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Genetic Characterization, phytochemical analysis and evaluation of the antioxidant and wound healing activities of *Phyllanthus angustifolius* Sw. and *Phyllanthus emblica* L

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Phyllanthus **species are widely recognized for their medicinal value, with *Phyllanthus emblica* being extensively studied, whereas *Phyllanthus angustifolius* remains comparatively under-investigated. This study aimed to comparatively characterize the genetic and phytochemical profiles, antioxidant capacity, and in vitro scratch closure activity of *P. angustifolius* and *P. emblica*. Genetic characterization was performed using primers targeting chalcone synthase (CHS), cinnamate 4-hydroxylase (C4H), phenylalanine ammonia-lyase (PAL), and D-galacturonate reductase (GalUR). While both species yielded positive amplicons for key biosynthetic genes, distinct differences in the PCR amplification patterns for the phenylalanine ammonia-lyase (PAL) locus were observed. These variations reflect genetic diversity rather than functional gene absence, underscoring the need for sequencing and expression analyses to clarify metabolic regulation. Colorimetric assays showed that *P. angustifolius* had a higher total flavonoid content, whereas *P. emblica* had a higher tannin content. HPLC-DAD analysis identified several phenolic acids and flavonoids, with gallic acid and quercetin among the predominant identified constituents. *P. emblica* exhibited higher antioxidant activity across the DPPH, ABTS, and FRAP assays and showed greater scratch closure than *P. angustifolius* under the tested conditions. Notably, the stronger antioxidant and scratch closure responses of *P. emblica* coincided with differences in its overall phytochemical profile, including its higher tannin content and markedly higher gallic acid content, whereas the higher total flavonoid content of *P. angustifolius* did not correspond to greater antioxidant or scratch closure activity. These findings suggest that the observed biological differences may reflect the combined contribution of the overall phytochemical composition rather than the abundance of a single compound class. Further mechanistic and in vivo studies are required to determine the constituents and pathways contributing to these activities.**

Keywords *Phyllanthus emblica*, *Phyllanthus angustifolius*, Genetic analysis, Phenolics, Antioxidant, Wound healing

Abbreviations

ABTS	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))
AIDS	Acquired immune deficiency syndrome
C4H	Cinnamate 4-Hydroxylase
CHS	Chalcone Synthase
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl

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ECM	Extracellular matrix
FRAP	Ferric reducing/antioxidant power
GalUR	D-galacturonate reductase
HSF	Human Skin Fibroblast
HPLC	High-performance liquid chromatography
NCBI	National Center for Biotechnology Information
PAL	Phenylalanine Ammonia-Lyase
PCR	Polymerase chain reaction
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
U	Units
WST	Water Soluble Tetrazolium Salts

Normal metabolism and factors like exposure to environmental oxidants are responsible for forming endogenous and exogenous free radicals. Reactive oxygen species (ROS) produced by the respiratory chain in mitochondria that include hydrogen peroxide H_2O_2, superoxide anion $O^-_{2,}$ or peroxide $O^{2-}{}_2$, are main contributors to cell damage disease, diabetes, AIDS, arthritis, immunological incompetence, neurodegenerative disorders, and Alzheimer's, inflammation, besides dysregulation of the healing process[1]. Wound repair is considered a complex but dynamic and arranged process, characterized by a series of overlapping phases, starting with coagulation, followed by the immune response, inflammation, proliferation, and finally remodeling. Disruption of this process affects healing, resulting in a chronic wound. The healing process also slows down by age, but the basic biology underlying chronic wounds and the influence of age-associated changes on wound healing are not understood[2]. Antioxidants can act not only as ROS scavengers by converting them into more stable molecules and protecting cells from oxidative damage, but they also reduce inflammation, and are crucial for the transitional process from the inflammatory phase to the proliferative phase of wound healing. Additionally, they support the proliferation and migration of fibroblasts and keratinocytes, responsible for tissue formation and wound closure[3–5].

P. emblica (*Emblica officinalis*, Amla, Indian Gooseberry) from the Phyllanthaceae family is highly valued in Ayurveda where all parts of *P. emblica*, fruits, flowers, seeds, leaves, and bark, have been extensively utilized in numerous traditional remedies[6]. Its fruits, seeds, and bark contain various beneficial compounds such as fixed oils, fatty acids, phenolic compounds, lignans and vitamin C[7,8] with wide range of biological activities as antioxidant effects, antitumor activity, immunomodulatory, cytoprotective properties, and anti-viral properties[9]. Other pharmacological properties include anti-jaundice, anti-dyslipidemic[10], anti-aging[11], and anti-apoptic[12], anti-inflammatory[13], liver protective[14], kidney protective[15] activities, and sugar-lowering or anti-diabetic activities[16]. However, its leaves remain underexplored despite their potential therapeutic value.

This study highlights the leaves of *P. emblica* as a new, accessible, and cost-effective source of bioactive compounds, contributing to sustainable practices by utilizing plant waste for potential use in medicine, as being previously reported for antioxidant and antibacterial properties[17,18].

Genetic diversity analysis provides valuable insights into the genetic basis of bioactive compound production. Molecular techniques such as PCR and sequencing offer a precise approach to understanding the genetic variation and ensuring the correct identification of plant species and cultivars[19]. Understanding the biosynthetic pathways of plants metabolites provide insights into their genetic and phytochemical diversity. From the key enzymes of flavonoid biosynthesis, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), and chalcone synthase (CHS), are leading the production of crucial flavonoid precursors. Similarly, the biosynthesis of vitamin C involves the conversion of methyl-D-galacturonate to D-galacturonate and further to L-galactono-1,4-lactone via D-galacturonate reductase (GalUR)[20–25].

Despite the extensive pharmacological and phytochemical literature on *P. emblica*, comparatively limited information is available regarding *P. angustifolius*, particularly concerning its phytochemical profile and biological properties in direct comparison with the better-characterized *P. emblica*. A comparative assessment under the same experimental conditions may therefore help distinguish species-specific chemical and biological characteristics and provide an initial scientific basis for further investigation of *P. angustifolius*. Accordingly, the present study was designed to compare the two species using complementary genetic, phytochemical, antioxidant, nutritional, and in vitro scratch closure assessments.

Materials and methods

Plant material collection and legal compliance

The collection of *Phyllanthus angustifolius* (Sw.) and *Phyllanthus emblica* L. leaves was formally authorized by the administration of the **Shehab Mazhar Botanic Garden**, located in El-Barageel, Giza/Cairo, Egypt (Latitude: 30.042689° N, Longitude: 31.245618° E). Sampling was carried out during the flowering stage in August 2020. Plant authentication was performed by Mrs. Therese Labib, Botanical Specialist and Consultant at the Orman and Qubba Botanical Gardens. The collection process strictly adhered to all relevant institutional, national, and international guidelines and legislation. Furthermore, field studies and sample collection complied fully with the International Union for Conservation of Nature (IUCN) Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES). The studied *Phyllanthus* species are not classified as endangered or threatened. Voucher specimens were deposited in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University (Voucher Nos. 18-05-2022I and 18-05-2022II). Fresh leaves from both species were immediately frozen and stored at – 80 °C until subsequent DNA extraction.

Chemicals and reagents

Folin–Ciocalteu's reagent from LobaChemie (Mumbai, India), 2, 2-Diphenyl1-picrylhydrazyl (DPPH), t-butylhydroquinone (TBH), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), tannic acid, vitamin A, and ascorbic acid were from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol used for HPLC analysis were of HPLC grade and were purchased from Sigma– Aldrich (Steinheim, Germany). o-Phosphoric acid was from Sdfine Chemlimited (Mumbai, India) and formic acid was from E-Merck (Darmstadt, Germany). Phenolic acids and flavonoids used in HPLC analysis (kaempferol, quercetin, apigenin, catechin, rutin, apigenin 7-glucoside, gentisic acid, chlorogenic acid, caffeic acid, ferulic, sinapic, and rosmarinic acid) were obtained from SigmaAldrich, St. Louis, MO, USA. The other chemicals used in this study were of analytical grade and were supplied by El-Gomhouria Company (GOMAC).

Human skin fibroblast cell line was obtained by Nawah scientific Inc., Cells were maintained in supplemented DMEM media of streptomycin 100 U/mL. Penicillin with 100 units/mL and heat-inactivated fetal bovine humidified serum with 10%, carbon dioxide atmosphere 5% (v/v) at 37 °C.

Plant extraction

The collected leaves were air-dried, powdered, and kept in tightly-closed amber containers at low temperature. For the biological assays, the powder of *P. angustifolius* and *P. emblica* (100 g) was macerated with 70% methanol using a solvent-to-drug ratio of 10:1 (v/w), The extraction was performed in five successive cycles, using 1000 mL of 70% methanol for each cycle. Each extraction cycle was performed by maceration for 24 h at room temperature with occasional shaking. After each extraction cycle, the mixture was filtered, and evaporated under reduced pressure till dryness. The obtained yield for *P. angustifolius* and *P. emblica* were 13.2 and 10.6 g/100 g DW respectively.

For HPLC analysis, a separate analytical sample preparation was performed using 2 g of the powdered plant material. The material was extracted with 70% methanol and the resulting extract was acidified with 4 N HCl, followed by liquid–liquid partitioning with ethyl acetate (50 mL × 4). The combined ethyl acetate fractions were dried over anhydrous sodium sulfate, filtered, evaporated to dryness, and reconstituted in 2 mL methanol before HPLC-DAD analysis. This additional fractionation step was used as an analytical sample-cleanup and enrichment procedure for the targeted phenolic constituents prior to chromatographic analysis[26].

Quantitative estimation of total flavonoid content

A volume of 1 mL of aliquots and 1 mL of standard quercetin solution (concentrations of 100, 200, 400, 600, 800, and 1000 µg/mL) was introduced into test tubes. Subsequently, 4 mL of distilled water and 0.3 mL of a 5% sodium nitrite solution were added to each tube. After a duration of 5 min, 0.3 mL of a 10% aluminum chloride solution was incorporated. At the 6-minute mark, 2 mL of 1 M sodium hydroxide was added. The final volume was adjusted to 10 mL with distilled water and thoroughly mixed. An orange-yellow hue was observed, and the absorbance was recorded using a spectrophotometer set to 510 nm using the UV-visible Jasco V-630 instrument. The blank was performed using distilled water[27]. The total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per milligram dry extract. Data are presented as mean ± SD (n = 3).

Estimation of Total Tannin Content

Tanin content was determined by the Folin-Ciocalteu method[28]. A mixture of 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu phenol reagent and 1 mL of a 35% sodium carbonate solution was added to a 10 mL volumetric flask. The mixture was then diluted with distilled water to a volume of 10 mL. After shaking, the solution kept at room temperature for 30 min. Using the same procedure, a series of reference standard solutions of tannic acid (20, 40, 60, 80, 100 µg/mL) were prepared. The absorbance of both the test and standard solutions was measured against a blank at a wavelength of 700 nm using a UV/visible spectrophotometer. The determination of tannin content was performed in triplicate, and the results were expressed as milligrams of tannic acid equivalents per gram of dried extract mean ± SD[29]. Data are presented as mean ± SD (n = 3).

Quantitative determination of phenolic compounds by HPLC-UV

The quantitative analysis of phenolic compounds was conducted utilizing HPLC equipment[26], specifically the Agilent Technologies 1260 Infinity II series liquid chromatograph, which was fitted with an auto sampler and a diode-array detector. An Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm) equipped with a C18 guard column was used for chromatographic separation. The mobile phases consisted of acetonitrile (solvent A) and 2% aqueous acetic acid (solvent B). The flow rate was maintained at 0.8 mL/min, and the total run time was 60 min. A 10 µL aliquot was injected for each analysis. Detection was performed at 280 nm, 320 nm and 360 nm for the corresponding phenolic acid and flavonoid classes. Samples were filtered through 0.45 µm syringe filters before injection[30].

The HPLC-DAD analysis was designed as a targeted analysis of selected phenolic acids and flavonoids for which authentic reference standards were available, with selection based on their reported occurrence in *Phyllanthus* species and their relevance to the phenolic composition of the investigated plants. Therefore, the HPLC analysis was not intended to represent an exhaustive characterization of all phenolic or bioactive constituents present in the extracts.

Identification of individual phenolic compounds was performed by comparison of the retention times and UV-DAD responses of sample peaks with those obtained from authentic reference standards analyzed under the same chromatographic conditions. The reference-standard chromatograms included the phenolic acids and flavonoids reported in Table 2. Quantification was performed using the external-standard method based on peak areas, and concentrations were expressed as µg/g extract. The analytical data files were processed using

ChemStation (Agilent) Software (http://www.chem.agilent.com/en-US/products-services/software-informatics/openlab-cdschemstation-edition/Pages/default.aspx)- Revision C.01.08/C.01.09.

Because the analyses were performed in separate chromatographic sequences, minor retention-time shifts were observed between standard and sample chromatograms. Therefore, compound identification was based on retention-time agreement with the corresponding authentic standard within the observed chromatographic variation rather than on an identical absolute retention time (see Fig. 1).

Determination of vitamin content

The presence of vitamin C in powdered plant samples of *P. angustifolius* and *P. emblica* was analyzed using an HPLC assay. The powdered plant material was homogenized in a cold environment with the addition of 4% metaphosphoric acid to enhance water content. Dehydroascorbic acid was converted to ascorbic acid through the addition of 2, 3-dimercapto-1-propanol. Following centrifugation and filtration of the homogenized sample, the extract was chromatographed via HPLC after adjusting to an appropriate concentration[31].

The standards and plant material underwent saponification in a basic ethanol-water solution to facilitate the determination of vitamin A. Following this process, the mixture was neutralized and diluted, resulting in the conversion of retinol esters to retinol. Quantification of retinol and alpha-tocopherol was performed on distinct liquid chromatography systems, employing UV detection at wavelengths of 313–328 nm for retinol, and fluorescence detection with an excitation wavelength of 290 nm and an emission wavelength of 330 nm for alpha-tocopherol. The concentrations of vitamins were determined by comparing the peak heights or areas of the vitamins in the test samples against those of the standards[32]. Data are presented as mean ± SD (n = 3).

Determination of mineral content

Approximately 1 gram of each dried plant sample was homogenized, then digested by adding HNO_3 dropwise with an optimized microwave digestion system. By the end of the digestion program, a twenty-minute ventilation step cooled the vessels. After digestion, the solution was evaporated, then the residue was dissolved by 3 mL of 5% HNO_3 and was transferred into a volumetric flask and made up to 50 mL with ultrapure water[33].

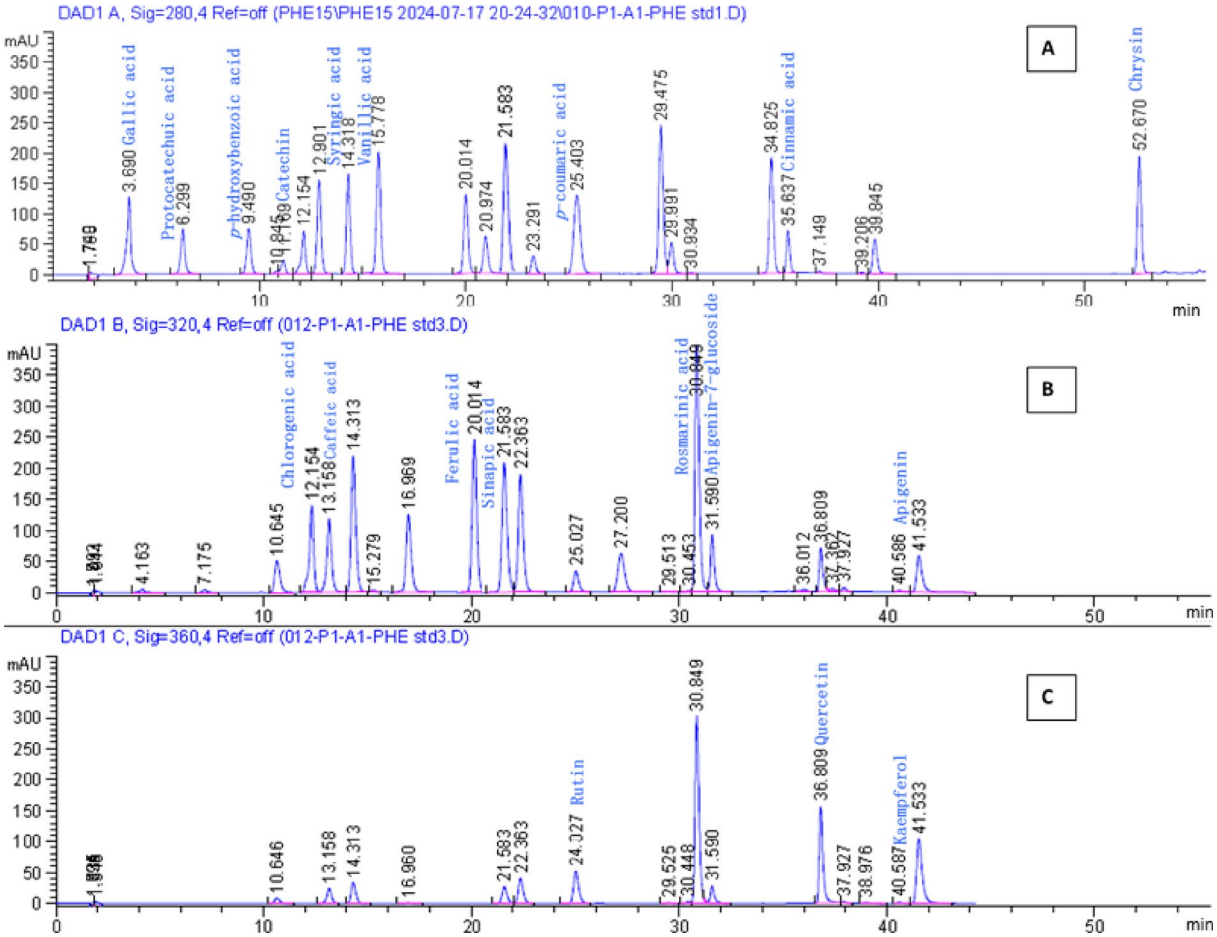


Fig. 1. HPLC chromatogram showing the standard identified phenolic compounds measured at 280 **(A)**, 320 **(B)** and 360 **(C)** nm.

Mineral concentrations were determined by the analytical laboratory using the validated routine procedure applicable to plant samples, with appropriate calibration standards and quality-control measures. The results were expressed on a dry-weight basis. Data are presented as mean ± SD ($n = 3$).

Determination of antioxidant activity

Three complementary antioxidant assays were employed to evaluate different aspects of antioxidant capacity. The DPPH assay was used to assess the ability of the extracts to scavenge the stable DPPH radical, whereas the ABTS assay evaluates radical-cation scavenging capacity and can provide complementary information regarding antioxidant activity across compounds with different polarity characteristics. The FRAP assay was used to assess the reducing capacity of the extracts based on their ability to reduce Fe^{3+} to Fe^{2+}. The combined use of these assays therefore provides a broader assessment of antioxidant potential than any single assay alone[34].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay was carried out according to the method of Boly et al.[35] with modifications to the DPPH and extract concentration ranges. Briefly, a 96-well microplates using extract concentrations from 50 to 800 µg/mL. Each well combined equal 100 µL volumes of the respective sample and a freshly prepared 0.1% methanolic DPPH solution. The mixture was incubated at room temperature in total darkness for 30 min before measuring the color attenuation spectrophotometrically at 540 nm[35]. Trolox was used as the reference antioxidant. Trolox was prepared in methanol at the same concentration range used for the reference antioxidant concentration–response curve, and the assay was performed under the same conditions as the extracts (100 µL Trolox solution + 100 µL freshly prepared DPPH solution, 30 min incubation at room temperature in the dark, followed by absorbance measurement at 540 nm). The Trolox IC_{50} was determined from the corresponding concentration–response curve and used as the reference value.

Data are presented as mean ± SD ($n = 6$) quantifing antioxidant capacity by calculating the percentage of radical inhibition relative to a control blank baseline[36].

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging assay

$ABTS^{\cdot+}$ chromophore was generated by combining 192 mg of aqueous ABTS with 140 mM potassium persulfate, that was stored in total darkness at room temperature for 24 h. Following this activation period, the radical stock was brought to a final volume of 50 mL by diluting it with methanol. After adding 10 µL of plant extract to 190 µL of diluted $ABTS^{\cdot+}$ solution, the mixture was left for 120 min, then the absorbance was measured at 734 nm. All the measurements were carried out at least three times. Trolox was used as a positive control. Antioxidant activity was calculated as moles of $ABTS^{\cdot+}$ quenched by 1 mol of Trolox[37].Data are presented as mean ± SD. The percentage of ABTS•+ scavenging was calculated according to the following equation:

$ABTS^{\cdot+}$ scavenging effect (%) = ((AB–AA)/AB) ×100 (2), where AB is absorbance of ABTS radical + methanol (Blank); AA is absorbance of ABTS radical + sample extract/standard (Test)[38].

Ferric reducing/antioxidant power (FRAP) method

The reducing power of the plant extracts was evaluated via a downscaled, microplate-adapted Ferric Reducing Antioxidant Power (FRAP) method[39]. The working reagent was generated by merging a 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (dissolved in 40 mM HCl), and 20 mM $FeCl_3$ at a volumetric ratio of 10:1:1. Inside a 96-well plate, a 190 µL aliquot of this freshly mixed reagent was introduced to 10 µL of the target sample in triplicate ($n = 3$). Following a 30-minute incubation period at room temperature in complete darkness, the generation of the blue-colored divalent Fe^{2+}- tripyridyltriazine complex was recorded spectrophotometrically at 593 nm. Data are presented as mean ± SD ($n = 3$) and expressed as Trolox equivalents against an iron sulfate ($FeSO_4$) linear regression curve, which was subsequently standardized relative to Trolox equivalents[40].

Wound Healing Assay

Cell proliferation (WST assay)

To evaluate metabolic activity, human skin fibroblasts were plated at a localized baseline density of 4×10^3 cells/well and maintained in culture for 48 h. Following this growth period, each well received a 10 µL volume of WST-1 cell proliferation reagent, after which the microplate matrix was incubated for 4 h at 37 °C in a humidified environment containing 5% CO_2. The final colorimetric changes and tracking values were captured utilizing an ELISA microplate reader, measuring sample absorbance against a control blank baseline at a wavelength of 440 nm[41].

Wound closure determination

Fibroblastic cultures were sustained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 units/mL of streptomycin under standard environmental conditions (37 °C, 5% CO_2). To prepare for scratch injury evaluation, cells were introduced onto a coated 6-well plate at a density of 3×10^5cells/well and grown overnight to form a uniform, confluent layer. Mechanical scratch wounds were introduced across the cell sheets the following morning, and non-adherent debris was cleared by washing thoroughly with PBS. While control groups received fresh normal medium, the experimental setups were replenished with drug-treated growth media. Microscopic monitoring was performed at specific intervals using an inverted microscope, returning the plates to the incubator between documentation steps. Scratch closure kinetics were calculated by evaluating the narrowing distance between the physical borders over time to determine velocity. Additionally, the scratch closure rate was calculated as a percentage of surface area reduction by manually tracing the cell-free boundaries across the timeline using Fiji/ImageJ software (https://imagej.net/

software/fiji/) -ImageJ v1.53k/Fiji v2.9.0, tracking the natural reduction of the open wound area relative to time zero[42]. Three replicates were used in this assay.

Determination of genes encoding flavonoids in *Phyllanthus* species

DNA extraction

Total genomic DNA was isolated from *Phyllanthus* leaf tissue using a modified Carlson lysis protocol, substituting the recovery phase with 0.5 volumes of NaCl alongside 0.7 volumes of isopropanol. The resulting nucleic acid yield and purity parameters were verified using both a Qubit 2.0 fluorometer and a NanoDrop 8000 spectrophotometric system[43].

Polyacrylamide gel electrophoresis of cellular protein

Denatured proteins were exposed to SDS-PAGE electrophoresis (biorad, USA)[44]. Afterwards, bromphenol blue, an indicator, was processed at 30 mA current until the gel ended. Finally, the gel was stained using Coomassie Brillant Blue R250 stain to give various colors to bacterial protein.

Supernatants were blended 1:1 with a Laemmli-type denaturation cocktail containing 64% Tris-HCl buffer (0.15 M, pH 6.8), 20% glycerol, 6% SDS, 10% 2-mercaptoethanol, and 0.1% bromophenol blue, then heated in boiling water for 3 min. Aliquots of 20 µL (equivalent to 40 µg protein) were loaded onto a discontinuous system comprising a 3.5% stacking matrix and a 7.5% polyacrylamide resolving gel containing 0.1% SDS. Electrophoretic separation was sustained at 10 °C for 4 h, applying sequential currents of 15 and 30 mA until the tracking dye front reached the gel base[44].

Electrophoresis was performed in a vertical slab mold (16 × 18 × 0.15 cm). Gels were stained with commasie blue R-250 for detecting the protein bands[45].

Polymerase chain reaction (PCR)

Target primers were identified utilizing the NCBI database alongside specialized medicinal plant repositories. Amplification reactions were executed in 25 µl total volumes consisting of 20 ng template DNA, 100 µM deoxyribonucleotide triphosphate, 20 ng decanucleotide primers, 1.5 mM $MgCl_2$, 1X Taq buffer and 0.5 U Taq DNA polymerase. Thermal cycling profiles on a Bio-Rad system initiated with a 2-minute denaturation at 94 °C, followed by 40 repetitions of 94 °C for 20 s, a 30-second primer-specific annealing window, and a 1-minute extension at 72 °C, concluding with a 10-minute final elongation at 72 °C. Amplicons were separated via 1% and 1.5% agarose gel matrices running in 1X TAE buffer alongside 100 bp and 1.0 kb plus DNA ladders. Gels were stained using ethidium bromide, imaged via a Bio-Rad Gel Doc 2000 setup under ultraviolet illumination, and fragment sizes were computed using Quantity One software analytics -Bio-Rad Quantity One 1-D Analysis Software- (https://www.bio-rad.com) -Quantity One v4.6.9-(see Table 1)[46].

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The number of replicates (n) is specified for each assay in the corresponding Methods section and figure legends. For descriptive comparison of the two *Phyllanthus* species, the mean values and corresponding variability were evaluated. Statistical significance was considered at $P < 0.05$ where inferential statistical analysis was applicable. The results of the scratch closure assay were evaluated based on the percentage of wound closure, wound width, wound area, and calculated closure rate over the observation period.

Results and discussion

The biological effects of plant extracts may arise from the combined contribution of multiple constituents; therefore, qualitative and quantitative characterization of phytoconstituents can provide insights into their possible relationship with biological activity[47].

Phytochemical analysis

Flavonoid and tannin quantitation

The quantitative assessment of flavonoids is conducted by precipitating them with aluminum chloride in an alkaline environment. The resulting color is measured colorimetrically. When comparing the flavonoid concentrations of the two extracts, *P. angustifolius* demonstrated a higher flavonoid content (128.1 mg QE/g dry extract (DE) in contrast to *P. emblica* (68.1 mg QE/g DE). Previous study on the fruits of *P. emblica* showed a

Gene	Primer
D-galacturonate reductase	TATCCCACATTCGATCCATTCATT GCTCATAATTCTTCGTCAACTTCC
Phenylalanine ammonia-lyase	PAL1FULLF 5′-ATGGCGGCGGAGAACGGTCATCACGAG-3′ PAL1FULLR 5′-CGAACTAGCAGATTGGCAGAGGTTG-3′
Cinnamate 4-hydroxylase	5′-ACTCCTAAGAGTGAGAACG-3′ 5′-CTCTGGTCTAAACTCTTCAG-3′,
Chalcone synthase	GT(G/C) GCTCC (T/A) GAAGAGCA forward CTC(A/G) GCAGT (G/T) GTGGT (G/A) AA reverse

Table 1. Design of the used primers.

flavonoid content of 101.99 ± 1.79 mg QE/g DE[48]. While leaves have shown a concentration of 3.04 ± 0.06 mg QE/g DE[49].

Tannins play a significant role in various biological activities, acting as antioxidants, anti-inflammatory, anti-aging, and antiproliferative agents[50]. In our study, *P. angustifolius* exhibited 34.62 mg TAE/g DE, while *P. emblica* showed a value of 36.75 mg TAE/g DE. Previous reports demonstrated the high phenolic content of *P. emblica*, with leaves showing a value of 95.5 ± 2.75 mg of Gallic acid equivalent/g DE[49,51], while no reports were found concerning *P. angustifolius*.

HPLC was employed to identify the major phenolic compounds in the hydromethanolic extracts of both species (Figs. 2 and 3 & S1). A total of 19 phenolic compounds were successfully identified and quantified, comprising 6 flavonoids and 13 phenolic acids. This qualitative and quantitative analysis provides valuable insights into the chemical composition of these plant species. (Table 2).

Gallic acid was the major phenolic acid identified, representing 2631.36 and 10300.42 µg/g (corresponding to ~ 0.26% and ~ 1.03% w/w) in *P. angustifolius* and *P. emblica*, respectively while quercetin was the major identified flavonoid, representing 598.56 and 184.5 µg/g (0.06% and 0.018% w/w), respectively. The high concentration of gallic acid (1.03%) compared to quercetin (0.009%) was previously reported in fruits of *P. emblica*[52].

Determination of vitamin content

Adequate consumption of beneficial nutrients, antioxidants, and vitamins allows the body to eliminate toxic components[53]. Vitamins play essential roles in the body's ability to repair and regenerate tissue. Vitamin C plays a great role as a radical scavenger, being the first line of antioxidant defense, promoting the regeneration of endogenous antioxidants GSH and catalase[54]. Additionally, vitamin C is involved in tissue repair mechanisms through collagen synthesis and angiogenesis, being able to quicken the recovery of injury four times, as well as increasing leucocyte ascorbic acid levels[55]. Vitamin A also plays an important role in all wound healing stages; it is crucial for epithelial growth, tissue granulation, angiogenesis, and collagen synthesis, besides its anti-inflammatory response[56].

Determination of vitamin content in both *Phyllanthus* species showed that *P. angustifolius* has a higher vitamin C content (82.49 mg/kg) while *P. emblica* has a higher vitamin A content (52.75 IU) (Table 3). Considerable vitamin C content was previously reported in fruits of *P. emblica*[24].

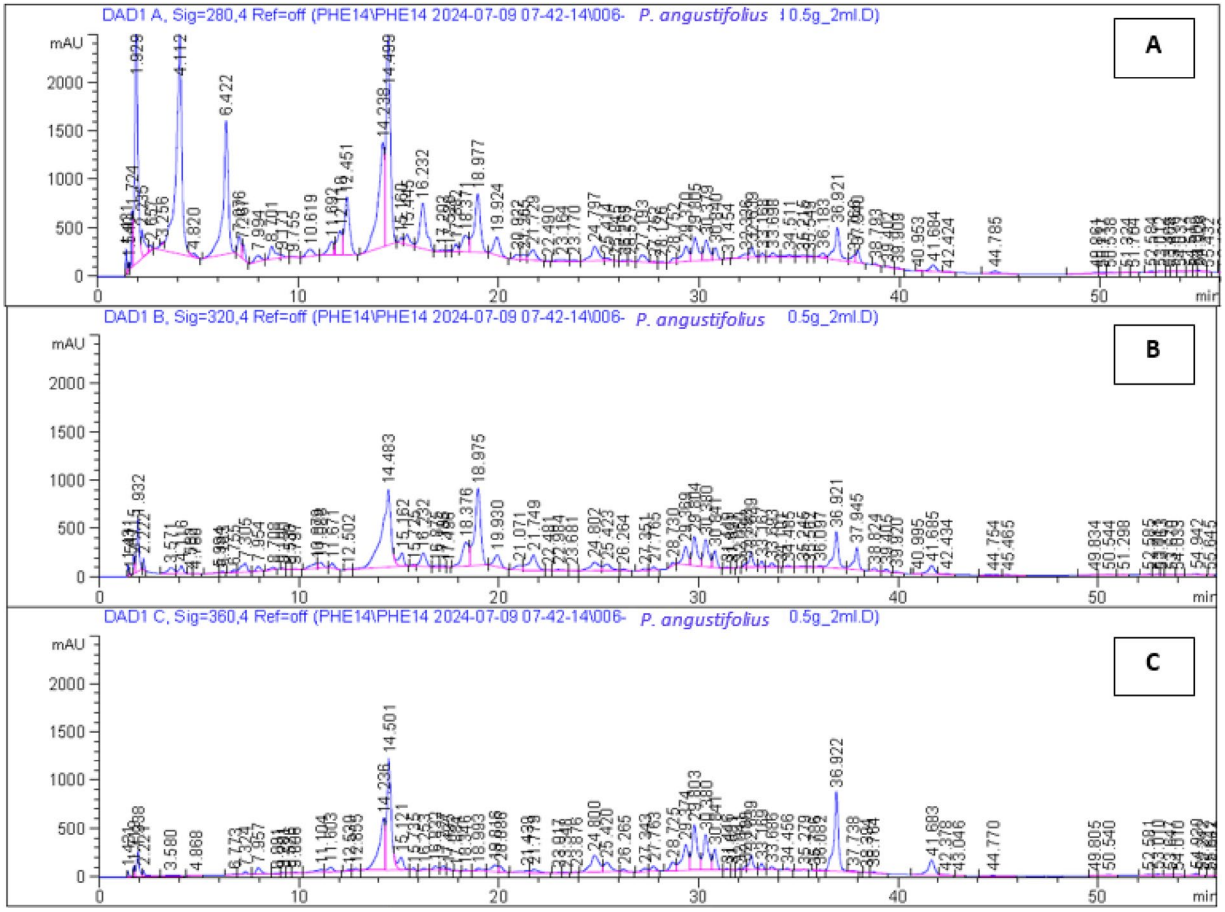


Fig. 2. HPLC chromatogram showing identified phenolic compounds in the methanolic extract of the leaves of *P. angustifolius* measured at 280 **(A)**, 320 **(B)** and 360 **(C)** nm.

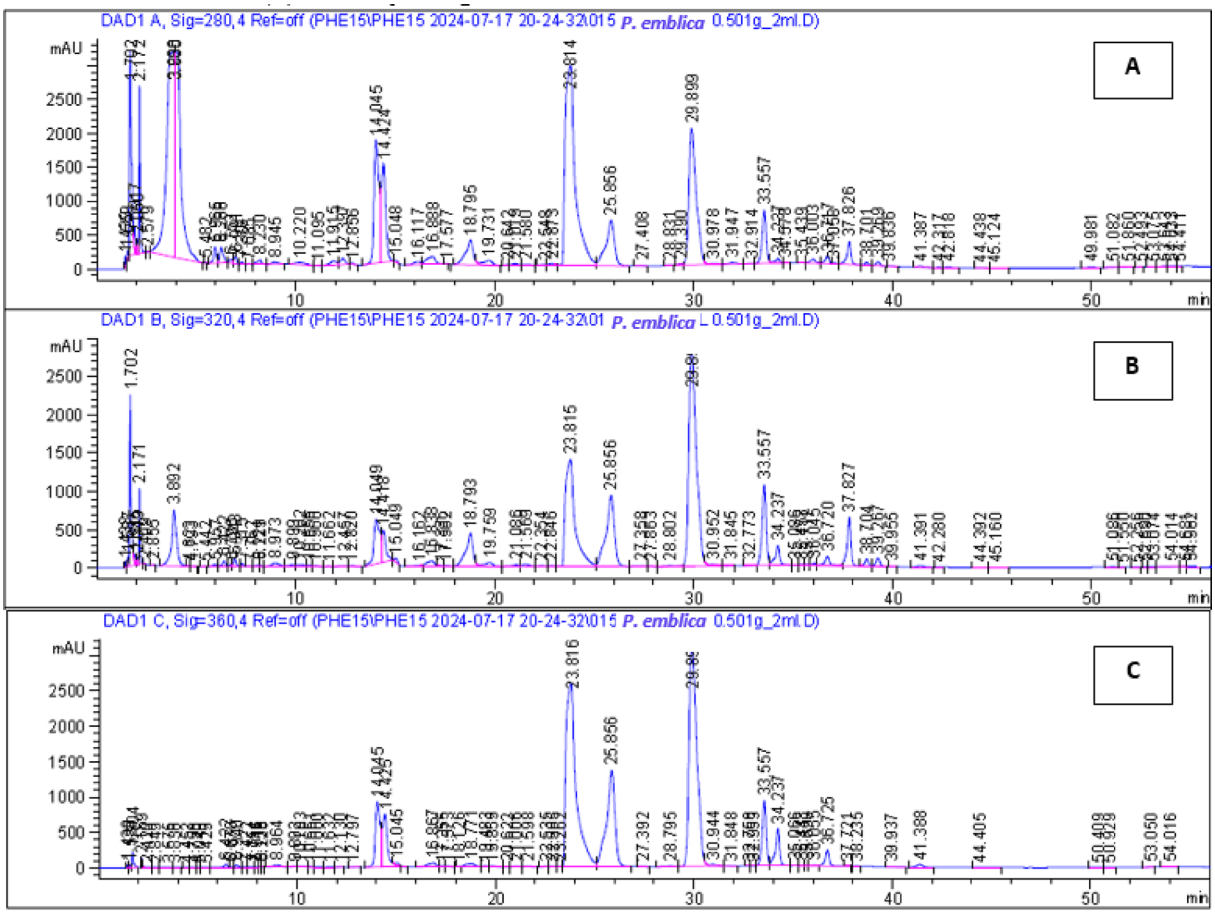


Fig. 3. HPLC chromatogram showing identified phenolic compounds in the methanolic extract of the leaves of *P. emblica* measured at 280 **(A)**, 320 **(B)** and 360 **(C)** nm.

	P. angustifolius		*P. emblica*	
Compound	**Concentration (µg/g)**	**Retention time**	**Concentration (µg/g)**	**Retention time**
Gallic acid	2631.36	3.58	10300.42	3.98
Protocatechuic acid	117.72	6.77	69.21	6.75
***p*-hydroxybenzoic acid**	160.43	9.81	159.59	10.22
Catechin	711.70	11.69	133.43	11.96
Chlorogenic acid	ND	-	15.81	16.16
Caffeic acid	774.37	14.24	456.08	14.05
Syringic acid	97.00	14.50	65.70	15.05
Vanillic acid	9.59	16.23	178.51	16.16
Ferulic acid	116.66	20.92	25.37	21.01
Sinapic acid	0.82	21.78	5.18	21.61
Rutin	246.29	23.88	ND	-
***p*-coumaric acid**	57.78	26.26	ND	-
Apigenin-7-glucoside	9.47	30.84	5.06	30.98
Rosmarinic acid	155.89	30.38	44.15	29.91
Cinnamic acid	14.03	35.56	26.19	35.69
Quercetin	598.56	36.18	184.50	36.06
Apigenin	1.65	40.99	ND	-
Kaempferol	183.79	40.95	48.11	39.96
Chrysin	2.84	53.01	5.60	53.08

Table 2. The phenolic content of *P. angustifolius* and *P. emblica*.

	Vitamins		Minerals			
	Vitamin A (IU)	Vitamin C (mg/kg DW)	Zn^{2+} (mg/kg DW)	Se (mg/kg DW)	Cu^{2+} (mg/kg DW)	Mg^{2+} (mg/kg DW)
P. angustifolius	21.61	82.49	29.117	4.881	6.610	2120
P. emblica	52.75	32.32	14.351	3.136	2.441	910

Table 3. Vitamins and mineral contents in *P. angustifolius* and *P. emblica*.

	P. angustifolius	*P.* emblica
IC_{50}(DPPH) µg/mL	15.73	9.18
µM TE/mg (FRAP)	1366.16 ± 101.10	1709.38 ± 86.033
µM TE/mg (ABTS)	2025.69 ± 78.44	3607.95 ± 286.78
Trolox (µM)	**24.42 ± 0.87**	

Table 4. Antioxidant activity of *P. angustifolius* and *P. emblica*.

Determination of mineral content

Minerals play a role in cellular homeostatic functions as an obligatory cofactor assisting enzymatic reactions. Macromolecular structures like proteins, lipid membranes, and nucleic acids are structurally stabilized by magnesium (Mg), a key metabolic cofactor[57,58]. Furthermore, Mg enhances systemic antioxidant defenses. This biological activity is vital for accelerating tissue repair mechanisms, especially when healing is compromised by diabetic foot ulcers or impaired immune functions[59].

Selenium is correlated with total antioxidant status[60]. Whereas copper plays a crucial role in blood vessel formation and hypoxia management[60], it is also involved in all stages of wound healing, modulating cytokines, and growth factors[61]. Furthermore, zinc has a role in several physiological and biochemical signaling cascades, including the immune system, enzymatic pathways, and cell structure; it promotes re-epithelialization, tissue generation, delayed wound healing, and weakened immune response[61]. On a dry-weight basis, *P. angustifolius* showed higher contents of zinc, selenium, copper, and magnesium than *P. emblica* (Table 3). Previous reports have demonstrated the high nutritional value of *P. emblica* with considerable mineral content of calcium, magnesium, and phosphorus[62].

Biological investigation

Determination of antioxidant potential

Oxidative stress is associated with aging and has a crucial effect on human total health[63]. Currently, there is no universally accepted method for determining antioxidant activity due to the diverse reaction characteristics, mechanisms, and varying phase localizations that are part of the process[64]. The low levels of ROS are favorable in protecting tissues against infection and stimulating wound healing by the production of cell surviving signals, which, when present in excess, produce oxidative stress leading to cell damage and a pro-inflammatory status. Redox imbalance dysregulates the healing process Therefore, a suitable balance between low or high levels of ROS is essential[65].

Several methods are used for the determination of antioxidant activity. The DPPH assay has high importance because it gives significant data on the capability of antioxidants to donate hydrogen atoms[66].

DPPH radical-scavenging activity was evaluated by determining the IC_{50} values of the two plant extracts. Trolox was used as the reference antioxidant under the same assay conditions. Because the extract IC_{50} values are expressed as µg/mL whereas the Trolox reference value is expressed as µM, the values were not directly compared on a numerical basis (Table 4). Radical scavenging activity was comparable in both species using the DPPH assay, where *P. angustifolius* showed an IC_{50} of 15.73 ± 0.02 µg/mL, while *P. emblica* showed an IC_{50} of 9.18 ± 0.02 µg/mL. The higher antioxidant activity observed for *P. emblica* may be associated with differences in its overall phytochemical composition, including its phenolic and flavonoid constituents. The antioxidant potential of phenolic acids and flavonoids relies on their ability to quench singlet, metal-chelating, and redox properties[67].

The antioxidant capacity is evaluated using ABTS assay by determining the ability of samples to quench ABTS radicals in comparison with Trolox. *P. emblica* exhibited pronounced activity, reaching 3607.95 ± 286.78 µM TE/mg.

Antioxidant potential is assessed by FRAP assay where the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions is quantifyed. Ferrous ions form a colored complex with 2,4,6-Tris (2- pyridyl)-s-triazine (TPTZ), exhibiting maximum absorbance at 593 nm. Reducing capacity is calculated using the linear regression equation derived from the Trolox standard calibration curve.and expressed as µM TE/mg sample. *P. angustifolius* and *P. emblica* showed values of 1366.16 ± 101.10 and 1709.38 ± 86.03 µM TE/mg, respectively.

Interestingly, the antioxidant activity did not parallel the total flavonoid content alone. Although *P. angustifolius* exhibited a higher total flavonoid content, *P. emblica* demonstrated stronger activity in the DPPH, ABTS and FRAP assays. This difference may reflect the contribution of the broader phytochemical composition of the extracts, including phenolic acids, flavonoids, tannins and other constituents, rather than the abundance of any single compound or compound class.

The use of DPPH, ABTS, and FRAP assays provides complementary rather than redundant information on the antioxidant potential of the two *Phyllanthus* extracts. Differences among the assays are consequently expected because they involve different reaction mechanisms and may respond differently to the diverse constituents present in plant extracts. The consistent trend observed across the three assays, with *P. emblica* showing greater antioxidant capacity than *P. angustifolius*, supports the reproducibility of the comparative antioxidant assessment, although the assays should not be interpreted as measuring an identical antioxidant mechanism.

The DPPH results were expressed as IC_{50} because the assay was performed over a concentration range that allowed concentration-dependent radical-scavenging responses to be evaluated. In contrast, ABTS and FRAP were expressed as Trolox-equivalent antioxidant capacity (μM TE/mg), which was the quantitative endpoint used for these assays. The FRAP assay measures reducing capacity rather than inhibition of a radical species and therefore is not directly represented by an IC_{50} value. Although IC_{50} can also be derived for ABTS under suitable concentration–response conditions, it was not used as the primary endpoint in the present study because the ABTS results were quantified relative to Trolox. Thus, the three assays were reported using their respective quantitative endpoints rather than forcing a common metric across assays with different reaction principles[68].

The lower numerical IC_{50} values obtained for the extracts should not be interpreted as evidence of greater intrinsic antioxidant potency than Trolox because the extract values are expressed on a mass-concentration basis, whereas the Trolox reference was expressed on a molar basis. Moreover, crude extracts contain multiple constituents that can collectively contribute to the measured radical-scavenging response. Consequently, the present results support the antioxidant capacity of the extracts under the tested conditions but do not establish superiority over the individual reference antioxidant[69].

Wound healing activity

Cell proliferation and cell viability measurement are considered to be key techniques in life science. The need for sensitive, reliable, fast, and easy methods led to the development of several standard assays. A proliferative assay assessed the cell viability percentage using a colorimetric assay designed to measure the relative proliferation rates of cells in culture after using different concentrations of the extract through the rate of reduction of the tetrazolium dye WST (Water Soluble Tetrazolium Salts) by an enzyme that is only functional in actively respiring, or, in other words, living cells[70]. *P. angustifolius* showed higher cell viability (70.43%) at 100 μg/mL compared to *P. emblica* (42.20%).

Wound healing is essential for the restoration of the barrier function of the skin. During this process, proliferation and migration occur to the cells at wound edges, leading to re-epithelialization of the wound surface. Numerous models of wound healing have been developed in recent years, such as scratch assay, stamp assay, thermal wounding, electrical wounding, and optical wounding using a laser. In contrast to acute wounds, chronic wounds will similarly begin the healing process but will have prolonged inflammatory, proliferative, or remodeling phases, resulting in tissue fibrosis and non-healing ulcers[71].

For understanding the wound healing capabilities of bioactive compounds, in vitro techniques like scratch assays are widely applied.

Based on the preliminary WST-1 viability assessment, 80 μg/mL was chosen as an appropriate sub-cytotoxic concentration ranging between the higher cellular viability, while avoiding the more pronounced reduction in viability observed at 100 μg/mL (70.43%).

The WST-1 assay was used to assess cellular viability and to inform selection of the extract concentration for the scratch assay; however, because proliferation was not specifically inhibited during the scratch experiment, the observed closure cannot be attributed exclusively to migration.

Cells were treated with 80 μg/mL of *P. angustifolius* and *P. emblica* extracts for 72 h. Cell migration at 0, 24, 48, and 72 h was recorded, and the wound area was quantified by tracing the cell-free area using Fiji/ImageJ software. Wound closure was calculated relative to the wound area measured immediately after scratching (0 h).

The results indicated that *P. emblica* leaf extract, at 80 μg/mL, closed the gap created by the scratch by 63.87% in 72 h compared to 56.72% for *P. angustifolius* (Fig. 4A) with wound widths of 0.75 ± 0.21 and 0.25 ± 0.07, respectively (Fig. 4B) and scratch closure rates of 0.02 and 0.02, respectively, after 72 h (Fig. 4C). The figures showed a time-dependent increase in the thickness of cells in the scratched area until the gap closed. Assay was conducted in three triplicates.

Despite the wider initial wound width, *P. emblica* achieved a higher percentage of wound closure. This may be contributed the ability of *P. emblica* to stimulate a more robust proliferative response, leading to rapid filling of the wound area with new cells correlated to a smaller wound area (Figs. 4D and 5). Additionally, the antioxidant properties of *P. emblica* may enhance extracellular matrix (ECM) production and remodeling processes more effectively, leading to quicker wound contraction and closure despite the initially larger wound width. Besides, the better effect of *P. emblica* compared to *P. angustifolius* may be correlated to the previously reported angiogenesis, leading to better vascularization and nutrient supply to the wound area, thereby supporting faster wound healing[72].

Historically utilized in traditional wound remedies, *Phyllanthus emblica* L. acts as a single agent or within multi-herbal mixtures. It was reported that in vivo rodent models validate its tissue repair efficacy, proposing that the underlying mechanisms rely on the upregulation of extracellular matrix (ECM) components, enhanced collagen synthesis, and the restoration of local antioxidant status[69].

A limitation of the present scratch closure assay is that the observed reduction in the cell-free area may reflect a combination of cellular migration and proliferation, as proliferation was not specifically inhibited during the assay. Future studies incorporating proliferation-controlled migration assays and mechanistic markers will be required to distinguish these processes.

Data are presented as mean ± SD; error bars represent SD. Scratch dimensions were quantified using ImageJ software. Statistical significance was not assigned where inferential statistical analysis was not performed.

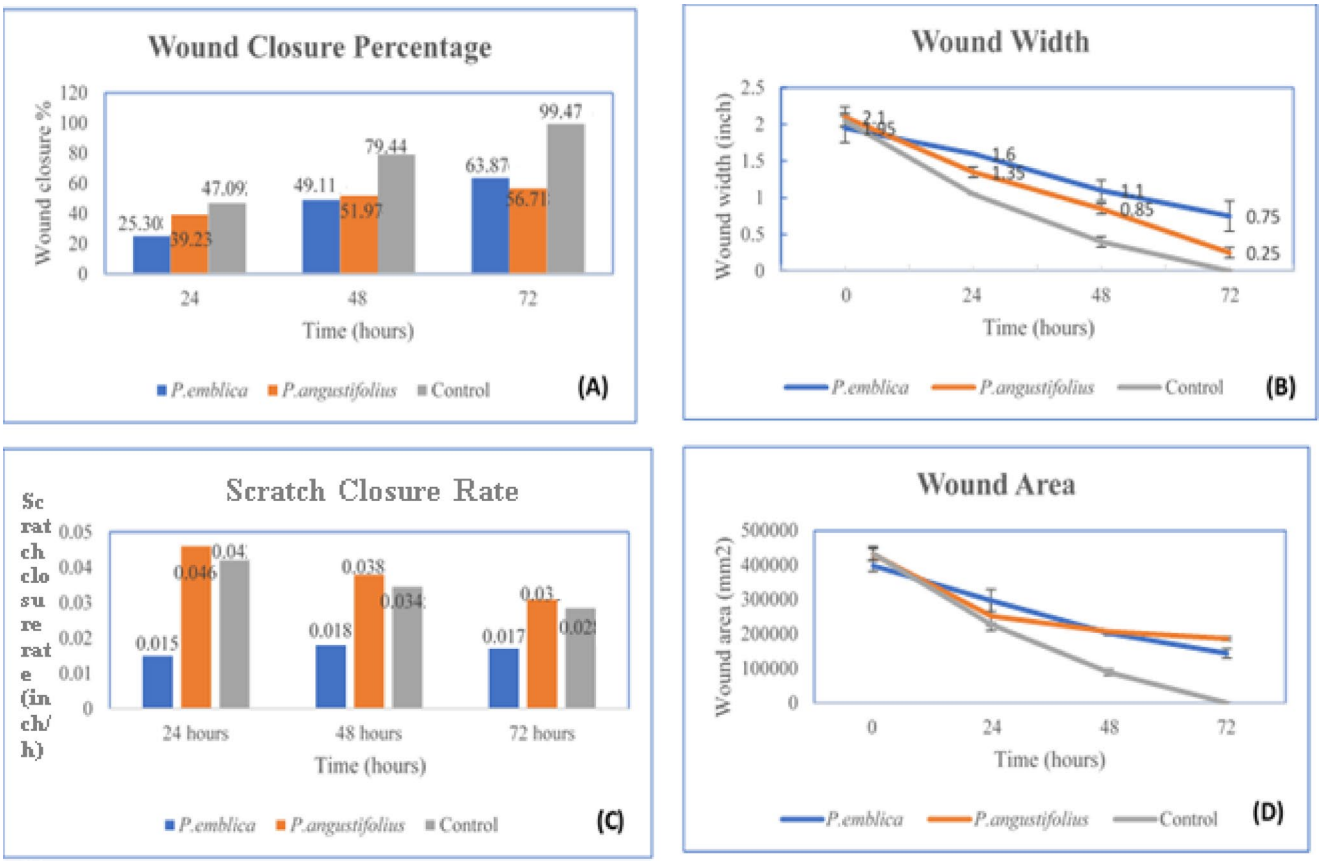


Fig. 4. In vitro scratch-closure assay of *P. angustifolius* and *P. emblica* leaf extracts. Cells were treated with 80 µg/mL of each extract and monitored at 0, 24, 48, and 72 h. (**A**): Wound closure percentage, (**B**): Wound width, (**C**): Scratch closure rate (**D**): Wound area.

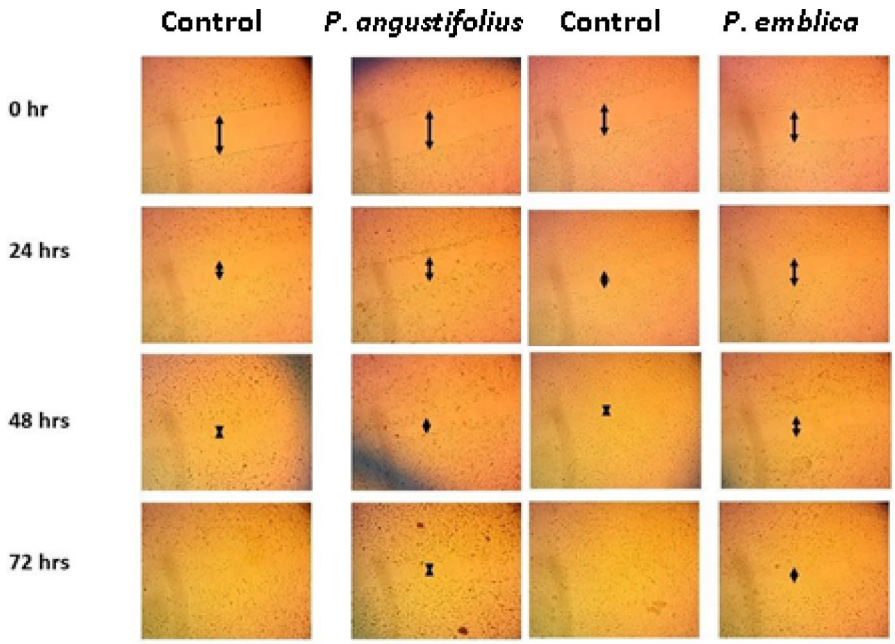


Fig. 5. Scratch assay analysis of *P. angustifolius* and *P. emblica* on Human Skin Fibroblast (HSF). Arrows indicate the scratched gap.

The HPLC-DAD analysis provided a targeted profile of phenolic constituents in the two species, including phenolic acids and flavonoids. Because the HPLC samples underwent an additional acidification and ethyl acetate partitioning step, the reported HPLC concentrations represent the analytically recovered phenolic fraction rather than the quantitative composition of the crude hydroalcoholic extracts used in the biological assays. Nevertheless, the detection of these constituents provides evidence for the presence of phenolic compounds in the plant material that may contribute to the antioxidant properties of the extracts. The stronger antioxidant and scratch closure responses observed for *P. emblica* may therefore be associated with differences in its overall phytochemical composition. The identified phenolic constituents provide a chemical basis for considering possible associations between the phytochemical profiles and the biological activities observed for the two species. However, the present data do not allow assignment of the observed biological activities to individual compounds.

Genetic characterization

Polyacrylamide gel electrophoresis of cellular protein

SDS-PAGE analysis of cellular proteins from *P. angustifolius* and *P. emblica* revealed broadly similar major protein profiles, together with several differences in the distribution and intensity of individual bands (Fig. 6). The shared major bands indicate similarities in the detectable cellular protein profiles of the two species, whereas the presence of species-specific bands indicates differences in their protein patterns. These differences may reflect variation in the cellular protein composition of the two species; however, the SDS-PAGE profiles were interpreted as qualitative protein-pattern differences rather than as evidence of differential gene expression or specific protein abundance.

Polymerase chain reaction (PCR)

The genetic analysis of *P. angustifolius* and *P. emblica* included the examination of genes encoding key enzymes such as chalcone synthase (CHS), phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and D-galacturonate reductase (GalUR). The annotation of high-confidence gene sets of *P. emblica* was performed using the NCBI non-redundant (nr) database; the protein sequences of all the available enzymes were downloaded from the Swiss-Prot or NCBI databases, while D-galacturonate reductase (GalUR) was obtained from strawberry plant species[73].

Investigation of both species for the primer for the GalUR gene (Fig. 7a) indicated the presence of distinct bands. A positive band appeared between the 100 and 150 bp markers. This band indicates the presence of the GalUR gene, which is a key gene in the biosynthesis of vitamin C. While for the PCR analysis of phenylalanine ammonia-lyase (PAL), sample 1 (*P. angustifolius*) showed a distinct amplification product, indicated by the presence of distinct bands at 50, 100, and 250 bp. In contrast, *P. emblica* showed bands at 75, 100, and 175 bp, indicating differences in the observed amplification profiles in PAL between the two species (Fig. 7b). For cinnamate 4-hydroxylase (C4H), both species (*P. angustifolius* and *P. emblica*) showed positive bands, indicating the presence of the C4H gene. However, the band intensity differed, where *P. angustifolius* showed a more intense amplification product; however, band intensity was not interpreted quantitatively (Fig. 7c). In the PCR analysis for chalcone synthase (CHS), both samples (*P. angustifolius* and *P. emblica*) showed positive bands (Fig. 7d). Two bands appear in *P. angustifolius* at 75 and around 125 bp. While bands at 100 and 200 bp were observed in

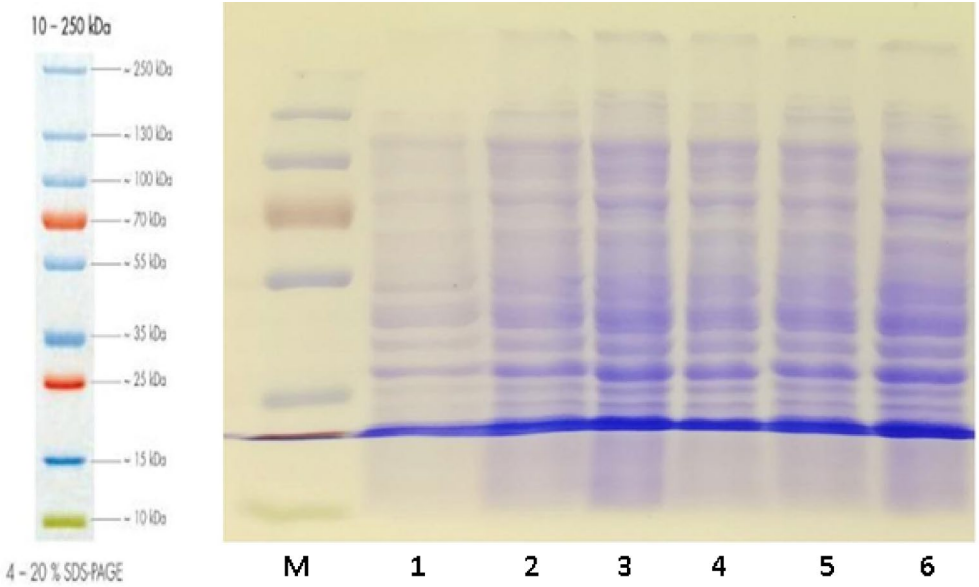


Fig. 6. SDS-PAGE protein profiles of *P. angustifolius* and *P. emblica*. Lanes 1–3 represent *P. angustifolius* samples, while lanes 4–6 represent *P. emblica* samples. M represents the molecular-weight protein marker (10–250 kDa). Proteins were separated using 4–20% gradient SDS-PAGE.

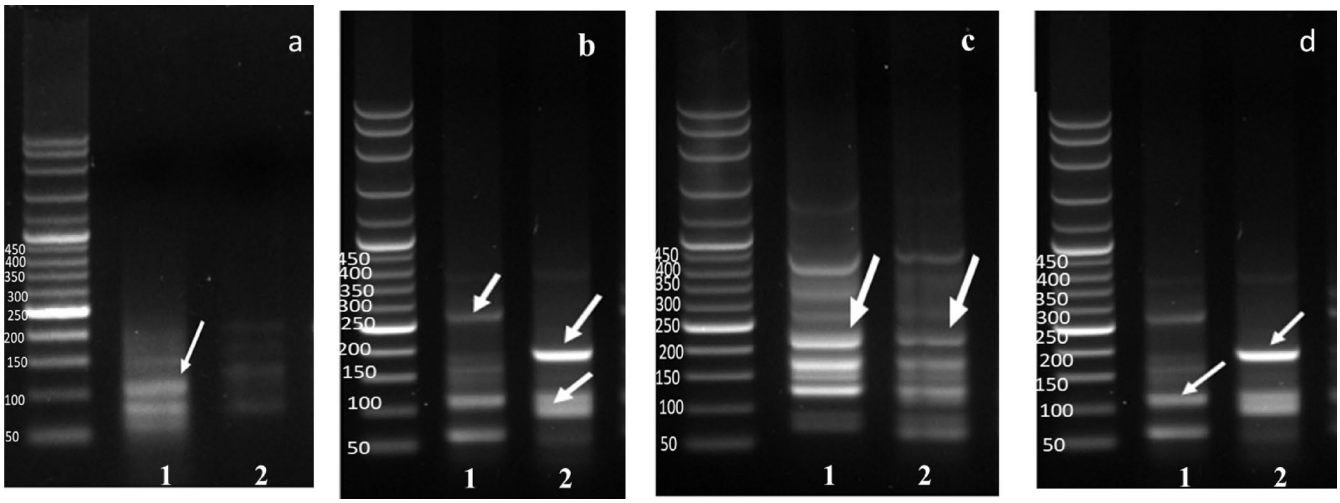


Fig. 7. PCR amplification of genes involved in the phenylpropanoid/flavonoid biosynthetic pathways in *P. angustifolius* and *P. emblica*. PCR products for **(a)** GalUR, **(b)** PAL, **(c)** C4H, and **(d)** CHS are shown. In each panel, lane 1 represents *P. angustifolius* and lane 2 represents *P. emblica*. The molecular-weight DNA marker is shown at the left of each panel, with fragment sizes indicated in base pairs (bp). Arrows indicate the amplified products.

P. emblica. Distinct amplification products or size variants were observed for CHS gene in *P. angustifolius* and *P. emblica*.

This genetic diversity may be related to insertions, deletions, or variations in the intronic or exonic regions of the gene, leading to PCR products of different lengths. Additionally, the presence of multiple bands in each species might also suggest that there are multiple copies of the gene or closely related paralogs in the genome, each producing a different PCR product[74].

These results demonstrate clear differences in the observed PCR amplification profiles for the PAL target sequence between *P. angustifolius* and *P. emblica* under the tested conditions. However, conventional endpoint genomic PCR indicates sequence amplification behavior rather than establishing gene absence, expression levels, or active biosynthetic flux. Thus, these amplification pattern variations should not be construed as definitive proof of alternative or compensatory biosynthetic pathways, which require future validation via transcriptomic and enzymatic studies[75].

Based on this, both *P. angustifolius* and *P. emblica* shared common ancestors (the same genetic pool), yet differences in environmental conditions and physiological cycles may lead to differences in some genes (proteins, or PCR amplification levels). But in this study, we were concerned with the proteins involved in phenolics biosynthesis correlated with their biological activities[76,77]. Despite the lower intensity of bands for phenylalanine ammonia-lyase (PAL) in *P. emblica*, the presence of chalcone synthase (CHS) and cinnamate 4-hydroxylase (C4H) indicates that the flavonoid biosynthesis pathway is still active. This suggests that *P. emblica* may utilize alternative pathways or regulatory mechanisms to bypass the need for PAL or to achieve similar metabolic outcomes. Those results indicate genetic diversity between *P. angustifolius* and *P. emblica*, which could be associated with differences in their biosynthetic pathways or regulatory mechanisms related to flavonoid and tannin synthesis. Further sequencing and analysis would be needed to pinpoint the exact cause of the size variation in the PCR products and the regulatory networks and alternative pathways that might compensate for the lack of PAL PCR amplification in *P. emblica*. Comparative studies on PCR amplification, enzymatic activity, and metabolic profiling under various environmental conditions could provide deeper insights into the underlying mechanisms and adaptive strategies employed by *P. emblica*.

Conclusion

This study provides a comprehensive comparative analysis of the phytochemical profile, in vitro biological activities, and genetic identification of *P. angustifolius* and *P. emblica*. Phytochemical analysis revealed the higher flavonoid content of *P. angustifolius* (confirmed by a high quercetin content) compared to a high tannin content in *P. emblica* (correlated with a high gallic acid content). Additionally, *P. angustifolius* exhibited higher vitamin C, selenium, and zinc contents, while *P. emblica* has a higher vitamin A content. The wound healing assays demonstrated that *P. emblica* leaf extract produced a greater percentage of gap closure than *P. angustifolius*. Those results are well correlated to the higher antioxidant activity, as confirmed by DPPH, ABTS, and FRAP assays. While both species express key enzymes involved in the biosynthesis of flavonoids, distinct variations in the PCR amplification patterns for the phenylalanine ammonia-lyase (PAL) gene were observed between the two species under the applied experimental conditions. These profile variations suggest alternative biosynthetic pathways or compensatory mechanisms in *P. emblica*. Both species expressed chalcone synthase (CHS) and cinnamate 4-hydroxylase (C4H), indicating their involvement in the flavonoid biosynthesis pathway. While both species exhibited positive PCR amplification for CHS and C4H, distinct differences in the amplification

patterns of PAL were noted. These profile differences demonstrate genetic diversity at the tested locus; however, functional and sequencing analyses remain essential to establish their metabolic implications. This research not only highlights the medicinal importance of *P. emblica* but also introduces *P. angustifolius* as a subject of pharmacological interest. By elucidating the chemical and biological characteristics of these species, we lay a foundation for future research to explore the underlying mechanisms and identify the active compounds responsible for their therapeutic effects.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Author contributions

AME, is the main supervisor of the research work and contributed to study selection and design, revision of the manuscript. ROB, conceptualization, methodology. MMO conceptualization, methodology. SKG performed the experiments and generated the data, data analysis, and drafting of the manuscript. HE, DNA extraction and genetic characterization and analysis. All authors have read and approved the final version of the manuscript and agree with the order of presentation of the authors.

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Declarations

Competing interests

The authors declare no competing interests.

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