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Original Article

Assessment of hydroalcoholic extract of seeds and leaves of *Moringa peregrina* on ileum spasm

H. Sadraei[1,*], G. Asghari[2], and F. Farahnaki[1,2]

[1]*Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[2]*Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*

Abstract

Seeds of *Moringa peregrina* (Forssk.) Fiori. (Moringaceae) is widely used in south east of Iran for gastrointestinal disorders. However, so far there is no pharmacological evidence for antispasmodic activity of this plant extract. Therefore, the aim of this research was to investigate antispasmodic activity of *M. peregrina* on rat isolated ileum contraction. Hydroalcoholic extract was obtained by percolation method from seeds and leaves of *M. peregrina* collected from Baluchestan province of Iran. A portion of isolated rat ileum was suspended under 1 g tension in Tyrode's solution at 37 °C and gassed with O_2. Effects of seeds and leaves extracts of *M. peregrina* were studied on ileum contractions induced by KCl (80 mM), acetylcholine (ACh, 250 µM) and electrical field stimulation (EFS). The seed extract of *M. peregrina* concentration dependently inhibited the response to KCl (IC_{50}=87 ± 18 µg/ml), ACh (IC_{50}=118 ± 18 µg/ml) and EFS (IC_{50}=230 ± 51 µg/ml). The extract of *M. peregrina* leaves also had inhibitory effect of ileum contraction induced by KCl (IC_{50}=439 ± 108 µg/ml), ACh (IC_{50}=365 ± 61 µg/ml) or EFS (IC_{50}=314 ± 92 µg/ml). From these experiments it was concluded that *M. peregrina* extract mainly had an inhibitory effect on ileum contractions but the seed extract was more potent than the leave extract in inhibiting KCl and ACh contractile responses.

Keywords: *Moringa peregrina*; Seeds; Leaves; Extract; Ileum

INTRODUCTION

Moringa. peregrina (Forssk.) Fiori. (Moringaceae) is a tree growing in Sistan and Baluchestan province of Iran and locally called "Gas-e-rowghan" or "Gaz Rokh" (1,2,3). *M. peregrina*, is a native plant in part of Asia and Africa (3-8). It contains 13 species that ranges in size from tiny herbs to massive trees (4,5). The most widely cultivated species is *M. oleifera* (9,10). Much of the plant including the seeds is edible by humans or by farm animals (11). The leaves are rich in protein, vitamins and minerals while the seeds contain 30 to 40% oil which is high in oleic acid (11-14)

Volatile constituents of the seeds of *M. peregrin*a includes isobutyl isothiocyanate, isopropyl isothiocyanate, sec-butyl isothiocyanate, n-butyl isothiocyanate and benzyl isothiocyanate while the volatile constitutes of the leaf are isobutyl isothiocyanate, isopropyl isothiocyanate, n-butyl isothiocyanate and sec-butyl isothiocyanate (15,16).

Ethnobotanical studies indicate that *M. peregrina* is used to treat headache, fever, gut pain, burns, back and muscle pain (7). Other indications include treatment of malaria, hypertension, stomach disorders, asthma and for labour (17,18). Furthermore, in animal model, *M. peregrina* has shown to have antihyperglycemic activity (8). *Moringa* seeds and its oil suggested having pharmacological properties such as antimicrobial, antitumor, anti-inflammatory, diuretic and larvicidal activities against mosquito that transmits dengue and yellow fever (17-21). In traditional medicine, *M. peregrina* has been used as antispasmodic, analgesic and antiinflammatory

*Corresponding author: H. Sadraei
Tel: 0098 31 37927072, Fax: 0098 31 36680011
Email: sadraei@pharm.mui.ac.ir

(7,19). Others suggested that *M. peregrina* may also have, antitumor activities including breast and colon cancers as well as antioxidant activities (22.23). It has been reported that the antioxidant activity might be due to the presence of a well-known flavonoid rutin (22). There is a pharmacological report about antispasmodic effect of *M. oleifera* (21). However, despite existence of a number of suggestions that *M. peregrina* has been used as antispasmodic agent in traditional medicine, the antispasmodic effect of *M. peregrina* extracts on gastrointestinal (GI) tract has not been studied by standard pharmacological techniques. Therefore, the aim of this research was to investigate the effect of *M. peregrina* seed and leaf extracts on ileum contraction, in order to evaluate their inhibitory effects on intestinal contraction.

MATERIALS AND METHODS

Plant materials

Seeds and leaves of *M. peregrina* were collected in 2013 from Nikshahr city, in Sistan and Baluchestan province of Iran. Voucher specimens identified by Dr. Iraj Mehregan and the seeds and leaves (NO: 2025) were deposited in the herbarium of School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

The leaves and the seeds were separated from branches and dried in shade. The plant materials (seeds or leaves) were powdered separately using electrical miller (Moulinex, France). The total hydroalcoholic extract was obtained by percolation (24) using 80% ethanol with solvent to plant powder ratio of 8:1. The solvent was evaporated and percentage yield of the dried extract was obtained.

In vitro contractility assessment

All animals were handled in accordance with the internationally accepted principles for laboratory animal use and care, as recommended by university authority (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010) (25). Male Wistar rats (180–220 g), bred in School of Pharmacy animal house were killed by a blow on the head followed by exsanguination. The abdomen was opened and a piece of ileum was dissected and placed in oxygenated Tyrode's solution at room temperature. Longitudinal strips of ileum, 2-3 cm long, were then prepared and mounted under 1 g resting force in an organ bath (Harvard, England) filled with Tyrode's solution at 37 °C and gassed with O_2. The tissues were washed several times with fresh Tyrode's solution and allowed to relax to a stable base line. Contractions were induced by KCl (80 mM), acetylcholine (ACh, 250 µM, 30 s contact) or electrical field stimulation (EFS, 6 V and 50 Hz for 1s duration) and recorded on a Harvard Universal Oscillograph (England) pen recorder device as described before (26-28).

After reproducible contractions were established, the extract was added directly into organ bath at 12 min intervals. Initially a number of pilot experiments were carried out for determination of effective concentration ranges of extract of *M. pereg*rina. Then full concentration response curves were obtained for each drug using 8-10 different concentrations of extracts.

In case of KCl drugs were added into the bath in a cumulative manner while for ACh and EFS noncumulative method was used. After maximum inhibitory effect was achieved, the tissue were washed with fresh Tyrode's solution and tested to see if the inhibition was reversible.

All experiments were performed alongside time-matched vehicle treated controls.

Drugs and solutions

Tyrode's solution composed of (mM): NaCl, 136.9; KCl, 2.68; $CaCl_2$, 1.8; $MgCl_2$, 1.05; $NaHCO_3$, 11.9; NaH_2PO_4, 0.42 and glucose, 5.55, were made up in distilled water. Unless stated, all chemicals and drugs were from Merck.

The following drugs were used in this research: Extracts of *M. peregrina*, acetylcholine hydrochloride (Sigma). The extracts were made up as 50 mg/ml stock solution in dimethyl sulfoxide (DMSO), dilution being made in 50% DMSO or distilled water (20 mg/ml, 2 mg/ml and 200 µg/ml).

KCl (2 M) stock solutions were prepared in distilled water. ACh was made up as 100 mM stock solution and acidified by 1% acetic acid, and further serial dilutions (250 µM) were made in distilled water.

Measurements and statistical analysis

Contractile response to KCl, ACh and EFS were measured as maximum amplitude from the baseline, just before addition of next concentration of the drugs and expressed as the percentage of the initial response in the absence of drugs for each tissue. All the values are quoted as mean ± standard error of the mean (SEM).

Statistical significance was assessed using one-way analysis of variance (ANOVA) for repeated measurements and comparing the appropriate ones with the control groups using unpaired *Student's t-test*. Differences were considered statistically significant for $P<0.05$.

Whenever appropriate, the IC_{50} value (drug concentration causing 50% of maximum response), was calculated. Sigma Plot computer program was used for statistical analysis, drawing the graphs and calculation of IC_{50} values.

RESULTS

The yield of seed and leaf extracts of *M. peregrina* was 7% and 23%' respectively. Dried leaf extract had dark greenish colour while the seed extract had yellowish oily appearance.

Rat isolated ileum suspended in the organ bath gradually relaxed to a stable baseline over 10 to 20 min. Addition of KCl (80 mM), caused a rapid phasic spasm followed by a stable sustained contraction maintained during the course of experiment. No increase in basal activity or tension of ileum was observed following addition of either seed or leaf extracts into organ bath. However, both extracts of seeds and leaves of *M. peregrina* concentration-dependently inhibited sustained contraction to KCl but the seed extract was more potent than the leaf extract (Fig. 1). At bath concentration of 320 µg/ml the seed extract removed the contractile response to KCl while the leaf extract only inhibited the response by 45%. At its highest used concentration (1.28 mg/ml) the contractile response to KCl was also totally removed by the leaf extract (Fig. 1).

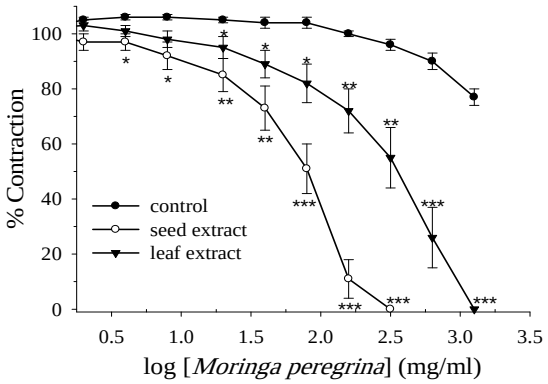

Fig. 1. Cumulative effect of *M. peregrina* seed and leaf extracts on tension development to potassium chloride (KCl, 80 mM), in rat isolated ileum. Lines drawn through the points, using two fold increments in concentration. The points are mean ± SEM (n=6). The reduction in ileum response in the control group is statistically significant ($P<0.001$, ANOVA). Stars shows statistical differences between each drug concentration with its corresponding vehicle treated control. Keys: $*P<0.05$, $**P<0.01$, $***P<0.001$ (*Student's t-test*). Maximum concentration of the vehicle (DMSO) in the bath was 0.4% for seed extract and 2.8% for leaf extract.

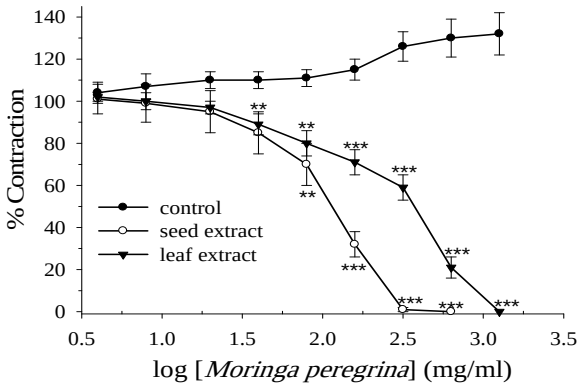

Fig. 2. Effect of *M. peregrina* seed and leaf extracts on tension development to acetylcholine (0.5 µM) in rat isolated ileum. Lines drawn through the points, using two fold increments in concentration. The points are mean ± SEM (n=6). The increase in the response of vehicle treated control tissues is statistically significant (ANOVA, $P<0.05$). Stars shows statistical differences between each drug concentration with its corresponding vehicle treated control. Keys: $**P<0.01$, $***P<0.001$ (*Student's t-test*). Maximum concentration of the vehicle (DMSO) in the bath was 1.28% for seed extract and 2.56% for leaf extract.

The IC_{50} values for seed and leaf extracts were 87 ± 18 µg/ml and 439 ± 108 µg/ml respectively. After washing the tissue with fresh Tyrode's solution the contractile response to KCl was gradually restored. In the vehicle treated time-matched control there are small but significant reduction in the contractile responses. The maximum concentration of DMSO for seed and leaf extracts was 0.4% and 2.8%, respectively. DMSO concentration less than 0.4% had no significant inhibitory effect on KCl induced contractions.

Addition of ACh into organ bath caused a rapid contraction in rat ileum during 30 s of the contact time. After washing the tissue with fresh Tyrode's solution the ileum tension quickly restored to the baseline. The seed (20-640 µg/ml) and the leaf extracts (20 µg/ml-1.28 mg/ml), in a concentration-dependent manner, inhibited ileum contraction induced by ACh (Fig. 2). The pattern of inhibition was relatively similar to the inhibitory effect seen on KCl induced contraction.

As it is presented in Fig. 2, the seed extract was more potent than the leaf extract. For comparison, the IC_{50} values with the seed extract was 118 ± 18 µg/ml while the IC_{50} values of the leaf extract was 365 ± 61 µg/ml. The inhibitory effect of both extracts was reversible following removal of the extract from organ bath. In the vehicle treated time-matched control group DMSO potentiated the ACh contractile responses and this increase in ACh response was statistically significant (P<0.05, ANOVA) (Fig. 2).

Rat's ileum contracted rapidly in response to EFS, reaching a peak followed by partial relaxation which was then followed by a second peak and then relaxed towards the baseline as described before (28,29). Relaxant effect of extracts of *M. peregrina* at the concentration ranges which inhibited the KCl and ACh responses were also examined on biphasic contractions induced by EFS. Both the seed (8 µg/ml-1.28 mg/ml) and the leaf extracts (8 µg/ml-1.28 mg/ml) in a concentration-dependent manner inhibited both first and second EFS contractile responses in rat ileum (Fig. 3a, 3b). The patterns of inhibition were relatively similar for both seed and leaf extracts. However, both extracts were more potent in inhibiting the second contractile response of EFS (Fig. 3b). The IC_{50} values of the seed extract for the initial and the secondary contractile response were 230 ± 51 µg/ml and 153 ± 68 µg/ml, respectively (n=7). The IC_{50} values of the leaf extract for the initial and the secondary contractile response were 314 ± 92 µg/ml and 106 ± 17 µg/ml, respectively (n=6).

Over the course of study, an increase in initial contractile response to EFS was observed in the time-matched vehicle treated control tissues (P<0.05, ANOVA) while, there was no statistically different change in secondary contractile response in the control tissues (Fig. 3). The inhibitory effect of the extract on KCl, ACh and EFS responses was reversible following washing the tissue with fresh Tyrode's solution.

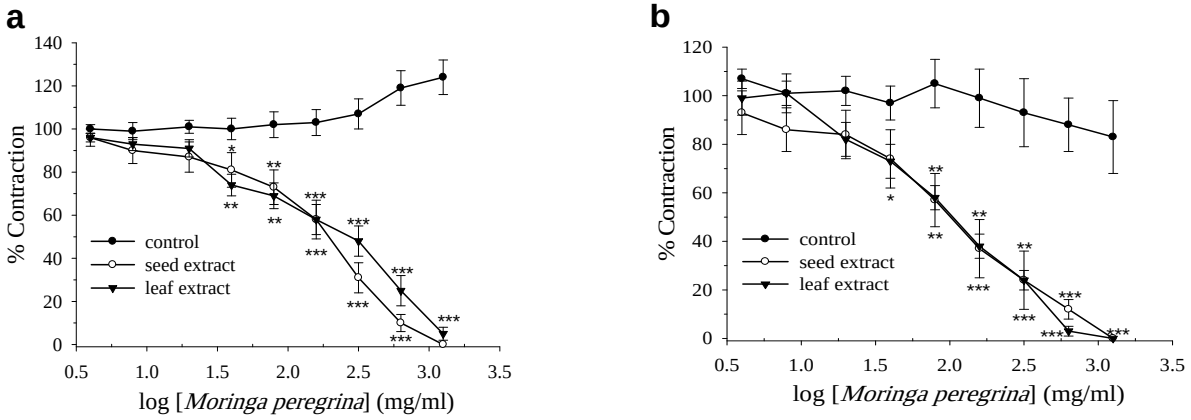

Fig. 3. Effect of *M. peregrina* seed and leaf extracts on tension development to a; first and b; second contractile responses to electrical field stimulation (EFS, 6V, 50 Hz, 1 s duration) in rat isolated ileum. Lines drawn through the points, using two fold increments in concentration. The points are mean ± SEM (n=6). The changes in the response of vehicle treated control tissues for first EFS response (a) is statistically significant (ANOVA, P<0.05). Stars shows statistical differences between each drug concentration with its corresponding vehicle treated control. Keys: *P<0.05, **P<0.01, ***P<0.001 (*Student's t-test*). Maximum concentration of vehicle (DMSO) in the bath was 2.56%.

DISCUSSION

The seeds of Gas-e-rowghan are traditionally used as an aliment for the treatment of GI disorders (1-3). However, so far there is no report on pharmacological activity of this plant extract on GI movement. Therefore, the objective of this research was to investigate antispasmodic effect of *M. peregrina* extract on rat isolated ileum. Drugs that affect lower GI function may act on smooth muscles and/or work by modulating the activity of the enteric nervous system (ENS). The ENS is embedded in the lining of the GI system. The neurotransmitters ACh, serotonin (5HT), and a number of peptides including opioid peptide are the important regulators of gut motility (30,31).

All ileum strips were contracted in response to addition of KCl into the bath and were maintained during the course of the studies in the control groups. The KCl induced contraction of ileum is due to K^+ depolarization of smooth muscle membrane potential, leading to an increase in the systolic free Ca^{2+} ions through activation of voltage gated calcium channels (32). Nifedipine totally blocks the KCl response indicating the involvement of dihydropyridine sensitive Ca^{2+} channels in contraction induced by KCl (32-34). Extracts of *M. peregrina* in a concentration-dependent manner completely inhibited the contractile response to KCl, indicating that voltage gated Ca^{2+} channels directly or indirectly are affected by the extracts.

Another spasmogen used in this study was ACh which caused a phasic contraction of rat isolated ileum. Contraction induced by ACh is mediated through muscarinic M_3 receptors which are coupled to phospholipase C and release of intracellular Ca^{2+} (35). The contractile response of ACh on rat ileum is totally removed by muscarinic receptors antagonist atropine or propantheline while nifedipine only partially inhibits the ACh response in rat ileum (27,28). In a similar fashion the extracts of *M. peregrina* reversibly inhibited contraction due to ACh. This indicates that the components exist in the extract are either blocking the muscarinic receptors or the active components are acting intracellularly to prevent myofibril contraction.

As contractile response to neuronal stimulation is closer to natural gut activity, we have applied EFS for stimulation and release of natural neurotransmitters. The parameters used for EFS mainly stimulate the ENS situated between the longitudinal and the circular muscle layers of the GI tract. This is supported by our previous studies that the local anesthetic lidocaine at selective concentration inhibits the EFS responses without affecting the ACh induced contraction (27,28). Atropine and propantheline did not inhibit the EFS responses completely. This is because excitatory transmitters other than ACh are released during neuronal stimulation. The remaining contraction is most likely due to the release of other excitatory neurotransmitters from the enteric plexus (27,28). These findings are in consistence with other reports that muscarinic antagonists only partially remove contractile response to neuronal stimulation of ileum (36). Nifedipine also partially inhibits the EFS responses (27,28) This is because some excitatory neurotransmitters including ACh is acting via production of second messenger system inositol triphosphate and release of internal Ca^{2+} (35). Extracts of *M. peregrina* totally removed both the initial and the secondary contractile responses to EFS on rat ileum. Total inhibition of KCl, ACh and EFS responses by the extracts indicate that the inhibitory effect is mainly post-synaptic but it is not like muscarinic receptor antagonists or Ca^{2+} channel blocker. On the other hand, as *M. peregrina* extracts contain many constituents, thus, there is a possibility that some compounds are acting as Ca^{2+} channel blockers while others acting intracellularly or antagonizing receptors for the neurotransmitters. The reversibility of response following washing the tissue with fresh Tyrode's solution indicate that no damage has been made to the tissue and after removal of the extract, normal activity of tissue could be restored.

Several different compounds are identified in ethanolic extract of leaf extract of *M. peregrina* which include lupeol acetate,

β-amyrin, α-amyrin, β-sitosterol, β-sitosterol -S-O-glucoside, apigenin, rhamnetin, neochlorogenic acid, rhamentin-3-O-rutinoside, 6-methoxy-acacetin-8-C-β-glucoside, chryseriol-7-O-rahmnoside, quercetin, and quercetin-3-orutinoside (8,18). Among these compounds α-amyrin and quercetin are reported to have spasmolytic activity on smooth muscles (37,38). It is also suggested that other compounds are responsible for antispasmodic activity but their effect are not proven by scientific research studies. Therefore, for identification of the lead compounds, further scientific investigation for determining the antispasmodic of these compounds are suggested. As the seed extract was found out to be more potent than the leaf extract, identification of active compounds of seed extract is also recommended.

CONCLUSION

The seed and leaf extracts of *M. peregrina* had inhibitory effect on the contraction of rat isolated ileum. The antispasmodic effect of the extracts was seen both before (ACh, EFS) and after (KCl) induction of contraction. The inhibitory effect of the extract of *M. peregrina* is in consistent with people beliefs that seeds of this plant are useful for abdominal spasm.

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