



An Experimental Exploratory Study for the Mechanism of Anti-Inflammatory Action of Mecca Myrrh (*Commiphora opobalsamum*)

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAAS performed the entire experimental study, collected and analyzed the data and performed the statistical analysis and wrote the first draft of the manuscript. Author MAAASA designed the study and developed the protocol and wrote the first draft of the manuscript. Authors MAAASA and LMK managed the analyses of the study.

Author LMK managed writing the final manuscript and gave the final shape to final. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i29A31574

Editor(s):

(1) Dr. Rahul S. Khupse, University of Findlay, USA.

Reviewers:

(1) Zeinab Hassanein Fahmy, Egypt.

(2) Wael Alaghawani, AlJazeera University, Syria.

(3) Dayaben L. Chothani, B. K. Mody Government Pharmacy College, India.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/68390>

Original Research Article

Received 10 March 2021

Accepted 17 May 2021

Published 20 May 2021

ABSTRACT

Background: Our recent past studies accomplished the target to investigate the anti-inflammatory effect as well as toxicological profile of *Commiphora opobalsamum* (CO), with almost identical potency in comparison to the contemporary anti-inflammatory drugs. This inspired us to explore its mechanism of action to further strengthen its efficacy.

Aim: To investigate the mechanism of anti-inflammatory action of CO by exploration of its correlation to its antioxidant activity as well as inhibitory effect on inflammatory mediators by interaction with MDA, NO, PGE₂ and TNF- α .

Methods: 10 weeks old male Swiss albino mice (30 to 40 g) were used. Carrageenan-induced paw edema method was used, pretreatment with CO alone in different doses and in combination with diclofenac was done prior to carrageenan administration, subsequently homogenate of the

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paw was used to quantify the levels of MDA, NO, PGE₂ and TNF- α by using their specific assays. In addition, Histological examination of edema paw was performed to evaluate the anti-inflammatory effect of CO extract versus diclofenac and control investigating their impact on the inflammatory cell migration and edema formation.

Results: CO extract in the dose of 500mg/kg demonstrated maximum reduction of MDA level; hence antioxidant activity of CO could be contributed to its anti-inflammatory effect. Furthermore, notable observation indicated that CO administration significantly suppress increment in NO level in response to carrageenan, unequivocally significant inhibition of PGE₂ accumulation at the site of inflammation by the pretreatment of CO extract was observed in the dose of 500mg/kg (0.001). Finally in contrast, the CO extract in the dose of 500mg/kg significantly accomplished the reduction of TNF- α in the paw in comparison to the control group ($p < 0.05$).

Conclusion: In this study we have illustrated a pioneering perspective to elucidate the mechanism of anti-inflammatory and antioxidant action of methanolic extract of CO, attributed through suppression of MDA, NO, PGE₂ and TNF- α at the site of inflammation.

Keywords: *Commiphora opobalsamum*; non-steroidal anti-inflammatory drugs; prostaglandin; prostaglandin E₂; malondialdehyde; tumor necrosis factor –alpha; nitric oxide.

1. INTRODUCTION

The basic essence of pathophysiology of inflammation reveals that our body respond by releasing certain chemicals in response to the noxious agents, infections, and physical injuries to counteract their deleterious effects, however, if inflammation is uncontrolled it can become a cause of suffering, leading to disabilities, contractures, disfiguring of body, and chronic pain. In such situations, the inflammation needs to be controlled or suppressed [1,2]. It is highly imperative to emphasize that the potent desirable anti-inflammatory effect produced by the cortisol and none of the undesirable effect of cortisol remains elusive using contemporary drugs. In essence, glucocorticoid inactivates the pro-inflammatory transcription factors such as NF-KB and activator protein1-AP-1, thus preventing the release of vital inflammatory mediators like prostaglandin, leukotrienes, cytokines, and platelet activating factor. [3-6].

A quite distinguishing as well as disheartening feature of currently used steroidal and Non-steroidal anti-inflammatory drugs (NSAIDs) therapy is characterized by major threatening and fluctuating grades of gastrointestinal, renal, cardiovascular, and hepatic hazards, consequently, the treatment guidelines basically recommend that the use of NSAIDs should be done in the lowest possible dose and long-term use should be restricted wherever applicable [7-10]. Hence, the development of novel anti-inflammatory agents is highly imminent, preferably from the natural herbal resources characterized with minimum side effects to substitute the current available drugs [7,8]. We tried to put together an extensive research effort

in the recent past to investigate the anti-inflammatory effect as well as toxicological profile of *Commiphora opobalsamum* (CO) with the intention to unearth a safe, protective, and potent alternative from the natural resources and these studies provide distinct characteristics for the basic requirement of an ideal anti-inflammatory drug [11,12].

It needs to be emphasized that causation is quite intricate to ascertain than correlation, our keen interest is to securitize and offer a notion of the fundamental anti-inflammatory mechanism action of CO, this motivates us to further extend our studies with the prime objective to investigate the mechanism of the recognized anti-inflammatory properties of CO by exploration of its interaction and correlation to antioxidant activity as well as inhibitory activity against the free radicals and inflammatory mediators such as Malondialdehyde (MDA), Nitric oxide (NO), Prostaglandin E₂ (PGE₂) and Tumor necrosis factor –alpha (TNF- α).

2. MATERIALS AND METHODS

Animals: This experimental work was performed by using 10 weeks old male Swiss albino mice (30 to 40 g), the animals were purchased from the King Fahad Research Center, King Abdulaziz University, Jeddah, Saudi Arabia, The animals were kept under the optimum laboratory environment (at the temperature 25 \pm 5 $^{\circ}$ C, relative humidity of 30-70% and 12/12h light and dark automated cycles) for a minimum period of one week prior to the commencement of experiments. The experimental animals were housed in transparent plastic cages (six rats in each cage) with water and food supply ad

libitum. The experimental procedures were approved by KFMRC and conducted according to their guidelines, and permission of the institutional ethical committee were acquired prior to the initiation of the experimental study.

2.1 Plant Material and Extraction

The aerial parts of CO was collected from Az-zemah, KSA (from a village located between the city of Taif and Makkah, KSA) (Herbarium number: 21.618466, 40.107040), its taxonomical recognition was performed in the department of natural product & alternative medicine, college of pharmacy, KAU, KSA. Extraction of the plant was executed by the procedure illustrated by Sawant S. B. et al 2014 [13]. Subsequently, the stem of the plant and its aerial part were stored carefully in a suitable ventilated dry place under controlled temperature of 30-40°C for a period of 5 days to allow natural drying and then mechanically grinded to powder form (900 gm) this was followed by subjecting it to soak in 99% w/v methanol at controlled room temperature (25±30°C) for a period of 48 hours followed by extraction four times. Then it was subjected to double filtration to remove the fine particles using cotton and filter papers. Additionally, by the utilization of rotator evaporator (Buchi®, Schweiz) evaporation of the filtrate was done, to get rid of even minor amount of methanol. Ultimately, the final product is acquired which is moist in nature and it was kept in the refrigerator at -80°C approximately for one hour, then it was subjected to overnight drying in a freeze vacuum dryer (Zirbus ®German) at 850C. The final product of approximately 100gm was stored carefully in a light protective container, kept in a refrigerator under controlled temperature. The administration of the extract was done by preparing a suspension in 0.9% saline solution immediately prior to administration and the volumes administered were adjusted to 10 ml/kg body weight of the mice.

2.2 Experimental Methods

2.2.1 Models used for evaluation of acute anti-inflammatory activity

2.2.1.1 Carrageenan-induced paw edema in mice

The anti-inflammatory effect of CO was evaluated against the experimental model of acute inflammation using the carrageenan-induced paw edema test performed according to the reported technique [14]. The edema effect

usually raised to a maximum during the 3rd to 5th h [15]. Therefore, the anti-inflammatory effect was evaluated 4h after carrageenan injection.

Procedure

The edema was induced by injection of 20 µL of 1% w/v carrageenan (in saline prepared 1h before the test) into the sub-plantar surface of the right hind paw of mice. A total of thirty male mice randomly selected and equally divided into five groups. After 12h fasting and 30 min before carrageenan injection, 0.9% saline solution (10mL/kg) was given intra-peritoneally (i.p.) to mice in the first group while CO extract was given i.p. to another two groups with doses of (250 and 500 mg/kg). The fourth group was treated i.p. with a combination of CO extract (125 mg/kg) and diclofenac (12.5mg/kg). For comparison, mice in fifth group were treated i.p. with diclofenac (25mg/kg). Hand screw steel micrometer was used to measure the paw edema immediately prior to the edema induction and 1, 2, 3 and 4h thereafter. The reading was expressed in millimeter (mm) and the increment in paw thickness at different intervals determined the inflammatory response observed as formation of edema in paw. The increase in paw thickness was calculated by subtracting the initial paw thickness to the paw thickness measured at each time point and considered as a criterion for assessment of the degree of edema.

2.3 Data Analysis

The anti-inflammatory effect and the enhancement activity of CO extract versus diclofenac and control were observed as percent inhibition of edema, calculated as the reduction in paw edema between control and treated groups. The inhibition of edema calculated by the following equation:

$$\% \text{ Inhibition} = 1 - (Tt / Tc) \times 100$$

Where, Tc and Tt represent mean increase in paw thickness in control and treated groups.

2.4 Histological Examination of Carrageenan-induced Paw Edema in Mice

Histological examination of paw edema was performed to evaluate the anti-inflammatory effect of CO extract versus diclofenac and control through investigating their impact on the inflammatory cell migration and edema

formation. The histological preparation and examination of edema paw was conducted according to method described in earlier research [16] (Ma et al., 2013). The intra-plantar injection of 1% carrageenan into the mouse paw produced an intense edema, characterized by epithelial and conjunctive tissue blisters and infiltrates of inflammatory PMNs, mainly neutrophils [14]. The edema usually raised to maximum during the 3rd to 5th h of carrageenan injection [15]. Therefore, the histological examination was conducted after 4h of carrageenan injection.

2.5 Methods of Mechanism of Action

The anti-inflammatory properties of methanolic extract of CO could be related to its antioxidant activity as well as its inhibitory effect on NO, PGE2 and TNF- α .

2.6 Investigation of Mechanisms Underlying the Anti-inflammatory of Action

The mechanism of the anti-inflammatory action of CO extract was investigated in carrageenan-induced paw edema model by evaluating MDA, NO, PGE2 and TNF- α levels. The mice used in carrageenan-induced paw edema model that treated i.p. with 0.9% saline, diclofenac (25 mg/kg), CO extract (250 mg/kg), CO extract (500 mg/kg) and combination of CO extract (125 mg/kg) and diclofenac (12.5mg/kg) were sacrificed 4h after the carrageenan injection and the right hind paw tissue was then dissected. The residual mice paw pieces were weighted, snap frozen in liquid nitrogen and homogenized on ice in (10% w/v) phosphate buffer saline (0.1 M PBS, pH = 7.4) by homogenizer. Then the homogenates were centrifuged at 9000 X g for 20 min at 4°C. The supernatants were obtained and stored at -20°C refrigerator for measuring of MDA, NO, PGE2 and TNF- α [16,17].

MDA assay

To examine the possible antioxidant activity of CO extract versus diclofenac and control, the level of MDA was measured. Among the available markers for oxidative stress detection, thiobarbituric acid-reactive substances (TBARS) assay was used to quantify the plasma lipid peroxidation. The effect of CO extract on MDA level in carrageenan-induced paw edema

model was evaluated by colorimetric method described by [16,18] using MDA assay kit.

TBARS are naturally present in biological specimens and increase in concentration as a response to oxidative stress. TBARS assay values are usually reported in MDA equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. This assay is based on the reaction of a chromogenic reagent, thiobarbituric acid (TBA), with MDA at 25°C. One molecule of MDA reacts with two molecules of TBA to yield a chromophore with absorbance maximum at 532 nm.

Procedure

The supernatant of right hind paw homogenate of the mice obtained from carrageenan-induced paw edema model was used to measure the MDA level. The assay was conducted according to manufacturer's instructions.

The TBA solution (600 μ l) was added into each vial containing 200 μ l standard or 200 μ l sample. Vials were incubated at 95°C for 60 min then cooled to room temperature in an ice bath for 10 min. Then, 300 μ l n-butanol was added to the extract MDA-TBA adduct from the 800 μ l reaction mixture and 100 μ l of 5M NaCl was added followed by vortexing and centrifugation (3 min at 16000 x g). The n-butanol was evaporated and the MDA-TBA adduct were dissolved in 200 μ l ddH₂O then placed into the 96-well microplate to measure the absorbance at 532 nm. The sample amount of MDA equivalents in nmol were determined by interpolation from the standard curve and the MDA concentration was calculated by the following equation:

$$\text{MDA concentration} = [(A/(\text{mg}))] \times 4 = \text{nmol/mg}$$

Where, A is the sample MDA amount from the standard curve (in nmol); mg is the original tissue amount used; 4 is the correction for using 200 μ l of the 800 μ l reaction mix.

Finally, the levels of MDA in CO-treated groups were compared with those in diclofenac and control groups.

$$\% \text{ inhibition} = [(Lc - Lt) / Lc] \times 100.$$

Where, Lc and Lt represent mean level of MDA in control and treated groups, respectively.

No assay

To clarify the mechanism of anti-inflammatory action of CO extract, the level of NO was determined by colorimetric method [15,19] using NO assay kit that measure the total nitrate/nitrite in a simple two-step process. The first step converts nitrate to nitrite utilizing nitrate reductase. The second step uses Griess reagents to convert nitrite to a deep purple azo compound. The amount of the azochromophore accurately reflects NO amount in samples.

Procedure

The supernatant of right hind paw homogenate of the mice obtained from carrageenan-induced paw edema model was used to determine the NO level. The assay was conducted according to manufacturer's instructions.

The assay buffer (115 μ l) was added to each sample blank (85 μ l) and then, 5 μ l of the nitrate reductase mixture was added to each well followed by 5 μ l of the enzyme cofactor. The plate was covered and incubated at room temperature for 1h to convert nitrate to nitrite and 5 μ l of the enhancer was added to each well and incubate 10 min. After that, 50 μ l of Griess reagent R1 and 50 μ l of Griess reagent R2 were added to each well and incubated for 10 min at room temperature. Then, the absorbance was measured at 540 nm. The sample amount of nitrate equivalents in nmol was determined by interpolation from the standard curve and the NO concentration was calculated by following equation:

$$\text{Nitrate concentration} = Sa / Sv = \text{nmol}/\mu\text{L}$$

Where, Sa is sample amount from standard curve (in nmol); Sv is sample volume added to the assay well (in μ L). Finally, the levels of NO in CO-treated groups were compared with those in diclofenac and control groups.

$$\% \text{ inhibition} = [(Lc - Lt) / Lc] \times 100.$$

Where, Lc and Lt represent mean level of NO in control and treated groups, respectively.

PGE₂ assay

To further clarify the mechanism underlying the anti-inflammatory action of CO extract, the level of PGE₂ was measured by colorimetric method reported by [15] using PGE₂ ELISA kit in which a mouse IgG antibody has been precoated onto 96-well plates.

Procedure

The supernatant of right hind paw homogenate of the mice obtained from carrageenan-induced paw edema model was used to measure the PGE₂ level. The assay was conducted according to manufacturer's instructions.

Standards or samples were added to the wells, along with an alkaline phosphatase (AP) conjugated-PGE₂ antibody. After incubation, the excess reagents were washed away and pNpp was substrate is added and is catalyzed by AP to produce a yellow color. Then, the absorbance was measured at 405 nm. The intensity of the yellow coloration is inversely proportional to the amount of PGE₂ captured in the plate. Finally, the levels of PGE₂ in CO-treated groups were compared with levels in diclofenac and control groups.

$$\% \text{ inhibition} = [(Lc - Lt) / Lc] \times 100.$$

Where, Lc and Lt represent mean level of PGE₂ in control and treated groups, respectively.

TNF- α assay

To further investigate the inquisitive mechanism underlying the anti-inflammatory action of CO extract, the level of TNF- α was measured by colorimetric method reported by [14] using TNF- α ELISA kit. This assay employs an antibody specific for mouse TNF- α coated on a 96-well plate.

Procedure

The supernatant of right hind paw homogenate of the mice obtained from carrageenan-induced paw edema model was used to measure the TNF- α level. The assay was conducted according to manufacturer's instructions.

Standards and samples were pipetted into the wells and TNF- α present in a sample bounded to the wells by the immobilized antibody. The wells were washed, and anti-mouse TNF- α antibody was added. After washing away unbound antibody, HRP-conjugated streptavidin pipetted to the wells. The wells washed again, a TMB substrate solution added to the wells and color develops in proportion to the amount of TNF- α bound. The "stop solution" changes the color from blue to yellow, and the intensity of the color was measured at 450 nm. Finally, the levels of TNF- α in CO-treated groups were compared with levels in diclofenac and control groups.

$$\% \text{ inhibition} = [(Lc - Lt) / Lc] \times 100.$$

Where, Lc and Lt represent mean level of TNF- α in control and treated groups, respectively.

2.7 Statistical Analysis

The statistical analysis of the results of this study was done by using statistical analyzer social science software (SPSS) version 16 (IBM®, USA). Multiple evaluation was performed by the one-way analysis of variance ANOVA), repeated one-way ANOVA and Tukey's *post adhoc* test. Moreover, the computation of statistically significant difference among the mean value was measured at p value of less than 0.05 ($p < 0.05$) and 0.001 ($p < 0.001$). The values revealed in the text and tables were characterized as \pm SEM. The graphs were prepared by the software of GraphPad Prism, version 5.

3. RESULTS

3.1 Paw edema induced by Carrageenan in Mice

Carrageenan induced edema was remarkably diminished by prior administration of CO in the dose of 250 and 500mg/kg body weight. (Fig. 2) Moreover, when CO in the dose of 250mg/kg was compared with control constantly after 1 hr

to 4 hrs *p* value significance was demonstrated as 1 h *p* value < 0.001 , 2 h ($p < 0.05$), 3 h *p* value < 0.001 and 4 h ($p < 0.001$) after carrageenan injection. Candid reflection of the result was equally illustrated CO in the dose of 500 mg/kg. Interestingly, these observation of repression of paw swelling were remarkably significant when compare to effect of diclofenac at 1st ($p < 0.001$) and 2nd h ($p < 0.05$) on prior administration of carrageenan injection.

Moreover, treatment with CO plus diclofenac group (125 and 12.5 mg/kg, respectively) considerably restrained the paw swelling compared with control after 1 h, 2 h, 3 h and 4 h of carrageenan injection ($p < 0.001$) mean % inhibition of 72.72, 70.00%, 73.25 and 74.22%, respectively.

It is also noteworthy to emphasize that the shrinkage in the development of the swelling in the paw were quite evident in contrast to diclofenac at 1st ($p < 0.001$) and 2nd h ($p < 0.05$) after carrageenan administration.

3.2 Histological Examination of Carrageenan-induced Paw Edema in Mice

Histological examination of carrageenan-induced paw edema is revealed in Fig 2. The section 3A revealed that following injection of carrageenan distinct dilation of the capillaries

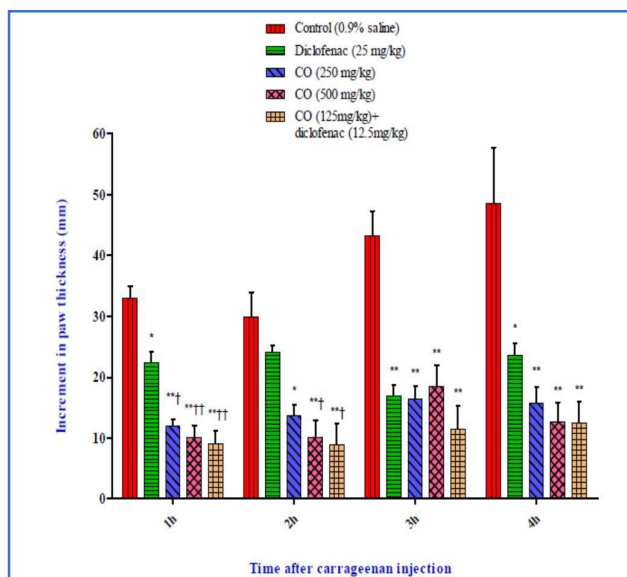


Fig. 1. Effect of CO extract (i.p.) versus diclofenac (i.p.) on carrageenan induced paw edema in mice. * $p < 0.05$, ** $p < 0.001$ compared with the corresponding control group values; † $p < 0.05$, †† $p < 0.001$ compared with the corresponding diclofenac group values; Data are expressed as means \pm SEM; 6 mice/group

and and congestion were observed with concomitant increase in polymorphnucleocytes (PMNs) both in intra and extravascular space. After treatment with CO extract in different doses, remarkably diminished inflammatory reactions, no dilatation of capillaries and congestion and devoid of PMNs cell infiltrate were perceived Fig. 2 C,D and E. Conversely, the dose of 500mg of CO extract, seemingly demonstrated the most potent anti inflammatory response depicting normalisation of both vasculare and connective tissue(Fig. 2D). However, it was pertinent to observe that diclofenac alone or in combination of reduced dose of diclofenac with CO extract revealed quite improvement but less than that observed with CO(500 mg/kg) alone (Fig. 2B, E).

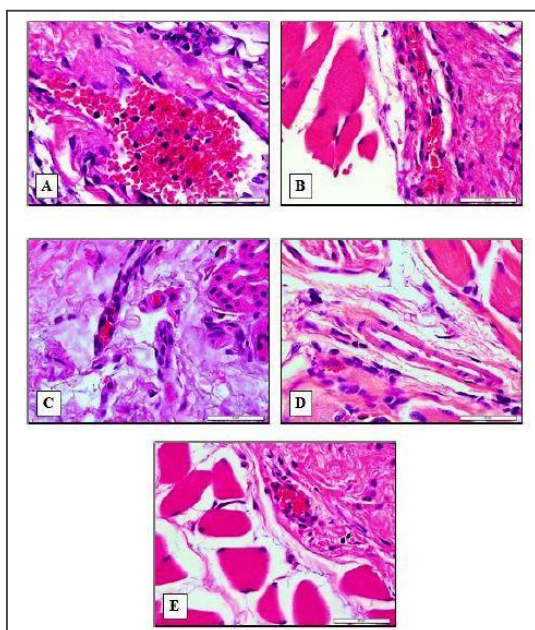


Fig. 2. Histological examination (H&E) of carrageenan-induced paw edema in mice 4h post induction

H&E-stained sections of paw harvested 4h after carrageenan injection. Obvious PMNs infiltration and swelling were seen followed carrageenan injection and congested dilated blood capillaries in paws of control group. After treatment with CO extract at doses of 250 and 500 mg/kg, the edema and PMNs infiltration were significantly reduced, and the paws returned almost normal at the dose of 500 mg/kg. The diclofenac (25mg/kg) group exhibited comparable effect to CO-treated group (250 mg/kg). However, slight improvements in edema and PMNs infiltration

were observed in the paw treated with combination of CO extract and diclofenac. A: Control (0.9% saline); B: Diclofenac (25 mg/kg/day, i.p.); C:CO(250mg/kg/day, i.p.); D:CO(500mg/kg/day, i.p.); E:CO(125 mg/kg/day, i.p.) + diclofenac (12.5 mg/kg/day, i.p.). Observation was made at 100 x magnification.

3.3 Investigation of Mechanisms Underlying the Anti-inflammatory Action

MDA assay

Fig. 3. demonstrated that the lipid peroxidation assay was performed to determine the MDA levels in mice paw homogenate of treated and control groups after 4h of carrageenan injection. MDA level was significantly decreased in paw tissue of mice which was that treated with diclofenac (25 mg/kg), CO (250 mg/kg), CO (500 mg/kg) and combination of CO (125 mg/kg) and diclofenac (12.5 mg/kg) as compared with control group after 4h of carrageenan injection ($p < 0.001$) with means values \pm SEM of 0.114 ± 0.004 , 0.120 ± 0.004 , 0.081 ± 0.008 and 0.123 ± 0.005 , respectively and mean % inhibition of 28.30%, 24.52%, 49.05% and 22.64%, respectively. The results also shown that the CO decreased the MDA level in a dose-dependent manner. Moreover, CO (500 mg/kg) significantly decreased the MDA level in paw tissue of mice 4h of carrageenan injection as compared with diclofenac group (25 mg/kg) ($p < 0.001$).

NO assay

Table 1. Illustrated that the lipid peroxidation assay was performed to determine the NO levels in mice paw homogenate of treated and control groups after 4h of carrageenan injection. NO level was significantly decreased in paw tissue of mice which was that treated with diclofenac (25 mg/kg), CO (250 mg/kg), CO (500 mg/kg) and combination of CO (125 mg/kg) and diclofenac (12.5 mg/kg) as compared with control group after 4h of carrageenan injection ($p < 0.001$) with means values \pm SEM of 0.00599 ± 0.0004 , 0.00678 ± 0.0004 , 0.00415 ± 0.0005 and 0.00619 ± 0.0007 , respectively and mean % inhibition of 35.72%, 27.25%, 55.47% and 33.58%, respectively. The results also shown that the CO decreased the NO level in a dose-dependent manner. Moreover, CO (500 mg/kg) significantly decreased the NO level in paw tissue of mice 4h of carrageenan injection as compared with diclofenac group (25 mg/kg) ($p < 0.001$).

PGE₂ Assay

Fig. 4. showed that the lipid peroxidation assay was performed to determine the PGE₂ levels in mice paw homogenate of treated and control groups after 4h of carrageenan injection. PGE₂ level was significantly decreased in paw tissue of mice which was that treated with diclofenac (25 mg/kg), CO (500 mg/kg) and combination of CO (125 mg/kg) and diclofenac (12.5 mg/kg) as compared with control group after 4h of

carrageenan injection ($p < 0.05$, $p < 0.001$ and $p < 0.05$, respectively) with means values \pm SEM of 3793 ± 529 , 2888 ± 486 and 3476 ± 503 , respectively and mean % inhibition of 22.25%, 40.80% and 28.75%, respectively. While a non-significant reduction of PGE₂ level in paw tissue of mice that treated with CO (250 mg/kg) as compared with control group ($p > 0.05$) with mean values \pm SEM of 4260 ± 354 and mean % inhibition of 12.68%.

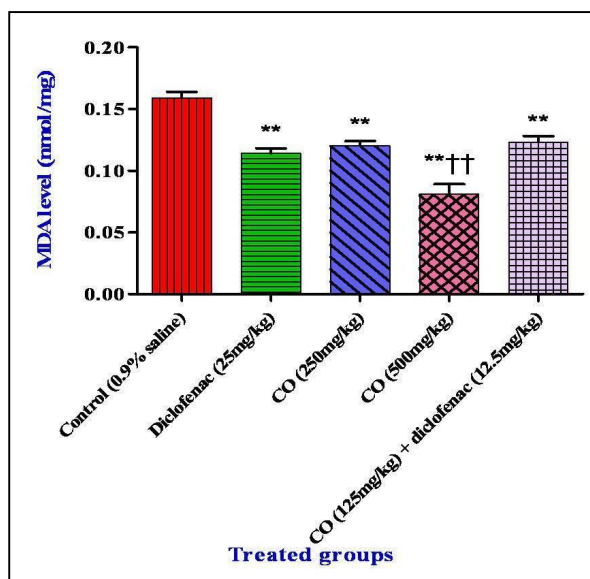


Fig. 3. Effect of CO extract (i.p.) versus diclofenac (i.p.) on MDA level in carrageenan-induced paw edema in mice. * $p < 0.05$, ** $p < 0.001$ compared with the corresponding control group values; † $p < 0.05$, †† $p < 0.001$ compared with the corresponding diclofenac group values

Data is expressed as means \pm SEM; n: Number of animals/ groups; CO: Commiphora opobalsamum; MDA: Malondialdehyde; *compared with control group; $p < 0.05$, ** $p < 0.001$ compared with the corresponding control group values; † $p < 0.05$, †† $p < 0.001$ compared with the corresponding diclofenac group values

Table 1. Effect of CO extract versus diclofenac on NO level in carrageenan-induced paw edema in mice

| Group n = 6 | Parameter measured | NO level (nmol/ μ L) |
|---|---|-------------------------------------|
| Control (0.9% saline, i.p.) | Mean \pm SEM % inhibition [†] | 0.00932 \pm 0.0003 - |
| Diclofenac (25 mg/kg, i.p.) | Mean \pm SEM % inhibition [†] | 0.00599 \pm 0.0004 ** 35.72 |
| CO (250 mg/kg, i.p.) | Mean \pm SEM % inhibition [†] | 0.00678 \pm 0.0004** 27.25 |
| CO (500 mg/kg, i.p.) | Mean \pm SEM % inhibition [†] | 0.00415 \pm 0.0005 ***†† 55.47 |
| CO (125 mg/kg, i.p.) + diclofenac (12.5 mg/kg, i.p.) | Mean \pm SEM % inhibition [†] | 0.00619 \pm 0.0007 ** 33.58 |

Data are expressed as means \pm SEM; n: Number of animals/groups; CO: Commiphora opobalsamum; NO: Nitric oxide; [†]compared with control group; $p < 0.05$, ** $p < 0.001$ compared with the corresponding control group values; † $p < 0.05$, †† $p < 0.001$ compared with the corresponding diclofenac group values

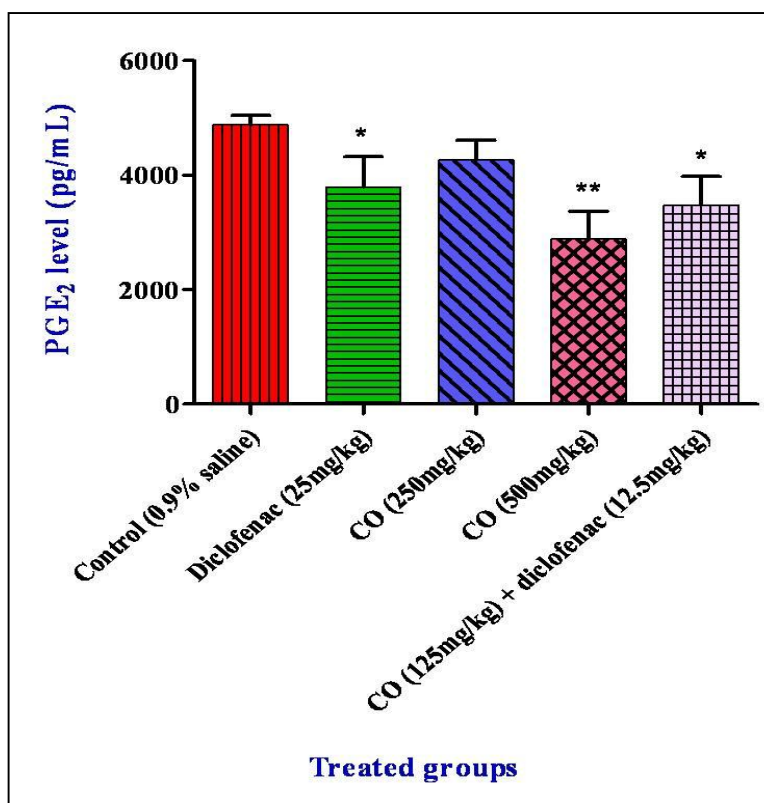


Fig. 4. Effect of CO extract (i.p.) versus diclofenac (i.p.) on PGE₂ level in carrageenan-induced paw edema in mice. * $p < 0.05$, ** $p < 0.001$ compared with the corresponding control group values. Data are expressed as means \pm SEM; n: Number of animals/groups; CO: *Commiphora opobalsamum*; PGE₂: Prostaglandin E₂; *compared with control group; ** $p < 0.05$, ** $p < 0.001$ compared with the corresponding control group values

TNF- α assay

Table 2. revealed that diclofenac (25 mg/kg), CO (250 mg/kg) and combination of CO (125 mg/kg) and diclofenac (12.5 mg/kg) were non-significantly decreased the TNF- α level in paw tissue of mice 4h of carrageenan injection as compared with control group ($p > 0.05$) with means values \pm SEM of 316.57 \pm 62.02, 334.67 \pm 23.71 and 328.50 \pm 10.37, respectively and mean % inhibition of 12.70%, 7.73% and 9.39%, respectively. Moreover, CO (500 mg/kg) significantly decreased the TNF- α level in paw tissue of mice 4h of carrageenan injection as compared with control group ($p < 0.05$) with mean values \pm SEM of 293.33 \pm 13.70 and mean % inhibition of 19.06%.

4. DISCUSSION

The exclusivity of mecca myrrh – *Commiphora opobalsamum* (CO) is characterized by its

capability of producing a resinous exudates, which possess a distinct qualities of diverse biological activities, few to illustrate as cytotoxic, analgesic, anti-inflammatory and antimicrobial effects and it is noteworthy to enlighten that its erstwhile phytochemical analysis revealed a series of terpenoids, flavonoids and sugars [20]. This essentially inspired us to execute a comprehensive experimental exploration of its anti-inflammatory and its toxicological study to put forward an effective substitute for NSAIDs [11,12]. Assessment of the anti-inflammatory effect of CO extract is one of the objectives of present study and was evaluated using experimental animal model of inflammation like carrageenan-induced paw edema because of its common utility in evaluating the anti-inflammatory activity of potential active component anti-inflammatory properties [21,22]. The results of this model in our study indicated that the CO extract act on both early and late phases of acute inflammation induced by

carrageenan probably involving pro-inflammatory mediators and PMNs migration (Figs. 2 and 3). These findings correlate well with results reported by former investigators [23-26]. Furthermore, combined group of reduced doses of CO and diclofenac (125 and 12.5mg/kg, respectively) produced a non-significant inhibition of edema compared with diclofenac alone (25mg/kg) ($p>0.05$) with almost constant percentage inhibition over 4h (72.72%). The present results (Fig.1) strongly support that the CO enhances the anti-inflammatory effect of diclofenac. The results demonstrated that the injection of carrageenan cause fundamental obvious swelling and migration of PMNs. However, treatment with CO extract at the doses of 250 and 500 mg/kg or with combination of CO and diclofenac (125, 12.5 mg/kg, respectively) significantly inhibit the edema and PMNs infiltration as compared to control group. This inhibitory effect was like that of diclofenac (25mg/kg). Combined with the results of carrageenan-induced paw edema model (Fig. 2.), the study fully justified that CO showed a significant anti-inflammatory effect at both phases of the edema development that might be related to inhibition of inflammatory mediators.

Seemingly, based on the outcome of the present work, it can be strongly emphasized that the methanolic extract of CO possess a significant dose-dependent anti-inflammatory effect on acute phases of inflammation with the greatest effect shown at a dose of 500 mg/kg. Moreover, a noteworthy recognition of our study is that the

CO extract could exert a significant anti-inflammatory effect of diclofenac as revealed by many past investigative research work [21,24,27].

Interestingly, the current study clarified the possible mechanism involved in the anti-inflammatory action of CO for first time through assessed the antioxidant activity of CO and its effect on various parameters of inflammation like NO, PGE₂ and TNF- α due to its possible related effects on mediators and cytokines. This study comprises of acquisition of the right hind paw of the mice 4 hour after carrageenan injection, multiple past studies have convincingly demonstrated that a variety of inflammatory mediators like PGE₂, NO, interleukins and oxygen reactive species are released into the mouse paw tissues in response to carrageenan induced paw edema corresponding to acute inflammation [17,28,29]. The resultant released free radicals in turn assault the plasma membrane to produce MDA, because of inflammatory response a higher concentration of MDA gets accumulated. Furthermore, precedent studies have also revealed that the local amount of TNF- α co – administered with carrageenan are associated with the paw edema and hence regarded as a distinct indicator of inflammatory response and apparently IL-1 β and IL-6 were realistically regulated by TNF- α [17,28-29].

Therefore, it is reiterated that the model of carrageenan induced paw edema finds its selectivity in our study to elucidate the anti-inflammatory mechanism of CO extract, where it

Table 2. Effect of CO extract versus diclofenac on TNF- α level in carrageenan-induced paw edema in mice

| Group n = 6 | Parameter measured | TNF- α level(pg/mL) |
|---|---|--|
| Control (0.9% saline, i.p.) | Mean \pm SEM % inhibition [♦] | 362.17 \pm 15.81 - |
| Diclofenac (25 mg/kg, i.p.) | Mean \pm SEM % inhibition [♦] | 316.57 \pm 62.02 12.70 |
| CO (250 mg/kg, i.p.) | Mean \pm SEM % inhibition [♦] | 334.67 \pm 23.71 7.73 |
| CO (500 mg/kg, i.p.) | Mean \pm SEM % inhibition [♦] | 293.33 \pm 13.70 [*] 19.06 |
| CO (125 mg/kg, i.p.) + diclofenac (12.5 mg/kg, i.p.) | Mean \pm SEM % inhibition [♦] | 328.50 \pm 10.37 9.39 |

Data is expressed as means \pm SEM; n: Number of animals/groups; CO: *Commiphora opobalsamum*; TNF- α : Tumor necrosis factor-alpha; [♦]compared with control group; ^{*} $p<0.05$ compared with the corresponding control group values

has been well recognized as a valid model to explore inflammatory mediators and cytokine generation in paw tissue in inflammatory conditions [30,31].

As regards MDA assay, the homogenate of the right-hand paw of the mice was utilized to determine the level of MDA in the inflamed paw tissue. The result of our study (Fig. 3.) illustrated credible correlation that the administration of carrageenan caused accumulation of MDA in the untreated group, remarkably pretreatment with CO extract in the doses of 250 and 500 mg/kg produced significant reduction in MDA level in comparison with the control group ($p < 0.001$). Furthermore, CO extract in the dose of 500mg/kg also demonstrated maximum reduction of MDA level and was effectively significant as compared with diclofenac group (25mg/kg) ($p < 0.001$). Thus, our results formidably substantiate that the CO extract possess significant antioxidant activity that could be contributed in the anti-inflammatory effect. Similar results were reported by many former investigators [25,32].

The comprehension of multiple action of NO in the regulation of inflammation, [33] necessitates the NO assay in the present work to validate the mechanism of anti-inflammatory action of CO, our results revealed a high level of NO after sub-planter carrageenan administration (Table 1), the important aspect of the results demonstrated that CO in the dose of 250 and 500mg/kg revealed a significant ability to suppress the increment in NO level caused by carrageenan injection when compared with control group ($p < 0.001$). Moreover, in the dose of 500mg/kg produced a significant reduction of NO level as compared with diclofenac in the dose of 25mg/kg ($p < 0.001$). This notable observation in our findings indicates that the NO was involved in the mechanism underlying the anti-inflammatory effect of CO extract, and substantially draw a parallel with the results cited in multiple past studies [34-37].

It is well established fact that PGE₂ is a recognized bioactive lipid, and it is one of the definite hallmark and potent mediator of inflammation [38,39], obviously it finds an important parameter in this study to further corroborate the evaluation of the anti-inflammatory action of CO. As expected, the results of PGE₂ assay in our study thoroughly illustrated (Fig. 4.) accumulation of PGE₂ in the paw of the mice subjected to carrageenan induced paw edema model. Interestingly, our

results unequivocally revealed significant inhibition of PGE₂ accumulation at the site of inflammation by the pretreatment of CO extract in the dose of 500 mg/kg(0.001).The present finding strongly bear a resemblance obtained in a recent study [36].

In view of the same perspective, we used similar procedures in the present work to determine the possible role of CO extract in modification of TNF- α in the carrageenan induced inflammation of the paw in mice. TNF- α is a pleotropic cytokine – a pro-inflammatory protein used by the immune system secreted by the macrophages and plays an important role in carrageenan induced acute paw edema by acting as an inducer of PGE₂ and NO secretion and activator of NF- κ B signal transduction. The present results explicitly demonstrated that carrageenan notably leads to accumulation of TNF- α in the control group and most strikingly, the CO extract in the dose of 500mg/kg was significantly accomplished the reduction of TNF- α in the paw in comparison to the control group ($p < 0.05$), (Table 2) interestingly analogous studies also displayed similar supportive findings [37,40].

Astoundingly, momentous ultimate outcomes can be observed in this study by combining the results of histological examination of mice paw in the carrageenan induced paw edema model, the study justified that CO possess a significant anti-inflammatory effect related to the inhibition of free radicals and suppression of NO, PGE₂ and TNF- α at the site of inflammation.

5. CONCLUSION

A distinctive feature is that this is the first type of study illustrating the pioneering perspective and the positive evidences for CO extract to exert its anti-inflammatory effect through suppressing NO, PGE₂ and TNF- α at the site of inflammation, in addition to possess the antioxidant activity. Further research is needed to confirm these results in large, prospective, randomized, placebo-controlled studies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The present experimental work was officially endorsed by the ethical committee of King Abdulaziz University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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