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Optimization of phenolic yield of *carthamus oxyacantha* (bieb.) leaves and flowers extracts pertaining to the antioxidant and antidiabetic effect by response surface methodology

Hosh Muhammad Lashari[*1], AH Memon[1], Sana Sarfraz[2], Qurat ul Ain[2], Muhammad Akram[3], Madan Lal Maheshwari[4] and Sana Javaid Awan[5]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Sindh, Jamshoro, Pakistan
[2]Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Karachi, Pakistan
[3]Department of Pharmacology, Faculty of Pharmacy, University of Sindh, Jamshoro, Pakistan
[4]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sindh, Jamshoro, Pakistan
[5]Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan.

Abstract: The study was designed to establish the pharmacognostic and pharmacological validation of *Carthamus oxyacantha* (*C. oxyacantha*) leaves and flowers crude extracts, the optimization of crude extracts as per Box-Behnken's experimental design, indicated that ethanolic leaves and flowers extracts of *C. oxyacantha* prepared at extraction conditions of run # 3 and 8 (ELE-3 and EFE-8) respectively, presented the highest level of dependent variables, while, the alpha-amylase IC_{50} value (5.976 µg/mL) of ELE-3 was relatively closer to that of standard acarbose (3.524 µg/mL). Chromatographic fingerprinting of ELE-3 resulted in the identification of bioactive phenolic compounds (quantitatively; 11.47 µg/mL of gallic acid and 8.27 µg/mL of quercetin). Furthermore, acute oral toxicity of ELE-3 suggested the LD_{50} > 2000 mg/kg. The findings of an *in vivo* antidiabetic study indicated that ELE-3 (100 and 500 mg/kg) produced a significant reduction in alloxan induced hyperglycemia of diabetic rats. Thus, the present study demonstrated the pharmacognostic and pharmacological implications of *C. oxyacantha* leaves and flowers crude extracts and revealed the remedial role of optimized extract (ELE-3) for the management of diabetes mellitus owing to its oxidative stress-lowering tendency and presence of bioactive phenolic compounds.

Keywords: *C. oxyacantha*; Optimization, Chromatographic fingerprinting, Acute oral toxicity, Antidiabetic.

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INTRODUCTION

Since the prehistoric times plants have been pursued as a frequently accessible solution to human illnesses. As per WHO estimates, around 21000 plant species have been recognized for exhibiting therapeutic potential (Parfait and Lawrence, 2023). *C. oxyacantha*, a flowering species of the family Asteraceae, is well known for the wide range of therapeutic and commercial uses. Several bioactive compounds have been recognized in aerial parts of this plant (Baban *et al*., 2023).

The prerequisite for the attainment of a biologically active extract is the extraction process; the absoluteness of this step tends to capitalize the extract with the content of targeted constituents (Phull and Gupta, 2023). Since various studies have shown that extraction efficiency is influenced by multiple parameters (particle size of the powdered plant material, ratio of the solvent to powdered plant material, extraction time, extraction temperature and type of the extraction solvent) (Wu *et al*., 2021). Considering this purview of interdependency of these parameters, it is quite difficult to recommend the conditional scale for any of them in their individual capacity, so the optimum interaction of these parameters during the entire extraction process is pivotal to rule out the effectiveness of extraction conditions. (Phull and Gupta, 2023, Turker and isleroglu, 2023). RSM (Response-surface methodology) has been frequently employed to achieve the simultaneous interaction of several response-influencing independent variables in a specific set of experiments (Feudjio *et al*., 2022). The present study involves the exploration of optimum extraction conditions yielding maximum amount of crude extract (CE) yield, total phenolic content (TPC), total flavonoid content (TFC) and showing amplified capacity of antioxidants in *C. oxyacantha* extracts by using Box-Behnken's experimental design of RSM and antidiabetic potential of selected extract is analyzed thereon.

MATERIAL AND METHODS

Chemicals

Solvents of analytical grade; (Chloroform [CF], ethanol [EtOH], n-Hexane [HEX]) and HPLC grade; (Ethylacetate [EA], formic acid [FA], acetonitrile [ACN], toluene [TOL], methanol [MeOH] Riedel-de Haen, Seelze Germany, double distilled water (DDW) and chemicals of analytical grade; (3,5-dinitrosalicylic acid [DNSA], gallic

*Corresponding author: e-mail: hoshmuhammadlashari@gmail.com

acid [GA], ascorbic acid [AA], quercetin [QT], acarbose, rutin [RT], Folin-Ciocalteau [FC], carboxymethylcellulose and alloxan monohydrate Sigma/ Aldrich/ Merck, Germany, were utilized in this study.

Collection of plant material

The plant material was collected in February 2020 from Tehsil Noorpur Thal, District Khushab, Punjab, Pakistan and authenticated (Vide. Voucher Specimen# 154-AST Department of Botany, University of Gujrat, Punjab, Pakistan). Moreover, leaves and flowers of plant were washed, shade driedand then the powdered crude drug was stored for further use.

Preparation of extract

RSM experimental design Box-Behnken's method (table-1) was adopted for the preparation of ethanolic leaves and flowers extract of *C. oxyacantha.* Drug-solvent ratio (X1) [:], extraction temperature (X2) [ºC] and extraction time (X3) [h] were set as independent variables within the combination of different ranges for the preparation of extracts, which were then trialed for the optimization study in terms of CE yield, TPC, TFCand total antioxidant activity-reducing power assay (TAA-RPA) (Hasni *et al*., 2021).

Crude extract yield

The CE yield of *C. oxyacantha* (ELE and EFE) was determined by getting the mass of concentrated extract and then the formula: CE yield% = W_1 / W_2 × 100. Where W_1 = weight of concentrated crude extract, W_2 = weight of powdered crude drug sample, was used for the calculation (Nofita *et al*., 2022).

Total phenolic content

TPC of ELE and EFE of *C. oxyacantha* was assessed by using colorimetric (FC method) (Molole *et al*., 2022). An aliquot of 500 µL of each extract was added with FC reagent (1.5 mL), the reaction mixture was incubated for 5 minutesand afterward, Na_2CO_3 10% (1 mL) solution was added. Total volume was adjusted up to 5 mL with DDW, the reaction mixture was then placed in the dark for 30 minutesand absorbance was recorded with a UV-Vis spectrophotometer at λmax 760 nm. TPC was estimated by using the regression equation of the standard GA (2-10 µg/mL) calibration curve and expressed as mg GAE/g extract.

Total flavonoid content

The $AlCl_3$ colorimetric assay (Orsavova *et al*., 2023) was followed for the determination of TFC of *C. oxyacantha* ELE and EFE. 500 µL of each extract was added to EtOH (1.5 mL) followed by subsequent addition of $AlCl_3$ 5% (0.1 mL) and 1 M-CH_3COONa (0.1 mL), thus, adjusting the total volume up to 5 mL with DDWand then the reaction mixture was placed in the dark for 40 minutes. Absorbance of the reaction mixture was recorded with a UV-Vis spectrophotometer at λmax 415 nm. TFC was estimated by using the regression equation of the standard QT (2-10 µg/mL) calibration curve and expressed as mg QTE/g extract.

Total antioxidant activity-reducing power assay

The antioxidant tendency of ELE and EFE of *C. oxyacantha* was evaluated by following the method of reducing power assay (Sobuj *et al*., 2021). An aliquot of 500 µL of each extract was mixed with phosphate buffer pH 6.6 (1 mL) and $C_6N_6FeK_3$ 1% (1 mL), followed by incubation of the reaction mixture (50°C and 20 minutes). Afterward, $C_2HCl_3O_2$ 10% (1 mL) and $FeCl_3$ 0.1% (0.2 mL) were added to the reaction mixture, thus, adjusting the total volume up to 5 mL with DDW. Absorbance of reaction mixture was recorded with a UV-Vis spectrophotometer at λmax 700 nm. TAA-RPA was estimated by using the regression equation of the standard AA (2-10 µg/mL) calibration curve and expressed as mg AAE/g extract.

Alpha-amylase inhibitory assay

The alpha-amylase inhibition of *C. oxyacantha* (ELE and EFE) was evaluated as described by (Giri *et al*., 2023). Test dilutions of standard/sample (2-10 µg/mL) were mixed with alpha-amylase solution 13 units/mL (0.1 mL) and sodium phosphate buffer solution (0.5 mL). The reaction mixture was incubated at 37°C for 15 minutes, afterward, soluble starch 1% solution (1 mL) was added and mixture was incubated at 37°C for 15 minutes and then the enzymatic action was ended with the addition of DNSA solution (1 mL) and the test tubes containing solution were boiled at 100ºC for 5 minutes. Upon cooling, the absorbance was recorded with a UV-Vis spectrophotometer (λmax 540 nm); the preparation of the enzymatic control was almost similar, however, excluding the addition of inhibitor. The % of alpha amylase inhibition was calculated by the following formula;

% Inhibition = AC–AS / AC×100, Where AC = Absorbance of enzymatic control and AS = Absorbance of test sample/standard.

Chromatographic fingerprinting

Thin layer chromatography

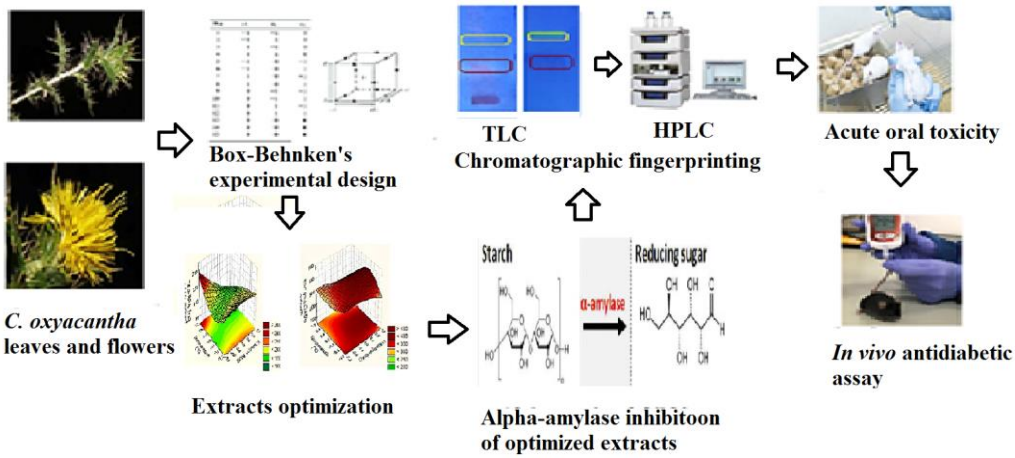
The identification of bioactive phenolics in the most potent *C. oxyacantha* extract was assessed by a planar chromatographic technique, i.e., thin layer chromatography (TLC) as described by (Giri *et al*., 2023, Kowalska and Sajewicz, 2022) with slight modifications.

Preparation of chromatographic plates

Aluminum-supported TLC plates packed with silica (Merck, Germany) of dimensions (10×10 cm) were purchased.

Preparation of working solutions

5 mL standard solution (GA and QT 1000 µg/mL) and 5 mL extract solution (5000 µg/mL) were prepared in EtOH, respectively.



Graphical abstract

Table-1: Experimental design for preparation of extracts at variable extraction conditions

Run#	X1	X2	X3
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

X1 indicates drug-solvent ratio, X2 indicates extraction temperature, and X3 indicates extraction time (-1, 0 and 1 encode the 1:10, 1:20 and 1:30 for X1, 30ºC, 45ºC and 60ºC for X2 and 12h, 18h and 24h for X3, respectively).

Table 2: Box-Behnken's experimental design and results of dependent variables for various ELE of *C. oxyacantha*.

Run#	Experimental design			Dependent variables			
	X1	X2	X3	CE yield (%)	TPC (mg GAE/g extract)	TFC (mg QTE/g extract)	TAA-RPA (mg AAE/g extract)
1	-1	-1	0	6.10	247.01 ±0.02	211.51 ±0.02	206.25 ±0.02
2	1	-1	0	8.60	286.93 ±0.03	213.62 ±0.05	221.22 ±0.03
3	-1	1	0	3.80	471.56 ±0.02	251.69 ±0.03	274.45 ±0.03
4	1	1	0	7.70	149.70 ±0.01	93.06 ±0.02	113.11±0.04
5	-1	0	-1	3.60	159.68 ±0.02	156.51 ±0.03	126.41 ±0.02
6	1	0	-1	5.40	164.67 ±0.02	126.90 ±0.03	143.05 ±0.01
7	-1	0	1	2.00	266.97 ±0.04	179.78 ±0.02	209.58 ±0.02
8	1	0	1	1.60	259.48 ±0.02	171.32 ±0.02	206.25 ±0.01
9	0	-1	-1	7.90	276.95 ±0.05	209.39 ±0.04	224.55 ±0.02
10	0	1	-1	0.30	284.43 ±0.04	184.01 ±0.06	207.92 ±0.02
11	0	-1	1	8.00	276.95 ±0.04	245.35 ±0.03	236.19 ±0.03
12	0	1	1	4.40	197.11 ±0.04	105.75 ±0.01	151.36 ±0.03
13	0	0	0	3.50	222.06 ±0.04	133.25 ±0.02	174.65 ±0.02
14	0	0	0	3.30	212.08 ±0.06	131.13 ±0.01	169.66 ±0.07
15	0	0	0	3.60	202.10 ±0.03	126.90 ±0.01	159.68 ±0.02

Each value of the dependent variables represents the mean ±SD of triplicates.

Table 3: Model coefficients and regression adjustment of ELE of *C. oxyacantha*.

	P-value				Regression coefficient			
	CE yield	TPC	TFC	TAA-RPA	CE yield	TPC	TFC	TAA-RPA
Mean intercept	0.001^{***}	0.001^{***}	$0.0001^{\#}$	0.001^{***}	3.467	212.076	130.429	167.997
X_1	0.044^{*}	0.912	0.028^{*}	0.475	0.350	-0.624	-9.518	3.327
X_1^2	0.021^{*}	0.099	0.009^{*}	0.818	0.542	15.282	17.273	1.040
X_2	0.001^{***}	0.068	0.002^{**}	0.022^{*}	-2.800	-18.089	-41.244	-25.366
X_2^2	0.001^{***}	0.007^{*}	0.001^{***}	0.013^{*}	2.542	61.440	44.769	34.722
X_3	0.005^{**}	0.048^{*}	0.023^{*}	0.098	1.050	-21.831	-10.575	-11.228
X_3^2	0.008^{*}	0.106	0.023^{*}	0.622	-0.858	-14.658	10.928	2.287
X_1X_2	0.044^{*}	0.003^{**}	0.002^{**}	0.007^{*}	0.350	-90.444	-40.186	-44.079
$X_1X_2^2$	0.007^{*}	0.010^{*}	0.006^{*}	0.018^{*}	1.250	-69.860	-29.611	-39.920
$X_1^2X_2$	0.003^{**}	0.030^{*}	0.011^{*}	0.104	2.000	39.920	21.151	15.386
X_1X_3	0.019^{*}	0.596	0.082	0.321	-0.550	-3.119	5.288	-4.990
$X_1^2X_3$	0.002^{**}	0.009^{*}	0.007^{*}	0.012^{*}	-2.400	72.355	27.496	47.821
X_2X_3	0.006^{*}	0.048^{*}	0.003^{**}	0.047^{*}	1.000	-21.831	-28.553	-17.049
MS Residual	0.023	99.601	10.438	58.100				
R^2	0.9994	0.9976	0.9994	0.9958				
R^2_{adj}	0.9964	0.9837	0.9957	0.9707				
$R^2_{Predicted}$	0.687	0.322	0.595	0.376				

P-value of <0.05 is considered significant, *indicates P-value<0.05, **indicates P-value<0.005 and ***indicates P-value<0.001 (independent variables V/S dependent variable).

Table 4: Box-Behnken's experimental design and results of dependent variables for various EFE of *C. oxyacantha*.

Run#	Experimental design			Dependent variables			
	X1	X2	X3	CE yield (%)	TPC (mg GAE/g extract)	TFC (mg QTE/g extract)	TAA-RPA (mg AAE/g extract)
1	-1	-1	0	5.80	359.28 ±0.04	88.83 ±0.03	211.24 ±0.04
2	1	-1	0	5.60	344.31 ±0.06	74.03 ±0.04	219.56 ±0.03
3	-1	1	0	3.40	309.38 ±0.03	103.64 ±0.03	201.26 ±0.02
4	1	1	0	10.40	242.02 ±0.02	84.60 ±0.03	158.02 ±0.03
5	-1	0	-1	3.90	334.33 ±0.01	107.87 ±0.07	264.47 ±0.04
6	1	0	-1	7.16	399.20 ±0.03	103.64 ±0.03	254.49 ±0.04
7	-1	0	1	4.50	341.82 ±0.04	179.78 ±0.03	272.79 ±0.04
8	1	0	1	4.60	471.56 ±0.04	156.51 ±0.03	297.74 ±0.03
9	0	-1	-1	5.30	381.74 ±0.04	114.21 ±0.05	257.82 ±0.03
10	0	1	-1	21.30	339.32 ±0.03	139.59 ±0.04	234.53 ±0.03
11	0	-1	1	6.50	404.19 ±0.04	109.98 ±0.02	254.49 ±0.05
12	0	1	1	8.50	311.88 ±0.02	101.52 ±0.03	209.58 ±0.04
13	0	0	0	4.00	396.71 ±0.03	103.64 ±0.04	239.52 ±0.04
14	0	0	0	4.60	404.19 ±0.03	93.06 ±0.03	236.19 ±0.02
15	0	0	0	5.00	374.25 ±0.03	78.26 ±0.04	211.24 ±0.04

Each value of the dependent variables represents the mean ±SD of triplicates.

Procedure

The bands of standard and sample were spotted on TLC plates using the thin capillary tube. After that plates were placed in a TLC tank containing mobile phase (10 mL). Moreover, the 5 cm distance on the TLC plate was considered as the solvent frontand the baseline was drawn at 0.5 cm. The TLC tank was saturated with mobile phase (Saturation time = 10 minutes). The locating reagent for visualization of developed spots was prepared with $AlCl_3$ in dilute $H_2SO_4$and spots were observed under visible light and UV light and compared with the standard in terms of R_f value.

High performance liquid chromatography

The sample solution of testing extract for quantification and authentication of bioactive phenolics was tested with the HPLC (High performance liquid chromatography) technique as described by (Khalid *et al*., 2023) with slight modifications.

Preparation of working solutions

The standard stock solution of GA, QTand RT (1000 µg/mL) was prepared in diluent; Water: ACN (70:30) and serially diluted to the concentration range of 2-20 µg/mL as necessary to plot the calibration curve of standards.

Moreover, the extract solution (100 mg/25 mL) was prepared in diluent; Water: ACN (70:30), homogenized at 40°C by using an ultrasonic water bathand filtered with the help of 0.20 μm PTFE filters before injection.

Procedure

The HPLC analysis of phenolic standards (GA, QTand RT) in sample extract was performed by using the C18 column (Merck, Germany, *150*4.6mm, 5 µm*) and mobile phase (ACN:FA:Water 30:0.2:70) (Park *et al*., 2024). The HPLC system Shimadzu (LC-20A) and the operational conditions: 10 µL injection (auto-sampler), 40°C (temperature)and the flow rate of 1 mL/min. Moreover, the components were detected by virtue of a UV-Vis detector (257 nm)and the entire procedure was commanded by using the lab-solution software. Furthermore, the retention time of separated components was compared with phenolic standardsand the assessment of quantification was done by plotting the standard calibration curve (Peak area vs. concentration).

Ethical approval for in vivo animal studies

All the Protocols performed for *in vivo* animal studies were approved by the Institutional Bioethical Committee of the University of Karachi, Karachi, Pakistan (Vide Reference No. IBC KU-255/2021).

Acute oral toxicity

Preparation of experimental animals

A group of female rats (albino outbred strain rats), 8-12 weeks old (weighing 140-200 g) was procured from F.S. (Faisal Scientific) Scientific and Animals, Karachi, Pakistan. The randomly selected test animals were marked with extra care to maintain the proper identification; furthermore, the animals were kept in spacious cages to acclimatize to laboratory conditions (Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Karachi, Pakistan) for a 5-day duration.

Experimentation and observation

The most potent extract was evaluated for acute oral toxicity as per Organization for Economic Co-operation and Development (OECD) guidelines 423.

The 5 groups (Normal control, group 1, 2, 3 and 4) of test animals were made considering n = 3 animals/per group. The untreated group was a normal control, while groups 1-4 were treated with the 5, 50, 300and 2000 mg/kg single oral dose of selected extract, respectively. Furthermore, the animals were kept on a fast for 24 hours before the dosing of the extract solution, while, the water access remained uninterrupted. After the keen observation of test animals for various toxicity parameters, the LD_{50} was assessed (Ouattara *et al*., 2021).

In vivo anti diabetic assay

Preparation of experimental animals

A group of rats (albino outbred strain) of either sex, weighing 150-200 g, was purchased from F.S. Scientific and animals, Karachi, Pakistan. The animals were kept in well spacious cages and given the standard pellet diet and access to water, thus, acclimatized to laboratory conditions for 2 days.

Experimentation and observation

The experimental animals were given an intraperitoneal injection of alloxan monohydrate (150 mg/kg) to induce diabetes. After the duration of two days, the animals projecting (200-300 mg/dL) blood glucose level were selected for an *in vivo* antidiabetic study (Malik *et al*., 2022). These animals were further divided into 5 groups (group 1- 3, Normal control and diabetic control), considering n = 6 animals/group. Groups (1-2) were extract treated (100 and 500 mg/kg) and group 3 was given standard drug Glibenclamide (GLC) 5 mg/kg, diabetic control was alloxan treated while normal control was retained as untreated, these groups (diabetic control and normal control) were given distilled water. Furthermore, the fasting blood glucose (FBG) level of all the animals was observed at different intervals of the study (Malik *et al*., 2022, Moharram *et al*., 2025).

STATISTICAL ANALYSIS

The data of RSM was assessed with STATISTICA six sigma (release 7), while, Graph Pad Prism 7.0 was used for the estimation of IC_{50} and one-way ANOVA followed by Tukey's multiple comparison test for the assessment of significance; all the results were articulated as mean ±SD of triplicates.

RESULTS

Optimization of extracts

The optimum extraction conditions of lab scale maceration were developed for the following dependent variables;

Crude extract yield

CE yield (%) of *C. oxyacantha* ELE and EFE was estimated as per the formula defined in (section 2.5)and the results are tabulated in tables 2 and 4, respectively.

Total phenolic content

TPC of *C. oxyacantha* ELE and EFE was estimated from the linear regression equation; y=0.0664x + 0.0029, R^2=0.9998 of the standard GA calibration curveand the results are tabulated in tables 2 and 4, respectively.

Total flavonoid content

TFC of *C. oxyacantha* ELE and EFE was estimated from the linear regression equation; y=0.0806x-0.0129, R^2=0.9983 of the standard QT calibration curveand the results are tabulated in tables 2 and 4, respectively.

Table 5: Model coefficients and regression adjustment of EFE of *C. oxyacantha*.

	P-value				Regression coefficient			
	CE yield	TPC	TFC	TAA-RPA	CE yield	TPC	TFC	TAA-RPA
Mean intercept	0.004**	0.001***	0.006*	0.002**	4.533	391.717	91.653	228.986
X_1	0.079	0.025*	0.394	0.676	0.840	48.653	-6.874	3.743
X_1^2	0.021*	0.089	0.334	0.921	-1.797	-25.262	8.372	0.901
X_2	0.003**	0.050*	0.575	0.158	4.500	-33.683	4.230	-17.049
X_2^2	0.005**	0.023*	0.206	0.057	3.563	-52.707	-12.250	-32.366
X_3	0.007*	0.887	0.239	0.457	-2.900	-1.248	-10.575	-7.069
X_3^2	0.013*	0.130	0.031*	0.034*	2.303	20.272	36.925	42.484
X_1X_2	0.019*	0.235	0.883	0.237	1.800	-13.099	-1.058	-12.891
$X_1X_2^2$	0.137	0.024*	0.877	0.372	0.860	-69.237	-1.586	-12.475
$X_1^2X_2$	0.008**	0.730	0.836	0.946	-3.900	-4.366	2.115	-0.832
X_1X_3	0.088	0.173	0.533	0.376	-0.790	16.218	-4.759	8.733
$X_1^2X_3$	0.021*	0.194	0.044*	0.209	2.410	21.208	41.772	19.960
X_2X_3	0.005**	0.250	0.316	0.557	-3.500	-12.475	-8.460	-5.406
MS Residual	0.253	242.777	162.536	238.858				
R^2	0.9981	0.9879	0.9724	0.9712				
R^2_{adj}	0.9871	0.9157	0.8071	0.7987				
$R^2_{Predidicted}$	0.608	0.669	0.643	0.839				

P-value of <0.05 is considered significant, *indicates P-value<0.05 and **indicates P-value<0.005 (independent variables V/S dependent variable).

Table 6: Experimental and predicted values of dependent variables for ELE of *C. oxyacantha*.

Dependent variables	Run#	Observed value	Predicted value	PI (-95%)	PI (+95%)	CI (-95%)	CI (+95%)
CE yield (%)	2	8.60	9.33	4.00	14.65	6.25	12.40
TPC (mg GAE/g extract)	3	471.56	326.22	85.16	567.29	187.04	465.40
TFC (mg QTE/g extract)	3	251.69	186.13	67.92	304.33	117.88	254.37
TAA-RPA (mg AAE/g extract)	3	274.45	202.72	71.06	334.38	126.71	278.73

CI=Confidence interval, PI=Predicted interval

Table 7: Experimental and predicted values of dependent variables for EFE of *C. oxyacantha*.

Dependent variables	Run#	Observed value	Predicted value	PI (-95%)	PI (+95%)	CI (-95%)	CI (+95%)
CE yield (%)	10	21.30	14.65	4.25	25.04	8.64	20.65
TPC (mg GAE/g extract)	8	471.56	410.12	294.65	525.58	343.45	476.78
TFC (mg QTE/g extract)	7	179.78	154.93	90.14	219.72	117.52	192.34
TAA-RPA (mg AAE/g extract)	8	297.74	272.79	221.16	324.42	242.98	302.60

CI=Confidence interval, PI=Predicted interval

Table 8: R_f values of separated components.

Sample	R_f values
ELE-3	0.52
	0.6
	0.65
	0.76
	0.9
	0.96
GA (Standard)	0.52
QT (Standard)	0.76

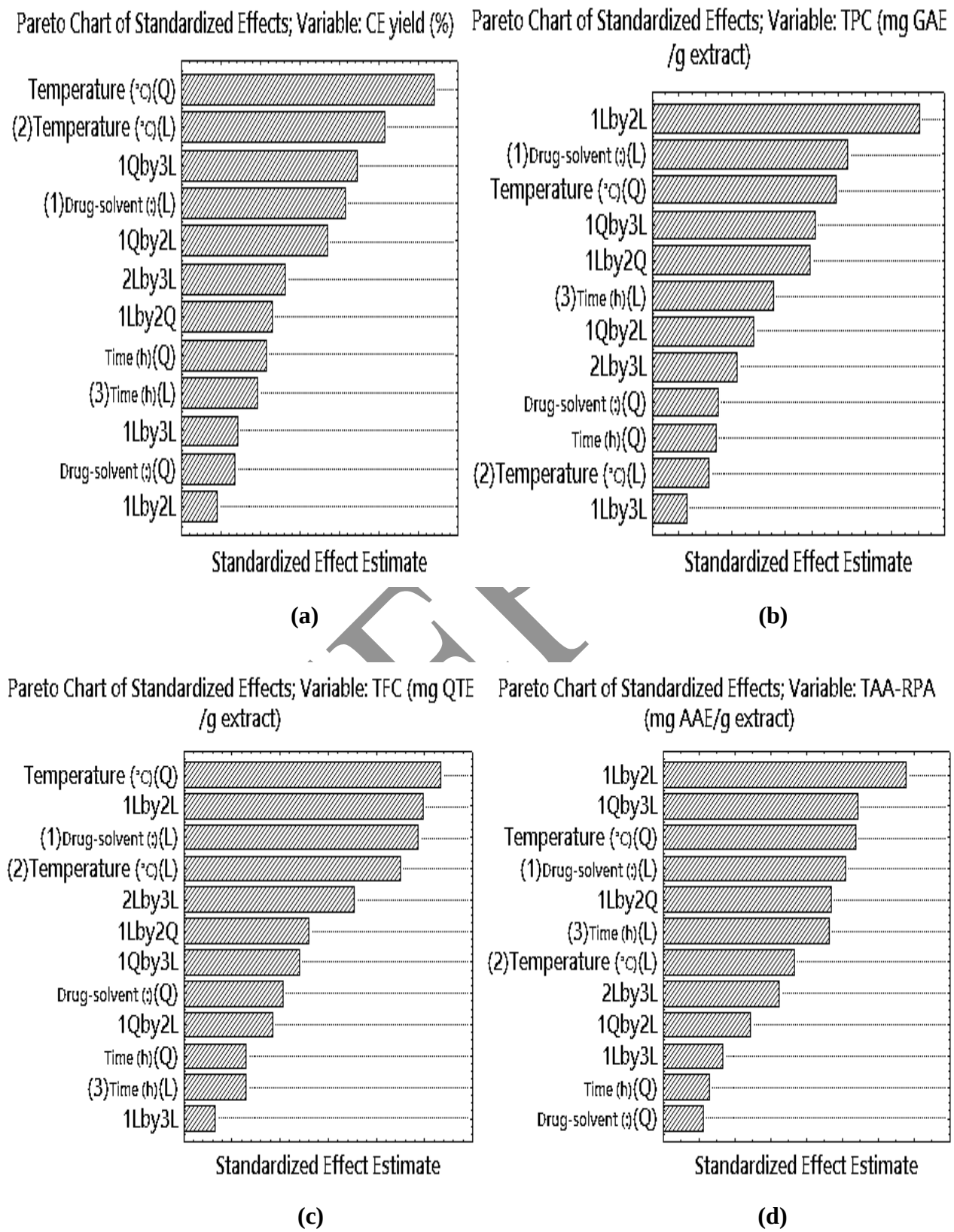


Fig. 1: Pareto chart showing influence of independent variables on dependent variables (CE yield, TPC, TFC, and TAA-RPA, respectively) for ELE of *C. oxyacantha*.

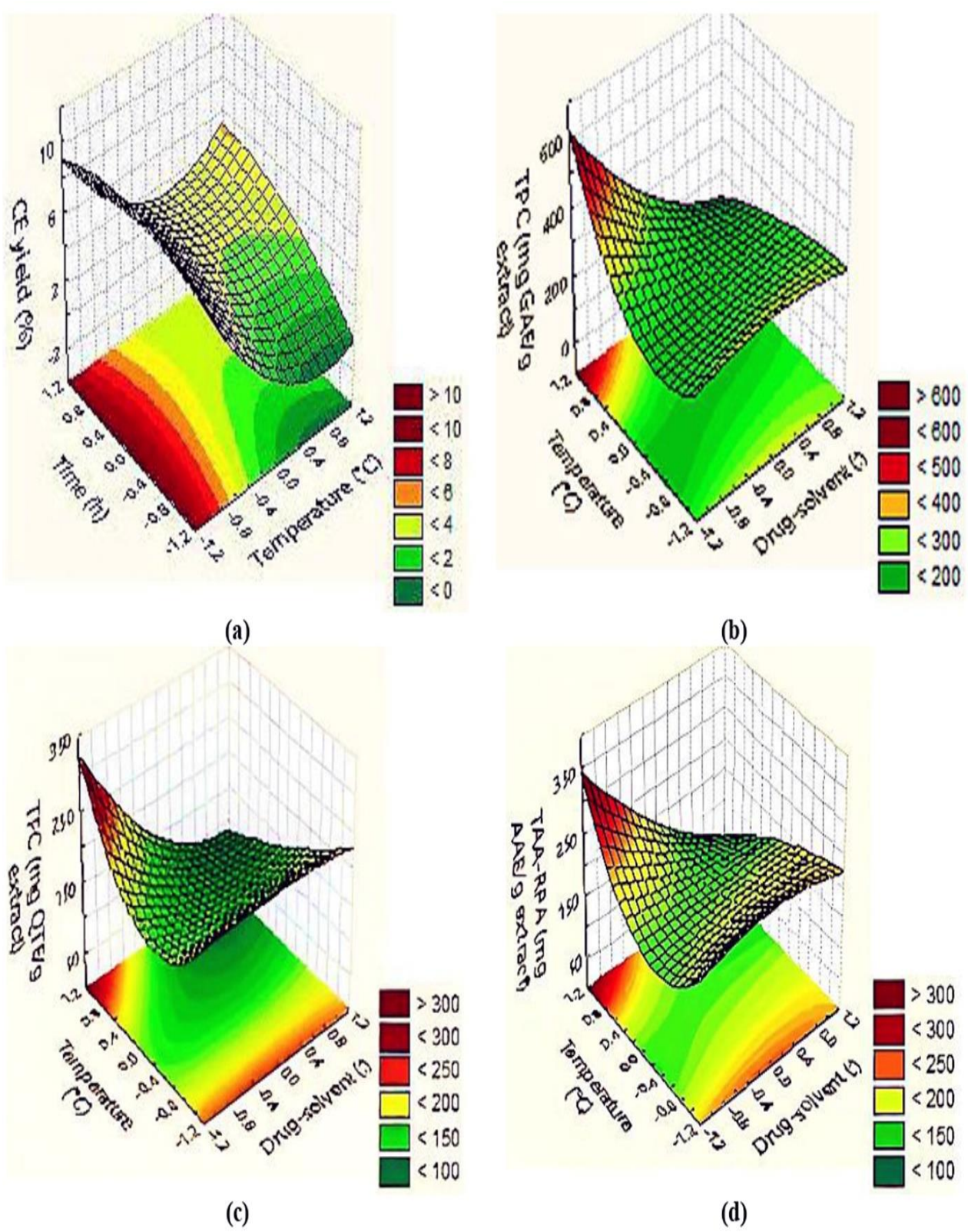


Fig. 2: The 3D response surface plots suggesting the best possible pattern of independent variables that may result in preparation of ELE of *C. oxyacantha* exhibiting the highest value of dependent variables (CE yield, TPC, TFC and TAA-RPA, respectively).

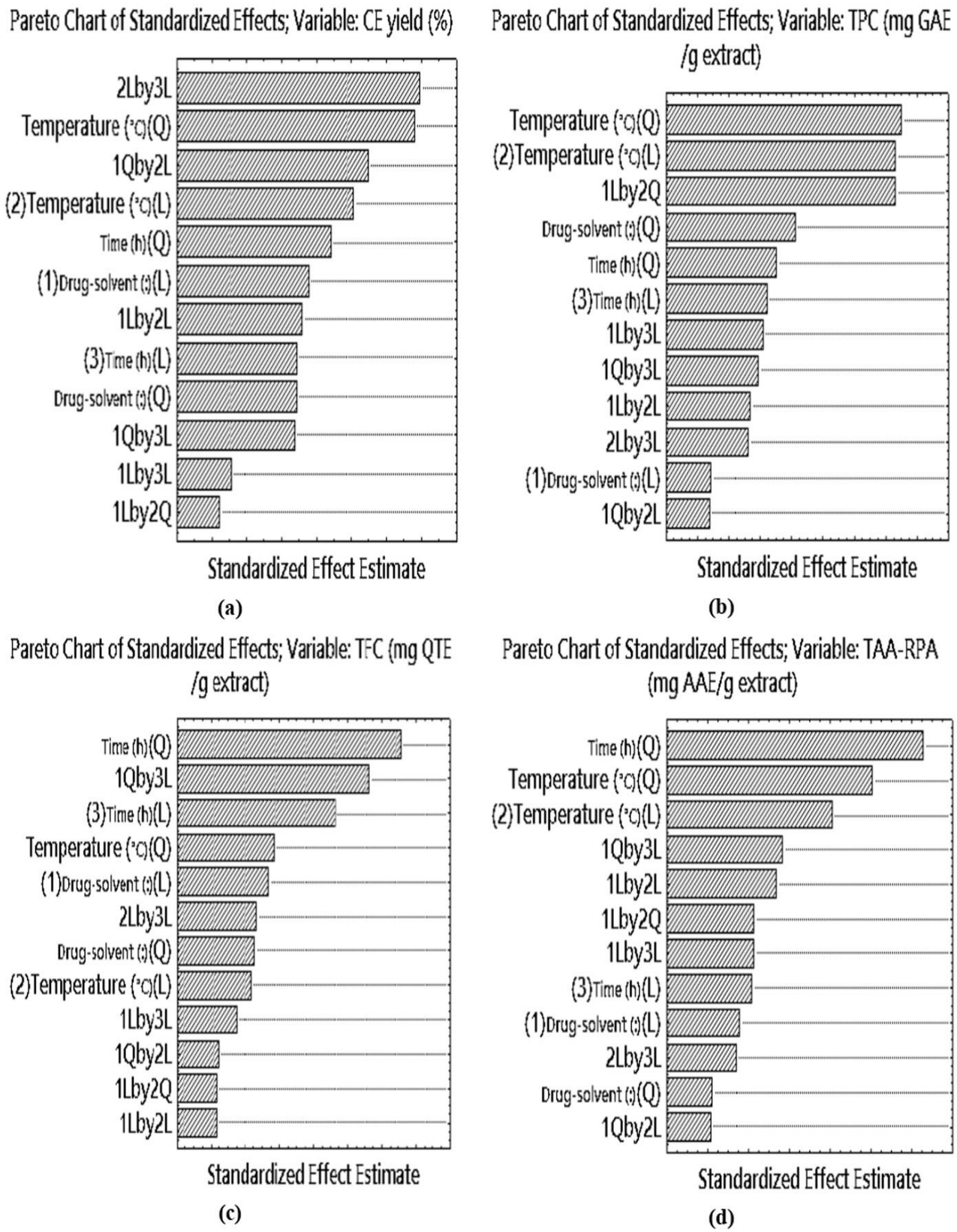


Fig. 3: Pareto chart showing influence of independent variables on dependent variables (CE yield, TPC, TFC, and TAA-RPA, respectively) for EFE of *C. oxyacantha*.

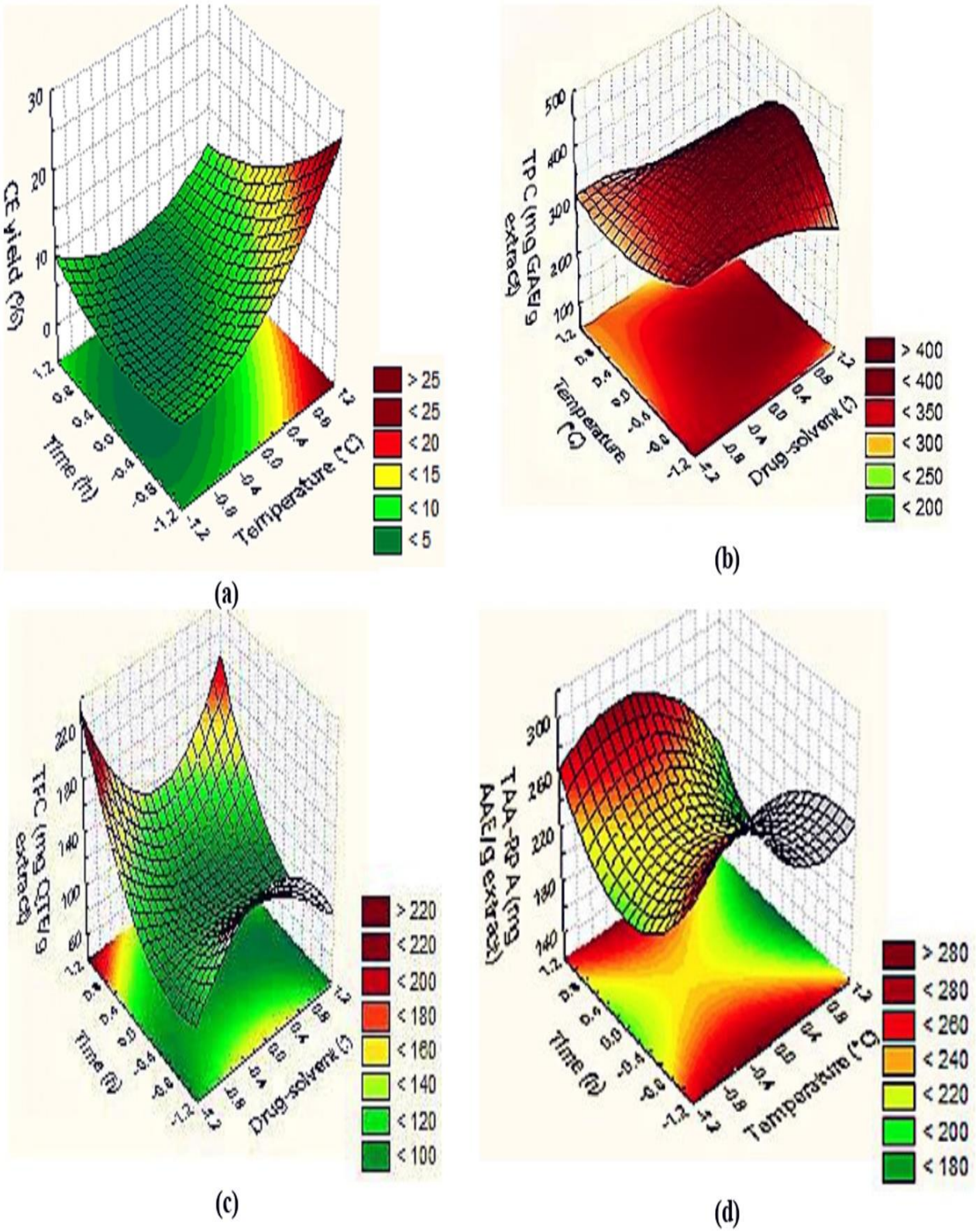


Fig. 4: The 3D response surface plots suggesting the best possible pattern of independent variables that may result in preparation of EFE of *C. oxyacantha* exhibiting the highest value of dependent variables (CE yield, TPC, TFC, and TAA-RPA, respectively).

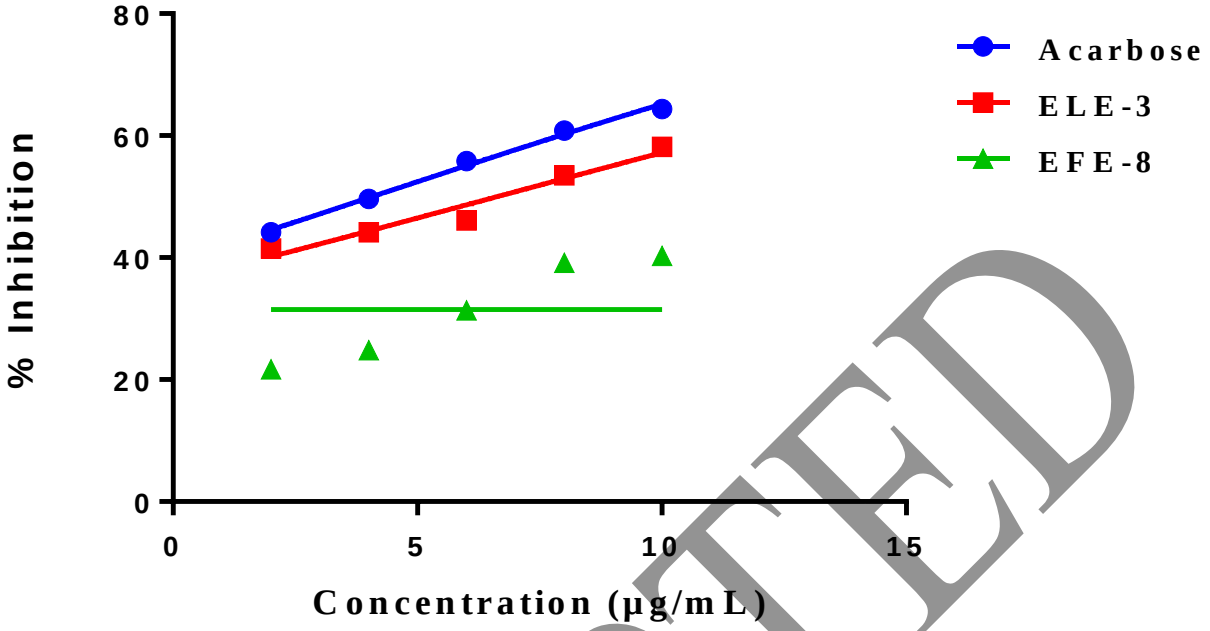


Fig. 5: Alpha-amylase inhibitory % of selected extracts and standard acarbose.

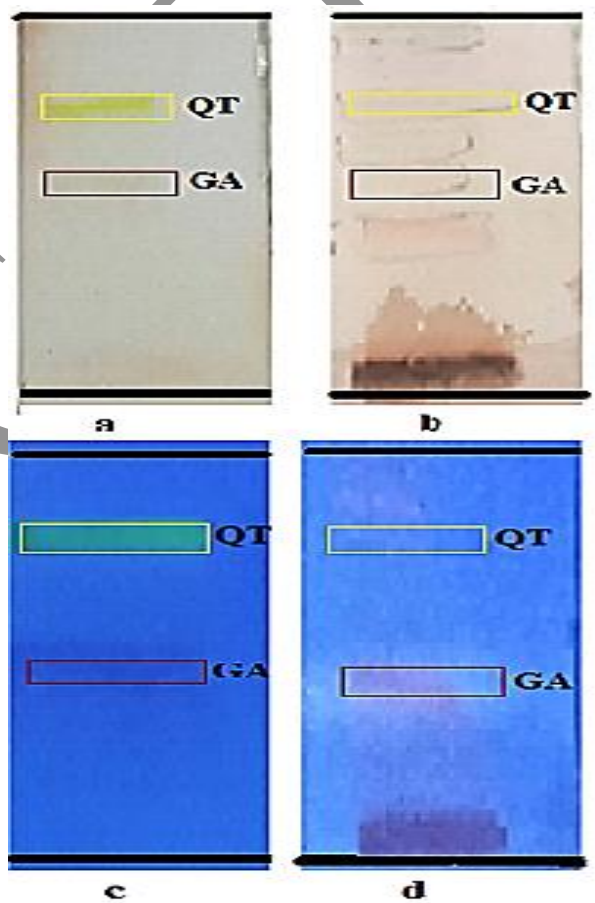


Fig. 6: TLC plate of ELE-3 and standards; a) standard GA and QT under visible light, b) ELE-3 under visible light, c)

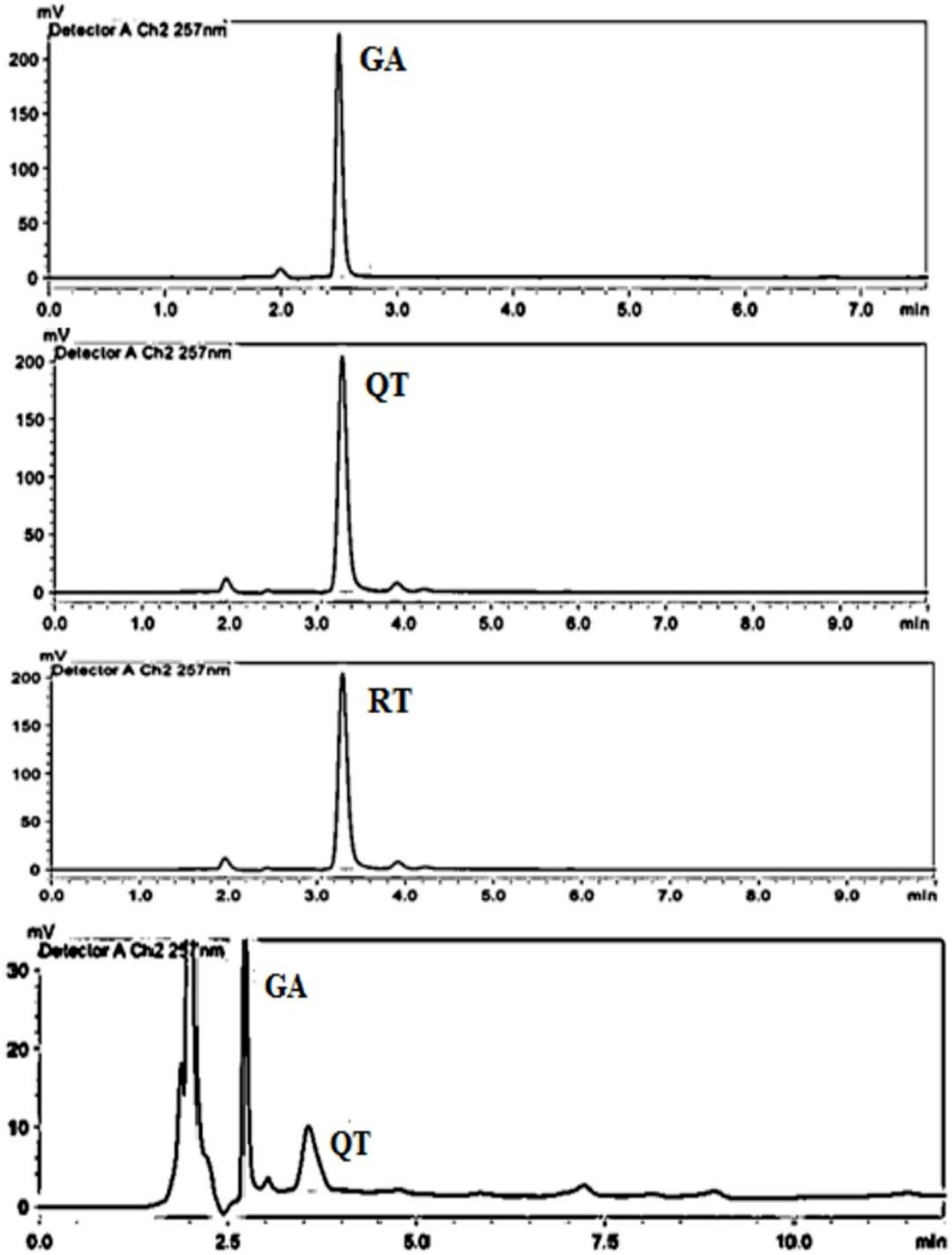
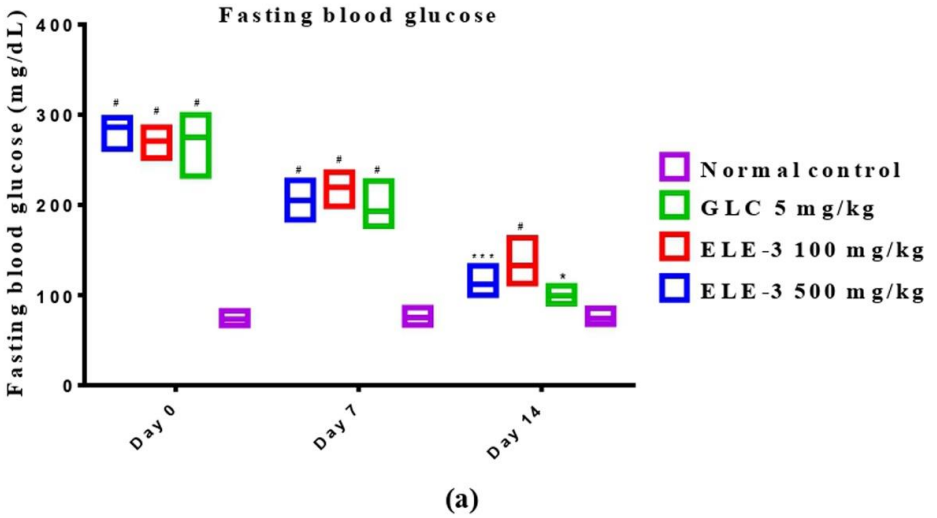
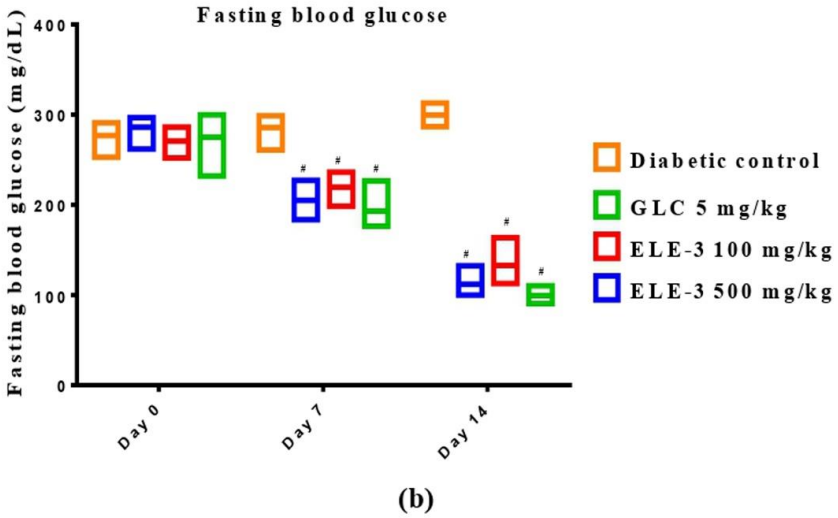


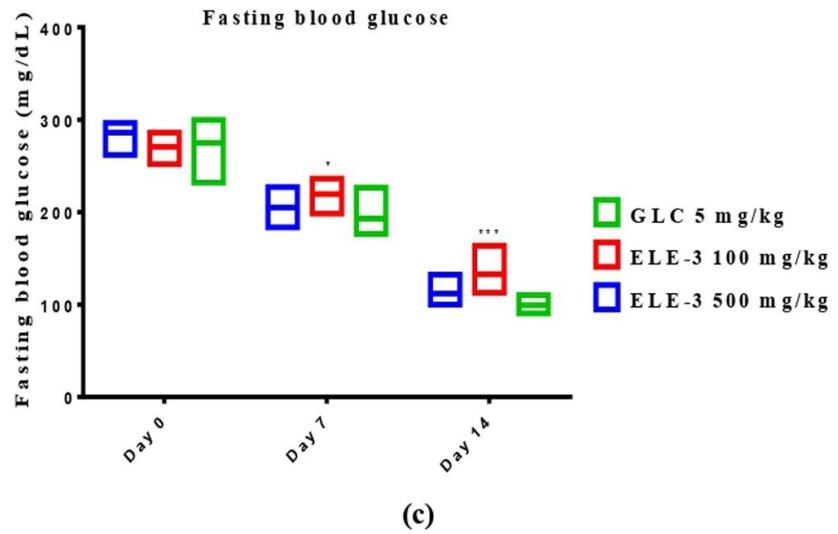
Fig. 7: HPLC chromatogram of standards (GA, QT and RT) and sample (ELE-3), respectively**.**



(a)



(b)



(c)

Fig. 8: Effect of ELE-3 and standard GLC on fasting blood glucose level of test animals. n=6, columns and vertical bars represents the % mean ±SEM, respectively. One way ANOVA analysis was performed followed by Tukey's multiple comparison test, #indicates P <0.0001, ***indicates P <0.001 and *indicates P <0.05 (ELE-3 100, 500 and GLC 5 mg/kg

Total antioxidant activity-reducing power assay

TAA-RPA of *C. oxyacantha* ELE and EFE was estimated from the linear regression equation; y=0.0984x + 0.0129, R^2=0.9989 of the standard AA calibration curve and the results are tabulated in tables 2 and 4, respectively.

Optimization of C. oxyacantha ELE

Combined effect of independent variables on dependent variables of various ELE of C. oxyacantha

The results of the dependent variables for the 15 runs of *C. oxyacantha* ELE are tabulated in table-2. Hence, it can be observed that combined independent variables (drug-solvent ratio, extraction temperatureand time) have shown a prominent impact on the resultant values of dependent variables (CE yield, TPC, TFCand TAA-RPA). Among these, the highest CE yield (8.60%) is witnessed for experimental run # 2, at the experimental conditions (1:30, 30ºC and 18h). However, the highest level of TPC (471.56 mg GAE/g extract), TFC (251.69 mg QTE/g extract)and TAA-RPA (274.45 mg AAE/g extract) is witnessed for experimental run # 3, at the experimental conditions (1:10, 60ºC and 18h), respectively.

Significant model of independent variables for the preparation of ELE of *C. oxyacantha* exhibiting maximum level of dependent variables.

Pareto chart illustrated the impact of independent variables, while, their regression values are tabulated in table-3; as shown in fig.-1a and table-3, temperature in its quadratic model influenced significantly (P <0.001) on CE yield. As shown in fig.-1b and table-3, the linear-linear interaction model of drug-solvent ratio and temperature significantly (P <0.003) influenced on TPC. As shown in fig.-1c and table-3 temperature in its quadratic model influenced significantly (P <0.01) on TFCand as shown in fig.-1d and Table-3 linear-linear interaction model of drug-solvent ratio and temperature influenced significantly (P <0.007) on TAA-RPA.

Optimization of C. oxyacantha EFE

Combined effect of independent variables on dependent variables of various EFE of C. oxyacantha

The results of the dependent variables for 15 runs of *C. oxyacantha* EFE are tabulated in table-4. Hence, it can be observed that combined independent variables (drug-solvent ratio, extraction temperatureand time) have shown a prominent impact on the resultant values of dependent variables (CE yield, TPC, TFCand TAA-RPA). Among these, the highest CE yield (21.30%) is witnessed for experimental run # 10, at the experimental conditions (1:20, 60ºC and 12h). However, highest level of TPC and TAA-RPA (471.56 mg GAE/g extract and 397 mg AAE/g extract respectively) is witnessed for experimental run # 8, at the experimental conditions (1:30, 45ºC and 24h) and the highest level of TFC (179.78 mg QTE/g extract) is witnessed for experimental run # 7, at the experimental conditions (1:10, 45ºC and 24h).

Significant model of independent variables for the preparation of EFE of C. oxyacantha exhibiting maximum level of dependent variables

Pareto chart illustrated the impact of independent variables while their regression values are tabulated in table-5; as shown in fig.-3a and table-5 linear-linear interaction model of temperature and time influenced significantly (P<0.005) on CE yield. As shown in fig.-3b and table-5, temperature in its quadratic model significantly (P<0.023) influenced on TPC. As shown in fig.-3c and table-5 quadratic model of time influenced significantly (P<0.031) on TFC and as shown in fig.-3d and table-5 quadratic model of time (P<0.034) influenced significantly on TAA-RPA.

Experimentally optimized C. oxyacantha leaves and flowers extracts depicting the highest values of dependent variables

The experimental values of dependent variables for the optimized *C. oxyacantha* ELE and EFE were compared to the predicted response values of Box-Behnken's experimental design (tables 6 and 7), the confidence interval of experimental values was within the range of predicted interval values, indicating the precision of the models.

Alpha-amylase inhibition of most optimized extracts of C. oxyacantha

C. oxyacnatha extracts; ELE-3 (ELE prepared at run # 3) and EFE-8 (EFE prepared at run # 8) possessing maximum concentration of dependent variables (table 6-7) were selected for *in vitro* alpha-amylase inhibitory activity. As shown in fig.-5, ELE-3 showed the highest % inhibition (58.14 ±0.04) followed by EFE-8 (40.31 ±0.05) at concentration of (10 µg/mL) respectively, while the standard drug acarbose at (10 µg/mL) showed 64.34 ±0.05. Similarly, the IC_{50} value obtained for ELE-3 (5.976 µg/mL) was close to that of acarbose (3.524 µg/mL) while EFE-8 demonstrated an IC_{50} value of 18.63 µg/mL.

Chromatographic fingerprinting

The maximum level of alpha amylase inhibition shown by ELE-3 indicated further, the appropriateness of this extract for the chromatographic analysis.

TLC analysis

After getting the trials with different proportions of solvents (MeOH, EtOH, EA, CF, TOLand FA), it was revealed that the mobile phase (EA:TOL:FA 4.63:3.45:0.15) resolved the better separation of components. The analysis of the sample solution of ELE-3 by using the developed mobile phase demonstrated that six TLC spots appeared on the chromatogram of ELE-3 (table-8), two spots projected the similar R_f values as those of

standards, thus confirming the presence of bioactive compounds (fig.-6).

HPLC analysis

The HPLC chromatogram of ELE-3 depicted the peak of GA (retention time 2.52 min) and QT (retention time 3.32 min), Moreover, the quantification of these phenolic compounds was done by using the linear regression equation of standard GA (y=27450x + 971035, R^2=0.9972) and QT (y=28526x + 931160, R^2=0.9971) calibration curve and the results revealed 11.47 and 8.27 µg/mL of GA and QT in sample extract (ELE-3) respectively. Furthermore, the standard peak of RT didn't match with the ELE-3 chromatogram.

Acute oral toxicity

The acute oral toxicity study at single oral dose administration (5, 50, 300and 2000 mg/kg) did not demonstrate any sign of toxicity for ELE-3, hence, depicted the >2000 mg/kg LD_{50}.

In vivo antidiabetic activity of ELE-3

The blood glucose lowering tendency of ELE-3 was evaluated in alloxan-induced diabetic rats, this assessment illustrated a significant rise in FBG level in diabetic rats group as compared to normal control group on day 0 (P <0.0001) fig.-8a; Furthermore these FBG levels were significantly reduced on day 7 (P <0.0001) and 14 (P <0.0001) in comparison of diabetic control group (fig.-8b). The study also revealed that ELE-3 100 mg/kg differed significantly from standard GLC 5 mg/kg on day 7 (P<0.05) and day 14 (P<0.001) while the results of ELE-3 500 mg/kg were very close to standard drug (fig.-8c).

DISCUSSION

It is evident in many studies that extraction factors; drug-solvent ratio, influence the efficacy of the extract by improving solvent-mediated cell permeability of crude drug sample and the tendency to dissolve the phenolic compounds present within (Ouattara *et al*., 2021). Consequently, increasing the extraction temperature 2-6 fold by the normal range for the suitable span of extraction time has been witnessed to be associated with significant improvement in the mass transfer through the plant matrix, thus improving the phenolic yield of crude extracts (Tomasi *et al*., 2023). This is the first ever study, focused on optimization of extraction parameters to increase the phenolic yield of *C. oxyacantha* leaves and flowers thus, it may be anticipated from the results illustrated in Pareto chart; extraction temperature has been observed as most influential parameter followed by drug-solvent ratio and time, moreover, the regression analysis (P-value) indicated that there was significant interaction between these variables that ultimately amplified the concentration of dependent variables for *C. oxyacantha* leaves and flowers extracts.

The analysis of variance; training R^2 and adjusted R^2 mentioned in table-3 and 5 indicated the accuracy of model, furthermore, the goodness of fit for ELE and EFE experimental model was compared in terms of MS residuals i.e. *C. oxyacantha* ELE; 0.023 (CE yield), 99.601 (TPC), 10.438 (TFC)and 58.100 (TAA-RPA)and EFE; 0.253 (CE yield), 242.77 (TPC) 162.536 (TFC) and 238.858 (TAA-RPA) thus the lower MS residual clued up the trending of lowest insights of residual errors for the *C. oxyacantha* ELE as compared to EFE. While, the low R^2 predicted value on the other hand for TPC 0.322 and TAA-RPA 0.376 suggested a room for future work to involve more regularization techniques that may address the limitations and may also improve the generalization of the model. These findings of the present study are very crucial for unveiling the optimized conditions, which might save lot of efforts and time for the researchers aiming to work on *C. oxyacantha* leaves on a large scale with the same likelihood.

Giving consideration to the alpha amylase inhibitory effect of ELE-3, a logical interpretation may be drawn based on the supporting conclusions of various studies corresponding to the effective interrelationship between polyphenols and inhibition of alpha-amylase (Li *et al*., 2022, Ćorković *et al*., 2022). This proportionality of results further strengthened the therapeutic significance of polyphenols and the pharmacological validity of ELE-3. Development of TLC mobile phase for the chromatographic fingerprinting of phenolic compounds and HPLC characterization and quantification of GA, QTand RT in ELE-3 is very crucial aspect of this study, owing to the considerations of WHO and pharmacopoeias for the standardization of botanicals (Khalid *et al*., 2021).
The acute oral toxicity study of ELE-3 revealed the safety of crude extract up to 2000 mg/kg; the toxicity study previously reported for the leaf and root part of this plant projected a similar range of LD_{50} >2000 mg/kg (Ikram *et al*., 2020, Aldossary and Khalil, 2019).

The *In vivo* antidiabetic effect of ELE-3 500 mg/kg on days 7 and 14 was very close to standard GLC (5 mg/kg) depicting the therapeutic strength of optimized extract and this as well generates a gap for further mechanistic rationalization of extract. Moreover, considering the fact that the diabetogenic tendency of alloxan monohydrate is associated with discrete mechanisms, including the inhibition of glucose-stimulated insulin secretion and abrupt formation of reactive oxygen species (Longkumer *et al*., 2021). Several studies have reported the rehabilitative role of polyphenols in alloxan-induced diabetic models via restoration of insulin levels towards normal and decreasing the degradation of insulin producing cells (Nie and Cooper, 2021). As reported by (Iftikhar *et al*., 2020) oral administration of *Caesalpinia bonduc* extract (Containing high levels of polyphenols, including gallic acid, caffeic acid, p-coumaric acid,

chlorogenic acid, protocatechuic acidand epicatechin) demonstrated the promising antidiabetic effect via beta cell regeneration in alloxan-induced diabetic rats. Furthermore, in another study, oral administration of QT in alloxan-induced diabetic mice normalized the hyperglycemia (Günal- Köroğlu *et al*., 2025). Based on these therapeutic findings of polyphenolic compounds, an encouraging correlation can be made between antidiabetic potential and chromatographic validation of polyphenols of ELE-3.

CONCLUSION

The present study highlighted the key insights regarding the pivotal role of extraction conditions in extracting the content of bioactive secondary metabolites that augments the therapeutic tendency of crude extracts. However, the extraction models obtained in this study by using the RSM experimental design demonstrated the best possible model for the preparation of bioactive extract, considering the antioxidant and antidiabetic potential of ELE-3 the extract may be analyzed further for underlying mechanisms pertaining to the activity guided isolation owing to the TLC and HPLC results of present study. Furthermore, chronic toxicity studies may be performed to ensure the safety of the crude extract.

Contributions

"All authors contributed to the study conception, design and writing of the manuscript. Material preparation and data collection were performed by Hosh Muhammad Lashari. Methodology design was devised by A.H Memon and Sana Sarfraz. Muhammad Akram and Madan Lal Maheshwari helped in the conceptualization and validation of the study. The first draft of the manuscript was written by Qurat ul Ain. Sana Javaid Awan helped in editing and reviewing the original draft. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical declarations

Ethical approval for in vivo animal studies

All the Protocols performed for *in vivo* animal studies were approved by the Institutional Bioethical Committee of the University of Karachi, Karachi, Pakistan (Vide Reference No. IBC KU-255/2021).

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