CHAPTER 5

Optimization of processing parameters of phytochemicals extraction from targeted plant material using physical and chemical treatments and study their anti-inflammatory properties

5.1. Introduction

The genus *Clerodendrum* Lindl. (Verbenaceae) is very widely distributed in tropical and sub-tropical regions of the world and is comprised of small trees, shrub sand herbs. Traditionally, Clerodendrum finds usage as medicine for lowering hypertension and for curing many skin diseases. Rural and urban people of Manipur (India) routinely grow CG in the kitchen garden with the leaves often sold in the market. Traditionally, cross sections of people across Manipur consume decoction of CG leaves for treating diabetes, obesity and hypertension (Jadeja et al., 2009).

Plants are rich and valuable resources of bioactive phenolics. Phenolic compounds, the most abundant secondary metabolites in plants, have received more and more attention in recent years because of their distinct bioactivities. Studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to the protective effects *in vivo* (Rice-Evans et al., 1997). PCs possess a common chemical structure comprising an aromatic ring with one or more hydroxyl substituents that can be divided into several classes, and the main groups of PCs include flavonoids, phenolic acids, tannins, stilbenes, and lignans (Alu'datt et al., 2017). In recent years, with the increasing recognition for their medicinal values, PCs have been found to help reduce the risk of many chronic diseases (Lin et al., 2016).

Emerging extraction technologies has advantages over conventional extraction methods and are being increasingly used for the recovery of bioactive compounds from plants, rich in phenolic compounds with antioxidant activity. Recently, new techniques such as supercritical fluid extraction (SFE), microwave assisted extraction (MAE), and ultrasound-assisted extraction (UAE) have been used for the extraction of phenolic compounds from plants. Among all these techniques, UAE was widely employed to extract bioactive compounds from plant materials due to the high extraction efficiencies that can

be achieved at relatively low temperatures (Yang et al., 2010). UAE is inexpensive so it is a good alternative to conventional extraction techniques. Ultrasound waves helped disrupt plant cell walls, improved the solvent penetration and enhanced mass transfer across cell membrane (Zhong et al., 2010). Supercritical Fluid Extraction (SFE) as a modern green extraction method has also garnered attention in recent years. Supercritical CO_2 is an inert, non-toxic, environmentally safe solvent and allows extraction at lower temperatures and relatively low pressures. The extracts obtained by SFE are of superior quality as compared to those obtained by conventional organic solvent extraction methods (Gomez et al., 1996). SFE extracts are also generally recognized as safe (GRAS) to be used in food products, therefore it may serve as a very promising technology in food processing (King, 2000). As for the experimental method, the response surface methodology (RSM) is a very helpful strategy to maximize the yields of the compounds from plants by optimizing operational factors (Roriz et al., 2017). The most advantageous feature of the RSM is that it reduces the number of experimental runs, saving energy, time, and raw materials as well as improving the quality of the information obtained from the results compared to the individual study of each variable (Belwal et al., 2019).

Angiotensin converting enzyme (ACE) inhibitors play a critical role in treating hypertension. Several reports showed the ACE inhibitory [ACE I] activity of medicinal plants (Khan et al., 2001; Loizzo et al., 2008; Patten et al., 2016). Angiotensin converting enzyme (ACE) is well known for its dual actions to convert inactive Angiotensin I to active Angiotensin II, and degrades active bradykinin (BK), which plays an important role in controlling blood pressure. Because it is the bottleneck step for the production of pressor Angiotensin II, it was targeted pharmacologically in the 1970s. Successful ACE inhibitors such as captopril were produced to treat hypertension. Studies on domain-specific ACE inhibitors are continuing to produce effective hypertension-controlling drugs with fewer side effects (Wong, 2021).

Response surface methodology (RSM) is a useful statistical technique for investigation of complex processes particularly in the fields of chemical and engineering processes, industrial research, biological investigations and agricultural processes, with emphasis on optimizing a process or a system (Khuri et al., 1987). Response surface methodology can determine the optimal settings of the experimental factors that give the maximum (or minimum) value of the response.

The aim of our study was to optimize UAE and SFE variables such as temperature, pressure and concentration of solvent as modifier for the maximum extract yield, total phenolic compounds, antioxidants and angiotensin I-converting enzyme ACE inhibition activity using an in vitro assay from *Clerodendron glandulosum* Lindl. leaves by using CCD design and response surface methodology. Further, MTT assays and its anti-inflammatory activities and analyze its causing genes. Above that, we have also explored its antioxidant activities and anti-hypertensive potential on human ovarian cancer cell line.

5.2. Materials and methods

5.2.1. Plant materials

The leaves of *Clerodendrum glandulosum* Lindl. were collected from the local market of Churachandpur district, Manipur, India and authenticated by the corresponding author. After collecting the samples were washed under running water to remove dust and rinse with distilled water to drain. Subsequently, they were dried in the shade, and afterwards the dried plant materials were finely grounded by mechanical grinders. The powder was stored in tightly closed glass containers in the dark at room temperature.

5.2.2. Chemicals

Angiotensin converting enzyme (ACE), hippuryl-L-histidyl-L-leucine (HHL), hippuric acid (HA) were purchased from Sigma-Aldrich Co. (England). HCl, acetonitrile, boric acid, *Trifluoroacetic acid* (*TFA*), bmethanol (HPLC grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, Na_2CO_3, gallic acid, butylated hydroxyl toluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), $FeCl_3$, NaCl, NaOH, Borax and dimethyl sulfoxide (DMSO) were also purchased from Sigma-Aldrich Co. (England). Ultrapure water was applied to prepare all the aqueous solutions. The cell culture specific chemicals and reagents were purchased from Sigma Aldrich (St Louis, MO, USA). Other required chemicals were procured from Himedia (Mumbai, India), Thermo Scientific, MA, USA, Merck Millipore (MA, USA) and penicillin/streptomycin was brought from Life Technologies (Gibco, USA).

5.2.3. Experimental Design

The central composite design (CCD) was created to produce optimum extraction conditions for greater total phenolic content, antioxidant antioxidant, and angiotensin converting enzyme (ACE) inhibitory activity of *Clerodendrum glandulosum* Lindl. CCD was used for this investigation because it offers more design points for each variable. The generalized second-order polynomial model used in the response surface analysis as shown in Eq. (5.1)

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{k} \beta_{ij} X_i X_j \qquad \text{Eq. (5.1)}$$

Where, Y is the response variable, X_i and X_j are the independent variables, and k is the number of tested variables (k=5). Regression coefficient is defined as β_0 for intercept, βi for linear, βii for quadratic and β_{ij} for cross product term. In this study, three independent variables were chosen: extraction time (A: 15-20 minutes), extraction power (B: 120-200 W), and solvent concentration (C: 30-70 ml), with total phenolic content (A^2), antioxidant inhibitory activity (B^2), and ACE inhibitory activity (C^2) as dependent variables for UAE extraction. The independent variables for SFE extraction were extraction time (A: 20-40 minutes), temperature (B: 50-70℃), and pressure (C: 200-250 bar). The dependent variables were total phenolic content (A^2), antioxidant inhibitory activity (B^2), and ACE inhibitory activity (C^2). The responses of total phenolic content were critical to be studied because the presence of both in plants has been reported to be reasons for treating chronic diseases, while antioxidant inhibitory activity and ACE inhibitory activity were determined using an in vitro enzyme assay, which has been widely used to determine the antihypertensive activity of plants. A statistical software application (Design Expert, version 13.0, Minneapolis, USA) developed the entire design to generate 3D response surface graphs, which consists of 20 combinations, six replicates at the centre point (Tables 5.1. and 5.2.).

Table 5.1: Experimental design for the extraction process using Ultrasound assisted extraction

Run order	A (extraction time) (min)	B (extraction power) (%)	C (solvent conc.) (ml)
1	17.5	160	70
2	17.5	160	50
3	15	120	70
4	17.5	120	50
5	15	200	70
6	17.5	160	50
7	20	120	70
8	20	200	70
9	20	160	50
10	15	200	30
11	20	120	30
12	15	160	50
13	17.5	200	50
14	17.5	160	50
15	17.5	160	50
16	17.5	160	50
17	17.5	160	50
18	17.5	160	30
19	15	120	30
20	20	200	30

Table 5.2: Experimental design for the extraction process using Supercritical fluid extraction.

Run order	A (extraction time) (min)	B (extraction temperature (℃)	C (pressure) (bar)
1	40	70	200
2	30	60	225
3	20	70	200
4	40	70	250
5	30	60	225
6	30	60	225
7	40	50	250
8	40	50	200
9	20	50	200
10	30	60	225
11	30	50	225
12	40	60	225
13	30	60	250
14	20	60	225
15	30	60	225
16	20	50	250
17	20	70	250
18	30	60	225
19	30	70	225
20	30	60	200

5.2.4. Validation of the model

The optimized conditions were validated for the maximum phenolic content (TPC) and antioxidant activities (DPPH) and Angiotensin converting enzyme (ACE) based on the values obtained using RSM. All the responses were determined under optimized conditions of the extraction. The experimental values were compared with predicted values based on CV% to determine the validity of the model.

5.2.5. Total Phenolic content

The total phenolic content was determined using a modified version of the Folin–Ciocalteu assay (Slinkard and Singleton, 1977). In brief, 20 µL of sample extract is mixed with 1.58

mL of distilled water and 100 μL of Folin–Ciocalteu reagent and incubated for 8 min at room temperature. Followed by addition of 300 μL of 10 % Na_2CO_3 and further incubation at 40 ˚C for 30 min in dark. The absorbance was taken at 765 nm.

5.2.6. Radical scavenging activity (DPPH)

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extracts was measured as described by Brand-Williams and his research group (Brand-Williams et al., 1995). Briefly, 1.4 mL of DPPH radical methanol solution (10^{-4} M) was allowed to react with 100 μL of extracts. Absorbance at 517 nm was measured after incubation for 30 min at room temperature. The results were expressed in terms of radical scavenging activity using the Eq. (5.2).

$$\text{Radical scavenging activity (\%)} = [(A_0-A_s)/A_0] * 100 \qquad \text{Eq. (5.2)}$$

where, A_0 is the absorbance of control blank, and A_s is the absorbance of sample extract. Ethanol extracted sample served as control for the study.

5.2.7. Determination of ACE inhibitory activity

The ACE inhibitory activity was assayed with RP-HPLC modified from the spectrophotometric method described by Wu et al., (2002). Commercial ACE (1 unit) was diluted in 50 mM Tris– HCl (pH: 7.5) containing 300 mM NaCl to obtain concentration of 100 mU/ml. Aliquots of 100 µL were then stored at -20 °C. A volume of 25 µL containing different concentrations of plant extracts or borate for the control were added to 100 µL solution containing 5 mM Hippuryl-L-Histidyl-L-Leucine (HHL). Plant extracts and HHL were prepared in 100 mM Na–borate buffer, pH 8.3, containing 300 mM NaCl. After incubation at 37 °C for 10 min, 10 ll of ACE (100 mU/ ml) was added and samples were incubated for 30 min at 37 °C with continuous agitation. The enzyme reaction was stopped by the addition of 100 µL of 1 M HCl. The solution was filtered through a 0.45 µm nylon syringe filter and injected directly onto a symmetry shield C_{18} column (4.6 mm 250 mm, 5 µm, Waters) to separate the product and hippuric acid (HA) from HHL. The column was eluted with 50% methanol in water (v/v) containing 0.1% TFA at a flow rate of 1 ml/min and the absorbance was measured at 228 nm. The evaluation of ACE inhibition was based on the comparison between the concentration of HA in the presence or not (control sample) of an inhibitor. After

injection in HPLC of the control and the assay with inhibitor, the HA peak areas obtained in the two cases were measured. The average value from three determinations at each concentration was used to calculate the ACE inhibition rate using Eq. (5.3).

$$\text{ACE inhibition } \% = (\text{B-A/B-C}) \times 100 \qquad \text{Eq. (5.3)}$$

where, A is the relative area of HA peak generated in the presence of ACE inhibitor component, B the relative area of HA peak generated without ACE inhibitors and C is the relative area of HA peak generated without ACE (corresponding to HHL autolysis during enzymatic assay).

5.2.8. FT-IR Spectroscopy

Using Nicolet Impact 410, Thermoscientific, UK's Fourier transform infrared spectroscopy, functional group presence in formulated pasta with *Clerodendrum glandulosum* Lindl. was identified. In a mortar and pestle, the samples were crushed into a fine powder (2 mg), completely combined with 50 mg of desiccated KBr, and then made into pellets using a hydraulic press. Scan wavenumbers ranged from 4000 to 400 cm^{-1} (Dutta and Mahanta 2012). Spectral analysis was performed by using Origin software 8.5.

5.2.9. Cell lines and culture conditions

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, USA) and grown in complete RPMI 1640 medium (Himedia, India) supplemented with 10% fetal bovine serum (Life Technologies, Gibco, USA) and penicillin/streptomycin (100 units/mL) (Life Technologies, Gibco, USA). Thermo provided a trizol and c-DNA conversion kit. Sigma Aldrich, in the United States, supplied phorbol-12-myristate-13-acetate (PMA). All the cultures were maintained in a humidified environment at 37 ˚C and 5 % CO_2 in cell culture incubator.

5.2.10. Cytotoxicity assay

This trial evaluated *Clerodendrum glandulosum* Lindl. extract's in vitro cytotoxicity. This was performed with a THP-1 cell line and the MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) (Mossman, 1983). To detect cell development, living cells must convert MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) into formazan crystals. The enzyme mitochondrial succinate dehydrogenase

transforms MTT to formazan, which can be used to tell if a cell is alive or dead. The experiment was conducted using a slightly modified version of Mosmann et al.'s methodology (Bisht et al., 2020a). Approximately 5 x 10^3 THP-1 monocyte cells were seeded in a 96-well plate and developed into macrophages with 5 ng/mL PMA for 48 hours. Cells were allowed to rest for 24 hours in full RPMI media before being treated with various concentrations of extract for 24 hours to determine cell viability using the MTT assay. The media was carefully removed, MTT dissolving solution was added, and absorbance was measured at 590 nm using a UV-Vis spectrophotometer (Multiscan Go, Thermo Scientific) (Bisht et al., 2020b).

5.2.11. Effect of *Clerodendrum glandulosum* Lindl. extract on pro-inflammatory marker

5.2.11.1. Cell Treatments

After determining the IC50 value, substances were further evaluated for anti-inflammatory efficacy. In a 6-well plate mm dish, approximately 0.5×10^6 - 0.75×10^6 THP-1 cells were differentiated to macrophages for 48 hours using 5 ng/mL PMA. Cells were allowed to rest for 24 hours in full RPMI media before being pre-treated with various concentrations of extract for 4 hours, followed by induction with lipopolysaccharide b4 (LPS) for 2 hours. All treatments were carried out in RPMI medium that contained 1% FBS. To investigate the compounds' anti-inflammatory activity, gene expression studies were conducted on pro-inflammatory genes such as TNF-alpha, IL-1 beta, and Cox-2 (Seo et al., 2020).

5.2.11.2. RNA isolation and Semi Quantitative-PCR

Total RNA was extracted and quantified from the cells using Trizol reagent according to the manufacturer's instructions. RNA was transformed to c-DNA using the Verso c-DNA kit (Thermo Scientific, USA). Gene-specific primers for numerous inflammatory genes were employed to investigate mRNA expression. The genes' primer sequences are as follows: COX-2 (78 bp) forward 5′- GTTCCAGACAAGCAGGCTAATA-3′, reverse 5′-CCACTCAAGTGTTGCACATAATC-3′; IL-1 beta (125 bp) forward 5′-GGTGTTCTCCATGTCCTTTGTA-3′, reverse 5′GCTGTAGAGTGGGCTTATCATC-3′; TNF-alpha (106 bp). Forward 5′- CCAGGGACCTCTCTCTAATCA-3′, reverse 5′-TCAGCTTGAGGGTTTGCTAC-3′, and Beta Actin (429 bp) forward 5′-CTCGCCTTTGCCGATCC-3′, reverse 5′- GAAGGTCTCAAACATGATCTGG-3′. The

resulting PCR products were resolved on a 1.5% agarose gel, and the intensity of the bands was assessed using the NIH Image J software (Yoo et al., 2013).

5.2.12. Microstructure analysis of treated samples

The dried powder of both UAE and SFE extracted optimized samples were analyzed for structural analysis using Scanning Electron Microscope (JEOL JSM 6390 LV, Singapore). The dried samples were sputter coated with platinum and their surface and shape morphological analysis were done at different magnifications at an accelerating voltage of 20 kV.

5.2.13. Statistical analysis

All the experiments were carried out in triplicates and calculation of mean, the standard deviation was carried out in OriginPro 8.0 (OriginLab Corporation, USA). All the analysis was statistically analyzed using SPSS Version at the 0.05 significance level.

5.3. Results and Discussion

5.3.1. Fitting the Response Surface Models for UAE process

Fitting the models is critical for understanding the accuracy of the RSM mathematical models for predicting TPC, antioxidant activity (DPPH), and angiotensin converting enzyme (ACE) inhibitory activity of *Clerodendrum glandulosum* Lindl. extract. In this work, CCD successfully established the association between the response functions (TPC, DPPH, and ACE inhibitory activity) and the independent variables (extraction time, extraction temperature, and pressure). Table 5.3. shows the responses for the 20 runs based on the experimental design. The TPC ranged from 47.83 to 68.98 mg GAE/g, and the antioxidant activity (DPPH) ranged from 50.40 to 77.89%. In terms of ACE inhibitory activity, the maximum was 43.02% and the lowest was 68.95%. The Design Expert software recommended a quadratic polynomial model, which fits well for all three independent variables and responses. In terms of coded values, the expected responses for TPC, DPPH, and ACE inhibitory activity might be described by the second-order polynomial equation using multiple regression analysis (Eq. 5.4, 5.5, 5.6)

$$Y_{TPC}\,(UAE) = 59.09 + 1.32A + 0.9770B + 2.84\,C - 0.5475AB + 0.1625\,AC + 0.1775\,BC + 1.58A^2 - 11.08B^2 + 5.41C^2 \quad \ldots (5.4)$$

Y_{AA} (UAE) = 66.32 + 2.91A – 2.42B + 0.9750C + 2.14AB + 0.9612AC – 5.97BC +2.91A^2 – 8.13B^2 + 5.41C^2 (5.5)

Y_{ACE} (UAE) = 56.55 + 1.75A – 0.1910 B + 7.25C – 0.4312AB – 0.4462AC – 2.43BC – 2.53A^2 – 2.82B^2 + 4.03C^2 (5.6)

where A, B, and C are the coded variables for extraction time, power and solvent concentration, respectively.

Table 5.3: Response surface central composite design (uncoded) and the results for total phenolic content (TPC), antioxidant inhibitory activity (DPPH) and Angiotensin converting enzyme (ACE) inhibitory activity for UAE process

Time	Power	Solvent Conc.	TPC	DPPH radical scavenging activity	ACE activity
(min)	(W)	(%)	(mg GAE/g)	(%)	(%)
17.5	160	70	68.98	72.55	68.95
17.5	160	50	59.44	66.08	55.39
15	120	70	54.85	74.49	62.82
17.5	120	50	47.83	58.96	55.06
15	200	70	58.39	50.4	58.9
17.5	160	50	59.21	66.08	56
20	120	70	58.24	77.89	66.69
20	200	70	59.9	67.31	60.35
20	160	50	63.25	66.76	56.59
15	200	30	53.38	64.98	48.12
20	120	30	53.29	64.73	47.98
15	160	50	58.53	70.62	53.3
17.5	200	50	48.62	56.33	54.24
17.5	160	50	59.3	66.25	55.99
17.5	160	50	59.13	66.21	57.02
17.5	160	50	58.27	65.23	55.21
17.5	160	50	58.27	70.23	55.99
17.5	160	30	61.14	69.83	54.05
15	120	30	50.24	60.24	43.02
20	200	30	53.93	73.11	52.05

Furthermore, the models' coefficients of determination (R^2) were 0.9825, 0.8817, and 0.9849, indicating that the projected model values matched the experimental data values by 98.25%, 88.17%, and 98.49%, respectively. Furthermore, the R^2 values were similar to adjusted R^2, indicating a good statistical model. The p-values for the lack of fit were 0.9979, 0.086, and 0.9276, indicating that the models' lack of fit was not statistically significant at $p > 0.05$ (Table 5.4-5.6).

Table 5.4: The regression coefficients and results of ANOVA for response surface quadratic model of total phenolic content using UAE

Source	Sum of squares	Df	Mean square	*F* value	*P* value
Model	469.52	9	52.17	62.41	< 0.0001
A-Time	17.48	1	17.48	20.91	0.0010
B-Power	9.55	1	9.55	11.42	0.0070
C-Solvent Conc.	80.54	1	80.54	96.35	< 0.0001
BC	0.2520	1	0.2520	0.3015	0.5950
A^2	6.87	1	6.87	8.22	0.0167
B^2	337.86	1	337.86	404.17	< 0.0001
C^2	90.95	1	90.95	108.80	< 0.0001
Lack of Fit	6.97	5	1.39	5.03	0.0504
R^2 0.9825					

Table 5.5: The regression coefficients and results of ANOVA for response surface quadratic model of Antioxidant activity (DPPH) using UAE

Source	Sum of squares	Df	Mean square	*F* value	*P* value
Model	690.06	9	76.67	8.28	0.0014
A-Time	84.51	1	84.51	9.13	0.0129
B-Power	58.47	1	58.47	6.31	0.0308
C-Solvent Conc.	9.51	1	9.51	1.03	0.3348
AB	36.77	1	36.77	3.97	0.0743
AC	7.39	1	7.39	0.7983	0.3926
BC	285.49	1	285.49	30.83	0.0002

A^2	23.31	1	23.31	2.52	0.1437
B^2	181.93	1	181.93	19.65	0.0013
C^2	80.53	1	80.53	8.70	0.0146
Lack of Fit	76.76	5	15.35	4.85	0.0540
Pure Error	15.83	5	3.17		
R^2 0.8817					

Table 5.6: The regression coefficients and results of ANOVA for response surface quadratic model of Angiotensin converting enzyme (ACE) activity using UAE

Source	Sum of squares	Df	Mean square	*F* value	*P* value
Model	673.19	9	74.80	72.38	< 0.0001
A-Time	30.62	1	30.62	29.63	0.0003
B-Power	0.3648	1	0.3648	0.3530	0.5656
C-Solvent Conc.	525.48	1	525.48	508.49	< 0.0001
AB	1.49	1	1.49	1.44	0.2578
AC	1.59	1	1.59	1.54	0.2427
BC	47.19	1	47.19	45.66	< 0.0001
A^2	17.59	1	17.59	17.02	0.0021
B^2	21.93	1	21.93	21.22	0.0010
C^2	44.57	1	44.57	43.13	< 0.0001
Lack of Fit	8.32	5	1.66	4.14	0.0725
Pure Error	2.01	5	0.4020		
R^2 0.9849					

5.3.1.1. Effect of UAE Parameters on Total Phenolic Content (TPC)

The whole model is significant (p-value < 0.0001), indicating that at least one of the independent variables has a substantial effect on the dependent variable. With a p-value of 0.0010, time has a substantial impact on extraction results. This could allude to the extraction process's power, which is likewise statistically significant (p-value=0.0070). Solvent Concentration is highly significant (p-value < 0.0001), indicating that so is an important element in the extraction process. The Interaction Terms (AB, AC, BC) are not significant, as evidenced by their p-values (0.1212, 0.6261, and 0.5950, respectively),

implying that the combined effect of these variables has no significant impact on the outcome. B^2 and C^2 are very significant (p-values < 0.0001), demonstrating a non-linear connection between power and solvent concentration on extraction outcome. A^2 is similarly significant (p-value = 0.0167), indicating a non-linear influence of time. Lack of Fit: Not significant (p-value=0.0504), indicating that the model fits the data well.

Figures 5.1. (a)-(c) depict 3-D response surface plots for the effects of extraction time, power, and solvent on total phenolic content. The constant term (59.09) indicates Y_{TPC}'s baseline value when all independent variables are zero. The coefficient of A implies that for every unit increase in A (potentially extraction time), Y_{TPC} rises by 1.32 units, provided all other variables remain unchanged. The coefficient of B implies that for every unit increase in B (potential ultrasonic power), Y_{TPC} increases by 0.9770 units, while other parameters remain constant. The coefficient of C indicates that for every unit increase in C (presumably solvent concentration), Y_{TPC} increases by 2.84 units, with all other variables remaining constant. The interaction term (AB) indicates that the combined rise in A and B has a less-than-additive effect on Y_{TPC}, lowering it by 0.5475 units for each unit increase in both. The positive interaction of AC (time and solvent concentration) term shows that increasing both time and solvent concentration results in a 0.1625-unit rise in Y_{TPC} for every unit increase in time (A) and solvent concentration (C). Similarly, this word indicates a positive interaction impact of ultrasonic power (B) and solvent concentration (C) on Y_{TPC}. The positive quadratic term for ultrasonic time (A^2) suggests a non-linear connection, with increases in time resulting in bigger increases in Y_{TPC}. The negative quadratic term for power (B^2) indicates a parabolic connection, in which increases in power first boost Y_{TPC} but subsequently decrease. The positive quadratic term for solvent concentration (C^2) implies that increasing solvent concentration has a gradually positive influence on Y_{TPC}.

In the context of ultrasound-assisted extraction (UAE), these parameters would be tuned to maximize phenolic compound extraction. Studies have revealed that extraction time, ultrasonic power, and solvent concentration all have a substantial impact on phenolic yield. For example, a study on the extraction of phenolics from *Curcuma Zedoaria* leaves using response surface methodology discovered that duration, temperature, and ethanol concentration combinations were best for maximum extraction (Azahar et al., 2017). Another study examined the effect of several solvent systems on total phenolics,

flavonoids, antioxidant, and antibacterial properties of medicinal plants, emphasizing the relevance of solvent selection during the extraction process (Mehmood et al., 2022). This research and the model offered highlight the need of knowing and optimizing the independent factors to increase the yield of total phenolic content, which is critical for the medicinal potential of plant extracts.

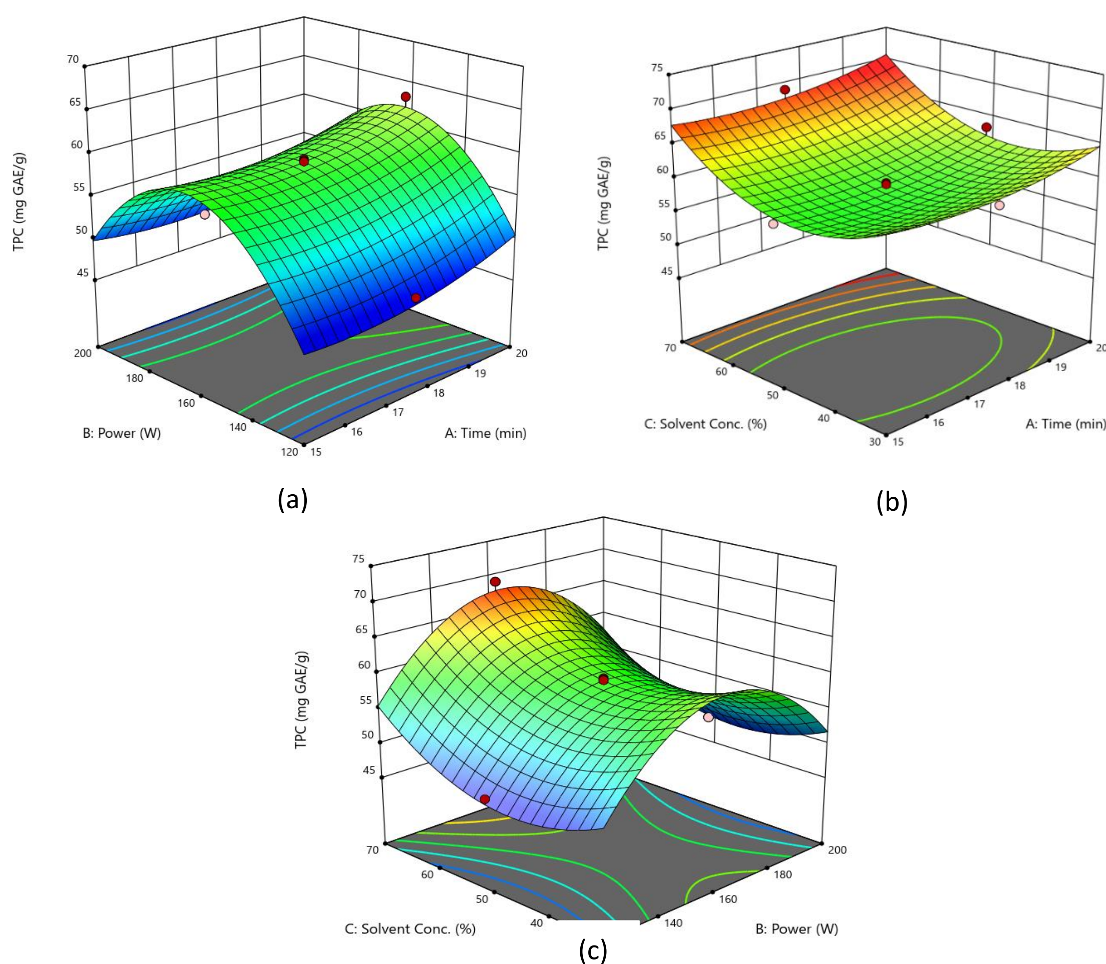


Figure 5.1.: Response surface plots of *Clerodendrum glandulosum Lindl.* showing the effect of (a) extraction time and power, (b) extraction time and solvent concentration, and (c) power and solvent concentration on total phenolic content using UAE. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

5.3.1.2. Effect of UAE Parameters on DPPH radical scavenging Activity

The overall model (Table 5.5) has an F-value of 76.67 and a low p-value (0.0014), showing statistical significance. Time has a significant effect on the extraction process (F-value = 84.51, p-value = 0.0129). B-Power (Ultrasound Power): Power has a substantial effect on

extraction, with an F-value of 58.47 and p-value of 0.0308. C-Solvent Concentration: The solvent concentration is not significant (p-value = 0.3348), indicating that it has little effect on extraction yield within the studied range. There is no significant interaction between time and power (AB) (p-value 0.0743), or between time and solvent concentration (AC) (p-value 0.3926). In a study on *Corchorus olitorius* leaves, UAE parameters such as temperature, time, solvent concentration, and liquid-solid ratio were optimized for total flavonoids (TFC) and total polyphenols content (TPC) extraction. The results showed that at the optimal circumstances, the best extraction yield was obtained with no significant interaction between the variables1. A similar work in *Melastoma malabathricum* research involved modeling and optimizing UAE with the goal of analyzing its phytochemical characteristics. The optimized settings resulted in 96 ± 1.48% DPPH scavenging activity, as well as recorded TPC and TFC values, with no significant interaction between factors. However, the interaction between power and solvent concentration (BC) is highly significant (p-value = 0.0002). The squared term for power (B^2) is significant (p-value 0.0013), demonstrating a nonlinear link between ultrasonic power and extraction yield. The residual represents the unexplained variation in the data after the model has been fitted. The lack of fit test compares the residual error to the pure error at replicated design locations. A non-significant lack of fit (p-value 0.0540) is good since it indicates that the model fits the data well.

Figure 5.2. (a)-(c) predicts antioxidant activity depending on the independent factors. It aids in determining the ideal conditions for optimum antioxidant extraction using supercritical fluid extraction. The coefficients' signs and magnitudes reveal how each factor and its interactions influence the antioxidant capabilities of medicinal plant extracts. The constant term, 66.32, represents the baseline level of antioxidant activity when all independent variables are at their central values (often the midpoint of their range). A, B, and C denote the coded levels of the independent variables (extraction time, temperature, and pressure). The coefficients (e.g., +2.91 for A, -2.42 for B) represent the magnitude of each independent variable's effect on antioxidant activity. Figures (a-c) show that a positive coefficient indicates that increasing the variable improves antioxidant activity, whereas a negative coefficient suggests the opposite. AB, AC, and BC represent the interaction effects of two variables. For example, +2.14AB indicates a synergistic effect on antioxidant activity when both variables A and B are increased simultaneously. Biswas et al., (2023) optimized ultrasound-assisted extraction parameters such as temperature,

duration, solvent concentration, and liquid-solid ratio to extract total flavonoids and polyphenols from leaves. In comparison to standard heat reflux extraction, the adjusted conditions greatly increased the yield of antioxidant chemicals. Another study used response surface methods to optimize parameters influencing the extraction of phenolic components and antioxidant capabilities from *Cortex fraxini*. The study emphasized the impact of temperature and solvent-material ratio on extraction efficiency (Huang et al., 2023).

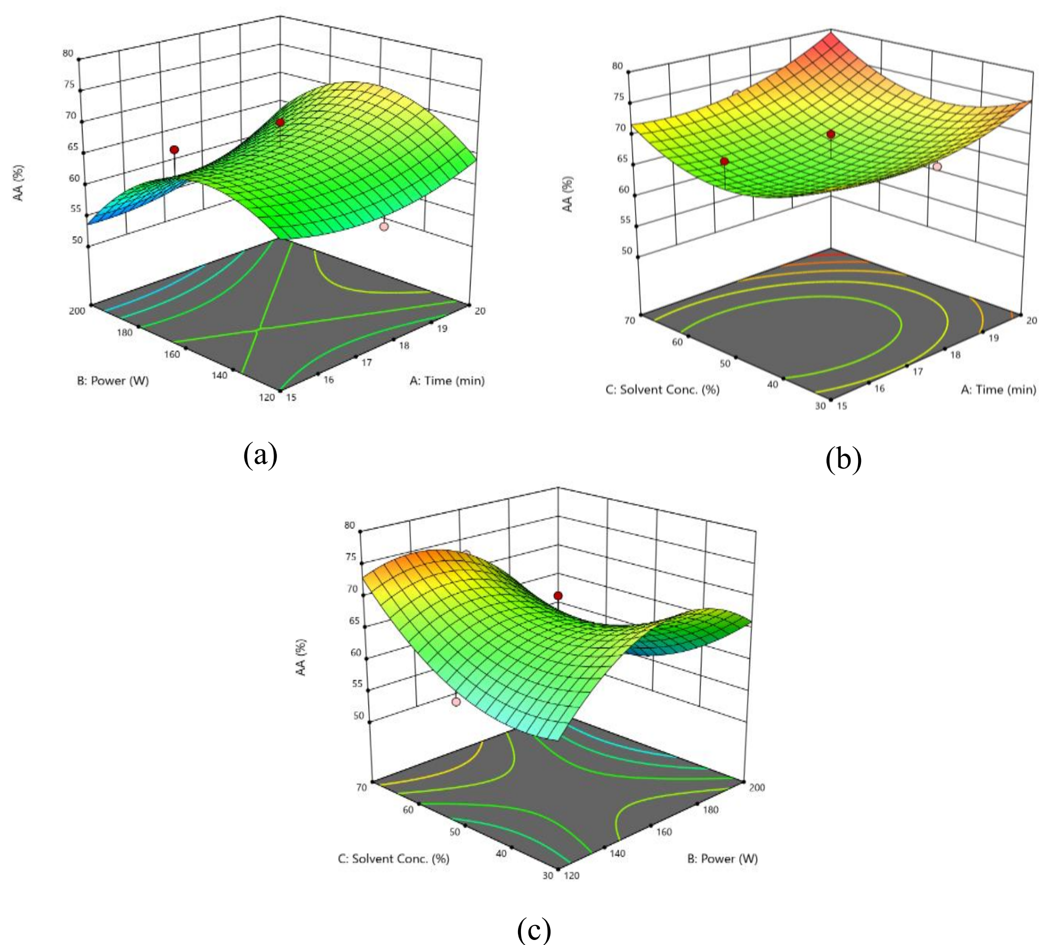


Figure 5.2. Response surface plots of *Clerodendrum glandulosum* Lindl. showing the effect of (a) extraction time and power, (b) extraction time and solvent concentration, and (c) power and solvent concentration on DPPH radical scavenging activity using UAE. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

5.3.1.3. Effect of UAE Parameters on Angiotensin converting enzyme (ACE) Activity

In the context of ultrasound-assisted extraction of medicinal plant extracts, the CCD model can be used to optimize extraction parameters to maximize bioactive ingredient yield and quality. When planning the extraction process, it is important to consider the major elements and their interactions. The total model's F-value of 673.19 with 9 degrees of freedom is statistically significant ($p < 0.0001$). This suggests that the model is accurate in predicting the response variable, which in this example could be the yield or concentration of bioactive chemicals recovered from medicinal plants using ultrasound-assisted extraction procedures. Time (A) has a significant effect on the extraction process (F=30.62, p=0.0003). This shows that changing the extraction period has a major impact on the extract's yield or quality. Ultrasound Power (B) has no significant effect on the extraction process in this model, as demonstrated by an F-value of 0.3648 and a p-value of 0.5656. This means that variations in ultrasonic power within the studied range have no meaningful effect on extraction results. Solvent Concentration (C) has a substantial influence (F-value = 525.48, p-value < 0.0001). This means that the concentration of the solvent used in the extraction process is an important component in determining extraction efficiency. In this model, only the interaction between B (Power) and C (Solvent Concentration) is significant ($p < 0.0001$), indicating that the combined effect of ultrasonic power and solvent concentration significantly influences the extraction process. The significant quadratic terms suggest that there is a non-linear relationship between these components and the extraction results, and adjusting these parameters may result in increased extraction efficiency. Lack of Fit: The lack of fit is not statistically significant ($p = 0.0725$), indicating that the model fits the data well and can predict the response variable. Extraction Time (A) has a positive coefficient of 1.75 suggests that the ACE inhibition increases with extraction time. However, the negative quadratic term ($-2.53A^2$) indicates that there is an optimal extraction time beyond which the ACE inhibition begins to drop. Power (B) has negative coefficient of -0.1910 indicates that increasing power reduces the ACE inhibitory activity. This is further reinforced by the negative quadratic term ($-2.82B^2$), which indicates that power has a greater effect on ACE yield at higher levels. Solvent Concentration (C) has a positive coefficient of 7.25 indicates that higher solvent concentrations result in higher ACE inhibition. The positive quadratic term ($4.03C^2$) indicates that this effect becomes stronger as solvent concentration increases.

Figures 5.3. (a-c) illustrate the interaction terms (AB, AC, BC), which suggest that the effect of one variable on ACE yield is reliant on the amount of another variable. For example, the negative AB interaction term (-0.4312) indicates that increasing extraction time and power has less than an additive effect on ACE yield. In a study by Salem et al. (2022), it was discovered that an 80% methanol extract had the strongest ACE inhibitory action, surpassing the conventional medication captopril. This shows that the concentration of ethanol in the solvent has a considerable effect on the extraction efficiency of ACE inhibitory drugs. The study's detailed analysis revealed that solvent selection is critical in bioactive component extraction. The 80% ethanol and 100% water extracts had the strongest in vitro ACE inhibitory activity of the solvents examined.

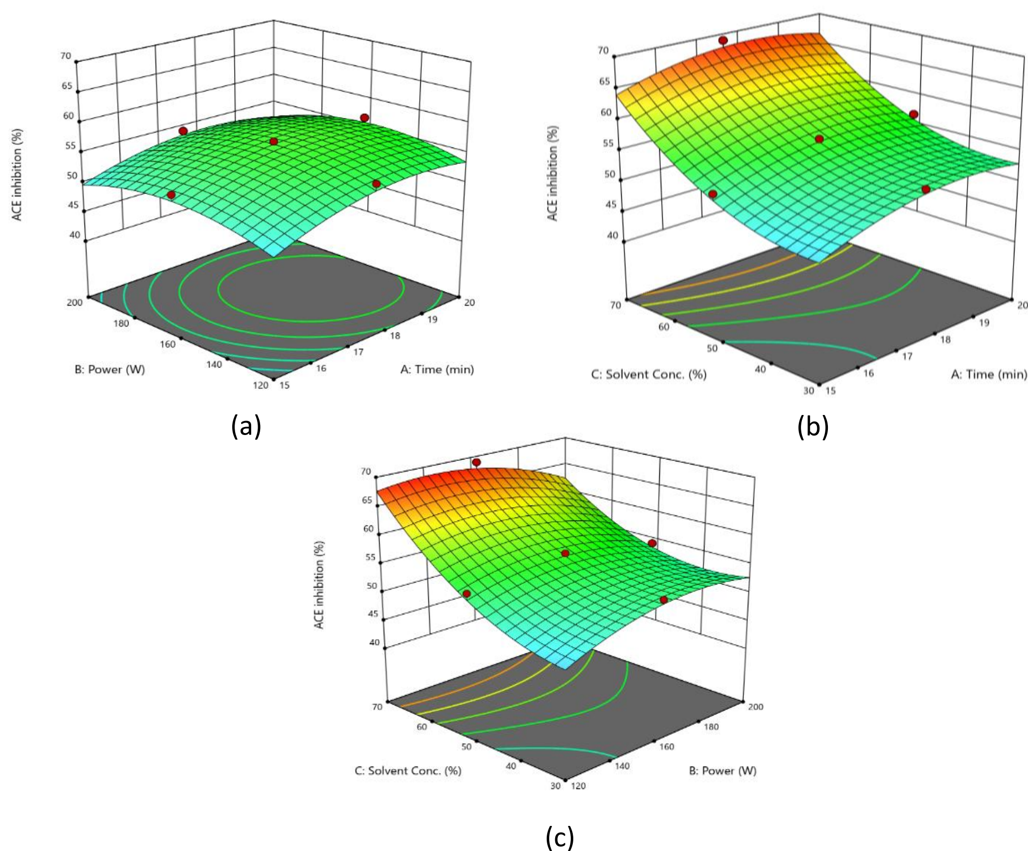


Figure 5.3: Response surface plots of *Clerodendrum glandulosum* Lindl. showing the effect of (a) extraction time and power, (b) extraction time and solvent concentration, and (c) power and solvent concentration on ACE activity using UAE. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

5.3.2. Fitting the Response Surface Models for SFE process

Fitting the models is critical for understanding the accuracy of the RSM mathematical models for predicting TPC, antioxidant activity (DPPH), and angiotensin converting enzyme (ACE) inhibitory activity of *Clerodendrum glandulosum* Lindl. extract. In this work, CCD successfully established the association between the response functions (TPC, DPPH, and ACE inhibitory activity) and the independent variables (extraction time, extraction temperature, and pressure). Table 5.7. displays the responses for the 20 runs conducted in accordance with the experimental design. The TPC ranged from 25.07 to 56.62 mg GAE/g, and the antioxidant activity (DPPH) ranged from 63.25 to 72.64%. In terms of ACE inhibitory action, the maximum was 95.92%, while the lowest was 55.11%. The Design Expert software recommended a quadratic polynomial model, which fits well for all three independent variables and responses. In terms of coded values, the expected responses for TPC, DPPH, and ACE inhibitory activity might be described by the second-order polynomial equation using multiple regression analysis (Eq. 5.7, 5.8, 5.9).

$$Y_{TPC}\,(SFE) = 33.43 + 1.38\,A + 3.01\,B + 10.29\,C + 0.1750\,AB - 1.10\,AC + 1.57\,BC + 8.53\,A^2 - 2.80\,B^2 + 2.19\,C^2 \quad (5.7)$$

$$Y_{AA}\,(SFE) = 68.94 + 0.3210\,A + 3.66\,C + 0.9613\,AB - 0.2818\,AC - 0.8387\,BC - 0.3127\,A^2 - 2.52B^2 + 2.20\,C^2 \quad (5.8)$$

$$Y_{ACE}\,(SFE) = 76.57 + 0.6820\,A + 6.62\,B + 10.16\,C + 0.3600\,AB - 0.2025\,AC - 3.70\,BC - 4.49\,A^2 - 2.97\,B^2 + 7.04\,C^2 \quad (5.9)$$

Where A, B, and C are the coded variables for extraction time, extraction temperature, and pressure, respectively.

Table 5.7. Response surface central composite design (uncoded) and the results for total phenolic content (TPC), radical scavenging activity (DPPH) and Angiotensin converting enzyme (ACE) inhibitory activity for SFE process.

Time (mins)	Temperature (℃)	Pressure (bar)	TPC (mg GAE/g)	DPPH radical scavenging activity (%)	ACE inhibitory activity (%)
20	50	200	27.34	63.74	55.11
40	50	200	31.79	63.45	56.05
20	70	200	30.12	67.27	75.60
40	70	200	35.22	68.34	78.49
20	50	250	46.87	72.52	82.12
40	50	250	46.87	68.62	82.76
20	70	250	55.87	70.21	88.32
40	70	250	56.62	72.64	89.89
20	60	225	40.25	66.51	72.12
40	60	225	43.75	70.41	72.90
30	50	225	28.09	64.18	69.06
30	70	225	33.25	68.32	79.01
30	60	200	25.07	63.25	72.16
30	60	250	46.25	78.7	95.92
30	60	225	36.05	69.3	76.41
30	60	225	32.87	68.36	75.22
30	60	225	35.37	70.31	76.30
30	60	225	32.87	66.35	77.51
30	60	225	30.02	70.15	75.16
30	60	225	33.25	69.84	77.09

A negative sign in each equation reflects an antagonistic influence of the variables, whereas a positive sign represents a synergistic effect (Azahar et al., 2017). Positive coefficients for A, B, and C were found in equation (2), showing that TPC increased with increasing extraction time (20-40 minutes), temperature (50-70°C), and pressure (200-250

bar). The same observation was made for TPC, where positive coefficients for A, B, and C were discovered in equation (3), showing that DPPH increased with increasing extraction time (20-40 minutes), extraction temperature (50-70°C), and pressure (1:10-1:20 ratio). The same pattern applies to the ACE inhibitory activity results. Analysis of variance (ANOVA) was used to validate the RSM model coefficients for the quadratic polynomial model, as shown in Tables 5.8. – 5.10. The models for TFC, DPPH, and ACE inhibitory activity were highly significant when computed F-values exceeded tabulated F-values and probability values were low ($p < 0.001$), indicating that individual terms in each response model were significant on the interaction effect. Furthermore, the coefficients of determination (R^2) for the models were 0.9849, 0.7526, and 0.9882, suggesting a 98.49, 75.26, and 98.82% match between the projected model values and the values obtained in the experimental data.

Furthermore, the R^2 values were similar to adjusted R^2, indicating a good statistical model. The p-values for the lack of fit were 0.9979, 0.0586, and 0.0951, indicating that the models' lack of fit was not statistically significant at $p > 0.05$. If the p-value for the lack of fit F-test is higher than the significance level (α), we cannot reject the null hypothesis. In other words, the gap in fit is not statistically significant. Thus, the findings show that the lack of fit is not substantial, implying that the model reasonably reflects the relationship between predictors and response.

Table 5.8: The regression coefficients and results of ANOVA for response surface quadratic model of total phenolic content using SFE.

Source	**Sum of squares**	**Df**	**Mean square**	***F* value**	***P* value**
Model	1557.75	9	173.08	72.25	< 0.0001
A-Time	19.04	1	19.04	7.95	0.0182
B-Temperature	90.72	1	90.72	37.87	0.0001
C-Pressure	1059.66	1	1059.66	442.35	< 0.0001
AB	0.2450	1	0.2450	0.1023	0.7557
AC	9.68	1	9.68	4.04	0.0722
BC	19.66	1	19.66	8.21	0.0168
A^2	200.03	1	200.03	83.50	< 0.0001

B^2	21.58	1	21.58	9.01	0.0133
C^2	13.17	1	13.17	5.50	0.0410
Lack of Fit	1.04	5	0.2087	0.0455	0.9979
Pure Error	22.91	5	4.58		
R^2 0.9849					

Table 5.9: The regression coefficients and results of ANOVA for response surface quadratic model of Antioxidant activity (DPPH) using SFE.

Source	Sum of squares	Df	Mean square	***F* value**	***P* value**
Model	193.12	9	21.46	3.38	0.0356
A-Time	1.03	1	1.03	0.1623	0.6955
B-Temperature	20.36	1	20.36	3.21	0.1036
C-Pressure	134.25	1	134.25	21.14	0.0010
AB	7.39	1	7.39	1.16	0.3059
AC	0.6328	1	0.6328	0.0997	0.7587
BC	5.63	1	5.63	0.8864	0.3686
A^2	0.2689	1	0.2689	0.0424	0.8411
B^2	17.50	1	17.50	2.76	0.1278
C^2	13.34	1	13.34	2.10	0.1779
Lack of Fit	52.24	5	10.45	4.64	0.0586
Pure Error	11.25	5	2.25		
R^2 0.7526					

Table 5.10: The regression coefficients and results of ANOVA for response surface quadratic model of Angiotensin converting enzyme (ACE) activity using SFE

Source	**Sum of squares**	**Df**	**Mean square**	***F* value**	***P* value**
Source	Sum of square	Df	Mean S	F-value	P-value
Model	1743.72	9	193.75	93.21	< 0.0001
A-Time	4.65	1	4.65	2.24	0.1656
B-Temperature	438.38	1	438.38	210.90	< 0.0001
C-Pressure	1032.26	1	1032.26	496.61	< 0.0001

AB	1.04	1	1.04	0.4988	0.4962
AC	0.3280	1	0.3280	0.1578	0.6995
BC	109.52	1	109.52	52.69	< 0.0001
A^2	55.47	1	55.47	26.69	0.0004
B^2	24.20	1	24.20	11.64	0.0066
C^2	136.24	1	136.24	65.54	< 0.0001
Lack of Fit	16.22	5	3.24	3.55	0.0951
Pure Error	4.56	5	0.9129		
R^2 0.9882					

5.3.2.1. Effect of SFE Parameters on Total Phenolic Content (TPC)

Phenolic compounds are a major type of secondary metabolite generated by all plants. Phenolic substances are secondary metabolites derived from the pentose phosphate, shikimate, and phenyl propanoid pathways in plants (Randhir et al., 2004). Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins, which primarily function as phytoalexins, pollinator attractants, plant pigmentation contributors, antioxidants, and UV light protectors (Gottlieb et al., 2000). The whole model is significant (p-value < 0.0001), indicating that at least one of the independent factors significantly affects the dependent variable, as demonstrated in the regression coefficients and findings of ANOVA Table 5.8 With a p-value of 0.1656, time is not statistically significant, indicating that it has little effect on the dependent variable in the range examined. Temperature has a substantial ($p < 0.0001$) effect on the dependent variable. Pressure has a substantial ($p < 0.0001$) effect on the dependent variable. A^2 is significant ($p = 0.0004$), suggesting a quadratic influence of time on the dependent variable. B^2 is significant ($p = 0.0066$), suggesting a quadratic effect of temperature. C^2 is highly significant ($p < 0.0001$), suggesting a quadratic influence of pressure. The residual is the variation that the model does not explain. A lower residual indicates better model fit. Lack of fit: Not significant ($p = 0.0951$), indicating that the model fits the data well.

Assuming all other variables remain constant, each unit increase in time (A) improves Y_{TPC} by 1.38, temperature (B) improves Y_{TPC} by 3.01 and pressure (C) increases Y_{TPC} by 10.29 respectively. AB signifies the combined effect of A and B on Y_{TPC}. The positive sign suggests that the interaction works synergistically to increase Y_{TPC}. Time and pressure

(AC) indicate an antagonistic relationship between time (A) and pressure (C), which reduces Y_{TPC}. Temperature and pressure (BC) represents a synergistic combination between temperature (B) and pressure (C), resulting in increased Y_{TPC}. A^2 (time) has a positive coefficient indicates that as A increases, so does the effect on Y_{TPC}. The value of B^2 (temperature) has a negative coefficient indicating that as B (temperature) increases, its influence on increasing Y_{TPC} slows, and at some time, it may begin to decrease Y_{TPC}. As with A^2 (time), rising C (pressure) has an increasing effect on boosting Y_{TPC}. This model shows that Y_{TPC} is influenced by the individual effects of A (time), B (temperature), and C (pressure), as well as their interactions and squared values, resulting in a non-linear relationship in which the impact of each variable can rise or decrease at various rates as their values change. This paradigm is very beneficial in disciplines like medicinal plant and bioactive ingredient research, where variable effects are rarely linear and straightforward.

Figs. 5.4 (a)-(c) exhibit 3-D response surface plots showing the effects of extraction time, temperature, and pressure on TPC. The 3D surface plot commonly demonstrated how TPC fluctuates with changes in two of these variables. Temperature and pressure have significant linear and quadratic effects on the dependent variable. The interactions between these variables are likewise essential, except for the interactions between time and temperature and time and pressure, which are not significant. The model fits the data well, as evidenced by the non-significant lack of fit. Longer extraction periods can enhance phenolic compound yields to a degree. However, overly extended extraction durations may result in the breakdown or modification of phenolic chemicals (Shi et al., 2022; Naczk and Shahidi, 2004) and may also enhance solvent loss by vaporization (Tan et al. 2013), which can have a direct impact on mass transfer losses during extraction. In general, higher temperatures enhance phenolic chemical solubility and extraction efficiency. Elevated temperatures, on the other hand, improve the extraction rate and hence shorten the time required to recover the maximum polyphenolic content (Cacace and Mazza, 2003). However, there is an ideal temperature range, typically between 60 and 80°C, beyond which phenolic compounds may degrade. Subcritical water extraction investigations have demonstrated high yields at temperatures ranging from 100 to 200°C, however this is dependent on the stability of the individual phenolic compounds present (Rahman et al., 2023). High pressure can improve phenolic chemical extraction by allowing more solvent to penetrate the plant matrix. For example, supercritical fluid extraction (SFE) with CO_2

is frequently performed at pressures around 350 bar and has been shown to be successful for removing phenolic chemicals (Shi et al., 2022).

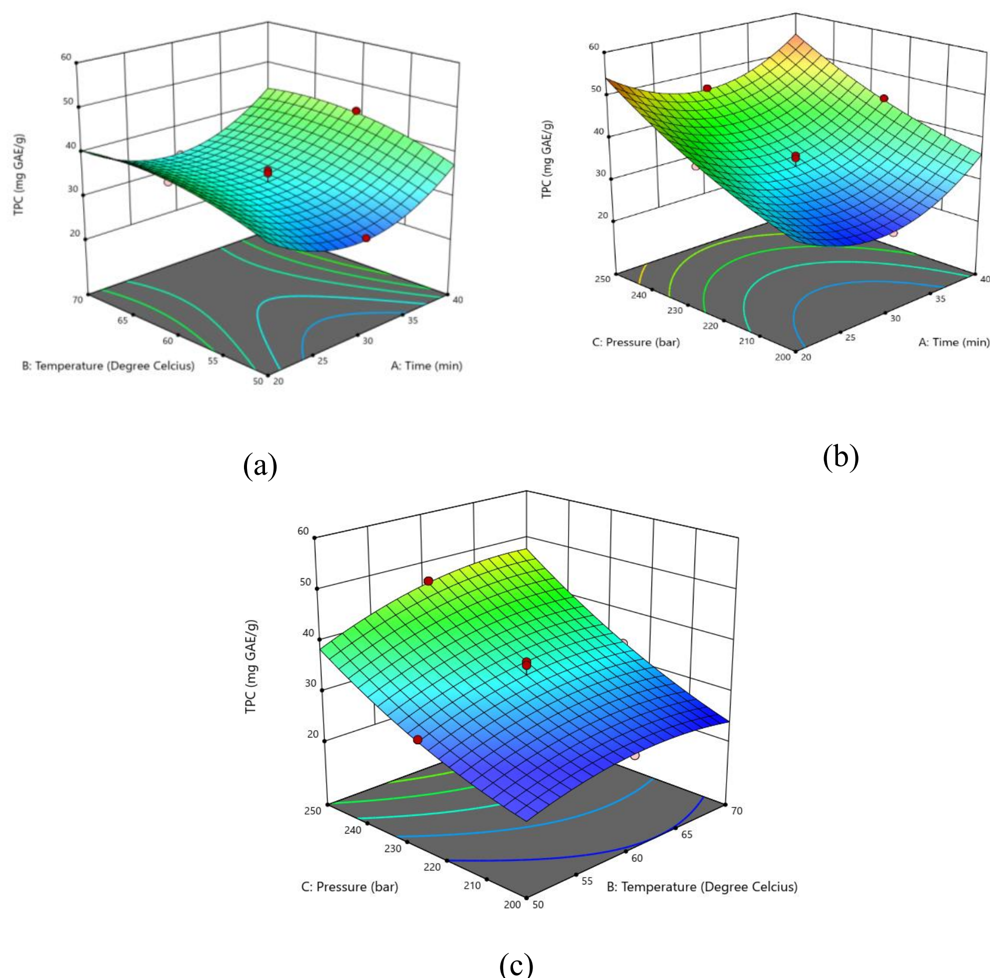


Figure 5.4.: Response surface plots of *Clerodendrum glandulosum Lindl.* showing the effect of (a) extraction time and power, (b) extraction time and solvent concentration, and (c) power and solvent concentration on total phenolic content using SFE. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

5.3.2.2. Effect of SFE Parameters on DPPH radical scavenging activity

The presence of bioactive chemicals, particularly phenolic compounds, in medicinal plants enhances their antioxidant activity, which may be measured using the DPPH assay. This approach is sensitive enough to distinguish between the antioxidant capabilities of different plant species. For example, a study found that antioxidant activity values quantified using DPPH were higher than those obtained using other assays such as ABTS and FRAP, and these values varied between species, highlighting the importance of using

the appropriate method to determine the highest antioxidant activity (Chaves et al., 2020). Another study found a link between antioxidant activity evaluated by DPPH and the total phenolic and flavonoid content of plant extracts. This shows that plants with high phenolic and flavonoid content have strong antioxidant activity, which is important for their potential anti-inflammatory and health-promoting properties (Diaz et al., 2012). In short, the DPPH assay is an important method for determining the antioxidant potential of medicinal plants, which is heavily impacted by their phenolic content and has a considerable impact on their therapeutic characteristics. AB (Time and Temperature): Not

significant (p-value = 0.3059). AC (Time and Pressure): Not significant (p-value = 0.7587). BC (Temperature and Pressure): Not significant (p-value = 0.3686). A^2 ($Time^2$): Not significant (p-value = 0.8411). B^2 ($Temperature^2$): Not significant (p-value = 0.1278). C^2 ($Pressure^2$): Not significant (p-value = 0.1779). Residual: The variation that is not explained by the model. A lower residual indicates better model fit. Lack of fit: The p-value of 0.0586 is not significant, indicating that the model fits the data well. The model is significant, but only one of the components and their interactions, pressure (C), has a substantial effect on the response variable. The lack of fit is not substantial, which is positive because it suggests that the model fits the data well. The non-significant lack of fit, when combined with the significant model, indicates that the model is appropriate for the data. However, this analysis shows that the parameters Time (A) and Temperature (B), as well as their interactions and quadratic terms, have no significant effect on the response variable.

Figs. 5.5 (a)-(c) show 3-D response surface plots showing the effects of extraction time, temperature, and pressure on AA. A has a positive linear coefficient (0.3210), indicating that when A increases, Y_{AA} tends to rise linearly. C has a higher positive linear coefficient (3.66), implying a more direct influence on Y_{AA} than A. The interaction term AB has a positive coefficient (0.9613), indicating that the combination of A and B raises Y_{AA}. However, the negative coefficients for AC (-0.2818) and BC (-0.8387) indicate that these interactions reduce Y_{AA}. The negative coefficients for A^2 and B^2 indicate a parabolic relationship in which increases in A and B initially enhance Y_{AA} but then decline.

The positive coefficient for C^2 indicates that Y_{AA} increases steadily as C values rise. In the context of medicinal plant supercritical fluid extraction, these variables are vital for

maximizing bioactive component extraction, such as antioxidants. Ghafoor et al., (2012) discovered that extraction temperature and pressure had a substantial impact on the yield of phenolic compounds and antioxidants from grape seeds using supercritical CO_2. Another study emphasized the necessity of adjusting these parameters to enhance flavonoid extraction and antioxidant activity (Li et al. 2016). The interaction and quadratic terms indicate that, while increasing these variables generally increases yield, there is an optimal range beyond which efficiency may decline. This is consistent with the idea that too high temperatures or pressures may destroy sensitive chemicals or result in the extraction of undesired components, lowering the quality of the extract. This is backed by several studies looking into the optimization of supercritical fluid extraction parameters for bioactive chemical recovery (Aman Mohammadi et al., 2024).

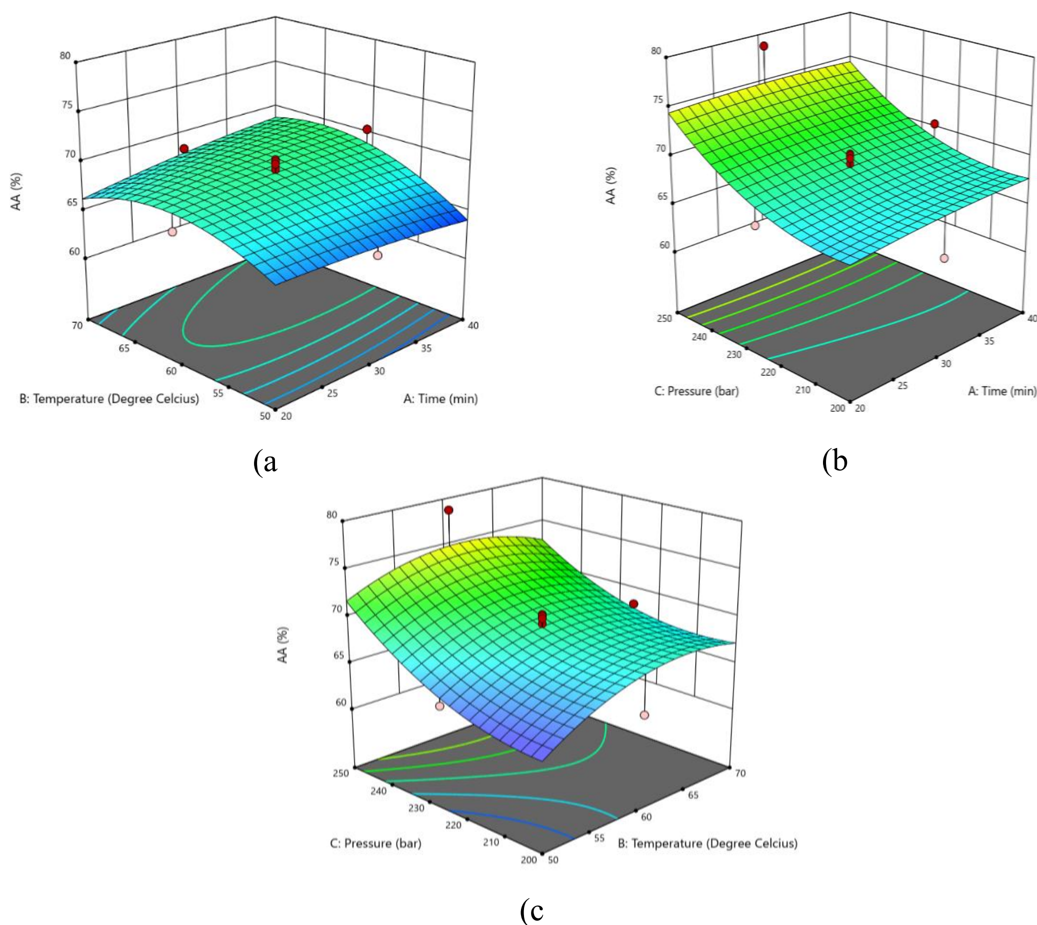


Figure 5.5: Response surface plots of *Clerodendrum glandulosum* Lindl. showing the effect of (a) extraction time and power, (b) extraction time and solvent concentration, and (c) power and solvent concentration on DPPH radical scavenging activity using SFE.

Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

5.3.2.3. Effect of SFE Parameters on Angiotensin converting enzyme (ACE) Activity

Medicinal plants that decrease ACE function may include chemicals that can help manage hypertension, a key risk factor for cardiovascular disease. These plants could be a significant resource for creating novel, natural ACE inhibitors with fewer negative effects than manufactured drugs12. For example, plant-derived peptides have been examined for their ACE inhibitory effects and have shown promise in both in vitro and in vivo assays2.In conclusion, ACE inhibitory activity in medicinal plants is significant because it represents an avenue for discovering new bioactive compounds that can contribute to the management and treatment of hypertension and cardiovascular diseases, which aligns with the global need for more effective and natural treatments (Fadahunsi et al., 2022; Dakaya-Dikmen et al., 2017).

The model is statistically significant (p-value < 0.0001), indicating that at least one component has a substantial effect on the response variable. The time component is not significant (p-value = 0.1656), indicating that time has little effect on its own. Temperature significantly affects the response variable (p-value < 0.0001). Pressure is very significant (p-value < 0.0001), indicating a strong effect. While there is no significant interaction between Time and Temperature (AB) or Time and Pressure (AC), the interaction between Temperature and Pressure (BC) is significant (p-value < 0.0001). This indicates that the combined effect of temperature and pressure is significant. Significant values for A^2, B^2, and C^2 suggest a non-linear relationship between the components and the response variable.
Figs. 5.6 (a)–(c) depict 3-D response surface plots showing the effects of extraction time, temperature, and pressure on angiotensin converting enzyme (ACE). A positive coefficient (0.6820) indicates that as variable A increases linearly, so does ACE inhibitory activity. B: Similarly, a positive correlation (6.62) suggests that increasing variable B relates to more ACE inhibitory action. C: With the greatest positive coefficient (10.16), variable C appears to have a significant positive impact on ACE inhibitory activity. The terms AB, AC, and

BC represent the interaction effects of the variables. For example, a positive AB term (0.3600) indicates that the combined rise in A and B has a synergistic effect on ACE inhibition. However, the negative AC and BC terms indicate that their combined rise may lower ACE inhibitory activity. The quadratic terms (A^2, B^2, C^2) indicate the presence of a non-linear relationship. The negative coefficients for A^2 and B^2 imply that there may be a point at which further increases in A and B result in a decrease in ACE inhibitory activity.

In contrast, the positive coefficient for C^2 indicates that increases in C continue to positively enhance ACE inhibitory activity at an increasing rate. In the context of medicinal plant research, these variables may refer to the conditions under which the plants are processed to extract ACE inhibitory chemicals. For example, higher temperatures (variable B) may improve the extraction of such compounds up to a point, after which the activity may decrease due to the destruction of heat-sensitive components. One study investigated at the ACE inhibitory activity of plant peptides and discovered that parameters including extraction process and peptide structural properties, such as chain length, content, and sequence, can have a substantial impact on their activity. Furthermore, these peptides' bioavailability, or ability to enter the circulatory system and demonstrate bioactivity, is critical (Daskaya-Dikmen et al., 2017). Another study, using molecular docking and dynamic simulation, looked at the ACE inhibitory effect of substances found in medicinal plants traditionally used to treat high blood pressure in southwestern Nigeria (Fadahunsi et al., 2022). This study emphasizes the significance of the structural interaction between plant chemicals and the ACE protein, which is affected by the extraction process and circumstances.

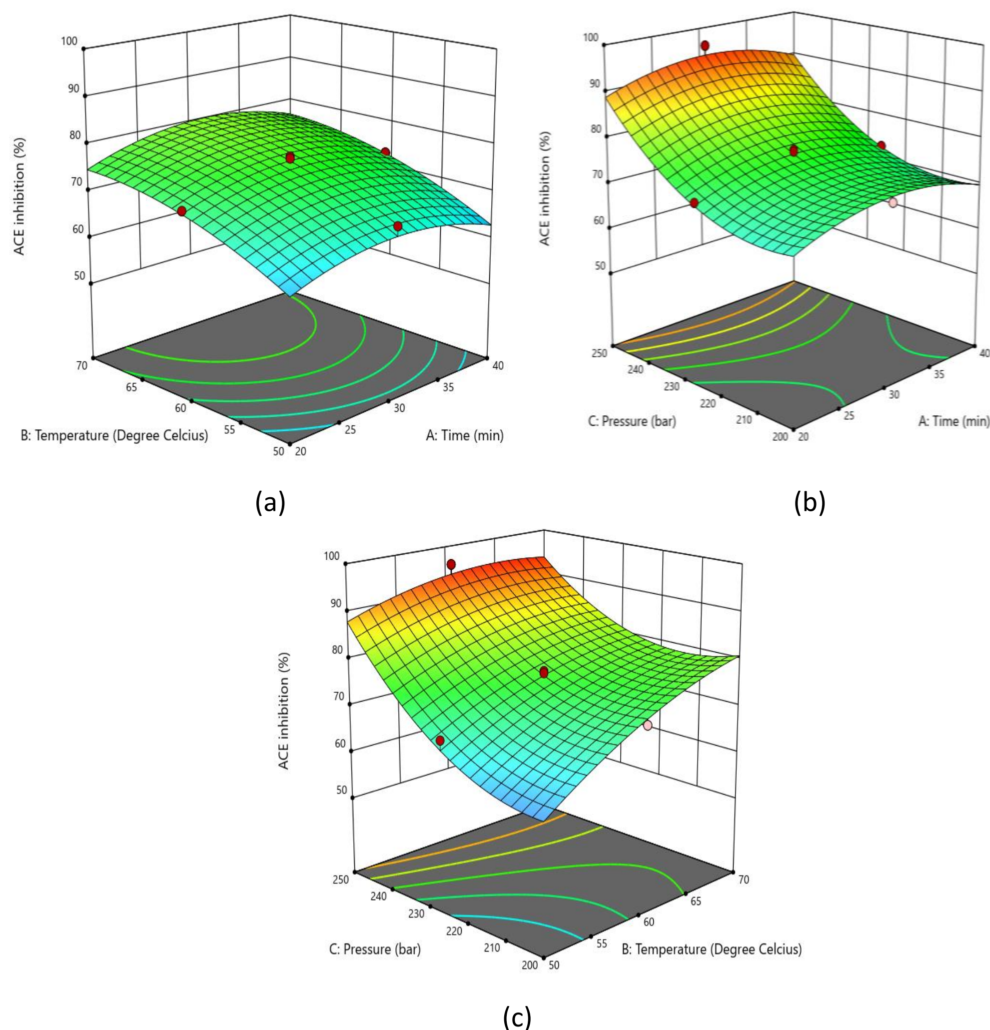


Figure 5.6: Response surface plots of *Clerodendrum glandulosum Lindl.* showing the effect of (a) extraction time and power, (b) extraction time and solvent concentration, and (c) power and solvent concentration on ACE activity using SFE. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

5.3.2.4. FT-IR spectroscopic analysis

The FT-IR spectra of both the UAE and SFE extracts are shown in Fig. 5.7 (a & b). In both extracted samples, the FT-IR banding around 3405-3375 cm^{-1} is commonly associated with the O-H stretch in hydrogen-bonded hydroxyl groups, which are found in alcohols and phenols. This band shows the existence of hydrogen bonding, which typically results in a shift to lower wavenumbers than free hydroxyl groups. This band indicates the presence of hydrogen-bonded hydroxyl groups, which are important in the study of plant extracts because they can indicate the presence of phytochemicals such as phenolics, which are

known to have health benefits, especially in relation to cardiovascular disease (Sudirman et al., 2023). In the context of plant extracts, the FT-IR banding at 2921-2933 cm^{-1} could be attributed to the presence of aliphatic hydrocarbons, which are found in many plant components. These bands indicate the presence of methyl (CH_3) or methylene (CH_2) groups, which are constituents of bigger molecules such as fatty acids, waxes, and other lipid-soluble components found in plant extracts. This is typical of methylene groups (-CH2-), and it frequently indicates the presence of lipids or fatty acids in a sample. This could indicate the existence of essential oils or other lipid-soluble chemicals with potential health effects, particularly in relation to cardiovascular illnesses (Krilov et al., 2009; Uysal and Boyaci, 2020). The banding at 1607-1613 cm^{-1} is commonly related with C=C stretching vibrations in aromatic compounds. This absorption band indicates the existence of an aromatic ring structure, which is prevalent among medicinal plants due to their complex phytochemical makeup. The FT-IR banding at 1382-1394 cm^{-1} could be attributed to specific functional group vibrations within a molecule. While the search results did not yield a concrete explanation for this range, absorption peaks in the vicinity are frequently associated with certain chemical structures or functional groups. In SFE, the FT-IR band at 1382-1394 cm^{-1} is commonly related with the bending vibrations of C-H bonds in methyl groups. This can indicate the existence of specific types of chemical compounds in a sample, but this banding was not seen in UAE samples. The banding at 1258-1270 cm^{-1} could be due to functional group vibrations within a molecule. While the search results did not provide a straightforward explanation for this precise range, analogous banding in the vicinity, such as a strong band at 1270 cm^{-1}, has been linked to C-O stretching modes in several compounds (Muthuselvi et al., 2018a; Muthuselvi et al., 2018b). The bands found at 1046-911 cm^{-1} could be attributed to different functional group vibrations inside a molecule. The FT-IR banding found around 811-592 cm^{-1} may relate to distinct functional group vibrations inside a molecule. In RSM experimental table in UAE, TPC was obtained maximum at std run no.14. And similarly, in FTIR spectra also, std run no.14 (UAE14) showed sharp bands in the range of 800-1600 cm^{-1} (wavenumber). Whereas in TPC is maximum at Std. run no.8 (SFE 8) as shown in RSM experimental table. Also, in FT-IR spectras also, Std. no.8 shows higher bands with sharp peaks from 800-1600 cm^{-1} (wavenumbers).

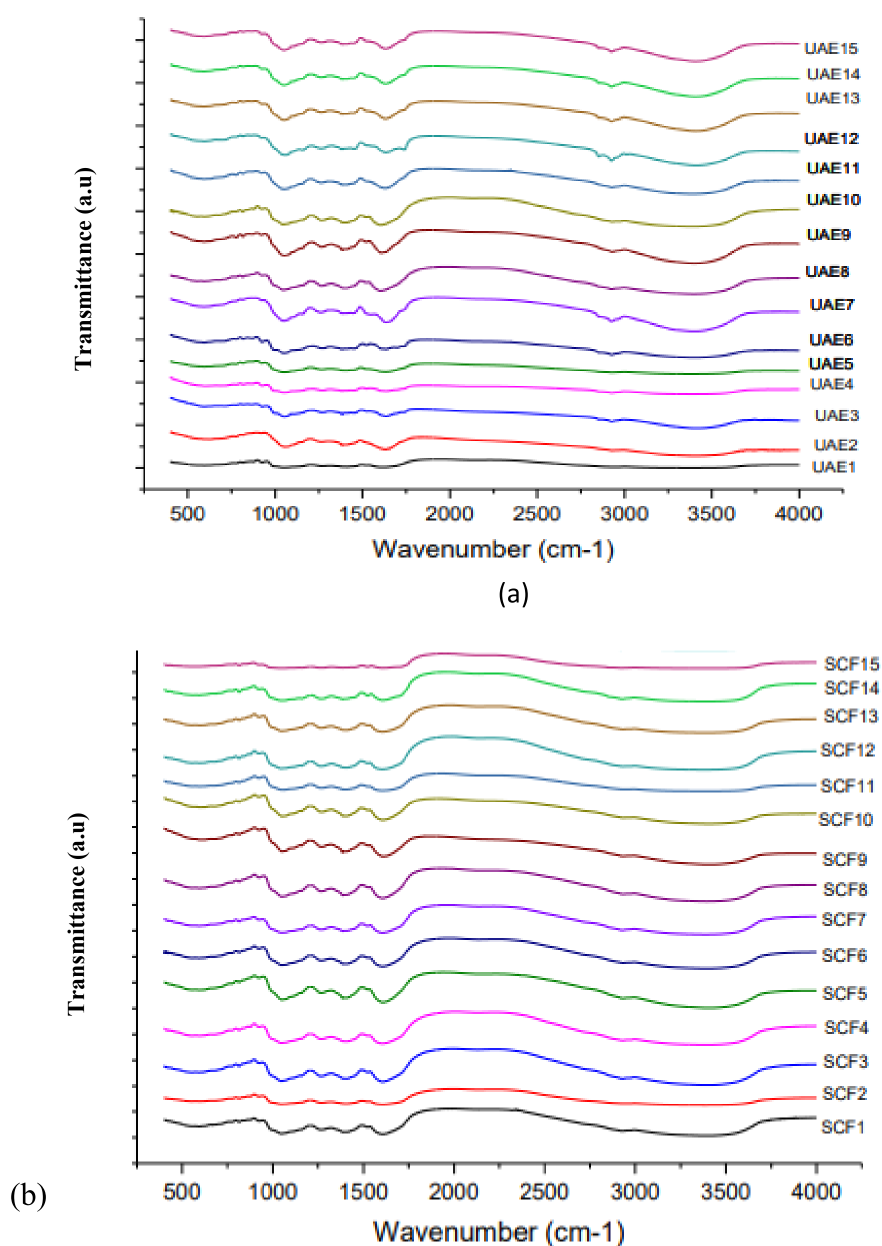


Fig. 5.7 (a): FT-IR spectra of UAE samples; (b) FT-IR spectra of SFE samples (The center points runs were not repeated as the conditions were same for all).

5.3.2.5. Optimization and model verification

The developed model in RSM was used for the optimization. By optimizing developed model for both the responses TPC, DPPH and ACE inhibitory activity best condition of the extraction process was determined in both UAE and SFE plant extracts. The predicted and actual values of TPC, free radical scavenging activity by DPPH radicals and Angiotensin converting enzyme (ACE) inhibition activity, as shown in Table 5.11**.** The optimum conditions for maximum TPC, DPPH ACE inhibition values in UAE sample i.e.

70.82±2.03 mgGAE/g, 79.88±1.49 mgGAE/g and 74.88±2.49 mgGAE/g were obtained using ethanol, extraction time of 19.27 min, power value of 152.52 W and 70% solvent concentration respectively as shown in Table 5.11. Under these optimal conditions the experimental values agreed with the predicted values with the CV range from 1.49 to 2.49%.

The predicted and actual values of TPC, free radical scavenging activity by DPPH radicals and Angiotensin converting enzyme (ACE) inhibition activity, as shown in Table 5.12. The optimum conditions for maximum TPC, DPPH ACE inhibition values in SFE sample i.e. 60.24±4.52 mg GAE/g, 71.07±1.49 mg GAE/g and 93.24±2.44 mg GAE/g were obtained using ethanol, extraction time of 40 min, extraction temperature of 65 °C and pressure of 250 bar respectively. The experimental values agreed with the predicted values with the CV range from 1.49 to 4.52%.

Table 5.11: Validation of optimized results of UAE sample (Mean ± standard deviation).

	Response obtained for TPC (mg GAE/g)	Response obtained for DPPH radical scavenging activity (%)	Response obtained for Angiotensin converting enzyme (ACE) inhibition (%)
Predicted value	68.98	77.89	67.93
Actual value	70.82±2.03	79.88±1.49	74.88±2.49
Mean difference	1.84	1.99	6.95

Table 5.12: Validation of optimized results of SFE sample (Mean ± standard deviation).

	Response obtained for TPC (mg GAE/g)	Response obtained for DPPH radical scavenging activity (%)	Response obtained for Angiotensin converting enzyme (ACE) inhibition (%)
Predicted value	56.36	74.69	90.64
Actual value	60.24±4.52	71.07±1.49	93.24±2.44
Mean difference	3.88	-3.62	0.03

5.3.3. Measurement of cell viability by MTT assay

THP-1 monocyte cells that had been differentiated into macrophages. Various concentrations of UAE and SFE were used for the toxicity analysis, exhibited reduction of cell viability with increasing concentration of the extracts. Fig. 5.8 (a and b). Treatment with 25, 50, 100 and 125 µg/ml extract induced cell viability by 99.57%, 96.19%, 88.07%, 84.47% for optimized samples UAE and 87.24%, 84.26%, 81.58%, 88.58% for SFE in 48 h incubation, respectively.

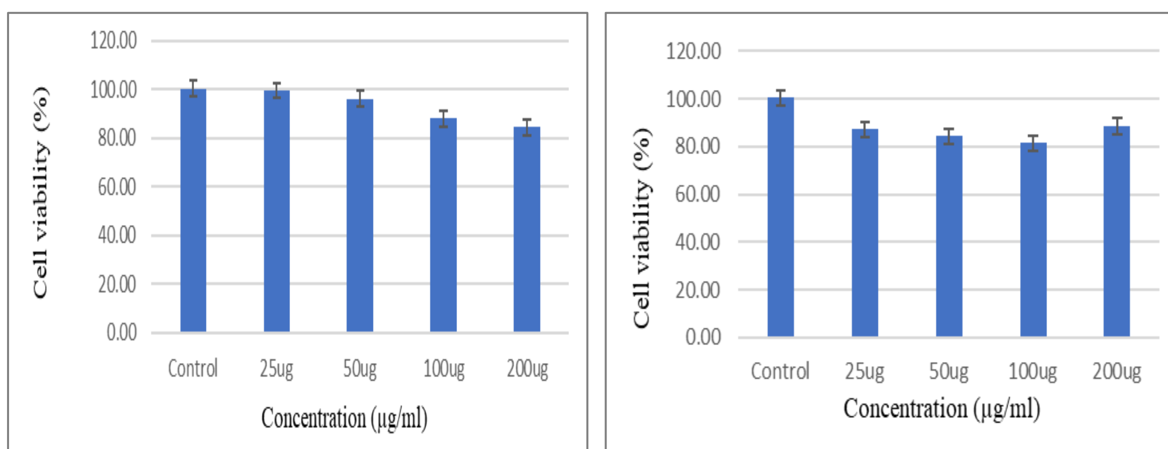


Fig.5.8: (a) Cytotoxicity study of *Clerodendrum glandulosum* Lindl. extract for UAE; (b) Cytotoxicity study of *Clerodendrum glandulosum* Lindl. extract SFE by MTT analysis

5.3.4. Effect of *Clerodendrum glandulosum* Lindl. extract on pro-inflammatory genes

Clerodendrum glandulosum Lindl. extracts (UAE3 and SFE3) were tested for their anti-inflammatory effects on mRNA expression of pro-inflammatory markers such IL-1β, TNF-α, and COX-2 in THP-1 cells stimulated by LPS (Fig. 5.9). The human THP-1 cell line and THP-1-derived macrophages are regarded as useful cellular models for evaluating anti-inflammatory medicines. In this investigation, THP-1 cells without any external stimuli were used as controls, and 100ng/ml LPS-stimulated THP-1 cells and *Clerodendrum glandulosum* Lindl. extract treated LPS-stimulated THP-1 cells were compared to the control. Three dosages (50, 100, and 200 μg/ml) of *Clerodendrum glandulosum* Lindl. extract was utilized to treat cells.

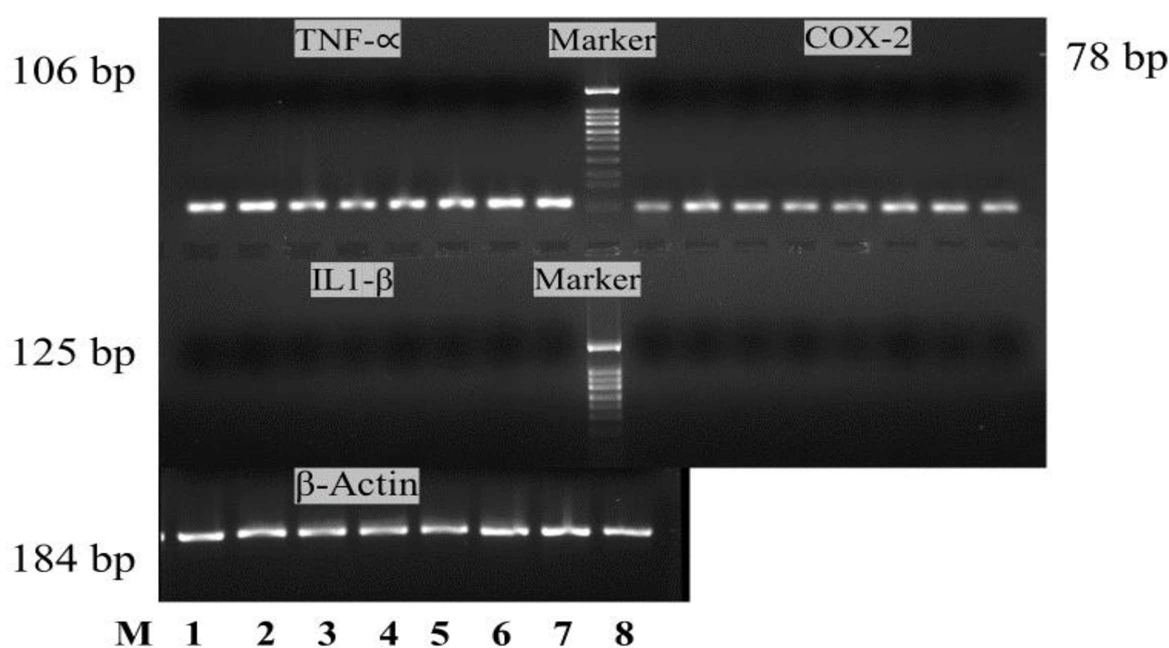
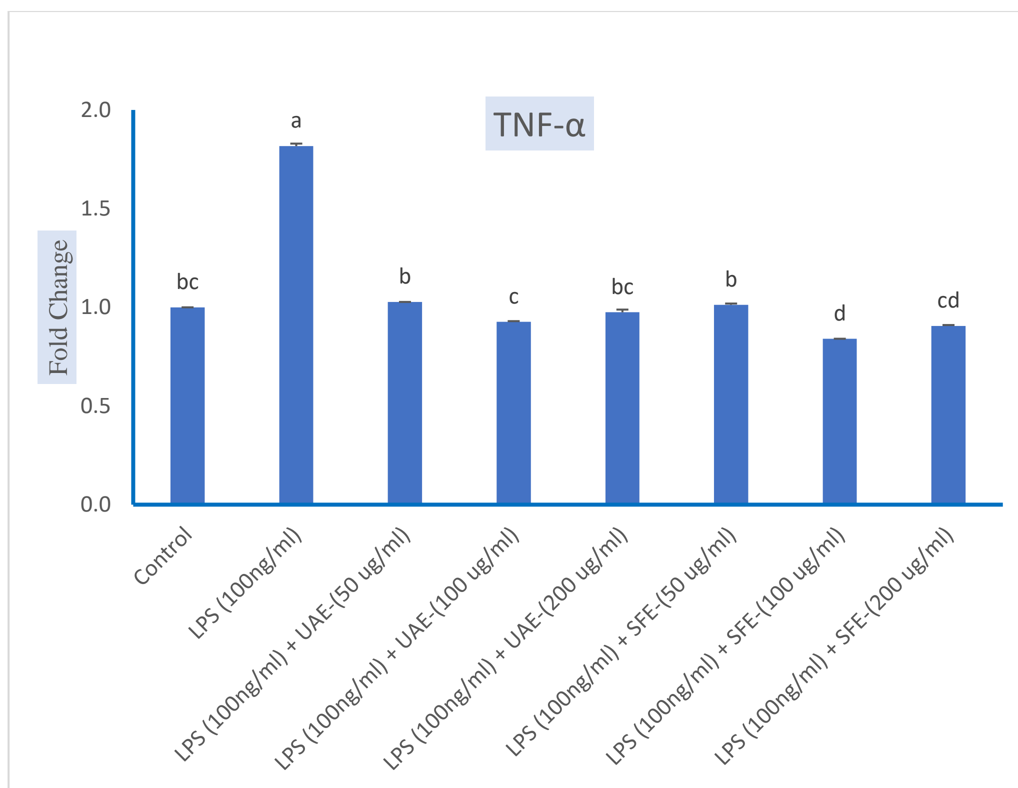
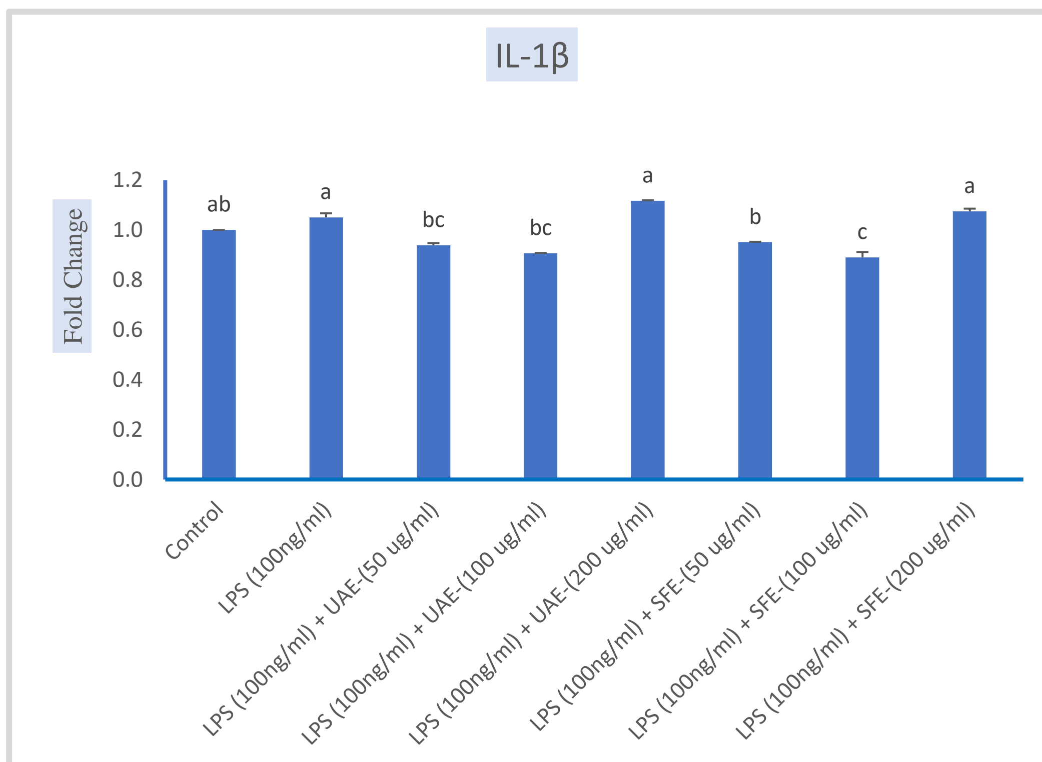


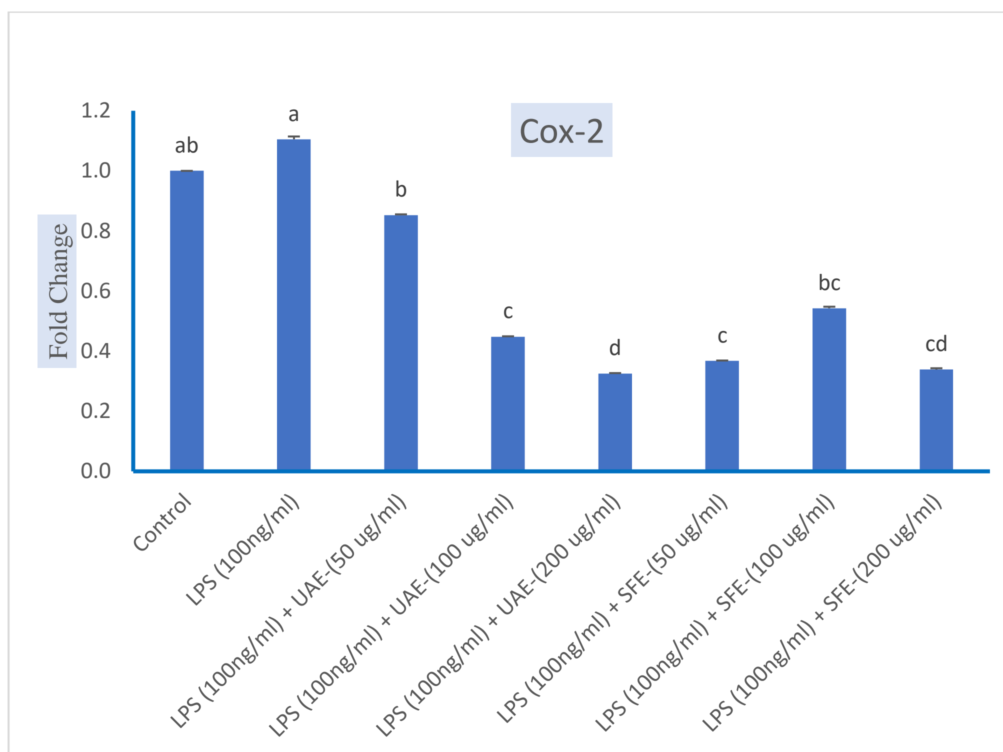
Fig. 5. 9 (a): Gel images for gene expression study : Lanes: M: Ladder (100 bp); 1: Control; 2: LPS (100 ng/ml); 3: LPS (100 ng/ml) + UAE-(50μg/ml); 4: LPS (100 ng/ml) + UAE-(100μg/ml); 5: LPS (100 ng/ml) + UAE-(200μg/ml); 6: LPS (100 ng/ml) + SFE-(50μg/ml); 7: LPS (100 ng/ml) + UAE-(100μg/ml); 8: LPS (100 ng/ml) + SFE-(200μg/ml)



(b)



(c)



(d)

Fig.5.9 (b-d): Gene expression studies for various pro-inflammatory markers.

Same letter for various pro-inflammatory markers with various concentrations denotes no significant differences at $p \leq 0.05$.

TNF-α is a key regulator of inflammation, and higher levels can lead to chronic inflammation-related illnesses. Using anti-TNF-α antibodies or TNF-α inhibitors can be a therapeutic method to address these disorders. Our investigation found that LPS treatment increased TNF-α expression by approximately 1.83±0.0018 fold. However, treatment with three different doses of the extract UAE and SFE reduced increased TNF-α expression to basal levels. UAE and SFE samples did not significantly lower TNF-α mRNA expression at 50 µg/mL doses. UAE extracts at concentrations of 100 µg/mL and 200 µg/mL showed stronger capacity to reduce TNF-α expression compared to a reference anti-inflammatory molecule, with fold change values of 0.92±0.005% and 0.961±0.020%, respectively. The relative fold change values for SFE extracts (100 µg/mL and 200 µg/mL) were 0.84±0.001% and 0.902±0.007%, respectively. Tseugeum et al., (2019) found that aqueous and methanolic extracts from *Paullinia pinnata* leaves can reduce TNF-α (35-68%) and IL-1β (31-36%) production in macrophages following LPS stimulation. The hydro-alcoholic extract (50, 200, and 500 mg/kg dose) of Bharangyadi preparation inhibited

carrageenan-induced inflammation by inhibiting the enzyme cyclooxygenase and, as a result, prostaglandin synthesis, which supports the plant's traditional use in bronchial asthma and other inflammatory conditions (Patel et al. 2014). This anti-inflammatory effect of *C. serratum* may be attributed to flavonoids and saponins, but other active compounds may also be involved, resulting in synergistic effects (Kajaria et al., 2012). IL-1β initiates persistent inflammation, which is essential for the host's response to infection. When activated by an inflammatory stimulus, they are released in mature form following inflammasome conversions via caspase-1 action. LPS treatment increased IL-1β mRNA expression by 0.94±0.012 times, which was considerably reduced with 50 µg/mL of UAE. Treatment with 100 µg/mL of UAE3 dramatically reduced IL-1β expression, similar to the positive control. UAE (100 µg/mL) and SFE (100 µg/mL) showed a relative fold change of 0.907±0.001% and 0.890±0.001%, respectively, compared to LPS treatments. When cells were treated with both extracts at 200 µg/mL doses, LPS-induced IL-1β mRNA expression remained unchanged. D'Eliseo et al. (2018) found that extracts from kiwi peel reduced the expression of IL-6, IL-1β, and TNF-α in both LPS-mediated and non-mediated THP-1 cells. Prakash et al. (2014) found that the monomer compound 3-hydroxy, 2-methoxy-sodium butanoate (HMSB, at doses of 25, 50, and 100 mg/kg, i.g.) isolated from *C. phlomidis* leaves had anti-inflammatory and anti-arthritic effects on carrageenan-induced inflammation and freund complete adjuvant (FCA)-induced arthritis in rat models. The findings revealed that HMSB may considerably lower paw edema, lysosomal enzymes, protein-bound carbohydrates, and acute phase protein levels. In addition, HMSB could considerably reduce pro-inflammatory cytokines TNF, IL-1, and IL-6 protein levels and mRNA expression in the joints in a dose-dependent manner (Wang et al., 2018). These findings suggested that HMSB had significant anti-inflammatory activity and a strong anti-arthritic effect.

Cycloxygenases (COX) are a group of enzymes that synthesize prostaglandins and play a crucial role in the progression of inflammation. COX2 expression causes tissue inflammation and is a primary target for the treatment of inflammatory disorders. LPS treatment increased COX2 mRNA expression by 1.105±0.013% fold as an inflammatory condition. Treatment with varying quantities (50 µg/mL, 100 µg/mL, and 200 µg/mL) in UAE and SFE samples significantly reduced COX2 mRNA expression compared to the standard. Treatments with UAE extract (200 µg/mL) and SFE showed a fold change of 0.326±0.002% and 1.114±0.013%, respectively, compared to LPS treatments

(1.114±0.013%). A screening study looked at the effects of several plant extracts on COX and LOX enzymes. Seven medicinal herbs were shown to inhibit both LOX and COX enzymes, indicating anti-inflammatory properties (Jacob, 2015).

5.3.5. Microstructure analysis of treated samples

Fig.5.10 (a-c). shows scanning electron micrographs of optimized samples freeze-dried using solvent, ultrasonic and supercritical fluid extraction. (a, b and c respectively). The solvent extracted sample (Control) had intact cell walls and tissues, whereas the cell wall and tissues of UAE-treated and SFE-treated samples displayed distorted surface morphology, as well as tissue and cell disintegration. The high cavitation bubble produced by ultrasonic waves aided in disruption and solvent penetration into the plant's matrix, leading in the release of phytochemicals (Xia et al., 2011). Another similar study investigates the UAE procedure for extracting thymol from *Plectranthus amboinicus* leaves. The SEM images demonstrate changes in the structural surface of the leaves following the UAE process, shedding light on the mechanism of UAE (Zahari et al., 2020). Similarly, SFE-treated sample demonstrates that supercritical CO2 extraction disrupted the cellular structure of the leaf sample. This disorganization is most likely the result of cellular rupture caused by the pressure and CO_2 flow used to extract volatiles, waxes, lipids, and other apolar substances. The ringed structures associated with cell walls appear to loosen, which could be due to the breakdown of hydrogen bonds and the extraction of a major portion of the cell wall's polar elements. Bittencourt (2019) observed similar results in a supercritical extract of *Psidium grandifolium* Mart. ex DC.

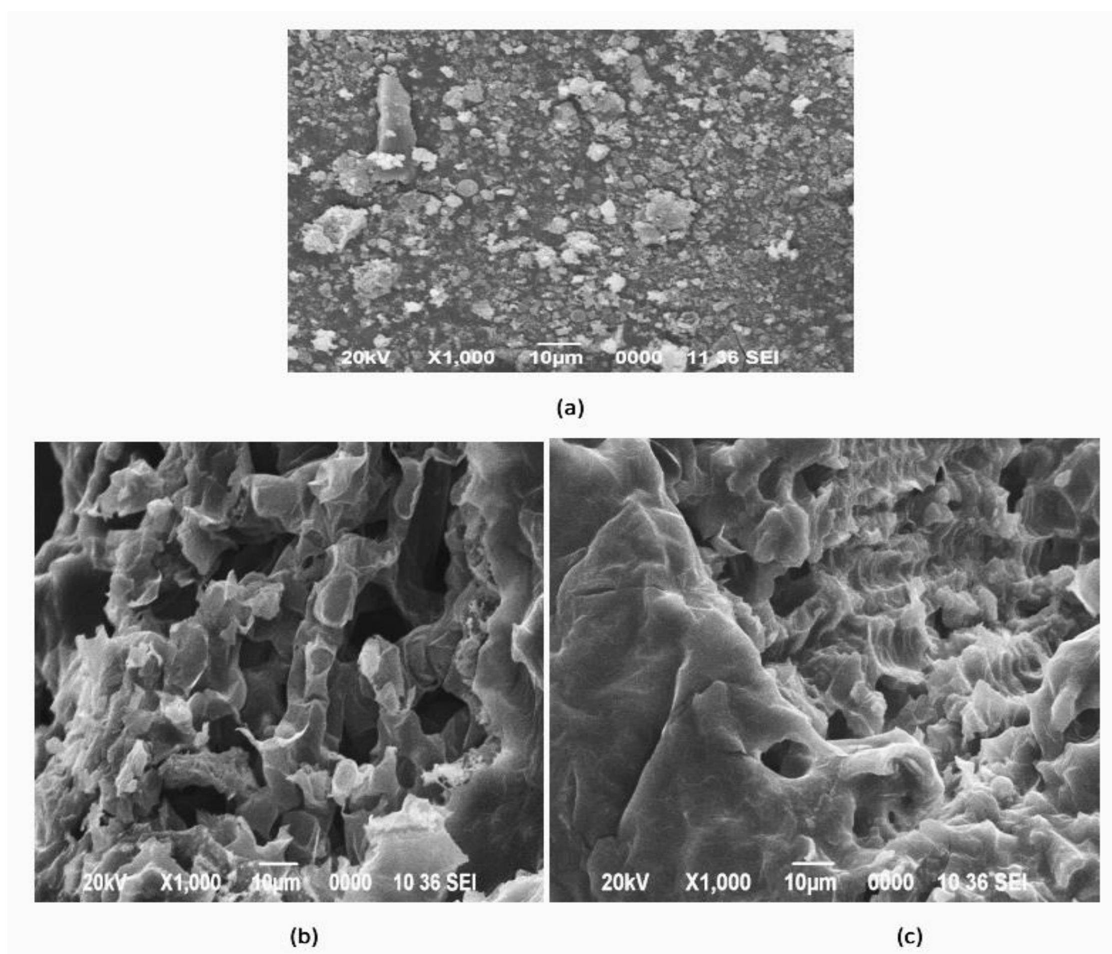


Fig. 5.10 (a-c): Scanning electron microscopic images of *Clerodendrum glandulosum* Lindl. leaves. a. Solvent extraction sample; b. Ultrasound assisted extraction sample; c. Supercritical fluid extraction sample.

5.4. Conclusions

RSM was successfully used to optimize the phytochemical components and antihypertensive action of *Clerodendrum glandulosum* Lindl, and CCD showed to be an effective method for optimizing these parameters. Second-order polynomial models for predicting responses were developed, and the optimal extraction duration, power, and solvent concentration in the UAE were found to be 19.27 min, 152.52 W, and 70% ethanol, respectively. For SFE, the optimal extraction time, temperature, and power were 40 min, 64.84℃, and 250 bar. FT-IR analysis of the secondary metabolites present in both the UAE and SFE extracts revealed the presence of O-H stretch in hydrogen-bonded hydroxyl groups, which are important in the study of plant extracts because they can indicate the presence of phytochemicals such as phenols. As a result, *Clerodendrum glandulosum*

Lindl. may be a natural source of polyphenol chemicals that can be employed as functional food ingredients or in medications to treat cardiovascular disorders.

This study shows the leaf extracts of *Clerodendrum glandulosum* Lindl. (using UAE and SFE) is an effective choice for treating a variety of inflammatory illnesses. When fed to THP-1 cells, the extracts were found to be non-toxic, and they showed potent anti-inflammaotory activity by inhibiting the production of inflammatory mediators such as IL-1β, TNF-α, and COX-2 in macrophages. As a result, it could be a promising option for further research into the creation of anti-inflammatory drugs or formulations as nutraceuticals or functional ingredients.