Isolation and Identification of a Flavone Apigenin from Marine Red Alga *Acanthophora spicifera* with Antinociceptive and Anti-Inflammatory Activities

Gihan A. El Shoubaky[1,*], Mohamed M. Abdel-Daim[2,*], Mohamed H. Mansour[1] and Essam A. Salem[1]

[1]Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt. [2]Pharmacology Department, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. *These authors contributed equally to this work.

ABSTRACT: Physicochemical investigation of the red alga *Acanthophora spicifera* (Vahl) Borgesen, collected from Al-Shoaiba coast, Red Sea, Saudi Arabia, led to the isolation of a flavone from the algal tissue with acetone. Preparative chromatography on silica gel thin-layer chromatography was used for the separation of the flavone and eluted with the methanol:chloroform:ethyl acetate (1:7:2) solvent system. The physicochemical analyses infrared, mass spectra, and ultraviolet spectra in addition to shift reagents (NaOMe, NaOAc, NaOAc + H_3BO_3, $AlCl_3$, and $AlCl_3$ + HCl) were used for the identification and elucidation of the structure of the flavone compound (4,5,7-trihydroxy flavonoids). The flavone compound was identified as apigenin bycomparing its physicochemical data with those in the literature. Analgesic and anti-inflammatory activities of apigenin were evaluated. Apigenin showed promising analgesic and anti-inflammatory activities in the hot plate test and writhing test in mice as well as tail-immersion tests and carrageenan-induced paw edema and cotton pellet-induced granuloma formation in rats. It is concluded that apigenin possesses potent analgesic, anti-inflammatory, and antiproliferative activities, which might be due to the inhibition of PGE_2 as well as proinflammatory cytokines such as interleukin-1β, interleukin-6, and tumor necrosis factor-α.

KEYWORDS: *Acanthophora spicifera*, apigenin, analgesic, anti-inflammatory, physicochemical analysis, infrared, mass spectra, ultraviolet spectra

CITATION: El Shoubaky et al. Isolation and Identification of a Flavone Apigenin from Marine Red Alga *Acanthophora spicifera* with Antinociceptive and Anti-Inflammatory Activities. *Journal of Experimental Neuroscience* 2016:10 21–29 doi:10.4137/JEN.S25096.

TYPE: Original Research

RECEIVED: December 9, 2015. **RESUBMITTED:** January 19, 2016. **ACCEPTED FOR PUBLICATION:** January 21, 2016.

ACADEMIC EDITOR: Lora Talley Watts, Editor in Chief

PEER REVIEW: Three peer reviewers contributed to the peer review report. Reviewers' reports totaled 526 words, excluding any confidential comments to the academic editor.

FUNDING: Authors disclose no external funding sources.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: abdeldaim.m@vet.suez.edu.eg; abdeldaim.m@gmail.com

Paper subject to independent expert single-blind peer review. All editorial decisions made by independent academic editor. Upon submission manuscript was subject to anti-plagiarism scanning. Prior to publication all authors have given signed confirmation of agreement to article publication and compliance with all applicable ethical and legal requirements, including the accuracy of author and contributor information, disclosure of competing interests and funding sources, compliance with ethical requirements relating to human and animal study participants, and compliance with any copyright requirements of third parties. This journal is a member of the Committee on Publication Ethics (COPE).

Provenance: the authors were invited to submit this paper.

Published by Libertas Academica. Learn more about this journal.

Introduction

More than 40,000 different species of phytoplankton, 680 species of marine algae belonging to seaweeds, and 71 mangrove plant species have been documented in the global marine biotope.[1] Marine floras are rich in biologically active and medicinally potent compounds. Polysaccharides and polyphenols are the most predominant groups, which are applicable for anti-inflammatory, anticancer, and antioxidant activities.[2–6] Their beneficial properties for plants, animals, and human beings were recognized in the past and are appreciated nowadays, in the development of new biotechnological products.[1,2]

In the past 10 years, the interest on the study of seaweeds as sources of bioactive compounds has increased. They provide bioflavonoids, ionic trace minerals, essential fatty acids, vitamins, enzymes, amino acids, and other nutrients.[1,2] Flavonoids are natural compounds with variable low molecular weight polyphenolics, found in many plants, including seaweeds.[7] Polyphenols are recognized by their strong antioxidant, anti-inflammatory, and anticancer activities.[8–10] Polyphenol compounds are produced from seaweeds, which protect them from external conditions such as stress and herbivores.[10] Seaweed extracts contain appreciable amounts of polyphenols, but their amount depends on the extraction method.[11,12] Different seaweed extracts have received increased attention due to their potent pharmacological effects, particularly hypolipidemic, antioxidant, analgesic, anti-inflammatory, immunological, and anticancer activities.[12] Apigenin, one of the most common flavonoids, has demonstrated anti-inflammatory, anticarcinogenic, and free radical-scavenging activities.[13]

The red alga *Acanthophora spicifera* is widely distributed in tropical and subtropical areas as an important seaweed in folklore remedies as well as a food supplement in the Gulf of Mannar, coastal region of Tamil Nadu (India).[14] It possesses antitumor, antioxidant activity, and the beneficial effect due to the presence of bioactive components such as flavonoids, terpenoids, and tannins.[15] Abundant halogenated compounds are the most frequently reported metabolites from the red algae.[16] Evidence of flavonoid has been reported only once from *A. spicifera*.[17] Zeng et al isolated two new flavonoids from *A. spicifera*, with three known compounds.[17] The main objective of this investigation was to separate and identify vital flavonoids from the red alga *A. spicifera* and determine their anti-inflammatory and analgesic effects.

Material and Methods

Collection and preparing of algal sample. *A. spicifera* (Vahl) Borgesen was collected from Al-Shoaiba coast, Red Sea, Saudi Arabia. The algal sample was abundant in summer 2011 to spring 2012. It was identified by referring to the studies by Papenfuss,[18] Cribb,[19] and Russell.[20] *A. spicifera* (spiny seaweeds) is an erect edible marine plant, which belongs to the largest family of Rhodophyta, Rhodomelaceae. The fresh collected samples were cleaned under running tap water to remove the sand, salt, and epiphytes. The samples were dried at room temperature and then ground into a coarse powder using an electric blender.

Sample extraction. A sample of dried seaweed powder (150 g) was extracted successively in Soxhlet apparatus using, first, petroleum ether to remove the algal chlorophyll, and then the algal tissues were extracted with acetone to elute the flavonoid content. The acetone extract was filtered through Whatman No. 1 filter paper and then concentrated to a suitable volume.

Separation and identification of flavonoids. The flavonoids, in the acetone crude extract fraction, were detected on thin-layer chromatoplates: 20 × 20 cm, silica gel 60 (Merck) and eluted with 20 mL of the solvent system: methanol:chloroform:ethyl acetate (1:7:2) for each chromatographic plate. The flavonoid zones were scratched with a spatula and eluted three times with methanol:chloroform (1:1) until complete exhaustion. The eluted flavonoid was concentrated using rotary evaporator, and then tested for purity using thin-layer chromatoplates. The physicochemical analysis (infrared [IR], ultraviolet [UV], and gas chromatography (GC)–mass spectrum) was carried out at Toxicology and Microanalytical Research Unit, Faculty of Science, Suez Canal University to elucidate the structure of the separated flavonoid.

The GC–mass spectrum was performed with gas chromatography (Hewlett Packard 5890 Series II, column HP-5 cross-linked 5% Ph Me Silicone, 12 m × 0.2 mm × 0.33 µM film thickness). The conditions were as follows: initial temperature at 100°C/6 minutes, level 1 rate at 3°C/min and final temperature at 120°C, level 2 rate at 6°C/minute and final temperature at 260°C, and using mass spectrum (Hewlett Packard 5970 Series). IR spectrum was carried out by using IR spectroscopy (Perkin-Elmer 1430 Ratio Recording Infrared Spectrophotometer). UV spectrum was determined by UV spectroscopy (T 90 + UV/Visible Spectrometer, PG Instruments Ltd) in methanol in addition to the shift reagents sodium methoxide (NaOMe), aluminum chloride ($AlCl_3$), hydrochloric acid (HCl), sodium acetate (NaOAc), and boric acid (H_3BO_3).[21]

In vivo animal study.

Chemicals. Glacial acetic acid was purchased from Sigma-Aldrich and diluted using pyrogen-free distilled water to obtain a 0.6% solution. Diclofenac sodium (declophen®) was purchased from local pharmacy and produced by Pharco Pharmaceutical.

Experimental animals. Male Wistar albino rats (125–175 g) and male Swiss mice (22.5–27.5 g) were used in the current study, which were purchased from the animal house of National Central Institute, and maintained in good hygienic conditions at the animal house of Pharmacology Department, Veterinary Medical College, Suez Canal University. Rats and mice were given a standard pellet and water ad libitum. The experimental animals were acclimatized for a period of seven days before the start of the experiments. Animal handling and experimental design were approved by the Research Ethical Committee of the Veterinary Medical College, Suez Canal University, Ismailia, Egypt (approval no. 20153).

Acute toxicity study. For acute oral toxicity, mice and rats ($n = 8$) were kept fasting for overnight and given water only. The isolated apigenin was given orally in different ascending doses (up to 5000 mg/kg bw) and observed for 72 hours to identify toxic signs and mortality.

Analgesic activities. The analgesic activity of apigenin was examined using acetic acid-induced writhing test and hot plate test in mice as well as tail-immersion test in rats.

Acetic acid–induced writhing test. Abdominal writhing behavior was induced by intraperitoneal injection of diluted acetic acid (0.6%, 10 mL/kg bw).[22,23] Four groups (eight mice each) of Swiss albino mice (25 ± 2.5 g) were used. The first group received saline and served as negative control. The second and the third groups were orally given apigenin at doses of 25 and 50 mg/kg bw, respectively, 30 minutes before diluted acetic acid injection. The doses were selected according to a previous study.[24] Diclofenac sodium was given orally for the fourth group (100 mg/kg bw) as a standard drug. The stretching and writhing number was determined over a period of 15 minutes, starting at 5 minutes postinjection of the acid.

The reduction of analgesic response by apigenin was determined for each mice group as follows:

$$\% \text{ Inhibition} = 100 \times (1 - \text{experimental/control}).$$

Tail-immersion test. Tail-immersion test was used as described.[23,25] Briefly, the apical 3 cm of the rat's tail was immersed in a bath containing water (55 ± 0.5°C). The rats withdraw their tails as a reaction. The tail withdrawal time was measured at one-hour interval (0–300 minutes) after administration of test material. Thirty-two rats were grouped as previously described. The rat doses of apigenin (25 and 50 mg/kg bw) were selected in accordance with those reported previously.[26] The fourth group was given diclofenac sodium (100 mg/kg) and kept as a standard group.

Hot plate test. The analgesic effect was evaluated using the hot plate test in mice according to the method described.[23,25] Thirty-two mice were grouped and given apigenin and diclofenac sodium as described in the writhing test. A large glass flask acted as hot plate (55 ± 0.5°C). The reaction time was determined when animals react by jumping or licking their paws. The reaction of each mouse in all the groups was taken

at one-hour interval (0–300 minutes) post apigenin administration. The cutoff time (in the case of absence of a response) was considered as 15 seconds in order to avoid burning of the animals.[27]

Anti-inflammatory activities. Apigenin anti-inflammatory activities were examined using carrageenan-induced paw edema and cotton pellet-induced granuloma in rats.

Carrageenan-induced paw edema. Paw edema and inflammation were produced by subplantar injection of 0.1 mL freshly prepared 1% carrageenan into the rats' right hind paws.[23,28] The thickness of the injected and contralateral paws was measured at one-hour interval (0–300 minutes) using skin caliber. Apigenin (25 and 50 mg/kg)[26] was administered to two animal groups, and the other two groups were given distilled water at 10 mL/kg (control) and diclofenac sodium at 100 mg/kg (standard).

Blood samples were collected from the orbital sinus in heparinized tubes, after the last measurement. The blood was centrifuged at 1200 *g* for 15 minutes, and the plasma was stored at –20°C until use for the evaluation of PGE_2 and pro-inflammatory cytokines.

Plasma interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and PGE_2 were evaluated using ready-made kits from Assay Designs Inc. using The Assay Max Mouse IL-1β, IL-6, TNF-α, and PGE_2 ELISA kit according to the manufacturer's protocols through a quantitative sandwich enzyme immunoassay technique.

Cotton pellet-induced granuloma. The chronic anti-inflammatory activities of apigenin were evaluated using cotton pellet-induced granuloma according to the method of Winter and Porter.[29] Sterile cotton pellets, weighing 10 mg, were implanted subcutaneously, under light ether anesthesia, into axillary region, in each rat. Animal grouping as well as apigenin and diclofenac dosing was done as previously described; all drugs were given daily for seven consecutive days from the day of cotton pellet implantation. On the eighth day, the animals were anaesthetized, and the pellets together with the granuloma tissues were carefully excised and made free from the surrounding tissues. The wet pellets were weighed, and then dried in an incubator at 60°C for two hours until a constant weight obtained, after that the dried pellets were weighed again. The exudate amount (mg) was calculated by deducting the constant dry weight from the immediate wet weight of the pellet. The granulation tissue formation (dry weight of granuloma) was calculated after subtracting the weight of cotton pellet (1 mg) from the constant dry weight of pellet and taken as a measure of granuloma tissue formation. The percent inhibitions of exudate and granuloma tissue formation were calculated.

Statistical analysis. All data were expressed as mean ± SEM and statistically analyzed using Minitab (version 16.1). Statistical significance of differences among the different study groups was evaluated by one-way analysis of variance. Tukey's multiple comparisons test was used to differentiate among the means. Statistical significance was acceptable to a level of $P \leq 0.05$.

Results

IR spectra. The IR spectra of the isolated compound (Fig. 1) showed a broad intermolecular OH stretch vibrations band at ~3333 cm^{-1}. There was an aromatic C–H stretch, 3040 cm^{-1}. There is a vibration band at 1646 cm^{-1} characteristic for flavone of conjugation between the C=O and double bonded of C2–C3, also, 1801 cm^{-1} for lactone ring.

The IR spectra showed three vibration bands (1466, 1497, and 1578 cm^{-1}) for the ring C=C, while 1466 cm^{-1} denotes the characteristic of C–O–H stretch. The intensive band at 1024 cm^{-1} was most probably the result of C–O–C stretch from the central heterocyclic ring.

Mass spectra. The mass spectrum of the isolated compound (Fig. 2) shows a molecular ion peak at *m/e* 270. Further fragments of the mass spectrum *m/e* 173, 145, 121, 105, 88, and *m/e* 94 are shown in Figure 3.

UV spectra. UV spectra in methanol in addition to the shift reagents are shown in Figure 4. The UV absorption spectra of the isolated compound in methanol exhibited two major peaks at 267 and 336 nm, characteristic of flavones and flavonols. The absorption spectra of methanol after the addition of shift reagents showed the presence of three hydroxyl groups in the 7, 5, and 4 positions. Table 1 shows that the UV absorption spectra of the isolated compound in methanol and the other shift reagents were in agreement with those reported for apigenin authentic sample.[21] Finally, the data obtained from IR, UV, and mass spectrum of the isolated compound from the red alga *A. spicifera* were typical with those reported for apigenin authentic sample.

Acute toxicity studies. The test for oral acute toxicity revealed no mortality or toxicity signs for apigenin at doses up to 5000 mg/kg either in mice or rats. Based on these results, and according to,[24,26] the doses of 25 and 50 mg/kg were

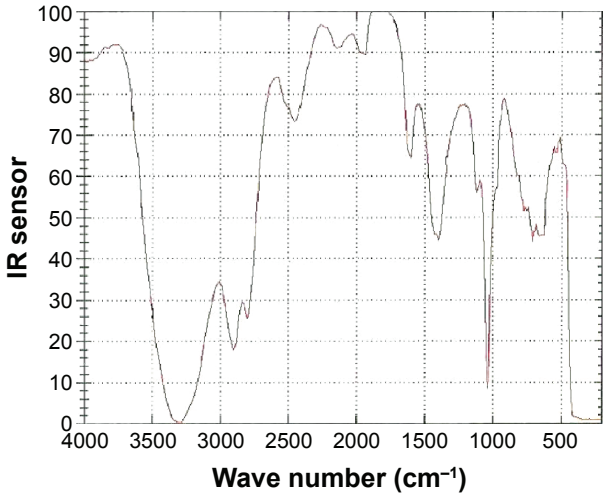

Figure 1. IR spectra of the isolated compound.

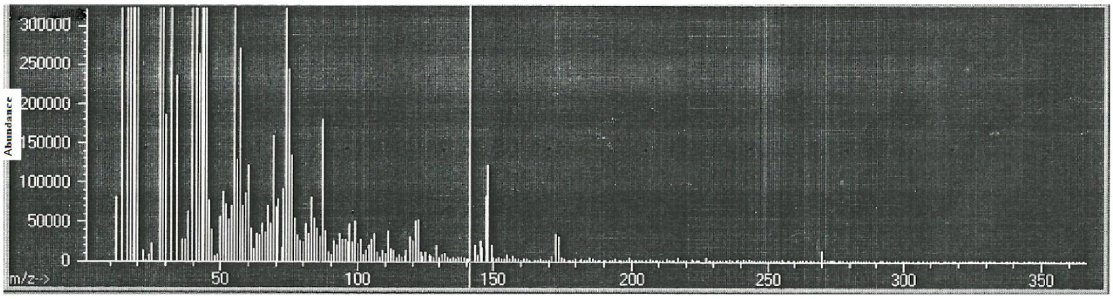

Figure 2. Mass spectra of the isolated compound.

selected for further evaluation of antinociceptive and anti-inflammatory activities.[30]

Analgesic activities. Using mouse writhing test, apigenin induced a significant ($P < 0.05$) dose-dependent decrease of stretching and writhing number, and the reductions were 42.35% and 62.24% for 25 and 50 mg/kg, respectively (Table 2). The reduction produced by the higher dose (50 mg/kg bw) was higher than that of diclofenac standard (45.41%). The analgesic activity of apigenin using tail-immersion test in rats is shown in Table 3. Apigenin induced a dose-dependent inhibition of pain responses throughout the experiment with the maximal activity at 120 minutes for 25 mg/kg bw and at 60 minutes for 50 mg/kg bw. Table 4 shows the analgesic effect using hot plate test in mice. Apigenin exerted a dose-dependent increase in reaction time with maximal activity at 180 and 120 minutes for the doses 25 and 50 mg/kg, respectively.

Anti-inflammatory activities. Oral apigenin pretreatment (25 and 50 mg/kg bw) markedly inhibited the rat paw edema in a dose-dependent manner (Table 5). In acute carrageenan-induced rat paw edema model, a dose of 50 mg/kg bw of apigenin showed a significant ($P < 0.05$) reduction at one hour up to the end of the experiment, which exerted a comparable activity to that of the diclofenac sodium. For understanding the mechanism by which apigenin exerts its anti-inflammatory effects, plasma level of IL-1β, IL-6, TNF-α, and PGE_2 were evaluated. Injection of carrageenan into rat right paws induced elevated plasma levels of IL-1β, IL-6, TNF-α, and PGE_2. Apigenin induced a significant ($P < 0.05$) dose-dependent reduction on these levels compared with carrageenan-induced inflamed non-treated group (Fig. 5).

Moreover, the anti-inflammatory activities of apigenin were examined in chronic model; effects of apigenin on the cotton pellet-induced granuloma in rats are given in Table 6. Apigenin doses significantly ($P < 0.05$) reduced the formation of inflammatory exudate and granuloma formation compared

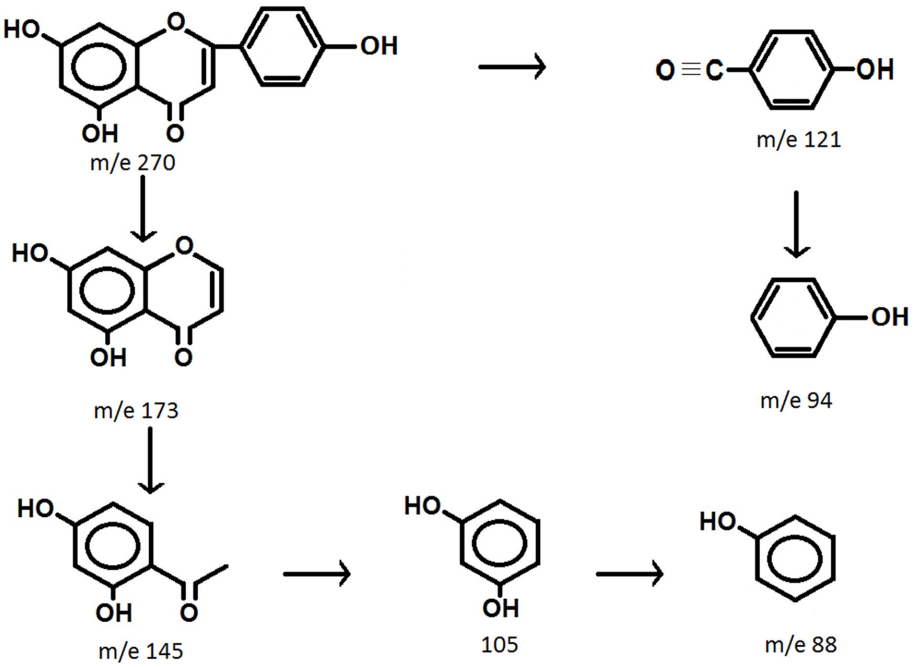

Figure 3. Scheme for fragmentation pattern of the isolated compound mass spectra.

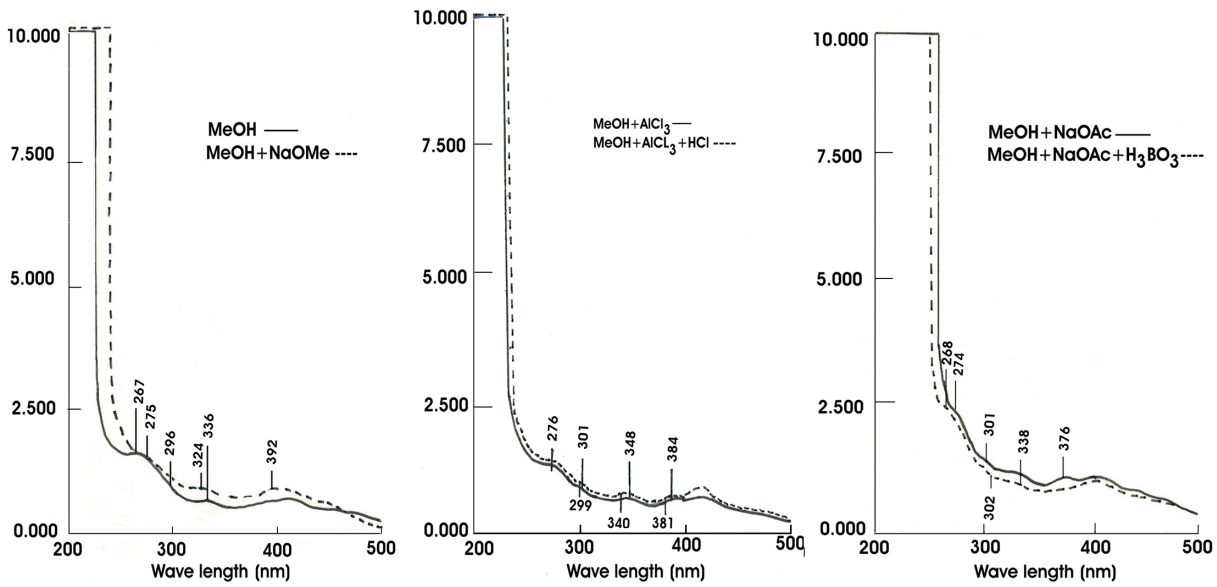

Figure 4. UV spectra of the isolated compound in methanol in addition to the shift reagents.

with controls. The effect of higher dose was comparable with that of diclofenac standard.

Discussion

Algae are considered as a rich source of flavonoids. *A. spicifera* is widely distributed in tropical and subtropical areas.[31] In the present work, the structure of the isolated compound from the marine red alga *A. spicifera* was determined using physico-chemical analysis; IR, mass spectrum, and UV spectra of the isolated compound from the red alga *A. spicifera* confirmed the structure of the flavonoid apigenin.[21] Zeng et al isolated two new flavonoids, namely, acanthophorin A and acanthophorin B, along with three known compounds tiliroside, catechin, and quercetin from the red alga *A. spicifera*. The structures of acanthophorin A and acanthophorin B were determined to be kaempferol 3-*O*-α-L-fucopyranoside and quercetin 3-*O*-α-L-fucopyranoside by spectroscopic methods. It showed significant antioxidant activity.[17]

Natural products have been widely used as good alternatives to produce new drugs and therapeutic agents for combating diseases.[5,6,32–34] In the current study, apigenin expressed potent analgesic activities in writhing and hot plate tests in the mice models as well as tail-immersion test in rats. Moreover, it revealed potent anti-inflammatory activities using carrageenan-induced rat paw inflammation as well as cotton pellet-induced granuloma formation models. This study has shown that apigenin has potent antinociceptive action, which might affect prostaglandin synthesis pathway. A significant reduction in stretching and writhing induced by acetic acid, as well as a significant prolongation of the reaction time for hot plate and tail-immersion tests, indicated analgesic activities via both central and peripheral mechanisms.

Carrageenan-induced rat paw edema is a common model to evaluate anti-inflammatory therapeutic agents and study inflammation.[35] The edema or swelling is one of the acute inflammation signs, and it is an important indicator to be considered when examining agents with potential anti-inflammatory activities.[36] In carrageenan-induced rat paw edema model, the swelling is a biphasic process. Serotonin and histamine release occurs in the first phase, while in the second phase, bradykinin (BK), lysosome, protease, and prostaglandin are predominate.[37] The role of PGE_2 in this model

Table 1. Comparison between the UV spectra of the isolated compound and that reported for authentic sample of apigenin.[21]

MeOH + MeOH SHIFT REAGENTS	U.V SPECTRA OF THE ISOLATED COMPOUND	U.V SPECTRA OF APIGENIN AUTHENTIC SAMPLE
Methanol	267, 336	267, 336
NaOMe	275, 324, 392	275, 324, 392
$AlCl_3$	276, 301, 348, 384	276, 301, 348, 384
$AlCl_3$ + HCl	276, 300, 340, 381	276, 299, 340, 381
NaOAc	276, 301, 376	274, 301, 376
NaOAc + H_3BO_3	268,302 sh, 338	268, 302 sh, 338

Table 2. Effect of apigenin on acetic acid-induced writhing in mice.

TREATMENT	DOSE (mg/kg)	NO OF WRITHES (15 MINUTES)	INHIBITION (%)
Control	–	$24.50^{a} \pm 1.56$	–
Apigenin	25	$14.13^{b} \pm 1.03$	42.35
	50	$9.25^{c} \pm 0.78$	62.24
Diclofenac sodium	100	$11.13^{b,c} \pm 1.01$	45.41

Notes: Results expressed as mean ± SEM. Within the same column, different letters mean statistical significance at $P < 0.05$.

Table 3. Effect of apigenin using tail-immersion test in rats.

TREATMENT	POST TREATMENT REACTION TIME (SECONDS)						
	DOSE (mg/kg)	0	60	120	180	240	300
Control		1.95 ± 0.06	1.94^{a} ± 0.08	2.05^{a} ± 0.10	1.99^{a} ± 0.17	1.93^{a} ± 0.07	1.91^{a} ± 0.09
Apigenin	25	1.93 ± 0.10	5.46^{b} ± 0.28	5.63^{b} ± 0.40	5.11^{b} ± 0.46	4.93^{b} ± 0.32	3.83^{b} ± 0.37
	50	2.09 ± 0.13	7.54^{b,c} ± 0.53	6.88^{b,c} ± 0.54	6.19^{b,c} ± 0.33	5.73^{b,c} ± 0.39	4.34^{b} ± 0.32
Diclofenac sodium	100	1.98 ± 0.16	7.53^{c} ± 0.45	7.85^{c} ± 0.50	7.71^{c} ± 0.92	6.63^{c} ± 0.71	4.11^{b} ± 0.31

Notes: Results expressed as mean ± SEM. Within the same column, different letters mean statistical significance at $P < 0.05$.

has been well clarified in many previous literatures.[35,38–40] BK and PGE_2 are responsible for the edema and the pain, which accompanies the inflammatory process, as both BK and PGE_2 are sensitizing the primary afferent neurons.[41,42] Therefore, the effect of apigenin in the reduction of edema might be due to the inhibition of cyclooxygenase enzyme and, in turn, the inhibition of PGE_2 synthesis.

Apigenin showed a reduction of the paw swelling induced by carrageenan all over the time of experiment and induced potent anti-inflammatory activity. The effectiveness of apigenin at one and three hours indicates its antagonistic effect on prostaglandin, BK, histamine, and serotonin. Histamine and serotonin production occurs at one hour, whereas BK and prostaglandin are released at two and three hours, respectively, after injection of carrageenan.[43] Apigenin at the high-dose level was comparable with the anti-inflammatory activity of diclofenac sodium. The obtained results are an indication that apigenin could be effective for acute inflammatory conditions. Since inflammation is also accompanied by pain, most anti-inflammatory agents have an antinociceptive potential. The peripheral analgesic activity of the drugs could be via inhibition of cyclooxygenases (COX) and/or lipoxygenases as well as other mediators, while the central analgesic action might be through inhibition of central pain receptors.

Cytokines are indigenous proteins, which are not produced in the case of usual physiological conditions. However, the inflammatory stimulus overexpresses cytokine genes, promoting the inflammatory reaction.[44] TNF-α is a primary cytokine included in the inflammatory process initiation. Its effects involve the production of other cytokines, including IL-6 and IL-1β, as well as an induction of arachidonic acid metabolism.[44] Prostaglandins (PGs), thromboxanes, and leukotrienes (LTs) are arachidonic acid metabolites via COX and lipoxygenase. PGE_2 could initiate alterations in blood flow and vascular tonus, leading to edema as a symptom of inflammation. Cox_2 and PGE_2 are cancer biomarkers, besides their activities as inflammatory mediators.[45–50]

To examine apigenin anti-inflammatory mechanism of action, IL-1β, IL-6, TNF-α, and PGE_2 and were further evaluated in the plasma in carrageenan-induced rat paw edema animal model. Our results indicate that apigenin caused a statistically significant ($P < 0.05$) reduction in plasma levels of IL-1β, IL-6, TNF-α, and PGE_2. These findings underline the anti-inflammatory mechanism of apigenin. TNF-α is a major cytokine, which promotes PGE_2 biosynthesis.[51] In the current study, the decrease of TNF-α level by apigenin was accompanied by PGE_2 reduction. The obtained results are in agreement with previous studies, which indicate that the anti-inflammatory effects of apigenin might be due to the inhibition of PGE_2 as well as the proinflammatory cytokines IL-1β, IL-6, and TNF-α.[24,52] Our results indicated that apigenin induced dose-dependent anti-inflammatory and antiproliferative activities by regression of PGE_2 and cytokines' production.

The cotton pellet-induced granuloma model has been widely used to assess the transudative, exudative, and proliferative components of chronic inflammation.[53] The fluid absorbed by the pellet mainly influences the wet weight of the granuloma, and the dry weight correlates well with the amount of formed granuloma tissue.[54] Fibroblast proliferation and monocyte infiltration rather than neutrophil infiltration occur in chronic inflammation.[55] In the current

Table 4. Effect of apigenin using hot plate test in mice.

TREATMENT	POST TREATMENT REACTION TIME (SECONDS)						
	DOSE (mg/kg)	0	60	120	180	240	300
Control		1.56 ± 0.09	1.65^{a} ± 0.08	1.64^{a} ± 0.10	1.63^{a} ± 0.08	1.65^{a} ± 0.07	1.66^{a} ± 0.05
Apigenin	25	1.58 ± 0.11	5.61^{b} ± 0.52	6.12^{b} ± 0.53	6.54^{b} ± 0.41	5.25^{b} ± 0.33	3.94^{b} ± 0.21
	50	1.57 ± 0.12	6.86^{b,c} ± 0.44	7.93^{c} ± 0.46	6.79^{b} ± 0.58	6.24^{b,c} ± 0.42	4.18^{b} ± 0.28
Diclofenac sodium	100	1.98 ± 0.08	8.19^{c} ± 0.42	8.43^{c} ± 0.60	7.33^{b} ± 0.93	6.98^{c} ± 0.68	4.18^{b} ± 0.30

Notes: Results expressed as mean ± SEM. Within the same column, different letters mean statistical significance at $P < 0.05$.

Table 5. Effects of apigenin on carrageenan-induced rat paw edema.

TREATMENT	POST TREATMENT PAW THICKNESS (cm)						
	DOSE (mg/kg)	0	60	120	180	240	300
Control		0.87 ± 0.04	0.86^{a} ± 0.03	0.88^{a} ± 0.02	0.89^{a} ± 0.05	0.89^{a} ± 0.04	0.87^{a} ± 0.02
Apigenin	25	0.89 ± 0.03	$0.78^{a,b}$ ± 0.02	0.71^{b} ± 0.03	0.66^{b} ± 0.02	0.61^{b} ± 0.03	0.56^{b} ± 0.02
	50	0.93 ± 0.05	$0.73^{b,c}$ ± 0.03	$0.65^{b,c}$ ± 0.02	0.59^{b} ± 0.01	0.57^{b} ± 0.02	$0.54^{b,c}$ ± 0.02
Diclofenac sodium	100	0.93 ± 0.06	0.69^{c} ± 0.02	0.61^{c} ± 0.01	0.58^{b} ± 0.01	0.56^{b} ± 0.02	0.49^{c} ± 0.02

Notes: Results expressed as mean ± SEM. Within the same column, different letters mean statistical significance at $P < 0.05$.

study, apigenin reduced both wet and dry weights of the cotton pellets compared with control groups. This may be due to the ability of apigenin in the reduction of fibroblast infiltration and synthesis of mucopolysaccharide and collagen, which are natural proliferative agents of granulation tissue formation, indicating the antiproliferative effects of apigenin.

Conclusion

The current study confirms that the elucidated structure of the biologically active flavone (apigenin) from the marine red alga *A. spicifera* was determined by using physicochemical analyses, IR, UV, and mass spectrum. Apigenin has antinociceptive and anti-inflammatory activities in writhing and hot plate tests in mice as well as tail-immersion test in rats and carrageenan-induced inflammation as well as cotton pellet-induced granuloma formation, respectively. Such activities may be derived from the inhibitory action on the peripheral synthesis and/or release of inflammatory mediators involved in these reactions, such as PGE_2, TNF-α, and inhibition of proinflammatory cytokine production, including IL-1β and IL-6, as well as inhibition of fibroblast infiltration and collagen synthesis. Further in vitro and in vivo studies are recommended to further investigate the detailed action and mechanism of actions as well as to increase the efficacy of apigenin for clinical application.

Author Contributions

Conceived and designed the experiments: MMAD, GAES, MHM, EAS. Analyzed the data: MMAD, GAES, MHM, EAS. Wrote the first draft of the manuscript: MMAD, GAES. Contributed to the writing of the manuscript: MMAD, GAES. Agree with manuscript results and conclusions: MMAD, GAES, MHM, EAS. Jointly developed the structure and arguments for the paper: MMAD, GAES. Made critical revisions and approved final version: MMAD, GAES, MHM, EAS. All authors reviewed and approved of the final manuscript.

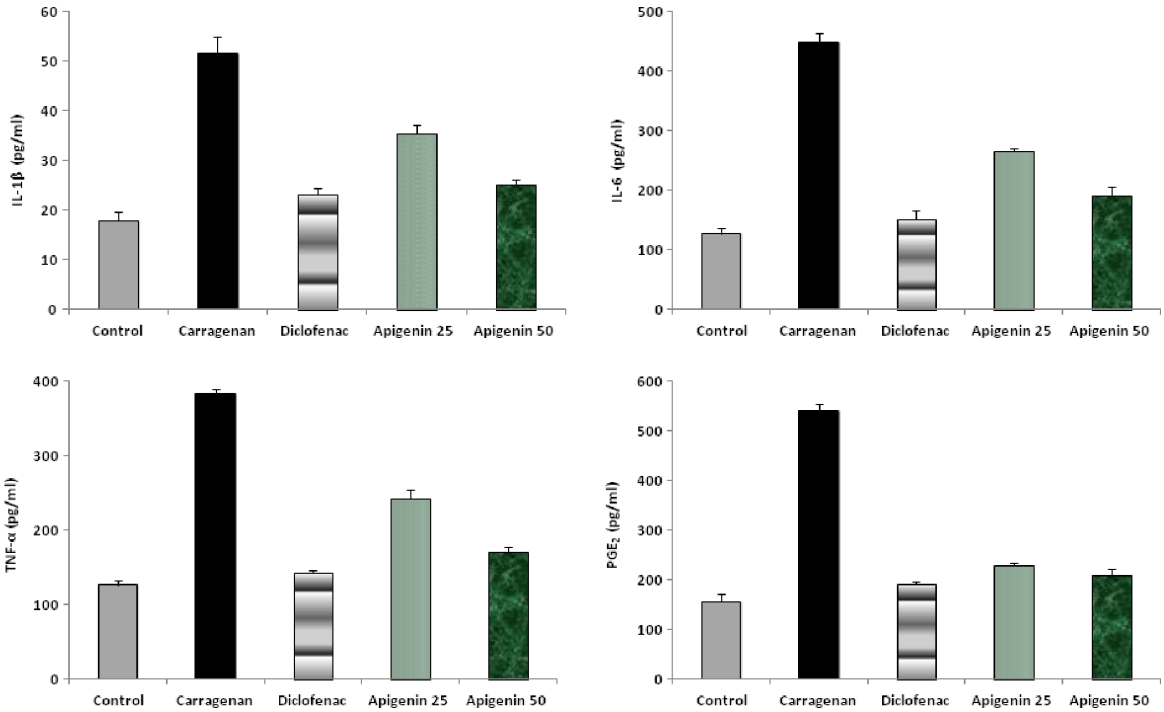

Figure 5. Effect of apigenin on plasma proinflammatory cytokine and PGE_2 production in rats with carrageenan-induced paw edema.

Table 6. Effect of apigenin on the cotton pellet-induced granuloma in rats.

TREATMENT	DOSE (mg/kg)	MEAN WEIGHT OF EXUDATE (mg)	% INHIBITION OF EXUDATE	MEAN DRY WEIGHT OF GRANULOMA (mg)	GRANULOMA INHIBITION (%)
Control		$112.75^{a} \pm 2.99$	–	$28.38^{a} \pm 1.46$	–
Apigenin	25	$75.75^{b} \pm 2.96$	32.82	$18.25^{b} \pm 1.08$	35.68
	50	$56.63^{c} \pm 2.55$	49.78	$9.75^{c} \pm 0.75$	65.64
Diclofenac sodium	100	$58.88^{c} \pm 3.34$	47.78	$11.13^{c} \pm 0.61$	60.79

Notes: Results expressed as mean ± SEM. Within the same column, different letters mean statistical significance at $P < 0.05$.

REFERENCES

1. Haefner B. Drugs from the deep: marine natural products as drug candidates. *Drug Discov Today*. 2003;8:536–544.
2. Sithranga Boopathy N, Kathiresan K. Anticancer drugs from marine flora: an overview. *J Oncol*. 2010;2010:214186.
3. Abdel-Daim MM. Pharmacodynamic interaction of *Spirulina platensis* with erythromycin in Egyptian Baladi bucks (*Capra hircus*). *Small Rumin Res*. 2014; 120:234–241.
4. Abdel-Daim MM, Abdelkhalek NK, Hassan AM. Antagonistic activity of dietary allicin against deltamethrin-induced oxidative damage in freshwater *Nile tilapia*; *Oreochromis niloticus*. *Ecotoxicol Environ Saf*. 2015;111:146–152.
5. Al-Sayed E, Abdel-Daim MM, Kilany OE, Karonen M, Sinkkonen J. Protective role of polyphenols from *Bauhinia hookeri* against carbon tetrachloride-induced hepato- and nephrotoxicity in mice. *Ren Fail*. 2015;37:1198–1207.
6. Abdel-Daim MM, Ghazy EW. Effects of *Nigella sativa* oil and ascorbic acid against oxytetracycline-induced hepato-renal toxicity in rabbits. *Iran J Basic Med Sci*. 2015;18:221–227.
7. Azab SS, Abdel-Daim M, Eldahshan OA. Phytochemical, cytotoxic, hepatoprotective and antioxidant properties of *Delonix regia* leaves extract. *Med Chem Res*. 2013;22:4269–4277.
8. Liu Y, Chou G. [Isolation and preparation of flavones from the leaves of *Lindera aggregata* using high speed counter-current chromatography]. *Se Pu*. 2007;25: 735–739.
9. Karimi E, Oskoueian E, Hendra R, Oskoueian A, Jaafar HZ. Phenolic compounds characterization and biological activities of *Citrus aurantium* bloom. *Molecules*. 2012;17:1203–1218.
10. Namvar F, Mohamad R, Baharara J, Zafar-Balanejad S, Fargahi F, Rahman HS. Antioxidant, antiproliferative, and antiangiogenesis effects of polyphenol-rich seaweed (*Sargassum muticum*). *Biomed Res Int*. 2013;2013:604787.
11. Zhao M, Yang B, Wang J, Liu Y, Yu L, Jiang Y. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn) pericarp. *Int Immunopharmacol*. 2007;7:162–166.
12. Kolanjinathan K, Ganesh P, Saranraj P. Pharmacological importance of seaweeds: a review. *World J Fish Marine Sci*. 2014;6:1–15.
13. Liu R, Zhang T, Yang H, Lan X, Ying J, Du G. The flavonoid apigenin protects brain neurovascular coupling against amyloid-beta(2)(5)(-)(3)(5)-induced toxicity in mice. *J Alzheimers Dis*. 2011;24:85–100.
14. Davis GD, Vasanthi AH. Seaweed metabolite database (SWMD): a database of natural compounds from marine algae. *Bioinformation*. 2011;5:361–364.
15. Yamamoto I, Maruyama H. Effect of dietary seaweed preparations on 1,2-dimethylhydrazine-induced intestinal carcinogenesis in rats. *Cancer Lett*. 1985;26:241–251.
16. Faulkner DJ. Marine natural products. *Nat Prod Rep*. 2002;19:1–48.
17. Zeng LM, Wang CJ, Su JY, et al. Flavonoids from the red alga *Acanthophora spicifera*. *Chin J Chem*. 2001;19:1097–1100.
18. Papenfuss GF. A history, catalogue, and bibliography of Red Sea benthic algae. *Israel J Bot*. 1968;17:1–118.
19. Cribb AB. Marine Algae of the Southern Great Barrier Reef, Part I. Rhodophyta. Australia Coral Reef Society, Brisbane; 1983.
20. Russell DJ. The ecological invasion of Hawaiian reefs by two marine red algae, *Acanthophora spicifera* (Vahl) Børerg. and *Hypnea musciformis* (Wulfen) J.Ag., and their association with two native species, *Laurencia nidifica* and *Hypnea cervicornis*. *ICES Mar Sci Symp*. 1992;194:110–125.
21. Mabry TJ, Markham KR, Thomas MB. *The Systematic Identification of Flavonoids*. Berlin: Springer Verlag; 1970.
22. Atta AH, Abo ELSK. The antinociceptive effect of some Egyptian medicinal plant extracts. *J Ethnopharmacol*. 2004;95:235–238.
23. Eldahshan OA, Abdel-Daim MM. Phytochemical study, cytotoxic, analgesic, antipyretic and anti-inflammatory activities of *Strychnos nux-vomica*. *Cytotechnology*. 2015;67:831–844.
24. Smolinski AT, Pestka JJ. Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (chamomile), ginsenoside Rb(1) (ginseng) and parthenolide (feverfew). *Food Chem Toxicol*. 2003;41:1381–1390.
25. Altawil HJA, Abdel-Rahman MA, El-Naggar MS, El-Khayat ZA, Abdel-Daim MM. Analgesic, antipyretic and anti-inflammatory activities of the Egyptian spitting cobra; *Naja nubiae* venom. *J Forensic Toxicol Pharmacol*. 2015;4:1–6.
26. Eldahshan OA, Azab SS. Anti-inflammatory effect of apigenin-7-neohesperidoside (rhoifolin) in carrageenin-induced rat oedema model. *J Appl Pharm Sci*. 2012;2:74–79.
27. Laughlin TM, Tram KV, Wilcox GL, Birnbaum AK. Comparison of antiepileptic drugs tiagabine, lamotrigine, and gabapentin in mouse models of acute, prolonged, and chronic nociception. *J Pharmacol Exp Ther*. 2002;302:1168–1175.
28. Esmat A, Al-Abbasi FA, Algandaby MM, et al. Anti-inflammatory activity of *Pistacia khinjuk* in different experimental models: isolation and characterization of its flavonoids and galloylated sugars. *J Med Food*. 2012;15:278–287.
29. Winter CA, Porter CC. Effect of alterations in side chain upon anti-inflammatory and liver glycogen activities of hydrocortisone esters. *J Am Pharm Assoc Am Pharm Assoc (Baltim)*. 1957;46:515–519.
30. Li R, Zhao D, Qu R, Fu Q, Ma S. The effects of apigenin on lipopolysaccharide-induced depressive-like behavior in mice. *Neurosci Lett*. 2015;594:17–22.
31. Vasanthi HR. Biomedical and Pharmacological Studies of Some Marine Algae of Gulf of Mannar South East Coast, Ph.D Thesis, Tamil University, India; 2002.
32. Abdelkhalek NK, Ghazy EW, Abdel-Daim MM. Pharmacodynamic interaction of *Spirulina platensis* and deltamethrin in freshwater fish *Nile tilapia*, *Oreochromis niloticus*: impact on lipid peroxidation and oxidative stress. *Environ Sci Pollut Res Int*. 2015;22:3023–3031.
33. Abdel-Daim M, Halawa S. Synergistic hepatocardioprotective and antioxidant effects of myrrh and ascorbic acid against diazinon-induced toxicity in rabbits. *Int Res J Humanit Eng Pharm Sci*. 2014;1:1–7.
34. Ibrahim AE, Abdel-Daim MM. Modulating effects of *Spirulina platensis* against tilmicosin-induced cardiotoxicity in mice. *Cell J*. 2015;17:137–144.
35. Nantel F, Denis D, Gordon R, et al. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. *Br J Pharmacol*. 1999;128:853–859.
36. Morris CJ. Carrageenan-induced paw edema in the rat and mouse. *Methods Mol Biol*. 2003;225:115–121.
37. Ouachrif A, Khalki H, Chaib S, et al. Comparative study of the anti-inflammatory and antinociceptive effects of two varieties of *Punica granatum*. *Pharm Biol*. 2012; 50:429–438.
38. Zanin MT, Ferreira SH. Relationship between oedema and plasma exudation in rat paw carrageenin inflammation. *Agents Actions*. 1978;8:606–609.
39. Mnich SJ, Veenhuizen AW, Monahan JB, et al. Characterization of a monoclonal antibody that neutralizes the activity of prostaglandin E2. *J Immunol*. 1995;155:4437–4444.
40. Portanova JP, Zhang Y, Anderson GD, et al. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J Exp Med*. 1996;184:883–891.
41. Williams TJ, Peck MJ. Role of prostaglandin-mediated vasodilatation in inflammation. *Nature*. 1977;270:530–532.
42. Calixto JB, Otuki MF, Santos AR. Anti-inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). *Planta Med*. 2003;69:973–983.
43. Di Rosa M, Willoughby DA. Screens for anti-inflammatory drugs. *J Pharm Pharmacol*. 1971;23:297–298.
44. Santos DR, Calixto JB, Souza GE. Effect of a kinin B2 receptor antagonist on LPS- and cytokine-induced neutrophil migration in rats. *Br J Pharmacol*. 2003; 139:271–278.
45. Abdel-Daim M, Funasaka Y, Kamo T, et al. Effect of chemical peeling on photocarcinogenesis. *J Dermatol*. 2010;37:864–872.
46. Abdel-Daim M, Funasaka Y, Kamo T, et al. Preventive effect of chemical peeling on ultraviolet induced skin tumor formation. *J Dermatol Sci*. 2010;60:21–28.

47. Funasaka Y, Abdel-Daim M, Kawana S, Nishigori C. Effect of chemical peeling on the skin in relation to UV irradiation. *Exp Dermatol*. 2012;21(suppl 1):31–35.
48. Elzagheid A, Emaetig F, Alkikhia L, et al. High cyclooxygenase-2 expression is associated with advanced stages in colorectal cancer. *Anticancer Res*. 2013;33: 3137–3143.
49. Karavitis J, Hix LM, Shi YH, Schultz RF, Khazaie K, Zhang M. Regulation of COX2 expression in mouse mammary tumor cells controls bone metastasis and PGE2-induction of regulatory T cell migration. *PLoS One*. 2012;7:e46342.
50. Perez-Ruiz E, Cazorla O, Redondo M, et al. Immunohistochemical expression of cyclooxygenase-2 in patients with advanced cancer of the larynx who have undergone induction chemotherapy with the intention of preserving phonation. *Clin Transl Oncol*. 2012;14:682–688.
51. Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: coordinators of immune and inflammatory responses. *Annu Rev Biochem*. 1990;59: 783–836.
52. Rithidech KN, Tungjai M, Reungpatthanaphong P, Honikel L, Simon SR. Attenuation of oxidative damage and inflammatory responses by apigenin given to mice after irradiation. *Mutat Res*. 2012;749:29–38.
53. Spector WG. The granulomatous inflammatory exudate. *Int Rev Exp Pathol*. 1969; 8:1–55.
54. Swingle KF, Shideman FE. Phases of the inflammatory response to subcutaneous implantation of a cotton pellet and their modification by certain anti-inflammatory agents. *J Pharmacol Exp Ther*. 1972;183:226–234.
55. Mohy El-Din MM, Senbel AM, Bistawroos AA, et al. A novel COX-2 inhibitor pyrazole derivative proven effective as an anti-inflammatory and analgesic drug. *Basic Clin Pharmacol Toxicol*. 2011;108:263–273.