Evaluation of the Anti-inflammatory and Antioxidant Parameters of Aqueous and Ethanolic Extracts of Roman chamomile in mice

Hoda Erjaee*[1], Hamid Rajaian[1], Saeed Nazifi[2]

[1]Department of Pharmacology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[2]Department of Clinical Studies, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Correspondence: H. Erjaee
Department of Pharmacology
School of Veterinary Medicine, Shiraz University, Shiraz, Iran
Shiraz University, Shiraz, P.O. Box: 1731- 71345, Iran
E-mail: hodaerjaee@shirazu.ac.ir
Tel: +98-713-2286940
Fax: +98-713-2286950

ABSTRACT

The injection of carrageenan in mouse paws causes inflammation and pain via enhancement of reactive oxygen species formation. In the present study, anti-inflammatory and antioxidant activities of the aqueous and ethanolic extracts of *Chamaemelum nobile* commonly known as Roman chamomile (RC) were assayed in carrageenan induced mice paw edema. Results showed that RC ethanolic extract is able to inhibit the development of paw edema. Moreover, oral pretreatment of RC ethanolic extract significantly decreased production of pro-inflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6. RC ethanolic extract showed significant radical scavenging effect by inhibition of lipid peroxidation and increasing antioxidant enzyme activities including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). However, the elevation of these enzyme activities by RC aqueous extract was not significant. Generally, RC ethanolic extract showed better inhibitory effect on inflammation, which is similar to the effect of the anti-inflammatory drug indomethacin. Thus, it is suggested that RC possesses anti-inflammatory effect by inhibiting the pro-inflammatory cytokines and reducing the oxidative damage. Therefore, this plant can be one of the alternatives for treating inflammatory conditions.

Keywords: Roman chamomile, Anti-inflammatory, Pro-inflammatory cytokines, Antioxidant enzymes, Malondialdehyde

INTRODUCTION

Inflammation is the primary response of body to the aggressive events such as traumatic injury, irritation or infection caused by pathogens (1). The inflammatory process involves numerous interactions between inflammatory cells (lymphocytes, neutrophils and monocytes/macrophages) and vascular cells (smooth muscle and endothelial cells) (2). Increase in blood flow and metabolism of cells with vasodilation and release of soluble mediators, extravasation of fluids and cellular influx are typical signs of inflammation (3). Carrageenan induced paw edema is one of the models widely used for determining the acute phase of inflammation (4). Various inflammatory mediators including neutrophil-derived active oxygen species and free radicals, such as hydrogen peroxide, superoxide and the hydroxyl radical, nitric oxide, prostaglandins and cytokines (IL-1β and IL-6), are released into the rat paw tissue during carrageenan induced acute inflammation. These could be expressed in macrophages, hepatocytes, and endothelial or smooth muscle cells (5, 6, 7, 8). The inflammatory mediators are responsible for perpetuation of inflammation and its consequences (9). In addition, reactive oxygen species (ROS) cause tissue damage during inflammatory process (10, 11, 12). Therefore, agents with capability to inhibit pro-inflammatory cytokines and antioxidant activity could represent a novel therapeutic approach in disorders associated with inflammation.

Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are the main therapeutic agents in inflammation, but they can cause serious side effects (13). Hence, the development of new drugs with comparable anti-inflammatory effects beside its antioxidant activity is required (14). Recently applications of herbal medicines derived from plant extracts have attracted many researchers in order to treat inflammation (15). As ROS play an important role in various diseases, there is a growing interest in plants having antioxidant activity. *Matricaria recutita* commonly known as "German chamomile" (GC) and *Chamaemelum nobile* commonly known as Roman chamomile (RC) are the two major types of chamomile used for health conditions. They are believed to have similar effects on the body (16). Most research worked on GC but, studies on RC is limited and stronger evidence to support its use for inflammation is required. RC belongs to the family Asteraceae and is widely distributed in Asia, Europe, Africa and Northern America. RC is known as a medicinal plant since the middle age (16). However, the provided clinical and non-clinical data for RC do not fulfill the requirements of a well-established medicinal use with recognized efficacy (17, 18). Herbal substance of RC has a small potential for sensitization and it possesses antibacterial, antifungal, anti-inflammatory, cytotoxic, brochodilatory, hypotensive, anti- platelet aggregation, antioxidant, and hypoglycemic effects (19, 20). The most characteristic constituents of this plant are volatile oil, sesquiterpene, hydroperoxides, catechins, coumarins, triterpenes and steroids, polysaccharides and phenolics including flavonoids (21). Studies on RC extracts demonstrated that both lipophilic and hydrophilic compounds possess therapeutic activity (22).

Although the anti-inflammatory properties of RC have been studied, however, the mechanisms involved are not yet known. Therefore, the present study was undertaken to determine the effects aqueous and ethanolic extracts of RC on the levels of pro-inflammatory cytokines including interleukin (IL)-1β, TNF-α and IL-6 and to evaluate the oxidative stress by quantifying malondialdehyde (MDA) and the antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT).

MATERIAL AND METHODS

Animals

Male albino mice weighing 20 ± 2 g were obtained from the Center for Comparative and Experimental Medicine (Shiraz, Iran) and maintained in a controlled temperature (21 ± 1 ºC) environment with 35% relative humidity and 12:12-h light-dark cycle with free access to tap water and standard food (*ad libitum*) for a week. All animal experiments were approved by the State Committee on Animal Ethics, Shiraz

University, Shiraz, Iran (IACUC no: 4687/63). The recommendations of European Council Directive (86/609/EC) of November 24, 1986 regarding the standards in the protection of animals used for experimental purposes were also followed.

Preparation of the plant

Fresh *C. nobile* plant was obtained from Shiraz area (South of Iran). The plants were identified by Dr. Bahadori from the Department of Agronomy, ShahreKord University, Shahrekord, Iran. A voucher specimen was deposited at the ShahreKord University, ShahreKord, Iran (herbarium no. 11042). The aerial parts of the plant was washed, dried in shade and powdered. The powder was used in order to prepare aqueous and ethanolic extracts.

Aqueous extract

Distilled water (500 ml) was added to 50 g of the dried powder plant and placed into an orbital shaker. The mixture was filtered using a number one Whatman filter paper after 6 h. The filtrate was freeze-dried and the residual aqueous extract was stored at -4 ºC for further studies.

Ethanolic extract

The plant powder (200 g) was soaked in 3 L of 90% ethanol and allowed to stand for two days at room temperature. The ethanolic extract was obtained using a vacuum rotary evaporator and then extract was freeze-dried and stored at -4 ºC.

Carrageenan induced paw edema

The inflammatory response was induced by carrageenan in mice reflected as paw edema based on Winter et al. (1962) method (23). Thirty mice were randomly divided into 5 equal groups:
Group I served as control group and was only pre-treated with normal saline;
Group II served as carrageenan group and was pre-treated with normal saline;
Group III was pre-treated with indomethacin (5 mg/b.w.);
Group IV was pre-treated with ethanolic extract of RC (15 mg/b.w.);
Group V was pre-treated with aqueous extract of RC (15 mg/b.w.).
Animals were given drugs by oral route one hour after treatment and the dose has been selected by the study of Lemhadri et al. (2007) (24). Carrageenan suspension (1%) was prepared by adding 100 mg of carrageenan powder on 10 ml of saline (0.9%) solution and set aside to soak for 1 h. A homogeneous suspension was then obtained by thorough mixing with a magnetic stirrer. Carrageenan suspension (0.1 ml) was injected into the subplanter region of left hind paw except group I. The paw volume was measured at 0 and 4 h after carrageenan injection using Vernier calipers (520-R, IITC, Life Science - USA).

The inhibition of inflammation was calculated using the following formula:

$$\% \text{ Inhibition of edema} = \frac{T - T0}{T} \times 100$$

Where T is the thickness of paw in carrageenan group; T_0, thickness of paw edema in the compound treated group.

Preparation of tissue for biochemical analysis

Animals were sacrificed under anesthesia using an ethical manner four hours after carrageenan injection. The soft paw tissues were harvested and weighed. Tissues were homogenized after the addition of phosphate buffer solution (0.1M, pH 7.4) (Yellow Line DI 18 Basic [IKA, Staufen, Germany]). The tissue suspension was subjected to centrifugation at 3000 x for 15 min at 4ºC. Supernatant of homogenate tissue for each animal was divided into aliquots and were kept at –80◦C for further experiments.

Biochemical analysis

Anti- inflammatory activity
The levels of IL-1β (ng/gr tissue), TNF-α (ng/gr tissue) and IL-6 (pg/gr tissue) in tissues were estimated by ELISA kit (CUSABIO, China) according to the manufacturer's protocol.

Superoxide dismutase (SOD) activity
The activity of SOD was assayed by RANSOD kit (Randox Lab., Crumlin, United Kingdom) by spectrophotometer (Shimadzu AA 670, Kyoto, Japan). SOD determination is based on xanthine and xanthine oxidase to produce superoxide radicals that interact with 2-(4- iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to generate red formazan dye. The inhibition level of this reaction shows the activity of SOD. The inhibition rate of reduction of INT by 50% is one unit of SOD expressed as unit per gram of tissue (U/g tissue).

Glutathione peroxidase (GPx) activity
GPx activity was evaluated with GPx detection RANSEL kit (Randox Lab., Crumlin, United Kingdom) using a spectrophotometer (Shimadzu AA 670, Kyoto, Japan). GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) will immediately be converted to the reduced form along with oxidation of NADPH to NADP+. The amount of enzyme that converts 1 μmol of NADPH to NADP+ per minute is defined as one unit (U) of GPX activity. The GPX activity is expressed as unit per gram of tissue (U/g tissue).

Catalase (CAT) activity
The activity of CAT was evaluated by the method described by Goth (1991) (25). In this method, hydrogen peroxide forms stable complex with ammonium molybdate that can be determined spectophetometrically at 405 nm wavelengths (Shimadzu AA 670, Kyoto, Japan). The values of enzymes were expressed as unit per gram of tissue (U/g tissue).

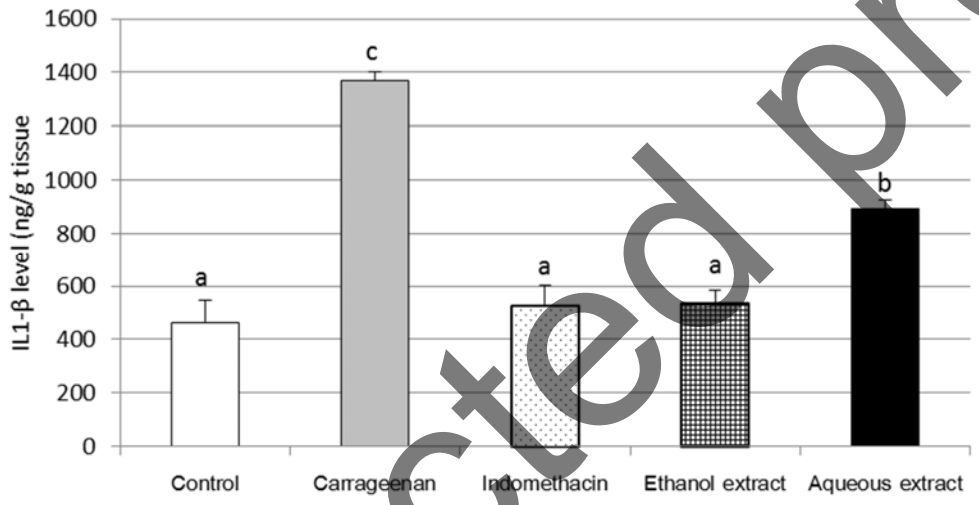
Lipid peroxidation (MDA)
Lipid peroxidation was determined in inflamed tissue by a modified HPLC method using the reaction of MDA with thiobarbituric acid (TBA) forming a colored MDA-TBA adducts (26). The values are expressed as mmol/g tissue.

Statistical analysis
The values for various parameters are expressed as the mean ± standard error of the mean (SEM). Data were analyzed by analysis of variance (one-way ANOVA) using Statistical Package for the Social Sciences (SPSS) software, version 20. The significance of differences in means between groups was evaluated by Duncan test. Significance level was set to be $p<0.05$.

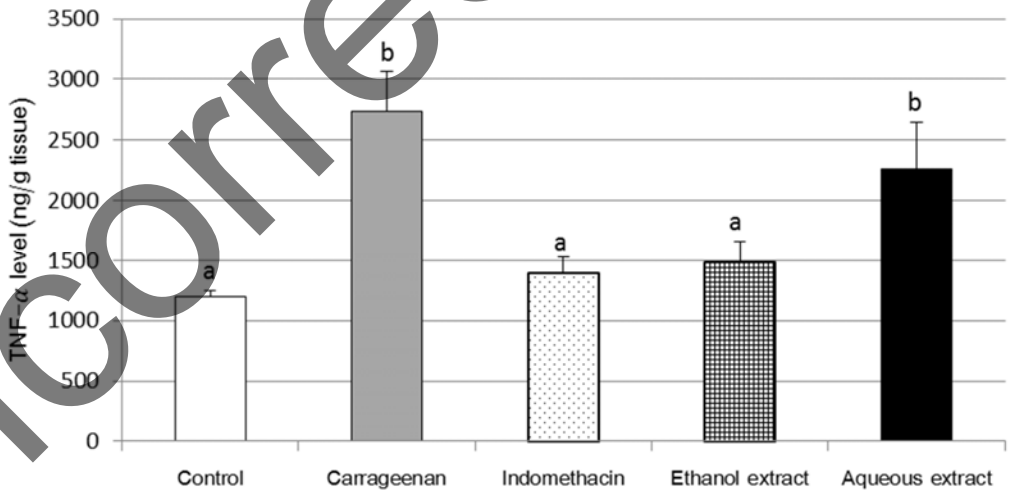
RESULTS
Administration of indomethacin and the ethanolic extract of RC prevented paw edema with respective percentage inhibition of 62.9 % and 54.1 % while aqueous extract of RC inhibited the paw edema by only 10.6 %.
Figure 1 shows the effects of both extracts of RC on pro-inflammatory cytokines. Injection of carrageenan increased the level of TNF-α, IL-1β and IL-6. Pre-treatment with RC ethanolic extract caused a significant decrease ($P<0.05$) in TNF-α and IL-1β and there were no significant differences between ethanolic extract, indomethacin and control groups. However, indomethacin had comparatively greater inhibitory action on IL-6. Moreover, the reduction of pro-inflammatory by aqueous extract of RC showed no significant difference compared with carrageenan group.
Figure 2 shows the antioxidant activity of RC extracts. The activity of SOD in carrageenan group was significantly reduced compared with that in the control group. Pretreatment with indomethacin and

ethanolic extract of RC elevated SOD activity in paw tissue. Pretreatment of animals with indomethacin and RC aqueous and ethanolic extract caused a significant increase in both paw GPx and CAT activities compared to the carrageenan group. However, RC aqueous extract had less effect on the reduction of GPx and CAT and there was no significant difference between this group and carrageenan group.

Sub-plantar injection of carrageenan significantly increased MDA level and pretreatment with indomethacin and RC ethanolic extract showed significant effect to attenuate MDA elevation after carrageenan injection. Moreover, there was no significant difference between carrageenan group and the group pretreated with RC aqueous extract.



(A)



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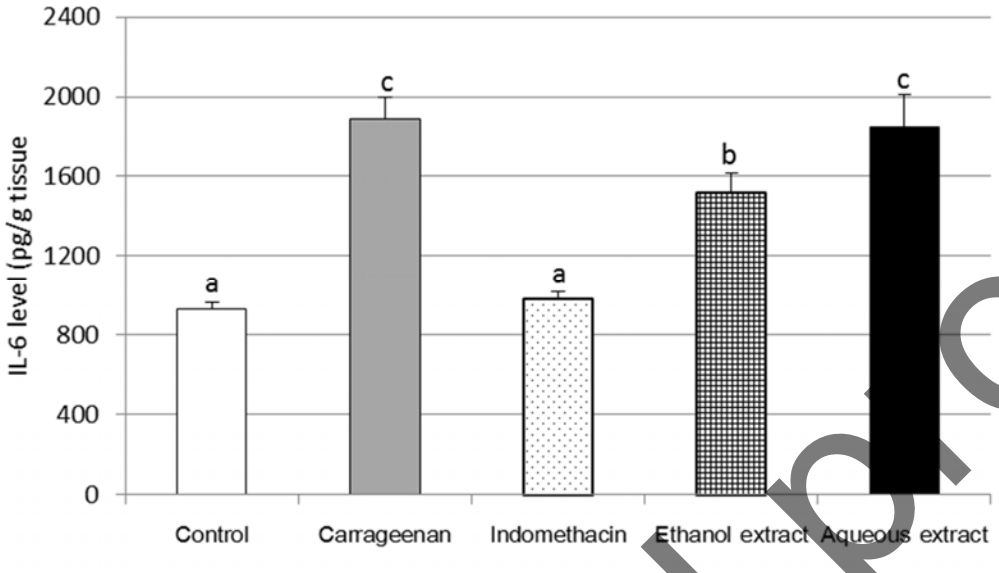
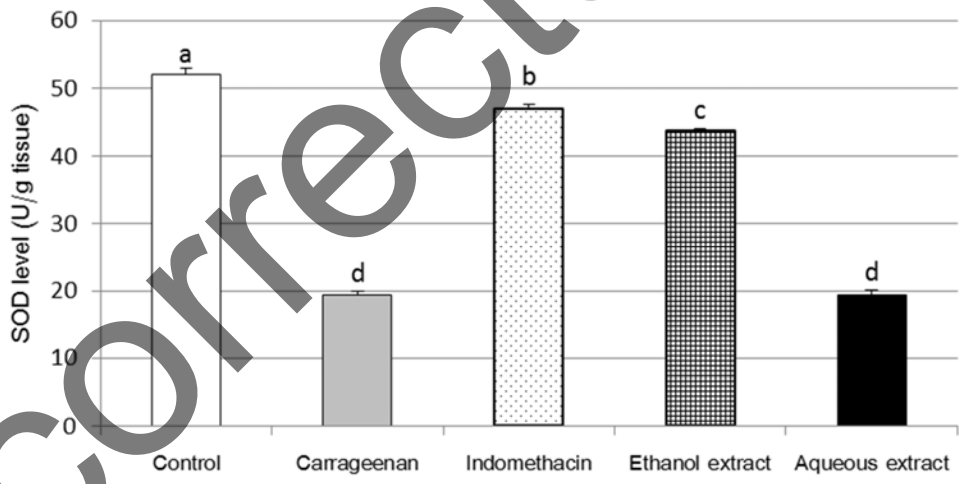


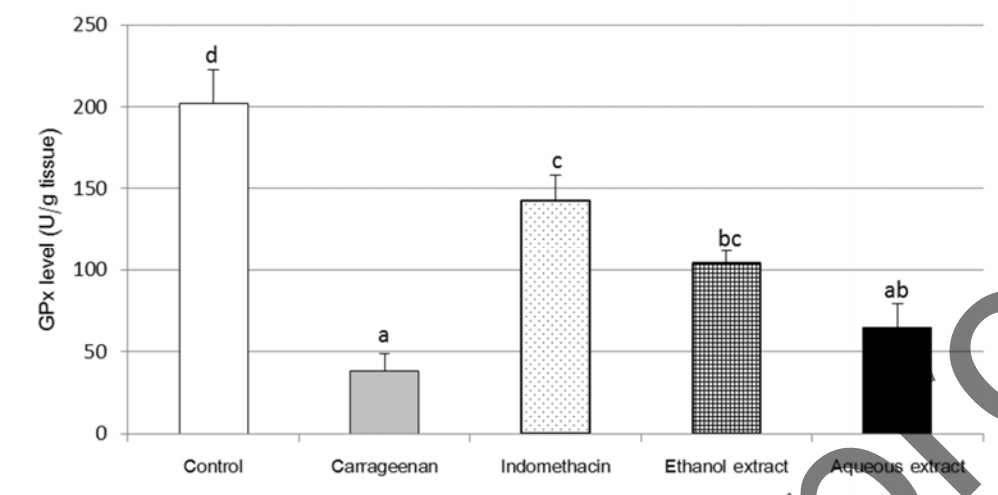
(C)

Figure 1: Effect of indomethacin, ethanol extract and aqueous extract on the levels of pro-inflammatory cytokines IL-1β (A), TNF-α (B) and IL-6 (C) in mice paw edema. Data are presented as Mean ± SEM (n=6).

Values which have a different superscript letter (a, b, c, d) are significantly different (P<0.05).



(A)



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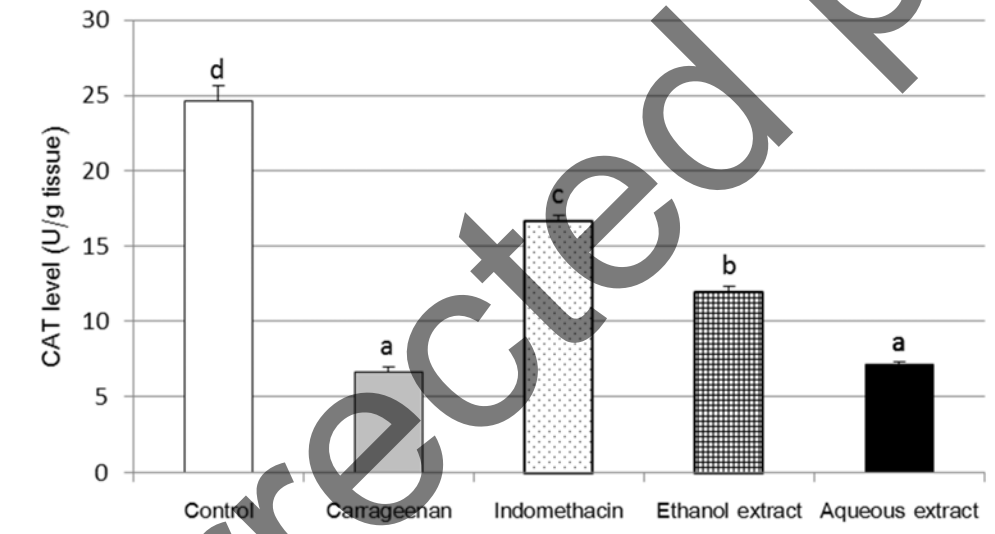

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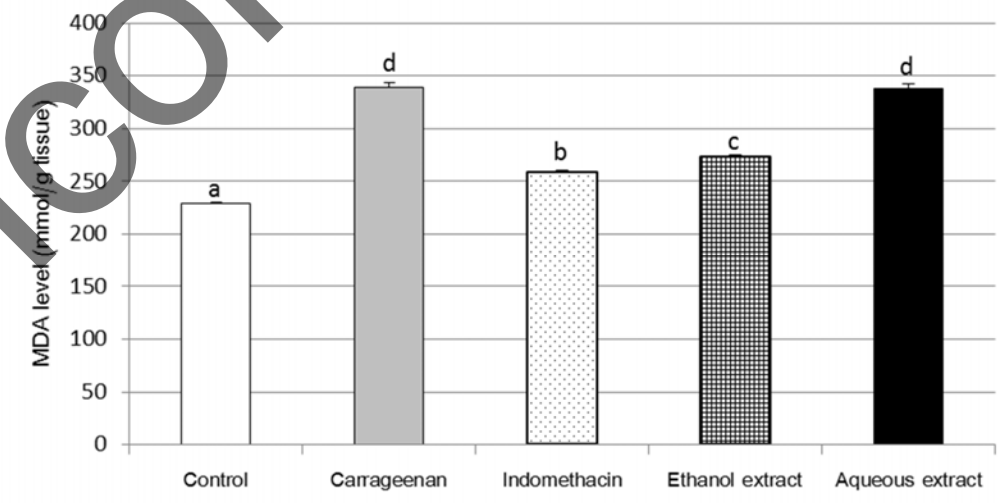

(D)

Figure 2: Effect of indomethacin, ethanol extract and aqueous extract on the levels of SOD (A), GPx (B), CAT (C) and MDA (D) in mice paw edema. Data are presented as Mean±SEM (n = 6).
Values which have a different superscript letter (a, b, c, d) are significantly different (P<0.05).

DISCUSSION

Inflammation is the vital response of the innate immune system and many cytokines, such as IL-1β, IL-6, TNF-α and PGE2, are involved in the inflammatory process (27, 28). Nonsteroidal anti-inflammatory drugs (NSAIDs) like indomethacin, naproxen and diclofenac are the main medications capable of inhibiting inflammatory cytokines and are the first choice for the treatment of inflammation. However, adverse effects of these drugs limit their use (29). In recent years, therefore, attention has been focused on plants with anti- inflammatory activity. Since ROS play an important role in the pathogenesis of inflammatory diseases, natural antioxidants, that can scavenge ROS, are expected to combat these disorders (30).

The flowers of RC contain 1–2% volatile oils including alpha-bisabolol, alphabisabolol oxides A & B, and matricin (usually converted to chamazulene and other flavonoids) which possess anti-inflammatory properties (31, 32, 33, 34). Few studies have been conducted specifically for RC, but the azulene compounds are reported to possess anti-inflammatory properties (35). Moreover, it has been reported that the polysaccharides isolated from the aqueous extract of RC flowers and herb have significant anti-inflammatory activity (36).

Another study in human volunteers demonstrated that RC flavonoids and essential oils penetrate below the skin surface into the deeper skin layers (37). This is important for their use as topical anti-inflammatory agents (38). Although the anti-inflammatory activity of RC, have been reported, but the exact mechanism of its inhibitory effect on paw edema has not yet been established. It is well known that vasodilatation causes the release of the inflammatory mediators during inflammation that initiates the acute inflammatory response (39, 40, 41, 42). The progress of edema in the mice hind paw following the injection of carrageenan has been characterized as a biphasic event. In the early stage serotonin, histamine, bradykinin and prostaglandins are involved. The late phase which is the systemic inflammation is mainly medicated by TNF-α, IL-1β and IL-6 (43). In the present study, RC ethanolic extract significantly reduced carrageenan-induced inflammation in the late phase of inflammation by inhibiting TNF-α, IL-1β and IL-6. The decreased levels of pro-inflammatory cytokines demonstrated that the anti-inflammatory activity of RC may be associated with inhibition on pro-inflammatory mediators such as TNF-α, IL-1β and IL-6. However, RC ethanolic extract showed greater anti-inflammatory effect when compared to the aqueous RC extract. In other words, indomethacin and RC ethanolic extract had similar anti-inflammatory activity.

Other researchers have demonstrated that ROS is one of the results of inflammatory process in carrageenan injection (44, 45). In the inflammatory reaction, production of ROS such as hydroxyl radicals, hydrogen peroxide and superoxide anion radicals are key factors in progressing cellular damage (46). Free radical generation releases MDA from plasma membrane. Therefore, the degree of inflammation can be determined by the MDA accumulation in tissues. Moreover, ROS disables antioxidant defense systems and thus indirectly causes cell damage. SOD, GPx and CAT are antioxidant enzymes that can protect cells by scavenging ROS (42). In the present study, RC ethanolic extract significantly increased the activity of GPx, SOD and CAT. Moreover, a significant decrease in the level of tissue MDA was observed after RC ethanolic extract application. These results show that RC can protect inflammatory oxidation because of its antioxidant activity. The suppression of MDA level is probably due to the activation of antioxidant enzymes including SOD, GPx and CAT.

Various classes of bioactive constituents exist in *C. nobile*, including sesquiterpenes, hydroperoxides, flavonoids, catechins, coumarins, polyacetylenes, phenolic acids, anthenobilic acid, triterpenes steroids and polysaccharides (21). Among these constituents only flavonoid and catechin are soluble in water.

Although, flavonoid and catechin have high antioxidant and anti-inflammatory activities, but the ineffectiveness of the aqueous extract may be due to its insufficient therapeutic dose to provide a protective effect against inflammation. Anti-inflammatory and antioxidant activities of coumarins, catechins, caffeic acid and phenolic compounds, such as flavonoid, have been reported by many researchers (28, 46, 47, 48, 49, 50, 51). Different constituents present in the ethanolic extract may have synergic activity in reducing the inflammation and, therefore, the ethanolic extract having more bioactive compounds was comparatively more effective than aqueous extract in reducing inflammation and oxidative stress.

CONCLUSION

It is generally concluded that RC ethanolic extract is effective in controlling inflammation and possesses anti-inflammatory activity by inhibiting IL-1β, IL-6 and TNF-α. It also improves antioxidant activity and lipid peroxidation during inflammation. Hence, RC extracts should be one of the alternatives for inhibiting the inflammation process as well as ROS production.

ETHICAL APPROCAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

ACKNOWLEGMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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