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IN-VIVO PHARMACOLOGICAL INVESTIGATIONS OF *CITRUS HYSTRIX*

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ABSTRACT

The present study was done to evaluate *in vivo* anti-pyretic, gastrointestinal motility, anti-nociceptive and acute toxicity effect of different leaf extracts of *Citrus hystrix* in Swiss albino mice following oral administration. *In vivo* anti-pyretic test of methanol and ethanol extracts of *Citrus hystrix* leaf was done brewer's yeast method; GI motility test was done by charcoal induced anti motility test, anti-nociceptive activity was tested by acetic acid induced writhing method and tail immersion method, acute toxicity study was done by investigating mortality/morbidity status of test animal. Statistically significant ($p<0.05$) result was found in case of i*n vivo* anti-nociceptive activity test for the 100 mg/kg methanol extract when compared to standard diclofenac-Na. None of the extracts showed any significant *in vivo* acute toxicity effect on mice. This plants leaf extracts exhibit some antipyretic activity and significant anti-nociceptive activity without inducing any discernible acute toxicity effect.

Key words: *Citrus histrix*, anti-nociceptive activity, gastro intestinal motility, anti-pyretic activity and acute toxicity.

INTRODUCTION

Citrus hystrix commonly known in English as kaffir lime, is a fruit native to Indochinese and Malesian ecoregions in India, Nepal, Philippines, Bangladesh, Indonesia, Malaysia and Thailand, and adjacent countries. It is used in Southeast Asian cuisine. *Citrus hystrix* is a thorny bush, 5-10m tall, with aromatic and distinctively shaped "double" leaves. The kaffir lime is a rough, bumpy green fruit. The green lime fruit is distinguished by its bumpy exterior and its small size (approx. 4 cm (2 in) wide). Bumpy, green, maturing to yellow skinned citrus fruit with a highly acidic flavor. The leaves are an important flavoring in Thai and other southeast Asian dishes Seeds are not available for the Kaffir Lime. Small tree, from 6-25ft in height. The Kaffir Lime is easily distinguished by its glossy, two-part leaves. Trees also usually contain some thorns. Trees are mildly frost hardy and grow best in areas that receive only short, mild frosts. Grow in full sun, provide water during growing months and protect from hard freezes. Fertilize at the beginning of growing season. Propagation is by seeds and grafts. In Bangladesh it is also known as Satkora. The leaves, which have a characteristic shape due to their winged petioles, which almost look like leaves themselves; if available, the fruits, especially the fruit skin, may also be used. The juice and rinds are used in traditional Indonesian medicine; for this reason the fruit is referred to in Indonesia as *jeruk obat* ("medicine citrus"). The oil from the rind has strong insecticidal properties. The juice finds use as a cleanser for clothing and hair in Thailand and very occasionally in Cambodia. Lustral water mixed with slices of the fruit is used in religious ceremonies in Cambodia. The leaves are aromatic, used as a spice, and for various flavoring purposes. The juice is also sometimes used in the preparation of food and beverages, although it is not consumed directly. Oil is also extracted from the rind for use in cosmetics and beauty products [1-5].

As a part of our continuing studies on medicinal plants of Bangladesh the organic soluble materials of the leaf extracts of *Citrus hystrix* were evaluated for anti-nociceptive activity, anti-pyretic activity, gastrointestinal motility and acute toxicity for the first time [7-12].

MATERIALS AND METHODS

Collection, identification and processing of plant samples: Leaves of *Citrus hystrix* was collected during December, 2013 from Govindaganj, Sylhet in June 2014 and was taxnomically identified with the help of the National Herbarium of Bangladesh, Mirpur-1, Dhaka (DACB; Accession Number-38758). Leaves were sun dried for seven days. The dried eaves were then ground in coarse powder using high capacity grinding machine which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

Extraction procedure: The powdered plant parts (22 gm) were successively extracted in a soxhlet extractor at elevated temperature using 250 ml of distilled Methanol (40-60)°C which was followed by ethanol. After extraction all extracts kept in refrigerator 4°C for future investigation with their necessary markings for identification.

Experimental animal: For the experiment Swiss albino mice of either sex, 4-5 weeks of age, weighing between 10-24 gm were collected from ICDDR, B, Dhaka. Animals were maintained under standard environmental conditions (temperature: (27.0 ±1.0) ºC, relative humidity: (55-65) % and 12 hour light/12 hour dark cycle) and free access to feed and water. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Gastrointestinal motility determination: Thirty six Swiss Albino mice, weighing between 10-20 g were selected and housed properly for 10 days before performing the experiment. On the test day, the animals were divided into eight groups of six mice each. They were weighed and deprived of food, with free access to water. Three hours after food deprivation, the animals in group 1 received orally by gavages 5 ml/kg body weight of 0.9% NaCl (normal saline) as control group, while those in group 2 received 5 mg/kg body weight of butapan (hyoscine butyl bromide) as standard group. The other four groups received methanol 100 & 200 gm and ethanol 100 & 200 gm respective doses. After 90 min, 0.3 ml of an aqueous suspension of 5% charcoal in normal saline was administered to each animal orally by gavages (time 90 min). Sixty minutes later they had free access to food (time 150 min). The animals were observed at 5 min intervals until feces with charcoal were eliminated (maximum time of observation was 300 min). Charcoal was observed on the feces using normal light when it was easily visible, or using a microscope to help the identification of the black spots. The results were based on the time for the charcoal to be eliminated [13].

Anti-pyretic activity: Thirty six Albino Swiss mice of both sexes (10-20 gm) were randomly divided into 6 groups and fasted overnight before the experiment with free access to water. The normal body temperature of each mouse was measured rectally at predetermined intervals and recorded. Fever was induced according to the method described by Smith and Hambourger (1935) [14]. A lubricated thermometer probe was inserted 3-4 cm deep into the rectum and fastened to the tail by adhesive tape. Temperature was measured on digital thermometer. After measuring the basal rectal temperature, animals were injected subcutaneously with 10 ml/kg of 20% w/v brewer's yeast in NSS in the dorsum of the mice. Mice were then returned to their housing cages. Eighteen hours after brewer's yeast injection, the animals were again restrained for rectal temperature recording, as described previously. Only mice that showed an increase in temperature of at least 10^0C were used for this study. The extracts at the doses of 100 & 200 mg/kg body weight were administered orally to four groups of animals. The control group received 1ml/kg body weight dose of vehicle (0.9% NaCl solution) and the standard group received paracetamol (50 mg/kg body weight) orally. Rectal temperature was measured at 1hr intervals for 4 hr after the extract/drug administration. The rectal temperature of normal rats (normothermic) was also measured at 1 hr. intervals for 7 hr. [15] The results are expressed as percentage of the pre-drug temperature recorded for the same animals using the formula of Makonnan *et al*., (2003) [16].

Anti-nociceptive activity test: Anti-nociceptive activity was evaluated by acetic acid writhing test and tail immersion test.

Acetic acid induced writhing test: The acetic acid writhing test in mice as described by Koster *et al*., (1959) [17], was employed with slight modification. Mice were divided into six groups containing six mice in each group. The first group was given 10 ml/kg of 1% Tween 80 i.p. and served as control. Group 2 was served as standard where diclofenac sodium has given to mice as dose of 50 mg/kg of body weight. Groups 3, 4 received methanol extract *Citrus hystrix* 100 mg/kg and 200 mg/kg of body weight. Groups 5, 6 received ethanol extract *Citrus hystrix* 100 mg/kg and 200 mg/kg of body weight. Thirty minutes later each mouse was injected i.p.

with 0.7% acetic acid at doses of 10 ml/kg of b.w. Full writhing was not always completed by the mice. Accordingly, two half writhing were considered as one full writhing. The no. of writhing responses was recorded for each mouse during a subsequent 5 min period after 15 min i.p. administration of acetic acid and the mean abdominal writhing for the each group was obtained and recorded.
The percentage inhibition of writhing was calculated using following equation:
% inhibition = [1 – (no. of writhing of standard or sample/ no. of writhing of control)] × 100

Tail immersion test: The tail immersion method was used to evaluate the central mechanism of analgesic activity. Here the painful reactions in animals were produced by thermal stimulus that is by dipping the tip of the tail in hot water [18]. On the test day, albino swiss mice were divided into 6 groups of 6 mice each. Here diclofenac Na (50 mg/kg) is used as standard drug as well. Animals were fasted for 16 hours with free access to water. After administration of standard and test drugs, the basal reaction time was measured by immersing the tail tips of mice (last 1-2 cm) in hot water of water bath, where temperature was previously adjusted at 51ºC. The actual flick response of mice that is time taken in second to withdraw it from hot water source was calculated and results were compared with control group. The latent period of the tail-flick response was determined at 0, 30, 60, 90 and 120 minute after the administration of drugs.

Acute toxicity test: The acute toxicity test in mice as described by Ecobichon, 1997 [19] was employed with slight modification. Mice were kept fasting for 1-2 hours but water was provided and were divided into 6 groups containing 6 mice in each group. All mice were weighed and kept separated using separate cage. The test samples i.e. methanol, ethanol and chloroform extracts were administered orally at different doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg of body weight of mice. After administration of the extract solutions mortality or sign of any toxicity was observed for one hour and kept under observation for 1 week.

Statistical analysis: Data was expressed as Mean ± SEM (Standard error of Mean). The results were analyzed statistically by ANOVA followed by Dunnet's test. Results below $p<0.05$ and $p<0.01$ are considered statistically significant.

RESULTS AND DISCUSSION

Gastro intestinal (GI) motility test: The gastro-intestinal motility test results are shown in **table 1**. In this test methanol and ethanol extract at doses of 100 and 200 mg/kg body weight were administered.

Abdominal cramping and pain is a frequent problem in the adult population of Western countries, with an estimated prevalence of ≤30%. Pharmacological studies have revealed that hyoscine butyl bromide is an anti-cholinergic drug with high affinity for muscarinic receptors located on the smooth-muscle cells of the GI tract. Its anti-cholinergic action exerts a smooth-muscle relaxing/spasmolytic effect. Blockade of the muscarinic receptors in the GI tract is the basis for its use in the treatment of abdominal pain secondary to cramping. However, because of its high tissue affinity for muscarinic receptors, hyoscine butyl bromide remains available at the site of action in the intestine and exerts a local spasmolytic effect [20]. But the results of the present study revealed both the doses of methanol and ethanol extract (100 and 200 mg/kg b.w) such no effect compared with the effect produced by standard.

Anti-pyretic test: The results of anti-pyretic test of *Citrus hystrix* leaf extracts using brewer's yeast induced pyrexia in mice have been shown in **figure 1** and **2**. In **figure 2** it is seen that the dose of 100 mg/kg b.w. of methanol extract showed maximum reduction of temperature among all the extracts.

In the present study, methanol extract showed some antipyretic activities in mice. Brewer's yeast-induced fever is called pathogenic fever. Its etiology includes production of prostaglandins, which set the thermo-regulatory center at a lower temperature [21]. So inhibition of prostaglandin synthesis could be the possible mechanism of antipyretic action as that of acetylsalicylic acid [22]. Alpayci, 2012 [23] suggested that there are several mediators or multi-processes underlining the pathogenesis of fever. Inhibition of any of these mediators may bring about anti-pyresis.

Anti-nociceptive activity test

Acetic acid induced writhing method: The result of acetic acid induced writhing method with leaf extracts of *Citrus hystrix* is shown in **table 2.** Acetic acid induced writhing model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipids [24]. The constriction response of abdomen produced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics. It has been associated with prostanoids in general, for example, increased levels of PGE2 and PGF2a in peritoneal fluids [25,26] as well as lipoxygenase or cyclo-oxygenases products [27,28] and acid sensing ion channels [29].

Table 2 represent the effect of different extracts of *Citrus hystrix* in acetic acid induced writhing test. Methanol extract inhibited writhes in a dose dependent manner. But ethanol extract at 100 mg/kg showed highest inhibition (58.38%).

Tail immersion test: Tail immersion method, the heat itself acts as a source of pain. The different concentrations of methanol and ethanol extract of plant (100 and 200 mg/kg) and diclofenac Na (50 mg/kg) were administered to mice and observed the basal reaction time in different time intervals. The basal reaction time increased with increasing the concentrations along with increasing the time. The basal reaction time was more for standard drug when compared to plant extracts (**table 3**).

Acute toxicity test: In the time of investigation of acute toxicity none of the extracts showed any sign of toxicity in the period of one week observation which is shown in **table 4**.

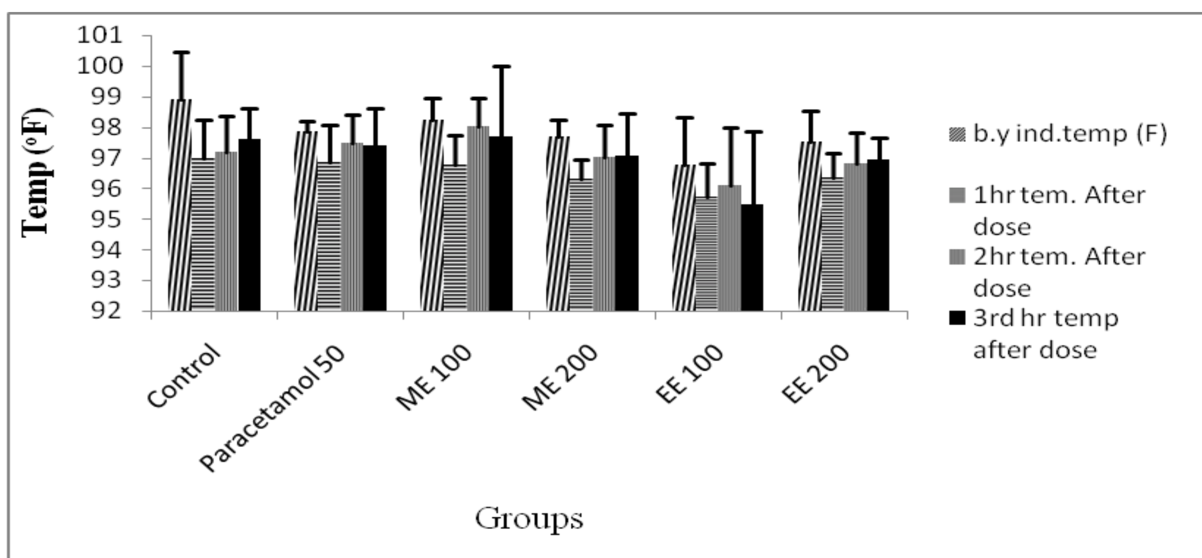
CONCLUSION

In this study, it can be concluded that, *Citrus hystrix* leaf extracts of respective solvents (methanol and ethanol) showed some level of anti-nociceptive effect as well as shows some level of % reduction of temperature. In GI motility test, extracts unable to exhibit any remarkable anti-motility effect compared with standard. None of the solvent extract of *Citrus hystrix* had shown any sign of toxicity in acute toxicity test during one week observation period. Our current work is suggestive to future works on *Citrus hystrix* with a consideration of compound isolation for particular activity and develop lead compound for therapeutic use.

Table 1: Gastrointestinal motility determination of different extracts of *Citrus hystrix*

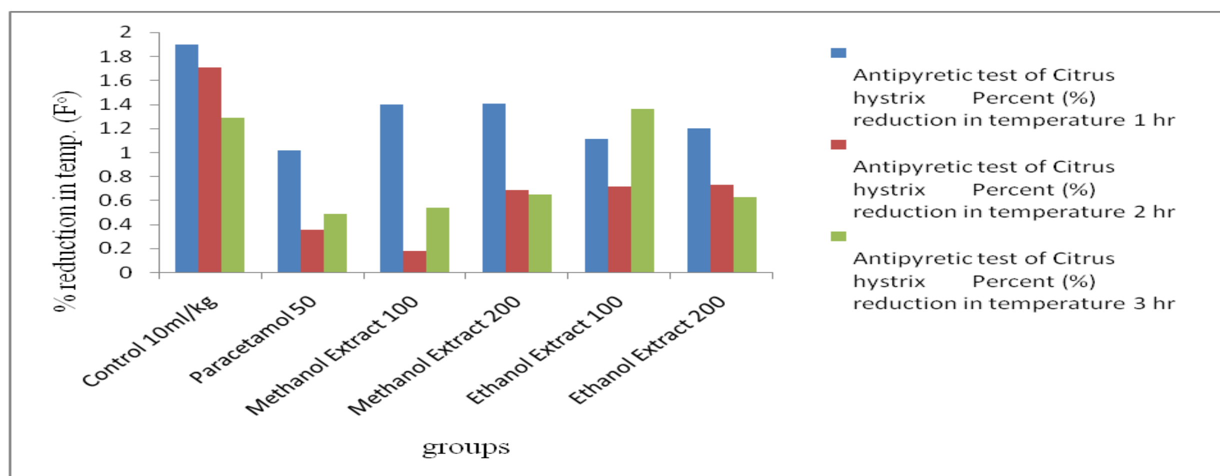
Groups	Treatment	Dose	Time of charcoal defecation(min)
G-1	Control (0.9% NaCl)	5ml/kg	410±59.160
G-2	Butapan (standard)	5mg/kg	154±32.098
G-3	Methanol Extract	100mg/kg	391±58.779
G-4	Methanol Extract	200mg/kg	401±48.140
G-5	Ethanol Extract	100mg/kg	365.2±52.608
G-6	Ethanol Extract	200mg/kg	450±40.728

Values are expressed as mean ± SEM (n=6)



Values are expressed as mean ± SEM (n=6)

Figure 1: Effect of leaf extract of *Citrus hystrix* in brewer's yeast induced pyrexia in mice



Values are expressed as mean ± SEM (n=6)

Figure 2: Comparative study of % reduction of temperature using leaf extracts of *Citrus hystrix*

Table 2: Writhing test of leaf extract of *Citrus histrix*

Groups	No. of writhing	% Inhibition
Control	29.8 ± 1.77	0
Standard	10.8 ± 1.24	63.75
Methanol Extract 100mg/kg	24.6 ± 0.50*	17.44
Methanol Extract 200mg/kg	12.8 ± 0.73	57.04
Ethanol Extract 100mg/kg	12.4 ± 0.50	58.38
Ethanol Extract 200mg/kg	12.8 ± 0.58	57.04

(Values are expressed as mean ± SEM (n=6), *p<0.05; significant when compared with the corresponding value of standard group)

Table 3: Tail immersion of different extracts of *Citrus hystrix* in tail immersion test

Treatment	Retention Time (sec)				
	0 min	15 min	30 min	45 min	60 min
0.9% NaCl	3.27±0.74	3.25±0.95	3.54±1.08	3.32±1.50	3.68±1.03
Diclofenac Na	2.75±0.98	2.798±0.44	2.892±0.85	3.476±0.98	3.188±0.41
Methanol Extract 100mg/kg	3.008±0.94	3.174±0.39	3.166±0.67	3.512±1.16	3.552±0.77
Methanol Extract 200mg/kg	2.776±0.37	2.52±0.78	2.65±0.50	3.03±0.34	2.96±0.87
Ethanol Extract 100mg/kg	2.536±0.38	3.33±1.16	3.146±1.00	2.848±1.63	3.348±0.78
Ethanol Extract 200mg/kg	3.308±1.78	3.556±1.24	3.728±1.99	3.8±1.58	3.726±1.42

Values are expressed as mean ± SEM (n=6)

Table 4: Results of acute toxicity test

Group	Administered substance	Doses (mg/kg of b.w)	Toxic effect
1	Methanol extract	500	None
2		1000	None
3		2000	None
4	Ethanol extract	500	None
5		1000	None
6		2000	None

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