The work embodied in this thesis describes the antimicrobial and anti-inflammatory activity of *Pedilanthus tithymaloides* (L) Poit. (Euphorbiaceae) leaf extract, used by tribes of Santal Parganas for the development of a cost effective, natural topical formulation. This work covers mainly three areas. (i) Extraction as well as bioactivity-guided isolation and structure elucidation of phytoconstituents from *P. tithymaloides* leaves. (ii) Toxicity study of leaf extracts and isolated compound(s) (iii) Evaluation of pharmacological and antimicrobial activities (Anti-inflammatory, antimicrobial and wound healing activity) of leaves extracts and isolated compounds.

**Chapter 1:** Brief Introduction of natural products.

**Chapter 2:** Extraction as well as bioactivity-guided isolation and structure elucidation of phytoconstituents from methanol and chloroform extract of *P. tithymaloides* leaves by various spectroscopic analyses.

**Chapter 3:** Toxicity profile of chloroform and methanol leaf extracts as well as isolated compound(s) administered orally in Swiss mice.

**Chapter 4:** The *in vitro* antimicrobial activity of leaf extracts and isolated compound(s) against different isolates of human pathogenic bacteria and fungus.

**Chapter 5:** Detailed anti-inflammatory, antinociceptive and antipyretic potential of bioactive constituents from *P. tithymaloides* leaves in standard laboratory animal models.

**Chapter 6:** Wound healing activity of methanolic extract of *P. tithymaloides* leaves and its isolated constituents in ointment form.

**Chapter 7:** The *in vitro* anti-HSV activity of crude methanolic extract of *P. tithymaloides* leaves and its isolated compound(s).
The present thesis embodies the results on “Studies on the antimicrobial and anti-inflammatory activity of Pedilanthus tithymaloides (L) Poit. (Euphorbiaceae) leaf extract, used by tribes of Santal Parganas for the development of a cost effective, natural topical formulation”.

The use of herbs as medicine is the oldest form of healthcare known to humanity and has been used in all cultures throughout history. Primitive people recognized their dependence on nature for a healthy life and since that time humanity has depended on the diversity of plant resources for food, clothing, shelter, and medicine to cure myriads of ailments. Indeed, in the 20th century, most of the pharmacopeia of scientific medicine was derived from the herbal lore of native people and laying the foundation for many systems of traditional medicine in both developing and developed countries. Considering the adverse effects of synthetic drugs, the Word population is looking for natural remedies which are easy available, economical and safe when compared to allopathic system of medicines.

Pedilanthus tithymaloides (L.) Poit. (family Euphorbiaceae) is a low tropical wild shrub growing in tropical and subtropical parts of India, as well as North and Central America (Fig. 1). It is known as Jew’s Slipper in English and Rang-chita in Bengali, and Brihatgokshura, Trikantaka, Gokantaka, and Bhakshantaka in Ayurveda system of medicine. This plant is half-woody containing fleshy branches which produce milky latex. This plant primarily ornamental border plant, but certain varieties are kept indoors and require a sunny area to grow.

The contemporary literature survey revealed that, P. tithymaloides is used for a wide range of healing properties including antiviral, antibacterial, anti-inflammatory, emetic, antihemorrhagic, antitumor, abortive, anticancer and anti-inflammatory. In folklore medicine, tea brewed with P. tithymaloides leaves has been used in asthma, mouth ulcers and venereal disease while some islanders of the Indian Ocean it used the leaf of this plant in headache, latex to cure venereal diseases, and the aerial part to cure skin disorder.

Earlier reports showed that, the plant contain several bioactive principles including cycloartenol, 1-Octacosanol, and friedelin, carotene derivatives azafrin and a cancer inhibitor diterpene pedilstatin. A bioassay-guided fractionation leads to the isolation of the main antioxidant principles of P. tithymaloides as quercitrin (Fig. 2),
kaempferol 3-O-β-D-glucopyranoside-6-(3-hydroxy-3-methylglutarate) (Fig. 3).

Interestingly, the tincture of American species reported to have in vivo anti-inflammatory and antioxidant activity. Another study with the ethanol extract of P. tithymaloides leaves and some of its phytoconstituents showed antimicrobial activity against Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa and Escherichia coli. Traditionally tribal peoples used a handful of leaves warmed on fire and tied around the affected wound and fire burns for relief and healing while its latex, is used in Maharashtra for healing wound.

Although most of the phytochemical and biological studies of the active components of P. tithymaloides were reported from the whole plant and its latex, there are no systemic phytochemical and biological evaluations of the constituents of its leaves. During a random drug-screening procedure chloroform (CHCl₃) extract and methanol (MeOH) extract of the leaves of the P. tithymaloides was found to possess strong anti-inflammatory, anti-microbial, antiviral and wound healing potentiality. Encouraged by this ideas, the present study aims to reports the isolation and structure elucidation of phytoconstituents from the CHCl₃ and MeOH extract and evaluation of anti-inflammatory, antimicrobial, wound healing as well as antiviral activity of crude extracts and isolated compounds.

Chapter 1 incorporates reviews dealing with brief introduction of natural products. This review covers the evolving role of ethnomedicine, problems and prospects of ethnomedicinal drug discovery. Phytoconstituents with their biological activities and identification of natural products by chromatographic and spectroscopic methods have been summarized in this review.
Chapter 2 deals with extraction, as well as bioactivity-guided isolation and structural elucidation of phytoconstituents from MeOH and CHCl₃ extract of *P. tithymaloides* leaves.

The shade dried powdered leaves of *P. tithymaloides* were first defatted with petroleum ether (60–80°C) and then successively extracted with chloroform and methanol at ambient temperature. The crude chloroform extract on removal of solvent under reduced pressure gave a greenish brown mass, while the methanol extract on removal of solvent under reduced pressure gave a dark brown mass. The preliminary phytochemical analysis showed the presence of steroids in petroleum ether extract while gum and tannins were found in chloroform extract. Methanol extract showed the presence of alkaloid, flavonoid, gum, reducing sugar, saponin and tannins.

The methanol extract was partitioned between *n*-Butanol and water saturated with *n*-Butanol. The organic layer was further washed with water for complete removal of inorganic impurities, free sugars and other water-soluble residues and then evaporated to dryness under reduced pressure using a rotary evaporator to yield a dark brown residue. The chloroform extract and *n*-Butanol fraction of methanol extract were separately chromatographed over a column of silica gel. Graded elution was carried out with petroleum ether (60–80°C), chloroform followed by various mixture of chloroform-methanol.

Repeated chromatographic purification of the all the fractions led to isolation of one known compound luteolin (compound-5) (for the first time from this plant), and one unknown compound 1,2-tetradecanediol, 1-(hydrogen sulfate), Na salt (compound-6) (first report of isolation from natural sources) from the MeOH extract and other four known compounds like epi-friedelanyl acetate (compound-1), friedelanol (compound-2), β-sitosterol (compound-3), ursolic acid (compound-4) from CHCl₃ extract (Fig. 4).
Chapter 3 describes the toxicity profile of leaf extracts and isolated compound(s) from *P. tithymaloides* on laboratory animals.

Acute toxicity study of crude chloroform and methanol extracts (0.1gm/kg to 5gm/kg body wt.) as well as isolated compound(s) (0.05 and 0.1 g/kg, body wt.) was performed by stair case method. Experimental animals (Swiss albino mice) were randomly divided into experimental and control group. Each group had 12 mice of 6 males and 6 females. The animals were observed continuously for the first 4 h after dosing and finally periodically monitored daily until fourteen day for mortality. Results showed that the dose of 5000 mg/kg body wt. did not cause death or any toxic signs in the both chloroform and methanol extracts treated male and female mice. Moreover, the isolated compound(s) dosing upto 100 mg/kg body wt. did not produce
any mortality or toxicity in any of the groups after treatment. All the mice were found to be normal throughout the study and survived until the end of the experiment period.

As none of the animals were died in oral acute toxicity study, thus, chloroform and methanol extracts of *P. tithymaloides* leaves at the doses of 0.25, 0.50, 1 g/kg body wt respectively administration for 28 days. One-fifth, one-tenth and one-twentieth of the maximum safe dose of the extract tested for acute toxicity was selected for the sub-acute toxicity study. During the study period, the animals were observed daily for abnormal clinical signs and symptoms such as weakness or aggressiveness, movements, food intake, loss of body weight, discharge from eyes and ears, noisy breathing and number of death if any, in each group of animals were monitored carefully. At the end of the study, all animals were fasted overnight (water was not restricted) and then weighed and anaesthetized with diethyl ether. Whole blood was collected immediately from mouse hearts for haematological (total RBC, WBC, Hb count) and biochemical parameters (SGPT, SGOT, total protein) analyses respectively. Oral Administration of chloroform and methanol extracts of *P. tithymaloides* leaves at doses of 0.25, 0.50, 1 g/kg body wt. daily for 28 day did not produce any mortality throughout the study. The animals did not show any changes in general behavior or other physiological activities. Although the absolute body weights and the weights of internal organs (liver, kidney, heart, lung and spleen) of male and female mice increased during experimental period, no significant difference in the body weight gain were recorded between treated male or female mice and the respective control groups every week and at the end of the experiment.

After 28 days of treatment with *P. tithymaloides* leaves extract, there were no significant changes in haemoglobin, red blood cells, total leukocyte count and the serum levels of total protein, SGPT, SGOT in either the control or treatment group. All these haematological and biochemical parameters were within the normal ranges. Microscopic examination of the liver, kidney, spleen obtained from mice after 28 day administration of the extract, showed normal cell structure and formation when compared with the control. No histological damage was detected in histological sections when viewed under the light microscope.

Based on acute and sub-acute toxicity study results, haematological parameters, serum biochemical parameters and histopathological examination, it can be conclude
that the *P. tithymaloides* leaf extract and isolated compound(s) is safe to use orally. Thus, the present study establishes the reliable safety profile of the chloroform and methanol extracts of *P. tithymaloides* leaves administered orally in Swiss mice.

Chapter 4 encompasses with the possible *in vitro* antimicrobial activity of leaves extracts and isolated compounds from *P. tithymaloides* against 77 strains of bacteria and 4 strains of fungi and the possible mechanism of action for its use in traditional medicine.

The present study deals with the determination of antimicrobial activity by disc diffusion method, as per NCCLS Standard 2004 and 2006 and minimum inhibitory concentration (MIC), minimum bactericidal–fungicidal concentration (MBC-MFC) of crude chloroform and methanol extracts as well as isolated compound(s) by agar and broth dilution methods as per NCCLS 2002 and 2006 protocol. Chloroform extract producing moderate antibacterial activity against *Vibrio cholerae* (MIC=312 µg/ml; and inhibition zone of 7.1 mm), *Staph. aureus*, *Bacillus Subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (MIC=256 µg/ml; inhibition zone ranges from 6.0 to 7.2 mm) and significant activity against *Escherichia coli*, *Shigella dysenteriae* (MIC=200 µg/ml; inhibition zone ranges from 7.0 to 7.2 mm). Methanol extract produced significant antibacterial activity with MIC ranging from 100-175 µg/ml against *Staph. aureus*, *Shigella dysenteriae*, *Vibrio cholera*, *Bacillus Subtilis*, *Escherichia coli* (inhibition zone diameter from 6.9 to 9.4 mm). The screening for antifungal activity showed that ME demonstrated significant activity against *Candida tropicalis* at MIC 128 µg/ml (inhibition zones 9.2 mm) while CE produced moderate activity to all the fungal strains (MIC= >500; inhibition zone diameter of 7.1 mm).

However, the antimicrobial activity study with isolated compounds revealed that compound-5 exhibited very promising activity against *Staph. aureus* (MIC=32 µg/ml), *Shigella dysenteriae* (MIC=64 µg/ml), *Klebsiella pneumoniae* (MIC=64 µg/ml) and *Candida tropicalis* (MIC=128 µg/ml), with zone diameter of 11.5, 9.4, 9.8, 9.1 mm, respectively; whereas compound-6 was the most active against *Staph. aureus* (MIC=64 µg/ml) and *Pseudomonas aeruginosa* (MIC=64 µg/ml) with inhibition zone of 9.8 mm Interestingly, compound 1-2 selectively showing significant activity against Gram negative *Escherichia coli*, *Shigella dysenteriae*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* with MIC between 125-256 µg/ml and inhibition zone diameter of 6.4 to 9.1 mm. The remaining compound 3-4,
presented in, produced moderate activity against both the Gram positive and Gram negative strains (MIC ranging from 176-312 µg/ml). The MBC/MFC results, revealed that the MBC/MFCs was 2- to 4-fold greater than the MIC values with all of the tested strains.

As compound 5 and 6 exhibited very promising antimicrobial activity, so that growth kinetic study was performed in Mueller-Hinton broth containing test sample (compound 5-6) with six different concentrations as 0.5, 1, 2, 4, 8 and 16 times the MIC for 24-48 hrs to evaluate the growth pattern of microbial cells. In case of fungi Saboraud dextrose’s broth used instead of Mueller-Hinton broth. No killing effect was observed at any time at 1/2×MIC of compound-5 and 6 while, at higher concentrations (4×MIC and 16×MIC) of compound-5, inhibition of Staph. aureus ATCC8532 cells was 100% in 24 h at 4×MIC and 4 h at 16×MIC. Moreover, the compound-6 exhibited superior killing rate at 4×MIC with complete bactericidal effect at 16×MIC in 8 hrs against P. aeruginosa 71. The killing activity of compound 5 against C. tropicalis and the fungicidal endpoint was achieved after 30 hrs and 20 hrs at 4×MIC and 8×MIC.

To achieve the goal, it was decided to exploit scanning electron microscope (S.E.M.) on compound treated bacterial cells at 2×MIC after 16 h of incubation in MHB at 37°C. The results showed that the untreated Staphylococcus aureus ATCC8532 and Pseudomonas aeruginosa 71 cells were normal round and rod shaped with smooth surface without any visible damage respectively. However, compound-5 treated Staph. aureus cells appeared to be empty of contents, flaccid with severe alterations of the cell wall (Fig. 5). Similarly, Compound-6 treated P. aeruginosa cells appeared to be shrinking and lost their normal morphology.
Fig. 5. Scanning electron micrographs of *Staphylococcus aureus* ATCC8532 (A) Untreated control (B) Treated with compound-5. White arrows in the picture indicate empty cell contents and breakage of the cell membrane.

To the best of our knowledge, this the first report *in vitro* antibacterial and antifungal activities of *Pedilanthus tithymaloides* leaves and its isolated compounds with possible mechanism of action. We may expect that *P. tithymaloides* extract or compound-5 and 6 may be used as phytotherapeutic drug for bacterial infections, validating the use of these plant products as non-conventional antibacterial agent, after appropriate studies that allow the design and rational planning of new drugs with new therapeutic properties with minimal side effects. Furthermore, *in vivo* experiments would be required to establish various limitations about their mammalian safety.

Chapter 5 deals with detailed anti-inflammatory, antinociceptive and antipyretic potential of bioactive constituents from *P. tithymaloides* leaves through bioassay-guided fractionation in standard laboratory models.

In the present study, the *in vivo* anti-inflammatory activity was assayed by carrageenan-induced hind paw edema, vascular permeability, and cotton-pellet mediated granuloma; antinociceptive activity by acetic acid-induced abdominal constriction, Hot plate test and Formalin test; and antipyretic activity assayed by brewer’s yeast-induced pyrexia.
The anti-inflammatory effect of chloroform and methanol extracts on carrageenan-induced paw edema in rats was depicted in Fig. 6. There was a gradual increase in paw edema of rat and reached its maximum after 2 h of carrageenan injection. The methanol extract significantly reduced carrageenan induced paw edema at all doses (100, 200 and 400 mg/kg) employed (P<0.001) after 3 h of carrageenan injection. Similarly, chloroform extract reduced paw edema significantly at 200 and 400 mg/kg (p < 0.05). The highest anti-inflammatory activities for both chloroform (49.29%) and methanol (62.28%) extracts were observed at the dose 400 mg/kg in comparison to standard indomethacin (65.80%) after 5 h of carrageenan injection. The anti-inflammatory effect of isolated compounds (10 mg/kg) was tested in carrageenan-induced paw edema model in mice. In the test groups, compound 3-5 showed a significant reduction in the edema paw volume between 3 and 4 h observed. After 5 h of carrageenan injection, compound 1, 2, 6 showed moderate activities with 30.55, 36.11 and 33.33% inhibition (p < 0.05).

Fig. 6. Effect of chloroform (CE) and methanol extract (ME) of *P. tithymaloides* leaves on rat paw edema induced by carrageenan.

The anti-inflammatory activity of the leaves of *P. tithymaloides* was investigated by the dose-related inhibition on vascular permeability. Methanol extract at 100, 200 and 400 mg/kg inhibited the acetic acid-induced extravasation of Evan's blue dye by
37.34%, 45.78% and 53.01% respectively as compared to the control ($P< 0.001$). In case of chloroform extract, no significant effect was observed at 100 and 200 mg/kg but it was significant at 400 mg/kg (34.97%).

In the cotton pellet induced inflammation studies in rats, Methanol extract at the doses of 100, 200 and 400 mg/kg (p.o), and chloroform extract at 400 mg/kg (p.o) produced a significant reduction on the granuloma tissue formation on implanted cotton pellets with inhibition of 29.93, 33.9 50.10 % and 36.89 % respectively. Again, the anti-inflammatory effect of isolated constituents (10 mg/kg) was tested in mice model of cotton pellet granuloma. It was observed that compound 2, 3, 4, 5 significantly decreased the dry weight of cotton pellet granuloma formation with 29.21, 30.37, 38.54, 36.98% ($P< 0.01$) after 5 days of continuous treatment, as compared to standard drug Indomethacine (5mg/kg) which produced 45.54% inhibition ($P< 0.001$). Oral administration of chloroform extract (100, 200, and 400 mg/kg) resulted in a significant dose-dependent reduction ($P<0.001$) in the number of acetic acid-induced writhing (22.0, 39.3 and 48.48 % inhibition) compared to the control group. Interestingly, diclofenac sodium (standard drug, 10 mg/kg), significantly reduced writhing by 90.90% inhibition which showed a similar effect with methanol extract (400 mg/ kg, 79.17% inhibition) and methanol extract at 100 mg/kg and 200 mg/kg doses showed inhibition by 36.36 and 66.67%. From the percentage inhibition of abdominal constriction it was observed that the antinociceptive activity of methanol extract was dose dependent, while compound 2, 4, 5 (10 g/kg), produced greater inhibition of acetic acid-induced writhing (45.83, 50.29 and 55.35% respectively; $P<0.001$).

As shown in Fig. 7, ME of PT leaves, at all doses tested, had significant effects on the pain latency as compared to the control group ($P<0.01$). The antinociceptive effect was dose-dependent as we observed stronger effect at 400 mg/kg dose than 200 mg/kg dose. In contrast, the standard drug Morphine showed significantly prolonged hot-plate latency and exhibited strong antinociceptive properties at all time points studied ($P<0.01$).

Results showed that in formalin test, oral administration of chloroform and methanol extracts (400 mg/kg) produced a significant reduction (36.81% and 58.01%) of the licking time in the late phase respectively ($P<0.01$). Similarly, treatment with compound 2, 4, 5 (10 mg/kg) showed significantly ($P<0.001$) reduced formalin-induced paw licking in both the phases. The standard drug, morphine (5 mg/kg), on the
other hand, significantly attenuated the neurogenic and inflammatory pain (both phases; 68.02% and 100%), whereas indomethacin (10 mg/kg) was only efficient in the late phase (97.60%).

![Graph showing hot plate latency for different extracts and treatments.](image)

**Fig. 7.** Effect of chloroform (CE) and methanol extract (ME) of *P. tithymaloides* leaves on hot plate test in mice.

The results of the antipyretic activity study by brewer’s yeast-induced pyrexia showed that oral administration of chloroform and methanol extracts at the doses of 100, 200 and 400 mg/kg decreased the rectal temperature of the rats in dose dependent manner and the effect started as early as 20 to 21 h after treatment. The antipyretic effect of compound-3 (10 mg/kg) was comparable with that of paracetamol (150 mg/kg, p.o) between 21 to 23 h after treatment.

This study for the first time demonstrated in vivo anti-inflammatory, antinociceptive and antipyretic activities of *Pedilanthus tithymaloides* (L.) Poit leaves and its isolated compounds, probably due to their ability to inhibit prostaglandin synthesis, its release and action, as well as inhibition of several mediators of inflammatory pathway. Thus, the result of the present study offers pharmacological evidence of the use of *Pedilanthus tithymaloides* as an anti-inflammatory drug from natural source. This plant could be a good candidate for further development of a new phytotherapeutic agent.
Chapter 6 describes the wound healing activity of methanolic extract of *P. tithymaloides* leaves and its isolated constituents in ointment form, compared to the standard formulation povidion iodide ointment.

Topical formulations were prepared, as per British Pharmacopoeia; 1980, to evaluate the efficacy of methanol extract (as used traditionally) and its constituents, in comparison with standard Povidion-iodine ointment, USP. Then, the stability of prepared ointment formulations was evaluated according to the guidelines of the International Conference on Harmonization (ICH), 1993; in terms of physical parameters (physical stability, centrifugation, spreadability) that may affect the stability and acceptability of the formulations. Storage of the formulations, at accelerated stability conditions does not influence the stability of formulation. Thus the formulations are satisfactory as far as physical parameters are concerned. Acute skin irritation test did not show any type of irritation and noticeable inflammation, swelling or any other change on the skin.

Excision, incision and dead space wound models were used to evaluate the wound-healing activity of methanol extract and its isolated constituents. The rats were divided into six groups (n=6) for excision and incision wound models. The animals of group I received ointment base (control), while group II were treated with a 5% w/w povidone-iodine ointment. The animals of group III and IV were treated with 2.5% and 5% w/w of methanol extract ointments, while group V and VI were treated with 0.25% w/w ointment of the isolated compound-5 and 6 respectively. For the dead space wound model, animals of group I (control) was treated tropically with simple ointment base. Group II was treated with 5% w/w povidone-iodine ointment; while group III and IV were treated with methanol extract ointments 2.5% and 5% w/w respectively. The rats were anaesthetized using ketamine hydrochloride (100 mg/kg) prior to and during infliction of the experimental wounds.

In the excision wound model, full thickness of excision wound of 500 mm$^2$ was created along the marking using toothed forceps, a surgical blade and pointed scissor and the entire wound was left open. Rate of wound contraction and epithelialisation period was assessed by tracing the wound on 1$^{st}$, 3$^{rd}$, 6$^{th}$, 9$^{th}$, 12$^{th}$, 15$^{th}$, 18$^{th}$, 21$^{st}$ post wounding days. The fastest healing of wound was observed in animals which received ointment of compound 5 and 6, along with complete healing (with 100% wound
contraction) within 18 days, as compared with the animals treated with standard 5% w/w povidone-iodine ointment. On the 18th post-wounding day, 5% extract treated animals showed significant reduction in wound area (95.88%) with faster rate of epithelialisation time (19.5±0.39) compared to the animal treated with 2.5% extract, which showed moderate degree of healing (93.85% wound area, 20.0±0.52 epithelialization time) (Fig. 8).

![Fig. 8. Effect of topical application of methanol extract of P. tithymaloides and its constituents expressed as percentage wound contraction.](image)

In the incision wound model, two paravertebral long incision of 6 cm length were made through the skin at a distance of about 1.5 cm from the midline on each side of the depilated back of the animals and the parted skin was stitched together at intervals of one centimeter using surgical thread. On 8th post-wounding day sutures were removed and treatment was continued. The skin breaking strength of healed wound was measured on the 10th day. It is interesting to note that 5% and 2.5% extract ointment exert nearly similar result on the tensile strength of the healing tissue. The tensile strength of wound treated with povidone-iodine ointment is comparable (572.50 g) to the group of animals that received ointment formulations containing compound 5 (565.10 g) and 6 (561.12 g).

Dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm × 0.3 cm), one on either side of the lumber region on the ventral surface of each rat. On the 10th post wounding day, granulation tissues
formed on the grass piths were excised from each group of animals carefully. The wet weight of the granulation tissues was recorded and dried (at 60°C for 12h in an oven) to obtain constant dry weight, simultaneously, dried granulation tissue was hydrolyzed with 5 ml of 6N HCl for 24h at 110°C in sealed glass tubes and subjected to hydroxyproline content estimation. A part of wet granulation tissue was preserved in 10% formalin for histopathological evaluation of the effect of the formulations on collagen formation. The dry and wet weight of control animals (13 and 85 g) was increased in 5% extract ointment treated group (19.1 and 177.66 g), whereas the standard drug treated group showed maximum weight (19.1 and 121.15 g). Significant increase in hydroxyproline content was seen in animals which received standard povidone-iodine (207.66 mg/g) and nearly comparable values were observed in animals that received the 5% extract ointment (192.33 mg/g).

Histopathology of granulation tissue sections from animals treated with 5% and 2.5% extract ointment showed proliferation of epithelial tissue covering the wound area with multiplication of fibrous connective tissue, without proper union, in dermis region. Interestingly the animals treated with ointment formulations of compound 5 and 6 showed fibrous connective tissue with well-collagenation and complete healing.

The present study for the first time demonstrated that topical application of methanol extract of Pedilanthus tithymaloides leaves and its isolated compounds 2- (3,4-Dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one and Tetradecanediol, 1- (hydrogen sulfate), sodium salt may promote wound healing activity in rats, probably due to their ability to scavenge free radicals, inhibite of several mediators of inflammatory pathway, along with its antibacterial activity. Thus, the result of the present study offers pharmacological evidence of the folk use of P. tithymaloides for wound healing.

Chapter 7 summarized the in vitro anti-HSV activity of crude methanolic extract of P. tithymaloides leaves and its isolated compound(s).

The in vitro antiviral activity was assayed by MTT assay of cytotoxicity, plaque assay, drug dose-response analysis, Time dependent activity study. Cell toxicity was monitored by determining the effect of the extract and two bioactive compounds on Vero cell morphology. Vero cells were cultured onto 96 well plates and different concentrations of methanolic extract (0-400 µg/ml) and isolated compounds 5 and 6 (0-300 µg/ml) were added to each culture wells. Data analysed as
the percentage of cell viability. In plaque assay, Vero cells seeded in 12-well plates, (5 × 10^5 cells per well) were treated with serial dilutions of extract and or test compounds 5 and 6 (0-100 µg/ml) for 15 min at 37°C and then challenged with HSV-1 (100 CFU/well) for 1 h. The inoculum and drugs were subsequently removed from the wells, and the cells were washed with PBS twice and overlaid again with different dilutions of the ME and compounds. Then, The 50% effective concentration (EC50) for antiviral activity, the concentration of antiviral compound(s) that produced 50% inhibition of the virus induced plaque formation was determined.

For dose-response assay, Vero cells seeded in 96-well plates were infected with HSV-1 (multiplicity of infection or MOI: 1) in the presence or absence of the isolated compounds at various concentrations (0, 5, 10, 20, 30, 60 and 100 µg/ml). After 2 days MTT assay was carried out to determine the inhibition of infection. Our results indicated that the methanolic extract and isolated compound 5 and 6 did not have apparent cytotoxic effects below 100 µg/ml in vero cells, while a dose-dependent cytotoxic effect was observed at 100 µg/ml. The 50% cellular toxicity indices (CC50) of methanol extract as well as compound 5 and 6 were 236.5, 178.6, and 146.4, respectively. In plaque assay, The results revealed that both ME and compound-5 could inhibit viral plaque formation, after inoculation of 100 PFU, in a dose-dependent manner, and their 50% effective concentration (EC50) were 42.4 and 14.8 µg/ml respectively. The selectivity index (SI), which measures the preferential antiviral activity of a drug in relation to its cytotoxicity, was calculated according to their CC50 and EC50. The SIs of methanolic extract, compound 5 and 6 were 5.58, 12.07 and 1.17, respectively. Out of the two isolated compounds tested, the SI value of compound 1 was >12 and thus, luteolin was chosen for subsequent analyses.

To obtain a more accurate dose-response curve for methanolic extract and compound-5, Vero cells were infected with HSV-1F (MOI: 1) in the presence of methanolic extract, and compound-5 and tested by MTT assay. Both extract and compound-5 displayed anti-HSV-1 activity in a dose-dependent manner, and compound-5 at 30 µg/ml and ME at 100 µg/ml provided near complete protection against the infection at an MOI of 5, were chosen for all subsequent experiments. Here compound-6 used as negative control.

To understand the antiviral mode of action and the stage of HSV-1 infection affected by methanolic extract and compound-5, we added the test samples at different times
of the virus life cycle (pre-entry, entry, and post-entry). In order to study pre-entry, i.e., the effect of the compound on Vero cell itself, prior to virus addition, Vero cells were pretreated with methanolic extract and compound-5 for 24 h (long) or 1 h (short) periods and then washed prior to HSV-1 infection. For the effects of test samples on the viral entry, virus and the drugs were simultaneously applied to the cells. To investigate events after virus entry, vero cells were first infected with HSV-1 for 1 h and then treated with the compound. Pretreating Vero cells with extract and compound-5 (both long and short term) did not protect the Vero cell against HSV-1 infection. Both extract and compound-5 were effective in preventing cell distraction when added during virus adsorption, immediately after viral entry, and throughout multiple cycles of virus replication (Fig. 9). The results indicate that HSV-1 infection is severely impaired only if the drug(s) was present at the time of infection or during viral spread and that it is unlikely that the antiviral activity is due to its direct effects on the cells (such as masking cellular receptors or entry factors for HSV-1).

In conclusion, our results of clearly demonstrated that methanolic extract of *P. tithymaloides* leaves containing luteolin (compound-5) as one of the major compound might be a potential therapeutic candidate against HSV infections, and can be further studied for its potential against an alternative anti-HSV candidate, through mechanistic and sterochemical studies.

![Fig. 9. Effect of time of addition of methanol extract (ME) and isolated compounds on plaque formation by HSV-1. After 3 days, inhibition was evaluated by MTT assay and expressed as the inhibition rate.](image)