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RESEARCH ARTICLE

# Anti-inflammatory activity of *Madhuca longifolia* seed saponin mixture

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## Abstract

The ethanol extract and saponin mixture of *Madhuca longifolia* L. (Sapotaceae) were evaluated for anti-inflammatory activity using acute (carrageenan-induced inflammation), sub-acute (formaldehyde-induced inflammation), and chronic (cotton pellet granuloma) models of inflammation in rats. Saponins alone seem to be responsible for the anti-inflammatory activity in the studied models. MLEE (*Madhuca longifolia* ethanol extract) at a dose level of 10 and 15 mg/kg and *Madhuca longifolia* saponin mixture (MLSM) at a dose level of 1.5 and 3 mg/kg significantly reduced the edema induced by carrageenan in acute model of inflammation, inhibiting both phases of inflammation. Both the extracts had a more effective response than the reference drug diclofenac sodium in the sub-acute inflammation model. Results indicated a significant anti-inflammatory activity by *M. longifolia* saponins in cotton pellet granuloma.

**Keywords:** Acute anti-inflammatory activity; chronic anti-inflammatory activity; ethanol extract; *Madhuca longifolia*; saponin mixture; sub-acute anti-inflammatory activity

## Introduction

*Madhuca longifolia* L. (syn. *Bassia longifolia* L.) (Sapotaceae) (Yoshikawa et al., 2000) is commonly identified as mahua together with its sister species, *Madhuca latifolia* Macb. (Shastri, 1962). Its bark, leaves, flowers, fruits, and seeds have various uses in the Indian indigenous system of medicine. The tree is valued for its oil-bearing seeds and flowers, which are used for alcoholic beverage production. Mahua seeds are of economic importance as they are a good source of edible fats (Ramadan et al., 2006). Its seed kernels are rich in saponins (Heywood & Kon, 1940). The distilled juice of the flower is considered a tonic, both nutritional and cooling, and also used in treatment of helminthes, acute and chronic tonsillitis, pharyngitis (Nadkarni, 1954) and bronchitis (Varier, 1995). In Indian folk medicine, LATTA is prepared by mixing and grinding equal amounts of mahua flower with roasted maize grain, which is claimed to be effective in arthritis to relieve pain. The astringent bark extract is used for dental-related problems, rheumatism, and diabetes. The medicinal properties

attributed to this plant are stimulant, demulcent, emollient, heating, and astringent (Shastri, 1962).

Previous phytochemical studies on this plant have revealed the presence of Mi-saponins A, B (Kitagawa et al., 1975), and C (Kitagawa et al., 1978). Four new oleanane-type triterpene glycosides, madlongisides A–D, were isolated from the seeds of *M. longifolia* (Yoshikawa et al., 2000).

Oleanolic and ursolic triterpenoids were found to inhibit croton oil-induced ear edema in mice. They were also effective in carrageenan and TPA-induced edemas in mice (Sun et al., 2006). Triterpenoids of the oleanane and ursane series were found to be active against formaldehyde-induced edema and formaldehyde-induced arthritis in rats (Bhargava et al., 1970). The diverse array of terpenoid structures and functions has provoked increased interest in their commercial use. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory, and immunomodulatory properties

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(Paduch et al., 2007). Because there is little information available, the present investigation evaluated the anti-inflammatory potency of *M. longifolia* seed saponins and also determined whether saponins alone were responsible for the activity.

## Materials and methods

### Plant material

*M. longifolia* seeds were collected from Konchavaram forest, Gulbarga, Karnataka during June-July 2006. Authentication was done by Y. N. Seetharam, faculty of Botany, Gulbarga University, Gulbarga, where a voucher specimen has been deposited in the herbarium (HGUG No. 723). The seed sample was air-dried and ground to fine powder. The powdered sample was defatted with petroleum ether (40–60°C) by Soxhlet. The defatted seed powder was extracted with ethanol to give *M. longifolia* ethanol extract (MLEE). The other portion of the defatted powder was used for saponin extraction to yield *M. longifolia* saponin mixture (MLSM) (Figure 1) (Yosioka et al., 1974). Both extracts were stored under refrigeration for further studies. MLEE was phytochemically screened according to Harborne (1973).

### Animals

Albino Swiss mice (20–30 g) and Wistar rats (150–300 g) were maintained in a group consisting of six animals each in standard cages under standard condition of 12 h dark/light cycles. The animals were fed with standard

rodent diet and water *ad libitum*. After one week of acclimatization the animals were used for the designed experimental studies. Approval from the institutional animal ethical committee for usage of animals in the experiments was obtained.

### Acute toxicity studies

*In vivo* toxicity was carried out according to Trease and Evans (1983). Albino mice of either sex were randomly divided into groups consisting of six animals each. Graded doses of MLEE and MLSM (dissolved in water) were administered orally. One group serving as control was treated with normal saline (vehicle, also used for drug administration in further studies). The animals were monitored for 24 h after drug administration for gross behavioral changes and mortality. Dose at which 50% mortality in a group was observed was considered as lethal dose<sub>50</sub> (LD<sub>50</sub>). Based on the results of preliminary toxicity studies, the doses for the further studies were fixed at 10 and 15 mg/kg for MLEE, and 1.5 and 3 mg/kg for MLSM.

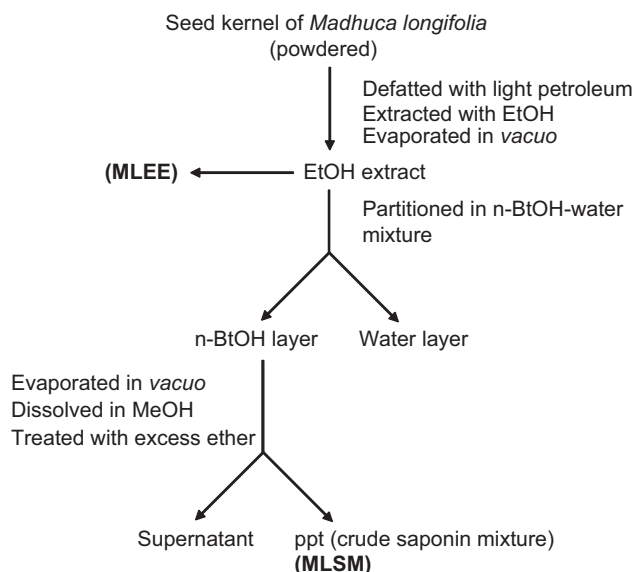
### Acute anti-inflammatory study

Carrageenan-induced hind paw edema was carried out following the method of Winter et al. (1962). The animals were divided into six groups (N = 6). In all the groups, acute inflammation was produced by sub-plantar injection of 0.1 mL freshly prepared 1% suspension of carrageenan in normal saline in the right hind paw of the rats and the paw volume was measured plythesmometrically at 0 (before carrageenan injection) and ½, 1, 2, and 4 h after carrageenan injection. Animals were pre-dosed with 10 and 15 mg/kg of MLEE, 1.5 and 3 mg/kg of MLSM. The vehicle (0.2 mL) and 50 mg/kg diclofenac sodium (reference drug) were administered intraperitoneally (i.p.) as negative and positive control, respectively, 30 min before the carrageenan injection. Mean increase in the paw volume was measured and the percentage inhibition was calculated with reference to negative control.

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

### Sub-acute anti-inflammatory study

Formalin-induced hind paw edema was carried out following the method of Brownlee (1950). The animals were divided into six groups (N = 6). In all the groups, sub-acute inflammation was produced by injecting 0.1 mL freshly prepared 4% formaldehyde into the foot pad of the right hind paw of the rats and the paw volume was measured plythesmometrically at 0 (before formaldehyde injection) and 2, 6, 12, 24, and 48 h after formaldehyde injection.



**Figure 1.** Preparation of ethanol extract and saponin mixture of *Madhuca longifolia* seeds. Powdered seed kernels (100 g) yielded approximately 53.84 g defatted seed powder, approximately 19.32 g ethanol extract, and approximately 2.69 g saponin mixture.

Animals were pre-dosed with 10 and 15 mg/kg of MLEE, 1.5 and 3 mg/kg of MLSM. The vehicle (0.2 mL) and 50 mg/kg diclofenac sodium were administered as negative and positive control, respectively, 30 min before the formaldehyde injection. Mean increase in the paw volume was measured and the percentage inhibition was calculated with reference to negative control as mentioned earlier.

### Chronic anti-inflammatory study

Cotton pellet granuloma in rats was carried out using the method of Swingle and Shideman (1972). Albino rats of either sex weighing 150–200 g were used. MLEE (10 and 15 mg/kg) and MLSM (1.5 and 3 mg/kg) were treated orally on a once daily dosage regimen for 7 days, while the positive and negative control groups received diclofenac sodium (50 mg/kg) and vehicle only, respectively. Two sterilized pellets of cotton weighing  $35 \pm 1$  mg were implanted subcutaneously, one on each side along the flanks of the animal, under anesthesia and sterile conditions. The rats were sacrificed on day 8. The implanted pellets were dissected out and recorded for wet weight. Pellets were dried at 60°C for 18 h and the dry weight was recorded. The transudative weight, granuloma formation, and the percentage granuloma inhibition of the test drugs were calculated.

### Statistical analysis

The data of the current experiment are presented as mean  $\pm$  SEM (standard error mean). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

## Results

### In vivo toxicity

Preliminary phytochemical screening of the MLEE extract showed the presence of saponins, flavonoids,

tannins, phenols, glycosides and alkaloids. Toxicity symptoms such as changes in gross animal behavior were visible with dose level of 300 mg/kg of MLEE and 60 mg/kg of MLSM within 6 h of dose administration. The LD<sub>50</sub> for MLEE was found to be 650 mg/kg body weight whereas it was 120 mg/kg body weight for MLSM.

### Carrageenan-induced edema

The anti-edematogenic response obtained by the administration of the MLEE, MLSM, diclofenac and vehicle on carrageenan-induced hind paw edema in rats is shown in Table 1. Both extracts showed a significant increase in percentage reduction of paw volume when compared to control, with anti-edematogenic activity seen as early as ½ h of induction of inflammation. The anti-inflammatory response of both extracts was comparable with that of the reference drug, though not exactly the same. Diclofenac sodium (50 mg/kg) exhibited highest inhibition of edema at all the studied time intervals, except for 1 h. A better anti-inflammatory response was observed with higher doses of both extracts when compared with their respective lower doses.

### Formaldehyde-induced edema

*M. longifolia* seed extracts exhibited an excellent inhibition of formaldehyde-induced paw edema in rats (Table 2). The anti-inflammatory response of the tested extracts was far better than the reference drug diclofenac sodium. A significant reduction of edema was seen from 6 h after edema induction with MLEE 10 mg/kg (16.57%), MLEE 15 mg/kg (21.48%), MLSM 1.5 mg/kg (23.71%) and MLSM 3 mg/kg (23.71%), whereas diclofenac sodium had a significant inhibition from the 12th (20%) after edema induction. MLSM at a dose level of 3 mg/kg had a much better potency in reducing the paw volume even at 48 h (24.35%) after edema induction when compared to the reference drug diclofenac sodium at dose level of 50 mg/kg, exhibiting only 18.83% reduction activity at the same period of time.

**Table 1.** Effect of ethanol extract and saponin mixture of *Madhuca longifolia* seeds on carrageenan-induced hind paw edema in rats.

Treatment	Dose (mg/kg)	Mean paw size in mm				% Inhibition			
		½ h	1 h	2 h	4 h	½ h	1 h	2 h	4 h
Vehicle	-	0.358 $\pm$ 0.030	0.342 $\pm$ 0.020	0.325 $\pm$ 0.025	0.317 $\pm$ 0.021	-	-	-	-
Diclofenac sodium	50	0.225 $\pm$ 0.021*	0.208 $\pm$ 0.027*	0.167 $\pm$ 0.024*	0.150 $\pm$ 0.025*	37.15	39.18	48.61	52.68
MLEE	10	0.250 $\pm$ 0.012*	0.200 $\pm$ 0.012*	0.200 $\pm$ 0.012*	0.167 $\pm$ 0.016*	30.16	41.52	38.46	47.31
MLEE	15	0.242 $\pm$ 0.015*	0.192 $\pm$ 0.015*	0.175 $\pm$ 0.011*	0.167 $\pm$ 0.016*	32.40	43.85	46.15	47.31
MLSM	1.5	0.233 $\pm$ 0.010*	0.217 $\pm$ 0.010*	0.208 $\pm$ 0.008*	0.183 $\pm$ 0.010*	34.91	36.54	36.00	42.27
MLSM	3	0.233 $\pm$ 0.010*	0.192 $\pm$ 0.015*	0.183 $\pm$ 0.010*	0.167 $\pm$ 0.016*	34.91	43.85	43.69	47.31

Values are mean  $\pm$  SEM; \*P < 0.01; MLEE, *Madhuca longifolia* ethanol extract; MLSM, *Madhuca longifolia* saponin mixture.

**Table 2.** Effect of ethanol extract and saponin mixture of *Madhuca longifolia* seeds on formaldehyde-induced hind paw edema in rats.

Treatment	Dose (mg/kg)	Mean paw size in mm					% Inhibition				
		2 h	6 h	12 h	24 h	48 h	2 h	6 h	12 h	24 h	48 h
Vehicle	-	0.275 ± 0.001	0.350 ± 0.018	0.375 ± 0.011	0.342 ± 0.015	0.308 ± 0.008	-	-	-	-	-
Diclofenac sodium	50	0.250 ± 0.018	0.300 ± 0.018	0.300 ± 0.018**	0.267 ± 0.016**	0.250 ± 0.022*	9.09	14.28	20.00	21.92	18.83
MLEE	10	0.242 ± 0.015	0.292 ± 0.015*	0.292 ± 0.015**	0.267 ± 0.021**	0.258 ± 0.015	12.00	16.57	22.13	21.92	16.23
MLEE	15	0.250 ± 0.018	0.275 ± 0.011**	0.267 ± 0.010**	0.258 ± 0.008**	0.258 ± 0.008	9.09	21.48	28.80	24.56	16.23
MLSM	1.5	0.233 ± 0.010	0.267 ± 0.010**	0.267 ± 0.010**	0.267 ± 0.010**	0.258 ± 0.015	15.27	23.71	28.80	21.92	16.23
MLSM	3	0.242 ± 0.15	0.267 ± 0.016**	0.267 ± 0.016**	0.242 ± 0.015**	0.233 ± 0.016**	12.00	23.71	28.80	29.23	24.35

Values are mean ± SEM; \*P < 0.05; \*\*P < 0.001; MLEE, *Madhuca longifolia* ethanol extract; MLSM, *Madhuca longifolia* saponin mixture.

### Cotton pellet granuloma

A significant anti-proliferative effect was seen in all tested extracts that were far superior to diclofenac sodium used as a standard reference drug at a dose level of 50 mg/kg. The mean weights of the dried pellets implanted under the skin in the rats from the control group were evaluated as 37.7 ± 4.16 mg, from the rats administered with diclofenac sodium (50 mg/kg) as 19.7 ± 2.69 mg and from the rats administered with MLEE (10 and 15 mg/kg), MLSM (1.5 and 3 mg/kg) as 18.2 ± 2.60, 16.8 ± 1.54, 18.7 ± 2.40, and 14.8 ± 2.12 mg, respectively (Table 3). Based on these results, the anti-proliferative effect (on dry pellet weight basis) of diclofenac sodium was found to be 47.74%, and of MLEE (10 and 15 mg/kg), MLSM (1.5 and 3 mg/kg) it was 51.72, 55.43, 50.39, and 60.74% respectively.

### Discussion

The acute toxicity studies indicated that the LD<sub>50</sub> for MLEE was 650 mg/kg when administered orally. The LD<sub>50</sub> for alcoholic extract of *Madhuca indica* flowers was 160 mg/kg (Dinesh, 2001). The LD<sub>50</sub> for the saponin mixture was 120 mg/kg orally. On the other hand, the LD<sub>50</sub> of purified seed saponins of *Madhuca latifolia* was 15 and 15–20 mg/kg for intravenous and intraperitoneal administration, respectively (Mulky & Gandhi, 1977). Cherian et al. (1996) have reported that the mechanism of toxicity by the parenteral route can be explained on the basis of massive hemolysis produced by the saponins, causing death due to anoxia. When administered orally, the saponin is perhaps not absorbed directly but causes destruction and sloughing of the superficial layers of the intestinal mucous membrane followed by intense inflammation and some degree of absorption into circulation through damaged hyperemic tissues. Histopathological examination revealed a gradation of damage from slight erosion of the tip of the villi of intestinal mucous membrane to complete necrosis and destruction of it, with increasing amounts of mahua seed meal in diets. The other significant change was a severe vacuolar degeneration of kidney tubular cells.

**Table 3.** Effect of ethanol extract and saponin mixture of *Madhuca longifolia* seeds on cotton pellet granuloma in rats.

Treatment	Dose (mg/kg)	Net dry pellets weight (mg)	Anti-proliferative effect (%)
Vehicle	-	37.7 ± 4.16	-
Diclofenac sodium	50	19.7 ± 2.69*	47.74
MLEE	10	18.2 ± 2.60*	51.72
MLEE	15	16.8 ± 1.54*	55.43
MLSM	1.5	18.7 ± 2.40*	50.39
MLSM	3	14.8 ± 2.12*	60.74

Values are mean ± SEM; \*P < 0.01; MLEE, *Madhuca longifolia* ethanol extract; MLSM, *Madhuca longifolia* saponin mixture.

In the present study, anti-inflammatory activity of ethanol extract and saponin mixture of *M. longifolia* seeds was evaluated by various inflammatory models in rats. Many investigators have reported that inhibition of carrageenan-induced inflammation in rats is one of the most suitable test procedures to screen anti-inflammatory agents (Vogel & Vogel, 1997). Although it has been described that the paw edema induced by carrageenan in mice is biphasic and can be detected up to 72–96 h after carrageenan administration (Henriques et al., 1987), a consistent hyperalgesic reaction can be measured 3–4 h after its intraplantar injection (Sammons et al., 2000). The present results clearly demonstrate a significant acute anti-inflammatory activity (induced by carrageenan injection) by both tested extracts. The present result also shows that saponins alone seem to be responsible for the anti-inflammatory activity since MLEE (which had saponins besides other secondary metabolites) exhibited more or less equal anti-edematogenic response when compared with MLSM. The tested extracts inhibited both phases of carrageenan-induced edema, since the inhibitory response was seen from ½ h of irritant injection till the 4<sup>th</sup> h indicating that saponins are inhibiting the release of inflammation mediators. Oleanane-type triterpenes have been shown to be reducing NO (nitrous oxide), PGE<sub>2</sub> (prostaglandin E<sub>2</sub>), and TNF-α production by inhibiting COX-2 (cyclooxygenase 2) and iNOS (inducible nitric oxide synthetase) activity and down-regulating nuclear factor (NF)-κB. All of these play critical roles in inflammatory processes (Paduch



et al., 2007). It has recently been shown that ursolic acid and oleanolic acids inhibit inhibitory- $\kappa\beta$ - $\alpha$  kinase and p65 phosphorylation, leading to suppression of NF- $\kappa\beta$  activation (Shishodia et al., 2003). Possibly *M. longifolia* saponins are exhibiting their anti-inflammatory effect by inhibiting COX-2, iNOS, down-regulating (NF)- $\kappa$ B, thus reducing NO, PGE<sub>2</sub>, and TNF- $\alpha$  production, and are able to modulate the NF- $\kappa\beta$ -activation involved in the increase in proinflammatory cytokines such as TNF- $\alpha$ . Diclofenac sodium is a widely used potent non-steroidal anti-inflammatory drug (NSAID) with pronounced analgesic and anti-pyretic activity (Tonussi & Ferreira, 1994). Therefore, diclofenac sodium was selected for this study as a positive control. Like MLEE and MLSM, diclofenac sodium also seems to act by inhibiting both phases of carrageenan-induced edema. But MLSM (3 mg/kg) seems to be a more potent anti-inflammatory compound than diclofenac sodium (50 mg/kg) for acute inflammation when compared with the amount of dose used. One of the major draw backs of NSAIDs is gastric mucosal damage by interfering with prostaglandin synthesis caused by non-selective inhibition of COX. Yoshikawa et al. (2005) have reported that triterpenes isolated from *Camellia sinensis* exhibited potent gastroprotective effects. Among the isolated saponins, theasaponins E1, E2, E5, and assamsaponin C showed an inhibitory effect on ethanol-induced gastric mucosal lesions at a dose of 5 mg/kg, and their activities were stronger than that of the reference compound omeprazole and cetraxate hydrochloride. Thus the anti-inflammatory effect of *M. longifolia* saponins could be due to selective inhibition of COX. Moreover, oleanolic and ursolic acids at low doses exert a hepatoprotective effect which can be due to their anti-oxidant and anti-inflammatory actions (Liu, 2005). Since low doses of MLSM (1.5 and 3 mg/kg) were used in the present study, it is likely that *M. longifolia* saponins may have anti-oxidant properties, thus possessing hepatoprotective and gastroprotective effects, making it a promising alternative for NSAIDs. However, the gastroprotective and hepatoprotective effect of *M. longifolia* saponins needs to be investigated.

It is known that formaldehyde-induced inflammation usually involves two distinct phases. It has been proposed that the early or first phase reflects the direct stimulation of nociceptors, while the later or second phase may be associated with inflammation mediators (Dray & Perkins, 1993). Some studies have shown that substance P receptor antagonists inhibit the later phase of formaldehyde-induced edema, and substance P has a role in this response (Rupniak et al., 1993). In the present investigation, saponins either as crude mixture or in ethanol extract effectively inhibited the edema induced by formaldehyde. Reduction in paw volume upon phlogistic injection could be due to effective inhibition of histamine/bradykinin or inhibition of prostaglandin

synthesis, the mediators of inflammation. *M. longifolia* seed saponins were effective in alleviating hyperalgesia, pain, and edema induced by formaldehyde as they were successfully inhibiting the mediators of these responses. It has been reported that triterpenoids of the oleanane and ursane series were found to be active against formaldehyde-induced edema and formaldehyde-induced arthritis in rats. *M. longifolia* seed saponins inhibited both phases of formaldehyde-induced edema more effectively than the reference NSAID (diclofenac sodium) used, indicating that they might be acting as an antagonist to substance P receptors.

The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation and is a typical feature of established chronic inflammatory reaction. The present results indicate a significant anti-inflammatory activity by *M. longifolia* saponins in cotton pellet granuloma and thus found to be effective in chronic inflammatory condition, which reflects its efficacy in inhibiting the increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during granuloma tissue formation (Recio et al., 1995). Oleanolic acid 3- $\beta$ -glucoside isolated from the seeds of *Randia dumetorum* (Rubiaceae) (25-500 mg/kg) showed a significant anti-inflammatory activity in the exudative and proliferative phases of inflammation in rats (Ghosh et al., 1983). *M. longifolia* saponins decreased both wet (results not shown) and dry weights of the cotton pellets when compared to control group. Reference drug diclofenac sodium also showed a significant anti-proliferative effect and reduction in the dry weights of the cotton pellets but was comparatively less potent than the tested extracts. It is possible that *M. longifolia* saponins also inhibit monocyte infiltration and fibroblast proliferation. The anti-inflammatory effect could also be due to the inhibition of intercellular adhesion molecule (ICAM-1) expression induced by TNF- $\alpha$ . Ahn et al. (2002) reported the inhibitory effects of oleanane-type triterpenoids from fabaceous plants on the TNF- $\alpha$ -induced expression of ICAM-1 on THP-1 human monocytic leukemia cells.

## Conclusion

It can be concluded that *M. longifolia* seed saponins have anti-edematogenic activities in different animal models such as carrageenan-induced inflammation, formaldehyde-induced pain, and cotton pellet granuloma. The anti-inflammatory potency of the saponins exceeded/equaled the reference drug diclofenac sodium (NSAID), thus they can be developed further for long term symptomatic treatment involving inflammation. Commercial therapeutic uses of *M. longifolia* seed saponins would be

economical since the seeds are rich in saponin content and are obtained as a by-product from the seed cake left after oil extraction. Further studies on the anti-inflammatory mechanism of the action of purified saponins will be carried out.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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