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Optimization of Total Flavonoid Content from Cardamom Fruits Using a Simplex-Centroid Design, Along with the Evaluation of the Antioxidant Properties

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ABSTRACT

The fruits of Cardamom (*Amomum compactum* Sol. Ex Maton; *Zingiberaceae*), commonly known as Queen species, which contained flavonoid compounds, have a variety of pharmacological properties and are used in traditional medicine. Therefore, this study aimed to employ solvent macerations to optimize the total flavonoid extraction from Cardamom fruits, as well as evaluate their antioxidant activity. The fruits were collected from a local farmer in Bogor, Indonesia, in August 2019. Then, different extraction solvents, namely water, acetone, methanol, and ethanol, alongside their binary, ternary, and quaternary combinations, were investigated using a simplex-centroid design. The optimized extracts were tested for their flavonoid content using an aluminium chloride reagent and their antioxidant activity compared by utilizing 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Water extracts showed the maximum flavonoid content of 88.01 mg/g DW with the highest reducing antioxidant activity at 1110.95 µmol TE/g DW by FRAP assay. Meanwhile, the radical scavenging activity by the DPPH method revealed the highest value for the water-ethanol binary mixture to be 153.17 µmol TE/g DW. Consequently, the water and water-ethanol are the optimal solvents for extracting cardamom flavonoid compounds with antioxidant properties.

***Keywords*:** *Amomum compactum* Sol. Ex Maton, DPPH, Flavonoid, FRAP, Mixture designs, Solvent extraction.

Introduction

Flavonoids are linked to several pharmacological activities, including anti-inflammatory, antioxidant, anti-apoptosis, anti-platelet aggregation, hepatoprotective, cardioprotective, neuroprotective, anti-diabetic, anti-depressant, anti-hypertensive, and anti-atherogenic activities.[1–3] They are synthesized in plants for growth, reproduction, biochemical, and physiological roles and are involved in interspecies interaction and the interplay with environmental stress.[4,5] Currently, flavonoids are an essential component in many pharmaceuticals, cosmetics, and medicines.[6] These compounds have also been studied in scientific literature and traditional medicine.

Natural flavonoid compounds are antioxidants that have greater consumer preference over those derived from the laboratory because of factors such as decreased toxicity and the absence of side effects.[7] Antioxidants can help inhibit chain reactions that lead to the production of reactive oxygen species (ROS), consequently preventing cancer and degenerative diseases.[8] Therefore, the discovery of new natural antioxidants is a necessary effort because these compounds are in high demand.

Flavonoids are widely found in Cardamom (*Amomum compactum* Sol. Ex Maton) fruits, from the *Zingiberaceae* family.[9]

Popularly known as the Queen species, Cardamom is famous globally for its economic value as an exotic and high-priced fruit.[10] It is used in folk medicine for nausea, digestive, gum infections, asthma, kidney disorders, and diarrhea.[11] Also, this fruit exhibits pharmacological activities that include anticancer, anti-inflammatory, anti-biofilm, antibacterial, antioxidant, atherosclerotic, and antimalarial activities.[10,12–16]

The process and solvent used to extract a flavonoid-enriched substance can have a significant impact on biological activity. Generally, the solubility of plant flavonoids in water and organic solvents, such as ethanol, methanol, and water-acetone solutions is greater.[17] However, the variety of flavonoids in plant materials pose a challenge to the standardization of the extraction process. Additionally, pure solvents are incapable of extracting all flavonoids due to their diverse polarity and structures.[18] One way is to select experimentally the mixture of solvents from which flavonoids are extracted, such as through a simplex-centroid design, by changing to binary, ternary, or even multi-component proportion systems. In contrast to trial-and-error methods, this design provides a time-saving and economic process because it uses statistical criteria to minimize the model error and the number of experiments required.[19] The ability to obtain compounds with different chemical characteristics in this system is possible because of the synergy between solvents.[20] Recently, the simplex-centroid design has been used in medicinal plants to extract their secondary metabolites and in the observation of synergistic or antagonistic impacts resulting from different extracted compounds.[21,22] A solvent-sample interaction greatly influences the extraction of flavonoids, where the solvent system's efficiency determines the structures and polarity of the compound obtained from a sample matrix.[18] Although maximum flavonoid extraction is required to meet the requirements of pharmaceutical and functional

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food industries, the selection of the extractive solvent should also be based on health and environmental risks. Water and ethanol are therefore preferred over methanol and acetone in this context for green extraction.[18] Additionally, no published research describes how to optimize solvents to obtain extracts rich in flavonoid compounds and their antioxidant properties from cardamom fruit.

In this study, the simplex-centroid design was used to determine the most appropriate solvent mixture for extracting flavonoid compounds with the best antioxidant activity from Cardamom fruits. The best extractor selection was based on the total flavonoid content (TFC) and DPPH and FRAP antioxidant responses.

Materials and Methods

Plant material preparation

Cardamom fruit samples (Figure 1) were collected in August 2019 from a local farmer in Bogor, Indonesia, at 6°43'30.4"S, 106°41'40.6"E, and an altitude of 1267 m. The plant was identified (BMK0472052020) in Tropical Biopharmaca Research Center, Bogor Agricultural University, Indonesia. Then, the samples were sun-dripped for five days to attain a constant weight before being milled into powder and prepared for extraction.

Figure 1: The fruit of cardamom used (a) dried and (b) powder.

Simplex-centroid design and extraction

According to Table 1, the simplex-centroid design employed mixtures of water, acetone, methanol, and ethanol for the solvent extraction, which were generated via Design-Expert program version 11.0. All the components in the mixture were examined from 0% to 100%, and the best-fitting model was selected for each response. Subsequently, the data responses were statistically analyzed using Design-Expert software version 11.0 (Stat-Ease Inc., Minneapolis, USA).

Then, 10 g of dry fruit powder was extracted with 100ml of the solvent mixture, protected from light, and stirred for 30 min at 140 rpm. The mixtures were macerated (2 x 24 h) in a dark room at room temperature, and each obtained solution was concentrated using a rotary vacuum evaporator (HAHNVAPOR, Korea). Extraction preparations were done in triplicates and randomized, and each extract was analyzed for total flavonoid and antioxidant capacity.

Total flavonoid content (TFC)

The TFC in the extracts was determined by the aluminium chloride colorimetric assay, as reported by Khumaida *et al*.[23] An extract volume of 10 µl was briefly mixed with 120 µl of distilled water, 60 µl of methanol, 10 µl of aluminium chloride (10%), and 10 µl of potassium acetate (1 M) in a 96-well microplate. After incubation for thirty (30) minutes at room temperature, the absorbance of the mixture was measured at 415 nm using the microplate reader (Epoch BioTek, USA), and the results expressed as equivalent of quercetin (QE) in mg per g (mg/g) fruits in dry weight basis (DW).

Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging

The DPPH radical scavenging in extracts was determined according to Calvindi *et al*.[24] A 100 µl aliquot of each extract was added to 100 µl or 125 µM DPPH solution in methanol in a 96-well microplate (Costar-USA). Subsequently, the mixture was incubated for thirty minutes at darkroom temperature, and the absorbance at 517 nm measured using a microplate reader (BMG Labtech, Germany). The DPPH radical scavenging activity was expressed as an equivalent of Trolox (TE) in µmol per g fruits on a dry weight basis (DW).

Determination of the ferric reducing antioxidant power (FRAP)

The FRAP of the individual extracts was determined using a previously documented method.[25] Here, 100 µl of extract was briefly added to the 300 µl FRAP reagent in the 96-well microplate (Costar-USA). The FRAP reagent was prepared by mixing 300 mM of pH 3.6 acetate buffer with TPTZ (10 mM, in 40 mmol HCl) and 20 mmol $FeCl_3$ with v/v/v ratio of 10:1:1 and incubated for thirty (30) minutes at 37°C. Then, the mixture of extract and FRAP reagent were incubated in the dark at 37°C for thirty minutes, and the absorbance at 593 nm measured using a microplate reader (BMG Labtech, Germany). The value of antioxidant activity was expressed as µmol TE per g DW.

Statistical analysis

The Design-Expert program (11.0 version, State-Ease Inc., MN, USA) was used to design and analyze the experiments. Data were analyzed using ANOVA with a confidence level of 95%, while the Pearson correlation graph between TFC and the antioxidant activity of DPPH and FRAP was generated in an R program using ggpubr and ggplot2 packages.

Results and Discussion

Optimization of sample extraction

The dry powder of the cardamom fruits was extracted using either four solvents, namely water, acetone, methanol, and ethanol, or a mixture produced from the simplex-centroid design, as shown in Table 1. This table also presents the results of the different solvent compositions that affect response variables, which are total flavonoid content (TFC) and antioxidant activity (DPPH and FRAP). Consequently, TFC ranged from 74.66 to 188.01 mg QE/g DW, with the maximum value provided by 100% water as the solvent for extraction, and the minimum was recorded by a mixture of 50% of methanol and ethanol each. The DPPH radical scavenging activity varied from 14.34 produced by 100% ethanol to 153.17 µmol TE/g DW obtained from 50% water-ethanol. Furthermore, the reducing antioxidant power (FRAP) was between 97.65 and 1110.95 µmol TE/g DW with the maximum obtained by 100% water. Therefore, this study concluded that water solvents and ethanol mixtures are best for extracting flavonoids with antioxidant properties from Cardamom fruits. This result is in line with the previous study, which showed that optimum extraction is possible in an aqueous solution with an ethanol concentration of between 35-90%.[18]

Model fitting

Variance analysis (ANOVA) for TFC, alongside the DPPH and FRAP antioxidant activity responses, were presented in Table 2. ANOVA was used to evaluate the fitted mathematical model to choose the best model for TFC, DPPH, and FRAP at a 95% confidence interval.[26] Meanwhile, the F-statistics were used to test the regression model derived from the ANOVA study. The model was considered significant if the calculated *F*-value was higher than the table's at a low *p*-value of < 0.05. From the results in Table 2, ANOVA showed a significant regression for the linear models for TFC (F=5.67, *p*=0.0135) and FRAP (F=11.08, *p*=0.0012) at the 95% confidence level. However, the special cubic model for DPPH was not significant (F=27.52, *p*=0.1482). The R^2 coefficient was used to evaluate the model performance. The R^2 value close to 1 indicated the experiment's data distribution is reliable.[22,27] The study showed the R^2 values of 0.6073, 0.9972, and 0.7513 for the linear TFC, special cubic DPPH, and linear FRAP models. These results indicated 61, 99, and 75% of data variability in TFC, DPPH, and FRAP models, respectively. The Adjusted R^2 values of TFC, DPPH, and FRAP models were 0.5001, 0.9610, and 0.6835, respectively.

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Table 1: Results of the simplex-centroid experimental design for TFC, DPPH, and FRAP responses

Run	Solvents (%)				Response variables*		
	Water (A)	Acetone (B)	Methanol (C)	Ethanol (D)	TFC (mg QE/g DW)	DPPH (μmol TE/g DW)	FRAP (μmol TE/g DW)
1	0.00	0.00	50.00	50.00	74.66f	25.75c	97.65f
2	50.00	0.00	50.00	0.00	138.42c	89.39b	879.09b
3	0.00	0.00	0.00	100.00	53.88g	14.34c	98.88f53
4	0.00	50.00	0.00	50.00	94.11e	26.58c	243.41e
5	50.00	50.00	0.00	0.00	92.18e	77.17b	423.09d
6	0.00	0.00	100.00	0.00	122.01d	49.30c	419.62d
7	50.00	0.00	0.00	50.00	131.45d	153.17a	719.11c
8	0.00	33.33	33.33	33.33	91.54e	43.28c	98.11f
9	0.00	50.00	50.00	0.00	99.02e	63.53b	32.46f
10	25.00	25.00	25.00	25.00	100.00e	25.74c	237.32e
11	100.00	0.00	0.00	0.00	188.01a	37.53c	1110.95a
12	33.33	33.33	33.33	0.00	146.07c	56.00b	525.75d
13	33.33	33.33	0.00	33.33	140.90c	66.89b	711.91c
14	0.00	100	0.00	0.00	168.65b	47.41c	456.59d
15	33.33	0.00	33.33	33.33	119.92d	39.98c	430.08d

*Values with different letters in the same column were significantly different at *p-value* < 0.05. TFC = total flavonoid content, DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP= ferric reducing antioxidant power.

Table 2: ANOVA results for TFC, DPPH, and FRAP responses

	TFC	DPPH	FRAP
	Linear model	Special Cubic model	Linear model
F	5.67	27.52	11.08
p	0.0135	0.1482	0.0012
R^2	0.6073	0.9972	0.7513
Adjusted R^2	0.5001	0.9610	0.6835

TFC = total flavonoid content, DPPH = 2,2-diphenyl-1-picrylhydrazyl, FRAP = ferric reducing antioxidant power.

Adjusted R^2 was used to compare the adequacy of the experimental and theoretical results.[28] R^2 value compared to adjusted R^2 showed that the linear TFC model, special cubic DPPH model, and linear FRAP model could not explain 10.72, 3.62, and 6.78% of the total variation, respectively. These results can be used to navigate the design space of the model investigated.[26]

Effects of the solvent system on total flavonoid content (TFC)

The linear model was generated to define the effect the interactions between water, acetone, methanol, and ethanol in the solvent system had on the total flavonoid content, which was defined as the coded value in Equation 1.

TFC = 172.91A + 131.21B + 106.24C + 59.19D ---------- (1)

Where A = water, B = acetone, C = methanol, and D = ethanol

All the linear terms were positive with the highest value from Equation 1 (+172.91), which presented water as the solvent that enhanced flavonoid extraction the most, followed by acetone (+131.21), methanol (+106.24), and ethanol (+59.19). As expected, these solvents were found to greatly assist in the recovery of flavonoid compounds from Cardamom fruits. To better interpret the adjusted mathematical model, a contour and three-dimensional plot were created from Equation 1, as shown in Figure 2. Additionally, the effects of the interactions between the independent variables were investigated, and Figure 2 indicated that a higher amount of flavonoid compounds were obtained via the water extraction. Flavonoids are various polyphenolic compounds, including anthocyanidins, flavanones, chalcones, flavones, isoflavones, and flavonol groups.[18] Due to the polarity of flavonoids, they are more easily extracted with a water-soluble solvent and their glycosides are known to have a high polarity.[4,29] This study indicates that cardamom may contain numerous flavonoid glycosides, leading to their extraction in the water solvent.[30]

Effects of the solvent system on antioxidant activity

An antioxidant is explained as a molecule that can slow down an oxidation process or prevent it altogether.[31] These molecules avoid macromolecular mutations by squandering reactive oxygen species (ROS) and reducing the oxidative damage caused by them.[32] Numerous studies have shown that oxidative damage plays a significant role in syndromic metabolic diseases, such as neurodegenerative, aging and age-related, and Parkinson's diseases, among others.[33-35] Antioxidant treatment also plays a crucial role in preventing diseases of metabolic disorders.[36] Besides, a natural source's antioxidant properties depend on the activity of the compounds produced during extraction hence, a variety of assays are required to determine these antioxidant activities.[37] In this research, DPPH and FRAP methods were used to evaluate the antioxidant properties of the Cardamom fruit extracts. The advantages of the DPPH method were rapidity, cheapness, and simplicity, whereas the FRAP method used affordable and straightforward tools, alongside good reproducibility and sensitivity.[38]

The DPPH special cubic model is presented by polynomial Equation 2:

DPPH = 37.45A + 47.33B + 49.22C + 14.26D + 141.63AB + 186.73AC + 511.77AD + 63.53BC – 14.35BD – 21.45CD – 927.13ABC – 1059.94ABD – 1917.52ACD + 30.62BCD --------- (2)

where A = water, B = acetone, C = methanol, and D = ethanol

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The Equation 2 terms show that the binary mixture generated the highest positive effect on DPPH scavenging activity than the pure solvents and ternary mixture. Subsequently, the binary mixture of water-ethanol (+511.77) was significantly more effective than water-acetone (+141.63), water-methanol (+186.73), and acetone-methanol (+63.53). However, there was a decrease in the radical scavenging activity of the binary acetone-ethanol (-14.35) and methanol-ethanol (-21.45) mixtures. Figure 3 demonstrates the response contour and three-dimensional plots for the interaction effects of water (A), methanol (C), and ethanol (D) on the scavenging activity while retaining acetone (B) at 25%. After analyzing the data from Figure 3 and determining the result from Table 1, 50% volume or 153.17µmol TE/g DW of water-ethanol was shown to be the best solvent for extraction in determining this ability. This result is in line with previously reported results in 72% ethanol in water presenting higher antioxidant compounds on *Empetrum nigrum* fruits.[39]
Meanwhile, the FRAP assay, due to its reasonable validity and approach, was used to reduce the antioxidant molecules' presence.[40] The positive terms for the linear model tested for the FRAP antioxidant reducing power are presented by polynomial Equation 3:

FRAP = 1124.85A + 239.64B + 254.66C + 109.92D. ---------- (3)
where A = water, B = acetone, C = methanol, and D = ethanol
This experiment showed that water was the solvent with the highest effect on reducing antioxidant power. A power of 1124.85 was found with water extraction, followed by methanol at 254.66, 239.64 with acetone, and ethanol at 109.92. Figure 4 presents the effect of solvent concentration on reducing antioxidant power. The ethanol solvent was set at 25%, and the reducing antioxidant power was more enhanced with the water solvent extraction than acetone and methanol.
Figure 5 shows the result correlation analysis between TFC and the DPPH and FRAP antioxidant activity. TFC showed a significantly positive correlation with FRAP, with the lowest value found with DPPH. This result indicated that the antioxidant mechanism of action of the flavonoid compound in Cardamom fruit presented was through the reducing power than radical scavenging activity. This finding corresponds with previous research concerning the correlation of the total flavonoid content with other assays of reducing antioxidant power (CUPRAC, cupric reducing antioxidant power).[41,42] The flavonoid compounds were polyphenol groups of plants associated with antioxidant activities, and the mechanism was influenced in the ring B phenyl by a hydroxy group.[43] Flavonoid compounds that have been reported to reduce oxidation include vitexin, rutin, cymaroside, quercetin, astragalin, and cyanidin-3-*O*-glucoside.[43] Consequently, further research must evaluate the antioxidants found in flavonoids of the Cardamom fruit extract.

Optimization solution of total flavonoid content and antioxidant activity of Cardamom fruits
The experimental set of variables evaluated was generally selected based on desirability, with 1.00 as the highest score.[44] At 0.75 desirability, the optimal solution was obtained from the mixture of 77.4% water and 22.6% ethanol as extraction solvents. The predicted TFC, DPPH, and FRAP values of this solution were 147.23 mg QE/g DW, 121.70 µmol TE/g DW, and 895.60 µmol TE/g DW, respectively.

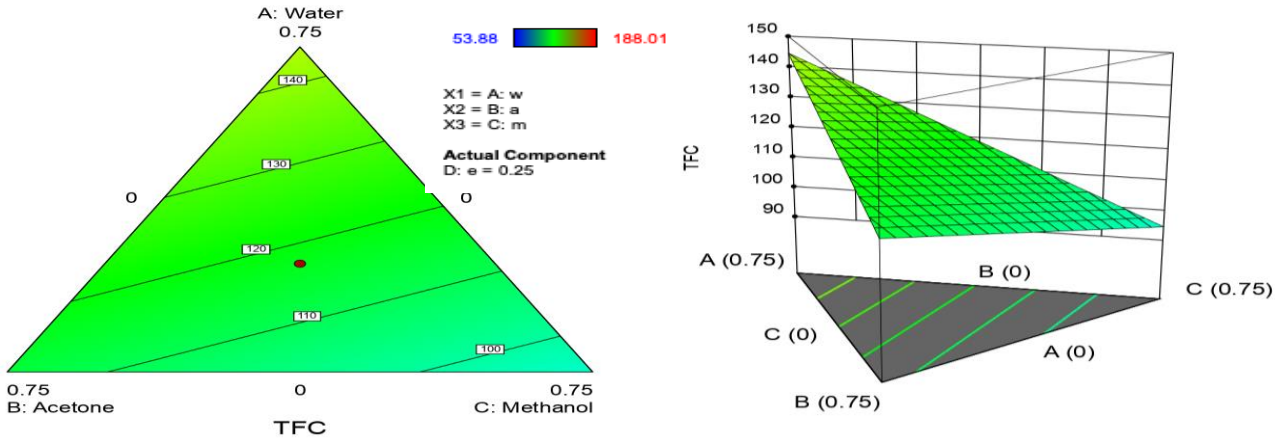

Figure 2: The contour and 3-D graph for the analysis of the linear model response surface predicted for the extraction of total flavonoid content (TFC) in water (A), acetone (B), and methanol (C) using 25% ethanol (D).

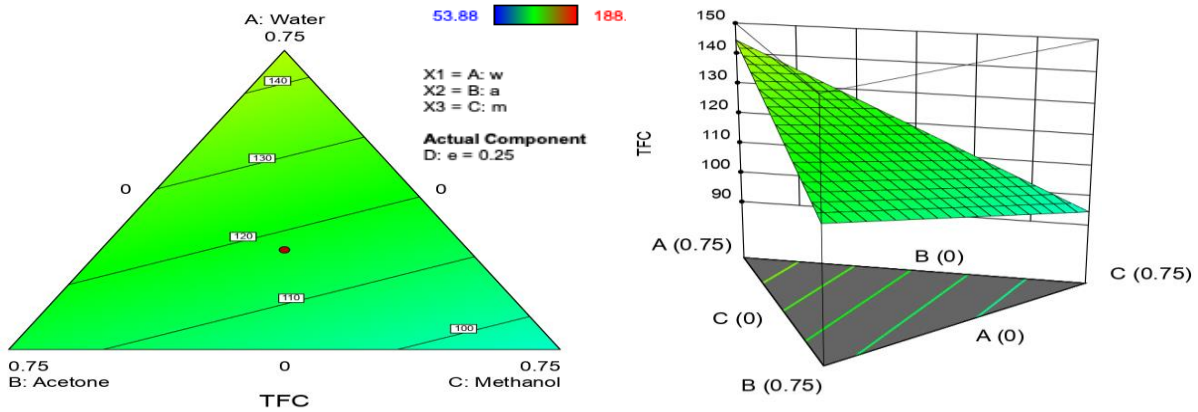

Figure 3: The contour and 3-D graph for the analysis of the special cubic model response surface predicted for the extraction of DPPH antioxidant in water (A), methanol (C), and ethanol (D) using 25% acetone (B).

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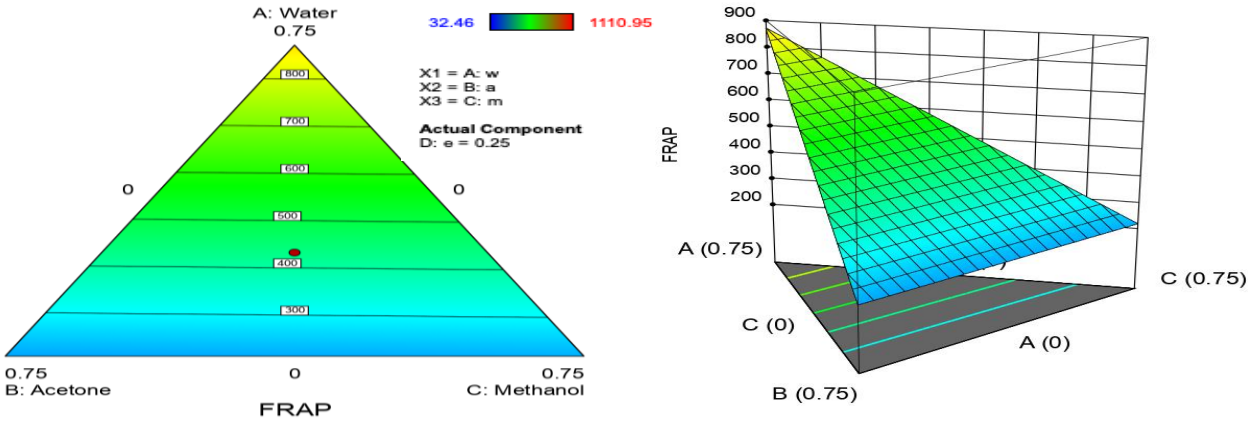

Figure 4: The contour and 3-D graph for the analysis of the linear model response surface predicted for the extraction of FRAP antioxidant in water (A), acetone (B), and methanol (C) using 25% ethanol (D).

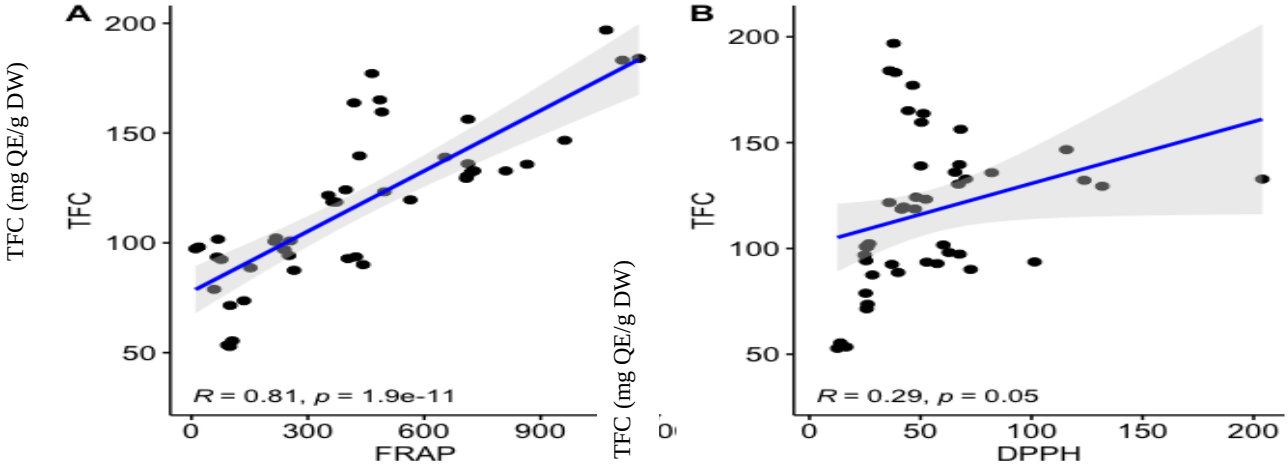

Figure 5: The simple correlation and regression analysis between the total flavonoid content (TFC) and antioxidant activity of the FRAP (A) and DPPH (B) assays in the extracts obtained from the simplex centroid design.

Conclusion

The statistical design was successful in maximizing the antioxidant capacity of the flavonoids in Cardamom fruits. Consequently, the maximum flavonoid content and reducing antioxidant power of 188.01 mg QE/g DW and 1110.95 µmol TE/ g DW, respectively, of the fruits were obtained from 100% water as the extraction solvent. Conversely, the radical scavenging activity of 153.17 µmol TE/ g DW was maximized by water-ethanol. The binary mixture containing 72% of water and 28% ethanol was a solution for optimizing flavonoid and antioxidant extraction in Cardamom fruits from the software with 0.75 desirability. Flavonoids of cardamom fruits were found to be more highly correlated with FRAP than with DPPH. Therefore, this research presented the potential of Cardamom fruits as a source of flavonoid antioxidants, using water and a binary mixture of water-ethanol as the solvents for extraction.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the contents will be borne by them.

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