Evaluation of anti-inflammatory activity of *Dracaena cinnabari* Balf. f. resin

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Dracaena cinnabari Balf. f. (Family- Dracaenaceae) resin is a traditional medicine since ancient times in many cultures. Most of the pharmacological investigations of this resin have been addressed to its anti-microbial, anti-viral and antioxidant activities. However, anti-inflammatory activity of *D. cinnabari* resin is not evaluated so far. The present study indicates that methanol extract of *D. cinnabari* resin (MEDC) and one of its components, 4'-hydroxy-7,8-methylenedioxyhomoisoflavan (MHF) inhibited nitrite, TNF-α and IL-6 productions in lipopolysaccharide-stimulated mouse macrophage cell line RAW 264.7 with increase in their concentrations. Anti-inflammatory activities of these treatments were further confirmed by the reduction of rat hind paw edema. These results suggest that MEDC and MHF have potential anti-inflammatory activity at the selected doses.

Introduction

Inflammation is an innate immunological process that occurs in response to trauma, infection, tissue injury or noxious stimuli[1]. It is characterized by the increase in the production of various inflammatory mediators like nitric oxide (NO), prostaglandin and cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ)[2]. Overproduction of these inflammatory mediators is implicated in many diseases, such as rheumatoid arthritis, atherosclerosis, chronic hepatitis and pulmonary fibrosis[3-6] and is the major target for the treatment of inflammatory disorders[7]. Thus, inhibitors of these mediators have been considered as potential candidates of anti-inflammatory drugs. Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) which have various negative side effects[8-9]. Hence, there is a need to develop new drugs that can generate desirable therapeutic effect with considerably less side effects.

Several natural products are being used for treating various diseases from time immemorial. In the recent past, there have been many research studies on natural products with anti-inflammatory activity, for example, *Polygonum tinctorium* Lour. (Polygonaceae)[10], *Melia azedarach* L. (Meliaceae)[11], *Cyperus rotundus* L. (Cyperaceae)[12], ginsenoside Rg3[13], sauchinone[14-15] and *Ruta graveolens* L. (Rutaceae)[16]. Herbal medicines derived from plant extracts are increasingly being used to treat a variety of pathological conditions. Several studies have suggested the inhibitory role of natural products on the NO production[17-19]. Natural product-based anti-inflammatory agents with a good efficacy and lower toxicity can offer promising treatment and prevention from inflammation driven pathological conditions. Dragon's blood is a name applied to many red resins described in the medical literature. A number of bioactive compounds belonging to the class of flavanoids, homoisoflavanoids, chalcones, sterols and terpenoids have been isolated from *D. cinnabari* resin[20]. It has a wide range of medicinal uses such as haemostatic, anti-diarrhetic, anti-ulcer, anti-microbial, anti-viral, wound healing, anti-tumor, anti-inflammatory, antioxidant[20-24]. Despite the wide use of *D. cinnabari* resin in folk medicine, no study has been reported about its anti-inflammatory activity.

The present study describes the effect of *D. cinnabari* resin and one of its homoisoflavans on the production of well established pro-inflammatory markers in LPS-stimulated mouse macrophages (RAW 264.7).

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Further, *in vivo* experiments using λ-carrageenan-induced acute paw edema rat model were carried out to demonstrate strong anti-inflammatory activities of resin extract and one of its constituent homoisoflavanoids.

Materials and Methods

Chemicals

Growth media [Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine] and antibiotic–antimycotic solution were purchased from HiMedia Laboratories (India). Fetal bovine serum, trypan blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell culture grade dimethylsulphoxide (DMSO), Griess reagent, lipopolysaccharide (LPS) and λ-carrageenan were the products of Sigma–Aldrich Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were procured from Diaclone (France). All other reagents, chemicals and solvents were of analytical grade and were obtained from Sigma Chemicals Co. USA. Phosphate buffered saline (PBS) and other reagents were prepared according to protocol.

Plant materials

Dragon's blood resin was purchased from whole sale supplier of traditional Unani medicine, Ballimaran, Delhi, India in August 2008 and identified as *Dracaena cinnabari* Balf. f. resin belonging to family Dracaenaceae. A voucher specimen (NISCAIR/RHMD/Consult/2008-09/1069/100) has been deposited at NISCAIR, New Delhi, India.

Preparation of plant extract and isolation of active compound

The resin extract was prepared as previously described[22]. Compound 4'-hydroxy-7,8-methylenedioxy-homoisoflavan (MHF); m.p. 140°C, was isolated as pure crystals in one of the active fractions of methanol extract of resin (MEDC) upon repeated column chromatography over silica gel as described by Suchý *et al*[25]. The structure of MHF (Fig. 1) was elucidated by spectral data (MS, NMR recorded at AnalytiCon Discovery, Germany).
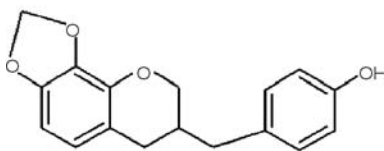

Fig. 1—Structure of MHF (4'-Hydroxy-7, 8-methylenedioxyhomoisoflavan) isolated from MEDC.

Macrophage cell culture

The mouse macrophage cell line RAW 264.7 (obtained from National Centre for Cell Science, Pune, India) was propagated in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum. The cells were incubated in a 37°C incubator with 5% CO_2 and subcultured when plates reached 90% confluence using fresh medium in the cell to medium ratio of 1:5.

Cell viability assay

Cell viability was determined in each experiment using MTT assay[26]. Cells (5×10^5 cells/well) in 96-well plates were incubated with various concentrations of MEDC (25-375 µg/mL) and MHF (2-10 µg/mL). After 24 h of treatment, MTT (100 µL, 5 mg/mL in PBS) solution was added to the medium in each well, and plates were incubated for 3 h at 37°C. The medium was then removed and DMSO (200 µL) was added to each well to solubilize the purple formazan crystals produced by mitochondrial dehydrogenase reduction of MTT. After 5 min of additional incubation, absorbance was measured at 570 nm on a microplate reader. The data were expressed as percent cell viability as compared with control (DMSO).

LPS stimulation of macrophages

Cells were seeded at a density of 5×10^5 cells/mL in 96-well plates 12 h prior to treatment. The cells were then treated with MEDC (5-50 µg/mL) or MHF (0.5-2 µg/mL) for 2 h before stimulating with LPS (0.5 µg/mL for 24 h). For every set of experiment, one positive control (cells treated only with LPS) and one negative control (cells without any treatment) were kept. Three independent replicates were made for both experimental and the controls.

Nitric oxide assay

As an indicator of NO production, nitrite concentration in the culture medium was determined by Griess reagent[27]. The culture supernatant (100 µL) was mixed with Griess reagent (100 µL) for 10 min, and then the absorbance of the chromophoric azo-derivative molecule was measured on a microplate reader at 550 nm. Fresh culture medium was used as the blank in all experiments. A range of dilutions of sodium nitrite was used to plot a standard curve.

Cytokine analysis

The levels of TNF-α and IL-6 in the cell free supernatants were quantified using ELISA kits according to the manufacturer's instructions. Culture

supernatants were collected after 4 and 6 h of LPS treatment for TNF-α and IL-6 analysis, respectively. The minimum detectable concentrations of ELISA kits for TNF-α and IL-6 were typically less than 25 and 10 pg/mL, respectively.

Animals

Wistar strain of albino rats (150-200 g) of either sex were used for animal experiments. The animals were housed in standard metal cages under controlled environment and provided with standard animal food (Amrut laboratory animal feed, Mumbai, India) and water *ad libitum*. Experimental rats were adapted to the standard animal house facilities. Animals were sanctioned and issued after the approval of the Animal Experimentation Ethics Committee of the Institute. Experiments were conducted strictly according to Indian National Science Academy ethical guidelines for use of animals in scientific research as prescribed by Institute of Genomics and Integrative Biology, CSIR, Delhi, India.

Rat paw edema study

Samples were administered in either oral (p.o.) or intraperitoneal (i.p.) mode. Rats were divided into eight groups, with each group containing five animals.
Group 1: Untreated control (50% DMSO, 0.5 mL)
Group 2: Indomethacin (10 mg/kg, p.o., standard)
Group 3: MEDC (250 mg/kg, p.o.)
Group 4: MEDC (500 mg/kg, p.o.).
Group 5: Indomethacin (5 mg/kg, i.p., standard)
Group 6: MEDC (100 mg/kg, i.p.)
Group 7: MEDC (250 mg/kg, i.p.)
Group 8: MHF (10 mg/kg, i.p)

Orally administered groups were treated with samples, 1 h prior to λ-carrageenan injection. While, intraperitoneally administered groups were treated with samples, 30 min prior to the λ-carrageenan injection. DMSO solutions of MEDC or MHF were diluted in normal saline for animal treatment.

Prior to the start of the experiment, body weight of animals was recorded individually for evaluating proper treatment dosage and animals were randomly divided into groups of five. Food was withdrawn 18-24 h before the experiment, water was allowed *ad libitum*. Initial paw sizes (basal volume) were measured by volume displacement method using a digital plethysmometer. Paw edema was induced by subcutaneous injection of 100 µL 1% λ-carrageenan solution (in 0.9% NaCl) in the plantar surface of the right hind paw of rat[28]. The paw volume was measured at 1, 3, 5 and 24 h after λ-carrageenan injection by dipping the paw into mercury column up to the knee joint to record water displacement using plethysmograph. After λ-carrageenan injection initial 1-3 h constitute early phase, while 3-5 h period was termed as late phase. A significant reduction in the paw volume compared to untreated control animals was considered as the *in vivo* anti-inflammatory response.

Statistical analysis

Statistical analysis was made by Student's t test using three independent sets of each experiment. Results are expressed as mean ± SD for n=3 (*in vitro*) or n = 5 (*in vivo*). $p < 0.05$ is considered statistically significant.

Results

Cytotoxicity of MEDC and its component MHF on RAW 264.7 cells

Cytotoxic effects of MEDC and its component MHF were evaluated by MTT assays in the concentrations range of 25 to 375 µg/mL and 2 to 10 µg/mL, respectively using RAW 264.7 cells. Experimental results indicated that the MEDC was not cytotoxic to this cell line up to 200 µg/mL (Fig. 2a) while its component MHF did not show cytotoxity even at 10 µg/mL concentration as shown in the Fig. 2b.

Anti-inflammatory activity of MEDC to LPS stimulated RAW 264.7 cells

RAW 264.7 mouse macrophage cell line was used as *in vitro* model of macrophage-mediated inflammatory events in response to LPS, to assess the effect of MEDC on nitrite synthesis. Nitric oxide (NO) synthesized by activated macrophage cells regulates the functions of other cells involved in the inflammatory process and appears to act as a secondary mediator of some actions of pro-inflammatory cytokines. When cells were stimulated with LPS for 24 h, the levels of nitrite, a stable oxidized product of NO, increased significantly in the culture medium whereas, unstimulated macrophages produced undetectable level of nitrite. MEDC was able to significantly inhibit the LPS induced nitrite production at 5, 10, 25 and 50 µg/mL by 35, 59, 62 and 65 %, respectively (Fig. 3a). However, at higher concentration MEDC did not show further increase in nitrite inhibition.

Anti-inflammatory activity of MEDC on LPS stimulated macrophages was further evaluated by
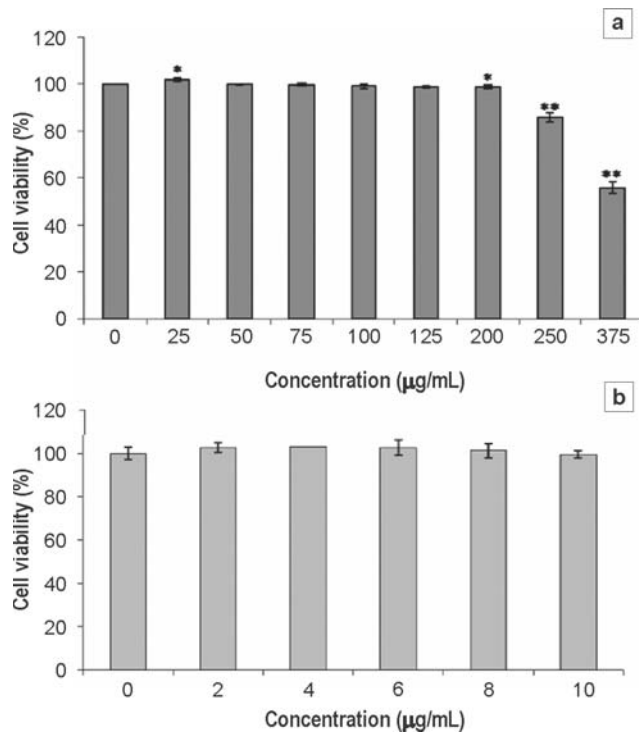
Fig. 2—(a) Effect of MEDC and (b) its component MHF on RAW 264.7 cell viability. Independent experiments using 5×10^5 cells/mL cells were repeated thrice. Results are presented as mean ± S.D. *p < 0.05 and **p < 0.005 represent statistically significant cell death (%) as compared with the control group.

estimating the released pro-inflammatory cytokines TNF-α and IL-6. Like nitrite, release of both of these cytokines decreased on increasing the extract dosage, however, the inhibition of TNF-α release was more prominent than that of IL-6 as shown in the Figs. 3b & 3c. Unstimulated cells produced 0.151±0.024 ng/mL of TNF-α and 0.005±0.002 ng/mL of IL-6 while on stimulation with LPS release of TNF-α and IL-6 were increased to 2.137±0.03 ng/mL and 0.714±0.029 ng/mL, respectively.

In the presence of MEDC at concentrations, 5, 10, 25 and 50 µg/mL, significant inhibition of TNF-α release by 26, 54, 74 and 77%, respectively (p <0.005) was observed (Fig. 3b). Similarly, inhibitions of pro-inflammatory IL-6 by MEDC with increasing concentrations (5, 10, 25 and 50 µg/mL) were observed to be 26, 29, 31 and 46% inhibition, respectively (p < 0.005) (Fig. 3c).

MHF as an anti-inflammatory component of MEDC

LPS induced nitrite and pro-inflammatory cytokines TNF-α and IL-6 production in cells was monitored, both in the presence and absence of MHF. Results in the Fig. 4 indicate that MHF is about 10 times more
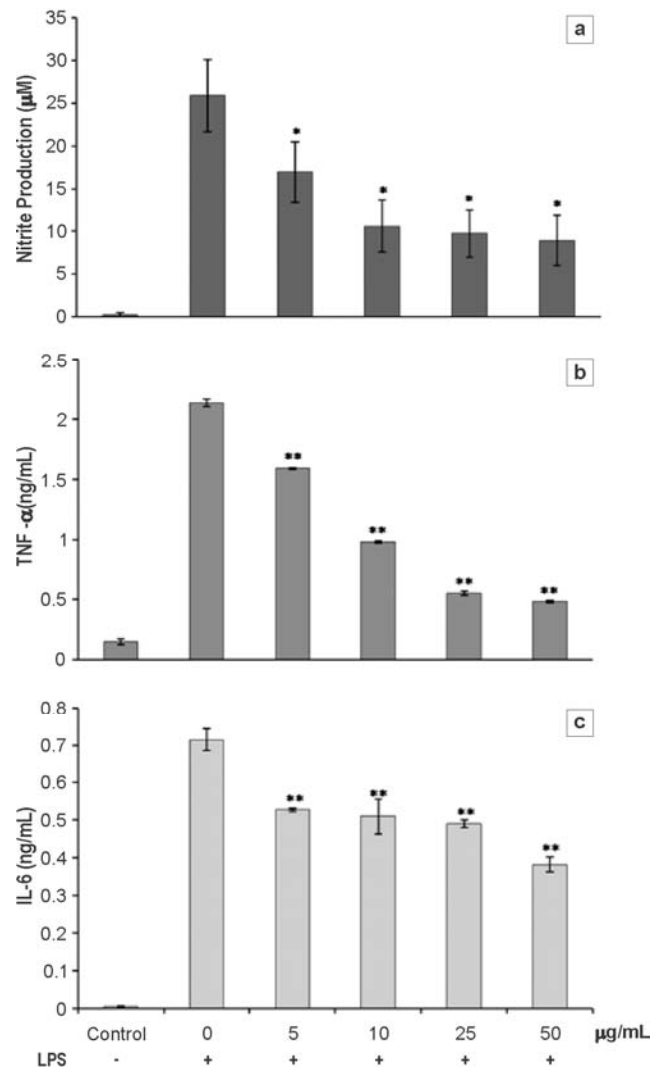

Fig.-3—*In vitro* anti-inflammatory properties of MEDC in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with indicated concentrations of MEDC for 2 h and then incubated with LPS (0.5 µg/mL) at 37º C in a 96-well plate. (a) Nitrite level in the medium of RAW 264.7 macrophages cultured with LPS for 24 h was monitored using the Griess reaction, (b) TNF-α and (c) IL-6 levels were measured in culture media after 4h and 6 h of LPS treatment, respectively. Data are expressed as the mean±S.D. of three individual experiments. *p<0.05 and **p<0.005 represent statistically significant change as compared with control groups without treatment.

potent inhibitor of nitrite, TNF-α and IL-6 than MEDC. These results suggest that MHF could be one of the active anti-inflammatory components of MEDC. MHF at concentrations of 0.5, 1.0 and 2.0 µg/mL inhibited nitrite production by 5, 38 and 69%, respectively (Fig. 4a). MHF at 2.0 µg/mL conc inhibited TNF-α and IL-6 productions by 90% and 79% (p < 0.005), respectively (Fig. 4b, 4c).
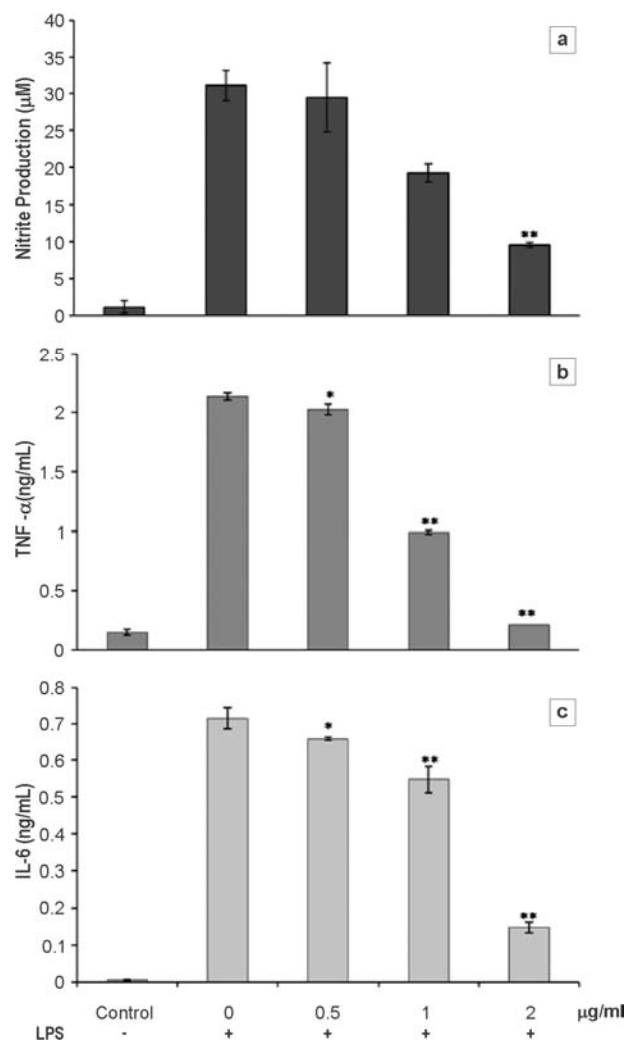
Fig. 4—*In vitro* anti-inflammatory activities of MHF in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with indicated concentrations of MHF for 2 h and then incubated with LPS (0.5 µg/mL) at 37º C in a 96-well plate; (a) Nitrite level in the medium of RAW 264.7 macrophages cultured with LPS for 24 h was monitored using the Griess reaction; (b) TNF-α and (c) IL-6 levels were measured in culture media after 4h and 6 h of LPS treatment, respectively. Data are expressed as the mean±S.D. of three individual experiments. *p<0.05 and **p<0.005 represent statistically significant change as compared with control groups without treatment.

In vivo **anti-inflammatory activity**

Anti-inflammatory activity *in vivo* is often characterized by the reduction of paw edema in experimental rat model. Indomethacin is an established drug used to resist λ-carrageenan induced paw edema of rats. Our results in Table 1 indicate that both MEDC and MHF effectively reduced hind paw edema of rats in dose and time dependent manner. Further, these results also indicate that anti-inflammatory effect of MHF is

Table 1—Anti-inflammatory effect of MEDC and isolated compound MHF on λ-Carrageenan induced paw edema

Treatment	Increase in paw volume (mL)		
	1h	3h	5h
Untreated control	0.32±0.04	0.82±0.04	0.87±0.03
Indomethacin (10 mg/kg, p.o.)	0.14±0.01**	0.21±0.02**	0.13±0.02**
MEDC (250 mg/kg, p.o.)	0.24±0.02*	0.82±0.03	0.62±0.03**
MEDC (500 mg/kg, p.o.)	0.22±0.01*	0.47±0.04**	0.41±0.01**
Indomethacin (5 mg/kg, i.p.)	0.10±0.01**	0.22±0.04**	0.14±0.03**
MEDC (100 mg/kg, i.p.)	0.16±0.01**	0.57±0.02**	0.46±0.03**
MEDC (250 mg/kg, i.p.)	0.07±0.01**	0.19±0.02**	0.10±0.03**
MHF (10 mg/kg, i.p.)	0.23±0.02*	0.43±0.01**	0.42±0.04**

Values are mean ± S.D. Sample size = 5; *p < 0.05; **p < 0.005; significance of any treatment was determined with respect to untreated control.

much higher than that of MEDC. Untreated control rats when injected with λ-carrageenan, respectively, 26, 67 and 71% increase in paw volume was observed after 1, 3 and 5 h post-treatment. As shown in Table 1, animals pre-treated with indomethacin (10 mg/kg, p.o.), 1 h before λ-carrageenan injection showed only marginal increase in paw edema, during both early as well as late phases of inflammation. MEDC when administered orally at 250 and 500 mg/kg, showed gradual increase in paw edema at 1 and 3 h. More than 60% inhibition of paw edema was observed in 500 mg/kg MEDC treated group, at 5 h.

Intraperitoneal administration of MEDC (100 mg/kg), 30 min prior to the λ-carrageenan injection, inhibited edema during first hour but was unable to sustain it for longer (Table 1). MEDC (250 mg/kg, i.p.) treated groups showed inhibition of paw edema greater than that of standard indomethacin (5 mg/kg, i.p.) which is highly significant (p < 0.005) when compared to control group of animals.

Isolated compound MHF (10 mg/kg, i.p.) also exhibited paw edema inhibitory activity (Table 1). MHF acted effectively in the early phase by inhibiting the inflammation by 79 %. MHF exhibited more than 60 % inhibition during 3-5 h of the inflammation process. All test groups recovered edema at 24 h.

Discussion

The inflammatory disorders are very much associated with the oxidative stress generated by the

immune cells present in the milieu. Excessive production of NO by inflammatory macrophages has been reported to mediate chronic and acute inflammatory diseases, such as rheumatoid arthritis and sepsis[29-30]. In our earlier study, MEDC of resin was observed to have superoxide and NO scavenging activities *in vitro*[21]. Whole resin methanolic extract significantly inhibited the nitrite and cytokine release, in LPS challenged murine macrophage cells. *In vitro* structure-related antioxidant activities of MHF and MEDC may also contribute to their anti-inflammatory properties. Previously, MHF was reported to exhibit a strong antioxidant activity due to its 4'-hydroxy and 7, 8-methylenedioxy groups[31].

Apart from oxidative stress, pro-inflammatory cytokines such as TNF-α and interleukins are produced by macrophages, monocytes and lymphocytes in response to inflammation, infection and injury. TNF-α plays critical role in activation and chemotaxis of leukocytes by inducting expression of adhesion molecules on neutrophils and endothelial cells and regulates secretion of pro-inflammatory cytokines[32]. Production of TNF-α is critical for the synergistic induction of NO synthesis in LPS-stimulated macrophages[33]. Further, TNF-α induces inflammation by stimulating IL-6 synthesis in several cell types. In the present study, MHF at 2 µg/mL showed marked decrease in the level of TNF-α, as compared to untreated LPS-stimulated cells, which is also supported by decrease in nitrite level. This behavior can be explained as pro-inflammatory cytokine TNF-α act as strong stimulator and/or co-stimulator of inducible nitric oxide synthase (iNOS) in certain cells[34].

Furthermore, IL-6 is often used as a marker for systemic activation of pro-inflammatory cytokines implicated in many infectious and inflammatory states[35-37]. Some of the regulatory effects of IL-6 involve inhibition of TNF-α production, providing negative feedback for limiting the acute inflammatory response. In this study, MHF exhibited great inhibition to IL-6 release at 2 µg/mL thus exerting anti-inflammatory effects.

It is well illustrated that the cytotoxicity of MEDC or MHF is much above than the highest dosages showing *in vivo* and *in vitro* anti-inflammatory activities. Therefore, inhibitory effects on nitrite production and cytokine release were not due to cytotoxicity, but rather due to anti-inflammatory properties of MEDC or its constituent MHF.

The λ-carrageenan-induced paw inflammation is a useful tool for investigation of systemic anti-

inflammatory agents and comprises of two phases. The early phase (1-3 h) of the inflammation is mediated by histamine, serotonin and similar substances; the late phase (3-5 h) is attributed to the activation of kinin-like substances, such as prostaglandins, proteases and lysosome[33]. The *in vivo* inhibitory effects on λ-carrageenan-induced paw edema in wistar rats using the methanolic extract and active compound isolated from the resin clearly demonstrate the anti-inflammatory activity of the resin and the isolated compound, with no obvious loss in growth or appetite in any group. In this study, the effect of mode of administration was also observed. MEDC was bioavailable when orally given in rats and showed pharmacological activity 5 h after λ-carrageenan injection. Intraperitoneally administered doses of MEDC inhibited λ-carrageenan induced inflammation during both early and late phases which could be explained on the basis of their rapid bioavailability. MEDC at dose of 250 mg/kg i.p. demonstrated similar efficacy to that of indomethacin *in vivo*, significantly reducing induced paw edema.

Flavonoids are known to target prostaglandins which are involved in the late phase of acute inflammation[38]. Therefore, we presume that the presence of flavonoids may be contributing to anti-inflammatory activities of MEDC. However, MHF, showed more inhibition to paw edema at early phase followed by reduction in inhibition at late phase. This action could be due to the degradation/transformation of the compound into inactive byproducts with time. These data indicate *D. cinnabari* resin may be an excellent candidate for development as a therapeutic agent for inflammation. Hence, the present study with *D. cinnabari* resin supports its traditional use as anti-inflammatory and wound healing agent.

MHF is one of the active principles of *D. cinnabari* resin and has great potential to be an anti-inflammatory therapeutic agent subjected to further exploration of the mechanisms of iNOS induction and cytokine release.

Acknowledgements

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