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Glycerol-Water Green Solvent for Bioactive Metabolites Extraction from *Ranunculus arvensis* and Extraction Optimization by Response Surface Methodology

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ABSTRACT

Ranunculus arvensis L. is a medicinal herb traditionally used for various purposes. The objective of the present research project was to find an effective and green method for obtaining bioactive natural products from it. Aqueous glycerol was employed as a green extracting medium, and the bioactive compounds were estimated as total flavonoid content (TFC) total phenolic content (TPC), antioxidant activity, and metal chelating activity (MCA). Response surface methodology (RSM) was applied to optimize the extraction process with temperature, time, and solvent (glycerol) concentration as input factors. The optimized conditions were 30 °C, 30 min, and 70% glycerol concentration at which the responses were TPC 7.15 mg gallic acid equivalents/g DW (dry weight), TFC 14.8 mg rutin equivalents/g DW, antioxidant 59.55%, and MCA 49.14%. The model was strongly supported by the validation study. The explored extraction process for bioactive natural products from *R. arvensis* is predictably applicable. Hence, 70% aqueous glycerol at almost room temperature and a minimum duration of 30 min can allow optimum extraction of phenolics, flavonoids, antioxidants, and metal chelators from *R. arvensis.*

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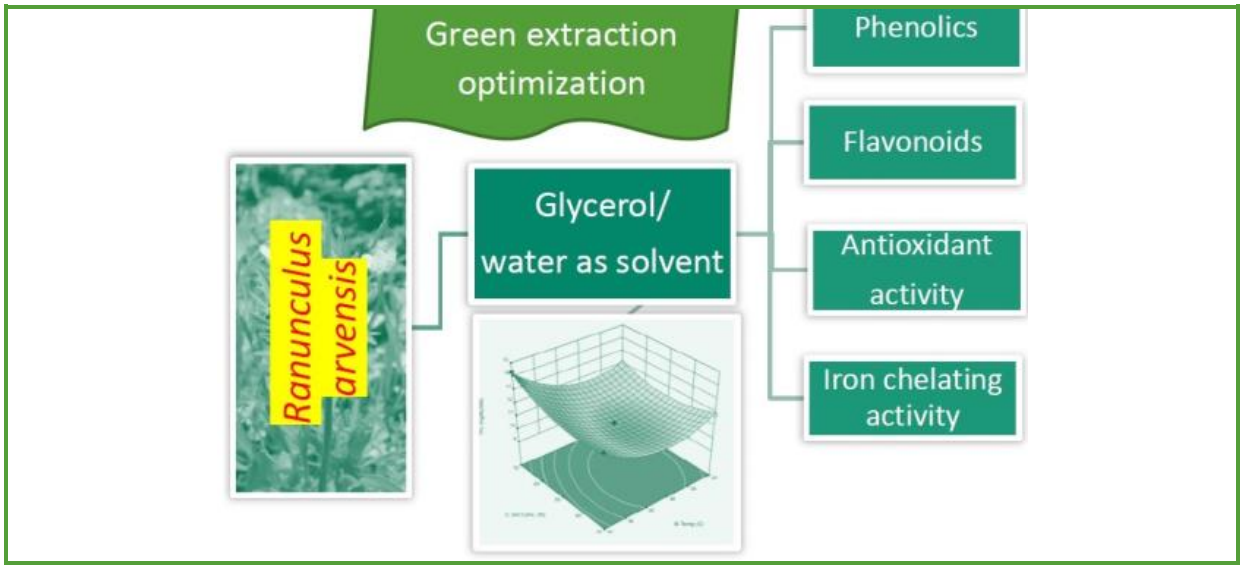
Graphical Abstract



Introduction

Plant-based medicines make up over 50 % of the medicines in clinical use. Medicinal plants contain compounds that are antioxidants and may inhibit or scavenge free radicals, thus providing efficacious, harmless, and low-capital treatments for degenerative diseases [1].

Phenolic compounds are natural products that exhibit antioxidant activity and can be evaluated by methods such as metal chelation and DPPH scavengers. Polyphenols are classified as flavonoids, phenolic acids, tannins, stilbenes, and lignans [2].

The human body essentially requires antioxidants against free radicals that form naturally after exposure to harmful radiation and carcinogens or food degradation [3].

Reactive oxygen species (radicals and non-radicals) are harmful by-products of aerobic respiration and lead to oxidative damage within the body [4]. Antioxidants help obliterate free radicals from the body [5, 6].

Ranunculus arvensis L. (*R. arvensis*; family Ranunculaceae) commonly known as "Corn Buttercup" is a medicinal herb abundantly available in Asian, African, and European countries [7].

Northern regions of Pakistan (Hindukush, Hazara, Kalash, and Chitral) are wellsprings of this imperative flora [8]. These are 10-50 cm high plant species distinctly recognized by their vibrant yellow flowers [9].

R. arvensis has been traditionally used to treat arthritis and asthma [10], myalgia and common cold [11], edema, blisters, jaundice, and pain [12]. Indeed, it has significantly exhibited anti-Cancerian, anti-mutagenic, anti-malarial, and wound healing properties [10, 13]. *R. arvensis* has shown the presence of many secondary metabolites such as alkaloids, coumarins, flavonoids, triterpenes, and tannins [14-16].

Specifically, polyphenolic compounds such as quercetin, gallic acids, rutin, isoorientin, and isovitexin have been characterized in *R. arvensis* extracts. Furthermore, aglycons, kaempferol, and isorhamnetin have been revealed [17].

Classical solvents (methanol, chloroform, water, hexane, and acetones) have previously been used to extract phenolic bioactive compounds from *R. arvensis* [7, 14]. However,

low yields have been acquired. This either could relate to the inefficacy of solvent or probably the overall low content of phenolics in the plant. Moreover, several conventional solvents have been identified as non-green and their usage is a potential threat to human health. Hence, this necessitates developing and using green solvents (environmental-friendly) that could also provide good yields of bioactive compounds [18].

Heat-assisted extraction is a traditional extraction technique that is still used in the industrial setting because it is more practical than other contemporary techniques like ultrasound or microwave-assisted extraction [19].

An environmentally friendly extracting solvent is glycerol. It may be utilized either alone or in conjunction with other liquids. In fact, glycerol is a desirable medium for the extraction of chemical compounds from plant biomass due to its relative affordability, great availability, water miscibility, and non-toxicity [20].

Glycerol with a high boiling point (290 °C) [21] is suitable for extracting moderately polar compounds like flavonoids since it has a dielectric constant of 42.5 at 25 °C [22, 23] and dipole moment of 2.62 D [24]. Its high viscosity (1.41 Pa. s) as an extraction medium is a disadvantage [25]. Glycerol's high viscosity precludes it from easily penetrating plant biomass and solubilizing its phytochemicals [26]. However, the problem can be easily fixed by combining with another suitable solvent, like water. Glycerol and water are miscible, and the resulting binary solvent system offers a practical means of extracting phytochemicals [20].

An effective technique used to compare responses to chosen parameters is called Response Surface Methodology (RSM). RSM can be applied through the designs known as the Central Composite design (CCD) or Box-Behnken design (BBD). BBD is less time-consuming than CCD since it requires, relative to the number of factors, fewer trials to explore the responses. The best extraction parameters for the extraction of antioxidant components from plant samples in a glycerol-water solvent system were examined for the study using BBD of Response Surface Methodology [27].

The data set is fitted in a second-order polynomial equation and models are predicted. The significance of the models is ascertained based on analysis of variance (ANOVA) [28].

The literature survey reveals that the efficacy of the aqueous-glycerol system to extract phenolics from *R. arvensis* and its optimized conditions as per RSM have yet not been investigated. As the glycerol-water solvent system is thought to provide a green extracting solvent system, it is expected that it would effectively extract phenolic components from *R. arvensis*. In addition, it is expected that an appropriate set of extraction parameters would be found using BBD of RSM.

A comparative analysis of the extraction efficiency of polyphenols between water-glycerol and water-ethanol systems was carried out. Optimum conditions were designed by response surface methodology. The higher concentration of polyphenols (51.91 mg GA/gDW) was attained at the optimum condition of 9.3 % aqueous glycerol and 80 °C. At the optimum conditions, the content of polyphenols acquired from aqueous glycerol was higher than the aqueous ethanol [20].

In 2016, Michail *et al.*, conducted an extraction of spent filter coffee to extract polyphenols using aqueous glycerol and compared it with pure water using an ultrasonic technique.

Response surface methodology was applied for optimizing the conditions by using the Box Behnken design. The optimum conditions were

3.6% aqueous glycerol and 175 min. The results showed that aqueous glycerol solvent is more effective for concentration than water because it provided 7.4% more phenolic concentration than water [29].

Different combinations of glycerol-water (15%, 32.5%, and 50%) were used to extract polyphenols of light molecular weight of Carménère grape pomace via HPLE. The optimum conditions were 50 % glycerol for stilbenes, phenolic acids, and flavonols, and 50 °C, 32.5% glycerol for flavonols. The glycerol water system showed more effective results than ethanol [30]. AbdElslam *et al.*(2013) determined the total phenolic, flavonoid, and saponins content of *R. arvensis* in different fractions of water, chloroform, n-hexane, and ethyl acetate using reported techniques. Chloroform and ethyl acetate showed the maximum quantity of alkaloids (0.1762 ± 0.011), flavonoids (1.3415 ± 0.0011), and saponins (2.414 ± 0.014) while water fraction exhibited the highest phenol (1.216 ± 0.011) concentration. Ethyl acetate and chloroform can be used for the extraction of phytochemicals [31]. Furthermore, Bhatti *et al.* (2015) examined total phenolic and flavonoid content on the methanol, water extracts, methanol: water, chloroform: methanol, acetone, chloroform, and methanol: acetone of *R. arvensis*. In the comparison of various extracts, water extract showed the highest concentration of polyphenols (1.43 mg/g GAE), and a remarkable amount of total flavonoid content was attained from methanolic extract (6.00 ± 0.02 mg RE/g). The methanolic extract exhibited notable antioxidant activity (IC_{50} 34.71 ± 0.02) in the DPPH free radical scavenging experiment. The presence of rutin (0.44 %) and caffeic acids (0.017 %) were detected via HPLC analysis [14]. Likewise, Khan *et al.* (2017) conducted the phytochemical screening of *R. arvensis* and found that the main components of *R. arvensis* are alkaloids, steroids, terpenoids, tannins, glycosides, saponins, and flavonoids based on both positive and negative test results. DPPH radical scavenging activity was carried out in different fractions of ethyl acetate and methanol to determine the antioxidant potential of *R. arvensis*. Methanolic fraction (75.25%) exhibited strong antioxidant activity than ethyl acetate [8]. In 2017, Boroomand *et al.*, detected caryophyllene oxide (7.1%), camphor (6.2%), Guaiol (8.81%), and spathulenol (6.73%) in GCMS analysis of *R. arvensis* [15].

The goal of the study was to evaluate the efficacy of green solvent of aqueous glycerol in the extraction of polyphenols and other antioxidants from *R. arvensis*. Extraction optimization of TPC, TFC, and DPPH radical scavenging activity (RSA), as well as iron metal chelating activity (MCA) according to response surface methodology.

It was presumed that the extraction of polyphenols and other antioxidant chemicals for *R. arvensis* would be effective using the glycerol-water binary solvent system, and an optimal procedure might be created using RSM used in accordance with BBD.

Experimental

Materials and Methods

Analytical-grade chemicals were used for experimentation. DPPH (2,2-diphenyl-1-picrylhydrazyl), Gallic acid, anhydrous sodium carbonate, Ferrozine, rutin, ascorbic acid, sodium hydroxide, aluminum chloride, methanol, ferrous sulfate heptahydrate, sodium nitrite, Folin-Ciocalteu reagent, glycerol, and EDTA (Ethylenediaminetetraacetic acid) were all acquired from Sigma-Aldrich (Steinheim, Germany). All extractions were conducted in VS-8480SN shaking incubator (Vision Scientific Co., Ltd., Korea). The Multi-Mode Micro-Plate

Reader Synergy HTX (Winooski, Vermont, US) was used to read the absorbances for antioxidant activity and content analysis.

Plant sample preparation

The plant material was a courtesy of Dr. Nighat Sultana of Hazara University, Mansehra, Pakistan, which was collected from the vicinity of university and was identified by Dr. Alia Gul, Department of Botany, Hazara University, Mansehra, Pakistan, with voucher number 2561. The herb was cleaned and allowed to dry for two weeks at room temperature. The plant was then manually crushed, and then ground into a fine powder using a high-speed multifunction comminutor. This fine powder was put through a filter with a mesh size of 100 and stored in the refrigerator in plastic seal bags.

Experimental design

To optimize the extraction process, RSM was applied as per the Box-Behnken design (BBD) [28]. The three factors were temperature, time, and solvent (glycerol) concentration. The responses were TPC, TFC, antioxidant activity, and metal chelating activity. The experimental design is shown in Table 1 along with the results.

Extraction process

Weighed amounts (1.0 g) of *R. arvensis* powder were macerated in 30 mL solvent system (aqueous glycerol with concentrations 30, 50 and 70 %) in 250-mL conical flasks as per the design (Table 1). The conical flasks were shaken in a shaking incubator at the shaking speed 200 rpm for a given time and temperature as per the design. After that, the extracts were filtered, and filtrates were collected and stored in glass vials with parafilm seals. The filtrates were used for the estimation of the responses.

Assays used for estimation of responses

For all the responses, reported protocols were used [28]. For TPC, the Folin-Ciocalteu reagent assay was used with gallic acid as a standard. For TFC, a colorimetric method based on complexation with aluminum chloride was used and rutin was the standard. For RSA (radical scavenging activity), the DPPH assay was employed. For MCA, an assay based on iron chelation was used and EDTA was the standard.

Statistical analysis

For statistical analysis, the Design Expert software program was used. All experiments were conducted three times and their statistical means with standard deviations were calculated. The software fitted the data into the 2nd degree polynomial equation and predicted the most viable models. ANOVA (analysis of variance) was carried out to determine the significance of the models and terms. The predicted models and terms with p-values < 0.0500 were considered significant. The adequacy of the predicted models was further assessed by the lack of fit p-values and coefficients of determination.

Results and Discussion

In the search for a green and efficient method to recover bioactive natural products from *Ranunculus arvensis*, aqueous glycerol in different concentrations was used as a solvent and the optimization was carried out as per the Box-Behnken design of RSM. The design of the experiment (DOE) and results are listed in Table 1.

Total phenolic content (TPC)

As indicated in Table 1, the highest TPC was 7.30 mg GAE/g DW, acquired at 50% solvent concentration (glycerol-water), kept at 60 °C for 30 min time (standard order 3), while the lowest TPC was 5.62 mg GAE/g DW, acquired at 50% solvent concentration (glycerol-water), kept at 60 °C for 90 min time (standard order 4). Thus, for both the highest and lowest TPC, solvent concentration and temperature are the same, only time durations were different. The longer time duration resulted in the TPC decrease, it may be due to degradation of phenolic compounds occurring on heating the sample for a long duration. The higher polyphenol yield was observed at a lower time duration in the experimental range.

Increasing the time period increases the exposure of polyphenols to heat for a longer time period, which may deteriorate them. Literature shows many examples of this trend. For instance, a study shows that the optimum time and temperature for extraction of phenolics from green tea was 20-30 min and 80 °C [32].

Table 1. Design of experiment (DOE) and experimental findings of the responses

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
		A	B	C	TPC	TFC	RSA	MCA
		Time	Temp	Sol Conc.				
		min	C	%	mg GA/DW	mg RE/DW	%	%
10	1	60	60	30	6.94 ± 0.02	11.88 ± 0.14	46.65 ±3.33	67.86 ± 0.69
13	2	60	45	50	5.74 ± 0.35	10.02 ± 0.07	47.19 ± 2.28	65.82 ± 0.54
3	3	30	60	50	7.30 ± 0.19	12.09 ± 0.23	49.56 ± 2.95	68.75 ± 0.78
6	4	90	45	30	5.90 ± 0.63	11.52 ± 1.11	46.54 ± 2.24	65.73 ± 0.57
14	5	60	45	50	5.95 ± 1.37	10.92 ± 1.01	46.83 ± 1.28	65.08 ± 2.16
7	6	30	45	70	6.97 ± 1.61	12.84 ± 0.89	52.63 ± 3.36	64.01 ± 1.31
11	7	60	30	70	6.92 ± 0.42	14.35 ± 0.85	55.12 ± 2.89	65.98 ± 1.83
12	8	60	60	70	6.13 ± 0.38	12.05 ± 0.51	51.27 ± 2.91	65.09 ± 2.33
17	9	60	45	50	5.70 ± 0.69	10.58 ± 1.22	47.19 ± 3.45	65.79 ± 2.12
15	10	60	45	50	5.70 ± 0.27	10.91 ± 1.75	46.00 ± 1.63	64.72 ± 1.19
2	11	90	30	50	6.81 ± 0.18	12.39 ± 0.45	49.91 ± 2.58	68.39 ± 2.97
8	12	90	45	70	5.64 ± 0.31	11.08 ± 0.73	47.13 ± 1.61	61.78 ± 1.47
5	13	30	45	30	6.36 ± 1.25	10.58 ± 0.06	45.71 ± 3.13	66.74 ± 3.86
16	14	60	45	50	5.91 ± 0.89	10.82 ± 0.72	50.15 ± 2.29	65.30 ± 2.27
9	15	60	30	30	6.22 ± 1.15	12.82 ± 0.98	50.93 ± 1.90	69.83 ± 1.88
4	16	90	60	50	5.62 ± 0.95	11.01 ± 0.22	49.26 ± 1.31	64.72 ± 0.90
1	17	30	30	50	6.52 ± 0.17	12.91 ± 0.37	55.77 ± 1.26	68.86 ± 1.14

**The highest values are shown in bold*

The increase of TPC in going from 30% to 50% glycerol concentration may be because increasing the ratio of glycerol decreased the dielectric constant or polarity of the solvent system which is more efficient to extract phenolics more compatible in polarity [33]. The reduced polarity of glycerol-water solvent is due to the reduced dielectric constant of glycerol [24]. However, further increasing the glycerol concentration increases the viscosity of the solvent, hence hindering the process of diffusion of polyphenolics during the extraction process [35]. The solubility of polyphenolics is

also impacted by the presence of hydrogen bonding and steric hindrance [36].

Previous studies show similar results. For instance, the extraction of bioactive compounds from apple peels and red grape pomace increased upon increasing the glycerol concentration up to 70 and 90%, respectively [37]. Increasing the temperature increases the phenolic extraction due to the increased kinetics. However, many polyphenolics are destroyed above 60 °C due to thermal degradation since low molecular weight polyphenol molecules are more sensitive to heat [38]. Optimized extraction up to 60 °C is observed in black rice, black currents, canola, and flax seeds [39]. The only difference in parameters for the maximum and the minimum TPC values is that of time.

Total flavonoid content (TFC)

The highest TFC was 14.35 mg RE/g DW, acquired at 70% glycerol concentration (in the glycerol-water mix), kept at 30 °C for 60 min time (standard order 11), while the lowest TFC was 10.02 mg RE/g DW, acquired at 50% glycerol concentration, heated at 45 °C for 60 min time (standard order 13). Increasing the glycerol concentration up to 70% significantly increases the flavonoid concentration. Literature shows that the extraction of antioxidant compounds from peppermint and nettle leaves in a glycerol-water system gives high values for TFC at high glycerol concentrations [40]. The aglycone flavonoids show a high affinity towards polar organic solvents like alcohols, while the glycosidic flavonoids are more soluble in non-polar solvents [41]. Hence, TFC is entirely dependent on the nature of flavonoids found in plants [42].

Increased TFC with time might be because a greater time duration produced greater damage to the cells and diffused flavonoids into the extraction system [43]. The flavonoids exposed to high temperatures will undergo thermal degradation if kept for a longer period of time [44]. Therefore, the optimal TFC is acquired at 30 °C, whereas increasing the temperature to 45 °C gives the lowest TFC. Higher temperatures are known to improve solvent permeability [45]. However, thermal effects trigger the loss of compounds and low molecular weight components [46].

A previous study reports a contrasting result. Extraction of flavonoids from seeds of Petai Belalang showed the highest TFC value observed at 60 °C, kept for 36 min time. Another study reported that ultrasound-assisted Soxhlet extraction of antioxidants from *Opuntia ficus-indica* peel gave the highest TFC and TPC for 17 min and 40 °C bath temperature [47].

Radical scavenging activity (RSA)

The highest RSA was 55.77%, acquired at 50% solvent concentration (glycerol-water), kept at 30 °C for 30 min time (standard order 1), while the lowest DPPH radical scavenging activity was 45.17%, acquired at 30% solvent concentration (glycerol-water), kept at 45 °C for 30 min time (standard order 5).

Higher RAS was observed for extracts with 50% glycerol-water concentration, which supports the finding of this study that greater TPC values were acquired with 50% glycerol-water concentration. Lower RSA was observed for extracts with 30% glycerol-water concentration and the temperature of 45 °C, which supports the lower TFC values at low solvent concentration and time above 30 °C. Hence, a good correlation is observed between RSA, TFC, and TPC, validating the fact that RSA is dependent on the nature of polyphenolic compounds in plant extracts [48] playing a significant part in their ability to transport electrons and having an impact on DPPH assay [49]. Slight variations in results are observed.

Metal chelating activity (MCA)

The highest MCA was 69.83%, acquired at 30% solvent concentration (glycerol-water), kept at 30 °C for 60 min time (standard order 9), while the lowest MCA was 61.78%, acquired at 70% solvent concentration (glycerol-water), kept at 45 °C for 90 min (standard order 8). MCA did not correlate with TPC and TFC strongly [50]. The highest MCA values are observed at parameter (30% glycerol-water concentration) which did not significantly enhance the TPC and TFC.

Table 2. The outcomes of analysis of variance (ANOVA) for all responses for optimization of numeric parameters for extraction of bioactive natural products from *Ranunculus arvensis*

	TPC	TFC	RSA	MCA
Sources	P values			
	Quadratic	Quadratic	Quadratic	Quadratic
Model	< 0.0001	0.0004	0.0027	<0.0001
A (time)	< 0.0001	0.0381	0.0169	0.0003
B (temperature)	0.2344	0.0007	0.0035	0.0007
C(concentration)	0.5361	0.0075	0.0022	<0.0001
AB	0.0001	0.4282	0.0579	0.0035
AC	0.0125	0.0050	0.0366	0.1827
BC	0.0007	0.0822	0.8680	0.2347
A^2	0.0120	0.6055	0.5983	0.5026
B^2	< 0.0001	<0.0001	0.0009	<0.0001
C^2	0.0150	0.0022	0.7487	0.0160
Residual				
Lack of Fit	0.3663	0.7006	0.9608	0.7209
		F-values		
Model	31.3627	19.1025	10.4639	42.9696
A (time)	74.2935	6.5042	9.7206	43.8892
B (temperature)	1.6927	33.0577	18.6173	32.3974
C (concentration)	0.4232	13.7814	22.0699	129.7113
AB	57.0241	0.7070	5.1295	18.5478
AC	11.1215	16.2696	6.6465	2.1866
BC	33.5027	4.1106	0.0297	1.6904
A^2	11.3067	0.2924	0.3045	0.4994
B^2	74.5198	69.4841	30.7929	151.9656
C^2	10.2735	22.0169	0.1110	9.9546
Residual				
Lack of Fit	1.3952	0.5029	0.0916	0.7209
		R^2 values		
R^2	0.9758	0.9609	0.9308	0.9822
Predicted R^2	0.9447	0.9106	0.8419	0.9594
AdjustedR^2	0.7835	0.7842	0.8277	0.9056

The lowest ICA values are given for extracts with 70% glycerol-water concentration, which contrarily gives greater TPC and TFC. This may be due to the other non-phenolic compounds such as vitamins and carotenoids present in plant extracts that interacted during the assay

conducted [51]. Therefore, a strong positive co-relation is not observed between TFC and MCA.

Model fitting and optimization

Table 2 provides the explanation of analysis of variance (ANOVA) for all analyzed responses for optimization of numeric parameters utilized in this research [52]. The ANOVA supported quadratic models for all the responses significant p-values ($p < 0.050$) [53]. All the responses were observed to have high correlation coefficient (R^2) values, and predicted R-squared and adjusted R-squared were close to R-squared values (Table 2).

These findings corroborate the adequacy of the predicted models [54]. As Table 2 presents, the linear effect of time (A) had a significant effect on all responses, while the linear effect of temperature (B) and glycerol concentration (C) had a significant effect on TFC, DPPH activity, and MCA. The interactive effect of time-temperature (AB) had significant effect on TPC and MCA, interactive effect of time-concentration (AC) had remarkable effect on TPC, TFC, and MCA, and the interactive effect of temperature-concentration (BC) had significant effect on TPC only. The quadratic effect of time (A^2) had notable effect on TPC only; quadratic effect of temperature (B^2) had significant influence on all response while quadratic effect of glycerol concentration (C^2) had significant effect on TPC, TFC, and MCA. P- and F-values showed the significance of coefficients for the responses analyzed [55]. All responses had a non-significant lack of fit F value [56]. This shows that there is no need for a model reduction and the model is fit [57]. The details of the predicted coefficients for different terms are presented in Table 3.

To obtain a regression equation for responses only the significant terms ($p < 0.05$) were included, eliminating non-significant terms [58].

ANOVA was used to find out the significance of the model and the terms. Regression equations for all the responses in this research are given in terms of A, B, C, AB, AC, BC, A^2, B^2,and C^2, where A, B and C are linear factors (time, glycerol-water solvent concentration, and temperature), AB, BC, and AC are interactive factors (time-temperature, temperature-solvent concentration, and time-solvent concentration), and B^2, C^2, and A^2 are quadratic factors, $(\text{temperature})^2$, $(\text{solvent concentration})^2$, and $((\text{time})^2)$. The non-significant terms ($P > 0.05$) are excluded from the regression equation [59].

Previously, no literature study reported the optimization of heat-assisted extraction of bioactive components from *R. arvensis* in glycerol-water binary solvent system via response surface methodology. Regression equations (Equations 1-4) of all responses containing only the significant terms ($p<0.0500$) are given in Table 4.

3D Surface plots

For TPC, TFC, RSA, and MCA, 3D surface plots are demonstrated in Figures 1, 2, 3 and 4, respectively. The plots show interactive effects of any two factors on the responses.

TPC 3D surface plots

Figure 1a exhibits the combined effect of temperature and time on TPC. The yield significantly increases as we increase the temperature from 30 to 50 °C, keeping the time less within the range 30-40 min.

The optimum values are observed for temperature and time range between 55-60 °C and 30-38 min, respectively. However, TPC notably decreases as we keep on increasing temperature and time with time more significantly reducing TPC in this interaction.

Table 3. Coefficient table

	Intercept	A	B	C	AB	AC	BC	A^2	B^2	C^2
TPC	5.8000	-0.3975	-0.0600	0.0300	-0.4925	-0.2175	-0.3775	0.2138	0.5488	0.2038
p-values		< 0.0001	0.2344	0.5361	0.0001	0.0125	0.0007	0.0120	< 0.0001	0.0150
TFC	10.6515	-0.3018	-0.6804	0.4393	-0.1407	-0.6750	-0.3393	0.0882	1.3596	0.7653
P-values		0.0381	0.0007	0.0075	0.4282	0.0050	0.0822	0.6055	< 0.0001	0.0022
DPPH	47.4719	-1.3544	-1.8743	2.0407	1.3914	-1.5838	0.1060	0.3304	3.3227	0.1995
P-values		0.0169	0.0035	0.0022	0.0579	0.0366	0.8680	0.5983	0.0009	0.7487
MCA	65.3425	-0.9672	-0.8310	-1.6628	-0.8892	-0.3053	0.2684	-0.1422	2.4809	-0.6349
P-values		0.0003	0.0007	< 0.0001	0.0035	0.1827	0.2347	0.5026	< 0.0001	0.0160

Table 4. Model regression equations for all responses

Response	Model Equation	Equation No.
TPC	$5.80 - 0.3975A - 0.4925AB - 0.2175\ AC - 0.3775BC + 0.2137A^2 + 0.5487B^2 + 0.2038C^2$	Equation 1
TFC	$10.65 - 0.3018A - 0.6084B + 0.4393C - 0.6750AC + 1.36B^2 + 0.7653C^2$	Equation 2
RSA	$47.47 - 1.35A - 1.87B + 2.04C - 1.58AC + 3.32B^2$	Equation 3
MCA	$65.34 - 0.9672A - 0.8310B - 1.66C - 0.8892AB + 2.48B^2 - 0.6349C^2$	Equation 4

The minimum values are observed for time greater than 50 min. Figure 1b represents the combined effect of time and solvent concentration on total phenolic content. This interactive effect did not significantly influence TPC. However, moderate yields are observed for short period of time between 30 to 35 min, increasing the solvent concentration from 30 to 70%. As aforementioned, increasing time notably decreases TPC in this interaction. The minimum yields are observed for time greater than 40 min, with increase solvent concentration having negligible effect on TPC. Figure 1c indicates the combined effect of temperature and solvent concentration on TPC. The yields significantly increase as we reduce the temperature lesser than 40 °C and increase the solvent concentration from 30 to 70%. Another pattern of combined effect is observed for this interaction. Increasing the temperature above 48 °C and decreasing the solvent concentration from 50 to 30% also increases TPC. Minimal values are observed within the temperature range 38-54 °C, with no notable effect of solvent concentration on TPC.

TFC 3D surface plots

Figure 2a exhibits the 3D surface plot for combined effect of temperature and time on TFC. The moderate values of TFC are observed for all sets of combinations. High TFC yields are observed for time and temperature range between 30-90 min and 30-37 °C, respectively.

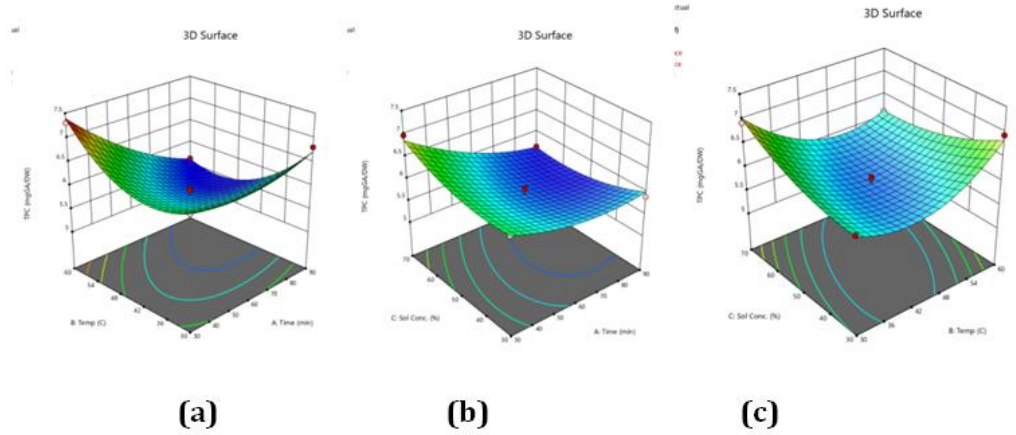


Figure 1. 3D surface plots showing the combined effect of (a) time and temperature, (b) time and solvent concentration, and (c) solvent concentration and time on TPC

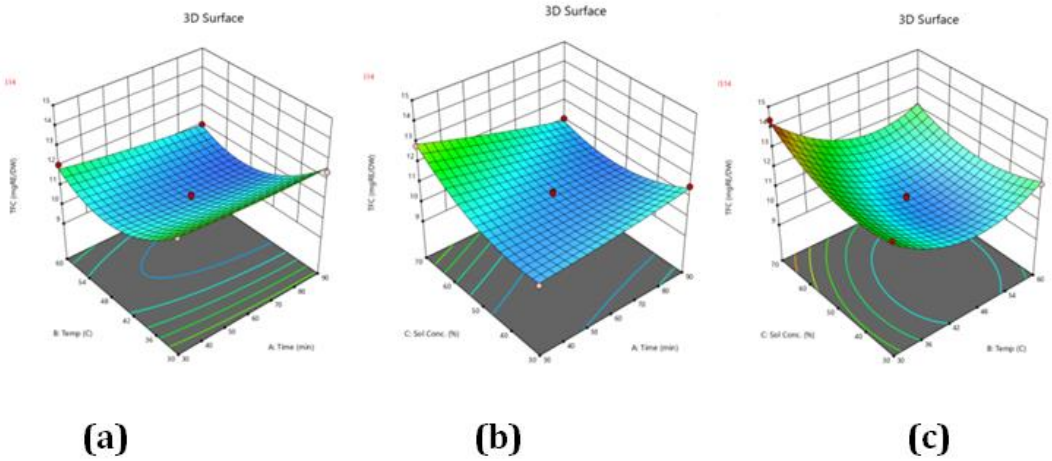


Figure 2. 3D surface plots for (a) combined effect of time and temperature, (b) time and solvent concentration, and (c) solvent concentration and time on TFC

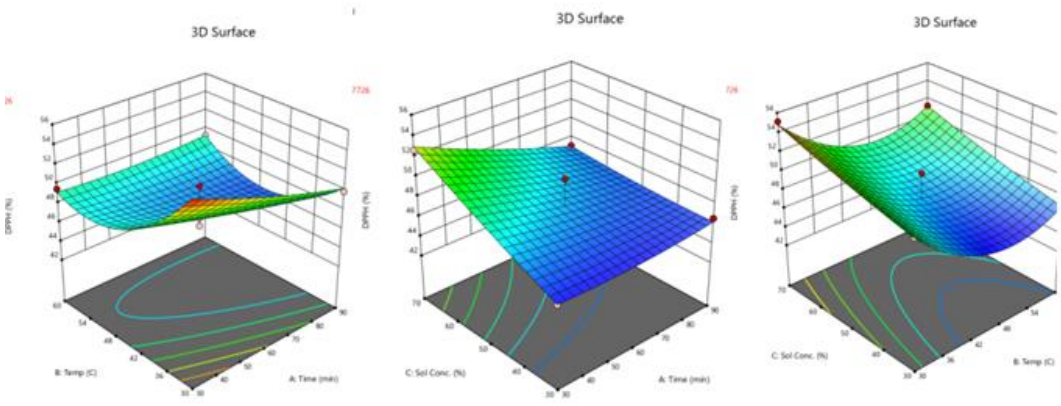


Figure 3. 3D surface plots for (a) combined effect of time and temperature, (b) time and solvent concentration, and (c) solvent concentration and time on RSA

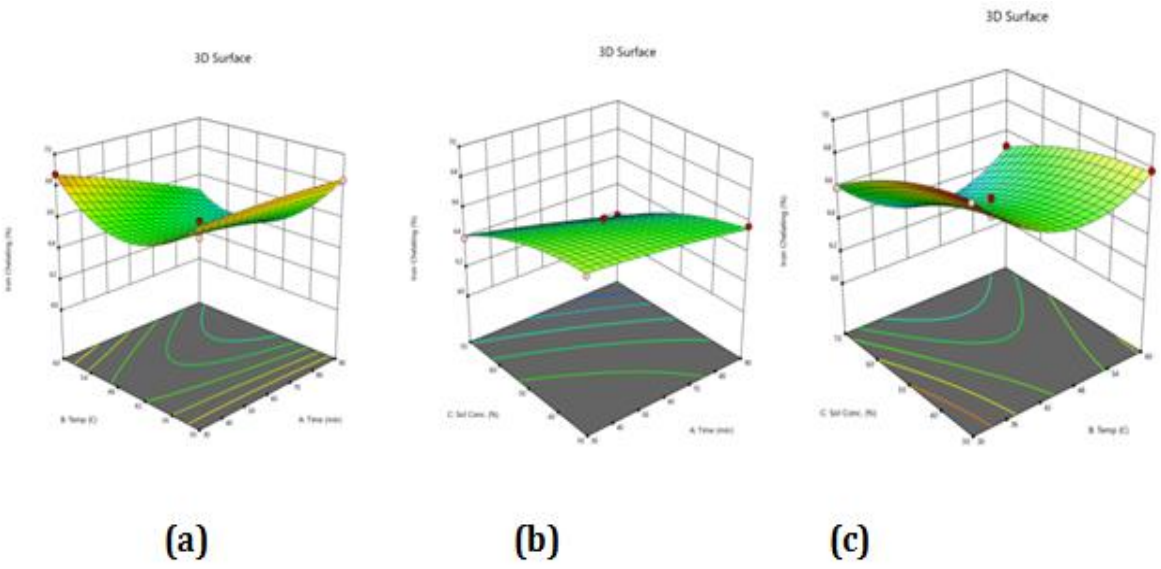


Figure 4. 3D surface plots for (a) combined effect of time and temperature, (b) time and solvent concentration, and (c) solvent concentration and time on MCA

However, further increasing the temperature above 40 °C, abruptly reduces TFC, providing us with the minimum TFC between 40 to 60 °C whilst a slight increase in TFC is observed upon the temperature above 55 °C, between 30 to 35 min. Figure 2b illustrates the combined effect of time and solvent concentration on TFC. Total flavonoid content significantly increases upon decreasing the time from 70 to 60 min and increasing the solvent concentration from 52 to 62%. The minimal yields are observed for time 70 to 90 min and 30 to 50% solvent concentration. The moderate and optimal TFC values are observed between the time range of 30 to 60 min and 60 to 70% solvent concentration. Figure 2c shows the combined effect of temperature and solvent concentration on TFC. TFC significantly increases on decreasing the temperature from 60 to 32 °C and increasing the solvent concentration from 30 to 60 %. The optimal TFC with insignificant increase is observed for temperature and solvent concentration range, 30-34 °C and 62-70%, respectively.

RSA 3D surface plots

Figure 3a displays the combined effect of temperature and time on RSA. A remarkable increase in RSA was observed upon decreasing the temperature and time from 60 to 34 °C and 90 to 55 min, respectively. The optimum values with no further significant increase in observed between range 30 to 45 min time and 30 to 34 °C temperature, reflecting that combination of lower temperature and time favored maximum RSA. The minimum activity is observed for a time range between 50 to 90 min and 42 to 52 °C temperature. Figure 3b shows the interactive effect of solvent concentration and time on RSA. A significantly high RSA is not observed for this interactive effect. However, a significant increase in RSA is observed upon decreasing the time from 90 to 50 min and increasing the solvent concentration from 30 to 60 %. The optimal and moderate RSA was observed for solvent concentration greater than 65 % and time between 30 to 35 min. The minimal value of RSA was observed for lower solvent concentration (30-40%) with increasing time having no effect on RSA. Moreover, increasing time above 60 min at high solvent concentration

(60-70%) provides us with minimal values. Hence, solvent concentration plays a major role in this interactive effect. Figure 3c depicts the interactive effect of temperature and solvent concentration on RSA. The optimal activity is observed at high glycerol concentration above 60% and low temperature between 30 to 34 °C. Minimal values are observed for temperature and solvent concentration in range 36 to 60 °C and 30 to 55%, respectively. The RSA significantly reduces as we increase the glycerol-water solvent concentration and decrease the temperature.

MCA 3D surface plots

Figure 4a demonstrates the interactive effect of temperature and time on MCA. Two sets of combinations are found for the optimum MCA in this interactive effect. High, optimal MCA with no further significant increase is observed when temperature is kept lower than 34 °C with change time not significantly affecting MCA. Moreover, the optimal MCA values are observed for temperature greater than 55 °C and time lesser than 35 min. The minimal values for MCA are observed for time and temperature in range of 80 to 90 min and 42 to 52 °C, respectively. A significant increase is observed as we increase temperature and reduce time. Figure 4b shows the interactive effect of time and solvent concentration. This interactive effect does not produce high, optimal MCA. There is a significant decrease in MCA as we increase time and solvent concentration in range 30-50 min and 30 to 60%. Minimal MCA is observed for long times (70-90 min) and high solvent concentration (55 to 70 %). Further increase in time and solvent concentration does not bring any notable change in MCA and provided low MCA. Figure 4c shows the interactive effect of glycerol-water solvent concentration and temperature. A significant increase in MCA is observed as the temperature is decreased from 60 to 36 °C and solvent concentration is reduced from 70 to 48%. Optimal MCA with no further significant increase is acquired at glycerol-water solvent concentration lower than 40% and temperature lesser than 33 °C. The minimal MCA is observed for temperature and solvent concentration in range between 42 to 54 °C and 62 to 70%, respectively.

Numerical optimization and validation studies

To find a joint model for all responses, numerical optimization was done [60]. To have the maximum desirability factor (that is 1), the constraints was set as “the maximum” for TPC, TFC, DPPH, and glycerol concentration, “the minimum” for time and temperature, and “none” for MCA [61]. The optimized conditions predicted by the optimization were 30 min time, 30 °C temperature, and 70 % solvent (glycerol) concentration.

Validation experiments were conducted under the predicted optimized conditions and the observed outcomes were compared with the predicted outcomes. The results are indicated in Table 5. Accordingly, the error rates between the observed and predicted values of TPC, TFC, and RSA were very small (0.15-2.80%), which shows that numerical optimization was successful and there is a better agreement between the observed and estimated results for the responses. However, the error rate for MCA was large and greater than 12 % (25.91%), which means that MCA could not be well predicted by regression model [62].

Table 5. Validation experiments and predicted and observed responses with relative errors

Responses	Predicted value	Observed value	Error rate (%)
TFC (mg RE/g DW)	15.160	14.800	2.37
TPC (mg GAE/g DW)	7.356	7.150	2.80
DPPH (%)	59.463	59.550	-0.15
MCA (%)	66.329	49.140	25.91

Conclusion

The main goal of the project was to investigate the efficacy of glycerol-water binary solvent to extract bioactive chemical components from *R. arvensis* according to response surface methodology. Three-factor Box-Behnken design was successfully applied. Quadratic model was obtained for each response. The optimum conditions suggested by numerical optimization were temperature 30 °C, time 30 min, and solvent glycerol concentration in its aqueous solution used as extractant 70% and the values for responses at these conditions were TPC 7.150 mg GAE/g DW, TFC 14.800 mg RE/g DW, RSA (59.550 %, and MCA 49.140%. All responses showed low error rate (< 3 %) except MCA (% error 26%). Thus, the validation experiments strongly supported the predicted model, showed it highly applicable to the extraction of polyphenolics and antioxidant compounds, and had the modest predictability for MCA. For TPC, TFC, and antioxidant activity, the model can be explored for a large-scale industrial application.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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