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# SUPPRESSIVE EFFECTS OF *MIMOSA PUDICA* (L.) CONSTITUENTS ON THE PRODUCTION OF LPS-INDUCED PRO-INFLAMMATORY MEDIATORS

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## ABSTRACT

The present study deals with the isolation of fourteen compounds from the active ethyl acetate (MPE) extract of *M. pudica* (L.) whole plant and their subsequent evaluation for the nitric oxide (NO), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) inhibitory activities in lipopolysaccharide (LPS) stimulated RAW 264.7 and J774A.1 cells. Among the tested compounds, L-mimosine (**12**; $IC_{50}$ = 19.23 to 21.15 μM)**,** crocetin (**4**; $IC_{50}$ = 23.45 to 25.57 μM)**,** crocin (**14**; $IC_{50}$ = 27.16 to 31.53 μM) and jasmonic acid (**11**; $IC_{50}$ = 21.32 to 29.42 μM) were identified as potent NO inhibitor when tested on the macrophages. Similarly, towards TNF-α and IL-1β inhibition, including these four compounds, and ethyl gallate (**3**)**,** gallic acid (**10**) and caffeic acid (**7**) were found to be more active with half maximal concentration, 17.32 to 62.32 μM whereas the other compounds depicted moderate and mild effects ($IC_{50}$ = 59.32 to 95.01 μM). Also, at a dose of 40 mg/Kg, L-mimosine (**12**), jasmonic acid (**11**), crocin (**14**) and its de-esterified form, crocetin (**4**) were found to significantly ($p$ < 0.05 and 0.001) reduce 60.7 %, 48.9 %, 48.4 % and 43.6 % respectively of TNF-α production in female Sprague Dawley rats. However, in case of IL-1β, with the same dose (40 mg/Kg), jasmonic acid (**11**) exhibited significant reduction with 54.2 % followed by crocin (**14**) (50.2 %) and crocetin (**4**) (39.8 %) while L-mimosine (**12**) was found to reduce only 16.3 %. Based on the results, it can be estimated that these compounds imparting greatly to anti-inflammatory effects of *M. pudica in vitro* as well as *in vivo* through reduction of LPS-induced pro-inflammatory mediators which affirm the ethno-pharmacological use of this plant for prevention of inflammatory-related disorders.

**Keywords:** *Mimosa pudica*, L-mimosine, crocin, crocetin, jasmonic acid, inflammation

## INTRODUCTION

Many pathological processes such as inflammation, necrosis, fibrosis and apoptosis normally required the cytokines. Binding of Gram negative bacterial endotoxin or lipopolysaccharide (LPS) to CD14 (a protein) initiates an inflammatory cytokine and other mediators which in turn, act on neutrophils, macrophages, endothelial cells and Kupffer cell to produce an array of pro-inflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6)

and nitric oxide (NO) (Good et al., 1998). Serious side effects of the approved synthetic antibodies (etanercept, infliximab and adalimumab) for inflammatory conditions providing impetus to alternative small molecule based therapies towards TNF-α inhibition (Palladino et al., 2003).

*Mimosa pudica* L. (Fabaceae) reported to have anti-diabetic (Marles and Farnsworth, 1995), hyperglycemic (Amalraj and Ignacimuthu, 2002), antidepressant (Molina et al., 1999), anticonvulsant (Bum et al., 2004), acetylcholinestrase inhibitory (Mukherjee et al., 2007), anti-ophidian, anti-venom (Mahanta and Mukherjee, 2001; Girish et al., 2004; Ambikabothy et al., 2011), wound healing (Kokane et al., 2009) and antifertility (Ganguly et al., 2007) activities.

In the continuous search for newer anti-inflammatory leads from natural products, many plant extracts, isolated compounds or their derivatives have been tested for their inhibitory effects towards pro-inflammatory cytokines (Patel et al., 2014; Patel and Bhutani, 2014; Bhandari et al., 2014a; b). Previously, we demonstrated that ethyl acetate extract (MPE) of *M. pudica* whole plant have significant inhibition of NO, TNF-α and IL-1β production in RAW 264.7 cells with an $IC_{50}$ of 34.4, 31.7 and 47.2 μg/mL respectively (Patel et al., 2014). Hence, the present study was carried out to isolate and characterize compounds from standardized MPE and then subsequent evaluated for NO, TNF-α and IL-1β inhibitory effects on RAW 264.7 and J774A.1 cells.

## MATERIALS AND METHODS

### Plant material

Whole plant of *Mimosa pudica* (L.) were collected from the region of Jabalpur, Madhya Pradesh, India in January, 2011 and authenticated by Dr. A. S. Sandhu (botanist) of the Department of Natural products (DNP), National Institute of Pharmaceutical Education and Research (NIPER) and a voucher specimen (NIP-NPM-CD-019) was deposited in the herbarium of DNP, NIPER, SAS Nagar, Punjab, India. The plant materials were shade dried for 10 days followed by pulverization and sieving (20-30 mesh).

### Instruments

Ultraviolet (UV) and Mass spectra were taken on a DU® 7400 spectrophotometer (Beckman, München, Germany) and mass spectrometer (Thermo Quest Finnigan, San Jose, CA, USA) respectively. [1]H and [13]C Nuclear magnetic resonance (NMR) spectra were recorded on Ultrashield 400 MHz and 100 MHz spectrometer respectively (Bruker DPX, Faellanden, Germany). High performance Liquid chromatography (HPLC) analysis for compound analysis was carried out on Novapak $C_{18}$ column (5 μm, 4.6 x 250 mm). Ultracentrifuge (Sigma, St. Louis, MO, USA), $CO_2$ incubator (WTC Binder, Tuttlingen, Germany), Biosafety cabinet (Clean air, Chennai, India), autopipettes, ELISA plate reader (Labsystems, Helsinki, Finland) and Neubauer chamber (HBG, Gießen, Germany) were used for the cell culture. All extracts and fractions were concentrated using a vacuum rotary evaporator (Buchi R-210, Flawil, Switzerland).

### Chemicals and reagents

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), phosphate buffered saline (PBS), antibiotic solution and other chemicals were purchased from Hi-media Limited (Mumbai, India). Silica gel (# 100-200 and # 230-400) from Loba Chemie (Mumbai, India), Diaion HP-20® resin from Supelco analytical (USA), Sephadex LH-20 from Amersham Pharmacia Biotech and GE Healthcare (USA), Polyamide 6 and $RP_{18}$ column from Sigma Chemical Co. (St. Louis, MO, USA) were used for column chromatography. Mouse and rat TNF-α and IL-1β ELISA kits were obtained from Krishgen Biosystems (Mumbai, India). Thin layer chromatography (TLC) plates pre-coated with silica gel 60 $F_{254}$ thickness 0.2 mm and solvents (laboratory grade) were received from Merck (Darmstadt, Germany). Dexa-

methasone, Lipopolysaccharide (*Escherichia coli* 026:B6) (LPS), dimethyl sulfoxide (DMSO), curcumin, L-$N^G$-Nitroarginine methyl ester (L-NAME), modified Griess reagent, L-mimosine, jasmonic acid and crocin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). For *in vivo* study, L-mimosine, jasmonic acid and crocin were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and crocetin was prepared by ester hydrolysis of crocin.

### Extraction and isolation

Dried and pulverized whole plant (3.2 kg, 20-30 mesh) subjected to sequential maceration with 6 L (3x) hexanes (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) for 72 h at 25 °C to yield hexanes (MPH, 15 g), dichloromethane (MPD, 11 g), ethyl acetate (MPE, 38 g) and methanol (MPM, 17 g) extracts respectively. Vacuum liquid chromatography (VLC) of MPE (37 g) over silica gel (230-400 mesh) column chromatography (CC) was performed using a stepwise gradient of hexanes containing increasing amounts of EtOAc (from 0 % up to 100 %) and then finally washed with 50 % MeOH. On the basis of similar thin layer chromatography (TLC) profiles, five pooled fractions (Fr); F1 (5.2 g, 0-25 % EtOAc/Hex), F2 (8.6 g, 25-50 % EtOAc/Hex), F3 (5.1 g, 50-75 % EtOAc/Hex), F4 (5.9 g, 75-100 % EtOAc/Hex), F5 (10.5 g, 100 % EtOAc to 50 % MeOH/EtOAc) were obtained and they were further subjected to CC using Diaion resin HP-20 for removal of chlorophyll. Purification of MPE-F2 (8.6 g) with CC over silica gel (230-400 mesh) and then with sephadex LH-20 in MeOH provided **1**(19 mg), **2** (13 mg) and **3** (27 mg). Similarly, following the same procedure, **4** (26 mg), **5** (18 mg), **6** (30 mg) and **7**(22 mg) were obtained from MPE-F3 (5.1 g). MPE-F4 (5.9 g) when subjected to repeated sephadex LH-20 chromatography in MeOH yielded **8** (13 mg) and **9** (32 mg). Polar fraction MPE-F5 (10.5 g) was fractionated by Polyamide 6 CC using $H_2O$/MeOH gradient to provide four sub-fractions (MPE-F5a-d). **10** (12 mg), **11** (10 mg) and **12** (19 mg) were obtained from MPE-F5a-c after CC with repetitive sephadex LH-20 in MeOH. Similarly, MPE-F5d was subjected to Sephadex LH-20 and $RP_{18}$ CC to give **13** (13 mg) and **14** (45 mg). All the compounds were found to be 97-99 % when analyzed by HPLC.

### HPLC condition

HPLC analysis was carried out on a Waters 600 Controller equipped with Waters 996 photodiode array (PDA) detector using a Novapak $C_{18}$ column (4.6 × 250 mm, 5 μm) included with 717-Autosampler & Inline degasser. The mobile phase consisted of solvent A, 0.1 % trifluoroacetic acid in acetonitrile and B, 0.1 % trifluoroacetic acid in water and performed gradient elution. The flow rate was 1 mL/min and chromatogram extracted at 290 nm. The mobile phase consists of solvent A, acetonitrile (ACN) and B, 0.4 % formic acid in water. Gradient elution was carried out starting from 95 to 90 % of B in 5 min, from 90 to 85 % of B in 3 min, from 85 to 80 % of A in 2 min, from 80 to 78 % of B in 2 min, from 78 to 76 % of B in 1 min, from 76 to 74 % of B in 1 min, from 74 to 73 % of B in 2 min, from 73 to 72 % of B in 2 min, from 72 to 65 % of B in 1 min, from 65 to 50 % of B in 3 min and finally from 50 to 40 % of B in 2 min. Fully dried MPE (5 mg/mL *w/v*) and **1**, **2**, **3**, **9** and **10** (1 mg/mL *w/v*) were dissolved in HPLC grade MeOH and then filtered through 0.22 μm membrane filter.

### Cell culture

RAW 264.7 and J774A.1 cells were obtained from the National Centre for Cell Science (NCCS, Pune, India) and were cultured in 250 ml culture flasks containing DMEM media supplemented with heat inactivated 10 % FBS, 10,000 units/mL penicillin and 10 mg/mL streptomycin in 0.9 % saline, in a $CO_2$ incubator (5 % $CO_2$ in air) at 37 °C. Test samples were dissolved in DMSO/Milli-Q water and stored at 4 °C until pharmacological analysis.

## Cell viability

Cell viabilities for RAW 264.7 and J774A.1 cells were performed according to the method recently used by us (Patel and Bhutani, 2014). Briefly, 10,000 cells per well/200 μL were incubated with DMEM (10 % FBS) for 24 h with test samples and then cell viability was measured using the MTT assay.

## NO inhibitory assay

NO inhibitory assay was performed using the Griess reaction according to method recently used by us (Patel et al., 2014). A NO release in the culture medium was quantified using Griess reaction. Briefly, cells were pre-incubated with different concentrations of the test samples for 1 h, and then were stimulated with 1μg/mL of LPS (*Escherichia coli* 026:B6) in a 96 well microtitre plate. After incubation at 37 °C for 24 h in an atmosphere of 5 % $CO_2$ and 95 % humidity, 100 μL of modified Griess reagent was added to 100 μL supernatant. After 30 min of incubation, the optical density (OD) was measured by using a 96 well microtitre plate reader at 540 nm and compared with the standards used (curcumin and L-NAME).

## TNF-α and IL-1β assays

The production of cytokines in the supernatants was quantified by sandwich immunoassays using the protocol supplied by the manufacturer (Krishgen Biosystems, Mumbai, India) and also as previously described by us (Patel and Bhutani, 2014). Briefly, cells was subjected with different concentrations of the test samples for 1 h, and then induced with 1μg/mL of LPS (*Escherichia coli* 026:B6) in a 96 well microtitre plate. In case of TNF-α and IL-1β, the cells were further incubated for 6 h and 12 h, respectively. Cytokines levels were measured in supernatants following the instruction manual supplied in sandwich ELISA kits at 450 nm (Krishgen Biosystems, Mumbai, India) and compared with the standards used (curcumin and dexamethasone).

## Measurement of TNF-α and IL-1β production in plasma of SD rats

The experimental study was approved by the Institute Animal Ethics Committee (IAEC) under approval number IAEC/13/46 and performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) given on animal experimentation. Female Sprague Dawley (SD) rats (200-250 g, $n = 5$ in each group) were issued from the Central Animal Facility (CAF), NIPER and separated into three different groups (control, test and a positive control group) and were permitted to have free water and normal diet *ad libitum*. Cytokines (TNF-α and IL-1β) concentrations were determined as per the method previously described by us (Patel and Bhutani, 2014). Briefly, a control group received *p. o.* vehicle (10 % Tween 80), test group received MPE (50, 100 and 400 mg/Kg), L-mimosine (**12**), crocin (**14**), crocetin (**1**) and jasmonic acid (**11**) (20 and 40 mg/Kg each) and a positive control group received dexamethasone (5 mg/Kg) respectively at an interval of 12 h. LPS (5mg/Kg) was administered *i. p.* after 2 h of the final dose. After LPS administration, blood samples were collected later 2 h and 6 h in case of TNF-α and IL-1β respectively. Animals were monitored for any unusual behavior and signs of toxicity at treated doses of test samples. Sandwich ELISA kits specific for rats were used to determine the cytokines (TNF-α and IL-1β) levels in the plasma.

## Statistical analysis

The results are shown as the mean ± SD and all experiments were performed in triplicates. Statistical treatment was carried by One-Way ANOVA preceded by a Dunnett's post test (commercially available software SigmaStat 3.5). A level of $p < 0.05$ or $0.001$ was used as the criterion of statistical significance.

## RESULTS AND DISCUSSION

### Characterization of isolated compounds

Chemical investigation of MPE led to the isolation of fourteen compounds (Figure 1) namely crocetin (**4**), crocin (**14**) (Van Calsteren et al., 1997), 6-hydroxy flavone (**2**), 2'-hydroxy flavanone (**1**) (Freeman et al., 1981), *p*-hydroxy benzoic acid (**5**), *p*-coumaric acid (**6**) (Durust et al., 2001), chlorogenic acid (**8**) (Ahmad, 2010), jasmonic acid (**11**) (Husain et al., 1993) and L-mimosine (**12**) (Nokihara et al., 2012) which were identified and characterized by comparison of their physicochemical and spectral data with that reported in the literature. Caffeic acid (**7**), ethyl gallate (**3**), catechin (**9**), gallic acid (**10**), and tyrosine (**13**) were identified by co-TLC with the authentic standards available in our laboratory and MS spectrometry. Further, HPLC profile for EtOAc fraction of *Mimosa pudica* (L.) whole plant showed the presence of major compounds. The compounds **1**, **2**, **3**, **9**, and **10** eluted at retention time ($R_t$) 32.32, 30.76, 20.19, 17.99 and 5.88 corresponds to 2'-hydroxy flavanone, 6-hydroxy flavone, ethyl gallate, catechin and gallic acid respectively (Figure 2).

### Inhibitory effects of isolated compounds on NO and cell viability in macrophages

With respect to NO inhibition, in comparison to other tested compounds and also to nitric oxide synthase inhibitor (L-NAME), L-mimosine (**12**; $IC_{50} = 19.23 \pm 1.53$ and $21.15 \pm 1.36$ μM) a toxic non-protein free amino acid, crocetin (**4**; $IC_{50} = 23.45 \pm 2.62$ and $25.57 \pm 2.47$ μM) and its ester crocin (**14**; $IC_{50} = 27.16 \pm 4.51$ and $31.53 \pm 1.25$ μM) and jasmonic acid (**11**; $IC_{50} = 29.42 \pm 1.65$ and $21.32 \pm 1.35$ μM) a plant hormone, exhibited the prominent activity on RAW 264.7 cells and J774A.1 cells respectively. However, Caffeic acid (**7**) exhibited highest NO inhibition on RAW 264.7 cells ($IC_{50} = 19.20 \pm 4.97$ μM) while found to be merely active ($IC_{50} = 78.01 \pm 3.94$ μM) on J774A.1 cells. Compounds, 6-hydroxy flavone (**2**), 2'-hydroxy flavanone (**1**), *p*-coumaric acid (**6**) and ethyl gallate (**3**) were also demonstrated moderate and modest inhibition towards NO release with an $IC_{50}$ ranging from $42.31 \pm 3.15$ to $69.21 \pm 2.77$ μM on the both cells. Tyrosine (**13**) being a primary metabolite was excluded from the study. All the tested compounds were found to be non-cytotoxic except L-mimosine (**12**) when tested at maximum concentration of 100 μM (cell viability > 80 %) (Table 1A).

### Inhibitory effects of isolated compounds on pro-inflammatory cytokines in macrophages

We further screened the isolated compounds for their inhibitory potential against pro-inflammatory cytokines (TNF-α and IL-1β). As shown in Table 1(B), on RAW 264.7 cells and J774A.1 cells, TNF-α inhibition follows the order: ethyl gallate (**3**; $IC_{50} = 17.32 \pm 4.61$ and $23.45 \pm 2.67$ μM) and crocetin (**4**; $IC_{50} = 20.36 \pm 2.48$ and $22.21 \pm 3.83$ μM) > crocin (**14**; $IC_{50} = 22.41 \pm 1.08$ and $25.21 \pm 2.56$ μM) > gallic acid (**10**; $IC_{50} = 23.81 \pm 2.14$ and $29.33 \pm 3.12$ μM > L-mimosine (**12**; $IC_{50} = 25.24 \pm 2.48$ and $25.21 \pm 2.56$ μM > jasmonic acid (**11**; $IC_{50} = 39.42 \pm 2.85$ and $41.36 \pm 1.67$ μM) > caffeic acid (**7**; $IC_{50} = 45.01 \pm 1.93$ and $49.61 \pm 1.33$ μM) respectively whereas the other compounds depicted moderate and mild effects with half maximal concentration grading from $59.32 \pm 1.76$ to $89.34 \pm 1.38$ μM. With respect to IL-1β inhibition, except for L-mimosine (**12**), similar activity pattern was accompanied for these compounds; ethyl gallate (**3**; $IC_{50} = 36.92 \pm 3.35$ and $39.52 \pm 2.86$ μM) and crocin (**14**; $IC_{50} = 29.23 \pm 3.69$ and $31.15 \pm 2.64$ μM) followed by crocetin (**4**; $IC_{50} = 35.43 \pm 2.34$ and $41.15 \pm 1.69$ μM), gallic acid (**10**; $IC_{50} = 42.11 \pm 3.62$ and $47.64 \pm 1.67$ μM, L-mimosine (**12**; $IC_{50} = 64.23 \pm 2.53$ and $65.76 \pm 1.86$ μM, jasmonic acid (**11**; $IC_{50} = 57.92 \pm 0.61$ and $59.24 \pm 2.25$ μM), caffeic acid (**7**; $IC_{50} = 59.34 \pm 2.94$ and $62.32 \pm 2.94$ μM) were found to be more active on RAW 264.7 cells and J774A.1 cells respectively as compare to other tested compounds.
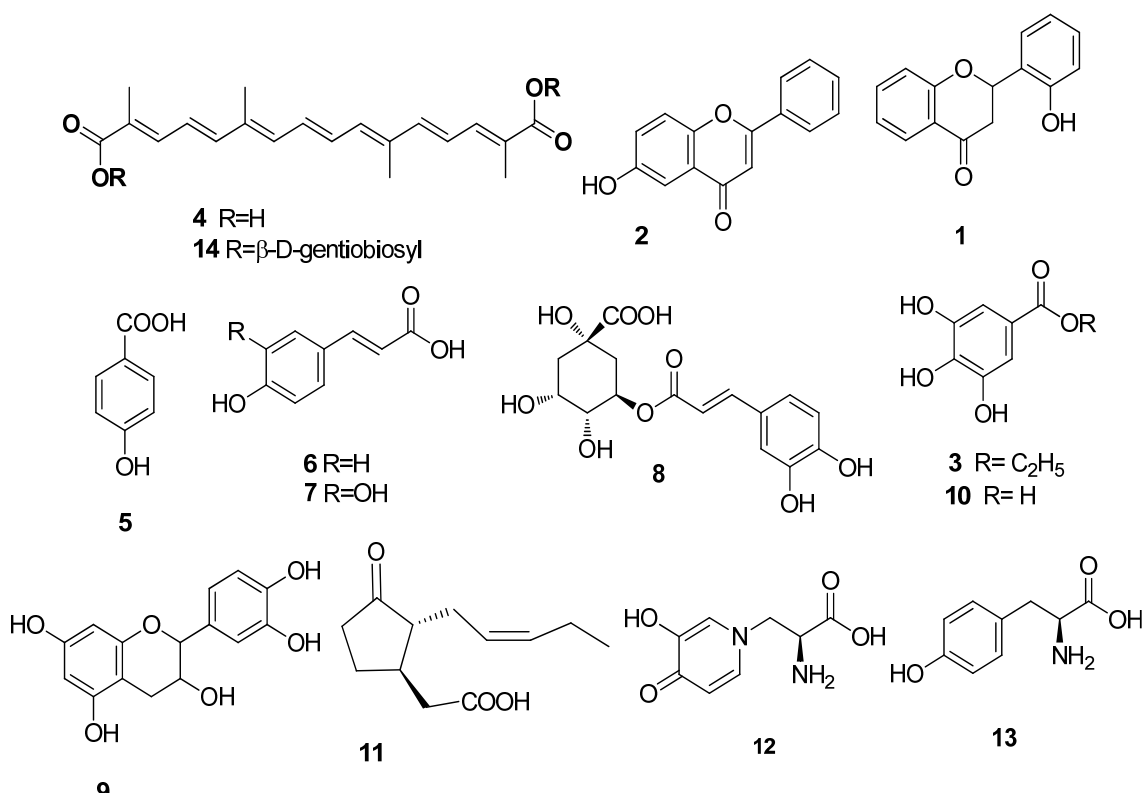
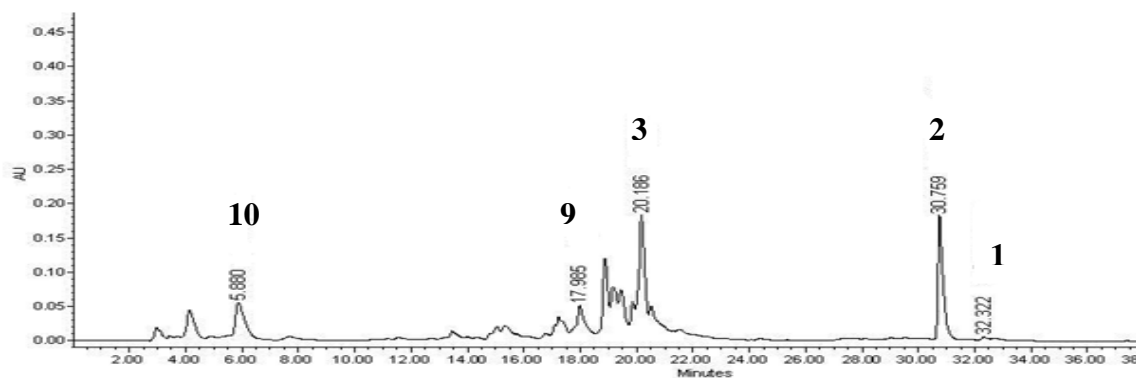**Figure 1:** Isolated compounds from MPE of *M. pudica* L. (whole plant)



**Figure 2:** HPLC profile for EtOAc fraction of *Mimosa pudica* L. leaves. The compounds **1**, **2**, **3**, **9**, and **10** eluted at retention time ($R_t$) 32.32, 30.76, 20.19, 17.99 and 5.88 corresponds to 2'-hydroxy flavanone, 6-hydroxy flavone, Ethyl gallate, Catechin and Gallic acid at 290 nm.

**Table 1A:** Cell viability and NO inhibitory effects of isolated compounds from *M. pudica*

| Compound | Cell viability[a] (% of control) | | IC$_{50}$ ( µM ) for NO inhibition | |
|---|---|---|---|---|
| | RAW 264.7 | J774A.1 | RAW 264.7 | J774A.1 |
| 2'-hydroxy flavanone (**1**) | 96.12 ± 3.23 | 91.14 ± 1.82 | 71.25 ± 2.41 | 74.06 ± 1.85 |
| 6-hydroxy flavone (**2**) | 92.33 ± 2.21 | 94.28 ± 3.36 | 61.15 ± 3.52 | 69.21 ± 2.77 |
| Ethyl gallate (**3**) | 94.12 ± 1.47 | 93.22 ± 2.35 | 67.20 ± 2.17 | 61.34 ± 2.34 |
| Crocetin (**4**) | 90.23 ± 1.01 | 92.84 ± 1.64 | 23.45 ± 2.62 | 25.57 ± 2.47 |
| *p*-hydroxy benzoic acid (**5**) | 81.34 ± 1.83 | 83.89 ± 3.71 | 43.11 ± 2.03 | 49.29 ± 1.72 |
| *p*-coumaric acid (**6**) | 94.62 ± 4.92 | 92.52 ± 1.14 | 42.31 ± 3.15 | 55.31 ± 2.17 |
| Caffeic acid (**7**) | 88.23 ± 1.35 | 87.47 ± 2.64 | 19.20 ± 4.97 | 78.01 ± 3.94 |
| Chlorogenic acid (**8**) | 87.23 ± 1.32 | 88.81 ± 1.34 | 82.45 ± 1.34 | 87.23 ± 2.56 |
| Catechin (**9**) | 95.31 ± 1.49 | 92.21 ± 3.21 | 98.81 ± 4.95 | 99.34 ± 2.38 |
| Gallic acid (**10**) | 91.13 ± 2.36 | 92.41 ± 3.75 | 78.81 ± 5.21 | 82.34 ± 1.46 |
| Jasmonic acid (**11**) | 83.19 ± 3.54 | 90.78 ± 1.48 | 29.42 ± 1.65 | 21.32 ± 1.35 |
| L-mimosine (**12**) | 73.24 ± 1.42 | 77.82 ± 2.81 | 19.23 ± 1.53 | 21.15 ± 1.36 |
| Crocin (**14**) | 92.32 ± 2.15 | 93.42 ± 1.43 | 27.16 ± 4.15 | 31.53 ± 1.25 |
| L-NAME[b] | - | - | 69.21 ± 1.31 | 73.18 ± 1.70 |
| Curcumin[b] | - | - | 11.02 ± 2.40 | 18.52 ± 2.89 |

Values are presented as mean ± S. D. of three different experiments in triplicates.
[a]Cell viability was measured at concentration of 100 µM.
[b]Standards used for the present study.


**Table 1B:** TNF-α and IL-1β inhibitory effects of isolated compounds from *M. pudica*

| Compound | IC$_{50}$ ( µM ) for inhibition of | | | |
|---|---|---|---|---|
| | TNF-α | | IL-1β | |
| | RAW 264.7 | J774A.1 | RAW 264.7 | J774A.1 |
| 2'-hydroxy flavanone (**1**) | 89.02 ± 1.42 | 84.30 ± 1.54 | 92.01 ± 1.32 | 95.01 ± 3.28 |
| 6-hydroxy flavone (**2**) | 71.12 ± 2.23 | 69.37 ± 2.57 | 75.31 ± 1.43 | 77.31 ± 2.37 |
| Ethyl gallate (**3**) | 17.32 ± 4.61 | 23.45± 2.67 | 36.92 ± 3.35 | 39.52 ± 2.86 |
| Crocetin (**4**) | 20.36 ± 2.48 | 22.21 ± 3.83 | 35.43 ± 2.34 | 41.15 ± 1.69 |
| *p*-hydroxy benzoic acid (**5**) | 81.31 ± 1.80 | 82.91 ± 1.83 | 90.81 ± 1.23 | 89.32 ± 1.73 |
| *p*- coumaric acid (**6**) | 59.32 ± 1.76 | 62.31 ± 2.43 | 67.15 ± 2.83 | 70.34 ± 1.42 |
| Caffeic acid (**7**) | 45.01 ± 1.93 | 49.61 ± 1.33 | 59.34 ± 2.94 | 62.32 ± 2.94 |
| Chlorogenic acid (**8**) | 67.91 ± 1.34 | 71.23 ± 1.45 | 78.21 ± 2.05 | 81.72 ± 1.76 |
| Catechin (**9**) | 81.48 ± 3.19 | 89.34 ± 1.38 | 83.73 ± 0.08 | 89.50 ± 2.83 |
| Gallic acid (**10**) | 23.81 ± 2.14 | 29.33 ± 3.12 | 42.11 ± 3.62 | 47.64 ± 1.67 |
| Jasmonic acid (**11**) | 39.42 ± 2.85 | 41.36 ± 1.67 | 57.92 ± 0.61 | 59.24 ± 2.25 |
| L-mimosine (**12**) | 25.24 ± 2.48 | 27.82 ± 1.18 | 64.23 ± 2.53 | 65.76 ± 1.86 |
| Crocin (**14**) | 22.41 ± 1.08 | 25.21 ± 2.56 | 29.23 ± 3.69 | 31.15 ± 2.64 |
| Dexamethasone[a] | 0.04 ± 3.10 | 0.42 ± 2.16 | 4.71 ± 1.52 | 22.95 ± 3.25 |
| Curcumin[a] | 3.41± 2.38 | 5.64± 0.25 | 17.51 ± 1.57 | 67.45 ± 1.31 |

Values are presented as mean ± S. D. of three different experiments in triplicates.
[a]Standards used for the present study.

***Inhibitory effects of crocetin (4), crocin (14), jasmonic acid (11) and L-mimosine (12) on pro-inflammatory cytokines in SD female rats***

In view of *in vitro* anti-inflammatory effects of crocetin (**4**), crocin (**14**), jasmonic acid (**11**) and L-mimosine (**12**), MPE and these compounds were further evaluated in female SD rats for *in vivo* inhibition of pro-inflammatory cytokines. Ethyl gallate (**3**) and gallic acid (**10**)) have also shown prominent inhibition of pro-inflammatory mediators *in vitro* (Table 1 B). However, these were already reported to have such activity in *in vivo* models and hence, excluded from the present *in vivo* study (Hsiang et al., 2013; Mehla et al., 2013). The subjection of LPS or/and test samples in animals was not found to cause any unnatural behavior and perniciousness at tested doses. As shown in Figure 3 (A & B) there is a substantial increase ($p < 0.001$) in the TNF-α (1623 pg/mL) and IL-1β (912 pg/mL) levels in the LPS induced groups with respect to the normal group (< 87 pg/mL). Dexamethasone (standard drug) at a dose of 5 mg/kg was found to suppress significantly ($p < 0.001$) TNF-α production by 75.8 % (394 pg/mL) and IL-1β production by 63.0 % (338 pg/mL) respectively. MPE at treated dose of 50, 100 and 400 mg/Kg manifested significant ($p < 0.05$ and 0.001) reduction of TNF-α levels by 18.4, 39.3 and 61.9 % and IL-1β levels by 10.5, 26.3 and 54.8 % respectively. Compounds, **4**, **14**, **11** and **12** were tested at two doses *viz.* 20 and 40 mg/Kg for pro-inflammatory cytokines inhibition. At dose of 40 mg/kg, L-mimosine (**12**) was found to be most active with significant ($p < 0.05$ and 0.001) reduction of TNF-α production by 60.7 % (639 pg/mL) preceded by jasmonic acid (**11**) 48.9 % (830 pg/mL), crocin (**14**) 48.4 % (836 pg/mL) and its de-esterified form, crocetin (**4**) 43.6 % (914 pg/mL). However, in case of IL-1β, with the same dose (40 mg/kg), jasmonic acid (**11**) exhibited significant reduction with 54.2 % (418 pg/mL) followed by crocin (**14**) (50.2 %; 456 pg/mL) and crocetin (**4**) (39.8 %; 549 pg/mL) while L-mimosine (**12**) was found to reduce only 16.3 % (763 pg/mL). Results indicated that these compounds imparting greatly to anti-inflammatory effects of MPE *in vitro* as well as *in vivo* through reduction of LPS-induced pro-inflammatory mediators.

Nam et al. (2010) showed that crocin (**14**) and crocetin (**4**) provide protection against neuroinflammation by reducing the production of LPS-induced NO release, TNF-α, IL-1β, and intracellular reactive oxygen species (ROS) in cultured rat brain microglial cells. Methyl jasmonate and its α-haloenone derivatives were also reported as anti-inflammatory agents (Dang et al., 2008). Yang et al. (2011) has reported the anti-asthmatic effects of *M. pudica* extract *via* suppression on airway inflammation associated with the regulation of IL-5 and IgE expression. Also, L-mimosine (**12**) depicted anti-inflammatory effect on TNF-α and IL-6 production in supernatant fluids of minced granulomas (Frydas et al., 2003). Recently, caffeic acid (**7**) present in Propolis demonstrated suppression of LPS-induced pro-inflammatory response by blocking nuclear factor-kappa B (NF-κB), c-Jun *N*-terminal kinase (JNK1/2) and p38 mitogen activated protein kinase (MAPK) activation in macrophages (Búfalo et al., 2013). Gallic acid (**10**) also reported to have inhibitory effects on LPS-induced inflammation in NF-κB transgenic mice (Hsiang et al., 2013). Taking the above findings together, it can be assumed that mostly crocetin (**4**) and crocin (**14**), jasmonic acid (**11**) and L-mimosine (**12**), caffeic acid (**7**), ethyl gallate (**3**) and gallic acid (**10**) present in the ethyl acetate fraction of whole plant of *M. pudica* imparted greatly to its anti-inflammatory effect *via* suppression of NO, TNF-α and IL-1β levels which may be partly due to their synergistic mechanisms.
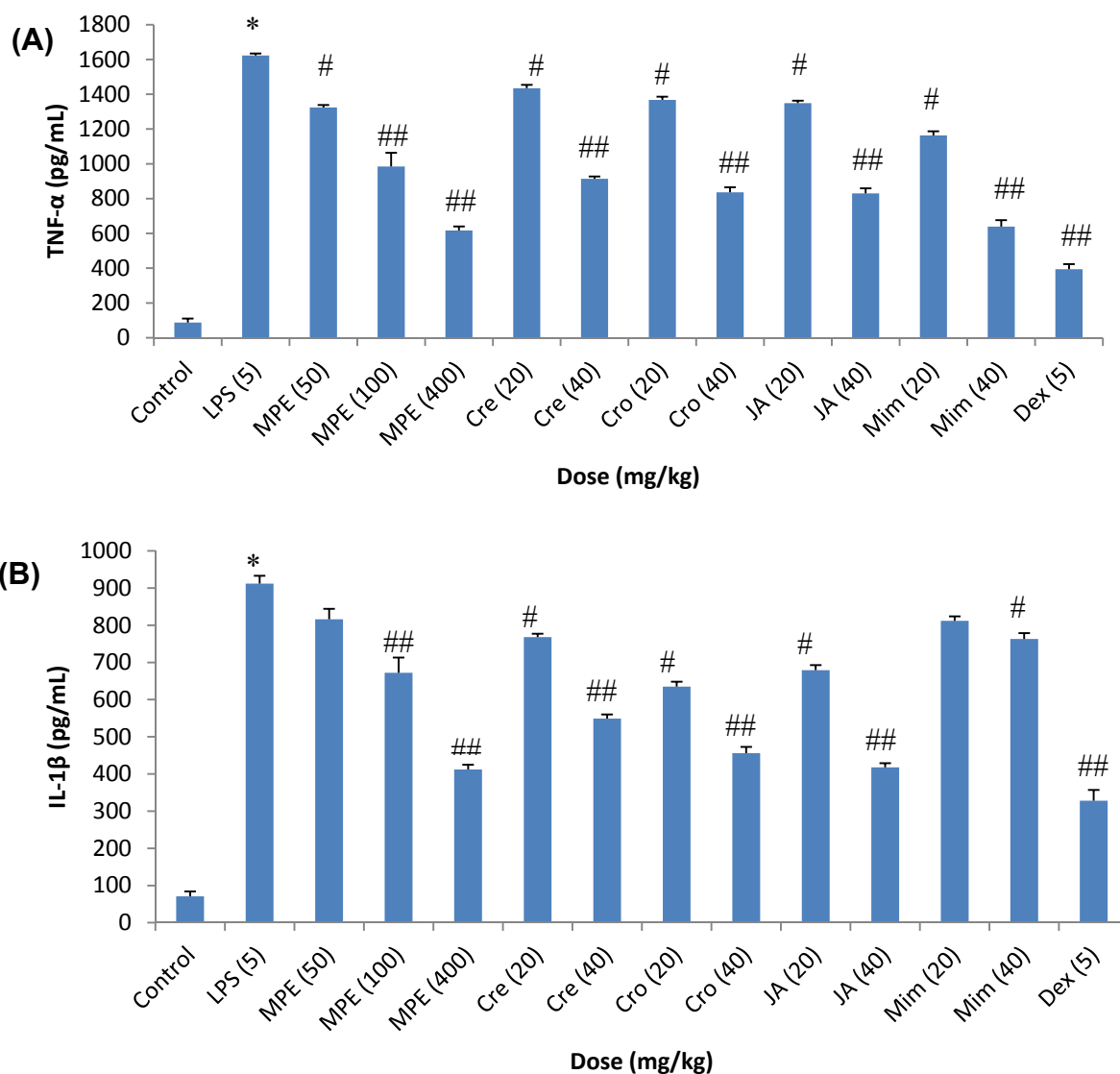
**Figure 3:** Inhibitory effects of tested samples on plasma levels of (**A**) TNF-α and (**B**) IL-1β in female SD rats {* ($p < 0.001$) = *vs* control; # ($p < 0.05$), ## ($p < 0.001$) = *vs* LPS}. Values are expressed as mean ± SD; *n* = 5 animals. The data was analyzed by One way of ANOVA test followed by Dunnett's test. LPS: lipopolysaccharide, MPE: ethyl acetate extract of *M. pudica*, Cre: crocetin (4), Cro: crocin (14), JA: jasmonic acid (11), Mim: L-mimosine (12), Dex: dexamethasone

In conclusion, the present investigation revealed the *in vitro* and *in vivo* anti-inflammatory effects of *M. pudica* through the suppression of pro-inflammatory cytokines and NO which affirm the ethnopharmacological use of this plant for prevention of inflammatory-related disorders.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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