

Research Article

Zataria multiflora Boiss and Carvacrol Affect β_2 -Adrenoceptors of Guinea Pig Trachea

Mohammad Hossein Boskabady,¹ Mahsa Kaveh,¹ Naeima Eftekhar,¹ and Ali Nemati²

¹Department of Physiology and Pharmaceutical Research Centre, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad 9177948564, Iran

²Department of Biology, Faculty of Science, Islamic Azad University, Mashhad Branch, Mashhad 918714758, Iran

Correspondence should be addressed to Mohammad Hossein Boskabady, boskabady@ums.ac.ir

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The stimulatory effect of *Zataria multiflora* Boiss (Labiatae) and carvacrol on β -adrenoceptors was examined on guinea pig trachea. The effects of three concentrations of aqueous-ethanolic extract, carvacrol, and propranolol (β -receptor antagonist) on β -adrenoceptors were tested in nonincubated (group 1, $n = 8$) and incubated tracheal chains with $1 \mu\text{M}$ chlorpheniramine (histamine H1 receptor antagonist) (group 2, $n = 5$). Isoprenaline (β -receptor agonist) curves obtained in the presence of all concentrations of the extract and carvacrol showed leftward shifts compared with that of saline in both groups. In both groups, the EC50 (the effective concentration of isoprenaline, causing 50% of maximum response) obtained in the presence of all concentrations of the extract and carvacrol was significantly lower compared to that of saline ($P < .01$ to $P < .001$). All values of (CR-1: (EC50 in the presence of active substances/EC50 obtained in the presence of saline)-1) obtained in the presence of concentrations of the extract and carvacrol in both groups were negative and significantly different from that of propranolol ($P < .001$ for all cases). The results indicated a stimulatory effect of *Zataria multiflora* Boiss extract on β_2 -adrenoceptors which is perhaps due to its constituent, carvacrol.

1. Introduction

Zataria multiflora Boiss L is a perennial plant with a woody, fibrous root, and its leaves are small, narrow, and elliptical, greenish-grey in colors. Identified constituents of this plant belong to the classes like terpenes, phenols, aliphatic alcohols, flavonoids, saponins, and tannins. Among the compounds, some classes have been previously identified as bioactive chemicals, particularly terpenes such as thymol and carvacrol. *Zataria multiflora* also contains apigenin, luteolin, and 6-hydroxyluteolin glycosides, as well as di-, tri-, and tetramethoxylated [1, 2].

The extract of this plant has been used to treat coughs due to colds, bronchitis, and pertussis, laryngitis and tonsillitis (as a gargle), the common cold, and disorders of the oral cavity and as an antibacterial agent in oral hygiene by traditional healers in Iran [3–5]. Both the essential oil and thymol are ingredients of a number of proprietary drugs including antiseptic and healing ointments, syrups

for the treatment of respiratory disorders, and preparations for inhalation [1]. It has also been used to treat pertussis, stomatitis, and halitosis [2].

Previous studies showed the relaxant effect of this plant in the ileum [6–8] and uterus [9] and another plant of this family (*Thymus vulgaris*) in tracheal smooth muscle [7, 8, 10]. The therapeutic effect of *Zataria* in respiratory disorders of chemical war victims [11], an antitussive effect for the plant [9], and relaxant effect of the plant constituent, carvacrol, on tracheal smooth muscle were also documented [12]. In addition, other effects have been also shown for the plant including antifungal and anticandida effects and effect on different parasites [13–18]. The antibacterial [19–21], anti-inflammatory, analgesic [22–25] and, antinociceptive effects [26] have been also shown for *Zataria multiflora*.

Our previous study showed a potent bronchodilatory effect for carvacrol [12] while our other study did not show any bronchodilatory effect for thymol [27]. Carvacrol is a terpene with phenolic structure. It is a volatile constituent of

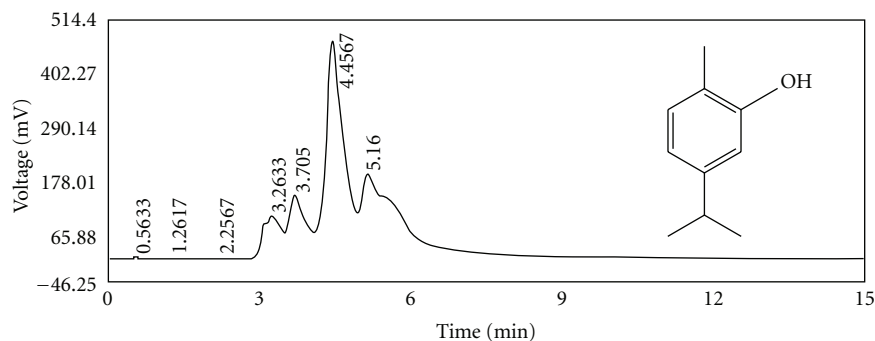


FIGURE 1: HPLC fingerprint of the the extract of *Z. multiflora* (500 $\mu\text{g}/\text{mL}$) illustrating carvacrol ($\text{C}_{10}\text{H}_{14}\text{O}$, MW = 150.217) and its chemical structure.

Zataria multiflora essential oil. Figure 1 illustrates chemical structure of carvacrol, its chemical formula, and molecular weight.

To examine the possible mechanism for the relaxant effect of the plant on smooth muscle, in the present study, the stimulatory effect of aqueous-ethanolic extracts of *Zataria multiflora* Boiss, and its constituent, carvacrol on β -adrenoceptors was examined on tracheal chains of guinea pigs. In fact, if *Zataria multiflora* Boiss and its constituents have stimulatory effect on β -adrenoceptors, they could have a potential therapeutic effect on obstructive pulmonary diseases such as asthma and chronic obstructive pulmonary diseases (COPD).

2. Material and Methods

2.1. Plant and Extracts. *Zataria multiflora* Boiss was collected From a mountain in the region between Tabas and Yazd, (centre east region of Iran), Fleurine mine, and identified by M. R. Joharghi. A voucher specimen was preserved in the Herbarium of the School of Agriculture, Ferdowsi University (Herbarium no: 35314, FUMH). The aqueous-ethanolic extract of the plant was prepared as follows: fifty grams of *Zataria multiflora* seeds were grinded and added to 700 mL of ethanol 50% (350 mL distilled water and 350 mL ethanol) using the Soxhlet apparatus. The solvent was then removed under reduced pressure. The extract concentration in the final extract was adjusted to 0.1 g/mL by adding distilled water to the dried extract.

2.2. Characterization of the Extract of *Zataria multiflora* by HPLC. The quality of the extract of *Zataria multiflora* was characterized by HPLC (Waters 474, Waters Corporation, MA, USA) fingerprint. The extract was dissolved in mobile phase and filtered through 0.22 μm membrane filter. An aliquot (20 μL) of sample (500 $\mu\text{g}/\text{mL}$) was injected to the reverse phase HPLC column (C18). The mobile phase consisted of phosphate buffer (pH = 4.8) : methanol : acetonitrile (40:30:30) with an isocratic elution at the flow rate of 1 mL/minute. The peaks were monitored at 280 nm (Figure 1). All solvents used were HPLC grade and supplied by Caledon Laboratories, Georgetown Ltd, Canada.

2.3. Tissue Preparations. Male Dunkin-Hartley guinea pigs (400–700 g) were sacrificed by a blow on the neck, and the tracheas were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle and sutured together to form tracheal chain [28]. Tissue was then suspended in a 10 mL organ bath (organ bath 61300, Bio Science Palmer-Washington, Sheerness, Kent UK) containing Krebs-Henseleit solution with the following composition (mM): NaCl 120, NaHCO_3 25, MgSO_4 0.5, KH_2PO_4 1.2, KCl 4.72, CaCl_2 2.5, and dextrose 11.

Krebs solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2 . Tissue was suspended under isotonic tension (1 g) and allowed to equilibrate for at least 1 hr while it was washed with Krebs solution every 15 min. This study was approved by the University's Ethics Committee. The allowance number of the relevant ethical committee for the animal experiments is 85301.

2.4. Protocols. The stimulatory effect of of *Zataria multiflora* Boiss and carvacrol on β_2 -adrenoceptors was examined by producing the cumulative log concentration-response curve of isoprenaline sulphate-induced (Sigma Chemical Ltd., UK) relaxation of precontracted tracheal chains by 10 μM methacholine hydrochloride (Sigma Chemical Ltd., UK) 10 min after the exposure of tissue to one solution. Different tested solutions were included: 10 nM propranolol (0.1 mL of propranolol hydrochloride with 0.1 μM concentration, Sigma Chemical Ltd., UK), three concentrations of aqueous-ethanolic extract from *Zataria multiflora* Boiss (0.5, 1, and 2 $\mu\text{g}/\text{mL}$), and carvacrol (Fluka, Italy, Catalogue no. C4915, purity 75%) (0.1, 0.2, and 0.4 $\mu\text{g}/\text{mL}$) or 0.2 mL saline. The consecutive concentrations of isoprenaline were added every 2 min (including 5 nM–1000 μM); and the percentage of relaxation due to each concentration in proportion to the maximum relaxation obtained in the presence of saline was plotted against log concentration of isoprenaline. The effective concentration of isoprenaline causing 50% of maximum response (EC_{50}) in each experiment was measured using the log concentration-response curve of the corresponding experiment.

The shift of cumulative log concentration-response curves obtained in the presence of different concentrations

of extract, carvacrol, and propranolol was examined by comparing the EC_{50} obtained in the presence of each solution with that of saline. In addition, the maximum responses to isoprenaline obtained in the presence of different concentrations of extract, carvacrol, and propranolol in all sets of experiments were compared with that of saline. To examine the parallel rightward shift, the slope of the isoprenaline-response curve of each experiment was measured and was compared with that of saline. In experiments with parallel shift in isoprenaline-response curve, the concentration-ratio minus one (CR-1) as an index of the competitive antagonism effect was calculated by the following equation:

$$\left(\frac{EC_{50} \text{ obtained in the presence of effective solutions}}{EC_{50} \text{ obtained in the presence of saline}} \right) - 1. \quad (1)$$

The stimulatory effect of *Zataria multiflora* on β_2 -adrenoceptors was tested on two different experimental conditions as follows:

- (a) nonincubated tracheal chains (group 1, $n = 8$),
- (b) incubated tracheal chains 30 min prior to the beginning and while obtaining the isoprenaline curve with $1 \mu\text{m}$ chlorpheniramine maleate (Sigma Chemical Ltd., UK) (group 2, $n = 5$).

Isoprenaline is β -receptor agonist which causes relaxation of airway smooth muscle. Propranolol is a β -receptor competitive antagonist, and chlorpheniramine is a histamine H_1 receptor competitive antagonist. Competitive antagonists bind to their specific receptor and prevent agonist binding to the receptor, causing a parallel shift in agonist concentration-response curve.

All of the experiments were performed randomly with 1-hour resting period of tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution. In all experiments contractions were measured using an isotonic transducer (Harvard APP LTD, 50-6360 SINO 0210) and measured using a software by computer (Acer model no.: G781) recording.

2.5. Statistical Analysis. All data were expressed as mean \pm SEM. The EC_{50} , slope, and maximum response obtained in the presence of extract, carvacrol, and propranolol were compared with those obtained in the presence of saline and (CR-1) obtained in the presence of extract and carvacrol, with those obtained in the presence of propranolol using the paired t -test. The comparison of the data of different concentrations of extract and carvacrol was performed using one-way analysis of variance (ANOVA) with Tukey-Kramer multiple posttest. The values of EC_{50} , the slope, (CR-1), and maximum response obtained in group 2 experiments were compared with those of group 1 using unpaired " t " test. Correlations between the concentrations of the extract and carvacrol with the values of EC_{50} and (CR-1) were examined using least square regression. Significance was accepted at $P < .05$.

3. Results

3.1. Characterization of the Extract. Carvacrol of the extract from *Zataria multiflora* was identified using HPLC method in the Department of Pharmacology, Medical School, Mashhad University of Medical Sciences (Figure 1).

3.2. The Extract and Carvacrol Stimulate β -Adrenoceptors. Cumulative log concentration-response curves to isoprenaline obtained in the presence of all concentrations of the extract and carvacrol showed clear leftward shift while the curve of propranolol showed clear rightward shift compared to isoprenaline curves produced in the presence of saline in both groups 1 and 2 (Figures 2(a)–2(d)).

Isoprenaline EC_{50} obtained in the presence of propranolol was significantly higher than that of saline in both groups of experiments ($P < .001$). However, the EC_{50} obtained in the presence of all concentrations of the extract and carvacrol was significantly lower than that of saline in both groups 1 and 2 ($P < .01$ to $P < .001$), (Figures 3(a) and 3(b)).

Maximum responses to isoprenaline obtained in the presence of all concentrations of the extract and carvacrol were not significantly different compared to that of saline in both groups 1 and 2 (Figure 4(a)). Slopes of isoprenaline-response curves obtained in the presence of all three concentrations of the extract and carvacrol were not significantly different from that of saline in both groups 1 and 2 (Figure 4(b)).

Values of (CR-1) obtained in the presence of all concentrations of the extract and carvacrol were negative and significantly different from those of propranolol in both groups 1 and 2 ($P < .001$ for all cases) (Figures 5(a) and 5(b)). There were significant negative correlations between the concentrations of the extract and carvacrol with the values of EC_{50} and (CR-1) in both groups ($P < .005$ to $P < .001$) (Table 1).

3.3. Differences between the Extract of *Zataria multiflora* and Carvacrol. Values of isoprenaline EC_{50} obtained in the presence of two higher concentrations of the extract were significantly lower than those of carvacrol in group 1 ($P < .05$ to $P < .001$) (Figure 3(a)). The values of (CR-1) obtained in the presence of two higher concentrations of the extract were significantly greater than those of carvacrol in group 1 ($P < .05$ to $P < .01$) (Figure 5(a)). In group 2 experiment, the values of isoprenaline EC_{50} obtained in the presence of all concentrations of the extract were significantly greater than those of carvacrol ($P < .05$ to $P < .001$) (Figure 3(b)). The values of (CR-1) obtained in the presence of two higher concentrations of the extract were significantly lower than those of carvacrol ($P < .05$ for both cases) (Figure 5(b)). However, there was no significant difference in maximum response to isoprenaline and slope between the extract and carvacrol in both groups (Figures 4(a) and 4(b)).

Values of isoprenaline EC_{50} obtained in the presence of all concentrations of the extract and only in the highest concentration of carvacrol in group 1 were significantly lower

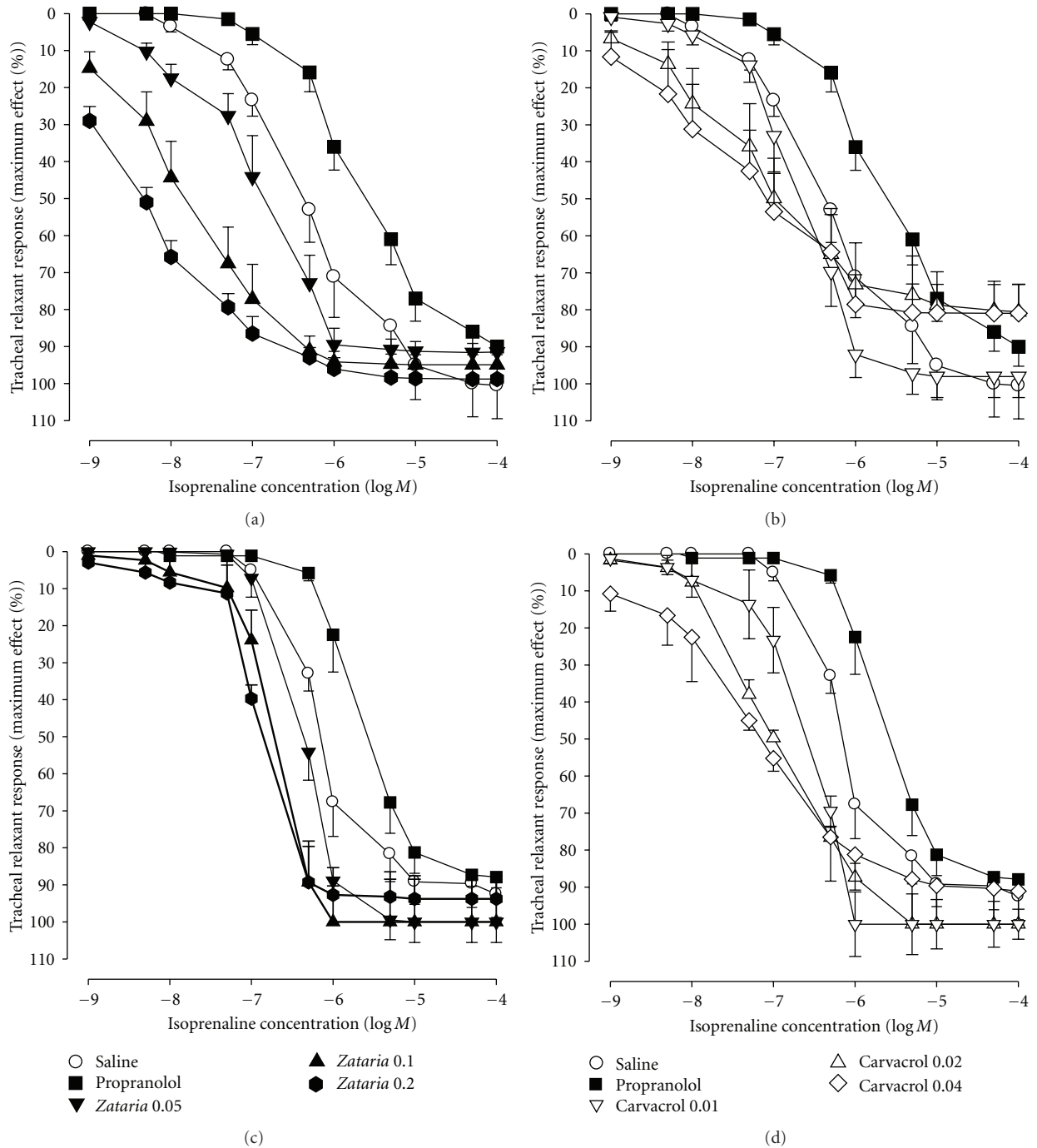


FIGURE 2: Cumulative log concentration-response curves of isoprenaline-induced relaxation of guinea pig tracheal chains, in the presence of saline, three concentrations of aqueous-ethanolic extract, three concentrations of carvacrol, and 10 nM propranolol in nonincubated trachea ((a) and (b); $n = 8$) and incubated tissues with chlorpheniramine ((c) and (d); $n = 5$).

TABLE 1: Correlation (r) between EC_{50} isoprenaline and (CR-1) with concentrations of the extract and carvacrol in two different experimental groups.

Solutions	Group 1				Group 2			
	EC_{50}		(CR-1)		EC_{50}		(CR-1)	
	R	P	R	P	R	P	r	P
Extract	-0.804	$P < .001$	-0.625	$P < .001$	-0.742	$P < .001$	-0.661	$P < .001$
Carvacrol	-0.755	$P < .001$	-0.590	$P < .005$	-0.810	$P < .001$	-0.688	$P < .001$

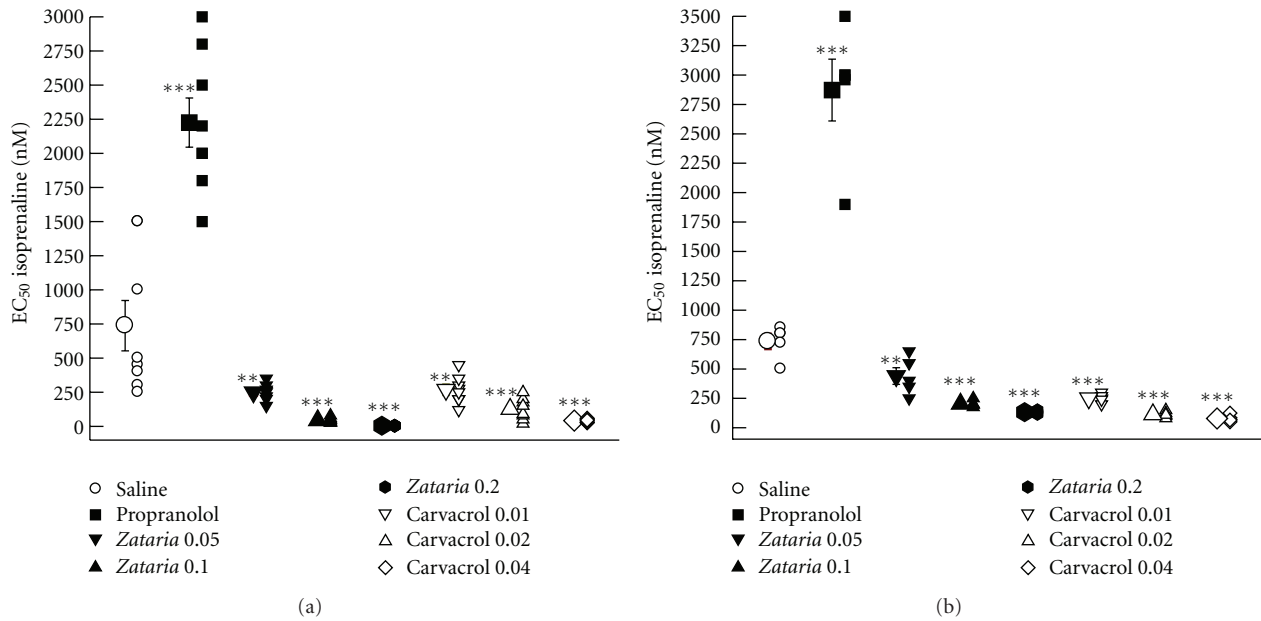


FIGURE 3: Isoprenaline EC₅₀ obtained in the presence of three concentrations of aqueous-ethanolic extract from *Z. multiflora* (0.5 Zataria 0.05, 1 Zataria 0.1, and 2 $\mu\text{g}/\text{mL}$, Zataria 0.2), carvacrol (0.1 Carvacrol 0.01, 0.2 Carvacrol 0.02, and 0.4 $\mu\text{g}/\text{mL}$, Carvacrol 0.04), 10 nM propranolol (Propranolol), and saline (O) in nonincubated trachea (a) (group 1: $n = 8$) and (b) incubated tissues with chlorpheniramine (group 2: $n = 5$). Statistical comparison between saline and other solutions. NS: nonsignificant difference, ** $P < .01$, *** $P < .001$. Statistical comparison between different concentrations of the extract and carvacrol $^+P < .01$, $^{++}P < .001$. EC₅₀ obtained in the presence of three concentrations of the extract in group 1 ($P < .05$ for low concentration and $P < .001$ for two higher concentrations) and only high concentration of carvacrol ($P < .01$) was higher than group 2. The comparison of EC₅₀ obtained in the presence of extract, carvacrol, and propranolol with that of saline and the data between two groups was done using the paired t -test. The comparison of the data of different concentrations of extract and carvacrol was performed using one-way analysis of variance (ANOVA) with Tukey-Kramer multiple post test.

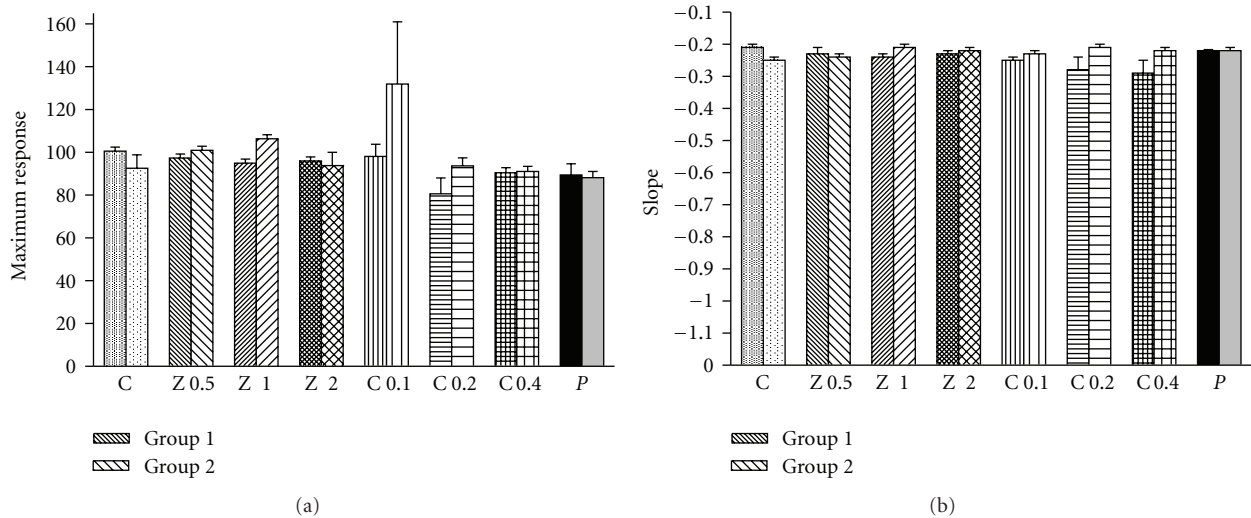


FIGURE 4: Values of maximum response to isoprenaline (a) and slope of isoprenaline log concentration-response curves (b) obtained in the presence of different concentrations of extract from *Zataria multiflora*, carvacrol, 10 nM propranolol, and saline in nonincubated trachea (fine filled bars) (group 1: $n = 8$) and incubated tissues with chlorpheniramine (medium filled bars) (group 2: $n = 5$). There was not significant difference between the data of the extract and between the data of the extract with those of saline and between the data of the extract with those of carvacrol. The comparison of slope and maximum response obtained in the presence of extract, carvacrol, and propranolol with those of saline and data of two groups were done using the paired t -test. The comparison of the data of different concentrations of extract and carvacrol was performed using one-way analysis of variance (ANOVA) with Tukey-Kramer multiple post test.

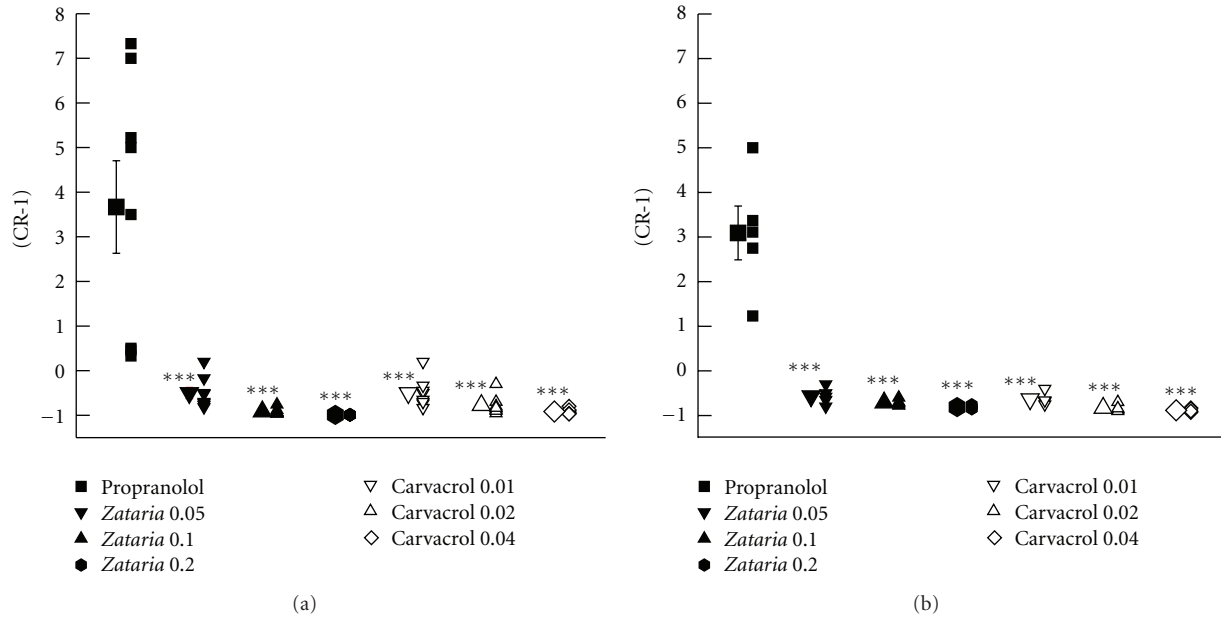


FIGURE 5: Values of (CR-1) obtained in the presence of three concentrations of aqueous-ethanolic extract from *Zataria multiflora* (0.5 *Zataria* 0.05, 1 *Zataria* 0.1, and 2 $\mu\text{g/mL}$, *Zataria* 0.2), carvacrol (0.1 Carvacrol 0.01, 0.2 Carvacrol 0.02, and 0.4 $\mu\text{g/mL}$, Carvacrol 0.04), and 10 nM propranolol (Propranolol) in nonincubated trachea (a) (group 1: $n = 8$) and (b) incubated tissues with chlorpheniramine (group 2: $n = 5$). Statistical comparison between chlorpheniramine and other solutions. NS: nonsignificant difference, $***P < .001$. Statistical comparison between different concentrations of the extract and carvacrol $*P < .05$, $**P < .01$. Values of (CR-1) obtained in the presence of two higher concentrations of the extract in group 1 were higher than those in group 2 ($P < .001$ for two higher concentrations). The comparison of (CR-1) obtained in the presence of extract and carvacrol with those of propranolol and data between two groups was done using the paired t -test. The comparison of the data of different concentrations of extract and carvacrol was performed using one-way analysis of variance (ANOVA) with Tukey-Kramer multiple post test.

than those in group 2 ($P < .05$ to $P < .001$) (Figure 3). The values of (CR-1) obtained in the presence of two higher concentrations of the extract in group 1 were significantly greater than those of group 2 ($P < .001$ for both cases) (Figure 5). However, there was no significant difference in maximum response to isoprenaline and slope between the two groups (Figures 4(a) and 4(b)).

4. Discussion

In the present study the stimulatory effect of the aqueous-ethanolic extract of the plant on β -adrenoceptors (as one possible mechanism responsible for the observed relaxant effect seen for the extract of *Zataria multiflora* Boiss and its constituent, carvacrol) on tracheal and other smooth muscles [6–10] was examined. In fact, the relaxant effect of tracheal smooth muscle due to stimulation of β -adrenoceptors is a well-known phenomenon [29]. The parallel rightward shifts in isoprenaline log concentration-response curves obtained in the presence of the different concentrations of aqueous-ethanolic extract and carvacrol and the achievement of maximum relaxation effect to isoprenaline compared to that of saline showed possible stimulatory effects of the hydroethanolic extract of the plant and carvacrol on β -adrenoceptors of guinea pig's trachea [29–31]. Lower values of EC_{50} obtained in the presence

of the different concentrations of aqueous-ethanolic extract and carvacrol compared to that of saline confirmed this effect. In addition the negative values of (CR-1) obtained in the presence of the extract and carvacrol are another indicator of stimulatory effect of the plant and its constituent on β -adrenoceptors of trachea smooth muscle.

The stimulatory effect of extract from *Zataria multiflora* and carvacrol on β_2 -adrenoceptors was also examined on incubated tracheal preparation with chlorpheniramine to block histamine H_1 receptors, (group 2 experiments). The group 2 experiment was done in order to evaluate the effect of the extract and carvacrol on β_2 -adrenoceptors more precisely. The results of group 2 experiments were very similar to those obtained in group 1, confirming the stimulatory effect of the extract and carvacrol on β_2 -adrenoceptors [29–31]. The dose response curves obtained in the presence of extract in group 2 (incubated with chlorpheniramine) showed smaller leftward shift compared to group 1, but for carvacrol the shift in the two groups was similar. In addition the values of EC_{50} obtained in the presence of concentrations of extract in group 1 were significantly lower than those of group 2. These results indicated an inhibitory effect for the extract on histamine (H_1) receptors in addition to stimulatory effect of the plant on β_2 -adrenoceptors.

The significant negative correlations between both values of EC_{50} and (CR-1) and the concentrations of the extract

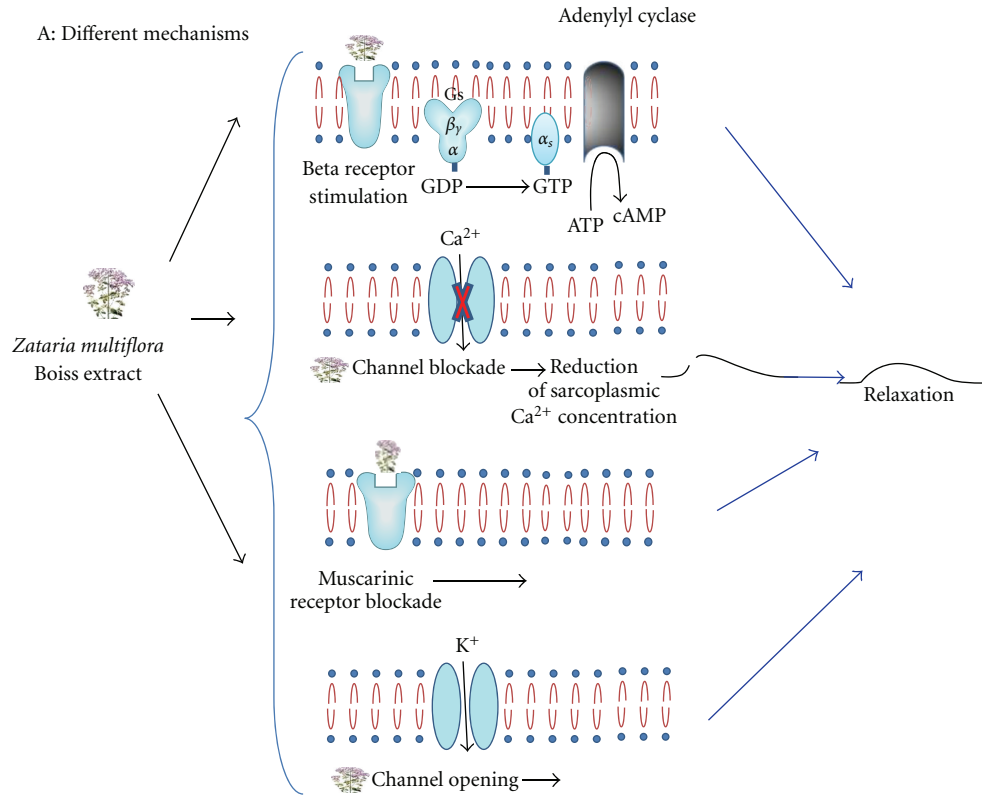


FIGURE 6: Suggested mechanism for the relaxant effect of the extract from *Zataria multiflora* on tracheal smooth muscle (A) including (a) beta-adrenergic stimulatory effect, (b) calcium channels opening effect, (c) muscarinic inhibitory effect, and (d) potassium channel opening effect.

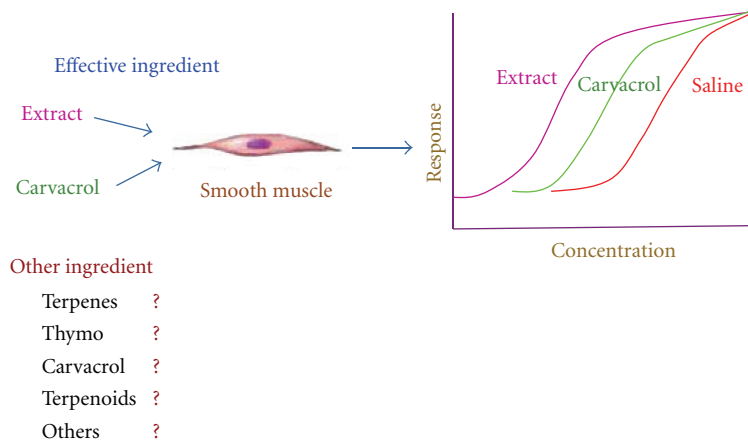


FIGURE 7: The contribution of carvacrol and suggested contribution of other constituents of the plant on β -adrenoceptors stimulatory effect of the plant (shifting concentration response curve of isoprenaline to the left).

and carvacrol indicated their concentration-dependent stimulatory effect on β_2 -adrenoceptors. However, the significant lower values of EC_{50} isoprenaline obtained in the presence of all concentrations of the extract in group 1 compared to those of group 2 and the higher values of (CR-1) obtained

in the presence of two higher concentrations of the extract in group 1 may indicate an inhibitory effect of the extract on histamine (H_1) receptors in addition to its stimulatory effect on β_2 -adrenoceptors. For carvacrol, the values of EC_{50} isoprenaline obtained in the presence of only the highest

concentration of carvacrol in group 1 were significantly lower than those in group 2, and there was not significant difference in the values of (CR-1) obtained in the presence of concentrations of carvacrol between the two groups which is an indicator of the absence of inhibitory effect of carvacrol on histamine (H_1) receptors. In fact, in our previous study the relaxant effect of macerated and aqueous extract of other species plant from this family was almost completely abolished in incubated tracheal preparations incubated with propranolol and chlorpheniramine [10] which confirms the result of the present study. However, the effect of both the extract and carvacrol on histamine (H_1) receptors should be examined in further studies more precisely.

In addition, the values of EC_{50} isoprenaline obtained in the presence of two higher concentrations of the extract in group 1 were significantly lower and those of its all concentrations in group 2 were greater than those of carvacrol. The values of (CR-1) obtained in the presence of two higher concentrations of the extract in group 1 were significantly greater and in group 2 were lower than those of carvacrol. These results also support the suggestion of an inhibitory effect on histamine (H_1) receptors for the extract in addition to its stimulatory effect on β_2 -adrenoceptors. These results indicated the differences between the effects of hydroethanolic extract of *Zataria multiflora* and its constituent carvacrol. Therefore, the pharmacological properties of the extract are not solely due to its constituent, carvacrol, which is a very important finding of this study; because in phytotherapy it is the extract that defines the efficacy not some analytical lead substance, even if it contributes to the overall effect.

The concentration of carvacrol in the essential oil of *Zataria multiflora* is reported between 17% and 40%, with a mean value of 30% [32–34] which should be lesser in the hydroalcoholic extract. Therefore, the concentrations of carvacrol used in the present study are equal to those of extract (the concentrations of carvacrol were one fifth of the extract). Thus the stimulatory effect of the extract of *Zataria multiflora* on β_2 -adrenoceptors could be due to its constituent carvacrol. Different suggested mechanism for the relaxant effect of the extract from *Zataria multiflora* on tracheal smooth muscle and the contribution of its constituent carvacrol was illustrated in Figures 5 and 6. The major limitation of the present study is that the selectivity of the stimulatory effect of *Zataria multiflora* Boiss and its constituent carvacrol on β_2 -adrenoceptors can not estimated from the present results precisely, and further studies needed to find out their selectivity on these receptors. In addition, the bronchodilatory and β_2 -receptor stimulatory effect should be examined in further studies.

5. Conclusions

In conclusion, these results indicated a relatively potent stimulatory effect for the extract from *Zataria multiflora* Boiss on β_2 -adrenoceptors which is perhaps due to its constituent, carvacrol. A possible inhibitory effect of the plant on histamine (H_1) receptors was also suggested.

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