SUPPLEMENTARY MATERIAL

Anti-inflammatory effect of methanolic extract of *Gmelina arborea* bark and its fractions against carrageenan induced paw edema in rats

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Abstract

Gmelina arborea has been traditionally used for treatment of abdominal pain, burning sensation and as stomachic and laxative. The anti-inflammatory effect of aqueous and methanol extracts of this plant has been studied but detailed investigations on anti-inflammatory activity of *G. arborea* stem bark are still not available. Therefore, the present study was designed to evaluate the anti-inflammatory activity of methanol extract and its fractions using carrageenan induced paw oedema model. Methanol extract at the dose of 500 mg/kg and its ethyl acetate fraction at 50 mg/kg showed significant reduction in paw oedema in comparison with standard drug diclofenac. Ethyl acetate fraction was further subjected to column chromatography which resulted in isolation of a new flavonoid (GM-01). Anti-inflammatory effect of methanol extract and its fractions can be attributed to the presence of the flavonoid GM-01. Further studies are in progress for evaluation of anti-inflammatory effect of GM-01 and identification of other active constituents present in ethyl acetate fraction.

Keywords: *Gmelina arborea*, flavonoid, anti-inflammatory.

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3. Experimental

3.1 *General*

The bark was obtained from Madurai district of Tamil Nadu in August 2011. The taxonomic identity of the plant was confirmed by Mr. Ram Prasad, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar and a voucher (# S.R.BotSci/96) has been deposited in department herbarium. Carrageenan (Sigma Chemicals, St. Louis, USA) and diclofenac sodium injection (Novartis India Ltd., Bombay) were used in the present study. All other solvents and reagents were of analytical grade. Melting point was determined with melting point apparatus. An IR spectrum was recorded using KBr pellets on Shimadzu 8400 S FT-IR Spectrophotometer. Mass spectra E_1 and ESI-methods were recorded on Shimadzu GCMS-QP-2000A. The ^{1}H-NMR was recorded on a 300 MHz FT-NMR and chemical shifts were reported in δ values using tetramethylsilane as internal standard.

3.2 *Preparation and fractionation of methanol extract*

Methanol extract was prepared by subjecting powdered stem bark to successive soxhlet extraction for not less than 48 hours using petroleum ether (60-80^{o}C), chloroform, methanol and water. Each extract was concentrated by distilling off the solvent using rotary evaporator and evaporated to dryness on the water-bath. Forty grams of the methanol extract was suspended uniformly in 100mL water and extracted with hexane. Further fractionation was done using ethyl acetate (3x150 mL) and butanol (3x150 mL). Ethyl acetate fraction (1g) was subjected to column chromatography using silica gel (# 60-120). A total of 300 fractions, each of 25 ml, were collected and pooled on the basis of TLC profile to get nine fractions F_1 –F_9. F_8 was eluted using mobile phase hexane: ethyl acetate (98:2) and it showed a single spot of yellow color on TLC which led to isolation of GM-01. Characterization of GM-01 was done by spectroscopic analysis which revealed that the phytoconstituent belongs to flavonoid family.

3.3 *Animal study*

Wistar rats (100-150 g) were obtained from Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. They were fed a standard rodent diet with water *ad libitum*. The protocol was duly approved by the Institutional Animal Ethics committee (16/1009/BT) of Guru Nanak Dev University, Amritsar. Carrageenan was used to induce inflammation (oedema) in paw. The linear paw circumference was measured at hourly interval for 4 h using digital vernier calliper. The animals were divided into nine groups, each containing 6 rats. The doses of the methanol extract for animal study was selected based on

the literature survey and in previous study, similar doses of 250 and 500mg/kg were given to animals for evaluating the anti-inflammatory activity. The doses of ethyl acetate and butanol fractions were selected based on their yield.

Group I: Control (saline *p.o*). Group II: Standard diclofenac sodium (10 mg/kg *i.p*).

Group III to V: Methanol extracts of bark *Gmelina arborea* (500, 250 and 125 mg/kg *p.o*).

Group VI and VII: Ethyl acetate fractions of methanol extract (25 and 50 mg/kg *p.o*)

GroupVIII and IX: Butanol fractions of methanol extract (25 and 50 mg/kg *p.o*).

The percentage (%) inhibition of oedema was calculated using formula:-

$$\% \text{ inhibition} = \frac{T_0 - T_t}{T_0} \times 100$$

Where T_t = Thickness of rat paw after giving standard/extract; T_o = Thickness of rat paw after injecting carrageenan

3.4 *Statistical analysis*

The data obtained from different groups were statistically analysed using one way analysis of variance followed by Tukey's test. Results were expressed as Mean ± SEM. $p<0.05$ was considered to be statistically significant. Instat software version 3 (Graph Pad Software Inc., San Diego) was used to carry out all statistical analyses.

3.5 *Compound Isolation*

Melting point of GM-01 is 231-232^{0}C.

^{1}H NMR (300MHz, $CDCl_3$, TMS=0); ™ = 7.86 (1H, d, *J* = 8.1 Hz, H-5); 7.04 (1H, dd, J_{12}= 1.5, J_{13} = 7.5 Hz, H-5'); 7.29-7.34 (2H, m, H-4',6'); 6.99 (1H, d, *J* = 7.5 Hz, H-5'); 6.90 (1H, d, *J* = 8.1Hz, H-6); 6.73 (1H, dd, J_{12} =1.2 , J_{13} =7.2 Hz, H-8); 6.70 (1H, d, *J* = 8.1Hz, H-3'); 5.12 (1H, m, H-2), 2.84-2.90 (2H, m, H-3a, 3b).

Infrared spectra (KBr; cm^{-1}): 2926(C-H stretch); 1665(C=0 stretch); 1590(C=C, aromatic); 1270(C-0 stretch). Mass spectra: m/z = 254(M^+).

The 1H NMR of the isolated molecule displayed three distinct doublets. The molecule showed positive test for flavonoids.

Table S1. Anti-inflammatory effect of methanol extract of stem bark of *G. arborea*

Treatment	Dose	Change in paw thickness (mm) ± SEM (%age inhibition)			
		1 hr	2hr	3hr	4hr

Control		3.90 ± 0.17[b]	4.16 ± 0.29[b]	4.23 ± 0.32[b]	4.68 ± 0.28[b]
Diclofenac sodium	10 mg/kg	2.00±0.44[a] (52.9%)	1.96±0.10[a] (63.6%)	1.90± 0.41[a] (69.8%)	1.52±0.10[a] (77%)
MEGA	125 mg/kg	3.83 ± 0.14[b] (6%)	3.20 ± 0.16[b] (26%)	2.76 ± 0.08[ab] (31%)	2.75 ± 0.13[ab] (43%)
	250 mg/kg	2.80 ± 0.17[a] (11%)	2.65 ± 0.12[ab] (33%)	2.52± 0.06[b] (52%)	2.37±0.08[a] (56%)
	500 mg/kg	2.48±0.25[a] (23.7%)	2.33±0.35[a] (33.6%)	2.20±0.25[a] (55.1%)	1.83±0.31[a] (67%)

MEGA = methanolic extract of *Gmelina arborea*

Values are expressed as mean ± SEM, statistical analysis was performed by one way ANOVA followed by Tukey test. [a]$p < 0.05$ vs control. [b]$p < 0.05$ vs standard.

Table S2. **Anti-inflammatory effect of butanol and ethyl acetate fractions of methanol extract**

Treatment	Dose	Change in paw thickness(mm)± SEM (%age inhibition)			
		1 hr	2hr	3hr	4hr
Control		3.90± 0.17[b]	4.16±0.29[b]	4.23 ± 0.32[b]	4.68 ± 0.28[b]
Diclofenac sodium	10 mg/kg	2.00±0.44[a] (52.9%)	1.96±0.10[a] (63.6%)	1.90±0.41[a] (69.8%)	1.16±0.19[a] (77%)
BFME	25 mg/kg	3.41±0.32[b] (11%)	3.35±0.10[b] (20.6%)	3.28±0.17[b] (25.6%)	3.08±0.15[ab] (30%)
	50 mg/kg	3.33±0.14[b] (24.6%)	3.25±0.25[b] (33.6%)	2.95±0.18[ab] (38.9%)	2.41±0.19[ab] (40%)
EFME	25 mg/kg	3.11 ± 0.17[b] (23.5%)	3.06±0.11a[b] (30.3%)	2.98± 0.25[ab] (37%)	2.55±0.28[ab] (40.8%)
	50 mg/kg	2.23± 0.21[a] (41.2%)	2.00± 0.17[a] (46.8%)	1.96±0.39[a] (58.5%)	1.43± 0.18[a] (69.5%)

BFME: butanol fraction of methanol extract

EFME: ethyl acetate fraction of methanol extract

Values are expressed as mean ± SEM, statistical analysis was performed by one way ANOVA followed by Tukey's test. [a]$p < 0.05$ vs control, [b]$p < 0.05$ vs standard.

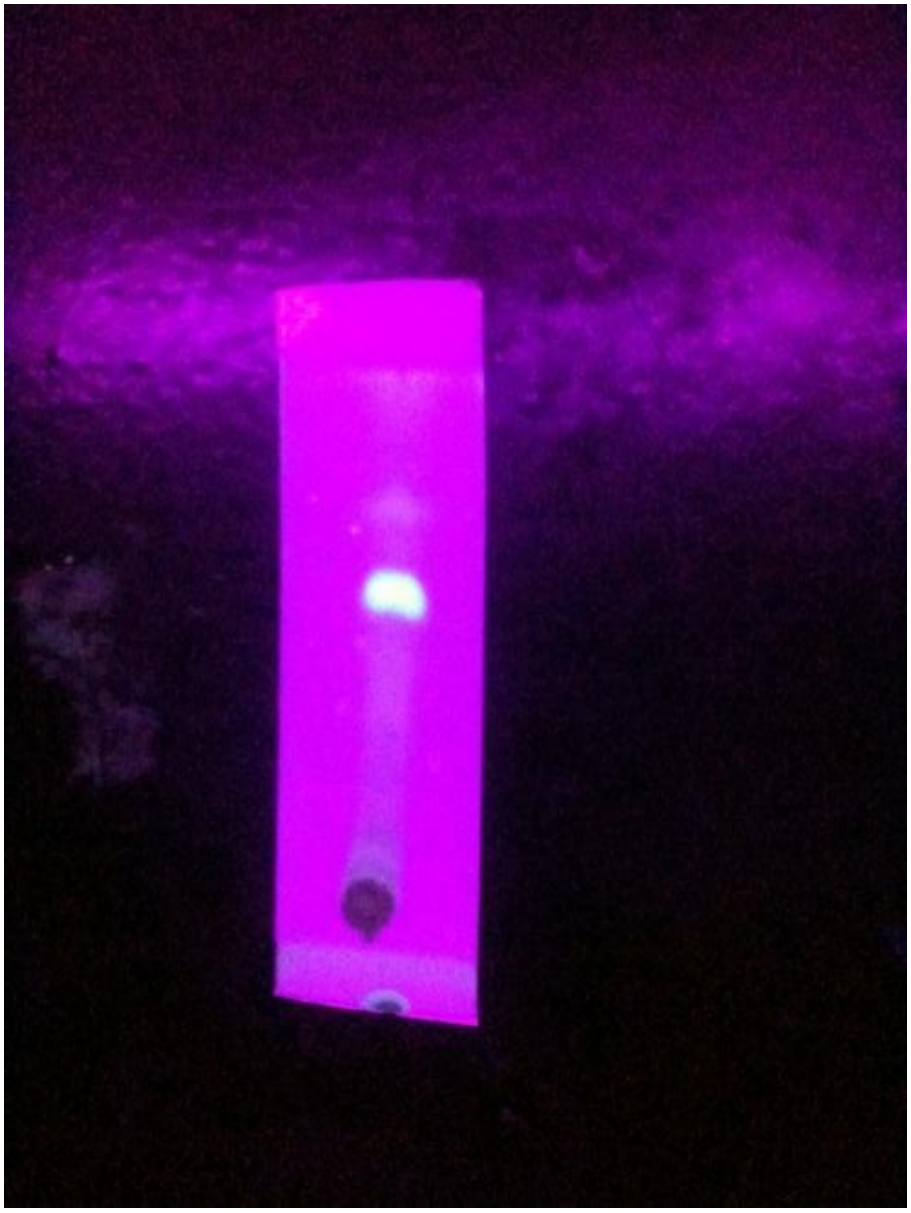

Figure S1. TLC of ethyl acetate fraction