Kelussia odoratissima Mozaffarian inhibits ileum contractions through voltage dependent and beta adrenergic receptors

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Abstract: The anti-spasm properties of *Kelussia odoratissima* Mozaffarian have been mentioned in Iranian traditional medicine and it is used to gastrointestinal disorders treatment. The plant leaf alcoholic extract cumulative effect of this plant on Wistar rats ileum contractions and expression of its probable mechanism was investigated in this research. Hydro-alcoholic extract was prepared by maceration method using 70% ethanol. Forty eight male Wistar rats (150-200 g) were randomly designated to 6 random groups with 8 rats in each, in this interventional research as following. Control group, cumulative concentrations of *Kelussia odoratissima* Mozaffarian extract receiving group, propranolol receiving group, naloxone receiving group, L-NAME receiving group, and sodium chloride receiving group. Ileum samples were taken from rat and subjected to 1gr tension in tissue-bath containing tyrode solution. Isotonic contractions was recorded following addition of potassium chloride (60mM), saline or 10% and 20% cumulative concentrations of *Kelussia odoratissima* Mozaffarian extract. In order to understand the above mechanism, ileum was incubated with L-NAME, naloxone or propranolol and also affected by different doses of calcium chloride. Then, the observed effect was recorded and the variation percentage was calculated. Statistical analysis was performed by parametric test, repeated measures, ANOVA and t test. Findings: 10% and 20% cumulative concentrations from alcoholic extracts of *Kelussia odoratissima* Mozaffarian could reduce contractions caused by potassium chloride ($P<0.001$). There was a significant different between extracts of 10% and 20% groups ($P>0.05$). Beta adrenergic receptor blocker (1µM propranolol), significantly decreased the contractions caused by potassium chloride (P-value=0.013) but nitric oxide inhibitor (100 µM L-NAME) and opioid receptor blocker (1µM naloxone) had no effect on this contraction. Calcium was also caused tissue contraction depolarized by potassium chloride and this contraction effect reduced by cumulative concentration ($P<0.001$). In general it can be concluded that Kelussia alcoholic extracts can inhibit ileum contractions of rat through the effect on voltage dependent and beta adrenergic receptors and it might be used to relieve intestinal spasms.

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Key words: Kelussia leaf, ileum, rat.

Introduction:

Plants can be a major source to supply drugs in traditional medicine (1). Among these plants, *Kelussia odoratissima* Mozaffarian from *Umbelliferae* family can be mentioned. Kelussia is a perennial, glabrous, erect and very aromatic and is harvested in April (2,3). Flavonoids have anti-inflammation (4), anti-allergy (5), anti-diabetes, anti-hyperlipidemic and anti-hypercholestrolemic (6,7) effects and improve spatial memory (8). Phthalides constitutes 70% of the plant essence with the effect of liver protection, prostaglandin F2α inhibition, cancer tumors prevention, epilepsy and liver disorders treatment and blood viscosity reduction (2, 10, 13, 14). The ligastilid extracted from Kelussia leads to relax rat's vessels (9). This plant is also used to cure some rheumatism disorders, common cold, cough, blood pressure, blood lipid and stomachache (15). But no research has been done concerning this plant effect on ileum motion activity. The smooth muscle motion activity can be changed by several factors. The contraction of smooth muscle can be affected by:

1. High increasing of extracellular potassium concentration and preventing the outward diffusion of potassium, depolarization, and subsequently contraction of smooth muscle.
2. Opening the slow sodium calcium channels and calcium entry into the cell and beginning the contraction.
3. Activating protein G binding receptors due to which, IP_3 and DG are produced. IP_3 affects sarcoplasmic proteins and results in releasing calcium.
4. Activating rhokinases (Rho) by arachidonic acid which dephosphorylate myosin phosphatase and pump calcium into sarcoplasmic reticulum.

In total it can be said, the factor that increases calcium concentration in cytoplasm and eventually deactivates myosin phosphatase, leads to contraction of the muscle and the factors that decreases cytosolic calcium and activates myosin phosphatase, leads to relaxation of the muscle (16, 17).

In addition to investigation of Kelussia hydroalcoholic extract anti contraction effect on motion activity in this study, the probable mechanism of the extract cumulative concentration anti contraction effect on motion activity of rats was examined including possible involvement of beta adrenergic receptors by propranolol (1µM) inhibitory effect, opioid receptors by naloxone (1µM) and nitric oxide synthesis inhibitory effect by L-NAME (100 µM). The effect of Kelussia hydroalcoholic extracts on ileum contractions of rats with the effect on voltage dependent receptors through calcium chloride (2 to 8mM) was studied cumulatively.

Materials and methods:

The applied materials:

The propranolol and L-NAME were prepared from Sigma Co.(USA), naloxone and all of the salts used in this research were prepared from Tolid Daru Co. (Iran) and Merk Co. (Germany) respectively.

The extraction method:

In order to prepare Kelussia extract the maceration method used in this study. After cleaning and separating extra parts, 1000 g of this plant was dried, powdered and 70% ethanol was added to it. After 72 hours, the obtained mixture filtered by Buchner funnel and the resulting solution was evaporated in 35 ˚C in a Rotary evaporator and dried completely in an incubator with maximum temperature of 40˚C (18). Finally, 35 g extract powder was obtained from 500 g of initial Kelussia. This powder was maintained in a refrigerator until the time of using.

Animals:

Forty eight male Wistar rats (150-200 g) were taken from the Animals Unit of Shahrekord University of Medical Sciences, and then randomly divided to 6 groups with 8 rats in each. The rats were kept in 12 h daylight situation in 24-20˚C and freely feed and watered. But the night before the experiment, they were deprived of food (19).

Ileum preparation and the procedure:

In the test day, the rats anesthetized with chloroform, a 2 cm piece was separated from their terminal part of ileum (except the last 2 cm) and the contractions of this piece was recorded under 1gr tension in tissue bath (500 ml) containing tyrode solution (pH 7.4, 37˚C), air bubble continuous flow and 60 minutes adoption period by potassium chloride (60 mM). The cumulative concentration of extract (10% and 20%) added to tissue bath when the contraction reached to pan mode (20). The obtained variation percentage was recorded by isotonic transducer (Harvard, UK) and a recording machine (Universal Harvard Osilloghraph). In order to study the involvement of beta adrenergic receptors, nitric oxide and opioid receptors, the effect of extract cumulative concentrations on potassium chloride induced contraction was recorded after incubating tissue with 1µM propranolol (21), 100µM L-NAME (22), or 1µM naloxone. To determine the role of extracellular calcium in extract performance, first tissue should be put in tyrode solution with no calcium and high concentration of potassium chloride (60mM) and calcium chloride added to the bath cumulatively (2 to 8 mM) (24). Then the extract was exposed to cumulative concentrations for 5 minutes and its effect was recorded on paper by a recording machine.

Composition of tyrode solution used in the bath (in mM) was:

NaCl (136), KCl (5), CaCl2 (2), NaHCO3 (11.9), MgCl2 (0.98), NaH2PO4 (0.36) and Glucose (5.55)

Data analyzing:

Contraction pan resulted from KCL then, the percentages of relaxation resulted from saline or extract were calculated as mean ± SD (standard deviation). Statistical analysis was performed by parametric test, repeated measures, ANOVA and t test.

Results:

Comparison the effect of Kelussia extract cumulative concentrations on contractions caused by potassium chloride in rat ileum.

Adding potassium chloride to tissue bath led to contraction due to potassium chloride on ileum in the entire period of experiment and after a short time contraction reached to pan mode in which contraction percentage of ileum was calculated. The variation mean of tissue contraction in response to saline was also calculated by adding saline after reaching tissue to the pan mode. Figure 1 shows that saline have low inhibitory effect on tissue contraction due to potassium chloride so that no significant difference observed between two groups. Additionally, although Kelussia hydroalcoholic extract cumulative concentrations (10% and 20%) has reduced ileum contraction caused by potassium chloride (60 mM) ($P<0.0001$), but no significant difference observed for contraction mean between 10% and 20% of Kelussia ($P>0.05$).

The effect of propanolol, L-NAME, and naloxone on extract inhibitory performance

The NO synthesis stimulation may cause reduction of extract contraction performance.

Incubating ileum in the presence of L-NAME (nitric oxide synthetase enzyme inhibitor) for 20 minutes was compared to incubating it in the absence of L-NAME with 15 minutes interval followed by washing tissue. But chart 2 indicates that the extract, has reduced the contraction effect of potassium chloride significantly (P-value=0.000) and there is no significant difference between the inhibitory effect of extract in the presence and absence of L-NAME (P-value=0.703).

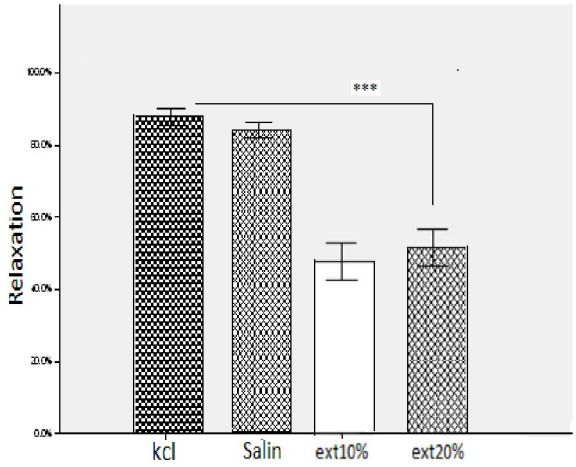

Figure 1: Comparison of ileum contractions between tested groups receiving saline and potassium chloride (60mM). No significant difference was observed between two groups. The relaxation effect of Kelussia extract cumulative concentrations (10% and 20%) on ileum contraction due to potassium chloride (60mM) in comparison with control group (*** P<0.0001, n=8, ANOVA).

Considering that the stimulation of opioid receptors makes intestinal movements reduced, so the inhibitory effect of extract on receptors in the absence of naloxone, was compared to the effect of extract on receptors when it was put in the presence of 1µM naloxone for 30 minutes with 15 minutes interval followed by washing tissue. Figure 2 shows that the contraction effect of potassium chloride significantly was reduced by the extract (P-value=0.000), but there is no significant difference between the inhibitory effect of extract in the presence and absence of naloxone (P-value=0.516).
Stimulation of beta adrenergic receptors also caused relaxation of small intestine. Effective materials may affect receptors and resulted in muscle relaxation. Incubating tissue without propranolol was compared to incubating it in the presence of 1µM propranolol for 30 minutes with 15 minutes interval followed by washing tissue. But figure 2 indicates that the contraction effect of potassium chloride significantly reduced by extract (P-value=0.000) and the inhibitory effect of extract in the presence and absence of propranolol was significantly different (P<0.001).

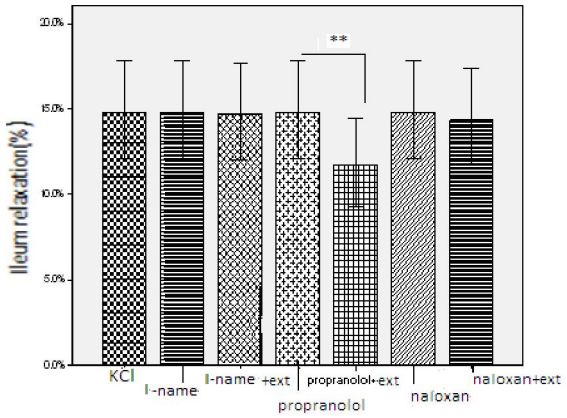

Figure 2: Comparison of the anti-contraction effect of Kelussia hydroalcoholic extract concentration (20%) on contraction caused by potassium chloride (60mM), in the presence and absence of L-NAME (1µM, 30 min, n=8), propranolol (1µM, 30 min, n=8) and naloxone (1µM, 30 min, n=8).
Propranolol+ext receiving groups and extract receiving group without propranolol was significantly different (**P<0/001, n=8).

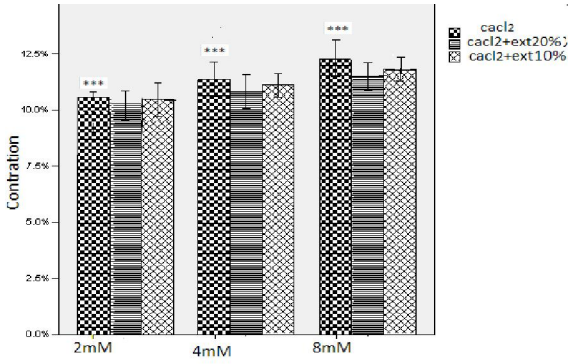

Figure 3: Comparison of calcium chloride cumulative concentrations (2, 4, 8 mM) on ileum in free calcium tyrode solution with high concentration of potassium chloride (60mM) in the absence of extract followed by incubating tissue in cumulative concentration of extract (10% and 20%) for 3 minutes.
Contraction responses of calcium chloride is dose dependent (** P<0.0001, n=8, ANOVA) and represents (P<0-001) significant different between groups with extract inhibitory response 10% and 20%) in the presence of calcium chloride.

The effect of Kelussia hydroalcoholic extract on contraction caused by depolarized calcium chloride through potassium chloride

Figure 3 shows that depolarized ileum of rat has contracted as of concentration dependant (2, 4, 8 mM) in the presence of cumulative concentration of calcium chloride, in tyrode solution with high concentration of potassium chloride (60mM) but without calcium (n=8,P<0.0001). After washing tissue in free calcium tyrode solution and tissue resting for 20 minutes, repeating these steps in the presence of cumulative concentration of extract (10% and 20%) for 3 minutes, decreased the contraction effect in ileum caused by calcium chloride (P<0.001).

Discussion:

This study was aimed to investigate the anti-contraction effect of Kelussia hydroalcoholic extract on ileum motion activity. The probable mechanism of the extract cumulative concentration anti-contraction effect on ileum activity of rats including possible involvement of beta adrenergic receptors, opioid receptors and nitric oxide synthesis inhibitory effect, as well as involvement of voltage dependent receptors in the effect of Kelussia hydroalcoholic extracts were also studied.

Cumulative concentrations of Kelussia hydroalcoholic extract, reduced contractions caused by potassium chloride in the ileum of rat and tissue washing and changing solution bath didn't remove the extract anti-contraction effect therefore; decreasing contraction in the presence of extract can't be due to exhausting muscle during contraction (25). Additionally, contraction occurred by potassium increasing out of smooth muscle cell along with depolarization despite adequate concentration of intracellular calcium ion. The major way for increasing calcium is opening voltage dependent calcium channels after depolarization. The existence of L type calcium channels has been proved in ileum smooth muscle of rat (26). Previous studies reported that contraction caused by potassium chloride is done by involvement of voltage dependent calcium channels (27). Considering the approval of L type calcium channels existence in smooth muscle of ileum, it seems that the observed extract effect is as a result of depolarization consequences inhibition and also increasing intracellular calcium prevention.

To understand the above mechanism, ileum was incubated with L-NAME, naloxone and propranolol and also affected by different doses of calcium chloride. Nitric oxide causes ileum relaxation through cAMP pathway (28). Incubating tissue with L-NAME was not enable to reduce inhibitory performance of extract which represents NO has no involvement in it. In addition, naloxone inability to reduce inhibitory performance of extract, demonstrates opioid receptors has no involvement in it.

Beta adrenergic receptors exist in rats ileum and their inhibitory effect has been proved (29). Propranolol has reduced extract inhibitory effect on contractions caused by potassium chloride in this research too, so probably the effective materials in Kelussia has activated receptors and relaxed muscle by affecting beta adrenergic receptors. Tissue depolarized by high concentration of extracellular potassium in free calcium tyrode, which is consistent with previous studies (30) and its contraction provided adding calcium to environment and the contraction level is depending on the environment calcium dose (31). The results also indicated that the extract inhibits calcium contraction performance in depolarized tissue so confirms calcium channels involvement in occurring extract inhibitory performance.

E-ligustilide Phthalide, 3- e -butyldiene phthalide and z-ligustilide are main chemicals in Koohrang Kelussia (9). Inhibitory and relaxant effects of butylidenephthalide and its z isomer were determined in tissues of many parts of body like different blood vessels, intestine, respiratory and genital system and it seems that inhibition of voltage sensitive calcium channels play a role in it (32). It has been determined in other studies that phtalids in Kelussia leaf have inhibitory effects on prostaglandin F2α (33). Contraction effects of prostaglandin F2α occurs through activation of EP1 receptors. Stimulation of these receptors activates phosphatidyl inositol pathway and increases intracellular calcium (34). So the phthalides in Kelussia extract stops activating phosphatidyl inositol pathway and reduction of cellular calcium as a result obviates contraction caused by potassium chloride in ileum by prostaglandin F2α. The anti-contraction effect of flavonoids on guinea pig was also reported (35). So it can be suggested that voltage dependant calcium channels have main involvement, beta adrenergic receptors have small involvement and nitric oxide synthesis and opiod receptors have no involvement in occurrence of this inhibitory effect and the extract anti contraction performance can be as a result of mentioned flavonoids and phthalides through voltage dependant calcium channels and beta adrenergic receptors.

General conclusion: Totally it can be concluded that alcoholic extracts of Kelussia leaf can inhibit ileum contractions of rat by affecting voltage dependant calcium channels and beta adrenergic receptors and can be used to relieve intestinal spasms.

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