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Antibacterial, antibiofilm, and wound healing activities of *Piper longum* root in multidrug-resistant *Staphylococcus aureus* infected micePooja Gaur^{1,2}, Pashupatinath Shukla^{1,3}, Jitesh Yadav¹, Ch. V. Rao¹✉¹Pharmacology Lab, CSIR–National Botanical Research Institute, Lucknow 226001, India²Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH, Government of India, New Delhi 110058, India³Academy of Scientific and Innovative Research, Ghaziabad 201002, India

ABSTRACT

Objective: To explore the effect of a hydrogel of *Piper longum* (*P. longum*) root against biofilm-forming multidrug-resistant (MDR) *Staphylococcus aureus* (*S. aureus*) through *in vitro*, *in silico*, and *in vivo* studies.

Methods: We isolated the *P. longum* root ethanolic extract and the compounds using p-HPLC. *In vitro* antibacterial and antibiofilm activities of *P. longum* root extract and isolated alkamide compounds against biofilm-forming MDR *S. aureus* (ATCC 33591) were assessed using agar diffusion and broth microdilution methods, respectively. *In silico* investigations were conducted to investigate the interaction of alkamide compounds with three target proteins glycogen synthase kinase 3 β (GSK3 β), matrix metalloproteinases-8 (MMP-8), and inducible nitric oxide synthase (iNOS). In addition, the wound healing effect of *P. longum* root extract 2% and 5% (*w/v*)-containing hydrogels was determined in mice.

Results: The ethanolic root extract of *P. longum* and its compounds exhibited *in vitro* antibacterial activity with minimum inhibitory concentrations between 50 μ g/mL and 700 μ g/mL, as well as significantly reduced biofilm formation. Piperdardine isolated from *P. longum* root extract had the best molecular docking score (–9.7, –9.8, and –9.2 kcal/mol) with target proteins GSK3 β , MMP-8, and iNOS. *In vivo* studies showed that *P. longum* hydrogels significantly lowered the number of colony-forming units ($P < 0.05$). The *P. longum* 5% (*w/v*) hydrogel-treated group showed enhanced wound healing activity, achieving a wound contraction rate of 99.34% on day 14. Furthermore, histopathological analysis confirmed increased re-epithelialization and reduced inflammation in mice treated with *P. longum* 5% (*w/v*) hydrogel.

Conclusions: *P. longum* root extract has pharmacological potential as an antibacterial and wound-healing agent, and further research is required to confirm its efficacy and clinical application.

KEYWORDS: *Piper longum* L.; Wound healing; Antibiofilm; Antibacterial; MDR *Staphylococcus aureus*; *In silico* docking

1. Introduction

A wound is an injury to the skin due to tearing, cutting, or

Summary

Question: Does *Piper longum* root hydrogel exhibit antibacterial, antibiofilm, and wound-healing properties against biofilm-forming multidrug-resistant *Staphylococcus aureus*?

Findings: *Piper longum* root extract and its alkamides exhibited antibacterial and antibiofilm activity (MIC: 50–700 μ g/mL). Piperdardine showed strong binding with GSK3 β , MMP-8, and iNOS. *Piper longum* hydrogel reduced bacterial load and enhanced wound healing *in vivo*, with 5% hydrogel achieving 99.34% wound contraction on day 14 and promoting re-epithelialization.

Meaning: *Piper longum* root extract and its hydrogel formulation possess potent antibacterial, antibiofilm, and wound-healing properties, supporting its potential as a therapeutic agent for multidrug-resistant *Staphylococcus aureus* infections. Further studies are required to validate its clinical application.

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puncturing. The wound-healing process involves epithelization, contraction, and connective tissue deposition[1]. The healing process involves producing and depositing new collagen at the wound site, leading to the damaged tissue's re-epithelialization. For the formation of new tissue, fibroblasts multiply and release a range of growth factors, such as type I collagen and vascular endothelial growth factor. *Staphylococcus aureus* (*S. aureus*) is a significant Gram-positive opportunistic pathogen of humans and many animal species[2]. *S. aureus* is the primary cause of skin and soft tissue infections, including abscesses, furuncles, and cellulitis. Patients often encounter significant challenges when managing tissue wounds infected with the drug-resistant bacteria *S. aureus*. Treating tissue wounds infected with the drug-resistant bacteria *S. aureus* often involves scar formation, granulation, and collagenation. The healing process becomes complicated due to antimicrobial resistance[3]. *S. aureus* is a common bacterium recognized for its capacity to form biofilms. It is a complex collection of microorganisms that adhere to surfaces encased in a self-produced matrix of extracellular polymeric substances. Biofilms protect the bacteria against antimicrobial agents and host immune defenses, facilitating chronic and recurrent infections. Inhibiting the formation of biofilms is a crucial strategy for managing *S. aureus* infections. These interventions aim to prevent the initial bacterial adhesion, disrupt established biofilms, and enhance the effectiveness of antibiotics.

According to the World Health Organization report, 80% of the global population uses plant-based medicines, especially for skin-related issues like wounds. In classical Ayurveda, 70% of plants are cited in texts like *Charaka Samhita* and *Sushruta Samhita* for their wound-healing properties[4]. *Piper longum* (*P. longum*) promotes wound healing[4] and exhibits different stages of wound healing based on its pharmacodynamic attributes, including inflammatory, proliferative, and remodeling phases.

The *Piper* genus has around 2000 species found in tropical regions with high commercial, economic, and medicinal importance[5]. *P. longum* L. (Piperaceae family), also known as Pippali, is prevalent throughout tropical and subtropical regions of Asia and the Pacific islands. Traditionally, *P. longum* fruit is used as a culinary spice, while the root is used for medicinal purposes such as treating digestive issues, bacterial diseases, and respiratory problems[6]. It exhibited the therapeutic activity “Kushtha,” which describes a category of skin diseases mentioned in Indian Pharmacopoeia Part 1, Vol. IV[7]. It treats inflammation, infectious skin diseases, lacerations, wounds, and sprains[8]. Diverse chemical constituents isolated from *P. longum* fruits and roots exhibited pharmacological activities, viz., anti-inflammatory, antimicrobial, antibiofilm, and antipyretic activities[9,10]. Numerous findings demonstrated the antibacterial properties of various *P. longum* solvent-based extracts[9,11,12]. As per the National Medicinal Plants Board of India, it is a medicinally significant plant subject to extensive trade acquired through cultivation. The high demand for pippali fruit in India and foreign markets has generated surplus roots and aerial parts[13]. Most studies

on the pharmacology and phytochemistry of this plant focus on its fruit rather than its root. Therefore, to explore the discarded part of pippali considered agricultural waste, the root was selected to search for new chemicals with wound healing potential and against potential biofilm-forming multidrug-resistant (MDR) bacteria. A previous study found that piperine, a major alkaloid in piper species, has been reported to possess antimicrobial properties[8,14]. In another study, a piperine-containing bioactive hydrogel system enhanced *in vivo* wound healing efficacy in the excision wound mice model[15]. However, there is a lack of studies on the *in vitro* antibacterial and antibiofilm activity of *P. longum* root against MDR *S. aureus* and its *in vivo* wound healing activity.

Numerous studies have demonstrated the potential of various plants as natural antibacterial agents, with investigations exploring the mechanistic interactions of their metabolites against MDR bacterial infections. However, comprehensive studies assessing their efficacy in facilitating *S. aureus* wound healing remain scarce. This study addresses this gap by assessing *P. longum*'s antibacterial, antibiofilm, and wound healing properties, advancing natural therapeutics in traditional medicine.

2. Materials and methods

2.1. Chemicals and reagents

For the *in vitro* experimental study, the Mueller Hinton broth (MHB) culture medium was obtained from Bioré (bioMérieux SA, Marcy l'Etoile, France), and the microbial growth indicator 2,3,5-triphenyl tetrazolium chloride (TTC) was obtained from Sigma-Aldrich (St. Louis, USA). Before its use, the water was purified using a Milli-Q water purification system (TGI Pure Water Systems, USA). *S. aureus* was obtained from the microbiology lab CSIR-NBRI, India, and stored in the deep freezer (−80 °C). Isoflurane, ketamine, and xylazine were purchased from Themis medicare Ltd. Eosin, hematoxylin, Ehrlich's reagent, and formaldehyde solution (37%–41%) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India, for histopathological analysis. Ethanol and DPX mountant were purchased from Merck Life Science Pvt. Ltd. Methanol (Sigma-Aldrich) and preparative high-performance liquid chromatography (p-HPLC) were used for the isolation of compounds. The study used exclusively analytical-grade chemicals and solvents.

2.2. Plant material and extract preparation

P. longum root material was collected from CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow. Roots were cut, washed with water, dried under shade, and stored in a freezer at −20 °C until use. The shade-dried root was ground into powder using a mixer grinder (Philips, India). A voucher specimen (CIMAP/8332) was deposited in the institute herbarium. The fine powder (<0.315 mm sieve size) of *P. longum* root (200.0 g) was macerated and

extracted in ethanol (2.5 L) for 48 h at room temperature. The crude ethanolic extract (20.0 g) was dried under vacuum (Rotavapor® R-210, BÜCHI, Switzerland) and concentrated.

2.3. Isolation of constituents from ethanol extract of *P. longum* root through p-HPLC

One gram of *P. longum* root ethanol extract was sonicated and dissolved in 5 mL of HPLC-grade methanol, then centrifuged at 10000 rpm to separate active compounds. Subsequently, p-HPLC was employed to separate and purify the chemical compounds. For the isolation of compounds, a C₁₈ column (10 cm × 10 mm, 10 µm) was used with an isocratic mobile phase consisting of a mixture of water and methanol (32:68) at a 5.0 mL/min flow rate. The elution of the compounds was monitored at a wavelength of 254 nm.

2.4. Antibacterial activity and antibiofilm activity

2.4.1. Antibiotic susceptibility testing and sample preparation

S. aureus (ATCC 33591) bacteria that were resistant to antibiotics (oxacillin, ceftriaxone, cefoxitin, ciprofloxacin, erythromycin, streptomycin and chloramphenicol) were used for antibiotic susceptibility testing. The ethanol extracts of *P. longum* root, fruit, and leaves stock solution (1 mg/mL) and compounds (0.5 mg/mL) were diluted to obtain a working solution range of 10-1000 µg/mL and 0.1-100 µg/mL, respectively[16].

2.4.2. Maintenance and preservation of *S. aureus*

S. aureus was grown on nutrient agar slants at 37°C for 18-20 h. Stock cultures of the bacteria were used to prepare the inoculum. A sterilized wire loop was used to transfer a small amount of the stock culture onto both nutrient agar slants and nutrient broth (30 mL). The inoculated cultures were incubated overnight at 37°C in a rotary shaker. After incubation, the inoculated cultures were stored at 4°C until further experimentation was needed. A glycerol stock of the bacteria was stored at -80°C.

2.4.3. Antibacterial susceptibility of *P. longum* root, fruit, and leaf ethanolic extract and six isolated compounds

The antibacterial activity of *P. longum* root, fruit, and leaves and six isolated compounds was determined against MDR *S. aureus* using the agar well diffusion method. A standardized suspension of the test microorganisms was prepared and incubated at 37°C for 17-18 h to create fresh cultures. These cultures were used to conduct antibacterial susceptibility testing. The microbial suspension, containing 1×10⁶ colony forming units (CFU) per milliliter, was then carefully spread as 100 µL on Muller-Hinton agar plates. Uniform wells were created on the dried agar plates using a sterile borer to assess the antibacterial activity of test samples. Subsequently, 100 µL of the prepared test samples were added to each well. After this, the microbial plates were incubated at 37°C for 24 h.

2.4.4. Determination of MIC of *P. longum* root, fruit, and leaf extracts

The MIC of the ethanol extracts of *P. longum* root, fruit, and leaves and six isolated compounds against *S. aureus* was determined by using the broth microdilution in accordance with the guideline of the Clinical and Laboratory Standards Institute[17]. Following the method, the MHB analysis was performed for bacteria[18]. A stock solution of 100 µL of sample was prepared at a concentration of 100 µg/mL by diluting compounds with dimethyl sulfoxide. To optimize, a concentration of 10⁶ CFU/mL was adjusted for bacteria from overnight suspensions by diluting with MHB media. *P. longum* extracts from different parts were analyzed using a two-fold serial dilution method with MHB media in a 96-well microtiter plate at concentrations of 10-1000 µg/mL. Then 100 µL of prepared bacteria cultures (10⁶ CFU/mL) were inoculated, and plates were kept at 37°C for 24 h. Vancomycin served as a standard control for bacterial susceptibility testing. After incubation at 37°C for 24 h, 5 µL of 0.5 mol/L TTC was added to each well. The plates were further incubated for 30 min to 1 h until optimal color development was achieved. The TTC assay identified the MIC as the lowest concentration without inducing color change. This indicates no visible microbial growth in the sample at that concentration. This experiment was performed in triplicate.

2.4.5. Antibiofilm activity of *P. longum* root, fruit, and leaf extracts

The microtiter plate technique was applied to determine the antibiofilm activity of *P. longum* root, fruit, and leaf extracts[19]. Each well was supplemented with 100 µL of *S. aureus* (10⁶ CFU/mL) and 100 µL of *P. longum* root, fruit, and leaf extracts. Following a 48-hour incubation period at a temperature of 37°C, the contents of the plates were discarded, and the wells were rinsed. The biofilm was stained using a 0.4% crystal violet solution for 15 min. Subsequently, it was rinsed and allowed to air dry. Finally, 200 µL of 95% ethanol was added to discolor the dye. *S. aureus* biofilm was estimated by determining the OD at 600 nm of each well using a UV/VIS spectrophotometer (Perkin Elmer, USA). The antibiofilm capabilities were quantified in percentage compared to control wells, which were bacterial wells without *P. longum* extracts. The calculation was done using the formula defined in Equation 1.

$$\text{Percentage inhibition} = (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100$$

2.4.6. Microscopic technique for antibiofilm activity

To study the formation of biofilms, we cultured biofilms on glass slides measuring 1 cm × 1 cm. The slides were placed into a 24-well microtiter plate with 100 µL of a standardized cell suspension in 1 mL of tryptic soy broth medium. The medium was enhanced by adding *P. longum* root, fruit, and leaf extracts, as well as without them. The microtiter plates were incubated at 37°C for 24 h. After incubation, biofilm slides were washed as described in section 2.4.5. The desiccated slides were examined using a light microscope at a

magnification of 100×, and the photographs were captured.

2.5. In vivo wound healing activity

2.5.1. Sample preparation and inoculation

To 25 mL of boiled distilled water, 2 g of powdered methyl cellulose was added and dissolved by 20 mL of cold distilled water with stirring. *P. longum* root extract dissolved in propylene glycol was inoculated to prepare *P. longum* 2% (w/v) and 5% (w/v) of the therapeutic hydrogel, respectively.

2.5.2. Animals and ethical approval

Swiss albino mice (male, 25–30 g) were acquired from the central animal house facility of CSIR. The animal experiments were carried out in the authorized animal house of the CSIR-NBRI Pharmacology Lab, Lucknow, India (Registration number 1732/GO/R/S/13/CCSEA). Healthy animals were housed and acclimatized in a carefully controlled laboratory environment with specific conditions: a room temperature (25 ± 3)°C, a relatively humid level (55 ± 5)%, and a 12/12-hour light/dark cycle. Before the commencement of experiment, they were given free access to commercial rodent water and diet. The experimental protocols for excisional and incisional wound models and a skin irritation study adhered to the Committee for Control and Supervision of Experiments on Animals guidelines. These protocols were approved by the Institutional Animal Ethics Committee at CSIR-NBRI, a government institution in India. Furthermore, all animal testing followed the ARRIVE guidelines, the principles outlined in the U.K. Animals (Scientific Procedures) Act of 1986, and relevant guidelines from the EU Directive 2010/63/EU. Age-matched animals were used for all treatments to minimize variability within experimental groups.

2.5.3. Skin irritation study

The acute dermal toxicity test of the blank and the optimized formulated hydrogel was performed in healthy Swiss albino mice as per the Organisation for Economic Co-operation and Development (OECD) guidelines 404[20]. Six male SA mice were divided into two groups of three each. Approximately 24 h before the test, the dorsal portion of the test animals was depilated using Veet (Reckitt Benckiser India, Pvt. Ltd., India). The experimental protocol involved using animals with healthy, unbroken skin to avoid any skin abrasions. Following the OECD guidelines, 0.5 mL of blank formulated hydrogel and an optimized hydrogel were applied to the test site, which covered an area of approximately 6 cm² of skin. These formulated hydrogels were secured with a gauze patch and non-irritant tape. A 1% saline solution was applied to another site on the dorsal skin as a positive control. All animals were closely monitored for signs of edema and erythema, with observations recorded at specific time intervals of 60 min after application, and then at 24, 48, and 72 h. The experiment followed OECD guidelines to evaluate skin irritation potential, aiming to assess the impact of formulated hydrogel on skin integrity and reactions[21].

2.5.4. Pilot study

This experiment used six animals to conduct a pilot excision wound model. The excision wounds which were 1.5 cm in diameter and 0.2 cm deep, were created on the dorsal side that had been shaven. The animals were then treated with ethanolic, hydroalcoholic, and aqueous extracts of *P. longum* root, standard povidone (5%, w/w) ointment, hydrogel base (control), or left untreated for 14 d. The wounds were treated topically once daily until the 14th day. Healing parameters were evaluated by calculating the wound contraction percentage.

2.5.5. Experimental design

Mice were divided randomly into five groups with six mice in each group as follows: G1-untreated *S. aureus*; G2-*S. aureus* + gel base; G3-*S. aureus* + 5% w/w povidone iodine standard ointment applied topically on the wound area; G4-*S. aureus* + *P. longum* 2% w/v applied topically as wound dressing, and G5-*S. aureus* + *P. longum* 5% w/v applied topically as wound dressing for 14 d. A bacterial suspension of 1 mL containing 1.1×10^6 CFU/mL was applied to the wounds to induce bacterial infection. This suspension was evenly spread on the skin using a cotton-tipped swab until no visible fluid remained. Swabs were collected before and after infection to assess the initial and subsequent bacterial load, ensuring consistent infection among groups. Mice were anesthetized using isoflurane (3%–5%), and a specific area on their dorsal skin was carefully shaved. Full-thickness excisional skin wounds were created in this shaved region, following a previously described method[22]. Treatment began 24 hours later with topical application of *P. longum* hydrogel (2% and 5% w/v) and standard (povidone iodine). The *P. longum* 5% w/v group received 500 mg of hydrogel for excised/incised wounds, while the *P. longum* 2% w/v group received 250 mg for the excised/incised wounds. Povidone iodine ointment hydrogel was applied on the wound area once daily for 14 d.

2.5.6. In vivo excision wound model

In this study, animals of each group were anesthetized using a combination of ketamine and xylazine [(90 + 10) mg/kg] before creating wounds[23]. Hair was removed from the dorsoventral region using a depilatory cream, before a sensitivity test to ensure skin tolerance was observed for 24 h. Following hair removal, the skin surface was cleaned with 70% ethanol. A 110 mm² area was marked, and a wound was created using a scalpel on the dorsoventral region. Then the wound was further infected with MDR *S. aureus* using a 1 mL suspension containing 1.1×10^6 CFU/mL in phosphate-buffered saline. Treatments were done as abovementioned for 14 d and the following assessments were conducted: determination of the epithelialization period, analysis of white blood cell (WBC) count, measurement of hydroxyproline content, and histopathological examinations[24].

2.5.7. In vivo incision wound model

The incision was created in the skin following the method described

previously[25]. The mice were categorized into the groups mentioned earlier, and all mice were anesthetized using ketamine and xylazine (100 mg/kg) based on their body weight. A 4 cm incision was made on each side of the vertebral column through the shaved skin. After ensuring complete hemostasis, the surgical wounds were closed with individual stitches of black silk surgical thread (number 000), spaced approximately 1.5 cm apart, and applied with a curved needle (number 11). Following the suturing, the wounds were left uncovered, and from the same day (day zero), 250 mg of each of the following preparations were topically applied to the sutured area for nine consecutive days: simple gel base formulated hydrogel *P. longum* (2% and 5%, *w/v*), and the standard drug (5% *w/w* povidone iodine ointment). On the 14th day, anesthetization was given to all mice, and the sutures were removed. The wound-breaking strength (WBS) was then measured using a previously used method, providing insights into the wound's mechanical integrity and healing progress[24].

2.6. Evaluation of wound healing parameters

2.6.1. Percentage of wound contraction and epithelialization period

The ointment was applied externally once daily until the wounds fully healed. The initial wound area, measured at day zero (110 mm²), was measured at three-day intervals following wound creation. After creating the full-thickness excisional wound, a transparent sheet of paper was placed over the wound to outline the affected area. Wound contraction was measured as the percentage decrease in the initial location of the wound. The wound contraction percentage was calculated using the measured surface areas at various post-wound creation time points, according to the formula in Equation 2[26].

Wound contraction = (Initial wound size – Specific day wound size) / Initial wound size × 100

The epithelialization period is defined as the period assessed for eschar detachment and complete wound healing. This period quantifies the number of days required for complete necrotic tissue removal from the wound site, with no remaining unhealed wounds[27]. Each animal's epithelialization time was individually recorded, and an average duration was calculated. This parameter is a critical indicator of the wound healing process, signifying the time taken for complete closure and recovery of the wounded area.

2.6.2. Bacterial burden in skin tissue

The bacterial burden in the skin was measured using a previously described method[28]. The *in vivo* assessment of wound healing enhancement was conducted on two concentrations of *P. longum* root hydrogels: 2% (*w/v*) and 5% (*w/v*). For the measurement of bacterial burden in skin tissue, mice were sacrificed on the 14th day post-infection using an intraperitoneal overdose of anesthesia (ketamine and xylazine, 100 mg/kg). The skin was excised under

sterile conditions and homogenized. The homogenated skin was diluted in a series of steps in the samples, and 500 µL of each dilution was applied to a Staph Selective Agar (SS agar) plate. The cultures were then kept at 37 °C for 24 h, and the number of bacteria per milliliter (CFU/mL) was determined by spreading the cultures on nutrient agar plates.

2.6.3. Measurement of WBS

On the 10th day, under anesthesia, sutures were aseptically removed, and a wound section was excised from the wound's area. Using No. 14 curved needles, three were inserted: one on the free end and two on the fixed sides. Two needles were attached to a rod, while the third needle was fixed to a hanging weighing pan. Graduated weights were added to the weighing pan until the wound was separated. The weight at which the wound ruptured served as an indirect indicator of WBS. The tensile strength of wounds treated with *P. longum* (2% and 5%, *w/v*) and standard treatment (povidone 5%, *w/w*) was compared to the disease control and control groups.

2.6.4. Hydroxyproline estimation

On the 14th day of wound creation, the animals were sacrificed, and tissue samples from the regenerated areas of the healed wounds were collected to estimate hydroxyproline content. Briefly, 30 mg of tissue samples were submerged in 2 mL of 6 mol/L hydrochloric acid (HCl), sealed without air removal, and hydrolyzed at 105 °C for 20 h. The resulting samples were filtered through a 0.22 µm syringe filter (Millex-GV), and then the pH was adjusted to 7.0. After filtration, the sample (100 µL) was mixed with distilled H₂O to obtain a final volume of 200 µL, and then 0.4 mL of 50% *v/v* isopropanol was added. The hydrolysate was mixed with reagent solution A (chloramine-T and acetate citrate buffer in a 1:4 *v/v* ratio) and incubated for 10 min for chloramine-T oxidation. After incubation, 2.5 mL of reagent solution B was added, followed by incubation at 58 °C for 30 min. Reagent solution B contained 3,3'-diaminobenzidine, such as Ehrlich's reagent, and 60% perchloric acid mixed in a 3:13 *v/v* ratio with isopropyl alcohol. The sample was cooled, and the absorbance was measured at 550 nm within 30 min. Hydroxyproline content in the regenerated tissue was quantified using a standard calibration curve[29–32].

2.6.5. Total DNA content

The mice were treated with the ointment, which was applied topically on the excision wound area once a day for 3 d. On the fourth day, tissue samples were collected. These tissues were then processed and extracted using the chloroform-isoamyl alcohol method[33]. The DNA quantity of each sample was determined in triplicate using a UV-visible spectrophotometer (PerkinElmer, Milan, Italy). The measurement was performed at a wavelength of 280 nm. This analysis evaluates the impact of the treatment on DNA content within the wound tissues, offering information about potential effects on cellular processes and tissue regeneration[34].

2.6.6. WBC count

Blood was collected in a heparinized tube, and the bioanalyzer Eurocount (Medsources Ozone; Biomedical Pvt. Ltd., India) was used to count WBC.

2.7. Histological studies

After 14 days, the animals were sacrificed by administering an overdose of ketamine and xylazine (100 mg/kg). Skin specimens from the wound areas were excised and preserved in a 10% buffered formalin solution. These specimens were subjected to tissue processing using a paraffin tissue processing machine to prepare them for hematoxylin and eosin (H&E) staining. The prepared tissue samples were visualized and captured using a Leica® microscope (Leica Microsystems, Switzerland) and processed with Leica® software (v. 4.2.0).

2.8. Molecular docking

2.8.1. Ligands and receptors

The 3D structure of proteins and Protein Data Bank (PDB) ID of the crystallographic structure of enzymes were obtained (<https://www.rcsb.org/>). The three target enzymes are glycogen synthase kinase 3 β inhibitor complex (GSK3 β , PDB ID: 3F88), matrix metalloproteinases-8 (MMP-8, PDB ID: 5H8X), and inducible nitric oxide synthase (iNOS, PDB ID: 3N2R).

A collection comprising six phytochemicals that can be extracted from *P. longum* was generated. The ligands pellitorine (PubChem: 5318516), piperanine (PubChem: 5320618), piperine (PubChem: 638024), piperdardine (PubChem: 10086948), piperlonguminine (PubChem: 5320621), and piplartine (PubChem: 637858) were obtained from the NCBI PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). The ligands were converted into PDB file format using Autodock Tools 1.5.7. software.

2.8.2. Molecular docking of *P. longum* isolated compounds

In computer-assisted drug design, molecular docking is the primary computational method for predicting stable ligand-receptor interactions when molecules are bonded to form a stable complex. For molecular docking interaction studies, we have used the AutoDock Vina integrated PyRx tool (<http://vina.scripps.edu/>). The PDB provided the protein crystal structures (<https://www.rcsb.org/>). Initially, the proteins were prepared using Autodock Tool 1.5.7 software, which removed the H₂O molecules and heteroatoms, added polar hydrogens, and saved them in PDBQT file format. The ligands were drawn using Chem Draw Professional 19.0, saved as MDL files, and converted to PDB format. Then, ligand energy (translational energy and rotational energy) will be minimized using the software Chem 3D Professional 19.0. To determine the docking score between the synthesized protein and isolated alkaloid ligands, the prepared protein and PyRx interface were loaded,

and then protein and ligand energies were reduced and transferred into PDBQT files. In this section, through the molecular docking simulations, GSK3 β (3F88), MMP-8 (3N2R), and iNOS (5H8X) were targeted to find the mechanistic interaction by which isolated phytomolecules from *P. longum* exert biological effects against these three proteins involved in wound healing activity and inflammatory activity. With the help of Discovery Studio 2021, the six identified major compounds docked with the target active site of three enzymes (GSK3 β , MMP-8, and iNOS).

2.9. Statistical analysis

The data was analyzed using GraphPad Prism 8 for statistical evaluation and Excel software. The results are presented as mean $\pm$ SEM ($n = 6$) and analyzed by one-way ANOVA analysis, followed by Tukey-Kramer's multiple comparison test. Significance levels were set as $P < 0.05$.

3. Results

3.1. Isolation of compounds from *P. longum* root using p-HPLC

We have isolated six alkaloids from the *P. longum* root through the p-HPLC analysis at 254 nm, represented in Supplementary Figure 1. Six targeted peaks were collected at retention times (Rt) of 3.61, 5.69, 9.41, 12.16, 15.03, and 20.42 min mentioned in Supplementary Figure 1. After isolation using p-HPLC, the following peaks were identified: piplartine (198.82 mg), piperlonguminine (46.07 mg), piperine (25.22 mg), piperdardine (2.91 mg), piperanine (14.17 mg), and pellitorine (49.76 mg). All isolated compounds have been checked for purity using the HPLC peak area normalization method and found to be $> 98.80\%$.

3.2. Antibacterial and antibiofilm activity of *P. longum* root, fruit, and leaf extracts

3.2.1. Antibacterial activity

The antibacterial efficacy was evaluated by measuring the diameter of the inhibition zones, as documented in Table 1. The ethanol extract from the root of *P. longum* exhibited the most potent activity, with a MIC of 700 $\mu\text{g/mL}$ against *S. aureus*. Of the six examined compounds, piperdardine demonstrated the most notable antibacterial activity, with the lowest MIC value of 50 $\mu\text{g/mL}$ and the highest inhibition zone [(13.33 $\pm$ 0.42) mm] (Table 1). Supplementary Figure 2 shows the zone of inhibition after different treatments. *P. longum* root extract demonstrated the highest inhibition zone [(10.66 $\pm$ 0.57) mm] among three plant extracts, therefore, it was used for further *in vivo* evaluation of wound healing activity.

Table 1. Zone of inhibition and minimum inhibitory concentration (MIC) of *Piper longum* (*P. longum*) extracts and isolated compounds against multidrug-resistant *Staphylococcus aureus*.

Extracts & compounds	Zone of inhibition (mm)	MIC ($\mu\text{g/mL}$)
PL root extract	10.66 $\pm$ 0.57	700
PL leaf extract	-	-
PL fruit extract	9.33 $\pm$ 0.89	800
Piplartine (1)	12.00 $\pm$ 1.00	150
Piperlonguminine (2)	12.33 $\pm$ 1.15	100
Piperine (3)	10.66 $\pm$ 1.23	>150
Piperdardine (4)	13.33 $\pm$ 0.42	50
Piperanine (5)	11.66 $\pm$ 1.52	>150
Pellitorine (6)	12.33 $\pm$ 0.95	>100
Vancomycin	13.00 $\pm$ 0.25	10

PL: *P. longum*.

3.2.2. Antibiofilm activity

Our findings revealed that *P. longum* root [(73.83 $\pm$ 3.71)%] and fruit [(58.59 $\pm$ 2.90)%] extracts exhibited better antibiofilm effects against MDR *S. aureus*. *P. longum* leaf extract did not exhibit any antibiofilm activity against MDR *S. aureus*. The microscopic study showed that 100 $\mu\text{g/mL}$ of *P. longum* root fruit significantly reduced biofilm and microcolony growth on the surface compared to the control samples, as seen in Supplementary Figure 3.

3.3. In vivo wound healing studies

3.3.1. Skin irritation study

We tested three extracts (ethanolic, hydroalcoholic, and aqueous) of *P. longum* root for skin irritation. Up to a concentration of 10%, they did not cause any irritation. Further tests revealed that *P. longum* is non-toxic and did not cause allergic reactions, even at 10% concentration when applied to shaved mice's backs.

3.3.2. Pilot study results

In a pilot excision study, three different extracts (ethanolic, hydroalcoholic, aqueous) of *P. longum* root were tested for *in vivo* wound healing activity. Among all the three extracts, the ethanolic extract of *P. longum* root exhibited the highest percentage of wound contraction, indicating the most significant wound healing effect

compared to hydroalcoholic (50/50, *v/v*) and aqueous extracts. Based on these findings, further investigation was carried out on *P. longum* root ethanolic extract to explore its *in vivo* wound healing activity.

3.4. Effect of *P. longum* root extract on wound healing parameter

3.4.1. Wound contraction and epithelization time

A statistically significant enhancement in wound contraction percentage was found in the group treated with the optimized formulated hydrogel. After three days post-wounding, the group receiving no treatment exhibited the least healing, as depicted in Supplementary Figure 4. At 14 days post-wounding, wounds in the infected group treated with *P. longum* 5% (*w/v*) were nearly completely healed, achieving a wound contraction rate of 99.34%, compared with the untreated control group with a wound contraction rate of 65.08%. These results suggest that *P. longum* 5% (*w/v*) treatment exhibited superior wound healing activity compared to the other groups (Figure 1).

The time required for complete epithelialization is important in assessing wound healing. Figure 2A shows the epithelization time following the topical application of *P. longum* root extract. The results indicate that *P. longum* 2% (*w/v*) and 5% (*w/v*) increased the percentage of wound contraction significantly ($P < 0.001$) from day 3. Significantly, an increase in wound healing pattern, characterized by nearly complete wound closure, was observed in the group of mice treated with povidone-iodine, as well as *P. longum* 5% (*w/v*) on day 14. Moreover, mice treated with *P. longum* 5% (*w/v*) demonstrated significantly shorter epithelization time. The epithelialization process extended beyond 14 days for the untreated control, placebo control and *P. longum* 2% (*w/v*) groups. The mean epithelialization period in the group receiving *P. longum* 5% (*w/v*) hydrogel was [(14.0 $\pm$ 1.5) days], comparable to the standard group treated with povidone iodine [(15.0 $\pm$ 0.7) days]. This suggests that the *P. longum* 5% exhibited a more efficient wound healing effect, as indicated by a shorter epithelization time compared with other groups.

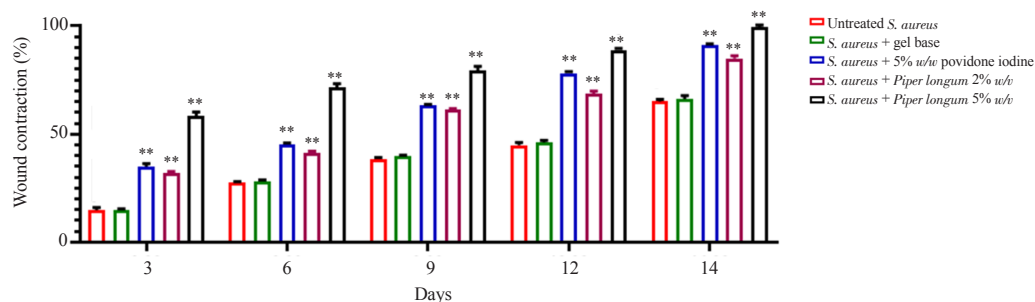


Figure 1. Effect of *Piper longum* (*P. longum*) 2% and 5% on wound contraction rate in mice infected with multidrug-resistant *Staphylococcus* (*S.*) *aureus* from day 3 to day 14. All values are represented as mean $\pm$ SEM ($n=6$) and analyzed by one-way ANOVA, followed by the Tukey-Kramer multiple comparisons test. ** $P < 0.001$ compared with the untreated control.

3.4.2. Bacterial burden in skin tissue

The effect of *P. longum* 2% (*w/v*) and 5% (*w/v*) on *S. aureus*-infected skin wounds was evaluated. After fourteen days of treatment, a notable reduction in bacterial burden was observed in mice treated with both concentrations of *P. longum* hydrogels ($P < 0.001$) (Figure 2B).

3.4.3. WBS

We determined the WBS on the 14th day using a weight suspension method. The animals treated with *P. longum* hydrogels exhibited higher WBS, compared with the untreated control group ($P < 0.001$) (Figure 2C).

3.4.4. Hydroxyproline estimation

The hydroxyproline content was (30.47 ± 1.25) $\mu\text{g}/\text{mg}$ and (30.43 ± 1.36) $\mu\text{g}/\text{mg}$ for the untreated control group and the placebo control group, respectively. *P. longum* (2% and 5%, *w/v*) significantly increased the hydroxyproline content $[(37.33 \pm 1.90)$ $\mu\text{g}/\text{mg}$ and (44.20 ± 2.86) $\mu\text{g}/\text{mg}$, $P < 0.05$] (Figure 2D).

3.4.5. Total DNA content

After 14 days post wounding, the total DNA content of the untreated control, placebo control, standard control, and *P. longum* 2% (*w/v*) and 5% (*w/v*) groups was 4.20 $\mu\text{g}/\text{mL}$, 4.33 $\mu\text{g}/\text{mL}$, 20.88 $\mu\text{g}/\text{mL}$, 16.09 $\mu\text{g}/\text{mL}$, and 21.15 $\mu\text{g}/\text{mL}$, respectively (Figure 2E). *P. longum* 2% (*w/v*) and 5% (*w/v*) obviously increased DNA content compared with the untreated control group, with the most significant effect observed at the higher dose ($P < 0.001$).

3.4.6. WBC count

After 14 days post wounding, the WBC count of the untreated

control, placebo control, standard control, and *P. longum* 2% (*w/v*) and 5% (*w/v*) groups was $(15.11 \pm 0.90) \times 10^3/\mu\text{L}$, $(13.39 \pm 0.67) \times 10^3/\mu\text{L}$, $(8.53 \pm 2.11) \times 10^3/\mu\text{L}$, $(9.41 \pm 1.12) \times 10^3/\mu\text{L}$ and $(6.53 \pm 0.54) \times 10^3/\mu\text{L}$, respectively (Figure 2F). The study revealed that *P. longum* treatment could lower the WBC count.

3.5. Histopathological results

Histological results are demonstrated in Figure 3. The untreated control group exhibited disrupted re-epithelialization, which indicates impaired wound healing. The *P. longum* 5% (*w/v*)-treated group displayed complete re-epithelialization in comparison to the untreated control group and placebo control group. Moderate inflammation was observed in both the untreated control and placebo control groups, which was markedly alleviated by *P. longum* treatment. Complete epithelialization was observed on day 14 in the group treated with *P. longum* 5% (*w/v*) or standard drug, with the development of skin appendages, such as the keratin layer, hair follicle, and sebaceous gland. This study found that *P. longum* 5% (*w/v*) hydrogel accelerated wound healing by increasing collagen and keratin production, reducing macrophages, and improving granulation tissue formation.

3.6. Molecular docking

Table 2 presents the results of the molecular docking study. The major identified six compounds in *P. longum* root had significant effects on the target enzymes, as indicated by their favorable docking scores. The GSK3 β enzyme was docked with the crystallized inhibitor (3HT), and the best docking pose was compared to its bound conformation (Supplementary Figure 5). The model was

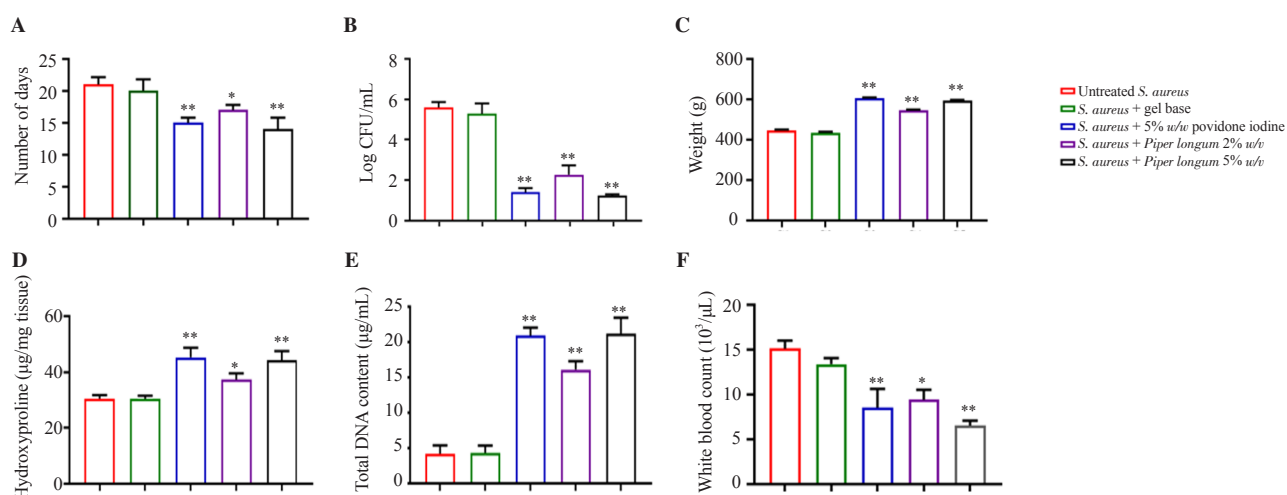


Figure 2. Effect of *P. longum* (2% and 5% *w/v*) on (A) epithelization period, (B) bacterial burden, (C) wound breaking strength, (D) hydroxyproline content, (E) total DNA content and (F) white blood count in mice with multidrug-resistant *S. aureus*-infected skin wound. All values are represented as mean $\pm$ SEM ($n = 6$) and analyzed by one-way ANOVA, followed by Tukey-Kramer multiple comparisons test. * $P < 0.05$ and ** $P < 0.001$ compared with the untreated control.

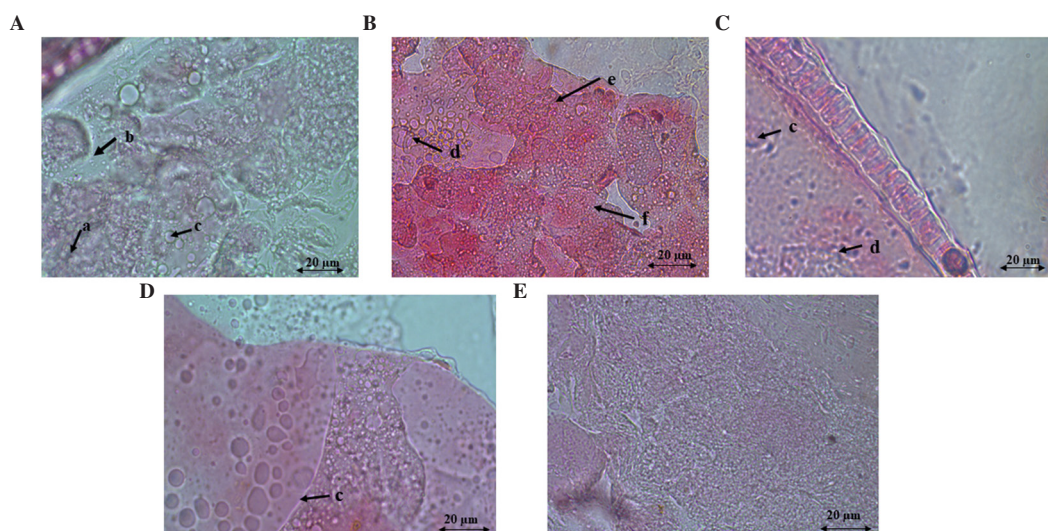


Figure 3. Hematoxylin & eosin stained histological sections of wound tissues after 14 days of treatment (scale bar: 20 µm; magnification: 100×): (A) Untreated control; (B) Placebo control (gel base treated); (C) *P. longum* 2% (w/v); (D) Standard treatment [povidone iodine 5% (w/w)]; (E) *P. longum* 5% (w/v). a: polymorphonuclear leucocytes, b: disrupted skin layer, c: fibroblast, d: platelets, e: mast cell, f: vacuoles.

further analyzed using the binding site and conformation with the highest docking score of piperdardine with GSK3β (Supplementary Figure 5). The root mean square deviation of the co-crystallized ligand and the retrieved docking poses were 0.79 Å, suggesting its potential for predicting the optimal binding mode. The alignment of the best components with co-crystallized BF471 (catechol inhibitor) is shown in Supplementary Figure 5B (left). A 2D diagram of co-crystallized ligand and all six molecules and their binding modes is shown in Supplementary Figure 5B-H (left). Out of the six compounds, piperdardine exhibited the most favorable docking scores of −9.7 kcal/mol and bonded in hydrogen bond interactions Val 135, and other interactions, *i.e.*, hydrophobic interaction alkyl: Lys 85, Cys 199, Ile 62; π -alkyl: Val70 and Phe 67 (π - π stacked) with protein (3F88), as depicted in Supplementary Table 1.

The appropriate co-crystallized ligand bound to a specific site of these enzymes. To validate docking, co-crystal ligands were perturbed within their respective binding sites, and the docking score of co-crystallized ligands with the best docking poses is mentioned in Table 2. The best docking score for MMP-8 was −9.8 kcal/mol, with hydrophobic interactions such as α -alkyl: Tyr 189, Leu 160; π -alkyl: Tyr 219, Val 194; and other interactions such as His 197 (π - π stacked) and Asn 218 (amide- π stacked) (Supplementary Table 2). Supplementary Figure 6 depicts the binding conformation and binding site of piperdardine with MMP-8.

Piperdardine showed the most remarkable docking score of −9.2 against iNOS with some hydrophobic interactions, such as alkyl: Ala 412, Arg 414, and π -alkyl: Cys 415 (Supplementary Table 3). The binding conformation and binding site of piperdardine with 3N2R are demonstrated in Supplementary Figure 7.

Table 2. The docking scores of six major compounds identified in *P. longum* root extract against the three target enzymes GSK3β, MMP-8 and iNOS (kcal/mol).

Compounds	GSK3β (3F88)	MMP-8 (5H8X)	iNOS (3N2R)
Pellitorine	−6.6	−6.5	−6.6
Piperanine	−8.5	−8.7	−8.7
Piperine	−8.5	−8.7	−8.6
Piperdardine	−9.7	−9.8	−9.2
Piperlonguminie	−8.0	−8.4	−8.0
Piplartine	−8.0	−7.9	−7.5
3HT	−9.5	-	-
7FY	-	−9.7	-
XJH	-	-	−9.2

−: not available.

4. Discussion

This study highlights the pharmacological potential of *P. longum* root, attributing its biological activity to secondary metabolites. Using p-HPLC analysis, six alkamides were isolated from the root extract. The extract and these alkamides exhibited significant antibacterial and antibiofilm activity. Identifying effective antibiofilm compounds is crucial, as biofilm-forming pathogens are particularly resistant to treatment and contribute to persistent infections.

We assessed the *in vivo* wound healing efficacy of *P. longum* root using incision and excision models. The *P. longum* 5% (w/v) hydrogel-treated group demonstrated superior wound healing. On day 14, the *P. longum* 5% (w/v) hydrogel exhibited enhanced wound closure, highlighting its effectiveness in tissue restoration. Wound contraction, a crucial phase in healing, was evaluated[35]. The *in vivo* studies confirmed improved wound stability in *P. longum* hydrogel-treated mice, as evidenced by increased tensile strength and collagen

turnover[36]. Elevated hydroxyproline levels further validated the role of *P. longum* in collagen synthesis and fiber stabilization. Notably, *P. longum* 5% (w/v) hydrogel exhibited superior efficacy over *P. longum* 2% (w/v), demonstrating greater epithelialization and wound contraction. These findings affirm the potential of *P. longum* in promoting effective wound healing. Collagen turnover plays a crucial role in tissue repair, with hydroxyproline levels serving as a reliable indicator of collagen viability and wound healing efficacy. Since hydroxyproline is abundant in collagen, its elevated levels indicate increased collagen maturation, epithelial and fibroblast migration, and extracellular matrix synthesis. The rapid synthesis of collagen post-injury facilitates tissue regeneration, making hydroxyproline assessment a valuable marker for the healing process.

In silico docking studies confirm that compounds isolated from *P. longum* root extract showed marked interactions with three key wound healing targets: GSK3 β , MMP-8, and iNOS. GSK3- β , a serine/threonine kinase, plays a crucial role in cellular processes, including proliferation, differentiation, and apoptosis. MMP-8 facilitates extracellular matrix remodeling by degrading type I collagen, aiding in tissue regeneration. However, excessive MMP-8 activity can impair healing by prolonging inflammation. iNOS produces nitric oxide, promoting angiogenesis, keratinocyte migration, and fibroblast activity. While iNOS supports wound repair, excessive production may cause oxidative stress and delay healing. Proper regulation of MMP-8 and iNOS is crucial for optimal wound recovery, particularly in chronic wounds. Docking results support that major phytoconstituents of *P. longum* root, particularly piperdardine, may aid wound healing by targeting these enzymes, thereby reducing inflammation and collagen degradation.

The histopathological analysis also provides valuable insights into the cellular dynamics of wound healing, particularly re-epithelialization, where epithelial cells proliferate and migrate to cover the wound bed[37]. This study showed that *P. longum* root extract obviously accelerated wound healing.

In conclusion, the results demonstrated that *P. longum* extracts effectively inhibited biofilm development and showed pronounced antibacterial activity. It also promoted wound healing by decreasing inflammation and accelerating wound contraction. However, further studies are needed to assess the efficacy of *P. longum* extract and its active compounds against other bacterial pathogens and to elucidate its precise wound healing mechanisms. Advancing this understanding will be crucial for validating the medicinal benefits of *P. longum* and supporting its clinical applications.

Conflict of interest statement

All authors declare that they have no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

PG carried out biochemical experiments, isolated compounds for p-HPLC analysis, and wrote and edited manuscripts. PS carried out microbial tests for antibacterial and antibiofilm activities. JY carried out both biochemical and histological analyses. CVR was involved in conceptualisation, supervision, data curation, research administration, validation, writing, review, and editing. All authors have approved the final manuscript.

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Supplementary Results

Characterization of compounds (1-6) isolated from *P. longum* root extract through NMR and HR-MS

Piplartine or piperlongumine (1)

Colourless crystals, HR-MS m/z (%): Observed m/z 318.133 9 $[M+H]^+$ (Calculated m/z 318.336 4, $C_{17}H_{19}NO_5$). It exhibited (U.V.) absorption at 322 nm. ^1H NMR spectrum (CD_3OD , 500 MHz): δ 2.49 (m, 2H, 5-H), 4.00 (t, $J = 6.5$ Hz, 2H, 6-H), 3.87 (s, 3H, OCH_3 's), 3.79 (s, 6H, $2 \times OCH_3$'s), 6.02 (d, $J = 9.5$ Hz, 1H, 3-H), 6.92 (s, 2H, 2', 6'-H), 7.09 (dt, $J = 9.4, 9.3$ Hz, 1H, 4-H), 7.38 (d, $J = 15.55$ Hz, 1H, 8'-H), 7.62 (d, $J = 15.55$ Hz, 1H, 7'-H). ^{13}C NMR ($CDCl_3$, 125 MHz) : δ 25.88 (C-5), 43.18 (C-6), 56.83 (3',5'-OMe) 61.34 (4'-OMe), 106.83 (C- 2' & 6'), 122.77 (C- 8'), 126.09 (C-3), 132.45 (C-1'), 143.7 (C-7', 4), 148.57 (C- 3', 5'), 154.94 (C-4'), 167.93 (C-2), 170.74 (C-9'). NMR data are matched with reported values in the literature[1].

Piperlonguminine (2)

Colourless powder, HR-MS m/z (%): Observed m/z 274.144 9 (Calculated m/z 274.326 9, $C_{16}H_{19}NO_3$), $[M+H]^+$, 296 $[M + Na]^+$, ^1H NMR spectrum (CD_3OD , 500 MHz): δ H 6.02 (d, $J = 9.5$ Hz, 3-H), 7.09 (dt, $J = 9.3, 4.2$ Hz, 1H, 4-H), 2.49 (m, 2H, 5-H), 4.00 (t, $J = 6.5$ Hz, 1H, 6-H), 6.92 (s, 2H, 2', 6'-H), 7.62 (1H, d, $J = 15.55$ Hz, 7'-H), 7.38 (d, $J = 15.55$ Hz, 1H, 8'-H), 3.87 (s, OCH_3 , 4'-H), 3.79 (s, $2 \times OCH_3$, 3', 5'-H).

^{13}C NMR ($CDCl_3$, 125 MHz) : δ 169.26 (C-1), 123.81 (C-2), 142.19 (C-3), 126.16 (C-4), 140.22 (C-5), 132.60 (C-1'), 106.89 (C-2'), 149.89 (C-3'), 145.93 (C-4'), 109.51 (C-5'), 124.45 (C-6'), 102.82 (- OCH_2O), 48.24 (C-1''), 29.69 (C-2''), 21.42 (C-3''), 20.63 (C-4''). NMR data are matched with reported values in the literature[2].

Piperine (3)

Pale yellow HR-MS m/z (%): Observed m/z 286.153 4 (Calculated m/z 286.337 6, $C_{17}H_{19}NO_3$)

$[M+H]^+$, ^1H NMR spectrum (CD_3OD , 500 MHz): δ 6.64 (d, 1H, $J = 14.65$ Hz, H-2), 7.34 (1H, m, H-3), 6.97 (1H, m, H-4), 6.78 (1H, m, H-5), 6.98 (1H, d, $J = 1.30$ Hz, H-2'), 6.89 (1H, d, $J = 8.05$ Hz, H-5'), 6.97 (dd, $J = 1.6, 8.05$ Hz, 1H, H-6'), 5.96 (2H, s, $-OCH_2O$), 3.63 (m, 2H, H-1''), 1.61 (m, 2H, H-2''), 1.70 (m, 2H, H-3''), 1.61 (m, 2H, H-4''), 3.63 (m, 2H, H-5'').

^{13}C NMR ($CDCl_3$, 125 MHz) : δ 167.90 (C-1), 120.80 (C-2), 144.71 (C-3), 126.56 (C-4), 140.30 (C-5), 132.57 (C-1'), 106.83 (C-2'), 149.90 (C-3'), 149.94 (C-4'), 109.51 (C-5'), 124.01 (C-6'), 102.86 ($-OCH_2O$), 44.69 (C-1''), 27.04 (C-2''), 25.70 (C-3''), 28.02 (C-4''), 48.27 (C-5'')[2].

Piperdardine (4)

Solid, HR-MS m/z (%) : Observed m/z 314.177 4 (Calculated m/z 313.390 8, $C_{19}H_{23}NO_3$)

$[M+H]^+$, ^1H NMR spectrum (CD_3OD , 500 MHz): 6.25 (d, $J = 15.0$ Hz, 1H, H-2), 7.20 (dd, $J = 15.0, 11.0$ Hz, 1H, H-3), 6.16 (dd, $J = 15.0, 11.0$ Hz, 1H, H-4), 6.05 (dt, $J = 15.0, 7.0$ Hz, 1H, H-5), 2.41 (dt, $J = 7.0, 7.5$ Hz, 2H, H-6), 2.65 (2H, $J = 7.5$ Hz, H-7), 3.46 (s, 2H, H-2'), 1.43–1.59 (m, 2H, H-3'), 1.60–1.69 (m, 2H, H-4'), 1.43–1.59 (m, 2H, H-5'), 3.60 (s, 2H, H-6'), 6.65 (s, 1H, H-2''), 6.59 (dd, $J = 1.5, 8.0$ Hz, 1H, H-5''), 6.69 (1H, d, $J = 8.0$ Hz, H-6''), 5.93 (s, 2H, $-OCH_2O$).

^{13}C NMR ($CDCl_3$, 125 MHz) : 166.5 (C-1), 120.1 (C-2), 143.2 (C-3), 130.1 (C-4), 142.5 (C-5), 35.5 (C-6), 35.6 (C-7), 47.3 (C-1'), 27.19 (C-2'), 25.0 (C-3'), 26.1 (C-4'), 43.6 (C-5'), 135.8 (C-1''), 108.5 (C-2''), 148.6 (C-3''), 147.0 (C-4''), 106.6 (C-5''), 122.4 (C-6''), 101.3 ($-OCH_2O$)[3].

Piperanine (5)

White amorphous solid, HR-MS m/z (%): Observed 288.159 9 (Calculated m/z 288.353 5,

$C_{17}H_{21}NO_3$) $[M+H]^+$, ^1H NMR spectrum (CD_3OD , 500 MHz): δ 6.22 (d, $J = 15.28$ Hz, 1H, H-2)

6.83 (m, 1H, H-3) 2.47 (m, 2H, H-4) 2.71 (t, J = 7.37 Hz, 2H, H-5), 6.69 (d, J = 1.23 Hz, 1H, H-2'), 6.74 (d, J = 7.89 Hz, 1H, H-5'), 6.64 (dd, J = 1.37, 7.91 Hz, 1H, H-6') 5.93 (s, 2H, OCH₂O), 3.50 (s, 2H, H-1'') 1.57 (m, 2H, H-2'') 1.65 (m, 2H, H-3'') 1.57 (m, 2H, H-4'') 3.50 (s, 2H, H-5'').

¹³C NMR (CDCl₃, 125 MHz): δ 167.93(C-1), 121.67 (C-2), 146.62 (C-3), 32.90 (C-4), 33.44 (C-5), 135.01 (C-1'), 106.41 (C-2'), 148.37 (C-3'), 149.58 (C-4'), 109.19 (C-5'), 122.53 (C-6'), 102.42 (-OCH₂O), 44.51 (C-1''), 26.93 (C-2''), 25.62 (C-3''), 23.86 (C-4''), 48.33 (C-5'')[2].

Pellitorine (6)

White amorphous solid, HR-MS *m/z* (%): Observed *m/z* 224.201 9 (Calculated *m/z* 224.354 4, C₁₄H₂₅NO) [M+H]⁺, ¹H NMR spectrum (CD₃OD, 500 MHz): δ 5.94 (d, 1H, J = 15.00 Hz, H-2), 7.14 (m, 1H, H-3), 6.19 (m, 1H, H-4), 6.07 (m, 1H, H-5), 2.19 (m, 2H, H-6), 1.47 (m, 2H, H-7), 1.34 (m, 2H, H-8) 1.34 (m, 2H, H-9) 0.92 (t, 3H, J = 7.05 Hz, H-10) 3.06 (t, 2H, J = 6.80 Hz, H-1''), 1.80 (m, 1H, H-2''), 1.17 (d, J = 8.60 Hz, 3H, H-3''), 1.17 (d, J = 8.60 Hz, 3H, H-4''), 4.60 (s, 1H, H-NH).

¹³C NMR (CDCl₃, 125 MHz): δ 169.37 (C-1), 123.16 (C-2), 142.31 (C-3), 129.93 (C-4), 144.18 (C-5), 34.01 (C-6), 29.82 (C-7), 32.66 (C-8), 23.64 (C-9), 14.45 (C-10) 48.17(C-1''), 29.88 (C-2''), 20.63 (C-3''), 20.63 (C-4'')[2,3].

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- [1] Rao GV, Rao KS, Mukhopadhyay T, Madhavi M. Alkamides and their biological activity from *Piper longum* Linn. *J Pharm Res* 2012; 5(1): 165-168.
- [2] Wu S, Sun C, Pei S, Lu Y, Pan Y. Preparative isolation and purification of amides from the fruits of *Piper longum* L. by upright counter-current chromatography and reversed-phase liquid chromatography. *J Chromatogr A* 2004; 1040(2): 193-204.

- [3] Huang XZ, Xu Y, Zhang YF, Zhang Y, Wong YH, Han Z, et al. Nontoxic piperamides and their synthetic analogues as novel antifouling reagents. *Biofouling* 2014; **30**(4): 473-481.

Supplementary Table 1. Binding interactions of *Piper longum* isolated compounds and co-crystallize ligand (3HT) against GSK3 β (3F88).

Compounds	H-bond	Conventional H Bond	Hydrophobic interaction	Others
Pellitorine	-	Asp 200	Alkyl: Ile 62, Val 70, Cys 199, Ala 83, Leu 188	Phe 67 (π -Sigma)
Piperanine	-	-	Alkyl: Cys199, Lys85, Ala83, Leu188; π -Alkyl: Val 70	Phe 67 (π - π stacked)
Piperine	-	Lys 85, Lys 183	Alkyl: Leu 88, Val 110, Val 70, Ala 83, Leu 132, Cys 199	Phe 67 (π - π stacked)
Piperdardine	Val 135	-	Alkyl: Lys 85, Cys 199, Ile 62; π -Alkyl: Val 70	Phe 67 (π - π stacked)
Piperlonguminie	-	Lys 85	Alkyl: Ile 62; π - Alkyl: Val 70, Val 110	Cys 199 (π -donar H-bond), Leu 132 (π -Sigma)
Piplartine	-	Lys 85	Alkyl: Cys 199, Leu 132; π -Alkyl: Val 110	Phe 67 (π - π stacked)
3HT	-	-	Alkyl: Met 101, Lys 85; π -Alkyl: Val 110, Ala 83	Leu 188, Leu 132, Val 70 (π -Sigma); Cys 199 (π -donar H-bond)

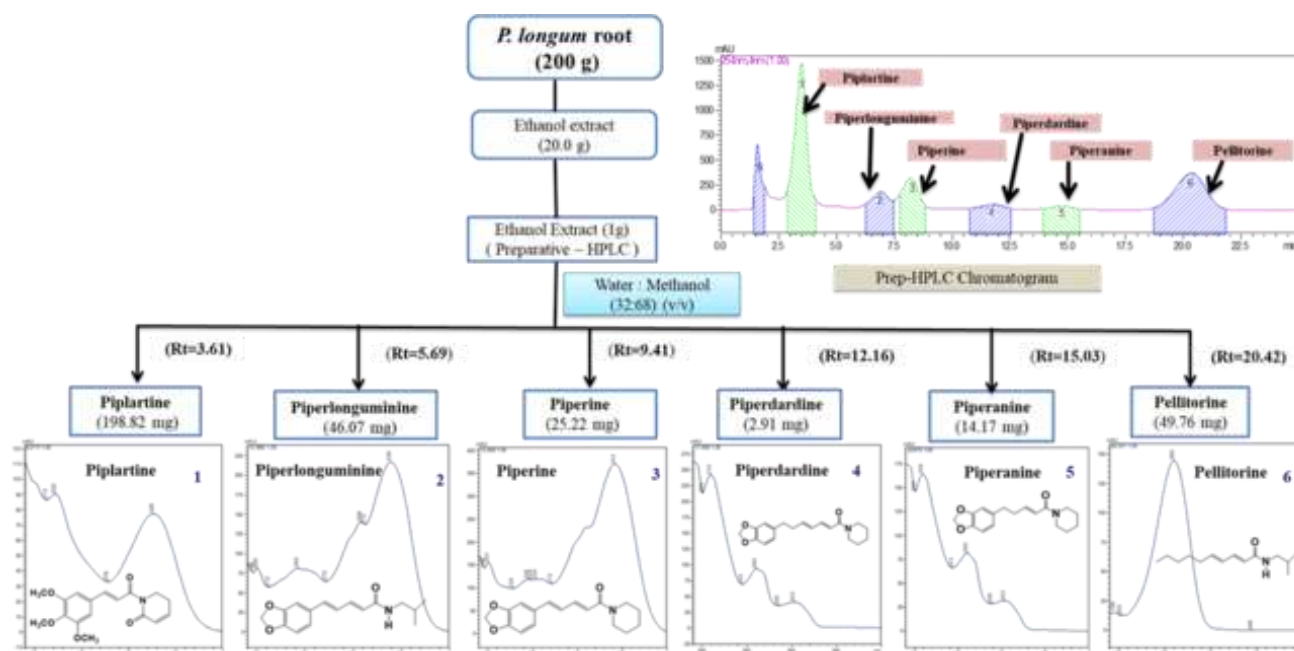
Supplementary Table 2. Binding interactions of *Piper longum* isolated compounds and co-crystallize ligand (7FY) against MMP-8 (5H8X).

Compounds	H-bond	Conventional H Bond	Hydrophobic interaction	Others
Pellitorine	-	Leu 160, Gly 18	Alkyl: Leu 214, Leu 193; π - Alkyl: Tyr 219; Alkyl: His 197, Val 194	-
Piperanine	-	-	Alkyl: Leu 160; π -Alkyl: Val 194	His 197 (π - π stacked), Asn 218 (Amide- π stacked)
Piperine	-	Arg 222, Leu 160	Alkyl: His 207, Ile 159; π - Alkyl: Val 194	His 197 (π - π stacked),
Piperdardine	-	-	Alkyl: Tyr 189, Leu 160; π - Alkyl: Tyr 219, Val 194	His 197 (π - π stacked), Asn 218 (Amide- π stacked)
Piperlonguminie	Glu 198, Ile	Arg 222,	π - Alkyl: His 201,	His 197 (π - π

	159, Ala 220	Leu 160	His 207, Val 194	stacked), Asn 218 (Amide- π stacked)
Piplartine	Asn 218, Leu193	-	Alkyl: Tyr 219; π - Alkyl: Val 194	-
7FY	Arg 222, Tyr 219	-	π - Alkyl: Val 194	Ile 159 (π - σ); Glu 198 (π -anion); His 197 (π - π stacked)

Supplementary Table 3. Binding interactions of *Piper longum* isolated compounds and co-crystallize ligand (XJH) against iNOS (3N2R).

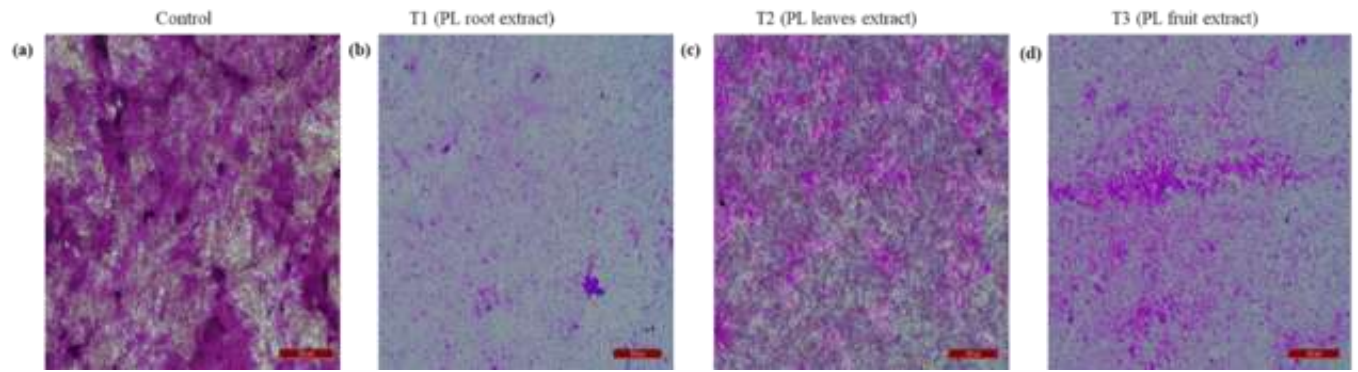
Compound	H-bond	Conventional H Bond	Hydrophobic interaction	Others
Pellitorine	-	-	Alkyl: Ile 459, Leu 424, Phe 704, Phe 584, Cys 415, Val 567, Pro 565; π -Alkyl: Trp 409	-
Piperanine	-	-	Alkyl: Cys 415, Met 570, Ala 412	Phe 584, Trp 409 (π - π stacked)
Piperine	-	-		Phe 584, Trp 409 (π - π stacked)
Piperdardine	-	-	Alkyl: Ala 412, Arg 414; π -Alkyl: Cys 415	Phe 584, Trp 409 (π - π stacked)
Piperlonguminie	-	Glu 592	Alkyl: Val 567; Pro 565	Phe 584, Trp 409 (π - π stacked)
Piplartine	Glu 592	-	Alkyl: Pro 565, Arg 414, Val 567, Trp 678, Phe 584, Leu 424; π - Alkyl: Cys 415	Trp 409 (π -Sigma)
XJH	-	Arg 414	π - Alkyl: Leu 424	Cys 415 (π -Sulphur), Trp 409, Phe 584 (π - π stacked)



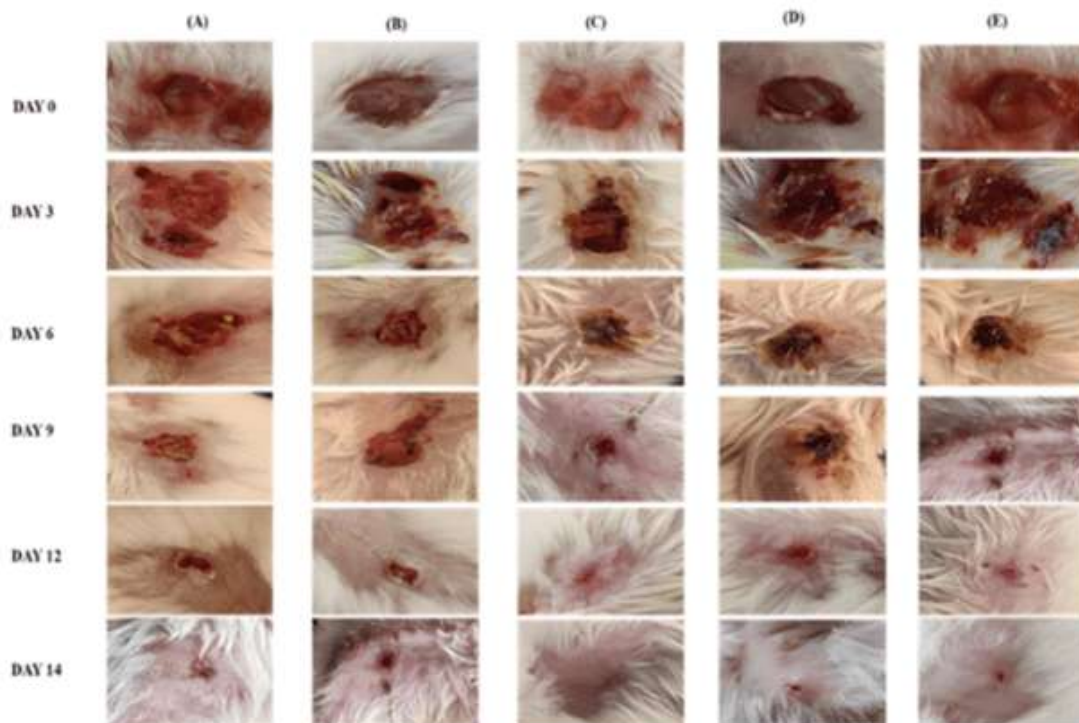
Supplementary Figure 1. Schematic display of extraction and isolation of six alkaloids compounds-Piplartine (PLT, 1), piperlonguminine (PLG, 2), piperine (PR, 3), piperdardine (PDD, 4), piperanine (PRN, 5), and pellitorine (PTR, 6) from *Piper longum* root.



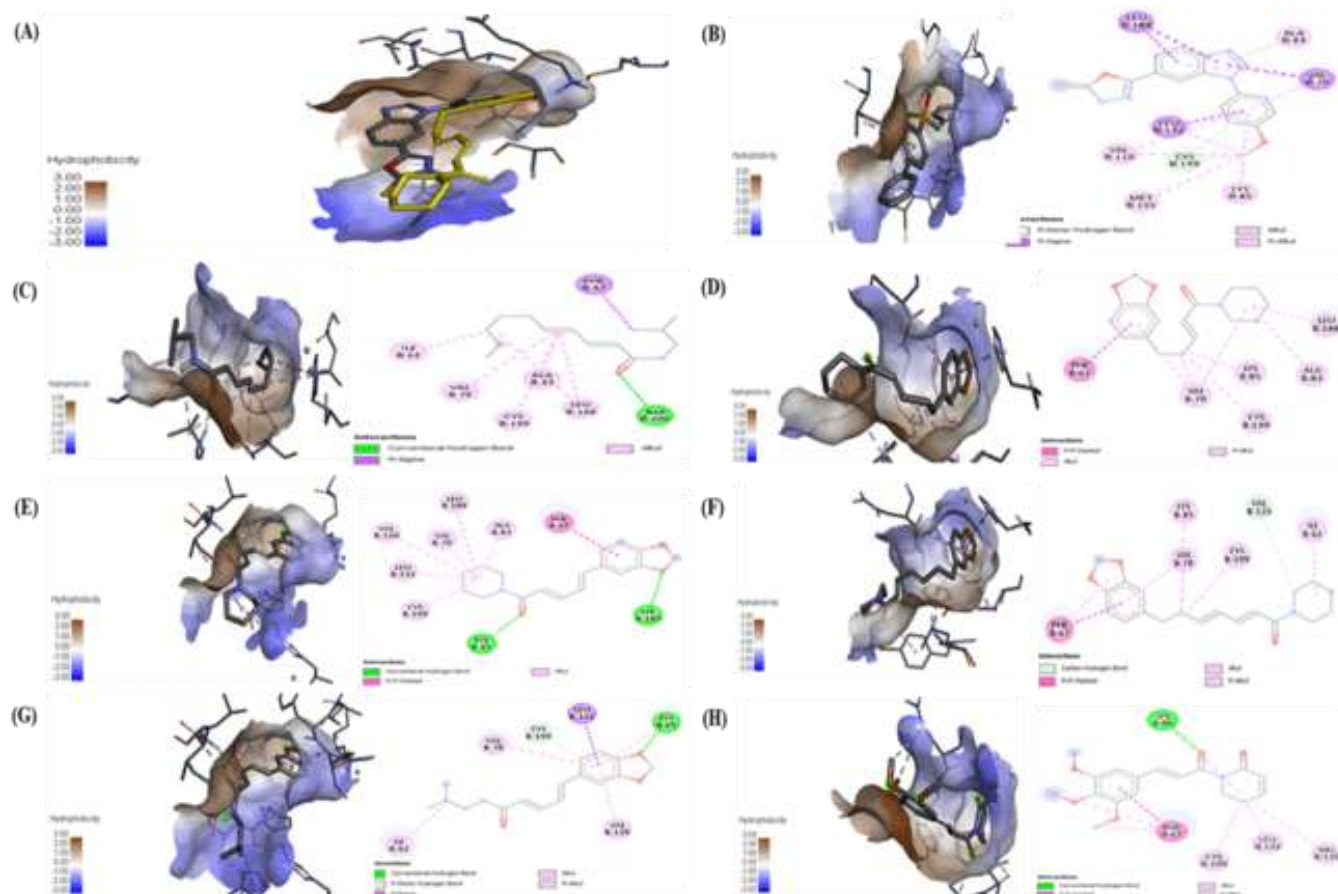
Supplementary Figure 2. *In-vitro* culture plates (well diffusion method) of standard drug vancomycin (Vm), negative control (-ve, ethanol), (A) ethanolic extracts of *Piper longum* root (PLR), *Piper longum* leaves (PLL), *Piper longum* fruit (PLF) at 10 mg/mL and (B) isolated compounds pellitorine (1), piperanine (2), piperine (3), piperdardine (4), (C) piperlonguminine (5), piplartine (6) from PLR showing zone of inhibition against *Staphylococcus aureus*.



Supplementary Figure 3. Biofilm images of *in-situ* visualization of *Staphylococcus aureus* by crystal violet staining under a light microscope (magnification: 100×). **(A)** Control-*Staphylococcus aureus* biofilm; **(B)** T1- Treated *Piper longum* root extract; **(C)** T2- Treated *Piper longum* leaf extract **(D)** T3- Treated *Piper longum* fruit extract.

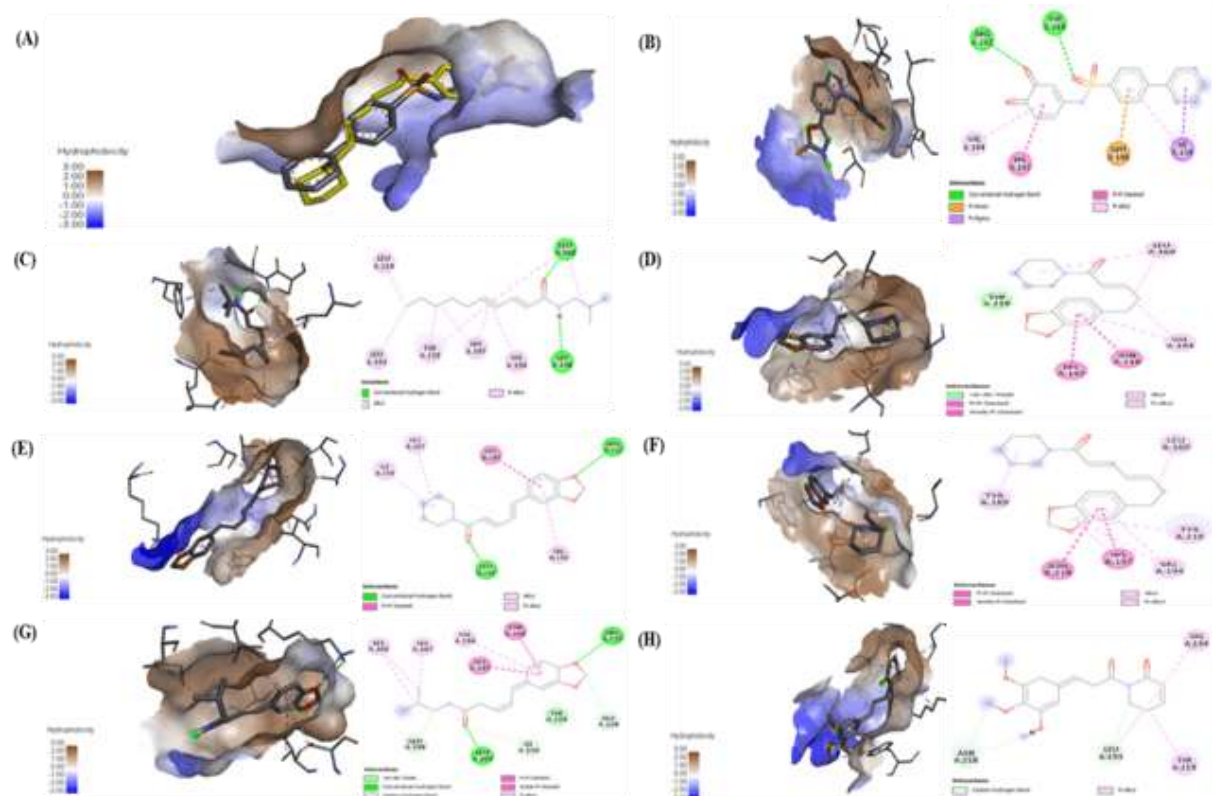


Supplementary Figure 4. Photographic representation of excision wounds. **(A)** Untreated control; **(B)** Placebo control (gel base applied topically); **(C)** Standard control (5% w/w povidone iodine ointment), **(D)** *Piper longum* 2% (w/v), **(E)** *Piper longum* (5% w/v).

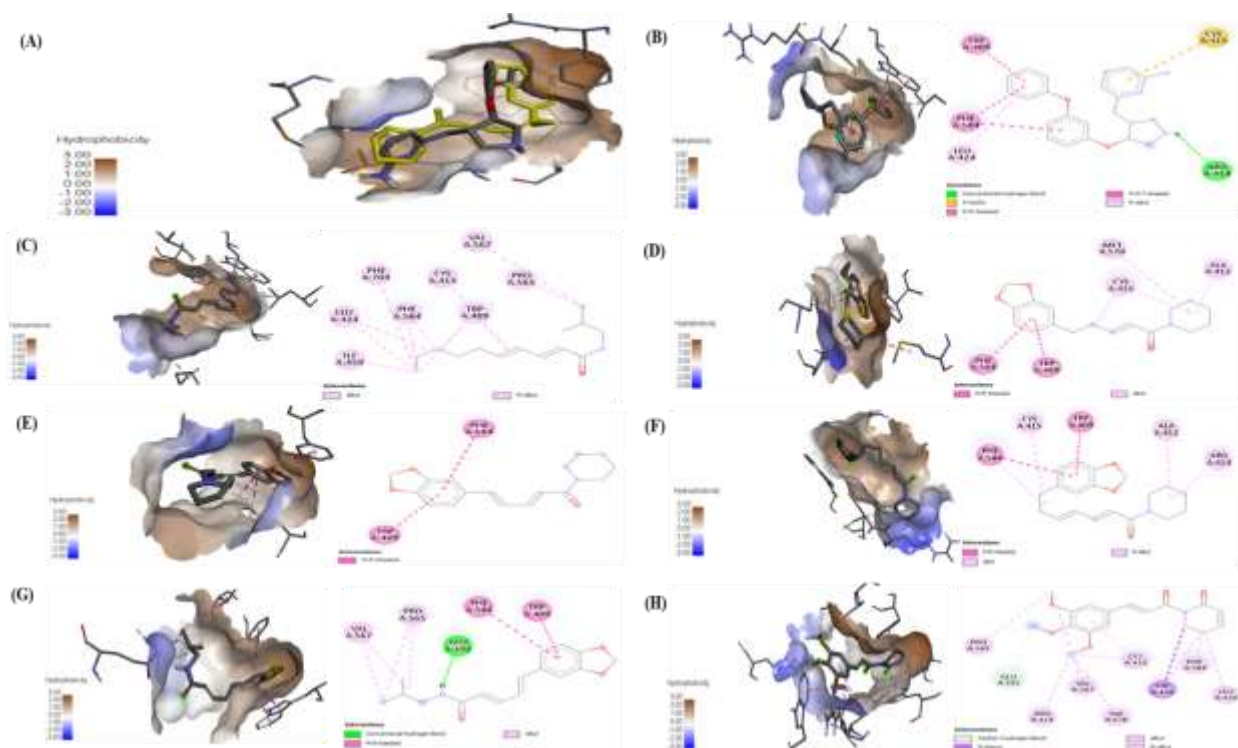


Supplementary Figure 5. Overlay of docked co-crystallised 3F88 (grey color) and piperdardine (yellow color) in the active site of GSK3β (PDB: 3F88) illustrates the interactions with the best-docked pose and hydrophobicity of the binding pocket **(A)**, **B-H** (right) The binding site of GSK3β illustrating the interactions with the best-docked pose of the co-crystallised ligand of 3F88 (3HT) **(B)**, pellitorine **(C)**, piperanine **(D)**, piperine **(E)**, piperdardine **(F)**, piperlonguminine **(G)**, and piplartine **(H)** to the active binding sites of GSK3β, B-H (left) surface

2D binding modes representations of the GSK3 β binding site and the *P. longum* isolated compounds, indicating the hydrophobicity of the binding pocket.



Supplementary Figure 6. Overlay of docked co-crystallised BF471 (grey color) and piperdardine (yellow color) in the active site of MMP-8 (PDB: 5H8X) illustrates the interactions with the best-docked pose and hydrophobicity of the binding pocket (A), B-H (right) The binding site of MMP-8 illustrating the interactions with the best-docked pose of the co-crystallised ligand of 5H8X (7FY) (B), pellitorine (C), piperanine (D), piperine (E), piperdardine (F), piperlonguminine (G), and piplartine (H) to the active binding sites of MMP-8, B-F (left) surface 2D binding modes representations of the MMP-8 binding site and the *P. longum* isolated compounds, indicating the hydrophobicity of the binding pocket.



Supplementary Figure 7. Overlay of docked co-crystallized 3N2R (grey color) and piperdardine (yellow color) in the active site of iNOS (PDB: 3N2R) illustrates the interactions with the best-docked pose and hydrophobicity of the binding pocket (A); B-H (right) the binding site of iNOS illustrating the interactions with the best-docked pose of the co-crystallised ligand of 3N2R (XJH) (B), pellitorine (C), piperanine (D), piperine (E), piperdardine (F), piperlonguminine (G), and piplartine (H) to the active binding sites of iNOS; B-F (left) 2D representations of the iNOS binding site and the *P. longum* isolated compounds.