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WOUND HEALING ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED FROM *PLEUROTUS* SPECIES AND THEIR FUSANTS USING WISTAR ALBINO MALE RAT

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Abstract: Renewed interest in silver only rekindled after nanotechnology has made it possible to produce pure silver particles on the nano-scale. In the case of exposing cells or tissue to silver nanoparticles (AgNPs), the active surface would be significantly larger than silver compounds and thereby exhibiting remarkably unusual physicochemical properties and biological activities. So, it was thought worthwhile to synthesize nanoparticles by bio-assisted pathway to provide better wound healing therapy to avoid the resistance-related problem. Thus this study aimed to provide a direction for future research in impaired wound treatment with reduced toxicity.

***Index Terms* - *Pleurotus* sp., AgNPs, Parent, Fusants, Wound healing.**

I. INTRODUCTION

According to the recent reports of Centre of disease control and prevention 2013, wound is the third largest cause of death after cardiovascular disease and cancer. In addition to above emergence of microbial resistance to antimicrobials makes it further difficult to improve healing potential. Conventional wound care products usually intended to produce localized antimicrobial activities. Clinical efficacies of those products largely depends on the antimicrobial activities of the drug and wound type and are usually found ineffective in wounds associated with pathological disturbances. Healing of wound is a complex and multiple-step process involving integration of activities of different tissues and cell lineages (Martin, P., 1997). Re-epithelization, a crucial process during the early phase of wound healing, occurs not only by the migration and proliferation of keratinocytes in the epidermal layer of skin from the wound edge, but also by differentiation of stem cells residing in the bulge of hair follicle. Rapid re-epithelization after wounding will provide an optimum environment, such as scaffold of cells and various growth factors, which are indispensable in wound healing. Further to re-epithelialization, wound contraction is another important process in the early phase of wound healing.

A large number of silver-containing dressings was being available on the market for the application of burns, either in the form of impregnated bandages or as a cream containing silver sulfadiazine as the active agent, a product that is still considered the benchmark silver product (Hussain, S. *et al.*, 2006). Renewed interest in silver only rekindled after nanotechnology has made it possible to produce pure silver particles in the nano-scale. It was thoughtful worthwhile to synthesize nanoparticles by bio-assisted pathway to provide better wound healing therapy to avoid the resistance related problem. Thus this study may provide a direction for future research in impaired wound treatment with reduced toxicity.

II. MATERIALS AND METHOD

2.1 Preparation of Hydroalcoholic Ointment or Gel Formulation

The synthesized silver nanoparticles (10g) were dissolved in alcohol before the incorporation of the specified amount of Vaseline (100gm). The burn wound healing effect of AgNPs against control (without AgNPs) alone was examined, and Vaseline was used as a base component of the ointment. The prepared gels were inspected visually for their color and homogeneity. The spreadability of the gel formulations was determined by measuring the spreading diameter of 1 g of gel between two horizontal plates (20 cm × 20 cm) after one min.

The pH was measured, at room temperature, in each gel sample using digital pH meter which was calibrated before each use with standard buffer solutions. The AgNPs incorporated gel (100 mg) was dissolved in 100ml of methanol stirred for 2 hrs on magnetic stirrer at 250 rpm to get complete solubility of the drug and estimated spectrophotometrically for the drug content. An unloaded gel was also subjected to a similar determination to observe the effect of excipients on the absorbance.

2.2 Experimental studies on animals

Twelve week-old wistar rats weighing approximately 160g - 180g body weight were used in the study. The rats were acclimatized to the laboratory environment for a period of 10 days. The protocol was approved by the Institutional Animal Ethical Committee, K. M. College of pharmacy, Madurai (KMCP/IAEC/353). Animals were cared and handled as per the guidelines set by the Indian National Science Academy New Delhi, India.

2.3 Grouping of animals and drug administration

The animals were randomly allocated into nine groups of six animals each for the three experimental animal wound models.
Group I received Dexamethasone, 0.17 mg/kg (Cadila Healthcare, Mumbai)
Group II received 1mg/kg Vaseline
Group III received 1mg/kg of AgNps of *P. eryngii* based ointment
Group IV received 1mg/kg of AgNps of *P. florida* based ointment
Group V received 1mg/kg of AgNps of *P. ostreatus* based ointment
Group V I received 1mg/kg of AgNps of *P. djamor* var. *roseus* based ointment
Group VII received 1mg/kg of AgNps of FST 1based ointment
Group VIII received 1mg/kg of AgNps of FST 2 based ointment
Group IX received 1mg/kg of AgNps of FST 3 based ointment and the AgNp based ointments were applied directly into the skin.

2.4 Excision wound model

The animals were anesthetized with anesthetic ether by open mask method and placed on an operation table in its natural position. An excision wound was inflicted on the dorsal thoracic region 1-1.5 cm away from the vertebral column on either side and 5 cm away from the ear. The skin impressed area was excised to the full thickness to obtain a wound area about 500 mm and 2mm diameter and 2mm depth. Hemostasis was achieved by blotting a wound with a cotton swab soaked in normal saline. The ointment topically applied once daily starting from the day operation till complete epithelialization. Wound areas were measured on days 3^{rd}, 7^{th}, 11^{th} and 15^{th}. The wound area was drawn with permanent marker on transparent sheet. The circular marked area of the transparent sheet was excised and measuring the weight for calculating the percentage of wound contraction. Percentage wound contraction was calculated as:

$$\text{Percentage of wound closer} = \frac{(\text{Initial wound size} - \text{specific day wound size})}{\text{Initial wound size}} \times 100$$

2.5 Incision wound model

Para vertebral straight incision of 6cm length was made through the entire thickness of the skin on either sides of the vertebral column with the help of a sharp scalpel. After complete hemostasis, the wounds were closed by means of interrupted sutures placed at approximately 1cm apart. Animals were treated daily with drugs, as mentioned above under excision wound model from 0^{th} day to 15^{th} day. The wound breaking strength was estimated at 10^{th} day by tensile tester (Instron 6021).

2.6 Visual observation

Healing scores for the test regions were obtained through assessing the extent of reduction in wound inflammation and edema based on the criteria reported in literature (Yu *et al.,* 1987). Wound healing property was evaluated based on the least closure time for percentage of wound contraction.

2.7 Histology of Skin

Microscopic evaluation of internal structure of treated skin was confirmed by histological analysis. In histological examination, treated skin area was excised; fat was removed, stored in 50 % (w/v) formalin and then dehydrated through graded series of alcohols. The skin was then treated with xylene and embedded in paraffin. Skin sections of 5mm thickness were cut from each sample and stained with haematoxylin–eosin for microscopic examination. The stained skin sections were observed under a light microscope to assess the histological changes in skin morphology (Jagetia *et al.,* 2003).

2.8 Statistical analysis of information

Statistical comparison was performed using one way analysis of variance (ANOVA) and for multiple comparison versus control group was done with Dunnett's test. All statistical analysis was performed using Graph pad prism version 5. *P< 0.05* was considered statistically significant.

III. RESULTS AND DISCUSSION

3.1 Physical Examination of Hydroalcoholic Ointment / Gel

The physical appearance of the gel was reddish brown in colour and transparent (Fig. 1) but the control appears colourless and transparent. The pH, spreadability, viscosity and drug content of the gel are shown in Table 1. It is important to say that there was no symbolic change in the pH values on time of formulation.

3.2 *In vitro* wound healing study

At the end of the experimental period, there was no significant changes in body weight of the animal when treated with AgNPs (Group III to Group IX) and commercial ointment treated group (Group I and II) (Table 2). Further the animals treated with AgNPs did not produce any significant change in behavior, water intake, food consumption and breathing responses.

3.3 Excision wound model

Wound contraction ability of excision model was evaluated at different time intervals till complete wound healing process. In the excision wound parameter, Group VIII show 88.92% of healing on the 15^{th} Day followed by Group VII – 82.56%, Group IX – 80.25%, Group V – 78.28%, Group IV - 76.22%, Group III - 75.49% and Group I - 68.67% whereas control group (Group II) shows 43.02 % of wound contraction. Group II alone takes 21 days for complete healing (Table 3). The Fig. 2 shows the period of epithelization of skin treated by different groups from 0^{th} day to 15^{th} day. The results were also displayed as photographs starting the treatment (0^{th} Day) and after finishing the treatment (15^{th} Day) in different groups as well as the control (Fig. 3).

According to Kumarasamyraja D and Swamivelmanickam M, 2014, silver nanoparticles of *Cassia auriculata* showed better wound contraction on 19th day than the aqueous extract of *C. auriculata*, control and wound treated with standard drug (Povidone-iodine) ointment. But in the present study, the period of epithelialization of dexamethasone has extended the epithelialization when compared to co-administration of silver nanoparticles along with Vaseline showed a significant increase in the wound contraction on 15th day. Hence, it is proved that silver nanoparticles synthesized from mushroom especially fusants have prohealing effect and AgNP's were also able to promote epithelialization either by facilitating the proliferation of epithelial cells or by increasing the viability of epithelial cells.

3.4 *In vivo* wound healing study

The histological studies pertaining to the wound healing process revealed that the structure of the skin tissue has been completely restored in the healed area after 15days of treatment with AgNPs. Optical microscope observation of the skin biopsies after hematoxylin eosin staining showed a major difference between the Standard, Control and the Samples. The histopathological result shows better cell attachment of fibrous connective tissue between the wound edges with regeneration of thicker keratin layer on wounds from 3rd day to 15th day of treatment (Fig. 4).

According to Somboonwong *et al.*, (2012), the untreated burn wound on day 16 showed a prominent fibrinoid necrosis in the subepidermal region, which was characterized by penetration of collagen with fibrin, re-epithelialization and additional degenerative changes were found incomplete. In the present study, the wounds of the AgNPs treated groups showed no fibrinoid necrosis in the sub-epidermis, fully arranged dermal region, fully developed epithelialization and keratinization. Skin appendages were also found almost normal. The wounds of the AgNPs treated groups showed no fibrinoid necrosis in the sub-epidermis, fully arranged dermal region, fully developed epithelialization and keratinization. Skin appendages were also found almost normal.

The histological studies made on the present attempt on the burn wound healing properties of 0.17 mg/kg concentration of commercial ointment revealed that the animals did not show any fibrinoid necrosis in the sub-epidermis. But the animal treated with 1mg/kg concentration of formulated mushroom AgNp based ointment, the wound tissues contained keratinocytes, which were clearly differentiated from the epidermal layer and accumulated in the basal lamina of epidermis. Collagen fibres were densely packed and arranged parallel. There was a greater accumulation of the collagen fibres in the extracellular matrix region with prominent thick bundles of collagen fibres embedded in the proliferating fibroblasts when compared to normal rat skin, whereas the control (Group II) showed a prominent fibrinoid necrosis in the sub-epidermal region, the collagen fibres were loosely packed with an irregular arrangement, and there were undifferentiated keratinocytes under the basal lamina layer.

According Rang *et al.*, (2013), after 16 days of AgNPs treatment, the skin morphology was quite normal, exhibiting an adequate thickness of epidermal layer and collagen fibrils. A high collagen deposition was observed along the granulation area in the AgNPs treated mice. Control animals did not exhibit complete skin tissue when compared to other groups. Granulation tissue was not observed in the untreated animals.

In the present study, the burn wound of rats treated with AgNPs of different individual species of *Pleurotus*, fusants and commercial ointment at the end of 15th day revealed that the animals treated with 1mg/kg b. w and commercial ointment 0.17mg/kg b.w showed better skin layers of uniform architecture of epidermis and densely parallel packed collagen fibres to that of the control group. The control group showed less collagen deposition and the collagen fibers were found disorganized. Further, the skin organization had not been re-established whereas AgNPs and commercial ointment treated animals skin showed the collagen, which was newly synthesized by fibroblasts.

3.5 Estimation of tensile strength

A key parameter in healing involves regaining strength of the regenerated dermal matrix. Tensile strength reflects the quality and speed of tissue regeneration. Tensile strength is directly related to collagen content of wounds (Ziv-Polat *et al.*, 2010).

In this study, the strength of skin tissues was investigated by tensile testing. The wound of rats treated with phytosynthesized AgNPs at 1mg/kg b. w of different groups and commercial ointments at 100 µg/kg b.w showed the tensile strength of healed wound (Table 4).

Hence, it was suggested that the AgNPs of dried mushrooms of individual and fused strains of *Pleurotus* species could modulated the collagen alignment and improved the ability in wound healing. The above investigation revealed the potential of phytosynthesized AgNPs to restore well-stratified epidermis, fibroblasts, collagen and tensile strength of burn wounds.

Table 1: Physical properties of the Gel Formulations

Drugs	Spreadability	Viscosity	pH	Drug Content
Control (Vasline)	52 ± 0.27	67291 ± 1360	7.2	99.2 ± 1.37
Pleurotus eryngii	58 ± 0.63	98984 ± 2328	7.1	98.2 ± 0.92
Pleurotus florida	79 ± 0.72	156281 ± 3562	7	99.1 ± 2.11
Pleurotus ostreatus	63 ± 0.84	106820 ± 4283	7.2	99.3 ±1.82
Pleurotus djamor var. *roseus*	54 ± 0.82	85699 ± 1572	7.1	97.4 ± 0.83
FST 1	80 ± 0.62	172942 ± 1653	7	98.82 ± 1.02
FST 2	81 ± 0.54	185365 ± 2743	6.9	99.1 ± 2.11
FST 3	79 ± 0.72	161650 ± 2157	7	99.2 ± 1.37

Table 2: Effect of different concentration of AgNPs on body weight of Wister albino rats

Group	Body weight (g)	
	Initial (0 days)	Final (15th days)
Group 1 Dexamethasone	169.33 ± 2.08	188.20 ± 1.08
Group 2 Control (Vaseline)	169.33 ± 2.88	185.33 ± 3.51
Group 3 *P. eryngii*	170.66 ± 2.50	189.10 ± 1.08
Group 4 *P. florida*	170.00 ± 2.00	190.10 ± 1.08
Group 5 *P. ostreatus*	169.00 ± 3.00	191.33 ± 0.57
Group 6 *P. djamor* var. *roseus*	170.23 ± 4.72	191.00 ± 1.08
Group 7 FST 1	170.46 ± 0.57	191.46 ± 0.70
Group 8 FST 2	169.43±2.32	186.98±1.93
Group 9 FST 3	169.12±1.04	191.43±0.54

Table 3: Effect of AgNPs on excision wound parameters

Drugs	Treatment (mg/kg)	Percentage of wound contraction				Period of Epithelialization (days)
		3rd day	7th day	11th day	15th day	
Group 1 Dexamethasone	0.17	23.4 ± 3.32	39.57 ± 3.58	55.85 ± 2.39	68.67 ± 1.28	17.75 ± 0.45
Group 2 Control (Vaseline)	0.17	10.75 ± 4.38	27.15 ± 5.25	39.45 ± 2.77	43.02 ± 1.12	21.25 ± 0.75
Group 3 *P. eryngii*	0.17 + 1.0	20.69 ± 3.52	49.69 ± 3.55	58.82 ± 2.24	75.49 ± 2.03	18.75 ± 0.77
Group 4 *P. florida*	0.17 + 1.0	28.28± 3.34	53.57 ± 3.58	61.86 ± 2.71	76.22 ± 2.05	18.37 ± 0.75
Group 5 *P. ostreatus*	0.17 + 1.0	35.25 ± 3.31	58.28 ± 2.75	67.81 ± 2.65	78.28 ± 2.90	19.48 ± 0.42
Group 6 *P. djamor* var. *roseus*	0.17 + 1.0	18.98 ± 3.36	37.54 ± 4.98	51.84 ± 2.35	72.32 ± 2.18	16.50 ± 0.49
Group 7 FST 1	0.17 + 1.0	53.87 ± 3.31	69.25 ± 7.35	78.83 ± 2.65	82.56 ± 2.22	17.51 ± 0.54
Group 8 FST 2	0.17 + 1.0	59.98 ± 3.35	72.25 ± 5.48	80.64 ± 3.43	88.92 ± 2.30	15.54 ± 0.78
Group 9 FST 3	0.17 + 1.0	46.99 ± 3.34	61.25 ± 2.55	73.70 ± 3.45	80.25 ± 2.32	17.57 ± 0.65

Table 4: Effect of AgNPs on Dead space and Incision wound parameters

Drugs	Treatment	Incision wound
		Tensile strength (N/cm^2)
Group 1 Dexamethasone	0.17	34.52 ± 7.2
Group 2 Control (Vaseline)	0.17	16.41 ± 7.32
Group 3 *P. eryngii*	0.17 + 1.0	32.04 ± 11.65
Group 4 *P. florida*	0.17 + 1.0	34.06 ± 7.52
Group 5 *P. ostreatus*	0.17 + 1.0	34.29 ± 8.13
Group 6 *P. djamor* var. *roseus*	0.17 + 1.0	34.07 ± 5.31
Group 7 FST 1	0.17 + 1.0	36.87 ± 6.52
Group 8 FST 2	0.17 + 1.0	37.16 ± 9.42
Group 9 FST 3	0.17 + 1.0	35.53 ± 5.20

Fig. 1: Physical appearance of formulated ointment

Fig. 2: Effect of AgNP's in Excision Wound healing

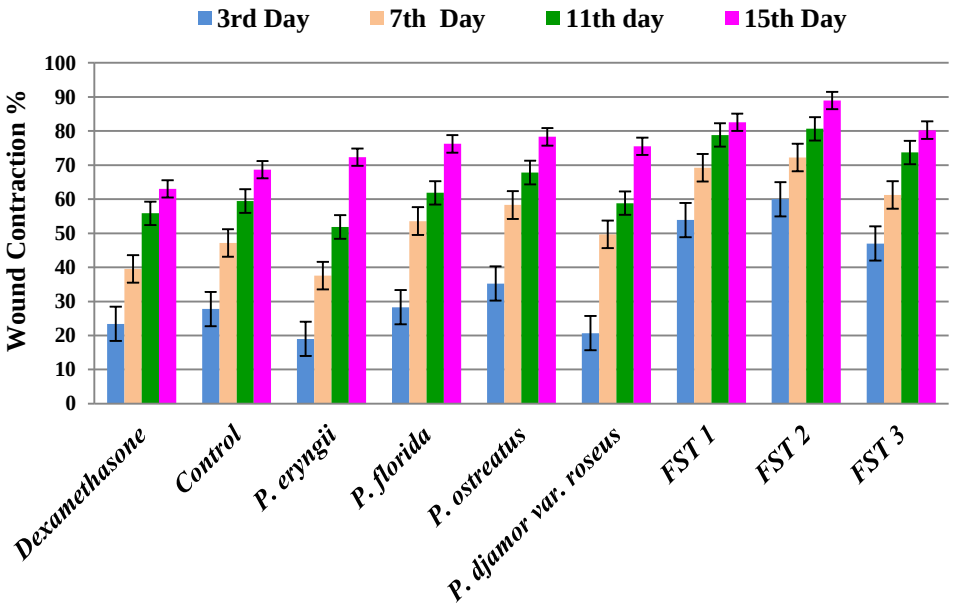


Fig. 3: Wound Healing Activity of AgNps with Standard and control

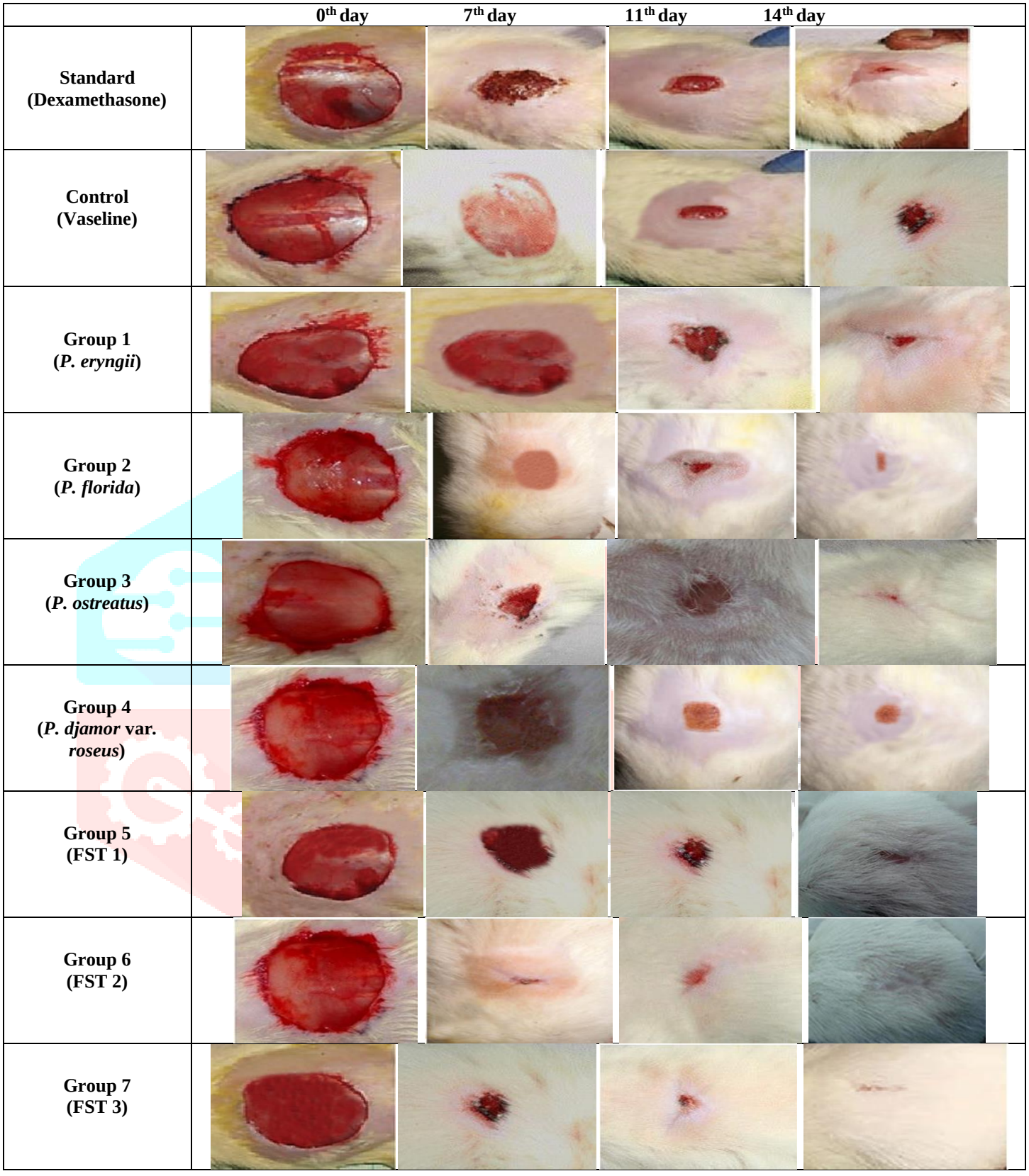
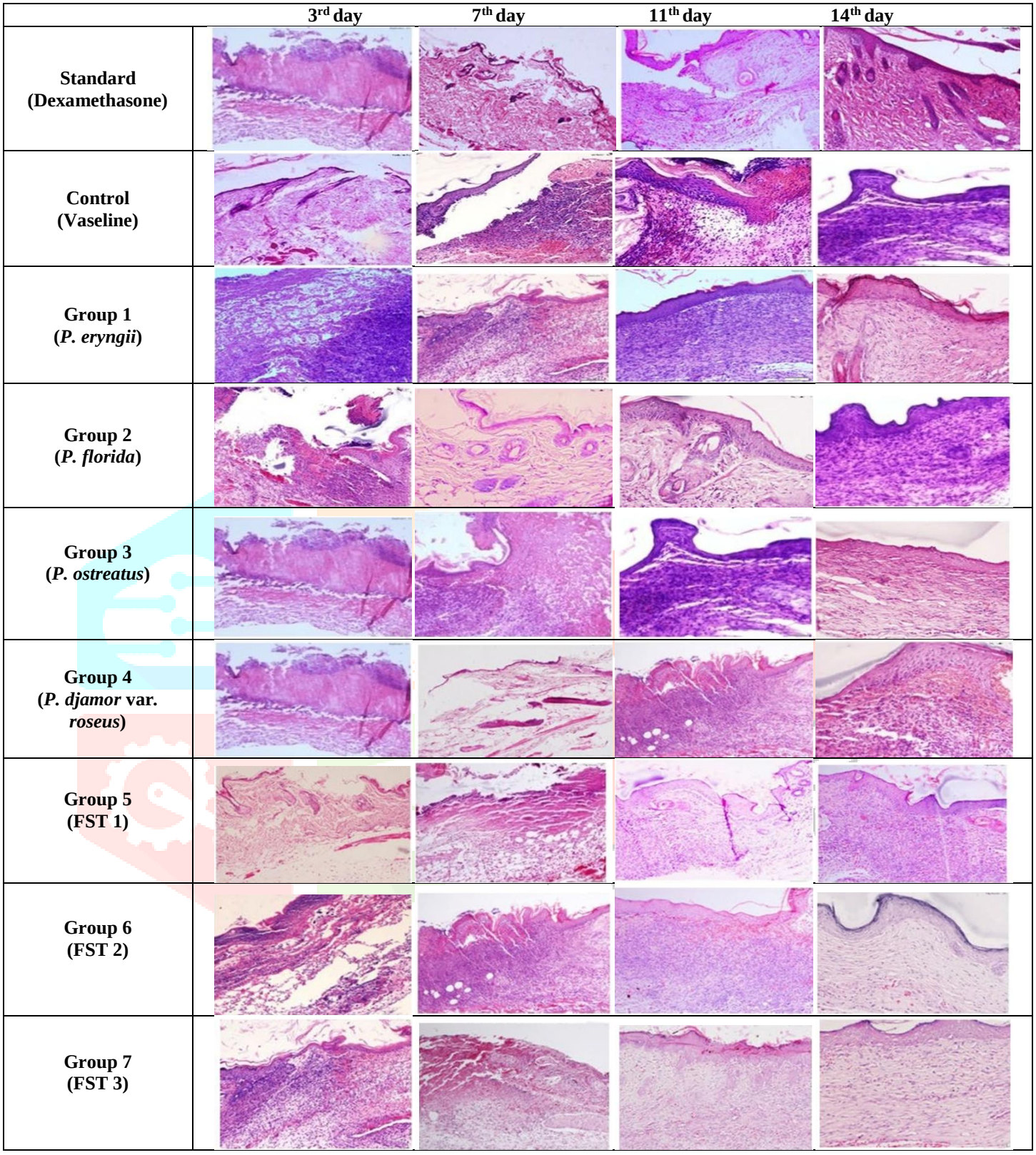


Fig. 4: Histopathological activity of AgNps with Standard and control



IV. SUMMARY

The present study was planned to develop an interspecific fusant between bigger, rich antioxidant, high nutritional value and especially longer storage shelf life species *P. eryngii* and fast growing and higher productivity with rich nutritional value possessing species *P. florida*, *P. ostreatus* and *P. djamor* var. *roseus* belonging to same generic group. Interestingly all the desirable characters from parent strains were inherit to the fused strains (FST 1, FST 2 and FST3). The somatic hybrids obtained through this study were used to serve as resource material for further studies that would give us insight about the basic genetics of fusants and in future it could be exploited as a commercial strain for cultivation throughout the year in tropical countries of the world.

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