



Fabrication, Characterization and Evaluation of *Glycosmis pentaphylla* gel Using Almond Gum for Wound Healing Activity

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Accepted on: 28-05-2016; Finalized on: 31-07-2016.

ABSTRACT

The present study aims to develop a suitable herbal formulation of the methanolic extract of *Glycosmis pentaphylla* for wound healing activity. The methanolic extract was obtained from the dried leaves of *Glycosmis pentaphylla* via solvent extraction method. Topical formulations were prepared by incorporating extract in a suitable almond gum gel base and evaluated for phytochemical investigation, pH, homogeneity, spreadability, *in vitro* antimicrobial activity, *in vivo* studies and stability studies. The results of phytochemical evaluation of *Glycosmis pentaphylla* herbal extract showed the presence of carbohydrate, flavonoids and tannins. The pH, viscosity and spreadability studies too demonstrated significant results. The microbiological studies indicated that optimized formulation possessed antimicrobial activity against tested organisms. Stability studies of the optimized formulation (GPM4) showed no significant changes over the study period. On the basis of the present study it was observed that the methanolic extract of *Glycosmis pentaphylla* at 15% concentrations exhibited significant wound healing activity than aqueous extract. From these results it can be concluded that the prepared herbal topical gel possesses a multifaceted approach in wound healing.

Keywords: *Glycosmis pentaphylla*; almond gum; methanolic extract; wound healing.

INTRODUCTION

Wound is a disruption of normal anatomic structure and function or it may be also defined as a break in the epithelial layer of the skin or loss and/or breaking of cellular and anatomical, functional continuity of living tissue. According to the Wound Healing Society, USA wounds are physical injuries that results in an opening or break of the skin that cause disturbance in the normal skin anatomy and function. This results in the loss of continuity of epithelium with or without the loss of underlying connective tissue. The wound may be of different types ranging from mild to severe to potentially fatal. It may be acute or chronic based on the nature of repair. Based on the nature of the wound and repair process it can be classified as chronic wounds and acute wounds.¹ Acute wounds are tissue injuries that heal within 8-12 weeks. The primary causes of acute wounds are mechanical injuries (friction contact between skin and hard surface), burns and chemical injuries.²

Chronic wounds heal slowly and leave serious scars. Wound healing process involve several steps including coagulation, inflammation, formation of granulation tissue, matrix formation, remodelling of connective tissue, collagenization, and acquisition of wound strength.^{3,4}

Topical formulations like creams, ointments, pastes, etc having a disadvantage like greasiness, need to apply with rubbing, and possess stability problems. Gels are getting more popularity now a day because they are more stable and can provide controlled release than other semisolid

preparations. The gel formulation can provide better absorption characteristics and hence the bioavailability of drug. It also provides better information regarding formulation and evaluation parameters of the novel herbal gel for wound healing activity and providing the better therapeutic effects to patient compliance. Gels provide a fast drug release compared to ointments and cream, provides a cooling effect on wound, minimise pressure required to apply gel for dermatological use.⁵ Gels have several properties such as thixotropic, greaseless, easy spreadability, easy removed, non-stickiness, non-irritant and are compatible with several excipients.⁶ Gels are water- or alcohol-based substances thickened without oil or fat. The skin does not absorb gels it absorbs preparations containing oil or fat. Thus, they are often most effective for conditions that require slow absorption, such as acne, rosacea, and psoriasis of the scalp.⁷

Glycosmis pentaphylla (Rutaceae) is extensively used in ayurveda for variety of condition. The genus *Glycosmis* of the family Rutaceae is represented by nearly 11 species. *Glycosmis pentaphylla* is a shrub or small (1.5-5 m) tree widely distributed from India, Malaysia, Southern China where it occurs in tropical forests at low altitudes. It is traditionally used for the treatment of fever, liver, complaints and certain other diseases.⁸ Juice of the leaves is used in fever and liver complaints and as vermifuge, leaves are considered as a good antidote for eczema and other skin problems and applied in the form of paste. Though the plant has potential traditional uses or the treatment of various diseases, there is no scientific study to validate their folkloric uses.⁹ The present study focuses



on the wound healing activity of *Glycosmis pentaphylla* in wistar rats using methanolic extracts.

MATERIALS AND METHODS

Plant Materials

Plants of *Glycosmis pentaphylla* were collected from Wayanad district Kerala, India during the month of May, in a quantity sufficient for all the experiments in a single batch and the plant materials were authenticated and identified by the botanist Dr. N. M. Naganandini from the Dept. of Pharmacognosy, JSS College of Pharmacy, Mysuru, India.

Chemicals Used

Propylene glycol, methyl paraben and propyl paraben were purchased from Merck Specialist Pvt. Ltd., Mumbai, India. Badam gum was procured from Lobachemie Pvt. Ltd., Mumbai. All other chemicals used were of analytical grade and obtained commercially.

Preparation of Crude Extract

The plant parts were washed under running tap water, and the leaves were cut into small pieces of 2-3 cm and shade dried (30 °C, 50±5% RH) for 15 days. The shade dried leaves were powdered using a dry grinder to get the coarse powder (sieve no. 22/8). The coarse powder of the *Glycosmis pentaphylla* leaves was subjected to solvent extraction process. 480 g of dried leaves powder was taken with the solvent in the ratio of 1:2. The temperature of the water bath was maintained at 40-45 °C and the extraction was carried out by successive

method, initially with chloroform, followed with methanol. The powder solvent mixture was heated on a water bath for about 8 hours, filtered and the remaining solvent from filtrate was removed under reduced pressure, thus formed a semisolid, which was then subjected for rotary flash evaporator apparatus (50-60 rpm), to get a solid residue. This residue was the Methanol Extract of *Glycosmis pentaphylla* (MEGP). The dried extract thus obtained was used for the formulation.

Preliminary Phytochemical Screening

The dried methanolic extract was analyzed for various phytoconstituents like alkaloids, proteins, steroids, saponins, flavonoids, phenolic compounds and tannins, and gums and mucilages.¹⁰

Phytochemical Investigation

The crude extract was also subjected to determination of ash value, total ash, water soluble ash, acid insoluble ash and residual moisture content in terms of loss on drying (LOD) method.¹¹⁻¹⁴

Formulation of Gel

Gels were prepared by dispersing the polymer in a mixture of water and glycerol with methyl paraben as the preservative. The aqueous and methanolic extracts were incorporated into it. The dispersion was then neutralized and made viscous by the addition of triethanolamine.^{15,16} The composition of gel formulations are given in **Table 1**.

Table 1: Gel Formulation Chart for Aqueous and Methanolic Extracts

Formulations	Aqueous/methanolic extract (g)	% badam gum	Glycerol (g)	Propyl paraben (g)	Methyl paraben (g)
GPA1	1.5	2.5	6.15	0.20	0.25
GPA2	5	2.5	6.15	0.20	0.25
GPA3	10	2.5	6.15	0.20	0.25
GPA4	15	2.5	6.15	0.20	0.25
GPM1	1.5	2.5	6.15	0.20	0.25
GPM2	5	2.5	6.15	0.20	0.25
GPM3	10	2.5	6.15	0.20	0.25
GPM4	15	2.5	6.15	0.20	0.25

Table 2: Results Obtained for Phytochemical Investigation

Chemical Constituents	Aqueous Extract	Methanolic Extract
Amino acids	–	–
Carbohydrates	+	+
Flavonoids	+	+
Glycosides	–	–
Proteins	–	–
Saponins	–	–
Sterols	–	–
Tannins	+	+

+: present; –: absent



Table 3: Appearance, pH, homogeneity, viscosity and spreadability of formulations

Formulation code	Appearance	pH*	Homogeneity	Viscosity (cps)*	Spreadability (g.cm/sec)*
Formulations containing aqueous extract of <i>Glycosmis pentaphylla</i>					
GPA1	Brown	6.5 ± 0.23	Good	11161.2 ± 2.39	1.2 ± 0.0716
GPA2	Brown	6.7 ± 0.22	Good	11216.1 ± 2.77	1.2 ± 0.0643
GPA3	Brown	6.7 ± 0.21	Good	12312.2 ± 0.93	1.3 ± 0.0722
GPA4	Brown	6.8 ± 0.24	Good	13002.3 ± 2.28	1.3 ± 0.0739
Formulations containing methanolic extract of <i>Glycosmis pentaphylla</i>					
GPM1	Green	6.6 ± 0.23	Good	13006.1 ± 1.64	1.2 ± 0.0756
GPM2	Green	6.7 ± 0.24	Good	13016.1 ± 1.43	1.2 ± 0.0816
GPM3	Green	6.6 ± 0.21	Good	14000.2 ± 1.25	1.3 ± 0.0783
GPM4	Green	6.8 ± 0.23	Good	14412.3 ± 1.62	1.3 ± 0.0801

*Mean ± S.D., n = 3

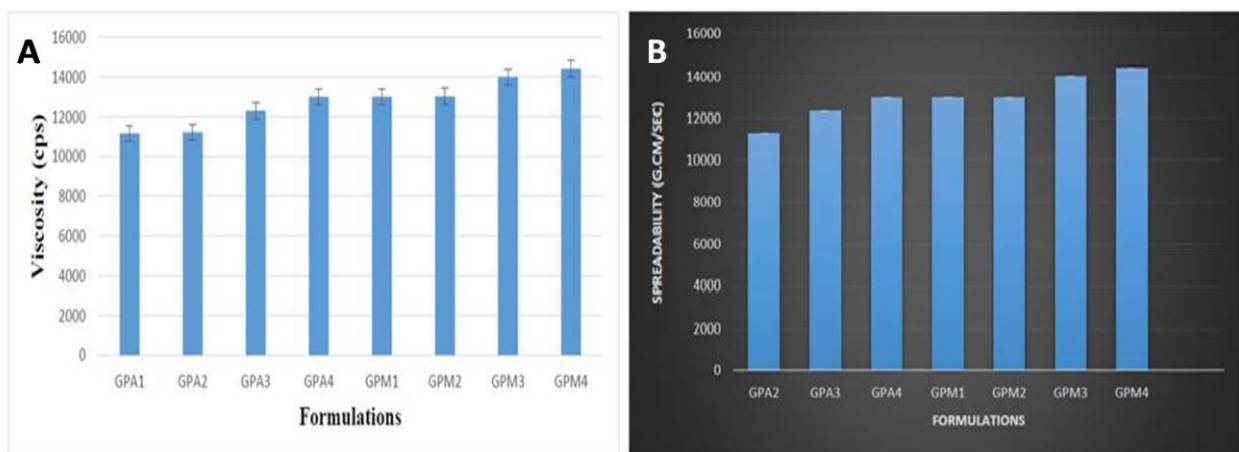
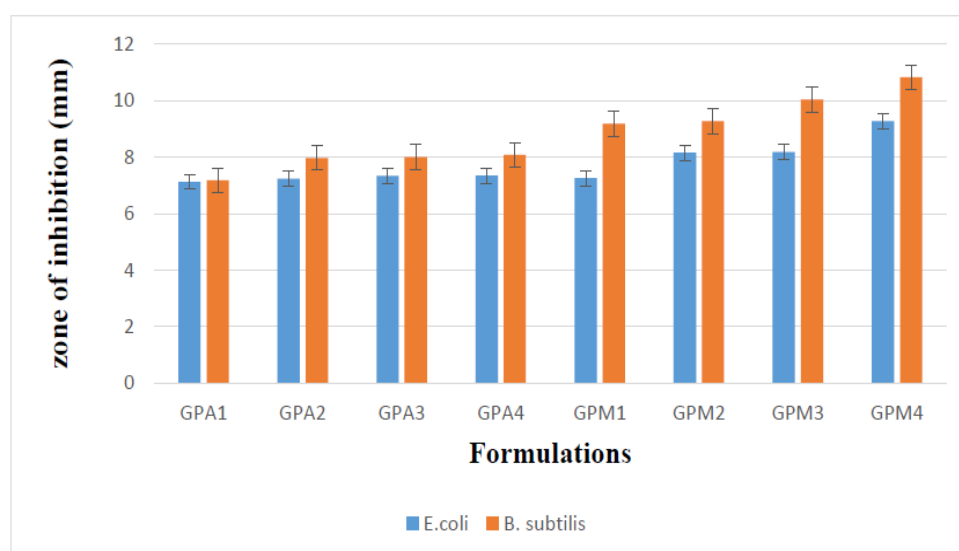
**Figure 1:** (A) Graphical representation of viscosity for the herbal formulations and (B) spreadability of the herbal formulations.**Figure 2:** Graphical Representation of Anti-microbial Activity

Table 4: Observations for Wound Contraction Study

Treatment	Day 1	Day 5		Day 10		Day 15	
	Wound (cm)	Wound (cm)	% wound contraction*	Wound (cm)	% wound contraction*	Wound (cm)	% wound contraction*
Control	1.5±0.62	1.14±0.12	24.12±1.87	0.85±0.09	43.35±1.63	0.44±0.05	71.13±1.68
GPM4	1.5±0.73	0.78±0.48	48.21±1.63	0.33±0.31	77.68±1.74	0.05±0.16	96.89±1.26
Marketed	1.5±0.49	0.99±0.11	33.76±2.56	0.58±0.11	61.41±1.3	0.26±0.03	82.34±1.17

*Mean±S.D., n = 6

Table 5: Stability Studies Data Optimized Gel Formulation

Stability Condition	Sampling (in days)	Physical Appearance	pH*	Report
30C/60% RH	0	No change	6.6 ± 0.23	Complies
	15	No change	6.7 ± 0.22	Complies
	30	No change	6.7 ± 0.20	Complies
	60	No change	6.8 ± 0.24	Complies
	90	No change	6.6 ± 0.25	Complies
40C/75% RH	0	No change	6.6 ± 0.28	Complies
	15	No change	6.7 ± 0.27	Complies
	30	No change	6.6 ± 0.23	Complies
	60	No change	6.8 ± 0.24	Complies
	90	No change	6.6 ± 0.22	Complies

*Mean±S.D., n=3

**Figure 3:** (A) Excision wound model for optimized herbal formulation and (B) Histology regeneration tissue of open wounds as on 16th day at 40X.

Evaluation of the Formulation

Organoleptic Parameters

Organoleptic parameters like colour, odour and texture of all the eight formulations were assessed.

pH Measurement

The pH was measured using a pH meter, which was calibrated before each use with standard buffer solutions at pH 4, 7, 9.

The electrode was inserted into the sample 10 min prior to taking the reading at room temperature.¹⁷

Homogeneity

All the developed gels were tested for their appearance and homogeneity by visual inspection.¹⁸

Viscosity

The viscosity of the formulations was checked using a Brookfield Viscometer (DVI PRIME, USA). The gels were rotated at 20 and 30 rpm using spindle no.64.

The viscosity of the gel was obtained by multiplying the corresponding dial reading with the factor given in the Brookfield Viscometer catalog.¹⁹

Spreadability

Spreadability is expressed in terms of time in seconds taken by two slides to slip off from the gel when placed in between the slides under the direction of a certain load.

The excess amount of sample was placed between the two glass slides and a definite amount of weight was placed on these glass slides to compress the glass slides of uniform thickness.²⁰

A fixed weight was added and the time required to separate the two slides was noted.

Spreadability was calculated using the formula

$$S = (M.L/T)$$

Where, S = Spreadability, M = Weight tied to upper slide, L = Length of glass slides and T = Time taken to separate the slides.

Antimicrobial Evaluation

In vitro Antibacterial Activity

Escherichia coli and *Bacillus subtilis* are the commonly occurring gram negative and gram positive bacteria causing skin infection, hence, were selected for the study.

Test Organism

Clinical microbial extracts of *E. coli* and *B. subtilis* were used as antibacterial agents.

Antibacterial Activity

In vitro antibacterial activity was evaluated using the agar well diffusion technique. Nutrient agar was used as the medium.²¹ The sterile agar was inoculated with the bacteria culture (*E. coli*, *B. subtilis* for 48 hours, at 37°C. Wells were bored by using a sterile borer, and standard formulations (1000 µg / ml was prepared by dissolving the test sample in DMSO) were placed into them. Plates were kept for two hours in the refrigerator to enable pre diffusion of the extracts into the agar. Next, the plates were incubated overnight (24 hours) at 37°C. The spectrum of activities of the extracts were compared with the marketed product *Aloe vera* gel.²²⁻²⁶

In vivo Studies

The study was conducted after obtaining approval from the Institutional Animal Ethical Committee of JSS College of Pharmacy, Mysuru. Either sex (male and female) of Albino wistar rats ($n = 18$) weighing between 200-250 g were used in the study.

The animals were kept in well-spaced, ventilated cages and maintained on a normal diet. The animals were divided into three groups: Group A-Control (topically treated with the gel base I.P. as a placebo control), Group B-Test (topically treated with 15% gel of the various extract of *Glycosmis pentaphylla* formulated in gel base I.P) and Group C-Marketed (topically treated with marketed *Aloe vera* gel). All animals were periodically weighed before and after the experiment. The rats were

anaesthetized by intraperitoneal injection of ketamine at a dose of 40 mg/kg body weight, prior to and during creation of the experimental wounds. Skin of the animals was shaved and disinfected using 70% (v/v) ethanol, and excision wounds of 1 cm x 1 cm were created using a punch-biopsy needle with a depth of about 1 mm on the dorsal aspect of the thoracolumbar region of the rats.

Till complete epithelisation, the wound closure rate was assessed by tracing the wound on post-wounding day 0, 5, 10 and 15 using transparency paper and a permanent marker. The wound areas recorded were measured using a graph paper. Number of days required for falling of eschar without any residual raw wound was noted as the period of epithelisation.^{27,28}

Measurement of Wound Area

The dynamic changes in wound area were monitored by a camera on foreordained days i.e., 0, 5, 10 and 15. Later on, wound area was measured by tracing the wound on a millimeter scale graph paper.

Measurement of Wound Contraction

Wound contraction, which also contributes to wound closure and restoration of the functional barrier.

This is studied on alternate days from the day of infliction of wound till the day of complete epithelisation by tracing the raw wound on a transparent sheet.

Wound contraction was calculated as percentage of the reduction in original wound area size.

$$\% \text{ wound contraction} = (A_i - A_f) / A_i * 100$$

Where, A_i = wound area on day zero, A_f = wound area on day of complete epithelisation

Period of Epithelisation

Falling of scab deserting no raw wound behind was taken as end point of complete epithelisation and the days required for this was taken as period of epithelisation.

Measurement of Wound Index

Wound index was measured daily with an arbitrary scoring system from 1 to 4 where "0" for complete healing, "1" for incomplete but healthy healing, "2" for delayed but healthy healing, "3" for healing has not yet been started but environment is healthy and "4" for formation of pus evidence of necrosis.

Histopathological Studies

The regenerated tissue previously collected and preserved in 10% buffered formalin was used for histopathological studies.

The tissue was removed from buffered formalin after a day or two, dehydrated in ascending grades of alcohol, cleared in chloroform, embedded in paraffin in tissue processor (Shandon, Citadel 1000) and were fine cut with rotary microton (Sipcon, India) getting sections of 3 to 5 µm in thickness. The sections were dewaxed by xylene



and later the xylene was removed by descending grades of alcohol to facilitate staining procedure.

Histopathological studies were carried out using light microscopy by taking tissue sections (4 mm) stained with haematoxylin and eosin (HE), and changes in the regenerated tissue which took place during the wound contraction or healing phase in both the test and control were observed for epithelisation, fibroblasts, collagen, cell infiltration (inflammation) and neo-vascularisation.

These parameters were qualitatively assessed and microphotographed under "40X" magnification.^{29,30}

Statistical Analysis

All the results obtained from various activities, as described above, were analysed statistically by using Student's t test and $p < 0.05$ were considered significant.

Stability Studies

Optimized gel formulation was selected for stability studies.

The formulation was packed in a screw capped bottle and short-term stability studies were carried out for a period of 3 months at $30 \pm 2^\circ\text{C}/65 \pm 5\%$ RH and $40 \pm 2^\circ\text{C}/75 \pm 5\%$ RH.

Samples were withdrawn on 0, 15th, 30th, 60th and 90th day and were analysed for change in visual appearance and pH.^{31,32}

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The preliminary phytochemical screening was done to establish a chemical profile of a crude drug.

The two extracts were screened for phytochemical contents by different phytochemical tests to check the presence of carbohydrates, tannins, flavonoids etc. and the results obtained for the same are mentioned in **Table 2**.

The methanolic solution (70%) was used in the present study for extraction.

After filtration, the methanolic extract had been subjected to evaporation so as to get alcohol free extract to avoid the harmful effect of methanol on liver.

Another rationale behind selecting methanolic extract was that it has been reported as the best solvent to separate/extract most of the active constituents from herbal source.

The extract obtained in the study was pale brown, oily, and sticky in nature.

Phytochemical Investigation

The phytochemical investigation was performed on crude drug to determine the diverse physicochemical constants. The dried leaves of *Glycosmis pentaphylla* showed higher percentage of loss on drying (16%) and total ash (4.5%

w/w). Water-insoluble ash and acid-insoluble ash was found to be only 1.75% w/w. All these values were found to be well within the Herbal Pharmacopeial limits.

Evaluation of the Formulation

Organoleptic Parameters

The prepared gels were inspected visually for clarity, colour and odour. All the prepared gels were appeared to be translucent. The aqueous extract containing gel showed pale brown colour (because of the absence of chlorophyll) whereas the methanolic extract containing gel showed pale green colour (due to presence of chlorophyll as it is soluble in methanol).

pH Measurement

The pH of all the formulations were recorded in the range of 6.5 ± 0.23 to 6.8 ± 0.23 (**Table 3**) enduring them most acceptable to avoid the risk of irritation upon application on to the wounds.

Homogeneity

All prepared gels were set in the containers, and tested for homogeneity and presence of any aggregates by visual inspection. All the formulations were observed to be uniform in consistency without any particles, lumps and aggregates.

Viscosity

The viscosity of all the herbal extract formulations was in the range 11161.2 ± 2.39 to 14412.34 ± 1.62 cps (**Table 3**). Results showed that, viscosity was a direct function of concentration and temperature dependant slight variation in the viscosity was also observed as depicted in **Figure 1A**.

Spreadability

All the prepared gel formulations containing different ratios of the herbal extract were easily spreadable on the wound surface. The gels resembled to semi-stiff gels in their consistency. The results of the spreadability varied from 1.2 ± 0.0643 to 1.3 ± 0.0816 g.cm/sec (**Table 3**). The results indicated that spreadability had been increased with the increase in the herbal extract concentration as depicted in **Figure 1B**.

Antimicrobial Evaluation

In vitro anti-microbial activity of all the prepared herbal formulations were assessed using *E. coli*, *B. subtilis* and the results obtained for the same are depicted in **Figure 2**. The figure represents that the formulations had antibacterial activity against both gram negative and gram positive bacteria. The results were encouraging with zone of inhibition ranging from 7.12 mm to 9.27 mm. The highest zone of inhibition was shown by GPM4 formulation having highest concentration of the methanolic extract of *Glycosmis pentaphylla*.

The results from this study justifies that the antimicrobial activity was dependent on the dose/concentration of test



material and as the concentration increased the zone of inhibition has increased (**Figure 2**). The incorporation of herbal extracts in gels helps in keeping the wound area sterile, in addition to the wound healing action. This fact does support a faster wound healing as compared to the conventional formulations.

In vivo Studies

Wound Contraction Studies

Taking into consideration the results of pH, viscosity, spreadability, antimicrobial studies etc., it was found that GPM4 exhibited promising results than all other formulation batches. Hence due to the superior results of GPM4 and animal constrain only GPM4 has been taken for *in vivo* studies.

The results of wound contraction studies in rats are reported in **Table 4**. The wound area was observed and measured at different time intervals of 5, 10, and 15 days post incision.

Reduction in percentage wound area was calculated at 5th, 10th and 15th days there with herbal extract of GP compared to the wounds treated with standard (marketed products), gel without drug and control (**Figure 3A**).

The results indicated that the percentage wound reduction rate was higher in the order of GPM4 (96.89 ± 1.26) > Aloe Vera gel (82.34 ± 1.17) > control (71.13 ± 1.68).

Period of Epithelialization

The period of epithelialization for the control, treated and treated with marketed formulation groups were noted to be 24.12 ± 2.02 , 12.16 ± 1.57 and 29.05 ± 0.12 . This period of epithelialization was found to be higher in the marketed product (Aloe Vera gel) as compared to the GPM4.

Wound Index

Wound index was measured daily with an arbitrary scoring system i.e. "0" for complete healing, "1" for incomplete but healthy healing, "2" for delayed but healthy healing, "3" for healing has not yet been started but environment is healthy, "4" for formation of pus evidence of necrosis. The wound index obtained for the study were 2.28 ± 0.05 , 1.18 ± 0.04 and 1.72 ± 0.02 for control group, GPM4 and aloe vera gel (marketed product), respectively.

Histopathological Studies

The results of the histopathological studies are shown in **Figure 3B**. Microscopic examination of the sections prepared from wounds of control, treated group as well as that from the normal tissue as on the 16th day indicated that all parameters that altered the wound healing viz., epithelialization, inflammation (cell infiltration), fibroblasts, collagen deposition and neovascularisation were all at an advanced stage of healing in the both treated groups when compared to the

control groups. The process of healing was almost completed by 16th day in the treated groups when compared to control.

The increase in the value of inflammation indicates that the fibroblasts have been directly accelerated to synthesize collagen.

The values of all the above parameters were found to be slightly higher in normal tissue as compared to both the treated groups.

This fact may be due to various active constituents present in the formulation that affect various phases of wound healing.

The same reason also holds good for the fact that parameters like inflammation, fibroblast, collagen and neo-vascularisation were higher in GPM4 formulation treated group when compared to gel with aqueous extract, whereas the other parameter viz., epithelisation was higher in treated group of GPM4 formulation.

These results hence justify that the process of wound healing was quicker in the treated groups as compared to the control group although some time interval will always be taken for the treated tissues to normalize.

Stability Studies

There was no marked change in the physical appearance of the GPM4 after 90 days of stability study (**Table 5**).

The pH of the gel was in the range of 6.6 ± 0.22 to 6.8 ± 0.24 during the period of study.

The drug was stable in gel base and also no significant change in physical appearance has been observed.

CONCLUSION

Glycosmis pentaphylla methanolic extract contains several active constituents. Wound healing activity of *Glycosmis pentaphylla* was investigated by formulating herbal gels using its methanolic extract.

To assess the efficacy of *Glycosmis pentaphylla* in wound healing, *in vivo* studies were performed on albino wistar rats.

Wound healing activity was evaluated, in which GPM4 was observed to have a shorter period of epithelization and greater rate of wound contraction.

It has been noted that wound contraction i.e. the process of wound area shrinkage was significantly greater in case of optimized formulation (GPM4) than that of marketed product (Aloe Vera gel).

Moreover, the wound closure time was lesser, as well as the percentage of wound contraction was much more with the 15% w/w methanolic leaf extract gel.

Thus, it was concluded that the *Glycosmis pentaphylla* methanolic extract based gel formulated using almond gum has promising wound healing action, when applied topically.



Acknowledgement: The authors express their gratitude toward the JSS University and JSS College of Pharmacy for providing necessary facilities and support in due course of the work.

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Source of Support: Nil, Conflict of Interest: None.

