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ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF AN ETHANOL EXTRACT OF *DUNALIELLA SALINA* TEOD. (CHLOROPHYCEAE)

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ABSTRACT

This study investigated the analgesic and anti-inflammatory effects of an ethanol extract of Dunaliella salina *Teod. (Chlorophyceae) (EDS) in Imprinting Control Region mice. Standard all-trans-β-carotene and the amount of all-trans-β-carotene in an EDS were analyzed by high-performance liquid chromatography (HPLC). In HPLC analysis, the fingerprint chromatogram of EDS was established. Both all-trans-β-carotene and EDS showed similar peaks at the retention time of 24 min. This implied that EDS contained the active ingredient all-trans-β-carotene.*

Treatment of animals with EDS significantly inhibited the numbers of acetic acid-induced writhing responses at doses of 0.5 g/kg ($P < 0.01$*),*

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*1.0 g/kg (*P < *0.001) and 2.0 g/kg (*P < *0.001). This inhibitory effect of EDS (1.0 and 2.0 g/kg) on acetic acid-induced writhings was similar to that of the positive control indomethacin (10 mg/kg) (*P < *0.001). EDS did not significantly inhibit the formalin-induced pain in the early phase; however, at doses of 0.1 g/kg (*P < *0.01), 0.5, 1.0 and 2.0 g/kg, EDS significantly inhibited the formalin-induced pain in the late phase (*P < *0.001). Finally, EDS at doses of 1.0 and 2.0 g/kg also inhibited the development of paw edema induced by λ-carrageenan (carrageenan). EDS (1.0 and 2.0 mg/kg) decreased the level of nitiric oxide (NO) in edematous paw tissue and in serum level, and diminished the level of serum tumor necrosis factor-α (TNF-α) at the fifth hour after carrageenan injection. Based on these findings, EDS probably exerts anti-inflammatory effects by suppressing TNF-α and NO. These results suggest that EDS might be a potential pharmacological analgesic and anti-inflammatory agent.*

PRACTICAL APPLICATIONS

Dunaliella salina (Dunal) Teod. is a valuable β-carotene source. *D. salina* is cultivated in a great quantity in the southern part of Taiwan. In order to find its potential application for this product, the analgesic and anti-inflammatory effects were investigated. It was found that *D. salina* could attenuate the acetic acid-induced writhings and formalin-induced analgesia. We also demonstrate that *D. salina* could lessen the carrageenan-induced paw edema. Also, the anti-inflammatory effects verified that nitric oxide and TNF-α could play an import role.

INTRODUCTION

Dunaliella salina, a unicellular halophilic green microalgae, is a well-known source of β-carotene. It is widely consumed in China, Japan and Taiwan. Several reports indicate that *Dunaliella* has anti-oxidative effects (Lavy *et al.* 2003; Chidambara Murthy *et al.* 2005; Murthy *et al.* 2005; Vanitha *et al.* 2007). *D. salina* exhibits potent hepatoprotective effects on CCl_4-induced liver damage in mice, and it is believed that these effects may be due to both the increase of anti-oxidant enzymes activities and inhibition of lipid peroxidation (Hsu *et al.* 2008). *In vivo*, it has been shown that anti-oxidative effect of algal carotenoid is similar to that of synthetic carotene (Chidambara Murthy *et al.* 2005). β-carotene-rich algae *D. bardawil* markedly inhibited spontaneous mammary tumorigenesis in mice by increasing the anti-oxidant function of β-carotene (Nagasawa *et al.* 1991). *Dunaliella*

bardawil promotes the growth of normal mammary gland cells, but inhibits neoplastic cells (Fujii *et al.* 1993). It has also been shown that an extract of *D. salina* could significantly inhibit NSAR-induced carcinogenesis (Xue 1993). Levin *et al.* reported that the antiperoxidative effect of 9-*cis*-β-carotene was more potent than that of all-*trans*-isomer in preventing malignant and cardiovascular diseases (Levin *et al.* 1997). In a recent study, *D. salina* exerted a protective effect against experimentally induced fibrosarcoma in Wistar rats (Raja *et al.* 2007). In our previous studies, we found that the ethanol extract of *D. salina* (EDS) inhibits proliferation and induces apoptosis in the human lung cancer cell line A549 (Sheu *et al.* 2008). However, little information is available on the analgesic and anti-inflammatory effects of *D. salina*. Therefore, we examined the analgesic effects of *D. salina* on acetic acid- and formalin-induced nociception. We also evaluated the anti-inflammatory effects of *D. salina* on paw edema induced by carrageenan in mice and investigated its related mechanisms.

MATERIALS AND METHODS

Materials

Acetic acid and formalin were purchased from Merck (Darmstadt, Germany). All-*trans*-β-carotene, carrageenan and indomethacin were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of *D. Salina*

Spray-dried algae material from *D. salina* cultured in outdoor cultivation pools were prepared by GONG BIH Enterprise Co., Ltd (Doo-Liu City, Taiwan). The composition of *D. salina* was analyzed and is shown in Table 1. *D. salina* (1.5 kg) was soaked in 70% ethanol (each 10 L) at 100C for 20 min.

TABLE 1.
COMPOSITIONS OF DS DRY POWDER

Compositions	mg/g
α-carotene	120/100
β-carotene	6,882/100
Xanthohyll	194/100
Zeaxanthin	275/100
Lycopene	7.18/100
Chlorophyll	1.124/100

Atio β-carotene ratio(9-*cis*/all-*trans*) 47:53.
Data are provided by GONG BIH Enterprise Co., Ltd.

The supernatants were collected, concentrated with a vacuum evaporator (Eyela N-N Serials, Rikakikai Co. Ltd., Tokyo, Japan) until the volume was reduced to 5 mL and stored at −20C in a refrigerator (Herrero *et al.* 2006). Samples were filtered with filter paper (Advantec No.1, Advantec MFS Inc., Tokyo, Japan) while the residue was further extracted under the same conditions thrice. The filtrates collected from these separate extractions were combined and evaporated to dryness under vacuum at 50C. The yield obtained was 4% (60 g) from the ethanol extract of *D. salina.*

Compositional analysis of β-carotene and EDS by High-Performance Liquid Chromatography (HPLC)

HPLC was conducted to analyze both the standard (all-*trans*-β-carotene) and EDS. The purity of the standard was more than 95% based on reverse phase HPLC analysis (Instrument: Jasco system; column: C18 reversed-phase column with particle size 5 μm, Vydac 201 TP54 stainless column [25 cm × 4.6 mm i.d.]; Mobile phase : methanol : acetonitrile [9:1, v/v]; mobile phase flow rate:1.0 mL/min).

Animals

Male ICR mice (18 g to 25 g) were obtained from BioLASCO Taiwan Co., Ltd. (Nankang, Taiwan). The animals were kept in plexiglass cages at a constant temperature of 22 ± 1C, relative humidity 55 ± 5% with 12-h dark–light cycle for at least 2 weeks before the experiment. They were given food and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. The control groups were given 0.1 mL/10 g saline intraperitoneally (i.p.) using a bent blunted 27-gauge needle connected to a 1-mL syringe. All tests were conducted under the guidelines of the International Association for the Study of Pain (Zimmermann 1983). This study was approved by the ethics committee of the Institutional Animal Care and Use Committee of China Medical University.

Acetic Acid-Induced Writhing Response

After a 2-week adaptation period, male ICR mice (18–25 g) were randomly assigned to six groups ($n = 8$) including a normal control, an indomethacin (Indo) positive control and four EDS-treated groups. Control group received normal saline and the positive control group received indomethacin (10 mg/kg, i.p.) 25 min before i.p. injection of 1.0% acetic acid (10 mL/kg body weight). EDS-treated groups received EDS (0.1, 0.5, 1.0, and 2.0 g/kg, p.o.) 55 min before i.p. injection of 1.0% acetic acid (10 mL/kg body

weight). Five minutes after the i.p. injection of acetic acid, the number of writhings during the following 10 min was recorded. Control mice received normal saline (Taber *et al.* 1969).

Formalin Test

The antinociceptive activity of the drugs was determined using the formalin test described by Dubuisson and Dennis (1977). Twenty microliters of 5% formalin was injected into the dorsal surface of the right hind paw 60 min after administration of EDS (0.1, 0.5, 1.0 and 2.0 g/kg, p.o.) and 30 min after administration of indomethacin (10 mg/kg, i.p.). The mice were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post-formalin injection is referred to as the early phase and the period between 15 min and 40 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch. The activity was recorded in 5 min intervals.

λ-Carrageenan (Carrageenan)-Induced Edema

Carrageenan-induced hind paw edema model was used for determination of anti-inflammatory activity (Winter *et al.* 1962). After a 2-week adaptation period, male ICR mice (18–25 g) were randomly assigned to five groups ($n = 8$) including control, carrageenan, positive indomethacin control and three EDS-treated groups. The control group only received normal saline. The carrageenan group received 1% carrageenan (50 μL). EDS at doses of 0.5, 1.0 and 2.0 g/kg were orally administered 2 h before the injection with 1% carrageenan (50 μL) in the plantar side of right hind paws of the mice. Indomethacin (10 mg/kg) was intraperitoneally administered 90 min before the injection with 1% carrageenan (50 μL) in the plantar side of right hind paws of the mice. Paw volume was measured immediately after carrageenan injection at 1, 2, 3, 4 and 5 h intervals using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio *a*/*b*, where *a* is the volume of the right hind paw after carrageenan treatment, and *b* is the volume of the right hind paw before carrageenan treatment. Indo was used as a positive control (Mascolo *et al.* 1989). After 5 h, the animals were sacrificed and the carrageenan-induced edema paw was dissected. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline four times their volume and homogenized at 4C. Then, the homogenate was centrifuged at $12{,}074 \times g$ for 5 min. The supernatant was obtained and stored at −20C refrigerator for the NO assays. Also, blood was withdrawn and kept at −80C for NO and TNF-α assay.

Total Protein Assay

The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

Determination of Nitric Oxide (NO)

Nitrite, a stable end product of NO, was then measured using the Griess reaction (Liao *et al.* 2007). Samples of 100 μL aliquots mixed with 100 μL of Griess reagent (0.1% N-(1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid), followed by spectrophotometric measurement at 550 nm. Nitrite concentrations in the supernatants were determined by comparison with a sodium nitrite standard curve.

Measurement of Serum TNF-α by Enzyme Linked Immunosorbent Assay (ELISA)

Serum levels of TNF-α were determined using a commercially available ELISA kit according to the manufacturer's instructions. TNF-α was determined from a standard curve for the cytokine. The concentrations were expressed as pg/mL (Chun *et al.* 2007).

Statistical Analysis

Data are expressed as mean ± SEM Statistical evaluation was carrageenanied out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests). Statistical significance is expressed as $*P < 0.05$, $**P < 0.01$ *and* $***P < 0.001$.

RESULTS

Compositional Analyses of β-carotene and EDS by HPLC

D. salina contains α-carotene, β-carotene, lutein, cryptoxanthin and zeoxanthin (Table 1). The composition of all-*trans*-β-carotene (standard) and that of EDS were analyzed by HPLC. The HPLC fingerprint demonstrated that EDS and the standard (all-*trans*-β-carotene) had similar peaks at the retention time of 24 min. The chromatogram indicated that EDS contained the active ingredient all-*trans*-β-carotene.

Acetic Acid-Induced Writhing Response

The cumulative amount of abdominal stretching correlated with the level of acetic acid-induced pain (Fig. 1). EDS treatment (1.0 and 2.0 g/kg) signifi-

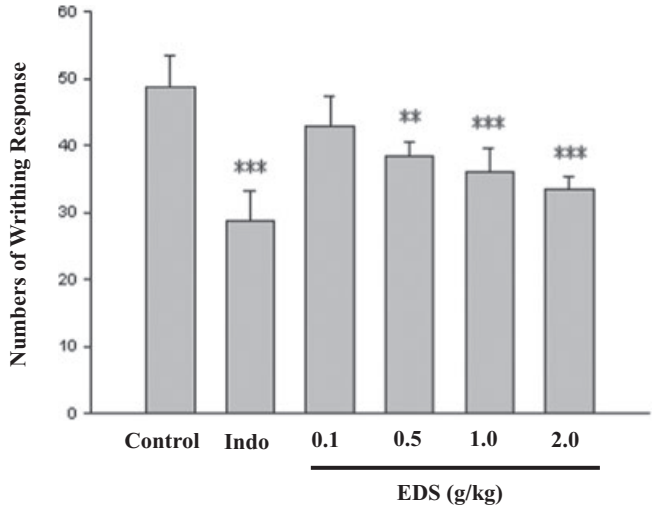

FIG. 1. The effect of EDS on 1% acetic acid-induced writhing response in mice. 1% acetic acid (10 mL/kg) was intraperitoneally injected to mice 55 min after administration of the EDS (0.1, 0.5, 1 and 2 g/kg, p.o.) and 25 min of indomethacin (Indo, 10 mg/kg, i.p.). Data are represented as mean ± SEM (n = 8). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. (One-way ANOVA followed by Scheffe's multiple range test).

cantly inhibited the number of writhings in comparison with the normal controls ($P < 0.001$). Also, 0.5 g/kg EDS inhibited the number of writhings in comparison with the normal ($P < 0.01$). This inhibiting effect of acetic acid-induced writhings by EDS (1 and 2 g/kg) was similar to that produced by a positive control indomethacin (10 mg/kg).

Formalin Test

EDS 0.1 g/kg ($P < 0.01$) and EDS (0.5, 1 and 2 g/kg) significantly ($P < 0.001$) inhibited formalin-induced pain in the late phase (Fig. 2); however, there was no inhibition in the early phase (data not shown). The positive control indomethacin (10 mg/kg) also significantly ($P < 0.001$) inhibited the formalin-induced pain in the late phase.

Carrageenan-Induced Edema

EDS (1.0 g/kg) ($P < 0.05$) and EDS (2.0 g/kg) ($P < 0.01$) significantly inhibited the development of carrageenan-induced paw edema after 3 h. of treatment; however, it only showed inhibition in the early phase. Indomethacin (10 mg/kg) significantly decreased the carrageenan-induced paw edema after 3 ($P < 0.01$), 4 ($P < 0.01$) and 5 h of treatment ($P < 0.001$) (Fig. 3).

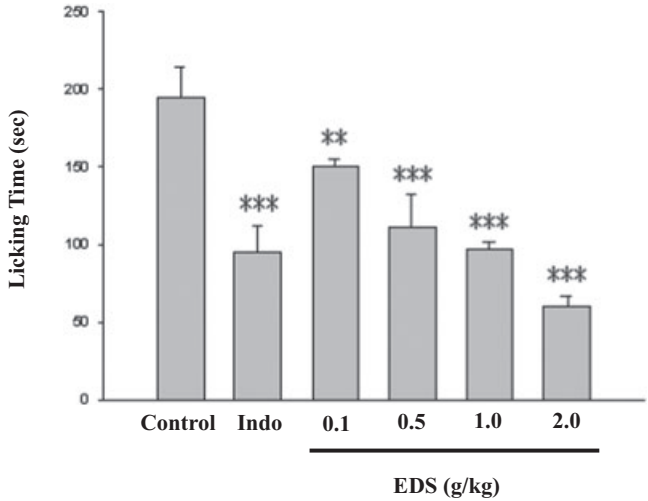

FIG. 2. The effects of EDS on the late phase B (15–40 min) on 1% formalin-induced inflammation in mice. 20 μL of 5% formalin was injected into the dorsal surface of the right hind-paw paw of mice 60 min after administration of the EDS (0.1, 0.5, 1 and 2 g/kg, p.o.) and 30 min of indomethacin (Indo, 10 mg/kg, i.p.). Data are represented as mean ± SEM (n = 8). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. (One-way ANOVA followed by Scheffe's multiple range test).

Effects of EDS on NO Measurement

EDS (1.0 g/kg and 2.0 g/kg) significantly decreased the NO level in edematous paw tissue ($P < 0.05$) and in serum ($P < 0.05$) (Fig. 4). Indomethacin (10 mg/kg) significantly decreased the NO level in the edematous tissue and in serum at the fifth hour after carrageenan injection ($P < 0.001$).

Effects of EDS on TNF-α Level

EDS (1.0 g/kg) and EDS (2.0 g/kg) decreased the TNF-α level in serum at the fifth hour after carrageenan injection ($P < 0.01$) (Fig. 5). Indomethacin (10 mg/kg) significantly decreased the TNF-α level in serum at the fifth hour after carrageenan injection ($P < 0.01$).

DISCUSSION

The anti-nociceptive effects of test samples were assayed using the acetic acid- and formalin-induced analgesic models. Our results indicate that EDS treatment significantly inhibited the number of writhings in comparison with

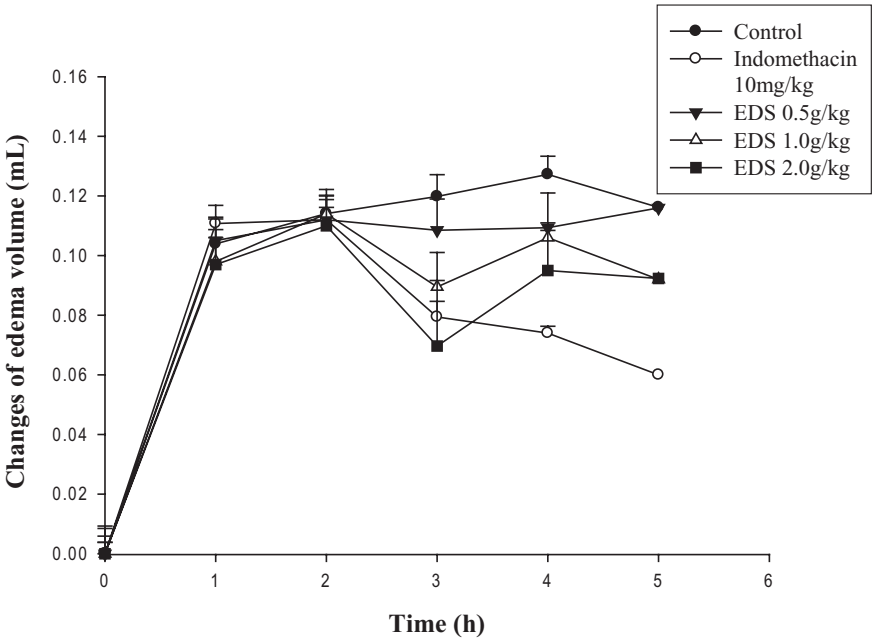

FIG. 3. The effects of EDS on mice hind-paw edema induced by carrageenan. 50 μL of 1% carrageenan was intraperitoneally injected into the plantar side of right hind paws of the mice 120 min after administration of the EDS (0.1, 0.5, 1 and 2 g/kg, p.o.) and 90 min of indomethacin (Indo, 10 mg/kg, i.p.). Data are represented as mean ± SEM (n = 8). $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the control group. (One-way ANOVA followed by Scheffe's multiple range test).

the normal controls ($P < 0.001$) (Fig. 1). Also, EDS significantly inhibited formalin-induced pain in the late phase (Fig. 2). The acetic writhing test is commonly used to study the peripheral analgesic effects of drugs and widely used for analgesic screening (Shibata *et al.* 1989). We found that EDS (0.5, 1 and 2 g/kg) exhibited an antinociceptive effect in acetic acid-induced writhing response.

The formalin test is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The formalin test produces a distinct biphasic response, and different analgesics may act differently in the early and late phases of this test. Therefore, the test can be used to clarify the possible mechanism of an antinociceptive effect of a proposed analgesic (Tjolsen *et al.* 1992). Centrally acting drugs such as opioids inhibit both phases equally (Shibata *et al.* 1989), but peripherally acting drugs such as aspirin, indomethacin and dexamethasone only inhibit the late phase. The inhibitory effect of EDS on the nociceptive response in the late phase of the

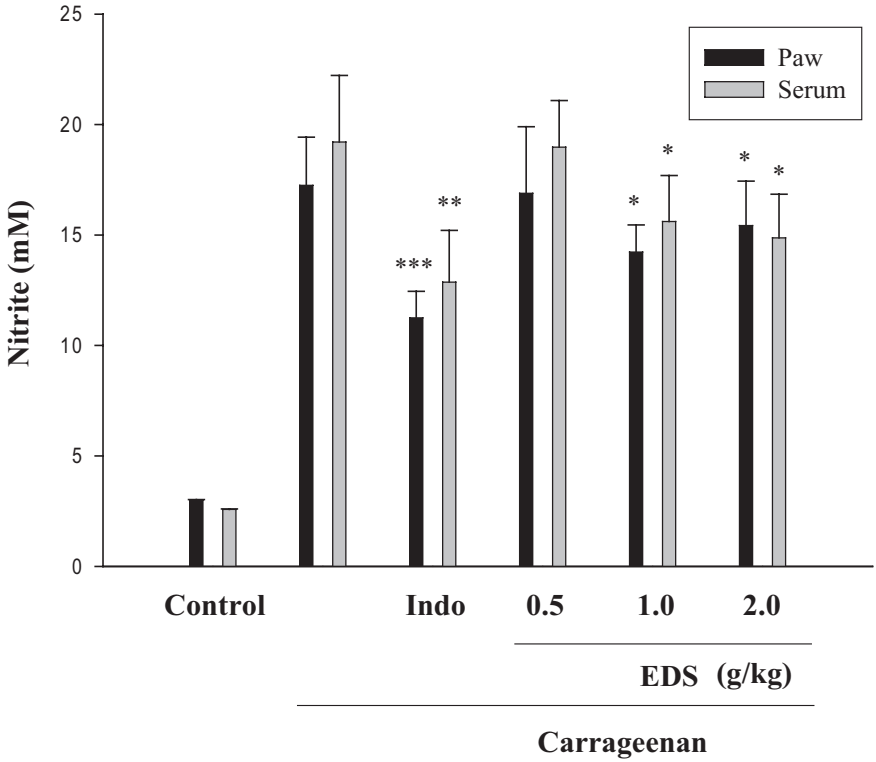

FIG. 4. Effects of the EDS and indomethacin on carrageenan-induced NO concentration of edematous paw tissue and serum at the fifth hour in mice. Each value represents as mean ± SEM ###$P < 0.001$ as compared with the control group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared with the carrageenan group (one-way ANOVA followed by Scheffe's multiple range test).

formalin test suggested that the anti-nociceptive effect of the EDS could be due to its peripheral action.

The carrageenan-induced edema test is highly sensitive to nonsteroidal anti-inflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs (Just *et al.* 1998). The degree of swelling of the carrageenan-injected paws was maximal 3 h after injection and the mean increase in volume at that time was about 100% in the control group. Statistical analysis revealed that EDS (1.0 g/kg) ($P < 0.05$) and EDS (2.0 g/kg) ($P < 0.01$) significantly inhibited the development of carrageenan-induced paw edema after 3 h of treatment (Fig. 3).

The L-arginine-NO pathway has been proposed to play an important role in the carrageenan-induced inflammatory response (Salvemini *et al.* 1996).

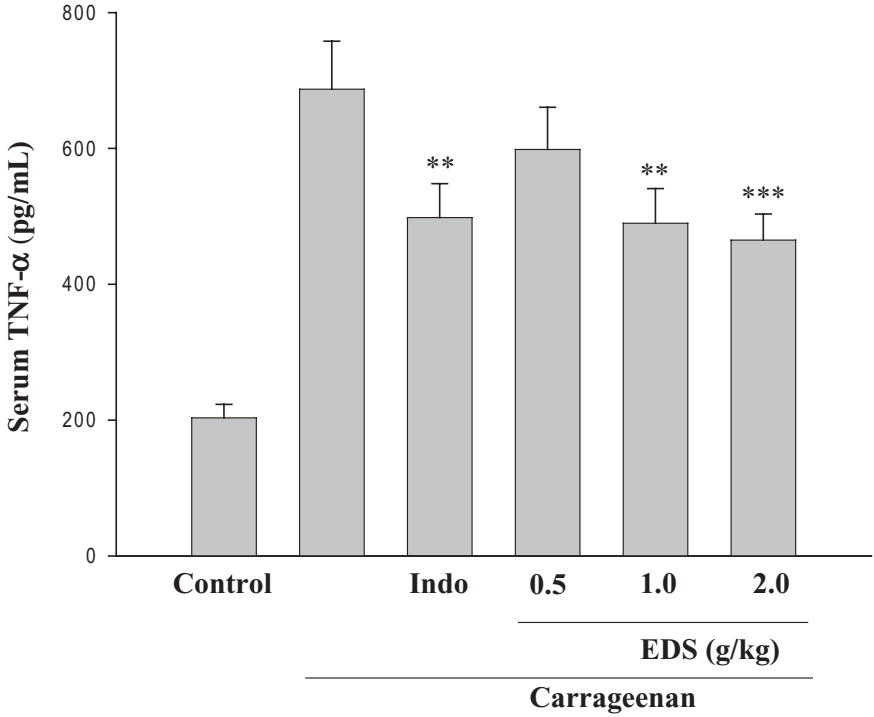

FIG. 5. Effects of the EDS and indomethacin (Indo) on carrageenan-induced TNF-α concentration of serum at the fifth hour in mice. Each value represents as mean ± SEM ###$P < 0.001$ as compared with the control group. *$P < 0.05$ and **$P < 0.01$ as compared with the carrageenan group (one-way ANOVA followed by Scheffe's multiple range test).

The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation (Cuzzocrea *et al.* 1997). In our study, EDS at 1.0 and 2.0 g/kg significantly decreased the levels of NO in edematous paw tissue and in serum, indicating that EDS elicits an anti-inflammatory response via the L-arginine-NO pathway (Fig. 4).

TNF-α is a major mediator in inflammatory responses. It induces innate immune responses by activating T cells and macrophages, and stimulates secretion of other inflammatory cytokines (Beutler and Cerami 1989). Also, TNF-α is a mediator of carrageenan-induced inflammatory incapacitation, and is able to induce the further release of kinins and leukotrienes, which are suggested to play an important role in the maintenance of long-lasting nociceptive response (Tonussi and Ferreira 1999). In this study, we found that EDS decreased the TNF-α level in serum after carrageenan injection (Fig. 5).

Although algae is a source of protein in certain human foods and animal feeds, the effects of the algae are not clear. Certain algae are believed to possess anti-inflammatory activity (Price *et al.* 2002). For example, *Spirulina* has been shown to modulate the Th profile in patients with allergic rhinitis by inhibiting the production of IL-4, thereby suppressing the differentiation of Th2 cells (Mao *et al.* 2005). A pharmacological study of hydrosoluble and liposoluble extracts of the marine microalgae *Chlorella stigmatophora* and *Phaeodactylum tricornutum* indicated that hydrosoluble components of both species show significant anti-inflammatory, analgesic and free radical scavenging activity. These activities were not detected in the liposoluble fractions (Guzman *et al.* 2001).

β-carotene is a bioactive molecule with anti-inflammatory activities (Bai *et al.* 2005). Our results show that *D. salina* contain 6% of β-carotene, 0.12% of α-carotene, 0.3% of zeaxanthin and scarce amount of lycopene and chlorophyll (Table 1). Study also showed that *D. salina* extract, a significant source of β-carotene, had significantly higher anti-oxidant activity than all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin in all anti-oxidant assays (Hu *et al.* 2008). *D. salina* could protect rats from tetrachloride-induced hepatotoxicity (Hsu *et al.* 2008). It has been proposed that the antihepatotxic activity of *D. salina* is mediated by the isomeric forms of beta-carotene. We propose that *D. salina* elicits an analgesic effect and anti-inflammatory activities. We suggest that the mechanisms of *D. salina* may be associated with the inhibition of inflammatory mediator overproduction, including NO and TNF-α. These findings suggest that *D. salina* may be therapeutically useful for mitigating inflammatory pain.

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