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Characterization of antiradical and anti-inflammatory activities of some cold pressed oils in carrageenan-induced rat model of acute inflammation

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ABSTRACT

Inflammation is a process involving multiple factors acting in a complex network, where a distinct cytokine cascade of pro-inflammatory and inflammatory mediators unfolds. Substantial levels of bioactive phytochemicals were found in cold-pressed oils (CPO). To evaluate the antiradical and anti-inflammatory potential of CPO including Nigella sativa oil (BCO), Coriandrum sativum oil (COO), and Syzygium aromaticum oil (CLO). Indomethacin was used as the reference standard. In vitro assays were undertaken to evaluate radical scavenging properties utilizing stable 1,1-diphenyl-2-picrylhydrazyl (DPPH˙) and galvinoxyl radicals. In vivo studies comprised carrageenan (CG)-induced rat paw edema as a model for acute inflammation. Biochemical estimations of tumor necrosis factor-α (TNFα), interleukin 6 (IL-6), and interleukin 12 (IL-12) were evaluated in paw exudates and sera of treated as well as untreated animals groups. Histopathological examination of rat paw sections was carried out. In vitro assays revealed strong radical scavenging potential of CPO against stable DPPH˙ and galvinoxyl radicals with CLO showing the highest radical quenching activity. Pro-inflammatory cytokine levels were markedly improved as compared to untreated CG group. CLO exhibited superior attenuation of pro-inflammatory release as compared to BCO, COO, and indomethacin. Rat paw sections of CPO-treated groups showed a marked improvement in histopathological alterations induced by CG injection. Attenuation of inflammatory mediators by bioactive constituents of the selected CPO, together with the antioxidant potential, might represent a possible prophylactic or therapeutic target against inflammation and subsequent free radical generation.

Keywords: Cold pressed oil, *Coriandrum sativum*, *Syzygium aromaticum*, *Nigella sativa*, anti-inflammatory, indomethacin.

INTRODUCTION

Inflammation is a complex biological response of the body against infections, irritations, cell damage, vascularized tissues and other injuries. It is critical for both innate and adaptive immunity [1, 2]. The migration of leukocytes into the site of inflammation is crucial in the pathogenesis of inflammatory conditions. Inflammation cardinal signs; edema, erythema and hyperalgesia result from action of pro-inflammatory agents such as bradykinin, histamine, tachykinins, complement, reactive oxygen and nitrogen species. Such agents can be generated *in situ* at the site of insult or by infiltrating cells. Iwalewa et al. [3] showed that neutrophils readily migrate to sites of inflammation and

can generate pro-inflammatory reactive oxygen species. During inflammatory response several pro-inflammatory mediators are released, including interleukin [(IL-6, IL-12, IL-1, (TNFα), interferon (INF-γ), and cyclooxygenase-2 (COX-2)]. These cytokines play major roles in the initiation, and amplification of inflammatory processes [4]. Gomes et al. [5] and Mequanint et al. [6] reported that inflammation induced by carrageenan, is non-immune, acute, highly reproducible, well researched and will continue to be useful in novel drug development. The response of inflammation is usually quantified by increase in paw size (edema) which is maximal around 4-6 h post-carrageenan injection and is modulated by inhibitors of specific molecules within the inflammatory cascade. Alwashli et al. [7] demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) exhibited adverse side effects, such as bleeding, and gastrointestinal discomfort. Ulcerative lesions of the gastrointestinal tract are one of the major side effects associated with the use of NSAIDs. Indomethacin, particularly, was reported to inhibit platelet aggregation, liver, and kidney toxicity [8].

Nigella sativa is a medicinal plant belonging to Ranunculaceae family. Ballero and Fresu [9] revealed that the seeds were used in the orient as condiments or flavourings, and also in traditional medicine. It was reported that *N. sativa* has bronchodilatory [10], antibacterial [11], hypotensive [12], immunopotentiating [13] and antioxidant properties [14]. *N. sativa* oil was potent analgesic, and anti-inflammatory drug in rats [15]. The extract or seed oil was considered as one of the newer sources of edible oils and found to have therapeutic properties [16]. Both oils and seeds are often used as nutritional supplement on account of its various health properties as they have been reported to possess antitumour [17], antioxidant [18] and anti-inflammatory activities [19].

Coriander (*Coriandrum sativum* Linn.), belonging to family Umbelliferae, is a highly reputed medicinal herb. It is used in the preparation of many household medicines to cure cold, stomach disorders, seasonal fever, vomiting, nausea and also as a drug for indigestion, helminthic infestations, rheumatism and joint pain. In some parts of India, it has traditionally been used for its anti-inflammatory properties [20]. In Ayurvedic system of medicine, it has been used to treat local swelling, pain, headache, burning sensation, lymphadenopathy, stomatitis, conjunctivitis, vertigo, syncope, memory loss, digestive disorders, bleeding disorders, cough, dyspnoea and as a diuretic [21].

Syzygium aromaticum L. (clove) is a nail-shaped dried flower bud belongs to Myrtaceae family [22]. The plant has a strong phenolic smell and sharp acrid taste. It contains essential oil (15-20%) and fixed oil (10%). Non-essential ether extract constitutes 6-12%. Essential oil of clove is a colorless or light yellowish fluid extracted from dried flower buds by steam distillation. The main constituents are eugenol (80-90%), β-caryophyllene (9%), eugenyl acetate (7%), ylangen, α-humulen, benzyl alchohol, benzaldehyde, metoxy benzaldehyde and cavychole. Clove is used in cooking, food processing, pharmacy, perfumery and cosmetics [23]. Clove has biological properties which have been reported, but little is known about its effect on the immune system. Kim et al. [24] declared that molecular evidence for its anti-inflammatory activity comes from the studies indicating COX-2 inhibition without affecting COX-1 in mice macrophage cell cultures. On the other hand, *in vitro* studies are validated only with consistent *in vivo* studies as many compensatory mechanisms may interfere with the drug effects resulting in weaker physiological response.

The current study was conducted to evaluate the antioxidant and anti-inflammatory potential of cold pressed oils (CPO) including *Nigella sativa* oil (BCO), *Coriandrum sativum* oil (COO) and *Syzygium aromaticum* oil (CLO), utilizing indomethacin as the reference standard. We hypothesize that edible CPO may be used to develop antioxidant/anti-inflammatory nutraceuticals for health promotion and disease prevention and might provide a possible preventive or therapeutic target.

MATERIALS AND METHODS

Materials

Female albino rats (150-180 g) were obtained from animal breeding house of the National Research Centre (NRC, Dokki, Egypt). Rats were housed in clean plastic cages with free access to food (standard pellet diet) and tap water *ad libitum*, under standardized housing conditions (12 h light/dark cycle, at 23 ± 2˚C and relative humidity of 44%) in the laboratory animal room. Animals received human care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". The Local Ethics Committee at NRC approved the experimental protocols and procedures. CPO were obtained from a local market (Cairo, Egypt). Indomethacin, tween 80, and carrageenan were obtained from Sigma Chemicals (USA). IL-12, IL-6, and TNF-α were quantified with rat enzyme linked immunosorbent assay (ELISA) kits.

Methods

In vitro assays

Radical scavenging activity (RSA) of CPO toward DPPH$^{\cdot}$

RSA of CPO was assayed with DPPH$^{\cdot}$ radical dissolved in toluene [25]. Toluene solution of DPPH$^{\cdot}$ radicals was prepared at a concentration of 10^{-4} M. Ten mg of CPO (in 100 µL toluene) was mixed with 390 µL toluene solution of DPPH$^{\cdot}$ radicals, and vortexed for 20 s at ambient temperature. Against a blank of toluene (without DPPH$^{\cdot}$), the decrease in absorption at 515 nm was measured in 1-cm quartz cells after 1, 30, and 60 min of mixing using a UV-260 visible recording spectrophotometer (Shimadzu, Kyoto, Japan). RSA toward DPPH$^{\cdot}$ radicals was estimated from the differences in absorbance of DPPH$^{\cdot}$ solution with or without sample (control) and the inhibition percent was calculated from the following equation:

% Inhibition = *[(absorbance of control – absorbance of test sample)/ absorbance of control] x 100.*

RSA of CPO toward galvinoxyl radical

A miniscope MS 100 ESR spectrometer (Magnettech GmbH; Berlin, Germany) was used [25]. Conditions of the experiment were: microwave power, 6 db; measurement at room temperature; centerfield, 3397 G, receiver gain 10, sweep width 83 G, and modulation amplitude 2000 mG. Ten mg of CPO (in 100 µL toluene) was reacted with 100 µL of toluene solution of galvioxyl (0.125 mM). The mixture was stirred for 20 s then transferred into a 50 µL micro-pipette. The amount of galvinoxyl radical inhibited was measured after 60 min of the addition of the galvinoxyl radical solution. Signal intensities were evaluated by the peak height of galvinoxyl signals against a control. Estimation of the radical concentration was calculated by evaluating the decrease of the ESR signals in arbitrary units after 60 min incubation using the KinetikShow 1.06 Software program (Magnettech GmbH, Germany). Reproducibility was *ca*. 5% as usual for kinetic parameters.

In vivo assays

Anti-inflammatory activity

Carrageenan-induced rat paw edema has been used for assessment of the anti-inflammatory activity of CPO [26]. Female rats were acclimatized for 7 days before treatment, and randomly assigned into six groups of eight rats each. Dosages of indomethacin, and CPO were freshly prepared in 7% tween 80 saline solution (0.9% w/v) and given *via* oral gavage. Animals were fasted overnight, assigned into groups, and orally given indomethacin (10 mg/kg, p.o.) [27] as well as CPO (400 mg/kg, p.o.) [28]. One hour following the treatments, paw swelling was induced by injection of 100 µL of 1% sterile lambda carrageenan suspension in saline into the plantar region of the right hind paw of animals in all groups [29]. An equivalent volume of 100 µL saline was injected in left hind paw of all groups.

Blood samples

Four hours following carrageenan injection, the rats were lightly anesthetized for blood sample collection from the orbital sinus in clean dry tubes and left to clot, then centrifuged at 3000 rpm for 10 min at 4˚C (Sigma Laborzentifugen, 2K15, Germany, GmbH) to separate sera. Sera aliquots were frozen at -80˚C for analysis of pro-inflammatory cytokines.

Paw weight

Rats were deeply anesthetized with ether and the two limbs (left and right paw) were excised by sharp surgical blade then weighed on an animal balance. The left hind paw weight represents basal value (as 100%) and right hind paw weight represents post-treatment value. The mean % of the basal value of the right hind paw was estimated for each group [30]. Rat hind paw tissue segments (normal, untreated, and treated) were cut and rinsed several times with ice-cold normal saline, and immediately placed in its four volumes of cold normal saline and homogenized at 4˚C. The homogenate was centrifuged at 12000 rpm for 5 min. The supernatant was stored at -80˚C for TNF-α, IL-6, and IL-12 assays.

Biochemical Analyses

Serum and paw exudate tumor necrosis factor-α (TNFα) was determined according to Seriolo et al. [31] by Enzyme-linked Immuno-Sorbent Assay (ELISA) kit (Orgenium Laboratories, Finland). Interleukin 6 (IL-6) and Interleukin 12 (IL-12) were determined with an ELISA kit (Endogen Inc, USA) [32].

Histopathological study

In another set of control and treated rats, animals were sacrificed. The paws were separated and fixed in 10% neutral buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 4-5u thickness. The sections were stained with Haematoxylin and Eosin **[33]** for histopathological examination.

Statistical analysis

Statistical analysis was done using Graphpad Prism (version 5), wherein the values were expressed as mean ± SEM. The statistical significance of differences between the means was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for comparison between different treatment groups. Statistical significance was set at $p \leq 0.05$.

RESULTS

***In vitro* assays**

CPO were compared using stable DPPH$^{\cdot}$ and galvinoxyl radicals as antioxidant activities. **Figure no.1A** shows CPO had high RSA against DPPH$^{\cdot}$. After 30 min of incubation with DPPH$^{\cdot}$ radicals, 18%, 13% and 10% of DPPH$^{\cdot}$ radicals were quenched by CLO, COO, and BCO, respectively. In addition, after 60 min of incubation with DPPH$^{\cdot}$ radicals, 25%, 16%, and 14% of DPPH$^{\cdot}$ radicals were quenched by CLO, COO and BCO, respectively. ESR measurements showed the same pattern. After 30 min of incubation with galvinoxyl radicals, 21%, 15%, and 8% of galvinoxyl radicals were quenched by CLO, COO, and BCO, respectively. While, after 60 min of incubation with galvinoxyl radicals, 24%, 18% and 15% of galvinoxyl radicals were quenched by CLO, COO and BCO, respectively **(Figure no.1B**).

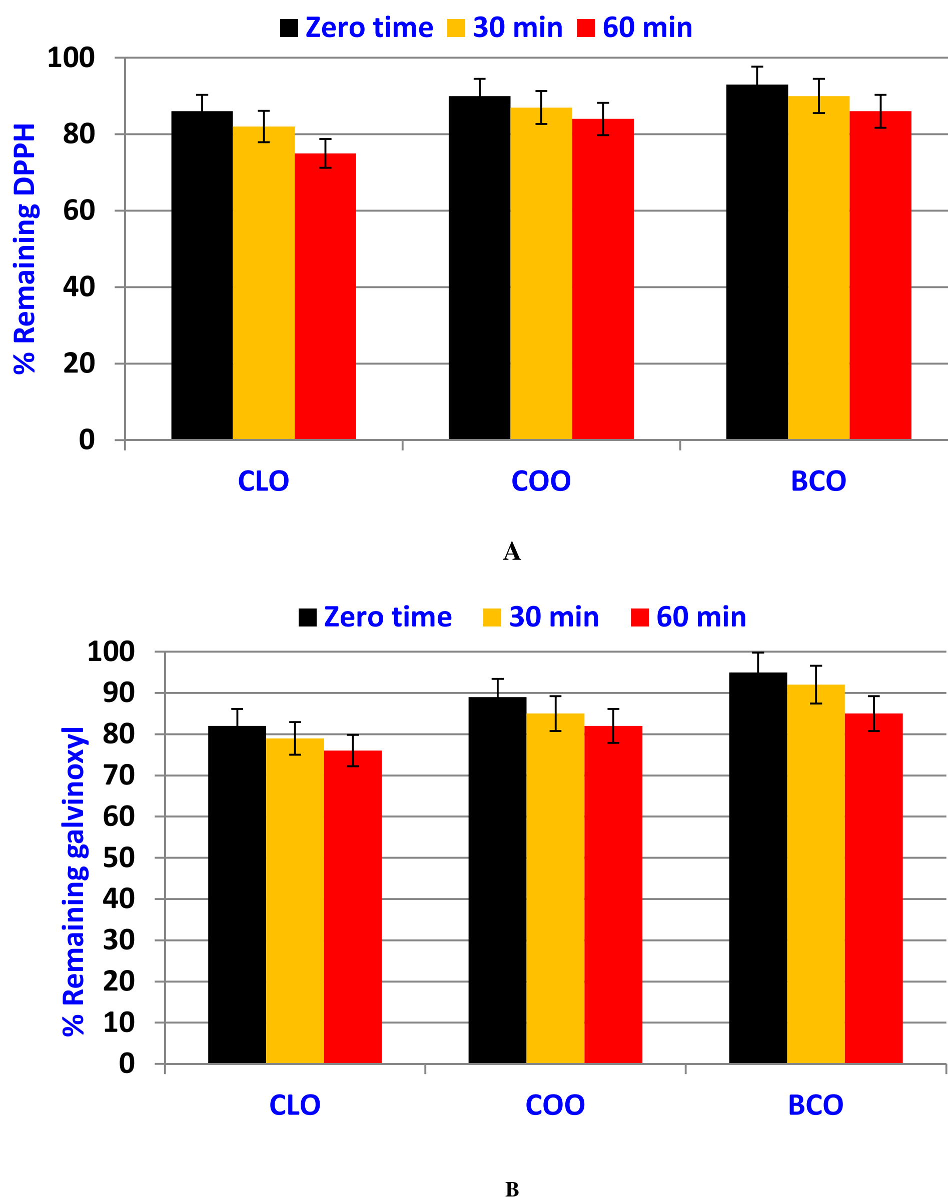

Figure no.1. Antiradical effect of CPO at different incubation times on DPPH$^{\cdot}$ (A), and galvinoxyl (B) radicals. Values given are the mean of three replicates and error bars show the variations of three determinations in terms of standard deviation.

***In vivo* study**
The current investigation was conducted to evaluate the effect of oral administration of indomethacin, and CPO on pro-inflammatory mediators TNFα, IL-6, and IL-12 in rat serum and paw exudates using the carrageenan model of acute inflammation. The present results revealed that the subplantar injection of CG-induced a significant increase in paw weight by 56.4% in comparison with that of the normal animals **Table no.1**. Additionally, oral administration of indomethacin (10 mg/kg) and CLO (400 mg/kg) normalized % basal paw weight, since they induced a marked reduction in paw weight by 26.5% and 19.0% respectively, as compared to CG-untreated group. Meanwhile, oral treatment with BCO and COO showed an insignificant improvement in their respective paw weights (13%, and 9.87%, respectively).

Table no.1. Effect of oral administration of indomethacin and CPO on paw weight, 4 h post-injection of CG-induced rat paw edema

Group	Dose	Paw weight (% basal value)
Normal	100 µL saline	$107.5^{a} \pm 0.33$
Carrageenan	100 µL (1%)	$168.2^{*b} \pm 3.79$
Indomethacin	10 mg/kg	$123.5^{a} \pm 5.22$
BCO	400 mg/kg	$146.1^{*} \pm 10.24$
COO	400 mg/kg	$151.6^{*} \pm 4.55$
CLO	400 mg/kg	$136.2^{a} \pm 7.59$

Data are shown as mean ± S.E.M. (n=8).
[*]significantly different from normal value (p<0.05),
[a]significantly different from carrageenan-treated group (p<0.05),
[b]significantly different from indomethacin-treated group (p<0.05)

Data present in **Table no. 2** revealed that the local injection of CG raised the TNFα in rat paw exudates by 129.6% as compared to the normal animals. On the other hand, oral administration of indomethacin (10 mg/kg) induced a reduction in exudate TNFα by 27.3%. Interestingly, oral administration of CLO (400 mg/kg) normalized TNFα levels in paw exdate, since it induced a 50.08% reduction, as compared to the CG-untreated group. Oral pretreatment with BCO and COO exhibited an insignificant decrease in TNFα in their respective paw exudates (13.6%, and 16.2%, respectively) in comparison with the CG-untreated rats. Similarly, the subplantar injection of CG elevated the serum TNFα 2.7 folds with respect to that of the normal animals. Meanwhile, oral administration of indomethacin (10 mg/kg) or CLO (400 mg/kg) induced a reduction in serum TNFα by 27.3% and 32.3%, respectively, in comparison with the CG-untreated group. A slight but insignificant improvement was observed in the serum TNFα following the oral administration of COO (17.6%) or BCO (14.0%) oils, 4 h post-injection of CG.

Table no. 2. Effect of oral administration of indomethacin and CPO on TNFα (pg/mL) in paw exudates and serum, 4 h post-injection of CG-induced rat paw edema

Group	Dose	TNFα (pg/mL)	
		Paw exudate	Serum
Normal	100 µL saline	$207.2^{ab} \pm 12.15$	$103^{ab} \pm 14$
Carrageenan	100 µL (1%)	$475.8^{*b} \pm 38.65$	$278^{*b} \pm 17$
Indomethacin	10 mg/kg	$345.6^{*a} \pm 36.1$	$202^{*a} \pm 8.7$
BCO	400 mg/kg	$\mathbf{411.0^{*} \pm 32.79}$	$239^{*} \pm 23$
COO	400 mg/kg	$\mathbf{398.4^{*} \pm 7.72}$	$229^{*} \pm 19$
CLO	400 mg/kg	$\mathbf{237.5^{a} \pm 23.30}$	$188^{*a} \pm 4.5$

Data are shown as mean ± S.E.M. (n=8).
[*]significantly different from normal value (p<0.05),
[a]significantly different from CG-treated group (p<0.05),
[b]significantly different from indomethacin-treated group (p<0.05).
Bold typed values denotes significance difference from CLO-treated group (p<0.05).

As shown in **Table no. 3**, injection of CG in rat hind paw elevated IL-6 level in paw exudates by 185.5% in comparison with normal group. Oral pretreatment with indomethacin, BCO or CLO exhibited a significant reduction of local IL-6 level in the paw exudates by 46.4%, 34.2% and 48.8%, respectively, as compared to that of CG group. Interestingly, CLO (400 mg/kg) induced a significant improvement similar to that induced by indomethacin. An insignificant decrease of IL-6 in paw exudate by 16.6% was detected in COO-treated animals. CLO normalized exudate IL-6, and exhibited a better effect than that of COO with a comparable effect to indomethacin locally.

In a similar pattern, the subplantar injection of CG increased the serum IL-6 to 1.9 folds as compared to the normal animals. Pretreatment of rats with BCO or CLO (400 mg/kg) significantly depressed the serum IL-6 by 26.7% and 45.8% as compared to the CG-untreated groups, respectively. Noteworthy, CLO prominently normalized serum IL-6, and exerted a significant improvement in serum IL-6 by 36.6%, as compared to indomethacin-treated animals.

Table no.3. Effect of oral administration of indomethacin and CPO on IL-6 (pg/mL) in paw exudates and serum, 4 h post-injection of CG-induced rat paw edema

Group	Dose	IL-6 (pg/mL)	
		Paw exudate	Serum
Normal	100µL saline	922[a] ±67	167[ab] ±16
Carrageenan	100µL (1%)	2633[*b]±216	325[*]±19
Indomethacin	10 mg/kg	1411[a]±83	**278[*]±15**
BCO	400 mg/kg	1732[*a]±117	238[a] ±9.9
COO	400 mg/kg	**2194[*b]±129**	**263[*] ±25**
CLO	400 mg/kg	1346[a] ±131	**176[ab]±8.3**

Data are shown as mean ± S.E.M. (n=8).
[*]significantly different from normal value (p<0.05),
[a]significantly different from CG-treated group (p <0.05),
[b]significantly different from indomethacin-treated group (p <0.05).
***Bold** typed values denotes significance difference from CLO-treated group (p<0.05).*

Data presented in **Table no.4** revealed that the subplantar injection CG recorded a significant local elevation to 1.8 folds in IL-12 of rat paw exudates with respect to the normal animals. However, oral pretreatment with indomethacin normalized IL-12 of paw exudates as it induced a significant decrease by 36.3% in comparison with the CG-untreated animals and exhibited superior reduction to CLO. On the contrary, all oils showed an insignificant reduction in IL-12 of paw exudates as compared to that of the CG-untreated group. Furthermore, the subplantar injection of CG caused a significant rise in serum IL-12 to 1.5 folds as compared to that of the normal rats. Pretreatment of animals with either indomethacin or CPO induced no marked changes in the serum IL-12 with respect to the CG group, four hours post-administration.

Table no. 4. Effect of oral administration of indomethacin and CPO on IL-12 (pg/mL) in serum and paw exudates, 4 h post-injection of CG-induced rat paw edema

Group	Dose	IL-12 (pg/mL)	
		Paw exudate	Serum
Normal	100 µL saline	120[a] ±5.2	53[a] ±1.8
Carrageenan	100 µL (1%)	223[*b]±18	79.4[*]±1.9
Indomethacin	10 mg/kg	142[a]±3.8	73.8[*]±1.7
BCO	400 mg/kg	176[*]±18	71.8[*]±5.6
COO	400 mg/kg	177[*]±8.4	74.3[*]±6.9
CLO	400 mg/kg	201[*b]±18	63.2±3.2

Data are shown as mean ± S.E.M. (n=8).
[*]significantly different from normal value (p<0.05),
[a]significantly different from CG-treated group (p<0.05),
[b]significantly different from indomethacin-treated group (p<0.05).

Histopathological examination of rat hind paw

Data presented in (**Table no.5 and Figure no.2**) illustrated that microscopical examination of rat paw sections injected with CG and treated with indomethacin revealed mild histopathological changes summarized as dermal edema associated with few inflammatory cells infiltration **(Figure no.2A)** and congestion of dermal blood vessels. However, examined sections from rats treated with BCO showed an improvement in the histopathological picture as the examined sections revealed mild dermal edema with few inflammatory cells infiltration **(Figure no.2B)**. Moreover, paw of rats treated with COO showed no histopathological changes except few inflammatory cells infiltration in the dermal layer **(Figure no.2C)**. Paw of rats treated with CLO showed no histopathological changes **(Figure no.2 D&E)**. Meanwhile, examined sections from rats injected with CG alone revealed severe histopathological changes as compared to normal rat paw sections, confined as marked dermal edema, massive inflammatory cell infiltration (mainly polymorphnuclear cells), and necrosis of cartilage **(Figure no.2F)**.

Table no. 5 Histopathological lesions score

Histopathological lesion	Indo + CG	BCO + CG	COO + CG	CLO + CG	Control	CG
Dermal edema	++	+	+	-	-	+++
Polymorphnuclear cells infiltration	++	+	-	-	-	+++
Necrosis of cartilage	-	-	-	-	-	++

(-) no change (+) mild (++) moderate (+++) severe

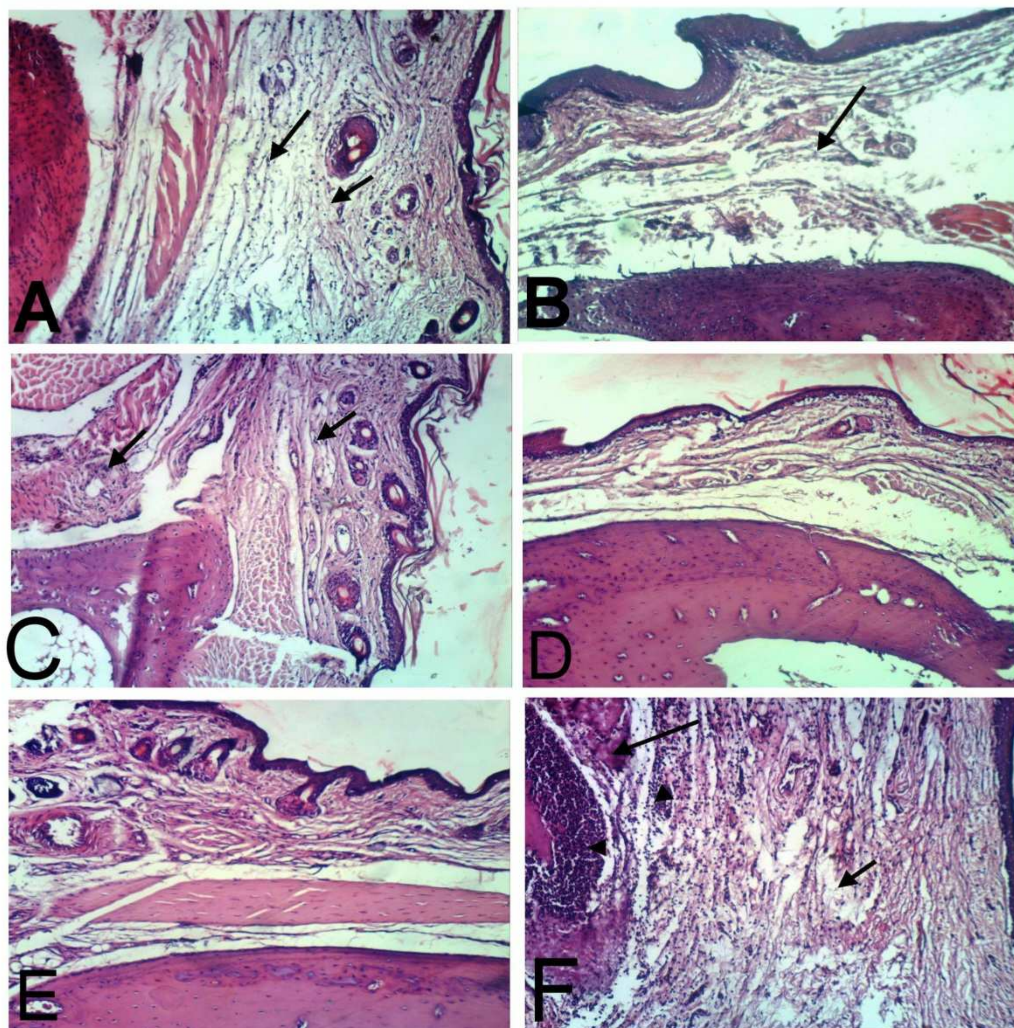

Figure no.2. Paw of rat treated with; A) Indomethacin + CG showing dermal edema (small arrow) and few inflammatory cells infiltration (large arrow). B) BCO + CG showing dermal edema with few inflammatory cells infiltration (arrow). C) COO + CG showing few inflammatory cells infiltration. D) CLO + CG showing no histopathological changes. E) normal control showing no histopathological changes. F) CG alone showing marked dermal edema (small arrow), massive inflammatory cells infiltration (large arrow), and necrosis of cartilages (arrow head) (H & E X 100).

DISCUSSION

Health-promoting impacts of vegetable oils can be attributed to its fatty acid composition (MUFA, PUFA and *omega*-6PUFA/*omega*-3PUFA ratio) and its richness in phenolic compounds, tocols, and sterols. CPO under study contains high amounts of PUFA and MUFA [28]. The health-promoting benefits of PUFA as well as MUFA, in inflammation, and other diseases were reported. Reifen et al. [34] and Song et al. [35] showed that *Omega*-3 PUFA provided anti-inflammatory properties due to the activity of eicosapentaenoic acid-derived eicosanoids and modulated precursors of inflammatory mediators, hence benefiting brain health. The results of the present study are in agreement with Reifen et al. [34] who declared that the administration of sage oil significantly reduced the inflammatory response with lower necrotic mucosal damage in rats.

BCO, COO, and CLO under study contained high amounts of tocols [28]. Tocol effects on disorders were reported in humans and animals. α-Tocopherol and tocotrienol reduced the increase in oxidative stress in models of gastric lesions [36, 37]. The CPO under study also contain high levels of total phenolic compounds, which is known exhibit a broad spectrum of functional properties in vegetable oils, including antioxidant, antiradical, and anti-inflammatory activities [38]. Moreover, Rosillo et al. [39] reported the anti-inflammatory and protective effects of olive oil phenolic compounds in a collagen-induced arthritis model.

The current *in vivo* study revealed that the subplantar injection of CG induced an increase in paw weight by 56.4% in comparison with that of the normal animals. This finding was consistent with Cunha et al. [40], [41]. The current result was further confirmed *via* histopathological examination of rat paw sections, where injection of CG revealed

severe histopathological changes confined as marked dermal edema, massive inflammatory cells infiltration (mainly polymorphnuclear cells) and necrosis of cartilage. These results were in agreement with Farshid et al. [42] who detected edema and infiltration of the paw tissue with neutrophils. Hence, CG is an inflammatory agent that increases the vascular permeability, paw edema, neutrophil infiltration and production of free radicals. On the other hand, oral administration of indomethacin and CLO induced a marked reduction and normalized % basal paw weight with respect to CG-untreated group. This could be explained by the high levels of polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA), tocols, phenolics and free radical scavenging power in CLO. In the meantime, oral pretreatment with BCO and COO showed an insignificant improvement in their respective paw weights. Our data is in line with Hashemi et al. [43], who claimed that volatile oil of COO possessed an insignificant anti-inflammatory effect on CG-induced paw inflammation. This contrasts a study which reported that *Nigella sativa* extract has an anti-inflammatory effect that was demonstrated by its inhibitory effect on CG-induced paw edema [44]. Moreover, Bhat et al. [45] stated that the high dose of *C. sativum* ethanol extract might act in the early phase of inflammation. Many studies attributed the edema effect to increased COX-2 levels with a simultaneous elevation of prostaglandin production [46, 47]. Another study suggested that TNFα together with other mediators, may be possibly involved in the sustained COX-2 activity in the CG model, since they promote COX-2 production that trigger infiltration of monocytes to the inflammation site [48]. Hence, prostanoids may play a fundamental role in COX-2 induction at early time points. This explains the result herein, where oral pretreatment with indomethacin (10 mg/kg) normalized % basal paw weight, as compared to CG-untreated group. This is in line with an investigation carried out by Nantel et al. [49], who declared that indomethacin blocks COX-2 induction in paw edema. It has also been reported that part of the PGE_2 produced following CG injection in the hind paw may be produced by the COX-1 enzyme [50]. Therefore, indomethacin, the inhibitor used in the current investigation as reference standard, might have an inhibitory action on both COX-1 and COX-2 equally. Interestingly, our data revealed that oral pretreatment with CLO exhibited an equipotent effect to indomethacin and normalized % basal paw weight as well.

The study of Ibrahim et al. [28] revealed a marked improvement in the CG model of acute inflammation after oral administration of cold pressed nigella (BCO), coriander (COO) and clove (CLO) oils. The release of various mediators by CG in the rat paw is triphasic. Histamine and 5-hydroxytryptamine (5-HT) are released in the initial phase of inflammatory response, followed by kinins that mediate the second phase. Finally, prostaglandins were proposed to be the key mediator in the third phase [51]. Poole et al. [52] explained the cytokine cascade that unfolds during inflammation, where agents derived from the injured tissue, such as bradykinin, induce the release of TNFα, which in turn stimulates interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). Both IL-1β and IL-6 activate cycloxygenase enzymes that convert arachidonic acid to prostaglandins [52].

Consistent with previous studies [40, 41], we detected a substantial increase in TNFα production in the hind paw during inflammation with the increase in paw weight, 4 h post-CG injection. Some authors explained the importance of TNFα in CG-induced hypernociception, which was completely abolished *via* pretreatment with antiserum anti-TNFα [53]. In the current investigation, oral administration of indomethacin (10 mg/kg) induced a reduction in exudate TNFα, possibly through the inhibition of prostaglandin synthesis. This finding is in accordance with Verri et al. [54] who performed mechanical hypernociception in rat hind paw. The authors suggested that indomethacin and atenolol (a sympathetic blocker) attenuated TNFα-induced hypernociception. Therefore, prostanoids and sympathetic amines could be two independent pathways that mediate TNFα-induced hypernociception in CG model. Interestingly, oral administration of CLO (400 mg/kg) in the current study normalized TNFα levels in paw exudate as compared to the CG-untreated group. Hence, CLO exhibited superior effect to indomethacin, BCO and COO in depression of local paw TNFα. A similar pattern was observed in serum TNFα. This could be attributed to the high levels of PUFA, MUFA, tocopherol and phenolic components found in CLO. These results are in agreement with a study carried out by Caughey et al. [55], who claimed that flax seed oil enriched diet could effectively lower the production of these cytokines. Moreover, Blok et al. [56] hypothesized that cell-mediated immune responses were suppressed by omega-3 PUFA, partially via inhibition of antigen presenting-cell function and alteration of the membrane protein expression [56].

Interleukin-6 (IL-6) is produced and released from various body cells such as subcutaneous adipose tissue, monocytes, lymphocytes, fibroblasts and endothelial cells [57]. It is the only cytokine that can promote the synthesis of all acute phase proteins of inflammatory response, including TNFα and IL-1 [58]. In the current investigation, injection of CG in the hind paw of rats substantially elevated the IL-6 level in paw exudates and serum in comparison with that of the normal group. This is in agreement with a study by Xu et al. [59], who emphasized the role of IL-6 in mice deficient in this cytokine. Thermal and mechanical inflammatory responses were markedly reduced following CG injection in their peripheral tissue. Thus, this reinforces the importance of IL-6 in inflammatory hypernociception. Similar to TNFα, IL-6 was claimed to mediate dose and time-dependent mechanical hypernociception in rats, and this action was clearly suppressed by indomethacin [53]. Our results revealed that oral

pretreatment with indomethacin, BCO or CLO exhibited a significant reduction of local IL-6 level in the paw exudates, with CLO being strikingly equipotent to indomethacin. Again one could assume that the PUFA content and antioxidants found in CLO and BCO may be responsible for the modulation of carrageenan induced IL-6 production. This assumption could be supported by an investigation by Khalfoun et al. [60], who examined the effects of PUFA (e.g. eisosapentaenoic and docosahexaenoic acids) on IL-6, and concluded that it was significantly lowered in human endothelial cells.

The mechanism of CLO suggested herein is probably different from that of indomethacin and needs further elucidation. Additionally, according to a study carried out by Sethi et al. [61], many anti-inflammatory mechanisms assigned to *Nigella sativa*, may be partially mediated via suppression of NF-κB activation. This transcription factor regulates the gene expression of many enzymes such as COX, iNOS and 5-lipoxygenase, as well as proinflammatory cytokines such as IL-1, IL-6 and TNF-α [62]. A similar effect to exudates was also exerted in the levels of serum IL-6 in the current study. Hence, CLO normalized exudate IL-6 and exhibited a better effect than that of COO with a comparable effect to indomethacin locally (exudates) and a superior effect systemically (serum) according to serum data provided in **Table no.3**.

Interleukin 12 (IL-12) is a cytokine that possesses pleiotrophic effects on T cells and natural killer (NK). It is produced by antigen-presenting cells and enhances interferon-γ (IFN- γ) production and cytotoxicity [63]. This cytokine is mainly produced by macrophages and dendritic cells. *In vivo* and *in vitro* IL-12 supports the generation of a T Helper (TH-1) immune response which is characterized by cell-mediated immunity and delayed-type hypersensitivity [64, 65]. IL-12 was claimed to induce pain in human studies, since patients that received intravenous administration of recombinant human IL-12 presented arthralgia in the shoulders and fingers [66]. Consistent evidence in animal studies reported that intraplantar injection of IL-12 exhibited mechanical hypernociception *via* endothelin receptor type B (ETB) receptors in rats [67]. Furthermore, inhibition of IL-12 hypernociception was achieved by administration of endothelin ETB receptor antagonist, whereas, indomethacin and anti-TNFα were proven ineffective [67]. This could explain the insignificant effects of CPO on exudate and serum IL-12 levels revealed in the current study, which may be attributed to the lack of substantial effect on the endothelin receptor.

One important aspect to consider in this model is the utilization of a single dose of the selected CPO, so it is possible that BCO and COO may possess a substantial effect on cytokine production in a longer term therapy, hence we propose these issues for future elaboration. However, CLO proved to be highly efficient in acute inflammatory response (within 4 h) and even better than indomethacin with respect to gastric safety, radical scavenging activity, overall cytokine reduction in addition to improved histopathological changes induced by CG injection. This could be attributed to the high PUFA, MUFA, phenolics, and tocols contents in CPO as well as the potent antioxidant potential which play a crucial role in anti-inflammatory process.

Conclusively, the studied CPO may possess constituents that have a significant role in reducing the expression and production of pro-inflammatory mediators. Therefore, one could recommend utilizing CPO, prophylactically or therapeutically, in the development of nutraceuticals or functional food for health improvement and disease prevention against inflammation and subsequent free radical generation.

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