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Main Manuscript

Optimization of microwave-assisted extraction to obtain a polyphenol-rich crude extract from duku (*Lansium domesticum* Corr.) leaf and the correlation with antioxidant and cytotoxic activity

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Abstract

The present study employed Microwave-assisted extraction (MAE) as a method to extract a crude extract high in polyphenols from the leaves of duku (*Lansium domesticum* Corr.), a medicinal plant indigenous to Indonesia. The study acquired data on the impact of various extraction parameters, such as ethanol concentration (X_1), microwave power (X_2), and extraction time (X_3), on the levels of total phenolics content (TPC), total flavonoids content (TFC), and antioxidant activity using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. The extraction process was optimized using a Box-Behnken design (BBD) and response surface methodology (RSM). The optimal conditions were achieved with an X_1 of 75%, an X_2 of 315 W, and an X_3 of 8.5 minutes. The aforementioned experimental settings yielded results of 86.176 mg GAE/g, 31.585 mg QE/g, and 75.850% for the TPC, TFC, and antioxidant activity, respectively. The extract included several significant active chemicals, including octadecanoic acid, undecane, 2-methyl-, 9-octadecenoic acid (Z)-hexyl ester, pentadecanoic acid, and 9-hexadecanoic acid. The cytotoxic activity against MCF-7, T47D, and 4T1 cell lines was found to be slowed when exposed to crude extract doses ranging from 7.81 to 500.00 µg/mL. The present study's results suggest that MAE is a viable technique for extracting a polyphenol-rich crude extract from duku leaf. This extract shows promise as a natural antioxidant and has potential as an anti-breast cancer agent.

Keywords: *Lansium domesticum* Corr, Response surface methodology, Total phenolics content, Total flavonoids content, Antioxidant, Anti-breast cancer

Introduction

In recent years, there has been an expansion in the pharmaceutical sector's focus on advancements in the medicinal plant industry(Salmerón-Manzano, Garrido-Cardenas and Manzano-Agugliaro, 2020). The knowledge of health issues is also rising due to consumers' increased demands and interest in bioactive products(Bortolini *et al.*, 2022). Currently, folk medicine continues to hold significant importance in the treatment of various diseases. Moreover, traditional medicine has demonstrated notable efficacy in combating cancer, particularly in adjuvant chemotherapy(Yang *et al.*, 2014; Okem *et al.*, 2023). The therapeutic approach employed in this study was derived from a diverse array of botanical sources renowned for their substantial concentration of bioactive constituents, particularly

polyphenols(Vergara-Jimenez, Almatrafi and Fernandez, 2017). Plants provide a diverse variety of natural products, encompassing several groups of molecules, among which flavonoids stand out as a particularly significant group of polyphenolic substances(Dias, Pinto and Silva, 2021). Flavonoids have been the subject of recent studies that have revealed their diverse biological and pharmacological attributes. These include their antioxidant, anti-inflammatory, antibacterial, and wound-healing activities(Zulkefli *et al.*, 2023).

Duku (*L. domesticum* Corr.) is a readily accessible plant, predominantly found in Southeast Asia, particularly in Indonesia(Lubis, P. A. Hasibuan, *et al.*, 2022). Multiple studies have documented the cytotoxic properties of duku leaf against various cancer cell lines, indicating their potential as viable candidates for the development of anticancer medicines(Abdallah, Mohamed and Ibrahim, 2022). Cytotoxic action has been found in many components of duku, including the fruit, peel, and seeds. The previous study showed anti-pancreatic cancer activity of duku leaf extract against PANC-1 cells(M F Lubis *et al.*, 2023). In order to enhance the anticancer efficacy of duku leaf extract, it is imperative to undertake endeavors aimed at augmenting the concentration of chemical constituents inside duku leaf, with particular emphasis on phenolic compounds and flavonoids(Tungmunnithum *et al.*, 2018). According to the findings, there was a direct correlation observed between the amounts of total phenols and total flavonoids, and their corresponding cytotoxic activity.

The preceding investigation employed traditional extraction techniques to generate extracts derived from duku leaf. In order to get a greater quantity of bioactive compounds derived from raw plant materials that are rich in phytochemicals, manufacturers have implemented effective ways to optimize the extraction procedures while keeping costs low(Gil-Martín *et al.*, 2022). The utilization of MAE has been documented as a superior extraction method in terms of efficiency when compared to conventional extraction techniques(Teslić *et al.*, 2019). Nevertheless, there is currently no known information regarding the optimal environment for using MAE to extracted duku leaf. To achieve an optimal phytochemical-rich extract, it is necessary to consider the variation in solvent concentration, extraction time, and microwave power(Ghasemzadeh *et al.*, 2018). The application of RSM was utilized to enhance the extraction conditions of duku leaf.

The RSM exhibits significant potential as a valuable tool. This methodology, which integrates statistical and mathematical methodologies, allows for the organization of experimental models, analysis of the impacts of different factors, and identification of optimal process variables to achieve favourable surfaces(Riswanto *et al.*, 2019). The RSM was effectively employed to develop and evaluate the model equations. This approach was utilized to estimate the ideal experimental values of extraction circumstances, considering several independent parameters(Yu *et al.*, 2019). To the authors' knowledge, RSM has not been previously utilized to optimize the extraction process of polyphenols and flavonoids from duku leaf. The primary aim of this study was to examine the impact of several combination parameters, including X_1, X_2, and X_3, on TPC and TFC, thus DPPH scavenging activity. Furthermore, the research aimed to enhance the efficiency of the extraction procedure for antioxidant compounds derived from duku leaf. The present study employed a RSM-BBD design to optimize a MAE technique, to maximize the extraction of bioactive chemicals from *Moringa oleifera*(Setyani *et al.*, 2023). Our primary focus was examining total polyphenols and flavonoids widely utilized in the pharmaceutical and food sectors.

Materials and methods

Material and chemical

Duku leaves used in this research were collected from the duku field, Medan, Sumatera Utara, Indonesia. The fresh leaves with the criteria include a length of 8 cm and a width of 3 cm were used in this study. The leaves underwent a process of being cleansed with purified water, after which they were further fragmented into smaller segments. The sliced leaves underwent a drying process using hot air at a temperature of 50 ºC until the moisture content decreased to -5 wt%. Subsequently, the leaves were ground into a coarse powder with a mesh size ranging from 5 to 10. The resulting powder was then stored in a freezer until it was ready for use.

Ethanol and methanol pro analysis, DPPH, Folin-Ciocalteau, aluminium chloride, quercetin, and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All reagents for cytotoxic test were obtained from Parasitology Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. In addition, The MCF-7, T47D, and 4T1 cell lines were used to determine cytotoxic activity of extract. The cell lines were obtained from Parasitology Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Microwave-assisted extraction procedure

The microwave (ME731K Samsung, Suwon, South Korea) with modification was used as a extraction tools. Approximately 30 g of dried duku leaf was placed in round-bottom flask. The powder was extracted using a condition that has been designed using design expert software v.13. The design factors include ethanol concentration (50%, 70%, and 100%), microwave power (180 W, 300 W, and 450 W), and extraction time (3 min, 7 min, and 14 min). The collected samples were subjected to evaporation using a rotary evaporator (DLAB RE100-Pro) at a temperature of 45 ºC and a pressure of 72 mPa until all solvent was removed. The resulting dry extracts were then stored at a temperature of -18 ºC for subsequent analysis(Tomasi *et al.*, 2023).

Convetional extraction procedure

In order to compare the standard extraction approach with MAE, maceration technique was performed. A mixture was prepared by blending 15 g of sample dry powder with ethanol that has a concentration of 70%. The solvent to solid ratio employed was 10 milliliters per gram. The mixture was thereafter subjected to extraction at a temperature of 60 °C for a duration of 4 hours, with continuous stirring at a rate of 350 rpm. Subsequently, the combination underwent centrifugation at a force of 4696 × g using the Sorvall Legend X1R centrifuge (Thermo Fisher Scientific, MA, USA), followed by filtration to isolate the liquid fraction. The sample was subjected to evaporation until complete dryness was achieved, ensuring the removal of any residual solvent. Subsequently, the sample was held at a temperature of approximately -18 °C in preparation for further examination(Hasibuan *et al.*, 2020).

Determination of total phenolic content, total flavonoid content, and antioxidant activity

The quantification of TPC was conducted by employing the Folin-Ciocalteu reagent, following the methodology outlined in a prior investigation, albeit with slight adjustments. The crude extract was diluted to a concentration of 250 mg/L using deionized water. A volume of 0.2 mL of this diluted extract was combined with 0.2 mL of Folin-Ciocalteu reagent and 2.6 mL of deionized water. The resulting mixture was allowed to react for a duration of 6 minutes at room temperature. Subsequently, a solution of sodium carbonate with a concentration of 7% (2 mL) was introduced, and the resulting combination was subjected to a period of 90 minutes of darkness. The color that was produced was measured at a wavelength of 750 nm using a spectrophotometer (Thermo Scientific, located in Madison, WI, USA). A standard curve was generated using a solution of Gallic acid, and the outcome was quantified in terms of mg GAE/g extract(Alara, Abdurahman and Ukaegbu, 2018; Lubis, Syahputra, *et al.*, 2022).

Based on previously study, the TFC of extracts was determined with minor adjustments. In summary, a series of steps were followed to prepare the sample solution. Firstly, 0.1 mL of a 10% (w/v) $AlCl_3$ aqueous solution was added, followed by the addition of 0.1 mL of a CH_3CO_2K solution with a concentration of 1 M. Subsequently, 4.3 mL of ultrapure water was added. The resulting mixture was then incubated for a duration of 30 minutes at room temperature. The measurement of the mixture's absorbance was conducted at a wavelength of 415 nm. In this study, a solution of ethanol at a concentration of 50% was employed as the negative control, whereas Quercetin was utilized as the positive control. The TFC of the sample was evaluated by comparing it to the quercetin standard curve ($y = 2.6326x + 0.1255$; $R^2 = 0.9976$, 0.01–0.64 mg/mL). The TFC was then represented relative to the equivalent standard concentrations in mg QE/g extracts(Alara, Abdurahman and Ukaegbu, 2018; Lubis, Syahputra, *et al.*, 2022).

The measurement of the DPPHº scavenging capacity was conducted in accordance with the previously published methodology, with minor adjustments. In summary, a 0.5 mL sample was introduced into a 3.5 mL solution of DPPHº (0.2 mM DPPHº solution diluted in ethanol pro analysis) and allowed to incubate at room temperature for a duration of 30 minutes. The absorbance values of the sample, ethanol pro analysis, and distilled water were determined at a wavelength of 517 nm. The sample absorbance (As), solvent absorbance (Ae), and blanko absorbance (Aw)(Lubis, P. A. Z. Hasibuan, *et al.*, 2022). The DPPHº scavenging capacity was determined by employing the subsequent formula:

$$\textbf{\textit{Scavenging capicity}}\ (\%) = \left(1 - \frac{\textbf{\textit{As} - \textbf{\textit{Ae}}}}{\textbf{\textit{Aw}}}\right) \textbf{\textit{x}}\ 100\%$$

Phytochemicals analysis using Gas chromatography-mass spectrophotometry (GC-MS)

This analysis was conducted on a crude extract that prepares using optimum conditions. The examination of the crude extract was conducted using a Shimadzu QP-2010 plus GC/MS instrument equipped with a TD 20 thermal desorption device. The column employed in the experiment was a Rtx-5 column with dimensions of 30 meters in length, 0.25 millimeters in diameter, and a particle size of 0.25 micrometers. The column temperature was initially set at 1000°C and then increased to 2800°C at a rate of 50°C per minute. This elevated temperature was sustained for a duration of 3 minutes. The temperature was subsequently raised to 2800°C at a rate of 150°C per minute, and maintained at this level for a duration of 35 minutes. The ion source of the mass spectrometer was maintained at a temperature of

2300°C, while the interface temperature was set at 2700°C. The detection process was conducted using full scan mode, spanning the mass-to-charge ratio (m/z) range of 40 to 650. The chemicals were identified by comparing the mass spectra of unknown peaks with those kept in the NIST and Wiley mass spectral electronic databases(Olivia, Goodness and Obinna, 2021).

Table 1. BBD for independent variables and observed responses. X_1 (ethanol concentration), X_2 (microwave power), X_3 (extraction time), TPC (total phenolic content), TFC (total flavonoid content), DPPH (2,2-Diphenyl-1-picrylhydrazyl)

Run	X_1 (%)	X_2 (W)	X_3 (min)	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (%)
1	70	300	7	80.53	31.53	73.67
2	70	300	7	78.35	30.96	70.35
3	50	300	14	30.12	8.35	45.15
4	70	450	3	75.64	23.52	63.35
5	100	300	3	52.51	6.73	52.04
6	70	180	3	69.35	20.53	64.36
7	100	450	7	43.21	5.97	50.63
8	70	450	14	60.34	18.36	64.67
9	70	180	14	81.05	15.32	62.45
10	50	180	7	26.34	4.68	46.61
11	70	300	7	79.56	27.46	75.13
12	50	300	3	22.64	5.96	42.67
13	70	300	7	89.90	29.57	69.01
14	50	450	7	20.57	5.94	40.85
15	100	300	14	36.35	7.35	50.09
16	100	180	7	32.61	5.88	52.06
17	70	300	7	83.46	32.96	80.53

Cytotoxic activity of extracts against MCF-7, T47D, and 4T1 cell lines

In this experiment, the cells were collected once they had reached a confluence level of 80%. Prior to commencing the MTT test, the cells were subjected to optimization by varying the seeding density within the range of 2.0×10^3 cell/mL to 1.0×10^6 cell/mL under light conditions. This was done to ascertain the most suitable seeding number for the experimental procedure. In this experiment, a 96-well microtiter plate was utilized. At a seeding density of 5×10^4 cell/mL, 100 L of cell suspension containing MCF-7, T47D, and 4T1 cells was dispensed into each well of the plate. The cell suspension was prepared in complete growth media. Following a 24-hour incubation period, cellular specimens were subjected to various doses of leaf extract, spanning from 7.8125 to 250 μg/mL. Each well had a total volume of 200 μL and was replicated for technical accuracy. The microtiter plates were subjected to an additional incubation period of 72 hours in the presence of plant extracts. Following a 72-hour incubation period, 20 μL of MTT added to each well. The plates were then incubated for a duration of 4 hours at a temperature of 37 °C. The medium from each well was meticulously extracted without causing any disruption. 1 M HCl and 100 mM isopropanol added to the plates for dissolved formazan crystals. The absorbance was quantified using a BioRad microplate reader at 575 nm (Shinagawa-ku, Tokyo, Japan) (Fitri *et al.*, 2023).

Conception of Experiments and Statistical Evaluation

This experiment was carried out utilizing independent variables, specifically X_1, X_2, and X_3, each set at three different levels. The experimental design employed for this purpose was the BBD. The comprehensive design comprised a total of 17 experimental points. The encodings and actual values of the experimental design components are shown in Table 1.

Multiple regression analysis was applied to the BBD's experimental data in order to find a good fit with the second-order polynomial model, as shown below.

$$Y = Z_0 \sum_{i=1}^{3} Z_i + \sum_{i=1}^{3} Z_{ii} X_i^2 + \sum_{j=i+1}^{3} Z_{ij} X_i X_j$$

In the given equation, Y is the response function which comprises of TPC (Y_1), TFC (Y_2), and antioxidant activity (Y_3). A_0 is a constant term, while Z_i, Z_{ii}, and Z_{ij} indicate the coefficients of the linear, quadratic, and interaction factors, respectively. X_i and X_j are the independent variables in the equation(Siddiqui and Aeri, 2016). An analysis of variance (ANOVA) was conducted to assess the lack of fit, determination coefficient (R^2), and the impact of linear, quadratic, and interaction terms on each response variable. The RSM was employed to construct three-dimensional charts representing the response surface. The experimental design, analysis of experimental data, model fitting, and optimization procedure were conducted using Design Expert V. 13 Software. In order to assess the distinction between the mean and the predicted value and observation value (by a one-sample t-test), as well as the Pearson correlation, the statistical software IBM SPSS Statistics (IBM Co., Ltd., America, United States) was employed.

Ethics approval

The attainment of the objectives of this study did not necessitate the permission of research ethics committees, as it did not involve any tests conducted on human or animal subjects, nor did it entail field studies involving plants.

Table 2. Regression coefficients (A) of the independent variables, coefficient of determination (R^2) and lack of fit of the backward second-order polynomial regression models. [a]X_1, X_2 and X_3 represented the ethanol concentration (%), microwave power (W), extraction time (min), respectively. [b]p value more than 0.05 is not significantly different at 5% level. TPC (total phenolic content), TFC (total flavonoid content), DPPH (2,2-Diphenyl-1-picrylhydrazyl)

Source[a]	TPC (mg GAE/g)		TFC (mg QE/g)		DPPH (%)	
	Coefficient	p value[b]	Coefficient	p value[b]	Coefficient	p value[b]
Model		< 0.0001		<0.0001		0.0003
Constant (A_0)	85.64		31.59		75.77	
X_1	7.60	0.0016	0.0826	0.9292	3.60	0.0278
X_2	-1.74	0.2939	0.8809	0.3619	-0.5174	0.7047
X_3	-2.47	0.1461	-0.9369	0.3273	-0.0521	0.9689
X_1X_2	4.09	0.0905	-0.3822	0.7645	0.8753	0.6381
X_1X_3	-5.49	0.0318	-0.1550	0.9016	-1.03	0.5764
X_2X_3	-6.98	0.0118	-0.0249	0.9843	1.05	0.5724
X_1^2	-47.57	< 0.0001	-19.41	<0.0001	-22.85	<0.0001
X_2^2	-7.86	0.0071	-6.46	0.0012	-5.00	0.0266
X_3^2	-2.76	0.2625	-4.91	0.0079	-5.43	0.0263
R^2	0.9872		0.9772		0.9628	

Adjusted R^2	0.9709		0.9479		0.9150		
Lack of fit		0.6389		0.2600		0.9256	

Results and discussion

The impact of X_1, X_2, and X_3 on TPC, TFC, and antioxidant activity

Table 1 shows how BBD extraction conditions affect TPC, TFC, and DPPH scavenging. TPC, TFC, and DPPH scavenging activities ranged from 20.57 to 89.90 mg GAE/g, 4.68 to 32.96 mg QE/g, and 40.85% to 80.53% were obtained. These findings first reported that extraction conditions would affect the TPC, TFC, and DPPH scavenging activity of duku leaf extract. Another study described by Waremfo et al., showed a similar result to this report that the variations in X_1, X_2, and X_3 affected the TPC, TFC, and DPPH scavenging activity of 47.25 – 89.39 mg GAE/g, 0.66 – 21.45 mg QE/g, and 22.93% – 79.76%, respectively(Weremfo, Adulley and Adarkwah-Yiadom, 2020). Meanwhile, the result reported by Le et al., explained that the variables, namely extraction time, ethanol concentration, microwave power, and solvent pH, significantly impacted the result of TPC, TFC, and DPPH scavenging of 18.6 – 33.6 mg GAE/g, 13.8 – 28.3 mg QE/g, and 22.0% – 33.2%(Le *et al.*, 2019). If compared to other research conducted to determine the TPC, TFC, and DPPH scavenging activity using reflux with variables, namely methanol concentration, extraction temperature, and liquid-to-solid ratio reported by Ghasemzade and Jaafar, have the lowest parameters than using MAE of 0.55 – 1.74 (mg GAE/g), 3.12 – 6.58 (mg QE/g), and 44.70% - 80.50%(Ghasemzadeh and Jaafar, 2014). The recovery efficacy of bioactive components and antioxidant activity derived from duku leaf is contingent upon several factors, including the cultivar variety, growing region, extraction methodology, solvent selection, and operational conditions(Klongdee and Klinkesorn, 2022).

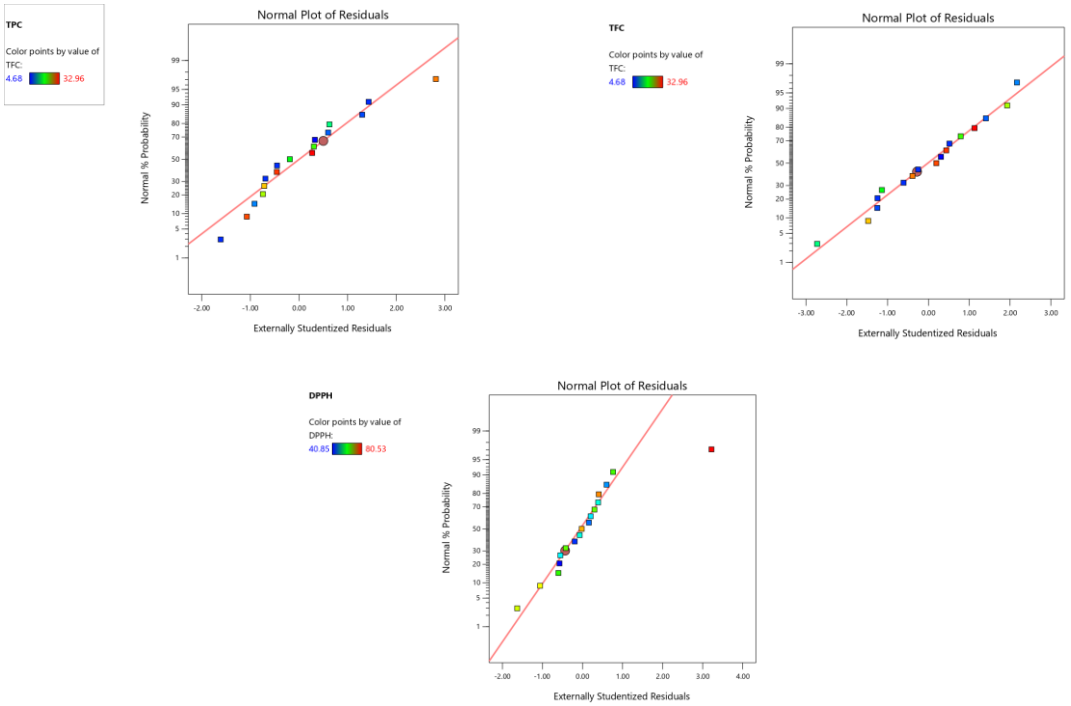

Fig. 1. Normal probability plots of residuals for the total phenolic content, total flavonoid content, and DPPH scavenging activity

Fitting the models

The experiment data (Table 1) was used for multiple regression. Table 2 shows the regression coefficients (A) for the independent variables and the backward second-order polynomial regression models' lack of fit and coefficient of determination (R^2). The models' adequacy and predictability were assessed using R^2, R^2_{adj}, and lack of fit. All models showed p-values between 0.0001 and 0.0003, indicating substantial statistical significance. The models exhibited a strong agreement with the experimental data, as evidenced by R^2 and R^2_{adj} values exceeding 0.96 and 0.91, respectively. The aforementioned figures indicate that the models possess the ability to interpret in excess of 90% of the variation seen in the response variables. A lack-of-fit test was undertaken to assess the adequacy and goodness-of-fit of each model to the data(Youse *et al.*, 2019). The lack of fit of the regression models for TPC, TFC, and DPPH scavenging of the duku leaf crude extract yielded p-values of 0.6389, 0.2600, and 0.9256, respectively, as shown in Table 2. The statistical analysis indicated that the lack of fit exhibited by the models was not statistically significant ($p > 0.05$), hence confirming their trustworthiness. The proposal made aligns with the observations from the normal probability plots (Figure 1). These plots demonstrate that the residuals, which represent the discrepancies between the observed and anticipated values, exhibit a normal distribution and display a linear pattern. The results of this analysis indicate that the regression models used in this study adequately captured the relationship between the variables and the experimental data.

Response surface plots

The utilization of surface plots was employed to examine the interaction effects of the independent factors on the response variables, in line with the regression equation. The present study employed response surface analysis, utilizing flexible regression models as outlined in Table 3.

Table 3. Regression models as a function of independent variables for the response variables of the *L. domesticum* leaves extract in terms of actual levels. X_1, X_2, and X_3 represented the ethanol concentration (%), microwave power (W), extraction time (min), respectively. TPC (total phenolic content), TFC (total flavonoid content), DPPH (2,2-Diphenyl-1-picrylhydrazyl)

Response variables	Regression model
TPC (mg GAE/g)	$Y_{TPC} = 85.64 + 7.60X_1 - 1.74X_2 - 2.47X_3 + 4.09X_1X_2 - 5.49X_1X_3 - 6.98\ X_2X_3 - 47.57X_1^2 - 7.86X_2^2 - 2.76X_3^2$
TFC (mg QE/g)	$Y_{TFC} = 31.59 + 0.0826X_1 + 0.8809X_2 - 0.9369X_3 - 0.3822X_1X_2 - 0.1550X_1X_3 - 0.0249X_2X_3 - 19.41X_1^2 - 6.46X_2^2 - 4.91X_3^2$
DPPH (%)	$Y_{DPPH} = 75.77 + 3.60X_1 - 0.517460X_2 - 0.0521X_3 + 0.8753X_1X_2 - 1.03X_1X_3 + 1.05X_2X_3 - 22.85X_1^2 - 5.00X_2^2 - 5.43X_3^2$

The study depicted the relationship between the response variables, namely TPC, TFC, and DPPH scavenging activity, with the independent variables of X_1, X_2, and X_3. The TPC in the crude extract exhibited a range of values, spanning from 20.57 to 89.9 mg GAE/g, as presented in Table 1. The lowest yield was seen when the X_1 of 50% and the X_2 of 450 W, with X_3 of 7 min. Whereas, the best yield was recorded when the X_1 of 70% and the X_2 of

300 W, also with X_3 of 7 min. According to the findings presented in Table 2, it can be observed that the X_1 exhibited a statistically significant positive impact on the TPC, as indicated by a $p < 0.05$. The quadratic terms (X_1 and X_2) exhibited a statistically significant ($p < 0.05$) impact on TPC in the presence of MAE. A statistically significant interaction was observed between X_1X_3, as well as X_2X_3, with a $p < 0.05$. The influence of the independent variables and their interplay on TPC may be observed by the probability plots of residual and three-dimensional response surface curves for the TPC, as depicted in Figures 1 and 2 (A – C). A comparable finding was documented with the mean absolute error of polyphenols derived from various sources, including the *Coriolus versicolor* mushroom(Maeng *et al.*, 2016), chokeberries(Ćujić *et al.*, 2016), *Myrtus communis* L. leaves(Dahmoune *et al.*, 2015), and blueberry leaves(Aliaño-González *et al.*, 2020). The observed quadratic effect of X_1 on TPC, as indicated by a statistically significant with $p < 0.05$ (Table 2), may be attributed to the increased susceptibility of sample cell membranes to breaking and the enhanced solubility of phenolic compounds resulting from the initial rise in ethanol concentration. Nevertheless, when the concentration of ethanol increases, there is a consequential alteration in the polarity of the solvent(Hikmawanti, Fatmawati and Asri, 2021). This change in polarity has the potential to enhance the extraction of impurities, consequently resulting in a decrease in the quantity of total phenolic compounds that are extracted. Furthermore, the extraction rate may be influenced by the increased diffusion resistance caused by protein coagulation at high ethanol concentrations, which hinders the solubility of polyphenols(Khoddami, Wilkes and Roberts, 2013; Shi *et al.*, 2022).

The ANOVA revealed that the combined influence of X_1X_3 had a statistically significant adverse effect ($p < 0.05$) on the TPC. As depicted in Figure 1B, the extraction of TPC exhibited an upward trend with the progressive increase in X_1 up to about 80%. Additionally, a reduction in X_3 from 14 to 3 minutes resulted in a modest elevation in TPC levels. The TPC of the crude extract exhibited a small reduction as the percentage of ethanol was increased beyond 80%. The impact of X_2 and X_3 on TPC is depicted in Figure 1C. The negative interaction seen in Table 2 is statistically significant ($p < 0.05$), which aligns with previous findings. The augmentation of X_2 within the range of 180 - 300 W, along with an extended X_3 of 1 - 9 minutes, leads to a rise in TPC. However, beyond this range, the TPC exhibits a minor reduction. The observed phenomenon can be attributed to the increased rate of mass transfer and solubility of phenolic compounds(Medina-Torres *et al.*, 2017). This can be attributed to the decrease in surface tension and solvent viscosity as the X_2 is increased to approximately 300 W. These changes contribute to improved wetting of the sample and enhanced penetration into the matrix, ultimately resulting in higher extraction efficiency(Yuan *et al.*, 2018). Nevertheless, when exposed to X_2 levels reaching 300 W, the prolongation of X_3 beyond 9 minutes resulted in a reduction in TPC, potentially attributed to the destruction of certain phenolic compounds.

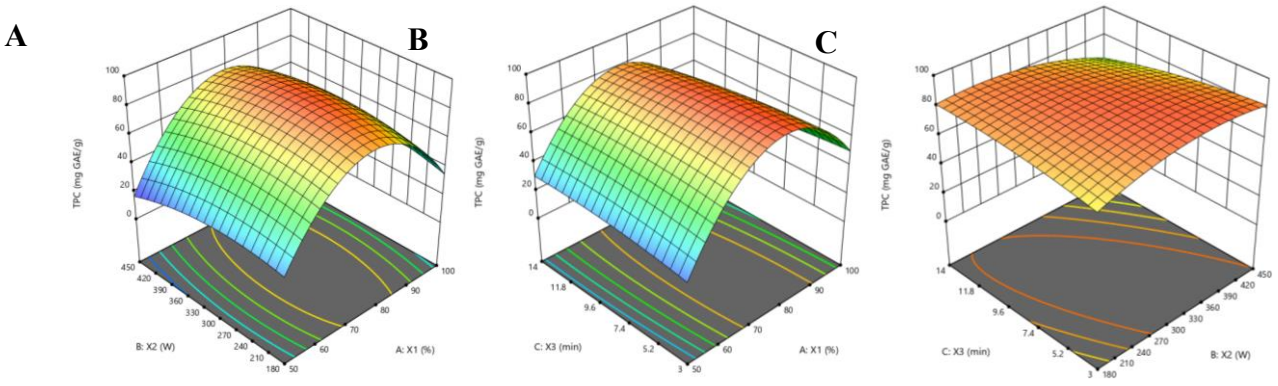

Fig. 2. Response surface plots as a function of independent variables on the total phenolic content. (A) ethanol concentration and microwave power, (B) ethanol concentration and extraction time, and (C) microwave power and extraction time

According to the findings presented in Table 2, it was observed that the quadratic terms related to X_1 had a very significant ($p < 0.0001$) negative linear impact. Similarly, the quadratic terms associated with X_2 and X_3 demonstrated a substantial ($p < 0.001$) negative influence on the extraction of TFC from duku leaf. Similar trends were identified in the extraction of TPC and TFC from duku leaf, indicating that comparable factors influenced the extraction of both compounds(Muflihah, Gollavelli and Ling, 2021). This outcome is anticipated, given flavonoids are a subset of polyphenols. In the present study, the statistical analysis revealed that the interaction of the dependent variables did not exhibit a significant effect ($p > 0.05$) on TFC extraction. The recovery of TFC exhibited an upward trend as the X_1 and X_2 rose, reaching a maximum of approximately 75% and 300 W, respectively. However, further increases in X_1 and X_2 resulted in a decline in the recovery of TFC. An observed rise in the TFC implies an enhanced solubility of flavonoid molecules in a 70% hydro-alcohol. The use of ethanol as a solvent has the potential to enhance extraction yields, whereas water has the ability to promote swelling of cell material(Che Sulaiman *et al.*, 2017; Kumar, Srivastav and Sharanagat, 2021). This can lead to a favorable increase in the contact surface area between the plant matrix and the solvent, ultimately resulting in an increase in the extraction yield(Wardatun *et al.*, 2020). The X_2 is a crucial factor in microwave-assisted extraction as it plays a significant role in facilitating the liberation of flavonoids from various matrices by the disruption of cell walls. Moreover, X_2 possesses the capacity to alter the equilibrium and mass transfer conditions involved in the extraction process(Yao *et al.*, 2021).

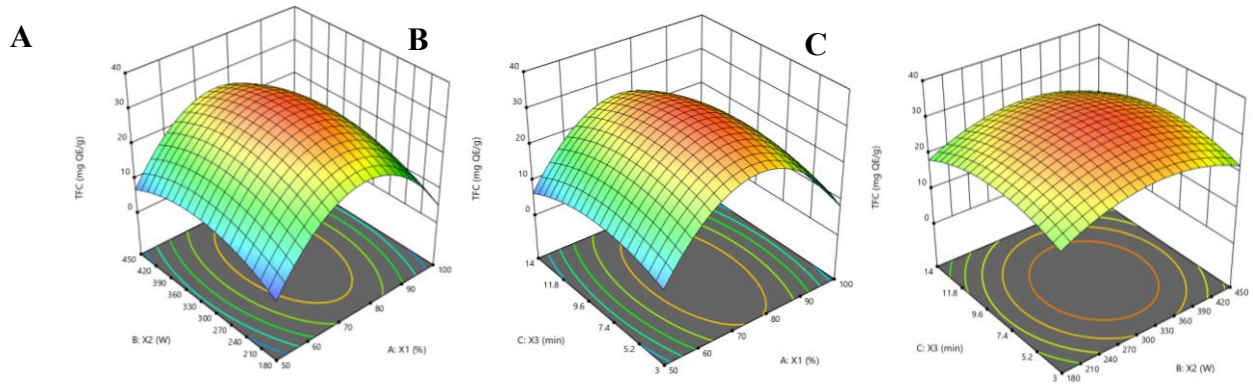

Fig. 3. Response surface plots as a function of independent variables on the total flavonoid content. (A) ethanol concentration and microwave power, (B) ethanol concentration and extraction time, and (C) microwave power and extraction time

The extraction of flavonoids was expedited by increasing the X_2 to approximately 300 W, as evidenced by Figure 3A. However, it was observed that the TFC reduced after the X_2 was increased to 300 W. The results presented in Figure 3B illustrate the impact of varying X_1 and X_3 on the total TFC. There is no substantial difference observed in the X_3 factor when considering various X_2 factors. The TFC exhibits a modest reduction after an extraction

period beyond 10 minutes, with the X_1 being effective at approximately 70 - 80%. This finding is consistent with a previous work, which also shown that the extraction of flavonoids using MAE requires precise control of the extraction duration(Moreira *et al.*, 2017; V. González-de-Peredo *et al.*, 2022). This study provides a comprehensive description of the relationship between X_2 and X_3. The results shown in Figures 3C demonstrate the impact of X_2 and X_3 on the TFC of duku leaf. The TFC exhibited an increase when the X_2 and X_3 were raised to approximately 300 W and 7 minutes, respectively. However, beyond this threshold, the TFC demonstrated a minor reduction. The length of the extraction process has the capacity to enhance the solubility of phenolic compounds and decrease the viscosity of the extraction solvent(Bonacci *et al.*, 2020). Consequently, this can accelerate the liberation and disintegration of these chemicals(Septiani, Kumoro and Djaeni, 2021). However, prolonged X_3 can contribute to the breakdown of specific flavonoid molecules due to the subsequent increase in extraction temperature(Liu *et al.*, 2022).

Table 4. Pearson's correlation coefficient between response variables. *Correlation is significant at the level of $p < 0.001$. TPC (total phenolic content), TFC (total flavonoid content), DPPH (2,2-Diphenyl-1-picrylhydrazyl)

Variables		TPC	TFC	DPPH
TPC	Correlation		0.904*	0.935*
	Sig. (2-tailed)		0.000	0.000
TFC	Correlation	0.904*		0.946*
	Sig. (2-tailed)	0.000		0.000
DPPH	Correlation	0.935*	0.946*	
	Sig. (2-tailed)	0.000	0.000	

The X_1 had a substantial ($p < 0.05$) impact on the measured antioxidant activity as determined by the DPPH assay. The quadratic coefficients associated with ethanol content demonstrated a statistically significant and extremely negative linear relationship ($p < 0.0001$) with the extraction of DPPH scavenging activity from duku leaf. Similarly, the quadratic coefficients of X_2 and extraction time revealed a statistically significant negative effect ($p < 0.05$) on the extraction process. The DPPH value exhibited a rise within the range of X_1 of 70-80%, followed by a subsequent drop at higher concentrations, as depicted in Figure 4. The correlation between X_1 and DPPH exhibited a resemblance to the patterns observed in total phenolic content (TPC), which can be attributed to a highly significant positive correlation ($p < 0.001$) between these phenolic compounds and DPPH, as illustrated in Table 4. The surface plot (Fig. 4A) demonstrated an interaction relationship between X_1 and X_2. The maximum DPPH activity was seen at specific X_1 of 70-80% and X_2 ranging from 240-360 W. At lower X_1, an increase in temperature resulted in a drop in the DPPH value. This can be attributed to the degradation or destruction of some bioactive chemicals that occur at higher temperatures(Alide, Wangila and Kiprop, 2020). In contrast, larger concentrations of ethanol resulted in an increase in the DPPH value. This can be attributed to the facilitation of compound extraction with lower polarity by organic solvents and elevated temperatures(Thouri *et al.*, 2017).

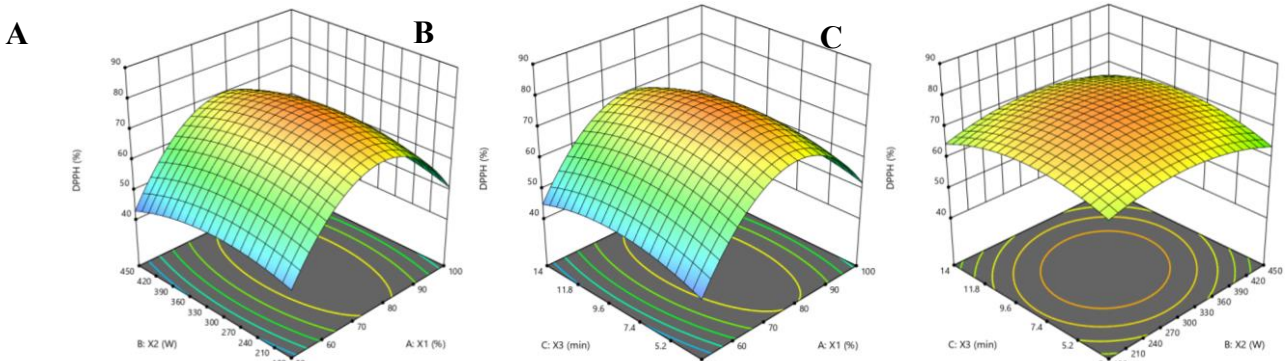

Fig. 4. Response surface plots as a function of independent variables on the DPPH scavenging activity. (A) ethanol concentration and microwave power, (B) ethanol concentration and extraction time, and (C) microwave power and extraction time

The optimization of processes and validation of models

Table 5 displays the outcomes of studies conducted under the optimal extraction conditions utilizing microwave technology. The best circumstances for maximum MAE efficiency, as determined by the stationary point, were achieved through a degree of experiment. The crucial values for these conditions were a 75% X_1, 315 W X_2, and anX_3 of 8.5 min. These parameters were found to yield the highest efficiency in terms of TPC, TFC, and DPPH scavenging activity. The adequacy of the model equation in forecasting the optimal response values was evaluated using the previously indicated specified ideal conditions. The anticipated extraction yield of total phenolic content (TPC), total flavonoid content (TFC), and DPPH scavenging activity were determined to be 86.176 mg GAE/g, 31.585 mg QE/g, and 75.850%, respectively. that was consistent with the experimental yield of 80.631 ± 3.562 mg GAE/g, 28.953 ± 4.672 mg QE/g, and 73.567 ± 3.351% for TPC, TFC, and DPPH scavenging activity, respectively. The predicted outcomes exhibited a high level of concordance with the empirical values and were determined to lack statistical significance with $p > 0.05$. The anticipated response values exhibited a minor deviation from the empirical data. Based on the initial data, the analysis of residuals suggests that there are no deviations from the expected distribution, indicating that the methodology employed is sound. The robust correlation observed between the actual and predicted outcomes provides empirical evidence that the regression model's response is sufficient in accurately representing the anticipated optimization(Chicco, Warrens and Jurman, 2021).

Table 5. Experimental and predicted values of the response variables at optimal extraction conditions of MAE along with the experimental value from conventional maceration method. TPC (total phenolic content), TFC (total flavonoid content), DPPH (2,2-Diphenyl-1-picrylhydrazyl). ^{ns} is representation a nonsignificant difference between experimental and predicted values ($p > 0.05$). ^{a,b}Values with different superscript letters between experimental values of MAE and conventional methods are representation a significant difference ($p < 0.05$)

Response variables	Microwave Assisted Extraction			Conventional maceration method
	Predicted value	Experimental value^{ns}	Relative error (%)	
TPC (mg GAE/g)	86.176	80.631 ± 3.562^{a}	6.435	66.343 ± 5.783^{b}
TFC (mg QE/g)	31.585	28.953 ± 4.672^{a}	8,333	20.056 ± 4.522^{b}
DPPH (%)	75.850	73.567 ± 3.351^{a}	3,009	65.436 ± 2.562^{b}

A comparison was made between the optimal mean absolute error (MAE) and standard maceration techniques, using similar extraction parameters except for variations in X_2 and X_3. The study revealed that the response variables, namely TPC, TFC, and DPPH activity of the crude extract obtained using MAE, exhibited considerably greater values compared to those obtained through the standard maceration process (Table 5). The findings suggest that the most suitable method for extracting crude extract from duku leaf, which is rich in polyphenols and has high antioxidant activity, is the use of MAE.

Cytotoxic activity of duku leaf crude extract

The determination of cell viability was conducted using the MTT test on cells treated with an optimized extract of duku leaf at doses ranging from 7.81 to 500.00 µg/mL. Fig. 5., displays the dose-response curves of three distinct cell types that were subjected to treatment with a refined extract derived from duku leaf. The viability percentage is expected to decrease as the concentration of the sample increases. The condition above was seen in all cell lines, with a statistical significance of $p < 0.001$. The test conducted at a greater dose (500.00 µg/mL) resulted in the lowest percentage of cell viability for three types of cancer cells: MCF-7 (25.903 ± 0.505%), 4T1 (30.830 ± 1.622%), and T47D (38.113 ± 0.575%). The results of this study provide confirmation that the optimized extract derived from duku leaf exhibits potential as an agent for the development of anti-breast cancer treatments.

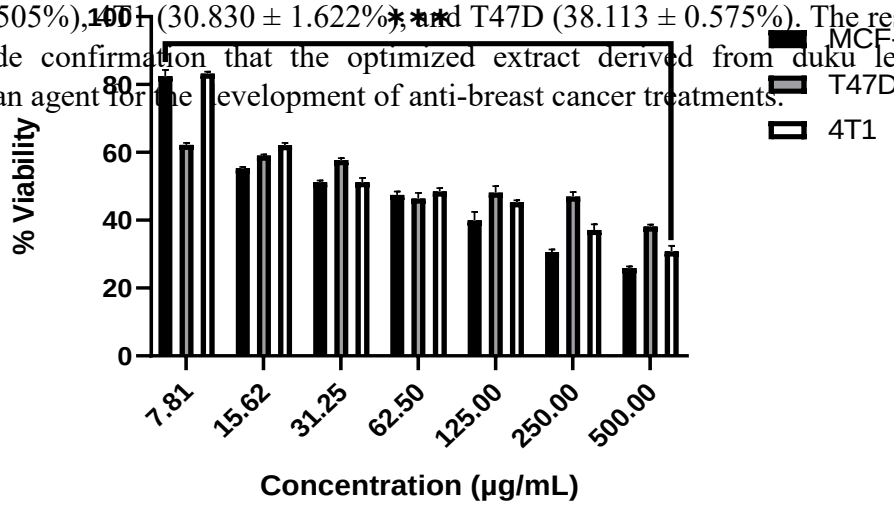

Fig. 5. Dose-response curves of MFC-7, T47D, and 4T1 cell lines treated with optimized extract of duku leaf from 7.81 to 500.00 µg/mL. *** The concentrations were significantly affected viability of cancer cell ($p < 0.001$) using one sample t-test

This study provides the phytochemicals information of optimized extract of duku leaf using GC-MS/MS. A total 5 mayor compounds were identified in duku leaf extract, as shown in Fig. 6., based on % peak area that can be seen in Table 6. The compounds were octadecanoic acid (33.16%), undecane,2-methyl- (7.10%). 9-octadecenoic acid (Z)-hexyl ester (6.63%), pentadecanoic acid (5.31%), and 9-hexadecanoic acid (5.19%). In the previous study, the octadecanoic acid and hexadecanoic acid that founded in the young fruit extract of duku(Manosroi *et al.*, 2012) and another reported in n-hexane extract of duku leaf(Fadhilah, Wahyuono and Astuti, 2021). According to reports, elevated quantities of specific fatty acids have been found to induce cellular demise through mechanisms including apoptosis or necrosis. (Khan *et al.*, 2012). The preceding statement was According to previous studies, palmitic acid, also known as hexadecanoic acid, has demonstrated the ability to trigger

apoptosis in tumor cells(Tan *et al.*, 2019). However, the anticancer activity of duku part was reported against HT-29 cells(Khalili *et al.*, 2014), HepG2(Muhammad Fauzan Lubis *et al.*, 2023), and A549 cells(Ramos *et al.*, 2022). For anti-breast cancer context, the fruit peel of duku was reported has cytotoxic activity against T47D cells and the compounds that responsible for this activity is Lamesticumin A(Wahyuono, Fadhilah and Astuti, 2021). In addition, this study will be provided the anticancer activity of optimized extract of duku leaf against several breast cancer cells.

Conclusions

The process used in the study involved a MAE technique to obtain an extract rich in polyphenols from duku leaves. This extract showed the highest DPPH scavenging activity. The Box-Behnken design and response surface methodology were effectively used to optimize and validate this technique. The best results were obtained using 75% ethanol concentration, 315 W microwave power, and an extraction time of approximately 8.5 minutes. The extract contained several phytochemical compounds such as octadecanoic acid, undecane, 2-methyl-, 9-octadecenoic acid (Z)-hexyl ester, pentadecanoic acid, and 9-hexadecanoic acid that were identified to prove the extract's cytotoxic activity against breast cancer cell lines. Further studies are required to explore the extract's mechanism of action and toxicity levels before it can be used as an anti-breast cancer agent.

Table 6. Mayor compounds identified in the extract of *L. domesticum* leaves by GC-MS

RT	Compound	Molecular formula	MW	Peak area (%)	Compound nature	Activity
36.504	Octadecanoic acid	$C_{18}H_{36}O_2$	284	33.16	Linoleic acid	Anti-inflammatory, hypocholesterolemic, cancer preventive, insectifuge, antiarthritic, hepatoprotective, antiandrogenic, nematicide, antihistaminic, antieczemic
22.581	Undecane,2-methyl-	$C_{12}H_{26}O$	170	7.10	Alkane hydrocarbon	Anti-inflammatory, antiallergic, and immunosuppressant effects
39.999	9-Octadecenoic acid (Z)-, hexyl este	$C_{24}H_{46}O_2$	366	6.63	Linoleic acid ester	Anti-inflammatory, antiandrogenic cancer preventive, dermatitigenic hypocholesterolemic, 5-alpha reductase inhibitor, anemiagenic, insectifuge
39.702	Pentadecanoic acid	$C_{17}H_{34}O_2$	270	5.31	Saturated fatty acid	Anticancer, anti-inflamatory, anti-anemia, antidyslipidemia, antifibrosis
40.090	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254	5.19	Palmitic acid ester	Antioxidant, hypocholesterolemic, nematicide, anti-

	androgenic, hemolytic, pesticide, lubricant, 5-alpha reductase inhibitor, antipsychotic

Credit authorship contribution statement

Ririn Astyka: Methodology, Formal analysis, Investigation, Writing an original draft. **Nur Aira Juwita:** Investigation, Writing original draft. **Sumaiyah Sumaiyah:** Conceptualization, Investigation, Formal analysis, Project administration, Writing review & editing. **Rony Abdi Syahputra:** Methodology, Investigation, Formal analysis. **Poppy Anjelisa Zaitun Hasibuan:** Conceptualization, Supervision, Writing – review & editing. **Muhammad Fauzan Lubis:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Husnarika Febriani:** Funding acquisition, Project administration, Writing original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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Revision 1

Response to Reviewers – OPEN PEER REVIEW

Title: **Optimization of microwave-assisted extraction to obtain a polyphenol-rich crude extract from duku (*Lansium domesticum* Corr.) leaf and the correlation with antioxidant and cytotoxic activity**

Manuscript number: KJS-D-23-01808
Revision Version: 1
Editor's Decision Received Date: April 15, 2024
Revision Submission Date: April 26, 2024

Author Response 1st revision

Reviewer
Reviewer Comments: Provide the equation for the calibration curve of standards used for TPC determination.

Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well. We have added the equation for the calibration curve for TPC determination. Please check the revised manuscript.

Reviewer Comments: "….potentially attributed to the destruction of certain phenolic compounds." Why? Provide more details.

Author Response: Thank you very much for your willingness to correct this article. Your corrections have followed well. We have added more details in the revised manuscript. Please get the revised manuscript and check it once again. Thank you.

Reviewer Comments: "This can lead to a favorable increase in the contact surface area between the plant matrix and the solvent,…" I recommend replacing this sentence "This can lead to a favorable increase in the penetration of solvent in the cells of the plant matrix,…" because the increase in contact surface area can be obtained by agitation.

Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well.

Reviewer Comments: "Table 6. Mayor compounds…" → "Table 6. Major compounds…"

Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well. Please check the revised manuscript.

Reviewer Comments: in Table 6. Provide references for the activity

Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well. The references to pharmacology claims of compounds were added in the revised manuscript. Please check the revised manuscript.

Reviewer Comments: Can authors provide LC-MS analysis. This could provide more active compounds of extracts.

Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well. The phytochemical compounds of the optimized extract were determined using LC-HRMS. Please check the revised manuscript for details.

Reviewer Comments: Check the grammar of the manuscript, number of errors throughout the text.

Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well.

Editorial

Editorial Comments: Please carefully review the provided editorial guidelines to confirm that your paper aligns with them. This will help expedite the review process for your submission.

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Author Response: Thank you very much for your willingness to correct this article. Your suggestion was followed well.

Revision2

Response to Reviewers – OPEN PEER REVIEW

Title: **Optimization of microwave-assisted extraction to obtain a polyphenol-rich crude extract from duku (*Lansium domesticum* Corr.) leaf and the correlation with antioxidant and cytotoxic activity**

Manuscript number: KJS-D-23-01808
Revision Version: 2
Editor's Decision Received Date: May 30, 2024
Revision Submission Date: June 06, 2024

Author Response 2nd revision

Reviewer
Reviewer Comments: The authors have addressed all the points and made additions accordingly. However, I strongly suggest rewriting the highlights. A bullet point must contain a maximum of 85 characters, including spaces. I advise the author to carefully respect the instructions of the journal.

Author Response: Thank you very much for your willingness to correct this article. About the highlights, We have checked that and it does not have more than 85 characters instead only 53 characters.

Reviewer Comments: Please include all the figures and tables at the corresponding sections of the manuscript.

Author Response: Thank you very much for your willingness to correct this article. Your corrections have followed well. We have added all the figures and tables at the corresponding section of the manuscript.

Revision 3

Response to Reviewers – OPEN PEER REVIEW

Title: **Optimization of microwave-assisted extraction to obtain a polyphenol-rich crude extract from duku (*Lansium domesticum* Corr.) leaf and the correlation with antioxidant and cytotoxic activity**

Manuscript number: KJS-D-23-01808
Revision Version: 3
Editor's Decision Received Date: July 25, 2024
Revision Submission Date: July 31, 2024

Author Response 3th revision

Reviewer
Reviewer Comments: Mention the reference (pages 5, line 150)

Author Response: Thank you very much for your willingness to correct this article. The reference has been added in the sentence.

Reviewer Comments: Mention the reference (pages 5, line 162)

Author Response: Thank you very much for your willingness to correct this article. The reference has been added in the sentence.

Reviewer Comments: Incomplete sentence (pages 5, line 166)

Author Response: Thank you very much for your willingness to correct this article. The sentence has been revised.

Reviewer Comments: Numbering the Equation (pages 5, line 168)

Author Response: Thank you very much for your willingness to correct this article. The equation has been numbered.

Reviewer Comments: Mention the full form (pages 5, line 185 and 188)

Author Response: Thank you very much for your willingness to correct this article. It has been revised.

Reviewer Comments: Numbering the Equation (pages 7, line 233)

Author Response: Thank you very much for your willingness to correct this article. The equation has been numbered.

Reviewer Comments: Remove this section (pages 7, line 246)

Author Response: Thank you very much for your willingness to correct this article. The section has been removed.

Reviewer Comments: Remove this section (pages 7, line 246)

Author Response: Thank you very much for your willingness to correct this article. The section has been removed.

Reviewer Comments: Mention the reference in the end of sentence (pages 7, line 246)

Author Response: Thank you very much for your willingness to correct this article. The reference was mentioned in the end of the sentence.

Reviewer Comments: Tabel 6, Saha et al. Please check the year

Author Response: Thank you very much for your willingness to correct this article. The reference related to Saha et al has been corrected.

Reviewer Comments: Wang and Wang, Please check the year (Pages 14, line 505)

Author Response: Thank you very much for your willingness to correct this article. The reference related to Wang and Wang has been corrected.

Revision 4

Response to Reviewers – OPEN PEER REVIEW

Title: **Optimization of microwave-assisted extraction to obtain a polyphenol-rich crude extract from duku (*Lansium domesticum* Corr.) leaf and the correlation with antioxidant and cytotoxic activity**

Manuscript number: KJS-D-23-01808
Revision Version: 4
Editor's Decision Received Date: August 14, 2024
Revision Submission Date: August 21, 2024

Author Response 4th revision

Reviewer
Reviewer Comments: Please follow a numbering pattern for all headings and subheadings.

Author Response: Thank you very much for your willingness to correct this article. Your advice has been followed in the revised manuscript.

Reviewer Comments: For references with the same author name and year, please differentiate them by mentioning, for ex: 2022a and 2022b.

Author Response: Thank you very much for your willingness to correct this article. Your advice has been followed in the revised manuscript.

Letter of Acceptance

View Letter

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Date: Sep 10, 2024
To: "Muhammad Fauzan Lubis" fauzan.lubis@usu.ac.id
From: "Kuwait Journal of Science" support@elsevier.com
Subject: Decision on submission to Kuwait Journal of Science

Ref.: Ms. No. KJS-D-23-01808R3
Optimization of microwave assisted extraction to obtain a polyphenol-rich crude extract from duku (Lansium domesticum Corr.) leaf and the correlation with antioxidant and cytotoxic activity
Kuwait Journal of Science

Dear Mr Lubis,

I am pleased to inform you that your paper has now been accepted for publication.

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Meanwhile, you will be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

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Thank you for submitting your work to Kuwait Journal of Science. We hope you consider us again for future submissions.

Kind regards,
Pooja Vijayan
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