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Optimization of microwave-assisted extraction of bioactive polyphenolic compounds from *Marsilea quadrifolia* L. using RSM and ANFIS modelling

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Extraction of bioactive compounds, rich in plant secondary metabolites as a form of polyphenolic compounds has gained utmost important in the food and pharmaceutical industries due to their antioxidant properties. Microwave-assisted extraction (MAE) was utilized for maximum extraction of bioactive polyphenolic compounds from *Marsilea quadrifolia* L. with consuming less toxic solvent. A central composite rotatable design (CCRD) based on response surface methodology (RSM) and adaptive neuro-fuzzy inference system (ANFIS) were followed to design and optimize the experimental parameters to get highest yield of bioactive polyphenolic compounds from *M. quadrifolia* L. The quantitative effects of experimental parameters such as methanol concentration (X_1), microwave power (X_2), irradiation temperature (X_3) and irradiation time (X_4) were investigated to obtain the maximum yields of total phenolic (TPC), total flavonoid contents (TFC) and antioxidant properties. The optimum conditions were observed at methanol concentration (X_1= 87.5 %), microwave power (X_2= 25 %), irradiation temperature (X_3= 60 ºC) and irradiation time (X_4= 15 min). Under these conditions, the highest yields of TPC (y_1)= 693.28 mg gallic acid equivalents (GAE)/g), TFC (y_2)= 84.86 mg rutin equivalents (RU)/g), % DPPHsc (y_3)= 81.06 %, %ABTSsc (y_4)= 71.34% and FRAP (y_5)= 68.09 µg mol (Fe (II)/g) has been attained. Further, the experimental results were highly acknowledged with predicted values of RSM and ANFIS. The analysis of LC-ESI-MS spectrum confirmed 6 major bioactive compounds, namely, Betasitosterol, Tridecyliodide, 2,3,7,8 tetracholorodibenzofuaran, Chlorogenic acid, Pentachlorophenylacetate and Triacontyl hexacosanoate in the optimized extract of *M. quadrifolia* L. The optimized extract can be used as an alternative of synthetic antioxidants for product manufacturing in food and pharmaceutical industries.

Keywords: ANFIS, Antioxidant activity, CCRD, *Marsilea quadrifolia* L., Polyphenolic compound, RSM.

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Introduction

Bioactive polyphenolic compounds are known to be natural chemical molecules obtained from plants, microorganism or animals which can confer beneficial effects on human health. Foods with the presence of these compounds are called functional-food[1]. For centuries these bioactive polyphenolic compounds from biological sources play important role in improving the quality of life. Research on bioactive polyphenolic compounds extraction from biological sources has increased due to the toxicity of synthetic compounds as well as the cost of synthesis in food, pharmaceutical and biochemical industries[2]. It is well known that free radical generation by normal cellular metabolism causes senescence of the cells[3]. However, the human body naturally has a system to neutralize or detoxify the free radicals generating reactive oxygen species (ROS)/reactive nitrogen species (RNS)[4]. The misproportion of free radicals producing ROS/RNS draws attention to the fact that there will be a graded response to oxidative stress and lead to irreparable damage and cell death. Hence, many researchers are intensively searching for highly effective, non-toxic and powerful antioxidants for neutralizing or scavenging the free radicals generating

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ROS/RNS from plant sources is currently a major scientific interest[3,5].

The secondary metabolites consist of antioxidants in the form of bioactive compounds such as polyphenols, glycosides and steroids which has become the area of interest for the researchers as it attributes to several biological properties such as anti-inflammatory, anti-diabetic, antibiotic and anticancer activity[6]. Among them, polyphenolics are particularly attractive because of their significant role in human health by acting as a free radical scavenger, inhibition of ROS/RNS generation and reducing intracellular damage of lipids, proteins, and deoxyribonucleic acid[7]. *Marsilea quadrifolia* L. is an aquatic fern widely distributed in southern and eastern parts of India. Several research reports demonstrated that the extract of *M. quadrifolia* L. has hypoglycemic, anti-epileptic and antioxidant properties[8-10]. Further, it is consumed as foodstuff by many regions of peoples in India[11]. The investigation of our research work was to view it not only as a food for sustenance but also as medicine or more specifically as a functional food. Several types of research revealed that phytochemical profile of *M. quadrifolia* L. extract has an abundance of polyphenols, alkaloids, C-glycosides, sterols, and fatty acids[12]. The separation and purification of polyphenolic compounds from *M. quadrifolia* L. is essential to follow suitable extraction methods that ensure the highest extraction yield, less consumption of toxic solvent and shorter extraction time. Obviously, very low quantity of these bioactive polyphenolic compounds present in plant source, so it is certainly the most difficult technological operations required to maximize the yield of these compounds.

Indeed, a microwave assisted extraction has recently gained a special attention and was described as an environmentally safe technology, using microwave and irradiation temperature to rupture the plant cells to get maximum yield[13]. Advantages of this technique included consumption of less toxic solvents, at low temperature, and in shorter extraction time[14]. The secondary metabolites of plants are naturally present in the cell wall or the cytoplasm. The applied microwave power enable to heats the solvent and lowering the surface tension of extraction medium which allows penetrating solvent into plant matrix for solubilization of the polyphenolics into the solvent[15]. Further, microwave irradiation allows cell disruption by forced heat and facilitating mass transfer of the solvent to the plant material, which eventually leads to effective extraction[16]. Group of researchers have successfully applied microwave to extract polyphenols from Clinacanthus[17], black tea powder[18], *Vitis vinifera*[19], olive leaves[20], etc.

The present work aimed to investigate the impact of extraction parameters on to maximize the polyphenolic compounds yield from *M. quadrifolia* L. using microwave-assisted extraction. The yield of polyphenolic compounds and its antioxidant activities judged by various parameters, namely, solvent concentration, microwave power, irradiation temperature, irradiation time, etc., intrinsically seem to be irrelevant to each other. Therefore, there is a need to optimize the extraction process parameters with the utilization of statistical tool. An optimum condition refers to operating condition at which maximum response can be attained in terms of a process or a product. Statistical tool decides the range of variables and consequently lessen the number of experimental runs for obtaining the highest yield of polyphenolic compounds. One of the important statistical tools is response surface methodology (RSM), a collection of statistical and mathematical tool that can be used to develop a relationship between the yield and the process variables from appropriate analytical experiments and simultaneously solve complex processes[21]. Several input variables or independent variables potentially influence the performance of a process. RSM can build an empirical model between these independent factors and dependent factors, based on a polynomial equation as well as can develop a symmetrical model for prediction and determination of the experimental optimum conditions[22]. RSM offers design of experiment tools that lead to peak processes performance. Commonly used designs for RSM are central composite rotatable design (CCRD) and Box-Behnken design (BBD) to obtain an optimized extraction of polyphenolic compounds[23]. CCRD comprises of an imbedded factorial or fractional factorial design with center points. The center points are expanded with a group of 'star points'. The star points display new high value and low value for each factor. The star points lie at a distance. $|\alpha| > 1$ from the center of the design space whereas the factorial factor is ±1 unit away from the center point for each factor[24]. The estimation of α relies upon specific properties desirable for the design and on the number of variables involved. Moreover, CCRD give prediction over the whole design space, however,

require factor settings outside the scope of the components in the factorial part[25]. While the BBD is a free quadratic outline in that it doesn't contain an implanted factorial or partial factorial plan. Here the treatment combinations are at the midpoints of edges of the process space and at the center. Further, the BBD is rotatable (or close to rotatable) and require three levels of each factor[26]. The designs have restricted functionality for orthogonal blocking in contrast to the central composite designs. This means CCRD is significantly easier than BBD. In our previous study, the CCRD was successfully applied to optimize and analyze the experimental data and to extract maximum polyphenolic compounds from *Azolla microphylla* Kaulf[27].

Along with RSM, we have validated the optimized process parameters through ANFIS (Adaptive Neuro Fuzzy Inference System). ANFIS is a promising soft intelligent computing method used for studying and validating optimization of polyphenolic compounds extraction recently[28]. ANFIS has the capability of anticipating the obtained optimal responses utilizing the computational power of the neural network and high level human-like thinking fuzzy systems and producing a best outcome with the merits of both for nonlinear processes[29]. Our present study deals with the optimization of the extraction conditions, i.e., solvent concentration, irradiation temperature, microwave power, and irradiation time with respect to TPC, TFC and maximum antioxidant scavenging activities from *M. quadrifolia* L. using RSM along with ANFIS model.

Materials and Methods

Materials

The compounds 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2, 4, 6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu's phenol reagent (FCR), Rutin and Gallic acid were purchased from Sigma-Aldrich Chemicals Ltd., Bangalore, Karnataka, India. Rest of other chemical and reagents were used in this study were of analytical grade or of the highest quality commercially available. The double distilled water was used throughout the experiments. The fresh *M. quadrifolia* L. water fern was collected from Jadavpur vegetable market, Kolkata, India. *M. quadrifolia* L. cleaned with household water and allowed to dry in shadow for 72 hours and the entire plant was reduced to a fine dried powder (60 mesh) using a mixer grinder and kept at water/airtight desiccator until analysis. The MAE process was carried out using a CATA R Microwave Extractor in closed vials which was provided by Catalyst Systems (Pune, India) with variable magnetron input voltage of 2450 MHZ, normal energy of 850W and a reflex unit working at 2455 MHz with 10 control levels, time controller, exhaust framework, beam reflector and a mixing gadget. The MAE used was capable of withstanding working pressures up to 30 bar and may vary based on the physicochemical properties of the reaction mixture as well as their operating parameters. Logical instruments, UV- visible spectrophotometer (Varian, Cary 50), Rotary vacuum dryer (Varian Rotavac), and LC-ESI-MS (A Liquid chromatography-electrospray ionization-mass spectrometry, 6200 series TOF, Q-TOF B.06.01 version, Agilent Technologies, Santa Clara, CA 95051, USA) comprising automatic liquid chromatographic sampler and an auto injection system were utilized.

Solvent selection for MAE

Selection of an appropriate solvent system is a major objective of the experiment as that could recognize the most noteworthy substance of TPC, TFC along with confirming maximum DPPH* radicals scavenging activity of *M. quadrifolia* L. extract. We employed various solvents like benzene, chloroform, methanol, diethyl ether, acetone, ethyl acetate and n-hexane in this experiment. The reaction mixture for every solvent system comprises of 2 g of finely ground *M. quadrifolia* L. sample added with 20 mL of constant solvent concentration (70 % v/v in water) at microwave power (20 %), irradiation temperature (50 ºC) and irradiation time (10 min).

Selection of process variables

In this study, RSM was introduced to optimize the process variables of MAE extraction to maximize the yield of polyphenolic compounds from *M. quadrifolia* L. Many parameters are effectively involved to maximize the yield of polyphenolic compounds. In our experiment, four process variables i.e., solvent concentration (%), microwave power (%), irradiation temperature (ºC) and irradiation time (min) had major effect on MAE extraction process. The selected process variables were investigated with respect to dependent variables, such as, TPC, TFC, DPPHsc, ABTSsc and FRAP in the extraction of *M. quadrifolia* L. On the basis of the preliminary experimental result (chloroform: 648 mg gallic acid equivalents (GAE)/g

(TPC), 58.67 mg rutin equivalents (RU)/g (TFC) and 62.06 (% DPPHsc); benzene: 595 mg gallic acid equivalents (GAE)/g (TPC), 58.98 mg rutin equivalents (RU)/g (TFC) and 61.56 (% DPPHsc); Diethyl ether: 652 mg gallic acid equivalents (GAE)/g (TPC), 61.14 mg rutin equivalents (RU)/g (TFC) and 63.36 (% DPPHsc); methanol: 688 mg gallic acid equivalents (GAE)/g (TPC), 65.36 mg rutin equivalents (RU)/g (TFC) and 71.88 (% DPPHsc); acetone: 681 mg gallic acid equivalents (GAE)/g (TPC), 63.24 mg rutin equivalents (RU)/g (TFC) and 69.62 (% DPPHsc); ethyl acetate: 596 mg gallic acid equivalents (GAE)/g (TPC), 61.18 mg rutin equivalents (RU)/g (TFC) and 59.36 (% DPPHsc); and n-hexane: 592 mg gallic acid equivalents (GAE)/g (TPC), 60.05 mg rutin equivalents (RU)/g (TFC) and 68.69 (% DPPHsc)) aqueous methanol has been used as an extraction solvent. The selected independent variables were investigated at five levels, coded as -2, -1, 0, +1 and +2 (as shown in Table 1) and the application of CCRD design was used to explore the optimal combination of extraction variables of *M. quadrifolia* L. sample. The ranges of independent variables, such as 60-80 % of methanol concentration, 15-25 % microwave power, 40-60 ºC of irradiation temperature and 10-15 min irradiation time were selected as per the preliminary experimental result for the highest content of TPC, TFC and DPPHsc.

MAE of polyphenolic compounds and antioxidants

The extraction of polyphenolic compounds and antioxidants from *M. quadrifolia* L. by MAE process with 2 g of accurately weighed fine powder of *M. quadrifolia* L. along with 20 mL of pre-selected solvent (aqueous methanol). It was placed in an extraction vessel and eventually, it was kept inside the microwave cavity. Sets of experiments were performed followed by varying concentrations of methanol (60-85 %), microwave power (15-25 %), irradiation temperature (40-60 ºC) and irradiation time (10-15 min) according to CCRD of RSM. The extracts were filtered through Whatman No.1 filter paper and concentrated using rotary vacuum dryer at 40 ºC to obtain TPC, TFC and antioxidant activities.

Table 1 — Experimental range of coded and actual values for central composite rotatable design (CCRD)

		Factor levels				
Independent variables (x_j)	Symbols	-2	-1	0	+1	+2
Methanol concentration (%)	X_1	50	60	70	80	90
Microwave cower (%)	X_2	10	15	20	25	30
Irradiation cemperature(˚C)	X_3	30	40	50	60	70
Irradiation time (min)	X_4	7.5	10	12.5	15	17.5

Determination of total polyphenolic content (TPC)

TPC of the *M. quadrifolia* L. extract was determined spectrophotometrically (Varian Cary 50 UV-Spectrophotometry) using Folin-Ciocalteu's phenol reagent (FCR) which was described by Singleton and Rossi with minor modifications[30]. Shortly, ~0.3 mL extracts were added with 1.8 mL of Folin-Ciocalteu's reagent and the mixture allowed to remain at room temperature for 5 minutes, then 1.2 mL sodium carbonate (7.5 %, w/v) solution was added to the mixture. The blank sample was prepared by replacing 0.3 mL of extract with 0.3 mL of distilled water. After standing for 60 minutes at room temperature, absorbance was measured at 765 nm using a spectrophotometer. The results were expressed as mg gallic acid equivalents (GAE)/g sample with gallic acid as a standard reference.

Determination of total flavonoid content (TFC)

The total flavonoid content of the *M. quadrifolia* L. extract was assessed using the colourimetric assay developed by Siddhuraju and Becker with slight modifications[31]. Briefly, aliquots of diluted extract (1 mL) were added to test tubes and mixed with 0.3 mL of 5 % (w/v) aluminium chloride. A small quantity of the extract, sodium nitrite solution and 4 mL of 80 % (v/v) methanol were added to prepare a mixture and was kept for 5 minutes. Subsequently 0.3 mL of 10 % (w/v) Al_2Cl_3 solution was added and mixed in an elapse of 6 minutes, 3 mL of 1 M sodium hydroxide solution was added. After making up the volume of reaction mixture to 10 mL using distilled water, the resultant mixture was vortexed and absorbance was recorded at 510 nm. The standard curve was prepared with rutin as a reference. The concentration of TFC was determined in terms of mg rutin equivalents (RU)/g samples.

Determination of antioxidant power

% DPPH Scavenging assay

The radical scavenging activity of the extracts in relation to the 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical was measured using the method of Brand-Williams *et al*. with slight modifications[32]. Exactly 0.1 mL of sample extract was added to 3 mL of an ethanolic solution of DPPH (0.1 μM) and the mixture was shaken vigorously. Then it was kept in the dark for 30 minutes, and the absorbance was measured at

517 nm against a blank. The free radical scavenging capacity was calculated in percentage of the sample (% $DPPH_{SC}$) with the following formula,

$$\% \ DPPH_{SC} = (A_0 - A_1) \times 100/A_0 \quad \ldots(1)$$

where A_0= Absorbance of the control; A_1 = Absorbance of the sample.

% ABTS Scavenging assay

Following the method of Re *et al*. with some modifications ABTS* radical scavenging activity assay was carried out[33]. The assay was based on incubation reaction which was carried out between 7 mM ABTS (2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) solution and 2.45 mM potassium persulphate solution in the dark maintained at room temperature for 16 hours to generate ABTS*. Before spectrophotometer examination, ABTS solution was diluted with 0.3 mL ethanol and mixed with 0.1 mL of the extracts was thoroughly mixed vigorously and the absorbance at 734 nm was adjusted to 0.700 (±0.0020). The absorbance of the reaction mixture incubated for 6 minutes was examined at 734 nm using UV-Vis spectrophotometer. The % ABTS scavenging activity was calculated using the standard curve using rutin in 80 % ethanol,

$$\% \ ABTS_{SC} = (A_0 - A_1) \times 100/A_0 \quad \ldots(2)$$

Where A_0= Absorbance of the control; A_1 = Absorbance of the sample.

Antioxidant activity by the iron reduction method (Ferric reducing antioxidant potential)

Antioxidant activity by the iron reduction assay of the extract was carried out according to Benzie and Strain[34] and a method modified by Pulido *et al*.[35] FRAP reagent was prepared using 300 mM acetate buffer (3.1 g sodium acetate, and 16 mL acetic acid) at pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM hydrochloric acid solution, and 20 mM $FeCl_3.6\ H_2O$ solution in distilled water. The acetate buffer (25 mL) and TPTZ (2.5 mL) were then mixed together with $FeCl_3.6\ H_2O$ (2.5 mL). The mixture FRAP solution was allowed to react with the plant extract (40 µL) in the darkroom, maintained at 37 ^oC for 30 min and subsequently absorbance recorded at 593 nm. The standard curve was linear through 200 and 1000 µM $FeSO_4$. Results calculated in µM Fe (II)/g dry mass was compared with ascorbic acid as a standard.

Experimental design and optimization

The CCRD of RSM was selected to optimize the process variables of MAE of maximum yield of bioactive polyphenolic compounds. The experimental design is comprised of 30 experimental runs with 16 and 8 factorial and axial points (α) respectively at a distance of ±2 from the center and 6 replicates of central points are shown in Table 1.

The number of experiments was calculated from equation (3)

$$N = 2^k \text{ (factorial points)} + 2k \text{ (axial points)} + n_0 \text{ (central points)} \quad \ldots(3)$$

where N is the total number of experiments, k is the independent variable number, and n_0 is replicate number of the central points, which resulted in an experimental design of 30 points. A second order polynomial regression model was used to correlate the relationship between independent variables and dependent variable responses (TPC, TFC, %DPPHsc, %ABTSsc and FRAP). The second order polynomial regression model was as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \varepsilon \quad \ldots(4)$$

Based on the value of four variables, the equation (4) could be converted as given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 \quad \ldots(5)$$

Where Y is the dependent variable responses (TPC (y_1), TFC (y_2), $DPPH^*$(y_3), $ABTS^*$ (y_4) and FRAP(y_5), β_0 is the model constant, β_i, β_{ii}, and β_{ij} are model coefficients, X_i and X_j are coded value of independent variables, and ε is an error. Subsequently, additional experiments were carried out to statistically verify the error in the process variables.

Optimization using adaptive neuro-fuzzy inference system (ANFIS) modelling

ANFIS is a framework of neuro-fuzzy model demonstrated to be competent and used in a variety of applications that range from medical to mechanical engineering, to business and economics[36]. It enables to express the relationship between the input and the output variables. For the modelling by ANFIS, we have used the same set of experimental data used for RSM for estimating the predicted individual response output of TPC, TFC and antioxidant activities. The multi-input (methanol concentration, microwave power, irradiation temperature and irradiation time)

single-output (TPC/TFC/DPPH/ABTS/FRAP) system of the first order Takagi-Sugeno-Kang (TSK) model has been used for this study. The architecture of ANFIS depicted in Fig.1 is basically multi-input and single-output of the neural network system. The ANFIS rule for effective extraction of polyphenolics determines the number of rules and antecedent membership functions and followed by linear least square estimation to determine each rule's consequent equations. If we consider a Sugeno Fuzzy Inference System (FIS), which has two inputs namely 'x' and 'y' and one output as 'z'. A first order Sugeno FIS has the rules as follows:

Rule1: If *x* is A_1 and y is B_1, then $f_1 = p_1x + q_1y + r_1$

Rule2: If x is A_2 and y is B_2, then $f_2 = p_2x + q_2y + r_2$

The number of membership function in each input variable is selected by trial and error method. The experimental data have been separated into training, testing and validation of the network model with the help of Fuzzy logic tool box in MATLAB v R2013a for predicting the response for extraction of polyphenolic compounds from *M. quadrifolia* L.

LC-ESI-MS analysis

The bioactive polyphenolic compounds present in the optimized extract was separated and identified by liquid chromatography-electron spray ionization-mass spectrum (LC-ESI-MS) according to previously described method[37]. For separations using, Zorbax-SB-C18 column (2.1× 50 mm, 1.8-µM particle size; Agilent Technologies, USA) maintained at temperature 25 °C and the optimally obtained extract was filtered through a membrane filter (Millipore, USA) and injected (3 µL). MS detection was performed in a 6200 series Q-TOF B.06.01 version mass spectrophotometer equipped with an electrospray ionization source (ESI) and obtained peak was detected. The MS was performed with negative mode polarity of ESI. Using online libraries, polyphenolic compounds identification was done to obtain molecular mass and structural formula of a compound.

Statistical data analysis

All experiments were conducted in triplicate and the mean was reported. Design expert software (version 8.0.7.1, Stat-Ease, Inc., 2021 East Hennepin Ave, Suite 480, Minneapolis, MN 55413, USA) was used for the experimental design, data analysis and quadratic model building. Further, the strength of analysis was assessed by one way analysis of variance (ANOVA) and statistically significant levels accepted at p <0.05, p <0.01 and p <0.001. Three dimensional (3D) response surfaces and two dimensional (2D) contour plots were obtained through the application of optimal extraction conditions.

Result and Discussion

Fitting the model

The experimental data based on RSM (Table 1) was used to calculate the coefficients of the second order polynomial by the least squares technique using Design Expert software. Each experiment was

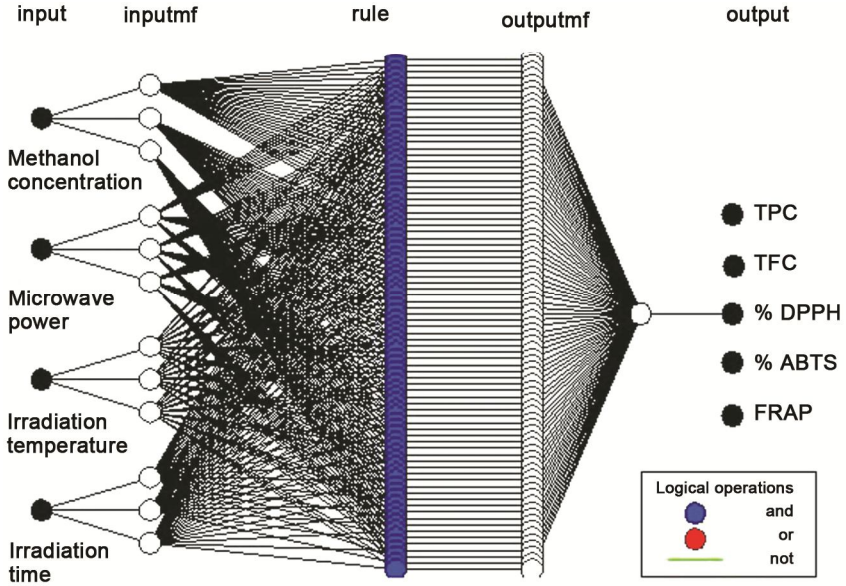

Fig.1 — The architecture of the ANFIS input and output model

assessed as a function of linear, quadratic and interactive terms of the independent variables including methanol concentration, microwave power, irradiation temperature and irradiation time. The experimental and predicted values of TPC, TFC and antioxidant activities, namely, % DPPHsc, % ABTSsc, FRAP in *M. quadrifolia* L. extracts obtained from all the experiments are illustrated in Table 2. According to the experimental design, we have obtained the maximum yield of TPC and TFC from *M. quadrifolia* L. extract at methanol 87.5 %, microwave power 25 %, irradiation temperature 60 ^{o}C and irradiation time 15 min. Under this circumstance, the optimal yields of TPC and TFC are 689.76 mg GAE/g and 83.84 mg RU/g of extract, and their antioxidant activities are 80.45 % DPPHsc, 71.25 % ABTSsc and FRAP value of 68.06 µg mol/g (Fe (II)).

The regression coefficient of second order polynomial is summarized in Table 3 by fitting the experimental results in the quadratic model. Analysis of variance (ANOVA) statistical tool was conducted to determine the significance of the coefficients. This was done by performing F-test which showed the significance of each coefficient. The significance of each coefficient is determined such that a greater value of F and smaller p value indicate that the corresponding response variable is more significant[38]. Further p >0.05 indicates the coefficient becomes statistically significant. Therefore, the F value (F= 7.45) and p value (p= 0.0001) obtained, implies that the model was highly significant for all the responses.

Table 2 — Central Composite Rotatable Design (CCRD) with experimental responses and predicted responses

S. No	Independent variables				Experimental value[a]					Predicted value				
	X_1	X_2	X_3	X_4	y_1	y_2	y_3	y_4	y_5	y_1	y_2	y_3	y_4	y_5
1	80	25	40	10	657.53	68.64	66.28	55.78	54.07	660.95	69.97	67.57	57.7	54.98
2	70	30	50	12.5	656.65	67.86	65.08	53.18	51.98	658.11	66.7	63.45	54.76	54.17
3	70	20	70	12.5	654.87	65.45	64.59	53.84	51.19	661.48	71.16	69.04	59.6	56.25
4	70	20	50	12.5	654.87	64.8	63.78	54.25	52.28	655.44	64.58	64.41	54	51.75
5	60	15	40	10	644.25	50.12	48.35	44.19	41.68	642.58	49.69	49.15	45.21	43.12
6	60	25	40	15	649.01	50.33	49.73	46.87	45.09	653.64	54.41	52.81	48.1	47.31
7	60	25	40	10	647.56	50.45	49.68	45.76	43.65	640.99	49.66	48.46	42.43	41.12
8	70	20	50	12.5	655.82	64.76	64.88	53.72	51.54	655.44	64.58	64.41	54	51.75
9	80	15	40	15	681.08	76.34	71.89	65.18	60.27	683.39	78.13	74.16	66.5	62.72
10	90	20	50	12.5	685.26	79.43	75.62	68.26	63.12	694.41	85.3	80.08	72.12	68.25
11	80	25	40	15	683.35	78.79	74.87	65.35	64.75	676.82	76.6	73.51	62.84	61.05
12	60	25	60	10	652.8	63.16	60.28	53.25	51.05	650.21	60.22	57.18	50.81	47.32
13	70	20	30	12.5	653.06	64.76	63.18	52.68	50.87	655.63	63.46	62.98	53.75	51.76
14	60	25	60	15	652.34	61.78	58.23	55.34	50.57	655.35	64.03	61.27	55.29	51.58
15	50	20	50	12.5	640.18	48.75	45.56	42.08	40.46	640.22	47.29	45.35	45.05	41.27
16	80	15	60	15	682.38	77.75	73.74	66.38	63.17	680.03	75.26	71.51	63.97	61
17	60	15	60	15	651.5	60.04	58.02	55.21	50.08	643.8	57.56	55.9	52.17	47.9
18	70	20	50	17.5	664.46	70.05	67.56	56.95	54.27	665.47	67.76	66.03	58.8	55.24
19	80	15	60	10	678.53	74.34	68.68	61.46	60.98	673.62	69.1	64.76	59.11	57.49
20	80	15	40	10	681.42	76.55	73.65	66.15	62.98	670.48	71.02	67.16	60.46	57.27
21	60	15	60	10	645.01	54.38	52.83	50.02	45.25	643.61	53.28	50.74	46.79	44.25
22	70	20	50	7.5	640.25	50.15	49.15	43.29	40.56	650.42	58.85	54.93	48.27	45.53
23	80	25	60	15	687.26	81.43	79.62	69.26	67.12	687.64	80.71	77.99	67.12	64.41
24	80	25	60	10	683.18	78.18	73.45	65.91	62.12	672.27	75.02	72.3	63.16	60.28
25	60	15	40	15	645.28	55.05	56.85	54.78	51.56	650.26	54.92	54.56	51.79	48.7
26	70	10	50	12.5	649.37	55.72	51.78	49.15	49.02	655.09	61.29	57.66	54.39	52.77
27	70	20	50	12.5	656.76	63.25	65.56	54.5	51.2	655.44	64.58	64.41	54	51.75
28	70	20	50	12.5	654.87	64.8	63.78	54.25	52.28	655.44	64.58	64.41	54	51.75
29	70	20	50	12.5	655.56	64.72	65.23	54.05	52.15	655.44	64.58	64.41	54	51.75
30	70	20	50	12.5	654.8	65.16	63.28	53.25	51.05	655.44	64.58	64.41	54	51.75

[a]All the experiments were repeated three times

Table 3 — Analysis of variance (ANOVA) for the quadratic polynomial mode

Source	Sum of squares	df[*]	Mean square	F Value[**]	p-value[***]
TPC[#]					
Model	5677.75	14	405.55	7.45	< 0.0001
X_1	4735.974	1	4735.974	87.05438	1.234E-07
X_4	340.0548	1	340.0548	6.250722	0.0245
X_1^2	283.8754	1	283.875	5.21806	0.0373
Residual	816.0372	15	54.40248		
Lack of Fit	813.0769	10	81.30769	137.3286	1.840E-05
Pure Error	2.960333	5	0.592067		
Cor Total	6493.785	29			
TFC[‡]					
Model	2594.75	14	185.3393	9.983877	3.35E-05
X_1	2167.33	1	2167.33	116.75	1.79E-08
X_3	88.81954	1	88.81954	4.78454	0.045
X_4	178.7058	1	178.7058	9.626545	0.007
Residual	278.4579	15	18.56386		
Lack of fit	276.2038	10	27.62038	61.26744	0.000135
Pure Error	2.254083	5	0.450817		
Cor Total	2873.208	29			
% DPPHsc[¥]					
Model	2229.095	14	159.2211	10.08651	3.14E-05
X_1	1808.391	1	1808.391	114.5599	2.03E-08
X_2	50.2572	1	50.2572	3.183746	0.09461
X_3	55.1157	1	55.1157	3.491528	0.08134
X_4	184.6485	1	184.6485	11.69731	0.003798
X_2X_3	50.73001	1	50.73001	3.213698	0.093211
Residual	236.7833	15	15.78556		
Lack of Fit	232.4973	10	23.24973	27.12234	0.000982
Pure Error	4.286083	5	0.857217		
Cor Total	2465.879	29			
%ABTSsc[†]					
Model	1420.752	14	101.4823	6.532779	0.000428
X_1	1099.042	1	1099.042	70.74929	4.62E-07
X_3	51.3045	1	51.3045	3.302656	0.089197
X_4	166.2687	1	166.2687	10.70332	0.00515
X_2X_3	BC	46.34206	1	46.34206	2.983205
Residual	233.0148	15	15.53432		
Lack of Fit	231.9964	10	23.19964	113.9099	2.93E-05
Pure Error	1.018333	5	0.203667		
Cor Total	1653.766	29			
FRAP[§]					
Model	1328.508	14	94.8934	6.396383	0.000483
X_1	1091.476	1	1091.476	73.57201	3.61E-07
X_4	141.3776	1	141.3776	9.529696	0.007514
Residual	222.5322	15	14.83548		
Lack of Fit	220.9738	10	22.09738	70.89764	9.44E-05
Pure Error	1.5584	5	0.31168		
Cor Total	1551.04	29			

[*]Degrees of freedom.
[**]Test for comparing model variance with residual (error) variance.
[***]Probability of seeing the observed F-value if the null hypothesis is true.
[#]Std Dev: 7.375; Mean: 659.97
[‡]Std Dev: 4.308; Mean: 64.89
[¥]Std Dev: 3.973; Mean: 62.83
[†]Std Dev: 3.941; Mean: 55.47
[§]Std Dev: 3.851; Mean: 52.87

The estimated multiple regression coefficients (R^2) and lack-of-fit determine the fitness and adequacy of the model. The experimental results of the CCRD showed the calculated coefficients of regression (R^2) of the models were 95 % for TPC, TFC and antioxidant activities, suggesting that 95 % of the actual levels can be matched with the model-predicted levels.

Analysis of the model

TPC

In Table 2 and equation (6) showed significant ($p << 0.05$) result indicated that the linear terms are significant. It was observed that the correlation coefficient (R^2) of microwave-assisted extraction of TPC value in predicting model was 0.874336 with p-value of lack of fit was 0.000018. The "Lack of Fit F-value" of 137.33 implies the Lack of Fit is significant. There is only a 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise. The observed values are applied to establish the model fitting. The second–order polynomial equation was established for the fitted quadratic model for TPC in coded variables is given in below eqn.

$$y_1=655.44+14.04X_1+0.755X_2+1.9641X_3 +3.7641X_4-1.735X_1X_2+ 0.7763X_1X_3 +1.5562X_1X_4+ 2.0462X_2X_3 + 1.2412X_2X_4-0.875X_3X_4 +3.217X_1^2+0.7895X_2^2+1.0283X_3^2+0.6258X_4^2 \ldots(6)$$

Fig. 2a shows that the normal percentage probability plot for studentized residuals of X_1, X_2, X_3

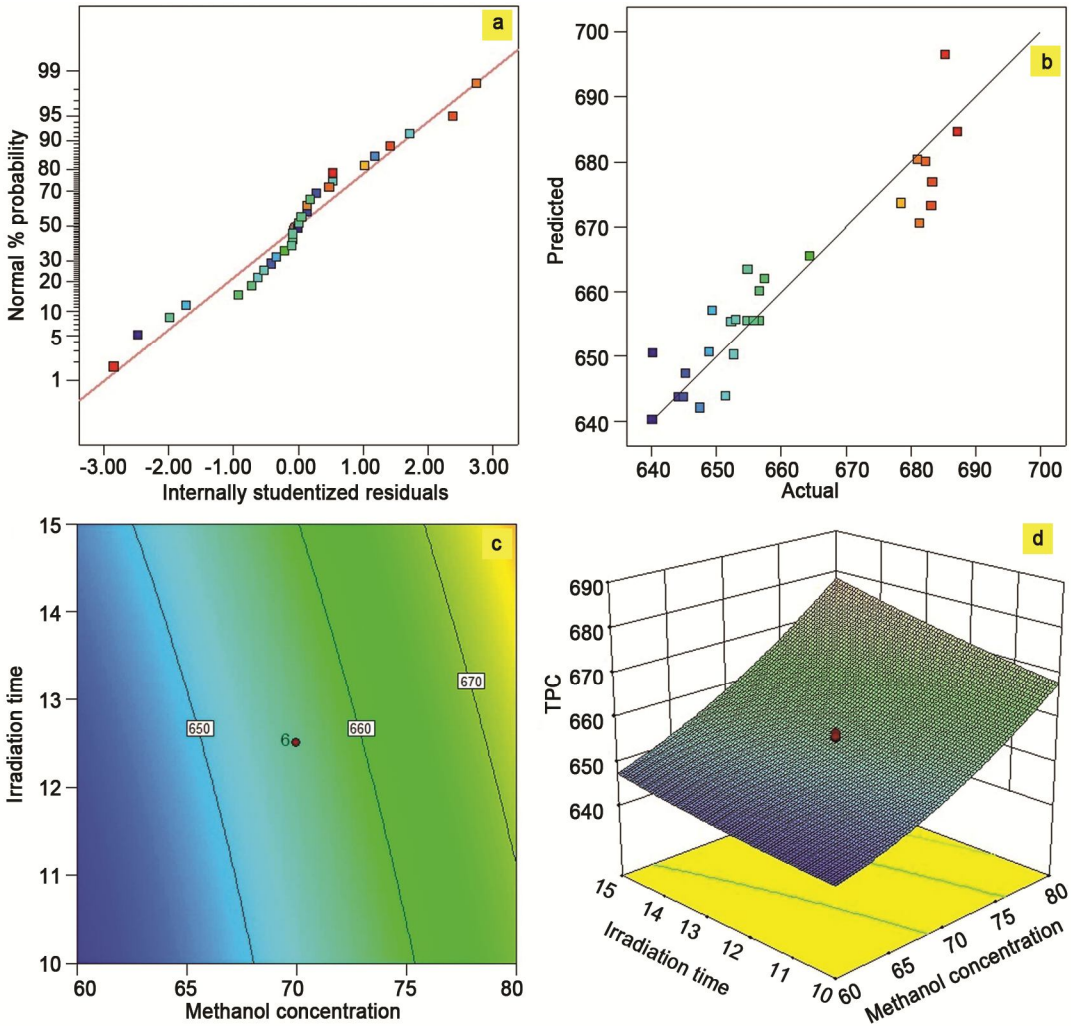

Fig.2 — Normal percentage probability plot for the studentized residuals for highest yield of TPC (a), Relationship between experimental and predicted value for highest yield of TPC (b), Response surface and contour plot showing the combined effects of methanol concentration (X_1) and irradiation time (X_4) for highest yield of TPC when microwave power and irradiation temperature were held at fixed level (zero level=20%, 50 ºC, respectively) (c) and (d).

and X_4 and these variants are normally distributed and have no deviation. Further, the predicted data against experimental data exhibited a higher R^2 value (0.8458) compared to RSM's adj R^2 value (0.757049). Fig. 2b and the high values of regression coefficient (R^2 >> 0.8) considered as a good fit. Fig. 2c and d show 3D response surface and 2D contour plot for representing the interaction among the different process variables. It reveals that there is a significant effect of methanol concentration and irradiation time in maximizing the yield of TPC with microwave power and irradiation temperature when held at a fixed level (zero level= 20 %, 50 °C respectively). The yield of TPC value in *M. quadrifolia* L. extract of various experiments was represented in Table 2 varied from 640.18 to 687.26 mg GAE/g. Lowest content of TPC yield was obtained at methanol 50 %, microwave power 20 %, irradiation temperature 50 °C and irradiation time 12.5 minutes while highest content was obtainable at methanol 80 %, microwave power 25 %, irradiation temperature 60 °C and irradiation time 15 minutes. Methanol concentration and irradiation time played a major role in obtaining the highest yield of TPC, increasing methanol concentration and decreasing irradiation time correlated to a higher content of TPC. Mild heating might soften the plant tissue, weaken the cell wall integrity and enhance the phytochemical solubility, allowing for more compounds to distribute to the solvent. However, prolonged microwave treatment at a higher temperature may induce phytochemicals degradation.

TFC

The second order polynomial equation (7) and ANOVA Table 3 shows the significant (p <<0.05) contribution of liner term X_1, X_4, interaction term X_1X_4 and quadratic term X_1^2, X_2^2, X_3^2, X_4^2 for maximum content of TFC in the *M. quadrifolia* L. extract. Also, the response surface analysis of TFC content in the extract indicated high regression coefficient value R^2= 0.903085 and p-value for lack of fit was 0.000135. The high values of the regression coefficient (R^2 >>0.8) indicate a good fit of the experimental model (Fig. 3b). The second–order polynomial equation for the fitted quadratic model for TFC as a function of the independent variable in coded variables are given in equation (7).

$$y_2 = 64.58167 + 9.502917X_1 + 1.352917X_2 + 1.92375X_3 + 2.72875X_4 - 0.25438X_1X_2 - 1.37688X_1X_3 + 0.469375X_1X_4 + 1.743125X_2X_3 - 0.118135X_2X_4 - 0.23813X_3X_4 + 0.429063X_1^2 - 0.14594X_2^2 + 0.682813X_3^2 - 0.56844X_4^2 \quad \ldots(7)$$

Fig. 3a shows that the normal percentage probability plot of studentized residuals of X_1, X_2, X_3 and X_4 and these variants are normally distributed and have no deviation. In addition, 3D response surface and the contour plot shown in Fig. 3c and d illustrate the significant effects of methanol concentration and irradiation time on the maximum recovery of TFC in the extract when microwave power and irradiation temperature were held at a fixed level (zero level= 20 %, 50 °C, respectively). Table 2 varied from 48.75 to 81.43 mg RU/g, the lowest content of TFC yield was obtained at methanol 50 %, microwave power 20 %, irradiation temperature 50 °C and irradiation time 12.5 minutes while highest content was obtainable at methanol 80 %, microwave power 25 %, irradiation temperature 60 °C and irradiation time 15 min.

Antioxidant activities

From the RSM analysis values in Table 2 and model equations (8) - (10) shows the linear term of methanol concentration (X_1), irradiation time (X_4), the interaction term of X_1X_2 and followed by the quadratic term X_1^2, X_2^2, X_3^2 has significant effects on all three antioxidant activities. The model equations have been established under different extraction conditions in Table 3 indicated that there is a significant effect of the linear term X_3, interaction term X_1X_4, X_3X_4 and quadratic term X_4^2 on % DPPH activity. The longer extraction time might have enhanced the polyphenolic extraction which has eventually resulted in increase in antioxidant activity. Similarly, quadratic term X_4^2 and interaction term X_2X_3 has significant (p <0.05) effect on FRAP. The correlation coefficient (R^2) value of the models in % DPPHsc, % ABTSsc and FRAP are 0.903976, 0.859101, 0.856527 respectively; with p-value of lack of fit were 0.000981864, 0.000029 and 0.000094 respectively. All the observed value has indicated that the model is a considerably a good fit for the experiment. The second–order polynomial equation for the fitted quadratic models for % DPPH, % ABTS, FRAP, represented in coded variables are given in eqn. (8) - (10).

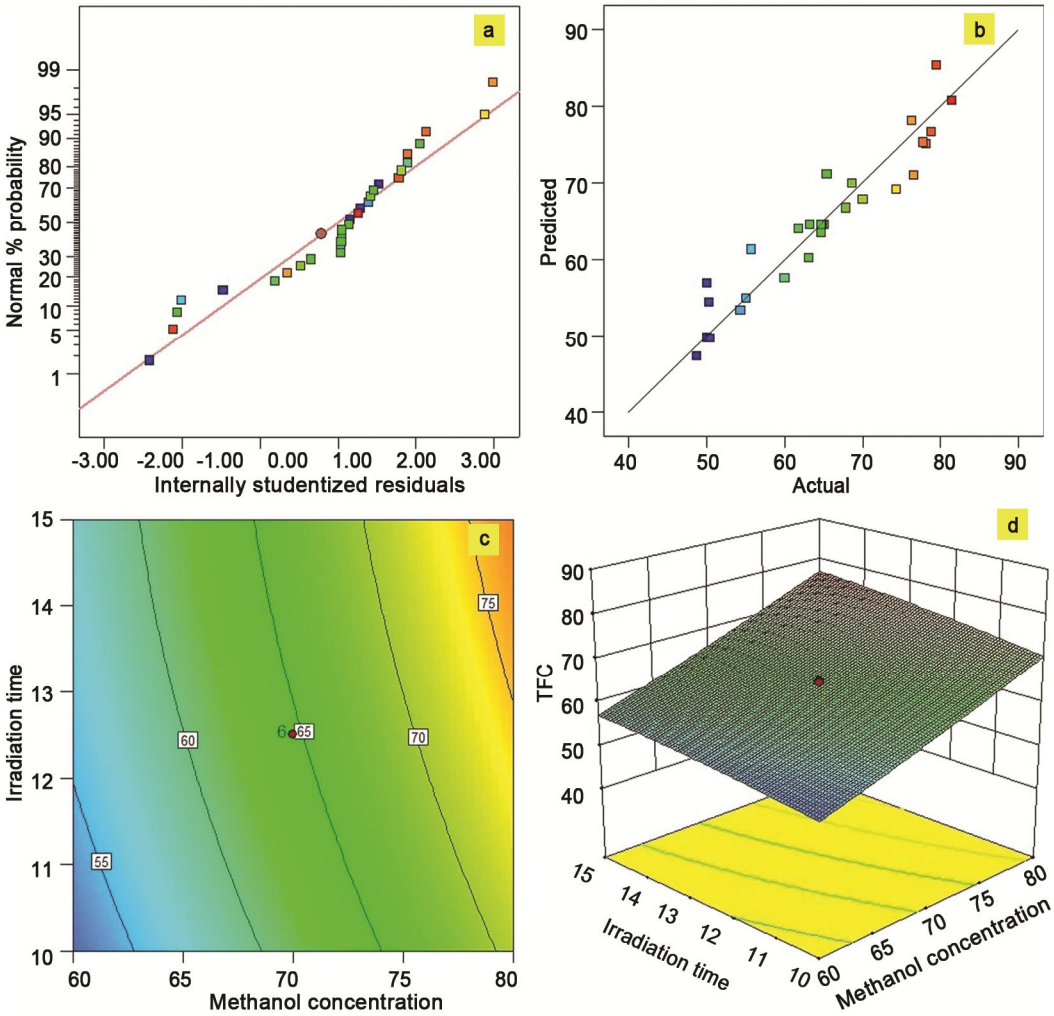

Fig.3 — Normal percentage probability plot for the studentized residuals for highest concentration of TFC (a), Relationship between experimental and predicted value for highest concentration of TFC (b), Response surface and contour plot showing the combined effects of methanol concentration (X_1) and irradiation time (X_4) for highest yield of TFC when microwave power and irradiation temperature were held at fixed level (zero level=20%, 50 ºC, respectively) (c) and (d).

$$y_3=64.41833+8.680417X_1+1.447083X_2+1.515417X_3+2.77375X_4+0.274375X_1X_2-0.99688X_1X_3+0.398125X_1X_4+1.780625X_2X_3-0.26438X_2X_4-0.06312X_3X_4-0.42469X_1^2-0.96469X_2^2+0.399063X_3^2-0.98344X_4^2 \quad \ldots(8)$$

$$y_4= 54.00333+6.767083X_1+0.092083X_2+1.462083X_3+2.632083X_4+0.006875X_1X_2-0.72938X_1X_30.13187X_1X_4+1.701875X_2X_3-0.22562X_2X_4-0.29687X_3X_4+ 1.146146X_1^2+ 0.144896X_2^2+0.668646X_3^2-0.11635X_4^2 \quad \ldots(9)$$

$$y_5 = 51.75+6.74375X_1+0.34875X_2+1.122083X_3+2.427083X_4-0.07062X_1X_2-0.22813X_1X_3-0.03187X_1X_4+1.269375X_2X_3+0.153125X_2X_4-0.48438X_3X_4+0.754271X_1^2+0.431771X_2^2+0.564271X_3^2-0.33948X_4^2 \quad \ldots(10)$$

Fig. 4a, 5a and 6a show that the normal percentage probability plot of studentized residuals of X_1, X_2, X_3 and X_4 and these variants are normally distributed and have no deviation of antioxidant activities. Further, the predicted data against experimental data for all three antioxidant activities gave a higher R^2 values (0.903976, 0.859101 and 0.856527) compared to RSM's adj R^2 value (0.814354, 0.727594 and 0.722619) and the high values of the regression coefficient ($R^2 >> 0.8$) is a good fit (Fig. 4b, 5b and 6b). The 3D response surfaces and 2D contour plot for antioxidant activities (% DPPH, % ABTS and FRAP) as a response functional variable of methanol concentration and irradiation time are shown in Fig. 4c and d, 5c and d, and 6c and d respectively. The Figures show that methanol concentration of (85 %),

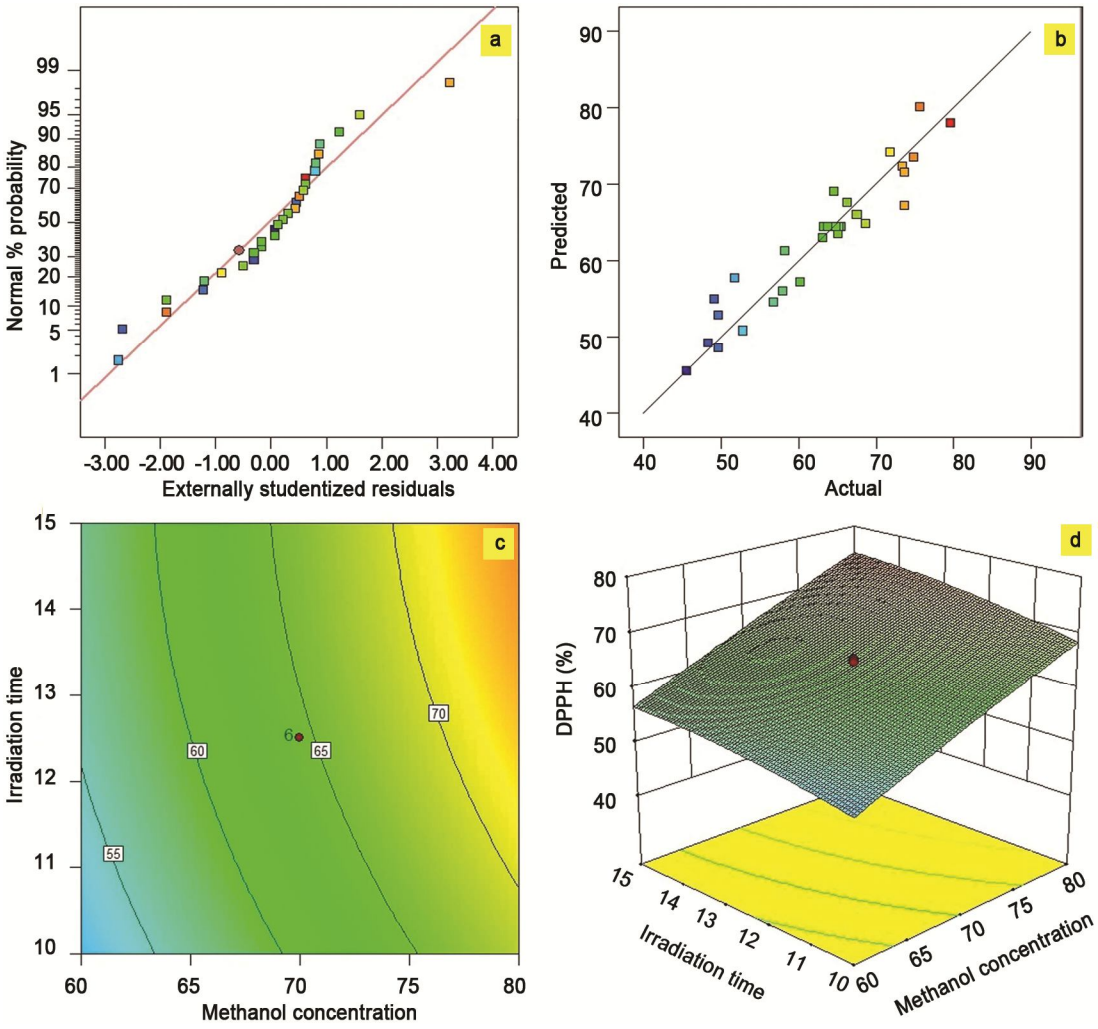

Fig.4 — Normal percentage probability plot for the studentized residuals for highest % DPPHsc activity (a), Relationship between experimental and predicted value for highest % DPPHsc activity (b), Response surface and contour plot showing the combined effects of methanol concentration (X_1) and irradiation time (X_3) for highest % DPPHsc activity microwave power and irradiation temperature were held at fixed level (zero level=20%, 50 ºC, respectively) (c) and (d).

and irradiation time of (15 min) correspond to the highest antioxidant activities (% DPPHsc, % ABTSsc and FRAP). The maximum yields of antioxidant or antioxidant activities are % DPPH: 79.62 %, % ABTS: 69.26 % and FRAP: 67.12 µg mol Fe (II)/g.

ANFIS model analysis

Adaptive neuro-fuzzy inference system (ANFIS) modelling was developed as a function of process parameters of bioactive polyphenolic compounds present in the *M. quadrifolia* L. by using 30 experimental data presented in Table 2. At first, all the obtained from input and output data was randomized. Then the data set was separated for training, testing and validation of the model. Fuzzy logic toolbox in Matlab v R2013a was utilized to train the ANFIS and get the results. Several parameters were tested as training parameters for obtaining accurate results and build in a fuzzy inference system (FIS) of ANFIS model comprises of four inputs, five output (one at a time) and membership functions. Each input variable is assigned by as low, medium and high three fuzzy sets such as solvent concentration, microwave power, irradiation temperature and irradiation time. Correspondingly, experimental data on predicted output responses were TPC (687.26 mg GAE/g), TFC (81.43 mg RU/g), DPPHsc (79.6 %), ABTSsc (69.6 %), and FRAP (67.12 µg mol (Fe (II))/g) were defined in five fuzzy sets namely very low, low, medium, high and very high. The fuzzy inference system consists of total 144 network nodes and 36 fuzzy rules. The fuzzy rules

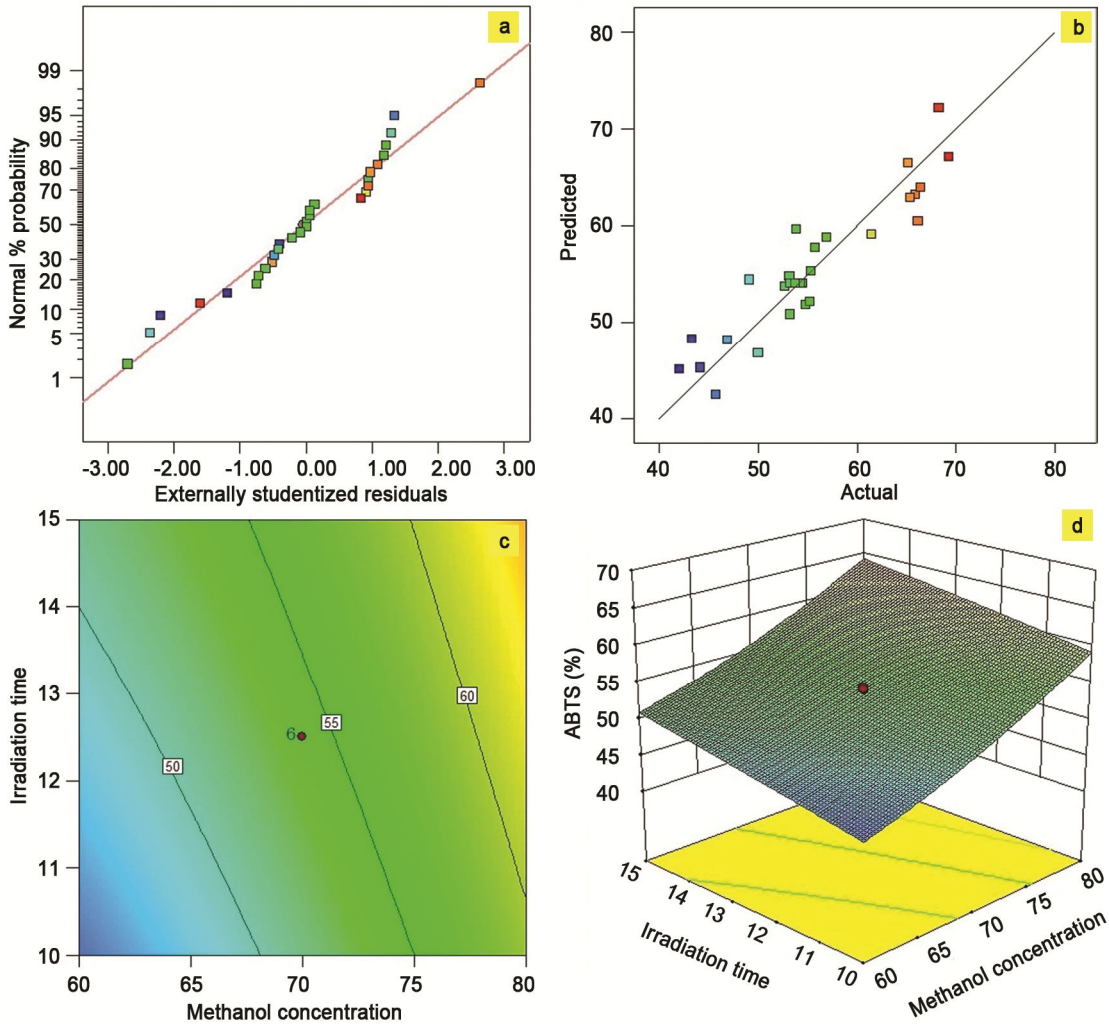

Fig.5 — Normal percentage probability plot for the studentized residuals for highest %ABTSsc activity (a), Relationship between experimental and predicted value for highest % ABTSsc activity (b), Response surface and contour plot showing the combined effects of methanol concentration (X_1) and irradiation time (X_3) for highest % ABTSsc activity microwave power and irradiation temperature were held at fixed level (zero level=20%, 50 ºC, respectively) (c) and (d).

were constructed on the basis of experimental data and human experiences. The predicted values of responses through RSM were also used to refine the fuzzy rules.

Validation experiments

The appropriateness of verification experiments was carried out to confirm the consistency of the experimental design. The experiments were carried out through Design Expert software, and it was able to identify optimum extraction parameters and their combinations. Further, the optimized parameters were also validated through ANFIS model using the same data. The experimental results showed that the methanol concentration and irradiation time had significant effects on the yields of polyphenolic compounds from *M. quadrifolia* L. Table 4 shows verification experiment under optimum conditions based on a combination of responses with minor variations. Based on those optimal conditions were methanol concentration of 80-85 %, a microwave power 25 %, irradiation temperature 60 ˚C and irradiation time 13.5-15 min. Under this condition, while the experimental values of TPC, TFC, % DPPHsc, % ABTSsc and FRAP were 687.26-693.28 mg GAE/g), 81.43-84.86 mg RU/g, 79.62-81.06 %, 69.26-71.34 % and 67.12-68.09 µg mol (Fe (II))/g respectively, predicted values from RSM's are TPC, TFC, % DPPHsc, % ABTSsc and FRAP were 687.64-695.99 mg GAE/g), 80.71-85.41 mg RU/g, 77.99-81.63 %, 67.12-71.51 % and 64.41-68.55 µgmol (Fe (II))/g respectively, Rule viewer plot (Fig. 7) provides

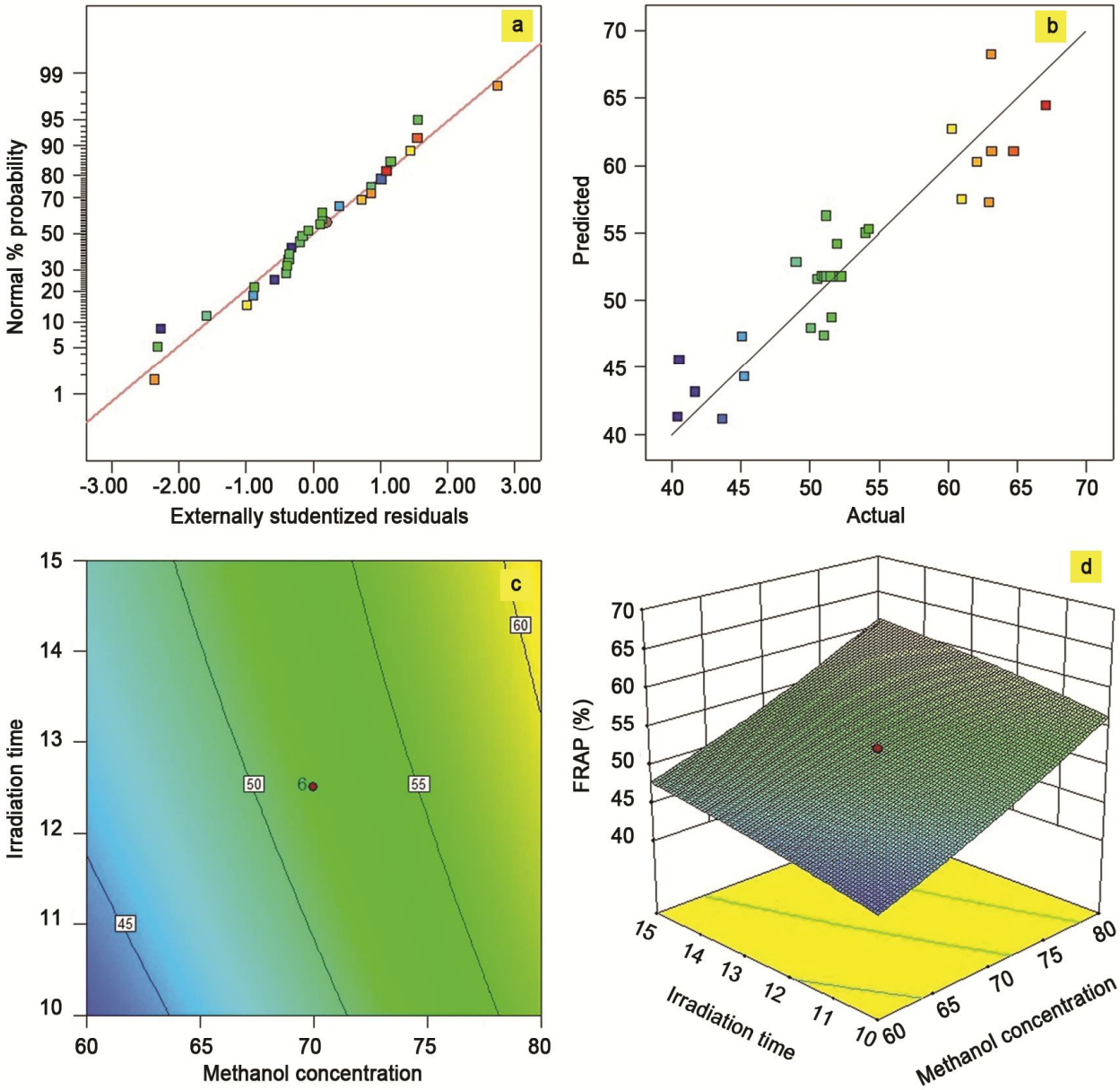

Fig.6 — Normal percentage probability plot for the studentized residuals for highest FRAP activity (a), Relationship between experimental and predicted value for highest FRAP activity (b), Response surface and contour plot showing the combined effects of methanol concentration (X_1) and irradiation time (X_3) for highest FRAP activity microwave power and irradiation temperature were held at fixed level (zero level= 20%, 50 ºC, respectively) (c) and (d).

Table 4 — Verification of experimental and predicted values under optimum conditions based on combination of responses

Run	Independent variables				Experimental value*					RSM Predicted value					ANFIS Predicted output value				
	X_1	X_2	X_3	X_4	y_1	y_2	y_3	y_4	y_5	y_1	y_2	y_3	y_4	y_5	y_1	y_2	y_3	y_4	y_5
1	82.5	25	60	15	689.76	83.84	80.45	71.25	68.06	695.99	85.41	81.63	71.51	68.55	690	83.4	80.3	70.5	67.5
2	87.5	25	60	15	693.28	84.86	81.06	71.34	68.09	702.26	87.85	83.38	73.92	70.77	692	84.8	81	71.8	68.4
3	80	25	60	13.5	678.26	78.26	76.39	65.24	63.25	680.71	79.48	77.1	66.03	63.45	680	79	76.8	66.1	64.1
4	80	25	60	16.5	687.26	81.08	80.34	68.24	66.19	689.03	81.53	78.16	68.13	65.11	689	81.8	80.8	69.2	67.1

*All the experiments were repeated three times

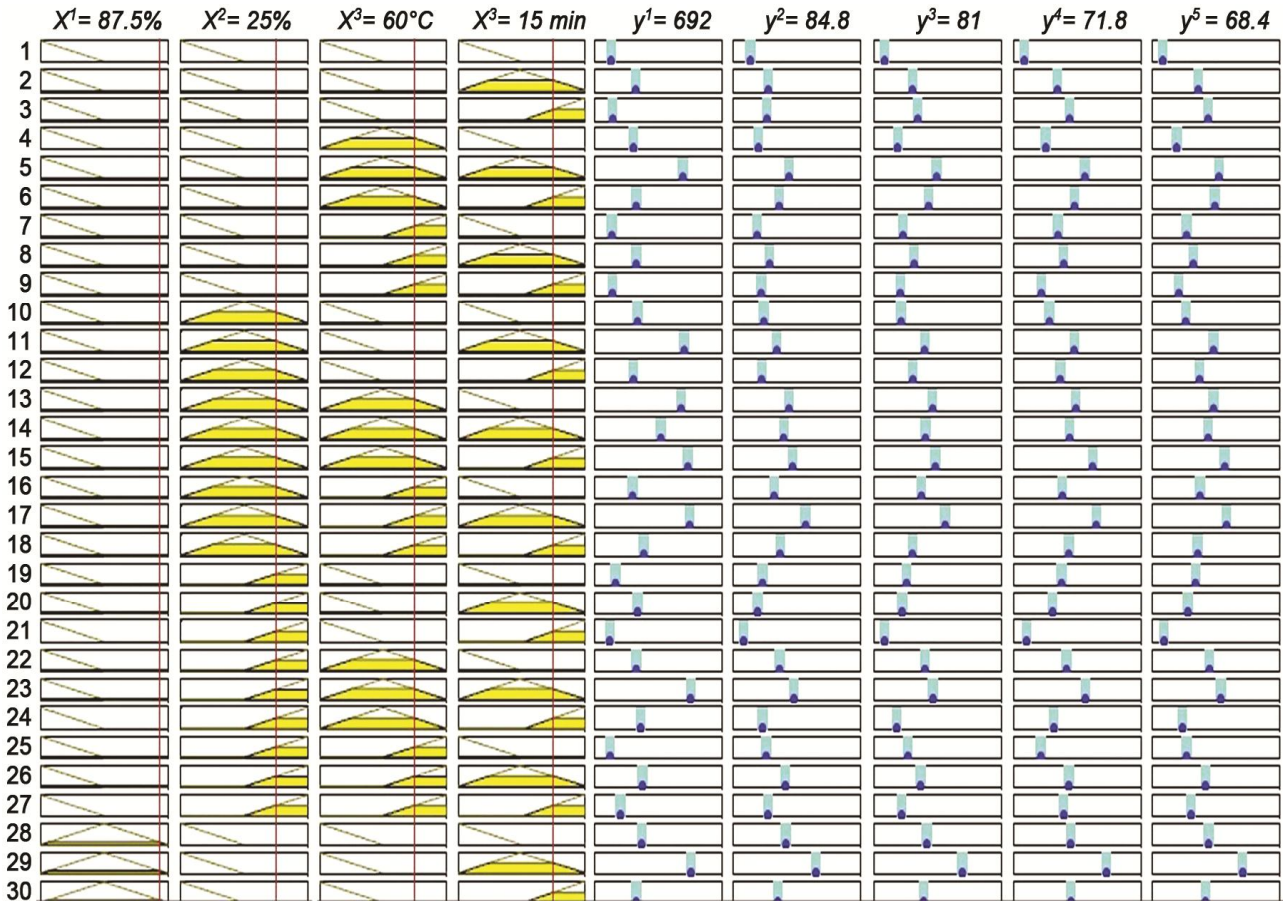

Fig.7 — ANFIS rule viewer for the effect of process variables on responses for extraction of TPC, TFC and antioxidants from *M. quadrifolia* L. extract.

the value of responses by varying the process variables. At the targeted optimized process conditions of process variables (methanol concentration= 87.5 %, microwave power= 25 % irradiation temperature= 60 °C, irradiation time=15 min), the responses obtained through ANFIS model were TPC, TFC, % DPPHsc, % ABTSsc and FRAP were 690 mg GAE/g), 83.4 mg RU/g, 80.3 %, 70.5 %, and 67.5 µg mol (Fe (II))/g respectively, in *M. quadrifolia* L. extract. There exists a close fit between the obtained experimental values, the regression model and the predicted values obtained from RSM and ANFIS modelling.

LC-ESI-MS analysis

The completed optimal extraction condition continued by LC-ESI-MS analysis to identify the presence of polyphenolic compounds in the extract. The obtained LC-ESI-MS chromatogram results of optimized methanolic extract of *M. quadrifolia* L. are depicted in Fig. 8a and b. Moreover, the chromatogram displayed different peaks at different retention times and indicates the presence of six phenolic compounds namely Betasitosterol, Tridecyliodide, 2,3,7,8 tetracholoro–dibenzofuaran, Chlorogenic acid, Pentachloro–phenylacetate and Triacontyl hexacosanoate. The identified phenolic compounds retention time and molecular weight were depicted in Table 5. The observed molecular mass of six phenolic compounds and its structural identification were confirmed by using an online library. The identified chemical compounds, Betasitosterol has long been known to control the blood cholesterol level and benign prostatic hyperplasia, and 1- triacontanol-cerotate ameliorates reactive oxidative damage on cells[39]. Based on these results, the optimally obtained methanolic extract of *M. quadrifolia* L. could be considered as one of the major sources to provide ingredients of antioxidant and antidiabetes therapies.

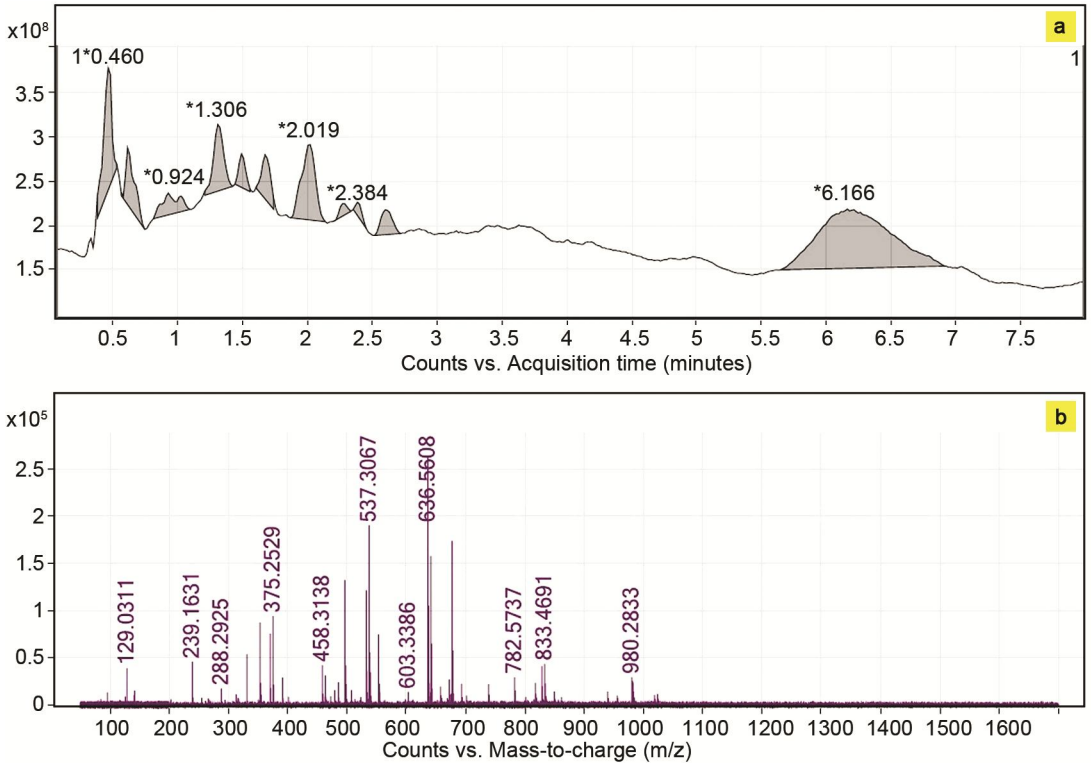

Fig.8 — LC-ESI-MS chromatogram of optimized methanolic extract of *M. quadrifolia* L. (a) Retention time (b) with mass peaks

Table 5 — Important compound identified from the optimized methanolic extract of *M. quadrifolia* L. by LC-ESI-MS

Retention time	Name of the compound	Molecular Weight
0.46	Betasitosterol	414.71
0.92	Tridecyliodide	310.26
1.30	2,3,7,8 tetracholorodibenzofuaran	305.97
2.01	Chlorogenic acid	354.31
2.38	Pentachlorophenylacetate	308.37
6.16	Triacontyl hexacosanoate	817.51

Conclusion

In this study, microwave-assisted extraction has been used to identify the optimal condition for maximum recovery of bioactive polyphenolic compounds from *M. quadrifolia* L. Aqueous methanol was found to be a suitable solvent for maximum extraction of polyphenolic compounds from *M. quadrifolia* L. The independent extraction parameters were successfully optimized and verified through RSM based on CCRD and together with ANFIS modelling. The independent variables of methanol concentration, irradiation time and interaction terms of methanol concentration, irradiation temperature, and irradiation time had significant extraction of bioactive polyphenolic compounds on MAE. The high R^2 value of the design demonstrated the consistency of the model. Hence, the optimal conditions were obtained as methanol concentration (X_1)= 87.5 %, microwave power (X_2)= 25 % irradiation temperature (X_3)= 60 °C, irradiation time(X_4)=15 min. Under optimized conditions, experimental and predicted values of RSM were well agreed with the values predicted by ANFIS modelling. Further, 6 major bioactive compounds were identified through LC-ESI-MS analysis in the optimized extract of *M. quadrifolia* L. Our study proves that microwave-assisted extraction is indeed superior to the conventional extraction methods in some aspects such as reducing extraction time, lowering extraction temperature, and reducing the use of organic solvents. The operational parameters under optimized conditions account for the low-cost extraction process thus, provides an efficient, rapid and cost-effective method for isolation and scale-up of these commercially viable flavonoids.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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