RESEARCH PAPER

Wound Healing Film Preparation using Saponins from Piper nigrum root extracts - Evaluating the Physio-chemical and Biological Characteristics

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ABSTRACT

Aim: The present research was aimed to determine the efficiency of wound healing film developed using saponins from Piper nigrum root extracts and to evaluate the physio-chemical and biological characteristics of the developed film.

Methods: Antibacterial and antifungal screening was tested to wound healing hydrogel films (PNHF). Characterization studies such as, swelling behaviour, degradation study, antibacterial activity, antifungal activity, tensile strength and solubility properties of the developed films were determined. In vitro and in vivo wound healing efficacy of the films were investigated using scratch assay and in animal models.

Results: Antibacterial and antifungal activity of the films for the higher concentration (50µg/ml) was recorded against test bacteria and fungi respectively. Swelling behaviour or water uptake ability of the developed films were recorded as 26.1%. During degradation studies, about 35.3% degradation of the films were recorded after 30 min incubation time period. Antibacterial activity of the PNHF samples exhibited good inhibitory zones size of about 36mm, 35mm, 37mm and 38mm respectively for the tested bacterial cultures. Antifungal activity of the PNHF samples exhibited good inhibitory zones size of about 34mm, 35mm, 34mm and 36mm respectively for the tested fungal cultures. Film thickness and tensile strength of the developed Piper nigrum saponin films (PNHF) were tested and results indicated that as the thickness increases, tensile strength was also increased. The total solubility percentage was recorded as 21.76% for the developed Piper nigrum saponin films (PNHF) after three hours of experimental time. In vitro wound scratch assay on L929 mouse fibroblast cells showed that after 24hours, positive cell migration and cell proliferation was evident indicating the wound healing ability of the PNHF sample. Wound healing efficiency using Excision wound model in Wistar albino rats exhibited, positive healing with indication of healed skin structures; well-formed epidermis and collagen tissues within the dermis.

Conclusion: The obtained results showed that the developed films would be suitable for wound healing in humans and animals; and the research can also be extended to investigate the wound healing efficiency even in diabetes foot ulcers which shall be considered as our future study.

Keywords: Piper nigrum root, saponin, film, antimicrobial, wound healing.

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INTRODUCTION

Human body is mostly covered with skin which represented the outermost defensive covering of the body. Skin protects against mechanical pressure, microbial contagion, and septicity [1] also reported to hold antimicrobial protective role due to its different composition such as, epidermis, dermis, adipose tissue, sweat and sebaceous glands, and hair follicles [2]. These layers are reported to be protected by different types of symbiotic bacteria and yeast like fungi against invasive

microbial pathogens such as, *Staphylococcus* spp, *Malassezia* spp and *Demodex* sp [3]. Wounds at skin site was reported such as, loss of histological composition due to internal and external factors, tissue damage or disturbance due to sequential loss of function in any layers of the skin. These damages may lead to permit the entry of different types of bacteria and fungi or some time virus and parasites [4]. Also, these microbial invasion leads to inflammation and infection in skin to cause wound associated systemic infection called septicaemia which is considered as a life-threatening condition [5].

Wound healing is a complex process which contains sequence of events [6], such as, bleeding, clotting, regeneration, migration, and proliferation of parenchyma cells and deposition of collagen [7], the collagen synthesis and cross-linking afford stability to the healing tissue [8]. Different mechanisms of wound healing process were reported as modulation, declining bacterial count, refining collagen deposition, increasing fibrocytes and fibroblasts, by [9]. Due to this complex wound healing process, only few treatment methods were found effective and hence a need for effective wound healing therapy is significant [10].

One such therapy found more protective in wound healing was application of medicinal plants; which are considered more effective healers because they could inherently facilitate recovery mechanisms [11]. There is report that, around 13,000 herbal plants were used for wound healing in the recent five years of time [12]. [13] highlighted that plant-based therapy enhance would healing process. About 70% of wound treatment are plant derived extracts and 30% of treatment are plant associated mineral based. [14] reported that, medically significant herbal extracts were used as antiseptic first aid at the wound site.

Many reports on wound healing and managing were described both in vitro [15] and in vivo [16] recently. Phytochemical constituents play a major role in repairing wound at different conditions (acute or chronic). These constituents having remedial features are flavonols, bromelain, flavanones, proanthocyanidins, isoflavones, β-glucans and flavonolignans [17] and [18]. Few plants containing these compounds were highlighted for topical treatment for wound repairing, such as aloe vera, banana leaves [19], turmeric, *Centella asiatica*, *Rosmarinus officinalis, Calendula officinalis* [20].

Saponins are used to enhance immune system, due to their antioxidant properties. They exhibit anti-inflammatory, hypocholesterolaemia, anti-cancer and other biological properties [21]. This may be due to the various elemental compositions of the crude saponins. Steroidal glycosides are predominant found in wild plants, mainly the monocotyledonous angiosperms and have been used as alternative medicine in combating different ailments, thus aiding health maintenance.

Based on these properties of medicinal plants, the present research was carried out to determine the efficiency of wound healing film developed using *Piper nigrum* extracts. As a primary aim, the study was designed to evaluate the physio-chemical and biological Characteristics of the developed film.

MATERIALS AND METHODS

Collection and Preparation of Plant Extract

Piper nigrum root was collected and extraction was prepared using soxhlet process as per the method described by [22]. Extracts were dried and added with DMSO solution before processing for other biological parameters.

Isolation of saponin content

The portion of the extract (50 g) was dissolved in 20 ml of ethanol (96% v/v). Acetone was added to the solution drop by drop until complete precipitation appeared. The precipitate was separated by decantation and washed with acetone. Finally, the saponins (8 g) were dried under reduced pressure using a rotary evaporator at 40˚C [23].

Preparation of Wound Healing Hydrogel-Films using *Piper nigrum* (PN_{HF})

About 1% of PVA (Fig) and 1ml of glutaraldehyde (Fig) mixture was prepared by heating at 80 to 100^0C till complete dissolving of PVA. The PVA+glutaraldehyde mixture was added to saponin solution at 1:1 ratio (10 ml:10 ml). The mixture was cooled to room temperature and aliquots of 20 mL were poured into round glass and plastic petridish with a diameter of 10cm (Fig). All the plates were incubated at 4^0C for 48 to 72 hours till films were completely dried. Developed films were detached from the petridish surface and pop out after specific incubation period (Fig). Similar protocol was carried out separately for films without saponin extract and PEG using PVA + glutaraldehyde (Plain films). All the films were treated for 15s into 4% (w/v) solution of NaOH and then rinsed in distilled water for 15s. The final products were stored at either room temperature or at refrigeration temperature (Fig).

Developed Hydrogel-Films (polymer + *Piper nigrum* mixture - PN_{HF})

Screening the antibacterial and antifungal concentration of film using well diffusion method

Antibacterial and antifungal activity for the developed extracts was tested using five different concentrations (10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml) against bacteria (*Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus luteus*) and fungi (*Candida albicans*, *Candida tropicalis, Candida glabrata* and *Candida krusei*) separately. All the test cultures were inoculated in a sterile Nutrient broth* and allowed to attain the growth for 24 to 48 hours. Sterile Mueller-Hinton Agar* plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of each test organisms were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 20µl of each herbal extract fractions were loaded into the well and the plates were incubated at 37ºC for 24h. The antibacterial and antifungal activity was evaluated in terms of zone of inhibition around the wells in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter.

CHARACTERIZATION STUDIES

Moisture absorbency (Swelling properties) of *Piper nigrum* saponin films (PN_{HF})

A known weight of *Piper nigrum* saponin films (PN_{HF}) was incubated in phosphate buffered saline (PBS, pH-7.4) to determine the swelling ability. The percentage water absorption of *Piper nigrum* saponin films (PN_{HF}) in the media was calculated as follows 60 seconds of time period:

$$E_{SW} = (W_e - W_0) / W_0 \text{ X } 100$$

Where, E_{SW} is the percentage water adsorption of PN_{HF} at equilibrium. W_e denote the weight of the PN_{HF} at equilibrium water absorption, and W_0 is the initial weight of the PN_{HF}. Each experiment was repeated 3 times, and the average value was taken as the percentage water absorption.

Biodegradation studies of *Piper nigrum* saponin films (PN_{HF})

Piper nigrum saponin films (PN_{HF}) were incubated at pH7.4 in phosphate-buffered saline (PBS) with 500-1000U/C.C. of lysozyme concentration in 6-well plate and kept at 37ºC. At required period of time (12 h), the films were taken out, washed with deionized water. The weights of the films were weighed and recorded

$$\text{Weight loss (\%)} = W_0 - W_f / W_0 \text{ X } 100$$

Where *Wo* denotes the initial weight of the PN_{HF} and W_f denotes the final weight of the PN_{HF} films after lysozyme treatment. The experiment was performed in triplicate, and the average value was taken as the percentage weight loss.

Antibacterial activity of PN_{HF} against four wound associated bacteria

3A. Antibacterial activity of *Piper nigrum* saponin films (PN_{HF}) against wound associated bacteria

The test specimens, *Piper nigrum* saponin films (PN_{HF}) were cut into pieces (20mm in diameter). Sterile Nutrient agar plates (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) were prepared and allowed to solidify. Using sterile 4mm inoculating loop, one loop full of culture (*Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus luteus*) was transferred by swabbing all around the surface of the agar plate and also covering the central area of the petridish. For each test organism, separate Nutrient agar plates were used in a sterile zone. PN_{HF} sample was placed over left side of Nutrient Agar plates and plain Hydrogel-Film Control sample was placed right side of the NA plates. All the inoculated plates were incubated at 37°C for 24 hours. The test plates were examined for the clear zone of inhibition around each samples separately. The average width of the zone of inhibition around each sample was calculated and presented in Table separately. The zone of inhibition was measured in millimeter (mm).

Antifungal activity of PN_{HF} against four wound associated Candida

3B. Antifungal activity of *Piper nigrum* saponin films (PN_{HF}) against wound associated bacteria

The test specimens, *Piper nigrum* saponin films (PN_{HF}) were cut into pieces (20mm in diameter). Sterile Nutrient agar plates (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) were prepared and allowed to solidify. Using sterile 4mm inoculating loop, one loop full

of culture (*Candida albicans*, *Candida tropicalis, Candida glabrata* and *Candida krusei*) was transferred by swabbing all around the surface of the agar plate and covering the central area of the petridish. For each test organism, separate Nutrient agar plates were used in a sterile zone. PN_{HF} sample was placed over left side of Nutrient Agar plates and plain Hydrogel-Film Control sample was placed right side of the NA plates. All the inoculated plates were incubated at 37°C for 24 hours. The test plates were examined for the clear zone of inhibition around each samples separately. The average width of the zone of inhibition around each sample was calculated and presented in Table separately. The zone of inhibition was measured in millimeter (mm).

Tensile strength

Tensile strength (TS) of the developed *Piper nigrum* saponin films (PN_{HF}) was determined using a texture analyzer (Model; TA. XT2i). The films were cut into strips of size 20 mm wide and 50mm long and these film strips were fixed between the grips of the texture analyzer. The initial grip separation was set at 30 mm and speed at 1.0 mm/s. The average value of three thickness measurements was recorded and tabulated

Solubility test

Solubility of the developed *Piper nigrum* saponin films (PN_{HF}) in water was determined by the method of [24]. Developed film samples comprising size of 20 mm × 20 mm dried were weighed in Precision balance. All film samples were then submerged in a flask containing 80 mL of distilled water, at 20C for 1h. The films samples were then collected gently and dried again at 50C for 1d. Dry weights of the films were recorded and subsequently, reduction in weight of films was noted. Percentage loss in weight in 1h is considered as, per cent solubility.

$$\text{Solubility (\%)} = W_0 - W_f / W_0 \text{ X } 100$$

Where *Wo* denote the initial weight of the PN_{HF} and W_f denotes the final weight of the PN_{HF} films after treatment. The experiment was performed for three hours and total solubility in percentage was calculated.

Wound healing efficiency of *Piper nigrum* saponin hydrogel films using scratch assay method

The migration rates of fibroblast cells were assessed by the scratch assay method. The cell density of 2×10^5 cells was seeded into each well of a 24 well plate and incubated with complete medium at 37°C and 5% CO_2. After 24h of incubation, the monolayer confluent cells were scrapped horizontally with a sterile P_{200} pipette tip. The debris was removed by washing with PBS. The cells were treated with the *Piper nigrum* hydrogel films (PN_{HF}) by dissolving using 0.5% of toluene. Dissolved samples were treated with serum-free Dulbeco modified eagle medium (DMEM). In parallel, the washed cells were treated with allantoin (Sigma Aldrich, Germany) which was used as positive control. The scratch induced as represented wound, was photographed at 0h using phase contrast microscopy at ×40 magnification. After 0^{th} hour, 6^{th} hour, 12^{th} hour and 24^{th} hour of incubation, the second set of images was photographed. Migration of cells between the scratch site and the distance traversed by cells migrating into the denuded area which emphasize the self-healing was observed using Phase contrast microscope for each time.

Wound healing studies in animal models

To investigate the wound healing potential of the developed *Piper nigrum* saponin Films (PN_{HF}), an in vivo animal model was used. Excision wound model in Wistar albino rats were investigated in a controlled condition. In Group-1 (control) the Silver Sulfadiazine antiseptic cream was applied onto the wound sites. In Group-2, *Piper nigrum* Films (PN_{HF}) was placed over the wound site area. The rats were inflicted with excision wounds as described by [25]. Wound healing was observed till 15 days. Significance in wound healing of the test groups was derived by comparing healed wound area on respective days with healed wound area of control group. The period of epithelialization, that is, day of fall of eschar and the scar area, was also noted. Wound area and wound contraction, epithelialization period were monitored.

Six Wistar Albino rats of 6-8 weeks old and 160-180g body weight was used. In each group three animals were used. All rats were kept at room temperature and allowed to accommodate in standard conditions at 12-hour light and 12 hour dark cycle in the animal house. Animals were fed with commercial pellet diet and water ad libitum freely throughout the study. All the animals were continuously observed to detect any changes in the behaviour in relation to posture, mood, and motor activity. Parameters such as alertness, grooming, restlessness, touch response, righting reflex, gripping strength, corneal reflex, writhing, urination, salivation, skin colour, skin irritation, food, and water intake will be continuously observed during the study.

RESULTS AND DISCUSSION

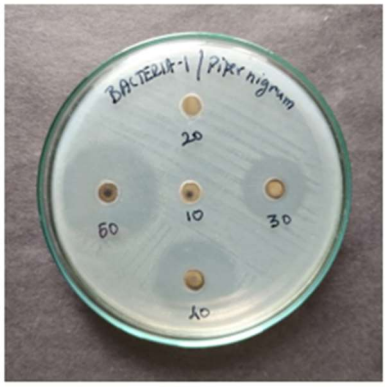
Screening the antibacterial and antifungal concentration of *Piper nigrum* saponin film using well diffusion method

Table-1: Minimal Inhibitory Concentration of *Piper nigrum* against test bacteria

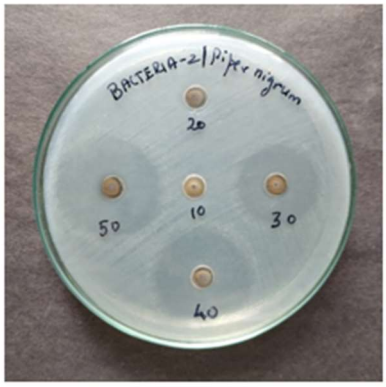
S. No.	Test Bacteria	Minimal Inhibitory concentration				
		10	**20**	**30**	**40**	**50**
1	*Escherichia coli*	NZ	NZ	13	16	21
2	*Staphylococcus aureus*	NZ	NZ	15	17	21
3	*Staphylococcus epidermidis*	NZ	NZ	15	17	22
4	*Micrococcus luteus*	NZ	NZ	14	15	19

NZ: No Zone, PC: Positive control (Streptomycin)

10: 10µg/ml, 20: 20µg/ml, 30: 30µg/ml, 40: 40µg/ml, 50: 50µg/ml



Escherichia coli



Staphylococcus aureus

Staphylococcus epidermidis

Micrococcus luteus

Antibacterial activity of the *Piper nigrum* saponin film sample, against *Escherichia coli* and *Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus luteus* showed GOOD inhibitory zone size for the higher concentrations (30, 40 and 50µg/ml). No Zone (NZ) was found evident for first lower concentrations (10 and 20µg/ml) against all test bacteria. Higher concentration of *Piper nigrum* extracts (50µg/ml) expressed maximum zone size of about 21mm, 21mm, 22mm and 19mm against all the test bacteria *Escherichia coli* and

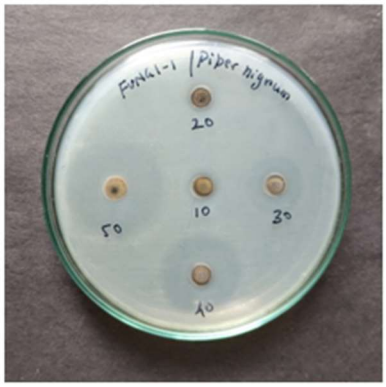
Staphylococcus aureus, Staphylococcus epidermidis and *Micrococcus luteus* respectively.

Table-1: Minimal Inhibitory Concentration of *Piper nigrum* against test fungi

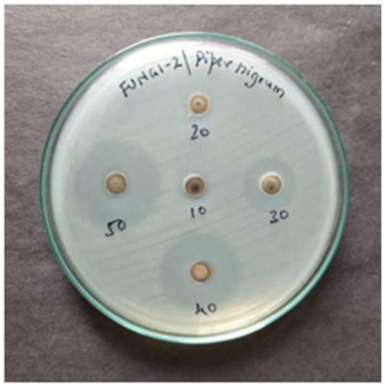
S. No.	Test Fungi	Minimal Inhibitory concentration				
		10	**20**	**30**	**40**	**50**
1	*Candida albicans*	NZ	NZ	11	15	20
2	*Candida tropicalis*	NZ	NZ	11	15	19
3	*Candida glabrata*	NZ	NZ	12	16	21
4	*Candida krusei*	NZ	NZ	12	15	19

NZ: No Zone, PC: Positive control (Streptomycin)

10: 10µg/ml, 20: 20µg/ml, 30: 30µg/ml, 40: 40µg/ml, 50: 50µg/ml

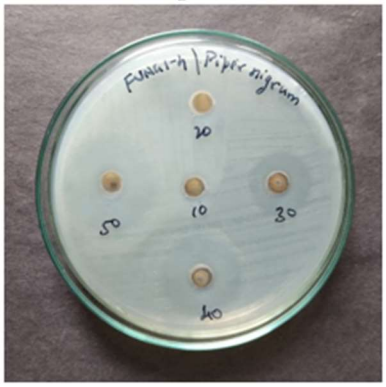


Candida albicans



Candida tropicalis

Candida glabrata



Candida krusei

Antifungal activity of the *Piper nigrum* extracts sample, against *Candida albicans* and *Candida tropicalis, Candida glabrata* and *Candida krusei* showed GOOD inhibitory zone size for the higher concentrations (30, 40 and 50µg/ml). No Zone (NZ) was found evident for first lower concentrations (10 and 20µg/ml) against all test fungi. Higher concentration of *Piper nigrum* extracts (50µg/ml) expressed maximum zone size of about 20mm, 19mm, 21mm and 19mm against all the test fungi *Candida albicans* and *Candida tropicalis, Candida glabrata* and *Candida krusei* respectively.

Characterization studies

Moisture absorbency of PN_{HF} (Swelling properties)

Table-4A: Moisture absorbency (Swelling properties) of *Piper nigrum* saponin films (PN_{HF})

S. No	**Swelling behaviour of PN_{HF}**		
	W_0	W_e	WU (%)*
1	1.21 mg	1.59 mg	23.2
2	1.14 mg	1.53 mg	25.1
3	1.08 mg	1.54 mg	29.9
Average WU (%)			**26.1%**

W_0 – Initial weight of the film, W_f – Final weight of the film after lysozme treatment,

WU – Water uptake in percentage, *Mean % value of three samples

Swelling behaviour or water uptake ability of the developed films were tested. The average swelling ability of the films were recorded as 26.1% (Table-4A).

Biodegradation studies of P_{HF}

Table-4B: Biodegradation studies of *Piper nigrum* saponin films (PN_{HF})

S. No	Weight Loss studies		
	W_0	W_f	WL (%)*
1	1.54 mg	0.98 mg	36.3
2	1.58 mg	1.04 mg	34.1
3	1.57 mg	1.01 mg	35.6
Average WL (%)			**35.3 %**

W_0 – Initial weight of the film, W_f – Final weight of the film after lysozme treatment,

WL – Weight loss in percentage, *Mean % value of three samples

After treating with lysozyme enzyme, 35.3% degradation of the films were recorded after 30 min incubation time period. This indicated that the films were 100% biodegradable in nature with in 48 hours or more. In Table-4B the values were presented; and in Fig. 4B three samples of degraded films were presented.

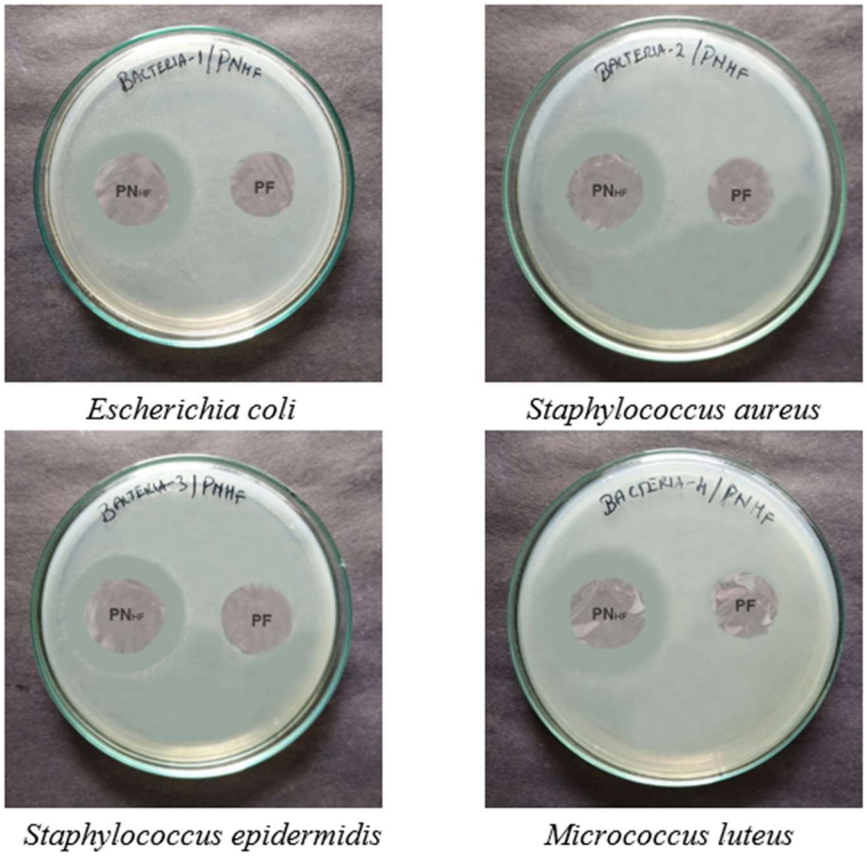
Antibacterial activity of PN_{HF} against four wound associated bacteria

Table-3A: Antibacterial activity of PN_{HF} samples

Test Bacteria	**Zone of Inhibition (mm)**			
	Escherichia coli	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Micrococcus luteus*
***Piper nigrum* films (PN_{HF})**	36	35	37	38
PF (Plain films)	0	0	0	0

In Table-3A, the antibacterial activity expressed in terms of inhibitory zones around the PN_{HF} samples were measured in millimetre and presented against all test bacteria. From the image, it was evident that, all PN_{HF} samples exhibited significant inhibitory zones; and plain films did not exhibit any zones due to absence of plant extracts. PN_{HF} samples revealed inhibitory zone size of about 36mm, 35mm, 37mm and 38mm respectively for the tested bacterial cultures, *Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus luteus* (Fig. 3A).

Fig. 3A: Antibacterial activity of PN_{HF} samples



Escherichia coli *Staphylococcus aureus*

Staphylococcus epidermidis *Micrococcus luteus*

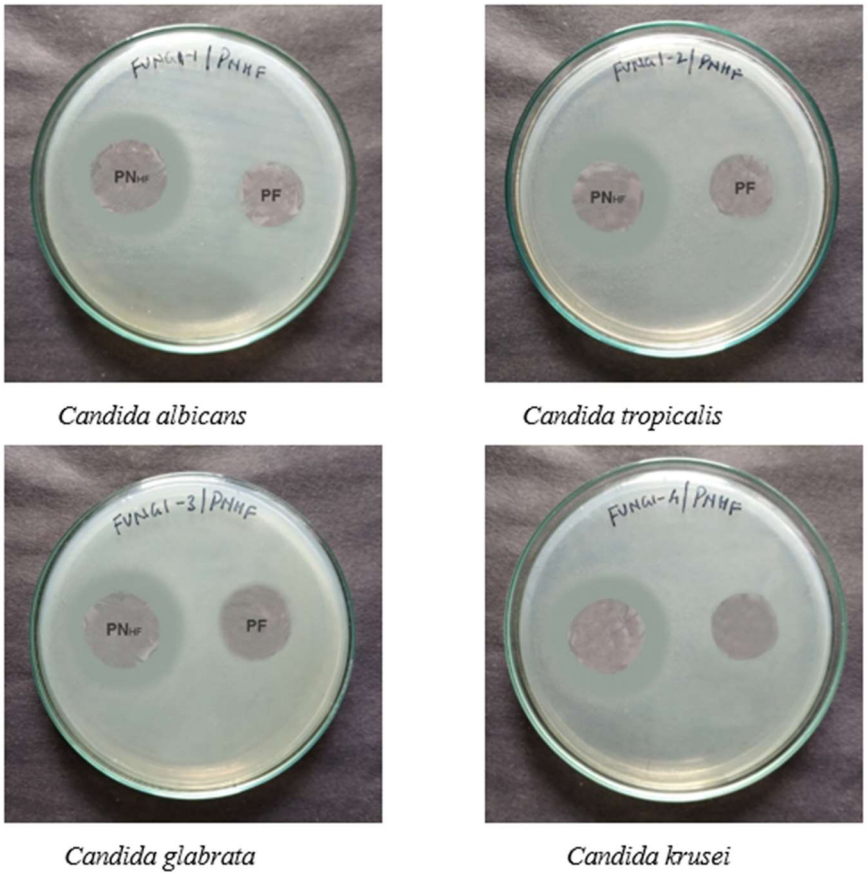
Antifungal activity of PN_{HF} against four wound associated Candida

Table-3B: Antifungal activity of PN_{HF} samples

Test Bacteria	**Zone of Inhibition (mm)**			
	Candida albicans	*Candida tropicalis*	*Candida glabrata*	*Candida krusei*
***Piper nigrum* films (PN_{HF})**	34	35	34	36
Control films	0	0	0	0

In Table-3B, the antifungal activity expressed in terms of inhibitory zones around the PN_{HF} samples were measured in millimetre and presented against all test fungi. From the image, it was evident that, all PN_{HF} samples exhibited significant inhibitory zones; and plain films did not exhibit any zones due to absence of plant extracts. PN_{HF} samples revealed inhibitory zone size of about 34mm, 35mm, 34mm and 36mm respectively for the tested bacterial cultures, *Candida albicans*, *Candida tropicalis, Candida glabrata* and *Candida krusei* (Fig. 3B).

Fig. 3B: Antifungal activity of PN_{HF} samples



Candida albicans *Candida tropicalis*

Candida glabrata *Candida krusei*

Tensile strength

Table-4C: Film thickness and Tensile strength test of *Piper nigrum* saponin films (PN_{HF})

S. No	Characterization studies of *Piper nigrum* films (PN_{HF})	
	Film thickness test* (mm)	Tensile strength test* (MPa)
1	1.1 mm	2.52 MPa
2	1.2 mm	2.67 MPa
3	1.1 mm	2.62 MPa
Average	**1.13 ± 0.3**	**2.6 ± 0.3**

*Mean value of three samples

Film thickness and tensile strength of the developed *Piper nigrum* saponin films (PN_{HF}) were tested using standard methods. As thickness and tensile strength are dependent each other, both test were done simultaneously. As thickness increases, tensile strength was also increased. This was evident in Table-4C.

Solubility test

Table-4C: Solubility test of *Piper nigrum* saponin films (PN_{HF})

S. No	Characterization studies of *Piper nigrum* films (PN_{HF})		
	Film Weight W_0	Film Weight W_f	Solubility* (%)

0th	1.53 mg	1.53	0
1st	1.53 mg	1.42 mg	7.18
2nd	1.42 mg	1.34 mg	5.63
3rd	1.34 mg	1.22 mg	8.95
Total solubility after 3h (%)			**21.76**

W_0 – Initial weight of the film, W_f – Final weight of the film after treatment,

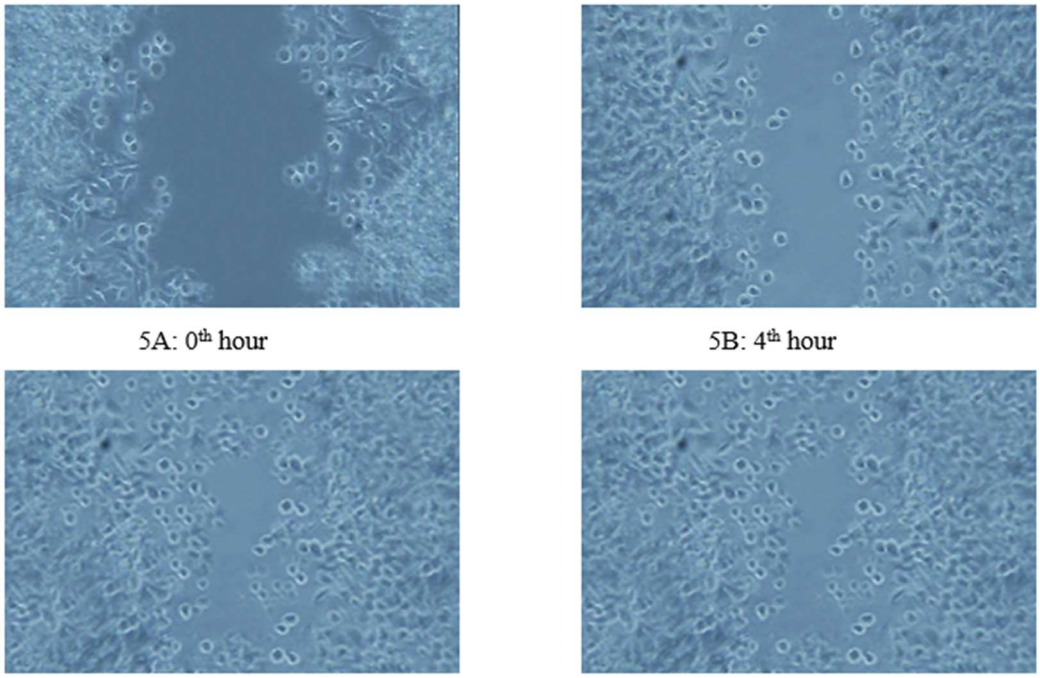
*Mean % value of three samples

The total solubility percentage was recorded as 21.76% for the developed *Piper nigrum* films (PN_{HF}) after three hours of experimental time.

Wound healing *In vitro* scratch assay using L_{929} cell lines

In this study, the PN_{HF} sample used for the cell adhesion studies was determined for its ability to improve wound healing by acting directly on L_{929} mouse fibroblast cells. In the below Fig. 5, the corresponding to wound healing ability of the PN_{HF} sample showed that, at 0th hour, no cell migration and proliferation was observed for the known concentrate (Fig. 5A). At 4th hour and 12th hour, showed positive cell migration and cell proliferation (Fig. 5B and 5C). After 24hours, more cell proliferation was evident and thus indicating the wound healing ability of the PN_{HF} sample (Fig. 5D).

Fig. 5: Wound healing *In vitro* scratch assay using L_{929} cell lines



5A: 0th hour

5B: 4th hour

5C: 12th hour

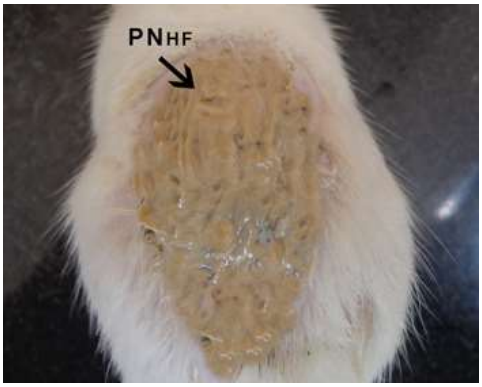
5D: 24th hour

Wound healing studies in animal models

Wound healing efficiency of the *Piper nigrum* saponin Films (PN_{HF}) was studied using Excision wound model in Wistar albino rats. During the analysis, it was found that wounds in control (Group-1 - Silver Sulfadiazine antiseptic cream treated) and test group (Group-2 - PN_{HF} treated) was successful. Healing stages in both control and test groups were started recorded from Day-1 (Fig. 1A and 1B). After creating wound on Day-1, slight ulceration with edema at wound site was evident with possible formation of mononuclear inflammatory cells. On Day-7 positive healing indicating the healed skin structures at wound site was found evident in Control and test groups (Fig. 2A and 2B). On 15th day complete healing of wounds in all the animals from both control and test group was evident (Fig. 3A and 3B). Images of wound healing on Day-1, Day-7 and Day-15th was presented in the images separately for each group. From the images it was clear that wound gets healed after 15 days; which was indicated from normal skin growth covering completely over wound surface and also from the hair growth (Fig. 14A, B, C).

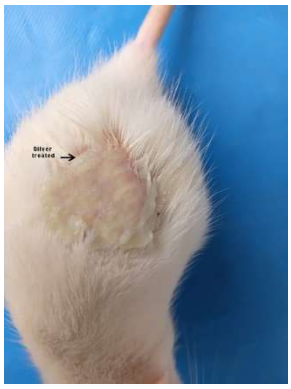
Fig. 1: Observation of wound healing stages in animal models treated with silver Sulfadiazine antiseptic cream and PN_{HF}

Fig. 1A: Day - 1



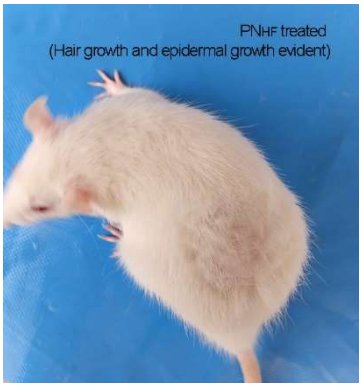
Group - (control) Group (PN_{HF} treated)

Fig. 1A: Day – 7



Group - (control) Group (PN_{HF} treated)

Fig. 1A: Day – 15



Group - (control) Group (PN_{HF} treated)

DISCUSSION

As repeated usage of antibiotics, bacteria gaining resistance stimulated the researchers to develop novel antibacterial agents from the plant and herbal sources; which significantly considered as best alternative to chemotherapeutic agents [26]. The antibacterial and antifungal activity exerted in the present research also revealed the same in terms of inhibitory zone size against all the test bacteria and fungi. The results obtained in the present research was found in correlation with [27]; as the

ethanol extract were found highly active towards the tested bacterial strain in terms of inhibitory zones. Much research works on *Piper nigrum* extracts exhibited that the presence of different phytochemical compounds such as, terpenoids, flavonoids, tannins, alkaloids, steroids and polyphenols were attributed for the antibacterial and antifungal activity [28] and [29]. *Piper nigrum* extracts (aqueous and ethanol) was found to contain phytochemicals like alkaloids, flavonoids, saponins, reducing sugars, steroids and tannins [30]. In another study, chloroform extracts of *Piper nigrum* leaves showed good antibacterial activity due to the presence of alkaloids and flavonoids [31].

Mode of action of these phytochemicals on the type of microorganisms was significantly correlated to the cell wall or cell membrane arrangements. One such essential compound piperine inhibits bacterial growth, biofilm formations and enzymes against Gram-Positive bacteria. Hence, the extracts of *Piper nigrum* disrupts bacterial cell membranes, which further leads to leakage of cytoplasmic contents such as proteins and nucleic acids. In another study, similar piperine compound present in *Piper nigrum* extracts showed excellent antimicrobial activity [32]. [33] reported that the presence of significant phytochemicals such as, sabinene, β-pinene and limonene contributed effective antimicrobial activity against different test organisms.

Antibacterial and antifungal activity of the developed films showed excellent inhibitory zones against all test bacteria and fungi. In comparison to the findings of our research, recent studies also exhibited similar antibacterial activity for the developed wound dressing films.

The study developed antimicrobial wound dressing films containing essential oils and oleoresins of *Piper nigrum* encapsulated with sodium alginate. Antibacterial inhibitory zone size of about 35.2 mm, 33 mm and 24.2 mm for the respective bacteria *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* was recorded [34]. In our study, *Escherichia coli* and *Staphylococcus aureus* exerted 36mm and 35mm of inhibitory zone which was almost like the findings of [34]. In another recent study, [35] used *Piper nigrum* extracts to develop a novel wound dressing material with the aid of carboxy methyl cellulose. The researchers studied the swelling degree, solubility, tensile strength, wound healing efficacy and antibacterial activity of the developed films. The results of swelling degree, solubility and tensile strength were found supportive to our findings. Antibacterial activity exhibited the clear zones from 8mm to 8.8 mm against *Staphylococcus aureus* depending on the concentrations of *Piper nigrum* used for developing the wound dressing materials.

The antibacterial activity and wound healing activity exerted in our present research also relies on the properties of other significant parameters such as swelling degree and solubility. Swelling and solubility increases the release of antimicrobial agents from the films at constant rate so that effective healing and antibacterial activity was attained.

In vitro wound scratch assay results of the PN_{HF} sample showed positive cell migration and cell proliferation from 4^{th} hour to 24 hours. Recently, similar in vitro wound scratch assay studied by researchers using the developed wound dressing films were found supportive to our present findings. [36] developed a wound dressing film using *Moringa oleifera* leaf extract coupled with graphene oxide and polyvinyl alcohol exhibited cell viability (83–135%) in mouse fibroblast cells after performing the similar wound scratch assay. In another study, [37] evaluated the efficacy of topical application of *Aloe vera* hydro-alcoholic leaf extract in addressing the various contributing elements to delayed wound healing in the diabetes through cell-based assays [38,39,40]. The extract displayed enhanced wound closure, achieving a remarkable 57.03 % closure rate at concentration of 0.1mg/mL compared to the untreated cells 10.01% in an *in vitro* scratch assay [41,42,43].

Wound healing efficiency of the *Piper nigrum* Films (PN_{HF}) was studied using Excision wound model in Wistar albino rats. When compared to control group, the test group with *Piper nigrum* films showed positive wound healing efficiencies. After wound healing healed skin structures with well formed, near to normal epidermis, restoration of adnexa, and extensive fibrosis and collagen tissue within the dermis was formed.

CONCLUSION

The present research was carried out to determine the efficiency of wound healing film developed using *Piper nigrum* saponin extracts. Different physio-chemical and biological characteristics of the developed film was studied. Antibacterial and antifungal activity of the *Piper nigrum* extracts showed significant inhibitory zones against all test organisms for the higher concentration (50µg/ml). Swelling behaviour and degradation of the films exhibited 26.1% and 35.3% respectively. Antibacterial and antifungal activity of the PN_{HF} samples exhibited significant inhibitory zones. Film thickness and tensile strength of the developed *Piper nigrum* films (PN_{HF}) were tested and results indicated that as the thickness increases, tensile strength was also increased. In vitro wound scratch assay revealed, positive cell migration and cell proliferation after 24hours. In vivo animal studies exhibited, healed skin structures; well-formed epidermis and collagen tissues within the dermis. The obtained results showed that the developed films would be suitable for wound healing in humans and

animals; and the research can also be extended to investigate the wound healing efficiency even in diabetes foot ulcers which shall be considered as our future study.

Ethics Committee Approval

The study was approved by (Approval No: TB/IAEC/2025/09/032) by lAEC of Trichy Research Institute of Animal Facility, Trichy – 620009, Tamil Nadu, India.

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Conflict of interest

The authors have no conflicts of interest to declare.

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