



Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

In vitro wound healing activity of 1-hydroxy-5,7-dimethoxy-2-naphthalene-carboxaldehyde (HDNC) and other isolates of *Aegle marmelos* L.: Enhances keratinocytes motility via Wnt/ β -catenin and RAS-ERK pathways

Lubna Azmi^{a,b}, Ila Shukla^{a,c}, Arti Goutam^a, Allauddin^a, Ch.V. Rao^a, Talha Jawaid^d, Mehnaz Kamal^{e,*}, Amani S. Awaad^f, Saleh I. Alqasoumi^g, Osama A. AlKhamees^h^a Pharmacognosy and Ethnopharmacology Division, CSIR-National Botanical Research Institute, Lucknow, Uttar Pradesh, India^b Department of Chemistry, University of Lucknow, Lucknow, India^c Academy of Scientific and Innovative Research, Ghaziabad, India^d Department of Pharmacology, Hygia Institute of Pharmaceutical Education and Research, Ghaila Road, Lucknow 226002, Uttar Pradesh, India^e Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam Bin Abdulaziz University, P.O. Box 173, Al-Kharj 11942, Saudi Arabia^f Director of Gateway to United Kingdom Education Ltd., Bradford, United Kingdom^g Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia^h Department of Pharmacology, College of Medicine, Al Imam Mohammad Ibn Saud Islamic University (IMSIU), P.O. Box 11623, Riyadh 11544, Saudi Arabia

ARTICLE INFO

Article history:

Received 24 October 2018

Accepted 27 January 2019

Available online 29 January 2019

Keywords:

Aegle marmelos

Akt signaling pathways

Cutaneous wound

Dermal fibroblasts HaCaT keratinocytes

ABSTRACT

Wound healing is a complex process in which injured skin and tissues repaired by interaction of a complex cascade of cellular events that generates resurfacing, reconstitution and restoration of the tensile strength of injured skin. It follows β -catenin, extracellular signal regulated kinase (ERK) and Akt signaling pathways. *Aegle marmelos* L., generally known as bael is found to act as anti-inflammatory, antioxidant and anti-ulcer agent. Furthermore, studies have demonstrated that this Indian traditional medicinal plant, *A. marmelos* flower extract (AMF) was used for wound injury. Henceforth, the current study was investigated to ascertain the effect of its active constituents *in vitro* wound healing with mechanism involve in migration of cells and activation of β -catenin in keratinocytes, inhibition of PGE₂ in macrophages and production of collagen in fibroblasts. We have taken full thickness wound of rats and applied AMF for 2 weeks. Cutaneous wound healing activity was performed using HaCaT keratinocytes, Hs68 dermal fibroblasts and RAW264.7 macrophages to determine cell viability, nitric oxide production, collagen expression, cell migration and β -catenin activation. Results shows that AMF treated rats demonstrated reduced wound size and epithelisation was improved, involved in keratinocytes migration by regulation of Akt signaling, beta-catenin and extracellular signal-regulated kinase (ERK) pathways. AMF and its active constituent's increased mRNA expression, inhibited nitric oxide, PGE₂ release, mRNA expression of mediators in RAW 264.7 macrophages and enhances the motility of HaCaT keratinocytes *in vitro* wound healing of rats.

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1. Introduction

A variety of inflammatory cells, parenchymal cells, soluble mediators and extracellular matrix molecules undergo complex

interactions to accomplish the process wound healing and it is constituted of three stages, first is inflammatory followed by proliferative and final stage is remodeling (Mendonca and Coutinho-Netto, 2009). Wound healing process create extracellular signal-regulated kinase (ERK) pathways Akt signaling and beta-catenin (Squarize et al., 2010; Mi, et al., 2018). Embryonic development majorly involves beta-catenin signalling pathway for migration and proliferation of cells (Widelitz, 2008). In wound healing process, both β -catenin dependent pathway and β -catenin independent pathway are important (Mendonca and Coutinho-Netto, 2009). Growth factors like fibroblast growth factor and epidermal growth factor activates ERK and Akt signaling pathways, which in turn induce

* Corresponding author.

E-mail address: m.uddin@psau.edu.sa (M. Kamal).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

<https://doi.org/10.1016/j.jsps.2019.01.017>

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keratinocytes motility and causes re-epithelialization in wound (Seeger and Paller, 2015; Perrone et al., 2018). Nitric oxide comes under the category of highly reactive free radicals. It is an important factor in signaling pathways of various physiological processes (Kurutas, 2015). Prostaglandin H₂ which is a precursor for various biologically active mediators like thromboxane A₂, prostaglandin E₂ and prostacyclin, is produced from arachidonic acid in presence of cyclooxygenase (COX) (Smith et al., 1996). Proinflammatory mediators e.g., tumor necrosis factors (TNF- α) and lipopolysaccharide (LPS) alleviate the production of COX-2 derived prostaglandin E₂ and NOS-derived nitric oxide (Kurutas, 2015). Wound healing process is further delayed by induced interleukin (IL)-1 β because it instigates additional injury to adjacent cell or tissues around wounded area. Hence PEG₂ suppression and production of nitric oxide by cyclooxygenase-2 and nitric oxide synthase inhibitors may aid in deterrence of the damage (Landen et al., 2016). This analysis presents the effect of *Aegle marmelos* L., commonly known as baelflowers extract (AMF) and its constituents in case of *in vitro* wound healing. This medicinal plant is widely found throughout the Indian subcontinent and in Bangladesh, Ceylon, Burma and Thailand. Chemical investigation on various parts of the tree have been carried out. More than 100 bioactive compounds including skimmianine, agelin, lupeol, cinenol, citral, citronellal, cuminaldehyde, eugenol, marmesinin, marmelosin, luvangetin, aurapten, psoralen, marmelide, fagarine, marmin and tannin have been proved to have biological activity (Mujeeb et al., 2014), generally this plant is used for the treatment of microbial infection, ulcer and viral infection (Azmi et al., 2017; Kim et al., 2017). The formation of new epidermis is a measure used here to measure the effect of the given flower extract on wound healing. Also we analyzed the role of given extract and the constituents present in it, on migration of cells and activation of β -catenin in keratinocytes, production of collagen in fibroblasts, and inhibition of PGE₂ in macrophages.

2. Material and methods

2.1. Extraction and isolation of bioactive compounds

2.1.1. Collection of flower material

Plants for the study were gathered from their natural habitats in and around Lucknow. The plants were authenticated and submitted to the department herbarium (Pharmacognosy and ethno pharmacology division) of NBRI, Lucknow.

2.1.2. Extraction

Dried flower of *A. marmelos* L was ground to powder and passed through 50 meshes. The powdered material was subjected to solvent extraction (SE). Various concentration of ethanol solution was applied solely as the solvent because the plant material yield with 60% ethanol was 5 times superior to that using pure distilled water. Also there is no major difference in yield in case of metha-

nol. Regulatory factors subjected as the variables of SE include: 1. the concentration of ethanol solution, 2. the time taken by the process and 3. The temperature maintained during extraction. Dried and powdered flowers (250 g) were defatted by petroleum ether. After that powder was extracted with ethanol for 50 h and filtered with Whatman filter paper No.4. Residue was re-extracted with 100 mL of methanol. Solvent was evaporated under reduced pressure 150 PSI and 40 \pm 2 $^{\circ}$ C in Buchi rotavapor (Buchi India Private Limited, Mumbai, India) and lyophilized (Free zone Freeze Dryer, Labconco, Kansas City, U.S.) to get dried extract.

2.1.3. Isolation

Liquid partition of the 60% hydroethanolic extract of flowers of *A. marmelos* produced four fractions of chloroform, butanol, hexane and water, we purified active chloroform soluble fractions (CF) by repeated column chromatography over silica gel and yielded eighteen pure compounds. The known compounds were recognized comparing their spectroscopic data with those previously reported in literature (Mujeeb et al., 2014). The chloroform fraction (80.0 g) of flower extract was subjected to column chromatography over silica gel (60–120 mesh) and eluted with a gradient of ethyl acetate-hexane (05:95) to methanol-ethyl acetate (05:95), 28 fractions of CF-AMF were collected (500 mL each) and their composition was monitored by thin layer chromatography (TLC), those fractions showing similar TLC profiles were grouped into 9 fractions of CF-AMF1 to CF-AMF9 (Table 1). Successive flash chromatography (BUCHI Sepacore[®], Buchi India Private Limited, Mumbai, India) of fractions yields isolated compounds. The chemical structure of compounds (Fig. 1) were determined by spectral data and compared with reported data (Maity et al., 2009; Manandhar et al., 2018).

2.1.4. HPLC analysis

The HPLC system Shimadzu LC-10A (Japan) was equipped with dual pump LC-10AT binary system, UV detector SPD – 10A, Phenomenex Luna RP, C 18 column (4.6 \times 250 mm) (Phenomenex India Pvt. Ltd, Bangalore, India) and data was integrated by Shimadzu Class VP series software (HPLC; Shimadzu Asia Pacific Pvt. Ltd, Singapore). Separation was attained with a two pump linear gradient program for pump A (Water containing 1% acetic acid) and pump B (Acetonitrile). Initially started with a gradient of 18% B changing to 32% in 15.0 min and finally to 50% in 40 min followed by washing for 25 min, and detection was at 254 nm. Concentration of sample was 10 mg/min. The HPLC spectra of AMF is shown in Fig. 1.

2.2. In vitro wound healing model

2.2.1. Cell culture

Human keratinocyte cell line (HaCaT), human foreskin fibroblast cell line (Hs68) and macrophage cell line of mouse (RAW 264.7) obtained from National Center for Cell Science, Pune, India were taken in this study. Cell lines were kept in penicillin and

Table 1
Elution of CF-AMF (10 g) column using solvent of increasing polarity.

Fraction	Mobile phase	Weight (g)	Remark	Compound isolated
CF-AMF 1 (1)	Hexane, EtOAc binary mixture	1.123	Brownish yellow mixture	Cineol was isolated through preparative TLC
CF-AMF 2 (2–5)	Hexane, EtOAc binary mixture	0.845	Brownish mixture	Eugenol was isolated through preparative TLC
CF-AMF 3 (6)	Hexane, EtOAc binary mixture	0.898	Brownish mixture	Cuminaldehyde was isolated through preparative TLC
CF-AMF 4 (7–10)	Hexane, EtOAc binary mixture	0.693	Brownish yellow mixture	Aegelin was isolated through preparative TLC
CF-AMF 5 (11–13)	Hexane, EtOAc binary mixture	1.634	Brownish yellow mixture of 3 compounds	HDNC was isolated through preparative TLC
CF-AMF 6 (14–17)	Chloroform, and hexane (90:10)	0.033	Yellowish green mixture of	–
CF-AMF 7 (18–20)	Hexane, EtOAc binary mixture	0.589	Brownish yellow mixture of	–
CF-AMF 8 (21–25)	Chloroform, and methanol (95:5)	0.762	Yellowish mixture 4 compounds	Luvangetin was isolated through preparative TLC
CF-AMF 9 (26–28)	Methanol: water (70:30)	0.678	Brownish yellow mixture of	–

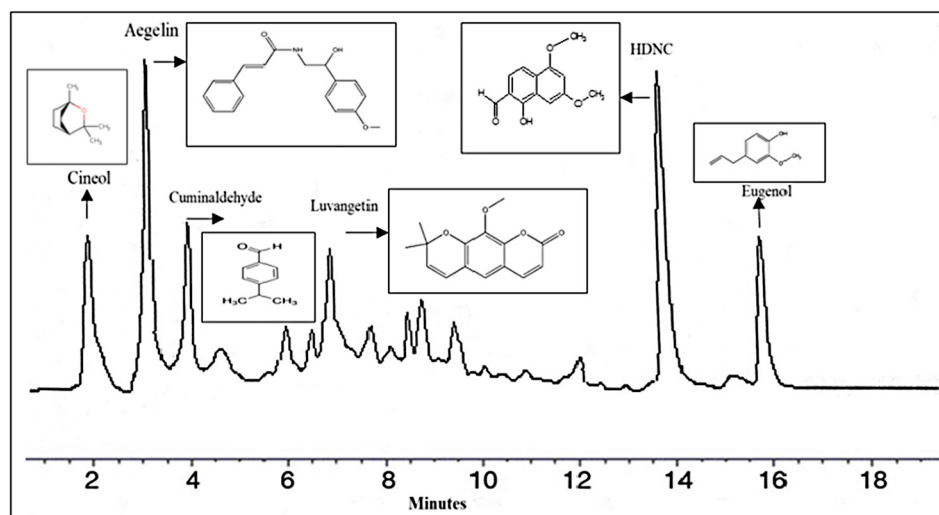


Fig. 1. HPLC spectra of chemical constituents isolated from *A. marmelos* flower extract.

Dulbecco's modified eagle-medium (DMEM: ThermoFisher Scientific, Bangalore, India) which was accompanied by 10% foetal bovine and 1% penicillin-streptomycin. All the analysis over cell culture was carried out in humid atmosphere with 6% CO₂ at 38 °C.

2.2.2. Cell viability assay

Cell viability was carried out by MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) colorimetric assay (Merck Ltd, Mumbai, India). HaCaT, Hs68 and RAW 264.7 cells were grown in 24 well plates (1 × 10⁵ cells/mL) for 24 h. Ethanolic extract AMF and its constituents were added to cell cultures for six hours. Once the treatment was over cells were properly washed & incubated with MTT (0.5 mg/mL) at a temperature of 37 °C for four hours. Again the cells were washed after incubation and stored in 200 μL of DMSO. This was used to solubilize the insoluble formazan products. Spectrophotometer was employed to measure the absorbance at a wavelength of 550 nm (Kim et al., 2017).

2.2.3. Cell migration assay

HaCaT cells were placed in the 24-well plates (1 × 10⁵ cells/well). Sterile pipette tips were employed to scrap the monolayers after 24 h. These cells were fixed in four percent formalin for 20 min and then colored with two percent crystal violet. For measurement of the wound closure rate Olympus microscope (magnification, ×200; Olympus India, Mumbai, India) was employed.

2.2.4. Reporter assay

TOP and FOP flash assays were carried out in HaCaT cells. HaCaT cells were placed in the 24-well plates (1 × 10⁵ cells/well). After 24 h duration co-transfection of cells was done by Eugene 6 (Promega Biotech India Pvt. Ltd, Mumbai, India) by employing Luciferase reporter construct (TOP or FOP Flash) and pRL-CMV-Renillareporter plasmid. Then AMF (5 or 10 μg/mL) and its constituents (10 or 20 μM) were added to the HaCaT cells for another 24 h. For all the procedures we strictly followed manufacturer's guidelines for the assays. Luminometer (BioTek Instruments Pvt. Ltd, Mumbai, India) was used to record the values of transcriptional activity.

2.2.5. Enzyme-linked immunosorbent assay (ELISA) and NO assay

RAW 264.7 cells were treated with ethanolic extracts of AMF (50, 100 or 200 μg/mL) and its constituents (20 or 40 μM) for one hour. These cells were then stimulated with LPS (*Escherichia coli*, serotype 0111 Merck Ltd, Mumbai, India) (1 μg/mL) for a

duration of 24 h. For determination of PGE₂ secretion ELISA was applied to the media from the cell cultures according to the manufacturer's guidelines (Sandwich ELISA kit, Merck Ltd, Mumbai, India) (Li et al., 2014). Nitrite (indicates Nitric oxide production;) levels were measured by putting Griess reagent (Thermo Fisher scientific, Gujarat, India) to the culture medium and mixing it thoroughly for 20 min (Bryan and Grisham, 2007). After this whole procedure absorbance was recorded at wavelength of 540 nm by spectrophotometer (PerkinElmer Privet Ltd, Delhi, India). Hs68 cells were incubated in serum-free Dulbecco modified eagle-medium with ethanolic extract of AMF (1, 5 or 10 μg/mL) and its constituents (20 μM) for 24 h. For quantification of type I procollagen cell culture media was collected and procollagen type I C-peptide enzyme immunoassay was applied to it.

2.3. Animal study

Experimental animals included seven-eight weeks old male Sprague-Dawley (SD) rats. These animals were acquired from CSIR-CDRI (Central Drug Research Institute, Lucknow, India) and housed under standard environment (24 ± 2 °C, 54% ± 5% relative humidity, 12 h light-dark cycle). Animals were allowed to acclimatize for one week and observed for any infection or disease. CPCSEA guidelines were strictly followed for all the animal experimentation (Reg. No. 1732/GO/Re/s/13/CPCSEA). Full-thickness incision wounds were created after 24 h of dorsal hair removal AMF respectively (1%, 2% or 3%) were applied topically to the wounds every day (n = 10), and wound sizes were measured 3 times a week. For collection of the lesion skins at the end of the experiment, animals were anaesthetized with Zoletil-Rompun mixture and stored them at -80 °C for later analysis.

2.4. Histological analysis

Skin collected from the lesions was embedded into the paraffin. Fine sections (4 mm thick) were made and after that the sections were deparaffinized in xylene. Deparaffinized sections were then rehydrated by a gradient of alcohol solution and stained with hematoxylin-eosin (H&E). These coloured slides were visualized using Olympus microscope (magnification, ×200; Olympus India, Mumbai, India). For scoring epithelisation, angiogenesis, fibrosis and collagen levels histological scoring system by Abramov was employed.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Trizol reagent (Invitrogen; Merck Ltd, Mumbai, India) was employed for extracting total RNA out of skin collected from the lesions and it was determined quantitatively by spectrophotome-

try at 260 nm. Synthesis of cDNA was accomplished by an equivalent quantity of whole RNA with reverse transcriptase premix. Reverse transcription was carried out at a temperature of 42 °C for 55 min and was concluded by incubation at 94 °C for 5 min. PCR premix and the primer pair was used for PCR amplification

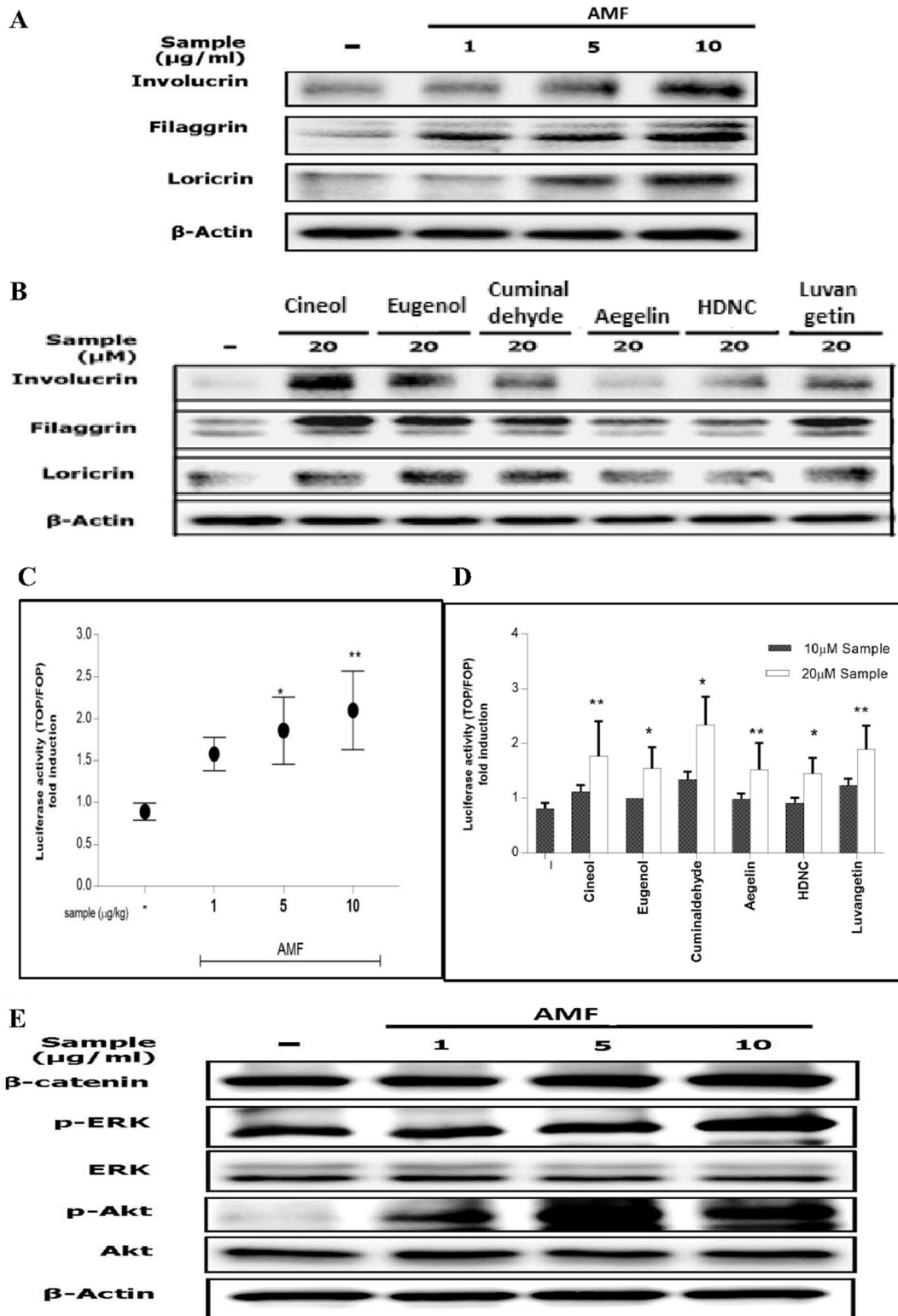


Fig. 2. Effects of AMF and its constituents on HaCaT keratinocyte migration, differentiation, and β-catenin activation. (A, B) Western blot analysis (C, D) AMF (1, 5, or 10 µg/mL) or various compounds (20 µM) were added to HaCaT cells for 24 h. Luciferase activity of HaCaT cells transfected with TOPflash/FOPflash was measured. (E, F) Western blot analysis. Results are expressed as mean ± SD of 3 independent experiments (**p < 0.01, *p < 0.05 and **p < 0.01).

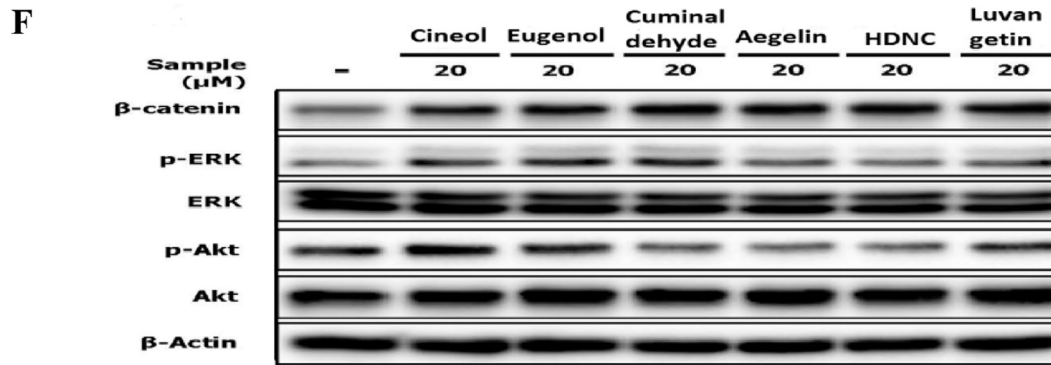


Fig. 2 (continued)

of cDNA products (3 μL). Primers were denatured at 94 °C for 5 min before the initiation of PCR amplification. PCR was carried out on a GeneAmp PCR System 2700 (ThermoFisher scientific, Gujarat, India). Separation of products of PCR was carried out by 1.5% agarose gel electrophoresis and seen with ethidium bromide. Data was normalized by employing β-actin.

2.6. Western blotting analysis

RIPA buffer consisting protease inhibitors were employed for lysis of HaCaT and Hs68 cells. Cells were incubated on ice for ten minutes after lysis. Bradford method (Bio-Rad Laboratories, Mumbai, India) was employed for normalizing the protein concentrations of the lysates. An equal amount of protein (20 μg) was determined by 10% SDS-PAGE and relocated tonitro cellulose membranes. ECL Western detection reagents were used for developing protein and enhanced chemiluminescence was employed for visualization.

2.7. Statistical analysis

All the experiments were done in triplet. All values are expressed as mean ± standard deviation. Differences between groups were analyzed using a one-way analysis of variance (ANOVA) followed by Scheffe's test with software Graphpad Prism

7. ^{##}*p* < 0.01, ^{*}*p* < 0.05 and ^{**}*p* < 0.01 were considered statistically significant.

3. Results and discussion

3.1. Compound identification

With the help of HPLC and LC-MS, six bioactive compounds were isolated from *A. marmelos* flowers: Cineol, Eugenol, Cuminaldehyde, Aegelin, 1-hydroxy-5, 7-dimethoxy-2 naphthalene-carboxaldehyde (HDNC), Luvangetin having purity >98% (Fig. 1).

3.2. *A. marmelos* flower wound healing activity

3.2.1. AMF (its active compounds) mediated increment in motility and differentiation of HaCaT cells

Treatment with AMF and its constituents for 24 h drastically improved the cell motility & keratinocyte expression in given cell lines. Furthermore, protein expression in loricin, filaggrin and involucrin (Keratinocyte differentiation markers) was enhanced by AMF and its active constituents (Fig. 2A and B). For correlating woundhealing and β-catenin signaling pathway the effect of AMF and its ingredients on β-catenin activation was observed. Protein expression and nuclear translocation in β-catenin was improved in presence of AMF and its constituents (Fig. 2C-D). Keratinocyte motility was enhanced by the ERK and Akt signaling pathways,

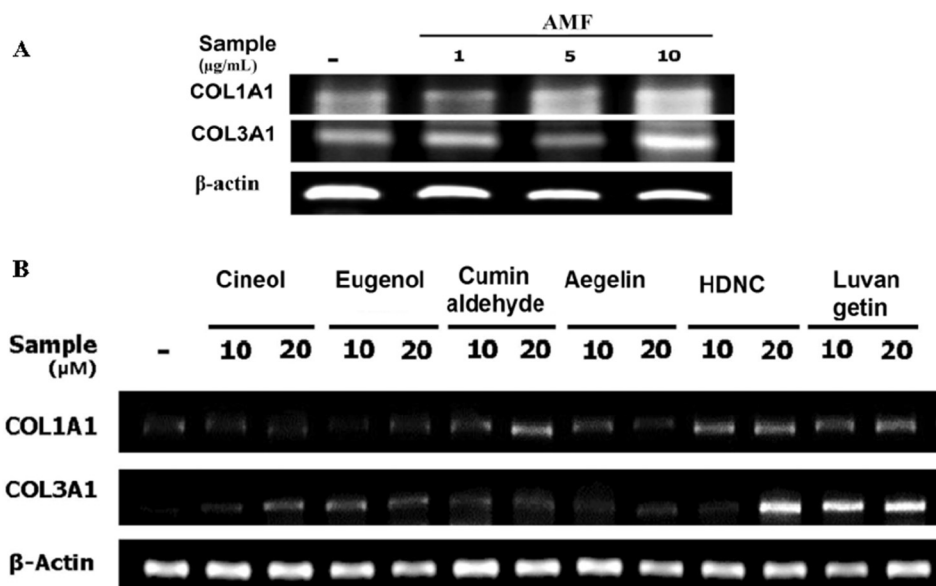


Fig. 3. Effects of AMF and its constituents on mRNA expression of collagen types I and III (COL1A1, COL3A1) and production of type I procollagen. (A, B) mRNA levels of COL1A1, COL3A1 were determined with RT-PCR. Results are expressed as the mean ± SD of 3 independent experiments (^{##}*p* < 0.01 and ^{**}*p* < 0.01).

Keratinocytes and dermal fibroblasts play an important role in skin structure formation and maintenance of homeostasis, including skin barrier construction and extracellular matrix (ECM) production (Lee et al., 2012, 2018). It was reported that the proliferation of keratinocytes is regulated by inhibition of apoptosis during early wound healing (Cheon et al., 2005) Hence, these pathways become an important target for study of wound healing process. Therefore, impact of AMF and its constituents on phosphorylation of ERK and Akt was analyzed. Results indicated significant increase in the phosphorylation of ERK and Akt (Fig. 2E and F).

3.2.2. AMF and its active compounds induce collagen expression in Hs68 cells

Levels of messenger RNA in COL1A1 & COL3A1 and production of type I procollagen were drastically amplified by AMF and several compounds (especially HDNC) as shown by RT-PCR and ELISA analysis (Fig. 3A, B). Transition of Fibroblast to Myofibroblast plays a critical role in cutaneous wound healing, Fibroblast-to-myofibroblast transition requires a combination of several types of factors, the most important of which are divided into humoural and mechanical factors, as well as cer-

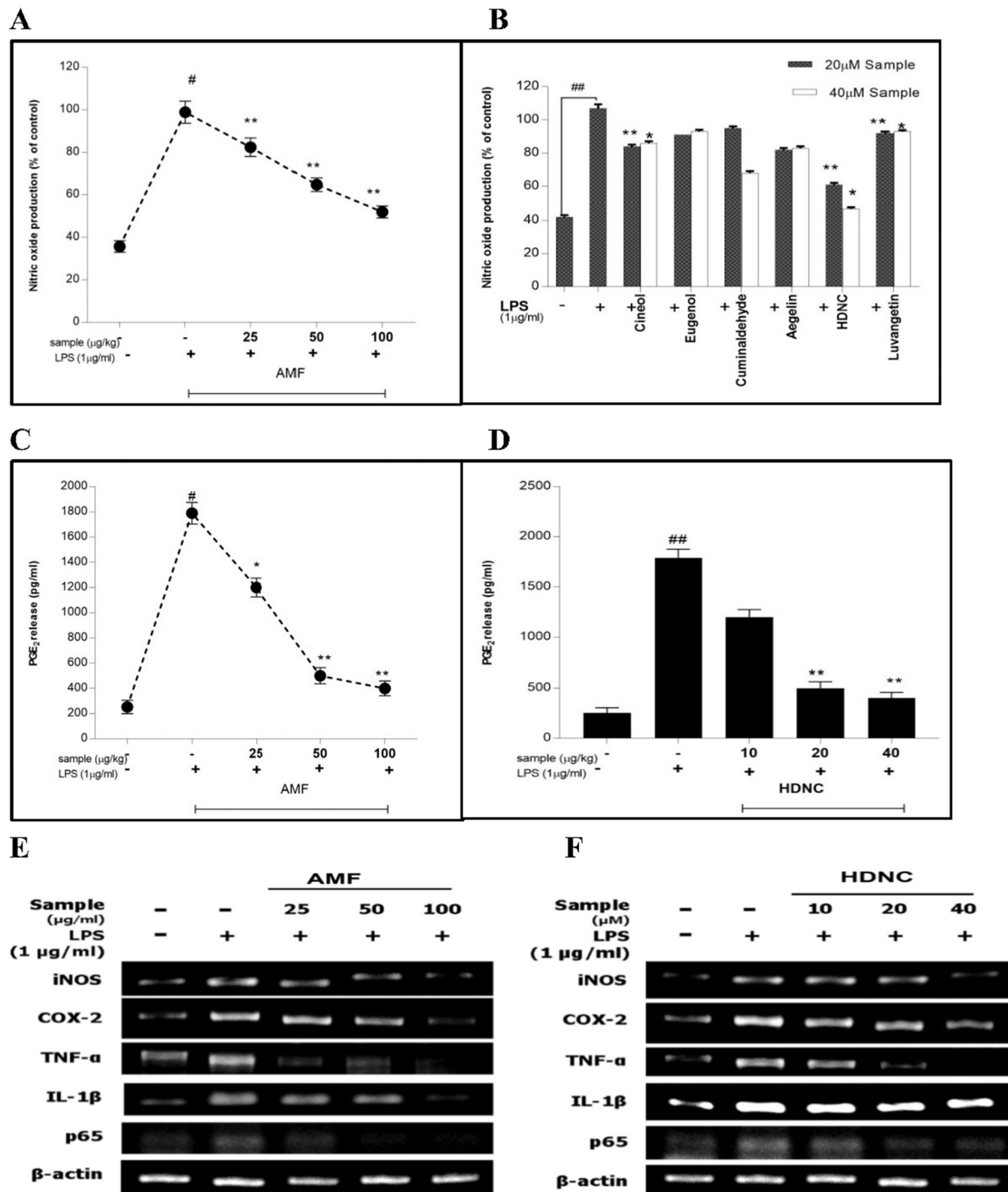


Fig. 4. Effects of treatment on RAW 264.7 cells. Dose of AMF (25, 50, and 100 µg/ml) or various compounds (20 or 40 µM). (A, B) Nitrite levels were measured by the Griess reaction. (C, D) PGE₂ levels were measured by the ELISA kit. (E, F) mRNA levels of inflammatory mediators were determined with RT-PCR (Kim et al., 2017). Results are expressed as the Mean ± SD of 3 independent experiments (^{##}*p* < 0.01, ^{*}*p* < 0.05 and ^{**}*p* < 0.01).

tain extracellular matrix proteins (Darby et al., 2014; Michalik et al., 2018).

3.2.3. AMF and HDNC inhibit NO and PGE₂ production and expression of inflammatory mediators in RAW 264.7 cells

LPS-induced NO production was reduced in presence of AMF and HDNC (Fig. 4A and B). While production of LPS-induced PGE₂ showed dose dependent decline when pretreated with AMF and HDNC (Fig. 4C and D). These are the results for LPS-stimulated macrophages. Since macrophages are responsible to secrete various types of inflammatory mediators (COX-2, IL-1 β , TNF- α , iNOS

and p6534) (Michalik, et al., 2018) Hence we analyzed those proinflammatory mediators which possessed nuclear factor- κ B-binding site in their promoter region (Kawai et al., 1999). Levels of these inflammatory mediators was measured using RT-PCR. Treatment with AMF and HDNC appreciably suppressed the production of the inflammatory mediators (Fig. 4E and F).

3.2.4. AMF promotes invitro wound healing

Topical application of AMF (1%, 2% or 3%) to the wound areas of rats largely decreased the size of wound. Fastened re-epithelialization of skin lesions was observed (Fig. 5). For

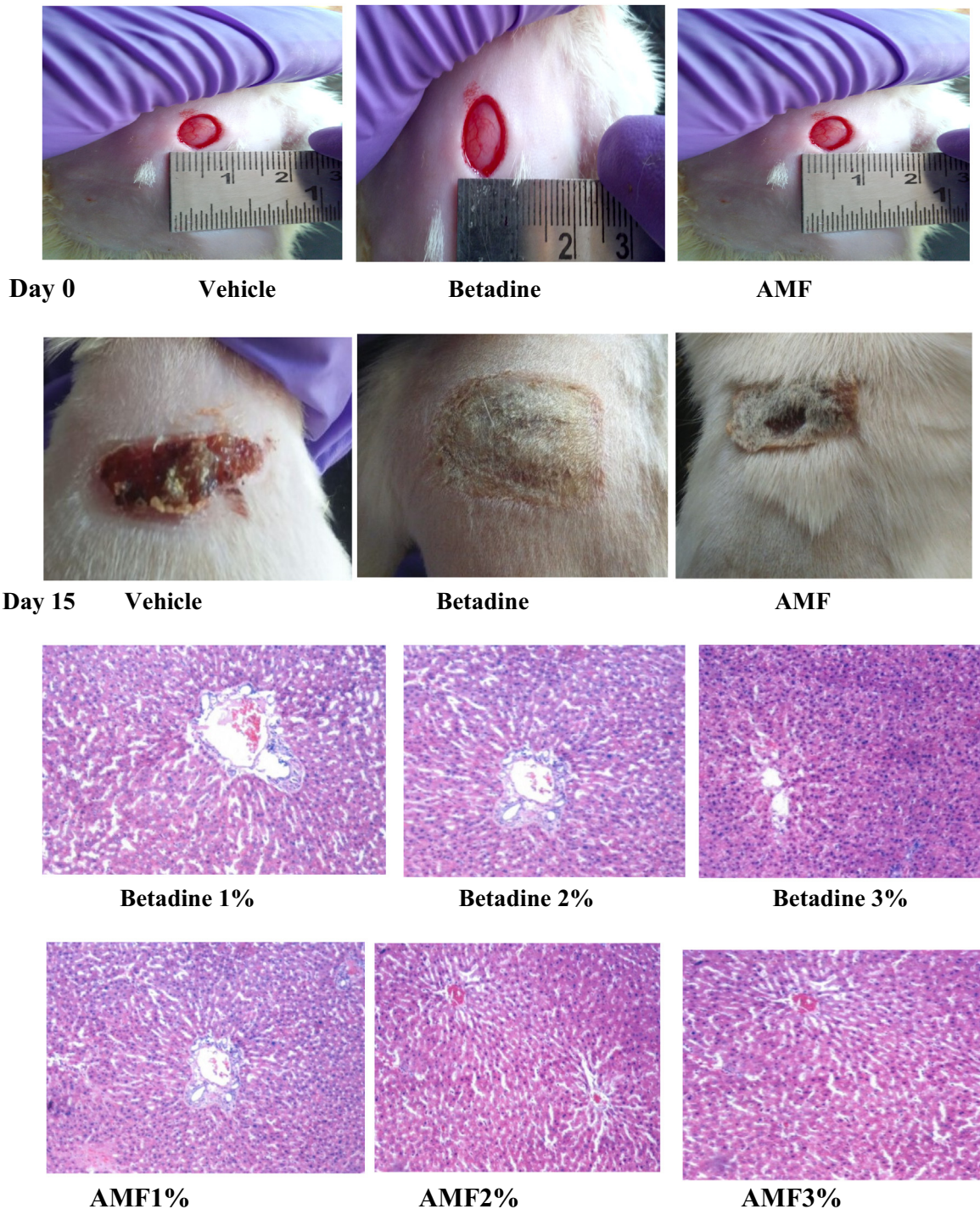


Fig. 5. Effect of AMF on wound healing (A) Representative gross images of wounded skin treated by AMF or Betadine for fifteen days. (B) Representative hematoxylin-eosin stained tissues of wounded skin treated with or without AMF or Betadine (original magnification \times 100).

histological analysis, Hematoxylin and Eosin staining revealed reversion to normal tissue structure in presence of AMF (Fig. 5). The proliferative phase of wound healing is associated with the re-epithelialisation process including collagen production and ECM remodelling. During wound re-epithelialisation stage, skin lesions close the wound and remodels the cytoskeleton by enhancing the proliferation and migration of keratinocytes (Gentleman et al., 2003). The process of tissue repair of experimentally induced cutaneous wounds in rats was evaluated in vivo based on the histopathological over time. Wound healing involves a complex and coordinated number of events which include inflammation, cell proliferation, and contraction of the wound and tissue remodeling (Albina et al., 1993).

4. Conclusion

Our study indicates that AMF constituents play a significant role in wound healing process by exerting their impact on various molecular elements involved in the process. Majority of the components of extract enhanced the migration of keratinocyte, their proliferation, and differentiation. Plant extract and its constituents caused activation of β -catenin in HaCaT keratinocytes. Hs68 fibroblast showed increased expression and production of collagen after HDNC treatment. Raw 264.7 macrophages decreased production of inflammatory mediators in presence of HDNC. Messenger RNA expression in collagen type I and III was enhanced and PGE₂ production was inhibited in presence of AMF and its constituents. Given flower extract and its constituents also reduced the production and expression of other inflammatory mediators. Re-epithelialisation process fastened in presence of AMF. Most of the drugs used for wound healing constitute synthetic molecules and growth factors which often cause severe side effects. While this didn't happen in case of AMF. Hence, AMF and its constituents may be beneficial in process of wound healing and can be a part of futuristic research for development of better alternatives for treatment of wounds.

Acknowledgements

Authors are thankful to the Director, CSIR-NBRI, Lucknow, India for providing necessary facilities. Author L.A. is also thankful to Department of Science and Technology, New Delhi. Author I.S. is also thankful to Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India for their support.

Conflict of Interest

Nil.

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