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Phytochemical Screening, Antioxidant Activity, GC–MS Analysis and Optimization Using Response Surface Methodology of *Pimenta dioica (L.) Merr.* Leaf Extracts

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Abstract

This study investigates the antioxidant potential, phytochemical profile, and extraction optimization of *Pimenta dioica* (L.) Merr. leaves using Response Surface Methodology (RSM). Extractions were conducted with acetone, chloroform, methanol, petroleum ether, and water. Qualitative screening identified key phytochemicals, including terpenoids, flavonoids, phenols, alkaloids, and phytosterols, with methanol and aqueous extracts showing the highest content. Antioxidant assays revealed strong radical-scavenging activity, with the aqueous extract achieving 95.08 % inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and 70.23 % nitric Oxide (NO) scavenging at 0.5 mg/mL, comparable to ascorbic acid. GC–MS analysis identified bioactive compounds, including dodecanoic acid, 1-hexadecane sulfonic acid, oxalic acid ester, and 2-chloro-5-iodobenzamide, many of which exhibit antioxidant and anti-inflammatory properties. The Box–Behnken Design was used to optimize extraction variables: extract concentration, extraction time, and temperature. Optimal conditions were found to be 60.45 ºC, 499.99 µg/mL, and 6 hours, resulting in 93.4 % DPPH activity, 54.46 % NO scavenging, a Total Phenolic Content (TPC) of 156.45 mg GAE g^{-1}, and an extraction yield of 9.65 %, with an overall desirability of 0.995. The findings highlight L. as a promising natural source of antioxidants and bioactive compounds for pharmaceutical, nutraceutical, and food applications.

Keywords: *Pimenta dioica* (L.) Merr; Phytochemical screening; Antioxidant activity; GC–MS analysis; Response surface methodology; Box–Behnken design optimization; Total phenolic content; Natural antioxidants.

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1. Introduction

Medicinal plants are an essential source of bioactive compounds used to prevent and treat diseases [1]. Although medicinal plants produce numerous bioactive compounds, they also produce a variety of secondary metabolites, including tannins, terpenoids, phenolics, flavonoids, and alkaloids, which contribute to plants' antioxidant and antimicrobial

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properties [2]. Increasing concerns about synthetic drugs' side effects and antimicrobial resistance have caused a growing interest in using natural plant-based therapies [3]. As a result, researchers have placed greater emphasis on the identification, characterization, and optimization of bioactive compounds derived from natural products for subsequent use in pharmaceutical, nutraceutical, and functional food applications [4].

L. Merr., or Allspice, belongs to the family Myrtaceae and is found in various tropical settings. Its leaves have been widely used in traditional herbal and folk medicine for treating respiratory conditions, metabolic disorders, pain, and infections [5]. *P. dioica* has had numerous phytochemicals characterized, including eugenol, phenolic acids, flavonoids, and tannins, which have had significant free-radical scavenging activity and antimicrobial activity [6]. Previous studies have reported significant phytochemical composition, antioxidant, and biological activities of medicinal plants using in vitro assays and formulation-based approaches. Pharmacognostical and antioxidant evaluations of *Actiniopteris dichotoma* and *Moringa oleifera* have demonstrated strong free radical scavenging potential and improved bioactivity through phytosome formulations. Similarly, incorporation of standardized *Curcuma longa* extracts into phytosomes showed enhanced anti-inflammatory activity. In addition, phytosome formulations of *Trigonella foenum-graecum* extracts were reported to exhibit notable antioxidant and anti-inflammatory properties. These findings support the relevance of exploring phytochemical profiles, antioxidant activity, and optimization strategies for medicinal plant extracts [7–11]. However, despite its extensive ethnomedicinal history, there is limited systematic investigation of the phytochemical profile, bioactivity, and extraction efficiency of *P. dioica* [12].

The extraction of phytochemicals from plant sources is essential to ensuring yields, purity, and stability. Key variables that affect the extraction of a compound include solvent concentration, temperature, extraction time, and the sample-to-solvent ratio [13]. Solvent polarity affects which phytochemicals will ultimately dissolve and be extracted, while temperature and time can influence the rate of diffusion of components into the solvent. However, heat and time can also degrade thermolabile components, leading to decreased antioxidant activity or chemical structure modification [14]. The sample-to-solvent ratio can also hinder extraction if it does not allow the active compounds to be efficiently solubilized. Therefore, the extraction conditions must be reproducible and optimal to ensure recovery of bioactive compounds, be cost-effective, and improve biological performance [15].

RSM is a powerful statistical optimization approach for assessing the influence of various independent variables on a response [16]. This is distinct from the standard one-factor-at-a-time experimental approach, which is labor-intensive and fails to capture interactions among factors. RSM can assess multiple parameters and investigate how those parameters may interact [17]. This is helpful in extraction studies because these studies encompass aspects such as solvent concentration, temperature, and time that are often non-linear and typically involve interactions that overlap or compound. Among RSM designs, the BBD is preferred because it can model a quadratic response surface and requires fewer

experimental runs [18]. In addition, RSM can provide a mathematical model across response contour plots that can be interpreted to show trends, define interactions, and optimize response contingencies to increase yields of phytochemicals and to optimize biological properties. RSM can be viewed as a valid and effective option for researchers seeking to design standardized protocols for extracting phytoactive compounds and improving their functional uses.

Although L. Merr. has been extensively used in traditional medicine and is recognized for its antioxidant and therapeutic potential. Available scientific reports are largely limited to qualitative phytochemical screening and preliminary in vitro antioxidant evaluations. Most previous studies do not systematically quantify bioactive compound recovery or investigate the effects of extraction parameters, such as extract concentration, extraction time, and temperature, on antioxidant activity and phenolic yield. Furthermore, there is a lack of statistically optimized extraction studies employing RSM to evaluate the interactive effects of these variables and to maximize extraction efficiency. To the best of our knowledge, no comprehensive investigation has integrated phytochemical screening, antioxidant activity assessment, GC–MS characterization, and RSM-based Box–Behnken optimization for L. leaf extracts. Therefore, the present study is justified as it aims to bridge this research gap by providing a systematic, statistically optimized extraction strategy and a detailed evaluation of the antioxidant potential of L., thereby enhancing its applicability in pharmaceutical, nutraceutical, and functional food applications. The objectives are (i) to quantitatively identify the main phytochemical compounds available in the leaf extracts of L. Merr. (ii) to evaluate the antioxidant activity of the extracts with standard radical scavenging assays (DPPH and NO), and to characterize the compounds with GC–MS analysis, and (iii) to optimize extraction conditions using RSM, to maximize the yield of bioactive compounds, and improve the biological activities associated with them.

This combined approach offers a systematic, scientific approach to establishing *P. dioica* as a promising source of a natural antioxidant for the development of phytopharmaceutical preparations, herbal medicines, and natural food preservation.

2. Materials and Methods

2.1. *Proposed methodology*

Fresh L. Merr. Leaves were gathered from their natural location and authenticated by a qualified botanist for plant identification. The leaves were then washed, shade-dried, ground to a fine powder, and extracted using a Soxhlet apparatus, yielding the raw extract. The yield of crude extract indicates the efficiency of the extraction. The response variables that could influence efficiency for extraction were selected as the variables for optimization, so variables included were extraction concentration, time, and temperature, and RSM was used to provide a plan for the experimental trials using an appropriate model design, and also to assess any individual and interaction effects of the variables. Model fitting and Analysis of Variance (ANOVA) were performed to analyse the generated data

and to test model assumptions. Based on response surface plots and desirability functions, optimal conditions for extracting L. were developed to achieve optimal phytochemical recovery and biological activity. Following optimization, the crude extract was processed and the treated extracts characterized by GC–MS for the major bioactive compounds. Fig. 1 shows the proposed methodology.

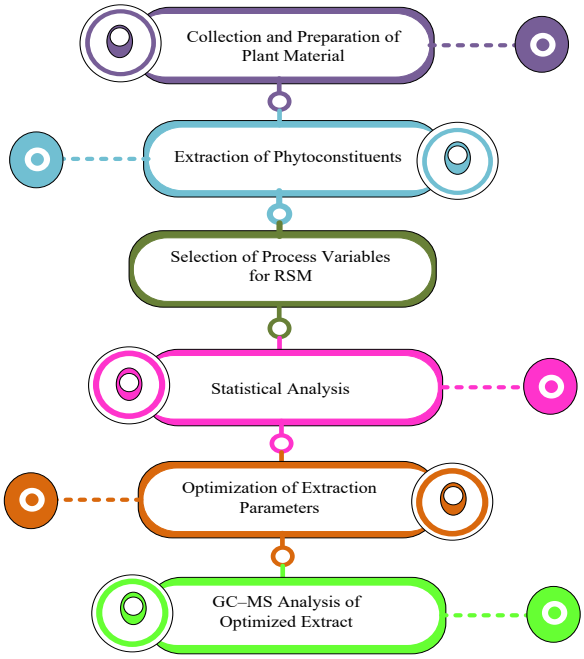

Fig. 1. Proposed methodology.

2.2. *Collection and authentication of plant*

Fresh and mature leaves of L. were harvested from their natural habitat in the Western Ghats of India at early morning to maintain maximum phytochemical integrity. The leaves collected had a healthy appearance, while damaged or unhealthy plant parts were not used. Following collection, the plant material was placed in clean, sterile bags and transported to prevent contamination. The plant species was identified and authenticated based on morphological traits and standard botanical keys by competent taxonomists from the Department of Botany, Nesamony Memorial Christian College, Marthandam. A voucher specimen of the authenticated plant material was deposited in the departmental herbarium under voucher number NMC–PD–2024–01 for future reference.

2.3. *Preparation of extracts*

The collected leaves were washed with running tap water, then rinsed with distilled water to remove dust, dirt, and microbial contamination. The rinsed leaves were then shade-dried at room temperature for 7-10 days to limit loss or degradation of heat-sensitive phytochemicals. Once completely dry, the leaves were ground to a fine, powder using a mechanical grinder and stored in an airtight container to minimize moisture absorption.

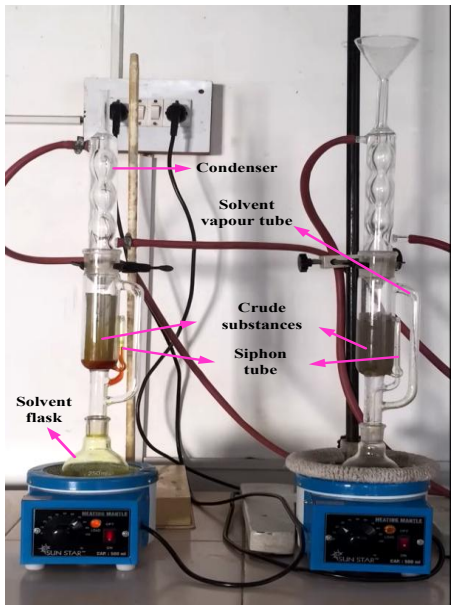

Fig. 2. Soxhlet extraction setup used for preparing leaf extracts.

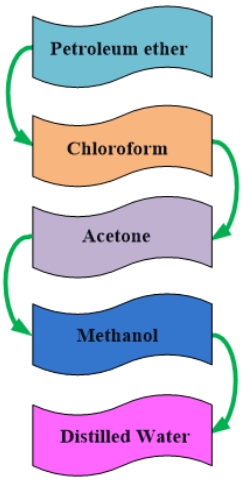

Fig. 3. Successive solvent extraction sequence used for Pimenta dioica (L.) Merr. leaf powder based on increasing solvent polarity.

For extraction, 50 g of dried leaf powder was placed into a Soxhlet apparatus, as shown in Fig. 2. Solvent extraction was carried out in succession with solvents of increasing polarity to achieve complete extraction of different classes of phytochemicals. The solvents used in the study are shown in Fig. 3.

Extraction was carried out in cycles until the solvent in the siphon tube became entirely colorless, indicating complete extraction. The extracts were passed through Whatman No. 1 filter paper to remove plant residues. The filtered extract was concentrated using a rotary evaporator at 45 ºC to remove the solvent, without altering the phytoconstituents. The concentrated crude extracts were stored in sterile, identifiable glass vials at 4 °C until evaluation for phytochemicals, antioxidant capacity, and GC-MS analysis.

2.4. *Calculation of extract yield*

To assess extraction efficacy, the yield of each solvent extract was determined. After evaporation by rotary evaporation, the concentrated extracts were gently dried to thoroughly remove residual solvent. The final weight of dried extract was noted. Extract yield was calculated according to Equation (1), [19].

$$Extract Yidd\,(\%) = \frac{weight of\ dried\ Extract(g)}{Weight of\ plant powder used(g)} \times 100 \quad (1)$$

In this research, the extraction involved 50 g of leaf powder, wherein the weight of each dried extract was measured separately from the varying solvent extractions. The determined percentage yields were included for solvent efficiency assessment, and solvent efficiency was then correlated to phytochemical richness and antioxidant activity.

2.5. *Preliminary phytochemical screening*

L. extracts were prepared using various solvents and used to conduct qualitative phytochemical screening to identify important classes of secondary metabolites. Standard phytochemical test procedures were used for to detect chemicals in their crude state, including Steroids, Reduced sugars, Alkaloids, Phenols, Flavonoids, Phytosterols, Quinones, Xanthoprotein, Saponins, Carboxylic acids, Coumarin glycosides, and Terpenoids. The presence of their corresponding phytochemical groups and their determination are evidenced by their characteristic colours/colour changes or precipitates. The obtained qualitative results indicate a potential therapeutic significance and guided the selection of extracts for subsequent quantification and antioxidant studies.

2.6. *Assessment of total phenolic content*

The TPC of the crude leaf extracts was determined by the Folin–Ciocalteu method. A 1 mL aliquot of extract was mixed with 5 mL of 10 % Folin–Ciocalteu reagent and allowed to stand for five minutes, after which 4 mL of 7.5 % sodium carbonate was added. The

blue solution was allowed to stand at room temperature for 30 min before measuring the absorbance at 765 nm using a UV–Visible spectrophotometer. A calibration curve of gallic acid was used to express the TPC as mg of gallic acid equivalent per gram of extract [20].

$$TPC = \frac{C_c \times V_E}{M_E} \quad (2)$$

Where, C_c denotes concentration from calibration curve (mg/mL), V_E represent volume of extract used (mL), M_E is the weight of extract (g).

2.7. *Antioxidant activity assays*

DPPH and NO were used to evaluate the antioxidant activity, where the ability of the extracts to neutralize free radicals was measured spectrophotometrically. A decrease in absorbance indicates a higher scavenging potential, reflecting the extract's stronger antioxidant capacity.

2.8. *DPPH radical scavenging assay*

The extract's antioxidant activity was determined using the DPPH method. Extracts were prepared at concentrations of 10, 50, 100, 250, and 500 µg/mL, which were mixed with 0.1 mM DPPH solution in test tubes and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. Ascorbic acid was the control antioxidant. [21].

$$\%\,DPPH\,Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (3)$$

Where, $A_{control}$ represents the reagent without NO extract and A_{sample} constitutes the absorbance of reagent with the extract.

2.9. *Nitric oxide radical scavenging assay*

The NO was evaluated using sodium nitroprusside and the Griess reagent. Sodium nitroprusside was mixed with the extract concentrations (10–500 µg/mL) and incubated for 30 min at room temperature. Absorbance readings were taken at 540 nm with a microplate reader after the addition of Griess reagent. The standard for comparison was ascorbic acid. [22].

$$\%\,NO\,Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (4)$$

Where, $A_{control}$ represents the reagent without NO extract.

2.10. *GC–MS analysis*

GC–MS was performed to determine the compounds in the selected leaf extract of *P. dioica* using a Perkin Elmer GC Clarus 500 system with an Rtx-5MS capillary column. The sample was injected into the GC/MS in split mode, and the injector temperature was held at 250 ºC to guarantee the complete vaporization of the extract. The oven temperature was programmed to gradually increase to affect the separation of compounds based on volatility. Helium was used as the carrier gas and delivered at a constant flow rate. The total runtime was 45 min, during which the separated compounds were detected and ionized in the mass spectrometer. The resulting spectra were compared to the NIST and Wiley mass spectral libraries for the identification of the phytochemical constituents. Furthermore, the relative percentage of each compound was calculated by dividing the area of each peak by the total chromatogram peak area. The analysis and identification of engaged phytochemical constituents enabled the understanding of the majority of bioactive compounds that were present and potentially contributing to pharmacological activity [23].

2.11. *Response surface methodology*

In the present study, RSM was applied to identify optimal extraction conditions to improve the phytochemical yield and antioxidant activities of L. leaf extract. A Box–Behnken Design (BBD) under the quadratic model was selected due to its efficiency, reduced number of experimental runs, and suitability for evaluating interactive effects among variables. The experimental design consisted of three independent factors, such as extract concentration, temperature, and time, each set at three levels as low, medium, and high. The design generated of 30 experimental runs, including center-point to estimate experimental error.

The response variable experimental data were analyzed using a second-order polynomial regression model, as indicated in Equation (5). The overall optimization process is shown in Fig. 4.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (5)$$

Where, Y is the predicted response, β_{ij} denotes interaction coefficients, β_0 indicate the intercept term, β_{ii} is the quadratic coefficients, $X_i X_j$ are the coded independent variables and β_i represent linear regression coefficients.

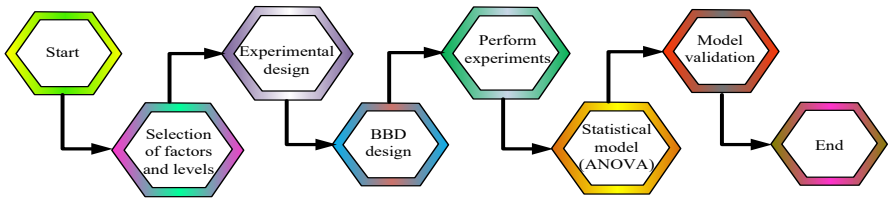

Fig. 4. Flow Chart of RSM.

To assess the significance of the and model parameters, data were using ANOVA. To visually represent the interaction effects of the variables, response plots were constructed. Extraction conditions deemed to produce the most optimal extract recovery and enhanced antioxidant potential were determined desirability function analysis.

4. Results and Discussion

4.1. *Phytochemical screening*

A detailed study of the chemical constituents of plant materials is useful to ascertain the medicinal as well as the toxic properties of a particular plant. The various concentrated extracts have been tested qualitatively. The test results are presented in Table 1. The phytochemical analysis of the leaf extracts shows the presence of steroids, reduced sugars, alkaloids, phenols , flavanoids, phytosterols, xanthoprotein, carboxylic acid, coumarin glycosides, and terpenoids in the acetone methanol, and aqueous extracts and reduced sugars, alkaloids,flavanoids,phytosterols, xanthoprotein, carboxylic acid, coumarin glycosides, and terpenoids in the petroleum ether and chloroform extracts. When a comparison is done, there are large quantities of bioactive components in methanol and aqueous extracts than in petroleum ether, chloroform extract, and acteone extracts.

Table 1. Phytochemical analysis of leaf extracts.

S.No	Phytochemicals	Petroleum ether extract	Chloroform extract	Acetone extract	Methanol extract	Aqueous extract
1	Steroids	+	+	+	+	+
2	Reduced sugars	+	+	-	+	+
3	Alkaloids	+	+	+	+	+
4	Phenols	-	-	+	+	+
5	Flavonoids	+	+	+	+	+
6	Phytosterols	+	+	+	+	+
7	Quinones	-	-	-	-	-
8	Xanthoprotein	+	+	+	+	+
9	Saponins	-	-	-	-	-
10	Carboxylic acids	+	+	+	+	-
11	Coumarin glycosides	+	+	+	+	+
12	Terpenoids	+	+	+	+	+

4.2. *Antioxidant activity*

The antioxidant of leaf extracts was evaluated using the DPPH radical scavenging assay over amounts ranging from 10 to 500 µg/mL as presented in Fig. 5. All extracts showed a concentration-dependent enhancement in scavenging activity. Among them, the aqueous extract exhibited the highest inhibitory effect, reaching 95.08 % at 500 µg/mL, which was

comparable to the reference standard, ascorbic acid (94.04%). The methanolic extract also showed considerable activity (63.2 8 % at 500 µg/mL), followed by acetone (49.63%), chloroform (47.67 %), and petroleum ether (34.60 %) extracts. The enhanced antioxidant potential observed in the aqueous and methanol extracts may be attributed to the higher solubility of phenolic and flavonoid constituents in polar solvents. Although ascorbic acid exhibited strong antioxidant activity, the aqueous extract of L. showed comparable DPPH radical-scavenging acttivity at higher concentrations, indicating its effectiveness as a natural antioxidant source.

The present findings are consistent with earlier reports on L., which attributed significant antioxidant activity to the presence of phenolic and flavonoid compounds. Similar concentration-dependent DPPH radical scavenging activity has been reported in previous studies, highlighting the strong free radical neutralizing potential of L. leaf extracts.

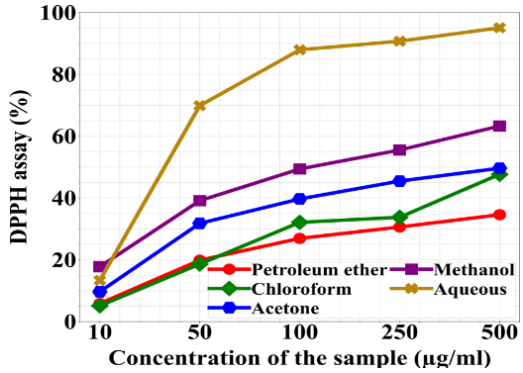

Fig. 5. DPPH radical scavenging activity (%) of *Pimenta dioica* (L.) Merr. leaf extracts at different concentrations (10–500 µg/mL) compared with ascorbic acid.

The NO scavenging activity of the leaf extracts of L. Merr. was assessed at concentrations ranging from (10-500 µg/mL), and the results are presented in Fig. 6. The inhibition of NO radicals increased progressively with the concentration in all extracts tested. Among the extracts, the aqueous exhibited the maximum scavenging activity (70.23 % at 500 µg/mL), followed by methanol (51.81 %), acetone (38.42 %), chloroform (41.74 %), and petroleum ether (46.53 %) extracts. Ascorbic acid, used as a standard antioxidant, showed 100 % inhibition at all tested concentrations. The pronounced NO scavenging potential of the aqueous and methanolic extracts suggests the presence of strong hydrogen-donating compounds, likely phenolic and flavonoid constituents, which effectively neutralise NO radicals. The relatively lower activity was observed in non-polar extracts. specifically chloroform and petroleum ether may be due to the limited solubility of such active compounds in these solvents. Overall, these results support that L. Merr. possesses notable NO scavenging potential, complementing the DPPH radical scavenging findings. When compared with the standard antioxidant ascorbic acid, the plant extracts showed lower NO

scavenging activity; however, the aqueous and methanolic extracts demonstrated appreciable inhibition, suggesting their potential as natural NO scavengers.

4.3. *Identification of components*

Selected portions from the column chromatography are subjected to GC-MS analysis. A database from the National Institute of Standards and Technology (NIST) is used for the interpretation of components. Unknown spectra were referenced against the NIST spectral database. Details such as compound name, structure, and molecular weight were obtained.

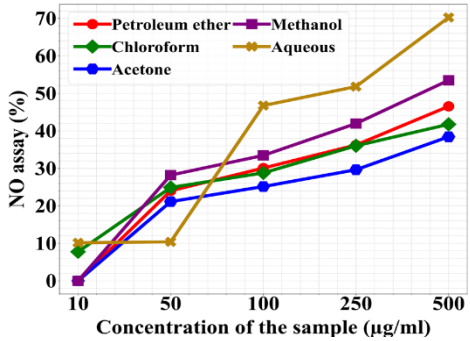

Fig. 6. Nitric oxide (NO) radical scavenging activity (%) of *Pimenta dioica* (L.) Merr. leaf extracts at different concentrations (10–500 µg/mL) compared with ascorbic acid.

The GC-MS analysis of leaf extract revealed several bioactive phytochemicals as listed in Table 2. The chromatograph showed separation of peaks attributed to the various functional compounds, such as dodecanoic acid, 1-hexadecane, oxalic acid, 6-ethyl oct-3-yl hexyl ester, sulphonic acid, and 2-chloro-5-iodobenzamide eluting in the retention time range of 15.33 - 38.26 min. These compounds exhibit a range of biological, including antioxidant activity, anti-inflammatory properties, and antihypercholesterolemic effects, indicating the pharmacological properties of the plant extract. Specifically, the presence of dodecanoic acid and its keratin derivatives suggests radical-scavenging and membrane-stabilizing effects; similarly, sulphonic acid derivatives suggest anti-inflammatory and hemolytic properties. The identification of halogenated benzamide derivatives such as 2-chloro-5-iodobenzamide is also an indicator of antiretroviral properties.

Table 2. GC–MS profile of bioactive compounds in Fraction 1.

Sl No.	Retention time (min)	Compound name	Molecular formula	Molecular weight (g/mole)	Active biological activity
1	15.33	Dodecanoic acid	$C_{24}H_{50}O_4Si_2$	458	Antioxidant, anti-inflammatory, hemolytic, hypocholesterolemic

2	38.06	1-hexadecane sulphonic acid	$C_{23}H_{39}C_{l2}NO_3S$	479	Anti-inflammatory, antioxidative
3	38.19	Oxalic acid,6-ethyloct-3-yl hexyl ester	$C_{18}H_{34}O_4$	314	Antimicrobial activity
4	38.26	2-chloro-5-iodobenzamide	C_7H_5ClINO	281	Antimicrobial, antiretroviral.

The second fraction of the leaf extract contained four main constituents, eluting between 19.42 and 35.77 minutes, as shown in Table 3. The compounds identified were dodecanoic acid, 11-octadecanoic acid, octadec-9-enoic acid, and butyl-9-octadecenoate, with molecular weights ranging from 200 to 338 g mol^{-1}. These compounds are fatty acid derivatives and esters which are known from many medicinal plants for their diverse pharmacological properties. Dodecanoic acid has antioxidant, anti-inflammatory, anticancer and cardio-protective properties. Butyl-9-octadecenoate has been reported to have antimalarial, antioxidant, and antihistaminic activities. The dominance of these fatty acid esters may indicate a significant contribution from the non-polar fractions of L. Merr. to the antioxidant potential observed in this study, possibly through radical-stabilizing and membrane-protective mechanisms. The findings from this study again reinforce the plant's traditional use and uniqueness in its prospectively valuable bioactive lipophilic compounds.

Table 3. GC–MS profile of fatty acid derivatives in Fraction 2.

Sl.no	Retention time (min)	Compound name	Molecular formula	Molecular weight	Active biological activity
1	19.42	Dodecanoic acid	$C_{12}H_{24}O_2$	200	Antioxidant, anti-inflammatory, hemolytic, hypocholesterolemic.
2	28.82	11-Octadecanoic acid	$C_{19}H_{36}O_2$	296	Anti-inflammatory, antiandrogenic, cancer preventive.
3	29.42	Octadec-9-enoic acid	$C_{18}H_{32}O_4$	282	Anti-inflammatory
4	35.77	Butyl 9-octadecenoate	$C_{22}H_{22}O_2$	338	Antimalarial activity, antioxidant, antihistaminic,

Table 4 displayed three different compounds with elution times of 4.20, 4.30, and 39.41 minutes. The compounds identified were Bis(flouromethyl)(dimethyl)silane, Dimethylsulfoxonium formylmethylide, 17-(acetyloxy)-3-(hydroxyamino)-4,4-dimethyl androstane, having molecular weights of 124, 120, and 433 g mol-1, respectively. Of these compounds, two derived from silane and sulfoxonium are known to have cytotoxic and antioxidant activities, while the androstane compound is a very potent anticancer /agent because it is a steroidal compound that interacts with cellular membranesw and nucleic acid molecules. The presence of volatile and non-volatile bioactive molecules in this fraction demonstrates the chemical variation from the extract. The presence of both an

antimicrobial and an anticancer compound underscores the plants pharmacological significance, and provides additional support for its traditional medicinal uses.

Table 4. GC–MS profile of major phytoconstituents in Fraction 3.

Sl.no	Retention time (min)	Compound name	Molecular formula	Molecular weight	Active biological activity
1	4.2	Bis(flouromethyl)(dimethyl)silane	$C_4H_{10}F_2Si$	124	Antimicrobial activity
2	4.30	Dimethylsulfoxonium formylmethylide	$C_4H_8O_2S$	120	Antioxidant and cytotoxic activities.
3	39.41	17-(acetyloxy)-3-(hydroxyamino)-4,4-dimethyl androstane	$C_{25}H_{39}NO_5$	433	Anticancer agent

4.4. *RSM results*

Table 5 reports the experimental matrix developed via BBD under RSM to optimize extraction parameters. The three independent factors investigated in the BBD matrix were extract concentration (A. 10–500 µg/mL), extraction time (B: 1–6 h), and extraction temperature (C: 30–66 °C). The corresponding responses measured were: DPPH radical-scavenging activity (%), NO scavenging activity (%), TPC (mg GAE g^{-1}), and extraction yield (%). Each experimental run represents a partial combination of the factor levels and resulting response values, which were used to develop predictive quadratic models. These are the basis for the subsequent statistical and optimization analyses.

Table 5. RSM experimental results for extract parameters.

Run	A:Extract conc. µg mL^{-1}	B:Extraction time h	C:Extraction temp. ºC	DPPH radical-scavenging activity %	Nitric-oxide scavenging activity %	TPC Mg GAE g^{-1}	Extraction yield %
1	10	1	59	11	6.7	28.36	1.65
2	500	1.43	61	66.62	42.5	125.12	7.84
3	500	1.99	66	69.88	46.53	128.76	8.71
4	50	6	30	23.4	18.73	66.17	5.29
5	10	2.9	39	10.27	2.82	35.24	2.75
6	500	1.62	48	63.11	42.84	117.13	6.44
7	100	6	46	31.02	17.41	66.37	5.87
8	10	5.28	36	15.87	9	42.58	3.69
9	100	2.05	52	20.57	7.23	51.7	3.64
10	500	6	66	95	52.42	150	9.5
11	295	2.1	36	34.98	22.96	84.77	5.44
12	171	3.01	44	28.6	18.85	61.96	3.83
13	345	3.5	44	54.11	31.45	104.71	6.86
14	391	3.81	62	58.85	34.69	108.36	6.94
15	80	3.55	34	12.88	11.28	45.99	3.82
16	358	3.5	44	61.4	32.91	118.93	7.31
17	293	5.3	46	49.44	31.85	101.65	7.05
18	403	4.2	66	62.55	37.62	121.6	7.28
19	378	3.5	63	60.87	36.62	119.9	7.77

20	440	4.19	66	74.01	49.01	139.87	8.2
21	482	3.65	62	64.12	44.52	126.92	7.82
22	435	3.5	61	62.8	40.76	121	7.75
23	258	5.08	52	51.1	32.8	94.44	7.25
24	145	3.64	37	35.15	15.98	63.19	5.08
25	112	6	45	34.6	22.73	63.89	6.59
26	241	1.61	66	27.79	18.97	62.18	4.88
27	13	1.35	44	11.33	2.89	36.09	2.73
28	273	1	50	29.88	19.38	80.29	5.2
29	302	3.88	44	39.6	28.74	89.51	6.06
30	230	2.77	35	30.99	24.49	71.35	4.66

4.5. *ANOVA for Quadratic model*

Table 6. ANOVA for RSM quadratic models.

Responses	Sum of Squares	df	Mean Square	F-value	p-value	model
DPPH	13969.38	9	1552.15	65.17	< 0.0001	significant
NO	5791.81	9	643.53	93.43	< 0.0001	significant
TPC	34137.61	9	3793.07	82.05	< 0.0001	significant
Extraction yield	106.38	9	11.82	48.18	< 0.0001	significant

Table 6 summarizes the ANOVA results for the quadratic regression models developed for each response parameter. The statistical parameters, including p-value, Sum of Squares, df, Mean Square, and F-value, were used to evaluate the significance and adequacy of the models. All four response models, such as DPPH, NO, TPC, and extraction yield, showed high F-values and particularly low p-values ($p < 0.0001$), indicating that the quadratic models were statistically significant. This confirms that the selected factors and their interactions had a substantial influence on the extraction efficiency and antioxidant properties of the samples.

4.6. *Fit statistics*

Table 7. Fit statistics for model adequacy.

Responses	Std. Dev.	Mean	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
DPPH	4.88	43.06	0.9670	0.9522	0.9378	29.5087
NO	2.62	26.82	0.9768	0.9663	0.9306	34.6664
TPC	6.80	87.60	0.9736	0.9618	0.9228	32.5933
Extraction yield	0.4953	5.93	0.9559	0.9361	0.9000	27.7014

RSM fit statistics for the developed models are provided in Table 7, including standard deviation, Mean, R^2, Adjusted R^2, Predicted R^2, and Adequate Precision. The R^2 values ranged from 0.9559 to 0.9768, suggesting a strong correlation between predicted vs. experimental values. Both the adjusted R^2 and predicted R^2 values were similar, again

demonstrating the model's good predictive reliability. Adequate precision values were all greater than 4 for all responses, indicating the models also had a good signal-to-noise ratio. In summary, this shows the adequacy of the developed quadratic models for process optimization.

4.7. *Regression equation in coded form*

$$\text{DPPH radical-scavenging activity} = +39.97 + 30.33\,A + 11.02B + 1.87C + 4.63\,AB - 0.1597\,AC + 0.4674\,BC + 5.11A^2 + 1.78B^2 - 2.08\,C^2 \quad (6)$$

$$\text{NO scavenging activity} = +24.12 + 20.53\,A + 6.36\,B + 0.1856\,C + 1.06\,AB - 2.18\,AC - 2.08\,BC + 2.85\,A^2 + 0.9643\,B^2 + 2.59\,C^2 \quad (7)$$

$$\text{TPC} = +84.77 + 51.11\,A + 12.37\,B - 2.15\,C + 7.56\,AB + 8.09\,AC - 5.20\,BC\ 0.5138A^2 + 1.94\,B^2 - 4.27\,C^2 \quad (8)$$

$$\text{Extraction yield} = +5.96 + 2.53\,A + 1.10\,B + 0.1580\,C + 0.0670\,AB + 1.21\,AC - 0.4570\,BC - 0.7856\,A^2 + 0.5243\,B^2 - 0.7940\,C^2 \quad (9)$$

The coded quadratic equations (6-9) generated from RSM indicate how extract concentration (A), extraction time (B), and extraction temperature (C) influence the response. The positive coefficients of the linear terms (A, B, and C) indicate that increasing extract concentration and extraction time generally increases all response values, with extract concentration (A) being the most significant factor due to a greater availability of bioactive compounds. Extraction time (B) also had a positive effect by improving diffusion and solubilization, while higher extraction temperatures (C) slightly lowered TPC due to possible thermal degradation. The positive interaction terms (AB and AC) show a synergistic effect between concentration–time and concentration–temperature, suggesting that heat (within moderate levels) and time increase extraction efficiency. Negative interaction terms (AC or BC) suggest that high concentrations and time with extreme combinations may promote instability of the compounds. The quadratic terms (A^2, B^2, and C^2) suggest non-linear behavior, indicating that each parameter can be optimized to an optimal level; beyond that, further increases may lower the response, particularly at elevated temperatures, since phenolic compounds are more prone to thermal degradation.

4.8. *Sensitivity analysis*

The Perturbation plots for DPPH and NO scavenging activities are plotted in Fig. 7. The disturbance plots display the sensitivity of each response by perturbing a single process variable at a time (with all other factors being constant). The steepness of the curve demonstrates the relative importance of that factor to the role. The disturbance plots for both response variables indicate that both extraction temperature and extract concentration had a significant effect on antioxidant activity, with DPPH and NO scavenging activities increasing with higher extract concentration and increased extraction temperature. Furthermore, this may demonstrate that as both extract concentration and extraction temperature increased, it the release of antioxidant compounds increased.

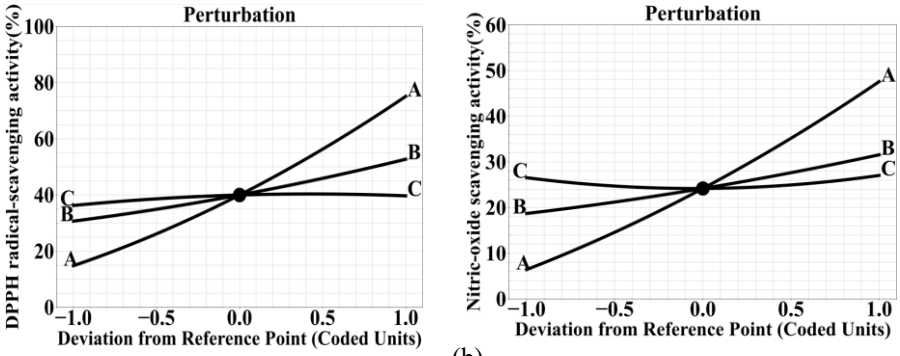

Fig. 7. Perturbation plots showing the effect of extraction parameters on (a) DPPH radical scavenging activity and (b) NO scavenging activity of Pimenta dioica (L.) Merr. leaf extracts.

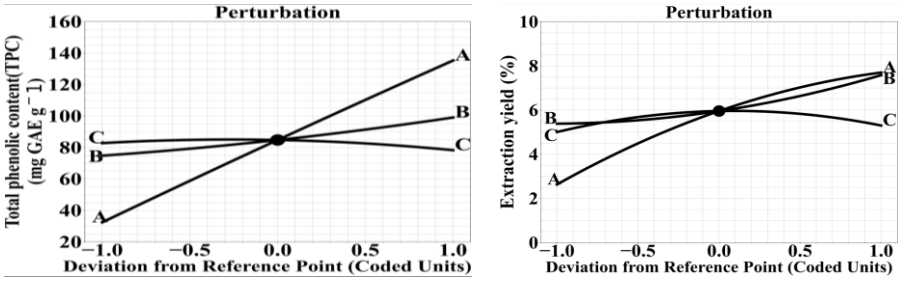

Fig. 8. Perturbation plots illustrating the influence of extraction variables on (a) TPC and (b) extraction yield of *Pimenta dioica* (L.) Merr. leaf extracts.

Using perturbation plots for TPC and Extraction Yield, Fig. 8 provides insight into the parameters that influence the respective responses. Each line within the plots displays the responses as the extract concentration, extraction time, and temperature were varied. The shapes of the curves imply that temperature and extraction time were important for TPC and yield, and a clear trend seems to propose that if higher values are used, the extraction process improves, until reaching a certain threshold, before degradation begins to occur from increasing the temperature and potential contamination from inactive phenolic compounds above elevated temperatures. These plots provide all the necessary information for understanding the impact of the parameters studied on TPC and extraction yields, as well as optimal conditions for extracting phenolic compounds.

4.9. *Desirability analysis*

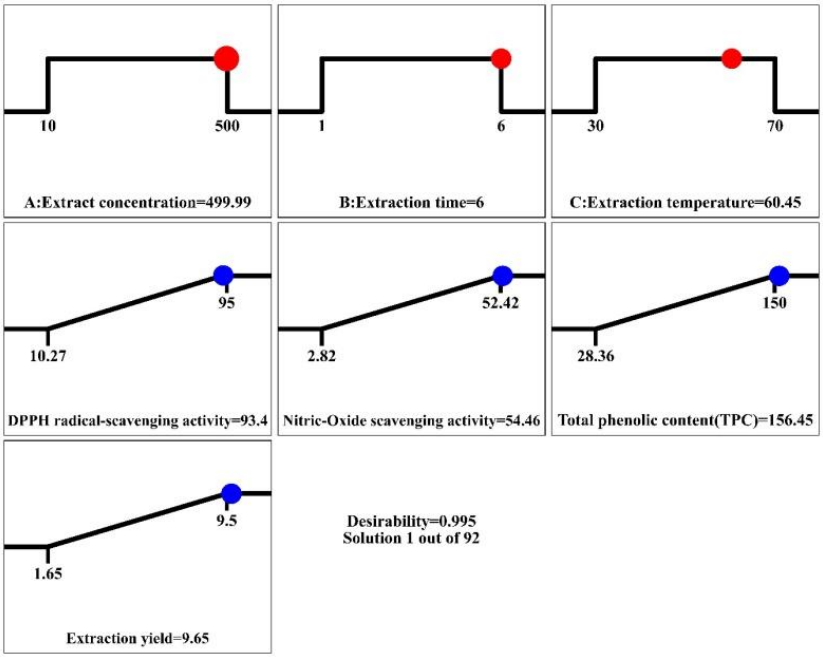

Fig. 9. Overall desirability plot obtained from multi-response optimization of extraction parameters for *Pimenta dioica* (L.) Merr. leaf extracts.

In Fig. 9, the combined desirability plot generated through multi-response optimization is provided. Each response parameter was converted to a desirability function, represented between 0 (least desirable) to 1 (most desirable). The combined desirability curve indicates the optimal region where the maximum predicted values of all responses occur simultaneously. The highest point of the desirability surface indicates the global optimum extraction conditions as an extract concentration of 499.99 µg/mL, extraction time of 6 hours, and temperature of 60.45 °C, which, resulted in the highest antioxidant activity (DPPH = 93.4%, NO= 54.46%), TPC (156.45 mg GAE g^{-1}), and maximum extraction yield of 9.65%. The overall desirability value of 0.995 indicated a good level of model optimization and predictive accuracy. This analysis demonstrated that the optimized parameters simultaneously balanced multiple endpoint responses, increased the extraction efficiency, and enhanced the antioxidant potential of **L.** Merr. leaf extracts.

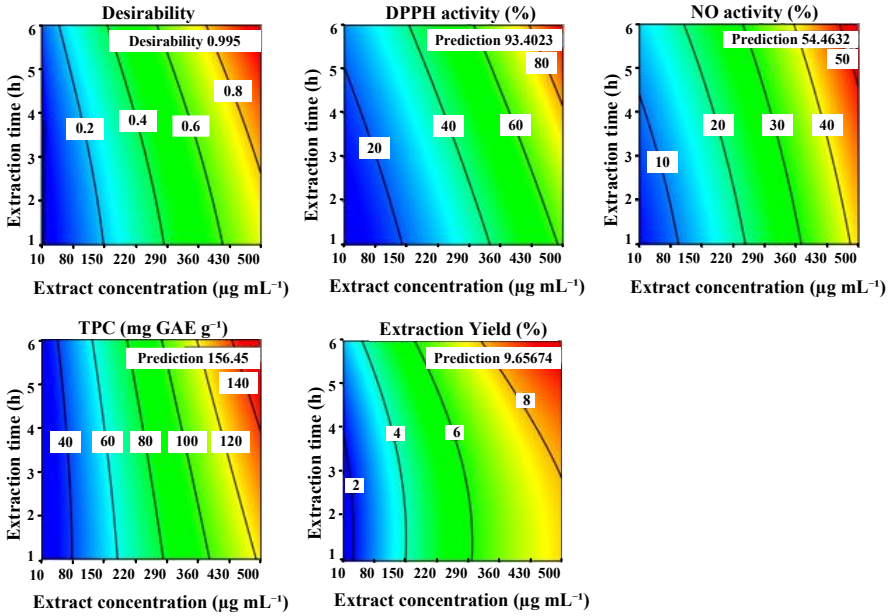

Fig. 10: Optimized contour plots showing the interactive effects of extraction concentration, extraction time, and temperature on antioxidant activity, total phenolic content, and extraction yield of Pimenta dioica (L.) Merr. leaf extracts.

The contour plots in Fig. 10 were optimized to display the interaction effects among pairs of independent variables, while the third variable was held constant, on the recorded responses. They present the interaction of two factors and the region of optimal response, along with variations depicted in color scales, where darker, or more saturated regions represent a higher response. Based on the contour plots, it is possible to identify optimal combinations of concentration, time, and temperature to maximize yield in extraction, while providing a visual representation of the interactions among the factors, and their effects on the responses.

4.10. *Comparison of existing literature with present study*

Table 8 provides an overview of recent research on the phytochemical profiles and antioxidant assessments of different medicinal plants, alongside our current investigation into L. Earlier studies focused on qualitative analyses and antioxidant or antimicrobial tests, with only a limited number using advanced optimization techniques or quantitative modeling. Conversely, this one is to incorporate phytochemical and antioxidant analyses with GC–MS profiling and RSM–BBD statistical optimization. This comprehensive approach not only identified major bioactive compounds but also optimised extraction parameters to improve antioxidant activity and increase phenolic yield, thereby offering greater methodological precision and extraction efficiency than previous studies. Although standard antioxidants showed higher activity, the present study indicates that L. leaf

extracts possess substantial antioxidant potential, supporting their potential application as safer, plant-based alternatives to synthetic antioxidants.

Table 8. Comparison of present study with existing study.

Study	Objective	Plant Used	Methods / Techniques	Findings	Limitation
[24]	Analyze phytochemicals, antioxidant and antimicrobial activity; support with molecular docking	*Portulaca oleracea* seeds	Phytochemical screening, Antioxidant & Antimicrobial assays, Molecular docking	Phenolics and flavonoids present; strong antioxidant and antimicrobial activity; docking confirmed bioactivity	No in vivo confirmation or isolation of individual compounds
[25]	Identify bioactive compounds and evaluate antioxidant and cytotoxic potential	*Barleria prattensis*	Phytochemical profiling, GC-MS, Antioxidant & Cytotoxicity assays	Several bioactive compounds; strong antioxidant and moderate cytotoxic activity	Cytotoxic activity not validated in vivo or at cell-line level
[26]	Compare GC–MS profiles and antioxidant activity from different regions	*Selaginella bryopteris*	GC-MS, Antioxidant assays (DPPH/FRAP)	Variation in phytochemicals and antioxidant activity with location	Only data collection; biological relevance not assessed
[27]	Evaluate phytochemical composition, antioxidant and antibacterial activity	*Nyctanthes arbor-tristis*	Phytochemical screening, DPPH, Antibacterial tests	Flavonoids, phenols, alkaloids present; significant antioxidant & antibacterial activity	No isolation or mechanism studies
[28]	Assess antibacterial and antioxidant activity; analyze secondary metabolites	*Aloe barbadensis* Miller	GC-MS, Antibacterial, DPPH assays	Significant antibacterial and antioxidant activity; multiple secondary metabolites identified	Active compounds not isolated; mechanisms not validated
[29]	Evaluate phytochemical composition and	*Guizotia abyssinica* L.	Phytochemical screening, Antimicrobial assays	Leaf and flower extracts contained bioactive compounds	No quantitative analysis or specific

	antimicrobial activity			with notable antimicrobial activity	compound identification
[30]	Analyze phytochemicals and in vitro pharmacological activities	*Alangium salviifolium* (Lf) Wang	GC-MS, In vitro pharmacology	Multiple bioactive compounds; extracts showed antioxidant and pharmacological activities	Limited to in vitro studies; no in vivo validation
[31]	Analyze phytochemicals and bioactivities of peel and seed extracts	*Tamarindus indica* L.	GC-MS, Antibacterial, Antifungal, Anti-inflammatory, Antioxidant assays	Multiple bioactive compounds; significant antibacterial, antifungal, anti-inflammatory, and antioxidant activities	Limited to in vitro studies; no in vivo or clinical validation
[32]	Profile phytochemicals and evaluate biological activities	*Neptunia prostrata* Linn	GC-MS, HPTLC bioautography, In vitro assays	Diverse bioactive compounds; significant antimicrobial and antioxidant activities	Limited to in vitro studies; no in vivo validation
[33]	Screen phytochemicals, quantify bioactive compounds, and characterize leaf extracts	*Costus pictus* D. Don ex Lindl.	Phytochemical screening, Quantitative analysis, FTIR, GC-MS	Multiple bioactive compounds; functional groups confirmed via FTIR	Limited to in vitro chemical analysis; biological activity not fully evaluated
Present study	Assess phytochemical profile, antioxidant activity, and optimize extraction by RSM	*Pimenta dioica* (L.) Merr.	Solvent extraction, Phytochemical tests, DPPH & NO assays, GC–MS, RSM–BBD	Identified key bioactives; aqueous extract showed high DPPH (95.08%) and NO (70.23%); optimized conditions (499.99 µg/mL, 6 h, 60.45 °C) gave 93.4% DPPH, 54.46% NO, 156.45 mg	Antimicrobial study not included; limited solvent and environmental parameters analyzed.

	GAE g^{-1} TPC, 9.65% yield.

5. Conclusion

The present study provides an extensive investigation into the phytochemical profile, antioxidant activity, and optimized extraction of *P. dioica* leaf extracts using successive solvent extraction, GC–MS profiling, and RSM. The results confirm that the plant has high bioactive potential and can serve as a useful source of natural antioxidants with therapeutic and industrial significance. The phytochemical screening of all extracts assured the presence of terpenoids, phenols, alkaloids, phytosterols, and flavonoids, which contribute to its powerful antioxidant capabilities. Among the extracts, the aqueous extract had the highest DPPH radical-scavenging activity (95.08 % at 500 µg/mL), which was similar to that of ascorbic acid (94.04 %). The extraction of higher amounts of polar antioxidant compounds was demonstrated by the strong antioxidant activity of the methanolic extract (63.28% inhibition at 500 µg/mL). The aqueous extract showed the greatest inhibition of NO (70.23% at 500 µg/mL), followed by the methanol extract (51.81%), further demonstrating the ability of polar solvents to extract active compounds. The analysis carried out by GC-MS identified several bioactive components, including dodecanoic acid, 1-hexadecane sulphonic acid, oxalic acid ester, and 2-chloro-5-iodobenzhamide, in addition to fatty acid derivatives such as butyl-9-octadecenoate, and 11-octadecanoic acid, which are reported to have antioxidant and anti-inflammatory activities. The RSM-BBD model was found to successfully optimize extraction factors, from which selections include: Extract Concentration (10-500 µg/mL), Extraction Time (1-6 hours), and Extraction Temperature (30-66 ºC), with respect to the model being very accurate (R^2 = 0.9559-0.9768). Through a desirability analysis, it was determined that the optimum extraction conditions were a concentration of 499.99 µg/mL, an extraction time of 6 h, and a temperature of 60.45 °C, resulting in DPPH of 93.4 %, NO scavenging of 54.46 %, TPC of 156.45 mg GAE g^{-1}, yield of extraction of 9.65 %, and a total overall desirability of 0.995. Optimizing these conditions results in significant improvements in phytochemical recovery and antioxidant activity; therefore, RSM could be a robust statistical tool for the standardization of processes.

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