Statistical Modelling of Ultrasonic-Aided Extraction of *Elaeis guineensis* Leaves for Better-Quality Yield and Total Phenolic Content

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Abstract: *The present study highlighted the statistical modelling of an ultrasonic-aided extraction (UAE) of* Elaeis guineensis *leaves extract for maximal extraction yield (EY) and total phenolic content (TPC). A Box-Behnken design investigated the effects of ethanol concentration (X_1: 0–100%), extraction time (X_2: 5–55 min), the solvent-to-solid ratio (X_3: 15:1–35:1 mL/g) and sonification amplitude (X_4: 20–100 %). Under optimized conditions, the highest EY of 14.38% was attained using 50% (v/v) ethanol:water ratio, 55 min, 35 mL/g solvent-to-solid ratio, 60% sonication amplitude, whereas maximum TPC was 209.70 mg gallic acid equivalent (GAE)/g [50% (v/v) ethanol:water ratio, 30 min, 25 mL/g solvent-to-solid ratio, 60% sonication amplitude]. Second-order polynomial models of EY and TPC showed the R^2 value corresponding to 0.9303 and 0.9500, respectively, indicating their significance ($p < 0.05$) to predict the responses. HPLC chromatograms revealed gallic acid and catechin were present in the UAE extracts. UAE technique afforded better EY (14.38%) and TPC (209.70 mg GAE/g) than maceration (3.73%, 85.23 mg GAE/g) and Soxhlet (6.86%, 102.13 mg GAE/g) extractions, as based on scanning electron micrographs of untreated, UAE, macerated and Soxhlet treated samples. Cell walls of ultrasonic-treated E. guineensis leaves were visibly disrupted to facilitate the higher release of bioactive plant materials, thus justifying the higher EY and TPC. The application of ultrasound appeared to remarkably increase the extraction efficiency of E. guineensis leaves to extract as compared to the conventional methods.*

Keywords: *ultrasonic-aided extraction;* Elaeis guineensis*; extraction yield; total phenolic content; response surface methodology*

■ INTRODUCTION

Elaeis guineensis Jacq. or commonly known as palm oil tree belonging to the family Arecaceae is an oil-producing crop that grows perennially in a tropical climate [1-2]. The carotenoid-rich orange-red colored oil is obtained from the thinly-skinned fleshy fruits, as well from the mesocarp and kernels [3]. Being the most important agricultural crop in Malaysia, palm oil is the fourth largest contributor to the nation's economy [4]. Nonetheless, biomass from oil palm industries generated from pruning, replanting and milling activities are currently underutilized, as apparent from the insignificant conversions of oil palm biomass into value-added products [5]. The biomass of the oil palm constitutes oil palm fronds, oil palm trunk, empty fruit bunches, palm kernel shells, mesocarp fibers and palm oil mill effluent [6]. In fact, oil palm leaves (OPL) make up the largest portion amounting to 53% dry weight of the total biomass [7]. As far as applicability of OPL is concerned, it is limited as ruminant feed and food flavoring agents [8]. Therefore, utilizing the cellulosic

materials in OPL can improve agriculture sustainability while reducing the quantity of this biomass [9].

Studies have shown that OPL extracts (OPLE) contain a myriad of beneficial water-soluble flavonoids and phenolic acids, as well as oil-soluble vitamin E, α- and β-tocopherols [10]. These compounds are recognized for their potency as antioxidants, anti-mutagenic, anti-inflammatory and anti-cancer agents, as well as several other nutritional and health benefits [11-12]. Other bioactive ingredients exhibiting anti-tyrosinase and anti-microbial activities are also present in the OPLE, whereas certain compounds can protect the human skin against ultraviolet (UV) radiation, implying their usability for topical application [13]. Specifically, catechins viz. epigallocatechin (0.08%), catechin (0.30%), epicatechin (0.01%), epigallocatechin gallate (0.28%) and epicatechin gallate (0.05%) are among the reported phenolic compounds existing in OPLE [14]. There are claims of glycosylated flavonoids, ferulic acid, gallic acid, protocatechuic acid and carotenoids being present, too [15].

Considering the high presence of beneficial phytochemicals in the OPLE, the study, therefore, emphasizes that they may be valuable as active ingredients in topical creams. This is in light of the alarmingly widespread use of synthetically produced anti-oxidants, for instance, butylated hydroxyanisole, butylated hydroxytoluene, tertiary butyl hydroquinone and propyl gallate as active ingredients in such creams. It is somewhat worrying as these substances are thought to be among the causal agents of skin cancer [16-17]. In this perspective, their replacement with natural plant-based antioxidants such as phytopolyphenols, flavonoids, vitamin C and carotenoids as active ingredients in creams, may offer a safer alternative. Moreover, these plant-based compounds are known as good scavengers of hazardous free-radicals [18].

To promote the use of plant bioactive compounds in cosmeceutical topical creams, their process recovery must be judiciously carried out to ensure retention of their bioactivity. This is because conventional extraction methods that rely on physical treatments, for example, heating, refluxing, boiling and Soxhlet extraction tend to result in major losses of bioactivity due to oxidation, ionization and hydrolysis of plant compounds during prolonged extraction time, alongside the undesirable employment of high quantities of solvents [19]. These issues may be circumvented using modern techniques, for instance, supercritical fluid extraction, subcritical water, and accelerated solvent extraction, high hydrostatic pressure processing, microwave-assisted and Ultrasonic-Aided Extraction (UAE) to extract plant materials [20-22]. Herein, the work reported here was focused on the use of UAE to extract valuable plant phytochemicals. In conjunction with being cheap and simple to operate, UAE is highly efficient and leads to good extraction efficiency [23]. Unlike the aforementioned simple physical treatments, UAE acoustically induced cavitation rigorously rupture plant cell walls and synergistically improve solvent penetrability into the plant matrix while reducing particles size. A higher surface of contact between trapped bioactive compounds and the solvent medium is achieved afterwards, facilitating better extraction [24-25].

In this study, the UAE based on the Box-Behnken Design (BBD) was optimized for parameters solvent concentration, extraction time, solvent-to-solid ratio and sonication amplitude to account for the highest yield and total phenolic content in the OPLE. Suitability of this mathematical and statistical tool has been established in a myriad of experimental trials for different processes. In fact, the BBD has been proven excellent for evaluating and observing influences, as well as interactions of multiple factors in any given process; benefits that are unseen in the one-variable-at-a-time technique [26-27]. Thus, the objective of this study was to seek the best aforementioned UAE conditions for maximizing the extraction yield and total phenolic content of *E. guineensis* leaves extract. For better comprehension of the UAE extraction efficacy, conventional extraction methods using Soxhlet extraction and maceration on *E. guineensis* leaves were also performed and the results were compared to that of UAE. Scanning electron micrographs were used to observe and accentuate the morphological differences between the untreated, ultrasonically treated and *E. guineensis* leave samples subjected to maceration and

Soxhlet extraction. It is hypothesized the efficient extraction of phytochemicals trapped in OPL by the UAE is due to the available higher contact area that stems from the acoustically ruptured plant cell walls. To the best of our knowledge, this is the first study detailing the use of a UAE of oil palm leaves for optimizing the extraction yield and total phenolic content.

■ EXPERIMENTAL SECTION

Materials

Plant materials

Fresh Oil Palm Leaves (OPL) were collected from a plantation on the grounds of Universiti Teknologi Malaysia, Johor in October 2017. The OPL was left to air-dry for a week before undergoing cutting and grinding into a powder. The powdered samples were kept in zip-locked plastic bags and stored at room temperature until further use.

Chemicals and reagents

Folin–Ciocalteu's phenol reagent, (+)–catechin hydrate, and sodium carbonate were purchased from Sigma–Aldrich (St. Louis, USA). Gallic acid, sodium nitrite, and aluminum chloride hexahydrate were purchased from Merck (Darmstadt, Germany). Analytical grade ethanol (99.86% mass fraction purity) used for the extraction was procured from Haymankimia (Essex, England), while HPLC grade ethanol was from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade. Millipore Milli–Q water purification system was used to produce 18 mΩ deionized water that was used in all analyses.

Procedure

Ultrasonic-aided extraction of OPL

Ultrasonic-Aided Extraction (UAE) of the OPL was performed using an ultrasonic probe Sonics Vibra Cell (America) equipped with a digital control system that controls sonication time and amplitude. In all experiments, ultrasonication on OPL was performed at 20 kHz frequency and constant power of 130 W under a constant temperature of 25 °C. This was to avoid structural alteration and thermal degradation of the biologically active compounds [28]. Dry OPL (1 g) was weighed and transferred each into centrifuge tubes (50 mL) containing designated volumes of ethanol (15, 25 and 35 mL) at varying concentrations (0, 50, 100% v/v). The suspensions were homogenized using a homogenizer IKA T18 Digital Ultra Turrax (Germany) at 10,000 rpm for 40 s prior to UAE. The samples were then sonicated and assessed for the effects of solvent concentration (0–100%), solvent–to–solid ratio (15:1, 25:1 and 35:1 mL/g), extraction time (5, 30, 55 min) and sonication amplitude (20, 60, 100%) for the responses, Extraction Yield (EY) and Total Phenolic Content (TPC). Sample collection was done by centrifuging each sample mixture for 15 min at 4,000 rpm, the supernatants were collected and filtered through a Whatman No. 1 filter paper. The solvent was removed using a rotary evaporator under reduced pressure at 40 °C to obtain the crude extract, and then lyophilized for 48 h. Each crude sample was accurately weighed using an analytical balance with a sensitivity of 0.1 mg by Shimadzu Philippines Manufacturing Inc. (Philippines) until a constant weight was attained. Samples were then stored at 4 °C until further analysis. Percentage of extraction yield (%) was determined using Eq. (1).

$$\text{Yield (\%)} = \frac{\text{Weight of sample after freeze drying (g)}}{\text{Weight of dried sample (g)}} \times 100\% \quad (1)$$

Determination of total phenolic content (TPC)

Folin-Ciocalteu (FC) phenol method with slight modifications was used to estimate the TPC of the OPLE. The readings were colorimetrically determined by a UV-Visible spectrophotometer (UV-1601PC, Shimadzu), based on an oxidation/reduction reaction [29]. OPLE (0.2 mL) was mixed with FC reagent (0.2 mL) and deionized water (1.8 mL), followed by further additions of 7% (w/v) Na_2CO_3 (2 mL) and deionized water (0.8 mL) after 5 min. The extracts were homogenized and left to stand for 30 min before the absorbance was read at 765 nm. Absolute ethanol, pure water and a mixture of ethanol and water were used as blanks for the different OPL samples extracted using absolute ethanol, pure water and a mixture of ethanol, respectively. Three different calibration curves for extracts of absolute ethanol, pure water and a mixture of ethanol and water (concentrations varying from

0–100 µg mL^{-1}) were prepared using gallic acid as the standard (Fig. 1(a-c)) and each analysis was triplicated. The results were expressed in mg gallic acid equivalent (GAE) per gram of extract (mg GAE/g extract) as shown in Eq. (2).

$$T = C \times \frac{V}{M} \quad (2)$$

where T is the total phenolic content in mg g^{-1} of the extract expressed as GAE; C is the concentration of gallic acid established from the calibration curve in mg mL^{-1}; V is the volume of the extract solution in mL, and M is the weight of the extracts in g.

Determination of total flavonoids content (TFC) of the highest TPC crude

TFC was determined spectrophotometrically using a colorimetric method based on aluminum chloride ($AlCl_3$) with minor modifications [29-31]. A sample (2 mL) was mixed with $NaNO_2$ solution (0.6 mL, 5%, w/v), followed by the addition of $AlCl_3$ (1 mL, 2%, w/v) after 5 min. The mixture was mixed thoroughly and incubated for 5 min. The mixture was then neutralized with NaOH solution (0.5 mL, 1 mol/L), left to stand for 10 min at room temperature and the absorbance read at 510 nm. The linearity range of the prepared calibration curve (Fig. 1(d)) was between 0.00–0.10 mg/mL and the TFC was estimated using a catechin hydrate standard curve, reported as mg catechin hydrate equivalents (CAE)/g of dried weight (DW) (Eq. 3). All absorbances were measured in triplicate;

$$C = \frac{CV}{m} \quad (3)$$

where C is the total content of flavonoid compounds in mg/g of plant extract as CAE; C is the concentration of catechin hydrate established from the calibration curve in mg^{-1} mL; V is the volume of the extract in mL; m is the weight of the crude plant extracts in g.

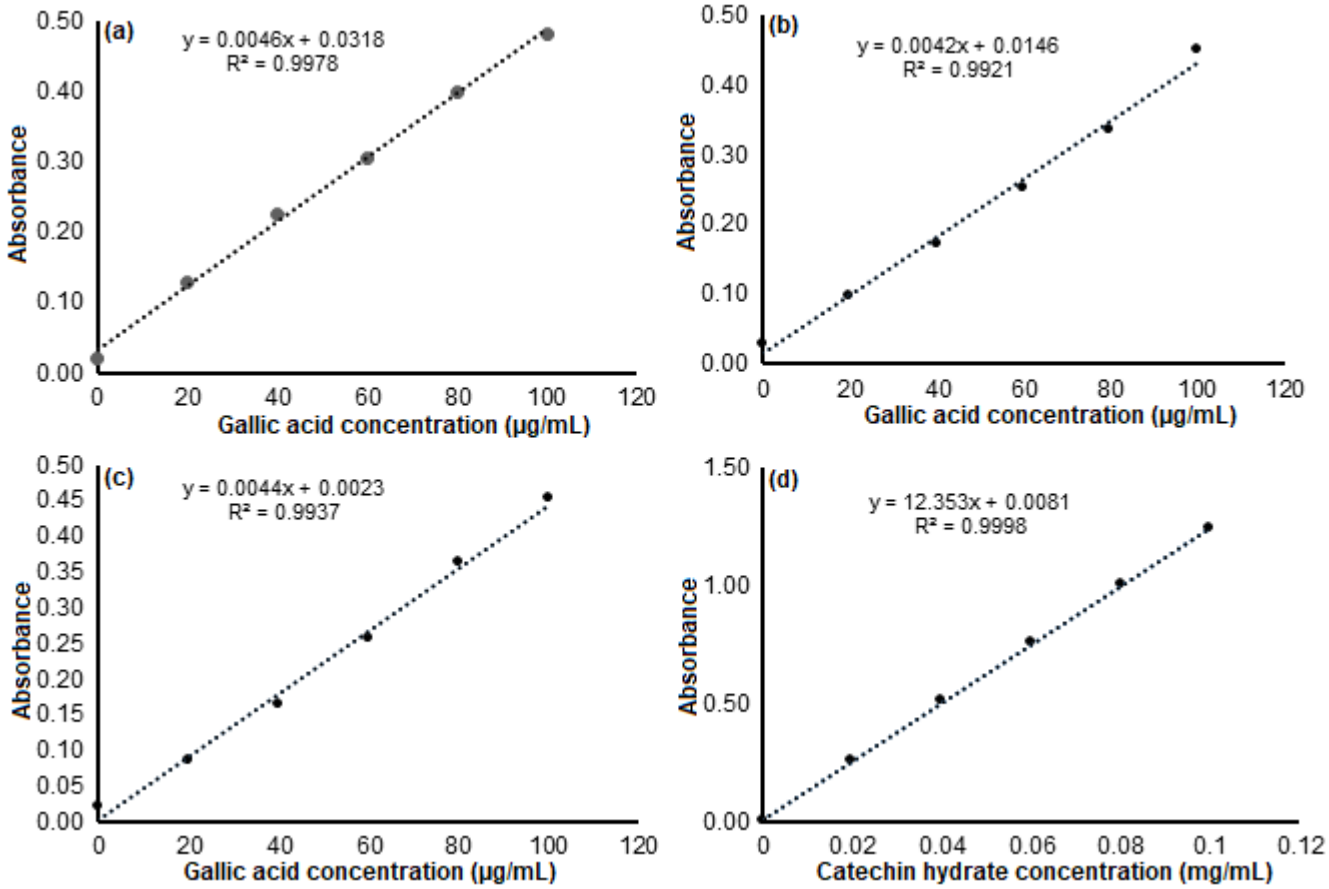

Fig 1. Calibration curve of (a) gallic acid in 100% water, (b) gallic acid in 100% ethanol, (c) gallic acid in 50% ethanol for determination of TPC, and (d) catechin hydrate in 50% ethanol for determination of TFC value in OPLE

Maceration extraction (ME) and soxhlet extraction (SE)

Ground dried OPL (1 g) was mixed with an ethanol solution (200 mL, 50%) and shaken on an orbital shaker (12 VDC 1.5 Amps 18 Watts, Seastar, China) at room temperature for 24 h. The extractant was collected, dried as specified in Section 2.3 and stored at 4 °C until further analysis. For the Soxhlet extraction, ground OPL (1 g) was placed over a Whatman filter paper and refluxed in ethanol (200 mL, 50%, v/v) at 95 °C for 24 h. The extractant was collected, dried (Section 2.3) and stored at 4 °C. Extraction yields and TPCs for both methods were calculated as per Eq. (1) and (2) [32].

Scanning electron microscopy (SEM)

Ground OPL samples either untreated or ultrasound-treated, as well as those obtained from the Soxhlet and maceration extraction samples, were subjected to SEM to observe changes in morphology of the plant materials, as the result from the above experiments. A SEM (JEOL JEM–6700F) operated at 5kV was used in the analyses, where the four differently treated dried samples of OPL were deposited on silicon wafers and were sputter-coated with a thin layer of gold to avoid charging under the electron beam [9].

HPLC analysis for gallic acid and catechin

HPLC analysis was carried out according to the manufacturer's protocol for the instrument Agilent Technologies Model 1123 equipped with a diode–array detector, Agilent Software and a C18 column (5.0 µm, 250 mm x 4.6 mm; Agilent). The mobile phase consisted of ethanol:water:orthophosphoric acid in the ratio of 20:79.9:0.1, respectively, moving isocratically at a rate of 1.0 mL/min. The injected volume was 5 µL and the detection wavelength of the UV detector was 270 nm. For detection of catechin, HPLC analysis on the extract was carried out using Waters 2695 series HPLC equipped with an autosampler, PDA detector, Empower Software and a C18 column (5.0 µm, 150 mm x 4.6 mm; Waters), operated at 30 °C. The mobile phase was comprised of solvent A (0.1% acetic acid) and solvent B (acetonitrile), and flowed under an isocratic program (95% A: 5% B) at 0.8 mL/min, whereas the injected volume and detection wavelength was 20 µL and 280 nm, respectively.

Box-Behnken experimental design

Prior to optimization, a screening study was performed to estimate the logic range of optimization experiments, as well as to determine factors that were relevant in affecting the two responses in the study. A four-factor-three-level Box-Behnken design (BBD) was used in the optimization experiment, in which only relevant factors that influence the efficiency of EY and TPC were assessed. The BBD experiment was carried out in random order and comprised of 29 combinations which include five replicates for the central points. Factors of ethanol concentration (%, X_1), extraction time (min, X_2), the solvent-to-solid ratio (mL/g, X_3) and sonification amplitude (%, X_4) were examined and their ranges were tabulated in Table 1. The temperature of the UAE was kept at room temperature to avoid degradation of temperature-sensitive compounds. The second-order (quadratic) polynomial response surface model which delineates the relationship between the experimental results is as follows (Eq. 4):

$$Y = b_0 + \sum_{i=1}^{n}(b_i x_i) + \sum_{i=1}^{n}(b_{ii} x^2_{ii}) + \sum_{ij=1}^{n}(b_{ij} x_i x_j) \quad (4)$$

where Y is the predicted variable (EY or TPC), b_0 is the constant; x_i stands for the coded levels of the design variable (solvent concentration, extraction time, solvent-to-solid ratio, energy of sonification amplitude and n is the number of tested variables (n = 4), b_i = linear effects, b_{ii} = quadratic effects and b_{ij} = interaction effects.

Table 1. Three levels of the independent variables in the BBD of the UAE

Independent Variables	Units	Symbols	Coded Levels		
			−1	0	+1
Ethanol concentration	% v/v	X_1	0	50	100
Extraction time	min	X_2	5	30	55
Solvent-to-solid ratio	mL/g	X_3	15	25	35
Energy of sonification amplitude	%	X_4	20	60	100

Statistical analysis

The regression analysis and the optimization of RSM were analyzed by the Design-Expert 7.1.6 software. The analysis of variance (ANOVA) was carried out to check the statistical significance ($p < 0.05$) of the independent variables. The coefficient of determination (R^2), lack of fit, adequate precision which measures the signal to noise ratio, adjusted coefficient of determination (adj. R^2), coefficient of variation (C.V), and Fischer's test value (F-value) were used to examine the adequacy of UAE models.

■ RESULTS AND DISCUSSION

Model Fitting and Process Optimization

Response Surface Methodology (RSM) is an effective analytical tool to measure the interaction and correlation process variables and corresponding responses, along with the effects of optimized multiple variables on the responses [33-34]. The current study was conducted to seek the optimal conditions for maximizing extraction yield (EY) and total phenolic contents (TPC) from *E. guineensis* leaves. The BBD used in this study was fitted to second-order polynomial equations (linear, two-factorial, quadratic and cubic), in which a quadratic model was found to best describe the ultrasonic-aided extraction (UAE) of *E. guineensis* leaves (OPL). Two models generated by the study were tested for statistical significances and adequacies based on their p- and F-values, the coefficient of determinations (R^2) and adjusted determination of coefficients (adj. R^2) from analysis of variances (ANOVA). For brevity, a model with a p-value of less than 0.05, indicates the model is significant and a p-value of below 0.0001 implies a highly significant model.

The range of the UAE processing variables, levels along with the experimental and predicted responses for the EY and TPC are tabulated in Table 1, 2 and 4, respectively. The regressed experimental data corresponding to ANOVA (Table 3 and Table 5) exhibited that EY and TPC, respectively, were well delineated by a quadratic polynomial model and equations. The experimental results of EY (Fig. 2(a)) and TPC (Fig. 2(c)) agreed well with the predicted values, as

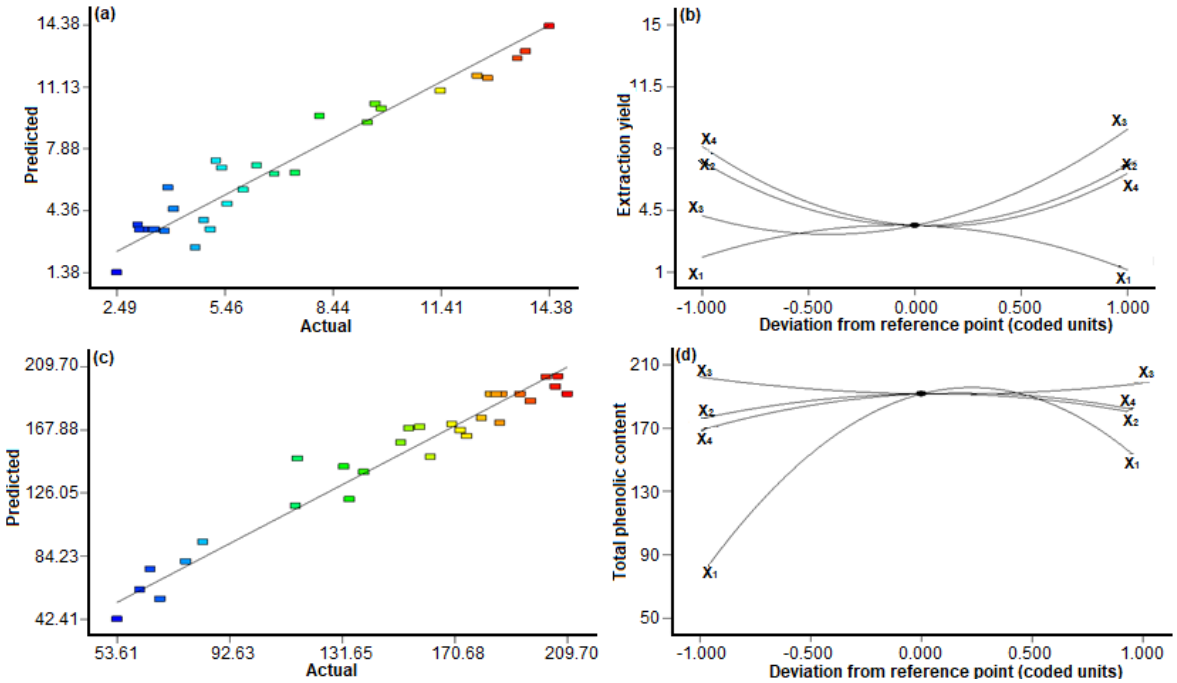

Fig 2. Comparison between the (a) predicted and actual values and (b) deviation of the reference point for EY, as well as (c) predicted and actual values and (d) deviation of the reference point for TPC, for the effect of (X_1) solvent concentration, (X_2) extraction time, (X_3) solvent-to-solid ratio and (X_4) sonication amplitude

Table 2. BBD experimental design and results for EY

Run	X_1	X_2	X_3	X_4	Experimental Yield (%)	Expected Yield (%)
1	50	5	15	60	8.07	9.60
2	50	5	25	20	12.40	11.71
3	50	30	35	20	13.73	12.99
4	100	30	25	100	5.53	4.98
5	50	30	25	60	5.07	3.64
6	50	55	35	60	14.38	14.32
7	0	30	25	20	5.22	7.23
8	0	5	25	60	7.39	6.63
9	50	30	25	60	3.27	3.64
10	50	30	35	100	13.51	12.63
11	0	55	25	60	4.88	4.11
12	50	30	15	20	9.39	9.26
13	50	5	35	60	11.39	10.92
14	50	30	25	60	3.22	3.64
15	100	5	25	60	3.81	3.57
16	50	5	25	100	9.60	10.24
17	50	55	25	20	12.69	11.59
18	50	30	25	60	3.12	3.64
19	0	30	35	60	6.34	7.00
20	100	30	35	60	5.38	6.88
21	100	30	15	60	2.49	1.38
22	0	30	25	100	3.07	3.89
23	100	30	25	20	4.06	4.70
24	50	55	15	60	3.90	5.84
25	0	30	15	60	4.65	2.70
26	50	55	25	100	9.76	10.00
27	100	55	25	60	5.97	5.73
28	50	30	15	100	6.83	6.56
29	50	30	25	60	3.53	3.64

reflected by their close scattering to the trend line, thus explaining the high R^2 of 0.9303 and 0.9500, respectively. The EY and TPC showed an adjusted R^2 of 0.9000 and 0.8606, respectively, which were the indication of accuracy and general availability of the polynomial model. Chua et al. [35] reported earlier that a well-fitted model should achieve a R^2 value of at least 0.80. Pertinently, the values of adequate precision which measures the signal to noise ratio were found high for EY (12.963) and TPC (14.360) (≥ 4), further supported the reliability of the models to predict the best UAE conditions for the responses, EY and TPC.

The perturbation graph (Fig. 2(b)) depicted that EY was influenced by the ratio of solvent-to-solid (C) where the yield increased steadily as this factor was increased. This observation highlighted that use of higher volumes of solvent could facilitate a higher EY. Increasing ethanol concentration (X_1) appears to adversely affect EY. Conversely, the concentration of ethanol appears to appreciably influence TPC in the OPL

Table 3. ANOVA of the quadratic model and lack of fit for EY

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	360.09	14	25.72	13.35	< 0.0001*
X_1	1.55	1	1.55	0.80	0.3852
X_2	0.097	1	0.097	0.05	0.8255
X_3	72.03	1	72.03	37.38	< 0.0001*
X_4	7.04	1	7.04	3.65	0.0767
X_1X_2	5.45	1	5.45	2.83	0.1147
X_1X_3	0.36	1	0.36	0.19	0.6721
X_1X_4	3.28	1	3.28	1.70	0.2133
X_2X_3	12.82	1	12.82	6.65	0.0218*
X_2X_4	4.23E-03	1	4.23E-03	2.19E-03	0.9633
X_3X_4	1.37	1	1.37	0.71	0.4135
X_1^2	30.19	1	30.19	15.67	0.0014*
X_2^2	80.55	1	80.55	41.81	< 0.0001*
X_3^2	58.44	1	58.44	30.33	< 0.0001*
X_4^2	89.65	1	89.65	46.53	< 0.0001*
Residual	26.97	14	1.93		
Lack of Fit	24.33	10	2.43	3.69	0.1101
R^2	0.9303				
Adj R^2	0.8606				
Pure Error	2.64	4	0.66		
Corr Total	387.06	28			
C.V.%	19.86				

* = significant ($p < 0.05$)

extracts (OPLE) represented by the highly concave line (X_1) (Fig. 2(d)). This is consistent with a previous report describing the use of 50:50 hydro-ethanolic mixture as a better extracting solvent over pure water and ethanol [36].

ANOVA data for EY (Table 3) and TPC (Table 5) indicated that both models were highly significant (p-value < 0.0001). The high F-values for models, EY (13.35) and TPC (19) as compared to the tabulated $F_{0.05(14,14)}$ of 2.48, implied that the degree of freedom at 95% confidence level relative to the residual was significant. The adequacy of the EY and TPC models was further proven by the high p-value for the lack of fit that corresponded to 0.1101 and 0.1929, respectively, as well as the corresponding low F-values at 3.69 and 2.52. These values were lower than the tabulated $F_{0.05(10,4)}$ (5.964), thus affirming that the lack of fit of each model was insignificant with regards to the pure error. The above data thus, conveyed that the model can adequately be used for optimizing the yield from OPL.

Effects of UAE Experimental Factors on EY and TPC

The percentage of EY from the UAE experiments varied from 2.49–14.38%, with the maximum EY attained when using 35 mL of 50% ethanol at 60% of amplitude of UAE for an extraction time of 55 min. The regression equation for EY and TPC are as follows (Eq. 5 and Eq. 6)

$$EY = 3.64 - 0.36X_1 - 0.09X_2 + 2.45X_3 - 0.77X_4 + 1.17X_1X_2 + 0.3X_1X_3 + 0.91X_1X_4 + 1.79X_2X_3 - 0.032X_2X_4 + 0.59X_3X_4 - 2.16X_1^2 + 3.52X_2^2 + 3X_3^2 + 3.72X_4^2 \quad (5)$$

$$TPC = 191.66 + 36.03X_1 + 1.43X_2 - 1.98X_3 + 6.01X_4 - 17.64X_1X_2 - 11.13X_1X_3 + 3.01X_1X_4 - 13.99X_2X_3 - 3.96X_2X_4 - 11.71X_3X_4 - 79.79X_1^2 - 14.36X_2^2 + 8.69X_3^2 - 17.07X_4^2 \quad (6)$$

Table 4. BBD experimental design and results for TPC

Run	X_1	X_2	X_3	X_4	Experimental TPC (mg GAE/g)	Expected TPC (mg GAE/g)
1	50	5	15	60	186.30	172.53
2	50	5	25	20	116.22	148.83
3	50	30	35	20	196.98	187.00
4	100	30	25	100	139.14	139.86
5	50	30	25	60	193.34	191.66
6	50	55	35	60	169.70	171.45
7	0	30	25	20	68.52	55.78
8	0	5	25	60	53.61	42.41
9	50	30	25	60	183.19	191.66
10	50	30	35	100	179.93	175.61
11	0	55	25	60	77.49	80.55
12	50	30	15	20	172.66	167.54
13	50	5	35	60	205.61	196.57
14	50	30	25	60	209.70	191.66
15	100	5	25	60	162.24	149.74
16	50	5	25	100	154.86	168.76
17	50	55	25	20	152.05	159.61
18	50	30	25	60	185.08	191.66
19	0	30	35	60	83.33	93.69
20	100	30	35	60	132.24	143.48
21	100	30	15	60	158.59	169.70
22	0	30	25	100	61.47	61.78
23	100	30	25	20	134.14	121.81
24	50	55	15	60	206.37	203.39
25	0	30	15	60	65.16	75.38
26	50	55	25	100	174.86	163.71
27	100	55	25	60	115.57	117.33
28	50	30	15	100	202.43	202.97
29	50	30	25	60	186.98	191.66

Based on the ANOVA, only the linear coefficient (X_3), cross product coefficient (X_2X_3) and the quadratic term coefficients (X_1^2, X_2^2, X_3^2, X_4^2) were significant in influencing EY by the UAE, as represented by their low p-values ($p < 0.05$) (Table 3). These results implied that the deduced model was applicable to EY.

Conversely, the ANOVA for the TPC indicated only linear coefficient, (X_1), interactive coefficients, (X_1X_2) and quadratic coefficients (X_1^2, X_2^2, X_4^2) were significant, corresponding to their low p-values ($p < 0.05$) (Table 5). The outcome implied the deduced model was satisfactorily usable for estimating the optimized conditions for TPC. The experimental data for TPC achieved values between 53.61–209.70 mg GAE/g, for which a maximum TPC was obtained under conditions; solvent concentration of 50%, extraction time of 30 min, a 25 mL/g solvent-to-solid ratio and sonication amplitude of 60%.

Table 5. ANOVA of the quadratic model and lack of fit for TPC

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	64613.48	14	4615.25	19.00	< 0.0001*
X_1	15576.49	1	15576.49	64.11	< 0.0001*
X_2	24.65	1	24.65	0.10	0.7548
X_3	46.89	1	46.89	0.19	0.6672
X_4	433.44	1	433.44	1.78	0.2030
X_1X_2	1244.33	1	1244.33	5.12	0.0401*
X_1X_3	495.51	1	495.51	2.04	0.1752
X_1X_4	36.30	1	36.30	0.15	0.7049
X_2X_3	783.44	1	783.44	3.22	0.0942
X_2X_4	62.65	1	62.65	0.26	0.6195
X_3X_4	548.03	1	548.03	2.26	0.1554
X_1^2	41291.80	1	41291.80	169.95	< 0.0001*
X_2^2	1338.24	1	1338.24	5.51	0.0342*
X_3^2	489.71	1	489.71	2.02	0.1776
X_4^2	1889.20	1	1889.20	7.78	0.0145*
Residual	3401.60	14	242.97		
Lack of Fit	2936.40	10	293.64	2.52	0.1929
R^2	0.95				
Adj R^2	0.90				
Pure Error	465.20	4	116.30		
Corr Total	68015.08	28			
C.V.%	10.45				

* = significant ($p < 0.05$)

The Mutual Interaction of Process Variables on the EY and TPC of *E. guineensis* Leaves

The mutual interaction of process variables of EY

Since the ANOVA indicated that the interaction between factors, extraction time vs solvent-to-solid ratio (X_2X_3) was the only significant interactive term (p-value = 0.0218) (Table 3), only the corresponding contour and 3-D response surface plots for the term were discussed in the following section. Interaction between the two factors can be better understood by holding the solvent concentration and sonication amplitude at their central values, corresponding to 50% and 60%. Fig. 3 illustrated that EY as high as 12.45% was obtainable when both factors, extraction time and solvent-to-solid ratio were set close to their upper limits, at 55 mins and 35 mL/g, respectively. It was evident that the factor of solvent-to-solid ratio, X_3 (F-value = 37.38) has a greater influence over extraction time, X_2 (F-value = 0.05) to maximize the EY of OPLE. Additionally, their synergistic interaction ($+1.79X_2X_3$) (Eq. 5) implied that elevating both factors to their maximum values can favor better yields of the OPLE. This might be due to the use of a larger solvent-to-solid ratio that tends to promote a greater mass transfer of trapped plant solutes in cells into the surrounding medium, as previously described by similar studies [34,37]. This, in turn, led to an improved EY from the powdered OPL.

As observed previously, increasing the solvent-to-solid ratio has been agreed to be one of the key factors to promote higher yields from plant materials [38]. Sheng [39] described that cavitational effect from microjets generated from collisions of acoustic bubbles was the

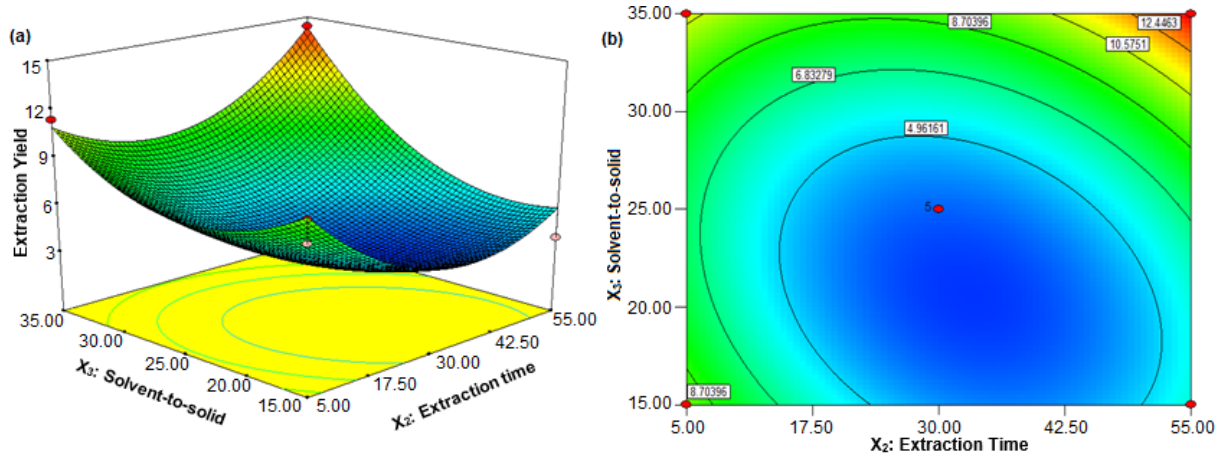

Fig 3. (a) Response surface (3D) and (b) contour plot (2D) showing the effects of extraction time and solvent-to-solid ratio on EY

trigger mechanism that caused the extensive swelling and disruption of plant cell structures that followed the ultrasonic treatment. This perpetually caused more solvent molecules from the surrounding extractive medium to diffuse in and out of the cells, thus liberating more bioactive compounds. Mass transfer of the solutes into the solvent is facilitated, thereby increasing EY.

The mutual interaction of process variables of TPC

Data on the interactive effect of varying extraction time and solvent concentration (X_1X_2) on TPC, investigated at a constant solvent-to-solid ratio of 25 mL/g and sonication of the amplitude of 60% is shown in Fig. 4. As seen here, the effect of solvent concentration on the TPC was more impacting than extraction time (Table 5), corresponding to an F-value of 64.11 as compared to 0.1 for extraction time. Their mutual interaction appears significant because of a small p-value (0.0401), but the factors were antagonistically related ($-17.64X_1X_2$) to affect TPC (Eq. 6). A TPC of as much as 191.65 mg GAE/g was possible using an ethanol concentration and extraction time of 50% and 30 min, respectively. It was clear that further increasing the factors beyond their optimal limits led to the general decline in the TPC of the OPLE.

López [36] reported obtaining the highest TPC when they used a mixture of 50% ethanol and water. This has to do with the polarity of plant phenolic compounds being higher than water but lower than absolute ethanol, thus a 50% ethanol:water mixed would provide adequate polarity to solubilize more of the

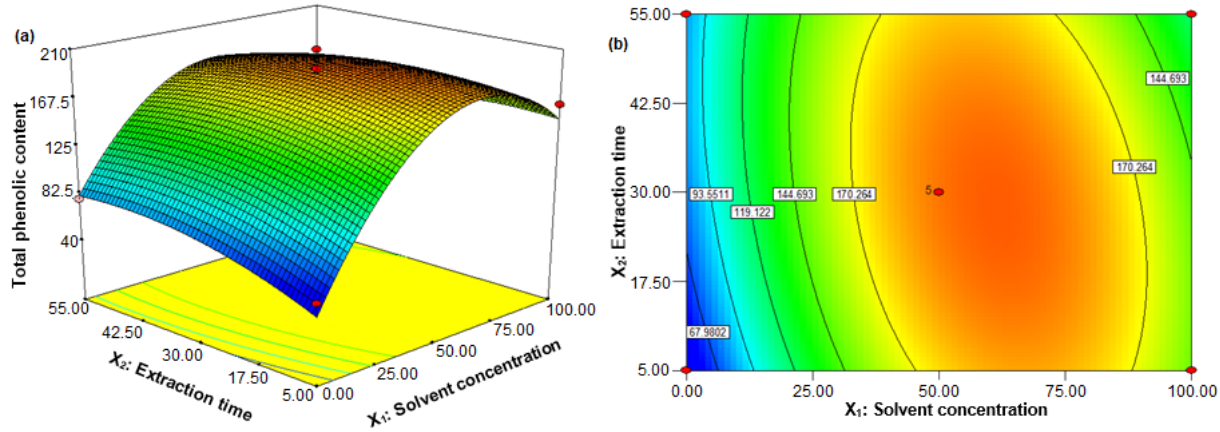

Fig 4. (a) Response surface (3D) and (b) contour plots (2D) showing the effects of solvent concentration and extraction time on TPC

extracted OPL compounds. Correspondingly, the same solvent mixture of ethanol:water can act a good swelling agent to cause the bursting of cell walls of OPL, permitting higher entry of the solvent mixture into the cells [37]. Contrariwise, a too high concentration of water would not improve the TPC of the UAE technique. This is because the naturally high viscosity of water can substantially lower the mass transfer of phenolic compounds out from the cell walls of the OPL into the extractive medium. The high dielectric constant of water can increase the polarity indices of ethanol with water [40] which would not help to improve the TPC of UAE process [41].

Whilst, an extraction time of 30 min was seen as sufficient to enhance extraction of phenolic compounds from powdered OPL, a longer extraction time can allow a higher cavitation effect to physically disrupt plant cells [37] of OPL, thereby releasing higher contents of phenolic compounds into the extractive medium. The study found the value of TFC was the highest at 181.46 mg CAE/g. This agreed with a previous study that showed quantities of extracted flavonoids tended to be maximum with increasing ethanol concentration in the solvent mixture of ethanol/water. Thus, directly influencing the quantity and composition of the flavonoids and polyphenolic compounds extracted from a plant material [42].

Comparison of Conventional Extraction Techniques with UAE

Comparison of UAE with Soxhlet extraction and maceration

As shown in Table 6, it was evident that the EY and TPC obtained from ultrasonically treated OPL samples were considerably higher than that obtained by Soxhlet extraction and maceration techniques. EY and TPC from Soxhlet extraction samples were 6.86% and 102.13 mg GAE/g, respectively, whilst maceration yielded the least EY and TPC corresponding to 3.73% and 85.23 mg GAE/g.

Table 6. Comparison of UAE of highest EY and TPC obtained by BBD with soxhlet extraction and maceration

Methods	EY (%)	TPC (mg CAE/g)
UAE	14.38	209.7
Soxhlet extraction	6.86	102.13
Maceration	3.73	85.23

The results conclusively showed that the UAE technique was efficient in releasing higher quantities of the plant solutes into the solvent layer, similar to observations by earlier studies [43-44]. The lower TPC values seen in the Soxhlet extracted sample was possibly due to a higher decomposition of phenolic compounds during reflux. Whereas, vigorous shaking alone, in the maceration process was ineffective in bringing out much of the bioactive compounds into the extractive liquid, hence explaining the lowest EY and TPC values [45].

In essence, the UAE technique is seemingly more attractive due to a short optimal extraction time of 55 and 30 min, as well as requiring lower quantities of solvents to give the highest EY (14.38%) and TPC (209.70 mg GAE/g). This is a marked difference from the Soxhlet extraction and maceration techniques that required 24 h to achieve their highest EY (6.86% and 3.73%) and TPC (102.13 and 85.23 mg GAE/g), thus proving that UAE is a more efficient in yielding better EY and TPC from powdered OPL.

Scanning electron microscopy (SEM) of untreated, Soxhlet extracted, macerated and UAE samples

SEM was employed to observe the physical consequence of sonication in altering cellular structures of OPL. Micrographs in Fig. 5(a-c) show cell walls of

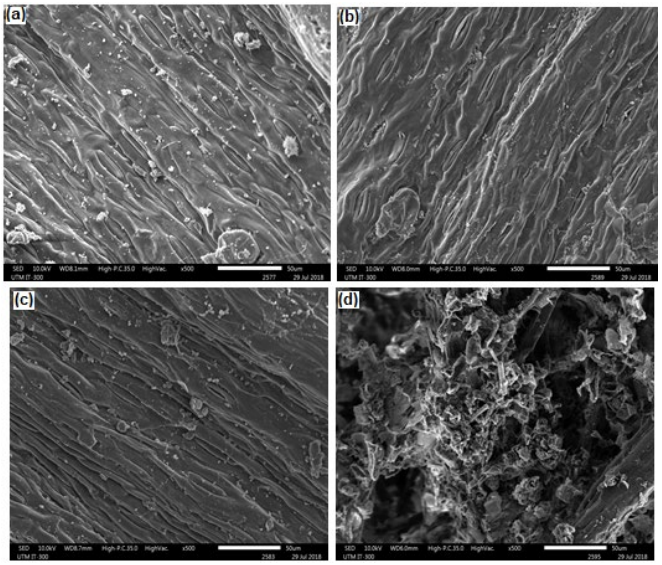

Fig 5. SEM micrographs of different OPL samples before and after extraction at 500x magnification. (a) non-extracted sample, (b) Soxhlet, (c) maceration and (d) UAE

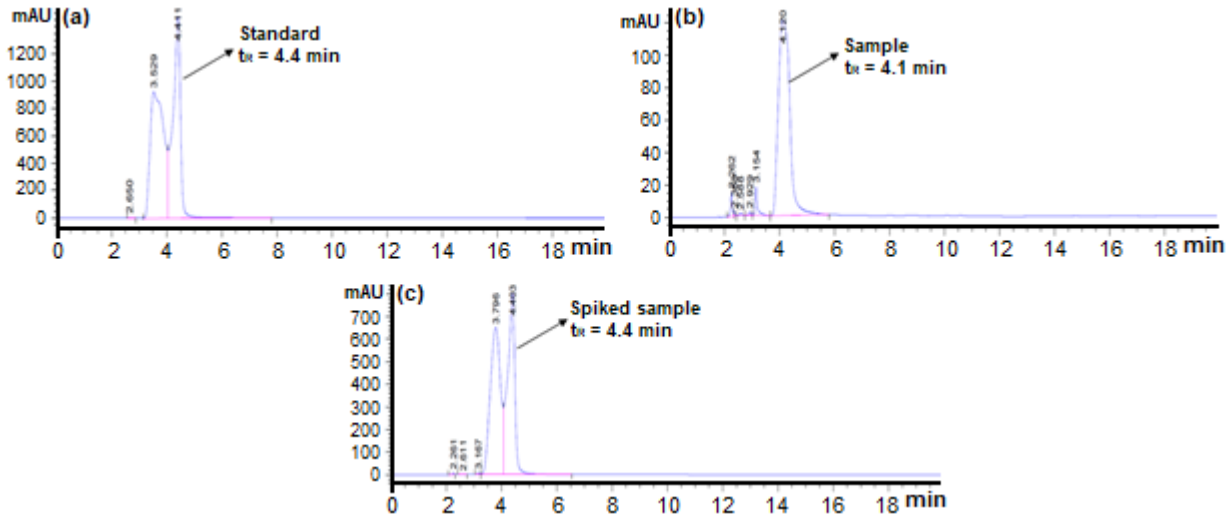

Fig 6. HPLC analysis of (a) gallic acid standard, (b) the highest TPC crude and (c) the highest TPC crude spiked with gallic acid standard

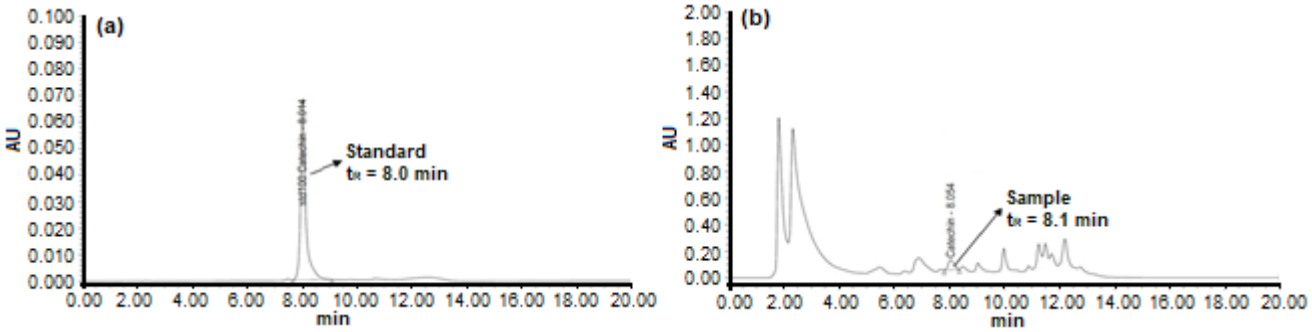

Fig 7. HPLC analysis of (a) catechin standard and (b) the highest TPC crude

untreated *E. guineensis*, Soxhlet extraction, and maceration. The cellular structures of Soxhlet and maceration treated samples did appear quite wrinkled but, in general, remained quite intact. This however, was very different for the UAE treated sample (Fig. 5(d)) that showed a widespread structure of burst cell fragments on the surface of OPL. The micrographs conclusively supported the theoretical acoustically-induced rupturing and disintegrating plant cell walls by ultrasonication [46], explicitly proving UAE as an efficient method to achieve the highest EY and TPC from OPL, as compared to techniques by maceration and Soxhlet extraction.

High-Performance Liquid Chromatography of OPLE

High-performance liquid chromatography (HPLC) analysis was performed on the highest TPC crude to reveal the presence of two major antioxidants extracted from the leaves of *E. guineensis*. The compounds were found to be gallic acid (Fig. 6) and catechin (Fig. 7), with the retention times, t_R for the gallic acid standard, crude TPC and the highest TPC crude spiked with gallic acid standard as 4.4, 4.1, and 4.4 min, respectively. The standard for catechin and the highest TPC crude were eluted at retention times, t_R 8.0 min and 8.1 min, respectively, which was in good agreement with a study by Ahmad [47] showing catechin as the main flavonoid (natural antioxidant) in OPLE.

■ CONCLUSION

In the present paper, RSM optimization was successfully employed for attaining the highest EY and TPC in OPLE. Influences of ethanol concentration, extraction time, solvent-to-solid ratio and sonication amplitude of UAE were evaluated using a four-factor-

three-level BBD, in which optimal UAE conditions yielded the highest EY at 14.38% [50% (v/v) ethanol-water ratio, 55 min, 35 mL/g solvent-to-solid ratio, 60% sonication amplitude] and TPC of 209.70 mg GAE/g [50% (v/v) ethanol-water ratio, 30 min, 25 mL/g solvent-to-solid ratio and 60% sonication amplitude], respectively, where compounds, gallic acid and catechin were the two main phytochemicals extracted from the OPL. The ANOVA suggested that the factor solvent concentration (F-value = 64.11) was the most influential in governing efficacy of the UAE of powdered OPL. Most importantly, the study envisages the UAE as a promisingly exceptional technique to extract high quantities of valuable plant-based bioactive compounds.

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