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Research Article

Analgesic, Anti-inflammatory and Diuretic Activity of Methanol Extract of *Flacourtia indica*

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Abstract

The present study was aimed at investigating the antinociceptive, anti-inflammatory and diuretic activities of methanol extract of *Flacourtia indica* (FIM). Administration of FIM, by gavage, at doses of 125 or 250 mg/kg body weight significantly reduced the number of acetic acid-induced writhing in mice by 29.78 and 44.12%, respectively, as compared to that of 66.18% inhibition by the positive control aminopyrene. FIM significantly reduced the volume of carrageenan-induced rat paw edema after 1 to 4 h, with maximum inhibitory effect observed after 3 hour (41.09 and 45.00% at doses of 125 and 250 mg/kg body weight, respectively), of carrageenan administration. The inhibitory effect of FIM was comparable to that of the positive control phenylbutazone. Pre-incubation of human keratinocyte HaCaT cells with FIM (25 or 50 µg/ml) decreased ultraviolet-B (UVB) radiation-induced expression of cyclooxygenase-2 (COX-2) and the phosphorylation of extracellular signal regulated kinase (ERK) and Akt. Intra-gastric administration of FIM at 125 and 250 mg/kg body weight to mice markedly increase the urine volume showing good diuretic activity as compared to standard diuretic drug furosemide. The observed analgesic, anti-inflammatory and diuretic activity of FIM support the use of the *Flacourtis indica* in traditional medicine. .

Keywords: Flacourtia indica, antinociceptive, anti-inflammatory, diuretic, mice, rat

Introduction

Herbal medicines hold a long history of therapeutic success. Many of the clinically used medicines are derived directly or indirectly from plants (De Smet, 1997). According to World Health Organization (WHO), about 80% of the population in some Asian and African countries depend on traditional medicine for primary health care. WHO also demonstrated that herbal preparations are the most popular form of traditional medicines in many developed countries (http://www.who.int/mediacentre/factsheets/fs134/en).

Herbs contain numerous secondary metabolites that are effective in the treatment and/or prevention of various chronic diseases, such as heart diseases (Vasanthi and Parameswari, 2010), diabetes (Chang *et al*, 2013), infectious diseases (Lin and Huang, 2009) and cancer (You *et al*, 2013). A vast wealth of information regarding the use of herbs and spices in traditional medicines has been recorded in early literature, for example, the Ayurveda. Although these traditional plant-based medicines have gained clinical success in the most cases, there have been many reports of health hazards upon use of herbal medication (Bauer, 2000). Standardization of herbal medicines through systematic evaluation of the biological as well as chemical characterization of medicinally important herbs and spices are essential steps in drug development. Many of the medicinal herbs are still being used in traditional therapy without being examined for their claimed therapeutic benefits. Thus, focus has been given to the screening of pharmacological activities of medicinal herbs for their appropriate use in the prevention and therapy of diseases.

Flacourtia indica (Burm F) Merr (Family-Flacourtiaceae; Bengali Name - Baichi) is a shrub available in the rural areas of Bangladesh. The plant has a very good folkloric reputation and is used as indigenous medicine for various purposes: fruits are used in the treatment of jaundice and enlarged spleen, seeds are used with turmeric to prevent rheumatic pain, bark is applied to the body during intermittent fever, and the root is used in the nephritic colic (Kirtikar and Basu, 1980). In the Comoros Islands, the aerial parts of the plant are used in traditional medicine to treat malaria (Kaou *et al*, 2010). Previous phytochemical studies on *F. indica* reported the isolation of phenolic glycosides, such as flacourtin (3-hydroxy-4-hydroxymethyl phenyl-6-O-benzyl-β-D-glucopyranoside) (Bhaumik *et al*, 1987). Madan *et al.* (2009) further performed phytochemical analysis of this plant and isolated several other phenolic glycosides with promising free radical scavenging activity. Kaou and colleagues (2010)

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isolated pyrocatechol, homaloside D and poliothrysoside from *F. indica*. Of these compounds, poliothrysoside showed strong antiplasmodial activity against *Plasmodium falciparum.*

In a recent study, Sashidhara *et al*. (2013) isolated several phenolic glycosides and mururin-A. The latter compound showed inhibitory effect on the growth of both chloroquine-sensitive and -resistant strains of *P. falciparum*. Methanolic extract of leaves (Tyagi *et al,* 2010) or fruit peel (Ndhlala *et al*, 2008) exhibited antioxidant properties. We have previously reported the isolation of scoparone, *trans*-cinnamic acid, *p*-hydroxybenzoic acid and β-sitosterol from this plant (Nazneen *et al*, 2002). Our study revealed that the petroleum ether, ethylacetate and methanol extracts of the whole plant parts inhibited *agrobacterium tumifaciencs*-induced crown gall tumor formation (Nazneen *et al*, 2002), and paracetamol-induced hepatotoxicity in rats (Nazneen *et al,* 2009).

In continuation of our work on the pharmacological characterization of different medicinal plants of Bangladesh, the present study has been designed to investigate the anti-inflammatory, analgesic and diuretic activity of the methanolic extract of *F. indica*.

Materials and Methods

Preparation of plant materials

The whole plant part(s) of *Flacourtia indica* was collected from Sripur of Gazipur district of Bangladesh and a voucher specimen has been deposited in the Herbarium of Department of Botany, University of Dhaka, Bangladesh. The plant was sun-dried for fifteen days and then pulverized. The coarse powder (700 g) was successively extracted with *n*-hexane, ethylacetate and methanol by cold extraction process.

All the extracts obtained were filtered off and evaporated to dryness *in vacuuo* at low temperature and reduced pressure by rotary evaporator. The bioactivity of only the methanol extract was investigated in the present study. The methanol extract of *F. indica* (FIM) was made into a suspension by using tween-80 as the suspending agent.

Experimental Animals

Swiss albino mice (20-25 g) and Long Evans rats (140-160 g) of either sex were obtained from the animal house of International Center for Diarrheal Disease and Research, Bangladesh (ICDDR,B). The mice were divided into six groups and the rats into five groups of five animals in each group. The animals were given standard animal feed developed by ICDDR,B and water *ad libitum* and kept in the laboratory environment for seven days. Animals were fasted overnight and weighed before the experiment.

Acetic acid-induced writhing assay

Swiss albino mice (6-8 weeks) weighing between 20 to 25 g were used to study the analgesic activity by recording acetic acid-induced writhing reflex according to the method described previously (Saha *et al*, 2005). Four groups of mice, each consisting of five, were taken. FIM was administered by gavage at a dose of 125 or 250 mg/kg body weight to two groups of mice. While one group received 0.1% tween-80 containing saline and served as control, a fourth group was treated with standard drug aminopyrene (50 mg/kg body weight). One hour after drug treatment, acetic acid (0.7%, 0.1 ml/10 g body weight) was administered intraperitoneally to all mice. After 10 min interval, the number of writhing was recorded for 10 minutes. The percent inhibition of writhing was calculated using the formula,

$$\text{Percent inhibition of writhing} = (1-W_F/W_C) \times 100$$

where, W_C and W_F represent the average number of writhing produced by the control and FIM-treated groups, respectively.

Carrageenan-induced rat paw edema assay

The effect of FIM on carrageenan (1%) induced inflammation in rat paw was investigated by following the method of Winter *et al* (1962) with minor modifications (Saha *et al*, 2005). Rats were randomly divided into four groups, each consisting of five animals, of which two groups were given FIM at 125 or 250 mg/kg body weight by gavage. One group of rats was treated with the standard drug phenylbutazone (100 mg/kg body weight), while the other group received saline with 0.1% tween-80 as the control group.

One hour after the administration of FIM or phenylbutazone, 1% carrageenan solution was injected to planter region of the right hind paw of each animal. The volume of paw edema was measured at indicated time intervals. For the measurement of paw volume, the inflamed paw was immersed into mercury-filled U-tube, which consists of a right cylindrical glass tube (8.0 cm X 2.2 cm) connected to a narrow side arm (10.0 cm X 0.72 cm) having a wall of uniform cross section and open upper end. The volume of mercury displaced was recorded by using traveling microscope (ELFO Scientific Apparatus, India). Prior to immersion into mercury, each of the inflamed right hind paw was labeled with permanent ink to ensure uniformity in the measurement of paw volume. The average percent increase in paw volume with time was calculated and compared against the control group.

Percent inhibition was calculated using the formula-

$$\%\ \text{inhibition of paw edema} = [(Vc- Vt)/Vc] \times 100$$

Where Vc and Vt represent average paw volume of control and FIM-treated animals, respectively.

Cell culture, UVB irradiation, and preparation of cell lysate

The immortalized human keratinocyte cell line HaCaT was obtained from American Type Culture Collections (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. In all the experiments, cells were seeded at 2×10^5 cells/ml and used for experiment at 50-60% confluence. The UVB light source was a 5*8 Watt tube, which emits an energy spectrum with high fluency in the UVB region (with a peak at 312 nm). Cells were incubated with FIM (25 or 50 μg/ml; in DMSO) for 30 min, and then media was replaced with phosphate-buffered saline (PBS) immediately before irradiation with UVB (50 mJ/cm^2) at

312 nm using the UVB chamber (Biolink BLX-312 UV crosslinker, Vilbert Lourmat, Marne-La Vallee, France). Upon exposure to UVB radiation, cells were further suspended in culture media and incubated in the presence of FIM (25 or 50 µg/ml) for additional four hours. Cells were then harvested and lysed with RIPA cell lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecylsulphate (SDS), 1% Triton X-100, 1% deoxycholate and 5 mM ethylene diaminetetraacetic acid (EDTA)] enriched with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), followed by centrifugation at 14,000 rpm for 15 min. The supernatant was collected as whole cell lysate and the protein concentration was measured by the BCA protein assay kit. Cell lysates were stored at -70 °C for biochemical assays.

Western blot analysis

The expression of inflammatory markers in UVB-irradiated HaCaT cells and its modulation by FIM were examined by Western blot analysis following the protocol described elsewhere (Kundu *et al,* 2008). Cell lysate (30-50 µg protein) was boiled in sodium dodecylsulfate (SDS) sample buffer for 5 min before electrophoresis on 10-12% SDS–polyacrylamide gel (PAGE). After transfer to polyvinylidene difluoride membrane (Gelman Laboratory, Ann Arbor, MI), the blots were blocked with 5% fat-free dry milk–PBST (phosphate-buffered saline containing 0.1% Tween 20) buffer for 1 h at room temperature. The membranes were incubated for 4 h at room temperature with 1:1000 dilution of primary antibodies for cyclooxygenase-2 (COX-2) (Cayman Chemical Co. Ann Harbor, MI, USA), pERK and ERK (SantaCruz Biotechnology, CA, USA), pAkt, Akt (Cell Signaling Technology Inc., Beverly, MA, USA) and β-actin (Sigma Chemical Co., St. Luois, MO, USA). Blots were washed three times with PBST at 5 min intervals followed by incubation with 1:5000 dilution of respective horseradish peroxidase-conjugated secondary antibodies (rabbit, or mouse) for 1 h at room temperature, and again washed in PBST for three times. The transferred proteins were visualized with an ECL detection kit according to the manufacturer's instructions.

Screening of diuretic activity

The diuretic activity of FIM was studied in *Swiss albino* mice following the method of Gujral *et al.* (1955) with minor modifications. Animals were divided into five groups, each containing five mice. Group I was provided only with saline solution containing 0.1% tween-80 i.e. control group. Group II was given urea at a dose of 500 mg/kg body weight and was considered as positive control group. Group III was provided with standard diuretic drug furosemide at a dose of 3 mg/kg body weight *per oral*. Mice in group IV and V were treated with FIM at doses of 125 and 250 mg/kg body weight by gavage, respectively. Animals were placed in to metabolic cages 24 h prior to the experiment. The urinary output of each group was recorded at different time intervals from the graduated urine chamber of metabolic cages. The volume of urine excreted during 4 h of study by each group was expressed as percent of the liquid administered giving rise to a measure of urinary excretion (U.E.).

U.E.= (Total urinary output/ Total liquid administered) x 100

The ratio of urinary excretion (U.E.) in test group and control group was denoted as diuretic action, which was used as the measure of degree of diuresis.

Diuretic action = U.E. in test group/ U.E. in control group

Diuretic activity= Diuretic action of drug / Diuretic action of urea

Statistical analysis

Data are expressed as means ± SEM. Statistical significance of changes was determined by the Student's *t*-test. Differences resulting in p values < 0.05 were considered to be statistically significant.

Results and Discussion

The antinociceptive, anti-inflammatory and diuretic activity of FIM obtained by successive cold extraction of coarse powder of whole plant parts of *F. indica* with *n*-hexane, ethylacetate and methanol was evaluated in this study. The effect of FIM on acetic acid-induced writhing was compared to that of aminopyrine (Table 1). FIM at a dose of 125 mg/kg body weight inhibited acetic-acid-induced writhing by 29.78% ($p < 0.001$), while the extract suppressed the writhing reflex by 44.12% ($p < 0.001$) at a dose of 250 mg/kg body weight. The inhibition of acetic acid-induced writhing by FIM was less than that observed with standard drug aminopyrene (66.18%, $p < 0.001$). The correlation co-efficient value 0.98 indicates that FIM showed significant analgesic activity in a dose-dependent manner. Acetic acid (0.7%) is a pain stimulus, which produces localized inflammation by releasing arachidonic acid from membrane phospholipids through the action of phospholipase A2 and other acyl hydrolases (Koster *et al,* 1959). The released arachidonic acid is metabolized by COX enzymes to produce prostaglandins (PGs), especially PGE_2, which produces pain sensation (Gyires and Knoll, 1975). Acetic acid, by stimulating this peripheral mechanism of pain perception, causes writhing movement. Substances that reduce the number of writhing function as analgesic agents by inhibiting the prostaglandin synthesis. Since increased prostaglandins release causes local tissue inflammation, we then examined the effect of FIM on carrageenan-induced rat paw edema formation.

The effect of FIM on carrageenan-induced rat paw edema was compared to that of control for the evaluation of anti-inflammatory activity on the basis of percent inhibition of paw edema volume. FIM at doses of 125 or 250 mg/kg body weight showed statistically significant inhibition of rat paw inflammation at 1, 2, 3 and 4 h of carrageenan administration. The maximum inhibition (41.09% and 45.00% with 125 and 250 mg/kg dose, respectively) of paw edema was noted at 3 h of carrageenan challenge, though FIM elicited significant inhibition of paw volume (20.21% and 21.97% with 125 and 250 mg/kg dose, respectively) at the 1st h of the study. The suppressive effect of FIM on rat paw inflammation was comparable to that of standard drug phenylbutazone (46.27 % at 3rd h, p <0.001) given *p.o.* at a dose of 100 mg/kg body weight (Table 2).

Table 1:
Flacourtia indica (FIM) inhibits acetic acid-induced writhing in mice

Treatment	Dose (mg/kg, *p. o.*)	No. of writhing Mean ± SE	*t* Value	Inhibition (%) of writhing reflex
Control	-	27.20 ± 0.60	----	----
FIM	125	19.10 ± 0.80*	8.100	29.78
FIM	250	15.20 ± 0.98*	10.405	44.12
Aminopyrine	50	09.20 ± 0.68*	19.757	66.18

* *p <0.001 ,* vs. control; Correlation coefficient (r) = 0.98.

Table 2:
Inhibitory effects of FIM on carrageenan-induced rat paw inflammation

Group	Dose	Edema volume (µl) (% inhibition)				
	(mg/kg, *p.o.*)	1 h	2 h	3 h	4 h	24 h
Control	-----	73.44 ± 0.90	93.50 ± 1.36	107.32 ± 1.62	116.56 ± 1.56	62.84 ± 1.79
FIM	125	58.60 ± 1.12	59.54 ± 0.58	63.22 ± 1.13	72.64 ± 2.18	55.80 ± 1.25
		(20.21) [a]	(36.32) [a]	(41.09) [a]	(37.68) [a]	(11.20) [c]
FIM	250	57.30 ± 1.33	57.70 ± 1.22	59.02 ± 0.80	69.44 ± 1.40	54.60 ± 1.12
		(21.97) [a]	(38.29) [a]	(45.00) [a]	(40.43) [a]	(13.11) [b]
PBZ	100	56.04 ± 1.10	57.00 ± 1.03	57.66 ± 1.53	67.40 ± 1.74	52.98 ± 0.90
		(23.69) [a]	(39.04) [a]	(46.27) [a]	(42.18) [a]	(15.69) [b]

The initial paw volume of rat was determined volumetrically. Each point represents the mean ± SEM of five rats. [a-c] Probability values (calculated as compared to control using student's t-test): a <0.001, b <0.01, [c] < 0.05.

The carrageenan-induced rat paw edema model is known to be sensitive to COX inhibitors and has been used to evaluate the effect of non-steroidal anti-inflammatory agents, which primarily inhibit the COXs involved in prostaglandin synthesis (Appleton *et al,* 1995, Seibert *et al,* 1994). The time kinetics of carrageenan-induced paw edema development in rats is represented by a biphasic curve. The first phase of inflammation, which occurs within an hour of carrageenan injection, is partly due to the trauma of injection and to the release of histamine and serotonin (Crunkhorn and Meacock, 1971). The second phase of inflammatory reaction that occurs after 3 h of carrageenan administration is largely contributed by PGs (Di Rosa and Willoughby, 1971). The presence of PGE_2 in the inflammatory exudates from the injected foot can be observed at 3 h and period thereafter (Vinegar *et al,* 1969). Therefore, the inhibitory effect of FIM on carrageenan-induced inflammation may result from the possible inhibition of the release of histamine or the expression and/or the activity of COX-2, a rate limiting enzyme that regulates the biosynthesis of PGE_2. We have previously isolated a coumarin compound scoparone from this plant (Nazneen *et al,* 2002). Scoparone elicited antiallergic effects in a mast cell-mediated allergy model (Choi and Yan, 2009). Thus, the observed anti-inflammatory effect of FIM may be due to scoparone present in this extract.

Since the exposure to UVB radiation induces strong inflammatory reaction in human skin, we examined the effect of FIM on UVB radiation-induced expression of COX-2 in human keratinocyte HaCaT cells. As shown in Fig. 1A, incubation of cells with FIM (25 or 50 µg/ml) markedly reduced UVB-induced COX-2 expression. Treatment with FIM at these concentrations neither induced cytotoxicity in HaCaT cells, nor increased COX-2 expression by itself (data not shown). Since UVB radiation-induced COX-2 expression is mediated through activation of several upstream kinases, such as mitogen-activated protein kinases (MAPK) and Akt in mouse skin (Bachelor *et al,* 2005), we examined the effect of FIM on UVB-induced activation of MAPKs and Akt. Our study showed that treatment with FIM attenuated UVB-induced phosphorylation of ERK (Fig. 1B) and that of Akt (Fig. 1C).

However, it is yet to be investigated whether FIM can also modulate UVB-induced activation of other members of MAPK family, such as p38 MAPK and c-Jun-N-terminal kinase. Moreover, the transcriptional activation of COX-2 in response to UVB irradiation is regulated by several transcription factors, such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) (Chun and Surh, 2004).

It would be worthwhile to examine if FIM-mediated suppression of UVB-induced COX-2 expression in HaCaT cells results from its possible inhibition of any of these transcription factors. However, the result of the present study that FIM attenuated UVB-induced COX-2 expression is well correlated with the finding that the extract diminished second phase of rat paw inflammation after 3rd hour of carrageenan administration. Jang *et al.* (2005) demonstrated that scoparone, a compound present in *F. indica* (Nazneen *et al.* 2002), inhibited lipopolysaccharide-induced expression of COX-2 and inducible nitric oxide synthase, two representative pro-inflammatory enzymes, in murine RAW264.7 macrophages.

We then attempted to validate the claim of the folkloric use of the plant *F. indica* as a diuretic therapy. The effect of

FIM on urination was investigated in mice. The urinary output at different hours of study has been presented in

Table-3:
Effect of FIM on the urine volume in mice

Group	Dose	Urine volume (ml)			
	(mg/kg, *p.o.*)	1 h	2 h	3 h	4 h
Control	----	0.6	1.1	2.4	2.8
Urea	500	0.8	4.0	4.5	4.5
Furosemide	3	2.0	4.0	5.5	7.0
FIM	125	1.5	2.3	2.8	4.7
FIM	250	2.2	3.1	4.2	4.6

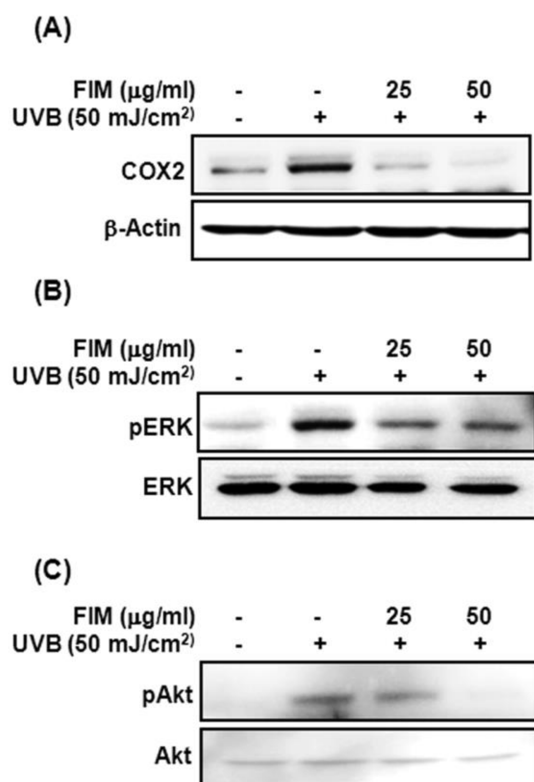

Figure 1.
Effect of FIM on UVB-induced expression of COX-2 and phosphorylation of ERK and Akt in HaCaT cells. Cells were incubated with FIM at indicated concentrations and irradiated with UVB radiation as described in Materials and methods. Cell lysate was subjected to Western blot analysis to detect the expression level of (A) COX-2, (B) pERK and (C) pAkt. Data are representative of three independent experiments showing similar pattern.

Results of the experiment revealed that FIM exhibited good diuretic activity, which is comparable to that of standard drug furosemide (Fig. 2). When administered at doses of 125 or 250 mg/kg body weight, FIM exhibited good diuretic activity, 1.88 and 2.76, respectively, at the first hour of study, indicating a rapid onset of diuretic activity by FIM. According to Gujral *et al* (1955), the diuretic activity of a drug is considered to be good if it is above 1.50, moderate if it is within 1.00 ~1.50, and little if it is between 0.72~1.00. Thus, our study revealed that FIM can promote diuresis, which justifies the traditional use of the plant as a diuretic remedy. The elucidation of mode of diuretic action, which may be the effect of FIM either on loop permeability or the reduction of antidiuretic hormone secretion or inhibition of carbonic anhydrase enzyme (Goodman and Gillman, 1975), needs to be examined in future study. Nonetheless, the observed diuretic property of FIM would justify the traditional use of the plant in nephritic colic and cardiovascular diseases (Kritikar and Basu, 1980).

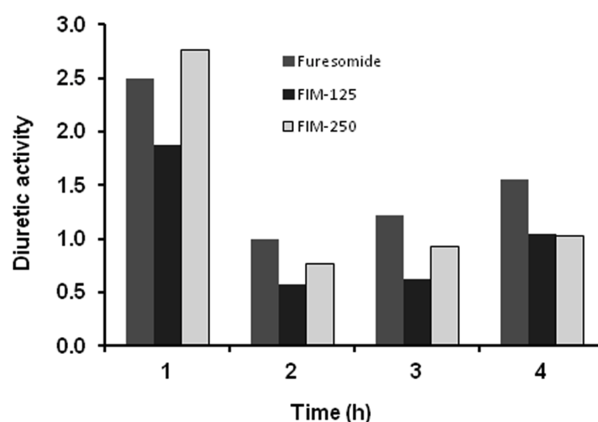

Figure 2. Diuretic activity of FIM. Mice (n = 5 per treatment group) were treated with FIM (125 or 250 mg/kg body weight) or furosemide or urea. Control animals received 0.1% tween-80 containing saline. Hourly urine volume was recorded and the diuretic activity was calculated according to the formula described in Materials and methods

In conclusion, the present study demonstrates the preliminary pharmacological activity of the methanolic extract of *F. indica*. Although our study provides the first time report that FIM can attenuate UVB radiation-induced COX-2 expression and activation of upstream kinases, such as Akt and ERK in cultured human keratinocytes, inhibit inflammation and pain perception, and induces diuresis in animal models, it is yet to be known that which of the constituents present in FIM is actually responsible for these biological activities. Thus, further experiments on the bioactivity-guided isolation of principal bioactive constituent(s) from FIM and extended biochemical studies would establish this medicinal plant or its active constituents in therapy.

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Conflict of interest
Authors declare that no conflict of interest exists.

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