EXPLORATION OF ANTI-INFLAMMATORY POTENTIAL OF *CITRUS LIMETTA* RISSO AND *CITRUS MAXIMA* (J. BURM.) MERR.

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Summary

Citrus fruits are rich in flavonoids and limonoids which are known to possess antitumor and anti-inflammatory activities. *Citrus limetta* Risso and *Citrus maxima* (J. Burm.) Merr. are two widely distributed indigenous plants found in Indian subcontinent. Methanol extracts of peel of *Citrus limetta* fruits (MECL) and leaves of *Citrus maxima* (MECM) were evaluated in two dose levels (200 mg kg^{-1} and 400 mg kg^{-1}) in histamine, carrageenan and dextran induced acute rat paw oedema models for their anti-inflammatory potential in Wistar albino rats. Both MECL and MECM were able to significantly ($p < 0.001$) reduce the inflammatory potential produced by different inflammatory mediators in different experimental models in a dose dependant manner. The effects were compared to reference drug and MECL was able to produce significant anti-inflammatory activity better than the reference drug used (phenylbutazone 100 mg kg^{-1} b.w. p. o.).

Key words: Anti-inflammatory, *Citrus limetta*, *Citrus maxima*, histamine, carrageenan, dextran.

Introduction

Medicinal plants are a rich source of compounds used in pain healing (1). Citrus fruits are very abundant in the Indian subcontinent and lemon, lime, orange, sweet lime, pomelo are the different variety of fruits that are cultivated in a large scale. Citrus fruits are very rich source of flavonoids like hespiridin, naringin (2). The peels of the fruits are richer sources of hydroxy cinnamic acid than juice and pectin (3). Flavonoids hespiridin and naringin are found to be present in the peel and inner part of the fruit of *Citrus limetta* (4).The essential oils α-pinene, β-pinene, sabinene, β-myrcene, *p*-cymene, limonene, γ-terpinene, neryl acetate, β-bisabolene ,α-bergamotene were isolated from the zests of *Citrus limetta* Risso (5). Limonene, γ-terpinene was isolated from the peel and their minimum inhibitory concentration (MIC) and maximal tolerated concentration (MTC) was estimated against *Pseudomonas putida* (6).

The major flavanones of *Citrus maxima* are neohesperidin and naringin, which are high in the seed than in unripe fruits (7). Hespiridin, naringin, caffeic, *p*-coumaric, ferulic and vanillic acid are present in the fruit juice (8). A C-C linked bisacridone alkaloid buntanbismine, was isolated from the stem bark of *C. grandis* (9). *C. maxima* essential oil is composed of α-pinene, sabinine, β-pinine, methyl heptenone , β-myrcene, hexanal, sabinine, DL-limonine, t-ocimine, linalool, 1-hexene, 4-methyl; 1-hexene,3,3-dimethyl; geranyl formate, Z-citral, geranyl formate, E-citral, geranyl acetate, β-farnesene (10). The chemical *in vitro* antioxidant assay showed that hesperidin significantly reduced the level of the DPPH, in a similar way to the antioxidant trolox (11).

The intestinal anti-inflammatory activity of hesperidin was evaluated in the acute stage of the trinitrobenzene sulfonic acid (TNBS) model of rat colitis. The results obtained showed that pre-treatment hesperidin (10 and 25 mg/kg) reduced colonic damage compared to TNBS control rats (12). Naringenin is a potent inhibitor of the pro-inflammatory cytokine response induced by lipopolysaccharide in both macrophages and in whole blood. Naringenin markedly inhibited the phosphorylation on serines 63 and 73 of Jun proto-oncogene-encoded AP-1 transcription factor in lipopolysaccharide-stimulated macrophages (13). Naringin and its aglycone naringenin showed high percentage edema reduction in carrageenan induced *in vivo* models (14).

In the concentration range 250-500 μM, hesperidin and its aglycone hesperetin showed potent inhibition of LPS-induced expression of the COX-2 gene in RAW 264.7 cells, suggesting the anti-inflammatory activity of these compounds (15). Naringenin , the aglycone of naringin, proved to be a potential immunomodulator for inhibiting bleomycin induced lung fibrosis and metastasis (16). Flavonoids are known to have protective effects against coronary heart disease, have anti-inflammatory, antitumor and antimicrobial effects (17). Citrus carotenoids, hydroxy cinnamic acid and pectin have lowered the risk of cancer. Pectin has the property of lowering the blood sugar and cholesterol levels (18). The current study involves the exploration of anti-inflammatory potential of the peel of sweet lime (*Citrus limetta*) fruits and the leaves of pomelo (*Citrus maxima*).

Experimental

Plant materials

Citrus maxima (J. Burm.) Merr. leaves and the *Citrus limetta* Risso fruits (Rutaceae) were collected from Nadia region of West Bengal, India in the month of March-April 2007. The plant materials were authenticated by the Botanical Survey of India, Shibpur, Howrah; and the voucher specimens (PMU-1/JU/2007 and PMU-2/JU/2007 respectively) are stored in our research laboratory for further references.

Extraction

The leaves of *Citrus maxima* were dried under shade and powdered by mechanical grinder. About 500 g of the plant material was successively extracted with petroleum ether and methanol in a Soxhlet apparatus. The methanol was then evaporated under reduced pressure to get the crude extract (MECM, yield 18.1%).

Fresh fruit peels of *Citrus limetta* were taken and grounded. About 500g of the grounded material was consecutively macerated for seven days each in petroleum ether, ethyl acetate, chloroform and methanol respectively. The methanol extract was then dried under reduced pressure to get the crude extract (MECL, yield 10.56%).

Chemicals

Carrageenan (S. D. Fine Chemicals Limited, Bombay, India), Dextran, Histamine (Sigma Chemicals Co., USA) and Phenylbutazone were used in the study.

Animals

Six to eight week old male Wistar albino rats (180 - 200 g) were procured from B. N. Ghosh & Co., Kolkata. The animals were housed in polyacrylic cages with six animals per cage. They were acclimatized to identical laboratory conditions prior to the study for seven days. The animals were kept at 25 ± 2°C and a relative humidity of 40 - 45% with alternative day and night cycles of 12 hours each. The animals had free access to commercial pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum.*

Phytochemical Screening

The plant extracts were subjected to screening for various phytochemicals employing standard protocol for determining the presence of steroids, alkaloids, tannins, flavonoids, glycosides etc (19).

Acute toxicity

The acute toxicity of the extracts was determined according to the OECD guideline No. 420 (20). Male albino mice weighing 27-30 g were used for this study. MECL and MECM were given to four groups (n = 5) of animals each at 5, 50, 300 and 2000 mg kg^{-1} b.w. p. o. The treated animals were under observation for 14 days, for mortality and general behaviour. No death was observed till the end of the study. The test samples were found to be safe up to the dose of 2000 mg/kg.

Anti-inflammatory activity

Carrageenan induced rat paw edema

The rats were divided into six groups (n = 6). Acute inflammation was produced by the subplantar administration of 0.1 ml of 1% Carrageenan in normal saline in the right hand paw of the rats. The paw volume was measured at 0 hr and 3 hrs after Carrageenan injection using plethysmometer (21,22). The first group received normal saline (3 ml kg^{-1} b.w. p. o.), while the second and the third groups were treated with MECL (200 and 400 mg kg^{-1} b.w. p. o. respectively). The fourth and fifth groups received MECM (200 and 400 mg kg^{-1} b. w. p. o. respectively). The sixth group received phenylbutazone (100 mg kg^{-1} b. w. p. o.). The animals were pre-treated with the drug 1 hour before the administration of carrageenan.

Mediator-induced inflammation

The anti-inflammatory activity of the extracts was measured with phlogistic agents (*viz*. histamine, dextran) which act as mediator of inflammation. The paw edema was induced in rats by subplantar injection of freshly prepared histamine (1 mg/ml) and serotonin (1 mg/ml) solutions respectively (23) and the paw edema was measured as mentioned earlier.

Calculation of final paw volume

Final paw volume (ml) = Mean paw volume after 3 hours of induction – Mean paw volume at the time of induction.

Calculation of % inhibition

$$\% \text{ inhibition} = \frac{final paw volume of control group - final paw volume of drug treated group}{final paw volume of control group} \times 100$$

Statistical Analysis

All the values were expressed as mean ± standard error of mean (SEM). One way ANOVA was performed followed by Dunnet's 't' test. The values of $p < 0.001$ were considered as statistically very significant and $p < 0.05$ was considered as statistically significant. The GraphPad Prism software ver. 5.0 was used.

Results

Sub-plantar injection of carrageenan exhibited increase in rat paw volume in a time dependent manner. The increase in rat paw volume in respect to the initial volume was recorded after 180 minutes for MECL and MECM. For the standard drug treated group also i.e. phenylbutazone the initial volume and the final paw volume at 180 minutes was measured. The percentage inhibition of the rat paw edema in comparison to carrageenan control and phenylbutazone is summarised in Table 1.

Table 1: % inhibition of carrageenan induced paw edema by MECL and MECM in rats (n = 6).

Treatment	Dose (mg/kg)	Final Paw volume (ml)	% inhibition	p value
Carrageenan control	0	0.470 ±0.015	-	-
MECL	400	0.180 ±0.006	61.70	< 0.001
MECL	200	0.208 ±0.004	55.74	<0.001
MECM	400	0.210 ±0.011	55.39	<0.001
MECM	200	0.264 ±0.005	43.83	<0.001
Phenylbutazone	100	0.182 ±0.004	61.27	<0.001

Percentage inhibition of dextran induced rat paw edema by MECM and MECL in comparison to dextran control and standard drug phenylbutazone is summarised in Table 2.

Table 2: % inhibition by MECL and MECM in dextran induced rat paw edema (*n* = 6).

Treatment	Dose (mg/kg)	Final Paw volume (ml)	% inhibition	*p* value
Dextran control	0	0.335 ±0.010	-	-
MECL	400	0.040 ± 0.005	88.05	< 0.001
MECL	200	0.188 ± 0.007	43.88	<0.001
MECM	400	0.207 ±0.012	38.21	<0.001
MECM	200	0.313 ±0.015	6.57	<0.001
Phenylbutazone	100	0.137 ±0.005	59.10	<0.001

Percentage inhibition of histamine induced rat paw edema as exhibited by MECL and MECM in comparison with histamine control and phenylbutazone is summarised in Table 3.

Table 3: % inhibition of histamine induced rat paw edema by MECL and MECM (*n* = 6).

Treatment	Dose (mg/kg)	Final Paw volume (ml)	% inhibition	*p* value
Histamine control	0	0.310 ±0.019	-	-
MECL	400	0.011 ±0.006	96.45	< 0.001
MECL	200	0.020 ± 0.003	93.55	<0.001
MECM	400	0.092 ± 0.016	81.29	<0.001
MECM	200	0.058 ± 0.015	70.32	<0.001
Phenyl butazone	100	0.109 ± 0.008	64.83	<0.001

MECL showed maximum inhibition of 61.7% at the dose of 400 mg kg^{-1} body wt. after 3 hrs of drug treatment in carrageenin induced paw edema whereas the standard drug produced 61.27 % of inhibition (Table 1). In case of dextran and histamine induced rat paw edema, MECL produced 88.05% and 96.45% of inhibition at the dose of 400 mg kg^{-1} whereas phenylbutazone produced 59.1% and 64.83% of inhibitions respectively (Tables 2 and 3).

Discussion

Carrageenan induced rat paw edema is commonly used as an experimental animal model for assessment of acute inflammation and is believed to be biphasic, of which the first phase is mediated by the release of histamine and 5-HT in the early stage followed by kinin release and then prostaglandin and bradykinins in the later phase (24, 25). From the present results it is evident that MECL exhibited maximum activity at the dose level of 400 mg kg^{-1} with respect to all other groups against all three phlogistic agents.

Preliminary phytochemical study indicated the presence of flavonoids, alkaloids, tannins and saponin in MECL and MECM. The flavonoids and limonoids present in citrus plants are postulated to be the cause of their antitumor and anti-inflammatory effects (26).

As discussed in the introduction section, the flavonoids hesperidin and naringin are found in C*itrus maxima* and C*itrus limetta* both. The flavonoids and their respective aglycones viz. hesperetin and naringenin have shown potent anti-inflammatory activity against different *in vitro* and *in vivo* models as discussed earlier.

The *in vitro* free radical scavenging activity of MECM and MECL was evaluated and the extracts were found to be possess significant free radical scavenging activity when tested against different free radicals (27).

The plants *Citrus limetta* and *Citrus maxima* both contain high quantity of flavonoids of proven anti-inflammatory potential. Therefore, it can be concluded that due to the presence of the flavonoids both MECM and MECL can act as potent anti-inflammatory agents.

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