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HPLC, GC-MS, XRD profiling, enzyme inhibition and cytotoxicity potential of *Phlomis Stewartii* extracts using response surface methodology

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Abstract

The mechanical extraction of various phytochemicals from *Phlomis stewartii* (*P. stewartii*) was modeled using response surface methodology (RSM). The Box-Behnken design (BBD) was used to optimize the three independent variables comprising of 17 experimental runs, with the experiments randomly arranged to minimize the effects of unexpected variation in the observed dependent variables {extraction yield, total phenolic content (TPC), and total flavonoid content (TFC)} due to systematic errors. The speed (X1, 100, 150 and 200 rpm), solvent volume (X2, 100 and 150 and 200 mL), and extraction time (X3, 2, 5 and 100 h) at 3-levels (high and low coded levels) were used to evaluate BBD of RSM. Results showed that the maximum value of yield, TPC and TFC in leaves ethanol extracts (LEE); flower ethanol extract (FEE), and whole plant ethanol extracts (WPEE) were observed at the following extraction conditions: A X1 of 150 rpm, X2 of 200 mL and X3 at 8 h. The minimum values were observed at an X1 of 150 rpm, X2 of 100 mL and X3 of 2 h. Analysis of variance (ANOVA) and the interaction effects of independent factors showed that X2 and X3 significantly positively influenced the response variables. These values closely matched the predicted yield, TPC and TFC. High-performance liquid chromatography (HPLC) analysis reveals that hydroxybenzoic acid, *p*-coumaric acid, gallic acid, chlorogenic acid, and salicylic acid, are the major secondary metabolites in the extracts. Gas chromatography-mass spectrometry (GC-MS) analysis showed the presence of different compounds such as thiazole, 2-ethylacridine, silicic acid, arsenous acid, 3,5-ethanoquinolin-10-one, and hexahydropyridine. All these identified compounds exhibit a wide range of various biological potential, including anti-viral, anti-inflammatory, anti-diabetic and anti-bacterial activities. The highest *α*-amylase inhibitory potential IC50 (53.33 ± 0.21 µg/mL), and *α*-glucosidase inhibitory potential IC50 (51.07 ± 0.17 µg/mL) were shown by LEE extracts. The study concludes that LEE extracts may have anti-inflammatory, immunomodulatory, antioxidant,

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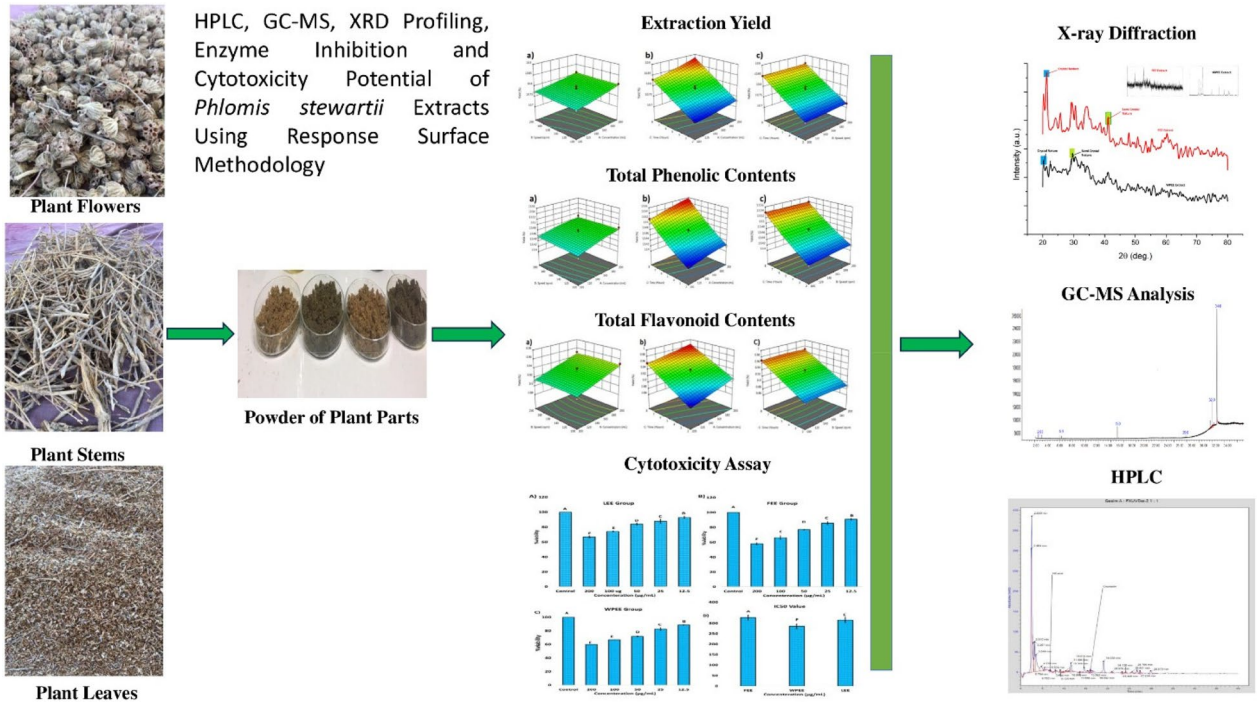
hepatoprotective, and nephroprotective potential, suggesting potential for further investigation in diabetes management.

Keywords *P. stewartii*, Phytochemicals, Enzyme inhibition, GC-MS, HPLC

Graphical Abstract



Introduction

Plant-based dietary antioxidants play an important role in maintaining human health and preventing chronic disorders because these antioxidants provide insufficient defense against constant reactive oxygen species (ROS) [1, 2]. These antioxidants, found abundantly in medicinal plants (MPs), include tocopherols, anthocyanins, and phenolic substances, as described by Nallusamy et al. [3]. MPs are employed in pharmacological investigations, with favorable potential for the healthcare system, such as the treatment of heart diseases, chronic cancers, aging, nerve degeneration diseases and diabetes mellitus (DM) and oxidative stress [4]. All of these defects can be combated or even cured by the use of exogenous components. The use of these components is limited due to their complexity in human health; thus, efforts have been made to investigate natural agents as a substitute for synthetic medications. The biological potential of phenols, flavonoids, alkaloids, ascorbic acids, amides, saponins, and other bioactive components from various plant sections to treat a wide range of illnesses makes them important for human health [5]. The method of extraction and the type of solvent employed have an impact on the plant extract's yield and biological activity. According to published studies, ethanol is an excellent choice for extracting a large number of bioactive chemicals [6, 7]. For the preparation of plant extracts, various ultrahigh extraction techniques, supercritical carbon dioxide extraction, microwave-assisted extraction, and shaking extraction techniques, have been employed [8, 9]. One of the easiest, least expensive, and least invasive extraction methods is mechanical shaking extraction, which, when the extraction parameters are optimized, can produce high yields [10]. RSM is used to maximise the extraction yield while using the least amount of solvent and shaking time [11]. One kind of RSM that is regularly used to optimise the technical parameters for extraction is the Box-Behnken design (BBD), which is also commonly utilised for other techniques needed to optimise a procedure [12].

The genus *Phlomis*, which includes over 100 species, is part of the Lamiaceae family and *P. stewartii* is one of these species. Strong medicinal substances from the Lamiaceae family are known to be used in medicines for the treatment of various disorders [13, 14]. Many varieties of Phlomis have been used in folk medicine for decades as a pain reliever and wound healer [15]. To the best of our knowledge, no studies have been conducted on the extraction of bioactive components from *P. stewartii*

using ethanol solvent. However, *P. stewartii* needs to be explored because few studies have covered evaluating and isolating phenylethanoids, caffeic acid, p-hydroxybenzoic acid, and notohamosin as α-glucosidase inhibitors [10].

We wonder if the optimized extraction conditions will yield higher concentrations of bioactive compounds with significant enzyme inhibition potential. In this study, the current objective is to optimize the extraction parameters of phytochemicals from different parts of *P. stewartii* whole plant ethanol extract (WPEE), leaves ethanol extracts (LEE), and flower ethanol extracts (FEE), determine the total phenolic contents (TPC), total flavonoid contents (TFC), gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) analysis, X-ray diffraction (XRD) was used to analyze the structure and composition of crystalline materials in extracts, and test the extracts obtained under the best optimized conditions for *in vitro* α-glucosidase and α-amylase inhibition potential.

Materials and methods

Collection of plant

From June to August 2017, a fresh *P. Stewartii* entire plant was taken from the Baluchistan desert region during June to August. The plant was authenticated using reference materials (voucher specimen number, GCUF-1243) from the herbarium of the Botany Department at Government College University, Pakistan, by Dr. Qaim Ali and University of Baluchistan, Botany Department in Quetta, Pakistan, identified the specimen. The whole plant (leaves, flowers, roots, stems, and bark) was transported to the Government College University Faisalabad, Faisalabad, Punjab, Pakistan to the research laboratory using polythene bags. Different parts of *P. Stewartii* (leaves, flowers, roots, stems, and bark) were washed with water and allowed to dry at room temperature and stored in the dark. Furthermore, a mechanical blender was used to make a fine powder of the whole plant. Then it was stored in air-proof plastic bags until the extraction process. Our research work complies with relevant institutional, national, and international guidelines and legislation.

Extract preparation

Ethanol solvent was mixed with the powdered dry plant samples in the following ratios: 1:10, 1:15, and 1:20 weight (W) of powder to solvent volume (V). To improve the conditions for the extraction of bioactive components from plants, a fixed amount of plant powder and varying solvent volumes were used. Whatman No. 1 filter paper (Cytiva, Marlborough, MA, USA) was applied for the filtration process of extracts, then placed in the rotary evaporator for evaporation of solvent. The extraction yield was calculated by the given Eq. 1.

$$Yield\,(\%) = \frac{\text{Weight of plant extract after evaporating of solvent (g)}}{\text{Weight of dry sample (g)}} \times 100 \quad (1)$$

Total phenolic contents (TPC)

The TPC was performed by the Folin-Ciocalteu method displayed in Naqvi et al. [16]. The mixture of 1 mL plant extract in 1 mL of Folin-Ciocalteu reagent was incubated for 5 min at room temperature, then 5 mL of Na_2CO_3 (1 M) was added. A 10 mL reactant mixture was prepared with distilled water and placed for 90 min. The absorbance was estimated at 760 nm and interpreted it as mg gallic acid equivalent (GAE)/g dry weight (DW) of plant extracts. The TPC was calculated using a gallic acid standard curve, assuming that the gallic acid was 100% pure.

Total flavonoid contents (TFC)

The aluminum chloride method, with slight modifications, was used to determine the TFC, as described by Naqvi et al. [17]. Took 0.75 mL of water and allowed it to mix with 0.25 mL of the sample having different intensities (0.25, 0.5, 1, 2, and 4 µg/mg). 0.15 mL of 5% $NaNO_3$ solution was added to the whole mixture and incubated for 5 min, then added 0.3 mL of $AlCl_3$ (10%). After 5 min of incubation, 1 mL NaOH was added and placed on the shaker. The absorbance was measured at 510 nm using a spectrophotometer while quercetin was kept as a standard. The results of the extracts were interpreted in mg quercetin equivalent (QE)/g DW.

HPLC analysis

The sample preparation for measurement of phenolic compounds in flowers, leaves, and whole plant parts of *P. stewartii* was conducted according to the guidelines outlined by Ying et al. [18]. A 0.5 mL mixture of standard phenolic contents and 0.5 g of dried powdered LEE, FEE, and WPEE extract were collected in a lidded flask. The extraction process was then followed by a 50 mL aqueous mixture of ethanol (50% v/v) for 30 min in an ultrasonic bath. The mixture was centrifuged for five minutes at 3000 rpm in a temperature-controlled centrifuge equipment set at 4 °C. A 0.46 µm thickness membrane filter was used to filter the supernatant and allow 20 µL of the mixture for aspiration through a microsyringe in an HPLC.

For identification of phenolic compounds Perkin Elmer Series 200 HPLC system (Rodgau company, Germany) fitted through a C-18 column of particle size (4.7 × 250 mm, 5 µm stationary phase), injection volume 10µL, temperature 30 °C with a UV Visible Double Beam Spectrophotometer (Lambda 25' PerkinElmer®) was used. A binary solvent mobile-phase solution was used for gradient elution. The two alphabets A and B were used to nominate methanol and water as mobile phases, respectively. 0.02%

trifluoroacetic acid (TFA) is used to acidify both stationary and mobile phases. The dilutions in gradient were maintained as follows: B25% for 0–4 min; B25-30% for 4–8 min; B30-50% for 8–12; B50% for 12–15 min; B50-80% for 15–18 min; B80% for 18–22 min. The wavelength was set at 275 nm and maintained 25 °C with a flow rate of 1.0 mL/min for detection.

GC-MS analysis

Characterization of the sample was carried out through GC-MS (Shimadzu QP2010, MS Detector SPD 20 A). The column Agilent (HP-5MS, 30 m × 250 µm 0.25 µm) was packed with 5% phenyl methyl siloxane as a stationary phase. As a mobile phase, helium gas was used. Initially, the temperature was at 50°C for at least 3 min and then it was increased to 10–18°C, over 15 min. In the second phase, the temperature was increased to 300°C, and the pressure and temperature of the oven were recorded as 9.05 psi and 360°C, respectively [13]. Helium gas (99.99%) was used as a carrier gas, flowing at a constant rate of 1 mL/min. The electron impact was used as a source for ionization at 250 °C. The run-up time was 68 min and GC–MS data was analyzed using NIST database software to cross-reference and correlate the detected compounds with available data in the database.

α-amylase inhibition assay

The *α*-amylase inhibition of extracts was performed as per explained in the research work of Bhutkar & Bhise [19]. A 96 well plate combined with 50 µL of plant extract and 150 µL of $(C_6H_{10}O_5)_n$, and 10 µL enzyme solution was used. The whole mixture was incubated for 30 min at 37 °C. The well is filled with color reagent and NaOH both measured as 20 µL; the plates were kept for 20 min in a water bath at 100 °C. The absorbance at 540 nm was used to set for reading. The extracts were substituted with 50 µL of dimethyl sulfoxide (DMSO) solution using it as a negative control. The plant extracts at varying doses (25, 50, 100, and 200 µg/mL) were used to produce the interference. The acarbose was used as a positive control along with the sample. The *α*-glucosidase potential of extracts was calculated and their IC50 values were determined. The IC50 value is determined after getting the trend line and R^2 value between 0.95 and 0.99. The equation for the inhibition percentage of *α*-amylase was estimated using the following Eq. 2.

$$I\alpha - Amylase = \frac{\text{A Control} - \text{A Sample}}{\text{A Control}} \times 100 \quad (2)$$

Where A Control = A Test − A Blank and A Sample = A Test − A Blank.

α-Glucosidase inhibitory potential

The inhibitory potential of α glucosidase assay was accomplished according to guidelines mentioned by Yin et al. [20]. 10µL solution of *α*-glucosidase (1 U/mL), phosphate buffer strength of 6.8 pH (100 mM) 50µL with sample concentrations at (25, 50, 100, and 200 µg/mL) in 20 µL were mixed and incubated for 15 min at 37 °C in a 96-well plate. Then, 20 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside (P-NPG) was added, and the reaction mixture was incubated for 20 min at 37 °C. The 0.1 M of Na_2CO_3 (50 UL) was added as a stop solution. Using a microplate reader, the absorbance of the released $C_6H_5NO_3$ was measured at 405 nm. The acarbose was used as a positive control along with the sample. The *α*-glucosidase potential of extracts was calculated and its IC50 values were determined using the software GraphPad prism. The *α*-glucosidase activity was measured using Eq. 3.

$$Inhibition\ ability\ (\%) = \frac{1 - \text{As}}{\text{Ac}} \times 100 \quad (3)$$

where As = Absorbance of the sample and Ac = Absorbance for control.

Cytotoxicity assay

According to the guidance given by the American Type Culture Collection (ATCC), the cell line ($HepG_2$) hepatocellular carcinoma was preserved. The duration of culture before the experiment to ensure consistency was about 24–48 h at 37 °C. The $HepG_2$ cell line was cultured in 10% fetal bovine serum and 0.1% streptomycin. Under the atmosphere containing $C0_2$ (5%) in a humidified incubator, these cells were incubated and subcultured at the pre-confluent densities by using trypsin-ethylenediaminetetraacetic acid (try-EDTA) (0.25%). The 2,5-diphenyl-2 H-tetrazolium bromide (MTT) assay was used to perform the cytotoxicity Properties activity of ethanolic extracts of *P. stewartii* plant [21]. The plate with a density of 5×10^4 cell were used for growth. After a 24-h incubation period, cells were treated with different sample concentrations and given another 48-h incubation period. The MTT solution (25 µL of 5 mg/mL) is added into each well and incubated for a few hours, then 100 µL DMSO is added to solubilize the formazan crystal. At the absorbance of 570 nm, the formazan crystal was measured using the spectrophotometer. The IC50 (concentration of extract that achieved a 50% of grown inhibition) was calculated, with results expressed in µg/mL.

XRD analysis

P. stewartii extracts were used to investigate the presence of non-crystalline and crystalline phases in the powder form of different extracts using XRD [22]. The machinal

shaking method was used for the extraction of samples, followed by syringe filtration for the fine filtration of the sample. The filtered sample was dried and changed into a fine powder by adding ethanol solvent. The XRD pattern of powder was noted by Bruker Advance XRD Cu-ka radiation and Ni filter in the angular ranges 2θ = 10–80° with the size of angular step Δ2θ = 0.05°.

Optimization design

Seventeen experimental runs, including 5 center points, were favored for different combinations in the statistical analysis of Box-Behnken Design (BBD). Five center points were chosen to estimate the experimental error and assess the adequacy of the model. The suitable prediction model for response calculation of *P. stewartii* leaf extracts under the following conditions. The parameters of extraction were optimized, including X1 (100, 150, and 200 rpm), X3 (2, 5, and 8 h), and X2 (100, 150, and 200 mL). Table 1 lists the actual and coded levels of extraction conditions. The rotary evaporator and shaker were used to optimize the extract. For additional analysis, rotary evaporators' extracts, including TFC and TPC, were gathered.

Statistical analysis

A BBD of the response surface methodology (RSM) with three independent variables was used to optimize the relationships between X1 (100, 150, and 200 rpm), X2 (100, 150, and 200 mL), and X3 (2, 5, and 8 h). The total number of experimental runs was 17. Five central points (C_1–C_5) were established to determine the response error. Using a Stat–Ease® software (version 11.1.2.0, Minneapolis, MN, USA), the extraction process was statistically tested for calculation of level of significance with 5% ($p \le 0.05$) as described by Montgomery et al. [23]. Analysis of variance (ANOVA) was performed to estimate the significance differences between independent variables and the model was validated using lack of fit tests and R^2 values.

Table 1 The independent variables for optimized forms are calculated from the actual and code levels through BBD

Independent Variables	Units	Coded levels		
		−1	0	+1
X1 = Speed	Rpm	100	150	200
X2 = Solvent volume	mL	100	150	200
X3 = Extraction time	H	2	5	8

Table 2 Effect of ethanolic extraction conditions on the response parameters

Run	Independent variables			Yield (%)		
	X1 (rpm)	X2 (mL)	X3 (h)	LEE	FEE	WPEE
1	150 (0)	200 (+1)	8 (+1)	13.95 ± 0.40^{i}	14.66 ± 0.34^{a}	18.92 ± 0.16^{d}
2 (C_1)	150 (0)	150 (0)	5 (0)	13.10 ± 0.24^{ij}	14.62 ± 0.19^{d}	18.85 ± 0.20^{g}
3 (C_2)	150 (0)	150 (0)	5 (0)	13.89 ± 0.28^{ij}	14.61 ± 0.36^{hi}	18.85 ± 0.13^{i}
4	200 (+1)	150 (0)	8 (+1)	13.15 ± 0.14^{k}	14.65 ± 0.11^{k}	18.91 ± 0.23^{gf}
5	200 (+1)	200 (+1)	5 (0)	13.11 ± 0.12^{ik}	14.63 ± 0.14^{b}	18.87 ± 0.31^{gh}
6 (C_3)	150 (0)	150 (0)	5 (0)	13.91 ± 0.19^{de}	14.60 ± 0.20^{hl}	18.84 ± 0.25^{b}
7 (C_4)	150 (0)	150 (0)	5 (0)	13.80 ± 0.27^{e}	14.59 ± 0.021^{s}	18.84 ± 0.022^{f}
8 (C_5)	150 (0)	150 (0)	5 (0)	13.70 ± 0.27^{ed}	14.59 ± 0.40^{m}	18.84 ± 0.13^{c}
9	150 (0)	200 (+1)	2 (−1)	13.30 ± 0.31^{hi}	14.55 ± 0.24^{mi}	18.81 ± 0.22^{j}
10	100 (−1)	150 (0)	8 (+1)	13.14 ± 0.29^{h}	14.65 ± 0.33^{n}	18.91 ± 0.26^{ba}
11	100 (−1)	150 (0)	2 (−1)	13.20 ± 0.41^{gf}	14.53 ± 0.28^{c}	18.8 ± 0.11^{df}
12	100 (−1)	100 (−1)	5 (0)	13.50 ± 0.24^{f}	14.57 ± 0.26^{c}	$18.83 \pm 0.30^{cf.}$
13	200 (+1)	100 (−1)	5 (0)	13.60 ± 0.29^{d}	14.58 ± 0.36^{f}	18.83 ± 0.15^{ij}
14	150 (0)	100 (−1)	8 (+1)	13.13 ± 0.17^{c}	14.64 ± 0.38^{hi}	18.81 ± 0.17^{kl}
15	150 (0)	100 (−1)	2 (−1)	13.10 ± 0.29^{d}	14.51 ± 0.25^{a}	18.79 ± 0.18^{b}
16	100 (−1)	200 (+1)	5 (0)	13.11 ± 0.37^{di}	14.63 ± 0.24^{c}	18.86 ± 0.25^{c}
17	200 (+1)	150 (0)	2 (−1)	13.30 ± 0.22^{kj}	14.54 ± 0.33^{f}	18.81 ± 0.25^{a}

X1: Speed; X2: Solvent volume; X3: Extraction time; C_1-C_5 represents the central points of BBD

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

[a–q]Means with different superscripts indicating the level of significant difference ($p \le 0.05$)

Results and discussion

Extraction of ethanolic extracts

The effect of the extraction conditions on the yield of the LEE, FEE, and WPEE is presented in Table 2. The X1, X2, and X3 values indicated that the highest yield (13.95 ± 0.40, 14.66 ± 0.34, and 18.92 ± 0.16%) were observed at 150 rpm, 200 mL, and 8 h, while the lowest yield (13.10 ± 0.29, 14.51 ± 0.25, and 18.79 ± 0.18%) were observed 150 rpm, 100 mL, and 2 h. The outcomes of the present study reveal that in comparison between these extracts, WPEE (Leaves, Stem, Flower, Roots, Bark) exhibited the highest extraction yield, 18.92 ± 0.16%, followed by FEE (14.66 ± 0.34%) and LEE (13.95 ± 0.40%). The value of the extraction yield increased with an increase in the X2 and X3, while the level of yield decreased with a decrease in X2 and X3. Our results agree with reported data that extraction efficiency increased with an increase in X2 and X3 [9, 24, 25]. An increase in X1 can improve extraction efficiency, but excessive speed can lead to solvent evaporation and compound degradation, ultimately decreasing yield [26].

Figure 1 displayed the 3D surface plots between X1 versus X2, X2 versus X3, X1 versus X3. The 3D surface plots of X1 and X2 indicated the decrease in extraction yield, as shown in Fig. 1A and D, and G. The combined impact of X1 and X3 can be seen in Fig. 1C, F and F, showing less efficiency in extraction yield. An increase in extraction yield was seen in Fig. 1B and E, and H when the combined

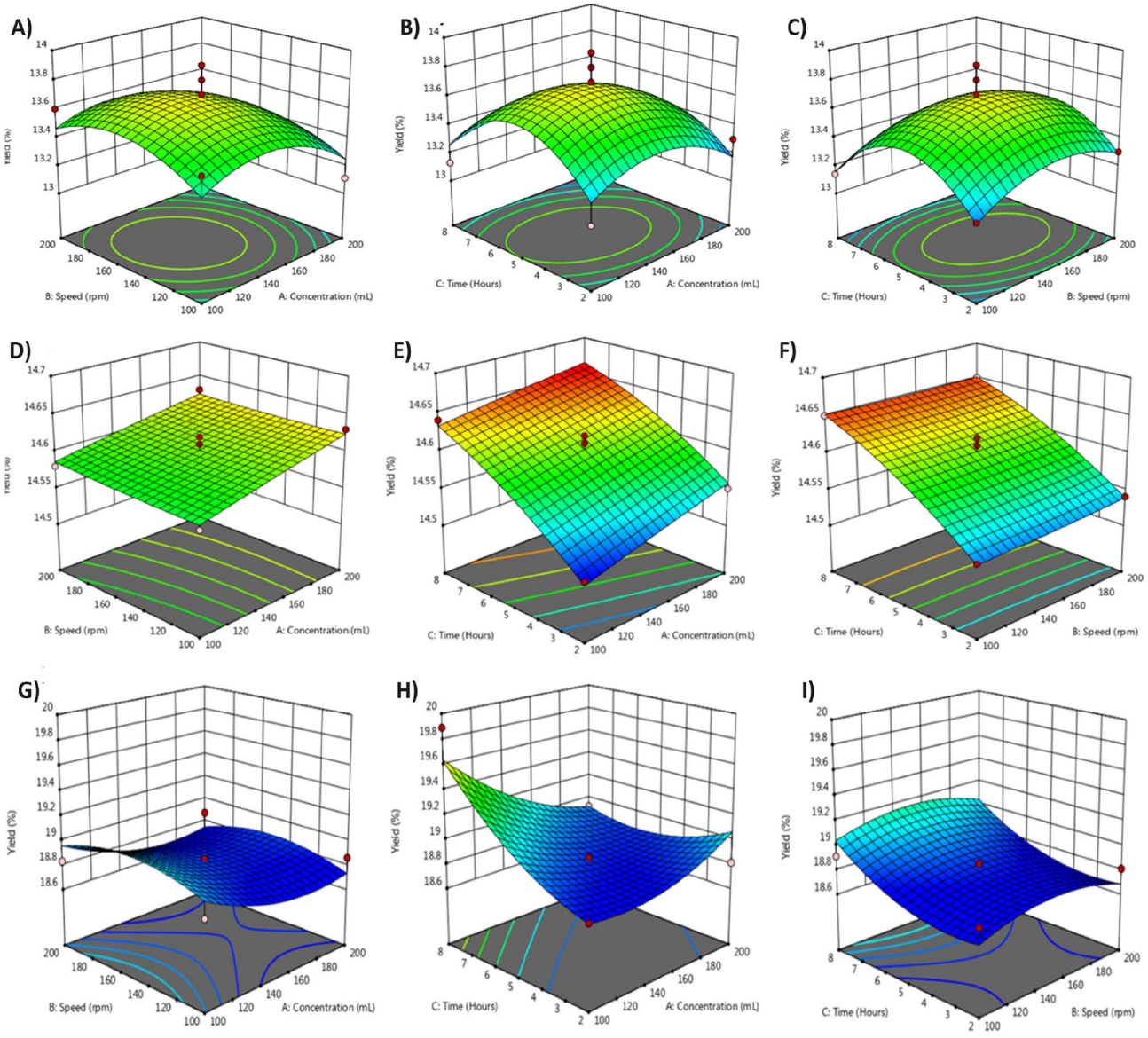


Fig. 1 Response surface plots (3-D) showing the effects of independent variables, **A** X1 versus X2 for LEE, **B** X2 versus X3 for LEE, **C** X1 versus X3 for LEE, **D** X1 versus X2 for FEE, **E** X2 versus X3 for FEE, **F** X1 versus X3 for FEE, **G** X1 versus X2 for WPEE, **H** X2 versus X3 for FEE, **I** X1 versus X3 for WPEE

impact of X2 and X3 was observed, which agrees with the previously published findings that X2 and X3 play an important role in increasing the extraction yield as reported by Mahmud et al. [25]. It has been reported in a previous study that X1 can improve extraction efficiency, but excessive speed can lead to solvent evaporation and compound degradation, ultimately decreasing yield [26]. It has been documented that independent parameters play an ample role in extraction yield. As for the biomolecule extraction from different parts of the plant, solvents are selected based on the polarity of the target solute, and to get a reproducible amount of yield from analogous compounds, multiple solvents can be used. A solvent having similar polarity to the solute will appropriately dissolve the solute. Previous studies also reported the impact of various solvents on the contents of secondary metabolites and their antioxidant potential [27, 28]. However, ethanol solvent proved to be best for the extraction of different phytochemicals from MPs with antioxidant and antimicrobial potential [29, 30]. Another study reported the potency and excellence of ethanol solvent for extraction of bioactive components from MPs [31]. Hence, multiple ethanolic extracts of *P. Stewartii* were employed for the biological evaluation.

A previous study reported that ethanolic (85%) extracts of *P. bovei De Noe* exhibited a 2.32% extraction yield at room temperature and 72 h with maximum agitation, while *P. Stewartii* resulted in a good extraction yield as compared to *P. bovei de Noe* plant [32]. Another study reported that *P. russeliana* exhibited a 14.12% extraction yield, these findings agreed with the present results of Alpay et al. [33]. A recent study reported that *Melissa officinalis L.* (MEL), *origanum majorana L.* (MAJ), *Calendula officinalis L.* (CAL), and *Achilea millefolium L* (MIL) exhibited 4.25%, 5.9%, 6.7%, and 5.1% extraction yield in ethanol solvent, respectively, which was comparatively less than our findings [34].

The linear coefficients (A, B, and C), the quadratic coefficients (A^2, B^2, and C^2), and the interactive coefficients (AB, AC, and BC) are noteworthy or non-significant, indicating the pattern of the interface among examined variables. The *P*-value indicating the significance level for each model was mentioned in Table 3. The BBD was highly significant and the lack of fit was statistically non-significant, suggesting that the BBD was suitable for yield. A high coefficient of calculation (R^2, which was 0.98 for LEE, 0.96 for FEE, and 0.95 for WPEE) and the difference between the adjusted R^2 and predicted R^2 were small, thus showing the reasonable fit of the model to the yield. Analysis of variance (ANOVA) showed that X2, and X3 were highly significant independent variables affecting the yield. This was confirmed by the significant linear and quadratic terms of the model. The high value of adj R^2 indicated the strong significance of the model. The signal-to-noise ratio, which contrasts the range of the expected values at the design points with the average prediction error, is sufficient. A ratio greater than four indicates adequate model discrimination [35].

The optimal precision signal for the model and its productive use in design navigation was revealed by LEE, FEE, and WPEE. In this research, the R^2 value was very close to the adj R^2, which was similar to the reported

Table 3 Analysis of variance (ANOVA) for second-order polynomial predicted pattern on different extraction conditions for ethanolic extracts and influence on response factors

Variation Sources		Yield (%)					
		DF	LEE		FEE		WPEE	
			MS	*P*-value	MS	*P*-value	MS	*P*-value
Model		9	0.0946	0.4589	0.0035	0.0002	0.0878	0.1296
Linear Effects	Concentration (A)	1	0.0544	0.4517	0.0036	0.0015	0.0968	0.1472
	Speed (B)	1	0.0055	0.8071	0.0001	0.5736	0.0001	0.9715
	Time (C)	1	0.0136	0.7022	0.0276	0.0001	0.2521	0.0339
Interaction Effects	AB	1	0.0025	0.8693	0.001	0.6890	0.0000	0.9798
	AC	1	0.0081	0.7675	0.0001	0.4315	0.2450	0.0358
	BC	1	0.0020	0.8822	0.0001	0.6890	0.0000	0.9798
Quadratic Effects	A^2	1	0.1500	0.2275	0.06	0.8689	0.0650	0.2235
	B^2	1	0.1095	0.2957	0.009	0.8047	0.0614	0.2355
	C^2	1	0.4345	0.0591	0.0005	0.1016	0.0759	0.1923
Residual		7	0.0858	–	0.0001	–	0.0365	–
Lack of Fit		3	0.0508	0.7292	0.0003	0.6295	0.0850	0.0001
Pure Error		4	0.1120	–	0.0002	–	0.0000	–
Cor. Total		16	–	–	–	–	–	–

A: Solvent volume; B: Speed; C: Extraction time; AB: Concentration * Speed; AC: Concentration* Time; BC: Speed * Time; A^2: Concentration^2; B^2: Speed^2; C^2: Time^2

Table 4 Displays the model regression equations for ethanolic extracts of yield (%) with actual and coded values using response methods

Response factor	Regression form	Regression equation
LEE	Coded	+ 13.68–0.0825 A + 0.0263B-0.0412 C-0.0250AB-0.0450AC-0.0225BC-0.1888A2-0.1613B2-0.3212C2
	Actual	+ 9.31264 + 0.024000Con + 0.022125Speed + 0.410694Time-0.000010 con*speed-0.000300 con*time-0.000150 Speed*time-0.000076 con^2 –0.000065 $speed^2$ –0.035694 $time^2$
FEE	Coded	+ 14.60 + 0.0213 A + 0.0025B + 0.0588 C-0.0025AB-0.0050AC-0.0025BC-0.0010A2 + 0.0015B2-0.0110C2
	Actual	+ 14.34678 + 0.000862Con + 0.000103Speed + 0.039306Time-1.00000E-06 con* Speed-0.000033 con*time-0.000017 speed*time-4.00000E-07 Con^2 +6.00000E-07 $speed^2$ –0.001222 $time^2$
WPEE	Coded	+ 18.84–0.1100 A + 0.0025B + 0.1775 C + 0.0025AB-0.2475AC-0.0025BC + 0.1242A2-0.1208B2 + 0.1343C2
	Actual	+ 18.04758-0.009010Con + 0.014473Speed + 0.160000Time + 1.00000E-06 con* speed-0.001650 con*time-0.000017 speed*time + 0.000050 con^2 –0.000048 $speed^2$ + 0.014917 $time^2$

A: Solvent volume; B: Speed; C: Extraction time; AB: Concentration * Speed; AC: Concentration* Time; BC: Speed * Time; A^2: $Concentration^2$; B^2: $Speed^2$; C^2: $Time^2$

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

Table 5 Phytochemical screening of different parts of *P. stewartii* plant extracts in ethanol

Phytochemicals	LEE	FEE	WPEE
Betacyanins	+	+	+
Quinones	+	+	+
Alkaloids	+	-	-
Flavonoids	+	+	+
Tannins	+	+	+
Phenols	+	+	+
Cardiac Glycosides	+	+	+
Phlobatannins	-	-	-
Terpenoids	+	+	+
Emodols	+	+	+

(+): Presence of phytochemicals; (-): Absence of phytochemicals

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

data of Zhang et al. [36]. LEE, FEE, and WEE expressed a high value of predicted R^2, which is established as its significant importance, as reported by earlier research of Mallieswaran et al. [37]. Table 4 reports the regression equation for LEE, FEE, and WEE.

Phytochemical analysis

Table 5 reports that LEE extracts showed the absence of phlobatannins from all the tested phytochemicals. Phlobatannins are well known to exhibit a wide range of biological effects, including anti-oxidant, anti-inflammatory, anti-viral and many other [38]. While FEE and WPEE extracts showed the absence of alkaloids and phlobatannins. Our findings are consistent with earlier information that the ethanolic extracts of *Phlomis* indicated the existence of such important phytochemicals, which were found to help impart antimicrobial, anti-analgesic, and antioxidant actions [14]. Another study has reported that *P. linearis Boiss* phytochemicals may be attributed to their antioxidant potential [39]. Many other investigations have reported that *P. olivieri* and *P. Persica Bioss* revealed the presence of bioactive components [40]. The diversity of various bioactive constituents present in ethanolic extracts suggests that *P. stewartii* could serve as a source of useful drugs.

Total phenolic contents (TPC) in ethanolic extracts

Run no. 1, where independent variables (X1 was 150 rpm, X2 was 200 mL, and X3 was 8 h) were set to study the response, recorded the highest TPC in LEE (17.79 ± 0.22 mg GAE/g DW). The lowest result for the response FEE (8.59 ± 0.26 mg GAE/g DW) was observed at run no. 15, where independent parameters (X1 was 150 rpm, X2 was 100 mL, and X3 was 2 h) were fixed to study the response. The efficiency of TPC increased with an increase in the X2, and X3, while the level of TPC decreased with a decrease in X2, and X3 (Table 6). A higher X1 can increase the extraction efficiency, but excessive speed results in compound degradation and solvent evaporation, which ultimately reduces yield. Our results are in agreement with reported data that TPC efficiency increased with an increase in X2 and X3 [9, 24–26].

Figure 2 illustrates the 3D surface plots between X1 versus X2, X2 versus X3, X1 versus X3. The effect of X2 and X3 is shown in Fig. 2B and E, and H. Figure 2C and F, and I displayed the combined impact of X1 and X3. The 3D surface plots of X1 and X2 indicated a decrease in TPC, as shown in Fig. 2A and D, and G. An increase in TPC was seen in Fig. 2B and E, and H when the combined impact of X2 and X3 was observed, which agrees with the previously published findings that X2 and X3 play an ample role in increasing the TPC [25]. The increase in X3, and X2 allows more of the desired bioactive components to diffuse and dissolve into the solvent. Similarly, greater solvent concentration can increase the extraction yield [9, 24, 25].

Figure 2A and D, and G recorded the lowest extraction of TPC under the influence of X1 and X2. As shown in Fig. 2B and E, and H, the highest extraction of TPC was observed under the impact of X2, and X3, which

Table 6 Effect of extraction parameters for response surface in TPC estimation of ethanolic extracts

Run	Independent variables			TPC (mg GAE/g DW)		
	X1 (rpm)	X2 (mL)	X3 (h)	TPC	FEE	WPEE
1	150 (0)	200 (+1)	8 (+1)	17.79 ± 0.22^{k}	9.39 ± 0.22^{g}	15.76 ± 0.34^{c}
2 (C_1)	150 (0)	150 (0)	5 (0)	17.32 ± 0.31^{c}	9.03 ± 0.41^{b}	15.33 ± 0.28^{da}
3 (C_2)	150 (0)	150 (0)	5 (0)	17.23 ± 0.19^{e}	9.02 ± 0.23^{hl}	15.31 ± 0.29^{b}
4	200 (+1)	150 (0)	8 (+1)	17.71 ± 0.12^{f}	9.33 ± 0.11^{s}	15.71 ± 0.27^{p}
5	200 (+1)	200 (+1)	5 (0)	17.36 ± 0.33^{c}	9.06 ± 0.18^{n}	15.43 ± 0.27^{ed}
6 (C_3)	150 (0)	150 (0)	5 (0)	17.21 ± 0.23^{i}	8.99 ± 0.35^{ef}	15.29 ± 0.10^{d}
7 (C_4)	150 (0)	150 (0)	5 (0)	17.22 ± 0.27^{g}	8.91 ± 0.40^{l}	15.22 ± 0.11^{v}
8 (C_5)	150 (0)	150 (0)	5 (0)	17.17 ± 0.12^{j}	8.96 ± 0.24^{m}	15.23 ± 0.13^{hi}
9	150 (0)	200 (+1)	2 (−1)	16.85 ± 0.25^{ba}	8.76 ± 0.34^{a}	14.87 ± 0.19^{fg}
10	100 (−1)	150 (0)	8 (+1)	17.67 ± 0.28^{z}	9.28 ± 0.16^{h}	15.68 ± 0.16^{a}
11	100 (−1)	150 (0)	2 (−1)	16.73 ± 0.26^{h}	8.63 ± 0.26^{t}	14.81 ± 0.26^{ba}
12	100 (−1)	100 (−1)	5 (0)	17.08 ± 0.11^{f}	8.91 ± 0.18^{c}	15.13 ± 0.11^{gf}
13	200 (+1)	100 (−1)	5 (0)	17.16 ± 0.25^{d}	8.94 ± 0.21^{d}	15.14 ± 0.22^{f}
14	150 (0)	100 (−1)	8 (+1)	17.63 ± 0.30^{p}	9.25 ± 0.26^{l}	15.63 ± 0.38^{h}
15	150 (0)	100 (−1)	2 (−1)	16.68 ± 0.27^{f}	8.59 ± 0.26^{c}	$14.75 \pm 0.30^{cf.}$
16	100 (−1)	200 (+1)	5 (0)	17.37 ± 0.15^{ij}	9.03 ± 0.36^{f}	15.39 ± 0.29^{d}
17	200 (+1)	150 (0)	2 (−1)	16.82 ± 0.14^{mn}	8.71 ± 0.31^{q}	14.83 ± 0.22^{fk}

X1: Speed; X2: Solvent volume; X3: Extraction time; C_1-C_5 represents the central points of BBD

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

[a–z]Means with different superscripts indicating the level of significant difference ($p \leq 0.05$)

conforms to the previously reported data that X2 and X3 have a significant influence on raising the TPC [25]. This finding was consistent with previous extraction results of the *Phlomis* in ethanolic solution, resulting in high contents of phenolic compounds, which contribute significantly to antioxidant potential. Additionally, a few previously studies were looked up on Lamiaceae families for comparison purposes, such as *P. bruguieri*, *P. persica*, and *Marrubium vulgare*, which have possessed good antioxidant action [41]. Quantitatively, it has been mentioned in a previous investigation that ethanol extract of *O. majorana* belonging to the Lamiaceae family recorded TPC 5.62 mg GAE/g DW, while ethanolic extracts of *P. stewartii* 8.59 mg GAE/g DW exhibited good TPC as compared to *O. majorana* [42].

The values of the determination coefficient *P*-value of all models have been described in Table 7.

The BBD was highly significant and the lack of fit was statistically non-significant, suggesting that the BBD was suitable for yield. A high coefficient of calculation (R^2, which was 0.9877 for LEE, 0.9877 for FEE, and 0.9874 for WPEE) and the difference between the adjusted R^2 and predicted R^2 were small, thus showing the reasonable fit of the model to the yield. Analysis of variance (ANOVA) showed that X2 and X3 were highly significant independent variables affecting the yield. This was confirmed by the significant linear and quadratic terms of the model. The recorded data display the response of LEE of TPC as Pred R^2 values of 0.9052 in a moderate association with Adj R^2 values 0.9720, which was less than 0.0668. Moreover, in FEE and WPEE of TPC all responses, Pred R^2 values of 0.9597 and 0.8816 showed agreement with Adj R^2 values of 0.9719 and 0.9712, as it was less than 0.0122 and 0.0896, respectively. An appropriate precision signal was directed by the models of TPC (25.64 for LEE, 25.86 for FEE, and 25.82 for WPEE) to be utilized effectively. In this research, the R^2 value was very close to adj R^2, which was similar to the data published in the research work of Zhang et al. [36]. The regression equation for LLE, FEE, and WPEE is given in Table 8.

Total flavonoid contents

The results of the present research work showed that ethanolic extracts of *P. Stewartii* LEE extract exhibited the highest TFC content (3.68 ± 0.38 mg QE/g DW) under the influence of independent parameter (X1 was 150 rpm, X2 was 200 mL and X3 was 8 h). It has been mentioned in a previous study that the leaves of *P. Stewartii* plants contain more bioactive constituents [13]. However, Table 9 shows the low level of TFC (1.24 ± 0.37 mg QE/g DW) obtained from the FEE extracts under the impact of independent parameters including X1 was 150 rpm, X2 was 100 mL and X3 was 2 h. The following order was obtained LEE > WPEE > FEE. The TFC increased with an increase

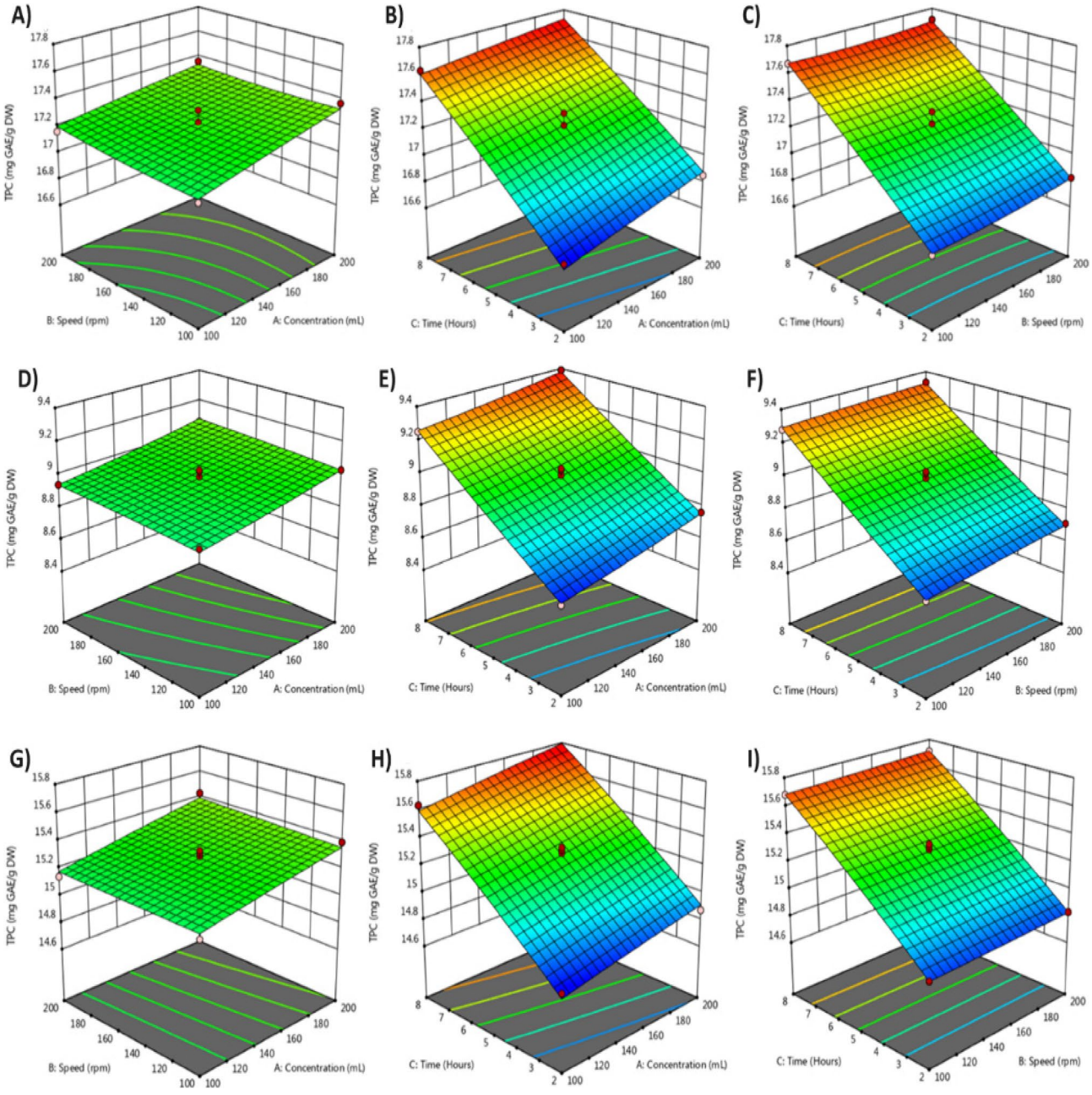


Fig. 2 Effect of independent variables, **A** X1 versus X2 for LEE, **B** X2 versus X3 for LEE, **C** X1 versus X3 for LEE, **D** X1 versus X2 for FEE, **E** X2 versus X3 for FEE, **F** X1 versus X3 for FEE, **G** X1 versus X2 for WPEE, **H** X2 versus X3 for FEE, **I** X1 versus X3 for WPEE

in the X2, and X3, while the level of TFC decreased with a decrease in X2, and X3. Our results agree with the reported data that TFC efficiency increased with an increase in X2, and X3 [9, 24, 25]. Another previous data reported that leaves of *Phlomis Lurestanica* had high value of total phenolic and flavonoid contents [43].

Figure 3A and D, and G displayed the combined effect of X1 and X2, which results in the lowest extraction of TFC. Figure 3C, F and I showed less TFC under the influence of X1 versus X3. As shown in Fig. 3B and E, and H, the highest value of TFC depends significantly on X2 and X3, which agrees with a previous study reporting that X2 and X3 play an important role in increasing the TFC [25]. Another study has reported that ethanolic extracts of Lamiaceae family possess good TFC, such as *Teucrium chamaedrys L*, *Origanum majorana L*, *Origanum*

Table 7 ANOVA for the predicted second-order polynomial pattern using extraction parameters for TPC through response parameters

Variation Source		TPC						
		DF	LEE		FEE		WPEE	
			MS	*P*-value	MS	*P*-value	MS	*P*-value
Model		9	0.1906	0.0001	0.0958	0.0001	0.1813	0.0001
Linear Effects	Concentration (A)	1	0.0648	0.0024	0.0378	0.0016	0.0800	0.0013
	Speed (B)	1	0.0050	0.2407	0.0045	0.1303	0.0012	0.5375
	Time (C)	1	1.64	0.0001	0.8192	0.0001	1.55	0.0001
Interaction Effects	AB	1	0.0020	0.4415	0.0000	1.0000	0.0002	0.7912
	AC	1	0.0030	0.3519	0.0002	0.7133	0.0000	0.9295
	BC	1	0.0006	0.6641	0.0002	0.7133	0.0000	0.9295
Quadratic Effects	A^2	1	0.0001	0.8930	0.0002	0.7436	0.0001	0.8775
	B^2	1	0.0011	0.5646	0.0001	0.8598	2.368E-06	0.9783
	C^2	1	0.0008	0.6247	0.0003	0.6519	0.0016	0.4924
Residual		7	0.0030		0.0015		0.0030	
Lack of Fit		3	0.0030	0.4808	0.0004	0.9052	0.0038	0.3259
Pure Error		4	0.0030		0.0024		0.0024	
Cor. Total		16	-		-		-	

A: Solvent volume; B: Speed; C: Extraction time; AB: Concentration * Speed; AC: Concentration* Time; BC: Speed * Time; A^2: Concentration^2; B^2: Speed^2; C^2: Time^2

Table 8 Model regression equations for ethanolic extracts with coded and actual values using the response surface methodology

Response Factors	Regression Form	Regression Equation
LEE	Coded	+ 17.23 + 0.0900 A + 0.0250B + 0.4525 C-0.0225AB-0.0275AC-0.0125BC-0.0038A2 + 0.0162B2-0.0137C2
	Actual	+ 15.80264 + 0.004517Con + 0.000317Speed + 0.206111Time-9.00000E-06Con*Speed-0.000183Con*Time-0.000083 Speed*Time-1.50000E-06Con2 + 6.50000E-06Speed2-0.001528Time2
FEE	Coded	+ 8.98 + 0.0688 A + 0.0238B + 0.3200 C + 0.0000AB-0.0075AC-0.0075BC + 0.0065A2-0.0035B2 + 0.0090C2
	Actual	+ 8.14817 + 0.000845Con + 0.001145Speed + 0.111667Time-7.59124E-19Con*Speed-0.000050Con*Time-0.000050 Speed*Time + 2.60000E-06Con2-1.40000E-06Speed2 + 0.001000Time2
WPEE	Coded	+ 15.28 + 0.1000 A + 0.0125B + 0.4400 C + 0.0075AB + 0.0025AC + 0.0025BC-0.0043A2 + 0.0008B2-0.0193C2
	Actual	+ 14.21269 + 0.001977Con-0.000373Speed + 0.163056 Time + 3.00000E-06Con*Speed + 0.000017Con*Time + 0.000017Speed*Time-1.70000E-06Con2 + 3.00000E-07Speed2-0.002139Tim2

A: Solvent volume; B: Speed; C: Extraction time; AB: Concentration * Speed; AC: Concentration* Time; BC: Speed * Time; A^2: Concentration^2; B^2: Speed^2; C^2: Time^2

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

vulgare L, *Teucrium chamaedrys L*, *Teucrium montanum L*, which showed TFC values of 5.87 mg GAE/g, 17.83 mg QE/g, 19.36 mg QE/g, 14.82 mg QE/g and 17.37 mgQE/g TFC, respectively [44].

The BBD was highly significant and the lack of fit was statistically non-significant, suggesting that the BBD was suitable for yield. A high coefficient of calculation (R^2, which was 0.8580, 0.9872, and 0.9944) and the difference between the adjusted R^2 and predicted R^2 were small, thus showing the reasonable fit of the model to the yield. Analysis of variance (ANOVA) showed that X2 and X3 were highly significant independent variables affecting the yield. This was confirmed by the significant linear and quadratic terms of the model (Table 10). In this research, the R^2 value was very close to R^2adj, which is similar to the data reported by Zhang et al. [36]. The regression equation of all ethanolic extracts is given in Table 11.

HPLC analysis

When examining the phenolic components found in MPs, one of the most popular applications is the HPLC. Characterizing every phenolic compound is impossible due to the rich diversity and complexity present in MPs. A typical HPLC chromatogram of different phenolic components of *P. stewartii* present in LEE, FEE, and WPEE extracts is shown in Fig. 4. LEE revealed the existence of important bioactive constituents including hydroxybenzoic acid (HB acid) and coumarin, as shown in Fig. 4A. *P*-coumaric, gallic acid, chlorogenic were detected in FEE extracts as shown in Fig. 4B, while WPEE showed the presence of *p*-coumaric acid, gallic acid, salicylic acid, HB acid, and coumarin as shown in Fig. 4C. Highest polyphenols p-coumaric (121.59) and HB acid (718.04) were reported in WPEE extracts. *P-Coumaric* acid has been shown to inhibit the proliferation and

Table 9 TFC response parameters in ethanol extracts under different extraction conditions

Run	Independent variables			TFC (mg QE/g DW)		
	X1 (rpm)	X2 (mL)	X3 (h)	LEE	FEE	WPEE
1	150 (0)	200 (+1)	8 (+1)	3.68 ± 0.38^{a}	1.62 ± 0.11^{fg}	2.92 ± 0.22^{m}
2 (C_1)	150 (0)	150 (0)	5 (0)	3.51 ± 0.19^{f}	1.51 ± 0.37^{kj}	2.73 ± 0.13^{f}
3 (C_2)	150 (0)	150 (0)	5 (0)	3.51 ± 0.19^{a}	1.51 ± 0.025^{c}	2.72 ± 0.18^{b}
4	200 (+1)	150 (0)	8 (+1)	3.65 ± 0.25^{a}	1.62 ± 0.27^{c}	2.91 ± 0.39^{a}
5	200 (+1)	200 (+1)	5 (0)	3.53 ± 0.22^{f}	1.54 ± 0.33^{bc}	2.75 ± 0.25^{s}
6 (C_3)	150 (0)	150 (0)	5 (0)	3.49 ± 0.31^{q}	1.51 ± 0.14^{mn}	2.71 ± 0.18^{d}
7 (C_4)	150 (0)	150 (0)	5 (0)	3.46 ± 0.11^{d}	1.48 ± 0.15^{ij}	2.71 ± 0.36^{f}
8 (C_5)	150 (0)	150 (0)	5 (0)	3.46 ± 0.28^{c}	1.46 ± 0.41^{f}	2.69 ± 0.39^{h}
9	150 (0)	200 (+1)	2 (−1)	3.26 ± 0.23^{i}	1.31 ± 0.25^{ba}	2.57 ± 0.31^{hi}
10	100 (−1)	150 (0)	8 (+1)	3.63 ± 0.33^{h}	1.62 ± 0.18^{n}	2.91 ± 0.27^{d}
11	100 (−1)	150 (0)	2 (−1)	3.51 ± 0.23^{df}	1.26 ± 0.38^{m}	2.53 ± 0.31^{c}
12	100 (−1)	100 (−1)	5 (0)	3.39 ± 0.19^{de}	1.43 ± 0.08^{ik}	2.65 ± 0.14^{f}
13	200 (+1)	100 (−1)	5 (0)	3.41 ± 0.34^{gh}	1.44 ± 0.023^{gz}	2.67 ± 0.027^{e}
14	150 (0)	100 (−1)	8 (+1)	3.62 ± 0.33^{h}	1.58 ± 0.31^{c}	2.88 ± 0.22^{fg}
15	150 (0)	100 (−1)	2 (−1)	3.48 ± 0.19^{ab}	1.24 ± 0.37^{ij}	2.51 ± 0.18^{e}
16	100 (−1)	200 (+1)	5 (0)	3.52 ± 0.22^{g}	1.52 ± 0.25^{i}	2.74 ± 0.28^{d}
17	200 (+1)	150 (0)	2 (−1)	3.24 ± 0.16^{d}	1.28 ± 0.20^{f}	2.55 ± 0.13^{i}

X1: Speed; X2: Solvent volume; X3: Extraction time; C_1-C_5 represents the central points of BBD

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

$^{a-z}$Means with different superscripts indicating the level of significant difference ($p \leq 0.05$)

migration of cancer cells and promote apoptotic cancer cell death, supporting its potential anticancer effects [45]. Gallic acid is a widely distributed phenolic acid in the plant kingdom, with antiviral and anti-tumor potential [46]. The current research supports the reported data that ethanolic extracts have a protective role and can prevent the phenolic components from being oxidized by enzymes, such as phenoloxidases [10]. A previous study revealed the presence of different phenolic acids, such as vanillic acids, *p*-coumaric acid, rosmarinic acids, caffeic acid, gallic acid, and sinapinic acid, in the *Phlomis* plants. To some extent, our results are in accordance with the reported data that the *Phlomis* plant has various phenolic compounds [47]. Another study reported the presence of p-coumaric acid, gallic acid in Phlomis Stewarttii plant. All these phenols have therapeutic uses, i.e., hydroxybenzoic acid and coumarin are commonly used in the treatment of renal cell carcinoma and they have the potential to counteract the side effects caused by radiotherapy [48]. Similarly, *p*-coumaric acid has anti-aging, anti-tumor, and many other pharmacological effects [49]. In several phytomedicines, gallic acid has been found with diverse pharmacological and biological potential, such as apoptosis of cancer cells, free radical scavenging, and interfering with cell signaling pathways [50]. Chlorogenic acid shows promise as an anti-hypertensive, anti-inflammatory, and anti-obesity agent [51]. Salicylic acid has anti-inflammatory and antibacterial activities and also acts as an exfoliant to remove skin cells [52].

Gas chromatography-mass spectroscopy (GC-MS) is a combined analytical technique used to determine and identify compounds present in a plant sample. GC-MS plays an essential role in the phytochemical analysis and chemotaxonomic studies of medicinal plants containing biologically active components.

GC-MS analysis

GC-MS analysis is an analytical technique used to determine the presence of various bioactive components in the extract. It plays an ample role in the phytochemical analysis and chemotaxonomic studies of MPs having biologically active constituents [13]. Various bioactive constituents were identified from the GC-MS investigation of ethanol extracts of *P. stewartii* extracts exhibiting various phytochemical activities. The chromatogram is presented in Fig. 5, while the chemical components with their molecular formula, concentration, retention time, and molecular weight are presented in Table 12. The data obtained from the present work showed the presence of different compounds, such as thiazole, 2-ethylacridine, 9,12–octadecadioic acid, arsenous acid, 3,5-ethanoquinolin-10-one, hexahydropyridine. It has been reported that thiazole exhibits a wide range of biological activities such as anti-cancer, anti-inflammatory and anti-bacterial

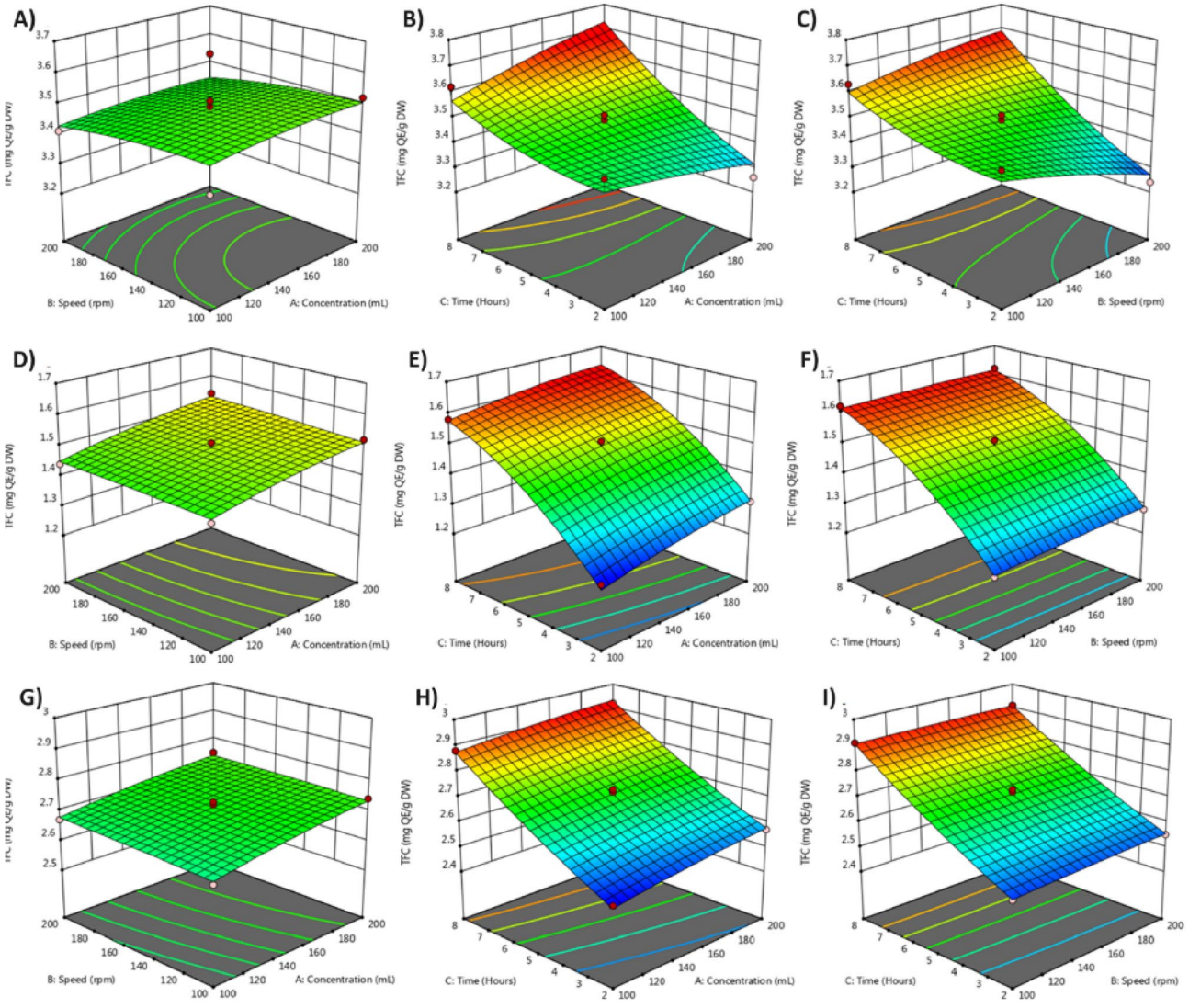


Fig. 3 Impact of independent variables, **A** X1 versus X2 for LEE, **B** X2 versus X3 for LEE, **C** X1 versus X3 for LEE, **D** X1 versus X2 for FEE, **E** X2 versus X3 for FEE, **F** X1 versus X3 for FEE, **G** X1 versus X2 for WPEE, **H** X2 versus X3 for FEE, **I** X1 versus X3 for WPEE

potential [53]. 2-ethylacridine and 9,12 octadecadioic acid possess anti-tumor, anti-microbial, anti-fungal, anti-viral and anti-diabetic effects [54]. All these identified constituents revealed their biological activities, such as antioxidant, anti-cancer, antiandrogenic, and anti-coronary potential [13, 54]. Some of these bioactivities reported in the literature were directly related to oral health, such as antipyretic and anti-inflammatory potential [55]. The results are comparable to those of the previous studies showing that the *Phlomis* plant present various phytochemicals, including linoleic acid, 9,12–octadecadioic acid, stigmasterol, pregnane, phytol, eicosanoic acid, and β-Sitosterol. The GC-MS analysis of *P. Stewartii* plant are in accordance with the reported data [13]. The obtained results confirm that *P. stewartii* is a powerful source of antioxidants and using different extraction conditions, and polar solvent plays an important role in the extraction of various compounds from the plant's extracts.

Enzyme inhibition

α-amylase enzyme inhibition assay

Enzyme inhibition of *α*-amylase potential by different fractions of *P. stewartii* and acarbose was observed at different (25 to 200 µg/mL) concentrations. A maximum 80.12, 80.88, 81.73, and 88.11% inhibition of the *α*-amylase assay was observed at 200 µg/mL concentration from LEE, FEE, WPEE, and acarbose, respectively.

Table 10 ANOVA of the predicted second-order polynomial pattern through different extraction conditions for ethanolic extracts and effect on response parameters

Variation Source		TFC						
		DF	LEE		FEE		WPEE	
			MS	*P*-value	MS	*P*-value	MS	*P*-value
Model		9	0.0225	0.0268	0.0277	0.0001	0.0308	0.0001
Linear Effects	Concentration (A)	1	0.0010	0.6592	0.0113	0.0017	0.0091	0.0004
	Speed (B)	1	0.0061	0.2976	0.0003	0.4387	0.0003	0.2743
	Time (C)	1	0.1485	0.0008	0.2278	0.0001	0.2665	0.0001
Interaction Effects	AB	1	0.0000	0.9444	0.0000	0.8230	0.0000	0.7471
	AC	1	0.0196	0.0825	0.0002	0.5085	0.0001	0.5238
	BC	1	0.0210	0.0741	0.0001	0.6564	0.0001	0.5238
Quadratic Effects	A^2	1	0.0005	0.7643	0.0004	0.3954	0.0002	0.3515
	B^2	1	0.0007	0.7110	0.0000	0.8542	0.0000	0.7658
	C^2	1	0.0050	0.3398	0.0093	0.0029	0.0010	0.0739
Residual		7	0.0048		0.0005		0.0002	
Lack of Fit		3	0.0103	0.0104	0.0004	0.5958	0.0002	0.4712
Pure Error		4	0.0006		0.0005		0.0002	
Cor. Total		16	-		-		-	

A: Solvent volume; B: Speed; C: Extraction time; AB: Concentration * Speed; AC: Concentration* Time; BC: Speed * Time; A^2: Concentration^2; B^2: Speed^2; C^2: Time^2

Table 11 Model regression equations for ethanolic extracts with coded and actual values using the RSM

Response factor	Regression form	Regression equation
LEE	Coded	+ 3.49 + 0.0112 A-0.0275B + 0.1363 C-0.0025AB + 0.0700AC + 0.0725BC-0.0105A2-0.0130B2C2
	Actual	+ 3.88200-0.000698Con-0.001257Speed-0.135417Time-1.00000E-06 con* Speed + 0.000467 con* Time + 0.000483 speed*time-4.20000E-06con2-5.20000E-06speed2 + 0.003833time^2
FEE	Coded	+ 1.49 + 0.0375 A + 0.0063B + 0.1688C + 0.0025AB-0.0075AC-0.0050BC-0.0095A2-0.0020B2-0.0470C^2
	Actual	+ 0.807444 + 0.001990Con + 0.000382Speed + 0.120972Time + 1.00000E-06 con* speed-0.000050 con*time-0.000033 speed*time-3.80000E-06con2-8.00000E-07speed2-0.005222time^2
WPEE	Coded	+ 2.71 + 0.0338 A + 0.0063B + 0.1825 C-0.0025AB-0.0050AC-0.0050BC-0.0073A2-0.0023B2 + 0.0153C^2
	Actual	+ 2.17219 + 0.001862Con + 0.000712Speed + 0.053889Time-1.00000E-06 con* speed-0.000033 con*time-0.000033 speed*time-2.90000E-06Con2-9.00000E-07speed2 + 0.001694time^2

A: Solvent volume; B: Speed; C: Extraction time; AB: Concentration * Speed; AC: Concentration* Time; BC: Speed * Time; A^2: Concentration^2; B^2: Speed^2; C^2: Time^2

LEE Leaves ethanol extracts, *FEE* Flower ethanol extract, *WPEE* Whole plant ethanol extracts

Among all the extracts, LEE exhibited the lowest IC50 at 53.33 µg/mL, followed by FEE at 55.65 µg/mL, WPEE at 58.88 µg/mL, and acarbose at 33.29 µg/mL, as shown in Table 13. Software GraphPad prism was used to find the IC50 value.

For comparison purposes, some previously reported data shows that *Phlomis* plants, such as *P. linearis boiss* ethanolic extracts, were evaluated for in vitro α-amylase potential, which inhibited the enzyme activity by 30.5 ± 1.4% at a concentration of 10 mg/mL [39]. Our findings are also in support of previously reported data that ethanolic extracts of *P. sieheana* showed inhibitory ability against α-amylase [56].

α-glucosidase enzyme inhibition assay

Diabetes Type II (DT2) can be managed by inhibiting the hydrolysis of carbohydrates and retaining the D-glucose absorption rate in the blood. The activity of various extracts to scavenge *α*-glucosidase enzyme activity was observed at different concentrations from 25 to 200 µg/mL. All the different fractions showed *α*-glucosidase inhibition activity in a dose-dependent way from 25 to 200 µg/mL. A maximum of 81.22, 80.22, 80.17, and 87.18% inhibition of *α*-glucosidase was observed at a maximum concentration of 200 µg/mL from LEE, FEE, WPEE, and acarbose, respectively, as shown in Table 13. Among all extracts, LEE exhibited the lowest IC50 value at 51.03 µg/mL, followed by FEE at 55.68 µg/mL, WPEE at 56.21 µg/mL, and acarbose at 37.25 µg/mL, as shown in Table 13.

In similar lines, another author reported that ethanolic extracts of genus *Phlomis* result in good *α*-glucosidase enzyme inhibition [57]. Furthermore, the result from the previous study revealed a direct correlation between

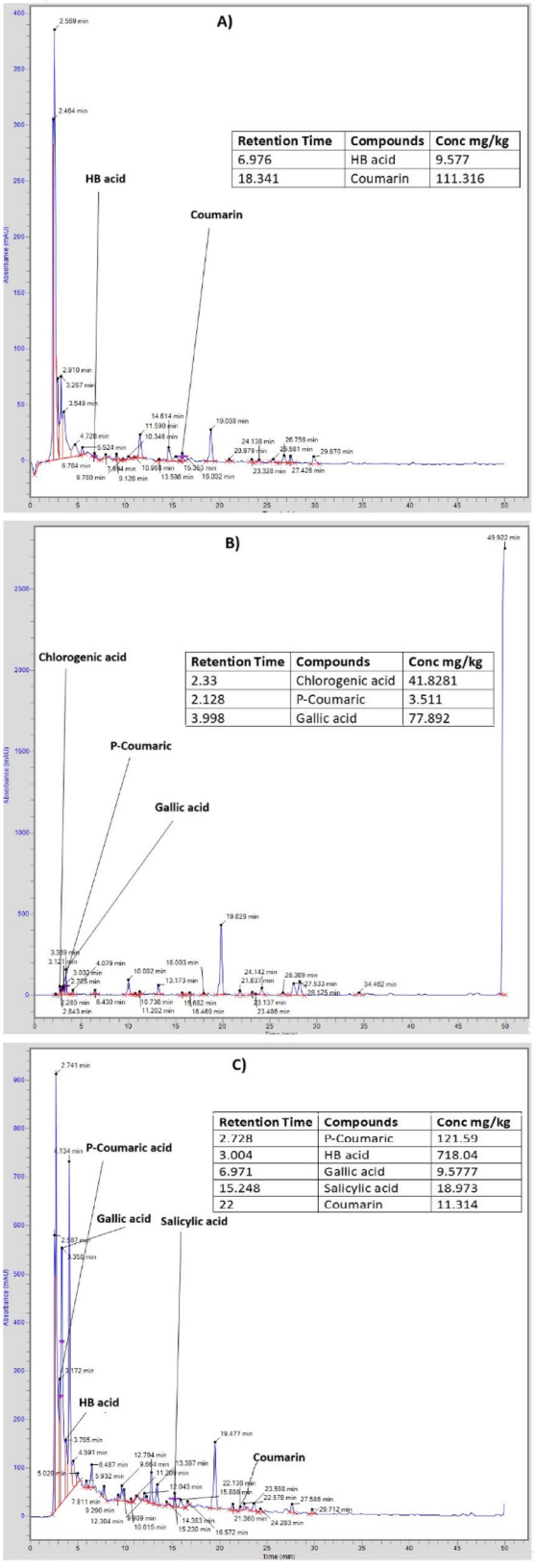


Fig. 4 Chromatograph of phenolic compound detected and their concentration from *P. stewartii* extract (**A**) LEE, **B** FEE, **C** WPEE

antioxidant potential and *α*-glucosidase inhibitory activity [58]. The small intestine's human pancreatic *α*- amylase potential has been linked to increasing postprandial glucose levels, the control of which is, therefore, an important factor in the treatment of diabetes. Different extracts from *P. stewartii* were reported to show antidiabetic potential, which includes the *α*- amylase and α-glucosidase potential of ethanol and methanol extracts [13]. Our results show that *P. Stewarttii* plant extracts are responsible for *α*- amylase and *α*- glucosidase inhibition; hence, further purification of the extracts was carried out. The ethanolic extract with the highest inhibitory potential was characterized by GC-MS, which confirmed the presence of a compound such as linoleic acid, 9,12–octadecadioic acid, stigmasterol, pregnane, phytol, eicosanoic acid, and β-Sitosterol. From the results, it was clear that the compound inhibited the *α*- amylase and *α*- glucosidase enzymes significantly. For instance, α-glucosidase and *α*-amylase are protein enzymes found in human secretions, fungi, and starch-containing seeds. They hydrolyse polysaccharides, such as starch and carbohydrates, to produce glucose, maltose, and glucoamylase. It serves as an important digestive enzyme that helps in intestinal absorption. These enzymes are responsible for the breakdown of long chain carbohydrates and the breakdown of diabetes. characterized by hyperglycemia. can be controlled through inhibition of *α*- amylase [59].

Screening of cytotoxicity effect of extracts

The screening of cytotoxicity Properties of extracts was investigated by using the MTT viability assay. $HePG_2$ cell line was incubated with different extracts of *P. stewartii* for 24 h and 48 h and the status of the cell growth was examined. The cytotoxicity assay uses $HepG_2$ cells because they are a dependable model for examining the harmful effects of substances on human liver cells. The human liver cancer cell line $HepG_2$ cells are stable, easy to work with, and have an endless lifespan [60]. The results showed all of the extracts expressed various concentrations (12.5, 25, 50, 100, 200 µg/mL) cytotoxicity impact against the treated cell line. The outcomes confirmed that FEE showed the most potent antitumor potential. At a concentration of 200 µg/mL, the maximum cytotoxicity properties were found in FEE, LEE, and WPEE, which showed 66%, 59%, and 58% in Fig. 6A, B, and C, respectively. Moreover, the results showed that the cytotoxicity properties of the extracts were recorded in the order FEE > LEE > WPEE. The IC50 value of the FEE, WPEE, and LEE were respectively 285 > 315 > 327 µg/mL, as shown in Fig. 6D. Various phenolic and polyphenolic components from the *Phlomis* plant have been reported to be efficient cytotoxicity agents. These include naringenin, verbascoside

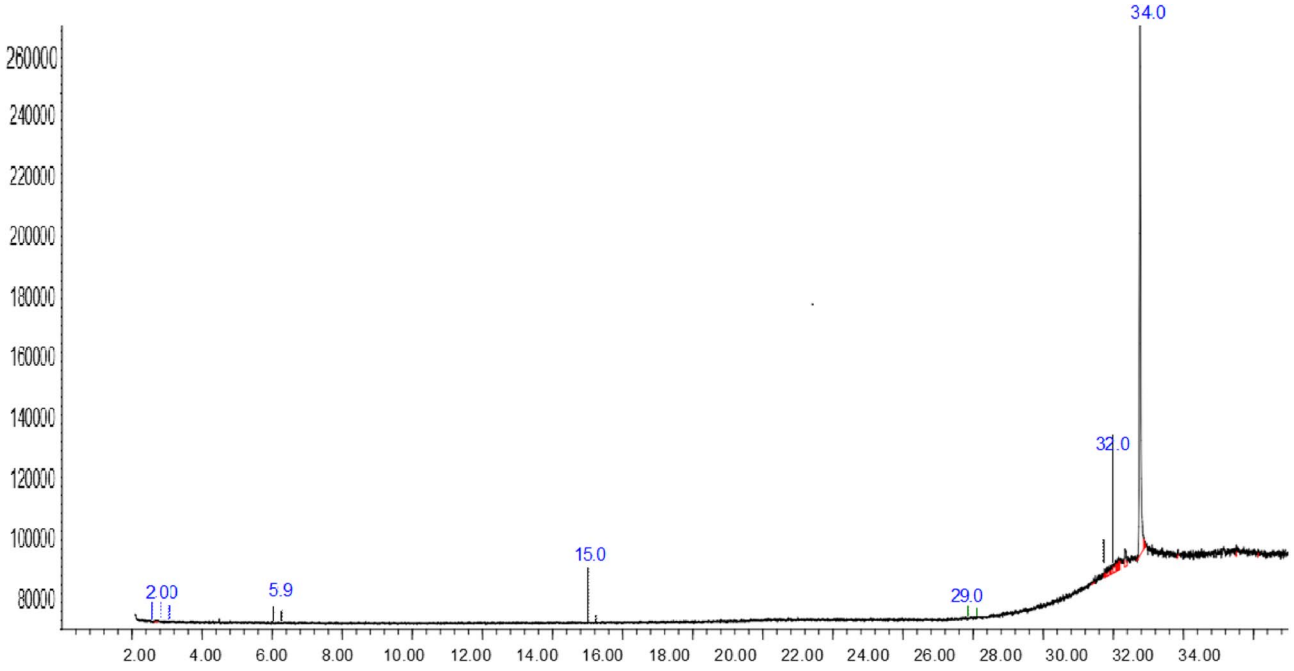


Fig. 5 GC-MS analysis WPEE extract

Table 12 Different bioactive components are present in WPEE extracts from GC-MS

Sr no	Peak Area	Retention Time (min)	Compounds Name	Molecular Formula	Molecular Weight
1	1.89	2.03	Thiazole	C3H3NH	85.13 g/mol
2	1.8	5.91	2-Ethylacridine	C15H13N	207.02 g/mol
3	4.89	15.00	9,12–octadecadioic acid	C18H32O2	78.091 g/mol
4	1.11	29.01	Arsenous Acid	H3AsO3	125.02 g/mol
5	15.23	32.00	3,5-Ethanoquinolin-10-one	C13H21NO	207.21 g/mol
6	30.94	34.00	Hexahydropyridine	C12H17NO2	193.24 g/mol

Table 13 The *α*-amylase and *α*-glucosidase inhibitory potential of *P. stewartii*

Sample	Concentration (µg/mL)	% of inhibition α-amylase	% of inhibition α-glucosidase	α-amylase IC50 value (µg/mL)	α-glucosidase IC50 value (µg/mL)
Acarbose	25	46.6 ± 0.02	45.71 ± 0.01	33.29 ± 0.34	37.25 ± 0.28
	50	55.01 ± 0.04	54.22 ± 0.02		
	100	66.22 ± 0.01	65.12 ± 0.04		
	200	88.11 ± 0.02	87.18 ± 0.03		
LEE	25	40.95 ± 0.02	41.75 ± 0.02	53.33 ± 0.21	51.07 ± 0.17
	50	51.11 ± 0.01	51.61 ± 0.04		
	100	62.47 ± 0.02	62.38 ± 0.04		
	200	80.12 ± 0.03	81.22 ± 0.02		
WPEE	25	39.81 ± 0.02	40.55 ± 0.03	58.88 ± 0.11	56.21 ± 0.30
	50	50.77 ± 0.01	50.65 ± 0.02		
	100	62.02 ± 0.03	61.19 ± 0.02		
	200	81.73 ± 0.02	80.17 ± 0.02		
FEE	25	40.11 ± 0.01	40.68 ± 0.02	55.65 ± 0.12	55.68 ± 0.16
	50	50.77 ± 0.03	50.75 ± 0.05		
	100	62.08 ± 0.01	62.19 ± 0.03		
	200	80.88 ± 0.02	80.22 ± 0.02		

LEE Leave Ethanol Extracts, *FEE* Flower Ethanol Extracts, *WPEE* Whole Plant Ethanol extracts

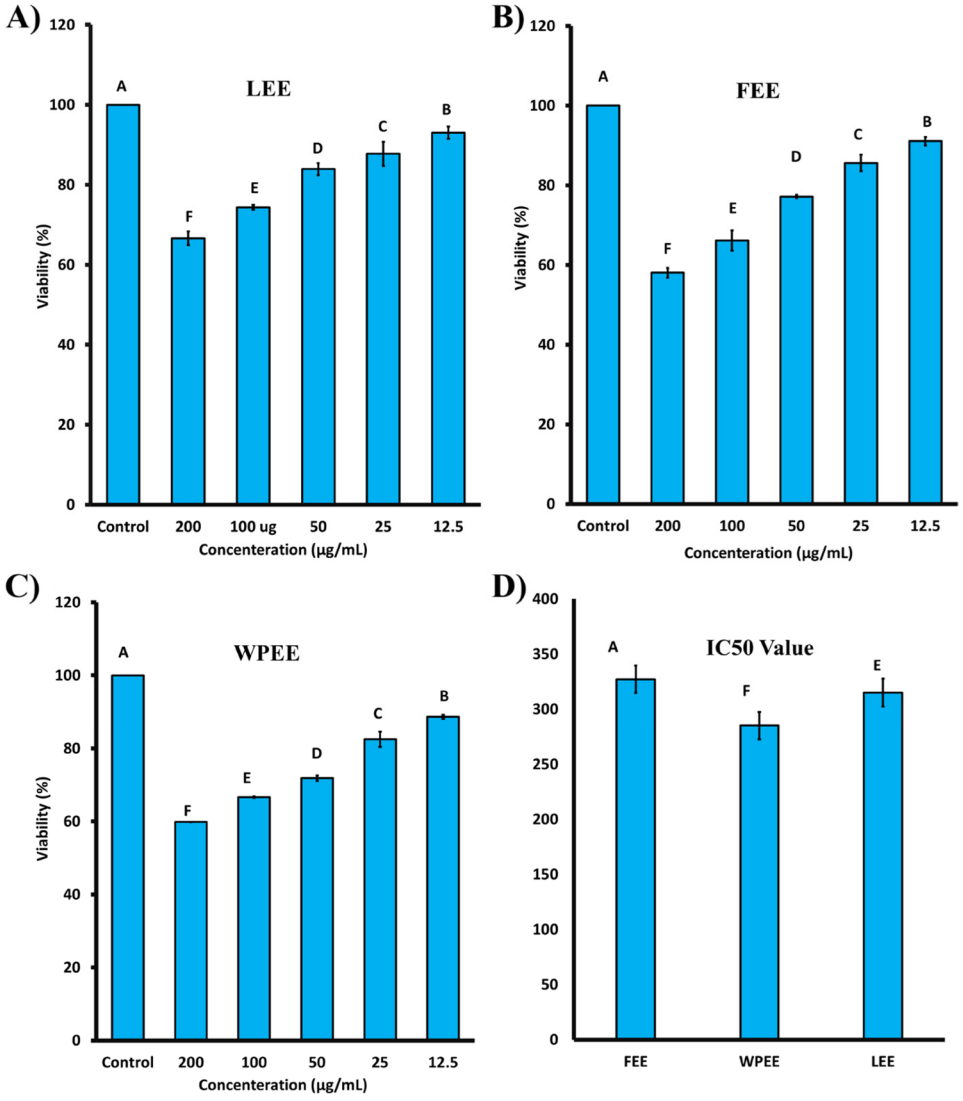


Fig. 6 Viability of *P. stewartii* plant ethanolic extract for anti-proliferative assay. **A** Leave ethanol extracts (LEE); **B** Flower ethanol extracts (FEE); **C** Whole plant ethanol extracts (WPEE); **D** IC50 value

(acetoside), chgenin, and kaempferol 3-O-glucoside [60]. Our findings are consistent with earlier research that demonstrated the ability of medicinal plant leaf extracts to stop the growth of human cancer cells.

X-Ray Diffraction (XRD) method of *P. stewartii*

The XRD method was used to show the crystalline nature of the sample plant of *P. stewartii*. XRD is particularly useful for identifying the crystalline structure of phytochemicals, including flavonoids, alkaloids, terpenoids, and other secondary metabolites [18]. The analysis of the X-ray diffraction pattern of *P. stewartii* is shown in Fig. 7. The few intense between 25°–40° with finite width, which indicates the presence of crystalline nature, and a few of them have small width peaks, which reflects the semi-crystalline nature of the plant sample. The peaks matched with the XRD differential pattern of phases of the carbon allotropes [18].

Conclusion

The various extraction factors of *P. stewartii* plant components were optimized using RSM. The extraction yield, TPC, and TFC increased with an increase in X3 and X2. The HPLC analysis revealed the existence of important bioactive constituents, including hydroxybenzoic acid, coumarin, *p*-coumaric acid, gallic acid, chlorogenic, and salicylic acid. GC-MS analysis showed the presence of different compounds such as thiazole, 2-ethylacridine, silicic acid, arsenous acid, 3,5-ethanoquinolin-10-one, and hexahydropyridine. The ethanol extracts exhibited a

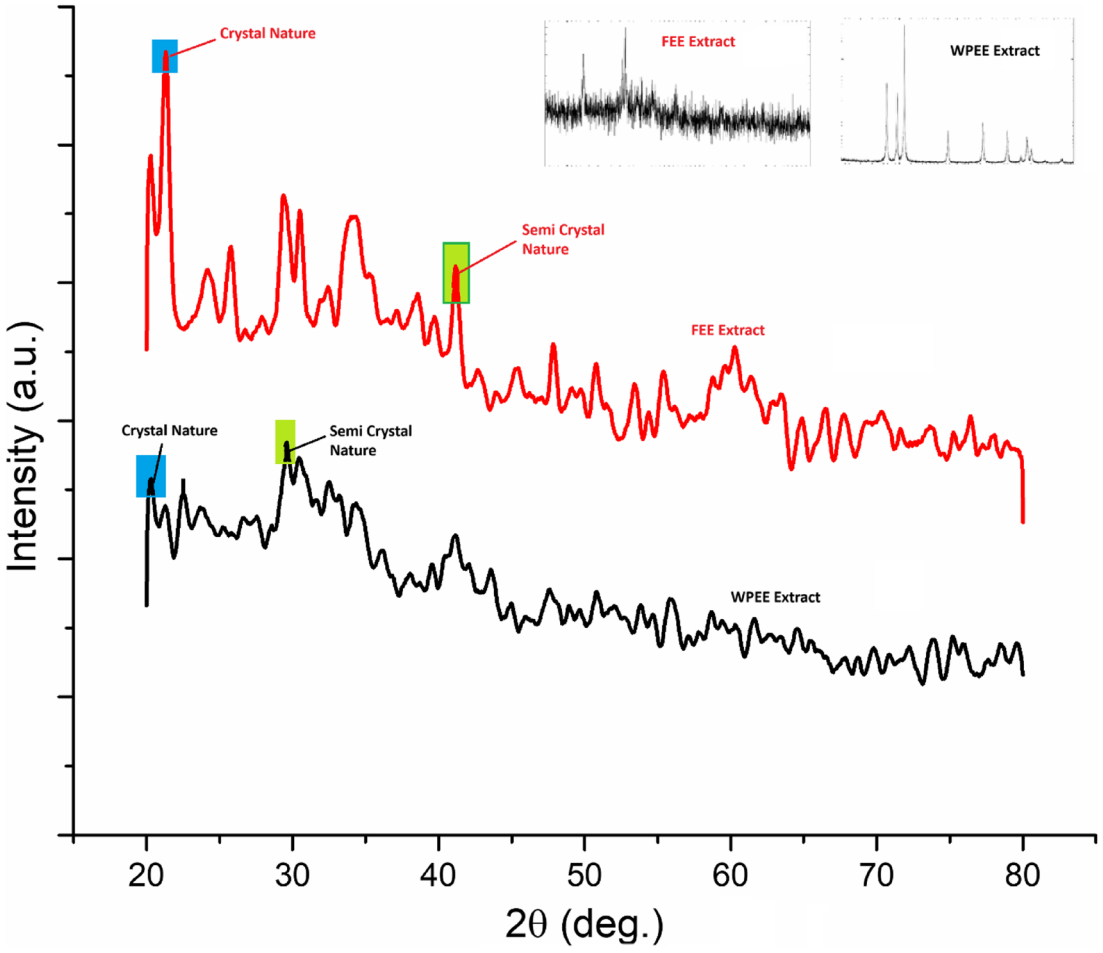


Fig. 7 The X-ray diffraction pattern of FEE and WPEE extracts

highly active profile against α-amylase and α-glucosidase. The anti-proliferative assay showed that all of the extracts expressed anti-proliferative impact against the treated cell line. The study finds that *P. stewartii* extracts may have hepatoprotective, nephroprotective, immunomodulatory, antioxidant, and anti-inflammatory qualities, suggesting that *P. stewartii* may be worth further research in the treatment of DM.

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Authors' contributions
Conceptualization, M.U.R.; methodology, H.R.; software, S.O.; validation, S.I, R.C.; formal analysis, M.U.R.; investigation, M.A.R.; resources, M.U.R.; data curation, M.A.R.; writing—original draft preparation, M.U.R., R.CM..; writing—review and editing, M.A.R. M.H.M. and R.C.M.; visualization, M.A.R. and E.Z.; supervision, H.R.; project administration, M.A.R.; funding acquisition, M.H.M. All authors have read and agreed to the published version of the manuscript.

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Data availability
All the data used in the manuscript are available in the tables and figures.

Declarations

Ethics approval and consent to participate
All authors have given their full consent to participate.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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