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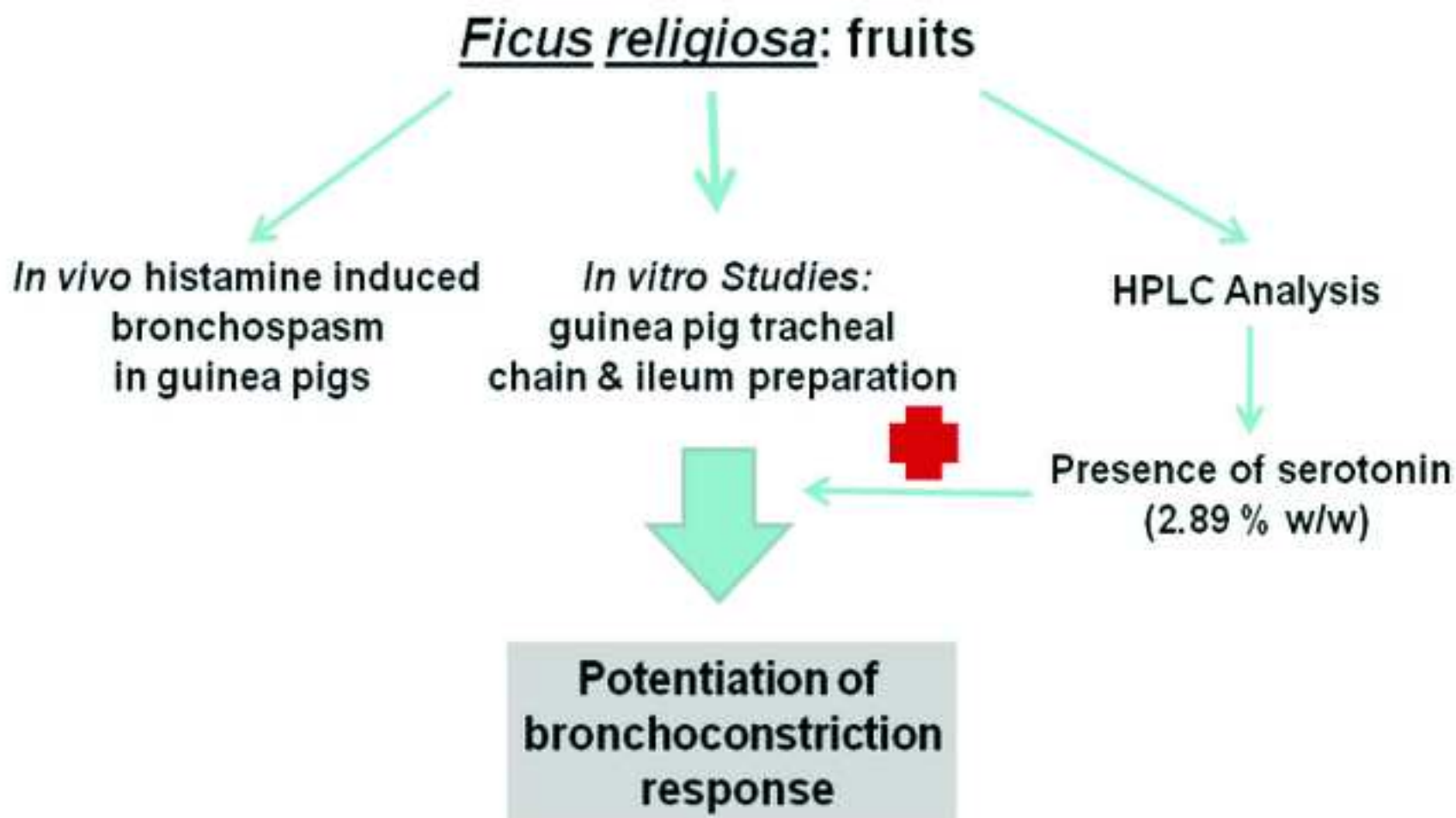
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Bronchospasm potentiating effect of methanolic extract of *Ficus religiosa* fruits in guinea pigs



Bronchospasm potentiating effect of methanolic extract of *Ficus religiosa* fruits in guinea pigs

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Abstract

Ethnopharmacological relevance: The sacred tree Peepal (*Ficus religiosa* family: Moraceae) has numerous therapeutic utility in folk medicine.

Aim of the study: It has been reported to be used in ethno medical treatment of asthma and also in epilepsy due to its high serotonin content, which has been implicated in pathophysiology of asthma, this led us to carry out the present study.

Materials and methods: The *in vivo* studies of histamine induced bronchospasm in guinea pigs and *in vitro* isolated guinea pig tracheal chain and ileum preparation.

Results: Pre-treatment of guinea pigs with ketotifen (1 mg/kg, p.o.) has significantly delayed the onset of histamine aerosol induced preconvulsive dyspnea, compared with vehicle control ($281.8^a \pm 11.7$ Vs 112.2 ± 9.8). The administration of methanolic extract (125, 250 and 500mg/kg, p.o.) did not produced any significant effect on latency to develop histamine induced preconvulsive dyspnea. On the other hand, methanolic extract of the fruits at the doses employed (i.e., 0.5, 1 and 2 mg/ml) has significantly potentiate the EC_{50} doses of both histamine and acetylcholine in isolated guinea pig tracheal chain and ileum preparation. In addition, HPLC analysis of the methanolic extract showed the presence of high amounts of serotonin (2.89 % w/w).

Conclusions: On the basis of data, it may be concluded that *Ficus religiosa* fruits have been found to be ineffective against histamine induced bronchospasm in guinea pigs. In addition, methanolic extract of the fruits have shown to potentiate the bronchoconstriction induced by both histamine and acetylcholine on guinea pig tracheal chain preparation.

Keywords: Asthma, Serotonin, guinea pig trachea, methanolic extract, *Ficus religiosa* fruits, ketotifen

1. Introduction

Ficus religiosa L. (Moraceae), popularly called peepal a sacred (bodhi) tree has got mythological, religious and medicinal importance in Indian culture for the treatment of asthma, cough and other respiratory disorders (Kala et al., 2006; Prasad et al., 2006; Ripu and Rainer, 2006). *Ficus* Linn being the largest genus of the family Moraceae comprises about 755 fig tree species worldwide (Van Noort et al., 2007). The potential therapeutic benefits of leaf juice and fruits of *Ficus religiosa* has been documented in folk medicine (Ripu and Rainer, 2006). The leaf juice has been used for the treatment of asthma, cough, sexual disorders, diarrhea, haematuria, ear-ache and toothache, migraine, eye troubles, gastric problems and scabies; leaf decoction as an analgesic for toothache; stem bark in gonorrhea, bleeding, paralysis, diabetes, diarrhoea, bone fracture, as antiseptic, astringent and antidote (Ripu and Rainer, 2006) and the fruits have been used for the treatment of asthma, other respiratory disorders and scabies (Bhattarai, 1993).

The *Ficus religiosa* fruits claimed to have potential anticonvulsant activity (Vyawahare et al., 2007). Antiepileptic activity of methanolic extract of the fruits has been documented due to the high amount of serotonin content (Bliebtrau, 1968; Singh and Goel, 2009). On other hand, an increased level of free serotonin has shown to be positively correlated with severity of bronchial asthma and negatively regulated pulmonary function (Lechin et al., 1994, 1996). Thus, it has been suggested that an increasing concentration of free serotonin in plasma may be detrimental to pulmonary function, particularly in asthmatic patients (Cazzola et al., 1995; Cazzola and Matera, 2000). No substantial scientific evidence is available to support that fruits of *Ficus religiosa* may be indeed useful for their ethanomedicinal uses. Therefore, the present study is designed to investigate the effect of fruits using *in vivo* histamine induced bronchospasm in guinea pigs and *in vitro* guinea pig tracheal chain preparation.

2. Materials and methods

2.1. Plant material

The fruits were collected from Chandigarh in the month of Nov-Dec and were authenticated by Prof. (Dr) Promila Pathak, Associate Editor, The Orchid Society of India, Deptt. of Botany, Punjab University, Chandigarh. The fruits were shade dried, coarsely powdered and stored in an airtight container. Specimen is preserved in herbarium vide specimen no. 17362 in Deptt of Botany, Punjab University, Chandigarh.

2.2. Preparation of the extract

The coarsely powdered dried fruits (750 gm) were first defatted with petroleum ether (40-60°C) and then dried marc was extracted with 90% methanol in soxhlet apparatus for 12 hours. The extract was concentrated and dried to constant weight (34.50 gm) in vacuum evaporator. Dried marc was re-extracted three times with distilled water at room temperature for 24h and the aqueous extract was dried to constant weight using rotary evaporator. Both extracts were stored in desiccators till further use.

2.3. Drugs and chemicals

Histamine and serotonin procured from Acrose, Belgium. Methanol and petroleum ether were of HPLC grade and all other standard chemicals used in this study were of analytical grade, purchased from Rankem Chemicals (Pvt) Ltd. India.

2.4. Animals

The experimental protocol used in the present study was approved by the Institutional Animal Ethical Committee. Age matched adult guinea pigs (600-800 g) were acclimatized in animal house with normal cycles of day and night under standard conditions at an ambient temperature $25 \pm 2^{\circ}\text{C}$ and 55-65% relative humidity.

2.5. *In vivo studies on histamine induced bronchospasm (asphytic reaction) in guinea pigs*

Guinea pigs of either sex were selected and randomly divided into six groups. The drugs were administered orally by suspending in 0.5% sodium carboxyl methyl cellulose in water. The single dose treatment was given one and half hour before challenged with 0.25% histamine hydrochloride aerosol. The time to onset of respiratory distress (pre-convulsion dyspnea) was noted (Sheth *et al.*, 1972). The time to onset of pre-convulsion dyspnea during challenge with these agents was recorded; the guinea pigs with pre-convulsion dyspnea more than 120 s were considered insensitive and discarded. The following experimental protocol was employed: **Group I:** Vehicle Control (0.5% CMC, p.o.) treated; **Group II:** Ketotifen (1mg/kg, p.o.) treated; **Group III:** Fruits powder of *Ficus religiosa* (1000 mg/ kg, p.o) treated; **Group IV:** The methanol extract of the fruits (125mg/kg, p.o.) treated; **Group V:** Methanol extract of the fruits (250mg/kg, p.o.) treated; **Group VI:** Methanol extract of the fruits (500mg/kg, p.o.) treated;

2.6. *In vitro studies on isolated guinea pig tracheal preparation*

Male guinea pigs (200–250 g) were starved overnight, with free access to water. Animals were sacrificed by exsanguinations and tracheae were immediately removed. The trachea was dissected and cut transversely into 2-mm segments between the segments of cartilage so as to give a number of rings of tracheal muscle. The tracheal rings were tied together with cotton thread to form a chain, which was placed in an organ bath containing Krebs bicarbonate buffer at 37°C, continuously aerated with 95% O₂ and 5% CO₂ (Seth *et al.*, 1972). The tissue was stabilized for 45 min and then isotonic contractions were recorded by exposing to graded doses of histamine. Firstly, EC₅₀ and E_{max} values of histamine in individual isolated preparations were established. Subsequently, contractile response of both EC₅₀ and E_{max} dose of histamine using a

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4 separate tissue preparation was recorded in the presence of varied concentrations (0.5, 1 and 2
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6 mg/ml) of methanol extract of fruits. The responses were expressed as the percentage of the
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8 maximum response induced by histamine.
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10 11 12 2.7. *In vitro* studies on isolated guinea pig ileum preparation

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15 The ileum was dissected and tied with cotton thread which was placed in organ bath
16 containing Tyrode buffer kept at 37°C. The tissue was stabilized for 45 min and then isotonic
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18 contractions were recorded by exposing to graded doses of histamine and acetylcholine to
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20 establish an EC₅₀ and E_{max} values in individual preparations. Subsequently, contractile response
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22 of both EC₅₀ and E_{max} doses of histamine and acetylcholine were again recorded in the presence
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24 of different doses (0.5, 1 and 2 mg/ml) of methanolic extract of the fruits. The responses were
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26 expressed as the percentage of the maximum response induced by histamine and acetylcholine,
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28 respectively.
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34 35 36 2.8. *Acute toxicity studies*

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39 *Ficus religiosa* is one of the oldest known human foods having a very high safety profile
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41 (Lansky et al., 2008). It has been reported that the intra methanolic extract of the fruits up to
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43 dose (1000 mg/kg, i.p.) did not produce any neurotoxic effects and mortality (Singh and Goel,
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45 2009).
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50 51 52 2.9. *Phytochemical screening*

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54 The fruits and methanolic extracts were subjected to phytochemical screening for the following
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56 classes of compounds such as alkaloids, steroids, carbohydrates, phenolic compounds,
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58 flavonoids, proteins and saponins (Harborne, 1998).
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60 61 62 2.10. *HPLC-UV detection of serotonin in methanolic extract of the fruits* (Han et al., 2006)

The standard solution of serotonin was prepared by dissolving 10mg of pure serotonin in 10 mL of methanol (1mg/ml). Sample solution of the fruit's methanolic (1 mg/ml) extract was prepared by centrifugation at 4500×g for 10min and the supernatant was used for further investigations. Both standard and sample solutions were filtered through a 0.45µm membrane filter. Prior to HPLC analysis, the optimized UV spectra of pure serotonin was determined at wavelength scanning between 200 and 400 nm using a standard UV system. The fruit extract solution was analyzed using HPLC system with a Photodiode detector. Separation was performed on a Thermohypersil C-18 column (150 × 4.6mm × 3µm) using 25mM phosphate buffer (pH 2.5): acetonitrile at 95:5 (v/v) at a flow rate of 1ml/min. The injection volume was 20µl and the peaks were identified by comparison with the retention times of the standard solution.

2.11. Statistics

All values were expressed as mean ± SEM. The data was analyzed by one way- ANOVA followed by Turkey multiple range test. The $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Phytochemical constituents

The preliminary phytochemical screening has revealed the presence of carbohydrates, amino acids, steroids and phenolic compounds in the fruit powder (data not shown).

3.2. Effect of the fruit powder and methnolic extract against histamine induced bronchospasm in guinea pigs

Pre-treatment of guinea pigs with ketotifen (1 mg/kg, p.o.) has significantly delayed the onset of preconvulsive dyspnea induced by aerosol exposure of histamine compared with vehicle

control. The administration of the fruits powder (1000 mg/ kg, p.o.) and the methanolic extract (125, 250 and 500 mg/kg p.o.) did not show any significant difference in latency to develop histamine exposure induced pre-convulsive dyspnea in guinea pigs as compared to vehicle treated control (Table 1).

3.3. *Effect of methanolic extract of the fruit on EC_{50} and E_{max} doses of histamine on guinea pig tracheal chain and ileum preparation*

The presence of fruit methanolic extract (i.e., 0.5, 1 and 2 mg/ml) significantly enhanced the contractile response of EC_{50} and E_{max} doses of histamine in guinea pig tracheal chain and ileum preparations (Fig. 2 and 3).

3.4. *Effect of various doses of methanolic extract of the fruits on EC_{50} and E_{max} dose of acetylcholine in guinea pig ileum preparation*

The presence of methanolic extract (i.e., 0.5, 1 and 2 mg/ml) has significantly enhanced the contractile response of EC_{50} dose of acetylcholine in guinea pig ileum. Further, methanolic extract (i.e., 0.5, 1 and 2 mg/ml) also significantly enhanced the E_{max} dose of acetylcholine in guinea pig ileum preparation (Fig. 4).

3.5. *The amount of serotonin presents in methanolic extract of the fruits*

The optimal UV spectrum for serotonin detection was calibrated at 275 nm. The HPLC chromatogram of standard serotonin solution showed an absorption peak with a retention time of 6.081min. A similar peak was observed in the HPLC chromatogram of the methanol extract at same retention time indicating the presence of serotonin in the extract (Fig.1). The HPLC analysis of the methanolic extract showed the presence of high amounts of serotonin (2.89 % w/w) of dried methanol extract of the fruit.

4. Discussion

An acute bronchospasm (asphytic reaction) in guinea pigs by exposing to histamine aerosol has been used in the assessment of anti-asthmatic potential of test compounds by measuring the time taken for the development of pre-convulsion dyspnea (PCD) as an end point (Mitra et al., 1999; Shah and Parmar, 2003). The guinea pig tracheal chain preparation is a widely accepted in-vitro model to screen the potential efficacy of compounds like antihistamines, leukotriene receptor antagonists, cholinergic antagonists and β_2 -adrenergic receptor agonists (Roseanna et al., 1987). It has been noted in the present study that the methanolic extract employed at various doses did not produce any spasmolytic effect. Interestingly, an exposure of methanolic extract of the fruits has showed incomplete recovery of normal previous basal response even after repeated and thorough wash out of the extract, indicating that methanolic extract of the fruits induced long lasting contractile effect. Therefore, it could not possible to plot complete CRC of histamine in the presence of extract. The contractile response for both EC_{50} and E_{max} dose of histamine was observed in the presence of the methanolic extract.

In present study, a single administration of fruits powder and the methanolic extract of the fruits produced no significant effect on the latency to develop of histamine-induced PCD as compared to vehicle control animals, suggesting that the fruits and the methanolic extracts at the doses employed in the present study are not effective to relieve bronchospasm induced by histamine. In contrast, data obtained from the present *in-vitro* studies using guinea pig tracheal chain preparation demonstrated that methanolic extract (i.e., 0.5, 1 and 2 mg/ml) significantly enhanced bronchocontraction induced by the EC_{50} and E_{max} doses of both histamine and acetylcholine. Similarly, methanolic extract (i.e., 0.5, 1 and 2 mg/ml) also significantly enhanced the contraction induced by EC_{50} and E_{max} of acetylcholine in guinea pig ileum preparation. It

indicates that the methanol extract produces non-specific potentiation of the acetylcholine and histamine induced contractions.

The symptomatic asthmatic patients are associated with increased levels of free serotonin, which has shown to be positively correlated with clinical status and negatively with pulmonary function (Lechin et al., 1994, 1996). Normally, less amount of serotonin is present in plasma of healthy humans, because it is mainly concentrated in platelets due to existence of active uptake mechanism. However, active serotonin transport is moderately disturbed in patients with asymptomatic exogenous asthma and dramatically altered in patients with symptomatic endogenous asthma, which may responsible for increased levels of plasma serotonin in these patients (Cazzola et al., 1995). Further, randomized clinical trail using children with asthma demonstrated that treatment with tianeptine, an antidepressant, has shown to improve pulmonary function, secondary to decreased plasma concentration of serotonin (Lechin et al., 1998). In addition, it has been suggested that an increasing concentration of free serotonin in plasma may be detrimental to pulmonary function, particularly in asthmatic patients (Cazzola and Matera, 2000). HPLC analysis carried out in the present study reveals the presence of high amount of serotonin (2.89 % w/w) in the methanolic extract, which is in agreement with previously reported data (Singh and Goel, 2009). Serotonin has shown to produce contractile response in guinea pig airways, which is either through direct activation of 5-HT₂ receptor on airway smooth muscles (Watts and Cohen, 1992) or indirectly via activation of 5-HT₃ receptor present on airway neurons to release of acetylcholine (Ach) (Takahashi et al., 1995). Thus, it can be speculated that the high amount of serotonin in methanol extract may be responsible for the observed potentiating effect in histamine and Ach induced contractile response. On this regard further studies are necessary to clarify the involvement of serotonin in potentiating effect of methanol

extract on histamine induced bronchoconstriction using suitable animal models of human bronchial asthma.

On the basis of the results obtained in the present study it may be concluded that *Ficus religiosa* fruits are ineffective against histamine induced bronchospasm in guinea pigs. In addition, methanolic extract of the fruit has shown to potentiate the bronchoconstriction induced by both histamine and acetylcholine on guinea pig tracheal chain preparation possibly due to the presence of high amount of serotonin. Hence, it is suggested that the fruits of *Ficus religiosa* should be used with caution in traditional system of the medicine for the treatment of epilepsy and bronchial asthma.

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Sr.No.	Treatment	Dose (mg/kg)	Preconvulsion time (s)
1	Vehicle control	--	112.8 ± 9.8
2	Ketotifen	1	281.8 ± 11.7 ^a
3	Powdered fruits	1000	98.4 ± 13.6
4	Methanolic Extract	125	103.3 ± 6.7
5	Methanolic Extract	250	102.1 ± 5.2
6	Methanolic Extract	500	97.2 ± 25.8

Figure 1

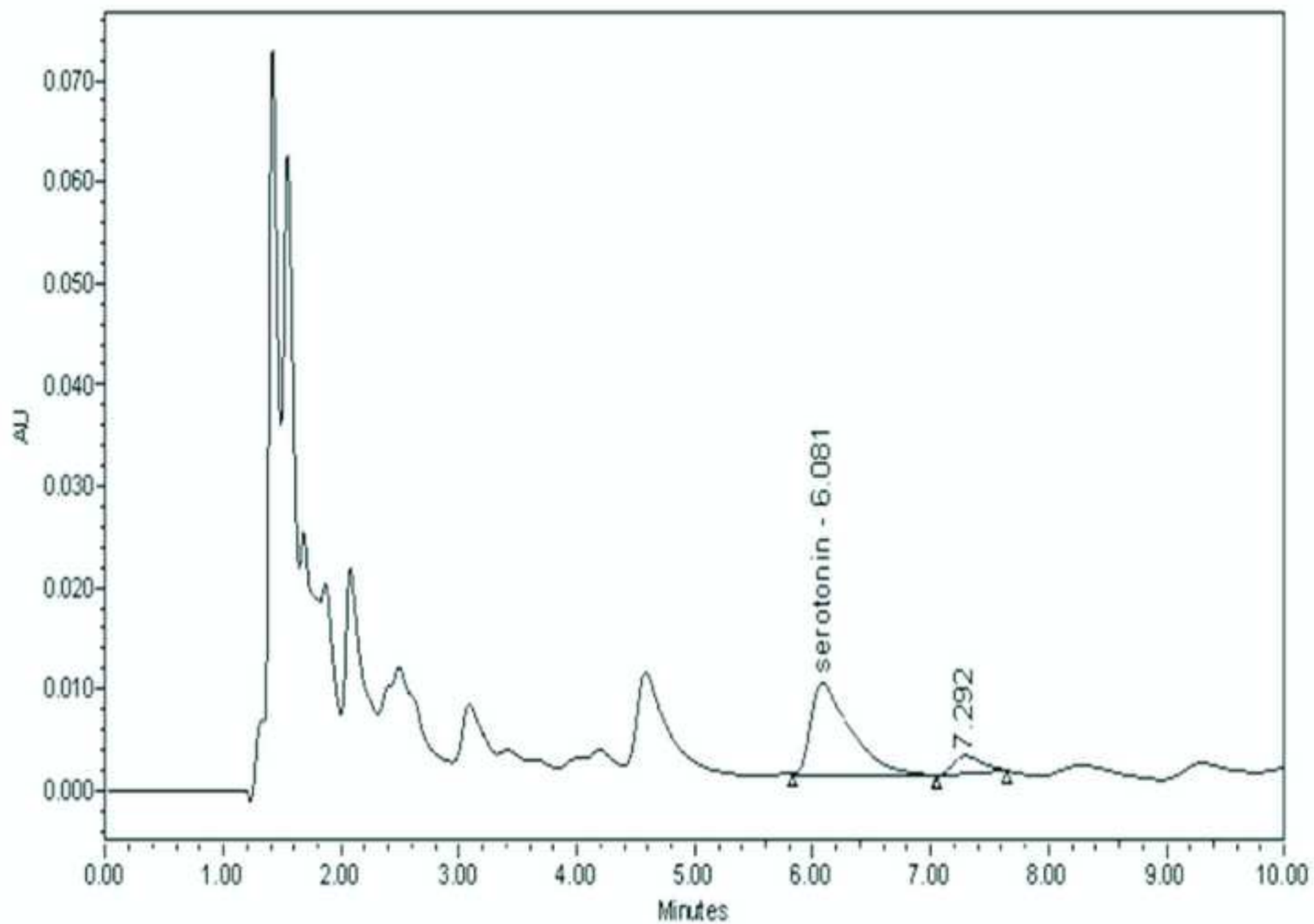


Figure 2

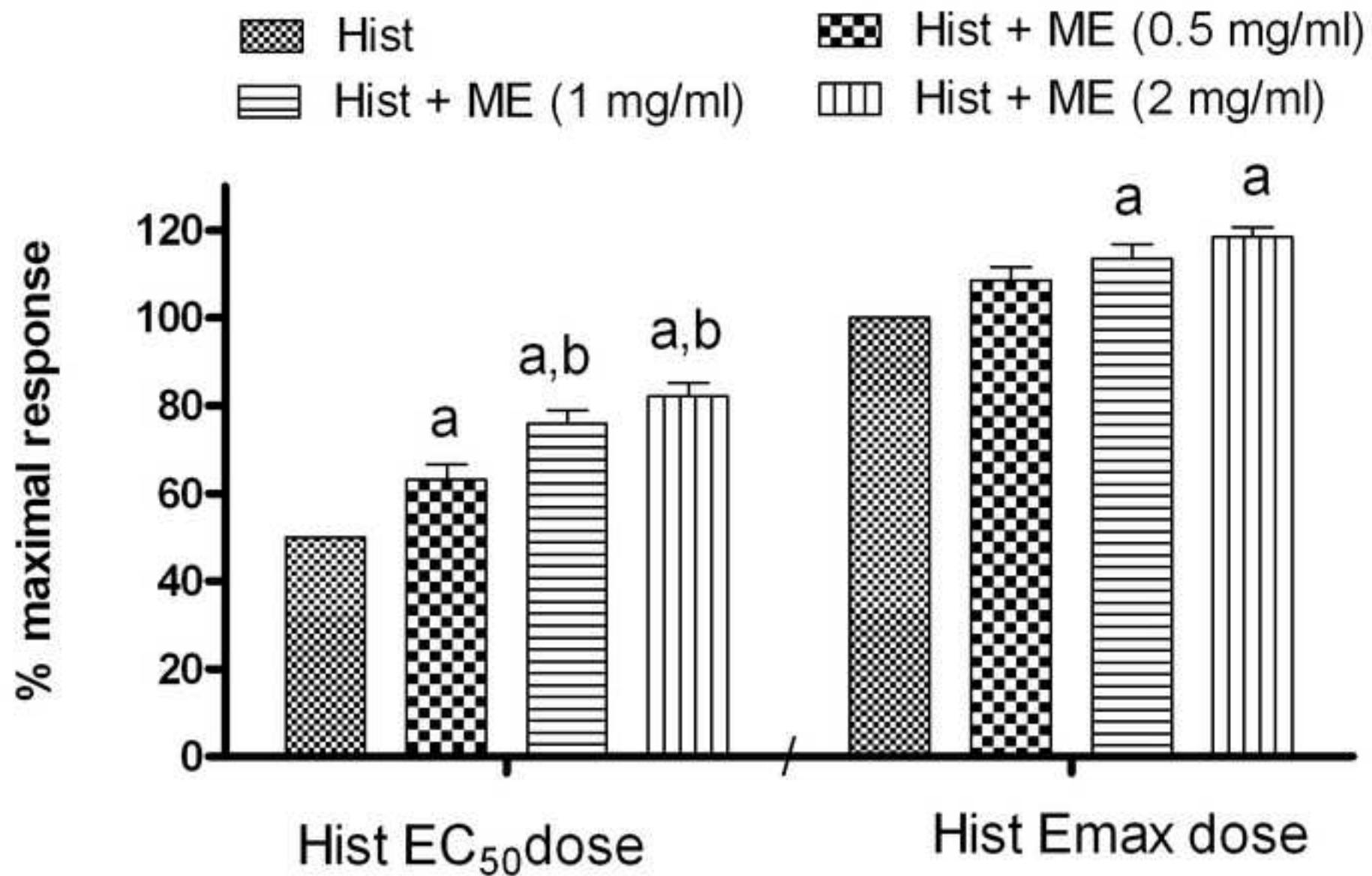


Figure 3

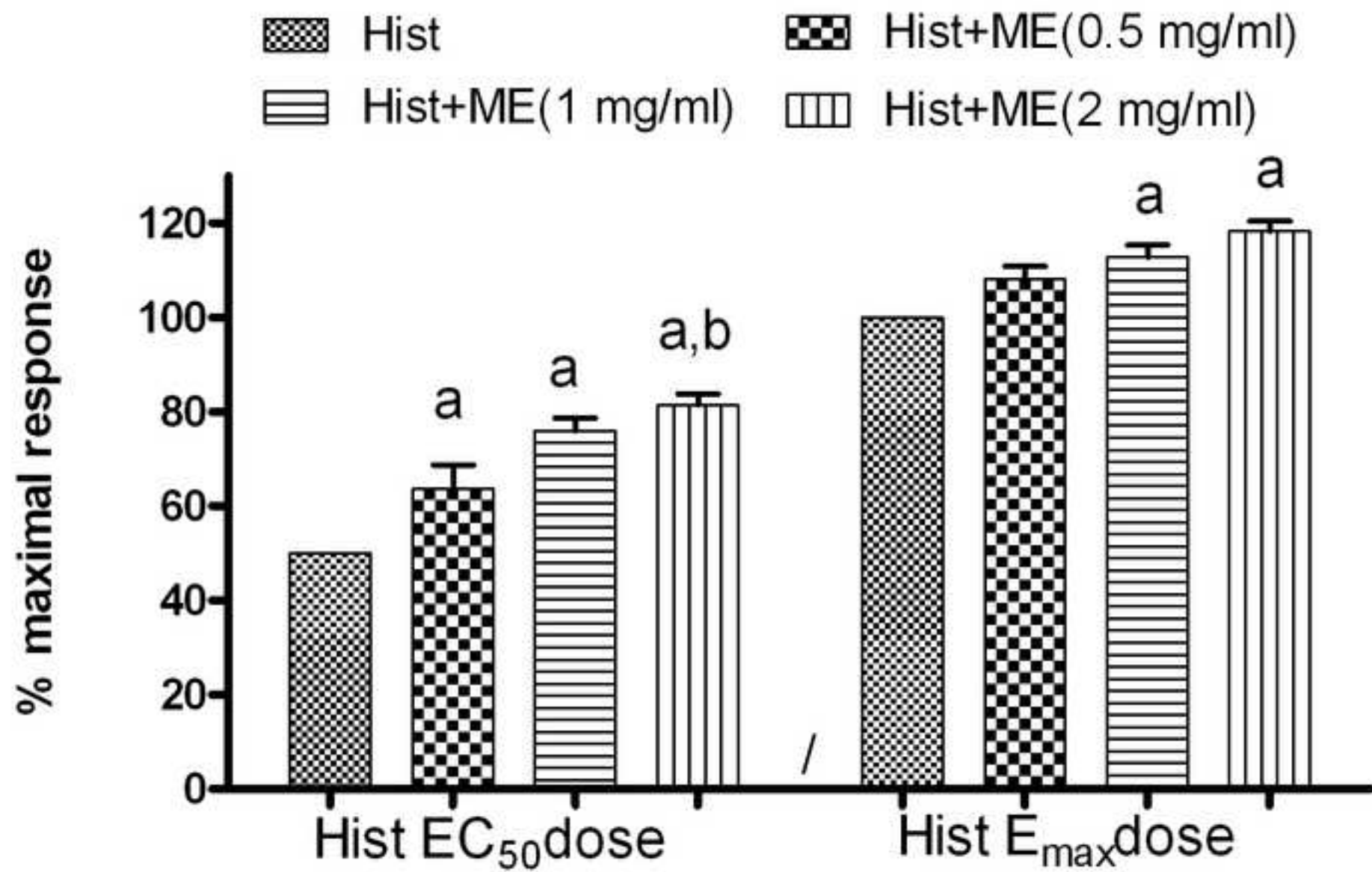


Figure 4

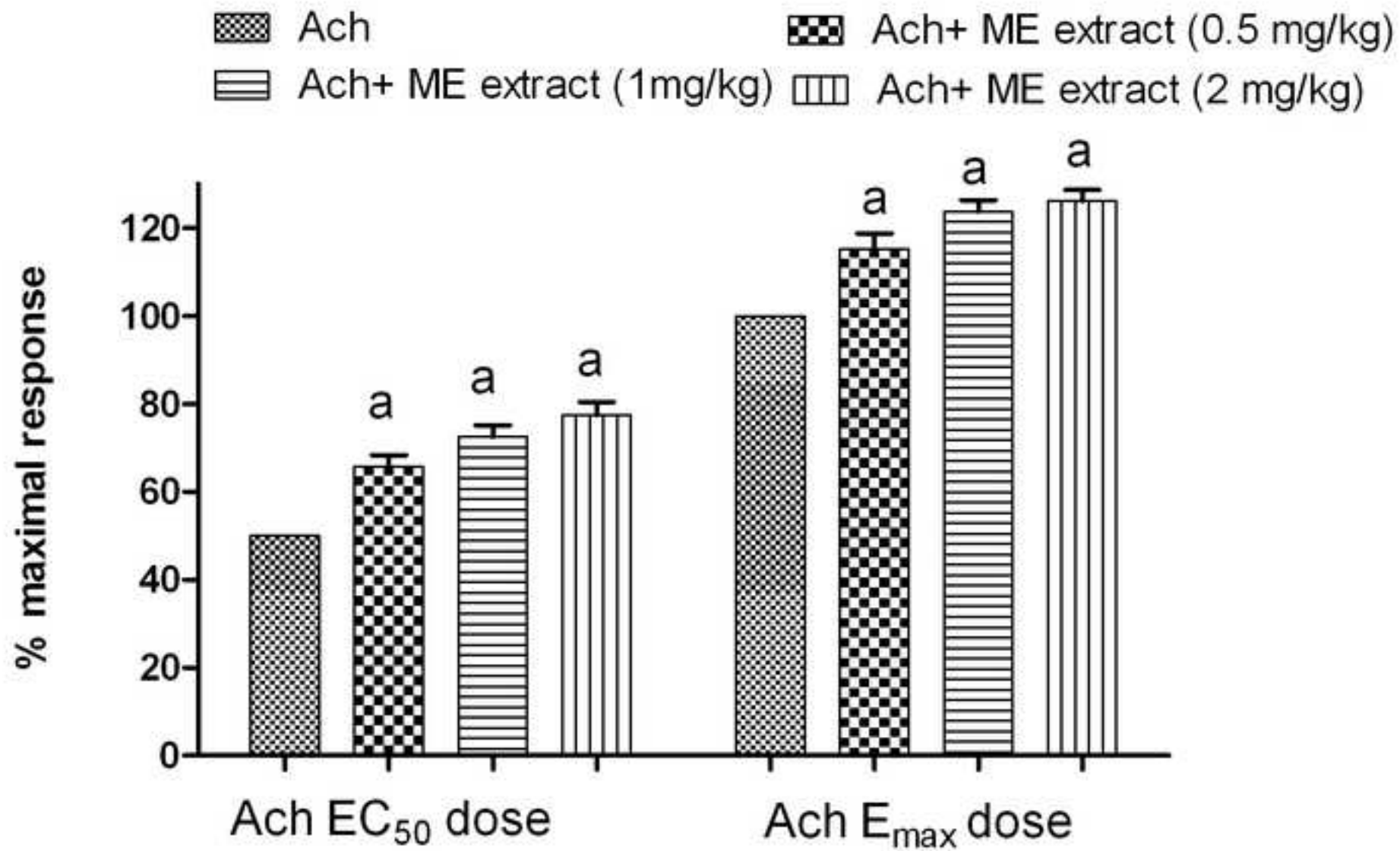


Table 1. The effect of *Ficus religiosa* fruits powder and its methanolic extract (ME) against histamine induced bronchospasm in guinea pigs in terms of dyspnoea. All the values were expressed as mean $\pm$ SEM. a = $p < 0.05$ Vs Vehicle control.

Fig. 1. Chromatogram of 90% methanolic extract of fruits of *Ficus religiosa*. Standard serotonin solution shows an absorption peak with a retention time of 6.081 min. A similar peak was observed in the HPLC chromatogram of the methanolic extract at same retention time indicating the presence of serotonin in the extract.

Fig. 2. Effect of various doses of methanolic extract (ME) of *Ficus religiosa* fruits on EC_{50} and E_{max} Dose of histamine(Hist) on guinea pig tracheal chain preparation. The responses were expressed as the percentage of the maximum response induced by histamine. All the values were expressed as mean $\pm$ SEM. A = $p < 0.05$ Vs. Hist; b = $p < 0.05$ Vs. Hist + ME (0.5 mg/ml).

Fig. 3. Effect of various doses of methanolic extract (ME) of *Ficus religiosa* fruits on EC_{50} and E_{max} Dose of histamine(Hist) on guinea pig ileum preparation. The responses were expressed as the percentage of the maximum response induced by histamine. All the values were expressed as mean $\pm$ SEM. A = $p < 0.05$ Vs Hist; b = $p < 0.05$ Vs. Hist + ME (0.5 mg/ml).

Fig. 4. Effect of various doses of methanolic extract (ME) of *Ficus religiosa* fruits on EC_{50} and E_{max} Dose of acetylcholine(Ach) on guinea pig ileum preparation. The responses were expressed as the percentage of the maximum response induced by acetylcholine. All the values were expressed as mean $\pm$ SEM. a = $p < 0.05$ Vs Ach.