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Role of Scopoletin Containing in an Aqueous *Morinda citrifolia* Fruit Extract on the Gastric Mucosal Integrity and Gastric Ulcer Healing Process

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Abstract: The antiulcer mechanisms of action of scopoletin (SCO), one of the biomarker compounds contained in an aqueous *M. citrifolia* fruit extract (AMFE), were evaluated and compared with those of AMFE. *In vitro* studies revealed that AMFE and SCO exhibited a low capacity in scavenging NO radical. Still, SCO possessed a prominent higher anti-inflammatory capacity in inhibiting the NO production in activated macrophage RAW 264.7 cell line. AMFE exerted a high and fast efficacy in accelerating the proliferation and migration of human gingival fibroblast and gastric epithelial cell lines. In contrast, SCO did not exhibit any significant wound-healing activity. The investigation on the mRNA expression of constitutive gastroprotective enzymes (nNOS and COX-1) and gastric pro-inflammatory enzymes (iNOS and COX–2) at the rat gastric ulcerated area induced by acetic acid demonstrated that SCO exerted a comparable efficiency to AMFE in up-regulating COX-1 and nNOS mRNA expression but down-regulating COX-2 and iNOS mRNA expression. Accordingly, the anti-inflammatory and gastroprotective properties of AMFE seemed to relate to the concentration of SCO in AMFE. On the contrary, the property of accelerating ulcer healing of AMFE might be accounted for by other contributions from some sources of ulcer healing activity contained in AMFE.

Keywords: *Morinda citrifolia*; scopoletin; anti-inflammatory; ulcer healing; nitric oxide synthase; cyclooxygenase.

1. Introduction

Scopoletin (SCO) (6-methoxy-7-hydroxycoumarin, $C_{10}H_8O_4$), one of the most important reported phenolic coumarin compounds, is ubiquitously found in all *Morinda citrifolia* fruit collected from any region and is present throughout the fruit's life [1]. It is recommended as a marker constituent for the quality control and pharmacokinetic study of *M. citrifolia* fruit juice [1]. SCO has been reported from *in vitro* and *in vivo* studies as a potent

anti-inflammatory agent in suppressing the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), inhibiting the activity of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) enzymes and regulating of glucocorticoid receptors [2-5]. Our previous pharmacological evaluation of gastric biochemical parameters and motility in rat models has indicated that SCO may be one of the important constituents of an aqueous *M. citrifolia* fruit extract claimed to prevent acute reflux esophagitis and gastritis formation. It has also been found that an oral administration of AMFE at the dose of 0.63–2.50 g/kg (equivalent to 0.5-1.5 mg SCO) twice daily for 10 consecutive days significantly accelerated the healing of acetic acid-induced chronic gastric ulcer in rats with an equal ulcer healing efficacy to those of standard antisecretory agents (ranitidine and lansoprazole) [6]. Although it was revealed that SCO exerted a significant antisecretory activity, its acid inhibitor activity was less potent than current standard antisecretory agents: ranitidine or lansoprazole. Additionally, the finding indicated that AFE and its biomarker, SCO, may exert antiulcer activity mainly through its inhibitory activity on free radicals and cytokine-mediated inflammation.

Gastric mucosal inflammatory pathways are categorized mainly into arachidonic acid (AA)-dependent pathway that is involved in inflammation dependent mainly on cyclooxygenase (COX)-2 enzymes [7,8] and AA-independent pathways that are involved mainly on NF–κB activation (a transcription factor necessary for the release of pro-inflammatory cytokines from activated inflammatory cells) [8-9], NO derived from iNOS in activated inflammatory cells and reactive radicals [10]. PGs synthesized via the constitutive COX–1 pathway have key roles in the cytoprotection of gastric mucosal surfaces via their ability to stimulate mucus and bicarbonate secretion, regulate acid secretion even in the presence of inflammation, elevate mucosal blood flow, suppress the recruitment of leukocytes into the mucosa and inhibit the release of pro-inflammatory cytokines from macrophages and leukocytes [7,11]. PGs derived from the inducible isoform COX–2 are pro-inflammatory PGs that potentiate other inflammatory mediators and modulate the activity of many immunocytes [7,8, 11-13]. However, they can also make an important contribution to the repair of ulcers in the later phase of inflammation through the formation of granulation tissue at the ulcer base and new blood vessels (angiogenesis), including the re-establishment of the glandular architecture [14-16]. At the GI level, NO derived from a constitutive neuronal NOS (nNOS) regulates gastric acid and mucus secretion [10,17,18]. Therefore, inhibition of the constitutive nNOS results in a disturbance to the GI blood flow, motility, and secretion.

On the contrary, NO derived from the inducible isoform NOS (iNOS) is a pro-inflammatory mediator likely to have a multifaceted role in inflammatory reactions [10, 19]. An excess amount of the produced NO from iNOS can interact with a superoxide anion to form a potent reactive nitrogen species (RNS) that amplifies the cytotoxic effect, induces oxidative stress on the DNA strand and inhibits enzymes involved in DNA repair. However, it has also been reported that some NO generated from iNOS is needed to produce a beneficial effect on ulcer healing by producing apoptosis in inflammatory cells in the regenerating mucosa, thereby eliminating iNOS–positive inflammatory cells during ulcer healing [20]. Likewise, NO derived from iNOS was found to accelerate ulcer healing in the late phase of inflammation by enhancing the expression of growth factors involved in promoting ulcer healing, angiogenesis, fibroblast proliferation and migration, and epithelial cell proliferation [21,22]. Accordingly, an excessive inhibition of iNOS induction during ulcer healing will increase the accumulation of inflammatory cells and delay ulcer healing.

There is still no study on the role of SCO in maintaining gastric mucosal integrity or accelerating the ulcer healing process. The present study, therefore, was carried out to evaluate mechanisms of action underlying gastric mucosal protecting and ulcer healing activity of SCO, compared to those of AMFE.

2. Materials and Methods

2.1. Plant material.

Fresh mature unripe *M. citrifolia* (Linn.) fruits were harvested from Songkhla province, Thailand. The plant material was authenticated, and voucher specimens (SKP 165130301) were kept at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The fresh mature fruits (4.53 kg) were cut into thick slices, dried at 50°C in a hot air oven, and ground into powder. The lyophilized powder of an aqueous *M. citrifolia* fruit extract (AFE) was prepared according to the method previously described [6] and then stored at -20°C until use. The dried powder of AMFE was dissolved in water before it was used. The percentage yield of AMFE was determined. The total content of phenolic compounds contained in AMFE was determined with the Folin-Ciocalteu reagent [23]. The content of SCO containing 1 g of AMFE powder was determined following the previous work of Mahattanadul et al. [6]. In brief, the sample solution was purified on C_{18} solid phase extraction (SPE) cartridges. The SPE procedure used consists of 0.01 M KH_2PO_4 (pH 7) followed by 100% methanol on conventional C18 sorbents. The SCO content was then analyzed using RP-HPLC assay on a C18 column using a mixture of 0.01 M sodium acetate (pH 3.0) and acetonitrile (80:20 v/v) as mobile phase at a 1.0 ml/min flow rate. More specificity was achieved by using high-wavelength UV detection at 350 nm.

2.2. Drugs and chemicals.

Dulbecco's modified Eagle's medium (DMEM), scopoletin (SCO), and trypsin-EDTA (0.05-0.02%) in Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} were purchased from Sigma-Aldrich, Missouri, USA. Fetal calf serum (FCS), penicillin, and streptomycin were purchased from Invitrogen, California, USA. Human gingival fibroblast (HGF-1)(CRL-2014) was purchased from the American Type Culture Collection, USA. All chemicals were of analytical grade.

2.3. Determination of in vitro antioxidant and anti-inflammatory activity.

2.3.1. Antioxidant activity in scavenging NO radical.

The 0.5 ml of 20 mM sodium nitroprusside volume dissolved in phosphate buffer saline (pH 7.4) was mixed with 1 ml of AMFE or SCO solution at various concentrations. The mixture was incubated under light at 25℃ for 2.5 h. After that, the incubated solution of 0.1 ml was withdrawn and mixed with 0.1 ml of Griess reagent (prepared by mixing 1% of sulphanilamide in 5% phosphoric acid and 0.1% of naphthylethylene diamine dihydrochloride immediately before use). The mixture was incubated at room temperature for 30 min. The amount of NO radical was measured at 546 nm using a microplate reader. Gallic acid was used as the positive control. The percentage of NO radical scavenging activity of the sample and gallic acid was calculated according to the following equation:

$$\%\ Inhibition\ =\ [(A546\ control\ -\ A546\ test\ sample)\ /\ A546\ control] \times 100 \quad (1)$$

2.3.2. Anti-inflammatory activity suppresses LPS-induced pro-inflammatory NO production in activated macrophage RAW264.7 cell line.

The macrophage RAW264.7 cell line was cultured in RPMI medium supplemented with 0.1% $NaHCO_3$, 1% penicillin and streptomycin, and 10% FCS. The cells were seeded at $1x10^5$ cells/well in a 96-well plate. The plate was then incubated in a humidified atmosphere containing a 5% CO_2 incubator at 37°C for 1 h to promote cell adherence at the bottom of the well. The medium in a 96-well plate was replaced with a fresh medium containing LPS (1 µg/ml) and test samples and incubated for 48 h. NO production was then evaluated by measuring nitrite accumulation in the culture supernatant using the Griess reagent. The density of the NO production was measured at 570 nm with a microplate reader.

Cytotoxicity was determined using the MTT colorimetric method. After incubating the samples in a 96-well plate for 48 h, 10 µl of MTT solution (5 mg/ml of MTT in PBS) was added to the well plate, and all the mixtures in a well plate were incubated for a further 4 h. The medium was then removed, and DMSO was added to dissolve the formazan production in the cells. The density of the formazan solution was measured at 570 nm with a microplate reader. The test sample was observed to be cytotoxic if the density of the sample-treated group was less than 80% of the vehicle-treated group.

L-nitroarginine (L-NA) (NOS inhibitor), caffeic acid phenethyl ester (CAPE) (NF-κB inhibitor), and indomethacin (COX and iNOS inhibitor) were used as standard positive controls.

% inhibitory activity on NO production was determined according to the following equation:

$$\%\ inhibitory\ activity\ =\ (A\ -\ B)/(A-C)\ x\ 100 \qquad (2)$$

A – C: nitrite concentration (µM)

A: LPS (+), sample (-); B : LPS (+), sample (+); C : LPS (-), sample (-)

2.4. Determination of wound healing activity.

2.4.1. Wound healing activity determination on the proliferation and migration of human gingival fibroblast (HGF) cell line.

The HGF cell line was cultured in a DMEM supplement. The cells were seeded at $1.5x10^4$ cells/well in a 12-well plate and incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 incubator to allow cell adhesion. The scratch wound healing assay was performed on native cells. A straight-line scratch was created in the native cell monolayer using a p200 pipet tip. The debris was removed by gently washing the cells once with PBS (1 ml). The medium was then replaced with 2 ml of a fresh medium containing test samples and incubated at 37°C in a humidified atmosphere containing 5% CO_2 incubator. 3 days later, the closure of the wound was observed and recorded using a phase-contrast inverted microscope. Relative wound closure (%) was measured from the images using computing software and compared with the value obtained before treatment (time 0). An increase in the relative wound closure indicated the cell migrations. The following equation estimated the % wound closure. The experiment was carried out in three replicates. AMFE was used as the positive control.

% Wound closure =

$$[(Wound\ distance\ of\ time0 - Wound\ distance\ of\ timen)/Wound\ distance\ of\ time0]\ x\ 100 \qquad (3)$$

$Time_0$ = initial time of migration; $Time_n$ = time of detection

2.4.2. Wound healing activity determination on the proliferation and migration of AGS human gastric epithelial cell lines.

AGS human gastric epithelial cell line (CRL-1739™, ATCC®) was maintained in Dulbecco's modified Eagle medium (DMEM, Gibco®) supplemented with 10% FBS, 50 Unit/ml penicillin and 50 µg/ml streptomycin. The cells were grown in 75 cm^2 plastic cell culture flasks and incubated in a humidified atmosphere with 5% CO_2 at 37°C. AGS cells with less than 20 passage numbers were used. Culture media was changed every alternate day. After 80% confluence, culture cells were trypsinized using trypsin-EDTA solution at 37°C. Cell suspensions were prepared at the desired density before determining the test samples' cytotoxicity and wound-healing effect. The migration of AGS cells was investigated using a wound healing method as previously described with some modifications. AGS cells (5×10^5 cells/ml) in media containing 2% FBS were seeded into each well of a 6-well plate and incubated at 37°C with 5% CO_2. After forming a confluent monolayer of cells, a wound was made by straight scratching the cells in a line with a sterile pipette tip. After washing any cellular debris with PBS three times, the cells were cultured with 1 ml of fresh medium with or without the presence of test samples. The time of the scratching wound was defined as time 0. Images were taken at a 4X magnification using a microphotograph on time 0, and then the cells were allowed to migrate into the wound for the next 5 h (time 5), 10 h (time 10), and 24 h (time 24) at 37°C with 5% CO_2 and subsequently taken the images at those determined time again.

Relative wound closure (%) was measured from the images using computing software and compared with the value obtained before treatment (time 0). An increase in the relative wound closure indicated the cell migrations. % wound closure was determined according to equation number 3. The experiment was carried out in three replicates. AMFE was used as the positive control.

2.5. Determination of COX and NOS mRNA expression at the gastric ulcerated area.

Due to our previous study on the significant ulcer healing efficacy of oral AMFE (at the dose of 0.63–2.50 g/kg, which is equivalent to 0.5-1.5 mg SCO) twice daily for 10 consecutive days on acetic acid-induced chronic gastric ulcer in rats [6], the RNA extraction of each gastric ulcerated mucosal sample of water treated-, vehicle-treated-, AMFE (1.25 g/kg) treated- or SCO (at the same equivalent dose containing in AMFE) treated-rats in acetic acid-induced gastric ulcer model (MOE 0521.11/1242) was purified using RNeasy mini kit. cDNA synthesis was performed using the one-step RT-PCR kit.

The PCR mixture of 20 µl composed of 5x FIREPol® Master Mix (Ready to load) (4 µl), RNase-free water (13 µl), forward primers (from working primer 5 pmol/µl)(0.5 µl), reverse primers (from working primer 5 pmol/µl)(0.5 µl) and DNA template (5 ng/µl)(2 µl). The RT-PCR program was carried out using Bioer Technology Co, Ltd. Thermal Cycler, Japan. The PCR program was started while PCR tubes were still on ice, and then there was a wait while the thermal cycler reached 60°C. After an initial denaturation for 2 min at 95°C, the 40 cycled Thermal cycler conditions were denaturation at 95°C, 2 min, annealing at 57°C, 30 s, and elongation at 72°C, 6 min. The PCR products were then electrophoresed on a 1.2% (%w/v) agarose gel in Tris-Acetate-EDTA (TAE) electrophoresis buffer (pH 8.0). The gel was then soaked with SYBR® Safe in TAE buffer for 30 min in the gel tank in the dark. The gel tank

was carried to the dark room, and the DNA bands were observed under a UV light box (GeneDirex, BLooK LED transilluminator, Taiwan). All bands were finally photographed.

A 20 µl of reaction components for qRT-PCR composed of 5x HOT FIREPol®EvaGreen®qPCR Mix Plus (4 µl), RNase-free water (13 µl), forward primers (from working primer 5 pmol/µl) (0.5 µl), reverse primers from working primer 5 pmol/µl) (0.5 µl) and DNA template (5 ng/µl) (2 µl). qRT-PCR condition was carried out using the following cycling conditions: initial activation at 95℃, 12 min, and 40 amplification cycles of denaturation at 95℃, 20 s, annealing at 57℃, 30 s, and elongation at 72℃, 50 s.

The forward and reverse rat COX-1 primers were 5'-AACCGTGTGTGTGAC TTGCTGAA-3' and 5'-GCATTTCTCGGGACTCCTTGATGA-3', respectively. The forward and reverse rat COX-2 primers were 5′-AGGTGTATCCTCCCACAGTCAAAG-3′ and 5′-TTTGGAACAGTCGCTCGTCATC-3′, respectively. The forward and reverse rat nNOS primers were 5′–ACCTGATCCTAACTTGCCTTGC–3′ and 5′–AGTGACATCACCGCAGA CAAAC–3, respectively. The forward and reverse rat nNOS primers were 5′-CGTCCTTTGA ATACCAGCCTGATC-3′ and TTCAGAGTCAACATGGGAGAGG-3′, respectively. The forward and reverse rat iNOS primers were 5'-CTTCAATG GTTGGTACATGGGCAC-3' and 5'-ACGTAGTTCAACATCTCCTGGTGG-3', respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The forward and reverse rats GAPDH were 5'-GAACGGGAAGCTCACTGGCATGGC-3' and 5'-TGAGGTCCACCACC CTGTTGCTG-3', respectively.

3. Results and Discussion

3.1. Preparation and quantification of an aqueous M. citrifolia fruit extract (AMFE).

AMFE 238.26 g was obtained from 4.53 kg of fresh mature unripe *M. citrifolia* fruits or 715.48 g of dried powder. The percentage yield of AMFE was 5.26 %w/w of fresh fruits weight or 33.3 %w/w of dried fruits powder weight. The dried AMFE powder after freeze-drying appeared to be hygroscopic dark brown. The number of total phenolics in AMFE determined with the Folin-Ciocalteu reagent was 29.79±0.33 mg GAE)/g dried extract. The amount of SCO in 1 g of AMFE powder was calculated to be 0.645 mg. For SCO quantity, solid phase extraction (SPE) and HPLC-UV technique were used to ensure the specificity and sensitivity of the compound determination. SPE was widely used as a sample clean-up and made more concentrated interest bio-active compounds from complex samples [24-26], including SCO from various herbal samples [4]. The extract from the high SCO recovery SPE technique [6] gave a clean chromatogram and identical SCO peak related to the SCO reference standard at the specified detection wavelength, as shown in Figure 1. The limit of detection (LOD) and limit of quantitation (LOQ) values of this rapid HPLC condition were 0.57 and 1.71 µg/ml, respectively, indicating that the method was sufficiently sensitive to be used over a wide range of SCO concentrations. As plants from different areas and different seasons may affect the obtained amount of active ingredients even though using the same extraction methods, the amount of total phenolic compounds contained in AMFE used in the study (29.79 ± 0.33 mg GAE/g dried AMFE) was found to be much higher than that reported in other previous study (2.10 mg GAE/g dried AMFE) [27]. On the contrary, the amount of SCO contained in AFE (0.645 mg/g dried AMFE) was found to be less than that reported in our previous study (0.85–0.87 mg/g dried AMFE) [6].

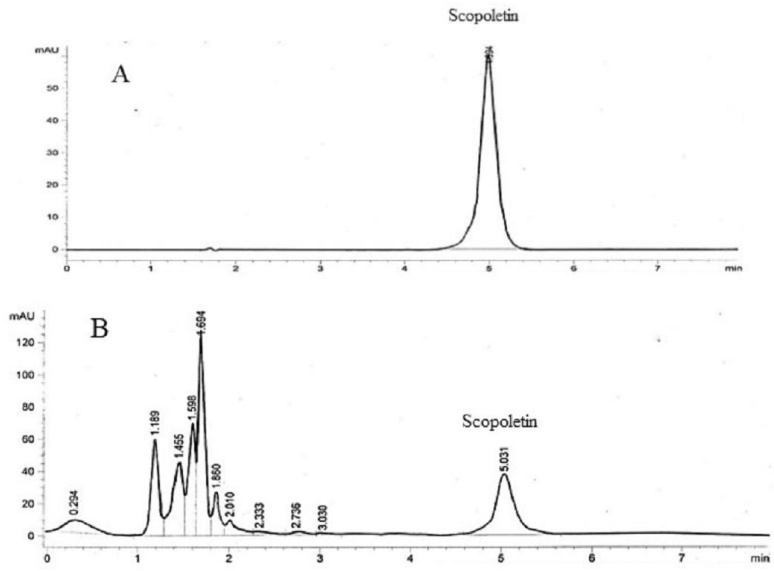

Figure 1. HPLC chromatogram of standard SCO and SCO content in AMFE **(A)** SCO reference standard 10 µg/ml; **(B)** SCO content in 4 mg/ml AMFE AMFE: an aqueous *M. citrifolia* fruit extract SCO: scopoletin.

3.2. In vitro antioxidant activity in scavenging NO radicals and anti-inflammatory activity inhibiting NO production of AMFE and SCO.

The results obtained from the present study showed that the half maximal NO scavenging concentration (IC_{50}) of AMFE and gallic acid on the released NO from the decomposition of sodium nitroprusside were 734.02 ± 2.56 µg/ml and 9.73 ± 0.06 µg/ml, respectively (Table 1). The antioxidant potency of AMFE in scavenging NO radical was about 80 times lower than that of a positive control gallic acid. The concentration of SCO containing the IC_{50} value of AMFE was equivalent to 0.47 µg/ml. Pure SCO at a dose of 45 µg/ml has been reported to scavenge DPPH, H_2O_2, superoxide, and OH-radicals about 63.79%, 70.21%, 68.98%, and 39.97%, respectively [28]. Therefore, it has been claimed as one of the antioxidant markers of *M. citrifolia* (Noni) fruit juice [28] and Noni bio-fermented drink [29]. However, the maximum NO scavenging capacity of pure SCO obtained from the present study was 34.30% at a 150 µg/ml dose.

On the contrary, IC_{50} values of anti-inflammatory activity in inhibiting LPS-induced NO production in activated macrophage RAW 264.7 cell line of AMFE was 221.70 ± 26.66 µg/ml, and the concentration of SCO containing at the IC_{50} value of AMFE was equivalent to 0.15 µg/ml. Pure SCO was found to inhibit LPS-induced NO production in activated macrophage RAW 264.7 cell line with IC_{50} values in the range of 6.54 ± 0.16 µg/ml. The IC_{50} values on NO production of positive controls at a series of CAPE (NF-κB inhibitor), L-NA (NOS inhibitor) and indomethacin (COX and iNOS inhibitor) were 2.66 ± 0.15, 11.43 ± 1.15, and 5.41 ± 0.91 µg/ml, respectively. CAPE was found to possess the highest inhibitory capacity, followed by indomethacin, SCO, L-NA, and AMFE. The survival of the macrophage RAW264.7 cell line was more than 80% at all concentrations of the tested samples. Comparing the efficiency against the production and the release level of pro-inflammatory NO mediator obtained from the present study, SCO possessed a higher efficiency as an anti-inflammatory agent in inhibiting the production of pro-inflammatory NO than as an anti-oxidative agent in scavenging the release of pro-inflammatory NO radical. It is possible that SCO may inhibit NO production through the inhibition of NO synthase activity and NF-κB transcription. The previous study had also reported that SCO (1–50 µg/ml) dose-dependently inhibited LPS-stimulated the release of PGE_2 through suppression of COX-2 and NF-κB transcription factor

activities leading to an inhibition on the release of TNF-α, IL-1β, and IL-6 including an inhibition on the production of NO derived from iNOS [3, 30-31]. The anti-inflammatory potency against NO derived from iNOS of AMFE seemed to relate to the concentration of SCO in AMFE. As it is widely known, phenolic compounds exhibit antioxidant properties through various mechanisms of action, including neutralization of free RNS; therefore, the antioxidant property of AMFE might be accounted for by SCO and other phenolic compounds contained in AMFE. It has been recently reported that rutin, 4-hydroxybenzoic acid, and gentisic acid are the major antioxidant compounds identified in commercial noni juice, and gentisic acid exerted the highest antioxidant capacity among these three major phenolic compounds [32].

Table 1. *In vitro* antioxidant activity in scavenging NO radicals and anti-inflammatory activity in inhibiting NO production of AMFE and SCO.

***In vitro* antioxidant activity in scavenging NO radicals**	
Compounds	**Half maximal NO scavenging concentration (IC_{50}) (µg/ml)**
AMFE	734.02 ± 2.56
SCO	NA
Gallic acid	9.73 ± 0.06
Pure SCO at a dose of 150 µg/ml exerted a maximal scavenging NO radicals capacity of about 34.30%	
***In vitro,* anti-inflammatory in inhibiting LPS-induced NO production in activated macrophage RAW264.7 cell line**	
Compounds	**Half maximal anti-inflammatory concentration (IC_{50}) (µg/ml)**
AMFE	221.70 ± 26.66
SCO	6.54 ± 0.16
CAPE	2.66 ± 0.15
L-NA	11.43 ± 1.15
Indomethacin	5.41 ± 0.91

Each value represented the mean ± SEM of three determinations: AMFE: an aqueous *M. citrifolia* fruit extract; SCO: scopoletin, CAPE: caffeic acid phenethyl ester L-NA: L-nitroarginine.

3.3. Wound healing activity of AMFE and SCO on the proliferation and migration of human gingival fibroblast (HGF) cell line.

The HGF cells were subjected to a scratch-induced wound. It was found that, after 3 days of the scratch wound creator, AMFE at a concentration of 100 µg/ml significantly induced fibroblast cell proliferation and migration at a comparable wound healing capacity with a percentage of wound closure of 73.95 ± 10.78 % (Table 2). In contrast, pure SCO at the same equivalent concentration containing AMFE (100 µg/ml) did not exert any significant wound healing capacity after 3 days of the scratch wound creator when compared with the control group.

Table 2. Effect of AMFE and SCO on HGF human gingival fibroblast cell proliferation and migration.

Treatment groups	**% Wound closure (Mean±SD)**			
	Day 0	**Day 1**	**Day 2**	**Day 3**
Control	0	8.63 ± 1.29	20.91 ± 1.79	42.12 ± 7.08
AMFE 100 µg/ml (SCO 0.06 µg/ml)	0	19.21 ± 5.26 ^{a,b}	53.04 ± 8.20 ^{a,b}	73.95 ± 10.78 ^{a,b}
SCO 0.06 µg/ml	0	9.02 ± 1.48	21.29 ± 6.27	45.76 ± 4.17

Each value represents the mean ± SD from four independent experiments; AMFE: an aqueous Morinda citrifolia unripe fruit extract; SCO: scopoletin; aP<0.05 when compared to control; bP<0.05 when compared to SCO 0.06 µg/ml. All measuring data were determined by the LSD test.

3.4. Wound healing activity of AMFE and SCO on the proliferation and migration of AGS human gastric epithelial cell lines.

The AGS human gastric epithelial cells were subjected to a scratch-induced wound, and the wound-healing effect of AMFE on AGS cells was evaluated in a 24-hour timing course. Interestingly, the closure of the wound was accelerated by the proliferation and migration of the human gastric epithelial cell line treated with 100 µg/ml of AMFE (equivalent to SCO 0.06 µg/ml) with a percentage of wound closure of 78.01 ± 1.78%. It was found that AMFE exhibits a higher wound healing efficacy than pure SCO at the same equivalent concentration, as shown in Table 3. Moreover, on time at 24 hours, the results showed that the cell line treated with AMFE, at a concentration of 100 µg/ml, had the fastest migrating of the cell line to close the scratch wound when compared with those of the control group (no treatment) and SCO-treated group.

The scratch assay is a useful method for gaining an insight into the potential of a sample to repair injured tissue by monitoring the proliferation and migration capacity of fibroblast cells, which is an important step in ulcer healing for tissue regeneration, including monitoring the proliferation and migration of epithelial cells in the mucosa of the ulcer margin to re-epithelialize the ulcer (re-epithelialization). AMFE was found to exert a high wound healing capacity and higher percentages of wound closure than pure SCO when compared at the same equivalent concentration contained in AMFE. In addition, SCO at the same equivalent concentration contained in AMFE did not exhibit any significant effect in the stimulation of the wound healing process compared to that of the control group.

Table 3. Effect of AMFE and SCO on AGS gastric epithelial cell proliferation and migration.

Treatment groups	% Wound closure (Mean±SD)			
	Time 0	Time 5 h	Time 10 h	Time 24 h
Control	0	2.96 ± 0.71	23.48 ± 1.70	56.27 ± 0.65
AMFE 100 µg/ml (SCO 0.06 µg/ml)	0	25.04 ± 0.60[a,b]	34.90 ± 1.19[a,b]	78.01 ± 1.78[a,b]
SCO 0.06 µg/ml	0	16.78 ± 2.62[a]	29.12 ± 1.51[a]	63.26 ± 0.90[a]

Each value represents the mean ± SD from four independent experiments; AMFE: an aqueous *Morinda citrifolia* unripe fruit extract; SCO: scopoletin; [a]*P<0.05* when compared to control; [b]*P<0.05* when compared to SCO 0.06 µg/ml. All measuring data were determined by LSD's test.

3.5. Effect of AMFE and SCO on COX and NOS mRNA expression at the gastric ulcerated area.

The mRNA expression of gastric COX (COX–1 and COX–2) and NOS (iNOS and nNOS) in the sample gastric tissues was analyzed using two-step RT–PCR analysis with specific primers and confirmed their expression level by quantitative real-time PCR reaction (qRT-PCR). G3PDH was used as a housekeeping gene. In normal rat gastric tissue, constitutive gastroprotective nNOS enzymes and a constitutive gastroprotective COX enzyme (COX–1), which maintains the integrity of gastric epithelium, were expressed, whereas gastric pro-inflammatory enzymes (iNOS and COX–2) remained in their quiescent state as shown in Figure 2. In ulcerated gastric tissue induced by acetic acid, it was found that acetic acid down-regulated the mRNA expression of constitutive gastroprotective COX-1 and nNOS enzymes but up-regulated the mRNA expression of pro-inflammatory gastric COX-2 and iNOS enzymes as found in the water and vehicle control-treated rats, compared to that in the normal gastric mucosa. An oral administration of 0.5% CMC was found in the present study as a mild gastroprotective agent in up-regulating the mRNA expression level of gastric cytoprotective

COX-1 (a powerful inhibitor of gastric acid and a stimulus of gastric mucus secretion and gastric microcirculation) and nNOS (an important regulator of gastric acid and mucus secretion) and a mild anti-inflammatory agent in downregulating the mRNA expression level of pro-inflammatory COX-2 when compared to those of the water control-treated group. However, CMC did not exert any significant inhibitory effects on the expression level of pro-inflammatory iNOS mRNA. Hence, it was not found to exert any significant curative efficacy on the chronic gastric ulcer induced by acetic acid [6]. The obtained result also confirmed our previous study that CMC exerted a mild anti-inflammatory and gastroprotective agent against indomethacin-induced acute gastric ulcer model in decreasing the production of pro-inflammatory PGE_2 and in up-regulating the expression level of gastric cytoprotective COX-1 and nNOS mRNA [33]. Treatment with AFE and pure SCO (at the same equivalent dose containing in AMFE) markedly up-regulated the mRNA expression of constitutive gastroprotective enzymes: COX-1 and nNOS ($p<0.05$) but significantly down-regulated the expression of gastric pro-inflammatory enzymes: COX-2 and iNOS into the normal level ($p<0.05$) with similar inhibitory efficacy. This indicated that AFE (at the dose of 1.25 g/kg, which equivalent to 0.8-1 mg SCO) twice daily for 10 consecutive days provided an optimal therapeutic dose in treatment of acetic acid-induced chronic GU as it exhibited a balance between gastric mucosal level of pro-inflammatory COX-2 and iNOS and gastric cytoprotective COX-1 and nNOS (which restored the gastric mucus and gastric mucosal blood circulation) leading to a decrease of gastric acid secretion, an enhancement of gastric mucosal integrity and a beneficial effect on ulcer healing in eliminating iNOS–positive inflammatory cells during ulcer healing including enhancing expression of growth factors involved in the promotion of ulcer healing, angiogenesis, fibroblast proliferation and migration and epithelial cell proliferation. Nevertheless, it was found in the present study that SCO (at the same equivalent dose contained in AFE) did not significantly stimulate the ulcer healing processes.

Apart from their antioxidant and anti-inflammatory activities, phenolic compounds, especially flavonoids, have been found to exhibit wound-healing activity [34-36]. Therefore, the property in accelerating the ulcer healing process of AMFE might be accounted for by other phenolic compounds contained in AMFE and probably other contributions of some sources of ulcer healing activity contained in AMFE. As a consequence, a sufficient amount of phenolic compounds and SCO in AMFE was important for the antioxidant, anti-inflammatory, gastroprotective, and ulcer healing activities of AMFE.

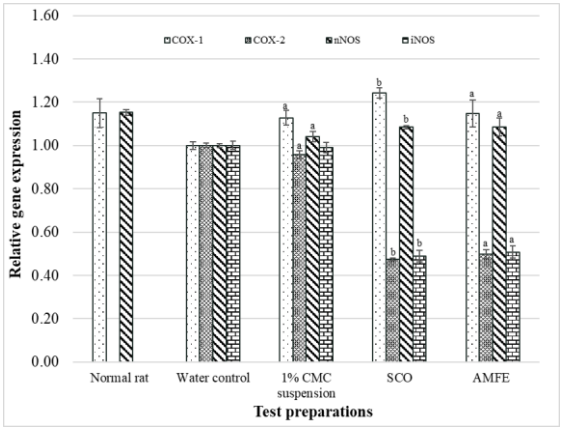

Figure 2. Expression of COX-1, COX-2, nNOS, and iNOS mRNA by quantitative real-time PCR reaction in the rat ulcerated gastric tissue induced by acetic acid on the 4th day after ulcer induction and treatment. Each value represents the mean ± SD from four independent experiments; AMFE: an aqueous Morinda citrifolia unripe fruit extract, SCO: scopoletin (at the same equivalent dose containing in AFE); [a]$p<0.05$ compared to the water control-treated rats (LSD's test); [b]$p<0.05$ compared to the vehicle (1% CMC) control-treated rats (LSD's test).

Currently, the pharmacological potential of SCO in the treatment of various chronic diseases (such as antihypertensive, anti-diabetic, anti-inflammatory, hepatoprotective, anti-cancer, *etc*.) has been evaluated in many studies due to its potential in simultaneously regulation of multiple signaling pathways and numerous target molecules [37]. SCO has been reported to relieve the symptoms and severity of these chronic diseases. In addition, it has a non-toxic nature and an excellent pharmacokinetic property [37, 38]. Thus, SCO can be considered a potential drug candidate, which warrants further investigation to develop it as an antiulcer potential drug in clinical settings.

4. Conclusions

The results obtained from the present study revealed that the anti-inflammatory and gastroprotective properties of AMFE seemed to relate to the concentration of SCO contained in AMFE. On the contrary, the property in accelerating the ulcer healing process of AMFE might be accounted for by phenolic compounds and probably other contributions of some sources of ulcer healing activity contained in AMFE.

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Conflicts of Interest

The authors declare no conflict of interest.

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