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Wound Healing Activity of *Premna latifolia* Roxb.

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Authors' contributions

Authors GP and VC performed the experimental work. Author PSC designed the experimental work. Author SY compiled the data. Authors CVR and SSG performed the animal experimental work. Authors AM and AKSR did the proof reading. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The study aims to understand the wound healing potentials of a 50% aqueous ethanolic extract of *Premna latifolia* stem using excision wound model.
Study Design: The wound healing potentials were simultaneously supported by observing the bacterial functional diversity of wound swabs using Biolog Eco plates. The antioxidant activity was performed using *In vitro* DPPH free radical scavenging assay.
Place and Duration of Study: CSIR-National Botanical Research Institute (NBRI), Lucknow, between May 2013 and November 2013.
Methodology: Wound healing activity of the plant was studied using excision wound model. Animals were divided into three groups of six male rats each as control group (GI) dressed with compound free simple ointment. Test group (GII) treated with 50% aqueous ethanolic extract of *P. latifolia* stem (10% w/w) in ointment vehicle and standard group (GIII) group treated Nitrofurazone ointment, Himedia (0.2%w/w). The wound healing potential was further supported by the DPPH free radical scavenging and antibacterial activity of the plant. The phytochemical estimations were done using standard methods.
Results: Sugar and starch content in the plant was 3.55% and 5.54% respectively. Total tannins, phenol and flavonoid content were estimated to be 0.18%, 0.54% and 2.73%.

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The 50% ethanolic extract of the plant showed moderate DPPH free radical scavenging activity with an IC_{50} of 188.02µg/ml. A 69.15% of wound closure was observed on 10th day post wounding of the rats treated with 200 mg/kg of extract. The results also indicated significant antibacterial activity of the extract.
Conclusion: The 50% aqueous ethanolic extract of *P. latifolia* shows significant wound healing activity.

Keywords: Premna latifolia; free radical; antibacterial; phytochemical; wound healing.

1. INTRODUCTION

Premna latifolia Roxb. (Verbenaceae) is widespread in India along the coastal regions in the plains and hills. Traditionally the paste of *P. latifolia* bark is applied to cure boils; tender leaves are diuretic, anti-inflammatory [1], anticancer [2] and are also used for treating rheumatism, intestinal edema and acute dropsy [3]. In Tamil Nadu it is regarded as a sacred tree and is used to cure swellings [4]. The major phytoconstituents of the plant are alkaloids, iridoids and oils. The stem bark shows the presence of iridoid glucosides and geniposidic acid [5]. Premnalatifolin A, a novel dimeric diterpene was isolated from the stem bark of *P.latifolia* [2] along with hexadecanoic acid and 1-octen-3-ol [6]. Healing of wound is an intricate process in which the skin (or another organ-tissue) repairs itself after injury [7]. The classical model of wound healing is divided into three or four sequential, yet overlapping [8], phases: (1) homeostasis, (2) inflammatory, (3) proliferative and (4) remodeling. The microbial infections are recognized as one of the many destructive processes that delay wound healing [9]. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and *Klebsiella pneumoniae* are some important organisms of wound infection [10]. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1-3% of modern drugs [11]. Researchers are now paying attention towards extracts of biologically active compounds isolated from plant species which can be used as herbal drugs. The present study was undertaken to evaluate the wound healing potentials of the *P. latifolia* using excision wound model.

2. MATERIALS AND METHODS

2.1 Plant Collection and Laboratory Animals

The stem of *P. latifolia* was collected in the month of May, 2013 from Uttarakhand, India. The plant specimen was pressed in a plant press to extract moisture in short time while preserving the morphological integrity of the plant and to yield material that can be readily mounted on a herbarium paper for long term storage. The specimen was identified by the taxonomists of Plant Systematics and Herbarium division of CSIR-National Botanical Research Institute, Lucknow and deposited at the same (voucher specimen LWG 98569).

For the study, colony bread, adult, male albino rats, (approximate weight of 150-160 g each) were used. The animals were kept in polypropylene cages under controlled conditions of temperature $26\pm2^{0}C$ and relative humidity of 44-56%, light and dark cycles of 10 and 14h respectively for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Amrut, India) and the feeding was stopped 18-24h before the experiment though water was allowed *ad libitum*. All experiments were performed in the

morning according to current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [12].

2.2 Preparation of Stem Extract

About 1000g of stem were washed vigorously with tap water to remove soil and dust and chopped to small pieces to remove soil and dust. The stem pieces were left in the shade to dry for 15-20 days. Dried stem were powdered coarsely using electric grinder to aid the extraction. Powdered samples were extracted by cold maceration technique at room temperature thrice with 50% aqueous ethanol, the crude drug and solvent ratio was 1:3 [13]. Finally, the extract was concentrated using rotary evaporator (4001; Heidolph Instruments, Schwabach, Germany) at a reduced pressure and temperature (50 ± 2^0C).

2.3 Phytochemical Analysis

Phytochemical screening of the stem powder of *P. latifolia* was carried out by using the standard protocols for the presence of carbohydrates, proteins, phenols, oil and fats, terpenoids, steroids, saponins, flavonoids, alkaloids and tannins [14]. The basic physico-chemical parameters like loss on drying, extractive and ash values, sugar and starch content were estimated using the standard methods mentioned in the Ayurvedic Pharmacopoeia of India. The tannin estimation in crude drug followed the method mentioned by [15]. Total phenol and flavonoids were estimated as mentioned by [16].

2.4 DPPH Free Radical Scavenging Activity

The effect of *P. latifolia* stem extract on DPPH radical was studied as described by Singh and Rajinia [17]. Briefly, 1.5ml of DPPH solution (0.1mM, in 95% Ethanol) was incubated with varying concentrations of the extract (100-350µg/ml). The reaction mixture was shaken well and incubated for 20 min in dark at room temperature and the absorbance of the resulting solution was read at 517nm against a blank. The standard antioxidant ascorbic acid was taken as control. The degradation of DPPH was evaluated by comparison with a control (0.5 ml of DPPH solution and 1.5ml of ethanol). Results were expressed as the proportion of DPPH degradation compared with the control.

2.5 Wound Healing Activity and Bacterial Count in Wound Swabs

Excision wound healing model was used to evaluate the wound healing activity of *P. latifolia*. Colony bread, adult, male albino rats (approximate weight of 150-160g each) were divided into three groups of six male rats each as follows:

Group I (GI): Control group were dressed with compound free simple ointment.
Group II (GII): Test group treated with 50% aqueous ethanolic extract of *P. latifolia* stem (10% w/w) in ointment vehicle [18].
Group III (GIII): Standard group treated Nitrofurazone ointment, Himedia (0.2% w/w).
All the ointments were topically applied once a day for 10 days (dose approximately 0.20g/wound).

2.5.1 Excision wound model

For excision wound study, rats were anesthetized with anesthetic ether and depilated at the dorsal thoracic region. A circular piece of full thickness ($500 mm^2$) was cut off from a pre-determined area on the dorsal back of rats [19]. The wound area was measured immediately by placing a transparent paper over the wound and tracing it out, area of this impression was calculated using graph sheet. The area of wound at the time of wounding was considered as 100%. The progressive change in wound area was monitored by tracing the wound margin on graph paper [20]. The degree of wound healing was calculated as percentage closure in wound area from original wound area using the formula:

%Inhibition = $(1 - A_d/A_o) \times 100$; A_o- wound area on day 0; A_d- wound area on corresponding day.

2.5.2 Bacterial count

The culturable population bacteria in wound swabs taken from Group I, II and III were determined by plating serial dilutions [21]. The microbial counts were determined using nutrient agar for heterogeneous bacteria, pseudomonas isolation agar for *Pseudomonas* species and Hi-Chrome ECC agar (Hi-Media) plates for *Escherichia coli.* The plates were incubated for 24h at 37°C and colony forming units were calculated (Cfu/ml).

2.6 Microbial Diversity Using Carbon Source Utilization Pattern

This experiment was conducted parallel to the wound healing experiment. Swabs taken from wound on 5th and 10th day were shaken in 10ml of sterile 0.85% saline MQW for 60 min and then made up to a final dilution to maintain the approximately 10^4 Cfu/ml bacterial counts. After incubation, 150µl sample was inoculated into each well of Biolog Eco plates (Biolog, Inc., Hayward, CA, USA) which contained 31 different carbon sources along with negative water control well in triplicate, and incubated at 28±2°C. Data were recorded for days 1-10 at 590nm as described earlier [22]. Microbial activity in each microplate, expressed as the average well color development (AWCD), was determined as described by Garland [23]. Diversity and evenness indexes and principal component analysis (PCA) were calculated on 5th and 10th day data divided by AWCD as described by Mishra and Nautiyal [22].

2.7 Statistical Analysis

Calculations and statistics were performed using GraphPad 5.0 software (GraphPad Software Inc., La Jolla, CA). Significance was determined Waller-Duncan Test and defined as $P<0.05$. Results are presented as mean ± the standard error of mean (SEM). All statistical analyses for Biolog study were performed by using SPSS 16.0 and Statistica 7.0.

3. RESULTS AND DISCUSSION

3.1 Extraction Yield and Phytochemical Estimation

Fresh 1000g of stem powder of *P. latifolia* yielded 225.5g of 50% aqueous ethanolic extract. The preliminary phytochemical screening showed the presence of alkaloids, flavonoids, phenols, tannins, terpenoids, saponins, glycosides, steroids and carbohydrates. The phytochemical screening was followed by chemical standardization of *P. latifolia* stem. The total ash and acid insoluble ash value of *P. latifolia* stem is 4.10% and 2.66% respectively.

The sugar and starch content is estimated to be 3.55% and 5.54%. The tannin content was 0.18% and phenol content was 0.54%. The *P. latifolia* stem has significant flavonoid content which was estimated to be 2.73%.

3.2 DPPH Free Radical Scavenging Activity

Total DPPH scavenging potential of the stem extract was measured at varying concentrations and the results are depicted in Fig. 1. Significant DPPH radical scavenging activity was evident at all tested concentrations of the extract. The scavenging effect increased with the increasing concentration of extract up to a concentration of 300µg/ml, after which the scavenging activity was leveled off. The IC_{50} for stem extract was 188.02±1.48µg/ml while that of ascorbic acid was 40.72±0.46µg/ml Table 1.

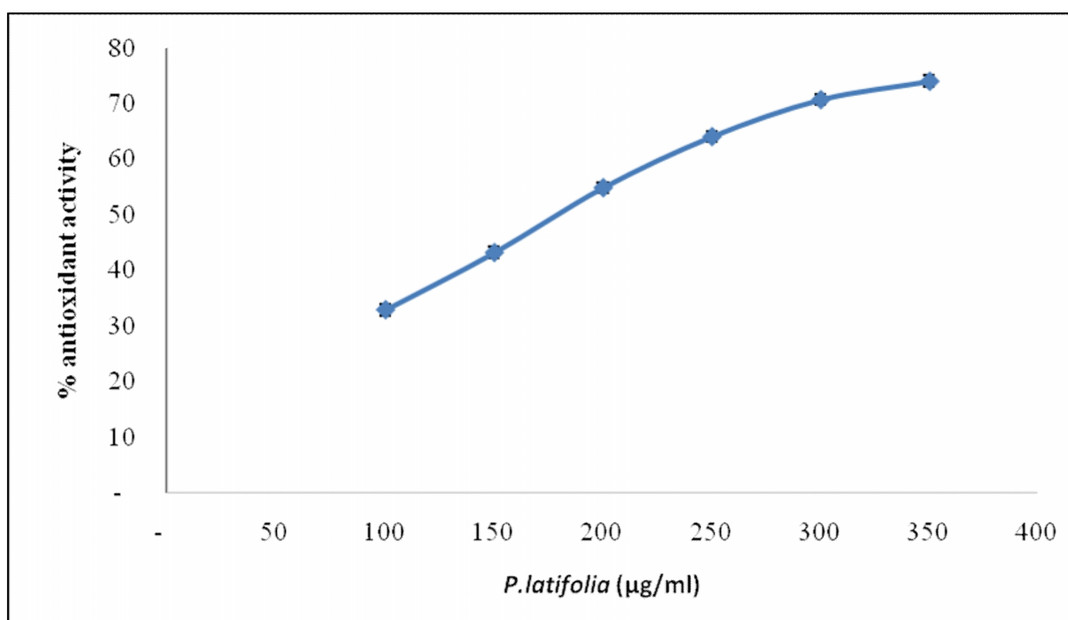

Fig. 1. Free radical scavenging activity of *P. latifolia* at varying concentrations (µg/ml)

Table 1. Inhibition of 1,1-diphenyl-2-picylhydrazyl (DPPH) by *P. latifolia* extract and ascorbic acid

P. latifolia		**Ascorbic acid**	
Conc (µg/ml)	**%Inhibition**	**Conc (µg/ml)**	**%Inhibition**
100	32.92±0.75	20	27.6±0.52
150	43.23±0.46	30	37.8±0.42
200	54.94±0.49	40	47.6±0.64
250	64.16±0.43	50	61.4±0.50
300	70.83±0.54	60	77.2±0.68
350	74.14±0.48	70	79.6±0.56

3.3 Wound Closure and Microbial Analysis

A significant increase in wound healing was observed in *P. latifolia* treated group GII in comparison to the control group GI, treated with simple ointment Table 2. The percentage closure of wound was taken as a parameter to measure the extent of wound healing. The wound closure on 5th day was 33.2% ($p<0.05$) for the control group GI, 45.3% ($p<0.05$) for the group GII, treated with *P. latifolia* extract and 46.6% ($p< 0.01$) for group GIII treated with Nitrofurazone. A high rate of wound closure for the groups GII and GIII was observed between 5th and 10th day post wounding. The wound closure on 10th day was 53.45% ($p<0.01$), 69.15% ($p<0.05$) and 75.45% ($p<0.01$) for groups GI, GII and GIII respectively Fig. 2. The bacterial count of wound swabs taken from all the three groups of rats (GI, GII, GIII) are given in Table 3. The results clearly demonstrate the antibacterial activity of *P. latifolia* extract as evident from least bacterial count in wound swabs taken from GII on the 5th day of wounding. However on the 10th day, least bacterial count, $1.80X10^5$ Cfu/ml, was observed in GIII wound swabs, as compared to the wound swabs taken from GII, $1.38X10^4$. The GI wound swabs showed maximum bacterial count $1.23X10^{12}$ and $1.20X10^{16}$ on 5th and 10th day of wounding respectively.

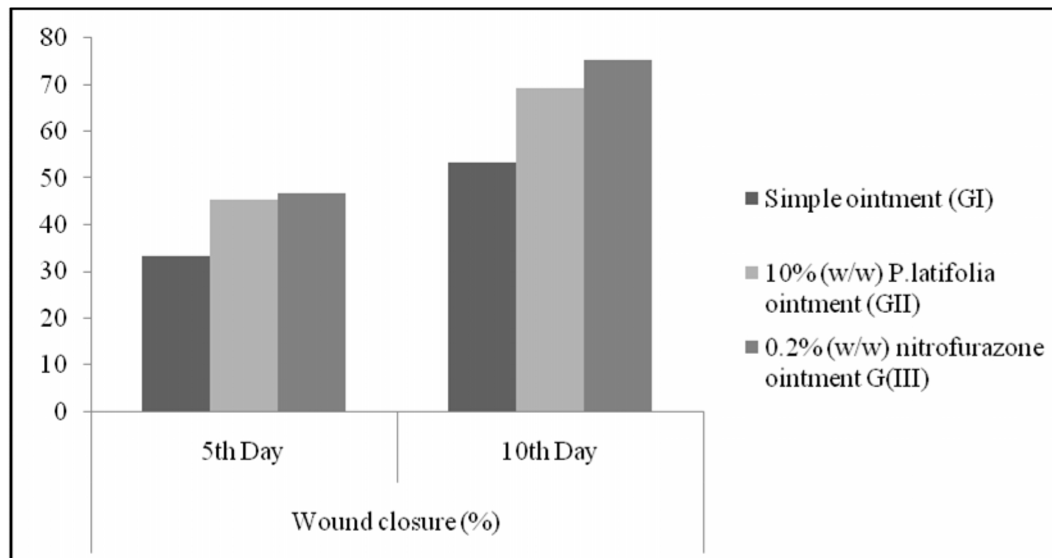

Fig. 2. Effect of *P. latifolia* extract on wound closure on 5th and 10th day of wound creation. Group GI was treated with simple ointment, GII group was treated with 10% aqueous ethanolic extract of *P. latifolia* ointment and GIII was treated with 0.2% Nitrofurazone ointment.

The values of bars represent mean±SEM.

Table 2. Wound closure on 5th day and 10th day of wounding

	Wound closure (%)	
	5th Day	10th Day
Simple ointment (GI)	33.2±2.15a	53.45±3.60b
10% (w/w) *P. latifolia* (GII)	45.3±3.67a	69.15±3.23a
0.2% Nitrofurazone (GIII)	46.6±3.02b	75.45±3.06b

Values (mean±SEM) were obtained for each group of six rats. $^a p< 0.05$ and $^b p<0.01$

Table 3. Bacterial count of wound swabs taken from rats treated with simple ointment (GI), 10% *P. latifolia* stem ointment (GII) and 0.2% Nitrofurazone ointment (GIII)

	Heterogeneous *bacteria* (Cfu/ml)		*Pseudomonas* species (Cfu/ml)		*Escherichia coli* (Cfu/ml)	
	5th day	10th day	5th day	10th day	5th day	10th day
GI	$1.23X10^{12}$	$1.2X10^{16}$	$1.3X10^{5}$	$2.6X10^{6}$	$1.6X10^{5}$	$1.5X10^{8}$
GII	$1.22X\ 10^{7}$	$1.38X10^{4}$	$4.2X10^{3}$	$2.4X10^{2}$	$3.1X10^{2}$	$1.8X10^{1}$
GIII	$1.29X10^{9}$	$1.80X10^{5}$	$1.6X10^{4}$	$1.4X10^{2}$	$2.1X10^{2}$	$1.2X10^{1}$

3.4 Community Level Physiological Profiling of Wound Swabs

Microbial functional diversity of wound swabs (GI, GII and GIII) was monitored on 5th and 10th day of wound creation by using Biolog Eco plates. The average well color development (AWCD) curve for 5th day showed least utilization of substrates in swab taken from GII, closely followed by GIII and GI. Substrate oxidation on 10th day post wounding showed least oxidation in swabs taken from GIII, a slightly higher utilization of substrates was recorded for swabs taken from GII while swabs taken from GIII showed significantly high utilization of substrates as evident from AWCD Fig. 3. Comparative results based on diversity and related evenness indices calculated on 5th day for GI, GII and GIII showed less bacterial diversity in GII treatment as compared to GI and GIII. However on 10th day, the diversity and related evenness indices of GII were recorded slightly higher as compared to GIII, while wound swab from GI demonstrated maximum diversity Table 4. Carbon substrates tested by Biolog Eco plates were categorized into six groups: (1) amine/amides, (2) amino acids, (3) carbohydrates, (4) carboxylic acids, (5) miscellaneous and (6) polymers. Significant differences were observed among all treatments for substrate utilization pattern in different categories Fig. 4. Carboxylic acid and miscellaneous substrates were most utilized in wound swabs from GII and amino acids and polymers were better utilized by in wound swabs from GIII. More amides/amines and carbohydrate utilizing communities were found in wound swab from GI. The substrate utilization pattern for 10th day was similar for GII and GIII wound swabs with higher utilization of polymers and carbohydrates as compared to other substrates. However, GI wound swab showed a significantly different pattern, with higher utilization of amino acids, amides and miscellaneous substrates Fig. 4.

Table 4. Diversity and related evenness indices based on carbon substrate utilization of wound swabs taken from rats treated with simple ointment (GI), 10% *P. latifolia* stem ointment (GII) and 0.2% Nitrofurazone ointment (GIII). Different letters showing significant difference at *p= 0.05* using Waller Duncan test

5th day			
	GI	GII	GIII
McIntosh diversity Index	0.926±0.001^{b}	0.905±0.002^{a}	0.935±0.005^{b}
McIntosh Evenness	0.941±0.001^{b}	0.914±0.000^{a}	0.953±0.003^{c}
Shannon Diversity Index	2.967±0.007^{b}	2.833±0.008^{a}	3.086±0.023^{c}
Shannon evenness	0.900±0.002^{b}	0.846±0.002^{a}	0.944±0.001^{c}
Simpson diversity Index	0.974±0.000^{b}	0.965±0.001^{a}	0.977±0.002^{b}
10th day			
	GI	GII	GIII
McIntosh diversity Index	0.977±0.003^{b}	0.936±0.013^{ab}	0.879±0.030^{a}
McIntosh Evenness	0.978±0.002^{b}	0.946±0.009^{ab}	0.909±0.020^{a}
Shannon Diversity Index	3.282±0.021^{b}	3.086±0.068^{ab}	2.884±0.095^{a}
Shannon evenness	0.959±0.004^{b}	0.923±0.010^{a}	0.914±0.010^{a}
Simpson diversity Index	0.993±0.001^{b}	0.978±0.005^{ab}	0.952±0.014^{a}

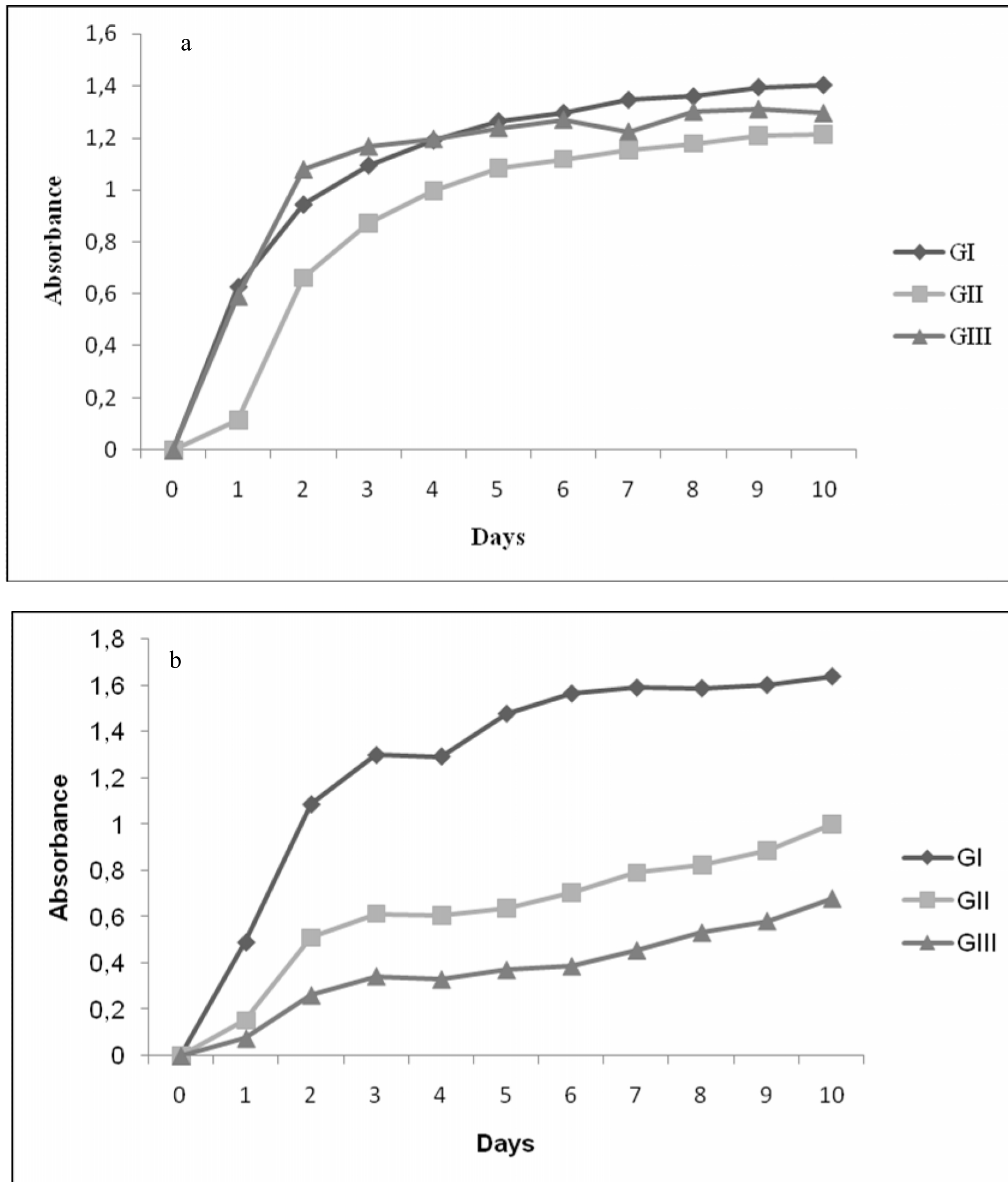

Fig. 3. Substrate oxidation curve for 5th (a) and (b) 10th day wound swabs taken from rats treated with simple ointment (GI), rats treated by 10% *P. latifolia* stem ointment (GII) and rats treated by 0.2% Nitrofurazone ointment (GIII)

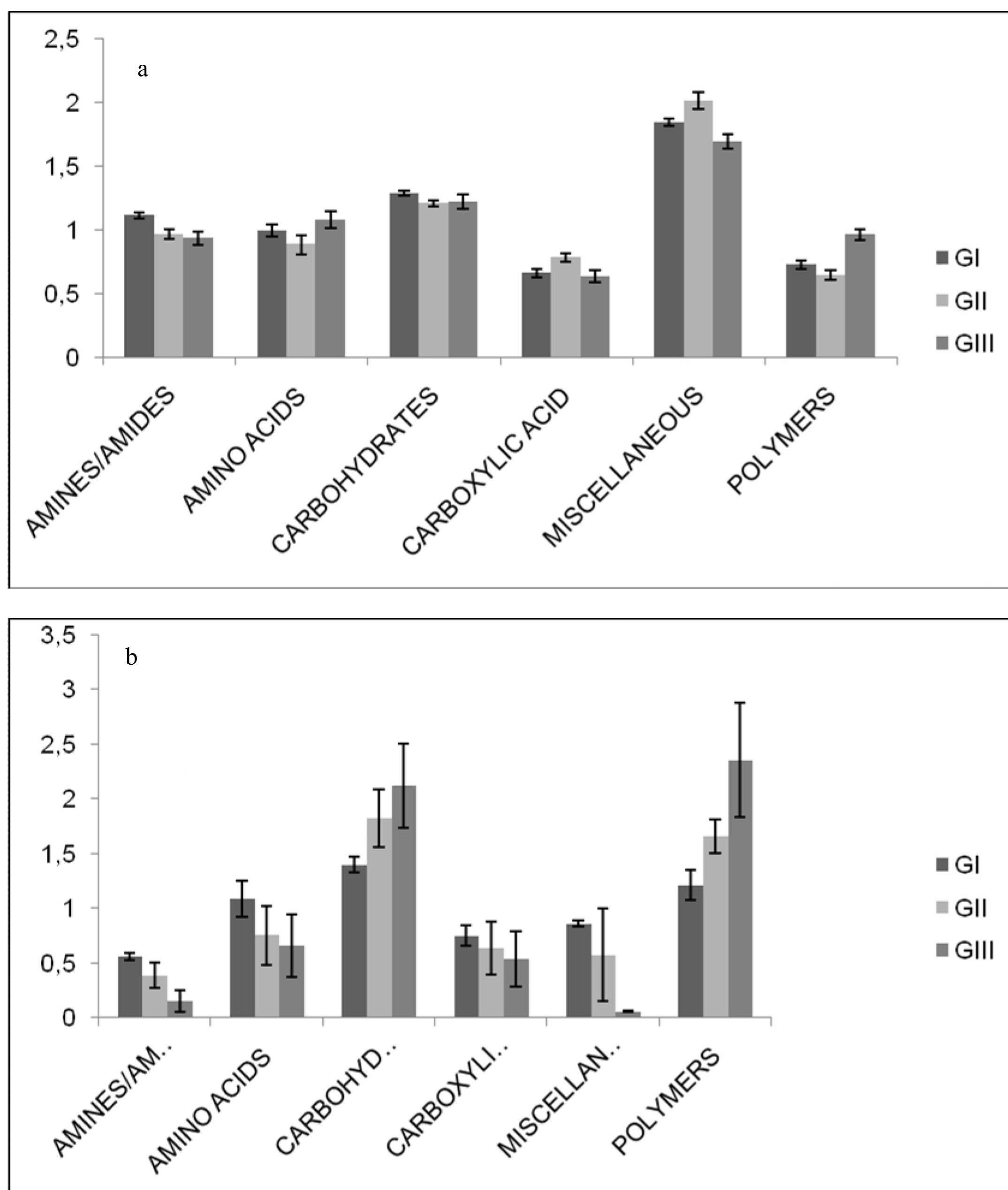

Fig. 4. Categorized carbon source utilization pattern for (a) 5th day (b) 10th day swabs taken from rats treated with simple ointment (GI), rats treated by 10% *P. latifolia* stem ointment (GII) and rats treated by 0.2% Nitrofurazone ointment (GIII)

The values of bars represent mean±SEM

The Principal components (PC) score plots describe the characteristics of the samples and help to understand their distribution and clustering. The PCs scores plot (PC-I and PC-II) shows the spatial distribution among the samples. Principal component analysis (PCA) of carbon source utilization pattern on Biolog Eco plates showed distribution of 57.83% and

31.43% on the PCA vector 1 and 2 axis. The principal component analysis for 5th day showed that GI, GII and GIII are clustered together, thus indicating that the all the treatments have similar microbial profile/diversity while in 10th day incubation sample clearly showed two clusters, one with GI and the second cluster with GII and GIII together based on carbon source utilization pattern Fig. 5.

There is an increasing interest in finding herbal extracts with wound healing efficacy although the use of such extracts for treating cuts and wounds is a common practice in traditional medicine [24]. Research on wound healing drugs is a developing area in modern biomedical sciences and the search for compounds derived from plants constitutes a significant part of such studies. The enhanced wound healing activity of various herbal extracts may be attributed to free radical scavenging action and antimicrobial property of the phytoconstituents present in extract and wound healing could be a function of either individual or synergistic effects of bioactive molecules [25]. Indeed, tannins, saponins and flavonoids promote wound healing due to their astringent, antioxidant and antimicrobial properties which appear to be responsible for wound contraction and elevated rate of epithelialization [26]. Sterols and polyphenols are responsible for wound healing due to free radical scavenging effect, known to reduce cell necrosis and improve vascularity. Plant steroids are known to be good immunomodulators and play role in curing allergy and inflammations [27]. The presence of terpenoids and alkaloids in phytochemical screening conforms to the earlier findings [2-5]. These classes of compounds are known to promote wound healing mainly due to their astringent and antimicrobial properties, which are responsible for wound contraction and increased rate of epithelialization [28]. The antioxidant activity of *P. latifolia* is also discernible in DPPH assay, which primarily evaluates proton radical scavenging activity. DPPH possess a proton free radical with characteristic absorption which decreases significantly on exposure to proton radical scavengers. In the present study *P. latifolia* showed a concentration dependent scavenging of DPPH radical, which may be attributed to its hydrogen donating ability [29]. Presence of significant concentration of flavonoids, phenols and tannins support its free radical scavenging effect. However, the extract was not as effective as the standard antioxidant ascorbic acid. Thus it can be suggested that the free radical scavenging effect may not be solely responsible for the wound healing activity of *P. latifolia.* Wound infections resulting from impaired immunity and exposure or poor hygiene is one of the most commonly encountered and clinically important impediments to wound healing. The injured skin remains vulnerable to invasive microbial infections of all kinds subsequent development of wound sepsis until epithelial repair has occurred. Injury becomes infected because wound area is an ideal medium for multiplication of the infected organism [30]. Thus a plant extract with antimicrobial properties may promote wound healing.

Community analysis using Biolog Microplates was originally described in 1991 by Garland and Mills [31]. Since then the method has been extensively used to compare the physiological profiles of microbial communities from diverse habitats [21,32-35]. The study requires less time and is repeatable. In the present study the technique is used for the primary categorization of microbial diversity in wound and also to study the effect of plant extract on bacterial communities. A comparison of the substrate utilization pattern for 5th and 10th day for groups GII and GIII indicates that there is a decrease in the bacterial communities thriving on amides/amines, amino acids, carboxylic acids and miscellaneous, however there is a rise in bacterial communities utilizing carbohydrates and polymers. Substrates like amides/amines and amino acids are most commonly used by Gram positive bacteria [36,37]. Initially chronic wounds will have predominantly Gram positive organisms, whereas wounds of several months duration, especially with deeper structure involvement, will have several pathogenic species within the wound bed [38]. Any natural extract that can cure the initial settlement of gram

positive colonies, would result in better wound healing. *P. latifolia* treatment on wounded animals showed significant wound healing activity owing to the presence of antioxidant and antibacterial activity of the presence of a mixture of phytoconstituents in the extract. Knowledge and usage of herbal medicine for treatment of various diseases and disorders in local villages is still a major part of life and culture. The results of current study revealed the potential use of *P. latifolia* as an external treatment for wounds. However, further study is needed to isolate the active components that promote wound healing.

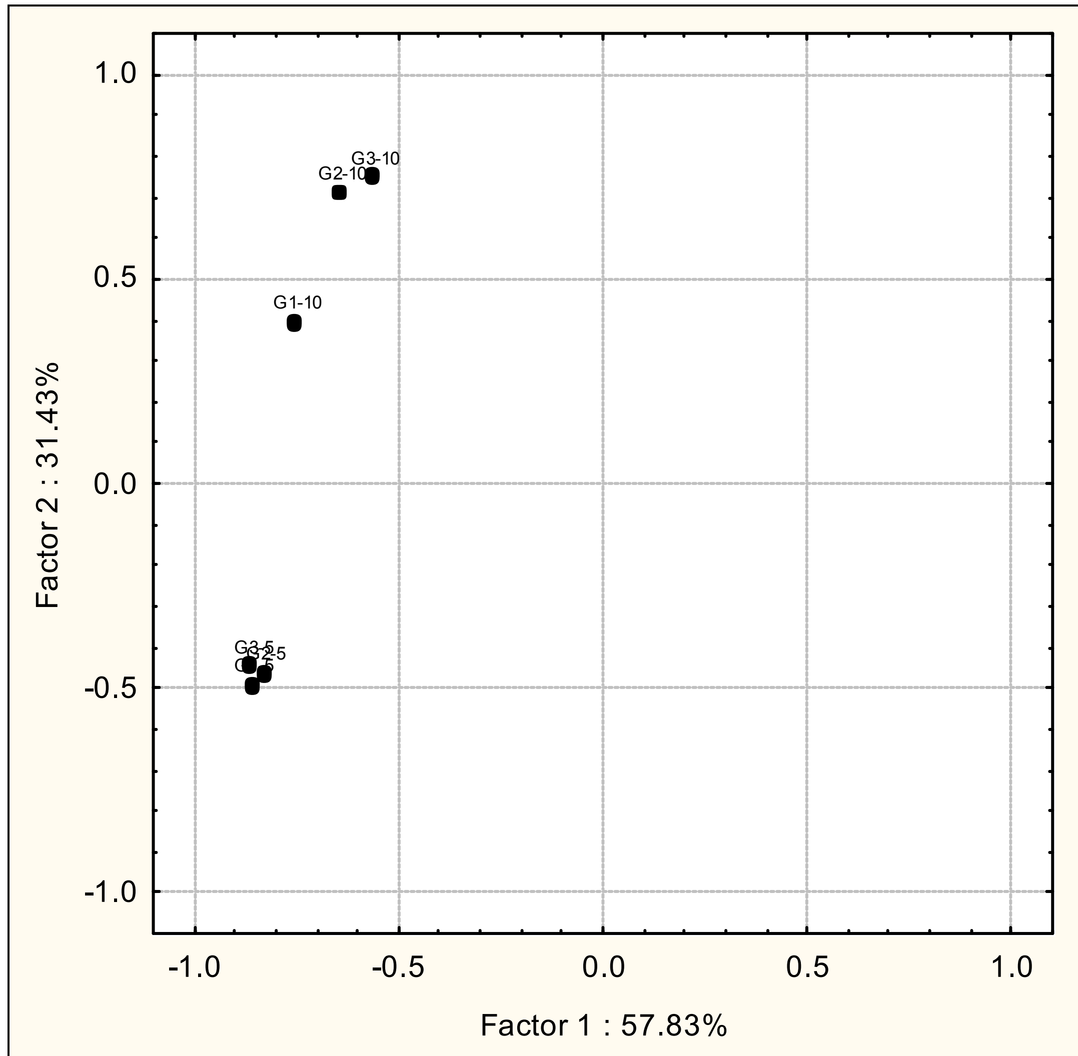

Fig. 5. Principal Component Analysis of 5th day and 10th day swabs taken from rats treated with simple ointment (GI), rats treated by 10% *P. latifolia* stem ointment (GII) and rats treated by 0.2% Nitrofurazone ointment (GIII)

4. CONCLUSION

The study suggests that *P. latifolia* has significant wound healing potential. The antioxidant and antibacterial activity of the plant contributes to its medicinal properties.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that the protocols were duly approved by Institutional Committee for Ethical use of Animals and Review Board (106/IAEC/RB/7-11), CSIR-NBRI, Lucknow.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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