

***In Vitro* Impact of Hydro-alcoholic Extract of *Rosa damascena* Mill. on Rat Ileum Contractions and the Mechanisms Involved**

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ABSTRACT

Background: The petal's hydro-alcoholic extract of *Rosa damascena* Mill. on ileum contractions of Wistar rats and its possible mechanism were investigated.

Methods: Forty-eight male Wistar rats were divided into six groups. Ileum was placed adjacent to propranolol (1 μ M), naloxone (1 μ M) and L-NAME (100 μ M) and also under the influence of different doses (2-8 mM) of calcium chloride.

Results: Cumulative extract of *R. damascena* Mill. (100, 500, and 1000 mg/L) decreased ileum contractions induced by KCl (60 mM) in a dose-dependent manner ($P < 0.0001$). Propranolol and naloxone significantly decreased the inhibitory effect of the extract on contractions induced by KCl ($P < 0.001$), but L-NAME was ineffective. Furthermore, calcium led to the contraction of depolarized tissue through KCl and this contractile effect decreased significantly induced by the cumulative concentrations of the extract ($P < 0.001$).

Conclusions: The results indicate that *R. damascena* Mill. dose-dependently (100, 500, and 1000 mg/L) decreases ileum movements of the rat probably through stimulating the β -adrenergic and opioid receptors and voltage-dependent calcium channels, and it may be used to treat digestive disorders.

Keywords: Ileum, L-NAME, rat, *Rosa damascena* Mill.

INTRODUCTION

Diarrhea is an important factor leading to morbidity and mortality, especially in third world countries.^[1] There are many drugs and compounds with different mechanisms to treat diarrhea. The bases of using are the increase in the absorption of water, food and electric from the intestine, the reduction in the secretion of the intestine and movements of small bowel.^[2] Although, there are effective synthetic drugs for this purpose, all have side-effects which necessitate the search for new drugs. In traditional medicine, medicinal plants have increasing applications and are used in producing the medicine with fewer side-effects.^[3-6]

The *Rosa* with the scientific name of *Rosa damascena* Mill. from the family of rosaceae and of the genus, *Rosa* grows in different climatic conditions. This plant of shrub nature is perennial, with a height near to 1.5 m, having cylindrical branches, with no grooves and shoulder composite flowers, pink in color and round or oval fleshy fruit.^[7] This plant is one of the most important fragrant genus which has grown wildly firstly and still exists and grows in automated in Europe, Asia and Morocco and Australia.^[7] The main components of plant anthocyanin is cyanidin 3, 5, D-glycoside and several other compounds such as kaempferol, quercetin, galactoside, arabinoside, azlyn, citronellol, linalool, geraniol and terpenes.^[8] Medicinal properties of quercetin and kaempferol falconoid are effective against viruses and cancer cells.^[9] The tetrahydroxy kaempferol falconoid combination which is derived from *R. damascena* Mill. extract is anti-acquired immunodeficiency syndrome (AIDS) through inhibiting the function of AIDS virus proteases.^[10] Rose is antiseptic, antispasmodic, antiviral, and antibacterial and is believed to assist conditions such as frigidity, chronic bronchitis, asthma, skin disease, cancer, ulcers, wounds, wrinkles, and infections.^[11-15] It may be used as antispasmodic for abdominal pain, but no research has been done on the therapeutic effects of the plant on ileum motor activity.

Motor activity of smooth muscle is affected by nerve agents, chemical mediators, drugs and muscle tension. These factors through affecting the cellular mechanisms of muscle activity cause muscle movement. The factors affecting the muscle ion channels, calcium homeostasis, and cytosolic enzymes change motor activity of the muscle.^[16]

In the previous studies, the impact of jonquil from rosaceae family on the contractile activity of ileum and boiled *R. damascena* Mill. *in vitro* effects on rat' jugenom activities were identified with considering a beneficial effect of its treatment in diarrheal disease.^[17,18] In this research, we aimed to determine the effect of *R. damascena* Mill. on the contractile activity of the rat's ileum which is mostly responsible for digestive movements and to investigate the possible mechanisms involved.

METHODS

Extraction methods

In this study, maceration method was used to obtain the extract.^[19] A total of 100 g of *R. damascena* Mill. was completely powdered after cleaning thoroughly. Then, in a suitable container, ethanol 70% was added to cover 3 cm above the powder. After 72 h, the mixture was filtered by a Büchner funnel and the obtained solution was evaporated in the temperature 35°C using a rotary evaporator. The concentrated solution was placed in the incubator with the temperature up to 40°C for 6 h so as to dispel the alcohol completely. Fifteen g of powder was derived from 500 g of the plant which was stored in a refrigerator until use.

Standardization of the extract

The extract was standardized by determination of total flavonoids and phenolic compounds as well as its antioxidant activity as follows:

The amount of total flavonoid compounds in the extract was determined using the method of Akhlaghi *et al.*,^[20] with a minor modification. In brief, to 0.5 mL of the extract or rutin (standard flavonoid compound) was added 1.5 mL of methanol, 0.1 mL of potassium acetate (1 M), 0.1 mL of aluminum chloride (10%) and 2.8 mL of distilled water. The mixture was left at room temperature for 30 min and then, the absorbance was measured at 415 nm using rutin solutions at concentrations of 25-500 ppm in methanol. Total flavonoids were expressed in terms of rutin equivalents (in mg/g).

The amount of total phenolic compounds in *R. damascena* extract was determined colorimetrically using the Folin-Ciocalteu reagent, as described by,^[21] with minor modification. To do this, 5 mL of the extract or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (1:10 diluted with distilled water) and aqueous Na₂CO₃ (4 mL, 1 M). The mixture was left at room temperature for 15 min, and the phenolic components were evaluated by colorimetric assay at 765 nm. To prepare the standard curve different concentrations (0, 50, 100, 150, 200, and 250 mg/L) of gallic acid in methanol:Water (50:50, vol/vol) were prepared. Total phenol contents were expressed in terms of

gallic acid equivalent (in mg/g). The experiments were repeated in triplicate.

To measure antioxidant activity of the extract, ferric thiocyanate method of^[22] was employed. In a suitable vial, 500 µg of the extract was dissolved in ethanol and added to a reaction mixture containing 2.88 mL of 2.5% linoleic acid and 9 mL of 40 mM phosphate buffer. The vial was incubated at 40°C for 96 h. Every 12 h (during incubation), 0.1 mL of the vial content was diluted with 9.7 mL of 75% ethanol, 0.1 mL of ammonium thiocyanate, and 0.1 mL of FeCl₂. Ethanol within the sample and without reagents was used as the negative control. The absorbance of the sample was measured at 500 nm, and the percentage inhibition (the capacity to inhibit the peroxide formation in linoleic acid) was determined using the following equation:

$$\text{Percentage of inhibition} = (1 - [\text{absorbance of sample} / \text{absorbance of control}]) \times 100$$

A high percentage inhibition indicates a high antioxidant activity.

Animals

Forty eight Wistar rats within the range of 150-200 g were prepared from the Research and Reproduction of Laboratory Animal Center of Shahrekord University of Medical Sciences. They were kept in 12/12 h light and dark conditions in a temperature 20-24°C while having easy access to food and water, but they were deprived from food over the night before the testing.

Materials used

Propranolol and L-NAME were prepared from Sigma Co., (USA), Naloxone from the Tolid-Daru Company (Iran) and fertilizer salts from Merck Company (Germany).

Ileum preparation and methodology

In the testing day, the rats were anesthetized with chloroform, and segments of 2 cm of length were isolated from the distal of their ileum (except the last 2 cm) and then were placed vertically in tissue baths (50 ml) including tyrode solution between the two stainless steel hooks. Fixed hook in the bottom of the bath and the upper hook by a thread were connected to the isotonic lever transducer (Harvard, UK) and data logger (Universal Harvard Oscillograph). Isotonic transducer was responsible for converting mechanical work of the tissue into electrical current and transferring it to the data logger

and then recording works on paper by the pen of data logger. Volume adjustment of 60 min during which each 15 min the solution of the bath was replaced, and the constant flow of air bubbles was pumped into the bath. Initial elasticity of the tissues was 1 g and the solution of tyrode bath was 37°C with pH = 7.4. After adjustment, the ileum was contracted using 0.9 ml of potassium chloride (60 mM). When the contractions reached to the pan mode, the cumulative concentration of the extract (100, 500, and 1000 mg/L) was added to the bath and the percentage of the change in contractile force was calculated in proportionate to the pan mode. KCl caused muscle contraction. The maximum contraction by 60 mM was considered as 100% contraction, and changes in contractions by saline or different concentrations of the extract were calculated.^[23,24]

Tyrode solution had the following composition: NaCl (136 mM), KCl (5 mM), CaCl₂ (5 mM), and NaHCO₃ (11.9 mM), MgCl₂ (0.98 mM), NaH₂PO₄ (0.36 mM), and glucose (5.55 mM).

In order to understand the mechanism of the extract's effect on the ileum (the involvement of β-adrenergic receptors, opioid receptors, or nitric oxide, on the contractions derived from KCl, after 30 min of incubating the tissue, propranolol) antagonist β-adrenergic receptor) with a concentration of 1 µM,^[25] naloxone) antagonist opioid receptors (with a concentration of 1 µM^[26] or L-NAME (inhibition NO) with a concentration of 100 µM^[27] were used. To investigate the role of extracellular calcium in the extracts' role, first the tissue was placed in calcium-free tyrode solution containing a high concentration KCl (60 mM) and the calcium chloride (2-8 mM) was added to the bathroom cumulatively. Then, for 5 min the extracts, with cumulative doses were added and the effect was recorded on paper using data logger.^[28]

Statistical methods

The data obtained were analyzed using Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., Chicago). Changes in contractile force induced by extracts and antagonists were calculated in the form of the mean ± standard error of the mean. Statistical test of ANOVA and least significant difference *post-hoc* tests were used to compare the different concentrations of the extract and Student *t*-test was employed to compare the two groups with each other. *P* < 0.05 was considered to be the significant difference.

RESULTS

Analyzing *R. damascene* Mill. factors showed that the amounts of total flavonoids and phenolic compounds in the extract were 48.5 mg/100 g and 109.1 mg/100 g (equivalent to gallic acid), respectively. The antioxidant activity of the extract was 78% equivalent to rutin.

At all stages of accomplishing the above testing, KCl solution (60 mM) led to the increase in ileum contraction and the contract reached to pan mode after a short time. At this point, the extent of ileum contraction was recorded and calculated on paper. Then the effects of saline and cumulative concentrations of *R. damascene* Mill. extract, the effect of incubating the tissue with antagonist drugs of β -adrenergic receptors (Propranolol 1 μ M), opioid (Naloxone 1 μ M) and inhibitors of the synthesis of nitric oxide (L-NAME 100 μ M) and the effect of calcium chloride on the channels dependent on voltage were investigated and recorded [Figure 1].

Comparison of cumulative concentrations of petal's hydro-alcoholic extract of *R. damascene* Mill. petal on KCl-induced contractions in the rat's ileum.

Each of the concentrations of 100 and 500, and 1000 mg/L of the extract significantly and

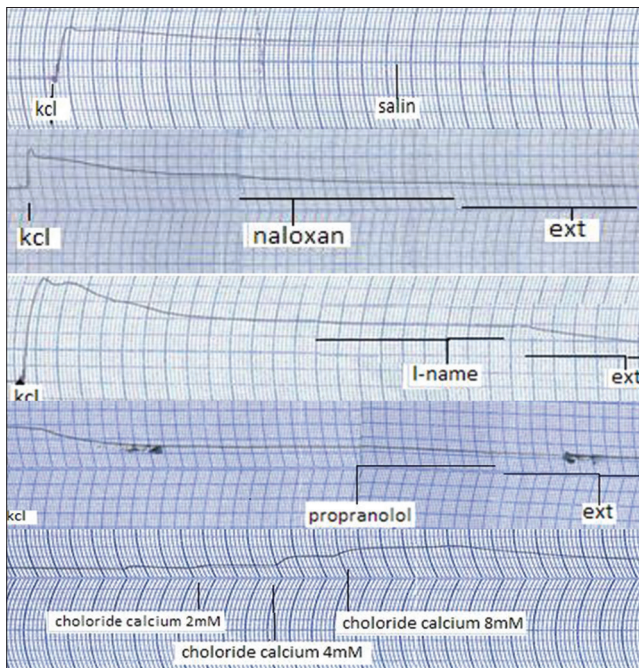


Figure 1: Examples of accomplished records of the rat's ileum in response to the increase in KCl and saline and also the effect of extract after incubating the tissue by chloride calcium, propranolol, L-NAME and naloxane

dose-dependently reduced the ileum contractions induced by KCl (60 mM) compared with the saline group ($***P < 0.0001$).

Figure 2 shows that accumulated extract doses of *R. damascene* Mill. (100, 500, and 1000 mg/L) inhibited ileum contractions induced by KCl ($P < 0.0001$, $n = 8$, ANOVA). Maximum effect was seen in the concentration of 1000 mg/L. The contraction derived from KCl was preserved throughout the testing and the inhibitive function of the extract was not removed after the repeated washing of the tissue within a 15 min period of relaxation. This may show the durable effect of the extract on the tissue, which is related to the extract and not to muscle's exhaustion [Figure 2].

The comparison of *R. damascene* Mill.'s petal extract on the KCl-induced contractions, before and after incubating the rat's ileum through propranolol.

Propranolol is likely to cause relaxation of the ileum and it is probable that the materials effective in the extract cause the inhibitory effect of the extract through affecting β -adrenergic receptors. After comparing the inhibitory effect of the extract on KCl contraction, the inhibitory effect of the extract within 15 min and the washing of tissue for 30 min in the presence of propranolol were compared.

The extract caused inhibition of the KCl contraction ($P < 0.0001$) and propranolol significantly reduced the inhibitory effect of the

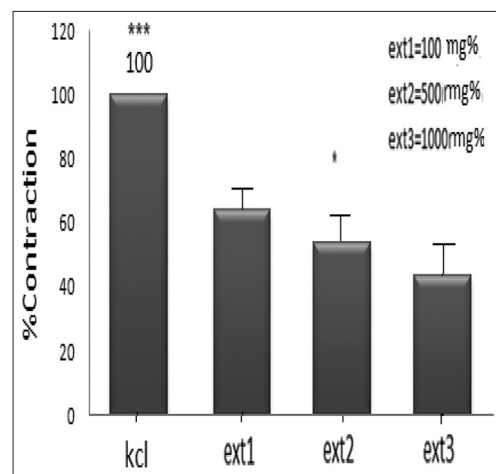


Figure 2: Effect of cumulative concentrations of *Rosa damascene* Mill. extract (100, 500, and 1000 mg/L) on the ileum contractions induced by KCl (60 mM). $***P < 0.0001$, in comparison to different concentrations of the extract. $*P < 0.05$, in comparison with ext1 and ext3, $n = 8$

contraction derived from the extract ($P < 0.001$, ANOVA test, $n = 8$) [Figure 3].

The comparison of *R. damascena* Mill.'s petal extract on KCl-induced contractions, before and after incubating the rat's ileum through L-NAME.

Nitric oxide causes relaxation of the ileum and it is likely that the extract has the inhibitory effect through nitric oxide synthesis. Thus, after comparing the inhibitory effect of the extract on KCl contraction, the inhibitory effect of the extract within 15 min and the washing of the tissue, in the presence of 20 min of L-NAME (an antagonist of nitric oxide synthesis) was compared. The extract caused the inhibition of the KCl contraction ($P < 0.0001$, $n = 8$), but there was no significant difference between the two modes of the extract in the presence and absence of L-NAME [Figure 4].

The comparison of *R. damascena* Mill.'s petal extract on the KCl-induced contractions, before and after incubating the rat's ileum through naloxone.

After comparing the inhibitory effect of the extract on KCl contraction, the inhibitory effect of the extract within 15 min and the washing of the tissue, in the presence of 30 min of naloxone (an antagonist of opioid receptors) were compared.

The extract caused inhibition of the KCl contraction ($P < 0.0001$, $n = 8$) and naloxone significantly reduced the inhibitory effect of the contraction derived from the extract ($P < 0.001$, $n = 8$) [Figure 5].

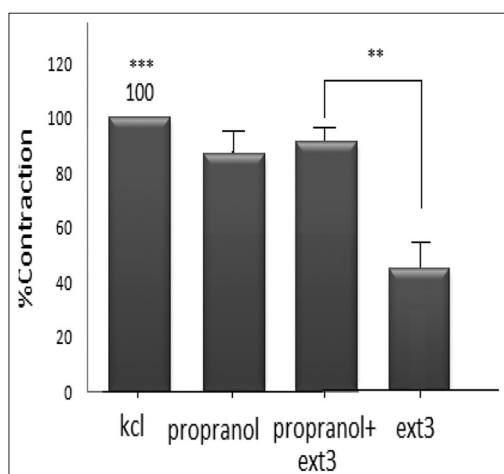


Figure 3: Comparison of the contractile effects of KCl on ileum and the inhibitory effect of the extract at a concentration of 1000 mg/L, and propranolol (1 μ M) on β -adrenergic receptors in the ileum ($n = 8$). $**P < 0.001$. $***P < 0.0001$, in comparison to other groups

The effect of the hydro-alcoholic extract of *R. damascena* Mill. on the CaCl-induced contractions in the depolarized ileum through KCl.

Ileum contractions induced by cumulative concentrations of calcium chloride (2-8 mM) in the depolarized tissue through KCl (60 mM) is related to the calcium chloride concentration ($***P < 0.0001$), and the contractile responses were reduced in the presence of cumulative concentrations of *R. damascena* Mill. extracts ($**P < 0.001$).

In calcium-free tyrode solution containing KCl (60 mM), the addition of cumulative concentrations of calcium chloride to the bath of tissue led to the calcium concentration-dependent contraction of the ileum (ANOVA, $P < 0.0001$, $n = 8$). Incubating the tissue for 3 min with different concentrations of the extract led to the inhibitory effect of the contraction derived from calcium chloride. This antispasmodic effect was concentration-dependent. Comparing the contractile effect of calcium chloride in the absence and in the presence of the extract in all calcium concentrations showed a significant difference ($P < 0.001$, $n = 8$, t -test) [Figure 6].

DISCUSSION

The results of the present research indicate an inhibitory effect of *R. damascena* Mill. on the contraction activity of ileum induced by KCl. The washing of the tissue and the replacement of the

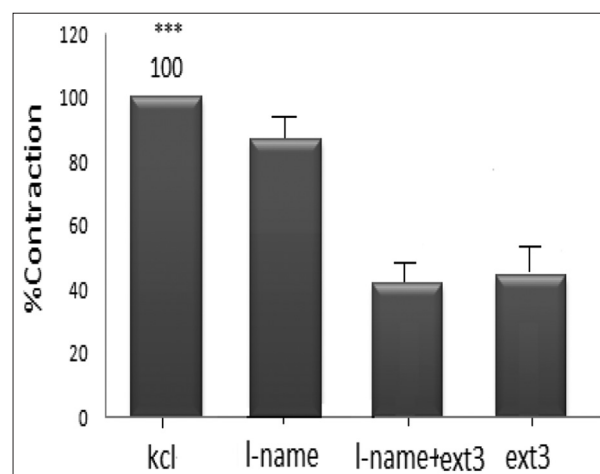


Figure 4: Comparison of the contractile effects of KCl and the inhibitory effect of the extract at a concentration of 1000 mg/L, and L-NAME (100 μ M) inhibitor of nitric oxide synthesis in the ileum ($n = 8$). $***P < 0.0001$, in comparison to other groups

bath solution did not diminish the anti-spasmodic effect of the extract. The reduction of contraction in the presence of the extract cannot derive from the muscle fatigue and is the result of the extract's effect on cellular mechanisms of the muscle and has changed the muscle's motor performance. However, due to lack of a positive control group, receiving a calcium channel blocker, in the present study, this is just estimation and should be clear in future studies.

The membrane of smooth muscle has calcium channels dependent on higher voltage

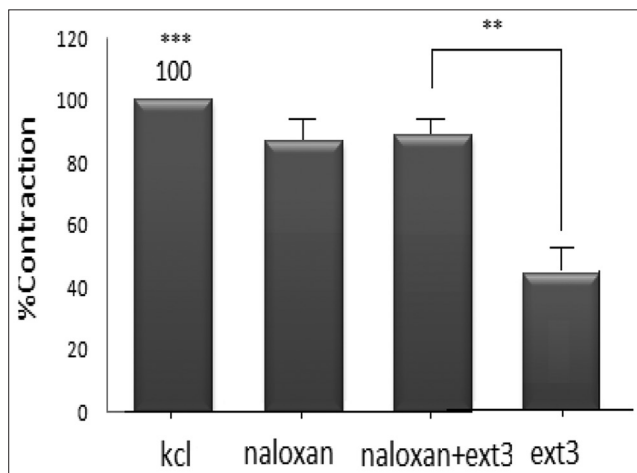


Figure 5: Comparison of the contractile effects of KCl and the inhibitory effect of the extract at a concentration of 1000 mg/L, and naloxone (1 μ M) on opioid receptors in the ileum ($n = 8$). *** $P < 0.0001$) compared to ext3

in comparison with skeleton muscle but has sodium channels dependent on much lower voltage; therefore, mainly the current of calcium ions through sodium and calcium slow channels into the fiber is responsible for the production of the contraction activity.^[17] It has been suggested that the compounds that inhibit the contractions induced by KCl act on voltage dependent calcium channels. Calcium channels are the main channels to cause muscle contraction and the contraction continues as far as these channels are open.^[28] The L-type channels have been identified in the rat's ileum.^[29] It seems that in the present experience, the entrance of calcium through these channels has been disrupted by the extract. As for investigating the mechanism of the extract's effect on the activity of the tissue, it can be said that the activation of opioid receptors leads to the ileum relaxation. The existence of opioid receptors of the kind of δ and μ and their inhibitory effect on ileum has been reported.^[30] β -Adrenergic receptor activation and subsequent increase in cyclic adenosine monophosphate and calcium active transport into the sarcoplasmic network has led to the inhibition of contraction activity of the ileum.^[31] Nitric oxide is also one of inhibitory neurotransmitters of the tract. This second messenger and gas molecule, through activating proteins cyclic guanosine monophosphate-dependent kinases and affecting on ion channels and calcium concentrations, can cause muscle relaxation.^[32] Incubating ileum

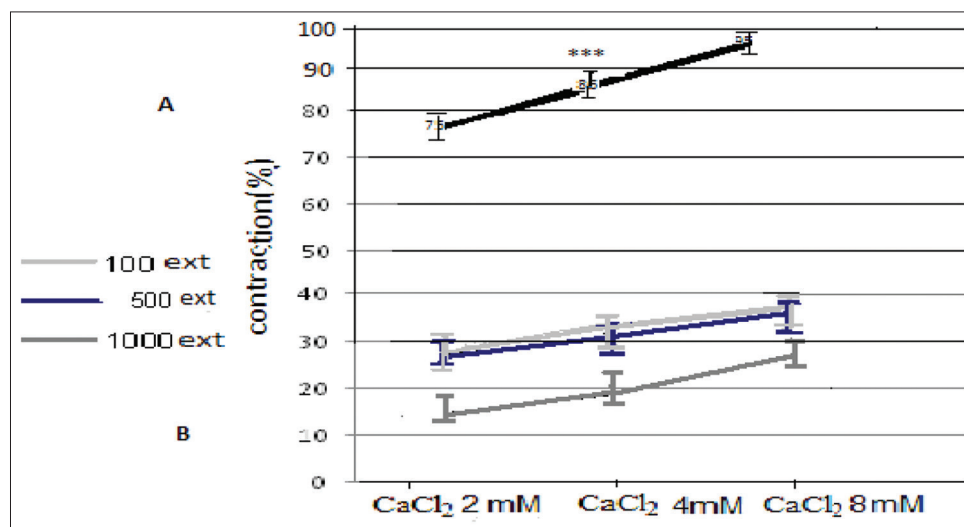


Figure 6: Comparison of the contractile effects of cumulative concentrations of calcium chloride (2-8 mM) and the inhibitory effect of the extract in cumulative concentrations of the extract (100, 500, and 1000 mg/L) ($n = 8$). *** $P < 0.0001$ (ANOVA) compared to other groups

segment with opioid receptor antagonist by naloxone and β -adrenergic receptor antagonist by propranolol has decreased the ileum contraction induced by KCl, indicating a significant difference between the effects of the extract in the presence and absence of propranolol or naloxone. Performance caused the extract to be inhibitory. It is probable that the effective materials in the extract cause the inhibitory activity of the extract through affecting β -adrenergic and naloxone receptor whose presence is determined in the ileum and their inhibitory effects has been determined.^[33] Incubating the ileum segment with antagonist nitric oxide synthesis through half – L medicine has led to the relaxation of the ileum tissue and there was no significant difference between two groups of the extract's reception in the presence and absence L-NAME which shows the inability of nitric oxide on the inhibitory function of the extract. In the present study, the addition of calcium chloride to the medium increased muscle contraction, but the extract led to the contractions induced by calcium chloride. In tyrode solution without calcium, the tissue was depolarized through a great deal of potassium extracellular^[34] and its contraction is dependent upon addition of calcium to the medium too.^[35] Here, the contraction created in the depolarized smooth muscle induced by KCl is dependent on the presence of calcium in the medium.^[36] After adding calcium chloride, the depolarized tissue is contracted through potassium chloride and by voltage-dependent calcium channel in muscle and the contraction is dose dependent. The results showed that the cumulative concentrations of extract inhibits the contractile function of calcium in the depolarized tissue which may indicate the involvement of calcium channels in the development of inhibitory function of the extract. This result is in agreement with the results of others who examined the effects of *R. damascena* on isolated guinea pig heart^[37] or evaluated the effect of hydroethanol extract of *Achillea millefolium* on β -adrenoceptors of guinea pig tracheal smooth muscle.^[38]

Quercetin is one of the flavonoids of *R. damascena* Mill. which can prevent a variety of cancers, including colon cancer^[39] and helping to lower blood pressure in people inflicted with this disease.^[40] Spasmolytic effects on intestinal movements and calcium antagonist properties have

been demonstrated.^[41] The quercetin flavonoid in the aorta led to the endothelial-dependent relaxation. The anti-contractile effects of flavonoids on vascular smooth of the muscle' sidewall and ileum contraction of guinea pig have been reported.^[42] The flavonoids found in marshmallow root of other plants cause relaxation of endothelial-dependent and non-dependent human arteries.^[43,44] It is likely that the inhibitory hydro-alcoholic extract of *R. damascena* Mill. in the concentrations of dose dependent affects voltage-dependent receptors, β -adrenergic and opioid ileum of the rat. Since calcium antagonist properties of the quercetin flavonoid have been proved and also the other inhibitory effects of flavonoids compositions on the smooth muscles of other tissues have been proved, it can be concluded that the present result is due to the main effect of flavonoids in particular quercetin of the extract on the voltage-dependent channels and other plant compositions on the β -adrenergic and opioid of the muscle. However, the illumination of the mechanism of the open extract's effect needs separating the plant's composition one by one and investigating their effect individually on receptors and other factors cause the relaxation of the muscle.

CONCLUSIONS

In general, it can be concluded that hydro-alcoholic extract of *R. damascena* Mill., through affecting the mechanism of the smooth muscle cells including voltage-dependent receptors, dose-dependently affects β -adrenergic and opioid receptors of the rat's ileum. These effects have mostly been attributed to the plants flavonoids. It may be used to treat digestive disorders.

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