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Evaluation of antimicrobial efficacy of *Psidium guajava* L. leaf extract in ketoconazole shampoo

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Despite their effectiveness, synthetic preservatives are often associated with adverse health effects, which has led to growing interest in natural alternatives. This study evaluated the antimicrobial activity of an ethanolic extract of *Psidium guajava* L. leaves incorporated into a shampoo formulation. The ethanolic extract of *Psidium guajava* L. leaves was tested at concentrations of 0.5%, 2.5%, 5%, 7.5%, and 10% (w/w) under accelerated conditions for three months. Phytochemical screening and HPLC analysis were conducted to identify key bioactive compounds (including phenols, flavonoids, and terpenoids), antimicrobial efficacy testing, and stability testing (such as pH, viscosity, density, and color) were evaluated. At concentration 10%, extract completely inhibited microbial growth over a 28-day period, in comparison with chemical preservatives. Stability testing conducting at accelerated temperatures (40 °C/75% RH) for three months confirmed the physical stability of the formulation and the chemical integrity of ketoconazole. Preservative efficacy tests, performed accordance with USP/BP guidelines against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus brasiliensis*, demonstrated that the extract reduced microbial counts beyond USP/BP acceptance criteria, achieving total inhibition of all strains within 28 days. Chemical and biological tests confirmed formulation stability and efficacy over a three-months period. The findings suggest that *P. guajava* L. extract is a promising natural preservative; however, further studies are needed to assess its long-term stability.

Keywords *Psidium guajava* L., Stability, Antimicrobial agent, Ketoconazole shampoo, Phytochemical analysis

Abbreviations

RH	Relative humidity
HPLC	High performance liquid chromatography
ATCC	American Type Culture Collection
O.D.	Optical density
CFU	Colony-forming units
SCDM	Soyabean Casein Digest Medium
CDSLP	Casein Digest- soy lecithin poly sorbate
FLM	Fluid lactose medium
SCDA	Soyabean Casein Digest Agar
TABC	Total aerobic bacterial count
TYMC	Total combined yeasts and molds count
ppm	Parts per million

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The use of medicinal plants in the pharmaceutical formulations has increased significantly due to their abundance of secondary metabolites[1,2], which exhibit a wide range of pharmacological activities, including antibacterial and antifungal properties[3–5]. There has been a notable increase in the use of plant extracts and essential oils as natural antimicrobial agents in food preservation[6] and pharmaceutical formulations[7]. This shift is largely driven by the potential adverse effects associated with synthetic chemical preservatives (such as methylparaben and propylparaben), particularly when used repeatedly or in high concentrations[8,9]. Regulatory authorities have responded by imposing restrictions on the use of parabens in cosmetics[10], while simultaneously encouraging the pharmaceutical industry to adopt natural ingredients for the development of safer cosmetic and pharmaceutical products[11,12].

In this context, a typical natural antibacterial preservative should also be non-toxic, cost-effective, and effective at low concentrations against a broad range of pathogenic and spoilage microorganisms[13]. The numerous medicinal and industrial applications of *P. guajava* extracts are increasingly recognized in sustainable agriculture, cosmetics, and healthcare[3,9,12]. This versatility is attributed to the presence of a wide spectrum of bioactive phytochemicals (such as alkaloids, sterols, phenols, flavonoids, tannins, and saponins) present in *P. guajava* that have therapeutic potential in the treatment of various ailments, including anorexia, cholera, laryngitis, wounds, ulcers, rheumatic pain, and skin disorders[14].

Psidium guajava, sometimes known as guava, is a tropical and subtropical plant that is a member of the *Myrtaceae* family. It is a medium-sized tree that usually starts to produce fruit after about four years. Because guava trees can grow in a variety of soil types and produce fruit quickly, they are widely distributed throughout the tropics[15–17].

It is crucial to investigate the incorporation of *Psidium guajava* L. (guava) extract into shampoos containing ketoconazole for several reasons. Ketoconazole is a synthetic antifungal medication that targets the *Malassezia* fungus and is commonly used to treat scalp disorders such as dandruff and seborrheic dermatitis. Prolonged use, however, may lead to reduced effectiveness, resistance, or scalp discomfort[18]. *P. guajava* possesses complementary antifungal, anti-inflammatory, and antioxidant properties and is rich in bioactive components, such as flavonoids, tannins, and terpenoids[3,9,12]. When used together, guava extract may improve the shampoo's therapeutic benefits, reducing the amount of ketoconazole needed and reducing adverse effects.

Furthermore, the anti-inflammatory properties of guava may help reduce scalp irritation caused by ketoconazole, while its antioxidants protect the scalp tissue from oxidative damage. This combination offers a safer, more sustainable, and multi-targeted method of controlling scalp health, which is in line with the rising consumer desire for natural adjuvants in dermatological solutions[19].

Accordingly, the main objectives of this study were, firstly, to evaluate the antimicrobial activity of the ethanolic extract of guava leaves. Secondly, to identify phenolic compounds and determine the minimum effective concentration of guava leaf extract using phytochemical screening and high-performance liquid chromatography (HPLC), respectively. Thirdly, to assess the potential of replacing synthetic chemical preservatives (methyl and propyl parabens) with guava leaf extract in shampoo formulations.

Methods

Sample collection and extraction

The mature (fully inflated) leaves utilized in this study were gathered from Qalqilya, Palestine (32.190°N, 34.969°E) in the early morning hours of March 2023 (spring), between 8:00 and 10:00 AM. The reference specimen code Pharm-PCT-280 was given to the plant after it was discovered and verified at the Arab American University. To reduce variation in phytochemical content, agronomic parameters such as clay-loam soil (pH 6.8), room temperature (22 °C), and shade-drying at 25 °C were standardized. After being carefully cleaned with water, all of the collected leaves were allowed to dry for 30 days at 25 °C in a dry, well-ventilated setting. After drying, the leaves were crushed and sieved through sieves with pore diameters between 0.1 and 0.6 mm.

The following materials and reagents were used in this study: 96% ethanol, chloroform, concentrated sulfuric (96%), concentrated hydrochloric acid (37%), glacial acetic acid, Iron (III) chloride, methanol (HPLC-grade), gallic acid (99%), catechin (99%), chlorogenic acid (98%), caffeic acid (99%), rutin (99%), Acetonitrile (HPLC grade), ammonium hydrogen phosphate (99%), and ketoconazole (99%) was used in this study. All materials used in the formulation of pharmaceutical preparations were of pharmaceutical grade.

An ultrasonicator set to 20 kHz was used to accomplish ultrasonic-assisted extraction (Model VCX 750, Sonics & Materials, USA, running at a frequency of 20 kHz and a power of 750 W). The extraction process involved combining 1500 g of *Psidium guajav*a L. leaves with 9000 mL of 96% ethanol and sonicating the mixture for three hours. A vacuum rotary evaporator was used to remove the solvent following extraction. After recovery, the resultant extract was kept at 4 °C in a freezer until it was needed again[20].

Phytochemical screening

Unless otherwise indicated, 1 mL of the produced extract solution (equal to 100 mg/mL of dry extract) was used as the test sample for each qualitative analysis (terpenoids, flavonoids, glycosides, phenols, and tannins).

Major secondary metabolites were identified by qualitative phytochemical screening of *Psidium guajava* L. leaf extract using conventional staining and precipitation techniques[21]. A reddish-brown tint at the chloroform–sulfuric acid interface suggested the existence of terpenoids[22]. The formation of an orange-to-pink hue following interaction with strong HCl and magnesium was used to identify flavonoids[23]. The presence of glycosides was verified by the development of a brown ring at the interface following treatment with strong sulfuric acid and glacial acetic acid containing $FeCl_3$[24].

High-performance liquid chromatography (HPLC) analysis

Phenolic chemicals (catechin, gallic acid, caffeic acid, chlorogenic acid, and rutin) were produced in standard solutions in methanol/water without adjusting the pH. For the phenolic analysis, 1% acetic acid (pH ~ 2.5–3.0) was used in the mobile phase. Before using, the mobile phase buffer (ammonium hydrogen phosphate) was brought to a pH of 7.2 ± 0.1 and ketoconazole standards were dissolved in a 75:25 ethanol/water ratio.

In all analyses, triplicate measurements were made using 20 µL injections in full-loop mode. It was confirmed that this volume offered the best sensitivity (LOD 0.1 µg/mL).

Purified water (B) containing 1.0% acetic acid (v/v) and HPLC-grade methanol (A) were used as analytical solvents for the liquid chromatographic separation, which was carried out at 40 °C. A Purospher® RP-18 Endcapped column (5 µm, 250 mm length) was used in the HPLC (Agilent 1260 Infinity II, Agilent Technologies, Waldbronn, Germany) procedure. Chromatography was carried out at a steady flow rate of 1.0 mL/min for 45 min. The gradient program was 100% B for 0–10 min, 70% B for 10–20 min, 10% B for 20–30 min, 70% B for 30–37 min, and 100% B for 37–45 min. The wavelength at which UV chromatograms were obtained was 280 nm.

After rotational evaporation and lyophilization, the freeze-dried powdered extract (about 0.6 g) was moved to a volumetric flask for HPLC sample preparation. Then, 90 mL of ethanol was added, and the mixture was shaken for 30 min. Purified water was added to adjust the volume, and the solution was mixed and filtered through a PTFE syringe filter (0.45 µm) prior to HPLC and spectroscopic analysis. Identification and qualitative analysis were performed by comparing the retention times and spectra with those of known standards[25,26]. The compounds detected at 280 nm included gallic acid ($C_7H_6O_5$), catechin ($C_{15}H_{14}O_6$), chlorogenic acid ($C_{16}H_{18}O_9$), caffeic acid ($C_9H_8O_4$), and rutin hydrate ($C_{27}H_{32}O_{17}$).

Quantification of phenolic compounds was performed using the external standard method. Calibration curves were constructed for gallic acid, catechin, chlorogenic acid, caffeic acid, and rutin, each prepared at five concentrations (10, 20, 40, 80, and 100 µg/mL). The concentration of each compound in the extract was calculated from its corresponding calibration curve based on the peak area at 280 nm. The linearity of each curve showed high correlation coefficients ($R^2 > 0.999$). All results were expressed as mg of compound per 100 g of dry extract.

Quantification of phenolic compounds was carried out using the external standard method. Calibration curves were constructed for each reference standard (gallic acid, catechin, chlorogenic acid, caffeic acid, and rutin) using five concentration points (10–100 µg/mL). The peak area of each analyte in the guava leaf extract was interpolated into its respective calibration curve, and results were calculated as mg of compound per 100 g of dry extract. All calibration curves showed high linearity with R^2 values > 0.998.

Preparation of ketoconazole shampoo containing *P. guajava* L. leaf extract

The shampoo formulation was prepared according to a documented protocol with modifications to address dandruff and itchy scalp[26]. The formula included various ingredients to achieve the desired characteristics are shown in Table 1 and quantity of each ingredient was calculated on weight basis. In this study, three ketoconazole shampoo preparations were prepared:

1. Test preparation: Contained the natural preservative (*P. guajava* leaf extract) at a concentration of 10% w/w.
2. Positive control: Contained chemical preservatives (0.12% methyl paraben and 0.08% propyl paraben).
3. Negative control: was preservative-free.

Chemical and physical analysis

Determination of ketoconazole content in the shampoo formula by HPLC

Before HPLC analysis, 1 g of shampoo was accurately weighed and diluted to 25 mL with ethanol: water (60:40, v/v). The mixture was vortexed, sonicated for 10 min, and centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 µm PTFE syringe filter, and 20 µL was injected into the HPLC system. Quantification

Item	Concentration (w/w%)	Material function
Ketoconazole (API)	2%	Antifungal
Coconut oil	1.4%	Emulsifying agent
Texapon N70	20%	Surfactant
Loramide	3%	Foam booster
Polyethylene glycol (PEG) 6000	3%	Solvent
Sodium Chloride	2%	Thickening agent
P. guajava L. leaves extract	10%	Antimicrobial Preservative
Blue color	0.25 mg/100 ml	Coloring agent
Kiwi mix	33 mg/100 ml	Perfuming Agent
Cremophor (RH 40)	1%	Emulsifying agent
Sodium hydroxide (0.1 N)	0.25%	pH adjusting agent
Purified water	57.32%	Solvent

Table 1. Composition and function of ingredients in the ketoconazole shampoo.

was performed using analytical grade ketoconazole (≥99% purity, Sigma-Aldrich, Germany) as the external standard.

The mobile phase used in the chromatographic study was made up of 35% acetonitrile and 65% buffer (5.7 g of ammonium hydrogen phosphate in 1 L of water), while the stationary phase was a 125 × 4 mm RP-8 column with a 5 µm particle size. Compounds were identified at a wavelength of 240 nm while the flow rate was kept constant at 1 mL/min. The HPLC system received an injection of a 20 µL aliquot of the sample. 75% ethanol and 25% filtered water made up the diluent. For ketoconazole, the retention period was around 12 min. Retention periods in comparison to a ketoconazole standard were used to identify ketoconazole.

For ketoconazole determination, standard stock solution of 1000 µg/mL ketoconazole was prepared in ethanol: water (60:40, v/v). The following calibration curve concentrations were prepared by dilution from the stock solution: 10, 20, 40, 60, 80, and 100 µg/3mL. The method was found to be linear from 10 to 100 µg/mL with a regression equation: y = 102.8x + 36.6 (y: peak area a x is the concentration in µg/mL), and correlation coefficient (R^2) of 0.9994, indicating excellent linearity[27].

Evaluation of the chemical and physical stability of the pharmaceutical preparations

The physical appearance, odor, and pH of the ketoconazole shampoo were evaluated over a three-month period under accelerated conditions (40 °C/75% relative humidity)[28,29].

The pH of the shampoo formulation was measured using a calibrated pH meter (Mettler Toledo S220-Kit). The pH meter was calibrated using buffer solutions within the expected measurement range. The pH electrode was immersed directly into the product, and measurements were taken in triplicate. The mean pH value was calculated from the three measurements. Moreover, viscosity was measured using a Brookfield DV2T viscometer. A 50 mL sample of the shampoo was placed in a 50 mL beaker, and viscosity was determined using spindle N4 at 100 rpm and a temperature of approximately 20–22 °C. Density was measured using a pycnometer. The density was calculated by dividing the weight difference between the filled and empty pycnometer by the volume of the pycnometer.

$$Density = \frac{\text{The weight difference (in grams) between the filled and empty pycnometer}}{\text{The volume of the pycnometer (in ml)}}$$

Preparation of microbial cultures for testing

The microorganisms selected in this study are pathogenic species commonly associated with infectious dermatitis. The strains included:

- Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538),
- Gram-negative bacteria: *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027),
- Fungi: *Candida albicans* (ATCC 10231, yeast) and *Aspergillus brasiliensis* (ATCC 16404, mold).

All bacterial and fungal strains were sub cultured from the original freeze-dried stocks and maintained in the Pharmacology and Phytomedicine Laboratory at the Arab American University (Jenin, Palestine)[30].

To conduct the tests, working stock cultures were first prepared by streaking the stored strains onto appropriate agar plates. To prepare the microbial suspension, growth surface was washed with a sterile saline solution. The resulting suspension was collected in a suitable container, and sufficient sterile saline was added to achieve a microbial density of approximately 10^8 CFU/mL. Cell density was measured using a spectrophotometer at 650 nm to obtain specific optical densities:

- **(A)** *Staphylococcus aureus*: 0.3–0.45 (corresponding to ~ $1–3 \times 10^8$ CFU/mL),
- **(B)** *Pseudomonas aeruginosa* and *Escherichia coli*: 0.2–0.3 (corresponding to ~ $1–3 \times 10^8$ CFU/mL),
- **(C)** *Candida albicans* < 1.0 (corresponding to ~ $1–3 \times 10^8$ CFU/mL).

Several dilutions (10^{-3} to 10^{-6}) were prepared and plated using the plate counting method (1 mL of each dilution in Sabouraud Dextrose Agar, in duplicate). The cultures were incubated for 24 h at 35 °C ± 2 for bacteria and for 2–3 days at 23 °C ± 2 for *Candida albicans*. Colony-forming units (CFUs) were counted on plates containing between 30 and 100 CFUs[45].

The surface of the *Aspergillus brasiliensis* culture was washed with sterile saline containing 0.05% polysorbate 80. Sterile saline was added to achieve a microbial count of approximately 10^8 CFU/mL. Several dilutions (10^{-3} to 10^{-8}) were prepared and inoculated using the pour plate method (1 mL of each dilution in Sabouraud Dextrose Agar, in duplicate). The plates were incubated for 2–4 days at 23 °C ± 2, and CFUs were counted on plates containing between 10 and 100 CFUs[31].

Microorganisms from the mother culture were grown in Soy-casein digestion broth or Sabouraud dextrose broth, as appropriate. The cells were harvested by centrifugation, washed, and resuspended in sterile saline to achieve a microbial concentration of approximately 10^8 CFU/mL. The viable count of each suspension was confirmed by plating under the appropriate culture conditions and incubation times to verify the initial CFU/mL count.

Evaluation of preservation efficiency of *Psidium guajava* L. extract

For every strain, the test was carried out independently in five sterile bacteriological containers. Twenty milliliters of the sample (*Psidium guajava* L.) extract in purified water was added to each container in varying percentages (0.5%, 2.5%, 5%, 7.5%, and 10% w/w). After that, one of the prepared, standardized inoculums was added to each

container. To reach a final concentration of 10^5 to 10^6 CFU/mL in the test preparation, the inoculum suspension volume varied from 0.5% to 1% of the product volume.

The containers that were inoculated were incubated at 23 °C ± 2. At predetermined intervals of 0, 7, 14, and 28 days, samples were drawn from each container. 1 mL aliquots from each container were used to create serial dilutions (1:10), which were then transferred to test tubes holding 9 mL of Letheen's broth medium to produce dilutions ranging from 10^{-1} to 10^{-5}. A sterile 90 mm Petri dish was then pipetted with 1 mL of each dilution, and each dish was filled with 15 mL of Letheen's agar medium (at 48 °C). To solidify, the plates were placed in a fume hood. Incubation times for bacterial and fungal plates were 35 °C ± 2 for two to three days and 23 °C ± 2 for five to seven days, respectively.

Colony-forming units (CFUs) were enumerated using the plate counting method at the specified time intervals. For each microorganism, the change in $\log_{10}$ values of CFU/mL values were calculated, and the results were expressed as logarithmic reduction. Logarithmic reduction was defined as the difference between the $\log_{10}$ value of the initial CFU/mL concentration and the $\log_{10}$ value of the surviving CFU/mL at each sampling point[10].

Antimicrobial efficacy criteria

In accordance with USP < 51 > and EP 5.1.3 recommendations[28,29], topical treatments must meet certain criteria for antimicrobial effectiveness. In short, yeasts and molds must not rise from baseline at days 14 and 28, whereas bacteria must exhibit at least one log reduction by day 7 and at least three log reduction by day 14.

Evaluation of total aerobic microbial count (TAMC) in shampoo

The purpose of this technique is to estimate the total aerobic microbial count (TAMC) and total combination yeasts and molds count (TYMC) both qualitatively and quantitatively in final shampoo products that contain extract from *Psidium guajava* L. The study evaluated preservation efficacy in accordance with the British Pharmacopoeia (BP) and United States Pharmacopoeia (USP) guidelines[29,32].

Disinfection of the tubes and caps with 70% ethanol was the initial stage of sample preparation. After that, the product was moved to a sterile vial. To enable additional testing, the vial was heated to 45 °C in a water bath to produce a consistent solution. A laminar flow hood was used to keep the vial sterile while it was being handled. To guarantee even dispersion, the suspension was shaken. An emulsifying agent was added to help dissolve products that were hard to dissolve.

For microbial enumeration (counts of Bacteria, Yeast, and Mold), samples were plated on Soybean Casein Digest Agar (SCDA) for bacterial counts after being diluted 1:10 in phosphate buffer. The results were reported as colony-forming units (CFU) per gram or milliliter after the plates were incubated for three to five days at 30 to 35 °C. If no growth was seen, counts < 10 CFU/g or mL were recorded. The same method, but with Sabouraud Dextrose Agar (SDA) or Broth (SDB) substituted, was used to determine total yeast and mold counts (TYMC), which were then incubated for five to seven days at 20 to 25 °C.

Acceptance criteria for finished products

According to USP/BP standards for cutaneous products, the total aerobic microbial count (TAMC) must be less than 200 colony-forming units per gram (< 200 CFU/g), corresponding to specific limit of 10^2. Similarly, the total yeast and mold count (TYMC) must be less than 20 colony-forming units per gram (< 20 CFU/g), with a specific result of 10^1. Additionally, the presence of *Staphylococcus aureus* or *Pseudomonas aeruginosa* must not be detectable. The USP/BP acceptance criteria interpretation is as follows:

- 10^1 CFU: maximum acceptable count = 20,
- 10^2 CFU: maximum acceptable count = 200.

Stability testing

Stability tests were carried out on ketoconazole shampoo formulations containing *Psidium guajava* L. leaf extract as a natural preservative. Several tests were performed at the end of each month for three months under accelerated conditions (40 ± 2 °C/75 ± 5% relative humidity). These included:

- Antimicrobial efficacy tests.
- Chemical tests to quantify the active ingredient (ketoconazole).
- Physical tests to evaluate appearance, color, pH, and odor.

Statistical analysis

The antimicrobial efficacy data, which is represented as a Log_{10} decrease in colony-forming units per milliliter (CFU/mL), was obtained via USP-approved preservative effectiveness testing. According to the methodology, the test was conducted in five replicate containers ($n = 5$) for every combination of guava leaf extract (GLE) concentration (0.5%, 2.5%, 5%, 7.5%, 9.5%, 10% w/w) and challenge microorganism (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, and *Aspergillus brasiliensis* ATCC 16404).

The software IBM SPSS Statistics 21 was used for all statistical analyses. All tests were conducted at a significance value of $\alpha = 0.05$. One-way analysis of variance (ANOVA) was used to examine the impact of extract concentration on microbial growth for selected strains as well as the effect of storage time.

Phytochemical tests	Phenols and tannins	Terpenoids	Flavonoids	Glycosides
Extract of *P. guajava* (L)	+	+	+	+

Table 2. Phytochemical constituents of *P. guajava* L. leaf extract. +: presence of the constituent.

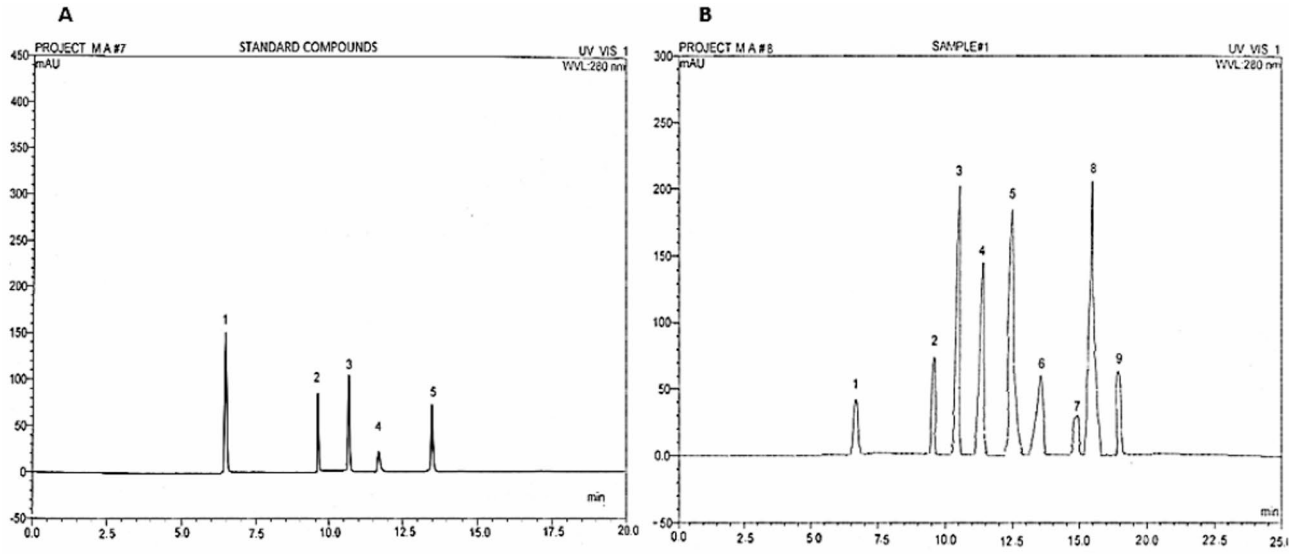

Fig. 1. (A) Chromatograms of standard phenolic compounds at 280 nm. Peaks: 1- gallic acid (RT = 6.523); 2- catechin (RT = 9.725); 3-chlorogenic acid (RT = 10.624);4 - caffeic acid (RT = 11.731); 5- rutin (RT = 13.524). B) HPLC chromatogram of ethanol extract of guava leaves. Peaks: 1- gallic acid (RT = 6.613); 2-catechin (RT = 9.614); 3- chlorogenic acid (RT = 10.611), 4-caffeic acid (RT = 11.491); 6 rutin (RT = 13.615). Peak 5, 7, 8, and 9 are unidentified.

Polyphenolic compound	Content (mg/100 g of dry extract)
Gallic acid	555.49 ± 2.9
Catechin hydrate	821.11 ± 1.3
Chlorogenic acid	42.04 ± 0.8
Caffeic acid	39.54 ± 1.0
Rutin	300.84 ± 1.5

Table 3. Chemical concentrations of phenolic compounds in the ethanol extracts of *P. guajava* L.

Results

Results of phytochemical screening

Qualitative phytochemical analysis of *Psidium guajava* L. leaves revealed the presence of several classes of secondary metabolites. The results indicated that *Psidium guajava* L. leaves showed a positive test for phenols, tannins, terpenoids, flavonoids, and glycosides (Table 2).

Although there are many phytochemicals in *Psidium guajava* leaves, gallic acid, catechin, and chlorogenic acid were chosen for measurement because (1) they were present in high concentrations in our extract (Table 2), (2) they have been shown to play roles in antimicrobial preservation, and (3) they were compatible with reference standards and HPLC resolution.

Determination of extracted powder by HPLC

High-performance liquid chromatography (HPLC) analysis confirmed that the ethanolic extract of *Psidium guajava* L. leaves contained several phenolic components including gallic acid, catechin, caffeic acid, rutin, and chlorogenic acid (Fig. 1).

The concentrations of the phenolic compounds detected in the ethanolic extract by HPLC analysis are presented in Table 3. The most abundant phenolic compound in the extract was catechin, while caffeic acid was the least abundant.

Determination of the antimicrobial activity of pure *P. guajava* L. extract

Preservation efficacy of *P. guajava* L. extract tested at concentrations of 0.5%, 2.5%, 5%, 7.5%, and 10% w/w in purified water as the solvent, are presented in Table 4. The tests were conducted against five representative microorganisms: *Pseudomonas aeruginosa* (Gram-negative non-fermenting bacillus, ATCC 9027), *Escherichia coli* (Gram-negative bacillus, ATCC 8739), *Staphylococcus aureus* (Gram-positive coccus, ATCC 6538), *Candida albicans* (yeast, ATCC 10231), and *Aspergillus brasiliensis* (mold, ATCC 16404).

For an antimicrobial agent to function effectively as a preservative, it must demonstrate efficacy against all tested microorganisms. Our results revealed that *P. guajava* L. extract at 0.5% concentration exhibited no antimicrobial activity against the tested microorganisms. At 2.5%, the extract was effective against Gram-positive bacterium (*Staphylococcus aureus*), reducing the count from 8 log to 2.3 log during storage. However, its activity against the remaining tested microorganisms was minimal.

At a 5% concentration, the extract completely inhibited *Staphylococcus aureus*, confirming that increasing the concentration enhanced its antimicrobial activity against Gram-positive bacteria. The extract at 5% and 7.5% concentrations showed partial antimicrobial activity against Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and achieved complete antimicrobial activity at a 10% concentration.

Microbiological counts in negative controls (no preservative) remained consistently high (> 9 log CFU/mL), which supports the necessity of a preservative and confirms that there was no natural decline in microbial activity under tested conditions. The antibacterial action was validated as the extract alone, not outside influences, was responsible for the observed microbial reductions in the treated samples.

Antimicrobial efficacy test for *P. guajava* L. extract in semi-solid pharmaceutical shampoo preparations

A 10% concentration of *P. guajava* L. leaf extract was selected as a natural preservative in the shampoo formulation to replace synthetic chemical preservatives. The preservative efficacy testing demonstrated exceptional results, with ketoconazole-containing shampoos showing complete inhibition of microbial growth for *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus brasiliensis*) at both 14 and 28 days (Table 5). Additionally, a satisfactory reduction (2.1 log) in the count of *Pseudomonas aeruginosa* was observed.

To evaluate the safety and suitability of *P. guajava* L. extract, the shampoo formulations underwent comprehensive analyses. This included stability testing of the active ingredient (ketoconazole) over three months under accelerated conditions (40 °C/75% relative humidity) using HPLC. The analysis indicated a slight reduction in the concentration of ketoconazole from 99.5% to 96% over the three-month period, confirming the stability of the shampoo formulation despite the incorporation of guava leaf extract.

Pharmaceutical preparations with natural preservatives (*P. guajava* L.) leaf extract 10%), chemical preservatives, and without preservatives

Three formulations of ketoconazole shampoo were prepared (Table 6). The first formulation contained a natural preservative (*P. guajava* L. leaf extract at 10%). The second formulation (positive control) included chemical preservatives (methyl paraben and propyl paraben). The third formulation (negative control) was preservative-free. Antimicrobial efficacy tests were conducted on all three formulations. As shown in the results for the formulations containing the natural preservative and the chemical preservatives were very similar. Both formulations met the established criteria and passed the tests successfully.

Ketoconazole shampoo containing *P. guajava* L. leaf extract (10%)

The concentration of the active ingredient (ketoconazole) was evaluated using HPLC (Fig. 2) over a three-month period. The results indicated a 3.5% reduction in the concentration of ketoconazole, from 99.5% to 96%, in the shampoo containing 10% *P. guajava* L. leaf extract during the storage period. These findings suggest that the ketoconazole shampoo remained stable and was not adversely affected by the incorporation of *P. guajava* L. leaf extract (Table 7).

The natural preservative (*P. guajava* leaf extract) maintained its antimicrobial efficacy over the three-month period (Table 7). The physical and chemical characteristics of the formulations remained consistent, confirming the stability of the shampoo containing *P. guajava* leaf extract as a natural preservative.

To assess the microbiological stability of the pharmaceutical preparation, direct inoculation and total aerobic microbial count were conducted over three months under controlled conditions (40 °C/75% RH). It is important to note that ketoconazole may influence the antimicrobial evaluation results from the initial samples, depending on the storage duration.

Antimicrobial test of ketoconazole shampoo

Microbial limit test– direct transfer (Broth Media) for shampoo containing 10% P. guajava L. leaf extract

Table 8 presents the results of the direct transfer (broth medium) test conducted at day zero and after one, two, and three months of storage. These results were compared with those of the positive control (ketoconazole shampoo containing methylparaben and propylparaben). The findings indicated no microbial growth in the ketoconazole shampoo containing 10% *P. guajava* L. leaf extract, consistent with the results of the positive control. In contrast, the negative control (preservative-free shampoo) showed bacterial growth but no fungal growth.

Microbial limit test– total count for ketoconazole shampoo

Table 9 presents the results of the total microbial count test for ketoconazole shampoo. The microbial counts for the shampoo containing 10% *P. guajava* L. leaf extract were comparable to those of the shampoo containing chemical preservatives (methylparaben and propylparaben). In contrast, the negative control (preservative-free

Microorganism CFU/ml	ATCC No.	Day	Test Dilution*						Control Dilution*				
			0.5% w/w)	(2.5% w/w)	(5% w/w)	(7.5% w/w)	(10% w/w)	p- values	0.5% w/w)	(2.5% w/w)	(5% w/w)	(7.5% w/w)	(10% w/w)
S. aureus	6538	0	7.8	8	7.2	7.2	6.3	$P<0.05$	>9	>9	>9	>9	>9
		7	7.6	5.5	<1	<1	<1	$P<0.05$	>9	>9	>9	>9	>9
		14	6.9	3.1	<1	<1	<1	$P<0.05$	>9	>9	>9	>9	>9
		28	6.8	2.3	<1	<1	<1	$P<0.05$	8.7	8.7	8.7	8.7	8.7
			$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$						
P. aeruginosa	9027	0	7.9	7.6	6.3	6.2	6	$P<0.05$	>9	>9	>9	>9	>9
		7	7.8	7.3	6.0	4.7	<1	$P<0.05$	>9	>9	>9	>9	>9
		14	7.3	7.0	5.8	<1	<1	$P<0.05$	>9	>9	>9	>9	>9
		28	6.9	6.8	4.7	<1	<1	$P<0.05$	8.8	8.8	8.7	8.7	8.7
			$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$						
E. coli	8739	0	7.8	7.4	6.3	6.8	6	$P<0.05$	>9	>9	>9	>9	>9
		7	7.6	7.1	6.0	5.0	<1	$P<0.05$	>9	>9	>9	>9	>9
		14	7.3	7.0	5.6	<1	<1	$P<0.05$	>9	>9	>9	>9	>9
		28	7.0	6.2	4.9	<1	<1	$P<0.05$	8.6	8.6	8.7	8.7	8.7
			$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$						
Candida albicans	10231	0	7.8	7.8	7.8	7.8	6.2	$P<0.05$	>8	>8	>8	>8	>8
		7	7.8	7.0	6.3	6.3	6.0	$P<0.05$	>8	>8	>8	>8	>8
		14	7.7	6.7	6.0	6.0	5.6	$P<0.05$	>8	>8	>8	>8	>8
		28	7.0	6.5	5.8	5.8	5.5	$P<0.05$	7.3	7.3	7.3	7.3	7.3
			$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$						
A. brasiliensis	16404	0	7.0	7.8	7.8	7.2	6.3	$P<0.05$	>6	>6	>6	>6	>6
		7	6.7	7.0	7.3	6.3	5.1	$P<0.05$	>6	>6	>6	>6	>6
		14	6.0	5.9	6.8	5.8	4.9	$P<0.05$	>6	>6	>6	>6	>6
		28	5.8	5.4	6.9	5.9	4.8	$P<0.05$	>6	>6	>6	>9	>9
			$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$						

Table 4. Antimicrobial activity of *P. guajava* L. leaf extract powder at concentrations of 0.5%, 2.5%, 5%, 7.7%, and 10% w/w in purified water against selected microorganisms. * $\log_{10}$ colony-forming units per milliliter ($\log_{10}$ CFU/mL), P-values < 0.05 within the same row denote statistically significant differences among the tested extract concentrations, whereas p-values < 0.05 within the same column denote statistically significant differences among sampling points throughout the storage period.

Microorganism	ATCC No.	Day	Tested product Log (cfu/ml) *	Control Log (cfu/ml) *
S. aureus	6538	0	4.9	> 9
		7	< 1	> 9
		14	< 1	8.7
		28	< 1	8.3
P. aeruginosa	9027	0	6.4	> 9
		7	5.0	> 9
		14	4.3	> 9
		28	< 1	8.8
E. coli	8739	0	5.3	> 9
		7	3.4	> 9
		14	< 1	8.9
		28	< 1	8.6
Candida Albicans	10231	0	6	> 7
		7	< 1	> 7
		14	< 1	> 7
		28	< 1	> 7
A. brasiliensis	16404	0	5.7	> 6
		7	< 1	> 6
		14	< 1	> 6
		28	< 1	> 6

Table 5. Preservative effectiveness of ketoconazole shampoo formulated Wit 10% w/w *P. guajava* L. leaf extract as a natural preservative. * $\log_{10}$ colony-forming units per milliliter ($\log_{10}$ CFU/mL).

Microorganism CFU/ml	ATCC No.	Day	With extract 10%*	Positive control*	Negative control*
S. aureus	6538	0	4	4	> 4
		7	< 1	< 1	> 4
		14	< 1	< 1	> 4
		28	< 1	< 1	> 4
P. aeruginosa	9027	0	> 4	> 4	> 4
		7	> 4	4	> 4
		14	3.8	3	> 4
		28	2.4	< 1	> 4
E. coli	8739	0	> 4	> 4	> 4
		7	3.4	3.1	> 4
		14	< 1	< 1	> 4
		28	< 1	< 1	> 4
Candida albicans	10231	0	> 4	4	> 4
		7	< 1	< 1	> 4
		14	< 1	< 1	> 4
		28	< 1	< 1	3.5
A. brasiliensis	16404	0	> 4	> 4	> 4
		7	< 1	< 1	> 4
		14	< 1	< 1	4
		28	< 1	< 1	3

Table 6. Antimicrobial test for ketoconazole shampoo with: (natural preservative, chemical preservative, no preservative) * $\log_{10}$ colony-forming units per milliliter ($\log_{10}$ CFU/mL).

shampoo) showed a significantly higher bacterial count compared to the formulations with natural and chemical preservatives.

The direct inoculation tests in Soybean-Casein Digest Medium (SCDM) and Sabouraud Dextrose Broth (SDB) showed no microbial growth. The total microbial load on Soybean Casein Digest Agar (SCDA) for bacteria (≤ 200 CFU/g) and on Sabouraud Dextrose Agar (SDA) for yeasts and molds (≤ 20 CFU/g) falling within

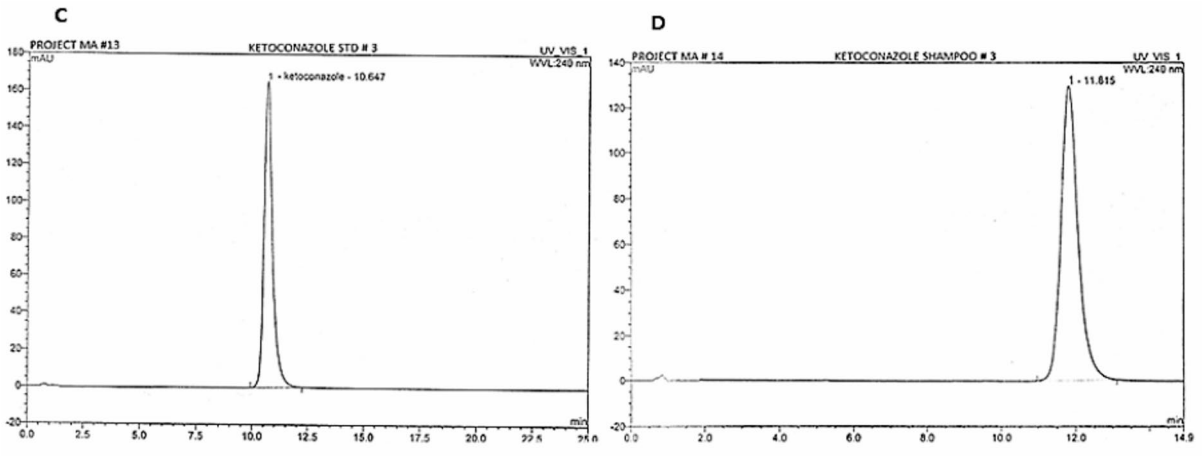

Fig. 2. **A**: Chromatogram of standard ketoconazole. **B**: Chromatogram of ketoconazole in shampoo preparation stored under accelerated conditions (40 °C/75% RH) for three months.

Time	Physical appearance	Odor	pH	Density (g/ml)	Viscosity (cp.)	Assay of Ketoconazole
Zero time	Dark green (oily color) clear thick shampoo	Kiwi	6.8 ± 0.1	1.03 ± 0.01	1500 ± 8	99.5%±1.2
First month	Dark green (oily color) clear thick shampoo	Kiwi	6.8 ± 0.1	1.03 ± 0.02	1509 ± 5	98.5%±0.8
Second month	Dark green (oily color) clear thick shampoo	Kiwi	6.5 ± 0.1	1.04 ± 0.01	1508 ± 7	97%±1.0
Third month	Dark green (oily color) clear thick shampoo	Kiwi	6.3 ± 0.1	1.04 ± 0.02	1520 ± 7	96%±0.7
P-values			$P < 0.5$	0.56	$P < 0.5$	$P < 0.5$

Table 7. Finished Preparation specifications of ketoconazole shampoo containing 10% *P. guajava* L. leaf extract at accelerated conditions (40 °C/75% RH). p-values < 0.05 within the same raw denote statistically significant differences among sampling points throughout the storage period.

Time	Direct transfer (broth media)	With 10% of guava leaves extract as a natural preservative	Positive control	Negative control
Zero time	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
First month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
Second month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear
Third month	Tryptic soy Broth	Clear	Clear	Turbid
	Sabouraud dextrose broth	Clear	Clear	Clear

Table 8. Antimicrobial limit (direct transfer test) for ketoconazole shampoo at accelerated conditions (40 °C/75% RH).

Time	Total count	With 10% of guava leaves extract as a natural preservative*	Positive control with chemical preservative (methyl and propyl paraben*	Negative control (Without Preservative) *
Zero time	Tryptic soy agar	< 10	< 10	< 120
	Sabouraud dextrose agar	< 10	< 10	< 10
First month	Tryptic soy agar	< 10	< 10	< 180
	Sabouraud dextrose agar	< 10	< 10	< 10
Second month	Tryptic soy agar	< 10	< 10	< 180
	Sabouraud dextrose agar	< 10	< 10	< 10
Third month	Tryptic soy agar	< 10	< 10	< 210
	Sabouraud dextrose agar	< 10	< 10	< 10

Table 9. Antimicrobial limit (total count test) for ketoconazole shampoo at accelerated conditions (40 °C/75% RH). * $\log_{10}$ colony-forming units per milliliter ($\log_{10}$ CFU/mL).

acceptable limits (Table 9). The results of the negative control underscored the importance of preservatives in all tested formulations.

Discussion

Our phytochemical analysis revealed the presence of several secondary metabolite classes-including phenols, tannins, terpenoids, flavonoids, and glycosides- in the ethanolic extract of *Psidium guajava* L. leaves. These results align with previous studies. For instance, Atik et al.[33] reported the presence of flavonoids, quinones, triterpenoids/steroids, alkaloids, tannins, and saponins in the ethanolic extract of *Psidium guajava* L. Similarly, Sherif et al.[34] identified 56 components in the ethanolic extract using HPLC, including gallic acid, catechin hydrate, caffeic acid, rutin, and chlorogenic acid, as well as other compounds not detected in our extract.

Psidium guajava L. contains a diverse array of chemical components, including carbohydrates, alkaloids, sterols, phenols, flavonoids, tannins, and saponins. It is important to note that the type and concentration of these phytochemicals can vary depending on factors such as the microclimate, soil conditions, plant tissue, and seasonal variations[35,36].

HPLC analysis identified catechin as the most abundant phenolic compound in the extract, while caffeic acid was the least abundant. These findings were consistent with those of Metwally et al.[37], who reported similar phytochemical compounds in their study.

P. guajava L. extract exhibited weak antifungal activity against *Candida albicans* and *Aspergillus brasiliensis* across all tested concentrations. Although a slight dose-dependent increase was observed, the overall activity remained low.

The antimicrobial activity of *P. guajava* L. extract can be attributed to several phytochemicals present in the extract. For instance, gallic acid has been shown to possess antibacterial and antifungal properties[38]. Additionally, Bae et al.[39] demonstrated that catechins exhibited antimicrobial activity. Dhiman et al.[40] found that the methanolic extract of *P. guajava* L. showed potent antibacterial activity against *E. coli* (MIC = 0.78 µg/mL) and moderate antifungal activity (MIC = 12.5 µg/mL).

Notably, a 10% w/w concentration of *P. guajava* L. leaf extract effectively replaced synthetic preservatives in shampoo, demonstrating complete inhibition of *E. coli*, *S. aureus*, *C. albicans*, and *A. brasiliensis* over 28 days, along with a 2.1 log reduction in *P. aeruginosa*. These results confirm its potential as a natural, safe, and effective preservative for pharmaceutical and cosmetic applications.

These findings align with work of Godebo et al.[41], who demonstrated that shampoo formulations incorporating certain plant-based biological preservatives exhibited strong antimicrobial activity. In their study, the minimum inhibitory concentration (MIC) qualitative antimicrobial test revealed good antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Aspergillus niger*.

This study investigated the use of medicinal plants, specifically *Psidium guajava* L. leaf extract, as a tool for effective antimicrobial preservation in the formulation of ketoconazole shampoo. The plant selected for this study is *Psidium guajava* L., specifically its leaves, which are known for their remarkable antimicrobial properties against a wide range of bacteria, including *P. aeruginosa*, *E. coli*, *Streptococcus faecalis*, *S. aureus*, and *Bacillus subtilis*[42]. Beyond its antimicrobial activities, *P. guajava* offers antioxidant and anti-inflammatory benefits due to its rich composition of phenolic acids, quercetin (a major flavonoid), triterpenoids such as betulinic acid, glycosides, and saponins[41]. Specific compounds, including gallic acid, chlorogenic acid, and rutin inhibit fungal growth by targeting key the cell membrane while tannins, act as bacteriostatic agents[43]. Furthermore, triterpenoids contribute to combating bacterial and fungal pathogens by modulating the inflammatory response and inhibiting bacterial colonization[44]. This antimicrobial profile makes *P. guajava* a promising candidate for use in cosmetic and pharmaceutical products, such as shampoos, where effective preservation against microbial growth is critical to ensuring product safety and quality.

Overall, the results confirmed the microbiological stability of the formulations. The shampoo containing 10% *P. guajava* L. leaf extract retained its antimicrobial efficacy over the three-month accelerated stability study (40 °C / 75% RH). The three shampoo formulations were subjected to accelerated storage conditions (40 °C/75% RH) to evaluate their stability, including odor, density, viscosity, and pH. The characteristics of the ketoconazole shampoo containing 10% *P. guajava* L. leaf extract as a natural preservative were similar to those of the shampoo containing chemical preservatives. These results fell within accepted limits and passed all tests, confirming the stability of the formulation. These findings align with those reported by Shukla et al.[45].

The *P. guajava* extract exhibited potent antibacterial activity for up to 28 days, in contrast to synthetic preservatives like parabens, which are known to retain antimicrobial efficacy for the duration of the usual shelf life of pharmaceutical and cosmetic formulations (which can range from 6 months to 2 years, depending on formulation and storage conditions). This demonstrates its short- to medium-term promise as a natural preservative. It is crucial to remember that these findings do not yet support equivalency to parabens' long-term effectiveness. Therefore, more research is needed to ascertain the extract's long-term preservation capability and potential as a full replacement for parabens, even if early stability testing indicates promising performance.

Conclusions

The results of this study show that ethanolic leaf extract from *Psidium guajava* L. works well as a natural preservative in shampoo formulations containing ketoconazole. The extract demonstrated broad-spectrum antibacterial action at a 10% (w/w) concentration, attaining a 2.1 log decrease in *Pseudomonas aeruginosa* over a 28-day period while totally inhibiting *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus brasiliensis*. These findings show that the extract has the potential to be a good substitute for synthetic preservatives because it not only meets but also surpasses the preservative effectiveness standards specified in USP/BP recommendations for topical products.

The antibacterial action of *P. guajava* extract was proven by phytochemical analysis to be derived from its high phenolic content, which includes rutin, gallic acid, and catechin. These compounds cause oxidative stress and damage microbial membranes. Furthermore, the extract-maintained shampoo stability in accelerated circumstances, preserving its physicochemical characteristics (pH, viscosity) and ketoconazole potency (96.0 ± 0.7%) for three months.

In conclusion, the use of *P. guajava* leaf extract as a natural preservative in shampoos with medicinal ingredients is well supported by evidence gathered from this study. Because of its stability, effectiveness, and other therapeutic advantages, it is a viable option for both clinical and commercial use, which will help dermatological goods move toward plant-based substitutes. Translational studies should be the main emphasis of future research to help industrial formulations embrace it.

In comparison to artificial preservatives such as parabens, *P. guajava* extract has antibacterial, antioxidant, and anti-inflammatory qualities along with the ability to reduce the risk of skin irritation. Although further study is needed to assess long-term stability, in vivo safety, and synergistic formulations for increased efficacy at lower doses, these advantages are in line with the growing desire for natural cosmetic ingredients.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Ethics approval and consent to participate

The current dataset does not meet the regulatory definition of human subject research. As such, an Institutional Review Board (IRB) review is not required.

Additional information

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