Journal of Traditional and Complementary Medicine 10 (2020) 345–353

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Gastroprotective and anti-inflammatory effects of *Prunus cerasus* phytochemicals and their possible mechanisms of action

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ARTICLE INFO

Article history:
Received 6 June 2018
Received in revised form
23 May 2019
Accepted 1 June 2019
Available online 5 June 2019

Keywords:
Prunus cerasus
Gastroprotective
Anti-inflammatory
Antinociceptive effects
Oxidative stress

ABSTRACT

Prunus cerasus (*P. cerasus*) is an alternative-medicine used traditionally for amelioration of chronic-ailments marked by elevation in oxidative-stress like neuropathy. The oxidative-stress control was reported to ameliorate the inflammatory-process. This study aimed to phytochemically-investigate *P. cerasus* most-active phytochemicals utilizing *in-vivo* biological models to explore their gastro-protective, anti-inflammatory, and antinociceptive potentials and their possible mechanisms of action. Sonication with EtAc was used to extract *P. cerasus* fruit (Scf), and seed (Scs). The phytochemical-investigation of Scf was performed by RP-HPLC, while that of Scs was explored utilizing GC-FID. A bio-guided-fraction and isolation method was done utilizing column-chromatography, and have shown that cyanidin-3-glucoside (Cy3G) was the most-active constituent in Scf, while linoleic-acid (LA) was the most-active constituent in Scs. Scf, Scs, Cy3G, and LA significantly ($p < 0.05$) protected the gastric-mucosa against HCl/EtOH-induced gastric-lesions. Scs (200 mg/kg) has shown the most gastroprotective-potentials, and had comparable-results to ranitidine (50 mg/kg). Scf, Scs, Cy3G, and LA have shown significant anti-inflammatory and antinociceptive potentials against carrageenan induced-edema and nociceptive-pain, respectively, where Scs (200 mg/kg) has shown the most anti-inflammatory and antinociceptive potentials, and had comparable results to ibuprofen (100 mg/kg). Scf, Scs, Cy3G, and LA have counter-acted carrageenan-induced oxidative-stress markers, with increased serum-catalase and reduced-glutathione levels, and decreased lipid-peroxidation. Histopathological-studies demonstrated gastroprotective potentials, regeneration and improvement of the spleen-structural architecture when treated with highest doses of Scs and Scf. The reduction of the pro-inflammatory TNF-alpha and IL-6, and elevation the anti-inflammatory factor IL-10 levels, spleen regenerative-capacity and oxidative-stress amelioration might be the main-mechanism responsible for *P. cerasus* anti-inflammatory potentials. *P. cerasus* appears to aid in ameliorating the inflammatory process, and reducing pain-thresholds while preserving the stomach.

1. Introduction

Complementary medicine and its active phytochemicals have long-standing profiles in prevention and treatment of a variety of disorders.[1] Their edible-species are characterized by their good safety profiles, and economical-prices which improve their patient-

Abbreviations: Scf, sour cherry fruit ethyl acetate extract; Scs, sour cherry seed ethyl acetate extract; *P. cerasus*, *Prunus cerasus*; Cy3G, Cyanidin 3-glucoside; LA, Linoleic acid; FID, flame-ionization detector; HAc, acetic acid; EtAc, Ethyl acetate; MeOH, methanol; EtOH, ethanol; Ib, Ibuprofen; LPO, lipid peroxidation; TBARS, Thiobarbituric acid reactive substances; GSH, reduced glutathione; PWT, paw withdrawal threshold; VEH, vehicle control; CTRL, vehicle control; NSAIDs, Non-steroidal anti-inflammatory drugs; TNF-alpha, Tumor necrosis factor alpha; IL-6, Interleukin 6; IL-10, Interleukin 10; H and E staining, Hematoxylin and Eosin staining; mu, mucosa; sm, sub-mucosa; er, erosions; h, hemorrhage; e, edema; ic, infiltration of inflammatory cell in the sub-mucosa.

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2019.06.001

adherence, when compared to conventional drugs.[2] Particularly, fruits and seeds have been studied as a good source of bio-active phytochemicals, especially for their antioxidant, anti-inflammatory, antimicrobial, and antitumor potentials.[3,4] Sour-cherry or *Prunus cerasus* (*P. cerasus*), from the family Rosaceae, is among the edible medicinal-plants which is used as a prophylactic agent against cardiovascular damage, Alzheimer's disorder, inflammatory diseases, and ameliorating a number of chronic ailments were marked by elevation in oxidative-stress like cancer, diabetes and neuropathy.[5] The fruit of *P. cerasus* is characterized by its content of polyphenols (like, anthocyanins and isoflavonoids), while its seed marked by its high content of fatty-acids (like, poly-saturated and poly-unsaturated fatty acids).[5,6] Bio-active poly-phenolic and fatty-acid compounds are known to present direct and/or indirect anti-inflammatory and antioxidant activities that aid in alleviating the oxidative-stress at the cellular-level.[7–9]

Gastric-ulcer has been a major reason for mortality and morbidity, with a prevalence of at least 10% of the world-population.[10] The gastric-ulcer occurs due to an imbalance between the stomach acids and enzymes, and its defensive (bicarbonate and mucus) components, which is affected by many exacerbating factors, like NSAIDs, stress, and *H. pylori*.[11,12] It has been found that gastric-ulcer is also associated with pro-inflammatory cytokines changes. Studies demonstrated that noxious-factors inducing gastric-mucosal injury resulted in the increase of TNF-alpha (tumor necrosis factor alpha) and IL-6 (Interleukin 6), the local pro-inflammatory cytokines, levels.[13] High levels of local pro-inflammatory cytokines intermediate the progress of local-inflammation and gastric-ulceration.[14] Conventional antisecretory drugs, like ranitidine, and proton-pump inhibitors, like omeprazole, were long used for the management of gastric-ulcers, though, their extended use might induce serious side-effects, including thrombocytopenia, hepato-toxicity, nephro-toxicity, impotence, and gynecomastia.[10,15,16] Moreover, there is increasing evidence that the ideal gastroprotective compound should possess antioxidant and anti-inflammatory potentials.[17] Consequently, alternative-medications meeting patient-safety coupled with gastroprotective and antioxidant efficacy are in need to prevent gastric-ulcer and its comorbidities.[18,19]

Inflammation is a normal bio-process exacerbated by various conditions such as microbial-infections, tissue-injury, and chemical-irritation.[20] It has been recognized as a contributing factor in a number of chronic health-diseases such as hypertension, tumor, diabetes, aging and neuro-degenerative disorders.[21,22] Oxidative-stress was reported to initiate the inflammatory-process resulting in synthesis and secretion of pro-inflammatory mediators.[23,24] Previous studies have demonstrated a regulatory role of native antioxidant defenses in the inflammatory-progression,[25,26] and inflammatory-pathologies.[27,28] Therefore, recent approaches aiming to prevent inflammatory-disorders often comprise biological models of oxidative-stress reduction to assess the anti-inflammatory mechanism.[29] Currently, the increasing number of inflammatory-disorders has urged the need for alternative-solutions.[20,29] Polyphenols and bio-active fatty-acids were found to be associated with strong antioxidant and anti-inflammatory activities.[30,31] Management of nociceptive-pain, which can be exacerbated by inflammation and reaction with noxious-stimuli, remains a major problem especially that conventional analgesics have many serious side-effects, including gastritis and gastric-ulceration.[32,33] Therefore, finding more safe phytotherapies preserving the stomach are of high demand.[34,35]

P. cerasus has been reported to have some *in-vitro* antioxidant and anti-inflammatory potentials.[36–38] However, no detailed *in-vivo* gastroprotective, anti-inflammatory, and antinociceptive studies have been performed.

Therefore, the aim of this work is to phytochemically investigate *P. cerasus* fruit and seed and identify their most bioactive phytochemicals utilizing *in vivo* biological models to explore their gastroprotective, anti-inflammatory, and antinociceptive potentials and their possible mechanisms of action.

2. Materials and methods

2.1. Chemicals

Milli-Q water was obtained by a Reagent-Water System (USA). All solvents, chemicals, and reference-standards were of analytical-grade obtained commercially (Sigma-Aldrich, Germany) and were utilized without any further-purification.

2.2. Plant materials

The sour-cherry (*Prunus cerasus*) fruits were obtained-commercially, and the genus has been verified by comparing to a reference-sample, and the seeds have been removed. Samples of both the fruit (PS-16-14) and the seed (PS-16-15) have been deposited in the Faculty herbarium. The seedless fruits were then sonicated (Clangsonic Sonicator, China) for one hour using EtAc and then carefully dried utilizing rotavapor (Buchi, Germany) at 40 °C under vacuum to form the sour cherry fruit EtAc extract (Scf). While the seeds were crushed homogenously with a mortar and then were carefully sonicated with EtAc for one hour and dried under vacuum to form the sour cherry seed EtAc extract (Scs). Both Scf and Scs were stored separately at −40 °C until further experimentation.

2.3. GC-FID of the sour cherry seed EtAc extract (Scs)

An Agilent gas chromatographic system (Japan) attached to a flame-ionization detector (FID), and an automated sampler and split-splitless injector, was used for evaluation of sour cherry seed EtAc extract (Scs). In each experimentation day, Scs samples were freshly dissolved in isopropanol and were examined in triplicates. At a split-ratio of 1:30, the injector (250 °C) was connected to HP-5MS column (30 m × 0.25 mm, 0.25 μm) and 1 ml/min carrier gas flow rate, and adjusting the temperature to 300 °C for the FID detector, while adjusting the column temperature to a ramp of 4 °C/min starting from 40 °C reaching 260 °C, and then kept at 260 °C for 10 min isothermally.

2.4. RP-HPLC of the sour cherry fruit EtAc extract (Scf)

An Agilent RP-HPLC system (USA) comprising a quaternary-pump and equipped with DAD-detector and an online-degasser was applied for evaluation of sour cherry fruit EtAc extract (Scf). Elution has been obtained in triplicates on an RP-C18 column (250 mm × 4.6 mm, 5.0 μm). The samples were freshly prepared and dissolved in the mobile phase, which consisted of trifluoro-acetic acid (TFA, 0.1%)/MeOH mixture (10:90, v/v), with injection volume adjusted to 20 μL, and flow rate 1 mL/min, and 200–600 nm detection range, focusing on 215 nm.

2.5. Bio-guided chromatographic fractionation and isolation of the most active compounds in Scf and Scs

The Scf and Scs have been fractionated separately using pre-parative column-chromatography technology in order to identify the most active compounds in each extract. For Scf, the column (50 × 100 cm) was loaded with silica gel as a stationary phase. The column was eluted with EtAc/formic acid/Milli-Q-water/hexane

(70:7.5:7.5:15, v/v/v/v) mixture as a mobile phase. For Scs, the column (50 × 100 cm) silica-gel was treated with one bed-full (bf) of an acetone/water mixture (7:1, v/v) and one bf hexane. After the column is loaded, the elution was done utilizing 2 bf of hexane/methyl *t*-butyl ether/HAc mixture (100:3.0:0.3, v/v/v), then one bf of hexane-chloroform-EtAc mixture (100:5:5, v/v/v), then with one bf of chloroform-2-propanol mixture (2:1, v/v), and finally with 2 bf of chloroform/MeOH/HAc mixture (100:2:2) as a mobile phase. In both fractionation procedures, the eluent has been sequentially and separately accumulated by time, and more than 100 fractions have been gathered. Various fractions were concentrated and chromatographically analyzed and similar fractions were combined. Furthermore, every fraction has been monitored for its gastroprotective, anti-inflammatory, and antinociceptive effects, in similar-ways as tested compounds using animal *in-vivo* models (Fig. S1).

2.6. Animals and biological-screening

Swiss-albino male mice (20–35 g) have been utilized throughout this work. The mice have been kept in an animal-house with free-access to food (unless indicated) and water, under standard temperature and light/dark cycles conditions. Animal-care and all procedures used in the study were done in agreement with the guidelines and regulations of BAU institutional review board (2015A-0012-HS-M-0088).

2.6.1. Gastroprotective effects of Scf, Scs, Cy3G, and LA against HCl/EtOH induced ulcer

To assess the gastroprotective activities of various-doses of Scf, Scs, Cy3G, and LA, HCl/EtOH induced ulcer method was adopted.[10] In brief, after 16 h of fasting, mice (n = 4/group) were orally treated with saline (vehicle control), ranitidine HCl 50 mg/kg (positive control), Scf (100, 150, 200 mg/kg), Scs (100, 150, 200 mg/kg), Cy3G (10, 15, 20 mg/kg), or LA (10, 15, 20 mg/kg), then after 60 min, 0.45 M HCl/EtOH (60%) were given by gavage for gastric-lesion induction. After 1 h, the animals were euthanized and an operation was done to remove the stomach and its greater curvature was exposed, photographed, and CAD-software was used to measure the area of the lesion as a % of the total-area of the stomach, and an average of each group was determined (mean ± SEM).

2.6.2. Stomach histopathological evaluation

Stomach tissue specimens were taken from the respective group of mice (normal, treated with ranitidine HCl 50 mg/kg, vehicle, Scf 200 mg/kg, Scs 200 mg/kg, Cy3G 20 mg/kg, or LA 20 mg/kg) after induction of lesions with HCl/EtOH. Samples were fixed in 10% buffered-formalin and paraffin embedded. Paraffin-sections have been cut to a thickness of five μm, before being stained utilizing H and E stain for histopathological-evaluation. These sections have been microscopically examined for severity in peptic histopathological-alterations, for instance, hemorrhage (h), erosions (er), infiltration of inflammatory cells in the sub-mucosa (ic), and edema (e).[39]

2.6.3. Antinociceptive effects of Scf, Scs, Cy3G, and LA against acute carrageenan-induced inflammatory-pain

To evaluate the antinociceptive effects of sour cherry extracts and their most active constituents, an acute carrageenan-induced inflammatory-pain method was performed.[40,41] In brief, 100 μL an intraplantar injection of 1% carrageenan-solution was injected into the left hind paw to induce acute inflammatory-pain, while the normal control mice had intraplantar injection with 100 μL saline only. As a positive control, ibuprofen (Ib) 100 mg/kg was orally administered 0.5 h before carrageenan-injection. The test extracts/compounds and the vehicle control group (saline) were orally administered 0.5 h before carrageenan-injection. Paw withdrawal thresholds (g) were observed for 2 h post carrageenan-injection.

2.6.4. Anti-inflammatory activities of Scf, Scs, Cy3G, and LA against carrageenan-induced paw edema

The potential anti-inflammatory activities of the tested compounds were evaluated similarly to the antinociceptive properties, but with measuring the change in carrageenan-induced mouse paw edema volumetrically with the aid of Labthai-Plethysmometer (Malaysia) for 4 h.[28] In brief, the tested compounds were orally administered, and 60 min later carrageenan was injected intraplantarly in the mice right paw, and the volume of the edema was recorded immediately (zero time), 1, 2, 3, and 4 h post-carrageenan injection. Ib 100 mg/kg has been utilized in this study as a positive-control.

2.6.5. Determination of the level of inflammatory mediators

The tissue-homogenates were centrifuged and the cytokines were detected in the supernatant utilizing commercial ELISA (enzyme-linked immune-sorbent assay) kits for TNF-alpha, IL-6, and IL-10 (Bio-Legend, USA).[17]

2.6.6. Assessment of oxidative-stress markers

Oxidative-stress was reported to trigger the inflammation resulting in regulating the secretion of pro-inflammatory mediators.[23,24] Thus, to determine the anti-inflammatory mode of action by which the tested extracts/active compounds work, the oxidative stress markers were monitored, while assessing the anti-inflammatory test, at zero time (predose) and 4 h post carrageenan administration. These markers include serum-catalase (CAT), lipid-peroxidation (LPO), and reduced-glutathione (GSH). CAT levels (kU/l) were evaluated utilizing the modified-method described before.[42] Furthermore, LPO has been measured by thiobarbituric acid reactive substances (TBARS) test (nM/100 g) that was modified from a method described before.[43] Moreover, GSH (μg/mg) was also monitored by a method previously described.[44]

2.6.7. Spleen histopathological evaluation

In order to explore the tested compounds' anti-inflammatory mechanism, spleen histopathological studies have been investigated. After eight days of Scf and Scs highest doses (200 mg/kg) treatment from carrageenan-induced inflammatory-edema, the animals were euthanized and spleens were harvested and fixed in buffered formalin (10%). The tissues have been dehydrated by serial dilutions of ethanol solution, cleared with xylene, and then inserted in paraffin. Paraffin blocks have been sectioned into 5 μm thickness using a microtome, and then hematoxylin and eosin (H and E) stained, followed by a light-microscope assessment.

2.7. Statistical analysis

The results interpreted as (mean ± SEM) were statistically-analyzed utilizing one-way ANOVA followed by Fisher post-hoc statistical test utilizing "Origin-Pro" statistical-program. Statistical differences (?? < 0.05) were considered significant.

3. Results

3.1. GC-FID assessing Scs fatty acids composition

The fatty acid composition of the Scs has been studied using GC-FID analysis (Fig. 1). The analysis of the GC-FID chromatograms has shown that Scs is mainly composed of monounsaturated fatty-acid including oleic acid (26.44 ± 0.90%), and polyunsaturated fatty-

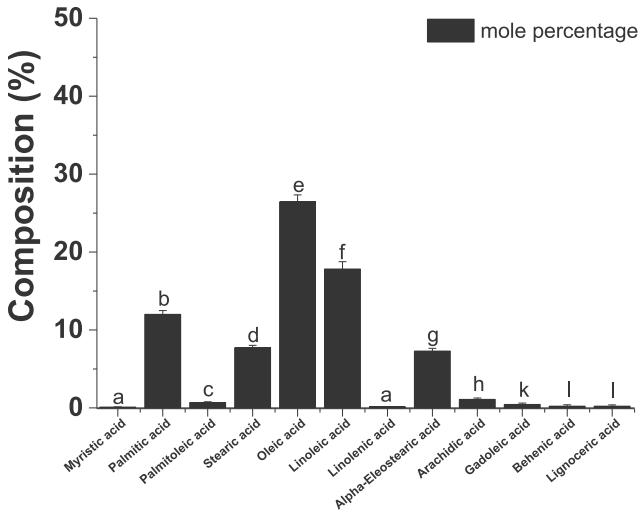

Fig. 1. GC-FID analysis showing the composition of fatty acids (mole percentage) of Sour cherry seed EtAc extract (Scs). Values are means of triplicate determinations ± S.E.M. Values with different letters are significantly different ($p < 0.05$).

acids including linoleic acid (LA, 17.81 ± 0.94%) and linolenic acid (0.13 ± 0.01%). Moreover, some saturated fatty acids, like stearic acid (7.70 ± 0.33%) and palmitic acid (11.99 ± 0.50%), was also present in Scs (Fig. 1). Other fatty acids like myristic-acid (0.10 ± 0.05%), palmitoleic-acid (0.69 ± 0.09%), stearic-acid (7.70 ± 0.32%), alpha-elostearic acid (7.29 ± 0.35%), arachidic-acid (1.10 ± 0.10%), gadoleic-acid (0.45 ± 0.10%), behenic-acid (0.22 ± 0.17%), and lignoceric-acid (0.23 ± 0.16%) were also detected (Fig. 1).

3.2. RP-HPLC assessing Scf polyphenolic composition

The polyphenolic composition of the Scf has been investigated using RP-HPLC analysis (Table 1). RP-HPLC chromatograms have shown that Scf is mainly composed of cyanidin glycosides using the peak area and standard-calibration curve methods, the major cyanidin glycosides identified in Scf were cyanidin derivative (27.28 ± 1.50%), cyanidin 3-glucosyl rutinoside (26.47 ± 1.81%); cyanidin 3-glucoside (Cy3G, 13.62 ± 0.52%), and cyanidin 3-rutinoside (14.12 ± 0.55%) (Table 1, Fig. S2).

3.3. Bio-guided chromatographic fractionation and isolation of the most active compounds in Scf and Scs

Using preparative column-chromatography technology and animal *in-vivo* models, Scf and Scs have been undergone bio-guided fractionation separately in order to identify the most-active constituents in each tested-extract. Scf most active fraction was injected in the RP-HPLC, while, the most active fraction of Scs was identified via GC-FID analysis. The bio-guided fractionation and the chromatographic steeping methods have shown that cyanidin 3-glucoside (Cy3G) is the most active compound in Scf (Fig. S3). On the other hand, linoleic acid (LA) has shown to be the most active compound in Scs. Therefore, Cy3G, and LA have been tested the same way as Scf and Scs to explore their gastroprotective, anti-inflammatory, and antinociceptive potentials.

Table 1
RP-HPLC major constituents of Sour Cherry fruit EtAc extract (Scf) (%, n = 3).

RT	Compound	%
28.3	Cyanidin derivative	27.28 ± 1.50
29.5	Cyanidin 3-glucosyl rutinoside	26.47 ± 1.81
32.4	Cyanidin 3-glucoside	13.62 ± 0.52
32.7	Cyanidin 3-rutinoside	14.12 ± 0.55

3.4. Assessment of the gastroprotective effects using HCl/EtOH induced ulcer model

To monitor the gastroprotective activities of the tested-extracts and their most active constituents, HCl/EtOH induced gastric-lesion method was performed. The HCl/EtOH oral administration, in the vehicle control group, caused extensive hemorrhagic gastric-lesion in the stomach glandular-mucosa. It was observed as red-bands and/or dark brown-patches of various sizes throughout the stomach-axis (Fig. 2B). Nevertheless, the ulcerated-area in the vehicle-control group was calculated as 71.25 ± 2.90%. When compared to vehicle treated group, the oral-treatment of mice with ranitidine HCl 50 mg/kg (positive control) has shown 77.1% reduction in gastric-lesion (Table 2). Moreover, Scf various doses (100, 150, 200 mg/kg) have shown 49.2, 71.8, and 74.9% reduction in gastric-lesion, respectively, when correlated to vehicle treated

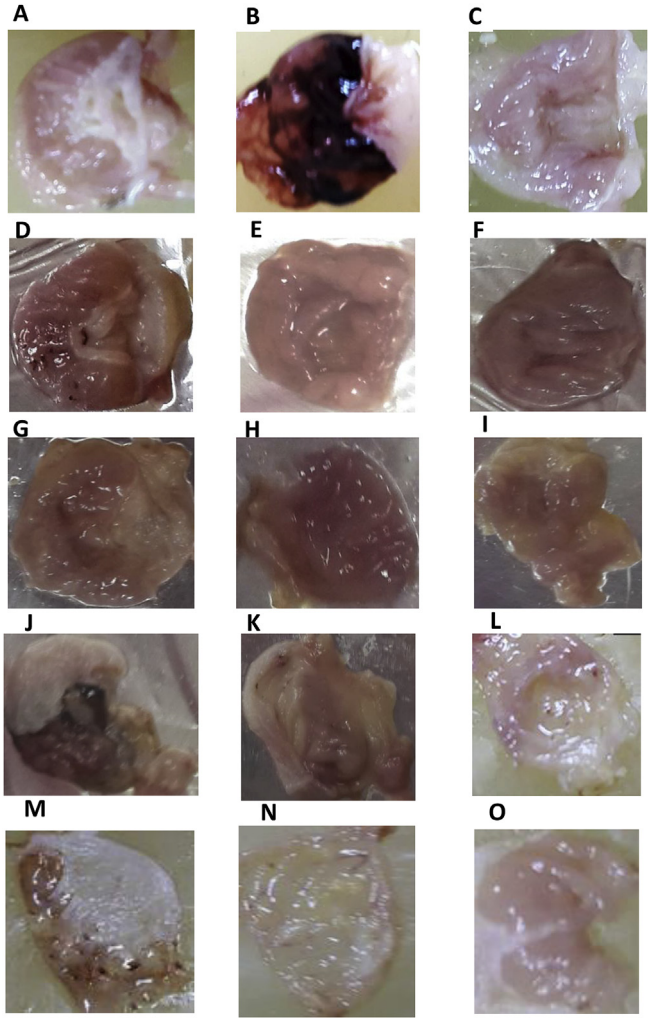

Fig. 2. Gastroprotective activities against HCl/EtOH-induced gastric lesion in mice: (A) Normal Control (Untreated) (B) Vehicle Control (C) Ranitidine-HCL (D) Sour Cherry Fruit 100 mg/Kg-HCL (E) Sour Cherry Fruit 150 mg/Kg-HCL (F) Sour Cherry Fruit 200 mg/Kg-HCL (G) Sour Cherry Seed 100 mg/Kg-HCL (H) Sour Cherry Seed 150 mg/Kg-HCL (I) Sour Cherry Seed 200 mg/Kg-HCL (J) Cyanidin-3-O glycoside (Cy3G) 10 mg/Kg-HCl (K) Cy3G 15 mg/Kg-HCl (L) Cy3G 20 mg/Kg-HCl (M) Linoleic acid (LA) 10 mg/Kg-HCl (N) LA 15 mg/Kg-Hcl (O) LA 20 mg/Kg-HCl.

Table 2
Effect of Scf, Scs, Cy3G, LA and ranitidine (as positive control) in HCl/EtOH induced gastric lesion in mice (mean ± SEM, n = 4/group).

Group	Dose (mg/kg)	Ulcerated area (%)	Gastro-lesion reduction (%)
Normal control	—	—	—
Vehicle control	—	71.25 ± 2.90	—
Ranitidine[a]	50	16.30 ± 2.55*	77.1
Scf[a]	100	36.22 ± 3.10*	49.2
Scf[a]	150	20.07 ± 2.64*	71.8
Scf[a]	200	17.90 ± 2.91*	74.9
Scs[a]	100	23.70 ± 1.90*	66.7
Scs[a]	150	17.77 ± 2.55*	75.1
Scs[a]	200	6.30 ± 0.30*	91.2
Cy3G[a]	10	63.90 ± 2.78*	10.3
Cy3G[a]	15	39.14 ± 2.56*	45.1
Cy3G[a]	20	22.42 ± 1.54*	68.5
LA[a]	10	27.44 ± 2.46*	61.5
LA[a]	15	19.56 ± 2.45*	72.6
LA[a]	20	8.87 ± 0.45*	87.6

SEM.: mean standard error.
*$P < 0.05$ significant from the vehicle control animals.
[a] Compared to vehicle control.

group (Table 2). In Scs treated mice (100, 150, 200 mg/kg) there were 66.7, 75.1, and 91.2% decline in gastric-lesions, respectively. Furthermore, Cy3G various doses (10, 15, 20 mg/kg) have shown 10.3, 45.1, and 68.6% reduction in gastric-lesion, respectively, while those of LA (10, 15, 20 mg/kg) have shown 61.5, 72.6, and 87.6% gastric-lesion reduction, respectively, when correlated to vehicle-treated control animals (Table 2, Fig. S4).

3.5. Stomach histopathological evaluation

Stomach specimen have shown histopathological alterations in various experimental groups (Fig. 3 and Table 3). Microphotographs from normal control animals demonstrated the normal appearance of mu (mucosa) and sm (sub-mucosa) (Fig. 3A). The positive control, ranitidine HCl 150 mg/kg, has shown also normal histological characteristics (Fig. 3B). Moreover, the vehicle-control group has shown severe gastric damage evidenced by er (erosions), h (hemorrhage), e (edema), and ic (infiltration of inflammatory cell in the sm) (Fig. 3C). Stomach specimen from mice treated with Scf 200 mg/kg 60 min before HCl/EtOH administration showed mild e, and absence of er, h and ic (Fig. 3D). Stomach microphotographs from mice treated with Scs 200 mg/kg before HCl/EtOH administration showed normal histology of the mu and sm (Fig. 3E). Nevertheless, mice specimen treated with Cy3G 20 mg/kg before HCl/EtOH administration, showed mild e and er, and absence of h and ic (Fig. 3F). Stomach from mice treated with LA 20 mg/kg before HCl/EtOH administration showed normal histology of the mu and sm (Fig. 3G).

3.6. Assessment of antinociceptive effects against acute carrageenan-induced inflammatory-pain

To assess the antinociceptive potentials of Scf, Scs and their most active constituents CY3G and LA, respectively, acute inflammation pain was induced using carrageenan paw-edema method. After 2 h of carrageenan-injection, mice have adopted a significant ($p < 0.05$, n = 7/group) nociceptive hypersensitivity pain (Fig. 4). This nociceptive hypersensitivity pain was evaluated through the paw withdrawal threshold (PWT, gm). Where the PWT of the normal mice (Normal) was reduced from 8.10 ± 0.24 g to 3.30 ± 0.10 g in

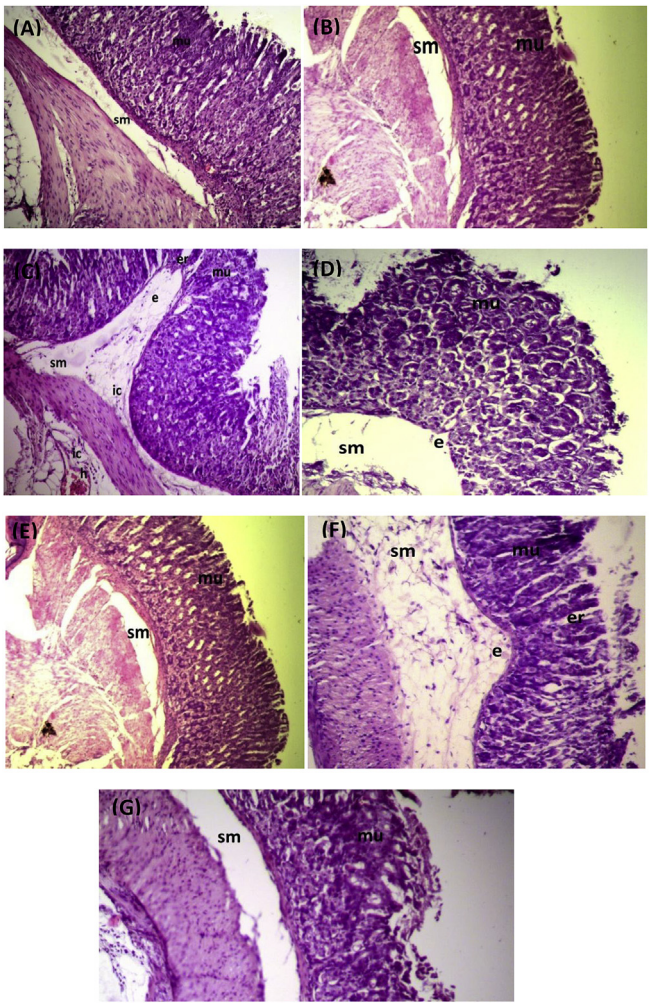

Fig. 3. Histological alterations in stomach sections of HCl/EtOH-induced gastric lesion in mice -induced gastric lesions in mice: (A) Microphotographs of normal control (NC) mice showing normal histological appearance of the mu (mucosa) and sm (sub-mucosa). (B) Stomach from mice treated with ranitidine-HCl, showing normal histological appearance of mu and sm. (C) Microphotographs of vehicle treated (vehicle control) HCl/EtOH, showing edema (e), severe-erosion (er), and hemorrhage (h) in the mu, and ic (infiltration of inflammatory-cells in the sm). (D) Stomach from mice treated with Scf 200 mg/kg 60 min before HCl/EtOH administration, showing mild e, and absence of er, h and ic. (E) Stomach from mice treated with Scs 200 mg/kg before HCl/EtOH administration showing normal histology of the mu and sm. (F) Microphotographs of mice treated with Cy3G 20 mg/kg before HCl/EtOH administration, showing mild e and er, and absence of h and ic. (G) Stomach from mice treated with LA 20 mg/kg before HCl/EtOH administration showing normal histology of the mu and sm.

vehicle treated mice (VEH) (Fig. 4). The Ib 100 mg/kg (positive control) has shown a 1.24 folds increase in PWT when correlated to VEH. When compared to VEH, Scf various doses (100, 150, and 200 mg/kg) have significantly increased PWT by 0.55, 0.70 and 0.82 folds, respectively, while those of Scs various doses (100, 150, and 200 mg/kg) has significantly increased PWT by 0.88, 1.03, 1.21 folds, respectively (Fig. 4). Moreover, Cy3G various doses (10, 15, and 20 mg/kg) has significantly increased PWT by 0.45, 0.61 and 0.76 folds, respectively, while those of LA various doses (10, 15, and 20 mg/kg) has significantly increased PWT by 1.00, 1.10, 1.15 folds, respectively, when compared to VEH (Fig. 4).

3.7. Assessment of anti-inflammatory activities against carrageenan-induced paw edema

The potential anti-inflammatory activities of the tested-

Table 3
Effect of Scf, Scs, Cy3G, LA highest concentrations and ranitidine in HCl/EtOH induced histopathological alterations in mice.

Histopathological alterations	Normal control	Vehicle control	Ranitidine	Scf (200 mg/kg)	Scs (200 mg/kg)	Cy3G (20 mg/kg)	LA (20 mg/kg)
Erosion in the mucosal area (er)	–	+++	–	–	–	+	–
Hemorrhage (h)	–	++	–	–	–	–	–
Edema in the sub-mucosa (e)	–	++	–	+	–	+	–
Inflammatory cell infiltration in the sub-mucosa (ic)	–	+	–	–	–	–	–

+++severe, ++ moderate, + mild, - nil.

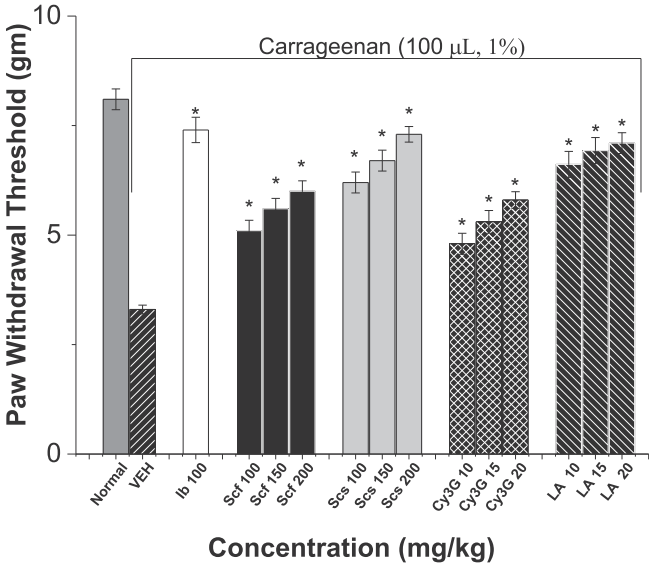

Fig. 4. Antinociceptive effects of Scf, Scs, Cy3G and LA against acute carrageenan-induced inflammatory-pain (mean ± SEM). Carrageenan (100 µL, 1%) was injected intraplantarly 2 h prior the pain threshold measurement by the paw pressure test. Sour cherry fruit EtAc extract (Scf), Sour cherry seed EtAc extract (Scs), Cyanidin 3-O-Glycoside (Cy3G), and Linoleic acid (LA) were administered 30 min before the test. Ibuprofen 100 mg/kg (Ib 100) was used as a positive control. "Normal" designates normal mice. "*" means $P < 0.05$, n = 7 compared with vehicle control (VEH).

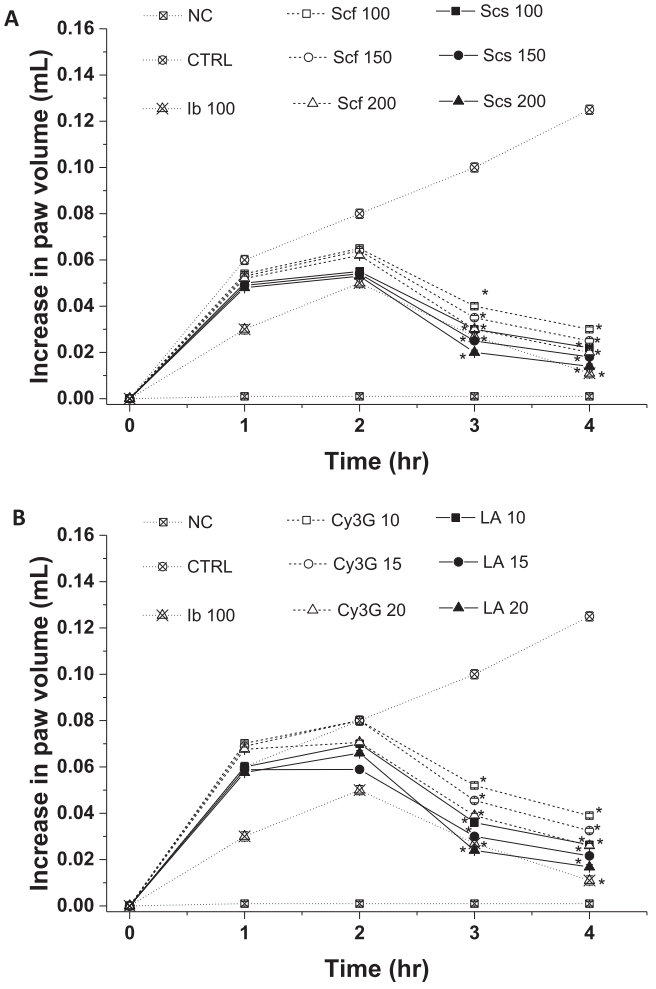

Fig. 5. The anti-inflammatory effects on mouse hind paw edema of: (A) Sour cherry Fruit EtAc extract (Scf) and sour cherry seed EtAc extract (Scs), and (B) cyanidin 3-glucoside (CY3G) and linoleic acid (LA). All doses were in mg/Kg. The actual edema volume increase was measured relative to that of standard drug ibuprofen 100 mg/kg. Values are presented as means ± SEM, n = 7. * denotes significant difference from control value at $P < 0.05$.

compounds have been monitored by the carrageenan-induced inflammatory paw-edema bioassay using Ib 100 mg/kg as a positive-control (Fig. 5 A and B). Ib 100 mg/kg has shown a 91.2% reduction in paw edema when correlated to vehicle control (CTRL). When compared also to CTRL, Scf various doses (100, 150, and 200 mg/kg) have significantly reduced paw edema by 76.0, 80.1 and 84.0%, respectively, while those of Scs various doses (100, 150, and 200 mg/kg) have significantly reduced paw edema by 82.4, 85.6, 88.8%, respectively (Fig. 5A). Moreover, Cy3G various doses (10, 15, and 20 mg/kg) has significantly reduced paw edema by 68.8, 74.0 and 79.2%, respectively, while those of LA various doses (10, 15, and 20 mg/kg) has significantly reduced paw edema by 78.9, 82.7, 86.6%, respectively, when compared to CTRL (Fig. 5B).

3.8. Determination of the level of inflammatory mediators

Scf, Scs, Cy3G, LA highest concentrations demonstrated immunomodulatory activities; as they reduced the levels of cytokines in the gastric tissue, diminished the levels of the pro-inflammatory cytokines TNF-alpha and IL-6, and elevated the anti-inflammatory factor IL-10 levels (Table 4).

3.9. Assessment of oxidative stress markers

To facilitate the investigation of the anti-inflammatory mechanism of the tested-constituents, oxidative stress markers have been monitored, while assessing the anti-inflammatory test, after 1 h of oral test administration (predose) and 4 h post carrageenan administration (Table 5). The CAT levels have been significantly ($p < 0.05$) declined in vehicle control from 28.71 ± 1.60 to 25.63 ± 1.30 kU/I 4 h post carrageenan administration (Table 5). When compared to vehicle control, Scf various doses (100, 150, and 200 mg/kg) have significantly counter-acted carrageenan induced oxidative stress and increased CAT levels by 36.2, 44.0 and 48.9%, respectively, while those of Scs various doses (100, 150, and 200 mg/kg) have significantly increased CAT levels by 39.4, 50.1, 56.0%, respectively (Table 5). Furthermore, Cy3G various doses (10, 15, and 20 mg/kg) have significantly increased CAT levels by 26.6,

Table 4
Effect of Scf, Scs, Cy3G, LA highest concentrations on the levels of TNF-alpha, IL-6 and IL-10 cytokines.

	Vehicle control	Scf	Scs	Cy3G	LA
		(200 mg/kg)	(200 mg/kg)	(20 mg/kg)	(20 mg/kg)
TNF-alpha	2012.80 ± 99.70	1565.35 ± 125.55*	140.70 ± 36.99*	1750.66 ± 88.95*	1267.79 ± 124.55*
IL-6	1201.83 ± 72.99	888.58 ± 78.58*	135.55 ± 25.60*	1040.22 ± 100.55	844.87 ± 91.66*
IL-10	1666.46 ± 155.56	2454.55 ± 100.53*	4628.84 ± 351.33*	1998.33 ± 94.56*	3662.72 ± 99.55*

The results are expressed as pg/mg of protein and reported as the mean ± SEM. *$P < 0.05$.

Table 5
In vivo assessment of the antioxidant activities of Sour cherry fruit EtAc extract (Scf), Sour cherry seed EtAc extract (Scs), Cyanidin 3-O-Glycoside (Cy3G), and Linoleic acid (LA) on CAT levels in serum, alterations in TBARS and reduced GSH (Mean ± S.E.M.).

Group	Dose (mg/kg)	Catalase level (kU/l)		TBARS Level (nM/100 g)		GSH (μg/mg)	
		Predose	4th hr	Predose	4th hr	Predose	4th hr
Normal control	__	28.50 ± 1.20	29.40 ± 1.53	0.85 ± 0.07	0.86 ± 0.05	60.70 ± 2.30	60.90 ± 2.30
Vehicle control	__	28.71 ± 1.60	25.63 ± 1.30	0.90 ± 0.05	3.10 ± 0.01	60.60 ± 2.10	54.90 ± 2.20
Ib[a]	100	28.90 ± 1.55	29.91 ± 1.90	1.10 ± 0.07	0.97 ± 0.04	60.10 ± 1.80	60.30 ± 2.40
Scf[a]	100	30.61 ± 1.12	34.90 ± 1.60*	0.70 ± 0.07	0.50 ± 0.02*	63.50 ± 2.00	65.90 ± 2.20*
Scf[a]	150	31.13 ± 1.70	36.92 ± 1.20*	0.65 ± 0.00	0.46 ± 0.01*	62.50 ± 1.80	66.80 ± 1.90*
Scf[a]	200	30.94 ± 2.10	38.16 ± 1.70*	0.63 ± 0.03	0.42 ± 0.03*	64.70 ± 2.40	69.80 ± 2.50*
Scs[a]	100	31.44 ± 1.60	35.73 ± 1.40*	0.69 ± 0.05	0.49 ± 0.01*	64.60 ± 1.80	67.90 ± 2.90*
Scs[a]	150	30.12 ± 1.90	38.47 ± 2.70*	0.64 ± 0.08	0.44 ± 0.02*	66.40 ± 2.10	70.80 ± 2.30*
Scs[a]	200	31.83 ± 2.20	39.99 ± 2.50*	0.60 ± 0.07	0.39 ± 0.01*	65.80 ± 2.00	71.90 ± 2.20*
Cy3G[a]	10	30.33 ± 2.00	32.44 ± 2.80*	0.88 ± 0.07	0.73 ± 0.03*	64.20 ± 2.20	65.80 ± 2.40*
Cy3G[a]	15	30.154 ± 1.70	34.92 ± 1.20*	0.78 ± 0.01	0.69 ± 0.02*	65.50 ± 1.90	68.80 ± 1.90*
Cy3G[a]	20	30.88 ± 2.30	35.86 ± 1.70*	0.70 ± 0.03	0.63 ± 0.01*	63.70 ± 2.40	69.10 ± 2.30*
LA[a]	10	31.44 ± 1.60	34.73 ± 1.40*	0.80 ± 0.05	0.60 ± 0.03*	63.30 ± 1.80	65.70 ± 2.40*
LA[a]	15	30.12 ± 1.90	35.47 ± 2.70*	0.72 ± 0.08	0.56 ± 0.04*	65.40 ± 2.10	69.30 ± 2.10*
LA[a]	20	31.89 ± 2.50	36.94 ± 2.60*	0.64 ± 0.03	0.45 ± 0.02*	64.80 ± 2.30	69.90 ± 2.00*

S.E.M.: mean standard error.
*$P < 0.05$ significant from the vehicle control animals.
[a] Compared to vehicle control.

36.3 and 39.9%, respectively, while those of LA various doses (10, 15, and 20 mg/kg) have significantly increased CAT levels by 35.5, 38.4, 44.1%, respectively, when correlated to vehicle-control (Table 5). Furthermore, the TBARS levels were significantly ($p < 0.05$) increased in vehicle-control from 0.90 ± 0.05 to 3.10 ± 0.01 kU/l 4 h post carrageenan administration (Table 5). When correlated to vehicle control, Scf various doses (100, 150, and 200 mg/kg) have significantly counter-acted carrageenan induced oxidative stress and reduced TBARS levels by 83.9, 85.2 and 86.5%, respectively, while those of Scs various doses (100, 150, and 200 mg/kg) have significantly reduced TBARS levels by 84.2, 85.8, 87.4%, respectively (Table 5). In addition, Cy3G various doses (10, 15, and 20 mg/kg) have significantly reduced TBARS levels by 76.5, 77.7 and 79.7%, respectively, while those of LA various doses (10, 15, and 20 mg/kg) have significantly reduced TBARS levels by 80.6, 81.9, 85.5%, respectively, when correlated to vehicle control (Table 5).

Additionally, The GSH-levels have been significantly declined in vehicle control from 60.60 ± 2.10 to 54.90 ± 2.20 kU/l 4 h post carrageenan administration (Table 5). When compared to vehicle control, Scf various doses (100, 150, and 200 mg/kg) have significantly counter-acted carrageenan induced oxidative stress and increased GSH levels by 20.0, 21.7 and 27.1%, respectively, while those of Scs various doses (100, 150, and 200 mg/kg) have significantly increased GSH levels by 23.7, 29.0, 31.0%, respectively (Table 5). Furthermore, Cy3G various doses (10, 15, and 20 mg/kg) have significantly increased GSH levels by 19.9, 25.3 and 25.9%, respectively, while those of LA various doses (10, 15, and 20 mg/kg) have significantly increased GSH levels by 19.7, 26.2, 27.3%, respectively, when correlated to vehicle-control (Table 5).

3.10. Spleen histopathological evaluation

Histopathological evaluation was done after eight days administration of the highest doses of Scs (200 mg/kg) and Scf (200 mg/kg) after carrageenan-induced inflammatory-edema, to aid the identification of *P. cerasus* anti-inflammatory mechanism (Fig. 6). The histological photomicrographs of the spleen sections of the untreated control mice (NC) showed normal spleen structure with clearly differentiated red pulps (RP) and white pulps (WP) (Fig. 6A). White pulps were well organized with normal rounded scattered follicles with central arterioles and with distinct germinal centers and marginal zones at the periphery (Fig. 6A). However, after the induction of inflammation in the vehicle control group (CTRL), spleen tissue displayed deleterious effects as evident from the atrophy of white pulp follicles (Fig. 6B). Treatment with both Scs (200 mg/kg) and Scf (200 mg/kg) led to regeneration and improvement in the structural architecture of the spleen with normal red and white pulps (Fig. 6C and D).

4. Discussion

The rich flora of eastern-Mediterranean region enriches the discovery of plant phytochemicals with high therapeutic values.[45] Moreover, combining phytochemical and biological studies are of increasing interest in order to bridge the indigenous knowledge, chemistry and phytotherapeutic activities of medicinal-plants with the ultimate goal of developing new-remedies, with good safety profiles. This work was the first detailed account of *P. cerasus* gastroprotective, anti-inflammatory, and anti-nociceptive properties,

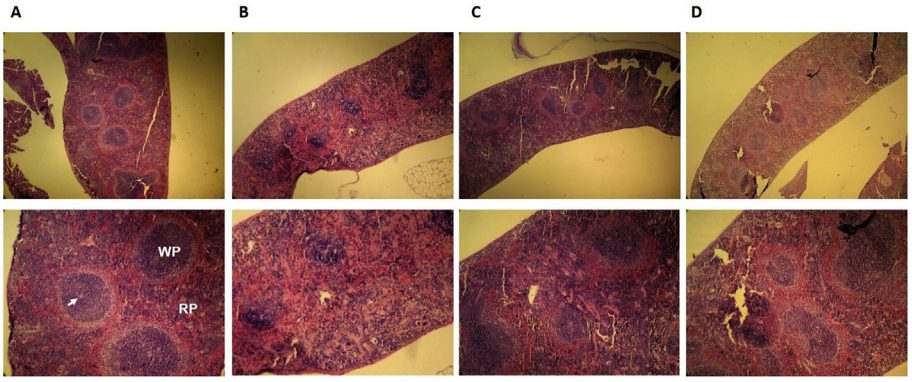

Fig. 6. Representative Hematoxylin and Eosin (H and E) stained sections of spleen-tissue from (A) Normal control (NC) (B) Vehicle control (CTRL) (C) Group administered 200 mg/kg Scs and (D) Group administered 200 mg/kg Scf. Top panel 4× magnification; Lower panel 10× magnification; red pulp (RP); white pulp (WP), central arteriole (arrow).

exploring their most active phytochemicals.

Many phytochemicals as polyphenols and bio-active fatty-acids were reported before to ameliorate the inflammation-processes.[24] In the present study, the abundance of the major polyphenols was studied by RP-HPLC analysis in Scf, and its fractions adopting bio-guided fractionation scheme utilizing *in-vivo* biological models of gastric-lesions, nociceptive pain, and inflammation. The bio-guided fractionation method has shown that cyanidin 3-glucoside (Cy3G) is the most active compound in Scf. In addition, the presence of bioactive fatty acids was investigated by GC-FID analysis in Scs. Similarly, the most bio-active compound utilizing bio-guided fractionation scheme utilizing *in-vivo* biological models was found to be linoleic acid (LA). Both bioactive phytochemical compounds, Cy3G, and LA, in various doses, were investigated along with Scf and Scs to explore their detailed gastroprotective, anti-inflammatory, and anti-nociceptive activities.

This study explored the gastroprotective-activities of the Scf, Scs, Cy3G, and LA in HCl/EtOH-induced gastric lesion model. Our results have shown that various doses of Scf, Scs, Cy3G, and LA present significant ($P < 0.5$) and dose-dependent gastro-protective activities. The Scs highest dose (200 mg/kg) has shown the highest gastro-protective properties compared to Scf, Cy3G, and LA, and had comparable results to classic anti-gastric ulcer drug, ranitidine (50 mg/kg), thus indicating Scs (200 mg/kg) gastro-protective potentials. The stomach histopathological investigation has strengthened these findings. As the highest concentrations of Scf, Scs, Cy3G, and LA have decreased the HCl-EtOH induced peptic ulcer. The tested compounds have significantly ameliorated HCl-EtOH induced Erosion in the mucosal area (er), hemorrhage (h), edema in the sub-mucosa (e), and decreased the inflammatory cell infiltration in the sub-mucosa (ic).

Furthermore, in order to investigate the antinociceptive properties of Scf, Scs, Cy3G, and LA, carrageenan-induced nociceptive-pain model was performed. Various doses of Scf, Scs, Cy3G, and LA have shown dose-dependent significant antinociceptive properties. The Scs highest dose (200 mg/kg) has shown the highest decrease in hypersensitivity to noxious-stimuli compared to Scf, Cy3G, and LA. Moreover, it had comparable results to conventional analgesic drug, ibuprofen (100 mg/kg), and suggesting Scs (200 mg/kg) ability to potentially control the acute nociceptive-pain.

To examine the anti-inflammatory potentials of the tested-constituents, carrageenan-induced inflammatory-edema model was adopted. Our results have shown that various doses of Scf, Scs, Cy3G, and LA present significant ($P < 0.5$) and dose-dependent anti-inflammatory activities. The Scs highest dose (200 mg/kg) presented the highest anti-inflammatory properties compared to Scf, Cy3G, and LA, and had comparable results to the classical anti-inflammatory drug, ibuprofen (100 mg/kg), indicating Scs (200 mg/kg) anti-inflammatory potentials.

TNF-alpha, a pro-inflammatory cytokine, is progressively secreted by macrophages during gastric-ulcer induction.[17] IL-6, a pleiotropic-cytokine, plays an important role in acute-inflammation and immune-regulation.[46] An elevated level of IL-6 stimulates lymphocytes, neutrophils, and macrophages at the inflammatory location, triggering the oxidative-pathways responsible for the local tissue-damage in gastric ulceration.[47] Pro-inflammatory cytokines TNF-alpha and IL-6 were found crucial in regulating gastric ulcer severity as they elevated the oxidative stress induced by mitochondrial ROS generation.[48] IL-10, anti-inflammatory and immune-suppressive cytokines abolishes the inflammatory response and inhibits TNF-alpha production.[17]

Highest doses of Scf, Scs, Cy3G, and LA demonstrated immunomodulatory activities; as they reduced the levels of cytokines in the gastric tissue, diminished the levels of the pro-inflammatory cytokines TNF-alpha and IL-6, and elevated the anti-inflammatory factor IL-10 levels.

To evaluate the anti-inflammatory mode of action, oxidative stress markers and spleen-histopathology were monitored. Various doses of Scf, Scs, Cy3G, and LA have significantly and dose-dependently counter-acted carrageenan induced oxidative stress, increased serum catalase and reduced-glutathione levels. Additionally, Scf, Scs, Cy3G, and LA various doses have significantly and dose-dependently counter-acted carrageenan induced oxidative stress and decreased lipid peroxidation levels. Scs (200 mg/kg) highest dose has shown the highest increase in serum catalase and reduced-glutathione levels, and a decrease in lipid peroxidation, indicating best control for inflammatory-induced oxidative-stress.

Since spleen serves as a cell and antibody repository which is involved in the inflammatory response,[49] histopathological-effects on spleen were monitored. Treatment with Scs and Scf highest doses led to regeneration and improvement in the structural architecture of the spleen with normal red and white pulps. Thus, the reduction of the pro-inflammatory TNF-alpha and IL-6, and elevation the anti-inflammatory factor IL-10 levels, spleen regenerative-capacity and amelioration of oxidative-stress might be the main mechanism responsible for *P. cerasus* anti-inflammatory potentials.

In conclusion, *P. cerasus* has shown potent gastro-protective, antinociceptive, and anti-inflammatory effects, utilizing a potential decline of the pro-inflammatory TNF-alpha and IL-6, and the elevation of the anti-inflammatory factor IL-10 levels, spleen-regenerative and anti-oxidative stress ameliorative mechanism. Thus, *P. cerasus* might have beneficial phytotherapeutic effects against nociceptive-pain, inflammatory-progression, while preserving the stomach, for further investigation.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

KR; is the main-author, and did most of the experimental part, data analysis, wrote and revised the manuscript. ND; wrote the introduction part and revised the manuscript. FS; did some measurements and revised the manuscript.

Acknowledgments

The authors would like to thank Dr. G. Onsy for the professional English-language scientific editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.06.001.

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