



Isolation of apigenin-7-O-(6''-O-E-caffeoyl)- β -D-glucopyranoside from *Leucas aspera* L. with anti-inflammatory and wound healing activities

[Aislamiento de apigenina-7-O-(6''-O-E-cafeoil)- β -D-glucopiranosido de *Leucas aspera* L. con actividad anti-inflamatoria y cicatrizante]

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Abstract

Context: *Leucas aspera* L. (Labiatae) is a common aromatic herb and grows generously in South India and in the broad area of South Asia. Traditionally, this species is taken orally for analgesic, anti-inflammatory, anti-bacterial and wound healing treatments.

Aims: To isolate compounds from *L. aspera* with anti-inflammatory and wound healing activities.

Methods: The chloroform extract was subjected to a column chromatography on silica gel 60 and their structures were established by spectral analysis (UV, IR, and NMR). The anti-inflammatory activity of the test compounds was evaluated in male albino rats. The acute inflammation was induced by the subplantar administration of 0.1 mL of 1% carrageenan in the right paw. The excision wound model was used to study the rate of wound contraction and the time required for complete epithelization of the injuries in rabbits.

Results: A flavonoid apigenin-7-O-(6''-O-E-caffeoyl)- β -D-glucopyranoside (**1**) was isolated from a chloroform fraction of *L. aspera*. The hydrolysis of compound **1** yield an apigenin (aglycone), caffeic acid and β -D-glucose. Assuming caffeoyl glucose linked to the 7-OH group of apigenin.

Conclusions: Compound **1** exhibited a significant anti-inflammatory activity compared with standard diclofenac sodium. The wound healing study revealed that decreased wound area and significant increase in epithelization in treatment groups was observed.

Keywords: Anti-inflammatory; flavonoids; *Leucas aspera*; wound healing.

Resumen

Contexto: *Leucas aspera* L. (Labiatae) es una hierba aromática común y crece generosamente en el sur de la India y en la zona amplia del sur de Asia. Tradicionalmente, esta especie se toma por vía oral para tratamientos analgésicos, anti-inflamatorios, anti-bacterianas y la cicatrización de heridas.

Objetivos: Aislar compuestos de *L. aspera* con actividades anti-inflamatoria y cicatrizante.

Métodos: El extracto clorofórmico de *L. aspera* se sometió a una columna cromatográfica de sílica gel 60 y las estructuras fueron establecidas por análisis espectral (UV, IR y RMN). La actividad anti-inflamatoria de los compuestos estudiados fue evaluada en ratas albinas machos. La inflamación aguda fue inducida por la administración subplantar de 0,1 mL de carragenano 1% en la aponeurosis plantar derecha. El modelo de escisión de la herida fue usado para estudiar la tasa de contracción de la herida y el tiempo requerido para la completa epitelización de las heridas en conejos.

Resultados: De la fracción clorofórmica de *L. aspera* se aisló un flavonoide apigenina-7-O-(6''-O-E-cafeoil)- β -D-glucopiranosido (**1**). La hidrólisis del compuesto **1** rindió apigenina (aglicona), ácido cafeico y β -D-glucosa. Suponiendo que la cafeoil-glucosa estaba unida al grupo 7-OH de apigenina.

Conclusiones: El compuesto **1** exhibió una actividad anti-inflamatoria significativa en comparación con diclofenaco sódico estándar. El estudio de la curación de las heridas reveló que hubo una disminución del área de la herida y un aumento significativo en la epitelización en los grupos de tratamiento.

Palabras Clave: Anti-inflamatorio; cicatrización de herida; flavonoides; *Leucas aspera*.

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INTRODUCTION

The herbal remedies traditionally used medicinal plants were increased attention from scientific and pharmaceutical communities Sekhar et al. (2012). Because of this resurgence of interest, the research on plants of medical importance is growing phenomenally in worldwide (Hammer et al., 1999).

A review of the literature has revealed that plant metabolite such as alkaloids, flavonoids, among others, play an important role in many of the activities including wound healing, anti-inflammatory, anti-oxidant, and anti-microbial activity (Noor Fathima et al., 2011). Flavonols are of particular interest to phytochemists as shown to have wide bioactive potential and regarded as one of the most numerous and widespread groups of natural polyphenols found in plants (Markham and Geiger, 1994).

Inflammation involves the action of the complement system and repair processes. It is both a free-radical generating and free-radical producing process (Miller, 1996).

Wounds or any damage to or, break of the skin or underlying tissues. The damage may be caused by accidents, incisions from surgery or other traumas (Ramzi et al., 1994). Medical treatment of wounds includes the administration of drugs either locally or systematically in an attempt to aid wound repair (Savanth and Shah, 1998).

Some reports concerning the anti-inflammatory and wound healing activities of various plants have appeared in the literature, but the vast majority has yet to be explored (Rupesh et al., 2011).

Leucas aspera L (Labiatae) is a common aromatic herb and grows generously in South India and in the broad area of South Asia. Traditionally, the decoction of the whole plant was taken orally for analgesic, antipyretic, anti-rheumatic, anti-inflammatory, anti-bacterial and wound healing treatments, and its paste has been applied topically to inflamed areas (Reddy et al., 1993; Sadhu et al., 2003).

The present studies to isolate further flavonoid namely apigenin-7-*O*-(6''-*O*-*E*-caffeoyl)- β -D-glucopyranoside (**1**), which occurs in chloroform fraction of *L. aspera* and the prevalence of flavonoids in daily life we decided to find out anti-inflammatory and wound healing activities of plant extracts.

MATERIAL AND METHODS

Plan material

Fresh flowers (3 kg) of *L. aspera* was collected during the October to March 2012 from the Ariyalur District in Tamil Nadu, India. Dr. N. Ramakrishnan authenticated this species, and voucher specimens (GACBOT-113) were deposited in the Herbarium of the Department of Botany, Government Arts College (Autonomous), Kumbakonam, Bharathidasan University, India.

Extraction and isolation

Air dried flowers of *Leucas aspera* (2.35 kg) was extracted with 90% methanol (MeOH) in a Soxhlet apparatus. The hydro-alcoholic solution was concentrated under reduced pressure to dryness, and the residue was dissolved in hot water (1000 mL) and kept in the cold overnight. After filtration, the clear solution was consecutively partitioned with petroleum ether, chloroform and ethyl acetate. In the previous studies (Prabakaran et al., 2013) on *L. aspera* flowers was reported on the identification of baicalein-7-*O*- β -D-glucuronide (baicalin) in the ethyl acetate (EtOAc) fraction with EtOAc/MeOH/H₂O (98:1:1). In this study was concentrated the chloroform (CHCl₃) extract (8.8 g) and was subjected to a column chromatography on silica gel 60 with a gradient of CHCl₃-MeOH of increasing polarity and further elution with *n*-butanol (BuOH)/acetic acid (AcOH)/H₂O (4:1:1) as eluents to yield compound **1** (5.7 g).

Acid hydrolysis

The compound **1** (10 mg) was refluxed with 5% HCl for 2 h. The hydrolysates were extracted with CHCl₃ and held extracts was washed with water, evaporated to dryness and resolved in MeOH. Identification of aglycone, apigenin (Rf: 0.29) (*n*-BuOH/AcOH/H₂O (4:1:5, organic phase). The sugars in the aqueous layer were identified by TLC (on silica gel 60) with reliable samples using different solvent system: D-glucose (*n*-BuOH/AcOH/H₂O, 4:1:5; Rf: 0.18) and caffeic acid (EtOAc/formic acid (HCOOH)/water, 80: 10:10; Rf: 0.60).

Spectroscopic methods

Melting points were determined on a Fisher Scientific melting point apparatus and are uncorrected. A UV spectrum was recorded in MeOH on an Ultraviolet spectrophotometer (UV/VIS 1601, Shimadzu, Japan). The IR spectrum was measured on FT-IR spectrograph (Perkin Elmer Spectrophotometer, USA) with KBr tablets from 4000 to 400 cm^{-1} with resolution 2 cm^{-1} . ^1H and ^{13}C -NMR (500 MHz, CDCl_3) spectra was recorded on AMX 500 NMR spectrometer (Bruker Company, Faelladen, Switzerland). Chemical shifts gave in δ (ppm) about TMS as internal standard materials, and the coupling constants (J) are in Hz. Column chromatography was performed on silica gel 60 as stationary phase (particle size 0.04 - 0.036 mm, 230-400 mesh, ASTM E. Merck, Germany) and activated by heating at 110°C for one hour before to use. TLC was carried out on 0.25 mm Brinkman percolated silica gel F254 plates (silica gel 60, 230-400 mesh, Merck, Germany). The different solvent systems were used for TLC analyses (MeOH/EtOAc/ H_2O , 7:2:1; n-BuOH/Ac-OH/ H_2O , 4:1:5; EtOAc/MeOH/ H_2O , 98:1:1; EtO-Ac/HCOOH/ H_2O ; 80:10:10) and the spots were visualized under UV light 365 nm, with NH_3 vapors and by spraying with 1% AlCl_3 in MeOH. A Shimadzu HPLC system (Columbia, MD), was used with UV detection at 2800 - 350 nm. A chromatographic system comprising a Spectra Physics P-200 series gradient pump (Fremont, CA, USA), a rheodyne injector fitted with a 20-FL loop, the C18 column (250 x 4.6 mm, phenomenex, Torrance, CA, USA) was used.

Animals

Healthy rabbits (New Zealand white rabbits) of both sex and nearly the same age, weighing about 2000 - 2200 g were used in the study. All animals were fed with the standard rodent pellet diet and water *ad libitum*. They were individually housed, kept in polypropylene cages under normal conditions. "All applicable institutional guidelines for the care and use of animals were followed." The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Trichirappalli,

Tamil-nadu, India (Approval No. BDU/IAEC/2011/31/ 29.03.2011).

Anti-inflammatory activity by carrageenan induced paw edema

The anti-inflammatory activity of the test compounds was evaluated in male albino rats (200 - 250 g). Animals were fasted overnight and divided into control; standard and different test groups, each consisting of six animals at the dose of 100 and 200 mg/kg of test compound 1 and 100 mg/kg diclofenac sodium was administered to the animals by the oral route. Control group animals were received 1% DMSO at the dose of 10 mL/kg body weight. They are housed in cages and kept under standard conditions at $26 \pm 2^\circ\text{C}$ and relative humidity 60 - 65%, 12 h lights, and 14 h dark cycles each day for one week before and during the experiments. Acute inflammation was induced by the subplantar administration of 0.1 mL of 1% carrageenan in the right paw. Paw volume was measured by using digital plethysmometer (Ugo Basile-Italy) before treatment (V_0). One hour after treatment, 0.1 mL of carrageenan was administered into the subcutaneous tissue of the plantar surface of the right hind paw. Then the volume of the paw was taken at 1, 2 and 3 h after carrageenan administration (V_T). The efficacy of different treated groups was tested on its ability to inhibit paw edema compared to control group. The edema was expressed as an increase in the volume of the paw (ΔV), and the percentage of inhibition (I %) for each treatment was obtained as follows:

Volume of edema (ΔV) = Final Paw Volume (V_T) - Initial Paw Volume (V_0)

The percentage inhibition of paw edema was calculated by the formula:

$$\% \text{ Inhibition of paw edema} = [(VC - VT) / VC] \times 100$$

Where,

VC = Paw edema of control group, and

VT = Paw edema of treated group.

Wound healing activity of excision wound model

The excision wound model was used to study the rate of wound contraction and the time needed for complete epithelization of the injuries (Nayak et al., 2007). The animals were assigned to four groups each group containing six animals. Group I: No treatment and served as controlled. Group II: Test group with the wound and treated with compound 1 (100 mg/day). Group III: Test group with the wound and treated with compound 1 (200 mg/day). Group IV: Test group with standard drug ointment (soframycin).

Animals were anesthetized with slight vapor inhalation of diethyl ether in the pre-determined area and the back of a rabbit was shaved. The excision wound, a circular piece of full thickness sized, nearly 500 mm² and 2 mm depth was made by cutting out the skin from the shaved area. The test formulation (10% w/w and 20% w/w ointment), standard drug (soframycin ointment) were applied to the wound twice daily, until complete recovery. The progressive changes in wound area were monitored by a camera every fourth day. The changes in the healing of wound were measured and recorded the wound area on graph paper (mm²) every four days until complete wound healing. Wound contraction was determined as a percentage of the decline in the wound area (Werner et al., 1994).

% wound contraction = [(initial wound area – specific day wound area) / initial wound area] x 100.

Histopathological examination

Small pieces of tissue were isolated from the healed skin of each group of rabbits for histopathological examination (Sadaf et al., 2006). Samples were fixed in 10% buffered formalin, processed and blocked with paraffin wax. Serial sections of 5 µm were prepared and light microscopic study of H&E and Masson's trichrome stained tissues for routine histopathological evaluation. Masson's trichrome stain was used to find out the degree of collagenization. All slides were examined satisfactorily by the surgical pathologist.

Detection of pH and the protease activity in the wound bed

Protease action and activity could be directly pH-dependant (Greener et al., 2005). Elevated protease levels were detected as soon as possible to prevent a wound, ending in a static state of persistent inflammation. However, there are no clinical signs that can specifically identify elevated protease levels in a wound bed. The most often used instrument is a top glass electrode attached to a meter when a probe used it has first calibrated in pH 4 and 7 or 9 buffers. The probe was rinsed with de-ionized water and then placed flat against the wound for 30 seconds and the result was displayed on the meter.

Gel formulation

The ointment of compound 1 was prepared by mixing with Vaseline in a concentration of 10% (100 mg compound 1/10 g Vaseline, w/w) and 20% (200 mg compound 1/10 g Vaseline, w/w) respectively.

Statistical analysis

The experimental results are expressed as multiple comparisons of mean ± SD carried out by one-way analysis of variance (ANOVA) followed by Dunnet Multiple Comparisons Test and statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Chemical identification

Some preliminary tests started with the crude flower extract of *Leucas aspera* that on major classes of chemical compounds were present. The chromatographic isolation of *L. aspera* showed the presence of flavonoid which showed UV absorption characteristic for 7-O-substituted flavone. The main monoglycosidic component baicalein-7-O-β-D-glucuronide was isolated and identified from the EtOAc fraction previously (Prabakaran et al., 2013). For further quantitative analyses and pharmacological evaluations of the major chemical compound (1), it was certain to isolate CHCl₃ fraction of the flowers

of *L. aspera* and to unfold by various methods, including HPLC, UV, IR and NMR-spectroscopy.

Compound **1** as a yellow amorphous solid; mp: 238-240°C. This compound also gave positive color reactions for a hydroxyl flavone with several reagents (Kaneta, 1971; Harborne et al., 1975). The UV spectrum λ_{\max} (MeOH) showed a band at 270 and 320 nm (sh), deducing its flavanone nature while on the addition of NaOAc showed to occupy the 7th position (Mabry et al., 1970). The addition of NaOAc and AlCl₃ pointed to the presence of free hydroxyl at C-4' and C-5. The IR spectrum displayed characteristic absorption band at 3280 cm⁻¹ for a hydroxyl group and 1690 cm⁻¹ for α , β -unsaturated carbonyl group, suggesting it flavonoid nature. The IR spectrum also displayed absorption bands for the aromatic ring at 1620 and 1510 cm⁻¹ (Silverstein and Webster, 1998). ¹H and ¹³C spectra presented particular resonance of a glycoside flavone. The ¹H NMR spectrum showed aromatic proton signals of two doublets at δ 6.52 and δ 6.63 (each 2H, $J = 2.1$ Hz) were attributed to the H-6 and H-8 of the A-ring, respectively. The two vicinal coupled doublets at δ 7.42 and δ 6.74 (each 2H, $J = 8.8$ Hz) was assigned to H-2'/6' and H-3'/5' of the B-ring. Also, two singlet signals at δ 12.88 and δ 8.47 which were assigned to 5-chelated OH group and H-3 of isoflavone (Harborne, 1988). However, additional resonances arising from a D-glucose unit with specific signals at δ 5.23 ppm (d, $J = 7.3$) for anomeric proton and the rest of the sugar protons appear in the range δ 3.30 - 4.32 ppm. Besides the ¹H-NMR spectrum also showed an ABX system for 1, 3, 4 - trisubstituted aromatic ring at δ H [6.86 (1H, d, $J = 3.2$ Hz, H-2'''), 7.82 (1H, d, $J = 8.0$ Hz, H-5'''), 6.98 (1H, dd, $J = 8.0, 3.2$ Hz, H-6''')] and two trans-olefinic protons at δ H [7.49 (1H, d, $J = 15.6$ Hz, H-7'''), 6.36 (1H, d, $J = 15.6$ Hz, H-8''')] suggesting the presence of caffeoyl group (Chen and Yang, 2007). The ¹³C-NMR resonance at δ C 165.0 ppm was attributed to C-7 and comparing this data with the published value (Markham, 1982; Liu et al., 1998; El-Sayed et al., 2001) and α , β -unsaturated carbonyl group at δ 181.3 (C-4, -C=O), and the olefinic carbons at δ 98.7 (C-6), 165.0 (C-7) of flavone moiety. Also, the ¹³C-NMR spectrum displayed a chemical shift of the anomeric carbon of β -D-glucose at δ 101.92 and the downfield chemical shift of C-6'' carbon at δ 62.9

suggested that the caffeoyl group is attached to the C-6'' of glucose. On the hydrolysis of **1**, yielded an aglycone apigenin, caffeic acid, and β -D-glucose was identified by TLC alongside with positive samples. Since Characteristic ¹H and ¹³C chemical shift values and coupling constant data pointed out the compound **1** were apigenin-7-O-(6''-O-E-caffeoyl)- β -D-glucopyranoside and it was presented in Fig. 1.

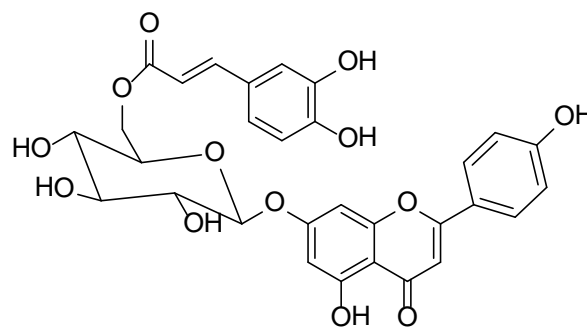


Figure 1. Apigenin-7-O-(6''-O-E-caffeoyl)- β -D-glucopyranoside.

Anti-inflammatory activity

Inflammation is a response of living tissue to injuries that involve activation of various enzymes, mediator release, cell migration, tissue breakdown and repair (Katzung, 2004). Carrageenan-induced hind paw edema is a suitable experimental animal model of acute inflammation (Turner, 1965). Carrageenan induced paw edema takes place in three phases, in the first phase (1 h after carrageenan induce) involves the release of serotonin and histamine from mast cells, in a second phase (2 h) was provided by kinins and the third phase (3 h) was mediated by prostaglandins, the cyclooxygenase and lipoxygenase products (Vinegar et al., 1969). As shown in the results (Table 1), restraint of paw edema (after 3 h) for test compound **1** (100 and 200 mg) with 3.83 ± 0.126 mL and 3.71 ± 0.019 mL paw volume, respectively. It shows the compound **1** had a significant anti-inflammatory effect, and the results were compared with standard diclofenac sodium 100 mg/kg and showed the paw volume reduction of 3.63 ± 0.126 without statistical significance ($p > 0.05$) between compound **1** and diclofenac sodium. Results were also reported in % restraint of edema (protection against inflammation) after three-hour treatment in comparison with the control group (Table 1). It showed a max-

imum percentage reduction (58.24%) in paw edema at 3 hours. Compound **1** at the dose of 100 and 200 mg/kg body weight showed the percentage of inhibition of paw edema at 4 h 36.26% and 49.45%, respectively. All the test and standard groups have reduced the thickness of edema of the hind paw in different percentages compared to the control group.

Wound healing activity

Also, our research report revealed an excision wounds healing by the recording the changes in the wound are at fixed intervals of time, like 4th, 8th, 12th and 16th days after treating with chloroform extract of *L. aspera* and reported in Table 2. The groups treated with 15% of ointment base with the soframycin cream were able to effect total wound closure on/before the 12th day of the studies, while the test groups treated with 10% and 20% ointment base effected complete wound closure on the experimental animals on/before the last day of the experiment (day 16). On 16th post wounding day, the period of epithelialization of test compound **1** (100 and 200 mg) were found to be 2 ± 1.10 and 3 ± 1.52 mm²/rabbit, respectively while in a standard group it was significantly reduced to 5 ± 0.69 mm²/rabbit. All readings were considered to be statistically significant ($p < 0.05$) and comparable to control (Table 2).

Also the surface pH measurement to prove compound **1** was better to wound healing activity.

Protease activity is a normally recognized part of this process. Proteases are produced by either activated inflammatory cells such as neutrophils and macrophages - these are known as endogenous proteases include collagens, gelatinase, and elastase (Walker et al., 2007; Gibson et al., 2009). Protease action and activity are pH-dependent. All the test groups became basic at pH 7.8 on the 4th day it will reduce to acidic (pH 4.3) on the 12th post wounding day. It is important to state that the results obtained are of surface pH and not tissue pH. Generally, non-healing wounds have a pH level of 8. If the pH level is reduced to a more acidic (approximately 4), the protease activity is decreased by approximately 80% (Greener et al., 2005). A few macrophages and lymphocytes were seen beneath the newly formed epidermis and seen fibrous tissue, which filled the defect in the dermis and surface pH measurement, depended on the elevated protease activity. It is a biochemical marker for predicting the condition of the wound bed.

The histopathological examination provided another evidence for the experimental wound healing studies (Fig. 2), which were based on the contraction value of the wound area. This study revealed a significant increase in epithelialization in drug treated group and granuloma showed increases in both the number of growing capillaries and a number of fibroblasts collagenous connective tissue when compared to granuloma of the control animal.

Table 1: Determination of paw volume and % inhibition on carrageenan-induced rats at different time for apigenin-7-O-(6''-O-E-caffeoyl)- β -D-glucopyranoside (**1**).

Treatment	Dose (mg/kg)	Initial paw volume (mL)	Time (h)			Inhibition (%)
			1	2	3	
Paw volume (mL)						
DMSO 1%	10 (mL/kg)	3.27 ± 0.164	3.66 ± 0.132	3.94 ± 0.241	4.15 ± 0.112	-
Compound 1	100	3.26 ± 0.089	3.48 ± 0.094	3.61 ± 0.115	3.83 ± 0.126	36.26
Compound 1	200	3.27 ± 0.113	3.43 ± 0.067	3.54 ± 0.069	3.71 ± 0.019	49.45
Diclofenac sodium	100	3.27 ± 0.089	3.40 ± 0.094	3.50 ± 0.115	3.63 ± 0.126	58.24

Values are expressed in mean \pm standard deviation (n = 6).

One-way ANOVA (Dunnett's method) Means for groups in homogeneous subsets are displayed.

There is no significant difference between standard and test drug at $p > 0.05$ significant level.

% Inhibition = at 3rd hour.

Table 2. Effect of oral treatment of apigenin-7-*O*-(6''-*O*-*E*-caffeoyl)- β -D-glucopyranoside (**1**) on excision wound in rabbits.

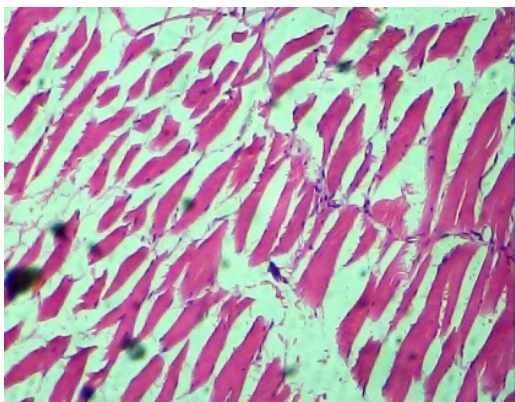
Treatment	Dose (mg)	Day				
		0	4 th	8 th	12 th	16 th
Wound contraction (mm ² /rabbit)						
Control	0	200 \pm 1.05 ^b	173 \pm 1.52 ^b	65 \pm 1.15 ^b	33 \pm 1.02 ^b	11 \pm 0.69 ^b
Compound 1	100	200 \pm 1.32 ^a	145 \pm 1.15 ^a	19 \pm 0.40 ^a	10 \pm 1.52 ^a	2 \pm 1.10 ^a
Compound 1	200	200 \pm 0.40 ^a	153 \pm 1.29 ^a	21 \pm 0.27 ^a	12 \pm 1.32 ^a	3 \pm 1.52 ^a
Soframycin ointment	100	200 \pm 1.32 ^a	160 \pm 1.02 ^b	26 \pm 1.15 ^a	14 \pm 1.15 ^a	5 \pm 0.69 ^a

Values are expressed by mean \pm standard deviation (n = 6)

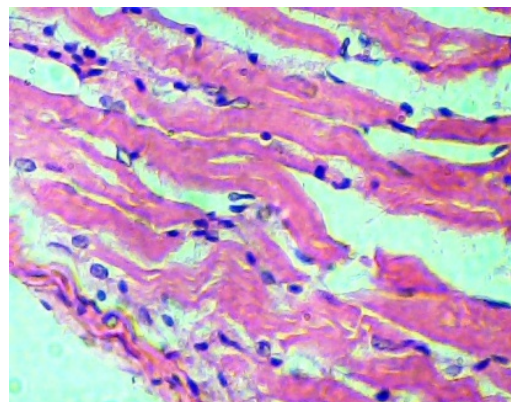
One-way ANOVA (Dunnett's method) Means for groups in homogeneous subsets are displayed.

Subset for alpha = 0.05 level. Different letters symbolize significant differences (p < 0.05).

A



B



C

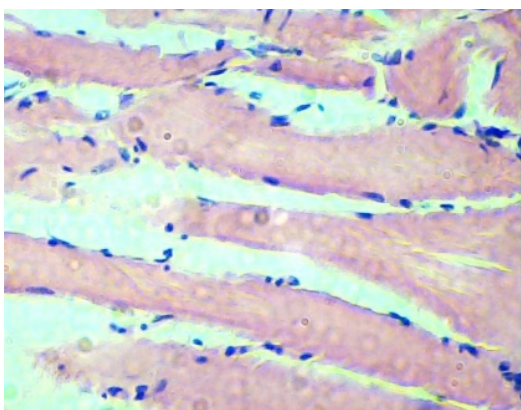


Figure 2. Histopathology of (A) control, (B and C) 100 and 200 mg/kg of compound **1** showing increased epithelialization on 12th day. (H & E stain)

CONCLUSIONS

The present investigation, we confirm that apigenin-7-O-(6''-O-E-caffeoyl)- β -D-glucopyranoside isolated from chloroform extract of *L. aspera* was better anti-inflammatory and wound healing activity it was concluded that the test formulation showed better and faster healing as compared to the control group. Bioactive substances from this plant were employed to develop drugs for treating inflammation and wound healing disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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