

# Toxicological Evaluation of *Emblica officinalis* Fruit Extract and its Anti-inflammatory and Free Radical Scavenging Properties

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## ABSTRACT

**Background:** *Emblica officinalis* (Euphorbiaceae), popularly known as Indian gooseberry or "Amla" in India, is used in Ayurveda as "rejuvenating herb" since ancient times. **Objective:** This study was carried out to estimate toxicity, anti-inflammatory, and antioxidative activities of the methanolic extract of *Emblica officinalis* fruit (MEO) in an animal model. **Materials and Methods:** Antioxidative property of MEO was assessed by *in vitro* assays such as phosphomolybdenum assay (total antioxidant capacity), free radical scavenging assays 1,1-diphenyl-2-picrylhydrazyl and 2,2'-azino-bis and 3-ethylbenzthiazoline-6-sulphonic acid (DPPH and ABTS method) and lipid peroxidation assay (LPO). The anti-inflammatory property was evaluated by carrageenan-induced acute inflammation in rats by measuring rat paw volume at different time intervals and toxicological analysis using mice. **Results and Discussion:** High performance liquid chromatography studies revealed the presence of gallic acid (2.10%), mucic acid (4.90%), ellagic acid (2.10%), quercetin (28.00%), rutin (3.89%), and  $\beta$ -glucogallin (1.46%). MEO showed highest antioxidant activities by using DPPH (17.33–89.00%), ABTS (23.03–94.16%), nitric oxide scavenging activity (12.94–70.16%), LPO (56.54%), and phosphomolybdenum assay ( $142 \pm 6.09 \mu\text{g/ml}$ ). The LD<sub>50</sub> was found to be approximately 1125 mg/kg (p.o). High dose of MEO showed significant reduction (72.71%) in the inflammation after 4 h of treatment, which was comparable to diclofenac (10 mg/kg) (61.57%) treated group. Significant reduction ( $P < 0.05$ ) in the inflammatory cytokine (interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ ) markers were also observed (57.25% and 35.41%, respectively) in serum of MEO treated animals as compared to control. **Conclusion:** Taken together, phenolic compounds of MEO may serve as a potential herbal drug for amelioration of acute inflammation due to their modulatory action on free radicals.

**Key words:** 1-diphenyl-2-picrylhydrazyl, acute inflammation, amla, antioxidant, *Emblica officinalis*, high performance liquid chromatography

## SUMMARY

- The methanolic extract of *Emblica officinalis* fruit (MEO) has potent antioxidant activity as assessed by DPPH, ABTS and LPO assays

- MEO has potent anti-inflammatory activity in carrageenan induced paw edema model
- The phenolic compounds of MEO might be a potential herbal drug for amelioration of acute inflammation.



**Abbreviations used:** ROS, reactive oxygen species; RNS, reactive nitrogen species, LPO, lipid peroxidation, NO, nitric oxide, IL, interleukin; TNF  $\alpha$  tumor necrosis factor alpha; NSAIDs, nonsteroidal anti inflammatory drugs; AA, ascorbic acid; MEO, methanolic extract of *Emblica officinalis* fruit; ABTS+; 2,2'-azino bis 3 ethylbenzthiazoline 6 sulphonic acid; DPPH, 1,1 diphenyl 2 picrylhydrazyl; HPLC, high performance liquid chromatography; MDA, malondialdehyde; DMSO, dimethyl sulphoxide; ELISA, enzyme linked immunosorbent assay.

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## INTRODUCTION

There is an escalating demand for herbal drug therapy, many plant species are being explored and are used in various human cultures around the world for their medicinal properties.<sup>[1]</sup> These natural drugs help in maintaining the homeostasis. When cells are under stress, they produce free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) that damage the cells in many ways such as lipid peroxidation (LPO), protein denaturation, and DNA damage.<sup>[2]</sup> Nitric oxide (NO) is an important mediator of many physiological functions and its role in the inflammatory process is gaining recognition.<sup>[3]</sup> Natural antioxidants have an important role in protecting the cells against damage by ROS/RNS, which can lead to several afflictions such as inflammation<sup>[4,5]</sup> and diabetes.<sup>[6]</sup>

Inflammation is one of the first responses to cell and tissue damage inflicted by diverse factors such as infections and chemicals and physical injuries. Inflammation is caused by the release of various inflammatory mediators such as cytokines ([interleukin [IL]-1 $\beta$ , IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ), prostaglandins, bradykinin, histamine, growth factors, neurogenic factors,

and followed by central sensitization and hypersensitivity.<sup>[7]</sup> During such conditions, nonsteroidal anti-inflammatory drugs (NSAIDs) are used to alleviate pain and reduce inflammation.<sup>[8]</sup> However, long-term use of these NSAIDs may lead to certain types of ulcers and other disorders,<sup>[9]</sup> and hence there is a prompt need to search for an alternative to these synthetic drugs where herbal medicines can be suitable candidates.

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*Emblica officinalis* Gaertn., syn. *Phyllanthus emblica* L., popularly known as Indian gooseberry, is a small to medium-sized deciduous tree, native to India and the Middle East, belonging to the family Euphorbiaceae. It is commonly known as "Amla" in India and is used in Ayurveda as "rejuvenating herb" since ancient times.<sup>[10]</sup> *E. officinalis* extract contains several antioxidants such as emblicanin A and B, gallic acid, ellagic acid, ascorbic acid (AA) (Vitamin C), which are used to treat several medical conditions.<sup>[11]</sup> It also possesses several attributes like antipyretic, analgesic, antimicrobial, antifungal, antitussive, chemopreventive, immunostimulatory, hepatoprotective, cardioprotective, radioprotective, and potential to increase hemoglobin.<sup>[12]</sup> Despite its wide-ranging medicinal uses, there are very few scientific evidence showing correlation between *in vivo* anti-inflammatory effects and antioxidant properties for this wonder berry. Hence, the present report emphasizes on *in vivo* anti-inflammatory effectiveness of methanolic extract of *Emblica officinalis* fruit (MEO) using carrageenan-induced rat paw oedema model and a wide spectrum of *in vitro* antioxidant studies which includes models like 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS+), 1,1-diphenyl-2-picrylhydrazyl (DPPH), molybdenum reduction method, NO determination, and LPO, along with quantitative analysis of potential antioxidant compounds of MEO by high performance liquid chromatography (HPLC).

## MATERIALS AND METHODS

### Chemicals and reagents

Acetic anhydride, ammonium hydroxide, AA, butylated hydroxytoluene, 2-deoxy-2-ribose, DPPH, ferric chloride, gallic acid, magnesium ribbon, quercetin, sodium nitrite, and trichloroacetic acid were obtained from HiMedia Laboratories, Pvt., Ltd., Mumbai, India. Thiobarbituric acid was obtained from Loba Chemical, Mumbai, India. Ammonium persulphate, benzene, chloroform, di-potassium hydrogen phosphate, ethanol, ethylenediaminetetraacetic acid, glacial acetic acid, hydrogen peroxide, methanol, 5% o-phosphoric acid, 0.01% naphthylethylene diamine, potassium di-hydrogen phosphate, potassium ferricyanide, potassium hydroxide, sodium hydroxide, and sulfanilamide were obtained from Rankem, Mumbai, India. ABTS and gallic acid (3,4,5-trihydroxy benzoic acid) were obtained from Sigma, USA. Conc. HCl, conc. H<sub>2</sub>SO<sub>4</sub>, Fehling's solution, Mayer's reagents, and sodium carbonate were procured from Merck, Mumbai, India. Folin-Ciocalteu reagent was from Sisco Research Laboratory, Mumbai, India. Aluminium chloride was obtained from SD Fine Chemicals Limited, Mumbai, India. All chemicals and solvents were of analytical grade.

### Preparation of plant material

Methanolic extract was prepared using 500 g of coarse dried fruit powder, which was extracted in Soxhlet apparatus with 2 l of methanol for 24 h, in a three cycle process. Then, the mixture was filtered, concentrated, and lyophilized (LyoQuest, Telstar, Spain).<sup>[13]</sup> The MEO was dissolved in double-distilled water in desired concentrations before analysis.

### *In vitro* assays

#### 3-ethylbenzthiazoline-6-sulphonic acid radical scavenging assay

ABTS radical cations were produced by mixing ABTS and MEO and incubating the mixture at room temperature for 16 h in dark condition. This was followed by addition of 20 µl of 10 mM phosphate buffer saline (pH 7.4), test solutions of various concentrations, and 230 µl of ABTS radical solution (0.238 mM).<sup>[14]</sup> The absorbance was measured immediately at 734 nm spectrophotometer (Elico, SL 210). A control reaction was carried out without the test sample. Linear graph of concentration against percentage inhibition was constructed, and IC<sub>50</sub> value was calculated.

#### 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay

Different concentrations of test solution and 50:l of DPPH (0.659 mM) solution was incubated at 25°C for 20 min, after which the absorbance was read at 510 nm using spectrophotometer (Elico, SL 210).<sup>[15]</sup> A control reaction was carried out without the test sample. Linear graph of concentration against percentage inhibition was constructed, and IC<sub>50</sub> value was calculated. The percentage inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = (A_0 - A_1)/A_0 \times 100$$

where A<sub>0</sub> was the absorbance of the control (blank, without extract), and A<sub>1</sub> was the absorbance in the presence of the extract.

#### Determination of total antioxidant capacity by phosphomolybdenum assay

The total antioxidant capacity of MEO was assessed by phosphomolybdenum method.<sup>[16]</sup> To 0.3 mL of extract solution (1 mg/mL), 3 mL reagent solution (28 mM sodium phosphate, 6 M sulfuric acid and 4 mM ammonium molybdate) was added and incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm against blank spectrophotometer (Elico, SL 210). The antioxidant capacity of the extract was evaluated as AA equivalents.

#### Lipid peroxidation assay

Malondialdehyde (MDA), a marker of LPO was estimated by the method of Okhawa *et al.*<sup>[17]</sup> and as modified by Middha *et al.*<sup>[18]</sup> using 1,1,3,3-tetramethoxy propane as the standard, measured at 532 nm and expressed in nM/mg protein.

### Quantitative analysis of antioxidant compounds

#### High-performance liquid chromatography analysis

HPLC (Waters Corporation, Singapore) protocol was followed as per Middha *et al.*<sup>[19]</sup> Active constituent in the MEO extract was dissolved in a mixture of methanol and water (6:4 v/v), and identified by comparison of the retention time in chromatogram with standard Vitamin C, gallic acid, rutin, quercetin, and ellagic acid (Sigma Chemical Co., St Louis, USA). Data were analyzed using Empower software (Waters Corporation, Singapore).

### *In vivo* assay

#### Animals

Male Wistar albino rats (140–160 g) and Swiss albino mice (25–30 g) were ordered from Indian Institute of Sciences (Bangalore, India), housed in groups of 4 and given 7 days to acclimate to the housing facility in polypropylene, polycarbonate, and stainless steel cages. Rats were randomly selected and sheltered under standard laboratory conditions of light and dark cycles of 7:00 am to 7:00 pm, temperature of 25°C ± 2°C, lighting of 350 lux, and 68% ± 1% relative humidity and given access to rat maintenance food (Lipton India Ltd., Bangalore, India) and tap water *ad libitum*. Cages (size 421 mm × 290 mm × 190 mm with a gap of 7 mm between wires) and corn cob bedding were changed every alternate day to ensure that animals are clean and dry as per the National Institutes of Health guidelines.<sup>[20]</sup> During housing, animal's health status was monitored twice daily, and no adverse events were detected. The study protocol was approved by Maharani Lakshmi Ammanni College Ethical Committee, clearance from Ethical Committee (1368/ac/10/CPCSEA), Bangalore, India.

#### Acute toxicity test

Swiss albino mice (25–30 g) of both sexes were divided into six groups of 10 each. Animals were housed 5 per cage and were starved overnight but were given water preceding the experiment. MEO was administered to each group at different dose levels of 0.5, 1.0, 1.5, 2.0,

2.5, and 3.0 g/kg BW/ml. The mice were kept under observation for 24 h, mortality was recorded, and LD<sub>50</sub> (median lethal dose) was determined as per Middha *et al.*<sup>[18]</sup>

### Anti-inflammatory activity

The carrageenan-induced rat paw edema test<sup>[21]</sup> was used as an experimental model for testing the anti-inflammatory activity of MEO. The rats were divided into five groups of six rats each as shown in Table 1. The investigated MEO, dissolved in dimethyl sulphoxide (DMSO) were administered orally in doses of 200 mg/kg and 400 mg/kg; these doses were selected based on an initial toxicity screening for up to 24 h. Diclofenac (10 mg/kg) (Sigma Chemical Co., St. Louis, USA) dissolved in DMSO, was used as a reference drug. The control animals were given the vehicle (1 ml/kg p.o. DMSO). 1 h after the oral administration of the extracts or diclofenac, carrageenan-saline solution (0.5% w/v) and saline were injected in a volume of 0.1 ml into the plantar surface of the right and left hind paw, respectively. Left paw served as the control (noninflamed) paw. The animals were observed for 0<sup>th</sup> h, 1<sup>st</sup> h, 2<sup>nd</sup> h, and 4<sup>th</sup> h; the paw volume was measured using plethysmometer (PanLab, Barcelona, Spain).<sup>[21]</sup> The anti-inflammatory effect was calculated using the equation below:

$$\text{Anti-inflammatory effect (\%)} = k - e/k \times 100$$

where *k* is the difference in the paw weight in the control group and *e* is the difference in the paw weight in the treatment group.

Finally, after drug treatments, rats were euthanized using 0.3 ml/100 g i.p ketamine (300 mg/kg) +0.15 ml/100 g xylazine (30 mg/kg) as recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals. After euthanization, the carcasses were placed in a non-PVC containing, sealable, and transparent plastic bag and were sent to incinerator. Blood samples were collected by heart puncture method, and serum was separated for biochemical estimations.

### Measurement of interleukin-1 $\beta$ and tumor necrosis factor- $\alpha$ levels

The serum concentration of IL-1 $\beta$  was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit method (Biosource, San Diego, California)<sup>[22]</sup> and TNF- $\alpha$  was measured with another ELISA kit (Endogen, Woburn, MA, USA).<sup>[23]</sup>

### Statistical analysis

All the measurements were repeated six times and expressed as a mean  $\pm$  standard error of means and Statistically analyzed by two way analysis of variance followed by Tukey's multiple ranges using GraphPad prism (GraphPad, San Diego, CA, USA)

**Table 1:** Paw volume variation due to anti-inflammatory effects of methanolic extract of *Embolia officinalis* fruit

Experimental animals	Dosages mg/kg	Percentage increase		
		1 <sup>st</sup> h	2 <sup>nd</sup> h	4 <sup>th</sup> h
NL		2.60 $\pm$ 0.09	2.90 $\pm$ 0.007	2.20 $\pm$ 0.019
IC	No treatment	48.13 $\pm$ 4.56	80.11 $\pm$ 9.01 <sup>a</sup>	92.84 $\pm$ 10.136 <sup>a</sup>
IC + LMEO	200	42.67 $\pm$ 1.93 <sup>NS</sup>	68.89 $\pm$ 3.50 <sup>b</sup>	89.86 $\pm$ 8.102 <sup>a</sup>
IC + HMEO	400	33.673 $\pm$ 4.06 <sup>NS</sup>	55.85 $\pm$ 6.012 <sup>c</sup>	72.71 $\pm$ 7.242 <sup>a</sup>
IC + diclofenac	10	18.68 $\pm$ 1.19 <sup>a</sup>	50.68 $\pm$ 0.012 <sup>c</sup>	61.57 $\pm$ 3.19 <sup>b</sup>

Different superscripts denote significant group at *P*<0.05 versus inflammatory group (IC). <sup>NS</sup>Not significant. IC + LMEO: Control rats treated with 200 mg/ml; IC + HMEO: Control rats treated with 400 mg/ml; IC + diclofenac: Control rats treated with diclofenac; NL: Normal; IC: Carrageenan injected control; SEM: Standard error of mean

## RESULTS

### Yield of the extract

The total yield of the extract was found to be 9.8%.

### In vitro activity

#### 3-ethylbenzthiazoline-6-sulphonic acid radical scavenging assay

A dose-dependent inhibition of scavenging activity was displayed by MEO to ABTS radical [Figure 1]. The percentage of ABTS scavenging by MEO was found to be at 23.03% and 94.16% at 10  $\mu$ g/ml and 100  $\mu$ g/ml, respectively.

#### 1-diphenyl-2-picrylhydrazyl radical scavenging assay

MEO showed radical scavenging activity as measured by DPPH, across concentrations ranging from 0.1 to 1.0  $\mu$ g/ml [Figure 2]. The values for maximum % inhibition at 0.1–1 mg/ml ranged from 7.33% to 83.33% in MEO, whereas that of standard (AA) was found to be 90%. The high DPPH scavenging activities of the methanolic extract have already been reported by other researchers.<sup>[24]</sup> IC<sub>50</sub> of MEO was found to be 0.628  $\mu$ g/ml.

#### Determination of total antioxidant capacity by phosphomolybdenum assay

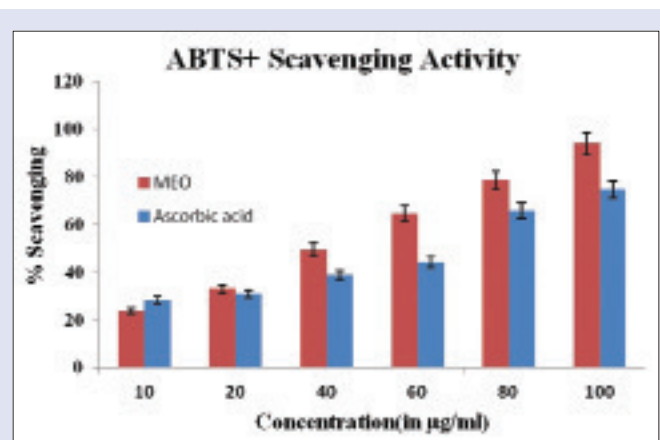
The principle includes the reduction of Mo (VI) to Mo (V) by the extract that contains antioxidant compounds. In this study, MEO was more effective in reduction of Mo (VI) to Mo (V) (142.0  $\pm$  6.09  $\mu$ g/ml). The reduction of Mo (VI) to Mo (V) by administration of reference chemical, AA (IC<sub>50</sub> 81.3  $\pm$  4.12  $\mu$ g/ml), suggested the presence of effective antioxidants in MEO.

#### Lipid peroxidation assay

Decrease in the MDA levels is a good indication of an effective antioxidant. There was a significant dose-dependent reduction in MDA levels by MEO. Maximum percentage reduction in MDA levels of 56.54% was observed at 300  $\mu$ g/ml concentration of MEO.

#### High performance liquid chromatography analysis

HPLC method has been reported to be simple, easy to use, and effective enough for the identification and quantification of major phenolic compounds found in aromatic plants,<sup>[19,25]</sup> despite the fact that the separation of procyanidins is not satisfactory with these phases.<sup>[25]</sup> To deduce a better separation of phenolic acids



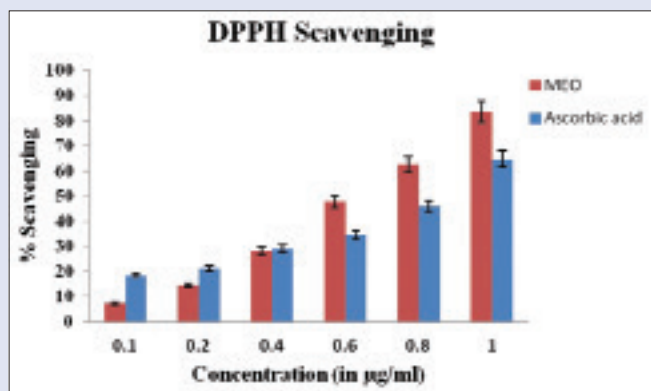
**Figure 1:** ABTS+ radical scavenging activity of methanolic extract of *Embolia officinalis* fruit compared to standard ascorbic acid

and flavonoids in aromatic plants, we have used Spherisorb C18 stationary phase, which provided a quite fine outcome. After extraction and acid hydrolysis, the content of phenolic substances was determined. Quantification was done *via* calibration with standards. The amount of phenolic acids such as quercetin (28.00 mg/100 g), rutin (3.89 mg/100 g),  $\beta$ -glucogallin (0.146 mg/ml), gallic acid (2.10 mg/100 g), and mucic acid (4.90 mg/100 g) were detected in the analyzed samples [Figure 3]. Quercetin has been reported to be present in a large number of herbals.<sup>[19,26]</sup> Rutin in some cases can be hydrolyzed to quercetin (aglycone). This could have happened in our sample of interest. Thus, hydrolysis was needed for the identification and quantitative determination of phenolic acids. Time of harvest and storing conditions might have an effect on the variations in the phenolic compounds.

## Assessment of pharmacological activity (*in vivo*)

### Acute toxicity

*In vivo* median lethal dose or LD<sub>50</sub> of the extract was 1125 mg/kg BW. Other parameters such as body weight and survival rate showed no side effects to MEO (data not shown).



**Figure 2:** DPPH scavenging activity of methanolic extract of *Embolica officinalis* fruit compared to standard ascorbic acid

### Anti-inflammatory activity

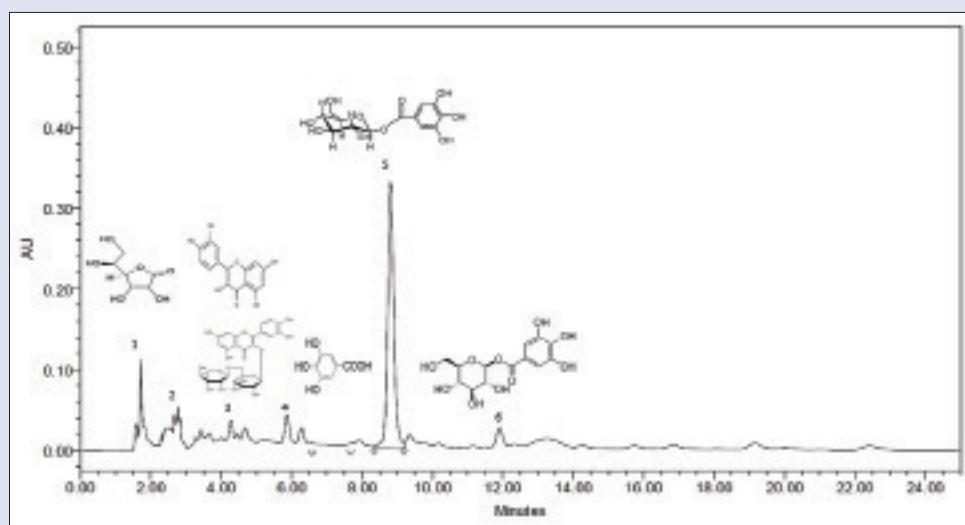
Carrageenan-induced paw edema: MEO significantly ( $P < 0.01$ ) exhibited anti-inflammatory activity in a dose-dependent manner at 4 h. The reduction in carrageenan-induced paw edema by high dosage of MEO (HMEO) after 4 h was 72.71% while edema reduction by the standard drug, diclofenac (10 mg/kg) was 61.57% [Table 1]. MEO produced a dose-dependent inhibition of paw edema, which was comparable to the effect of known anti-inflammatory drug, diclofenac.

### Effect of methanolic extract of *Embolica officinalis* on different cytokine levels

Cytokines are small glycoproteins counteracted in response to an antigen and initially defined as a mediator for regulating the innate and adaptive immune reactions. At the time of inflammation, IL-1 $\beta$  and TNF- $\alpha$  are the two major cytokines, which play a major role in leading mechanisms.<sup>[7]</sup> The levels of these cytokines should be suppressed to reduce the severity of the inflammatory reaction. Table 2 indicates that inflammatory reaction induced by carrageenan significantly increased the concentration of IL-1 $\beta$  and TNF- $\alpha$  ( $P < 0.05$ ). MEO treatment decreased the level of these cytokines significantly as compared to the inflammation-induced group.

## DISCUSSION

Quite a lot of present day diseases are due to the shift in the balance of pro-oxidant and antioxidant homeostasis in the body.<sup>[13,27]</sup> Inflammation is the response to injury of cells and body tissues through different factors such as infections and chemicals and mechanical injuries.<sup>[22,28]</sup> The pathogenesis of inflammation is brought about by free radicals.<sup>[22,29,30]</sup> Plants have many compounds which show different bioactivities, including antioxidant and anti-inflammatory, which are experimented on because of their numerous mechanisms of actions with non-toxic nature.<sup>[19]</sup> Antioxidants interfere with the oxidative processes by scavenging free radicals and chelating free catalytic metals.<sup>[31]</sup> There are numerous NSAIDs existing in the market, but these drugs have been shown to have certain side effects that may cause cardiovascular and gastrointestinal disorders; therefore, it is important to endorse the drugs from natural sources.<sup>[32]</sup>



**Figure 3:** High performance liquid chromatography chromatogram of ascorbic acid (1), quercetin (2), rutin (3), gallic acid (4), mucic acid (5), and beta-glucogallin (6) in methanolic extract of *Embolicea officinalis* fruit

**Table 2:** Cytokines levels IL-1 $\beta$  and TNF- $\alpha$  of experimental animals ( $n=6$ )

	IL-1 $\beta$ (pg/mg protein)	TNF- $\alpha$ (ng/ $\mu$ g protein)
NL	3.2 $\pm$ 0.02	0.2 $\pm$ 0.001
IC	12.4 $\pm$ 0.12	0.48 $\pm$ 0.002
IC + LMEO (200 mg/kg)	7.8 $\pm$ 0.04*	0.39 $\pm$ 0.005*
IC + HMEO (400 mg/kg)	5.3 $\pm$ 0.07**	0.31 $\pm$ 0.006**
IC + diclofenac	4.9 $\pm$ 0.04***	0.28 $\pm$ 0.008**

Values are in means $\pm$ SEM. \* $P$ <0.05 versus inflammatory group; \*\* $P$ <0.01 versus inflammatory group; \*\*\* $P$ <0.001 versus inflammatory group.

IC + LMEO: Control rats treated with 200 mg/ml; IC + HMEO: Control rats treated with 400 mg/ml; IC + diclofenac: Control rats treated with diclofenac; NL: Normal; IC: Carrageenan injected control. SEM: Standard error of mean; IL-1 $\beta$ : Interleukin-1 $\beta$ ; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$

*Embolica* has been used for the treatment of arthritis and other inflammatory diseases.<sup>[32]</sup> A wide variety of *in vitro* methods such as DPPH and ABTS are used to assess radical scavenging ability of certain agents from natural and synthetic sources. DPPH is a stable free radical, and its activity is based on its ability to decolorize from deep purple color at 517 nm to light yellow in the presence of antioxidants forming a stable DPPH molecule.<sup>[33]</sup> MEO showed an effective radical scavenging ability by this assay. ABTS is a blue colored chromophore and upon addition of MEO, it is reduced and this reduction process is concentration dependent. *E. officinalis* is a rich source of Vitamin C, alkaloids, phenolics, flavonoids, and tannins.<sup>[12]</sup> In agreement to this, our study revealed that the MEO has a high level of radical scavenging capacity as evidenced in both DPPH and ABTS assays. This might be because of more phenolic compounds in MEO, which help in scavenging of radicals in the two studied assays and hence attribute to their higher antioxidant activity.<sup>[13,33]</sup> The phosphomolybdate results further confirmed the potent antioxidant activity of MEO, which increased in a concentration-dependent manner.

*In vivo* toxicological estimation was performed to evaluate the extent to which a drug can cause deleterious effects to the cells; these tests are essential and widely accepted to know the optimum drug concentration to be administered. LD<sub>50</sub> of MEO was measured to check lethal toxicity and was found to be 1125 mg/kg BW. Treatment with MEO at a dose of 200mg/kg and 400mg/kg to their respective group of mice for 1 month did not show any toxic side effect. The selection of the 2 doses 200 mg/kg and 400 mg/kg was based on an initial toxicity study where until 1 month after administration of these doses of the extract, the animals were healthy without any visual signs or symptoms of illness (data not shown). Previously, Golechha *et al.*, 2014<sup>[34]</sup> showed that a 300–500 mg/kg dose of *Embolica* has not shown any adverse effect.

Carrageenan induced edema of rat paw is used widely as a working model of acute inflammation in search for new anti-inflammatory drugs.<sup>[35]</sup> During the first 2 h (Phase I), liberation of histamine and serotonin begins in rat paw after carrageenan injection, whereas in Phase II release of kinin, prostaglandin, bradykinin, leukotrienes, and polymorphonuclear cells in the damaged tissue in surroundings cells also increases depending upon the complement system for their activation.<sup>[34]</sup> Results of standard drug diclofenac sodium, normal saline, and two doses of test extracts (LMEO and HMEO) were compared. Both the tested doses of MEO have shown a significant reduction in paw volume as compared to control group; the efficiency of MEO is very much comparable to that of the standard drug ( $P < 0.05$ ).

Our *in vivo* results were strongly in accordance to previous studies.<sup>[36]</sup> In case of infection, inflammation, and immune activation, IL-1 $\beta$  and TNF- $\alpha$  play a major role. Sometimes, TNF- $\alpha$  can also stimulate the release of IL-1 $\beta$ .<sup>[4]</sup> The levels of IL-1 $\beta$  and TNF- $\alpha$  of experimental animals showed a significant decrease after administration of MEO as compared to the

saline-treated animal group. Rats treated with high dosage (400 mg/ml) showed more potent effect than lower dosage (200 mg/ml) which is very much comparable to the standard drug.

The role of phenolic compounds in inflammatory conditions is well established.<sup>[37,38]</sup> They also inhibit polymorphonuclear lipoxigenase, an enzyme involved in inflammatory conditions.<sup>[37]</sup> In this context, *E. officinalis* has caught the attention of the researchers as it is a potential source of phenolic compounds and flavonoids.<sup>[36]</sup> These effects may be due to high levels of quercetin, rutin, and other phenolic compounds found in the MEO.<sup>[23,28]</sup>

## CONCLUSIONS

In summary, the results of this study suggest that MEO not only is a storehouse of natural antioxidant but also possesses strong anti-inflammatory activity which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases. The anti-inflammatory activity of MEO may probably be due to the presence of several bioactive anti-inflammatory principals as elucidated by HPLC analysis. However, it needs isolation, structural elucidation, and screening of any of the various active principle (s) to point out the real activity of the drug. Even, an extensive clinical study needs to be hypothesized to ascertain the efficacy and safety of this agent to illustrate an improvement over currently available treatment.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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