

RESEARCH ARTICLE

Formulation Development and Evaluation of Herbal Antifungal Cream by using *Psidium guajava* Leaf Extract

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ABSTRACT

Fungal infection is one of the major health concerns globally and varies from superficial to systemic infections. *Psidium guajava* Linn. leaf extract showed antifungal activity against wide range of fungi. The present research focuses on the development and investigation of cream with guava leaf extract. Ethanolic, methanolic and hydroalcoholic leaf extract was evaluated for total flavonoid content, thin layer chromatography (TLC), high-performance thin layer chromatography (HPTLC) and antifungal activity against *Candida albicans*. Further cream-based extract was optimized using 3² factorial designs. Beeswax and tween 80 were selected as the independent variables whose impact was studied on viscosity and %cumulative drug diffusion. Optimized cream was characterized by appearance, pH, washability, spreadability, *in-vitro* drug diffusion study, antifungal test and skin irritation test on wistar rats. The hydroalcoholic extract showed the highest content of flavonoids and a better zone of inhibition (27 ± 1 mm). HPTLC results confirmed the presence of essential flavonoids. Cream with 6% beeswax and 5% tween 80 with a viscosity 10420 cP and 70% drug diffusion was considered as optimized batch. The prepared cream showed satisfactory results for all evaluated parameters. The antifungal test showed the highest zone of inhibition for cream (25 mm) in comparison to standard formulation (23 mm). No any sign of skin irritation was observed. The prepared formulation is the best suitable alternative for the available topical antifungal formulation.

Keywords: *Psidium guajava*, Antifungal, HPTLC, Topical formulation.

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INTRODUCTION

Psidium guajava Linn. (Guava) is a popular dietary plant used for medical purposes from several decades. It belongs to Myrtaceae family and is grown extensively in India, Bangladesh, Florida, and West Indies. Guava leaves has shown therapeutic benefits in many diseases and disorders. It is available in various dosages like capsule, tablet, liquid, and powder forms in market to treat diarrhoea, gastroenteritis, diabetes, dysentery, caries, hypertension, oral ulcers pain

relief, and cough to improve liver damage inflammation and locomotor coordination. It also been found to be effective in treating different conditions like lowering sugar level, boosting heart health, relieving the painful symptoms of menstruation, aiding of weight loss, and boosting immunity.

Recent study reveals antifungal potential of guava leaf extracts due to different types flavonoids *viz.* isoflavonoids, isoflavanones, isoflavane, flavonone, Flavonol (Rutin), quercetin, quercitrin, myricitrin, morin, guaijaverin,

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wogonin, kaempferol, baicalin, licoflavone C, catechin, gallic acid, dorsmanin and carvacrol. Extract showed antifungal potential against fungi like *Candida albicans*, *Cryptococcus neoformans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, *Trichophyton rubrum*, *Trichosporon Beigelii*, *C. parapsilosis*, *Aspergillus niger* etc fungi. Extract of guava leaf showed 21 to 30 mm zone of inhibition against *C. albicans* which is the causative agent for 'candidiasis'. Guava leaf extract has been found to exhibit antifungal properties through multiple mechanisms. These include inhibiting the synthesis of cell walls, impeding cell division, disrupting RNA and protein synthesis, and inducing failure in mitochondrial function.^{1,2}

Even though the guava extract has proved the involved in the management of fungal infection, through an exhaustive literature survey we have not found the research performed on the development of a suitable dosage form for its topical application. Henceforth; the proposed research provides more emphasis on the preparation of extract of guava leaf by using different solvents, conversion extract in suitable topical dosage form and its meticulous evaluation.

MATERIALS AND METHODS

Materials

Psidium guajava L. leaves are collected from the farms located at Gangapur, Nashik, and Maharashtra, India. Liquid paraffin was obtained from Merck Pvt. Ltd, Mumbai. Beeswax and Stearic acid were obtained from Research Lab Fine Chem. Mumbai. The remaining ingredients were of analytical grades.

Methods

Preparation of leaf powder for extraction

Collected leaves were thoroughly washed with water (3 times) and air dried for 72 hours under the sunlight. Leaves are incubated (24 hours; $37 \pm 2^\circ\text{C}$) and subjected for grinding using mixer. Prepared powder was passed from sieve number 120 and used for extraction.

Preparation of extraction and confirmation test for flavonoids

Prepared powder was subjected for extraction by the maceration process using three different solvents viz. methanol, ethanol and hydroalcoholic mixture. Powder (10 g) was placed in conical flask containing solvent (100 mL) and shaken for 24 hours. System was placed in dark for further 24 hours and then centrifugation was done for 15 minutes at 3000 rpm (Remi Centrifuge). The supernatant was collected in flask and the residue was again subjected for maceration as described previously. The process of maceration on same powder was repeated to obtain 300 mL extraction. The obtained solution was concentrated to 1/10th of its initial volume and used for further investigations. All extracts were confirmed for the presence of flavonoids by using standard chemical test. Test 1: Reaction between extract (1-mL) and ammonia solution (1%) forms the yellow color if flavonoids are present, whereas it can be confirmed by test 2: If red color formed when extract heated

with DMSO and further treated with magnesium chloride solution and concentrated hydrochloride acid.^{3,4}

Determination of total flavonoid content

To determine total content of flavonoids in extracts, 0.3 mL extract, 4 mL of 30% methanol, 0.15 mL of NaNO_2 (0.5M) and 0.15 mL of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (0.3M) were mixed in test tube. Sodium hydroxide (1-mL) was added in above mixture after 5 minutes. The solution was thoroughly mixed and samples were estimated at 506 nm against blank. Using std solution of rutin (0–100 mg/L) and following the previously stated steps, the calibration curve was plotted for total flavonoids. Total flavonoids in the dried fraction were measured in milligrams of rutin equivalents per gm.⁵

Antifungal test for extract

For confirm the antifungal activity of prepared extracts against the *C. albicans*, GMB media, i.e., glucose (2%), Mueller Hinton agar (M173) and 0.5 mcg/mL methylene blue dye was used. Zone of inhibition method was performed by using nystatin as the standard. Plates after sampling were placed $35 \pm 2^\circ\text{C}$ and examined for zone of inhibition after 24 hours.

Thin layer chromatographic study

To further confirm the flavonoids, present in extracts, Thin layer chromatographic (TLC) study was carried out. TLC plates of the desired dimension were purchased from the market. A line was drawn at 0.5 cm above to the bottom and plate was activated in hot air oven at 105°C for 30 minutes. Simultaneously, the mobile phase was transferred carefully in the glass chamber having lid and allowed it to stand for 10 minutes to cause the saturation of the compartment. Different compositions of mobile phases were tried viz. a. Chloroform: Methanol (9:1) b. Toluene: Ethyl acetate: Acetone (5:4:1) c. Chloroform: Acetone: Formic acid (10:2:1) d. Toluene: Ethyl acetate: Formic acid: ethanol (3:4:0.8:0.7) to obtain the proper resolution. The optimized extract was placed on TLC plate (above to 2 cm from the bottom) with the help of a capillary and placed in the chamber without direct contact between the sample spot and solvent. Plate was allowed to run solvent to its half height. After removing the plate, the solvent front was noted with a pencil. The further dried plate was visualized by applying anisaldehyde sulphuric acid reagent.⁶

High-performance thin layer chromatography

To perform the High-performance thin layer chromatography (HPTLC), standard stock solutions of quercetin, rutin and gallic acid of concentration 100 $\mu\text{g}/\text{mL}$ was prepared in methanol. The sample solution was prepared by dissolving the optimized extract (0.1 mL) in 10 mL methanol. Using a TLC semiautomatic sampler Linomat 5, sample and reference solutions (quercetin, rutin, and gallic acid) were applied to HPTLC plates as 8 mm bands, 4 mm apart, 10 mm from the lower edge, and 15 mm from the left and right edge of the plate (CAMAG, Muttenz, Switzerland). The chloroform: methanol (9:1) was used as the mobile phase which allowed traveling 8 cm distance on plate. All the procedure was performed as

Table 1: The matrix of the design including independent variables

Independent variables	Coded Level		
	-1	0	1
X1: Bees wax (%)	4	5	6
X2: Tween 80 (%)	5	6	7

per the manual given by the manufacturer. Plate was recorded and assessed at 366 nm under UV light. by using WinCATs software.^{7,8} The Rf value of extract and standard were compared with each other.

Formulation of antifungal cream

The optimized extract was further converted into creams to make it more convenient to apply topically. Oil phase contained liquid paraffin (5%), stearic acid (5%), beeswax (5%), cetostearyl alcohol (7%), tween 80 (6%), whereas the aqueous phase consisted of optimized extract (5%), sorbitol solution (5%), potassium hydroxide (5%), methylparaben (0.12%) in Water (q.s.). Both phases were heated and maintained at 70°C. The addition of aq phase to oil phase was done under continuous stirring and it was kept for cooling at RT to obtain extract loaded cream.

Experimental design (optimization)

Extract-loaded cream was optimized by using 3² full factorial design (9 batches) to obtain stable formulation. Percent concentration of bees wax (X1) and tween 80 (X2) were selected as the independent variables whose impact was studied on viscosity at 50 rpm (Y1) and %cumulative drug diffusion at the end of 180 minutes. As stated in Table 1, the independent variable was considered at three levels collected data was studied and assessed by ANOVA.

Evaluation of developed formulation

• Appearance and pH

Prepared all 9 batches of cream were checked for appearance by visual inspection. The pH is one of the important factors associated with topical preparation which can be related with the skin compatibility and ultimately to patient compliance. The prepared formulation was evaluated for pH using a precalibrated pH meter.⁸⁻¹⁰

• Washability

The washability of formulation from the skin under the running water was studied.¹¹

• Viscosity

The viscosity of the semisolid dosage form can be correlated with residence time, extrudability and spreadability. The prepared formulation was evaluated for viscosity by Brookfield Viscometer at 50 rpm with spindle 64.¹²

• Spreadability test

To determine 500 mg of the cream was applied between 2 slides to test its spreadability. The upper slide was loaded with a 100g weight. The extra formulation was scraped off and weight was reduced. The lower slide was attached to the apparatus's board,

and the upper slide was fastened with stiff string to which a 20 g load was applied. Time required to slip off for the upper slide to slip off was recorded.

• In-vitro drug diffusion study

Cellophane membrane was utilized to execute *in-vitro* diffusion on Franz diffusion cells. Prior to study, the cellophane membrane was dipped in pH 6.8 phosphate buffer (release medium) for soaking purpose for 24 hours. The release medium was charged into the diffusion cell and carefully clamped between the donor and receptor compartments. The donor compartment was charged with the formulation (1 g), and the assembly was subjected on the magnetic stirrer. At 37°C and 50 rpm, respectively, the temperature was maintained. A sample of 3 mL was withdrawn at 60 minute time intervals for 180 minutes and analyzed by using a UV spectrophotometer at 370, 258 and 273 nm. The sink condition was maintained throughout the experiment.¹³

• Antifungal test

Optimized cream was investigated for its antifungal activity as per the procedure described in the antifungal test for extract.

• Skin irritation test

Optimized cream was evaluated for skin irritation study on albino wistar rats as per OECD -404 guidelines. Rats (n = 6) of weight 200 to 250 gm of either sex were placed on normal food and water intake prior to study. The formulation was applied on shaved skin and observed for the presence of any sign of skin irritation.

RESULT AND DISCUSSION

Preparation of Extract and Confirmation Test for Flavonoids

Powder of guava leaves was subjected to maceration by using three different solvents to identify a suitable solvent system which can isolate the maximum concentration of phyto constituent. Prepared extracts were subjected for a confirmation test of flavonoids. Results are shown in Table 2. It was confirmed that flavonoids were present in all prepared extracts.

Table 2: Flavonoids test

Extract test	Methanolic extract	Ethanollic extract	Hydroalcoholic extract
Test 1	Yellow color	Yellow color	Yellow color
Test 2	Red color	Red color	Red color

Table 3: Total flavonoid Ccontent for *Psidium guajava* extract

S. No.	Type of extract	Concentration of rutin (mg/L)
1	Methanolic extract	11.51 ± 0.2
2	Ethanollic extract	5.25 ± 0.2
3	Hydroalcoholic extract	22.12 ± 0.2



Figure 1: Anti-fungal test results for isolated extract of *Psidium guajava*

Table 4: Zone of inhibition of prepared extract

S. No.	Type of extract	Zone of inhibition (mm)
1	Methanolic extract	20 ± 1
2	Ethanolic extract	18 ± 1
3	Hydroalcoholic extract	27 ± 1

Determination of Total Flavonoid Content

All prepared extracts were investigated for total flavonoid content against the calibration curve of rutin as a standard. Results are summarised in Table 3 and it indicated that hydroalcoholic extract contains the highest amount of rutin.

Antifungal Test of Extract

After confirmation and determination of total content of flavonoids in prepared extracts, an antifungal test was carried out by zone of inhibition. Test was performed to check effectiveness against *Candida albicans*. Results are shown in Figure 1 and summarized in Table 4. A hydroalcoholic extract found to be more effective as the highest zone of inhibition than other prepared extracts.

Based on the obtained results, the hydroalcoholic extract showed maximum flavonoid content and highest zone of inhibition and henceforth it was considered as optimized extract and used for further evaluation.

Thin Layer Chromatography

The optimized extract was further analyzed by TLC method to check the presence of flavonoids. Different solvent systems were tried to obtain proper separation of flavonoids present in the extract. TLC plates with different mobile phases are shown in Figure 2. Better resolution was observed with Chloroform: Methanol.

HPTLC Study of an Extract

HPTLC was studied for fingerprint analysis of standards with the extract. The mobile phase used for the analysis was Chloroform: Methanol which was optimized in the previous step. The Rf values of single spot of standards was compared with the Rf values of spots of optimized extract (Figure 3). The optimized extract showed Rf value closer to the standard (Table 5). Results confirmed the presence of essential flavonoids which can be converted in suitable form to make it convenient for topical application in order to treat fungal infections.

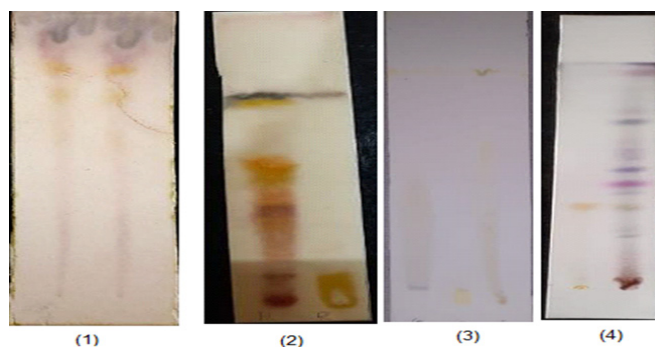


Figure 2: TLC plates with different mobile phases (1) Chloroform: Methanol (9: 1), (2) Toluene: Ethyl acetate: Acetone (5: 4: 1), (3) Chloroform: Acetone: Formic acid (10: 2: 1), (4) Toluene: Ethyl acetate: Formic acid: Ethanol (3: 4: 0.8: 0.7)

Table 5: Comparative Rf value

S. No.	Substance	Rf value for standard	Rf value for extract
1.	Gallic acid	0.34 ± 0.01	0.35 ± 0.01
2.	Rutin	0.06 ± 0.02	0.05 ± 0.02
3.	Quercetin	0.95 ± 0.01	0.94 ± 0.01

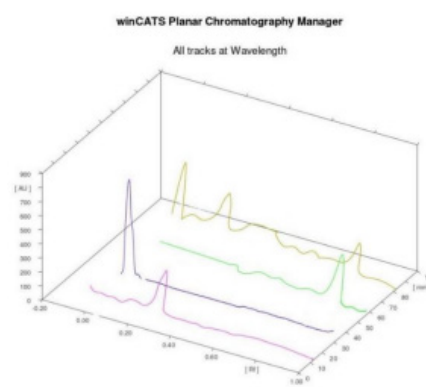


Figure 3: HPTLC spectrum of extract, quercetin, gallic acid and rutin

Formulation of Cream

Optimized extract was further converted into cream by using bees wax. A total nine batches were prepared and optimized by using the desirability search approach.

Experimental Design

In order to formulate stable antifungal cream, 3² full factorial design was selected. Beeswax and Tween 80 concentrations (X1 and X2) were independent variables that were examined at three levels using the responses viscosity (Y1) at 50 rpm and percent cumulative drug diffusion (Y2) at the conclusion of 180 minutes. Table 6 displays the design points along with their coded and existing values.

The response Y1 was found to be in the range of 6430 to 10950 cp, whereas %drug diffusion (Y2) was in the range of 70 to 85%. The findings are mentioned in Table 6 which were recommended by Design Expert.

Table 6: Design matrix, formulation components, parameters for experiment and formulation characterization

Batch no.	Beeswax (%): X1	Tween 80 (%): X2	Viscosity (cP): Y1	%Cumulative drug diffusion: Y2
F1	4	5	6430	85
F2	4	6	8670	84
F3	4	7	9360	82
F4	5	5	7850	80
F5	5	7	9510	78
F6	5	6	8950	76
F7	6	6	10510	75
F8	6	7	10950	72
F9	6	5	10420	70

Table 7: Regression analysis and ANOVA

Response	Model	F value	p-value	R ²	Significance
Y1	2FI	5.81	0.0045	0.9236	Significant
Y2	Liner	24.44	0.0013	0.8907	Significant

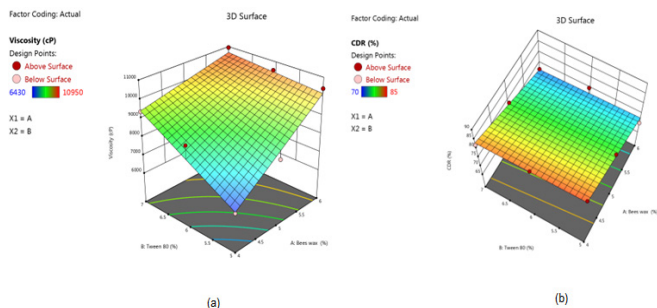


Figure 4: 3D surface response plot (a) Viscosity (Y1), (b) %CDR (Y2)

2FI model was suggested for Y1 and Y2 factor. ANOVA study (Table 7) confirmed that model was statistically significant with *p-value* less than 0.05.

The polynomial equations were recommended by software; $Y1 = +9183.33 + 1236.67 X1 + 853.33 X2 - 600 X1X2$ (1)
 $Y2 = +78.60 - 5.67 X1 - 0.500 X2$ (2)

From equation 1, the bees wax as well as the tween 80 have a positive effect on viscosity. The viscosity rises with rise in the concentration of X1 and X2. But it was discovered that X1 had a more significance than X2. However, the drug release was negatively impacted by both of the independent variables. Bees wax showed significant control on drug release than Tween 80.

As illustrated in Figure 4, it is possible to study the effect of independent variables on response.

The “Desirability search approach” was utilized to choose the optimum batch. Several solutions were suggested by software having a desirability value equal to one. One solution was batch F9 used for optimization (Figure 5) and henceforth it was considered as the optimized batch and used for further evaluation.

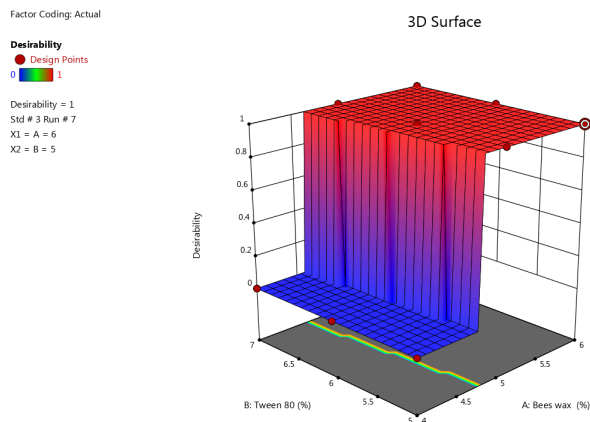


Figure 5: Selection of optimized batch

Table 8: Characterization of cream

Batch no	Appearance	pH	Washability
1	Greasy	5.8 ± 0.3	Not easily washable
2	Greasy	5.8 ± 0.2	Not easily washable
3	Greasy	6.0 ± 0.2	Not easily washable
4	Smooth	5.7 ± 0.5	Washable
5	Smooth	5.8 ± 0.4	Washable
6	Smooth	5.7 ± 0.2	Washable
7	Smooth	6.1 ± 0.3	Washable
8	Smooth	5.5 ± 0.4	Washable
9	Smooth	5.6 ± 0.2	Washable



Figure 6: Skin irritation test with free from irritation

Characterization of Cream

*Appearance, pH and washability*¹⁴

Prepared nine formulations were checked for its appearance by visual inspection and pH by using a digital pH meter. Results are summarized in Table 8. The formulation showed a satisfactory appearance with pH in the range of 5.5 ± 0.4 to 6.1 ± 0.3 which can be easily tolerated by skin buffer system. Batch 1, 2 and 3 were not easily washable under the running water while the rest of formulated batches showed easy washability.

Spreadability test

The spreadability of the optimized cream was studied using the spreadability apparatus. The cream showed good spreadability as 3.45 ± 0.5 g.cm/sec.

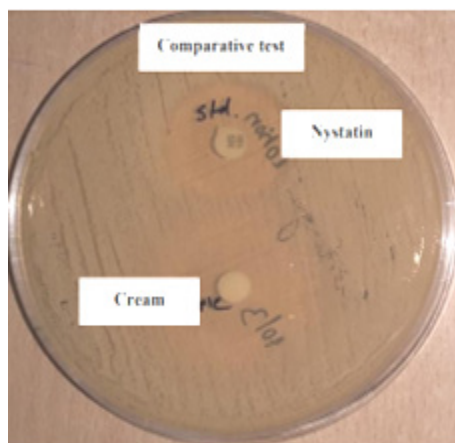


Figure 7: Antifungal test with zone of inhibition

Drug diffusion study

The optimized batch was investigated for drug diffusion in order to determine %release of rutin, quercetin and gallic acid. At the end of 180 minutes, $70.2 \pm 2\%$, $72.35 \pm 1\%$ and $69.49 \pm 2\%$ release of rutin, quercetin and gallic acid, respectively was observed. Upon mathematical treatment to the obtained data, the Krosmeier-peppas model was found to be suitable.

Skin irritation study

Prepared formulation tested for skin irritation on mice. No any sign of redness, or swelling was observed on mice skin (Figure 6). Henceforth; prepared formulation can be considered as safe for topical application.

Antifungal test

Optimized cream was tested for antifungal activity by using a zone of inhibition. As demonstrated in Figure 7, the developed formulation had a larger zone of inhibition (25 mm) in contrast to the normal formulation (23 mm). Consequently, it can be inferred that the developed formulation has excellent potential for the treatment of the fungus infection.¹⁵

CONCLUSION

In the present study guava leaf extract was successfully converted in stable cream in order to make it more convenient to apply the infected area. After confirmation of the presence of flavonoids in the extract of guava leaf, it was loaded in the cream prepared by using beeswax and tween. Cream passed all the essential test of the semisolid dosage form. The antifungal study showed more prominent results than the standard. Furthermore; no any sign of skin irritation was observed during the skin irritation study on Wistar Rats. Based on available evidence, the prepared formulation is the feasible alternative for available standard antifungal treatment.

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