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Research Article

Screening of Variables Influencing Extraction Yield of *Cotyledon orbiculata*: 2^3 Full Factorial Design

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ABSTRACT
Various ethnobotanical studies, carried out in different parts of the world, have emphasized the critical role played by plant-based compounds in the prevention and/or treatment of many health conditions. Investigations into the ethnopharmacological properties of any given plant require an effective extraction procedure. The aim of this study was to use 2^3 factorial designs to assess the effect of different variables and their interactions on the percentage yield of extracts from *Cotyledon orbiculata* L. using aqueous and methanol extraction processes. Eight experimental runs were carried out for each solvent. In each case, three varying parameters were used. For the aqueous design, extraction temperature, pH and extraction time were selected while for the methanol design, solvent composition, technique and extraction time were selected as the primary design variables. The extraction yield in both cases were used as the driver for optimal output. The results showed that only pH and extraction time had a significant influence on the percentage yield for the aqueous extraction and that their second-order interaction [pH x extraction time] did not produce a statistically significant increase in yield. The optimum conditions for the aqueous extraction design were extraction temperature 30°C, pH 8.99 and extraction time 240 minutes. On the other hand, the methanol design indicated that both extraction technique and extraction time contributed to an increase in yield and that two interactions, namely [methanol composition x extraction time] and [technique x extraction time] also influenced extraction yield significantly. The low percentage yields and the lower than expected predicted R^2 values for the regression models using methanol suggests that perhaps other variables should be considered to achieve a greater yield.

Keywords: *Cotyledon orbiculata*, extraction yield, 2^3 factorial design.

INTRODUCTION

Plants produce a wide array of phytochemicals that may act individually, additively, or synergistically in improving animal and human health[1,2]. Phytochemicals, the biologically active compounds in plants, have been identified and used by different civilizations for thousands of years. Some examples include aspirin (a salicylate from *Salix alba*), atropine (an alkaloid from *Atropa belladonna*), digitoxin (a cardiac glycoside from *Digitalis purpurea*), and taxol (a terpenoid from *Taxus brevifolia*). In addition, phytochemicals are also known to have antioxidant[3,4], antimicrobial[5,6], antifungal[7], antidiabetic[8,9], anti-inflammatory[10,11] and anticonvulsant[12] characteristics. The ethnopharmacological properties of any given plant can only be ascertained if the phytochemicals are extracted[13].
An extraction procedure allows for the recovery of solubilized phytochemicals with similar polarities to that of the solvent enabling the formation of a crude extract. The crude extract can then be screened for active compounds that could lead to the development of novel modern medicines, biopharmaceuticals, cosmeceuticals, nutraceuticals or food supplements[14]. Various factors such as the choice of solvent, solvent composition, pH, solvent-to-solid ratio, extraction temperature, extraction technique and extraction time may significantly influence the recovery of the crude extract[15]. Until recently a “one factor at a time” approach was used to optimise parameters involved in improving the yield of plant extracts. However, this approach is expensive, time consuming and labor intensive requiring many experiments. This approach also limits the interpretation of the results since no interaction between different factors are considered[16].
A factorial design, on the contrary, is a valuable screening methodology for experiments where many input variables (factors) can be investigated simultaneously. This multivariate tool allows for ascertaining the impact of each independent variable (main effect) and all combinations of variables on the response(s) of an experiment. Factorial designs are more cost effective and involve less experimental runs without compromising the accuracy of the data[17]. The factorial design has been used to study the impact of various independent variables (factors) on the extraction and yield of bioactive components from medicinal plants. Shah and Gary[18] employed a 2^k full factorial experimental design to optimize the extraction of essential oils from ginger. In addition, a factorial design

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was used to improve the extraction of phenolics, flavonoids and antioxidants from *Mangifera pajang* Kosterm[15], phytochemicals from *Garcinia indica* Choisy[19], and polyphenol from espresso coffee residues[20]. A factorial design may prevent the real effects from being obscured by experimental errors[21]. However, it is important to emphasise that a factorial design does not suggest or guarantee achieving the optimal value for each of these factors[15].

In this study, a 2^3 full factorial design was used to screen for variables that may influence the yield of crude extracts from *Cotyledon orbiculata.* Traditionally, *C. orbiculata* has been used in different ways to treat numerous ailments[12,22,23]. For instance, the peeled leaves have been used to facilitate easy removal of corns, warts, or plantar warts. Warmed peeled leaves have been used as a poultice for boils, skin eruptions and abscesses. The consumption of fresh leaves has been used as a vermifuge. Fresh leaf juice of *C. orbiculata* has been used to treat epilepsy when taken orally and as a lotion for acne, earache or toothache. Lastly, a leaf decoction, used as an enema, has also been used to treat syphilis. According to the South African National Biodiversity Institute, *C. orbiculata* has not been the subject of many *in vitro* or *in vivo* studies despite its popularity as a traditional medicinal plant. The objective of this study was to investigate the effects that independent variables such as temperature, pH, time and extraction techniques may have on the yield of crude aqueous and methanol extracts from *C. orbiculata* using two 2^3 full factorial designs.

MATERIALS AND METHODS

Chemicals

Chemicals used in this study included methanol (Sigma-Aldrich, St. Louis, MO) hydrochloric acid and sodium hydroxide (Rochelle Chemicals, Johannesburg, South Africa). Ultrapure water was collected from a Millipore Direct-Q water purifier system (Merck Millipore, Darmstadt, Germany).

Collection of plant material

A composite sampling design was employed during the collection of the plant material used in this study. Healthy thick leaves of *C. orbiculata* were collected from multiple populations from different locations around the Vanderbijlpark area from February to May 2016. The composite samples were obtained by mixing the randomly collected leaves, and reselecting a subset for analysis. A plant from each population was collected for identification and authentication. All the plants were identified and authenticated as *C. orbiculata* by Professor Stefan Siebert, Curator of the AP Goossens Herbarium, Northwest University, Potchefstroom. Dried voucher specimens were archived in the Herbarium under the voucher numbers PUC0014495, PUC0014496, PUC0014497, PUC0014498 and PUC0014499.

Preparation of the plant material

The freshly collected leaves of *C. orbiculata* were washed with tap water, rinsed twice in deionized water and then cut into 1 cm^3 cubes. The diced leaves were frozen at -20°C and lyophilized in a Scanvac CoolSafe™ (LaboGene, Lynge, Denmark) at – 57°C for 3-4 days. The dried plant material was ground to a fine powder in a Pulverisette 14, FRITSCH (Idar-Oberstein, Germany) and stored at -20°C in a dark container until needed.

The 2^3 Factorial design

Two 2^3 full factorial designs were constructed to investigate the efficiency of two solvent (aqueous and methanol) extraction processes on the yield of crude extract (%) obtained from *C. orbiculata*. Ultrapure water from a Millipore Direct-Q water purifier system and methanol were used as solvents, respectively. The 2^3 full factorial design was carried out with independent variables and levels of each variable were selected. The independent variables were chosen based on Tiwari's[24] recommendations. Tiwari[24] suggested that variations in different extraction methods had an effect on the quantity and secondary metabolite composition in plants. These variations were dependent on six factors: the type of extraction, extraction time, temperature, nature of solvent, solvent concentration and polarity of the solvent[24]. For the two solvent extraction procedures, three independent variables were selected using two levels for each variable (high (+) and low (-)) as shown in Table 1 (aqueous extraction design) and Table 2 (methanol extraction design). A 1:20 ratio of finely ground plant material to solvent was used in all experimental runs that were randomly executed and the response variable (percentage extraction yield) was recorded. Randomizing the experimental runs "averages out" the effect of extraneous factors or errors that may be present preventing the violation of independence[17]. Following each experimental run, all crude aqueous extracts were filtered through a Whatman No. 1 filter paper. The filtrate was then frozen at -20°C, lyophilized at -57°C for 2-3 days, weighed, and stored at -20°C in a dark container until needed. All methanol extracts were concentrated under rotary evaporation (HB 10 basic, IKA®-Werke GmbH & Co. KG, Staufen, Germany) and dried until a constant weight was achieved. The extracts were then stored at -20°C in dark containers until needed. The extraction yield (%) was expressed as shown in equation 1:

$$\%\ Extraction\ yield = \frac{amount\ (g)\ of\ dried\ crude\ extract\ obtained}{amount\ (g)\ of\ finely\ grounded\ plant\ material\ used}\ x\ 100$$

(1)

To minimize external variation, experimental runs were done in triplicate (aqueous extraction) and in duplicate (methanol extraction).

Statistical analysis

ANOVA was used to test the statistical significance of the different independent variables on the percentage extraction yield. A factorial ANOVA of the sum of squares (SS) of the treatment variance ($SS_{between}$) was used to cover all the permutations of possible group combinations. Equations 2, 3, 4 and 5 represent formulas that were used to determine the sum of squares for each of the groups.

$$SS_T = SS_{between} + SS_{within}$$

(2)

$SS_{between} = n\sum(M_g - M_{tot})^2$
(3)
$SS_{within} = \sum\sum(x - M_g)^2$
(4)
$SS_T = SS_A + SS_B + SS_C + SS_{AB} + SS_{AC} + SS_{BC} + SS_{ABC} + SS_{within}$ (5)

In equations 2, 3, 4 and 5, x represented each individual observation, n was the number of observations in each level of a variable, M_g represented each level mean, M_{tot} represented the grand mean or total mean and $SS_{A,} SS_{B,}$ etc. represent the sum of squares for each independent variable and the interactions between variables. Each sum of squares had different degrees of freedom. In a factorial experiment, the number of degrees of freedom associated with each variable is equal to the number of levels associated with that variable minus 1[25]. Therefore, the number of degrees of freedom associated with a three variable interaction used in this study A x B x C, was equal to the product of the degrees of freedom of the variables involved. The F-ratio was used to determine if there was a relationship between the response and a subset of the independent variables based on the Fisher's statistical test (F-test) at a p<0.05 level of significance. The F-ratio was calculated by dividing the mean squares *between*-groups ($MS_{(between)}$) by the mean squares *within*-groups ($MS_{(within)}$). A large resultant value was an indication of a greater likelihood that the differences between the means were due to something other than chance alone[17]. Assuming normality, the degrees of freedom of the $MS_{(between)}$ (called $df_{(num)}$) and the degrees of freedom of the $MS_{(within)}$ (called $df_{(denom)}$) were used to determine the $F_{critical}$ value from the F-distribution based on the formula shown in equation 6.

$F(df_{(num)}, df_{(denom)}) = value,\ p < 0.05$ (6)

A regression model was used to determine the statistical significance, direction and magnitude of the relationship between a variable and the response. A regression formula shown in equation 7 was used to predict the effect of the variables on the response.

$y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AC + \beta_{13} AB + \beta_{23} BC + \beta_{123} ABC + \varepsilon$ (7)

In equation 7, y represented the response, whereas β_n represented the regression coefficient associated with variable *n*. The values for *n* in equation 7 were obtained from the analysis of the experimental results. A, B and C represent the main variables, AB, AC and BC denote the two-way interactions, ABC represents the three-way interaction and ε represents the experimental error. The adequacy of regression models obtained in this study was examined by ANOVA, coefficient of determination (R^2), adjusted R^2 and predicted R^2. R^2, which has a value from 0 to 1, was used to measure the global fit of a model according to the formula shown in equation 8 where SS_T and SS_{within} are the total sum of squares and error sum of squares, respectively.

$R^2 = \frac{SS_T - SS_{within}}{SS_T} = 1 - (\frac{SS_{within}}{SS_T})$ (8)

In this study the value of the predicted R^2 as opposed to the calculated and adjusted R^2 values was used to determine the quality of the model and to confirm that the final regression model did not over-fit the observed data points. The experimental data were processed using the Design Expert 6.0.6 software (Stat-Ease Inc., MN).

RESULTS AND DISCUSSION

Experimental observations and outliers

This study was designed to assess the influence of three different independent variables on the extraction yield (%) of two different extraction processes. The evaluation of three independent variables at two levels required 8 experiments. The aqueous extraction process was done in triplicate, resulting in 24 observations while the methanol extraction process was done in duplicate, resulting in 16 observations. The design matrix with the values of the independent variables along with the response values (extraction yield (%)) for each of the experimental runs are given in Tables 1 and 2 for the aqueous and methanol extraction processes, respectively.

Statistical outliers are data points that sit on the upper and lower periphery of the dataset and have the potential of skewing the statistical conclusions drawn from the model. It was therefore important to test for outliers, and where applicable, exclude these data points from any further analysis. One commonly used tool to measure and ascertain the influence of possible outliers, is the Cook's Distance. Cook and Weisenberg[26] suggested that a Cook's Distance of $D_i > 1$ should indicate statistical outliers[26]. However, Bollen and Jackman[27] argued that $D_i > 4/n$ should be sufficient in most instances[27]. The results (Figure 1) showed that, some data points (run 7 for the aqueous extraction process (Figure 1a) and runs 3 and 14 for the methanol extraction process (Figure 1b) could be interpreted as outliers when considering the Bollen and Jackman interpretation, whilst none of the data points exceeds a Cook's Distance of 1. For this reason, the author chose to use all data points in further analysis.

Half normal probability plots

Half normal probability plots (Figure 2) were used to compare the magnitude and statistical significance of the independent variables and their interaction terms in two-level factorial designs. All insignificant effects, with zero means and variances, were distributed around the straight line, whereas significant effects, with non-zero means, were displayed on the right side of the straight line in the plots. Based on the analysis of the aqueous extraction process, two significant effects emerged, the main effects of B (pH) and C (extraction time) (Figure 2a). There was no significant interaction between the independent variables. Based on the distance from the straight line it was evident that the main effect of extraction time (C) was more significant than that of pH (B) (Figure 2a).

Figure 2b, the half normal plot resulting from the methanol extracts, showed that the percentage yield of the crude extract was influenced by a greater number of variables and their interactions. In decreasing order, the main- and interaction terms that influenced the extraction yield (%) were found to be B (technique) > C (extraction time) > AC

Table 1: Analysis of extraction yield data as a 2^3 factorial design for the aqueous extraction process of *Cotyledon orbiculata* .

Variable				Low (-)			High (+)	
extraction temperature (°C)				30°C			60°C	
B = pH				7			9	
C = extraction time (minutes)				30 min			240 min	
RUN	A	B	C	A	B	C	Response % (y)	
							Actual	Predicted
1	-	-	-	30	7	30	38.60	39.73
2	-	-	-	30	7	30	41.40	39.73
3	-	-	-	30	7	30	40.00	39.73
4	+	-	-	60	7	30	40.20	39.73
5	+	-	-	60	7	30	37.80	39.73
6	+	-	-	60	7	30	39.10	39.73
7	-	+	-	30	9	30	42.60	41.49
8	-	+	-	30	9	30	41.10	41.49
9	-	+	-	30	9	30	42.10	41.49
10	+	+	-	60	9	30	39.50	41.49
11	+	+	-	60	9	30	42.20	41.49
12	+	+	-	60	9	30	42.70	41.49
13	-	-	+	30	7	240	42.80	42.64
14	-	-	+	30	7	240	42.90	42.64
15	-	-	+	30	7	240	43.00	42.64
16	+	-	+	60	7	240	43.40	42.64
17	+	-	+	60	7	240	42.20	42.64
18	+	-	+	60	7	240	42.80	42.64
19	-	+	+	30	9	240	44.30	44.41
20	-	+	+	30	9	240	43.40	44.41
21	-	+	+	30	9	240	44.70	44.41
22	+	+	+	60	9	240	43.70	44.41
23	+	+	+	30	9	240	43.80	44.41
24	+	+	+	60	9	240	45.30	44.41

Table 2: Analysis of extraction yield data as a 2^3 factorial design for the methanol extraction process of *Cotyledon orbiculate.*

Variable				Low (-)			High (+)	
A = solvent composition (%)				60%			100%	
B = technique				M = Maceration			P = Percolation	
C = extraction time (hours)				24 hours			48 hours	
RUN	A	B	C	A	B	C	Response (y)	
							Actual	Predicted
1	-	-	-	60	M	24	21.1	22.56
2	-	-	-	60	M	24	24.0	22.56
3	+	-	-	100	M	24	23.9	23.14
4	+	-	-	100	M	24	22.4	23.14
5	-	+	-	60	P	24	27.1	26.19
6	-	+	-	60	P	24	26.4	26.19
7	+	+	-	100	P	24	28.4	30.36
8	+	+	-	100	P	24	31.2	30.36
9	-	-	+	60	M	48	27.8	28.46
10	-	-	+	60	M	48	29.1	28.46
11	+	-	+	100	M	48	28.1	27.89
12	+	-	+	100	M	48	27.7	27.89
13	-	+	+	60	P	48	29.6	31.14
14	-	+	+	60	P	48	33.8	31.14
15	+	+	+	100	P	48	27.9	26.96
16	+	+	+	100	P	48	24.9	26.96

M = Maceration: P = Percolation

(solvent composition*extraction time) > BC (technique*extraction time) and ABC (solvent composition*technique*extraction time).

Main effects and interactive plots

Main effect and interaction plots refer to simple line graphs obtained from connecting the mean values of each treatment. The graphs in Figures 3a - 3c, showed that there were two main effects: pH (B) and extraction time (C). As the magnitude of these variables increased there was a corresponding increase in the percentage yield. The main effect plot of extraction temperature (A) in Figure 3a showed that it had no effect on the percentage yield of the aqueous crude extracts, irrespective of the other treatments.

The almost parallel graphs observed in the interaction plots (Figures 3d – 3f), suggests a non-significant interaction between the independent variables in the aqueous extraction process. On the contrary, the percentage extract yield with methanol was influenced by the main effects as well as the interactions between the variables (Figures 4a – 4f). The gradients of the graphs due to the technique (Figure 4b) and extraction time (Figure 4c) showed that these variables had significant effects on the percentage yield when methanol is used. On the contrary, the solvent composition did not have any significant effect on the percentage yield with methanol (Figure 4a).

The merging graphs for the interaction of BC (technique*extraction time) implies that there was a rather strong two-way interaction between these main effects (Figure 4f). This interaction would not have been detected in a one-factor-at-a-time (univariate) statistical analysis[28]. The interactions between the other main effects were relatively weak (Figure 4d and 4e).

Aqueous extraction design: Regression model analysis and ANOVA

The effect of temperature (30 – 60°C), pH (7 - 9) and time (30 - 240 minutes) on the percentage yield of aqueous crude extracts from *C. orbiculata* was investigated using a 2^3 factorial design. Two regression models to predict the percentage yield of aqueous crude extracts from *C. orbiculata* was developed (Table 3).

The full regression model consisted of both the significant ($p<0.05$ level) and non-significant model terms (Table 3a), whereas the reduced model only shows significant ($p<0.05$ level) terms (Table 3b). The F- values for the full and reduced regression models (Table 3) were 9.68 and 36.73, respectively. The low p-values of the pH (B) and extraction time (C) suggest that they made a significant contribution on the percentage yield of the crude aqueous extracts. A regression model and adequacy checking was done based on the values of the R^2, R^2 adjusted and R^2 predicted. The full and reduced regression models had calculated R^2 values of 0.5703 (full) and 0.7096 (reduced). To test the model stability and confirm that we did not over-fit the model to the given data, there was a need to compare the adjusted and predicted R^2 values. The predicted R^2 is a measure of how well the model would predict actual observations by fitting existing data points and comparing the predicted values with the actual values. A high R^2 predicted value indicates a good fit. Since the predicted R^2 value cannot be greater than the R^2 adjusted value, it implies that a predicted R^2 that is less than or close to the adjusted R^2 is a desirable outcome[17]. In the full model, there was a large difference between the adjusted (0.7255) and predicted (0.5703) R^2 values. This may probably be due to the number of terms in the model as suggested by[29]. In the reduced model (Table 3b) the adjusted (0.7565) and predicted (0.7096) R^2 values produced a more reliable fit. After discarding the insignificant model terms, an equation based on the reduced regression model to predict the percentage extraction yield of crude aqueous extracts was developed (equation 9).

$$\hat{y} = \beta_0 + \beta_2 B + \beta_3 C \qquad (9)$$

$$\hat{y} = 33.125 + 0.883 * pH + 0.014 * Extraction\ Time$$

In equation 9, $\hat{y}$ represents the predicted extraction yield, B represents the pH and C represent the extraction time. β_0, β_2 and β_3 are the regression coefficients. β_0 was the mean value of the responses of all experiments, β_2 was a measure of the expected change in *y* per unit change in the pH (B) when C is held constant and β_3 was a measure of the expected change in y per unit change in extraction time (C) when B is held constant. The regression coefficients are a measure of the potential strength of each independent

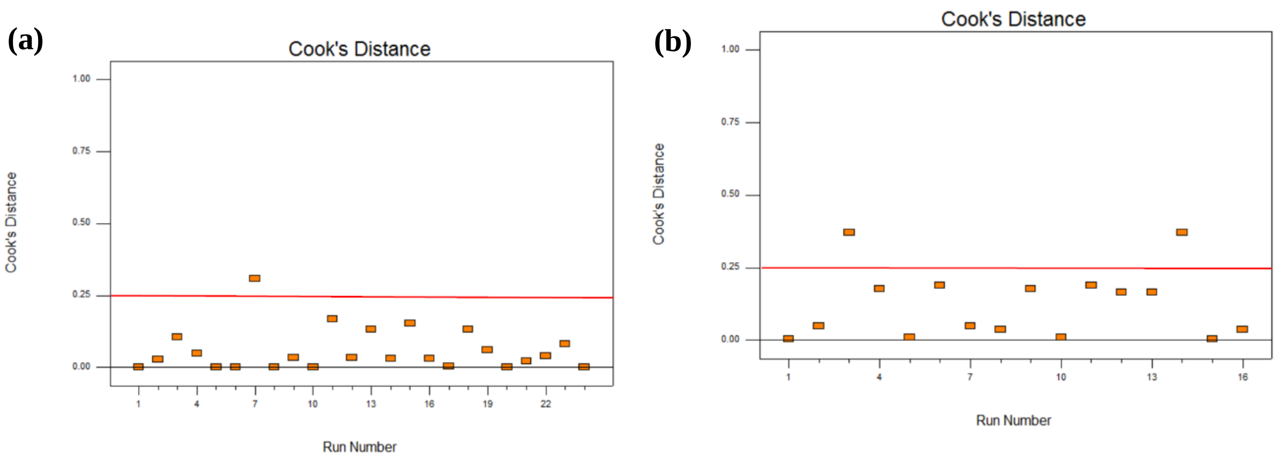

Figure 1: An illustration of Cook's Distance on an outlier versus run number plot for the (a) aqueous extraction and (b) the methanol extraction processes.

Table 3: ANOVA report for the (a) full and (b) reduced regression model for the aqueous extraction process.

(a)

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	72.58	7	10.37	9.68	<0.0001
A	0.74	1	0.74	0.69	0.4196
B	18.73	1	18.73	17.49	0.0007
C	51.04	1	51.04	57.67	<0.0001
AB	0.20	1	0.20	0.19	0.6701
AC	0.81	1	0.81	0.75	0.3983
BC	1.04	1	1.04	0.97	0.3387
ABC	0.027	1	0.027	0.025	0.8766
Pure error	17.13	16	1.07		
Cor total	89.71	23			

SD = 1.03; R^2 = 0.8090; R^2(adj) = 0.7255; R^2 (pred) = 0.5703; Adeq precision = 8.76

(b)

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	69.77	2	34.88	36.73	< 0.0001
B	18.73	1	18.73	19.72	0.0002
C	51.04	1	51.04	53.74	<0.0001
Residual	19.94	21	0.95		
Pure error	17.13	16	1.07		
Cor total	89.71	23			

SD = 0.97; R^2 = 0.7777; R^2(adj) = 0.7565; R^2 (pred) = 0.7096; Adeq precision = 13.59

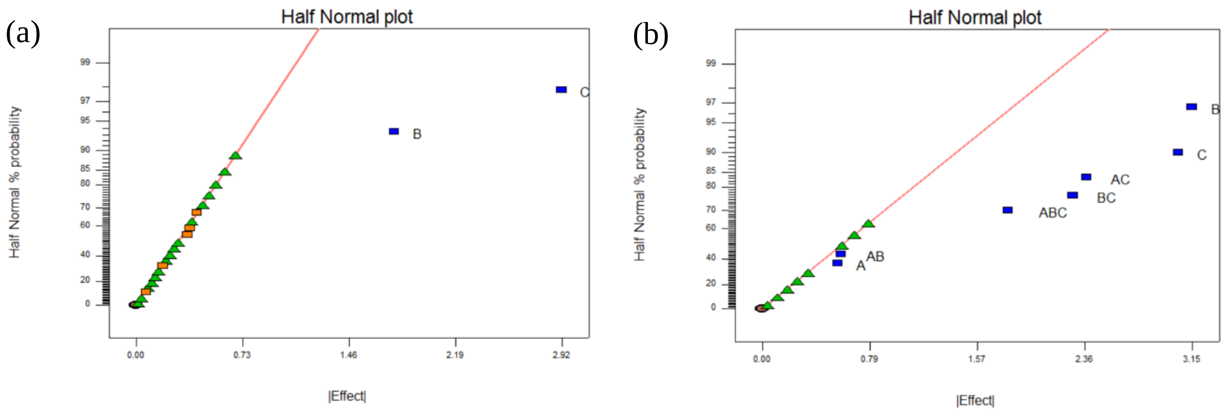

Figure 2: Half normal probability plot showing the effect (% yield) for (a) water extraction and (b) methanol extract.

variable or predictor on the dependent variable[17]. The qualitative outcome of this model is more favorable than those reported by Amabeoku, Green and Kabatende[12] when they investigated the anticonvulsant activity of *C. orbiculata* in mice. Their extraction method was to reflux 63 g of dried powder in 1 L boiled water that was cooled for over 24 h. After filtration, the filtrate was frozen at -80°C and freeze-dried to obtain a yield of 15.9 g of dried plant extract. The 2^3 factorial design approach demonstrates a higher percentage yield (37.8% – 45.3%) as compared to the 25.2% yield obtained by Amabeoku, Green and Kabatende[12]. The production of a higher yield by manipulating simple and inexpensive variables (pH and extraction time), holds great promise for improved commercial production on an industrial scale.

Methanol extraction design: Regression model analysis and ANOVA

A 2^3 factorial design was used to develop the regression model for yield of extracts using methanol and included the effect of methanol composition (60 – 100%), technique (maceration and percolation) and extraction time (24 – 48 hours). Analysis of the 16 experimental runs, showed that the methanol composition and the interaction between the methanol composition and technique had no significant effect on the yield of the crude extract (Table 4). The F-values for the full (Table 4a) and reduced regression (Table 4b) models were 6.53 and 10.13, respectively. The low p-values for technique (B), extraction time (C) and the interaction between methanol composition and extraction time (AC) and as well as that between technique and extraction time (BC) suggests that they made a significant ($p<0.05$ level) contribution to the percentage yield. The calculated R^2 values of the full (0.8511) and reduced (0.8352) regression models indicates that 85.1% (full) and 83.5% (reduced) of the total variability in the data is

Table 4: ANOVA report for the (a) full and (b) reduced regression model for the methanol extraction variables.

(a)

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	135.66	7	19.38	6.53	0.0084
A	1.21	1	1.21	0.41	0.5410
B	39.69	1	39.69	13.37	0.0064
C	37.21	1	37.21	12.54	0.0076
AB	1.32	1	1.32	0.45	0.5232
AC	22.56	1	22.56	7.60	0.0248
BC	20.70	1	20.70	6.98	0.0297
ABC	12.96	1	12.96	4.37	0.0700
Pure error	23.74	8	2.97		
Cor total	159.40	15			

SD = 1.72; R^2 = 0.8511; R^2(adj) = 0.7207; R^2 (pred) = 0.4043; Adeq precision = 7.512

(b)

Source	Sum of squares	Degree of freedom	Mean squares	F-values	P-value
Model	133.13	5	26.63	10.13	< 0.0011
B	39.69	1	39.69	15.11	0.0030
C	37.21	1	37.21	14.16	<0.0037
AC	22.56	1	22.56	8.59	0.0150
BC	20.70	1	20.70	7.88	0.0186
Residual	26.27	10	2.63		
Pure error	23.74	8	2.97		
Cor total	159.40	15			

SD = 1.62; R^2 = 0.8352; R^2(adj) = 0.7528; R^2 (pred) = 0.5781; Adeq precision = 8.63

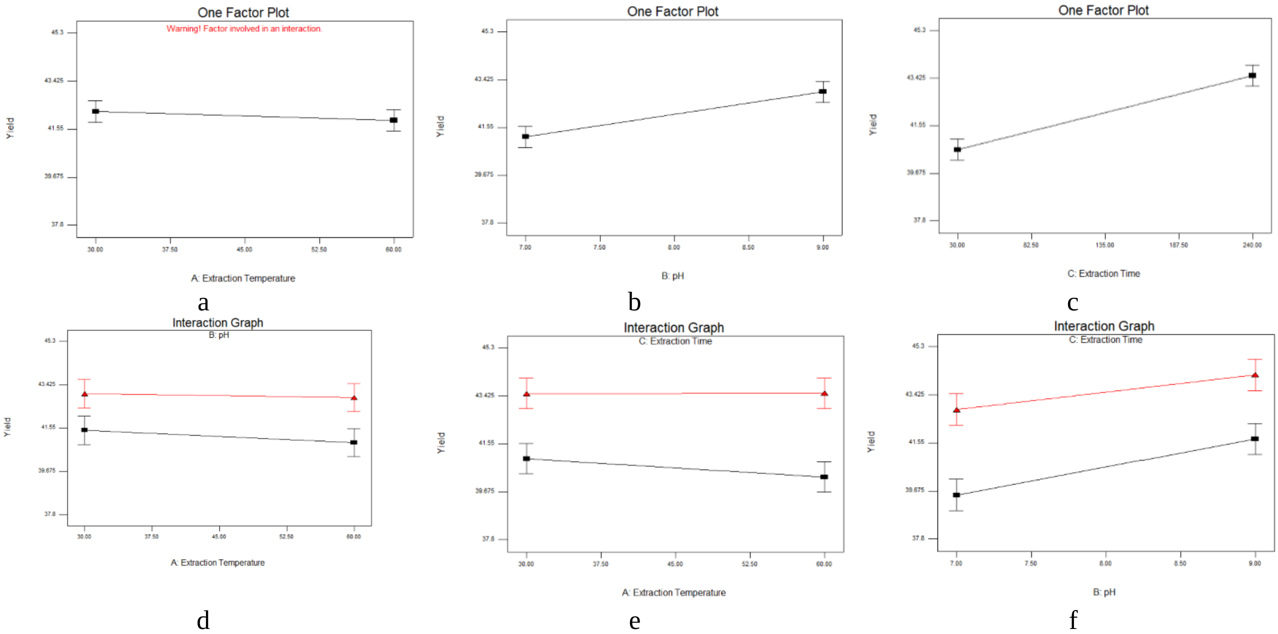

Figure 3: One Factor (a – c) and Interaction (d – f) plots on the effect (% yield) on the aqueous extraction process.

explained by ANOVA. The relative large difference (13.04%) between the calculated (0.8511) and adjusted (0.7207) R^2 values, for the full model suggests that insignificant terms were included in the model. Upon removal of the insignificant terms (A, AB and ABC) from the model, the difference between the predicted (0.8352) and the adjusted (0.7528) R^2 values decreased slightly (8.24%).

The full and reduced regression models had predicted R^2 values of 0.4043 and 0.5728, respectively. The low predicted R^2 values suggest that (i) the significant variables did not contribute to the model, (ii) that other variables needed to be considered, or (iii) that the model could have reached an over-fit and as a result noise was incorporated into the model [17]. After discarding the insignificant terms in the model, an equation based on the reduced regression model to predict the yield of extracts using methanol was

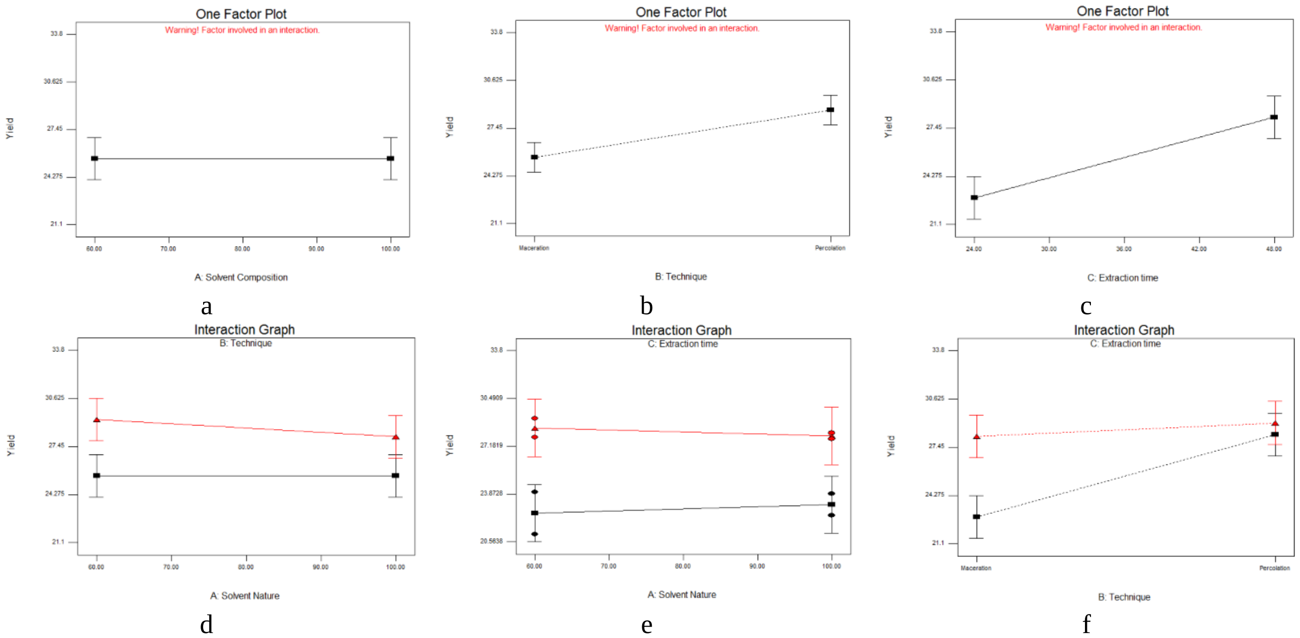

Figure 4: One Factor (a) – (c) and Interaction (d) – (f) plots on the effect (% yield) on the methanol extraction process.

developed according to the categorical variables, namely maceration and percolation. The reduced regression model (equation 10) for predicting the extraction yield using maceration was:

$$\hat{y} = \beta_0 + \beta_1 A + \beta_3 C - \beta_{13} AC \quad (10)$$
$$= 14.025 + 0.04375 * Solvent\ composition + 0.31771 * Extraction\ time$$
$$-1.1979\ x\ 10^{-3} * Solvent\ composition * Extraction\ time$$

Whereas that for percolation (equation 11) was:

$$\hat{y} = \beta_0 + \beta_1 A + \beta_3 C - \beta_{13} AC \quad (11)$$
$$= 4.7000 + 0.25800 * Solvent\ composition + 0.72812 * Extraction\ time$$
$$-8.69792\ x\ 10^{-3} * Solvent\ composition * Extraction\ time$$

In both these equations $\hat{y}$ represents the predicted extraction yield. The letters A, C and AC represents the methanol composition, extraction time and the interaction between methanol composition and extraction time, respectively. β_0, β_1, β_3 and β_{13} represents the regression coefficients. The positive sign of the regression coefficients for A and C is indicative of their synergistic effects on extraction yield while the negative sign of β_{13} denotes an antagonistic effect. Comparing the yield obtained by Amabeoku, Green and Kabatende[12] in the preparation of their methanol extract using 800.6 g of dried powder in a soxhlet extractor over 72 hours, the 35.4% yield (283.8 g) was slightly higher than that obtained in the 2^3 factorial design (29.6% – 33.8%; Table 2; Run 13 and 14). For the methanol extraction design, we believe that better results could be achieved by considering alternative variables in the 2^3 factorial design. This warrants further research.

CONCLUSION

The screening of variables that may influence the aqueous and methanol extraction processes of phytochemicals from *C. orbiculata* were achieved through two 2^3 full factorial experimental design. The effects of temperature (30 – 60°C), pH (7-9) and time (30 – 240 min) on the percentage yield for the aqueous extraction process showed that temperature had no effect on the yield. The absence of a significant effect from temperature could be beneficial in extraction experiments since less energy will be used and denaturation of proteins would not occur. Increasing pH or extraction time on the other hand resulted in an improved yield. The interaction between increasing both pH and extraction time did not produce a statistically significant increase in yield. Numerical optimization of the aqueous extraction design was determined by means of a desirability plot using Design Expert 6.0.6. The optimum values of the selected variables, namely, extraction temperature, pH and extraction time are 30°C, pH 8.99, and 240 minutes, respectively, with an expected yield of 44.13%.

With regards to methanol extraction both extraction technique and extraction time has a positive effect on yield. Similarly, the interaction between methanol composition and extraction time, as well as the extraction technique combined with the extraction time had a significant influence on the extraction yield. Considering numerical optimization, a predicted yield (highest) of 31.7% could be obtained with percolation, using a 60% methanol solution and an extraction time of 48 h. The low percentage yields, as well as the lower than expected predicted R^2 values for the regression models using methanol suggests that other variables should be considered to possibly achieve a higher yield.

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CONFLICT OF INTERESTS
The authors declare that there is no conflict of interests.

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