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Original Article

RELAXANT EFFECTS OF *LIMONIA ACIDISSIMA* LINN (PULP) ON GUINEA PIG TRACHEAL CHAINS AND ITS POSSIBLE MECHANISM(S)

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ABSTRACT

Objective: The study was done to evaluate the antiasthma activity of Macerated and Soxhlet extracts of (pulp) of plant *Liomnia acidissima* Linn on tracheal chains of guinea pigs.

Methods: The (pulp) of fruits of plant *Liomnia acidissima* Linn was extracted with 95% ethanol at 55°C and evaluate for their antiasthma activity on tracheal chains of guinea pigs by using three cumulative concentrations of macerated and soxhlet extracts (0.5, 0.75, and 1.0 g %) in comparison with saline as negative control and 3 cumulative concentrations of theophylline (0.5, 0.75, and 1.0 mM) as positive control were examined on precontracted tracheal chains of three groups of 6, 6 and 4 guinea pigs by 60 mM KCl (group 1) and 10 µM methacholine (group 2) and tissues incubated with 1 µM propranolol contracted tracheal chains by 10 µM methacholine (group 3) [2] [3] [4] [5] [6].

Results: Decrease in contractile tone of tracheal chains was considered as relaxant effect. Theophylline exhibits potent antiasthmatic activity at (1.0 mM) after administration and in compare with (1.0 g %) soxhlet extracts of 95% ethanol. Whereas (1.0 g %) soxhlet extracts of 95% ethanol exhibits significant antiasthma activity. In Group 1 experiments (contracted by 60 mM KCl) only the higher two concentrations (0.75, and 1.0 mM) of theophylline and the highest concentration (1.0 g %) of Soxhlet extract showed significant relaxant effect compared to that of saline ($P<0.001$ and $P<0.05$ for theophylline and Soxhlet extract respectively). The effects of two higher concentrations (0.75, and 1.0 mM) of theophylline in this group were significantly greater than those of (0.75, and 1.0 g %) Soxhlet and Macerated extracts ($P < 0.01$). In group 2 (Contracted by 10 µM methacholine) theophylline and both Macerated and Soxhlet extract showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$). In group 3 (Incubated with 1µM propranolol contracted by 10 µM methacholine) all concentrations of both Macerated and Soxhlet extract showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.001$). The relaxant effects of Macerated and Soxhlet extracts in group 1 were significantly lower than those of groups 2 and 3. In group 3 experiment potent relaxant effect was observed.

Conclusion: These results showed a potent relaxant effect (pulp) of fruits of plant of *Liomnia acidissima* on tracheal chains of guinea pigs which were lower than theophylline at concentrations used, but it was found that due to the presence of flavanoids, glycosides, saponins, tannins, alkaloids, polyphenol and sterols in ethanolic extracts of the plants *Limonia acidissima* (pulp) might be responsible bronchodilatory and antiasthmatic effect on tracheal chains of guinea pig.

Keywords: *Limonia acidissima*, Wood apple, Bronchodilatory Guinea pig, Trachea.

INTRODUCTION

Bronchial asthma is a disease characterized by increased responsiveness of the trachea, bronchi and bronchioles to be various stimuli and is manifested by wide-spread narrowing of the airways in allergic asthma; bronchoconstriction and bronchial secretion are the results of an immediate hypersensitivity reaction [7]. Bronchial asthma is one of the most disabling diseases, affecting nearly 7-10% of world population [8]. Bronchoconstriction plays a very important role on the physiopathology of asthma and compounds that relax respiratory smooth muscles such as β2 -agonists and cholinergic antagonists are usually used in symptomatic treatments of the disease [9]. Bronchodilators help to stop asthma attacks after they have started or can help prevent recurrent attacks. The clinical management of acute asthma is with bronchodilators like β2 receptor agonists, antimuscarinics, and anti-inflammatory therapy with corticosteroids and administration of oxygen if necessary [8]. *Limonia acidissima* Linn is a well-known plant drug in Ayurvedic and Unani medicine. *Limonia acidissima* Linn synonym Feronia limonia (Rutaceae) is a moderate sized deciduous tree grown throughout India. Its fruits are woody, rough and used as a substitute for bael in diarrhoea and dysentery while the bark and leaves are used for vitiated conditions of vata and pitta [10]. The fruits are used for tumors, asthma, wounds, cardiac debility and hepatitis. Fruit shells of *Limonia acidissima* have been reported to have antifungal compounds [11]. The leaves have hepatoprotective activity [12]. The stem bark of the plant was found to possess antimicrobial activity. A tree found in dry-deciduous forests and cultivated in gar-dens. Fruit pulp is edible. It is also used to prepare cool drinks by adding jaggary and flavours. Pulp as well as powder of woody rind is applied externally to treat insect bites. Leaves are aromatic and are used to treat digestive trouble in children [14]. *Limonia acidissima* is a large tree growing to 9 metres (30 ft) tall, with rough, spiny bark. The leaves are pinnate, with 5-7 leaflets, each leaflet 25–35 mm long and 10–20 mm broad, with a citrus-scent when crushed. The fruit is a berry 5–9 cm diameter, and may be sweet or sour. It has a very hard rind which can be difficult to crack open, and contains sticky brown pulp and small white seeds.

The fruit looks similar in appearance to fruit of Bael (Aegle marmelos) [13]. Wood apple is an erect, slow-growing tree with a few upward-reaching branches bending outward near the summit where they are subdivided into slender branchlets drooping at the tips. The bark is ridged, fissured and scaly and there are sharp spines 3/4 to 2 in long on some of the zigzag twigs [1] [15]. The deciduous, alternate leaves, 3 to 5 in long, dark-green, leathery, often minutely toothed, blunter notched at the apex, are dotted with oil glands and slightly lemon-scented when crushed. Yellowish green flowers, tinged with red, 1/2 in across, are borne in small, loose, terminal or lateral panicles [1]. The tree is mostly known for its hard woody fruit, size of a tennis ball, round to oval in shape. The pulp is brown, mealy, odorous, resinous, astringent, acid or sweetish, with numerous small, white seeds scattered through it. It is native in the Indomalaya ecozone to Bangladesh, India, Pakistan, Sri Lanka, and in Indochinese ecoregion east to Java and the Malesia ecoregion. Kawista grow naturally in areas of Sri Lanka, India, Myanmar and Indochina, and then spread to Malaysia and Indonesia. Hence, the present study has been made to investigate the relaxant effect of

macerated and soxhlet extracts of the pulp of *Limonia acidissima* Linn and its possible mechanism(s) on guinea pig tracheal chains were examined.

Fig. 1: (on right), mature *Limonia acidissima* tree in fruiting season. The tree has sharp spines and reaches an average height of 9-12 m. The fruit is harvested with curved knives (sickles) attached to the ends of long bamboo poles

Fig. 2: Ripe *Limonia acidissima* fruit is round to oval, 5-12 cm in diameter. The fruit is hard, woody, and grayish-white in color with a 6-mm thick scurfy rind. The pulp in the ripe fruit is brown and separates readily from the rind (inset)

MATERIALS AND METHODS

Plant and Extracts

Pulp of *Limonia acidissima* Linn was collected from the chandaka jungle, Bhubaneswar. The Fruits of the plant was authenticated by Prof P K Sahu, Taxonomist, Botany Dept, Utkal University, Bhubaneswar. The soxhlet extract was prepared as follows: Fifty grams of the chopped, dried plant pulps were extracted with 300 ml distilled water by soxhlet apparatus. For the preparation of the macerated extract, the same amount of plant was macerated with 300 ml distilled water (on a shaker) for 48 hr. The solvent of both extracts were then removed under reduced pressure at 50^{o}C and distilled water were added to residues in such a way that plant ingredient concentration in the final soxhlet extracts were 10% W/V.

Tissue Preparations

Male guinea pigs (400-700g) were killed by a blow on the neck and tracheas were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). The cartilages of all rings were then cut open opposite to the trachealis muscle, and sutured together to form a tracheal chain [5] [6]. Tissue was then suspended in a 10 ml organ bath (Pinnacle Biomedical Research Institute (PBRI) Syamala Hills Bhopal (M.P) India) containing Krebs-Henseliet solution of 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. The Krebs solution was kept at 37^{o}C under stream of 95% O_2 and 5% CO_2 gases. Tissue was suspended under an isotonic tension of 1 g and allowed to equilibrate for at least 1 h while it was washed with Krebs solution every 15 min [2] [16].

PROTOCOLS

The relaxant effects of three cumulative concentrations of macerated and soxhlet extracts of *Limonia acidissima* (0.5, 0.75, and 1.0 g/100 ml), three cumulative concentrations of theophylline anhydrous (S.D Fine, Mumbai, India) (0.5, 0.75, and 1.0 mM) as positive control, and saline as negative control were examined. The consecutive volumes were added to 10 ml organ bath at 5 min intervals. In each experiment the effect of three cumulative volumes of each extract, three cumulative volumes of theophylline, and saline on contracted tracheal smooth muscle were determined after exposure of tissue to the solution for 5 min. A decrease in tone was considered as a relaxant (bronchodilatory) effect and expressed as positive percentage change in proportion to the maximum contraction and an increase in tone was considered as a contractile (bronchoconstrictory) effect which was expressed as negative percentage change [17] [18]. The relaxant effect of different solutions ware tested with three different experimental designs as follows.

1. On tracheal chains contracted by 60 mM KCl (group 1experiments N = 6).

2. On non-incubated tracheal chains contracted by 10 µM methacholine hydrochloride (S.D Fine, India) (group 2 experiments N = 6).

3. On incubated tracheal chains with 1 µM propranolol hydrochloride 30 min prior to beginning and during the testing relaxation of different solutions. In this series of experiments, tracheal chains were also contracted by 10 µM methacholine hydrochloride (Group 3 experiments, N = 4).

The relaxant effect of theophylline was examined only on groups 1 and 2. The relaxant effects in three groups of experiments were examined in three different series of tracheal chains. All of the experiments were performed randomly with a 1 h resting period of tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution [19]. In all experiments responses were recorded on a kymograph and were measured after fixation.

Statistical Analysis

All data were expressed as mean ± SEM. Data of relaxant effects of different concentrations of extracts were compared with the results of negative and positive control using ANOVA. The data of relaxant effect obtained in three groups of experiments were also compared using ANOVA. The relaxant effect of two extracts and theophylline were related to the concentrations using least square regression. Significance was accepted at $P<0.05$.

RESULTS

Acute Toxicity Test (Determination of LD_{50})

The result of acute toxicity study (LD_{50}) of the ethanol extract of *Limonia acidissima* was calculated to be 2262.7 mg/kg by oral route. During the acute toxicity study, animals were observed for gross behavioral and morphological changes (respiratory distress, immobility, convulsion, loss of righting reflex etc.). Based on the results of acute study, doses were then selected. The plant extracts did not produce any significant changes in the normal behavior of the animals, and no toxic symptoms were seen at the dose levels studied [20].

Table 1: Relaxant effect of two different extracts from *Limonia acidissima* in comparison with negative control (saline) and positive control (theophylline) in group 1 experiment (contracted tracheal chains with 60 mM KCL)

S. No.	Conc	Saline	Macerated Extract	Soxhlet Extract	Theophylline
1	0.5	0	2.12 ± 0.76 NS, ns, nS	5.93 ± 2.12 NS, ns	13.65 ± 6.15 NS
2	0.75	0	16.57 ± 0.14 NS, +++, nS	32.41 ± 3.24 NS, ++	49.00 ± 5.77 **
3	1	0	32.54 ± 0.12 NS, +++,	71.45 ± 16.20 *, +++	85.83 ± 6.39 ***

Table 2: Relaxant effect of two different extracts from *Limonia acidissima* in comparison with negative control (saline) and positive control (theophylline) in group 2 experiments (contracted tracheal chains by 10 µM methacholine)

S. No.	Conc	Saline	Macerated Extract	Soxhlet Extract	Theophylline
1	0.5	0	20.51 ± 17.94 NS, ns	29.59 ± 11.44 *, ns, nS	11.97 ± 3.47 NS
2	0.75	0	32.31 ± 13.51 ***, ns	36.91±7.27 ***, ns, nS	39.56 ± 6.35 **
3	1	0	44.94 ± 12.31 ***, ns	57.83 ± 6.10 ***, ns, nS	76.99 ± 6.74 ***

Table 3: Relaxant effect of two different extracts from *Limonia acidissima* in comparison with negative control (saline) in group 3 experiments (incubated preparation with 1µM propranolol contracted tracheal chains by 10 µM methacholine)

S. No.	Conc	Saline	Macerated Extract	Soxhlet Extract
1	0.5	0	69.10 ± 16.67 **, nS	64.19 ± 10.33 **
2	0.75	0	84.47 ± 12.69 ***, nS	81.70 ± 10.59 ***
3	1	0	88.54 ± 10.31 ***, nS	93.57 ± 6.61 ***

Relaxant (Bronchodilatory) effect

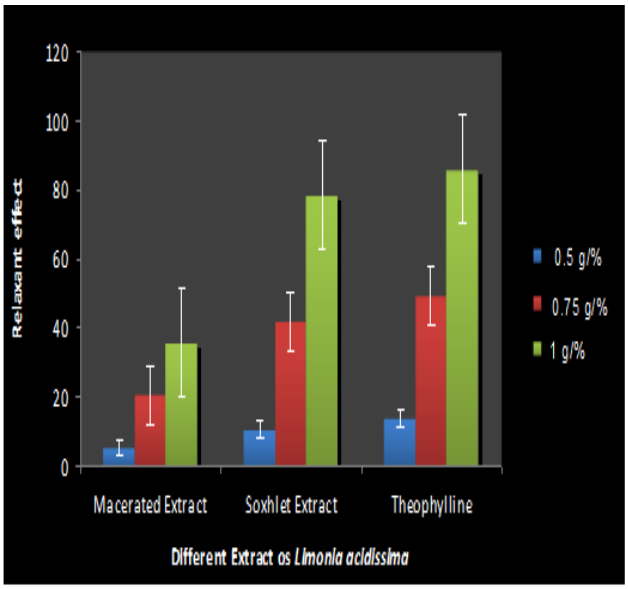


Fig. 3: Histogram representing relaxant effect of two different extracts from *Limonia acidissima* in comparison with negative control (saline) and positive control (theophylline) in group 1 experiment (contracted tracheal chains with 60 mM KCL)

Values are presented as mean ± SEM. Statistical differences between the effect of extracts and negative control (saline); NS: non-significant difference, *: P<0.05, **: P<0.01, ***: P<0.001. Statistical differences between the effect of extracts and positive control (theophylline); ns, non-significant difference, + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between the effect of two extracts; nS, non-significant difference; P<0.05; and P<0.01.

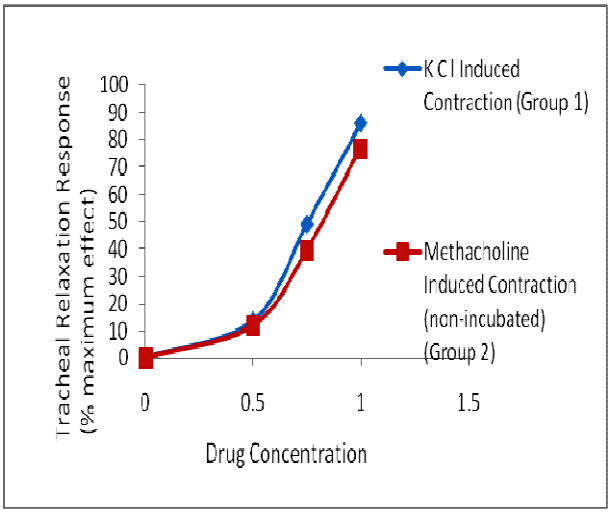


Fig. 4: Concentration response curves of the relaxant effects of theophylline

Values are presented as mean ± SEM. Statistical differences between the effect of extracts and negative control (saline); NS: non-significant difference, *: P<0.05, **: P<0.01, ***: P<0.001. Statistical differences between the effect of extracts and positive control (theophylline); ns, non-significant difference, + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between the effect of two extracts; nS, non-significant difference; P<0.05; and P<0.01.

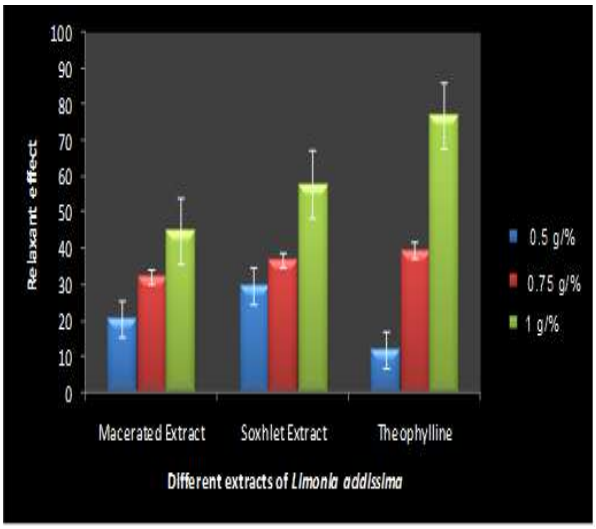


Fig. 5: Histogram representing relaxant effect of two different extracts from *Limonia acidissima* in comparison with negative control (saline) and positive control (theophylline) in group 2 experiments (contracted tracheal chains by 10 µM methacholine).

Values are presented as mean ± SEM. Statistical differences between the effect of extracts and negative control (saline); NS: non-significant difference, *: P<0.05, **: P<0.01, ***: P<0.001. Statistical differences between the effect of extracts and positive control (theophylline); ns, non-significant difference, + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between the effect of two extracts; nS, non-significant difference; P<0.05; and P<0.01.

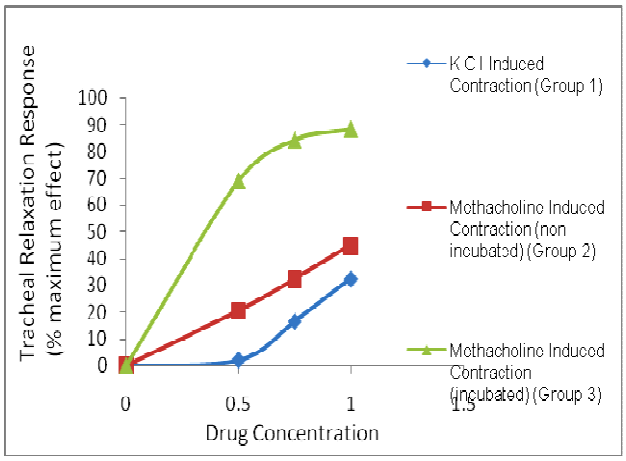


Fig. 6: Concentration response curves of the relaxant effects of macerated extract.

Values are presented as mean ± SEM. Statistical differences between the effect of extracts and negative control (saline); NS: non-significant difference, *: P<0.05, **: P<0.01, ***: P<0.001. Statistical differences between the effect of extracts and positive control (theophylline); ns, non-significant difference, + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between the effect of two extracts; nS, non-significant difference; P<0.05; and P<0.01.

Fig. 7: Histogram representing relaxant effect of two different extracts from *Limonia acidissima* in comparison with negative control (saline) in group 3 experiments (incubated preparation with 1 µM propranolol contracted tracheal chains by 10 µM methacholine).

Values are presented as mean ± SEM. Statistical differences between the effect of extracts and negative control (saline); NS: non-significant difference, *: P<0.05, **: P<0.01, ***: P<0.001. Statistical differences between the effect of extracts and positive control (theophylline); ns, non-significant difference, + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between the effect of two extracts; nS, non-significant difference; P<0.05; and P<0.01.

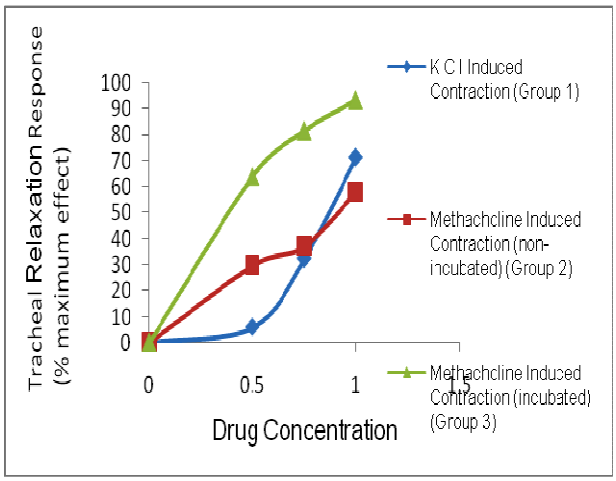


Fig. 8: Concentration response curves of the relaxant effects of soxhlet extract.

Values are presented as mean ± SEM. Statistical differences between the effect of extracts and negative control (saline); NS: non-significant difference, *: P<0.05, **: P<0.01, ***: P<0.001. Statistical differences between the effect of extracts and positive control (theophylline); ns, non-significant difference, + P<0.05, ++ P<0.01, +++ P<0.001. Statistical differences between the effect of two extracts; nS, non-significant difference; P<0.05; and P<0.0

Table 4: Correlation (r) between the relaxant effects of two different extracts from *Limonia acidissima* and theophylline with concentration in three groups of experiments.

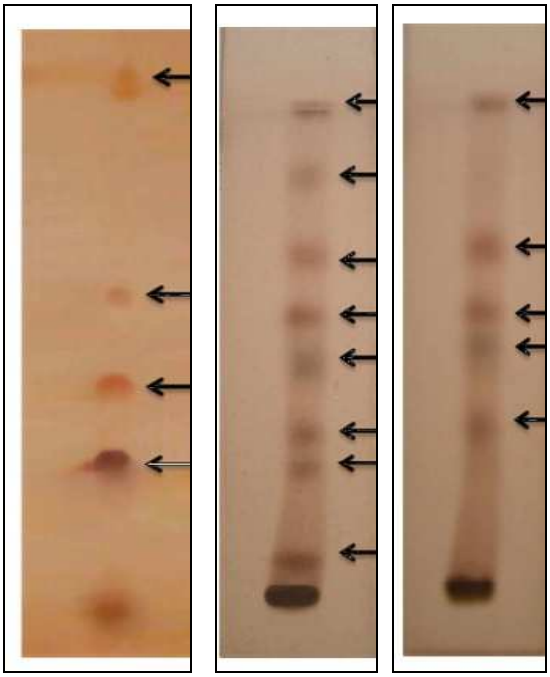
Different Substances	Group 1	Group 2	Group 3
Soxhlet extract	0.993***	0.963***	0.993***
Macerated extract	0.999***	0.999***	0.948***

Theophylline	0.999***	0.996***	-

Statistical significances; NS: non-significant difference, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

Table 5: Thin layer chromatography of ethanolic extract of *Limonia acidissima*

Phytoconstituent	Solvent System	Detecting Reagent	Numbers, color, and R_f values of spots
Alkaloids	Tolune:ethyl acetate: (85:15)	Dragendorff reagent	Three orange- brown with R_f values; 0.30, 0.587 were seen
Flavonoids	Ethyl acetate:formic acid:glacial acetic acid: water (10:1,1:1,1:206)	Sprayed with anisal dehyde sulphuric acid and heated at 110^0C for 5 minutes.	Seven light brown spots with R_f values; 0.09, 0.260, 0.346, 0.466, 0.560, 0.693, and 0.853 were seen.
Saponins	Chloroform:glacial acetic acid:methanol:water (64:32:12:08)	Sprayed with 1% $AlCl_3$ in methanol and observed under UV light Sprayed with Lieberman Burchard reagent and heated at 100^0 C for 5 minutes.	No spots were observed Four light brown spots with R_f values 0.320, 0.474, 0.551, and 0.666 were seen.



A B C

Fig. 9: TLC plates showing presence of (a) alkaloids, (b) flavonoids, and (c) saponins spots in ethanolic extract of *Limonia acidissima*

RESULTS

Relaxant (Bronchodilatory) effects

In groups 1 theophylline and Macerated and Soxhlet extract from *Limonia acidissima* showed concentration-dependent relaxant effects on tracheal chains of guinea pig. The relaxant effects of all concentrations of Macerated and Soxhlet extract and theophylline were significantly higher than those of saline (p<0.01 to p<0.001), (Table 1). The effects of two higher concentrations of theophylline in this group were significantly greater than those of Soxhlet and Macerated extracts (P<0.01). In group 2 (Contracted tracheal chains by 10 µM methacholine) theophylline and both Macerated and Soxhlet extract showed significant relaxant effects compared to that of saline (p<0.05 to p<0.001), (Table 2). In group 3 (Incubated with 1 µM propranolol contracted tracheal chains by 10 µM methacholine) both Macerated and Soxhlet extract showed significant relaxant effects compared to that of saline (p<0.05 to p<0.001). The relaxant effects of Macerated and Soxhlet extracts in group 1 were significantly lowers than those of groups 2 and 3, (Table 1, 2 & 3).

Comparison of the relaxant effects between three groups of experiments

The relaxant effects of most concentrations of Macerated and Soxhlet extract in groups 1 and 2 were statistically lower than those of group 3 experiments (p<0.001 to p<0.01) (Fig 6 & 8). The relaxant effects of most concentrations of extracts in group 1 were also significantly lower than those of group 2 (p<0.001 to p<0.05), (Fig 6 & 8). In addition, there were no significant differences in the effect of all concentrations of theophylline between groups 1 and 2 (Fig 4).

Correlation between concentrations of solutions and their relaxant effect

There were significant positive correlations between the relaxant effects of theophylline and Macerated and Soxhlet extract with solutions of different concentrations in groups 1 & 2 of experiments (p<0.001 for all cases), (Table 4).

DISCUSSION

In this study the relaxant (bronchodilatory) effects of Macerated and Soxhlet extracts of 95% ethanol from *Limonia acidissima* in comparison with saline as negative control and theophylline as positive control were studied. In group 1 experiment (contracted tracheal chains by 60 mM KCl) the highest concentration (1.0 g %) of Soxhlet extract from *Limonia acidissima* and higher two concentrations (0.75 & 1.0 mM) of theophylline showed relatively potent and concentration-dependent relaxant effects on tracheal chains of guinea pig. In group 2 experiments (contracted tracheal chains by 10 µM methacholine), two higher concentrations (0.75 & 1.0 mM) of theophylline and two higher concentrations (0.75 & 1.0 g %) of Soxhlet extract and one higher concentrations (1.0 g %) of Macerated extract showed significant relaxant effects compared to those of saline. However, the effects of two higher concentrations of Macerated and Soxhlet extract were significantly lower than those of theophylline. In group 3 experiments the 95% all ethanolic Macerated and Soxhlet extract of *Limonia acidissima* show potent relaxant effect. The effect of theophylline was not examined in group 3 experiments. The relaxant effects of Macerated and Soxhlet extract and theophylline was concentration dependent. There were positive correlations between increasing concentrations and the relaxant

effects of Macerated and Soxhlet extract in groups 1 and 2 experiments. The relaxant effects of all concentrations of Macerated and Soxhlet extracts in group 1 were lower than those of groups 2. In addition, the effects of Macerated and Soxhlet extract in group 1 experiments were comparable to that of theophylline. The relaxant effects of Macerated and Soxhlet extract from *Limonia acidissima* on tracheal chains of guinea pigs might be produced by different mechanisms including stimulation of β-adrenergic receptors, inhibition of histamine H1 receptors or an anticholinergic property of this plant, because the relaxant effect of β2-stimulatory [17], [21] histamine H1 receptor inhibitory [21], and anticholinergic drugs [23] have been shown in previous studies. To evaluate the contribution of β-adrenergic stimulatory, H1 histamine and muscarinic blocking effect of extract, the effects of these extracts on tracheal chains inhibited β-adrenergic, muscarinic and histamine H1 receptors by propranolol, was re-examined in group 3 experiments. The relaxant effects of all concentrations of the Macerated and Soxhlet extract from *Limonia acidissima* obtained in the group 3 experiments were significantly higher than those of group 2 and group 1. These findings suggest probable ß-adrenergic stimulatory, muscarinic and/or histamine H1 blocking properties of the plant extract that may contribute to their relaxant effect on tracheal chins of guinea pig. While KCl affects calcium channels [24] and because calcium channel blockers have bronchodilatory effect [25], [26]. The relaxant effect of Macerated and Soxhlet extract from *Limonia acidissima* in group 3 experiments may be due to blocking effects of the calcium channels. Another explanation for these findings is an opening effect of the extract on potassium channels [27]. The weak relaxant effects of extract on tracheal chains group 1 (contracted by 60 mM KCl) and high relaxant effect on tracheal chain contracted by group 3 (Incubated with propranolol and contracted tracheal chains by 10 µM methacholine) may support opening of potassium channels. In addition, the effects of the different concentrations of soxhlet extract in this group were nearly same than those of macerated extracts, which were statistically significant ($p<0.05$), (Table 3) & (Fig 7 & 8). Since the plant showed a potent relaxant effect on group 3 which was completely blocked in tissues Incubated with propranolol and contracted tracheal chains by methacholine, the most possible mechanisms of the relaxant effect of *Limonia acidissima* might be due to its inhibitory effects on muscarinic receptors. Howevere, the inhibitory effect of the plant on histamine (H1) receptors and its stimulatory effect on β-adrenoceptors can not ruled out with these results and should be reexamined in further studies. Therefore, flavonoids [28], saponins [29], tannins [30], [31], coumarins [32], [33], content of the fruits of plant *Limonia acidissima* may be responsible for its relaxant effects on tracheal chains.

CONCLUSION

In this case also, ethanolic extract of *Limonia acidissima* at 1.0 g/100 ml was found to be most effective as compared to other treatment groups. Even effect of ethanolic extract of *Limonia acidissima* at 1.0 g/100 ml was comparable to standard anti-asthmatic drug Theophylline used in present investigation. Therefore, the extract contained compounds such as alkaloids, flavonoids, saponins, tannins, polyphenol antioxidant and coumarins like biologically active compounds that might be responsible for the anti asthmatic activity.

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