Phytoconstituent optimization by response surface methodology and pharmaceutical activities of *Pelargonium graveolens* L'Hér acetone extract

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Abstract

Context: *Pelargonium graveolens* L' Hér is an evergreen shrub, cultivated principally for the medicinal essence and decoction in Southern Africa for the treatment of menopausal problems, tonsillitis, poor circulation, ringworm, and cervical cancer. **Objective:** We aimed to optimize the extraction of phenolics and flavonoids from *P. graveolens* by response surface methodology with particular attention on the proliferative and cytotoxic effects on human keratinocytes, as well as the antioxidant and antibacterial activities. **Materials and Methods:** The optimization was achieved by Box–Behnken design. Extract, metabolite yields, and minimal inhibitory concentrations (MIC) were determined by gravimetric, spectrophotometric, and microdilution methods, respectively. The antiradical potentials were evaluated using the phosphomolybdate, 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and lipid peroxidation assays. The kinetics of the lipid protective activity was studied and fitted into models. The proliferative and cytotoxic effects were evaluated using the CellTiter® Blue cell viability and lactate dehydrogenase assay. **Results:** The regression coefficient $r^2 \geq 0.9775$ indicated a close correlation between actual and predicted values of the responses. The ideal parameter for the extraction of phenolics and flavonoids by macerations was determined as an extraction time: 9.63–12.01 h, material mass: 2.78–2.62 g, agitation speed: 143.11–191.37 rpm, and solvent volume: 68.06–69.87 mL. Total antioxidant capacity and reducing power were comparable to standard gallic acid, while the antiradical activity has IC50 values of 0.18 ± 0.03–4.98 ± 0.15 mg/mL. Further, the lipid protective revealed a dose-dependent activity fitting into a pseudo-second-order kinetic model. MIC value of 1.56 mg/mL was registered against *Staphylococcus aureus* and *Salmonella typhi* compared to chloramphenicol. There was a significant ($P < 0.05$) increase in cell proliferation and viability when the extract was administered at concentrations of ≤ 50 μg/mL. However, at ≥ 100 μg/mL concentration, there was significant cytotoxicity in comparison to the untreated cells. **Conclusion:** These biological activities are confirmation of the phytomedicinal application and possible source of pharmaceutical compounds. However, administration of the decoction should take into cognizance the antiproliferative effect at doses ≥ 100 μg/mL as well as the potential to induce and maintain keratinocyte proliferation at low concentration with an eye on the antiproliferative effect at concentrations ≥ 100 μg/mL.

Key words: Antibacterial, antioxidant, cell viability, flavonoids, lipid peroxidation, maceration, phenolics

INTRODUCTION

Plant-derived bioactive compounds in the past few decades have become of great interest owing to their rich ethnomedicinal and nutraceutical potentials and versatile pharmaceutical applications.[1] The most acceptable method of assessing the bioactive metabolite is through extraction, which is the separation of secondary principles through standard protocols from plant or animal sources using appropriate solvents. The resulting secondary metabolites are dried by various methods and applied as galenicals.[2] Since the early 21st century, different extraction techniques have been developed for the collection of therapeutic principles, which are usually a complex mixture of many medicinal metabolites such as alkaloids, glycosides, terpenoids, flavonoids, tannins, and saponins.[3] Some of the commonly applied techniques for the extraction of medicinal herbs according to literature reports include maceration, Soxhlet, countercurrent, microwave-assisted, ultrasound, supercritical fluid, and phytonic extractions.[4–6] The quality of the medicinal metabolite recovered is dependent on the type of technique, plant part, and solvent used. Furthermore, the choice of the extraction technique employed influences the yield of the secondary metabolite and availability of thermolabile compounds.[7,8] A comparative review of literature of the various extraction techniques revealed that maceration is most suitable for the extraction of thermolabile compounds, conservation of metabolite integrity, and cost-effective.[8] However, the major setback of maceration is the poor yield of the desired principle.

The best attempt to optimize an extraction process was done using a one-factor-at-a-time experiment which has many pitfalls such as wastage of resources, low precision, and inestimable interaction effects.[9,10] In the field of engineering, many researchers are currently applying design experiment for the optimization of process engineering, but no attempt has been made to optimize the extraction of bioactive principles specifically from *Pelargonium graveolens*. *P. graveolens* is a shrubby perennial member of the Geraniaceae family of the *Pelargonium* genus indigenous to South Africa.[11] Conventionally, it has been used for the treatment of wounds, eczema, ulcers, acne, bruises and broken capillaries, liver, kidney functions, blood regulation, and depression.[12] Other ethno-application includes analgesic, anticancer, antifungal, and treating some hormonal problems.[13]

Optimization of extraction conditions to improve the quality of secondary metabolite, yield crude extract, total phenolic, and flavonoid from herbs has seldom been investigated. The solubility of extract, total phenolic, and flavonoid compounds, as well as the phytoconstituent functionality, may be affected by various independent variables, such as length of the extraction period, solvent polarity, temperature,[14] plant tissue sizes, pH, and solvent-to-sample ratio.[15,16]

Under these circumstances, when many factors and interactions affect desired phytochemical yields and

biological integrity, design experiment such as response surface methodology (RSM) will be an ideal tool for the optimization of the process.[17,18] RSM is a useful statistical method that uses a minimum of resources and quantitative data from the appropriate experimental design to determine and simultaneously solve a multivariate equation.[19] It usually uses an experimental design such as central composite or Box–Behnken Designs (BBD) to fit an empirical, full second-order polynomial model, which generally provides an adequate representation of the most continuous response surfaces over a relatively broad factor domain.[20]

Our focus in this study is to optimize the independent variables such as the mass of plant material, agitation speed, solvent volume, and contact time for the optimal extraction of specific principles from *P. graveolens* by maceration method with the aid of RSM. The effects of these variables on the extract, total phenolic, and flavonoids yields, as well as the antioxidant, antibacterial, cell proliferation, viability, and cytotoxicity, were evaluated [Figure 1].

In this study, acetone was chosen because of the capacity to dissolve hydrophilic and lipophilic phytoconstituents, low toxicity,[21] and the high solubility of phenolics and flavonoid compounds.[22]

MATERIALS AND METHODS

Chemicals and Equipment

All chemicals and reagents used in this study were of analytical grade. The optical density was measured on a spectrophotometer (Agilent Technologies Cary 60 UV-Vis, Santa Clara, CA).

Collection and Preparation of *P. graveolens*

Aerial parts were collected from the Sebokeng (26.5634° S, 27.8339° E), Gauteng Province, South Africa. A sample of the plant was authenticated, and voucher number: BP01026
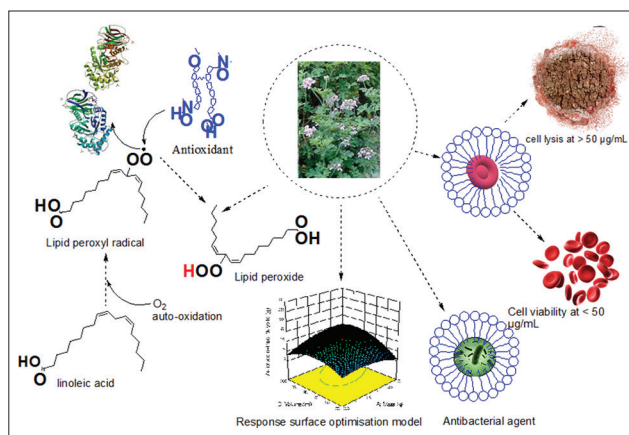

Figure 1: Graphical abstract

assigned at the South African National Biodiversity Institute, Pretoria, South Africa. The leaves were carefully separated, washed with distilled water, dried in open air for 14 days, and crushed on an S4 Drosky grinder to give powder material. The powdered sample was sieved on an ASTM standard sieve to obtain a 100 µm size material. For the maceration process, 3 g of the powder material was used at the desired volume of acetone, agitation speed, and contact time. The obtained menstruum after each of the experimental run was filtered using a Whatman filter paper No 2 and then evaporated to a dark green semi-solid extract under reduced pressure.

Total Phenolic Content (TPC)

TPC of the acetone extract was estimated according to the protocol of Ainsworth and Gillespie.[23] Extract (about 1000 µg) was taken in a test tube, and 1 mL of acetone, 3.16 mL distilled water, and 500 µL Folin–Ciocalteu reagent were added. After incubating for 15 min at 20°C in a test tube, 1.5 mL of saturated Na_2CO_3 solution was introduced, covered with Al-foil, and further incubated for 30 min at 25°C. Standard gallic acid solution (10–100 mg/mL) and a blank were prepared and treated using the same procedure. After that, the absorbance of the sample, standard, and blank was determined on a spectrophotometer at 300 nm. Then, the TPC was expressed as milligram of gallic acid equivalent per gram of sample.

Total Flavonoid Content (TFC)

The TFC was determined using the reported protocol of Chang *et al.*,[24] with slight modification. To 3 mL extract (0.3 mg/mL in acetone) in a test tube, 20 mL acetone was added to obtain a clear solution. Then, 150 µL of aqueous $NaNO_3$ solution (0.5 M) was added followed by 2 g of $AlCl_3$. After 5 min, 1 mL NaOH solution (1 M) was added, and the content was mixed well before measuring its absorbance at 412 nm on a spectrophotometer against a blank, which was prepared by the same procedure. Similarly, a calibration curve of quercetin was obtained (for concentrations ranging from 10 to 100 mg/mL), and the TFC of each extract was expressed as milligram of quercetin equivalent (QE) per gram of sample.

Experimental Design

The percentage crude extract (Y%) content, TPC, and TFC obtained by acetone extraction of *P. graveolens* were optimized through the aid of RSM.[25,26] The BBD consisting of 30 experimental runs with three replicates at the center point was used to investigate four influential parameters: Mass of plant material (X1), agitation speed (X2), contact time (X3), and solvent volume (X4) [Table 1].

The unexplained variability in the observed response due to extraneous factors was minimized by randomizing the order of experiments. At the end of the quantitative studies for the three responses, the data were fitted into a second-order polynomial equation (1):

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ii} X_i X_j \quad (1)$$

Where Y is the predicted responses, while o, i, ii, and ij are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and X_i and X_j are the independent variables.

Data Analysis

Design-Expert software version 11 was used to analyze the experimental data obtained from the investigation of the influence of the independent variables on the response factors.[26] Then, the software was used to generate response surfaces and contour plots according to BBD while holding a variable constant in quadratic polynomial second-order model.

Antioxidant Properties

The antioxidant activity of the *P. graveolens* acetone extract was evaluated by the following established protocols: Reducing power assay, total antioxidant capacity (TAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS·+) decolorization assay, and lipid peroxidation inhibitory assay. Gallic acid and quercetin were used as antioxidant controls.

Reducing Power Assay

Antioxidant capacity as per reducing power assay was measured according to a method reported by Oyaizu,[27] with slight modification. In a test tube, 2.5 mL plant extract (1 mg/mL in acetone), 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 mL potassium ferricyanide (1% w/v in distilled water) were added and mixed well. The mixture was incubated in a water bath for 20 min at 50°C. Then, 2.5 mL trichloroacetic acid (10% w/v in distilled water) was added, and the mixture was centrifuged at 650 rpm for 10 min. The supernatant (5 mL) was taken into a test tube, and 5 mL of distilled water and 1 mL ferric chloride (0.1% w/v in distilled water) solution were added and mixed well. Absorbance was measured at 700 nm. Blank for each

Table 1: Independent variables and the coded and actual values used for optimization

Independent variable	Units	Symbol	Coded levels		
			−1	0	1
Mass	g	X_1	0.5	1.75	3.0
Agitation speed	rpm	X_2	100	150	200
Contact time	h	X_3	12	18	24
Solvent volume	mL	X_4	50	75	100

solvent was run using the same procedure but replacing the plant extract with an equal volume of solvent. Gallic acid was used as a standard, and the total reducing power is expressed as milligram of QE/g.

Total Antioxidant Capacity (TAC)

According to the protocol of Prieto *et al.*[28] and Jan *et al.*,[29] with slight modification; the TAC of the *P. graveolens* acetone extract was determined using the phosphomolybdate assay. About 0.25 mg of plant extract was dissolved in 1 mL of warm methanol to get a homogeneous mixture. A stock solution of quercetin (1 mg/mL) was prepared in methanol, from which dilutions were made ranging from 0.8 mg/mL to 0.2 mg/mL. In a vial, 0.3 mL extract solution was mixed with 3 mL phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The vial was covered and incubated at 95°C for 90 min. The mixture was then allowed to reach ambient temperature; then, the absorbance was recorded at 765 nm. Blank was prepared and run using the same procedure. The antioxidant capacity was reported as mg QE/g.

DPPH Radical Scavenging Activity

The DPPH free radical scavenging activity of the extract was determined according to the method reported by Brand-Williams *et al.*,[30] The 0.05 mM DPPH solution was prepared by dissolving 1.97 mg DPPH in 100 mL methanol. In a vial, 3.5 mL DPPH working solution was mixed with various concentrations (1 mg/mL–0.2 mg/mL) of 1 mL extract solution or the standard solution. The absorbance was measured at 517 nm after incubating for 30 min in the dark. The percentage radical scavenging activity was calculated using the equation (2):

$$\text{Radical scavenging activity} \% = \left(\frac{Ac - As}{Ac} \right) \times 100 \tag{2}$$

Where Ac and As are the absorbance of DPPH control and *P. graveolens* acetone extract, respectively.

ABTS·+ Decolorization Assay

Antioxidant activity of *P. graveolens* extract as per ABTS·+ decolorization assay was measured using the method reported by Siddhuraju and Manian,[31] with some modifications. The working solution of ABTS•+ radical was made by reacting ABTS (9.5 mL, 7 mM) with potassium persulfate (245 µL, 100 mM) and raising the volume to 10 mL with distilled water. The solution was stored in a dark bottle in a refrigerator for 48 h and then diluted with phosphate buffer solution (0.05 M, pH 6.8) to an absorbance of 0.8577 at 734 nm. Extract concentrations were prepared in methanol with dilutions 0.2–1 mg/mL. A sample (500 µL) was introduced into vial and mixed thoroughly with 3.7 mL ABTS·+ working solution. Absorbance of the resulting clear mixture was recorded at 734 nm. The percentage antioxidant activity of the sample was determined using the equation:[3]

$$\text{Antioxidant activity} \% = \left(\frac{Ac - As}{Ac} \right) 100 \tag{3}$$

Where Ac and As are the absorbances of the absorbance of DPPH control and *P. graveolens* acetone extract, respectively.

Lipid Peroxidation Inhibitory Assay

The lipid peroxidation inhibitory activity of the extract was determined using the method described by Mitsuda *et al.*[32] Substrate emulsion was prepared by mixing 155 µL linoleic acid and 175 µg Tween-20 in 50 mL phosphate buffer (0.05 mM pH 6.8). To 100 µL of 5 mg/mL extract solution, 2.4 mL phosphate buffer and 2.5 mL linoleic acid emulsion were mixed. The mixture was then incubated for 30 min at 37°C and stored away in the dark for 10 days. A 100 µL of the mixture was taken at a pre-determined time interval of 24, 48, 72, 144, and 192 h and mixed with 3.7 mL ethanol and 100 µL of 20 mM ferrous chloride solution in 3.5% HCl. The contents were shaken for 10 min before the addition of 100 µL potassium thiocyanate solution (30% in distilled water). The absorbance of the resulting clear solution and blank was recorded at 500 nm. The kinetics of the inhibition was determined from the pseudo-first and pseudo-second-order models as presented in the equations 4 and 5, respectively.

$$\log(A_{UT} - A_t) = \log A_{UT} - \left(\frac{k_1}{2.303} \right) t \tag{4}$$

$$\frac{t}{A_t} = \frac{1}{k_2 A_{UT}^2} + \frac{t}{A_{UT}} \tag{5}$$

Where AUT is the absorbance of the untreated medium at a given time, t is the time of evaluation, A_t is the absorbance of extract treated medium at a given time, while k_1 and k_2 are the rate constants for pseudo-first and second-order kinetics, respectively.

In this study, we assume that there is a linear relationship between absorbance and concentration at a fixed path length, according to Beer-Lambert approximation.[33,34] Hence, the concentration term of the kinetic equation can be substituted in the equations 4 and 5.

Antibacterial Activity

Preparation of Resazurin-based indicator and plant extract

Exactly 1.5 mg of sodium,10-oxido-7-oxophenoxazin-10-ium-3-olate was dissolved in 100 mL of distilled water, vortexed, filtered, sterilized, and stored at 4°C for a maximum of 14 days before being applied as a growth indicator. The stock solution of the extract (50 mg/mL) was prepared in sterilized 2% dimethyl sulfoxide (DMSO)-lysogeny broth.

Test Microorganisms

Test bacteria were obtained from the clinical strains stored and maintained at 4°C in the Biotechnology Laboratory, Vaal University of Technology. The selected bacteria strains were found be closely associated with skin and wound infections, which include the following standard strains: *Staphylococcus aureus*, *Escherichia coli, Enterococcus faecalis,* and *Salmonella typhi*.

Preparation of Standardized Inoculum

Isolated colonies from a 24 h culture agar plate were collected with a loop and transferred to a tube containing lysogeny broth. The various suspensions were incubated at 37°C and the size adjusted to 1.5×10^6 CFU/mL.[35]

Antimicrobial Assay of the Extract

The antibacterial activity of the extract was evaluated against the selected bacterial strains by the microdilution method. Columns 1–11 was filled with 50 µL LB, followed by the addition of 50 µL of extract to well 1. A multichannel pipette was then used to transfer 50 µL of extract from the first well to the remaining well up to well 10, resulting in 50 µL extract per well. Well 11 contained 100 µL of standardized inoculum, while well 12 contained 100 µL of LB as sterility control. Further, 50 µL of the microorganism suspension was then added to all wells 1–11. The microtiter plate was incubated for 24 h at 37°C, followed by the addition of 30 µL of resazurin-based indicator solution and incubated further another 2 h for the observation of color change. On completion of the incubation, columns with blue indicator color were registered as the minimum inhibitory concentration (MIC) (mg/mL) values.

Cell Line

A human keratinocyte cell line HaCaT (Cellonex, Johannesburg, South Africa) was grown and maintained in complete cell culture medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and antibiotics (10,000 U/mL penicillin G and 10 mg/mL streptomycin) at 37°C in a 5% CO_2 incubator (ESCO, Horsham, PA). The culture medium was replaced with fresh medium every 3 days until the cells were 80% confluent. The cells were washed, trypsinized, and prepared for the cell proliferation assay.

Cell Proliferation Assay

The effect of the acetone extract on the proliferation of human keratinocytes was evaluated using the CellTiter® Blue Cell Viability Assay (Promega, Cat. No. G8081) according to the manufacturer's instructions with few modifications. The

HaCaT cells were seeded in a 96-well microtiter plate at a concentration of 1×10^4 cells/mL at a volume of 200 µL per well of complete cell culture medium and incubated at 37°C in a 5% CO_2 humidified CO_2 incubator. After 48 h of incubation when confluence had reached 80%, the medium was replaced with fresh complete cell culture medium containing different concentrations of acetone extract dissolved in 0.75% DMSO (1, 25, 250, 100, 250, 500, and 1000 µg/mL). Four control groups were set up and these consisted of (i) cell culture medium only (negative control to determine the background absorbance), (ii) untreated cells (vehicle control), (iii) medium with 0.75% DMSO (used as solvent for the extracts), and (iv) cell treated with hydrogen peroxide (H_2O_2) (used as negative control). After treatment, the cells were incubated for different time intervals (24, 96, and 168 h) at 37°C in a humidified 5% CO_2 incubator. At the end of each incubation period, 20 µL/well of CellTiter® Blue Reagent was added to each well containing the remaining culture medium. The plate was then agitated for 10 s and incubated at 37°C for 4 h in a humidified 5% CO_2 incubator. At the end of each incubation period, 50 µL supernatant was transferred to a new 96-well plate for lactate dehydrogenase (LDH) assay. Then, 20 µL/well of CellTiter® Blue Reagent was added to each well containing the remaining culture medium, and the plate was then agitated for 10 s and incubated at 37°C for 4 h in a humidified 5% CO_2 incubator. Thereafter, 100 µL medium containing CellTiter® Blue reagent was transferred into a 96-well microtiter plate, and the absorbance of the dissolved resorufin dye was determined at 570 and 600 nm using an EPOCH 2 (BioTek, Vermont) plate reader. Thereafter, the stained plates were used to determine the number of attached cells using crystal violet assay.

Cell Viability Assay

The viability of the HaCaT cells in the presence of the extract was evaluated by further examining the previously stained HaCaT cell plates. The remaining cell culture media were aspirated, and then the 96-well plates were washed twice using distilled water. Afterward, 50 µL of 0.5% crystal violet dye was added to each well, and the plates were placed in a shaking incubator at 20°C for 20 min. Thereafter, the plate was washed 4 times with distilled water and air-dried at 25°C. After 24 h, 200 µL of methanol was added to each well and the plate was incubated for another 20 min at the same temperature. Thereafter, the absorbance was measured at 570 nm.

LDH Assay

The 96-well microtiter plates obtained from the proliferation study were evaluated further to determine the level of LDH activity. The LDH assays consisted of two control groups; these were (i) spontaneous LDH release (consisting of medium from cells that had been treated with 10 µL of ultrapure water) and (ii) maximum LDH release (consisting

of medium from cell that had been treated with 10 µL of lysis buffer). To each of microtiter plate well, 50 µL of the reaction mixture was added and incubated at 25°C for 30 min, and the reaction was stopped. Thereafter, the absorbance was immediately read at 490 and 680 nm.

Statistical Analysis

All the determinations were conducted at least 3 times ($n = 3$); the statistical mean ± SD was calculated using OriginLab EULA for OriginPro 2015 (Northampton, MA 01060 USA) and the optimization study was performed by the use of Design-Expert Version: 11.0.6.0 (Stat-Ease, Inc., Hennepin, MN).

RESULTS

Fitting into Models

In this study, the relationship between the responses and the four independent variables was identified by factors inscribed into the BBD. The percentage of crude extract was determined by gravimetric analysis, while the TPC and TFC were determined spectrophotometrically and expressed as mg gallic acid and QE/g of sample, respectively.

Table 2 expresses the results of the predicted and actual responses for 30 runs according to the design of experiment. The crude extract yield ranged from 2.088% to 25.08% on dry weight mass, and the maximum yield was obtained on the 9th run under the experimental conditions of $X_1 = 0.50$ mg, $X_2 = 200$ rpm, $X_3 = 24$ h, and $X_4 = 50$ mL. The maximum yield obtained for the TPC and TFC on the 11th and 3rd runs was from 2.46 to 55.52 mg GAE/g and 0.869 to 6.037 mg QE/g, respectively, at an experimental conditions of $X_1 = 3.00$ mg, $X_2 = 200$ rpm, $X_3 = 24$ h, and $X_4 = 50$ mL.

The fitting of the model was generated through multiple linear regression by the exclusion of suggested insignificant terms at $P > 0.05$ from each model on the second-order polynomial equations [Table 3].

The application registered a close correlation between the experimental and predicted values for each of the responses, indicating the development of a satisfactory model, and the relationships are plotted in Figure 2.

Optimization Effect of the Independent Variables on the Studied Responses

The three-dimensional (3D) surface plots reflect the impact of the independent variables (X_1, X_2, X_3, and X_4) on the Y%, TPC, and TFC of the acetone extract, obtained by maceration as shown in Figures 3-5. The linear contact time primarily influences the Y%, (X_3), followed by the quadratic of agitation

speed (X_2^2) and the linear term of agitation speed (X_2), while TPC is influenced by the linear agitation speed (X_2), followed by the interaction effect of the mass of plant material and contact time (X_1X_3) and the quadratic contact time (X_3^2).

However, the extraction of TFC is dependent on quadratic contact time (X_3^2), followed by the agitation speed (X_2^2) and the interaction effect of the mass of plant material and agitation speed (X_1X_2)

Verification of Results

The suitability of the model equations for predicting the optimum responses was tested using the recommended optimum conditions. The experimental values were found to agree with the predicted ones [Table 4]. Extracts obtained were dried and subjected to further biological evaluation.

Antioxidant Effect of the Extract

The antioxidant activities of optimized acetone extract from *P. graveolens* and controls were evaluated by DPPH, ABTS scavenging, and ferric reducing power *in vitro* assays. The IC$_{50}$ values for the different assays were obtained from the plots of the % radical inhibition against concentration with $r^2 \geq 0.8992$, and the results are given in Table 5. The presence of reducing agents in the test extract caused the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+};[36] however, the controls showed a significant reducing power compared to the extract.

Lipid Protective Activity

The optimized extract was evaluated by the lipid peroxidation assay for the capacity to protect linoleic acid from autoxidation,[37] and the result is presented in Figure 6. In this study, the reagent ferrous ions are oxidized to ferric ions by the radicals produced during the autoxidation of linoleic acid. The resulting ferric ions immediately form a complex with thiocyanide ions within the medium and are monitored spectrophotometrically at 500 nm.[38]

The kinetics of the inhibitory process was studied by measuring the concentration of the ferrous ions formed at a given period. The obtained parameters are fitted into pseudo-first and second-order models, resulting in R^2 values of 0.7785 and 0.9934, respectively [Figure 7].

Antimicrobial Effect of the Optimized Extract

The extract was screened against two Gram-positive and two Gram-negative organisms, and the result is presented in Table 6. Our study focuses on the determination of the antibacterial activity as well as establishing the MIC of the extract as compared to chloramphenicol and ampicillin

Runs	Coded				Response extract (Y%)		Response TPC (mg/g GAE)		Response TFC (mg/g QE)	
	X_1	X_2	X_3	X_4	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	0	0	0	0	11.12	10.97	41.48	35.69	2.406	2.685
2	1	−1	−1	−1	2.088	2.81	32.45	32.73	3.569	3.669
3	0	0	−1	0	8.360	7.57	34.68	34.93	6.037	5.796
4	0	1	0	0	20.75	21.38	53.97	55.41	0.869	0.951
5	−1	−1	1	−1	9.12	8.89	36.72	36.84	3.598	3.754
6	0	0	0	0	10.49	10.97	40.48	41.35	2.703	2.875
7	−1	−1	−1	−1	1.160	1.06	35.68	35.63	3.526	3.319
8	1	0	0	0	8.577	9.71	44.14	44.81	2.397	2.766
9	−1	1	1	−1	25.08	20.40	37.14	44.81	1.541	1.648
10	1	1	1	1	20.84	18.27	48.96	47.78	2.628	3.420
11	1	1	1	−1	19.06	18.42	55.52	56.57	2.607	2.388
12	−1	1	−1	−1	10.13	9.97	42.23	42.17	1.014	1.308
13	−1	−1	1	1	6.452	6.36	27.35	28.03	4.010	3.961
14	1	−1	1	1	17.06	17.49	29.86	30.49	1.856	1.780
15	−1	0	0	0	8.183	7.76	40.49	42.08	2.703	2.393
16	0	0	1	0	13.74	14.77	36.54	37.21	4.990	5.184
17	0	−1	−1	−1	4.46	4.53	2.46	2.69	2.461	2.478
18	−1	−1	−1	1	6.740	7.65	35.61	35.12	3.025	3.378
19	1	−1	−1	1	20.30	19.02	22.27	22.08	3.526	3.420
20	1	1	−1	−1	2.940	2.74	47.62	46.44	4.107	4.158
21	0	0	0	0	11.12	10.97	41.49	40.54	2.703	2.822
22	0	0	0	0	11.40	10.97	41.49	41.35	2.703	3.011
23	0	0	0	1	8.412	8.91	32.88	33.21	1.655	1.737
24	0	−1	0	0	15.98	15.46	45.21	43.49	1.654	1.493
25	1	1	−1	1	13.36	13.85	45.54	45.95	4.584	5.184
26	0	0	0	−1	5.00	4.62	38.41	37.86	1.649	1.488
27	0	0	0	0	11.12	10.75	41.49	41.35	2.703	2.685
28	−1	−1	1	−1	10.65	10.33	50.00	49.43	1.783	1.780
29	0	0	0	0	11.12	10.97	41.49	40.55	2.703	2.677
30	−1	1	1	1	19.06	18.27	38.97	38.16	2.550	2.389

Table 2: BBD with the actual responses and predicted values for Y%, TPC, and TFC

% Y: Percentage yield of acetone extract, TPC: Total phenolic content, TFC: Total flavonoid content, Y%: Percentage crude extract, BBD: Box–Behnken design, QE: Quercetin equivalent

(positive controls). Results from the antimicrobial screening indicated that the extract has good-to-moderate activity against the tested microorganisms compared to the controls.

## Effect of Optimized Extract on Cell Proliferation and Viability

Stimulation of the HaCaT cells for a day with the extract at concentrations of 1, 25, and 50 µg/mL caused a significant ($P < 0.05$) increase in cell viability [Figure 8a and b]. Crude extract at a concentration of 50 µg/mL resulted in a high significant ($10.66 \pm 11.02\%$; $P < 0.05$) increase in cell viability compared to the untreated cells (100%). Extract administered at ≥100 µg/mL resulted in a significant ($76.96 \pm 3.16\%$; $P < 0.05$) reduction in cell viability in comparison to the untreated cells after day 1. When the HaCaT cells were stimulated for 4 days with crude extract at 1 µg/mL, a significant increase was observed ($134.89 \pm 5.81\%$), but at 25 and 50 µg/mL, there was a slight but non-significant effect of $105.13 \pm 1.84\%$ and $96.45 \pm 11.02\%$ on cell viability, respectively. However, 4 days' stimulation of the HaCaT cells with extract at a concentration of ≥100 µg/ml resulted in a significant ($\leq 25.77 \pm 3.16\%$; $P < 0.05$) decrease in cell viability compared to the untreated cells [Figure 8a]. After 7 days of stimulation with extract at concentrations
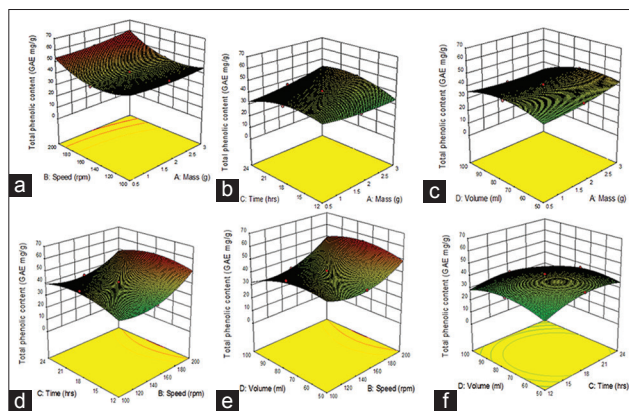
Table 3: The fitted models for crude yield, TPC, and TFC and the coded units (X_1, X_2, X_3, and X_4)

Responses	Second-order polynomial equations	*P* value	R_2	Adjusted R_2	Lack of fit
% Y	$10.97+0.9737X_1+2.959X_2+3.599X_3+2.144X_4-$ $2.248X_1X_2+2.424X_1X4+2.042X_2-$ $1.294X_2X_4-2.282X_3X_4-2.242\,X_1^2+7.443\,X_2^2+0.1993$ $X_3^2-4.206\,X_4^2$	<0.0001	0.9904	0.9815	0.089
TPC (mg GAE/g)	$41.35+1.6793X_1+5.961X_2+0.7635X_3-$ $2.326X_4+1.792X_1X_2+3.873X_1X_3-2.533X_1X_4-$ $1.643X_2X_3+2.538X_2X_4-2.075X_3X_4+1.778\,X_1^2+8.097$ $X_2^2-5.656\,X_3^2-5.819\,X_4^2$	<0.0001	0.9952	0.9906	0.167
TFC (mg QE/g)	$+2.685+0.1865X1-0.2710X_2-$ $0.3057X_3+0.1227X_4+0.6595X_1X_2-0.5812X_1X_3-1.464$ $X_2^2+2.805\,X_3^2-1.071\,X_4^2$	<0.0001	0.9775	0.9566	0.375

TPC: Total phenolic content, TFC: Total flavonoid content, QE: Quercetin equivalent
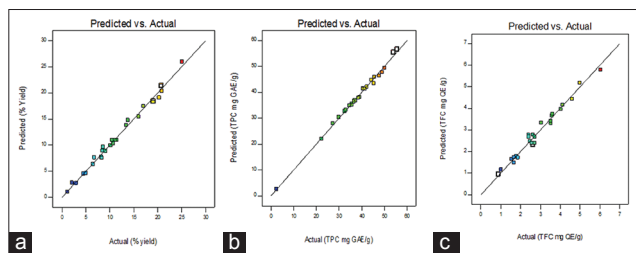
Figure 2: Relationship between predicted and observed (a) percentage crude extract, (b) total phenolic content, and (c) total flavonoid content
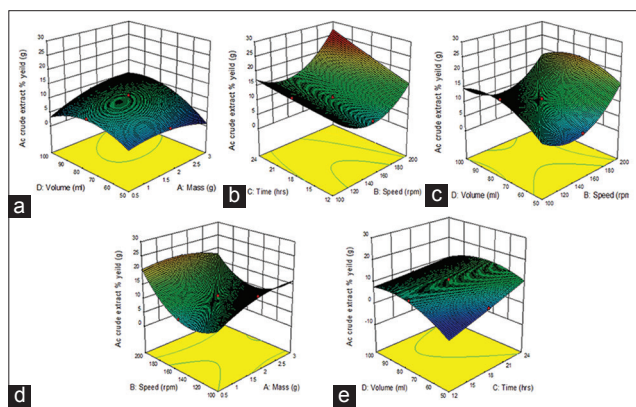
Figure 3: Three-dimensional graphic surface optimization of percentage crude extract versus (a) X_4 and X_1, (b), X_3 and X_2 (c), X_4 and X_2, (d) X_2 and X_1, and (e) X_4 and X_3

Figure 4: Three-dimensional graphic surface optimization of total phenol content versus (a) X_2 and X_1, (b) X_3 and X_1, (c) X_4 and X_1, (d) X_3 and X_2 (e), X_4 and X_2, and (f) X_4 and X_3
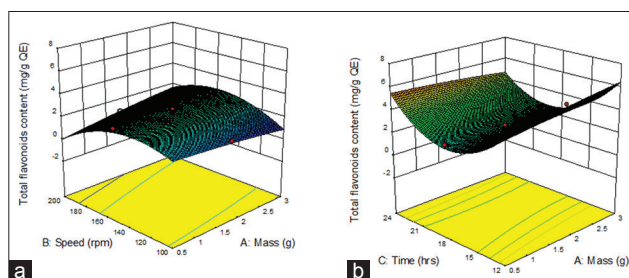
Figure 5: Three-dimensional graphic surface optimization of total flavonoid content versus (a) X_2 and X_1, (b) X_3 and X_1

of 1, 25, and 50 µg/mL, percentage cell viability remained slightly higher than that observed in the untreated cells. On the contrary, the percentage survived cells stimulated with extract at concentrations of 1–1000 µg/mL was significant ($P < 0.05$) lower than that observed in the untreated cells for the period of study [Figure 8b]. However, 1 day of stimulation with extract at a concentration of 25, 50, and 100 µg/mL resulted in a highly significant ($P < 0.05$) decrease in % cell survival compared to that observed after 4 days and 7 days and in the untreated cells. A highly significant ($P < 0.05$) decrease in the percentage cell

viability and percentage cell survival for 1, 4, and 7 day(s) was observed when the extract was administered at concentrations of 100 µg/mL and 250 µg/mL in comparison to the untreated cells, respectively.

The cytotoxic result presented in Figure 9 agrees with the proliferative and viability results. The stimulation of the cells at concentrations >50 µg/mL causes the cell membrane to lysis rapidly on the 1st day, but the effect decreases significantly ($P < 0.05$) over the study period compared to those of the control group.

Table 4: Predicted and experimental values of Y%, TPC, and TFC at the optimum conditions

Responses	Independent variable				Value	
	Mass (g)	Agitation speed (rpm)	Contact time (h)	Solvent volume (mL)	Predicted	Experimental
Y%	2.13	197.47	23.79	75.42	25.42	24.91±0.15
TPC (mg GAE/g)	2.78	191.37	143.11	68.06	56.28	55.63±0.21
TFC (mg QE/g)	2.62	143.11	12.01	69.87	6.16	5.98±0.87

Mean value±SD (*n*=3), QE: Quercetin equivalent, Y%: Percentage crude extract, TPC: Total phenolic content, TFC: Total flavonoid content

**Table 5:** Scavenging activity of the optimized acetone extract

Compounds	DPPH assay		ABTS assay		Reducing power (mg QE/g)	TAC (mg QE/g)
	IC$_{50}$ (mg/mL)	R²	IC$_{50}$ (mg/mL)	R²		
Crude extract	4.98±0.15	0.9108	0.18±0.03	0.8992	1.17±0.05	0.83±0.05
Quercetin	3.80±0.12	0.9767	0.11±0.06	0.9849	-	-
Gallic acid	-	-	-	-	0.58±0.01	0.35±0.10

Mean value±SD (*n*=3), QE: Quercetin equivalent, ABTS: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-Diphenyl-1-picrylhydrazyl

Table 6: MIC (mg/mL) of the optimized extract

Samples	MIC (mg/mL)			
	Staphylococcus aureus	*Escherichia coli*	*Enterococcus faecalis*	*Salmonella typhi*
Extract	1.56	6.25	3.13	1.56
Chloramphenicol	0.20	0.39	0.20	0.39
Ampicillin	-	-	0.10	-

MIC: Minimum inhibitory concentration
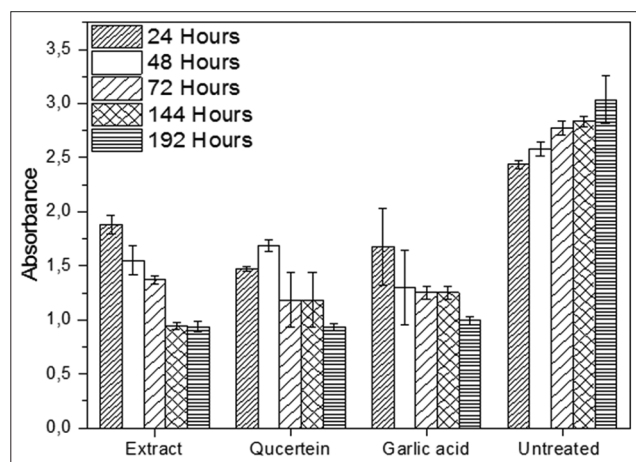
Figure 6: Lipid peroxidation inhibitory activity of extract and controls as a function of time (*n* = 3)

DISCUSSIONS

P-value and the quality of the models based on the regression coefficients indicated the suitability of the models to predict the variations accurately [Table 2 and Figure 2]. Furthermore, the coefficient of variance (CV) for the responses is small (CV ≤8.60%) and within acceptable range, an indication of a slight variation in the mean value and satisfactory suitability of the developed models.[39] The appropriateness of the models was determined using the statistical coefficient of r2 ≥ 0.9566; furthermore, there was no lack of fit at *P* < 0.05.

Figure 3a-e showed the interactions between the independent variables in the estimation of extract yield. The 3D surface plots showed that the extract yield is dependent on the quadratic effect of the mass plant material and agitation speed [Figure 3a-c]. By implication to increase the crude recovered by maceration, doubling the mass of the material and agitation speed with a proportionate increase in the contact time and solvent volume [Figure 3d-e], will exert a quadratic effect on extract yield. Therefore, the proposed optimized conditions for the quantitative extraction of 25.42% crude extract require a material mass of 2.13 g, agitation speed of 197.47 rpm, contact time 23.79 h, and solvent volume of 75.42 mL.

In Figure 4a-f, the independent variables such as the interaction between the solvent volume with agitation speed and the quadratic effect of the contact time influence the total phenol content [Figure 4a,e,f]. However, the TPC is dependent on the direct effect of the mass of the material with
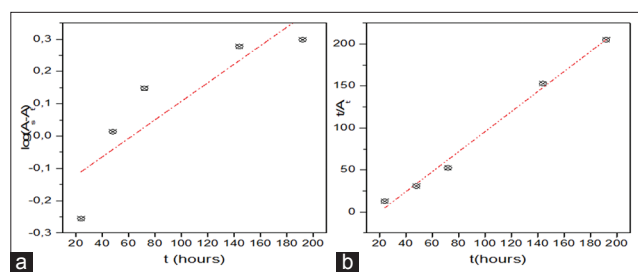
Figure 7: (a) Pseudo-first and (b) second-order kinetics for the lipid peroxidation inhibitory models
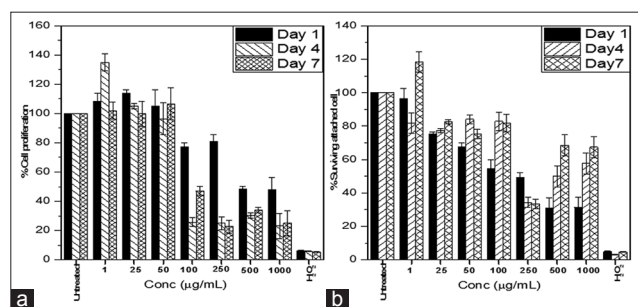

Figure 8: Percentage (a) cell viability and (b) surviving attached cell following the stimulation of HaCaT cells with acetone extract at 1, 25, 50, 100, 250, 500, and 100 μg/mL for 1, 4, and 7 days. The untreated cells were used as a negative control, whereas the H_2O_2 was used as a positive control
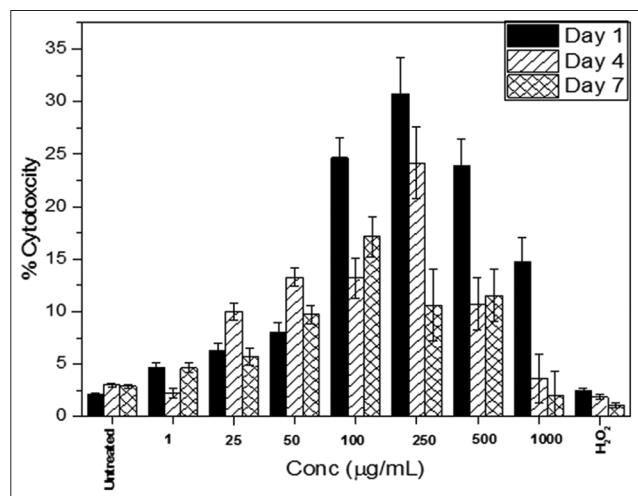

Figure 9: Percentage cytotoxicity following the stimulation of HaCaT cells with acetone extract at 1, 25, 50, 100, 250, 500, and 100 μg/mL for 1, 4, and 7 days. The untreated cells were used as a negative control, whereas the H_2O_2 was used as a positive control

contact time or solvent volume [Figure 4b-d]. The extraction conditions for TPC were proposed from the models as a material mass of 2.78 g, agitation speed of 191.37 rpm, contact time 19.63 h, and solvent volume of 68.06 mL with a yield of 56.28 mg GAE/g. The optimal extraction of TFC by maceration is dependent on only two significant interactions as illustrated on the 3D surface plots [Figure 5a and b]. The 3D surface plots showed that the TFC is influenced by a proportional increase in the sample mass, and also, a quadratic

increase in the agitation speed and contact time [Figure 5a and b]. The 3D model proposed optimal conditions for TFC extraction were a material mass of 2.62 g, agitation speed of 143.11 rpm, contact time 12.01 h, and solvent volume of 69.87 mL with an extraction yield of 6.16 mg QE/g. The set conditions obtained by RSM optimization approach were verified experimentally to validate the suitability of the predicted value of the responses [Table 4].

In general, our study revealed that the upward concaved 3D plots express a condition where the responses display an increasing trend with increasing interaction of X_1X_2, X_2X_3, and X_2X_4, whereas the convex interactions of X_1X_3, X_1X_4, and X_3X_4 are indications that the responses decrease beyond the optimal mass time, volume time, and volume mass of plant material. This may be that the extraction process has reached an equilibrium state with the extract ant.

Molybdenum (VI) was reduced to green phosphomolybdate (V) complex by electron transfer when in contact with the extract, this property of the extract was measured as TAC.[40] The extract and gallic acid (control) both showed closely related electron donating potential of 0.83 ± 0.05 mg QE/g and 0.35 ± 0.10 mg QE/g, respectively [Table 5]. This is an indication that the total antioxidant activity is a function of the phenolic (56.28 mg GAE/g) and flavonoids (6.16 mg QE/g) contents.

Evaluation of the scavenging activity of the extract against DPPH radical registered an IC_{50} value of 4.98 ± 0.15 mg/mL compared toquercetin (3.80 ± 0.12 mg/mL). However, the extract displayed a lower IC_{50} value (0.18 ± 0.03 mg/mL) against $ABTS^{•+}$, similar to quercetin (0.11 ± 0.00 mg/mL) [Table 5]. Consequently, the relatively weak radical scavenging activity against the picrylhydrazyl radical implies that the acetone-soluble metabolites are probably weak scavenger by proton or electron donation. Various studies have shown that most scavenging processes are accomplished either by proton or electron donation to unstable radical;[41] however, the effective activity against $ABTS^{•+}$ and weak anti-DPPH radical activity is suggestive of an alternative scavenging mechanism of the extract.

The lipid protective activity of the extract improved with time and is more effective than the control over 192 h [Figure 6]. Consequently, the low absorbance obtained over the study period corresponds to the high concentration of the ferrous ion and, hence, high peroxidation inhibitory activity of the extract compared to the control. This may result from the ability of the lipid inhibitory agent(s) to neutralize the radicals formed during the initial autoxidation reaction and the continuous prevention of a further reaction between linoleic acid and oxygen.

The inhibition-time study confirmed the suitability of the second-order model in the description of the kinetics of the radical [Figure 7]. The turnover numbers such as k_1 (pseudo-first-order) and k_2 (pseudo-second-order) were estimated at 625.43 h^{-1} and 0.0341 h^{-1}, respectively. Thus, the small turnover number, k_2,

further confirmed the suitability of the pseudo-second-order kinetic model[42] and the dependence of the inhibitory activity on the concentrations of both the extract and substrate.

The extract showed a spectrum of activity against all the tested organism [Table 5]. In general, the extract exhibited excellent activity against *S. aureus* and *S. typhi* (MIC 1.56 mg/mL), whereas the microorganisms were not sensitive to the presence of ampicillin except on *E. faecalis* (MIC 0.10 mg/mL). The sensitivity of *S. aureus* and *S. typhi* toward the extract but not ampicillin is suggestive of the inhibition of β-lactamase, an enzyme produced by bacteria to inactivate ampicillin and penicillin-related antibiotics.[43,44] However, the controls (ampicillin and chloramphenicol) showed potent antibacterial activity against the *Escherichia* and *Enterococcus* spp. compared to the extract.

The proliferative and viability studies showed that extract at low concentrations particularly at 1 µg/mL was able to significantly increase the number of viable [Figure 8a] and surviving attached keratinocyte cells [Figure 8b] after 4 and 7 days, respectively. Increase in cell viability could have been as a result of an increase in mitochondrial activity which could have resulted from the proliferation or self-renewal of the keratinocytes. The link between an increase in mitochondrial activity and cell proliferation has been established by various studies.[45,46] Clearly, the results showed that extract could act as a keratinocyte growth factor although the exact mode of action is not yet established because of the complexity of the chemical composition. However, it is possible that the extract could have stimulated the keratinocyte to increase the production of growth factors. as well as survival such as macrophage colony-stimulating factor and cytokines Il-4 and Il-34.

Phytomedicine and ethnomedicinal usage of the natural product is becoming widely acceptable by the world population.[47,48] Hence, it has become a matter of urgency to study the cytotoxic effect of these natural products extensively. In this study, the cytotoxic effect was expressed as percentage LDH release to confirm the integrity of the viable HaCaT cell membrane compared to the maximum release of LDH from lysis buffer-treated cells [Figure 9]. In principle, this is based on the fact that any damage to the cell membrane is widely determined by LDH release into the culture media. The detection of LDH released from the lysed cell membranes is due to the conversion of pyruvate to lactate by oxidized NAD, indicating a compromised cell membrane.

At concentrations >250 µg/mL, the cytotoxic effect monitored by LDH release after stimulating the HaCaT cells with the extracts reduces from 30%, 23%, to 3% for days 1, 4, and 7, respectively. Therefore, this may be due to unspecific inhibition of regulatory or feedback loops in the signal pathways, observed mostly at a very low drug dose when targeting a specific enzyme. Then, the overall outcome causes a revival of all the regulatory pathways, resulting in the cell proliferation and perhaps more survival.[42]

CONCLUSION

The optimization of the extract yield, TPC, and TFC was achieved using Design-Expert version 11 software. The four independent variables involved in the optimization are mass of plant material (X_1), agitation speed (X_2), contact time (X_3), and solvent volume (X_4). The 3D models indicated that the variable with the most significant effect on the extract and flavonoids yields is the mass of the plant material, while the phenolic is majorly dependent on the interaction between the solvent volume and agitation speed. From the RSM results, the optimal extractable crude, flavonoids, and phenolic are 25.42%, 6.16 mg QE/g, and 56.28 mg GAE/g, respectively. The MIC reported for the antibacterial activity of the optimized extract was 1.56–6.25 mg/mL against the microorganisms compared to ampicillin (MIC 0.10 mg/mL) and chloramphenicol (0.20–0.39 mg/mL); hence, this underscores the importance of optimizing maceration technique. Furthermore, the antioxidant activity at IC_{50} values of 0.18 ± 0.03–4.98 ± 0.15 mg/mL and proliferative effect at ≤50 µg/mL is a confirmation of the efficacy of the decoction from *P. graveolens* applied as a traditional herb. However, due to the cytotoxic effect at ≥100 µg/mL, the ethnotraditional practitioners must carefully administer the usage of the plant.

Supplementary Materials

Raw and treated data generated during the study are available from the corresponding author on reasonable request.

REFERENCES

1. Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin : Current methods and future trends. Afr J Biotechnol 2008;7:1797-806.
2. William L, Remington. The Science and Practice of Pharmacy. 20-21th ed. Easton, PA: In: Mack Publishing Company, Lippincott Williams and Wilkins; 2000. p. 773-4.
3. Pichersky E, Gang D. Genetics and biochemistry of secondary metabolites in plants: An evolutionary

perspective. Trends plant Sci 2000;5:439-45.

4. Handa SS. An Overview of extraction techniques for medicinal and aromatic plants. In Extraction Technologies for Medicinal and Aromatic Plants. Trieste, Italy: International Centre for Science and High Technology; 2008. p. 21-52.

5. Raynie DE. Modern extraction techniques. Anal Chem 2010;82:4911-6.

6. Camel V. Extraction Methodologies: General Introduction. Chichester, UK: In: Encyclopedia of Analytical Chemistry, John Wiley and Sons, Ltd.; 2014. p. 1-24.

7. Gil-Chávez GJ, Villa JA, Ayala-Zavala JF, Heredia JB, Sepulveda D, Yahia EM, *et al*. Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: An overview. Compr Rev Food Sci Food Saf 2013;12:5-23.

8. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: A comprehensive review. Chinese Med (United Kingdom) 2018;13:20.

9. Sakkas VA, Islam MA, Stalikas C, Albanis TA. Photocatalytic degradation using design of experiments: A review and example of the Congo red degradation. J Hazard Mater 2010;175:33-44.

10. Czitrom V. One-factor-at a time versus designed experiments. Am Stat 1999;53:126-31.

11. Carmen G, Hancu G. Antimicrobial and antifungal activity of *Pelargonium roseum* essential oils. Adv Pharm Bull 2014;4:211-514.

12. Asgarpanah J, Ramezanloo F. An overview on phytopharmacology of *Pelargonium graveolens* L. Indian J Tradit Knowl 2015;14:558-63.

13. Saraswathi MS, Kannan G, Uma S, Thangavelu R, Backiyarani S. Improvement of banana cv. Rasthali (Silk, AAB) against *Fusarium oxysporum* f.sp. Cubense (VCG 0124/5) through induced mutagenesis: Determination of LD50 specific to mutagen, explants, toxins and *in vitro* and *in vivo* screening for *Fusarium wilt* resistance. Indian J Exp Biol 2016;54:345-53.

14. Wettasinghe M, Shahidi F. Evening primrose meal: A source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. J Agric Food Chem 1999;47:1801-12.

15. Das K, Tiwari RK, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agents: Current methods and future trends. J Med Plants Res 2010;4:104-11.

16. Yurika I, Ju OB, Oliveira LW De, Se R. Response surface methodology for extraction optimization of pigeon pea protein. Food Chem 2000;70:259-265.

17. Gao L, Mazza G. Extraction of anthocyanin pigments from purple sunflower hulls. J Food Sci 1996;61:600-3.

18. Cacace JE, Mazza G. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. J Food Sci 2003;68:240-8.

19. Theodore K, Panda T. Application of response surface methodology to evaluate the influence of temperature and initial pH on the production of [beta]-1, 3-glucanase and carboxymethyl cellulase from *Trichoderma harzianum.* Enzyme Microb Technol 1995;17:1043-9.

20. Deming SN. Quality by design-part 5. Chemtech 1990;20:118-20.

21. Eloff JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J Ethnopharmacol 1998;60:1-8.

22. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564-82.

23. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using folin-ciocalteu reagent. Nat Protoc 2007;2:875-7.

24. Chang CC, Yang MH, Wen HM. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 2002;10:178-82.

25. Montgomery DC. Design and Analysis of Experiments. 5th ed. New York: Wiley; 2001.

26. Myers RH, Montgomery DC. Process and product optimization using designed experiments. In: Response surface methodology. 2nd ed. New York: Wiley; 2002.

27. Oyaizu M. Antioxidative activities of browning products of glucosamine fractionated by organic solvent and thin layer chromatography.pdf. Nippon Shokuhin Kogyo Gakkaishi 1988;35:771-5.

28. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a complex: Specific application to the determination of Vitamin E. Anal Biochem 1999;269:337-41.

29. Jan S, Khan MR, Rashid U, Bokhari J. Assessment of antioxidant potential, total phenolics and flavonoids of different solvent fractions of *Monotheca buxifolia* fruit. Osong Public Heal Res Perspect 2013;4:246-54.

30. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT Food Sci Technol 1995;28:25-30.

31. Siddhuraju P, Manian S. The antioxidant activity and free radical-scavenging capacity of dietary phenolic extracts from horse gram (*Macrotyloma uniflorum* (Lam.) Verdc.) seeds. Food Chem 2007;105:950-8.

32. Mitsuda H, Yasumoto K, Iwami K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. EiyoToShokuryo 1966;19:210-4.

33. Curtis RG, Houghton CD, Waters WA, Brown SM, Thomson GH, Braude EA, et al. Fluorescence and the beer-lambert law. A note on the technique of absorption spectrophotometry. J Chem Soc 1950;11:1017-21.

34. Kocsis L, Herman P, Eke A. The modified Beer-Lambert law revisited. Phys Med Biol 2006;51:N91-8.

35. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2017. P. M100.

36. Ferreira IC, Baptista P, Vilas-Boas M, Barros L. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual

cap and stipe activity. Food Chem 2007;100:1511-6.

37. Adaramoye OA, Odunola OA. Lipid peroxidation inhibition and antiradical activities. 2011;68:23-9.

38. Maqsood S, Benjakul S, Balange A. Effect of tannic acid and kiam wood on lipid oxidation and textural properties of fish emulsion sausages. Food Chem 2012;130:408-16.

39. Daniel W. A foundation for analysis in the health sciences. 5th ed. Biostatistics. New York: Wiley; 1991.

40. Mashwani ZU, Khan MA, Irum S, Ahmad M. Antioxidant potential of root bark of *Berberis lyceum* royle. from Galliyat, western Himalaya, Pakistan. Pak J Bot 2013;45:231-4.

41. Liang N, Kitts DD. Antioxidant property of coffee components: Assessment of methods that define mechanism of action. Molecules 2014;19:19180-208.

42. Murdoch JR. What is the rate-limiting step of a multistep reaction? J Chem Educ 1981;58:32.

43. Hauser AR. Antibiotic Basics for Clinicians: The ABCs of Choosing the Right. Alan R, editor. Hauser-Google Books. Baltimore, MD: Lippincott Williams and Wilkins; 2013.

44. Akova M. Sulbactam-containing β-lactamase inhibitor combinations. Clin Microbiol Infect 2008;14 Suppl 1:185-8.

45. Arciuch VG, Elguero ME, Poderoso JJ, Carreras MC. Mitochondrial regulation of cell cycle and proliferation. Antioxid Redox Signal 2012;16:1150-80.

46. van den Bogert C, Spelbrink JN, Dekker HL. Relationship between culture conditions and the dependency on mitochondrial function of mammalian cell proliferation. J Cell Physiol 1992;152:632-8.

47. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environ Health Perspect 2001;109 Suppl 1:69-75.

48. Liu Q, Wen J, Peng Z, Liu F, Tong X. Review of the powder and decoction formulae in traditional Chinese medicine based on pharmacologically active substances and clinical evidence. J Tradit Chin Med 2015;35:355-60.