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Original Article

Antispasmodic Effect of *Zataria multiflora* Boiss. Leaf Extract on the Rat Uterus

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Abstract

Zataria multiflora Boiss. is used in Iranian traditional medicine to treat gastrointestinal disorders and menrrhalgia. The inhibitory effect of this herb on rat ileum contractions is also reported. The aim of this study was to investigate the effect of *Zataria multiflora* Boiss. hydroalcoholic leaf extract (ZHLE) on isolated rat uterus in presence of some known uterus stimulants. Pieces of virgin adult rat uterus were mounted in an organ bath containing Tyrode or De Jalon solutions. Uterus contraction was induced by KCl, oxytocin and $BaCl_2$ in presence and absence of certain concentrations of ZHLE. In the oxytocin studies, animals received an injection of oestradiol valerate (5mg/kg, S.C.) 24h prior to the experiment. ZHLE (0.125, 0.25, 0.5, 1 and 2 mg/ml) relaxed the uterus precontracted by KCl (60mM) in a dose-dependent manner ($P<0.0001$) and at 2mg/ml attenuated the $BaCl_2$ (4mM)-induced uterus contraction ($P<0.001$). The inhibitory effect of ZHLE on KCl-induced uterus contraction was unaffected by propranolol (1μM). In normal De Jalon solution, ZHLE (0.125, 0.25, 0.5, and 1mg/ml) reduced the oxytocin (10mU/ml)-induced contraction dose-dependently ($P<0.0001$) but in Ca^{2+}-free De Jalon solution, the stimulatory effect of oxytocin was lesser nevertheless, the inhibitory effect of ZHLE was higher. In presence of atropine (0.5μM), acetylcholine (0.5μM) was failed to induce contraction but KCl (30mM) evoked contraction and the extract diminished the contractile response of KCl. The spasmolytic effect of extract (2mg/ml) on KCl-induced contraction was unaffected by naloxone (1μM). From the obtained results it may be concluded that, the ZHLE may induce the inhibitory effect through blockage of the voltage dependent calcium channels and releasing calcium from intracellular stores in rat uterus smooth muscle. The ineffectiveness of propranolol and naloxone on ZHLE inhibitory effect indicates that adrenergic and opioid agonist substance(s) does not exist in the extract. It seems that there is no anticholinergic substance(s) in the extract. The results support the usage of this plant in traditional medicine.

Keywords: *Zataria multiflora* Boiss.; Spasmolytic effect; Rat; Uterus.

Introduction

Zataria multiflora Boiss. from Labiatae grows in Pakistan, Afghanistan and Iran (1). Different parts of this herb has the same medicinal effect as origanum and traditionally used for convulsian, relief of menstruation pain (2) and its antinociceptive and anti-inflammatory effects has already been reported(1). It's reported that the antinociceptive effect is antagonized by naloxone (1). some antifungal (4-6) and antimicrobial

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effects have also been reported (7) for this herb. The main constituents of *Zataria multiflora* are carvacrol, thymol, linalol and *p*-cymene (7). It has been shown that the hydroalcoholic extract of this herb reduces the acetylcholine and KCl induced ileum contractions in rat (8). These results suggested that the voltage dependent calcium channels (VDCCs) are probably influenced by the herb extract. Since one of the major uses of this herb is to relief dysmenorrhea, therefore, the present study was designed to investigate the effect of *Zataria multiflora* Boiss. leaves hydroalcoholic extract on isolated rat virgin uterus and possible mechanism(s) of its action.

Experimental

Extract preparation

The herb was purchased from local herbal shop in Ahwaz City (Khuzestan province) and identified by Dr. Naanaie from Khuzestan Agriculture and Natural Resources Research Center. The leaves were cleaned, powdered by electric blender and the powder was extracted with 70% alcohol for 72 hours using macerated method. The mixer was filtered with Whatman No 1 filter paper. The solvent of filtrate was evaporated at ambient temperature and extract powder (13.1% of leaf powder) was kept at 4ºC until used.

Animals and tissue preparation

Adult virgin female rats (Sprague Dawley) weighting 185 to 225g were kept in Research Center & Experimental Animal House of Ahwaz University of Medical Sciences under standard conditions (12/12 light-dark cycle, 20-24ºC) with free access to food and water. The procedure followed was in accordance with Committee of Ethics in research of Ahwaz University of Medical Sciences. The animals were killed by a blow on the head, after laparatomy, a piece of uterus (1.5cm) was excised and mounted in an organ bath (10ml) containing Tyrode's or De Jalon solutions (29ºC) between two hooks. The lower hook was fixed at the bottom of the organ bath and the upper hook was connected to an isometric transducer (UF1 Harvard transducer, UK). The equilibrium period was 60 min in which, the bath solution was exchanged every 15min. Under 0.5g resting tension, a pen recording system (Harvard Universal Oscillograph, UK) recorded the uterus contractions. The composition of Tyrode's solution (9) was NaCl (137 mM), KCl (2.68 mM), $CaCl_2$ (1.8 mM), $NaHCO_3$ (11.9 mM), $MgCl_2$ (1.05 mM), and NaH_2PO_4 (0.42 mM) and glucose (5.55 mM). The De Jalon solution was used for oxytocin experiments (10) with following composition: NaCl (154 mM), KCl (5.6 mM), $CaCl_2$ (0.3 mM), $NaHCO_3$ (1.7 mM), $MgCl_2$ (1.4 mM), and glucose (5.5 mM). Because of two reasons De Jalon solution was used in oxytocin experiments: a) the uterus contraction induced by oxytocin in the De Jalon solution was more stable than in Tyrode solution and b) to prevent superimposing the extract inhibitory effect on the rhytmic contractions induced by oxytocin. In the oxytocin protocols, the rats were pretreated with estradiol valerate (5mg/kg, S.C.) 24h prior to the experiment (10). The salts were purchased from Merck (Germany), acetylcholine, propranolol, atropine from Sigma-Aldrich, estradiol valerate from Aborihan (Iran), naloxone from Tolidaru (Iran) and oxytocin from Weimer Pharma (Germany). The mean±SEM of contraction forces (g/100mg tissue) were calculated for each group. The extract was applied to tissue 2-3min after or before applying stimulants and then, the spasmolytic effect was calculated after 3min. The results were statistically analyzed with *t-test* and ANOVA and *P* values less than 0.05 were considered as significant. The (*n*) represents the number of animals used in each protocol.

Results and Discussion

Effect of extract on KCl-induced contraction

KCl (60mM) induced uterus contraction and after 2min and during the plateau, the extract was added at 0.125, 0.25, 0.5, 1, 2 and 4mg/ml non-cumulatively to the organ bath with 10 min intervals and several refreshing bath solution. As shown in figure 1, the KCl-induced contraction forces in the several stages of this protocol are not significantly different. The KCl-induced contractions however, are reduced by extract in a dose-dependent manner (ANOVA, $P<0.0001$, n= 9-12). As shown in figure 2 the extract had the same spasmolytic effect if it was applied before adding KCl to the tissue (n=8-10).

Effect of propranolol on spasmolytic effect of extract

Firstly, the extract (2mg/ml) was applied to the precontracted uterus induced by KCl (60 mM). After 10min and refreshing the bath solution, the same protocol was carried out in the presence of propranolol (1μM, 1min). As shown in figure 3, in both conditions, the extract induced the spasmolytic effect but this activity was unaffected by propranolol (n=9).

Effect of extract on barium chloride-induced contraction

Applying $BaCl_2$ (4mM) to the organ bath induced the sustain contraction in rat uterus and the extract (2mg/ml) reduced this contraction (P<0.01, n=6). Moreover, applying extract (2mg/ml) before adding $BaCl_2$ also reduced the uterus contraction as presented in figure 4. However, the Ba^{2+}-induced contraction in presence of extract (2 min) is less than the inhibitory effect of extract on Ba^{2+}-induced contraction (P<0.05, n=6).

Effect of extract on oxytocin-induced uterus contraction and the role of calcium

a) In normal calcium De Jalon solution:

To show the contractile response of uterus to oxytocin, this hormone (10mU/ml) was added to the bath for 3min and then the tissue was washed. In next stage, extract (0.25mg/ml) was added for 3min and then in the presence of extract, oxytocin was applied to the bath for more 3min. This protocol was repeated for 0.5 and 1mg/ml of extract with 15 min interval and tissue washing. Figure 5 demonstrates that in the presence of normal calcium solution, the contractions induced by oxytocin were reduced by the extract in a dose dependent fashion (ANOVA, P<0.0001, n=8).

b) In Ca^{2+}-free De Jalon solution:

This protocol was the same as previous one, except that, the De Jalon solution was prepared without calcium. Figure 5 also shows that in the absence of both calcium and extract, oxytocin induces contraction, which is not as potent as contractions in the presence of calcium. Extract, however, reduces the oxytocin contractions dose-dependently (ANOVA, P<0.001, n=6). Statistical analysis indicates that the extract dose responses in these two

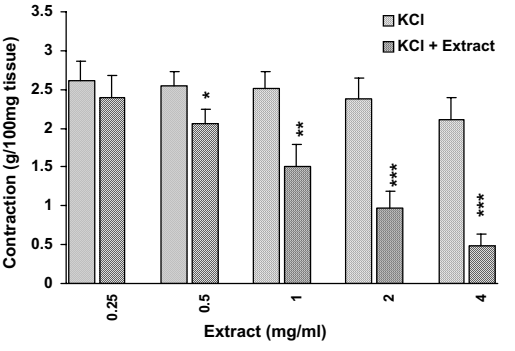

Figure 1. Spasmolytic effect of *Zataria multiflora* leaf extract on KCl (60 mM) induced rat uterus contraction. The repeated contractions induced by KCl are not significantly different but the inhibitory effect of extract is dose-dependent (ANOVA, P<0.0001, n = 9-12). The spasmolytic effect for each extract concentration against KCl-induced contraction were compared (* P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001).

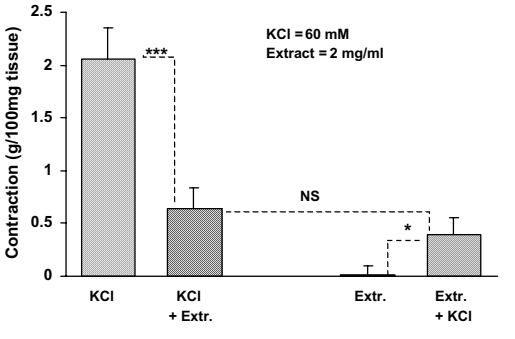

Figure 2. The comparison of spasmolytic effect of *Zataria multiflora* leaf extract on rat uterus after and before inducing uterus contraction by KCl. This figure shows that adding extract to the organ bath before or after applying KCl, inhibited the stimulatory effect of KCl (* P<0.05, NS= not significant, n=7-9).

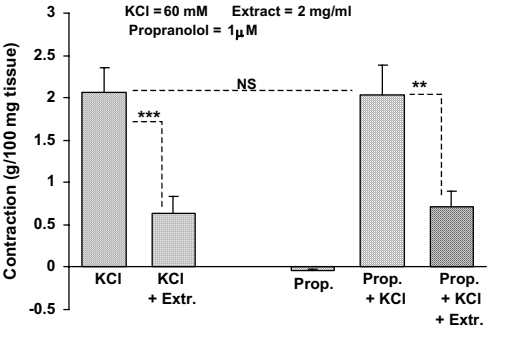

Figure 3. Spasmolytic effect of *Zataria multiflora* leaf extract on rat uterus precontracted by KCl in the absence and in the presence of propranolol. This figure indicates that propranolol as a non-selective β-adrenoceptor antagonist did not alter the extract spasmolytic effect. Moreover, the KCl-induced contraction was unaffected by propranolol (** P<0.01, *** P<0.001, NS = not significant, n=9).

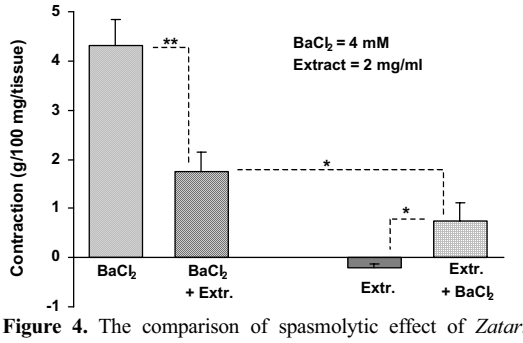

Figure 4. The comparison of spasmolytic effect of *Zataria multiflora* leaf extract on rat uterus after and before inducing contraction by $BaCl_2$ (4mM). As shown in this figure, presence of the extract before adding $BaCl_2$ induces a more potent inhibition which, could be due to longer presence of extract in the bath comparing to the first procedure (* P<0.05, ** P<0.01, n= 6).

conditions are significantly different (P<0.05) except for 0.5mg/ml extract concentration. The comparison of reproducibility of oxytocin-induced contractions before applying extract revealed that the contractions in the presence of calcium are more sustained than in the absence of calcium (results are not shown).

Inhibitory effect of extract in the presence of naloxone

Effect of the extract on the KCl-induced uterus contraction was investigated in the absence and in the presence of naloxone as a non-selective opioid receptor antagonist. KCl (60 mM) induced contraction was reduced by the extract (2mg/ml). After at least 15min recovery period and several tissue washing, the same protocol for KCl and extract was carried out but in the presence of naloxone (1μM, 5min). As demonstvated in figure 6, the spasmolytic effect of extract is not affected by naloxone (n=7-9).

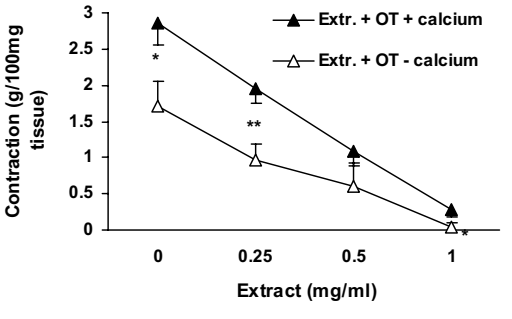

Figure 5. Spasmolytic effect of *Zataria multiflora* leaf extract on rat uterus contracted by oxytocin (OT, 1omU/ml) in normal (n = 8) and Ca^{2+}-free (n = 6) De Jalon solutions. As the figure shows, in the absence of the calcium, the induced contractions were significantly lower. In addition, the antispasmodic effect of the extract (conc. 0.25 mg/ml and 1 mg/ml) in these two solutions were also significantly different (* P<0.05, ** P<0.01).

Study of anticholinergic activity of extract

Uterus contraction induced by acetylcholine (0.5μM) was superimposed on the contraction induced by KCl (30mM) and then atropine (0.5μM) was added to the bath. As shown in figure 7, the uterus contraction was reduced (P<0.0001, n=7). Bath solution was exchanged several times and the tissue left to rest for 30min. Then, atropine (0.5μM) was applied to the bath for 5min and in the presence of atropine, KCl and acetylcholine were added with the previous concentrations. The figure 7 shows that the uterus responded to the KCl but not to the acetylcholine. Continuing this procedure (2mg/ml) extract reduced the uterus contraction (P<0.0001). The inhibitory effect of extract is more potent than the atropine effect (P<0.01).

This study demonstrated that *Zataria multiflora* Boiss hydroalcoholic leaf extract (ZHLE) can induce spasmolytic effect on KCl-, $BaCl_2$ - and oxytocin-induced contractions in rat uterus. Although, the antifungal (4-6), antimicrobial (7), antinociceptive (1), anti-inflammatory (3) and antispasmodic effects on ZHLE on rat ileum (8) have already been reported but its spasmolytic activity on uterus is poor investigated. However, the spasmolytic effect of *Thymus vulgaris* from the same family (Labiatae) on guinea pig trachea and ileum were reported (11-12). Extracellular high potassium is well known as smooth muscle stimulant and a calcium channels (VDCCs) opener (13). Since, verapamile (L-type VDCCs blocker) deminished the high K^+ -induced contraction in smooth muscle, it is therefore suggested that those substances that decrease the K^+-induced contraction can also block the VDCCs (14). The existence of L-type VDCCs in rat uterus is also documented (15). Our results indicated that ZHLE inhibited the KCl-induced uterus contraction. This part of results is in accordance to spasmolytic effect of ZHLE on rat ileum (8). It has already been shown that β-adrenoceptors activation in uterus causes relaxation (16, 17) but the ineffectiveness of propranolol on the extract relaxatory effect suggested that β-adrenoceptors are not involved in ileum study (8). Another study showed that barium chloride could induce smooth muscle contraction by blocking the potassium channels followed by depolarization and contraction (18)

although, it has also been reported that Ba^{2+} promotes Ca^{2+} release from intracellular pools in uterus (19) and stomach (20) smooth muscles. Our results showed that ZHLE reduced the Ba^{2+}- induced contraction which, results from prevention either calcium efflux or calcium release but, as mentioned above, the effect of ZHLE should be on the surface of the cells since this effect was disappeared by washing the tissue as is discussed in the following.

In next part of the study, the effect of ZHLE on oxytocin-induced uterus contraction was investigated in De Jalon solution with low calcium concentration which prevents the spontaneous motility in uterus and superimposing these contractions with oxytocin-induced contraction (21). Oxytocin binds to its receptors and increases inositol triphosphate (IP_3) production followed by promoting calcium release from intracellular pools (22, 23). Therefore, oxytocin may contract the uterus in Ca^{2+}-free solution (24). Oxytocin also activates the L-type VDCCs (25). In the present study we demonstrated that ZHLE reduced the oxytocin-induced contractions in De Jalon solution with and without calcium. From first part of the obtained results, it may suggest that ZHLE blocks the L-type VDCCs and therefore relaxes the contracted uterus. On the other hand, in Ca^{2+}- free solution it maybe suggested that extract interacted with oxytocin receptors and/or prevented the calcium release from intracellular pools. In these three main procedures (uterus contraction by K^+, Ba^{2+} and oxytocin) the voltage sensitive calcium channels are involved as these contraction were reduced by the extract. Therefore, it may postulated that ZHLE acts through blockade of these channels.

It has already been reported that naloxone antagonized the antinociceptive effect of *Zataria multiflora* (1), but the present study showed that the ZHLE spasmolytic effect was unaffected by naloxone which may suggest that the opioid receptors are not involved in this activity. Whether the extract has any anticholinergic activity or not, our results showed that atropine (as a non-selective muscarinic receptor antagonist) reduced the superimposed contractions of KCl and ACh. In the presence of atropine, only KCl but not Ach could induce contraction. The inhibitory effect of the same extract on ACh-induced contraction in

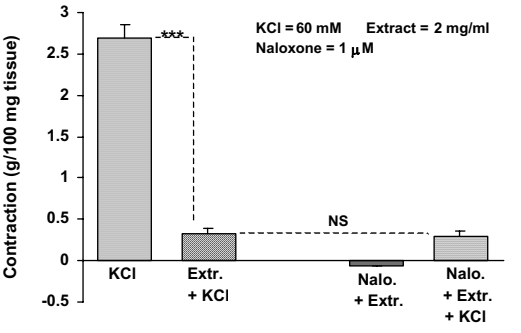

Figure 6. Spasmolytic effect of *Zataria multiflora* leaf extract on KCl-induced contractions in the absence and in the presence of naloxone. As this figure shows, naloxone alone induced a small reduction in basal tone. Moreover, the extract spasmolytic activity was unaffected by naloxone (*** P<0.0001, NS = not significant, n = 7-9).

rat ileum has also been reported (8). From this results we concluded that in ZHLE there is no anticholinergic substance(s) and the inhibitory effect of extract is due to the action on the common pathway that ACh and KCl cause the contraction. This common path way is the Ca^{2+} influx from extracellular fluid. This part of study also proved the suggested mechanistic effect of the extract on K^+-induced contraction through blockade of VDCCs. Therefore, the inhibition of the ACh-induced contraction in rat ileum by the extract (8) may indicate the ACh related consequences of the later events rather than the involvement of the extract anti-muscarinic property.

In the all procedures (except for oxytocin effect in Ca^{2+}-free solution) the spasmolytic effect of extract was reversible, becouse the induced relaxation was disappeared by washing the affected tissue. We may firelly conclude that,

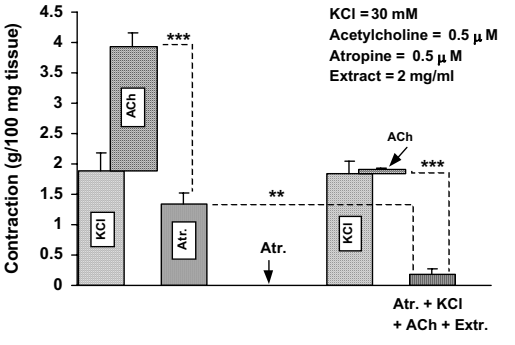

Figure 7. Superimposed contractions induced by KCl and acetylcholine before and after inducing atropine in rat uterus. Moreover the presence of atropine, the *Zataria multiflora* leaf extract diminished this superimposed contractions. In addition, in the presence of atropine, the KCl-induced contraction was not affected but the ACh-induced contraction was inhibited (** P<0.01, *** P<0.0001, n= 7).

the extract effective substance(s) induce their effect on the surface of the smooth muscle cells.

Our results also demonstrated the involvement of calcium channels in spasmolytic effect of *Zataria multiflora* Boiss in the rat uterus, which may support its use in folk medicine to relief dysmenorrhea.

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