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In Vitro Evaluation of the Angiogenic Potential of *Plantago Major* Extract in Enhancing the Wound-Healing Process

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ABSTRACT

Plantago major extracts have demonstrated considerable efficacy in promoting wound healing. However, there is limited research evaluating the angiogenic potential of *P. major* extract using Matrigel-based assays alongside gene expression analysis of key angiogenic markers such as vascular endothelial growth factor A and vascular endothelial growth factor receptor 2. This study evaluated the angiogenic effects of *P. major* extracts obtained by several extraction techniques: ultrasound-assisted extraction for leaves and both ultrasound-assisted extraction and maceration for non-leaf components using *in vitro* human umbilical vein endothelial cells. Aucubin content was analyzed using an HPTLC-densitometer, revealing the highest aucubin content in the non-leaves extract obtained from ultrasound-assisted extraction (16.75%). Furthermore, an *in vitro* experiment with human umbilical vein endothelial cells was conducted to assess *P. major* extract's effect on cell viability, migration, and the formation of capillary-like structures (tube formation). All extracts maintained cell viability above 80% at concentrations below 250 µg/mL. The leaves extract obtained from ultrasound-assisted extraction at 31.25 µg/mL showed the greatest wound closure (80.29%) and the highest branching length (2756.41 cm). However, gene expression of vascular endothelial growth factor A and vascular endothelial growth factor receptor 2 showed no significant upregulation in the leaves extract obtained from ultrasound-assisted extraction-treated group, and low replication numbers limited some assays. These findings suggest that *P. major* leaf extract may promote angiogenesis through mechanisms beyond vascular endothelial growth factor signaling, but further studies with higher statistical power and broader mechanistic approaches are warranted.

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by the body's inability to metabolize and control blood glucose, resulting from either excessive insulin secretion by the pancreas or the ineffectiveness of insulin in regulating blood glucose levels [1]. One of the complications experienced by diabetes patients is diabetic foot ulcer (DFU). DFU is a significant consequence in people with diabetes, impacting between 19% and 34% of the 537 million people with the disorder. Approximately 20% of DFU patients require lower extremity amputation (LEA), either minor (below the ankle) or major (above the ankle), causing a decrease in the patient's quality of life and increasing mortality rates [2, 3].

Delayed wound healing in diabetic patients is primarily caused by impaired angiogenesis [4]. Several studies have shown that prolonged hyperglycemia, hyperlipidemia, and oxidative stress in diabetic patients can disrupt angiogenesis in diabetic wounds. This leads to reduced oxygen and nutrient supply to the wound site, as well as a diminished influx of reparative cells through neovascularization, thereby delaying wound healing [5]. While current angiogenic therapies such as growth factors or stem cell-based treatments show potential, their clinical application remains limited by high cost, instability, and safety concerns [6,7]. These limitations underscore the need for safer, more accessible alternatives, particularly natural products with pro-angiogenic potential. Among various medicinal plants, *Plantago major* L., a perennial herb belonging to the Plantaginaceae family, has long been used in traditional medicine across Europe and Asia to treat wound and skin problems [8,9]. In several Asian, European, and American countries, both leaves and the whole plant of *P. major* are traditionally applied topically or as a decoction for wound healing, indicating that bioactive components are present in several parts of the plant [9,10]. *In vitro* and *in vivo* investigations have shown that the leaves and seeds of *P. major* exhibit anti-inflammatory, antioxidant, and wound-healing properties [11]. Different plant components, including leaves, seeds, roots, and flowers, possess bioactive substances such as flavonoids, terpenoids, phenolics, iridoid glycosides, and polysaccharides, which are believed to enhance their wound healing capabilities. Plantaginin, baicalein, and hispidulin are flavonoids that serve as antioxidants and free radical scavengers, facilitating wound healing [12]. Aucubin, an iridoid glycoside, is acknowledged for its antioxidant and anti-inflammatory qualities that may promote wound healing [13].

The extraction technique substantially affects the quantity and bioactivity of botanical extracts. Traditional maceration techniques, while prevalent, typically necessitate extended processing durations and may compromise heat-sensitive chemicals [14]. Conversely, ultrasound-assisted extraction (UAE) has benefits like decreased extraction durations, lower energy use, and maintenance of bioactive chemical integrity [15]. Notwithstanding these developments, few studies have systematically examined the bioactivity of extracts derived from various plant sections and extraction methods, especially concerning angiogenesis and wound healing.

P. major is widely used in traditional medicine and has been proven to have wound-healing properties. Its multi-component bioactive compounds offer a promising alternative to target angiogenesis. However, the molecular mechanisms that contribute to angiogenesis remain unexplored. There are study gaps in the study, particularly regarding the effect of extracts from different plant parts and extraction procedures on angiogenesis in wound healing. Furthermore, there is inadequate data on *P. major*'s angiogenic activities using Matrigel.

This study assesses the angiogenic capabilities of *P. major* leaf and non-leaf extracts obtained via maceration and UAE. It aims to elucidate the role of various extraction methods and plant components in promoting wound healing by examining their effects on endothelial cells using viability, migration, and tube formation assays. The findings will help shed light on the molecular basis of *P. major*'s wound-healing abilities and support its potential as a phytotherapeutic agent.

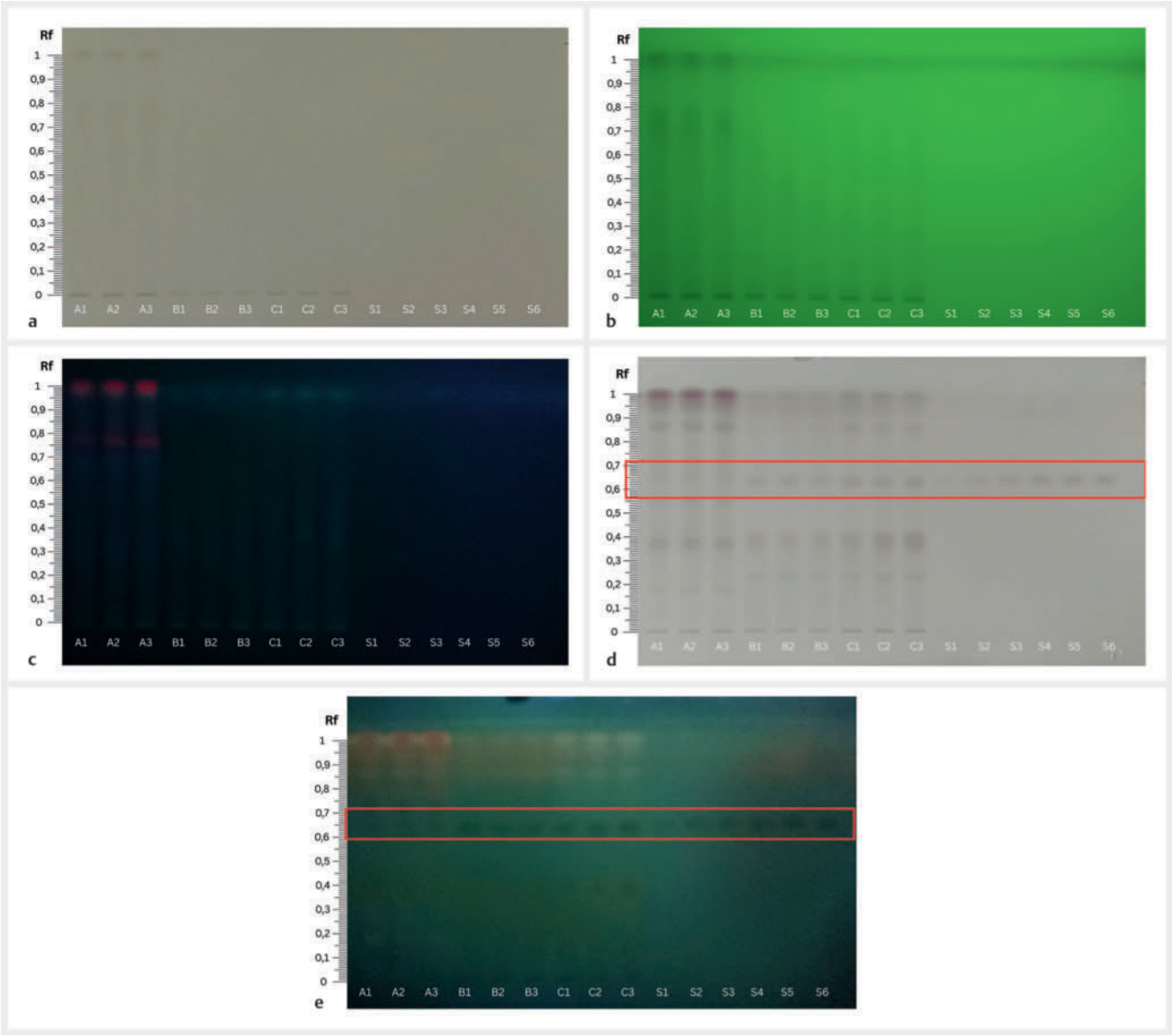
Results

Determination of aucubin content in the non-leaves extract obtained from maceration (NLC), leaves extract obtained from ultrasound-assisted extraction (LU), and non-leaves extract obtained from ultrasound-assisted extraction (NLU) was carried out using the HPTLC-densitometry, which showed that the chromatogram profiles of all the extracts have purplish brown spots in visible light and $UV_{366\,nm}$ after being sprayed with anisaldehyde and the same retention factor (Rf) as the aucubin standard at Rf 0.67 (▶ **Fig. 1**). NLU has the highest aucubin content (16.75 ± 1.66%), followed by NLC (14.17 ± 5.38%) and LU (5.98 ± 2.43%), with NLU showing a statistically significant difference compared to LU ($p < 0.05$) (▶ **Fig. 2**).

Following the determination of aucubin content in the extracts, a cell viability assay was conducted to evaluate the cytotoxic potential of each extract on human umbilical vein endothelial cells (HUVECs). Aucubin standard, used as a positive control at 34.63 µg/mL due to its known wound-healing properties [16], was compared alongside NLC, LU, and NLU extracts across concentrations of 15.625 to 250 µg/mL. Aucubin, NLC, and LU at a concentration of 250 µg/mL significantly reduced HUVEC viability ($p < 0.0001$) compared to control cells. Meanwhile, NLU showed the highest viability at 62.5 µg/mL concentration ($p < 0.05$) compared to control cells. These data imply that extracts with a concentration of 15.625–31.25 µg/mL have no cytotoxic effects on cells and can be examined for their potential therapeutic properties. ▶ **Fig. 3** depicts the percentage of cell viability for extracts on HUVEC.

The effect of the extracts on cell migration was then evaluated using the scratch assay. Extract concentrations of 7.81 and 31.25 µg/mL were used in the migration test based on cell viability tests, which produced cell viability of not less than 90%. Aucubin with a 34.63 µg/mL concentration was used as a positive control. After 24 hours, it was seen that the cells migrated to close the scratches. LU extract at 31.25 µg/mL significantly increased wound closure compared to the control cell ($p < 0.0005$) and showed the highest wound closure percentage (80.29%). In contrast, except for the LU extract, the aucubin and extract groups at a concentration of 7.81 µg/mL did not exhibit a significant increase in wound closure compared to the cell control. The NLU extract at 31.25 µg/mL demonstrated a significant enhancement in wound closure percentage relative to the NLC extract at 7.81 µg/mL ($p < 0.05$). The LU extract at a 31.25 µg/mL concentration demonstrated a statistically significant enhancement in wound closure percentage compared to the NLC and NLU extracts at 7.81 µg/mL ($p < 0.05$) (▶ **Fig. 4**).

The effect of *P. major* extract on angiogenesis was evaluated using the tube formation assay. LU extracts with 7.81 and 31.25 µg/mL concentrations were selected based on the migration assay results. The results of the tube formation test can be seen in ▶ **Fig. 5**. The results of the tube formation assay showed that the *P. major* leaf extract using the UAE (LU) method can stimulate the formation of a "tube-like structure" in the Matrigel ma-

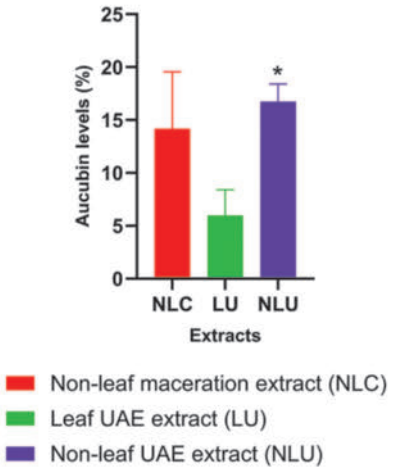


▶ **Fig. 1** Chromatogram profiles of NLC, LU, and NLU extracts with mobile phases of butanol: methanol: water (70 : 5 : 10) v/v. **a** Visible light before spraying anisaldehyde, **b** UV254 nm before spraying anisaldehyde, **c** UV366 nm before spraying anisaldehyde, **d** Visible light after spraying anisaldehyde, **e** UV366 nm after spraying anisaldehyde. A1–3 (UAE leaf extract), B1–3 (non-macerated leaf extract), C1–3 (UAE non-leaf extract), S1–6 (aucubin standard 1–12 µL)

trix. The extract concentration of 31.25 µg/mL produced the highest total branching length, 2756.41 cm, after being treated for 24 hours. Meanwhile, the 7.81 µg/mL extract produced a total branching length of 1843.87 cm. The positive control aucubin produced a lower total branching length than the extract group, which was 405.407 cm. Furthermore, gene expression of vascular endothelial growth factor A (VEGF-A) and vascular endothelial growth factor receptor 2 (VEGFR-2) was analyzed to investigate the underlying mechanism of angiogenesis. LU extract increased VEGF-A mRNA expression by 1.74 times (7.81 µg/mL) and 1.95 times (31.25 µg/mL), though not statistically significant ($p > 0.05$). Aucubin significantly upregulated VEGF-A expression by 4.84 times ($p = 0.0068$) compared to cell control (▶ **Fig. 6**). Similarly, VEGFR-2 expression increased 1.68 times and 1.22 times with LU at 7.81 and 31.25 µg/mL, respectively, without significance ($p > 0.05$), while aucubin induced a significant 2.46 times increase ($p = 0.02$) compared to cell control (▶ **Fig. 7**).

Discussion

P. major comprises various bioactive components, such as flavonoids, alkaloids, terpenoids, phenolics, iridoid glycosides, fatty acids, polysaccharides, and vitamins, which are known to contribute to its pharmacological properties such as antioxidant, anti-inflammatory, and wound healing effects [12, 17, 18]. Among these, aucubin is a well-known chemotaxonomic and pharmaceutical



▶ **Fig. 2** Aucubin levels of NLC, LU, and NLU extracts. Data are shown as mean percentage ± SD (n = 3), with *p < 0.05 against LU

marker, attributed to its pharmacological properties such as wound healing, antimicrobial, antioxidant, and anti-inflammatory effects [19].

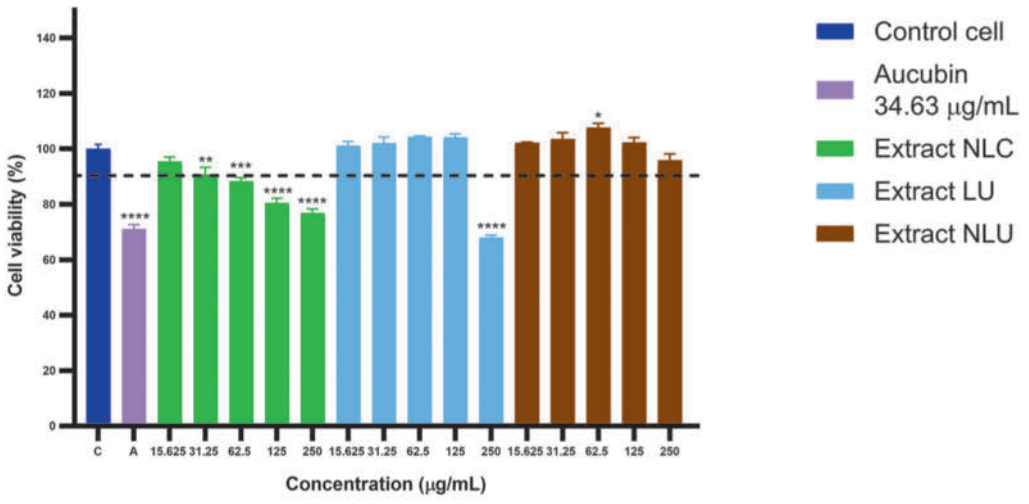
The analysis of aucubin levels in NLC, LU, and NLU extracts (▶ **Fig. 2**) demonstrates that aucubin content was highest in non-leaf extracts (NLC and NLU), while LU exhibited a minimal aucubin level. These findings correspond with previous research indicating that aucubin concentrations could vary based on the plant parts and extraction technique [20–22]. The UAE approach presumably improved aucubin extraction efficiency by ultrasonic cavitation [21], hence supporting its application in maximizing extract yield.

The result from cell viability assay demonstrated that NLC, LU, and NLU extracts were non-toxic at lower concentrations (15.62 and 31.25 µg/mL), maintaining cell viability above 90%. The findings are consistent with prior research indicating that *Lithospermi Radix* and *Mitragyna speciosa* extracts exhibit non-toxic effects on HUVEC cells at low concentrations. Based on these results, NLC, LU, and NLU extracts at 7.81 and 31.25 µg/mL concentrations were selected for further cell migration evaluation using the scratch assay [23, 24].

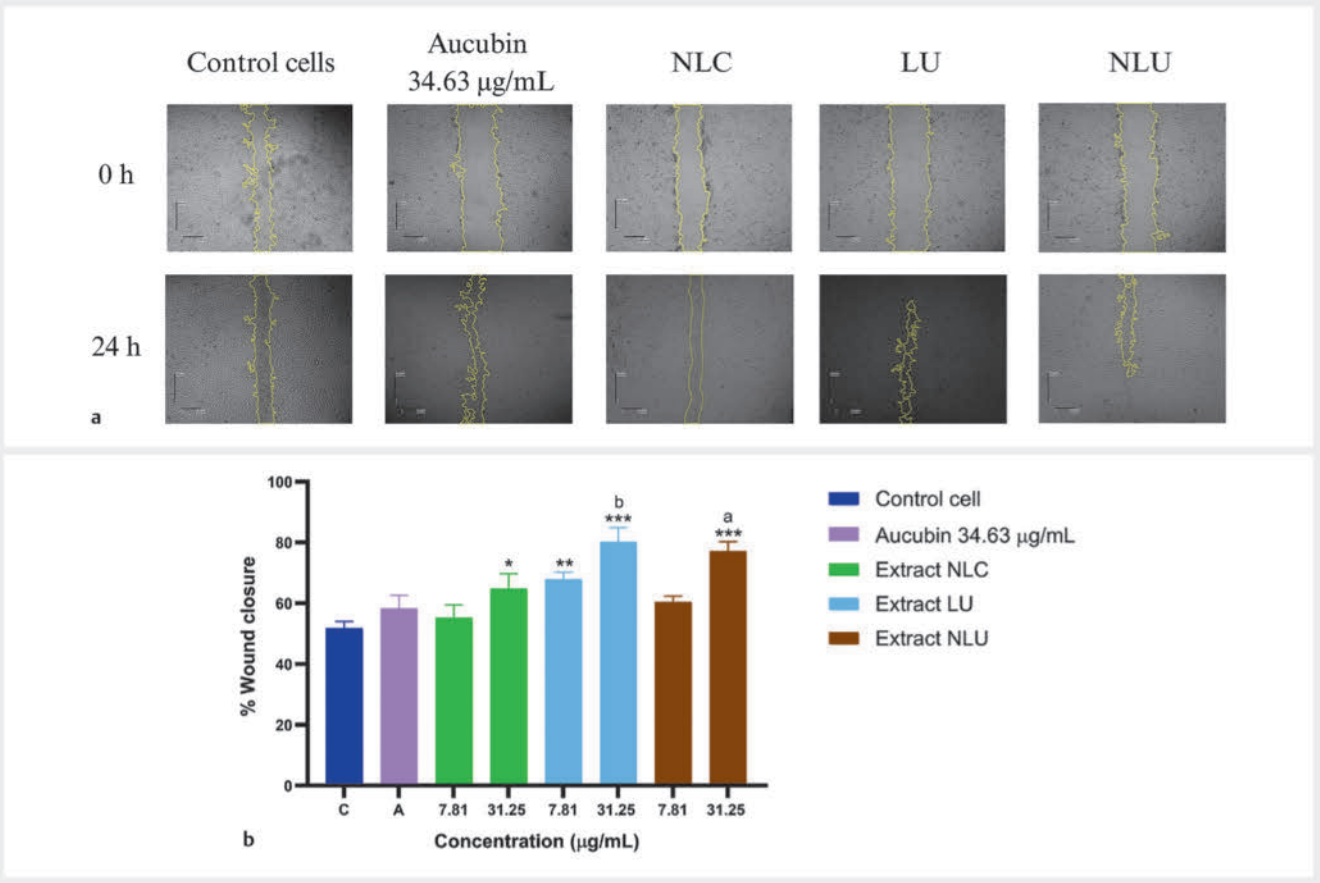
This research utilized a scratch assay to assess the impact of NLC, LU, and NLU extracts on the migration of HUVEC cells. The LU extract exhibited the highest percentage of wound closure compared to all other extracts and aucubin control at 34.63 µg/mL. Aucubin was a positive control due to its established capacity to enhance angiogenesis. Aucubin at concentrations of 5, 10, and 20 µM significantly enhanced the migration ability of injured HUVEC cells treated with SU5416 after 12 hours [16]. The results of migration assay indicate that the wound-healing effects of *P. major* extracts cannot be exclusively ascribed to aucubin.

Polyphenols such as plantamajoside and polysaccharides in leaf tissues have been linked to wound healing [25]. Other bioactive compounds in *P. major* leaves, such as Lupeol, β-amyrin, β-sitosterol, and α-amyrin are known to have antioxidant activities that can reduce oxidative stress, facilitating cell proliferation and migration in wound healing [12, 26]. The increased percentage of wound closure linked to the LU extract is due to its higher concentration of bioactive compounds. Consequently, the LU extract was selected for further evaluation in the tube formation assay to assess its capacity to promote capillary-like structure formation, a hallmark of angiogenesis.

The angiogenic potential of the LU extract was assessed through the tube formation assay, which relies on the capacity of endothelial cells to differentiate on a basement membrane matrix like Matrigel. Endothelial cells first adhere to the matrix and sub-



▶ **Fig. 3** Effect of *P. major* extract on HUVEC cell viability. Effect of *P. major* extract at various concentrations on HUVEC cell viability after 24 hours. Data are shown as mean percentage ± SD (n = 3). *p < 0.05; **p < 0.005; ***p < 0.0005; ****p < 0.0001 against the control group of cells.



▶ **Fig. 4** Effect of *P. major* extract on HUVEC cell migration. **a** Scratch assay illustrates the effect of NLC, LU, and NLU extracts at a concentration of 31.25 µg/mL on HUVEC cell migration. Cells were observed under a microscope with a digital camera at 4× magnification. **b** Graph of % wound closure. Data are shown as mean percentage ± SD (n = 2). *p < 0.05; **p < 0.005; ***p < 0.0005 for the control group of cells, ap<0.05 for NLC, NLU extracts 7.81 µg/mL; bp<0.05 for NLC extracts 7.81 µg/mL

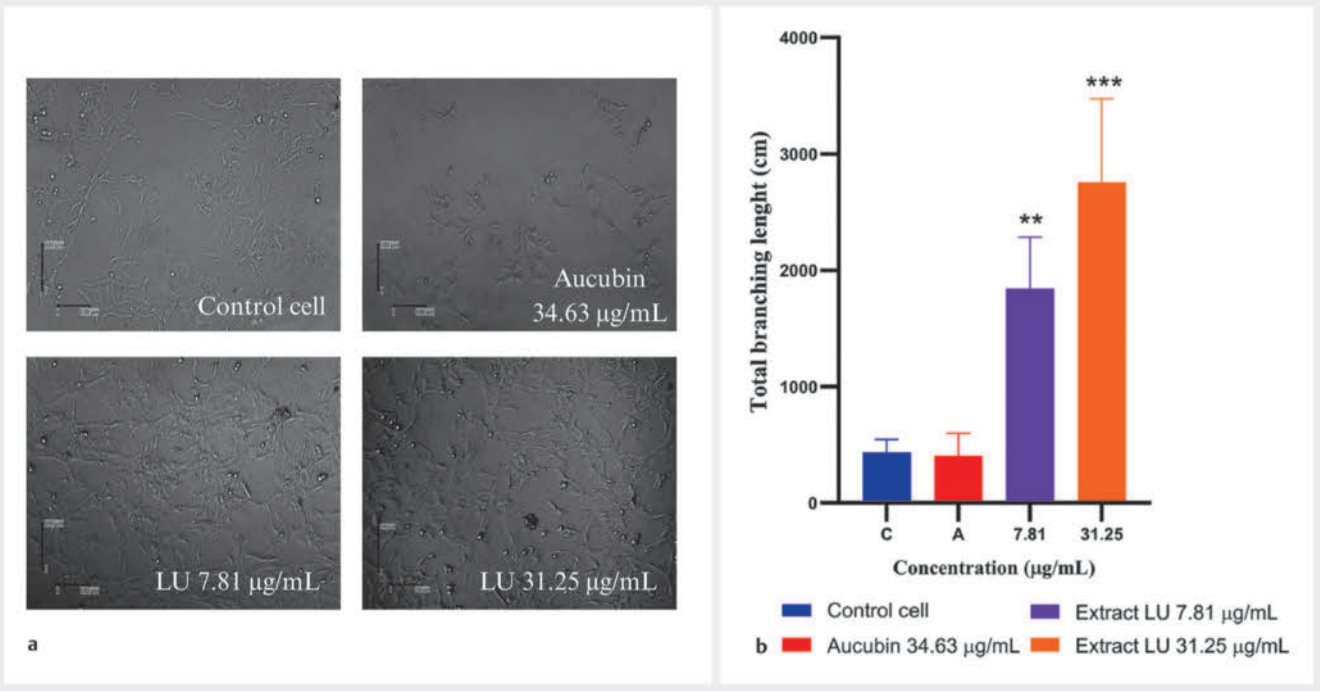
sequently migrate to create tubular structures akin to small blood vessels, signifying the process of angiogenesis [27, 28].

The tube formation assay results indicated that the LU extract at a concentration of 31.25 µg/mL produced the most significant total branching length when compared to the LU extract at 7.81 µg/mL, the untreated control, and the aucubin control at 34.63 µg/mL. The untreated control exhibited the inherent ability of HUVECs to develop capillary-like structures without the extract or aucubin. Aucubin at 5 to 20 µM concentrations may enhance capillary network formation by 49.82 ± 2.18% in SU5416-damaged HUVECs [16]. Those results suggest that the enhanced activity of the LU extract cannot be solely attributed to its aucubin content. Other phytochemicals present in the extract, such as phenolics, flavonoids, or other iridoid glycosides may also contribute to the observed biological effects.

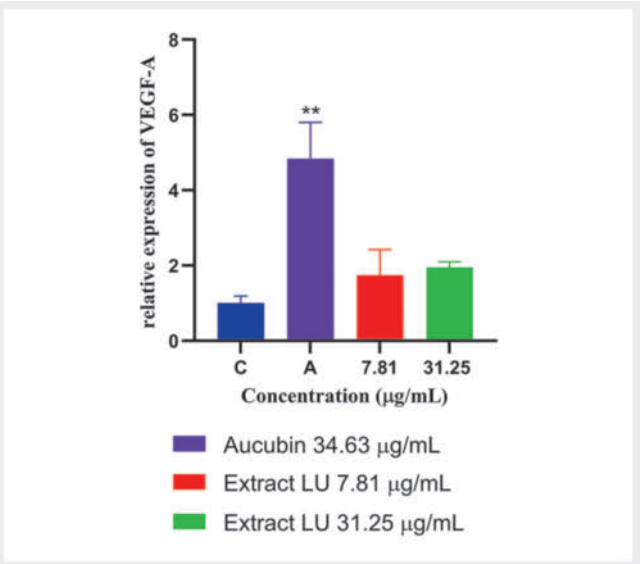
LU extract, which had the highest total phenolic (27.69 ± 0.81 mg GAE/g) and flavonoid (14.93 ± 0.33 mg QE/g) content (**Table 2S**, Supporting Information), showed superior wound closure in the scratch assay and promoted the most extensive capillary-like structures in tube formation. This suggests the key role of phenolics and flavonoids in its angiogenic activity. Interestingly, aucubin as a reference compound exhibited lower activity than LU, NLU, and NLC in the MTT, scratch, and tube formation assays. However, it induced higher VEGFA and VEGFR2 mRNA expression in RT-qPCR compared to LU. This result indicates that LU may act via additional or alternative pathways, supporting the idea that whole extracts may produce broader biological effects, possibly due to the contributions of other bioactive compounds present in the extract. Nevertheless, these findings should be interpreted as preliminary and approached cautiously, as they are based on duplicate samples (n = 2) and only two angiogenesis-related genes were evaluated.

While VEGFA/VEGFR2 are central to angiogenesis, the lack of significant gene expression changes with LU and the limited scope of our gene panel highlight the need for caution. Additional regulatory factors, such as bFGF, PDGF, IL-8, HIF-1α, and TGF-β and pathways including MAPK, PI3K/Akt, and AGE-RAGE, are also important in wound healing-related angiogenesis [29–31]. Broader transcriptomic or proteomic profiling would strengthen mechanistic understanding and should be pursued in future studies.

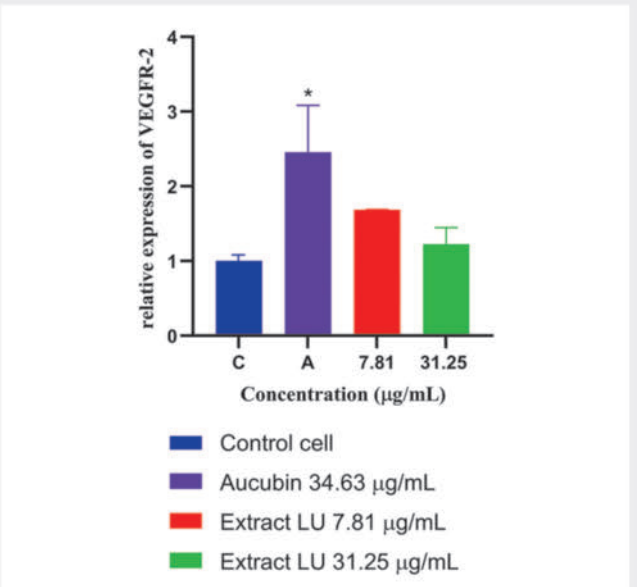
The present study offers significant *in vitro* evidence regarding the pro-angiogenic potential of *P. major* extracts. However, caution is warranted in interpreting these findings for clinical application. *In vitro* assays, such as scratch wound and tube formation



▶ **Fig. 5** Effect of *P. major* extract on HUVEC cell tube formation. **a** Effect of *P. major* leaf extract using UAE method on HUVEC cell tube formation after 24 hours. **b** Graph of total branching length. Data are shown as percentage of mean ± SD (n = 3). **p < 0.005; ***p < 0.0005 against aucubin control group.



▶ **Fig. 6** Effect of *P. major* extract on VEGF-A mRNA expression in HUVEC cells. Relative expression of VEGF-A mRNA to GAPDH from each treatment group (n = 2 ± SD). **p < 0.0068



▶ **Fig. 7** Effect of *P. major* extract on VEGFR-2 mRNA expression in HUVEC cells. Relative expression of VEGFR-2 mRNA to GAPDH from each treatment group (n = 2 ± SD). *p < 0.02

tests, provide preliminary insights and do not capture the full complexity of *in vivo* wound-healing environments, which encompass immune responses, extracellular matrix remodeling, and systemic factors. Future studies should incorporate *in vivo* models of wound healing or diabetic ulceration to validate the efficacy, safety, and pharmacodynamic behavior of the optimized extracts. The outlined steps are essential for assessing the translational potential of *P. major* as a therapeutic agent for angiogenesis-related disorders.

This research possesses several limitations. The scratch assay and RT-qPCR were conducted with merely two biological replicates (n = 2), while tube formation assays utilized three replicates (n = 3), hence constraining statistical robustness and heightening the likelihood of false positives or negatives. DMSO (0.125%) served just as a solvent control in the MTT experiment and was verified as non-cytotoxic (**Table 1S**, Supporting Information). However, it was excluded from the migration, tube formation, and RT-qPCR assays due to restricted cell availability. These factors decrease confidence in the accuracy of the findings and necessitate replication in subsequent research. Furthermore, scratch and tube formation assays exhibit intrinsic variability due to human techniques and subjective assessment. Thus, the integration of automated image analysis or real-time monitoring would improve objectivity and repeatability.

In summary, our findings indicate that optimized extracts of *Plantago major*, specifically LU, show significant pro-angiogenic activity *in vitro*, as evidenced by increased cell viability, migration, and tube formation in HUVECs. These effects are likely attributable to the combined contributions of multiple phytochemicals, including phenolics, flavonoids, and iridoid glycosides. However, the specific roles of individual constituents remain unclear. Therefore, future studies should include comprehensive chemical profiling and bioactivity-guided fractionation to better identify the active components and clarify their mechanisms of action. Given the inherent limitations of *in vitro* assays and the low replication observed in specific tests, additional investigation is required to validate these findings. Future research should evaluate the efficacy of these extracts in *in vivo* models of angiogenesis and wound healing, elucidate their molecular mechanisms through comprehensive gene expression profiling, and develop stable formulations for therapeutic application. These steps are crucial for transitioning *P. major* from a conventional remedy to a scientifically substantiated therapeutic agent.

Materials and Methods

Materials

The *P. major* used is three months old, during its reproductive phase, and sourced from Ballitro, West Java, Indonesia. EndoGRO HUVEC, aucubin standard (purity ≥ 98%), dimethyl sulfoxide (DMSO), and Triypsin-EDTA 0,25% were purchased from Sigma-Aldrich Co.; Sodium Dodecyl Sulfate (SDS), HCl 1 N, n-butanol, and methanol were purchased from Merck; ready-to-use Media for Cell Differentiation and Basal medium 131 (MCDB131) was purchased from Dermama Biotechnology Laboratory; Dulbecco's phosphate-buffered saline (dPBS) was purchased from Elabscience Biotechnology; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Bio Basic; sterile water for injection (SWFI) was purchased from OneMed; Matrix Matrigel was purchased from Corning; Quick-RNA Miniprep Kit was purchased from Zymo; and SensiFAST cDNA Synthesis Kit and SensiFAST SYBR No-ROX Kit were purchased from Meridian Bioscience.

Preparation of *P. major* extract

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The extraction procedure for *P. major* used a previously refined approach based on Box-Behnken Response Surface Methodology [32]. The optimisation, conducted using Design Expert software, assessed four variables–temperature, extraction duration, solvent-to-sample ratio, and ethanol concentration–over 27 experimental trials, with total phenolic content (TPC) and total flavonoid content (TFC) as response variables.

Utilising the defined optimal parameters (50 °C, 20 minutes, 71.41% ethanol, and a 1 : 5 sample-to-solvent ratio), both foliar and non-foliar components (root, petiole, and seed) of *P. major* were extracted employing two techniques: traditional maceration and ultrasound-assisted extraction (UAE). The UAE was conducted in an ultrasonic bath under optimal conditions, whereas maceration was executed using the same parameters without ultrasonic assistance. The extracts were subsequently filtered and concentrated utilising a rotary evaporator. This procedure produced four optimal extracts: macerated leaf extract (LC), UAE leaf extract (LU), macerated non-leaf extract (NLC), and UAE non-leaf extract (NLU). Prior publication [33] documented the results of the migration assay for the LC extract. This study concentrates on the remaining three extracts to prevent data redundancy.

Determination of aucubin in the *Plantago major* extracts

Aucubin was determined in NLC, LU, and NLU using the high performance thin layer chromatography (HPTLC) method, based on published procedure with minor adjustments [34]. A TLC silica gel 60 F_{254} plate was used as the stationary phase. The mobile phase was butanol: methanol: water (70 : 5 : 10) v/v. NLC, LU, and NLU with a concentration of 10 000 µg/mL were diluted in methanol, while aucubin standard with a concentration of 100 µg/mL was diluted in water for injection (WFI). The samples were spotted using the CAMAG linomat 5 applicator on the TLC plate. The development method used 5 mL of mobile phase in a twin chamber (Camag) pre-equilibrated with the mobile phase at room temperature for 20 minutes. After the development process, the plates were dried with heated air and sprayed with anisaldehyde sulfuric acid. The outcomes were examined utilizing visible light and UV light at 254 nm and 366 nm.

Sample preparation

Extracts were formulated as stock solutions at a 100 000 µg/mL concentration with dimethyl sulfoxide. Extracts were diluted in the culture medium to achieve serial concentrations of 250, 125, 62.5, 31.25, and 15.62 µg/mL.

▶ **Table 1** Primers used for quantitative RT-PCR.

Gene Name	Sequence	Reference
VEGF-A	F : GGGCAGAATCATCACGAAGT R : TGGTGATGTTGGACTCCTCA	[35]
VEGFR-2	F : GGCCCAATAATCAGAGTGGCA R : CCAGTGTCATTTCCGATCACTTT	[36]
GAPDH	F : GTCATCCATGACAACTTTGG R : GAGCTTGACAAAGTGGTCGT	[23]

Cell culture

HUVEC were cultured in MCDB131 medium and incubated at 37 °C in a humidified incubator with 5% carbon dioxide (CO_2) (Thermoscientific Series 8000 DH). The culture medium was replaced with a fresh medium every 48 hours. Cells were considered suitable for seeding and treatment upon reaching a confluence level of 70–80%.

Cell viability assay

A viability test was performed using the MTT method to determine the concentration of extracts to be used in scratch and tube formation assay. Cells were seeded in 96-well plates (Corning) at a density of 1×10^4 cells/well in 100 µL of MCDB131 medium and incubated for 24 hours. After removing the culture medium, 100 µL of LU, NLU, or NLC extract was added to each well. The extracts were initially dissolved in DMSO to prepare stock solutions and then serially diluted in MCDB131 medium to obtain final concentrations of 250, 125, 62.5, 31.25, and 15.62 µg/mL. The final DMSO concentration in all treatment wells, including the solvent control, was 0.125%. Following 24 hours of treatment, the extract-containing medium was removed, and the cells were gently washed once with dPBS to eliminate any residual extract. Then, 100 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated at 37 °C for four hours. Then, 100 µL of stopper solution (100 µL of 10% SDS in 0.01 N HCl) was added. The absorbance value was detected with a microplate reader (Bio-Rad iMarkTR) at a wavelength of 570 nm. Untreated cells were negative controls, aucubin standard was positive controls, wells containing only media were used as blanks, while wells containing media and DMSO were used as solvent controls. Cell viability is calculated using the following formula:

$$\text{Cell viability } (\%) = \frac{\text{absorbance test sample} - \text{absorbance blank}}{\text{absorbance negative control} - \text{absorbance blank}} \times 100$$

A detailed dataset of solvent control viability and absorbance values is provided in Supporting Information, **Table 1S**.

Wound scratch assay

The effects of extracts on HUVEC migration were assessed through the scratch assay, a method that demonstrates cell migration during the wound-healing process in living organisms. HUVECs at a density of 1×10^5 cells/well were seeded in 24-well plates (Iwaki) and grown to nearly confluent cell monolayers in a culture media. The cell monolayer was wounded using a sterile P200 pipette tip, and the cells were then washed twice with dPBS to remove residual cell streaks. The wounded monolayers were then incubated in a culture medium containing various concentrations of extracts or without extract as a negative control. At 0 and 24 hours, the scratched area was photographed using an inverted microscope at × 4 magnification. The wound closure area was recorded using ImageJ software. The percentages of the closed region were compared pre- and post-treatment. Two replications of the scratch assay were conducted due to the limited number of HUVECs available during the experiment. The percentages of wound healing were assessed by quantifying the decrease in scar area at a specific time point.

$$\text{Wound healing } (\%) = \frac{(\text{scratch area at 0 h} - \text{scratch area at 24 h})}{\text{scratch area at 0 h}} \times 100$$

Tube formation assay

HUVECs with a density of 1.2×10^5 cells/well were planted in 24-well plates (Iwaki) coated with 200 µL Matrigel/well. Additionally, extracts at different concentrations were added to the wells. After 24-hour incubation, the cells were examined microscopically and analyzed with the Angiogenesis Analyser in ImageJ to determine the total length.

Total RNA extraction, reverse transcription, and real-time quantitative PCR (qPCR)

The total RNA of HUVECs was extracted using the Quick-RNA Miniprep Kit. Isolated RNA was then reverse-transcribed into cDNA using the SensiFAST cDNA Synthesis Kit. The qPCR assay was conducted using SensiFAST SYBR No-ROX Kit with the 7500 Fast Real-Time PCR System (ThermoFisher). The amplification parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Each sample underwent duplicate analysis, and the relative mRNA expression was determined following normalization to elongation factor 1 alpha (elfα). RT-qPCR was performed in duplicate due to the limited number of HUVECs available during the experiment. The primer sequences employed are listed in ▶ **Table 1**.

Statistical analysis

Results are expressed as mean ± SD derived from at least two independent experiments. Data were analyzed utilizing GraphPad Prism version 8.3.0. Statistical significance was evaluated using One-way ANOVA, followed by post-hoc Dunnett's with a p-value of less than 0.05 ($p < 0.05$) deemed significant. Statistical significance is indicated in the figures using asterisks as follows: $p < 0.05$ (*), $p < 0.005$ (**), $p < 0.0005$ (***), $p < 0.0001$ (****).

Contributors' Statement

Data collection: L. A. Latifa Amalia, K. R. P. Kurnia Rahayu Purnomosari; design of the study: T. H. Triana Hertiani, R. M. Retno Murwanti; statistical analysis: L. A. Latifa Amalia, K. R. P. Kurnia Rahayu Purnomosari; analysis and interpretation of the data: L. A. Latifa Amalia, T. H. Triana Hertiani, R. M. Retno Murwanti; drafting the manuscript: L. A. Latifa Amalia; critical revision of the manuscript: L. A. Latifa Amalia, T. H. Triana Hertiani

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Conflict of Interest

~~The authors declare that they have no conflict of interest.~~ *okay?*

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