dorsal horn of the central nervous system [25,26]. The significant inhibition of nociceptive response induced by acetic acid clearly suggests that quercetin 3,7-O-dimethyl ether may be involved in the inhibition of the release or functions of these mediators and it will be effective against inflammatory pain.

In order to further clarify if the antinociceptive action of quercetin 3,7-O-dimethyl ether is centrally or peripherally, the formalin test was conducted. Intraplantar injection of formalin in the paw was found to produce a biphasic nociception [27]. Pain in the early phase (0-5 min, acute neurogenic phase) results from direct activation of primary nociceptors afferent entering dorsal horn of the central nervous system, while in the late phase (15-30 min, chronic inflammatory) it reflects a combination of an inflammatory reaction in peripheral tissue and functional changes in the dorsal horn of the spinal cord [23]. Centrally acting drugs inhibit both phases of pain, while peripherally acting drugs inhibit mainly the second phase [24]. In this study, quercetin 3,7-O-dimethyl ether caused significant inhibition of both phases of formalin-induced nociception in rats in a dose-dependent manner. It is worth noting that decreasing effect is similar in the chronic phase (late phase response) and the acute phase (early phase response).These results suggest that the antinociceptive activity of quercetin 3,7-O-dimethyl ether in the formalin test could be attributed to the action of both central and peripheral antinociceptive effect (neurogenic and anti-inflammatory mediators).

The nociceptive behavior induced by acetic acid and late phase of formalin test are considered due to an inflammatory response and any agent that suppresses the above responses may be considered to be useful in alleviating inflammatory pain. This proposal is supported by previous report on the effect of quercetin 3,7-O-dimethyl ether on certain inflammatory mediators. Quercetin 3,7-O-dimethyl ether effectively inhibited the levels of pro-inflammatory cytokines and the protein expression of COX-2 in colon epithelial cells, HT-29 cells. Furthermore our results is supported by several studies that showed that many flavonoids can suppress the intracellular Ca^{2+} level elevation, as well as the release of pro-inflammatory mediators such as TNFα [28]. Mansour abadi et al. further reported that flavonoids extracted from *S. officinalis* reduce inflammation in the mouse carrageenan model

The hot plate and formalin test using naloxone indicated that antinociceptive action of quercetin 3,7-O-dimethyl ether was partially

reversed by naloxone. These data strongly suggest that the antinociceptive effect of quercetin 3,7-O-dimethyl ether may occur through opioid receptors.

To further investigate whether the antinociceptive action of quercetin 3,7-O-dimethyl ether involves the cGMP pathway, ODQ (a nonspecific inhibitor of guanylyl cyclase) was used. It has been reported that cGMP can act on ion channels directly or may activate protein kinase and phosphodiesterases [30]. The results demonstrated that pretreatment with ODQ significantly reduced the antinociceptive effects of quercetin 3,7-O-dimethyl ether in both hot plate and formalin test. This suggests that the antinociceptive effect of quercetin 3,7-O-dimethyl ether probably involves cGMP pathway. Our results are agree with several studies showing that cGMP are important for the antinociceptive activity of different analgesic drugs [31-33]. To anticipate the possible mechanism(s) of antinociceptive action of quercetin 3,7-O-dimethyl ether. We evaluated the effect of naloxone, a non-selective opioid receptor antagonist, against the antinociceptive effect of quercetin 3,7-O-dimethyl ether. The results of [29]. These data along with present results support the contention that quercetin 3,7-O-dimethyl ether might be useful against inflammatory pain.

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Table 1. Effect of pretreatment of mice and rats with naloxone (5mg/kg, i.p) on the antinociceptive action of quercetin 3,7-O-dimethyl ether against hotplate test and formalin test.

Treatment	Increase over baseline (%) (Hotplate test)	Inhibition (%) of licking time (early phase)	Inhibition (%) of licking time (late phase)
Vehicle (0.9%NaCl) +naloxone (5mg/kg)	5.2±2.1	3.2±1.1	4.7±1.2
Quercetin 3,7-O-dimethyl ether (9mg/kg)	80.2±5.6	52.4±6.2	59.6±7.3
Quercetin 3,7-O-dimethyl ether +naloxone (5mg/kg)	32.1±3.8*	20.6±4.2*	25.4±2.9*
Morphine (5mg/kg)	149.2±7.2	84.2±5.2	90.5±7.5
Morphine (5mg/kg)+ naloxone	22.1±3.5	13.5±2.7	15.2±3.1

* Significant differences from quercetin 3,7-O-dimethyl ether (9mg/kg) alone.

Table 2. Effect of pretreatment of mice and rats with ODQ (10mg/kg i.p) on the antinociceptive action of quercetin 3,7-O-dimethyl ether against hotplate test and formalin test.

Treatment	Increase over baseline (%) (Hotplate test)	Inhibition (%) of licking time (early phase)	Inhibition (%) of licking time (late phase)
Vehicle (0.9%NaCl) + ODQ (10mg/kg i.p)	4.3±1.9	4.8±2.3	2.9±1.1
Quercetin 3,7-O-dimethyl ether (9mg/kg)	80.2±5.6	52.4±6.2	59.6±7.3
Quercetin 3,7-O-dimethyl ether + ODQ (10mg/kg i.p)	37.9±4.1*	31.5±2.9*	34.6±2.6*

* Significant differences from quercetin 3,7-O-dimethyl ether (9mg/kg) alone.

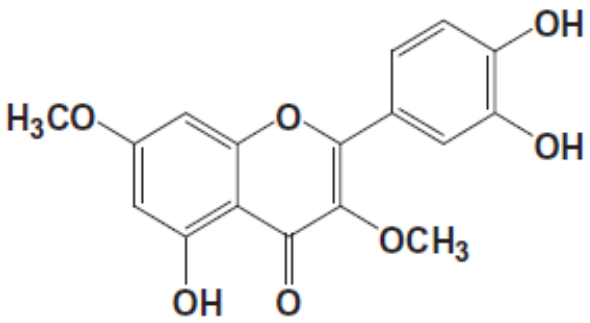

Figure 1: Structure of quercetin 3,7-O-dimethyl ether

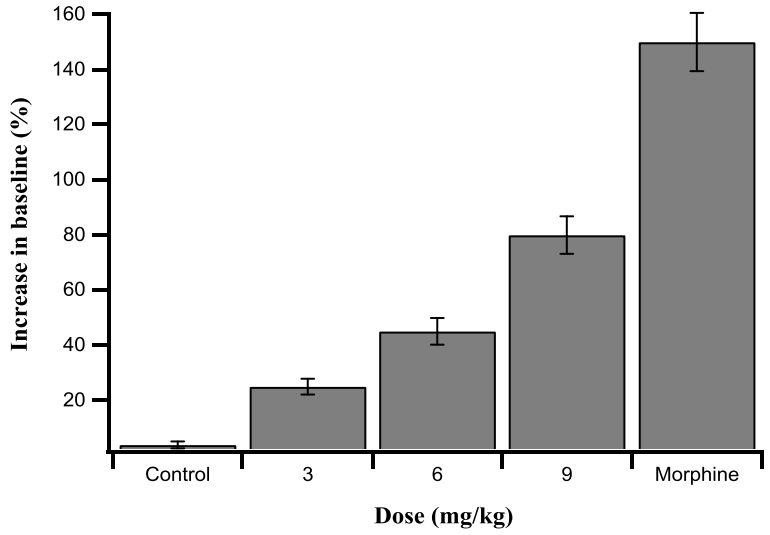

Figure 2. Effects of quercetin 3,7-O-dimethyl ether and morphine on the latency of mice subjected to the hotplates test. Each column represents the mean of six replicates mice and error bar represents the SEM.

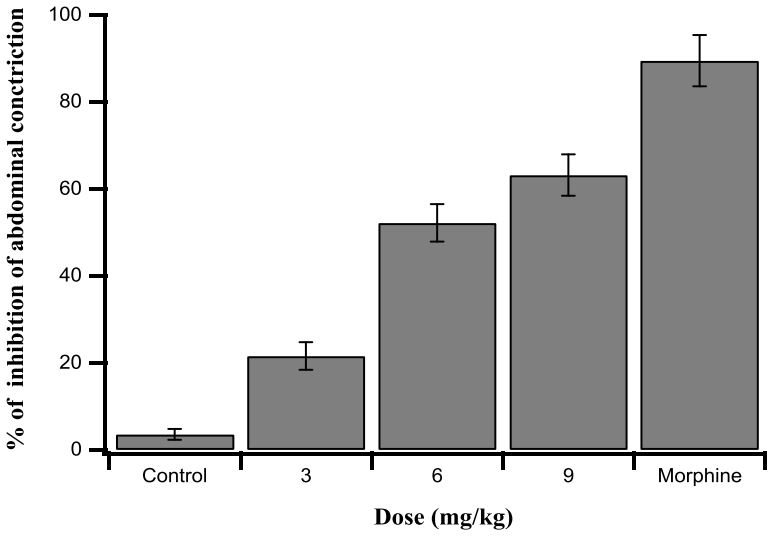

Figure 3. Effects of quercetin 3,7-O-dimethyl ether on acetic acid induced writhing response in mice. Each column represents the mean of six replicates mice and error bar represents the SEM.

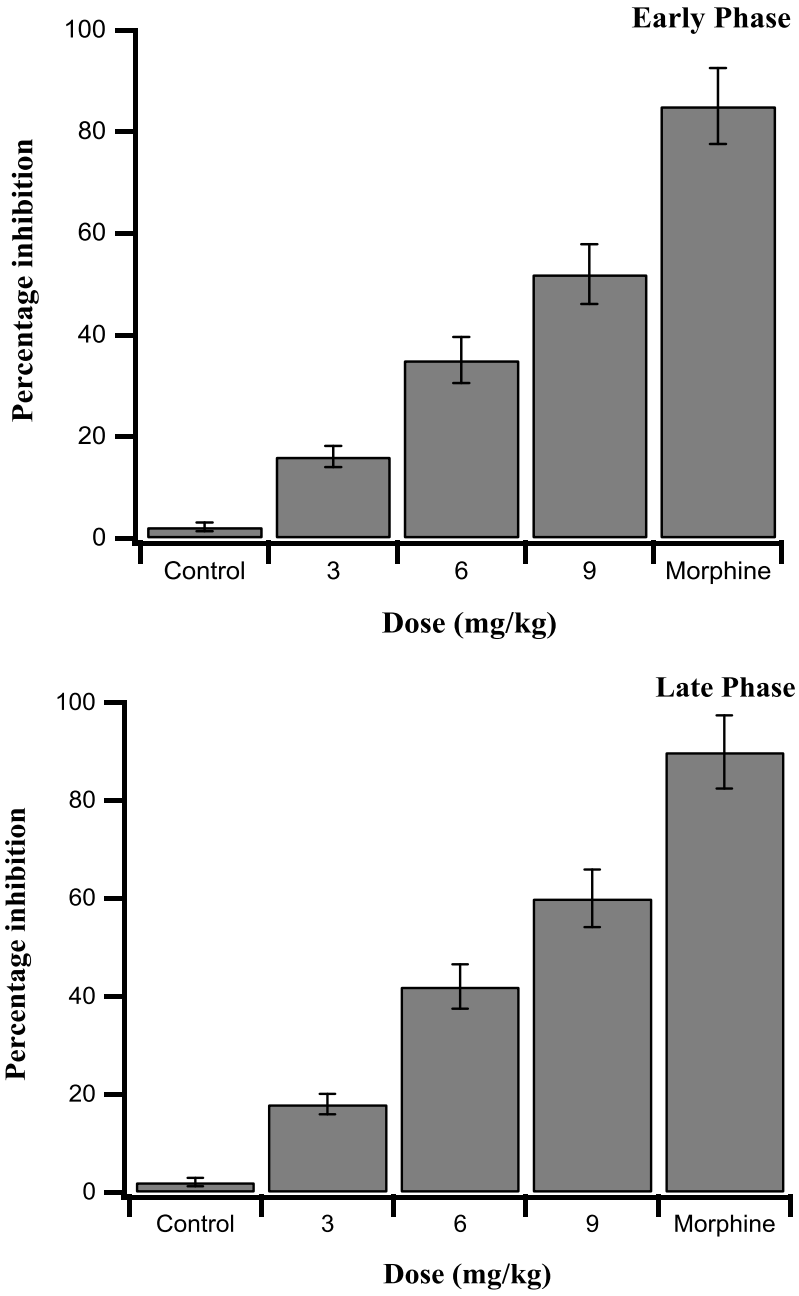

Figure 4. Effects of quercetin 3,7-O-dimethyl ether and morphine on early and late phase of formalin nociception in rat.