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Green extraction of bioactive compounds from *Azadirachta indica* in aqueous glycerol and modelling and optimisation by response surface methodology

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ABSTRACT

Development of efficient and green methods for extracting bioactive phytochemicals has great industrial value. Increasing environmental sensitivity at the global level has tremendously enhanced the demand for such methods. *Azadirachta indica* is a well-known medicinal tree. As glycerol has emerged as a green and safe extraction solvent for bioactive phytochemicals, this study aimed to investigate the efficacy of a glycerol–water solvent system to extract bioactive compounds from *A. indica* leaves. Modelling and optimisation were carried out by using response surface methodology (RSM) as per the Box–Behnken design with three variables, namely, solvent concentration, time and temperature. The responses were total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and metal chelating activity (MCA). The optimum conditions found by numerical optimisation were a solvent concentration of 69.713%, a time period of 38.328 min and a temperature of 32.114 °C with the predicted values of TPC, TFC, %DPPH and %MCA as 5.27 mg gallic acid equivalents · g^{-1} DW (dry weight), 9.869 mg rutin equivalents · g^{-1} DW, 73.8% and 54.366%, respectively. The validation experiments showed almost the same results for each response with very low% errors (5.431–7.661). Increasing glycerol concentration in the extracting medium favoured the extraction of TPC, TFC and antioxidant phytochemicals, but for MCA, the trend was the opposite. In conclusion, 70% aqueous glycerol is an effective medium for the extraction of polyphenolic and antioxidant phytochemicals from *A. indica* leaves. Extraction models suggested by RSM have high prospects to be used on a large industrial scale.

Keywords: antioxidant, Box–Behnken design, model fitting, neem, phytochemicals, polyphenols

Abbreviations: *A. indica, Azadirachta indica;* BBD, Box–Behnken design; CCD, central composite design; DoE, design of experiment; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, ethylenediamine tetraacetic acid; MCA, metal chelating activity; RSM, response surface methodology; TFC, total flavonoid content; TPC, total phenolic content.

INTRODUCTION

Medicinal plants constitute a large and continuous source of bioactive phytochemicals for pharmaceutical and other applications. Amongst the various classes of phytochemicals, polyphenols that consist of different types of flavonoids and other phenolics are especially known for their antioxidant and other bioactivities (Shahidi and Zhong, 2015; Gouda et al., 2016). In general, phytochemicals reside within plant cells that are enclosed by cell walls and membranes making it difficult for encaged phytochemicals to be released

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from the cells. A solvent that can solubilise the desired phytochemicals and strategies that can damage cell walls and membranes are required for an efficient extraction of chemical constituents from a given plant material (Jha and Sit, 2021). The growing realisation of the impact of industrial processes and products on environmental sustainability has added another important component to the method development. Thus, a method should not only be efficient and cost-effective, but it must also be environmentally friendly or green. The current study explored the extraction of bioactive phytochemicals from a well-known medicinal tree *Azadirachta indica*.

The plant *Azadirachta indica* L. (*A. indica*; family Meliaceae) is widely recognised for its diverse therapeutic applications (Ahmad et al., 2019). It is abundantly found in South Asia and Africa and has been successfully planted in many other regions of the world (Ascher, 1993). It has a rich phytochemical profile and contains compounds belonging to various classes of natural products such as polyphenols, terpenoids and alkaloids (Hossain et al., 2013; Shewale and Rathod, 2018). In addition, secondary metabolites and active compounds of saponins, tannins and steroids have been characterised in neem leaves extracts (Seriana et al., 2021). *Azadirachta indica* leaves contain polyphenolic antioxidant phytochemicals such as quercetin and kaempferol (Arivazhagan et al., 2000), rutin (Sunarwidhi et al., 2014), quercetin 3-O-β-D-glucopyranoside (Tatke et al., 2014), quercetin 3-galactoside (Rao et al., 2019), ellagic and ferulic acids (Pandey et al., 2014). Furthermore, polyphenols such as trifolin, phloretin, myricetin 3-O-galactoside, p-coumaric acid and isorhamnetin have been identified in neem (Maran et al., 2021).

The plant, particularly its leaves, has several applications in the traditional system of healthcare, including antibacterial, antifungal, antiviral and antipyretic (Subapriya and Nagini, 2005). The neem leaf paste has also been found effective to treat small pox and chicken pox (Al-Hashemi and Hossain, 2016). Notably, *A. indica* leaf extracts have been found effective against lipid peroxidation and tend to exhibit significant radical scavenging activity (Chattopadhyay, 1998).

While dealing with the extraction of phytochemicals from plants and other organisms, the choice of solvent is of fundamental importance (Chemat et al., 2017). The solvents commonly used for extraction include methanol (Ogidi et al., 2021), ethanol, acetone, hexane, chloroform, ethyl acetate and dichloromethane (Saxena et al., 2021). Many of the conventional organic solvents are, however, volatile and are, therefore, bound to spread to the atmosphere during extraction process (Bubalo et al., 2015). Their large-scale application in teaching/research laboratories and industry is hazardous to the environment (Clarke et al., 2018). Many of the organic solvents also suffer from health-related safety issues (Jordan et al., 2022). These aspects necessitate search for green and safe solvents for recovery of bioactive compounds from bio-sources (Farjaminezhad and Garoosi, 2020).

Glycerol has become known as a green extraction solvent in recent years (Behr et al., 2008). The literature shows an increasing number of studies promoting glycerol as a safe, sustainable and green solvent for chemical processes (Huamán-Castilla et al., 2020; Makris and Lalas, 2020). Glycerol is moderately polar with a dielectric constant of 42.5 at 25 °C (Han, 2013) and, thus, suitable for extracting moderately polar substances such as flavonoids (Wolfson et al., 2007). One drawback of it as an extracting media is its high viscosity (1.41 Pa · s^{-1}) (Singh et al., 2020). The high viscosity of glycerol prevents its easy penetration into the plant biomass and solubilise its phytochemicals (Huang et al., 2019). The issue, however, can be conveniently resolved by using it in combination with another suitable solvent such as water (Khupse and Kumar, 2009). Glycerol is miscible in water, and the resulting binary solvent system provides a viable medium for the extraction of phytochemicals (Apostolakis et al., 2014). Recently, the efficacy of glycerol–water has been studied as an extracting medium for polyphenolic extraction from *Artemisia* species (Shehata et al., 2015), *Hypericum perforatum* (Karakashov et al., 2015) and common nettle (Kowalska et al., 2021).

Furthermore, when developing an extraction process for large-scale industrial application, it is imperative to find out the optimum conditions for the highest possible output. One of the most employed strategies for optimisation of a process is the so-called response surface methodology (RSM). It is efficient, robust and comparatively economical. To apply RSM for modelling and optimisation, either a Box–Behnken design (BBD) or central-composite design (CCD) is commonly used, both of which have their merits and demerits (Nde and Foncha, 2020). The BBD is more economical than CCD as it requires a fewer number of trials per defined number of factors are required (Shehata et al., 2021). The second-order polynomial equation is employed for optimisation as follows (Eq. 1) (Parvez et al., 2022):

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

In this equation, Y is the predicted response, β_0 is the intercept coefficient; β_i, β_{ii} and β_{ij} represent regression coefficients of linear, quadratic and cross-products, respectively; X denotes independent factor; *i, ii* and *ij* indicate linear, quadratic and cross-product terms, respectively; and k shows the number of response factors and regression coefficients determined by analysis of variance (ANOVA) (Chao et al., 2014). Models' adequacy is determined by p value and the lack of fit p value of the suggested models and their coefficients of determination (R^2) and the adjusted coefficients of determination (AjdR^2) (Babaoğlu et al., 2022).

Based on the literature survey and environmental considerations, the current project was designed with the objective to explore an efficient and green model for extraction of polyphenols and other antioxidant phytochemicals from *A. indica* leaves using the glycerol–water solvent system and applying optimisation through the BBD of RSM and to evaluate the pertinence of glycerol–water as an extracting medium for bioactive compounds from leaves of *A. indica*. It was assumed that the planned strategy would be efficient, green and cost-effective. Since glycerol is a well-known skin moisturiser (Breternitz et al., 2008) and *A. indica* leaves have been shown to have antioxidant properties (Saroyo and Arifah, 2021), a glycerol extract of its leaves may have marketability as a skin care product.

MATERIALS AND METHODS

Chemicals and equipment

Analytical grade chemicals were utilised for experimentation. 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ferrozine, rutin, ascorbic acid, sodium hydroxide, sodium nitrite, aluminium chloride, methanol, anhydrous sodium carbonate, ferrous sulphate heptahydrate, Folin–Ciocalteu reagent, glycerol and ethylenediamine tetraacetic acid (EDTA) were all supplied by Sigma-Aldrich (Steinheim, Germany).

All extractions were carried out in a VS-8480SN shaking incubator (Vision Scientific Co., Ltd., Daejeon, Korea). The absorbances for antioxidant activities were read on a Multi-Mode Micro-Plate Reader Synergy™ HTX (Winooski, VT, USA).

Preparation of plant sample

Azadirachta indica leaves were obtained from New Officers Colony, Lahore, Pakistan, in November 2020. The leaves were washed, followed by drying at room temperature (around 20 °C) for 2 weeks. The desiccated leaves were manually crushed, followed by grinding in a high-speed multifunction comminutor to acquire a fine powder. This fine powder was sieved through a 100-mesh-size sieve and refrigerated in plastic seal bags.

Single-factor experiments

To find out the most effective levels of the independent factors, single-factor experiments were performed for total phenolic content (TPC) and total flavonoid content (TFC) assays with the following parameters at 200 rpm: solvent concentration, that is, concentration of glycerol in its aqueous solution (30%, 50%, 70%), temperature used for extraction (30 °C, 60 °C, 90 °C) and time used for extraction (30 min, 60 min, 90 min). The graphical results of single factor experiment for TPC and TFC are mentioned in Figure 1.

Extreme conditions (90 min, 90 °C) were deduced to be unfavourable due to a significant drop in output; hence, the parameters (time and temperature) were reset by attenuating the maximum value, thereby readjusting the three coded levels for BBD of RSM.

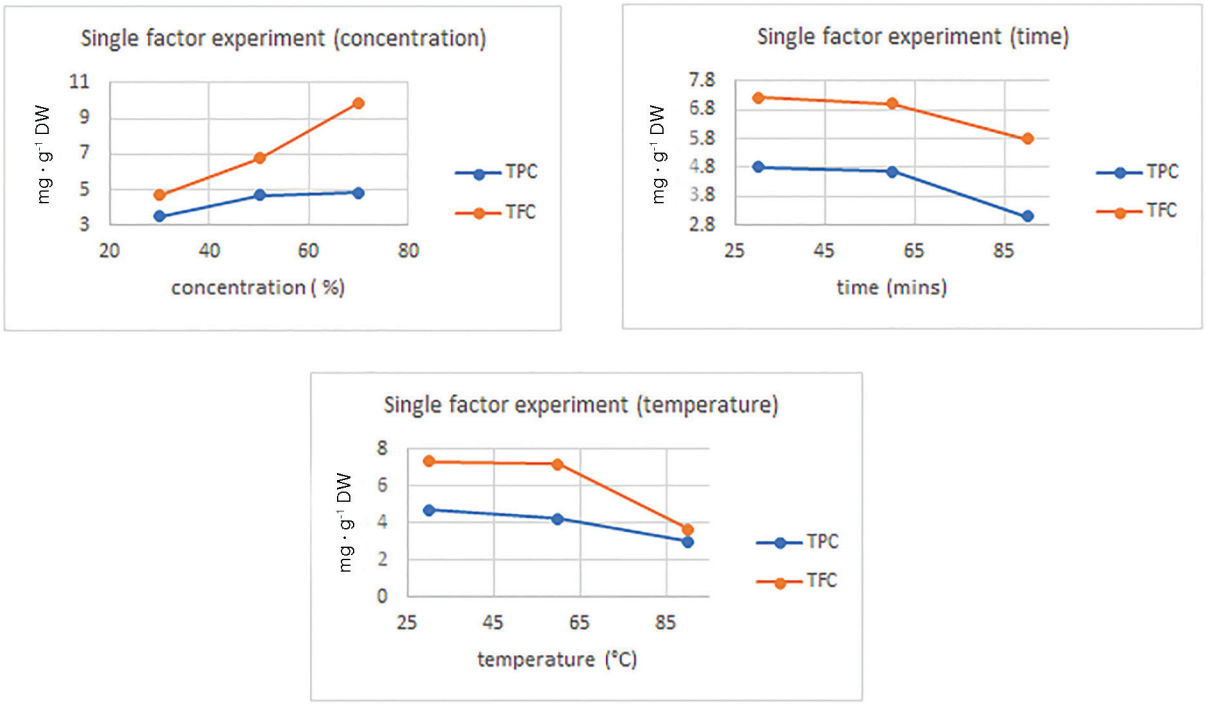

Figure 1. Single-factor experimental result for TPC and TFC. DW, dry weight; TFC, total flavonoid content; TPC, total phenolic content.

Design of experiment (DoE) as per RSM

Response surface methodology was employed according to BBD for DoE (Czyrski and Sznura, 2019). Based on a single-factor study, three independent factors were selected each with three levels (–1, 0, +1): temperature (30 °C, 40 °C, 50 °C), extraction time (20 min, 40 min, 60 min) and solvent percentage concentration (30%, 50%, 70%), and the details are shown in Table 1. Response factors were TPC, TFC, radical scavenging activity (DPPH) and metal chelating activity (MCA).

Extraction process

Measured amounts (1.0 g) of the *A. indica* leaf powder were soaked in 50 mL of solvent (of various concentrations) in 250-mL conical flasks. The conical flasks were placed in a shaking incubator for a given time and temperature at 200 rpm. Filtration of extracts through a Whatman filter paper #42 by vacuum filtration was performed. The filtrates were then collected in glass vials and used for determination of response factors.

Estimation of TPC

A reported procedure was used (Herald et al., 2012). Briefly, 25 µL of standard gallic acid dilutions (50–400 ppm) or plant extract filtrates were placed in a 96-well microplate. Then, 75 µL of deionised water and 25 µL of 10% FC reagent was added. Then, 100 µL of 7.5% Na_2CO_3 solution was added via a micro-pipette after 6 min. All the steps were carried out in the dark. The 96-well microplate was then incubated for 30 min at 37 °C. After incubation, absorbances were noted at 765 nm on a microplate reader. The standard gallic acid calibration curve was plotted with absorbance versus concentration, and an equation (y = 0.0046x + 0.1031) was obtained that was employed to assess the phenolic content (mg GAE · L^{-1}) of the *A. indica* extracts from the absorbances measured. The final results (mg GAE · g^{-1} DW) using Eq. (2) were obtained:

$$Y_{TPC} = (C_{TPC} \times V) \cdot m^{-1} \quad (2)$$

where V represents the solvent volume (L) used, C_{TPC} is the TPC (mg gallic acid equivalents · L^{-1} (mg GAE · L^{-1}) as obtained from the gallic acid calibration curve and m is the mass of the dried plant material (g).

Estimation of TFC

A reported procedure was employed to estimate the TFC of the neem leave extracts with rutin as a standard (Herald et al., 2012). Briefly, 25 µL of rutin dilutions (50–400 ppm) or plant extract filtrates were placed in a 96-well microplate. Then, 100 µL of deionised water, followed by 10 µL $NaNO_2$ solution, was added. Afterwards, 15 µL of $AlCl_3$ solution was added 5 min. Then, 50 µL 1 of M NaOH solution was added, followed by the addition of 50 µL of deionised water. After 15 min, absorbances were noted at 510 nm on a microplate reader. The absorbances of standard rutin dilutions were used to plot a rutin calibration curve. The equation (y = 0.0007x + 0.052) was used to assess the content of flavonoids (mg rutin · L^{-1}) of the *A. indica* extracts from the absorbances measured. The final results (mg RE · g^{-1} DW) using Eq. (3) were obtained:

$$Y_{TFC} = (C_{TFC} \times V) \cdot m^{-1} \quad (3)$$

where V represents the volume of the extraction solvent (L), C_{TFC} is the TPC (mg rutin equivalents · L^{-1} (mg RE · L^{-1}) as obtained from the rutin calibration curve and m is the mass of dry plant material (g).

Table 1. BBD and experimental values of response factors obtained in the analysis.

Standard order	Run order	Input factors			Responses			
		A (%)	B (min)	C (°C)	TPC	TFC	MCA (%)	ARA (%)
4	1	70	60	40	4.92 ± 0.32	10.05 ± 0.55	58.21 ± 5.00	67.14 ± 0.51
3	2	30	60	40	3.72 ± 0.15	3.67 ± 0.04	91.38 ± 0.27	23.16 ± 0.51
5	3	30	40	30	4.13 ± 0.07	5.45 ± 0.30	89.94 ± 1.30	33.94 ± 0.40
1	4	30	20	40	4.11 ± 0.08	5.67 ± 0.36	84.63 ± 1.32	41.97 ± 0.20
11	5	50	20	50	4.95 ± 0.32	8.07 ± 0.47	77.19 ± 4.66	57.85 ± 0.22
7	6	30	40	50	3.80 ± 0.25	4.55 ± 0.04	78.19 ± 3.37	26.64 ± 0.71
10	7	50	60	30	5.10 ± 0.63	7.33 ± 0.18	78.39 ± 1.48	51.25 ± 5.41
8	8	70	40	50	4.84 ± 0.91	8.88 ± 0.15	60.31 ± 0.90	74.85 ± 0.40
14	9	50	40	40	4.83 ± 0.24	7.52 ± 0.36	68.13 ± 7.14	50.97 ± 1.41
9	10	50	20	30	5.11 ± 0.29	6.81 ± 0.36	73.73 ± 8.50	58.78 ± 0.54
2	11	70	20	40	5.20 ± 0.41	10.10 ± 0.76	59.02 ± 4.24	73.91 ± 0.51
15	12	50	40	40	4.60 ± 0.72	7.07 ± 0.43	65.50 ± 10.57	48.17 ± 1.55
13	13	50	40	40	5.01 ± 0.30	6.98 ± 0.55	68.04 ± 6.61	45.30 ± 1.51
6	14	70	40	30	5.39 ± 0.29	10.14 ± 0.31	55.80 ± 5.34	74.51 ± 0.51
12	15	50	60	50	4.67 ± 0.34	7.21 ± 0.07	69.98 ± 10.83	43.23 ± 0.22

A, B and C are solvent concentration, time and temperature, respectively; units of TPC and TFC are mg GAE · g^{-1} DW and mg RE · g^{-1} DW, respectively.

ARA, anti-radical activity; BBD, Box–Behnken design; DW, dry weight; GAE, gallic acid equivalents; MCA, metal chelating activity; RE, rutin equivalents; TFC, total flavonoid content; TPC, total phenolic content.

Evaluation of radical scavenging activity

Anti-radical activity of *A. indica* leaf extracts was assessed according to a reported method (de Ancos et al., 2002; Herald et al., 2012). Briefly, 10 µL each of the extracts was placed in a 96-well microplate. Then, 90 µL of deionised water was pipetted out into each, with the addition of freshly prepared DPPH solution (0.078 mg · L^{-1}). The incubation period was 30 min maintained at room temperature (around 25 °C) in the dark, and absorbances were read at 517 nm. The control contained DPPH solution and solvent, instead of the sample. Eq. (4) was used to calculate the results, expressed as % anti-radical activity:

$$\% \text{ activity} = \left(1 - \left(\frac{Abs(sample)}{Abs(control)}\right)\right) \times 100 \quad (4)$$

Ascorbic acid was used as a positive control, and antiradical activity was also calculated in terms of mg ascorbic acid equivalents per gram of DW (mg AAE · g^{-1} DW). For this purpose, a calibration graph of ascorbic acid was plotted with % activity versus concentration, and mg AAE · g^{-1} DW was calculated from its regression equation (y = 0.2308x + 12.303).

Evaluation of MCA

A reported method was used to determine the MCA of plant extracts (Carter, 1971). Briefly, 25 µL of *A. indica* extracts were placed in a 96-well microplate. Then, 100 µL of 0.30 mmol · L^{-1} ferrous sulphate solution, followed by 0.80 mmol · L^{-1} of ferrozine solution 5 min afterwards, were added to each. After 15 min, absorbances were read at 562 nm. Ferrozine + ferrous sulphate was used as a control. Eq. (5) was used to calculate the final results, which are expressed as % activity:

$$\% \text{ MCA} = \left(1 - \left(\frac{Abs(sample)}{Abs(control)}\right)\right) \times 100 \quad (5)$$

For MCA, EDTA was used as a standard, and MCA was also calculated in terms of mg EDTA equivalents · g^{-1} DW (mg EDTAE · g^{-1} DW). For this purpose, a calibration curve of EDTA was plotted with% activity versus concentration, and mg EDTAE · g^{-1} DW was calculated from its regression equation (y = 1.0513x + 3.8956).

Analysis of data

All experiments were performed in triplicate, and means were calculated with ± standard deviation (SD). Design Expert software (Stat-Ease, Inc., Minneapolis, MN, USA) was used for statistical analysis and optimisation.

RESULTS AND DISCUSSION

Extraction of polyphenols, flavonoids, antioxidant contents and metal chelating contents from *A. indica* leaves was carried out in the green solvent aqueous glycerol, which has been used for the first time for this plant. Optimisation of extraction was performed according to the BBD of RSM. The results are shown in Table 1 and are discussed later.

TPC

The highest TPC of the neem leaves was 5.39 mg GAE g^{-1} DW, with a solvent concentration of 70%, a time period of 40 min and a temperature of 30 °C (run order 14), whereas the lowest TPC was 3.72 mg GAE · g^{-1} DW that was at a concentration of 30%, a time period of 60 min and temperature 40 °C (run order 2). The solvent concentration factor primarily contributed to this result as increasing the glycerol concentration (%) significantly increased the TPC of the extracts. The trend may possibly be explained based on polarity. Phenolics, in general, are moderately polar substances with low solubility in water; thus, as the ratio of water decreased and the ratio of glycerol increased, extraction of phenolics increased (Bao et al., 2021). However, after a glycerol concentration of 60%, the TPC extraction retarded and the curve levelled off near 70%. This behaviour can be attributed to the high viscosity of glycerol as it tends to hinder the diffusion of the solvent into the plant material, thereby affecting mass transfer and, hence, extraction (Shehata et al., 2015). Moreover, with increase in time and temperature beyond a certain threshold, the amount of phenolics decreased. This could be mainly due to the degradation of thermal-sensitive polyphenols that may occur upon a prolong exposure of the plant material to the solvent at high temperatures. It has been noted that polyphenols tend to degenerate at prolonged exposure to heat; hence, their extraction yield is reduced (Khemakhem et al., 2017). Higher TPC values for greater glycerol concentrations (20%–90%) were observed for *Artemisia* species (Shehata et al., 2015).

Earlier studies on neem leaves reported different values of TPC in other solvents (Shewale and Rathod, 2018). The results of TPC in methanol, ethyl acetate, n-butanol, deionised water and ethanol were 13.54 mg GAE · g^{-1} DW, 12.66 mg GAE · g^{-1} DW, 10.99 mg GAE · g^{-1} DW, 4.70 mg GAE · g^{-1} DW and 11.95 mg GAE · g^{-1} DW, respectively. The results denote the importance of solvent polarity for the extraction of polyphenols. In a recent optimisation study on Nigerian neem using deionised water as a solvent, TPC at optimised conditions (extraction time 2.79 h, process temperature 40.54 °C), solid–liquid concentration (0.01 g · mL^{-1}) was 1.27 mg GAE · g^{-1} DW TPC (Oke et al., 2020). The finding was much lower than the 70% aqueous glycerol used in the present study. The difference in results in different studies may be due to a host of factors, including solvent and extraction conditions (Kowalska et al., 2021).

TFC

The highest TFC was 10.14 mg RE · g^{-1} DW, which was obtained under the same parameter that gave the highest TPC, that is, a solvent concentration of 70%,

a time period of 40 min and a temperature of 30 °C (run order 14). The lowest TFC was 3.67 mg RE · g^{-1} DW, which was also at the same parameters as for the lowest TPC (solvent concentration 30%, time 60 min and temperature 40 °C; run order 2). The solvent concentration factor highly contributed to this result as increasing the glycerol concentration (%) significantly increased TFC of the extracts. Reasons like those given for TPC can be extended for TFC as flavonoids are a subclass of polyphenolics (Rupasinghe et al., 2011). Moreover, with the increase in time and temperature, the flavonoid content only negligibly decreased. This could be mainly due to the relative thermal stability of the flavonoids present in *A. indica* leaves (Chaaban et al., 2017). Nonetheless, the decrease did occur, and that may be the decomposition of flavonoids (Naczk and Shahidi, 2006). Another also substantiated the results that reported that anthocyanins (a type of flavonoids) extracted from black currants showed a depleted yield at temperature >30 °C–35 °C, thereby indicating temperature sensitivity (Cacace and Mazza, 2003). Notably, TFC showed a significant correlation with TPC as the maximum and minimum yields for both were obtained at the same conditions. The efficacy of glycerol–water as an extracting medium for flavonoids has been shown in an erstwhile study as well as that proclaimed that hydro-glycerol extracts compared to aqua-alcohol extracts yielded higher TFC values for peppermint leaves (Kowalska et al., 2021). In a recent study, the TFC of neem leaves was 26.09 mg RE · g^{-1} DW when using deionised waster as a solvent under the optimised conditions (Oke et al., 2020). When compared with the results of the present study, this may be taken to mean that deionised water is more efficient for the TFC than for the TPC.

DPPH radical scavenging activity

The highest DPPH radical scavenging activity of the neem leaf extract was 74.85% (271.00 mg AAE · g^{-1} DW), which was obtained at a solvent concentration of 70%, a time period of 40 min and a temperature of 50 °C, whereas the lowest % activity obtained was 23.16% (7.04 mg AAE · g^{-1} DW), which was at a solvent concentration of 30%, a time period of 60 min and a temperature of 40 °C. Here again, the solvent concentration factor appeared primarily contributing to this result as increasing the glycerol concentration (%) significantly increased the % activity of the extracts. A greater % concentration of glycerol provided a more suitable and efficient medium for the extraction of polyphenols, which exhibit high DPPH radical scavenging activity (Phuyal et al., 2020). An increase in the extraction time decreased the activity mainly because of the degradation of compounds under the interaction of solvent (Khemakhem et al., 2017). However, the increase in extraction temperature had a negligible effect probably due to the thermal stability of phenolic compounds, which gradually decomposed at high temperatures (Chaaban et al., 2017).

The radical scavenging activity showed a positive correlation with the TPC and TFC. The highest TPC and TFC values were obtained at run order 14, whereas for DPPH activity, run order 14 provided the second highest activity. The highest activity was provided by run order 8 with an insignificant difference between both the activity values. The difference between 8 and 14 run orders was that of temperature. Run order 8 had +1 coded temperature value, whereas run order 14 had −1 coded temperature value. However, temperature had a negligible effect on the activity. Moreover, the least values for TFC, TPC and DPPH activities were provided by run order 2.

2,2-diphenyl-1-picrylhydrazyl has been widely used to investigate the antioxidant capacity of plant extracts. Many previous studies reported DPPH to have a positive correlation with TPC and TFC; for instance, antioxidant properties of flour from pearl millet varieties showed a similar trend (Siroha et al., 2016).

The results of a study on Nigerian neem leaves showed different values of DPPH radical scavenging activity in different solvents (acetone (85.52%) > methanol (84.13%) > ethyl acetate (82.10%) > ethanol (81.49%) (Anokwuru et al., 2011). The findings demonstrated the role of solvents in extracting the biologically active natural products from plants. The issue with most organic solvents, however, is their impact on the ecosystem. Being volatile, they are bound to diffuse into the atmosphere, causing environmental hazards. Moreover, many organic solvents, such as methanol, also have high toxicity levels and, thus, should be avoided.

MCA

The MCA of neem leaf extracts was investigated in terms of iron chelating activity. The highest % MCA was 91.38% (83.22 mg EDTAE · g^{-1} DW), which was at a solvent concentration of 30%, a time period of 60 min and a temperature of 40 °C run order 2), while the lowest % MCA was 55.8% (49 mg EDTAE · g^{-1} DW), which was at a solvent concentration of 70%, a time period of 40 min and temperature 30 °C (run order 14). The solvent concentration factor primarily contributed to this result as increasing the glycerol concentration (%) significantly decreased % MCA. Furthermore, greater time durations and temperatures supported % MCA, despite the decrease at the mid-point values. All in all, MCA showed a opposite correlation with the TFC and TPC since run order 2 gave the maximum value for MCA but a minimum value for the TPC and TFC, whereas run order 14 gave a minimum value for MCA but a maximum value for the TPC and TFC. Lower glycerol concentrations favouring greater % MCA could be due to the non-phenolic extraction, showing chelating activity (Tungmunnithum et al., 2018). Their extraction could have been more efficient as solvent polarity increased with the increase in the water ratio. Furthermore, the increase in MCA with the increase in time and temperature towards the +1 coded level

could be primarily due to the high molecular weight compounds, which were facilitated with enough energy to dissolve and diffuse into the extraction medium (Sharma et al., 2015).

Previous study have reported that *Moringa oleifera* extracts did not show a good value of MCA in comparison to *A. indica* but showed good correlation with the TPC and TFC. The *Azadirachta indica* ethanolic leaf extract showed high values of MCA but reported poor correlation with the antioxidant content (Ekaluo et al., 2015).

ANOVA

With software (Design Expert), ANOVA was carried out for model fitting and optimisation, and the outcomes of the analysis are displayed in Table 2.

Model fitting and optimisation (TPC)

The regression equation for TPC while considering only the significant terms ($p < 0.05$) is given in Eq. 6:

$$TPC = 4.81 + 0.5737A - 0.1837C - 0.3717A^2 \quad (6)$$

A quadratic polynomial model was, thus, suggested. For it, R^2 was 0.9699, and there was a good correspondence amongst R^2, predicted R^2 and adjusted R^2, which showed high fitness of the model (Table 2). Analysis of variance showed a significant p value (0.0027) and nonsignificant lack of a fit p value (0.8729), indicating that the model is well fitted. As the p values of these terms implied, the TPC was significantly influenced by A (solvent concentration) and C (temperature), while the quadratic effect of solvent concentration (A^2) was also significant. The 3D surface plots for TPC are shown in Figure 2, which show how the TPC (the response variable) relates to any of the two independent variables. The peaks and valleys on the surface plots correspond with combinations of the independent variables (shown on x and y axes) that result in local maxima or minima.

Figure 2A shows the combined effect of solvent concentration (%) and time on TPC. The 3D surface plot reflects that the increasing solvent concentration from 30% to 70% within a time frame of 20–30 min, which significantly increases the TPC. The maximum TPC is found between solvent concentrations of 50% and 70%. Very low yields are observed at a glycerol–water concentration of 30%, with a change in extraction time having no effect on the TPC at low solvent concentrations. As the extraction time increased, the TPC decreased, which showed that high solvent concentrations at low extraction time provide optimal results.

Figure 2B demonstrates the combined effect of temperature (°C) and solvent concentration (%) on the TPC. At high glycerol concentrations of 50%–70% and low temperatures of 30 °C–35 °C, optimal yields of TPC are observed. An increase in the solvent concentration and temperature enhances TPC, but as the temperature increased above 40 °C, a drop in TPC is observed. Minimal values are observed at low glycerol–water concentrations of 30%–40%, which reflects that the solvent concentration plays a crucial role in this interaction.

Figure 2C shows the combined effect of temperature (°C) and extraction time (min) on the TPC. It indicates that the TPC decreased as the extraction time and

Table 2. Results of the ANOVA for model fitting and optimisation of extraction as per the three-factor BBD of RSM.

Source	*p values*			
	TPC (quadratic)	TFC (linear)	MCA (quadratic)	DPPH (linear)
Model	0.0027*	<0.0001*	0.0002*	<0.0001*
A-concentration	0.0001*	<0.0001*	<0.0001*	<0.0001*
B-Time	0.0736	0.18	0.5736	0.0007*
C-Temperature	0.0181*	0.5536	0.0828	0.1511
AB	0.7295		0.1162	
AC	0.4973		0.0095*	
BC	0.4104		0.0308*	
A^2	0.0051*		0.3132	
B^2	0.5835		0.0051*	
C^2	0.2644		0.0494*	
Residual				
Lack of fit	0.8729	0.1821	0.3174	0.4079
		R^2 values		
R^2	0.9699	0.9288	0.989	0.9621
Predicted R^2	0.9156	0.9094	0.9692	0.9517
Adjusted R^2	0.8274	0.8513	0.8578	0.9313

*p values < 0.05 were considered as significant.
ANOVA, analysis of variance; BBD, Box–Behnken design; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MCA, metal chelating activity; RSM, response surface methodology; TFC, total flavonoid content; TPC, total phenolic content.
For each response, the optimisation analysis is discussed here.

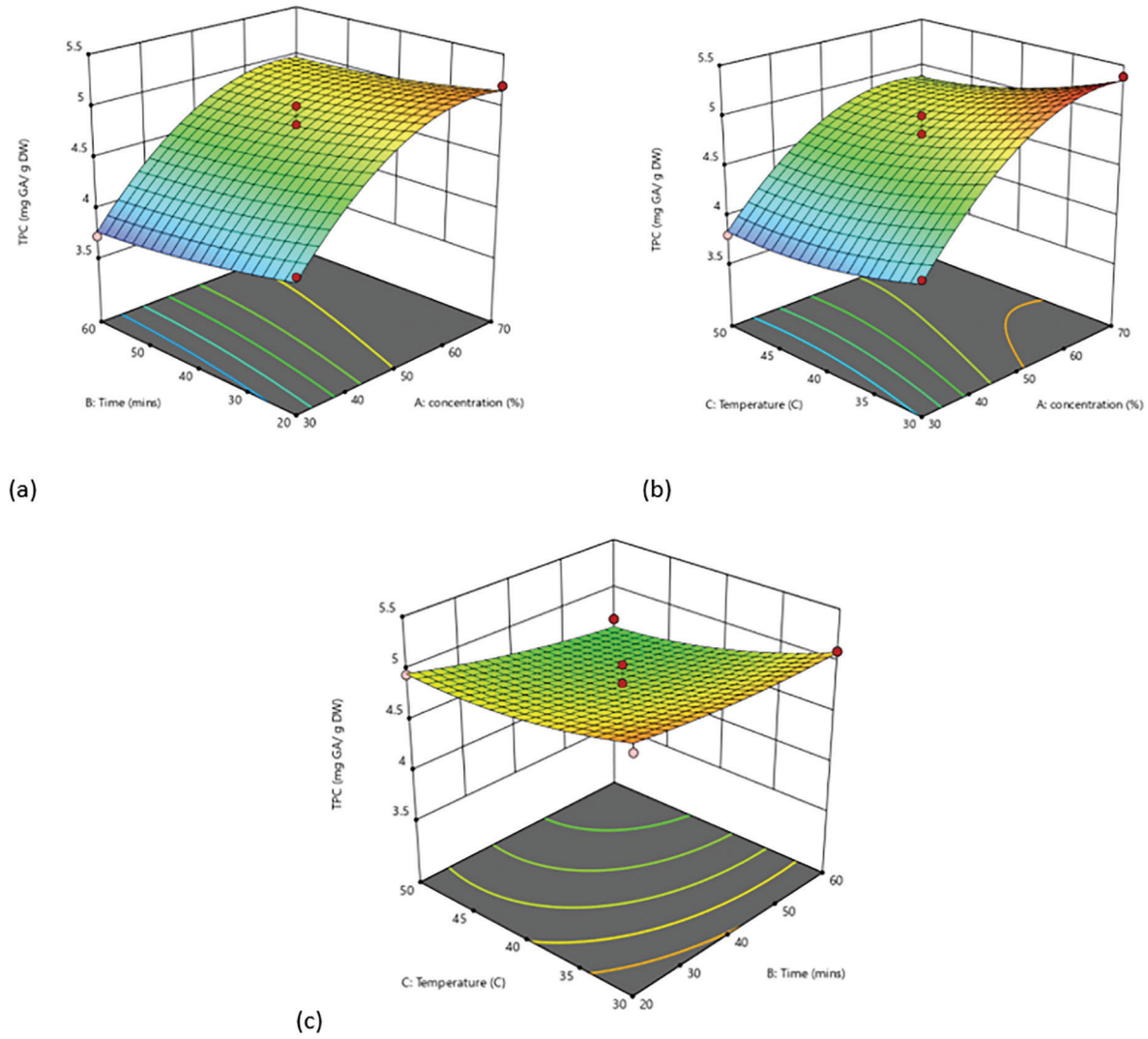

Figure 2. TPC 3D surface plots for interactive effect of (A) solvent concentration and extraction time, (B) solvent concentration and extraction temperature and (C) extraction temperature and extraction time. TPC, total phenolic content.

temperature increased in the range between 40 min and 60 min and 30 °C–50 °C.

Model fitting and optimisation (TFC)

The regression equation for the TFC while considering only the significant terms ($p < 0.05$) is given in Eq. 7:

$$TFC = 7.30 + 2.48A \quad (7)$$

Thus, it is a linear model with only A as significant. It had a high value of R^2 (0.9288), and there was a good correspondence amongst R^2, predicted R^2 and adjusted R^2, which showed high fitness of the model (Table 2). Furthermore, the model was significant having a p value of < 0.0001 and lack of fit p value 0.1821. Terms with $p < 0.05$ were indicated significant, and their coefficients were considered for optimisation. For TFC, A (solvent concentration) was the significant term. The 3D surface plot for TFC is shown in Figure 3.

Figure 3 shows a combined effect between extraction time (min) and solvent concentration (%) on TFC. An increase in the solvent concentration from 30% to 70% significantly enhances the TFC with optimal values observed in the range between 65% and 70% glycerol concentrations and a 20–50 min time. Thus, solvent concentration is indicated to play a major role in enhancing the TFC, while time is not a significant factor.

Model fitting and optimisation (radical scavenging activity)

The regression equation for DPPH radical scavenging activity while considering only the significant terms ($p < 0.05$) is given in Eq. 8:

$$\%\ DPPH = 51.44 + 20.59A - 5.97B \quad (8)$$

A linear model was suggested with R^2 0.9621. Analysis of variance showed a significant model p value (< 0.0001) and nonsignificant lack of fit p value (0.4079),

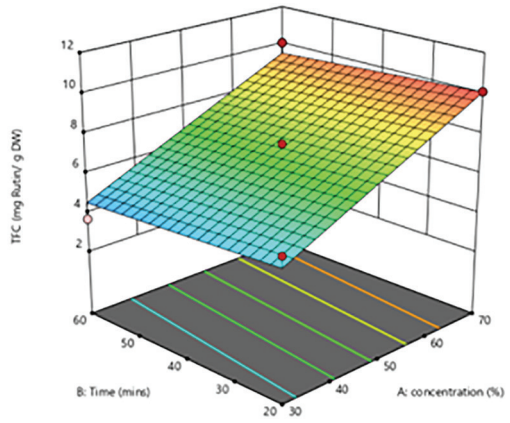

Figure 3. TFC3D surface plot for the interactive effect of solvent concentration and extraction time. DW, dry weight; TFC, total flavonoid content.

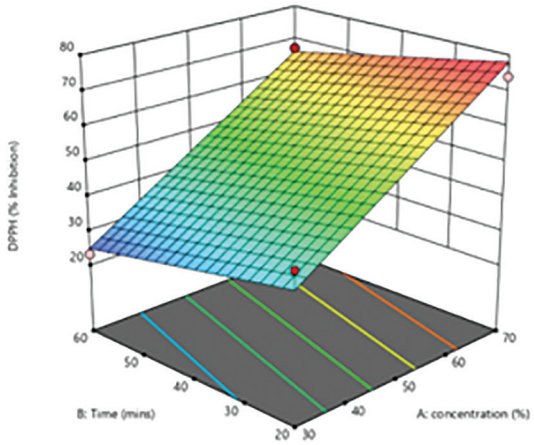

Figure 4. ARA 3D surface plot for interactive effect of solvent concentration and extraction.

indicating the model to be well fitted. Terms A (solvent concentration) and B (time) were found to be significant. The 3D surface plot for DPPH radical scavenging activity is shown in Figure 4.

As Figure 4 shows solvent concertation is heavily influencing the extraction of antioxidant compounds from the neem leaves, while the activity slightly decreases with time. Thus, high solvent concentration at a low time duration produces a better result than high solvent concentration at a long duration. It might be because of a possible degradation of antioxidant compounds upon a long exposure to solvent (Che Sulaiman et al., 2017).

Model fitting and optimisation (MCA)

Regression equation for MCA while considering significant terms ($p < 0.05$) is given in Eq. 9:

$$\% \text{ MCA} = 67.22 - 13.85A + 4.07AC - 2.97BC + 4.92B^2 + 2.67C^2 \quad (9)$$

A quadratic polynomial model was suggested with a significant p value (0.0002) and nonsignificant lack of a fit p value (0.3174). The high coefficient of determination (R^2 0.9890) and its high correspondence with the adjusted and predicted R squared values also supported the suggested model. MCA was significantly influenced by linear, quadratic and interactive factors, and A, AC, BC, B^2 and C^2 were the significant terms. The 3D surface plots for MCA are shown in Figure 5.

Figure 5A shows the combined effect of solvent concentration (%) and time. It shows that MCA is significantly decreased as glycerol concentration is increased from 30% to 70%. Optimal values are observed at a solvent concentration of 30%–35% and an extraction time of 55–60 min; however, time is not a significant factor for extracting metal chelating compounds in this solvent system.

Figure 5B demonstrates the combined effect of temperature (°C) and solvent concentration (%) on MCA. It resembles the aforementioned trend with minimal values observed in a range between 55% and 70% glycerol–water and 20–60 min extraction time. However, optimal MCA is observed at a low glycerol–water concentration of 30%–35% and a decreased extraction time of 30–35 min. It shows that MCA significantly decreased as the extraction time and solvent concentration are increased in the range between 20 min and 60 min and 30%–70%, respectively.

Figure 5C shows the combined effect of temperature (°C) and extraction time (min) on MCA. It demonstrates that MCA was insignificantly influenced by this interactive effect. Varying time and temperature in the range between 20 min and 60 min and 30 °C–50 °C did not significantly enhance MCA and shows a constant MCA with minute variations.

Numerical optimisation and validation study

A set of optimised conditions for all the response factors was determined by a statistical operation called the numerical optimisation (Jabraili et al., 2021; Santiago et al., 2021; Ozguven and Ozturk, 2022). The operation was done on the Design Expert software referred to above by setting on the software the input factors at "in range" goal and all the responses at "none" goal with the importance value as 3. The targeted "desirability" was 1, which is the highest value. The operation provided a series of solutions, and the one with the highest desirability was selected as per the suggestion made by the software.

As per the selected solution, the optimum conditions were a solvent concentration of 69.713%, an extraction time of 38.328 min and temperature of 32.114 °C. The predicted values of all the responses under the selected solution are shown in Table 3.

To verify the reliability of optimisation and model validation, experiments were performed under the predicted optimised conditions, and the results are shown in Table 3. As the table shows, there was a strong correspondence between the predicted and

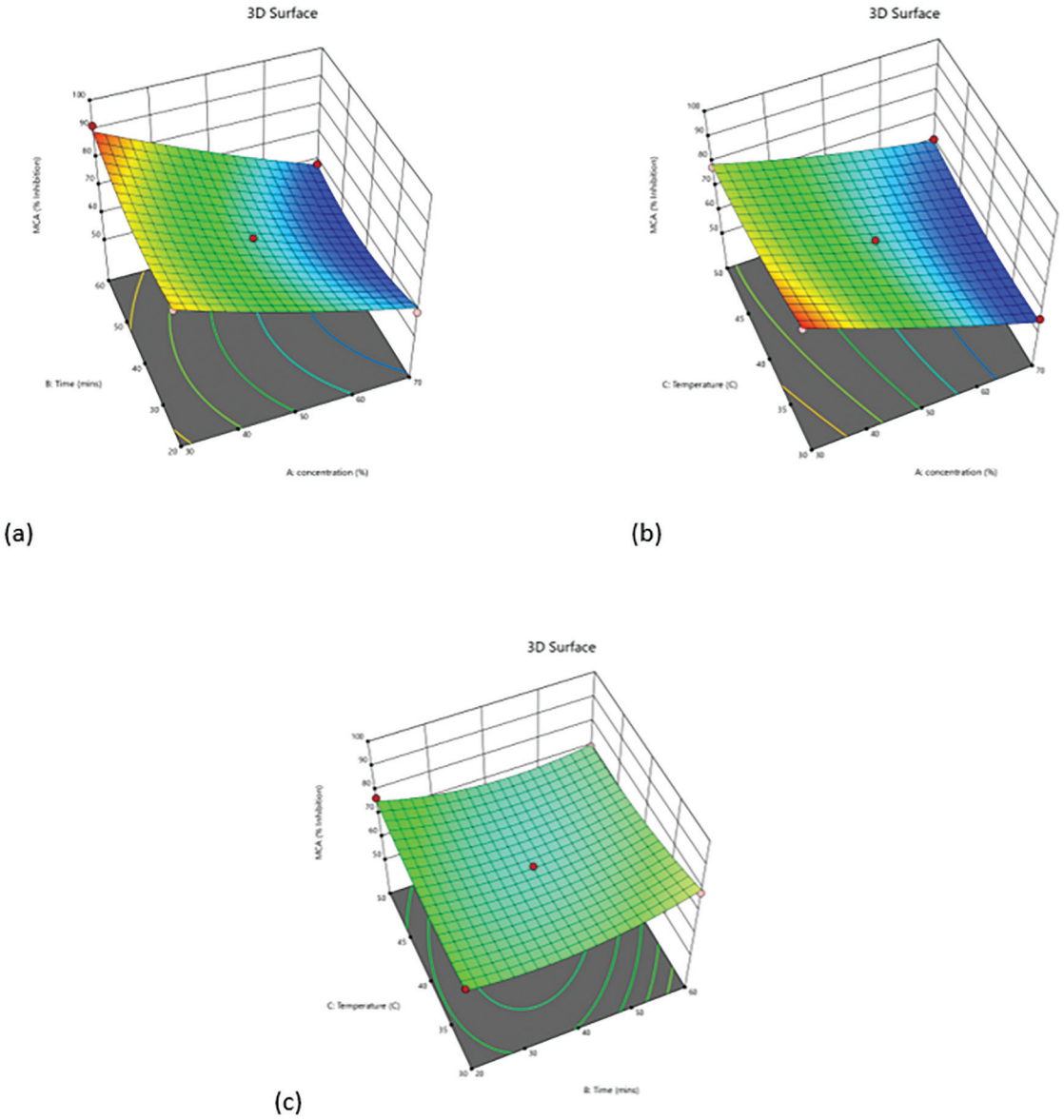

Figure 5. MCA 3D surface plots for interactive effect of (A) solvent concentration and extraction time, (B) solvent concentration and extraction temperature, and (C) extraction temperature and extraction time. MCA, metal chelating activity.

Table 3. Predicted and observed response values and their corresponding percent errors.

Responses	Predicted value	Observed value	Error (%)
TPC (mg GAE · g^{-1} DW)	5.27	4.937	6.318
TFC (mg RE · g^{-1} DW)	9.869	9.333	5.431
MCA (%)	54.366	50.378	7.335
DPPH (%)	73.804	79.458	-7.661

DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; GAE, gallic acid equivalents; MCA, metal chelating activity; RE rutin equivalents; TFC, total flavonoid content; TPC, total phenolic content.

observed result values with small% errors (5.431–7.661). Therefore, the proposed model was strongly validated by the experimental data (Jiskani et al., 2021).

CONCLUSIONS

The extraction optimisation of polyphenols, antioxidant and metal chelating bioactive compounds from *A. indica* leaves in the glycerol–water solvent system via heat-assisted maceration was conducted by RSM. For TPC, TFC and antiradical activity, higher glycerol concentration was significant, while for MCA, the water ratio was more important. The validation experiments showed almost the same results, as predicted by optimisation for each response with very low% error.

The study supports glycerol–water to be a low cost, efficient and green solvent system for extracting bioactive compounds. The validated model (maceration of dried powdered neem leaves in 70% glycerol at 32 °C for 38 min with 200 rpm shaking) can be projected

as a viable strategy for extraction of bioactive natural products from *A. indica* leaves for industrial applications.

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AUTHOR CONTRIBUTIONS

M.A. contributed to methodology, experimentation, literature review and data interpretation and wrote the manuscript. D.A. designed the research and supervised it and contributed to data analysis and manuscript writing. N.A. carried out data interpretation, statistical analysis and optimisation. All the authors approved the final version.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have exerted an influence on the study reported in this paper.

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