ORIGINAL ARTICLE

Formulation and evaluation of herbal gel from tannin-enriched fraction of *Psidium guajava* Linn. leaves for diabetic wound healing

S. Jaya Kumari, M. Sangeetha, Sajjad Ali

Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels Institute of Science Technology and Advanced Studies, Chennai, Tamil Nadu, India

Abstract

Aim: The ethno botanical studies and folklore claiming showed that the leaves of Psidium guajava Linnare used for the treatment of wounds. From the literature review, we found that tannins have significant wound healing activity by promoting the cicatrisation of wounds and also have antimicrobial property which will speed up the wound healing process. As the wound healing process is delayed in the diabetic condition, the present work is focused on the evaluation of gel formulated at different concentrations from tannin-enriched fraction of *Psidium guajava* leaves for diabetic wound healing. **Materials and Methods:** The tannin-enriched fraction was isolated from *P. guajava* leaves and phytochemically evaluated by qualitative chemical test, thin-layer chromatography, and high-performance thin-layer chromatography. The total phenolic content and tannin content were estimated by colorimetric technique (Folin–Ciocalteu and Folin-Denis method). The gel was formulated and evaluated for various physiochemical properties and diabetic excision wound healing activity using male albino Wistar rats. **Results and Discussion:** The statistical analysis showed that there is a significant difference between diabetic control group and the group of animals treated with gel formulation (5% and 10%). The histopathological evaluation showed that the 10% gel applied a group of animals has a significant effect on diabetic wound healing activity. **Conclusion:** It is concluded that the gel of tannin-enriched fraction of *P. guajava* Linn. showed significant wound contraction at the tested concentration in diabetic condition due to the presence of gallic acid.

Key words: Diabetes, excision, fraction gel, *Psidium guajava* Linn., tannin, wound healing

INTRODUCTION

The use of plants as a source of medicine has been an ancient practice and is an important component of the health-care system in India. Traditional use of medicine is recognized as a way to learn about potential future medicines. Researchers have identified a number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources.[1] *Psidium guajava* L. known as guava is a medicinal plant belonging to the family Myrtaceae. It is a well-known traditional medicinal plant used in various indigenous systems of medicine. It is widely distributed throughout India.[2] The leaves and bark of *P. guajava* tree have evergreen medicinal uses, which are still employed today. It is found to contain antibacterial, wound healing property which was mainly due to its phytoconstituents, namely, tannin. With the above reviews, our research work is focused to isolate tannin-enriched fraction from the leaves of *P. guajava* Linn, which is then formulated into gel and evaluated for diabetic excision wound healing activity.

MATERIALS AND METHODS

Collection and Authentication

The plant specimen (leaves) for the proposed study was collected during December 2015 from Pallavaram, Chennai. It was identified and authenticated by Dr. P. Jayaraman, Director

Address for correspondence:
Dr. S. Jayakumari, Department of Pharmacognosy, Vels Institute of Science Technology and Advanced Studies, VELS University, Chennai, India.
Phone: +91-9840282617.
E-mail: nisajayaa@gmail.com

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of Plant Anatomy Research Center (PARC), Tambaram, Chennai. A voucher specimen No. PARC/2016/3217 has been deposited for further reference.

Isolation of Tannin-Enriched Fraction

The leaves of *P. guajava* Linn. were shade dried and coarsely powdered. About 200 g of powder was defatted with petroleum ether. The defatted leaf powder was extracted with acetone (70% v/v) by cold maceration method, and this fraction was evaporated in a rotary evaporator under reduced pressure, freeze-dried, and used for the study.[3] The leaf powder and tannin-enriched fraction were subjected to qualitative phytochemical test.[4] The observations are tabulated in Table 1. Total phenolic content was estimated by Folin–Ciocalteu method.[5] The test samples were performed in triplicates. Blank solution was prepared by adding all the solutions except gallic acid. After incubation, the absorbance was measured at 750 nm spectrophotometer using UV visible Jasco *V-630* instrument.[6,7] The percentage of total phenolic content is tabulated in Table 2. Tannin content was estimated by Folin denis method.[8,9] It was estimated for *P. guajava* leaf powder, tannin-enriched fraction, and gel formulations (5% and 10%).

Fresh solution of standard tannic acid with different concentrations (20, 40, 60, 80, and 100 µg/ml) and 20% sodium carbonate was prepared. The test samples were performed in triplicates. Blank solution was prepared by adding all solutions except tannic acid. The absorbance was measured at 700 nm. The percentage of tannin content in respective components is tabulated in Table 2.

Thin-layer chromatography (TLC) was done in the mobile phase petroleum ether:ethyl acetate:formic acid (5:5:1). After development, plate was dried and visualized using UV chamber derivitization agent - 5% ferric chloride solution. TLC chromatogram is shown in Figures 1 and 2. High-performance thin-layer chromatography (HPTLC) was performed using stationary phase - Silica gel 60F 254 and mobile phase - petroleum ether:ethyl acetate:formic acid (5:5:1). The results are shown in Figures 3-5.

Formulation of Gel

Gels were formulated in two concentration, namely, 5% and 10%. Accurately weighed carbopol 940 was dispersed in sufficient quantity of water with gentle stirring. Then, glycerine was added to it and pH was adjusted between 6.8 and 7.4 by addition of the sufficient quantity of triethanolamine. The required quantity of tannin-enriched fraction was dissolved in small amount of water and added to the above solution with gentle stirring for uniform mixing. Methylparaben was dissolved in water and added to the

Table 1: Percentage of total phenolic content

Component	Concentration of solution (µg/ml)	Concentration of gallic acid (µg/ml)	Percentage of total phenolic content
Powder	10	1.22	12.2

Table 2: Percentage of tannin content

Components	Concentration of solution (µg/ml)	Concentration of tannic acid (µg/ml)	Percentage of tannin content
Powder	10	0.644	6.44
Tannin-enriched fraction	10	6.32	63.2
5% gel	10	5.901	59.01
10% gel	10	6.078	60.78

Table 3: Wound healing activity of the herbal gel of tannin-enriched fraction from *Psidium guajava* leaves in alloxan-induced diabetic rats

Groups	Wound area (mm²) (mean±SEM)			
	Day 0	Day 4	Day 8	Day 12
I (normal saline)	81.51±11.93	66.33±10.75	52.72±9.56	40.69±8.39
II (diabetic control)	92.50±12.90	76.27±11.72	68.74±11.13	61.62±10.54
III (standard) *Aloe vera* gel 90% w/w	76.27±11.72	48.94±9.36	25.38±4.46	10.72±12.83
IV (5% w/w tannin fraction gel)	76.27±11.72	48.54±9.36**	37.02±8.18***	18.70±5.80***
V (10% w/w tannin fraction gel)	110.29±14.08	61.62±10.54**	18.71±5.82***	6.67±3.47***

The values are shown as mean±SEM from 6 animals in each group. ** and *** show significant as compared to diabetic control (P<0.01 and P<0.001). SEM: Standard error of the mean

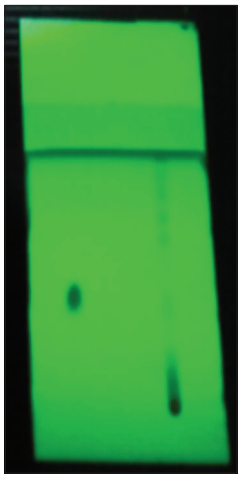

Figure 1: Before derivitization

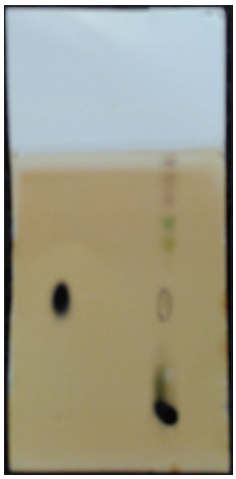

Figure 2: After derivitization

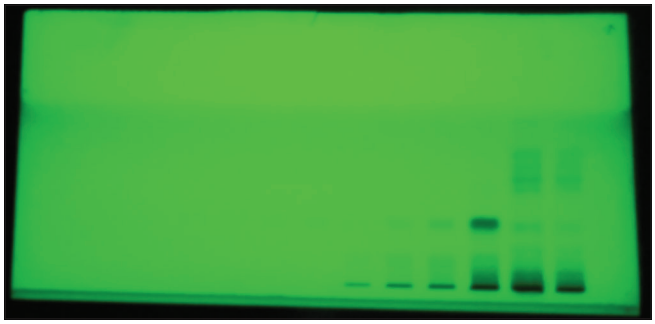

Figure 3: High-performance thin-layer chromatography fingerprint

above formulation. Finally, a sufficient amount of Tween 80 was added to the formulation.[10] The ingredients used for the gel formulation are tabulated in Table 3.

Blank gel was formulated with and without the tannin-enriched fraction. Physiochemical properties of gel physical appearance, pH, spreadability, viscosity, extrudability, and stability studies were evaluated by the ICH guidelines.[11-18] Stability studies of the formulated gels were performed at 40° ± 2°C/75% ± 5% RH for 3 months. After 90 days,

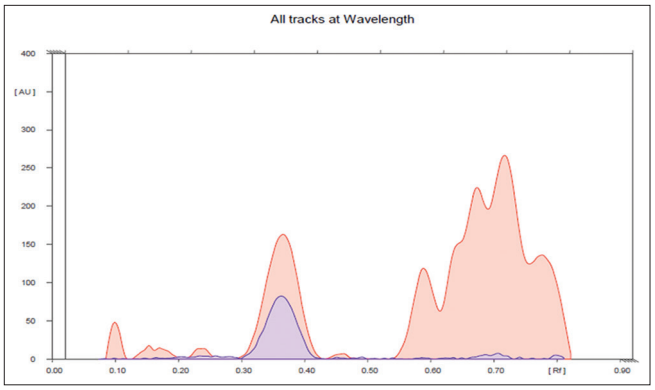

Figure 4: High-performance thin-layer chromatography UV-superimposed spectrum of tannin-enriched fraction with standard (gallic acid)

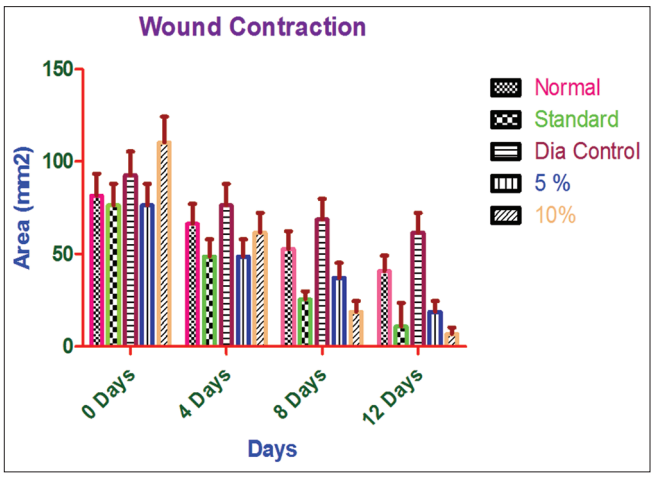

Figure 5: Wound contraction area (mm^2)

the evaluation parameters were studied again for the gel formulation. The evaluation results are tabulated in Table 4.

Evaluation of Gel for Diabetic Wound Healing

The animal studies were carried out with the institutional animal ethical committee clearance. The diabetic wound healing activity was performed on healthy male albino Wistar rats weighing 175–250 g. Animals were allowed to acclimatize for 1 week before the start of experiment.

Skin Irritation Test[19]

Hair on the dorsal side of Wistar albino rats was removed by clipping 1 day before experiment. The rats were alienated into four groups ($n = 6$). Group I served as the control (blank gel without drug), Groups II and III applied with gel 5% and gel 10%. Group IV applied with 0.8% v/v aqueous solution of formalin as a standard irritant.[20] The control formulations, gel formulation (5% and 10%), and formalin solution were applied daily for 7 days. Finally, the application sites were graded according to a visual scoring scale, always by the same investigator.

Table 4: Physiochemical properties of gel formulation

Properties	5% gel	10% gel
Physical appearance	Brownish transparent and homogenous	Brownish transparent and homogenous
pH	7.01	7.1
Spreadability	4.85 cm	3.95 cm
Viscosity	3004 cp	3250 cp
Extrudability	Good	Good
Stability	Stable	Stable

Induction of Diabetes[21]

A single dose of 84 mg/kg of alloxan monohydrate dissolved in sterile phosphate buffer saline was used for the induction of diabetes in an overnight fasted male albino Wistar rats through intraperitoneal injection. Phosphate-buffered saline was prepared freshly before administration. After 3 days, diabetes was confirmed by checking the fasting blood glucose level and only animals with fasting blood level 11–20 mmol/ml were considered diabetic used for the experimental study. The animals were allowed for the free access to drinking water and pellet diet and maintained at room temperature in clean iron cage.

Excision Wound Model[22]

All animals were given anesthesia before wound creation. Hairs were removed from the back and impression of 100 mm^2 was made. The skin of the impressed area was excised carefully. Animals were kept in separate cages with undressed wound. The day on which the wound was made considers as day 0 (zero). The normal group was left as such without any treatment. The diabetic control group was applied with blank gel, i.e., gel formulation without tannin fraction. The standard group was applied with marketed herbal gel (*Aloe vera* gel). The test I group was applied with 5% gel formulation. The test II group was applied with 10% gel formulation. The application was carried out daily for 12 days and area of wound contraction was measured on day 4, day 8, and day 12. The results are shown in Table 3.

Statistical Analysis

Results obtained from the animal model has been expressed as average mean ± standard deviation. The significant difference between the groups was analyzed using two-way ANOVA.

Histopathological Examination

A specimen sample of skin tissues of each group of rats was taken out from the healed wounds of the animals in the above excision wound model for histopathological examinations.

RESULTS

In the present study, tannin-enriched fraction was isolated from the leaves of *P. guajava* Linn. and the gel was formulated and evaluated for diabetic wound healing using male albino Wistar rats by excision wound model.

The percentage yield, color, and consistency of isolated tannin-enriched fraction were found to be 24.5%, brownish color, and greasy consistency as shown in Table 1.

Qualitative phytochemical analysis of the leaves powder showed the presence of saponin, phenolic compound, tannins, and flavonoids. The tannin-enriched fraction showed the presence of tannins as tabulated in Table 2. TLC findings showed that the isolated tannin fraction was found to contain gallic acid as shown in Table 2 and Figures 1 and 2. HPTLC was carried out for the estimation of gallic acid content in the tannin-enriched fraction. The HPTLC fingerprint of the test sample showed one band at the distance similar to that of the standard confirming the presence of gallic acid [Figure 5]. The HPTLC chromatogram of standard (gallic acid) showed peak at the Rf value of 0.35 at 289 nm, and the HPTLC chromatogram of test sample (tannin enriched fraction) showed nine peaks with Rf values 0.08, 0.14, 0.15, 0.22, 0.35, 0.57, 0.65, 0.70, and 0.76 at 289 nm. Hence, the fifth peak of the test sample was similar to that of the standard. Superimposed spectrum of standard and test sample of HPTLC showed one clear peak at the Rf value ranging from 0.30 to 0.40. From the results of HPTLC chromatogram [Figures 6-10], the test sample, i.e., the tannin-enriched fraction was found to contain 113.69 ng/µl of gallic acid. There was no indication of any irritation on the skin applied with gel formulation at the end of skin irritation test period. Hence, the prepared topical gel formulation was free from skin irritation. The various sections of tissues of normal rats, diabetic control rats, rats treated with standard (marketed *A. vera* gel), and rats treated with 5% gel and 10% gel are shown in Figures 11-15. The histopathological examination presented in Figures 6-10 showed that the tissue regeneration was relatively greater in 10% gel-treated group than diabetic control, standard, and 5% gel-treated rat tissues without any edema, congestion, or inflammation. More relative fibrous cell was observed in diabetic control, standard, and 5% gel-treated rat tissues when compared to 10% gel-treated rat tissue. Hence, 10% gel-treated rat tissue showed good healing effect.

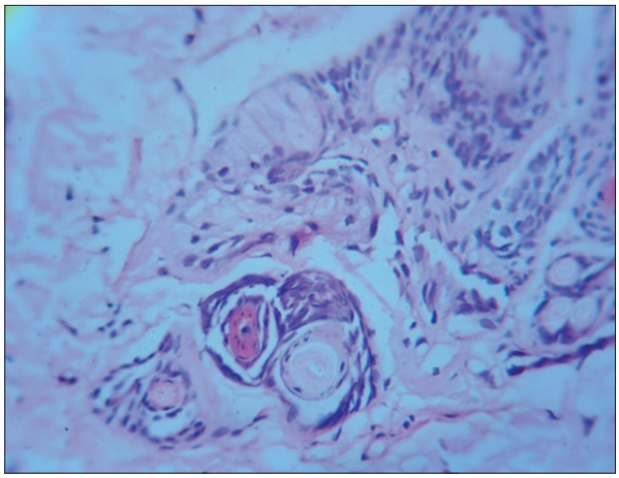

Figure 6: Normal tissue section

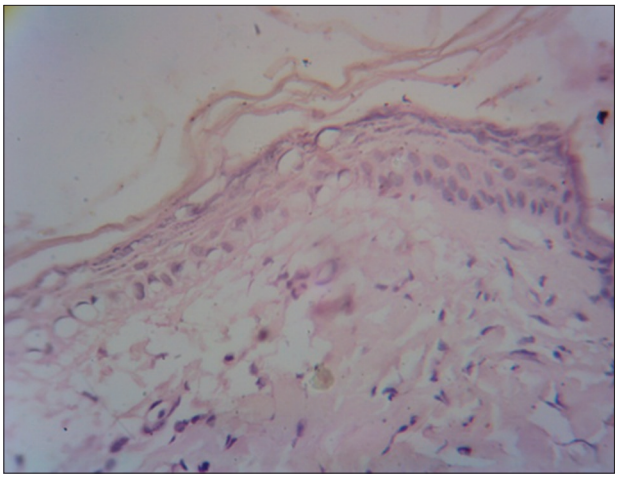

Figure 9: 5% gel treated tissue section

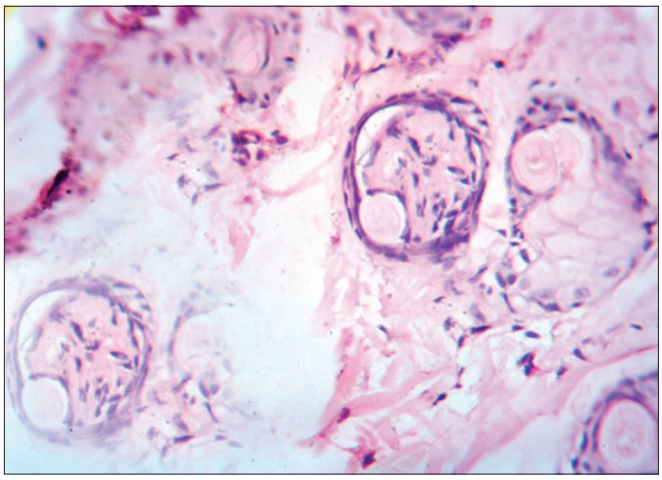

Figure 7: Diabetic control tissue section

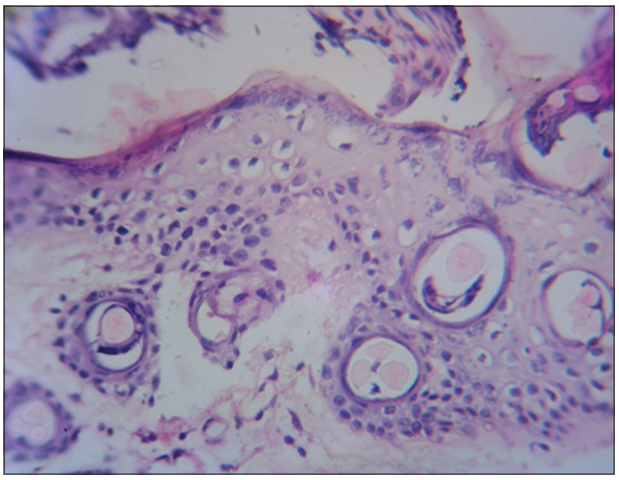

Figure 10: 10% gel treated tissue section

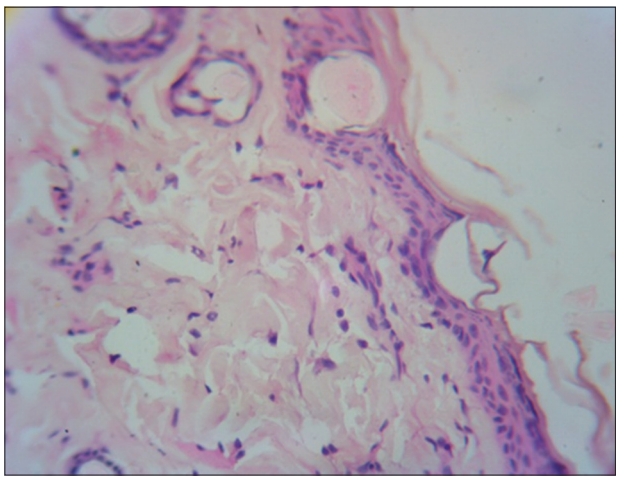

Figure 8: Standard treated tissue section

DISCUSSIONS

P. guajava Linn. has a long history of traditional use, and a good proportion of which has been validated by scientific research.[23] The ethnomedicinal uses include the crushing of the leaves and the application of the extract on wounds, boils, skin, and soft tissue infectious site.[24] Polyphenolic compound tannin has astringent, promotes wound healing by chelating free radicals, contracting injured tissues, and increases the formation of capillary vessels and fibroblast. Wound healing process is delayed in diabetic condition due to high blood glucose level, poor blood circulation, infection, diabetic neuropathy, and immunodeficiency. Hence, the present research work was focused to isolate tannin-enriched fraction and formulate the gel and evaluation of wound healing activity diabetic condition in animal model**.** With the support of the chemical test, TLC of tannin-enriched fraction was carried using gallic acid as standard where the test sample showed the one clear spot with Rf value similar to that of standard, and hence, it was found to contain gallic acid. HPTLC was carried out for the estimation of gallic acid content in the tannin-enriched fraction. The HPTLC fingerprint of test sample showed a band at the distance similar to that of the standard confirming the presence of gallic acid. The HPTLC chromatogram of the test sample showed a fifth peak similar to that of the peak of standard. Superimposed spectrum of standard and test sample of HPTLC showed one clear peak at the Rf value ranging from 0.30 to 0.40. From the results of HPTLC chromatogram, the test sample, i.e., the tannin-enriched fraction was found to contain 113.69 ng/

µl of gallic acid. Gel at two concentration (5% and 10%) was formulated with appropriate gelling agent and evaluated for its physiochemical parameters. The physical appearance was brown, transparent, and homogenous in nature. pH of the formulation ranged between 6.8 and 7.3. Spreadability ranged between 4 and 5 cm. The extrudability was good. The viscosity was determined by Brookfield viscometer where gel formulation had appropriate viscous nature. Stability test was performed as the ICH guidelines, and the gel formulation was found to be stable. The skin irritation test showed that the topical gel was free from irritation. These formulations were evaluated for diabetic wound healing activity. The wound healing involves different phases including contraction, the formation of epithelialization, and fibrosis.[25] Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue.[22] These processes are delayed in diabetic condition. The diabetic excision wound model was carried out to study the effect of topically applied gel of *P. guajava* leaves on wound healing and contraction. The results of the present study indicate that tannin-enriched fraction gel of *P. guajava* leaves at both strengths (5% and 10%) exhibited significant diabetic wound healing activity. However, this effect was found to be concentration-related fashion where 10% gel promotes significant diabetic wound-healing activity by increasing cellular proliferation, formation of granulation tissue, synthesis of collagen, and by increase the rate of wound contraction as compared to the diabetic control animals. Further phytochemical studies are needed to isolate and characterize the individual tannins and identify the compounds which are responsible for diabetic wound healing activity.

CONCLUSION

From this study, it is concluded that the gel of tannin-enriched fraction of *P. guajava* Linn. showed significant wound contraction at the tested concentration in diabetic condition. It may be probably due to the presence of gallic acid. Further studies are needed to isolate individual tannins and explore its biological potency by various preclinical and clinical trials of the isolated compounds.

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