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Research Article

DESIGN OF EXPERIMENTS-GUIDED EXTRACTION OF BIOACTIVE FLAVONOIDS FROM *PSIDIUM GUAJAVA L.* LEAVES: ANTIOXIDANT AND CYTOTOXIC EVALUATIONS

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ABSTRACT

Objectives: The objectives of the study are to optimize the Soxhlet extraction of flavonoids from guava leaves and evaluate the antioxidant and anticancer potential of the extract.

Methods: Extraction parameters such as solvent volume, temperature, and time were optimized using response surface methodology based on a Box–Behnken design. Phytochemical profiling was conducted using preliminary screening, thin-layer chromatography, ultraviolet-visible spectrophotometry, and high-performance liquid chromatography to confirm the presence of quercetin. Antioxidant activity was assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, reduced glutathione (GSH), glutathione peroxidase (GPx), and glutathione S-transferase (GST) assays. Cytotoxicity against HeLa cervical cancer cells was evaluated using the MTT assay and DNA fragmentation analysis.

Results: Optimization improved flavonoid extraction efficiency. Phytochemical analysis confirmed the presence of quercetin. The extract exhibited strong antioxidant activity, evidenced by high DPPH scavenging and increased levels of GSH, GPx, and GST. The MTT assay showed significant antiproliferative activity against HeLa cells, with an IC_{50} of 9.36 µg/mL. DNA fragmentation indicated apoptosis induction.

Conclusion: The optimized Soxhlet extraction effectively enhances flavonoid yield from *Psidium guajava* leaves. The extract demonstrates notable antioxidant and anticancer properties, highlighting its promise as a candidate for pharmaceutical and nutraceutical applications.

Keywords: *Psidium guajava* L., Flavonoids, Soxhlet extraction, Cytotoxicity, Design of experiments, Box–Behnken design.

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INTRODUCTION

Natural products continue to serve as a prolific source of pharmacologically active compounds, with plant-derived metabolites playing a pivotal role in drug discovery and development [1]. Among the diverse flora investigated for therapeutic potential, *Psidium guajava L.* (Guava), a member of the Myrtaceae family, has garnered considerable attention due to its broad spectrum of bioactive constituents [2,3]. Traditionally employed in ethnomedicine for the treatment of gastrointestinal, inflammatory, and infectious conditions, guava leaves are particularly rich in flavonoids, phenolics, tannins, and alkaloids – classes of compounds known for their antioxidant and cytoprotective properties [4]. Quercetin, a prominent flavonoid in guava leaves, has been extensively studied for its potent radical scavenging activity and potential anticancer effects [5,6]. However, the efficacy of phytoconstituent extraction is inherently dependent on various physicochemical parameters, including solvent composition, extraction temperature, and duration [7]. Optimizing these parameters is crucial for maximizing yield and ensuring the reproducibility of bioactivity in downstream applications [8]. Design of experiments (DOE), particularly the response surface methodology (RSM) employing Box–Behnken design (BBD), offers a robust statistical approach to model and optimize multi-variable processes in a systematic and efficient manner [9,10]. In the present study, Soxhlet extraction of guava leaves was optimized using BBD to enhance the extraction efficiency of flavonoids [11]. Subsequently, the phytochemical profile of the optimized extract was characterized using thin-layer chromatography (TLC), ultraviolet (UV)-visible spectrophotometry, and high-performance liquid chromatography (HPLC) [12-14]. The extract's antioxidant potential was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, while its cytotoxic efficacy was assessed *in vitro* using the MTT assay on HeLa cervical cancer cell lines [15]. Additional enzymatic assays, including those for glutathione peroxidase (GPx), glutathione S-transferase (GST), and reduced glutathione (GSH), were conducted to elucidate the extract's antioxidant mechanisms [16]. This study aims to establish an optimized extraction protocol for *P. guajava* leaves and validate their pharmacological potential through integrated phytochemical and bioactivity assessments, thereby supporting their use in therapeutic and nutraceutical applications [17].

METHODS

Chemicals used in this study

All the chemicals used in the study were of analytical or molecular biology grade and were procured from reliable sources. Ethanol, dimethyl sulfoxide (DMSO), Ellman's reagent (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced GSH, MTT reagent, penicillin G, and ethidium bromide were purchased from Sigma-Aldrich (Merck India). Water, ethylenediaminetetraacetic acid (EDTA), disodium hydrogen phosphate, L-glutamine, amphotericin B, agarose, phenol: chloroform:isoamyl alcohol mixture, isopropanol, and TE buffer (Tris-based) were obtained from Thermo Fisher Scientific India. Formic acid, glacial acetic, and ethyl acetate acid were procured from Prakash Chemicals International Pvt. Ltd. Anisaldehyde and phosphate buffer were sourced from CDH Fine Chemicals. Sulfuric acid was supplied by Aarti Industries, while silica gel was obtained from Sorbead India and boric acid from Organic Industries Pvt. Ltd. Acetonitrile was sourced from Jindal Speciality Chemicals, and methanol was procured from Vinati Organics. Quercetin was purchased from Manus Aktteva Biopharma LLP and DPPH from Sisco Research Laboratories. Trichloroacetic acid was obtained from Sujan Chemicals, while sodium azide and sodium dodecyl sulfate (SDS) were sourced from Loba

Chemie. Chloroform and isoamyl alcohol, used as part of the extraction mixture, were also supplied by Thermo Fisher Scientific.

Plant material collection and preparation

The plant sample was collected from home-grown trees in Chennai during 2025. The specimen was authenticated based on morphological characteristics and standard taxonomic references. Expert identification was performed by Ms. Vandhana, Lecturer, Department of Pharmacognosy, Saveetha College of Pharmacy. A voucher specimen has been deposited at the herbarium of Saveetha College of Pharmacy for future reference. Fresh guava leaves (*P. guajava*) were collected and carefully washed with distilled water to remove surface impurities and then shade-dried at room temperature to preserve their phytochemical integrity. Once fully dried, the leaves were pulverized into a coarse powder using a mechanical grinder. A total of 50 g of powdered guava leaves were used for extraction.

Inclusion and exclusion criteria

Only mature, healthy *P. guajava* L. leaves collected from organically grown home gardens in Chennai were included. Samples were authenticated by a Ms V Vandhana, Lecturer, Department of Pharmacognosy, Saveetha College of Pharmacy, and a voucher specimen was deposited. Leaves were shade-dried at ambient temperature to preserve phytoconstituents. Leaves showing signs of disease, pest infestation, mechanical damage, or discoloration were excluded. Samples exposed to agrochemicals or dried under direct sunlight or heat were not considered. Non-leaf parts were also excluded.

DOE

In the present study, guava (*P. guajava*) leaf extraction efficiency was optimized and assessed employing the BBD of the response surface approach. A total of 16 experimental runs, including four replicates at the center point to estimate experimental error, were performed [18,19]. The experimental design and subsequent statistical analyses were carried out using Design-Expert software (version 7.0.0; Stat-Ease Inc., Minneapolis, MN, USA). The extraction efficiency was calculated using the following equation:

$$\frac{Initial\ mass\ of\ plant\ material}{Mass\ of\ extract\ obtained} \times 100$$

A fit summary was generated to evaluate the adequacy of different models for predicting the extraction efficiency of guava leaf extracts (Table 1). Based on the statistical parameters obtained, including the p-values, adjusted R^2, and predicted R^2, the quadratic model was suggested as the most suitable. The quadratic model exhibited a significant p-value (0.0002), an acceptable lack of fit (p=0.0005), a high adjusted R^2 (0.9019), and a moderately acceptable predicted R^2 (0.3747), indicating a good fit to the experimental data. In contrast, the linear and two-factor interaction (2FI) models were found to be statistically insignificant, with higher p-values (0.6667 and 0.9894, respectively) and negative values for both adjusted and predicted R^2, indicating poor model performance. The cubic model, despite a significant p-value (0.0005), was aliased and thus deemed unreliable due to overfitting and confounding effects among the terms.

Soxhlet extraction and experimental design

The Soxhlet extraction process was conducted using varying solvent concentrations, temperatures, and extraction durations to optimize flavonoid yield [20]. The BBD under RSM was employed using design-expert software to determine the most effective extraction conditions [21]. Three independent variables were selected: solvent volume (120 mL, 150 mL, 170 mL), temperature (30°C, 50°C, and 70°C), and extraction time (4 h, 6 h, and 8 h). Experimental runs were conducted as per the software-generated design matrix (Table 1). The Soxhlet apparatus was used for extraction, where 50 g of coarse guava leaf powder was introduced into the thimble, and solvent was continuously refluxed over the sample.

Table 1: Extraction efficiency of flavonoids under varying solvent volume, temperature, and time conditions

Run	Volume of solvent (mL)	Temperature (°C)	Time (h)	Extraction efficiency (%)
1	150	50	6	88.7
2	150	50	6	89
3	150	50	6	88.9
4	150	30	8	82
5	120	70	6	78
6	120	30	6	79
7	150	30	4	81.8
8	180	50	8	86
9	120	50	8	83
10	180	50	4	80
11	150	70	8	84.6
12	180	70	6	79
13	150	70	4	81.3
14	180	30	6	79
15	120	50	4	78
16	150	50	6	88.9

Phytochemical analysis

To find whether the guava leaf extract consists of flavonoids, phenolic chemicals, tannins, saponins, and alkaloids, the crude extract was put through a first phytochemical screening. Standard qualitative tests were conducted, such as the lead acetate test for tannins, the ferric chloride test for phenol compounds, the Shinoda test for flavonoids, and the sodium hydroxide test for flavonoids [22,23]. Based on distinctive color changes or precipitate development, observations were made.

Separation of phytoconstituents by column chromatography

Flash column chromatography was conducted using a pipette-based column setup. A clean glass pipette (10 mL capacity) was used as the chromatography column. A small piece of cotton was inserted at the tip to serve as a support for the stationary phase. Silica gel 60 (70–230 mesh) or neutral alumina was used as the stationary phase, packed to a height of approximately 5–6 cm. A layer of fine sand (~0.5 cm) was added above the stationary phase to protect the surface during sample loading and elution. The column was pre-wetted with eluent before sample loading. After loading the sample onto the top of the silica/sand bed, the column was eluted under gentle pressure. Fractions were collected sequentially in clean test tubes placed beneath the column. Under UV light, TLC was used to track fractions on silica gel plates. A rotary evaporator was used to mix and concentrate the appropriate fractions that contained the required component at lower pressure [24].

Identification of phytoconstituents by TLC

TLC was performed to identify quercetin in the guava leaf extract. TLC plates were prepared using silica gel-G as the stationary phase. The plates were activated by heating at 105°C for 30 min. A small amount of the extract was spotted 2 cm from the base of the plate using a capillary tube. The mobile phase consisted of hexane: ethyl acetate (70:30, v/v). Before the plate was introduced, the development chamber was saturated with the mobile phase for half an hour. The plates were taken out and left to air dry after development. Under UV light, visualization was carried out at 254 and 366 nm. To verify identification, spots in the sample were matched to standard quercetin.

UV-visible spectrophotometry

The UV-visible absorbance profile of the guava leaf extract was recorded using a calibrated UV-visible spectrophotometer (Labmate). Two sample preparations were analyzed:

- Sample A (fractionated extract): 1 mL extract diluted in 10 mL ethanol: water (70:30, v/v)
- Sample B (crude extract): 1 mg dried extract dissolved in 10 mL ethanol: water (70:30, v/v).

Serial dilutions were prepared to yield concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL. Ethanol was used as a blank. Absorbance was measured across 200–400 nm. Boric acid served as a reference standard [25]. The wavelength of maximum absorbance (λ_{max}) was recorded, and calibration curves were generated for quantitative evaluation.

HPLC

HPLC (Shimadzu) was used to quantify the amount of quercetin present in the extract. The system included a C18 column (250 mm × 4.6 mm, 5 µm), a binary pump, and a UV-visible detector. Acetonitrile and methanol (50:50, v/v) made up the mobile phase, which was filtered through a 0.45 µm membrane and degassed. The detector was set at 256 nm, and the flow rate was kept constant at 1.0 mL/min. Both the sample and the reference solutions had an injection volume of 20 µL. Peak regions were used to create a calibration curve after a standard quercetin solution was made in ethanol. Under the same circumstances, the test extract was injected. To ascertain the quercetin content, the sample's retention time (RT) and peak area were contrasted with those of the standard [26,27].

DPPH radical scavenging assay

To assess the guava leaf extract's antioxidant capacity, the DPPH radical scavenging technique was employed. In short, several test sample concentrations (20–100 µg/mL) were made. While the control contained 3.5 mL of ethanol and 0.3 mL of DPPH, each tube was filled with 3 mL of ethanol and 0.3 mL of a 0.5 mM DPPH solution. For half an hour, the mixes were allowed to sit at room temperature in the dark. At 517 nm, absorbance was measured with a UV-visible spectrophotometer (Shimadzu UV-1900i). Every test was run in triplicate [28-30].

Reduced GSH assay

GSH content was determined using DTNB (DTNB-5,5'dithiobis[2-nitrobenzoic acid]). A 100 µL aliquot of each sample was mixed with 5% trichloroacetic acid and centrifuged at 10,000 rpm for 10 min. The supernatant (100 µL) was mixed with 1 mL phosphate buffer (0.1 M, pH 7.4) and 2 mL of DTNB. After 10 min of incubation at room temperature, absorbance was measured at 412 nm. GSH concentration was expressed in µg per mL using a standard curve of reduced GSH [31].

GPx assay

Spectrophotometric measurements were made of GPx activity. 0.4 mL of 0.4 M phosphate buffer (pH 7.0), 0.2 mL of 0.8 mM EDTA, 0.1 mL of 10 mM sodium azide, 0.2 mL of 4 mM reduced GSH, and 0.1 mL of 2.5 mM hydrogen peroxide were combined with each 100 µL sample. 0.5 mL of 10% TCA was added to terminate the reaction after it had been incubated for 10 min at 37°C. Next, 1 mL of DTNB and 3 mL of 0.3 M disodium hydrogen phosphate were added. At 420 nm, absorbance was measured. Using bovine serum albumin (BSA) as the standard, GPx activity was computed as enzyme activity (min/mg protein) [32].

GST assay

CDNB was used as the substrate to assess GST activity. 100 µL of material, 1 mL of 0.3 M phosphate buffer (pH 6.5), and 0.1 mL of 30 mM CDNB were all included in each reaction mixture. After using distilled water to adjust the volume to 2.5 mL, the mixture was incubated for 10 min at 37°C. The reaction was then started by adding 0.1 mL of 30 mM reduced GSH. At 340 nm, absorbance was measured. Using a standard curve produced with BSA, enzyme activity is measured in units per min/mg protein [33].

MTT assay for *in vitro* anticancer activity

The MTT assay was used to assess the test sample's cytotoxic potential on HeLa cervical cancer cell lines. L-glutamine, antibiotics (penicillin, streptomycin, and amphotericin B), and 10% fetal bovine serum were added to the minimal essential medium in which the cells were cultivated. After being seeded onto 96-well plates, HeLa cells (1×10^5 cells/well) were incubated for 72 h at 37°C in an environment with 5% CO_2. Cells were cultured for 48 h more after being treated with different doses of the test chemical (7.8–1000 µg/mL) dissolved in 0.1% DMSO. Following treatment, each well received 20 µL of MTT reagent (5 mg/mL in phosphate-buffered saline [PBS]), which was then left to incubate for 4–6 h in the dark. A microplate reader (Thermo Scientific, 2021) was used to detect absorbance at 540 nm after the formazan crystals were dissolved in 100 µL of DMSO [34,35]. Three duplicates of each experiment were conducted, and the IC_{50} value was calculated.

DNA fragmentation assay

DNA fragmentation, an indicator of apoptosis, was assessed using agarose gel electrophoresis (Orange India). HeLa cells were seeded into 12-well plates (1 mL/well) and incubated at 37°C with 5% CO_2 for 72 h. One milliliter of the test substance (10 µg/mL) was added after cell attachment, and the mixture was incubated for 24 h. Trypsinization was used to collect the cells, and PBS was used for washing. 100 µL of 10% SDS was used to lyse the cell pellet after it had been resuspended in 200 µL of 1X TE buffer. After 20 min of incubation at 50°C, 300 µL of phenol: chloroform:isoamyl alcohol (25:24:1) was added, and the mixture was centrifuged for 10 min at 10,000 rpm. After transferring the aqueous phase to a fresh tube, 1 mL of isopropanol was used to precipitate the DNA, which was then cleaned with 70% ethanol and allowed to air dry. Twenty microliters of TE buffer were used to dissolve the DNA pellet. 20 µL of DNA along with loading dye was added to each well of an agarose gel (1.2%) containing ethidium bromide. At 50 V, electrophoresis was performed. A UV transilluminator was utilized to view bands of DNA. A distinctive ladder pattern was used to identify apoptotic DNA fragmentation [36].

RESULTS AND DISCUSSION

The analysis of variance table indicates that the overall model is statistically significant (p=0.0015), meaning the combination of extraction variables significantly affects the response, likely plant extract yield (Table 3). Among the linear terms, only extraction time (C) has a significant effect (p=0.0078), while volume of solvent (A) and temperature (B) do not. Interaction effects between variables (AB, AC, BC) are also not significant, suggesting they do not influence the response jointly. However, the quadratic terms A^2, B^2, and C^2 are all significant, indicating that non-linear effects play a key role in optimizing the process. The significant lack of fit (p=0.0005), however, suggests that the model does not perfectly describe the data, possibly due to missing higher-order terms or other unaccounted factors, even though the pure error is low, reflecting good experimental precision.

Effect of volume of solvent on extraction efficiency

Plant constituents were extracted using varying solvent volumes (120 mL, 150 mL, and 170 mL). The extraction efficiency increased notably with an increase in solvent volume, reaching its peak at approximately 150 mL, after which a gradual decline was observed (Fig. 1). This trend aligns with previous studies, which suggest that insufficient solvent volume can limit solute diffusion and mass transfer, thereby reducing extraction efficiency. Conversely, excessively high solvent volumes may dilute the concentration gradient or alter solvent-solute interactions, negatively impacting the extraction rate and overall effectiveness [37].

Effect of temperature

Plant constituents were extracted at different temperatures (30°C, 50°C, and 70°C). The extraction efficiency increased markedly with rising temperature, reaching an optimum at approximately 50°C, followed by a gradual decline at higher temperatures (Fig. 2). This observation is consistent with prior studies, which indicate that lower temperatures may hinder the solubilization and diffusion of phytochemicals, resulting in reduced extraction efficiency. However, elevated temperatures beyond the optimal range can lead to the degradation of thermolabile compounds or alter solvent properties, thereby adversely affecting the extraction rate and overall efficiency [38].

Effect of time

Plant constituents were extracted over varying durations (4 h, 6 h, and 8 h). As depicted in the figure, extraction efficiency increased

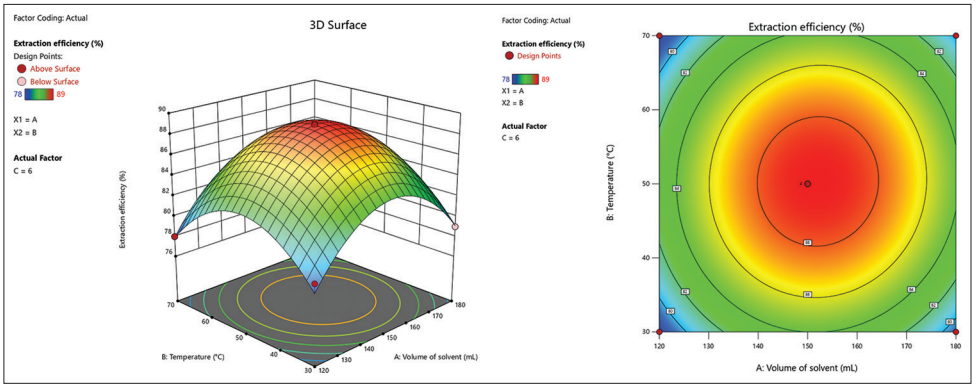

Fig. 1: Effect of volume of solvent on extraction efficiency

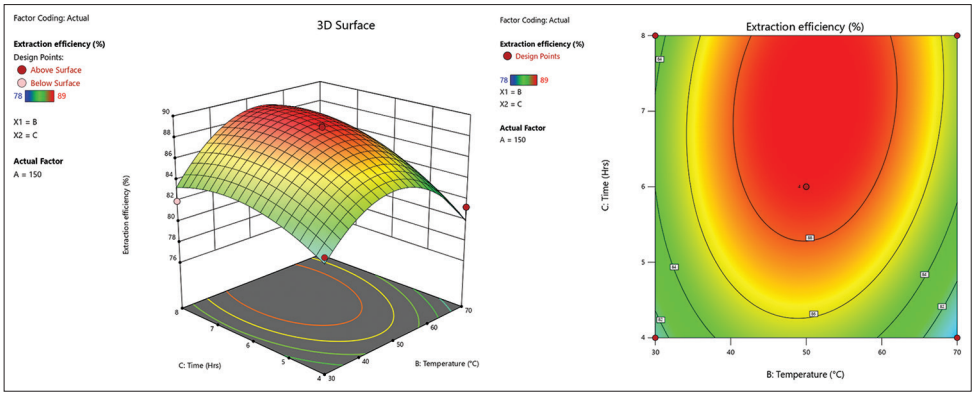

Fig. 2: Effect of temperature on extraction efficiency

substantially with time, reaching its maximum at approximately 6 h, followed by a gradual decline with prolonged extraction (Fig. 3). This trend is supported by experiments, which indicate that shorter extraction times may not allow sufficient diffusion and solubilization of phytoconstituents, resulting in lower yields. However, extended Soxhlet extraction durations can lead to the degradation of sensitive compounds or solvent saturation, thereby negatively impacting the efficiency and rate of extraction [38].

Phytochemical screening

Preliminary phytochemical screening of *P. guajava L.* leaf extract revealed the presence of key secondary metabolites including flavonoids, tannins, phenolic compounds, saponins, and alkaloids. The presence of flavonoids was indicated by the development of a pink-magenta coloration in the Shinoda test, while the ferric chloride test yielded a dark green color for phenolics. A white precipitate observed in the lead acetate test confirmed tannins. The foam test indicated moderate saponin content [22,23]. These results underscore the extract's therapeutic potential and justify further chromatographic profiling.

Column chromatography

The separation and purification of the target compound were successfully achieved using flash column chromatography with a pipette column. TLC analysis was used to monitor the elution process and confirm the purity of collected fractions. As shown in Fig. 2, initial TLC plates indicated multiple spots in the crude sample, suggesting the presence of at least two components. After chromatographic separation, the collected fractions were analyzed, and a single distinct fluorescent spot was observed in the purified samples. This is evident from the TLC images, where the purified fraction (right lane) shows a single, well-resolved spot under UV light, whereas the crude sample (left lane) displays multiple spots corresponding to a mixture. The purified compound was consistent across fractions, indicating a successful separation from impurities. The use of an appropriate eluent system (e.g., hexane: ethyl acetate in a suitable ratio) enabled clear resolution. The presence of distinct, non-overlapping bands suggests good column performance and effective elution under mild pressure. The reproducibility of the TLC results across different plates (Figs. 2-4) further confirms the reliability of the purification method [24]. The bright and compact nature of the spot in the purified lane supports the compound's purity, which was further confirmed by the absence of trailing or tailing.

TLC

To further verify the existence of flavonoids, particularly quercetin, in the ethanol extract of guava leaves, TLC analysis was performed. The developed TLC plates, when visualized under UV light at 366 nm, exhibited distinct fluorescent bands, indicating the presence of phytoconstituents. As shown in the TLC plate images (Figs. 4 and 5), multiple bands with varying Rf values were observed, prominently including a bright pink band that matched the R_f value of the quercetin standard. The components were successfully separated using the solvent system of ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:26 v/v/v/v). The existence of quercetin in the guava leaf extract was suggested by the test extract's main fluorescent spot's Rf value of around 0.72, which closely matched the standard quercetin under the same conditions. Post-derivatization with anisaldehyde-sulfuric acid and heating resulted in intensified coloration of the spots, further confirming the presence of flavonoids. The developed pink to violet zones, which are characteristic of flavonoid derivatives under such visualization, supported these findings.

Interpretation of TLC results

The observed TLC profile supports the successful extraction of quercetin and potentially other flavonoid compounds (Fig. 4). The presence of multiple spots of differing R_f values in the extract sample also implies the existence of other phenolic or flavonoid compounds that co-exist in *P. guajava* (Fig. 5). The intensity and clarity of bands under UV light

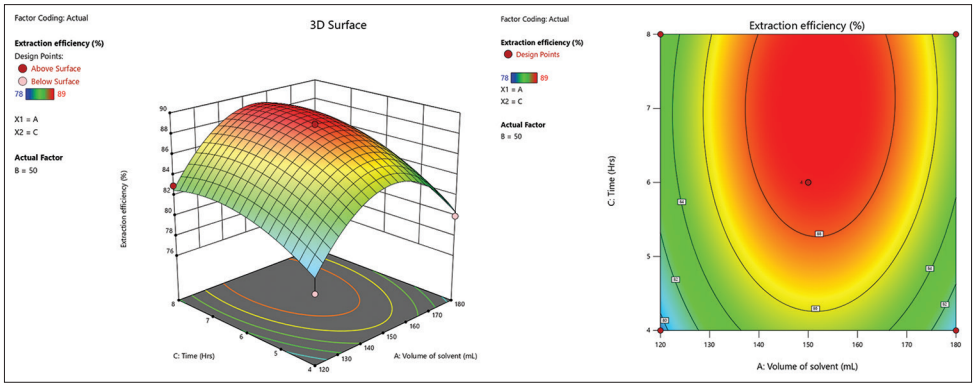

Fig. 3: Effect of time on extraction efficiency

validate the efficiency of the Soxhlet extraction method optimized through BBD. These chromatographic results corroborate the findings of HPLC and UV spectrophotometry, providing a reliable, cost-effective, and visual confirmation of the phytochemical richness of the extract. TLC thus proved to be a valuable preliminary technique to authenticate and qualitatively assess bioactive compounds in plant-based extracts.

Extract analysis by UV spectroscopy

The present study employed UV-Visible spectrophotometry to investigate the photochemical properties of guava (*P. guajava*) leaf extract obtained through Soxhlet extraction. Both crude and fractionated extracts were analyzed across a concentration range of 0.2 to 1.0 mg/mL using a 70:30 (v/v) ethanol-water solvent system. Boric acid was used as a reference standard to facilitate comparative assessment. The absorbance data demonstrated a concentration-dependent increase in UV absorption for both the crude extract and the standard, suggesting conformity with Beer-Lambert's law and indicating the feasibility of using UV spectroscopy for quantitative estimation of extract constituents (Fig. 6). The crude extract consistently exhibited higher absorbance values compared to the standard, particularly at elevated concentrations (0.8 and 1.0 mg/mL) (Fig. 7). This trend implies a higher density of UV-absorbing phytoconstituents, potentially flavonoids, tannins, or phenolic acids, which are known to be abundant in guava leaves (Figure. Notably, a sharp increase in absorbance was observed at concentrations above 0.6 mg/mL, reflecting the extract's rich composition of chromophoric groups capable of absorbing in the UV region (Fig. 8). Although the wavelength of maximum absorbance (λmax) was not specified in the current dataset, previous literature indicates that polyphenolic compounds typically show λmax values between 250 and 350 nm, aligning with the expected spectral behavior of guava leaf phytochemicals. These findings underscore the utility of UV-visible spectroscopy as a rapid, cost-effective preliminary tool for phytochemical screening and standardization of plant-based extracts. Further analytical techniques such as HPLC or LC-MS are recommended to isolate and characterize individual bioactive constituents responsible for the observed UV absorbance profile.

Identification of phytoconstituent by HPLC

The HPLC analysis was conducted to determine and quantify the quercetin content in *P. guajava L.* leaf extract. The chromatographic separation was performed using a UV detector set at 256 nm, and the elution profiles of both the standard quercetin and the guava extract were recorded. The standard quercetin solution exhibited a sharp, well-defined peak at a RT of 1.672 min, confirming the chromatographic behavior of pure quercetin (Fig. 1). In comparison, the chromatogram of the guava leaf extract showed a major peak at 1.560 min (Fig. 2), closely aligning with the standard. This minor difference in RT is acceptable and may be attributed to matrix effects or slight differences in solvent interaction during elution. The presence of quercetin in the guava extract is strongly suggested by the similarity in RT and UV detection wavelength. In addition, the extract chromatogram displayed

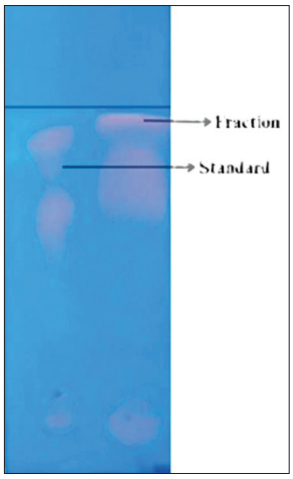

Fig. 4: TLC chromatogram of standard and fraction

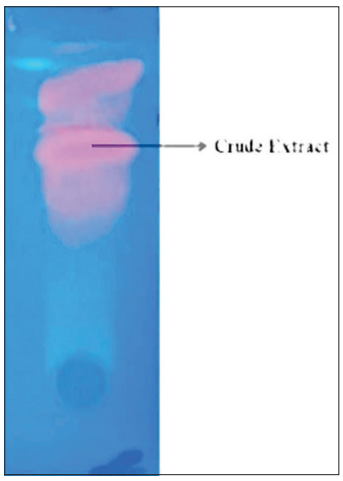

Fig. 5: Thin-layer chromatography chromatogram of crude extract

several minor peaks, suggesting the presence of other phenolic and flavonoid compounds co-extracted with quercetin. These secondary peaks highlight the chemical complexity and richness of the guava leaf phytochemical profile. A quantitative analysis of peak areas demonstrated that the quercetin content in the standard (Fig. 9) was significantly lower than that in the guava leaf extract (Fig. 10), especially after adjusting for injection volume differences. These results are in line with earlier research showing that *P. guajava* leaves contain significant levels of flavonoids, especially quercetin. Overall, the HPLC analysis confirms that guava leaf extract is a potent source of quercetin

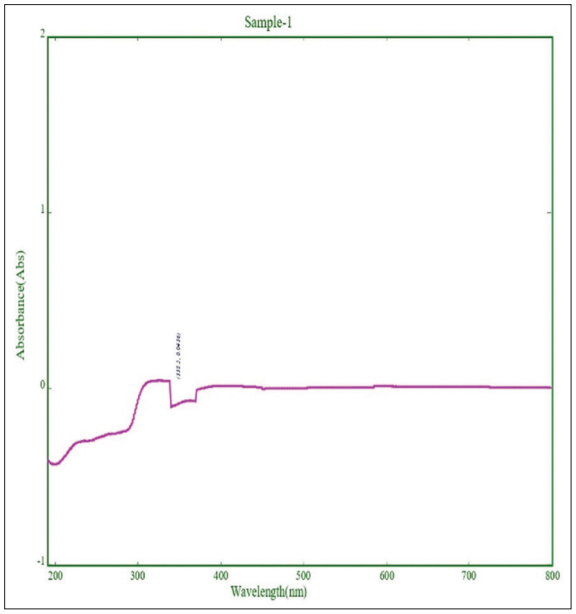

Fig. 6: Reference ultraviolet-visible absorption spectrum of standard quercetin

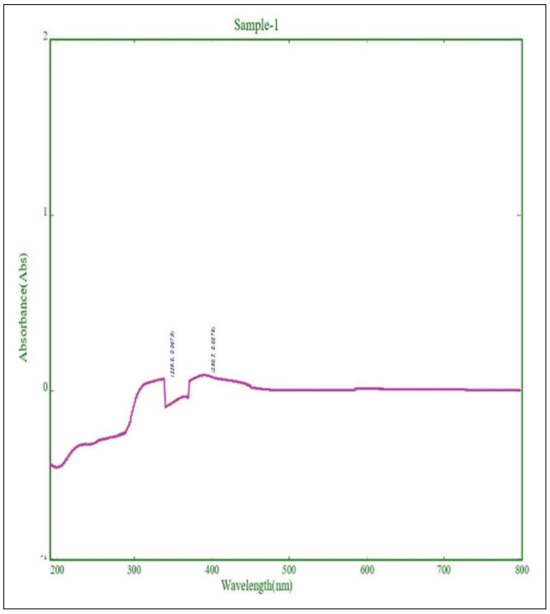

Fig. 8: Ultraviolet-visible absorption spectrum of fraction isolated through column chromatography

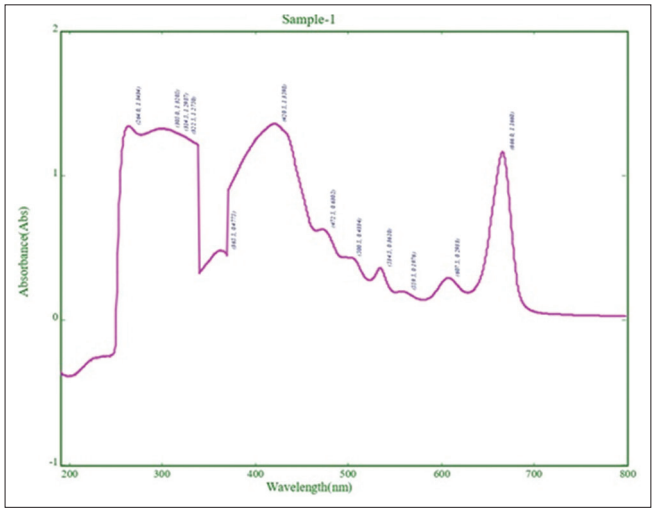

Fig. 7: Ultraviolet-visible absorption spectrum of crude ethanolic extract of guava leaves

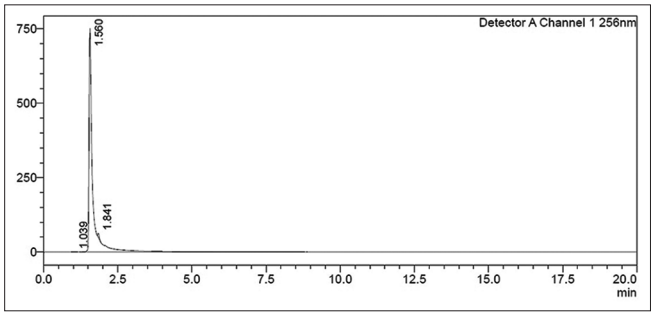

Fig. 9: High-performance liquid chromatography chromatogram of standard quercetin

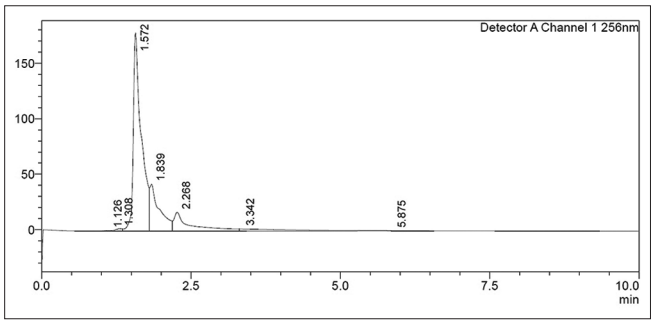

Fig. 10: High-performance liquid chromatography chromatogram of a fraction of *Psidium guajava*

and other phenolic constituents. The presence of a dominant quercetin peak along with secondary bioactive compounds underscores the therapeutic potential of guava leaf extract, particularly in antioxidant, anti-inflammatory, and cytoprotective applications.

DPPH radical scavenging assay

The antioxidant activity of *P. guajava L.* leaf extract was evaluated using the DPPH radical scavenging assay and compared to the synthetic standard, butylated hydroxytoluene (BHT). The results showed a clear concentration-dependent increase in % inhibition for both the sample and standard, indicating progressive free radical scavenging with increasing concentration. At the lowest tested concentration (20 µg/mL), the guava leaf extract exhibited a higher inhibition percentage (73.0±1.0%) compared to BHT (63.1±1.0%). This trend persisted across all tested concentrations. At 40 µg/mL, the extract achieved 85.8±0.9% inhibition, while BHT reached only 71.1±1.0%. At 60 µg/mL, the extract showed 88.4±1.0% inhibition, whereas BHT recorded 75.3±0.6%. The extract's inhibition peaked at 100% at 100 µg/mL, equal to that of BHT, although the extract consistently showed stronger inhibition at lower doses. A bar chart with standard deviation (Fig. 1) clearly demonstrates the superior antioxidant activity of the guava extract across all tested concentrations, emphasizing its efficiency in neutralizing free radicals [28]. In addition, the statistical distribution demonstrates the constant and repeatable antioxidant action of the extract. The high concentration of polyphenolic components, especially flavonoids like quercetin, which were verified by HPLC analysis, may be responsible for the extract's strong action. Quercetin is known for its high radical scavenging activity, and its abundance in guava leaf extract provides a strong basis for the observed results. Previous studies have similarly reported that *P. guajava* contains a wide

spectrum of phytoconstituents with antioxidant potential, supporting the findings of this study. The results affirm that *P. guajava* leaf extract demonstrates significant DPPH radical scavenging activity, surpassing that of BHT in most concentrations tested. This positions guava leaf extract as a promising natural antioxidant source for potential use in nutraceutical and pharmaceutical applications.

Reduced GSH

The study used a modified DTNB-based colorimetric test to estimate the amount of reduced GSH in a sample. The results showed a concentration-dependent rise in GSH content, with a linear association between absorbance and standard GSH concentrations. The GSH content increased gradually with concentration, from 40 µg/mL (5 µg GSH) to a maximum of 40 µg at 100 µg/mL sample concentration. This suggests that the sample contains bioactive substances that can preserve or increase reduced GSH availability. The increasing amounts of reduced GSH may indicate the sample's inherent antioxidant capacity. The findings are consistent with the DPPH assay results, which showed substantial antioxidant activity [39]. These results highlight the sample's potential as a therapeutic agent to strengthen cellular antioxidant defense systems. To completely comprehend the sample's antioxidative efficiency and method of action, more investigation is required.

Glutathione peroxidase

The study evaluated the activity of GPx, a crucial antioxidant enzyme involved in detoxifying hydrogen peroxide and organic hydroperoxides. The results showed that the test sample's GPx activity increased in a concentration-dependent manner. The standard curve used was derived from BSA, with optical density values ranging from 0.10 to 0.50 for concentrations between 20 µg and 100 µg. The sample components effectively upregulated or stabilized GPx expression, demonstrating the sample's capacity to strengthen enzymatic antioxidant defense systems. This suggests that the sample may offer cytoprotective effects under oxidative stress conditions, as GPx is essential in shielding cells from oxidative damage by lowering peroxides [40]. The results support the sample's excellent antioxidant qualities, which are in line with the findings of the DPPH and reduced GSH assays. Future studies should focus on identifying bioactive compounds causing this impact and assessing their applicability *in vivo* in models linked to oxidative stress.

GST activity

The study measured GST activity spectrophotometrically using reduced GSH as a co-substrate and CDNB as the substrate. The enzyme's efficiency was measured in units per minute per milligram of protein. The assay's linearity was confirmed when the BSA concentration increased, and the absorbance increased proportionately. The detected GST concentrations varied from 18 µg to 40 µg, indicating the enzyme's existence and activity in the samples. The results support the hypothesis that protein concentration and enzyme activity are related, possibly due to increased availability of CDNB and GSH. The assay's accuracy is supported by standard deviations and triplicate readings. Future research could use more replicates or different techniques. The GST activity assay effectively measured enzyme activity at different concentrations, validating its precision.

***In vitro* anticancer activity on cervical cancer (HeLa) cell line**

The calculated IC_{50} value of 9.36 µg/mL indicates that the test compound possesses potent cytotoxic activity against HeLa cells (Fig. 12). These results suggest that the compound could effectively inhibit cervical cancer cell proliferation at relatively low concentrations, making it a promising candidate for further exploration in cancer therapeutics. Moreover, the observed morphological changes and reduction in MTT conversion to formazan may reflect loss of mitochondrial function and cellular integrity, indicative of apoptotic or necrotic pathways being triggered by the compound. While this study confirms the compound's cytotoxicity, further studies including mechanistic evaluations such as flow cytometry, caspase activation assays, and gene expression profiling are warranted to elucidate the precise mode of cell death (Fig. 13) [41,42].

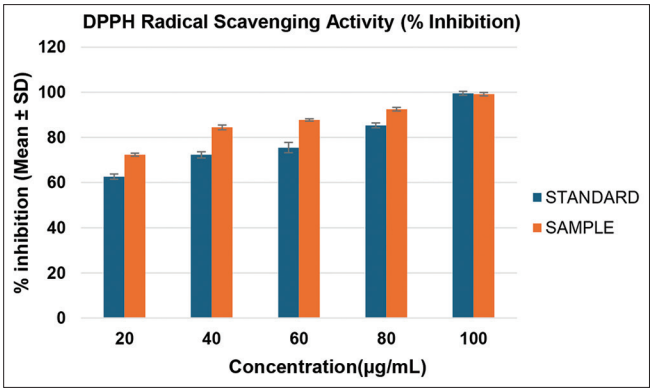

Fig. 11: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of guava leaf extract

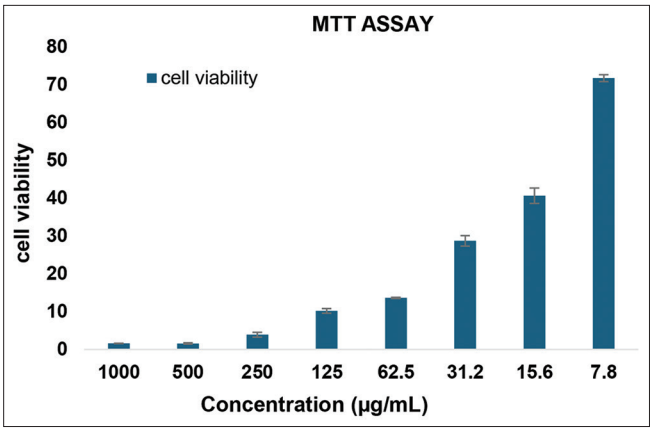

Fig. 12: *In vitro* anticancer activity of the extract on HeLa (cervical cancer) cell line

DNA fragmentation assay

DNA fragmentation analysis was performed to evaluate the induction of apoptosis in HeLa cells following treatment with the test compound. The results, as visualized through agarose gel electrophoresis, demonstrated clear differences between treated and untreated cells. DNA isolated from the treated HeLa cells (Lane 1) showed a distinct laddering pattern, characterized by multiple discrete bands ranging approximately from 1000 bp to 6000 bp. This pattern is indicative of internucleosomal DNA cleavage, a biochemical hallmark of apoptosis, confirming that the treatment effectively triggered programmed cell death. In contrast, the untreated control cells (Lane 2) displayed a single high-molecular-weight DNA band without any evidence of fragmentation, reflecting intact genomic DNA and the absence of apoptosis. The 1 KB DNA ladder (Lane 3) served as a molecular size marker, facilitating the estimation of fragment lengths. Collectively, these findings substantiate the apoptotic potential of the tested formulation in HeLa cells and underscore its promise as a therapeutic agent.

DISCUSSION

The current study illustrates the effective use of BBD within the framework of RSM to optimize the Soxhlet extraction of flavonoids from *P. guajava* L. leaves. The statistical model exhibited a significant p-value (p=0.0002) and an adjusted R^2 of 0.9019, indicating high model reliability in predicting extraction efficiency. This approach has proven instrumental in reducing experimental workload while maximizing optimization efficiency, consistent with recent phytochemical extraction studies employing BBD for natural products [21]. Optimal extraction conditions such as 150 mL solvent volume, 50°C, and 6 h were identified. These conditions align with thermodynamic principles,

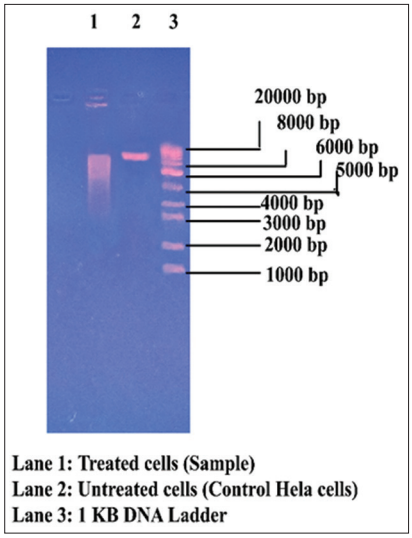

Fig. 13: DNA fragmentation assay indicating apoptosis in HeLa cells treated with the extract

where moderate heat promotes solute solubilization without degrading thermolabile flavonoids. Similar findings reported an efficiency drop at higher temperatures due to compound degradation and matrix breakdown [37,38].

Qualitative phytochemical analysis confirmed the presence of flavonoids, tannins, alkaloids, and saponins, correlating with *P. guajava*'s traditional ethnomedicinal use and previous phytochemistry reports [22,23]. Further confirmation of quercetin was achieved through TLC and HPLC, where the extract's RT (1.560 min) closely matched the standard (1.672 min). This correlation is in agreement with studies confirming quercetin as a principal bioactive flavonoid in *P. guajava* leaves [26]. UV-Visible spectrophotometric analysis revealed a concentration-dependent absorbance increase, validating the extract's flavonoid richness and supporting its quantifiability through Beer–Lambert's law. Similar trends were observed in flavonoid-rich extracts of other medicinal plants [8]. Antioxidant activity assessed by the DPPH radical scavenging assay showed strong inhibition (up to 100% at 100 µg/mL), outperforming the synthetic standard BHT at lower doses. This superior activity is attributed to quercetin and related polyphenols known for high radical-neutralizing potential [28]. Complementary enzymatic assays showed elevated GSH, GPx, and GST levels, suggesting enhancement of intracellular antioxidant systems [16].

The extract's cytotoxicity against HeLa cervical cancer cells, reflected by an IC_{50} of 9.36 µg/mL, aligns with previous literature reporting flavonoid-induced growth inhibition through mitochondrial-dependent apoptosis NA cleavage, often associated with caspase-mediated cell death [41]. Overall, this work successfully demonstrates that DOE-guided extraction of *P. guajava* leaves yields a potent flavonoid-rich extract with significant antioxidant and anticancer activity. The integration of chemometric optimization, phytochemical validation, and bioactivity profiling offers a holistic approach, supporting the extract's potential for pharmaceutical and nutraceutical development. Future research should focus on isolating bioactive constituents, elucidating molecular targets, and evaluating *in vivo* pharmacological effects.

The DNA fragmentation assay clearly demonstrates the induction of apoptosis in HeLa cells treated with the test formulation, as shown by the characteristic laddering pattern on agarose gel electrophoresis. This internucleosomal DNA cleavage, a hallmark of apoptosis, is typically mediated by caspase-activated DNase during the execution phase of cell death. In contrast, untreated control cells exhibited intact genomic DNA without fragmentation, confirming the specificity of the apoptotic response to the treatment. Likewise, the DNA fragmentation observed indicates effective apoptosis induction in HeLa cells. This aligns with the findings of *P. guajava* extract triggered apoptosis in MCF-7 cells by increasing the Bax/Bcl-2 ratio. Their study confirmed mitochondrial pathway involvement and DNA fragmentation [43]. These results support the potential of plant-derived compounds in cancer therapy. Flavonoids such as quercetin and guavinoside C triggered DNA fragmentation and mitochondrial disruption. These phytochemicals demonstrated selective cytotoxicity, sparing normal cells [44,45]. Taken together, these studies validate the use of DNA fragmentation as a reliable biomarker of apoptosis and support the potential of the present formulation as a promising anti-cancer agent.

CONCLUSION

This work describes a methodical strategy to maximize the extraction of *P. guajava* L. leaves by combining Soxhlet extraction with a Box–Behnken experimental design. The methodological framework applied enabled the effective identification of key variables influencing extraction efficiency and provided a structured basis for process optimization. The extraction method's appropriateness for downstream phytochemical analysis and biological assessments was validated by subsequent analytical methods. The overall approach demonstrates the potential of combining statistical modeling with classical extraction techniques to enhance the quality and reliability of natural product research. Further studies are recommended to explore compound-specific activities, mechanisms of action, and scalability of the optimized extraction process.

ETHICAL APPROVAL

Not applicable. This study does not involve any animal or human participants requiring ethical approval.

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AUTHOR'S CONTRIBUTIONS

Sam Helinto J: Designed and executed the experimental methodology, performed Soxhlet extraction, statistical modeling using DOE, and analytical characterization. Prena D: Contributed to literature review, manuscript drafting, and data organization. Arockiya Rabin A: Executed the experimental methodology and analytical characterization helped in the data acquisition. Gohulram S, Joyal S, and Thrisha S: Data collection, biological assay, and editing the manuscript. Dr. Renukadevi J*: Provided conceptualization, overall supervision of the research work, ensured methodological validation, critically revised the manuscript, and approved the final version for submission. All authors have read and approved the final version of the manuscript

CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this study. The research and its outcomes were not influenced by any personal or financial relationships.

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CONSENT STATEMENT

Not applicable. No human participants, personal data, or identifiable images were used in this study.

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