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Bioassay-Guided Isolation and Characterization of Wound Healer Compounds from *Morus nigra* L. (Moraceae)

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Abstract: Leaves and fruits of *Morus nigra* L. (Moraceae) are used for the treatment of wounds especially mouth sore in Turkish traditional medicine. The present study was designed to investigate wound healing activity of *M. nigra* by using incision and excision wound models. Furthermore, anti-inflammatory activity was assessed by Whittle method. Lyophilized fruit extract (MNF) displayed significant wound healing activity, while aqueous leaf extract of *M. nigra* (MNL) did not. Through biological activity guided fractionation technique, MNF was subjected to successive solvent extraction. Among the subextracts obtained, *n*-butanol (MNF-*n*-BuOH) subextract was found to possess wound healing activity. MNF-*n*-BuOH was subjected Sephadex LH-20 column chromatography to obtain three fractions, which then applied to the same biological activity tests. Compounds **1** and **2** were isolated from the active fraction and their structures were identified as quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside, respectively. The isolates were investigated for their *in vitro* enzyme inhibitory activities.

Keywords: Anti-inflammatory; Moraceae; *Morus nigra***;** Tensiometer; Wound healing.

1. Introduction

The mulberry belongs to the genus *Morus* from the family Moraceae. There are 24 *Morus* species and one subspecies, with at least 100 known varieties [1].

In Turkey, traditional products such as ‘dut pekmezi’ and ‘dut pestili’ are made with the fruits. The red-coloured fruits are eaten fresh and are also used in marmalades, juices, liquors, natural dyes and in the cosmetics industry [2]. In folk medicine, leaves and fruits of *Morus nigra* L. (Moraceae) are used for the treatment of various kinds of diseases i.e., solution prepared from fruits used for mouth and throat diseases, against dysentery and as laxative, odontalgic, anthelmintic, expectorant, hypoglycaemic and emetic; root or cortex is used as abortifacient, laxative and anthelminthic infusion prepared from the leaves used as diuretic and against fever and also decoction of the leaves used to heal diabetes mellitus [3].

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Morus species have been shown to exhibit anti-HIV, antioxidant, antihyperglycemic, antihypotensive and cytotoxic activities [4-7].

Deep-coloured fruits are good sources of phenolics, including flavonoids, anthocyanins and carotenoids [8-9]. Especially, phenolics possess a wide range of biological activities such as antioxidant, antiviral, antimutagenic and anticarcinogenic properties, as well as the ability to modify gene expression [10-11].

The aim of the present study was to investigate the *in vivo* wound healing and anti-inflammatory activities of *M. nigra* through biological activity guided fractionation and isolation technique, in order to verify the traditional use of this plant from the scientific point of view. Furthermore, *in vitro* hyaluronidase, collagenase and elastase enzyme inhibitory activities of the isolated compounds were investigated.

2. Materials and Methods

2.1. Plant material

Morus nigra L. furit and leaves were collected from Beypazarı, Ankara, Turkey in 2008 and identified by Prof. Dr. Ekrem Sezik from Gazi University.

2.2. Extraction, fractionation and isolation procedures for the bioassays

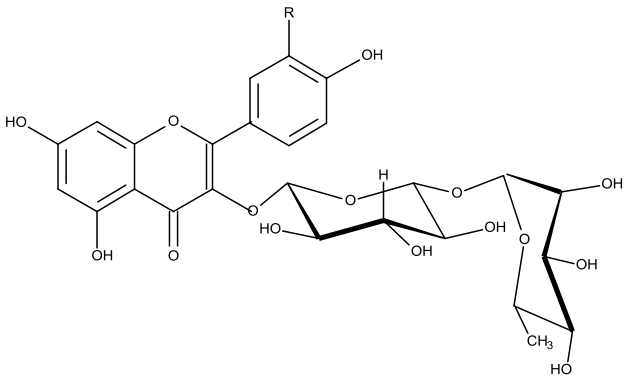
Powdered leaves (100 g) were extracted twice by continuous stirring for 5 h with distilled H_2O (500 mL) at room temperature. Each extracts were then lyophilized to yield 'Aqueous leaf extract' (20.42 g). Fruits (100 g) were pressed, and then filtered through filter paper. Filtered juice was then lyophilized to yield 'Liyophilized fruit extract' (17.65 g).

The fruit extract was then dissolved in 400 mL of methanol/H_2O (9:1) and transferred to a separator funnel and extracted with *n*-hexane. Combined *n*-hexane subextracts were evaporated under reduced pressure to give "MNF-*n*-Hexane". The remaining methanol phase was evaporated and the residual methanol extract diluted with distilled H_2O to 400 mL and successively extracted with chloroform and ethyl acetate. Each solvent extract was evaporated to dryness under reduced pressure to give "MNF-$CHCl_3$" and "MNF-EtOAc" respectively. The remaining aqueous extract was further extracted with *n*-butanol saturated with water and evaporated to dryness at 40°C under reduced pressure to give "MNF-*n*-BuOH". The final aqueous phase was also evaporated to dryness "MNF-R-H_2O".

2.3. Fractionation of the fruit extract and isolation of the active constituents

The active MNF-*n*-BuOH subextract was subjected to fractionation on a Sephadex LH-20 column, using MeOH as eluent. Fractions of 5 mL were collected and grouped into Fr. 1-7, Fr. 8-14 and Fr. 15-32 by TLC analysis on silica 60 F_{254}. The Fr. 8-14 was concentrated under reduced pressure and applied to preparative TLC (Si 60) using $CHCl_3$:MeOH:H_2O (61:32:7) as a mobile system, leading to isolation of two compounds **1** and **2** (Figure 1).

The structures of the compounds were elucidated as quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside, respectively by spectroscopic methods (UV, ^{1}H- , ^{13}C- and 2D-NMR and mass spectrometry) and comparison of their spectroscopic data with those of published in related literatures [12, 13].



	R
1	OH
2	H

Figure 1. Structures of the isolated compounds (Compounds **1** and **2**).

2.4. In vivo biological activity tests

2.4.1. Animals

160–180 g male, Sprague–Dawley rats and 20–25 g Swiss albino mice provided from the animal breeding laboratory of Saki Yenilli, Ankara, Turkey.

The animals were were housed in polysulfone cages at 21-24°C, 40-45% humidity, and light-controlled (12 hours light/12 hours dark) conditions given *ad libitum* access to food and water during the throughout the experiment. A minimum of six animals were used in each group. The study was was performed according to the international rules considering the animal experiments and biodiversity right.

2.4.2. Preparation of test samples for bioassay

In order to evaluate anti-inflammatory effect, test samples were given *per os* to the animals after suspending in a mixture of distilled H_2O and 0.5% sodium carboxymethyl cellulose (CMC). The vehicle solution was applied to the control group animals. Indomethacin (10 mg/kg) in 0.5% CMC was administered to the reference group animals.

Test samples were prepared by using glycol stearate, 1,2 propylene glycol, liquid paraffin (3:6:1) in 1% concentration for the *in vivo* wound models. Each test ointment (0.5 g) was applied topically on the wounded site. Vehicle group animals were treated with the ointment base, whereas reference group animals were treated with 0.5 g of Madecassol® (Bayer). [14].

2.4.3. Wound healing activity

2.4.3.1. Linear incision wound model

The animals were anaesthetized with 0.05 cm^3 Xylazine (2% Alfazine®) and 0.15 cm^3 Ketamine (10% Ketasol®). The back hair of the rats were shaved and two linear-paravertebral incisions were created with a sterile surgical blade in 5 cm length from the midline of each side of the vertebral column. The wounds were closed with surgical sutures of 1 cm apart. Test ointments were topically applied once in a day throughout 9 days. Negative control group animals were not treated

with any material. All the sutures were removed on the 9th post wound day. On day ten all the animals were sacrified. One incised treated tissue was measured using tensiometer (Zwick/Roell Z0.5, Germany) for its tensile strength, the other one was sent for histopathological examination [14, 15].

2.4.3.2. Circular excision wound model

Each group of animals (six animals in each) was anaesthetized with 0.02 cm^3 Xylazine (2% Alfazine®) and 0.08 cm^3 Ketamine (10% Ketasol®). The back hairs of the mice were depilated by shaving. The circular wound was created on the dorsal interscapular region of each animal by excising the skin with a 5 mm biopsy punch. Test ointments were applied topically once a day till the wound completely healed. The progressive changes in wound area were monitored by a camera (Fuji, S20 Pro, Japan) every other day. Wound area was evaluated by using AutoCAD program. Wound contraction was calculated as percentage of the reduction in wounded area. A specimen sample of tissue was isolated from the healed skin for the histopathological examination [14, 16].

2.4.3.3. Histopathology

The cross-sectional full-thickness skin specimens were fixed in 10% buffered formalin, processed and blocked with paraffin and then sectioned into 5 micrometer sections and stained with hematoxylin & eosin (HE) and Van Gieson (VG) stains. For epidermal or dermal re-modeling, the tissues were examined by light microscope (Olympus CX41 attached Kameram® Digital Image Analyze System) and scored as mild (+), moderate (++) and severe (+++). Re-epithelization or ulcus in epidermis; fibroblast proliferation, mononuclear and/or polymorphonuclear cells, neovascularization and collagen depositions in dermis were analyzedç At the end of the examination, obtained results were combined and staged for wound healing phases as inflammation, proliferation, and re-modeling [14].

2.4.4. Anti-inflammatory activity

2.4.4.1. Acetic acid-induced increase in capillary permeability (Whittle method)

Effect of the test samples on the increased vascular permeability induced by acetic acid in mice was determined according to Whittle method with some modifications [17]. Each test sample was administered orally to a group of 10 mice in 0.2 mL/20 g body weight. Thirty minutes after the administration, tail of each mice was injected with 0.1 mL of 4% Evans blue in saline solution (i.v.) and waited for 10 min. Then, 0.4 mL of 0.5% (v/v) AcOH was injected i.p. After 20 min. incubation, the mice were killed by dislocation of the neck, and the viscera were exposed and irrigated with distilled water, which was then poured into 10 mL volumetric flasks through glass wool. Each flask was made up to 10 mL with distilled water, 0.1 mL of 0.1N NaOH solution was added to the flask, and the absorption of the final solution was measured at 590 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). A mixture of distilled water and 0.5 % CMC was given orally to control animals, and they were treated in the same manner as described above [14].

2.5. In vitro biological activity tests

2.5.1. Determination of hyaluronidase inhibitory activity

The inhibition of hyaluronidase was assessed by the measurement of the amount of *N*-acetylglucosamine released from sodium hyaluronate [18, 19]. 50 µl of bovine hyaluronidase (7900 units/mL) was dissolved in 0.1M acetate buffer (pH 3.6). Then this solution was mixed with 50 µl of different concentrations of the extracts dissolved in 5% DMSO. For the control group 50 µl of 5%

DMSO was added. After incubation at 37°C for 20 min, 50 µl of calcium chloride (12.5 mM) was added to the mixture and incubated for 20 min at 37°C. 250µl sodium hyaluronate (1.2 mg/mL) was added and incubated for 40 min at 37°C. Afterwards, the mixture was treated with 50µl of 0.4 M NaOH and 100µl of 0.2 M sodium borate and then incubated for 3 min in the boiling water bath. *p*-Dimethylaminobenzaldehyde solution (1.5 mL) was added to the reaction mixture after cooling to room temperature and was incubated at 37°C for 20 min when colour developed. The absorbance was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). Tannic acid (100 µg/mL) was used as a reference [14].

2.5.2. Determination of collagenase inhibitory activity

The samples were dissolved in DMSO. The sample solution and *Clostridium histolyticum* collagenase enzyme (ChC) were dissolved in 50 mM Tricine buffer (with 0.4M NaCl and 0.01M CaCl2, pH 7.5) and pre-incubated at 25°C for 5 min. Then, 2 mM N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was prepared in the same buffer. 25 µl buffer, 25 µL test sample and 25 µL enzyme were added to each well and incubated for 15 minutes. 50 µL substrat was added to the mixture to immediately measure the decrease of the optical density (OD) at 340 nm using spectrometer. Epigallocatechin gallate (100 µg/mL) was used as a reference.
The ChC inhibition activities were calculated according to the following formula:

$$\text{ChC inhibition activity (\%)} = \frac{OD_{Control} - OD_{Sample}}{OD_{Control}} \times 100$$

where ODcontrol and ODsample represent the optical densities in the absence and presence of sample, respectively [14, 20].

2.5.3. Determination of elastase inhibitory activity

The sample solution and human neutrophil elastase enzyme (HNE) (17 mU/mL) were mixed in 0.1M Tris-HCl buffer (pH 7.5), then incubated at 25°C for 5 minutes. N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MAAPVN) was added to the mixture and incubated at 37°C for 1 hour. Afterwards, the reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/mL) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibition activities were calculated as in the ChC inhibition activity. Epigallocatechin gallate (100 µg/mL) was used as a reference [14, 21].

2.6. Statistical Analysis of the data

The data on percentage wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of $p \leq 0.05$ were considered statistically significant. Histopathological data were considered to be nonparametric; therefore, no statistical tests were performed.

3. Results

The entire wound healing process is a complex series of events that begins at the moment of injury and can continue for months to years. The agents, which provide a rapid and better healing are required especially when wound healing is accompanied by some chronic diseases. Wound healing comprises of inflammation, proliferation, and remodeling stages. The active compounds stimulate the healing process in one or more phases due to their anti-bacterial, anti-inflammatory, antioxidant and proliferative mechanisms of action. After the damage of the skin barrier, wounded tissues are more vulnerable to microbial diseases. That is why an inflammatory response occurs immediately after wounding. However, a chronic inflammatory period aggravates acute wound healing. Thus, the anti-inflammatory active agents help the healing process while preventing excessive inflammation. In the acute phases of the wound healing, antimicrobial treatment is also gaining importance. In addition, antioxidant compounds improve the wound healing process by the inhibition of the lipid peroxidation cell damage [22]. In the present study, *in vivo* anti-inflammatory activity model, circular excision and

linear incision wound models were used for the confirmation of the proposed anti-inflammatory and wound healing activities of *Morus nigra*.

Linear incision wound model was used for the determination of the tensile strength values. High tensile strength value is an indication of increase in collagen levels [23]. As shown in Table 1, treatment with the lyophilized fruit extract (MNF) increased the tensile strength of the incised wounds within 10 days. The activity was significantly greater when compared to the negative control and the vehicle groups with the value of 31.2% ($p < 0.01$). All these results have obviously indicated that MNF has a remarkable effect on healing of wounds. Effects of subextracts and fractions from MNF were also assessed in the linear incision wound model. The results revealed that, MNF-*n*-BuOH and Fr. 8-14 were highly effective in linear incision wound model.

The circular excision wound model was used for the assessment of the activities of the test samples on wound contraction and time of epithelialization. This is essential, since the active extract/s should provide a rapid contraction. Wound contraction was measured every two days for this model. As shown in Table 2, the wound contracting ability of MNF on circular excision wound model was significantly greater than that of the negative control and the vehicle itself, with the contraction values 41.2% ($p < 0.01$) and 59.6% ($p < 0.01$) on day 10 and 12. As shown in Table 2, among the subextracts obtained from MNF, MNF-*n*-BuOH provided significant contraction with the value of 51.4%. Fractions obtained from MNF-*n*-BuOH also applied to the same wound healing models and Fr. 8-14 found to provide significant contraction with the value of 42.5%, whereas the other fractions did not show any significant activity in this model.

Table 1. Effects of the extracts of *M. nigra* fruits and leaves, subextracts and fractions of *M. nigra* fruit extract on linear incision wound model.

Material	Statistical Mean ± S.E.M.	(Tensile strength%)
Vehicle	18.07 ± 2.74	17.3
Negative Control	15.40 ± 2.11	-
MNL	22.35 ± 2.54	23.7
MNF	23.71 ± 2.08	**31.2****
MNF-*n*-Hexane	17.23 ± 2.15	-
MNF-$CHCl_3$	16.39 ± 1.85	-
MNF-EtOAc	21.15 ± 1.83	17.0
MNF-*n*-BuOH	24.16 ± 1.75	**33.7****
MNF-R-H_2O	16.57 ± 1.91	8.30
Fr. 1-7	20.61 ± 1.68	14.1
Fr. 8-14	23.41 ± 1.06	**29.6****
Fr. 15-32	20.19 ± 1.92	11.7
Madecassol®	28.28 ± 1.96	**56.5*****

** : $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

Percentage of tensile strength values: Vehicle group was compared to negative control group; Extracts and the reference material were compared to vehicle group.

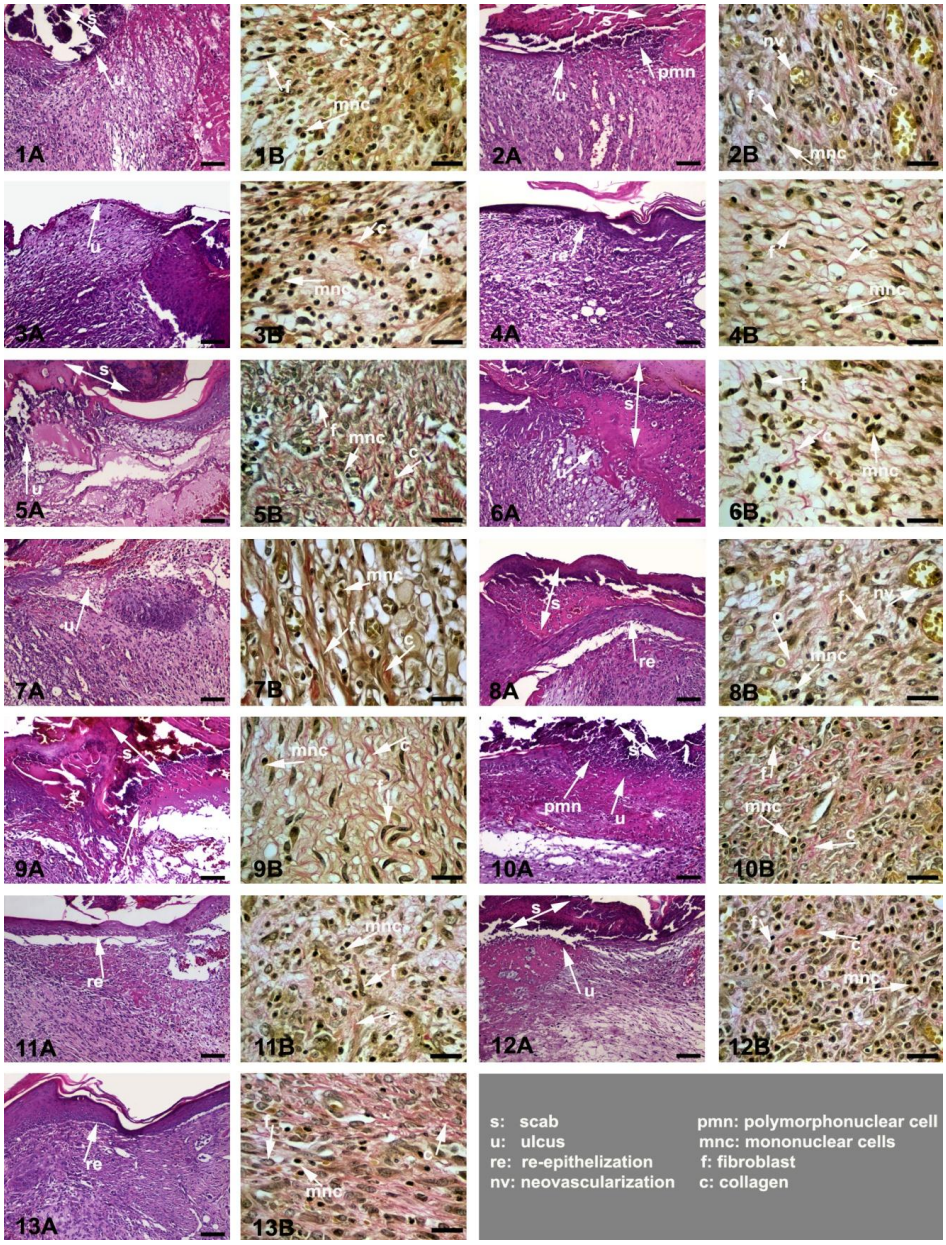
Table 2. Effects of the extracts of *M. nigra* fruits and leaves, subextracts and fractions of *M. nigra* fruit extract on circular excision wound model.

Material	Wound area (mm^2) ± S.E.M. (Contraction%)						
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Vehicle	19.44±1.96	17.75±2.39 (1.4)	16.13±1.93 (3.5)	13.92±1.54 (5.5)	9.43 ±1.19 (11.7)	5.27 ±0.87 (13.6)	2.80 ±0.14 (6.9)
Negative Control	19.47±2.15	18.01±2.01	16.71±1.29	14.73±1.16	10.68±1.64	6.10±1.59	3.01±0.81
MNL	19.05±2.06	17.19±2.55 (3.2)	16.09±2.11 (0.2)	13.15±2.08 (5.5)	8.83±1.12 (6.4)	4.15±1.92 (21.3)	2.12±0.53 (24.3)
MNF	19.21±1.76	17.31±1.99 (2.5)	15.94±2.20 (1.2)	12.61±1.79 (9.4)	7.01±1.49 (25.7)	3.10±0.41 **(41.2)****	1.13±0.28 **(59.6)****
MNF-*n*-Hexane	19.39±1.77	17.14±1.92 (3.4)	16.03±1.65 (0.6)	13.28±1.71 (4.6)	9.10±1.54 (3.5)	4.98±1.41 (5.5)	2.69±0.76 (3.9)
MNF-$CHCl_3$	19.25±1.68	17.51±1.74 (1.4)	15.98±1.46 (0.9)	13.20±1.52 (5.2)	9.02 ±1.08 (4.3)	5.07 ±0.85 (3.8)	2.51 ±0.37 (10.4)
MNF-EtOAc	19.51±1.79	17.22±1.60 (5.6)	15.70±1.49 (2.7)	11.96±1.28 (14.1)	7.91±0.75 (16.1)	4.26 ±0.38 (19.2)	2.13±0.24 (23.9)
MNF-*n*-BuOH	19.43±1.82	16.98±1.65 (4.3)	15.61±1.38 (3.2)	12.30±1.44 (11.6)	7.68 ±1.20 (18.6)	4.14±0.41 (21.4)	1.36 ±0.11 **(51.4)****
MNF-R-H_2O	19.62±1.70	17.39±1.73 (2.0)	15.88±1.86 (1.5)	12.87±1.51 (7.5)	8.25±1.17 (12.5)	4.66±0.24 (11.6)	2.45±0.31 (12.5)
Fr. 1-7	19.33±1.48	16.92±1.63 (4.7)	15.01±1.20 (6.2)	11.37±1.25 (18.3)	7.28±1.13 (22.8)	4.22±1.15 (19.9)	2.09±0.29 (25.4)
Fr. 8-14	19.49±1.85	17.05±1.71 (3.9)	15.85±1.28 (1.7)	12.26±1.19 (11.9)	7.09±1.04 (24.8)	3.49±0.51 **(33.8)***	1.61±0.09 **(42.5)****
Fr. 15-32	19.27±1.91	17.25±1.58 (2.8)	16.01±1.81 (0.7)	12.74±1.73 (8.5)	8.06±1.68 (14.5)	4.38±1.29 (16.9)	2.38±0.63 (15.0)
Madecassol®	19.50±2.40	17.04±3.43 (4.0)	14.13±2.23 (12.4)	10.56±2.12 (24.1)	5.46±0.87 **(42.1)****	1.63±0.37 **(69.1)*****	0.00±0.00 **(100)*****

* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

Percentage of contraction values: Vehicle group was compared to negative control group; Extracts and the reference material were compared to the vehicle group.

Following *in vivo* experiments, histopathological examination were conducted on the test materials applied tissues. For demonstrating of wound healing process, represantive figures (Figure 2), which stained with HE and VG were added. Phases in wound healing processes (inflammation, proliferation, and remodeling) were observed and recorded successfully within the experimental groups.



Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in A, and the dermis stained with Van Gieson (VG) in B. The original magnification was x 100 and the scale bars represent 120 µm for figures in A, and the original magnification was x 400 and the scale bars represent 40 µm for B. Data are representative of 6 animal per group. 1) Vehicle group; 2) Negative control group (untreated); 3) MNL; 4) MNF; 5) MNF-*n*-Hexane; 6) MNF-$CHCl_3$; 7) MNF-EtOAc; 8) MNF-*n*-BuOH; 9) MNF-R-H_2O; 10) Fr. 1-7; 11) Fr. 8-14; 12) Fr. 15-32; 13) Madecassol®

The vehicle and the negative control groups demonstrated incomplete healing in comparison to the other groups. On the other hand, faster re-modeling were noticed in extracts treated groups. The best re-modeling, in particular, re-epithelization were detected with the Madecassol®, MNF, MNF-*n*-BuOH and Fr. 8-14 groups, respectively. Weak foreign body reaction, superfluous process in wound healing, characterized with a few foreign body giant cells, which generally localized in peripheral

sides of some hair follicles were detected in all groups except for the reference drug Madecassol® group.

For the determination of the anti-inflammatory effects of *M. nigra* extracts and fractions, Whittle method, based on the inhibition of acetic acid induced increase in capillary permeability was used. As shown in Table 3, a dose-dependent inhibitory activity was observed for the lyophilized fruit extract at the dose of 200 mg/kg with the highest inhibitory value of 39.0%. The anti-inflammatory effect of the active extract was quite comparable to the reference compound indomethacin (53.2 % inhibition). However, the aqueous leaf extract did not show a siginificant inhibitory activity in this model. The results of the subextracts and fractions from MNF were also presented in Table 3, which were in accord with the wound models. MNF-*n*-BuOH and Fr. 8-14 showed significant anti-inflammatory activity with the values of 37.9% and 31.2% respectively.

Table 3. Effects of the extracts of *M. nigra* fruits and leaves, subextracts and fractions of *M. nigra* fruit extract on increased vascular permeability induced by acetic acid in mice.

Material	Dose (mg/kg)	Evans blue concentration (µg/mL) ± SEM	Inhibition (%)
Control		10.28 ± 0.74	
MNL	100	9.02 ± 0.61	12.3
	200	8.93 ± 0.56	13.1
MNF	100	7.94 ± 0.45	22.8
	200	6.27 ± 0.31	**39.0****
MNF-*n*-Hexane	100	11.04 ± 0.97	-
	200	9.25 ± 0.86	10.0
MNF-$CHCl_3$	100	9.82 ± 0.63	4.5
	200	8.79 ± 0.66	14.5
MNF-EtOAc	100	8.19 ± 0.43	20.3
	200	7.36 ± 0.34	**28.4***
MNF-*n*-BuOH	100	8.01 ± 0.47	22.1
	200	6.38 ± 0.36	**37.9****
MNF-R-H_2O	100	8.84 ± 0.27	14.0
	200	8.49 ± 0.41	17.4
Fr. 1-7	100	10.01 ± 0.75	2.6
	200	9.06 ± 0.42	11.9
Fr. 8-14	100	8.97 ± 0.53	12.7
	200	7.07 ± 0.44	**31.2****
Fr. 15-32	100	10.93 ± 0.72	-
	200	9.91 ± 0.46	3.6
Indomethacin	10	4.81 ± 0.33	**53.2*****

**p<0.01; *** p<0.001 significant from the control; S.E.M.: Standard error of the mean

Table 4. Hyaluronidase, collagenase and elastase enzyme inhibitory activity of the isolated compounds from *Morus nigra.*

Material	Concentration (µg/mL)	Hyaluronidase inhibition (%) ± S.E.M.	Collagenase inhibition (%) ± S.E.M.	Elastase inhibition (%) ± S.E.M.
1	50	28.12 ± 1.01	20.15 ± 1.78	7.40 ± 1.24
	100	**35.68 ± 0.52***	**27.17 ± 0.92***	9.15 ± 1.41
2	50	24.23 ± 1.05	**31.01 ± 0.84***	22.23 ± 1.16
	100	**46.11 ± 0.42****	**38.13 ± 0.71****	25.41 ± 1.27
Epigallocatechin gallate	100	-	**49.13 ± 0.96****	**86.13 ± 0.70*****
Tannic acid	100	**83.17 ± 0.31*****	-	-

* : p < 0.05; ** : p < 0.01; *** : p < 0.001; S.E.M.: Standard error of the mean

Through biological activity guided fractionation and isolation technique, compounds **1** and **2** were isolated from the active fraction. The isolates were investigated for their hyaluronidase, collagenase and elastase enzyme inhibitory activities by using *in vitro* methods. Both compounds were found to have inhibitory effect on hyaluronidase and collagenase enzymes, whereas none of the compounds showed elastase enzyme inhibitory activity (Table 4).

4. Discussion

Inhibition of collagenase, elastase and hyaluronidase enzymes could be beneficial for the wound healing process due to the prevention of the destruction of collagen, elastin and hyaluronic acid [14]. These extracellular matrix metalloproteins are known to support the cells and provide elasticity and humidity that cells need [19]. The results of the present study suggest that quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside could contribute wound healing by their hyaluronidase and collagenase enzyme inhibitory effects.

The fruits of *M. nigra* were reported to be rich in phenolic compounds, which exhibit a wide range of biological activities including antimicrobial, antioxidant, and anti-inflammatory effects. In previous studies, flavonoids, anthocyanins and carotenoids were determined as major compounds from this plant [9,24].

Yigit, et al., (2007) found out that the aqueous and methanolic extracts obtained from *M. nigra* fruits exhibited antifungal, antibacterial and antiviral properties, whereas the methanolic extract of leaves found to possess no activity against *Candida* species [25]. Kuwanon G, leachianone, 2-arylbenzofuran type compound chalcomoracin and moracin and prenylated flavonoids isolated from *Morus* species were reported to be antimicrobial active constituents [26, 27]. The presence of these compounds in fruit extract should probably provide a barrier against the microbial attacks in the first periods of the healing.

Agents which inhibit prostaglandin biosynthesis and nitric oxide production have anti-inflammatory potential. Chung et al. reported that the anti-inflammatory activity of *M. alba* may be attributed to the inhibition of iNOS and COX-2 [28]. In the present study, it was found out that lyophilized fruit extract of *M. nigra* demonstrated dose dependent anti-inflammatory activity, which may be ascribed to its phenolic constituents, especially anthocyanins and flavonoids.

The flavonol glycosides including rutin, isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin and kaempferol 3-(6-acetylglucoside) isolated from *M. nigra* fruit extract were reported to possess anti-inflammatory and antioxidant properties [25, 29, 30]. In previous studies, it was stated that, anthocyanins have strong antioxidative potential and their production influences the antioxidative capacity of the species studied. Therefore, especially the fruits of *M. nigra*, due to the rich anthocyanin content, demonstrated higher antioxidant activity among the other *Morus* species [31]. According to our results, it is much probable that *M. nigra* fruit extract promote wound healing by preventing persistent inflammatory condition and lipid peroxidation due to its phenolic components.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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