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Optimization of the extraction of bioactive compounds from *Pouzolzia zeylanica* using Box-Behnken experimental design and application in the preparation of mixed juice

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Abstract

Pouzolzia zeylanica grows naturally in the forest and is quite popular in Vietnam. This study was designed to investigate the influence of extraction parameters and their effective application in the processing of mixed fruit juice from *P. zeylanica* and orange juice. A three-factor [i.e., solvent ratio and material (5:1–6:1), extraction temperature (85–95 °C), and extraction time (10–15)] and three-level Box-Behnken design consisting of 18 experimental runs with 6 center points was used for the extraction of *P. zeylanica*. The optimal conditions (maximum extraction yield) based on both individual and combinations of all responses (ratio of water: materials is 5.4:1 v/w, extraction temperature 91 °C, and time 13 min) were found. At these optimum conditions, the TAC and TPC were found to be 13.55 mg/L and 88.26 mgGAE/L with a desirability value of 0.91. The experimental values are in close agreement with the corresponding predicted values (R^2=0.942). In addition, by adding orange juice as a natural ingredient that provides additional vitamin C, this study attempted to improve the mixed juice product quality and increase antioxidant capacity and organoleptic value. The mixed juice with the ratio of *P. zeylanica* extract and orange juice of 80% and 20%, respectively, for a product with high TPC and TAC contents (240.61 mgGAE/L and 10.84 mg/L), significantly improved vitamin C content (255.96 mg/L) and antioxidant activity (67.55% DPPH).

Keywords: *Pouzolzia zeylanica*; extraction; bioactive compounds; mixed juice; optimization.

Practical Application: *Pouzolzia zeylanica* (L.) Benn., a medicinal plant, was operated to extract the bioactive compound. The extraction was then combined with orange juice to produce high-quality mixed juice, which could increase the income of local farmers.

1. Introduction

Many medicinal plants often contain large amounts of antioxidants and play an important role in the prevention of many diseases, of which *Pouzolzia zeylanica* (L.) Benn. (*Family: Urticaceae*) is considered one of the several plants with medicinal properties found throughout Vietnam with the local name "Bọ Mắm" or "Thuốc giòi." It is an herbaceous perennial, which is usually 15–30 cm tall.

Pouzolzia zeylanica contains high content of bioactive compounds such as polyphenol, flavonoid, tannin, isoflavone, glycoside, phyllanthin, vitexin, and carotenoids (Ghani, 2003; Saha and Paul, 2012; Tan et al., 2019). According to oriental medicine, the medicinal plant maggots have a sweet, light, cool taste, have a generalizing effect, dissolve sputum, and are used to treat long-term coughs, coughs caused by sore throats, bronchiolitis, dry coughs, anti-inflammatory, urinary tract infections, stomach, enteritis, and urinary tract infection (Saha and Paul, 2012; Tan et al., 2019). In Vietnam, this plant is often used as shrimp paste so that there are no maggots. Some places use crushed leaves to cure tooth decay. The anti-inflammatory effect of *P. zeylanica* has also been handed down and used by people for a long time. "Cool water" is a folk name used to cool water cooked from medicinal herbs with the effect of clearing heat, making the body cool, reducing fever, often cooked, and used in the family in the hot season. In particular, *P. zeylanica* is one of the raw materials used in combination with other ingredients. Fluid balance is important when the body is battling a respiratory illness, and dehydration can thicken respiratory secretions and make them difficult to clear from the lungs. Keeping hydration levels high helps immune system to function even when not feeling thirsty (Thompson and Manore, 2017). Therefore, drinking forms from plant sources have medicinal properties capable of meeting the body's needs in disease conditions, in which *P. zeylanica* can be used in this situation for their beneficial properties. Furthermore, consumers are becoming more aware of the need to adopt a healthy, balanced diet by consuming functional, whole foods derived from vegetables. The combined study of *P. zeylanica* and orange juice also followed this criterion.

The selection of materials with good properties to process products and support patients or people in the recovery period

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is a matter of concern. A number of studies have been carried out, which can provide a useful source of information for the selection of good documents that are suitable for research purposes. The qualitative analysis of maggot medicinal plants with different cultivars was published by Tan et al. (2019). Research on the combination of fruits and vegetables with high bioactive compounds in juice production has been carried out in several previous studies (Islam et al., 2014; Thuy et al., 2022a). Fruit juices are rich in nutrients, vitamins, and minerals and often have a low energy value. A variety of fruits and vegetables can be used in blends to combine the benefits of each ingredient and improve the palatability and organoleptic properties of the product (Malik et al., 2019). *P. zeylanica* is a promising vegetable for use in such juices, mainly due to its high anthocyanin, total polyphenol content (TPC), and antioxidant capacity (Tan et al., 2018). Orange (*Citrus cinensis*) belongs to the citrus genus of the family *Rutaceae* and is a widely consumed fruit with a particularly delicious taste. The fruit pulp contains high levels of vitamin C, folic acid, potassium, and phytochemicals, which are components with high antioxidant capacity and good support for human health (USDA, 2014; Vasavada, 2003). Orange juice is also considered the most common and easy to drink. Mixed juice production aims to combine the benefits of a variety of vegetables and fruits, resulting in a product with improved nutritional value and good acceptance. Therefore, the aim of this study was to optimize the extraction parameters to maximize the recovery of bioactive compounds in *P. zeylanica* using response surface methodology (RSM) and application in the processing of mixed fruit juice (*P. zeylanica* and orange juice) to improve the diverse nutritional value of this new mixed juice product.

2. Materials and methods

2.1. Selection and preparation of ingredients

Pouzolzia zeylanica (L) Benn. selected for this study is the purple stem. The tree was planted at the campus of the College of Agriculture, Can Tho University. Approximately 3 months after planting (October to November), the plants can be harvested.

The fresh orange (*Citrus sinesis*) was collected from the local market.

2.2. Selection of variables and the extraction process

Extraction was carried out at liquid/solid (water/*P. zeylanica*) ratios (v/w) in the range of 5:1–6:1 (X_1), temperature from 85 to 95 °C (X_2), and time from 10 to 15 min (X_3) (Table 1). The fresh *P. zeylanica* was used for this study, and the initial moisture content was 93.23%. The selection of these values was made based on preliminary studies and previous studies (Linh and Thuy, 2014). The dependent (response) variables were determined, including TPC and total anthocyanin content (TAC). A total of 50 g of *P. zeylanica* was chopped and added to Erlene with different water volumes, temperatures, and extraction times. Extraction was performed on the SH-3 Lab Magnetic Stirrer. After each determined treatment, the mixture was immediately cooled in ice and then filtered with Whatman filter paper (Merck). The filtrate was analyzed for TPC and TAC immediately or stored at −70°C until analysis. Each treatment was performed three times.

Table 1. Coded and three variable levels.

Coded	Factors	Unit	Levels		
			-1	0	1
X_1	Water/*Pouzolzia zeylanica* ratio	v/w	5	5.5	6
X_2	Temperature	°C	85	90	95
X_3	Time	min	10	12.5	15

2.3. Experimental design

Experimental optimization was performed using RSM for extraction (TPC and TAC). The Box-Behnken design consisted of 18 test runs, of which 6 were at the central points (Table 2). The general quadratic polynomial model used in response surface analysis is as follows (Equation 1):

$$Y = b_o + \sum_{n=1}^{3} b_n X_n + \sum_{n=1}^{3} b_{nn} X_n^2 + \sum_{n \neq m=1}^{3} b_{nm} X_n X_m \quad (1)$$

where:

b_o: the Y-intercept (constant);

b_n: the regression coefficient for the linear effect of X_n on Y;

b_{nn} and b_{nm}: the regression coefficients for the quadratic effect on Y;

X_n and X_m: independent values.

2.4. Production of orange juice mixed with Pouzolzia zeylanica extract

Oranges (*Citrus sinensis* L.) were purchased from a local supermarket. The orange juice was obtained, after suitable washing and hygienization of the fruits, by using a squeezer (FMC juice extractor with a 2-mm diameter perforated plate), and it was then filtered with a 0.23-mm pore diameter filter. The juice was mixed with *P. zeylanica* extract at different ratios such as 90:10, 80:20, and 70:30 (v/v), and the control sample used 100% orange juice. The comparison of nutritional qualities and overall acceptance was investigated.

2.5. Determination of total polyphenol content, total anthocyanin content, and antioxidant activity

The Folin-Ciocalteu colorimetric method (Singleton et al., 1999) was used to determine TPC. The analyses were repeated three times. Calibration curves were established using gallic acid as standard. The results of TPC determination are expressed in milligrams of gallic acid equivalents per liter of extract (mgGAE/L).

TAC was analyzed using the differential pH method described by Giusti and Wrolstad (2001) with the pH-difference method based on the reaction at pH 1 and 4.5, allowing accurate and rapid measurement of total anthocyanins.

The antioxidant activity (AA) of orange juice was determined by the method of Demarchi et al. (2013).

2.6. Statistical analysis

The experimental data obtained from the Box-Behnken design were analyzed by using RSM to fit the second-order polynomial model (Equation 1) and obtain regression coefficients. The STATGRAPHICS Centurion version XV software (USA) was used for the regression and graphical analysis of the experimental data. The model fitting was evaluated through coefficients of determination (R^2) and analysis of variance (ANOVA).

3. Results and discussion

3.1. Effect of extraction conditions on bioactive compounds in Pouzolzia zeylanica extract

3.1.1. Total anthocyanin content

Response surface and contour plots (Figure 1) showed the influence of solvent/material ratio, extraction temperature, and time, together with their interactions on anthocyanin extraction from *P. zeylanica*. At a certain solvent/material ratio, the interaction between time and temperature to TAC is shown in Figure 1A. It was observed that increasing temperature reduced the TAC but it

Table 2. Box-Behnken experimental design.

No.	Ratio of water/*Pouzolzia zeylanica*	Temperature (X_2)	Time (X_3)	N_o	Ratio of water/*Pouzolzia zeylanica*	Temperature (X_2)	Time (X_3)
1	5	95	12.5	10	5.5	90	12.5
2	5.5	90	12.5	11	6	90	10
3	6	85	12.5	12	5.5	85	15
4	5.5	85	10	13	5	90	15
5	5.5	95	10	14	5.5	90	12.5
6	5.5	95	15	15	6	95	12.5
7	5	85	12.5	16	5.5	90	12.5
8	5.5	90	12.5	17	6	90	15
9	5	90	10	18	5.5	90	12.5

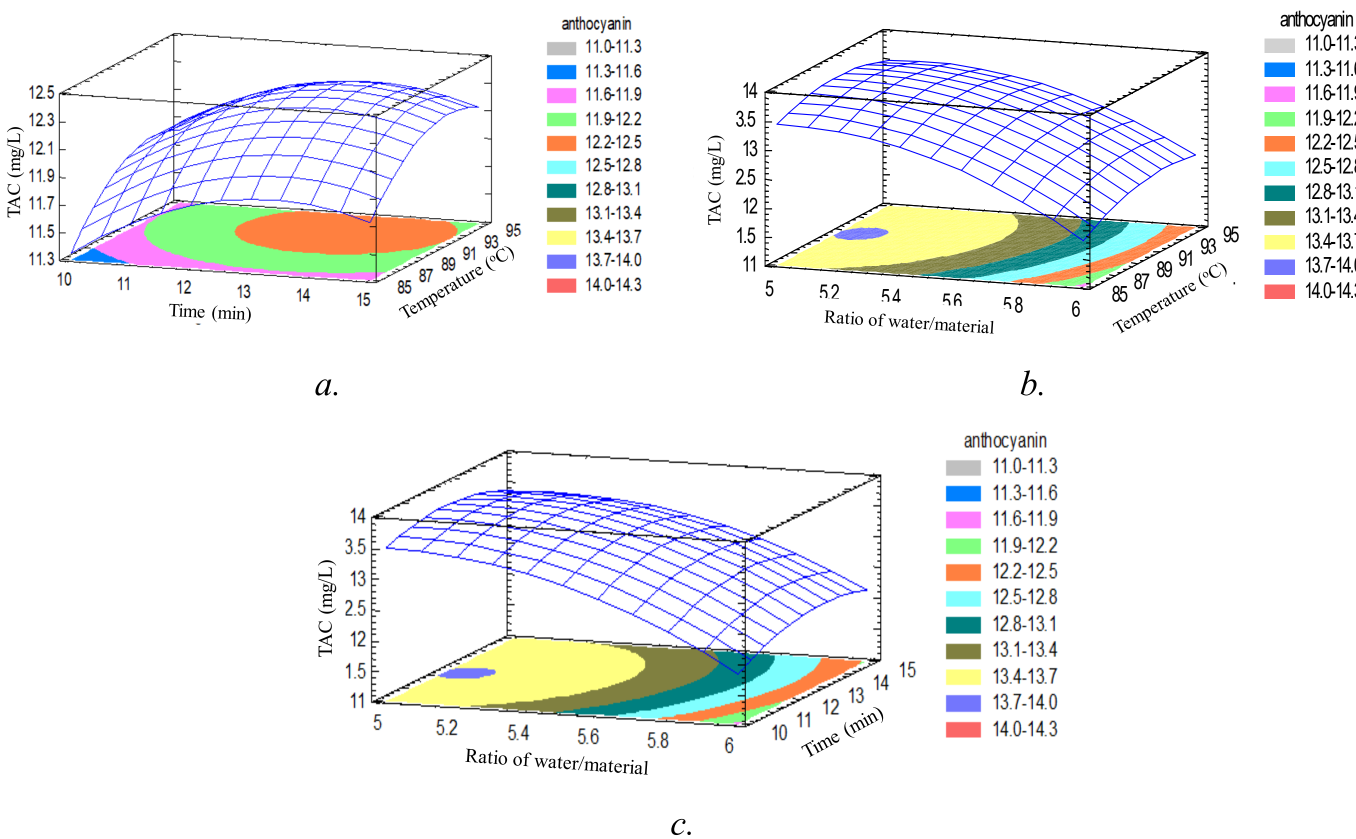


Figure 1. Estimated response surface plots for the effect of extraction variables on TAC (mg/L). (A) At a solvent/material ratio of 6:1; (B) At an extraction time of 12.5 min; (C) At an extraction temperature of 90 °C.

was not clear. At high temperature, anthocyanin easily degraded or polymerized to form brown or colorless pigments, so the TAC was also reduced. TAC increased from 13.05 to 13.4 mg/L as extraction time increased from 10 to 12.5 min. When the time increased past the optimal point, the anthocyanin content decreased due to the longer extraction time, possibly due to the influence of other factors (i.e., temperature, light, oxygen, etc.) (Van Tai et al., 2021).

The interaction effect of water/solvent ratio and temperature at a fixed extraction time of 12.5 min for TAC is shown in Figure 1B. The maximum anthocyanin content was extracted at 90.54 °C and a solvent/material ratio of 5.07:1. A higher solvent ratio induces a higher density gradient and a higher distribution coefficient, thus resulting in a faster release from the maggot tissue and thus higher extracted anthocyanin content. This trend continues until the solvent ratio reaches more than 5.2 mL/g. As the solvent ratio increased, the solute weight ratio was lower and the extracted anthocyanin density decreased, thus resulting in a decrease in the TAC in the extract. At constant temperature (Figure 1C), TAC increased as the extraction time increased from 10 to 12.30 min, and TAC loss occurred as the extraction time increased from 12.32 to 15 min, presumably also due to the effect of temperature on longer time extraction. Increasing solvent ratio also extracted more TAC but only to about 5.1 ratio, and then TAC also decreased.

3.1.2. *Total polyphenol content*

The response surface plot for the influence of independent variables on TPC during extraction is shown in Figure 2. Similar trends were observed with an elevation of TPC during extraction. Moreover, high TAC also resulted in high TPC. Response surface and contour plots of each pair of factors show that at constant values of extraction time (Figure 2A) and extraction temperature (Figure 2B) of 12.5 min and 90 °C, respectively, when the solvent/material ratio increased from 5:1 to 5.54:1, the TPC increased; however, when the solvent/material ratio exceeded the optimal point, the TPC decreased.

Similarly, at a constant solvent/material ratio (Figure 2C), the TPC increased as the extraction temperature increased from 85 to 90.9 °C and the time increased from 10 to 12.5 min. Heat treatment increases the solubility and diffusion of compounds, which in turn increases mass transfer and solvent penetration into cells (Nguyen et al., 2022). On the contrary, Mohamad et al. (2010) suggested that high temperature can weaken cell walls and membranes, facilitate solvent contact with components, and increase extraction capacity. However, when increasing past this optimal point, the polyphenol content tends to decrease. This is mainly due to the long extraction time, and the phenolic compounds are also destroyed by oxidation (Thuy et al., 2020; Yilmaz and Toledo, 2006). Our results showed that the TPC varied from 41.5 to 93.3 mgGAE/L, in which TPC was lowest (41.5 mgGAE/L) when the solvent/material ratio, extraction temperature, and time were 5:1 (v/w), 85 °C, and 12.5 min, respectively. In contrast, the highest TPC (93.3 mgGAE/L) was found when the solvent/material ratio, extraction temperature, and time were 5.5 (v/w), 90 °C, and 12.5 min, respectively.

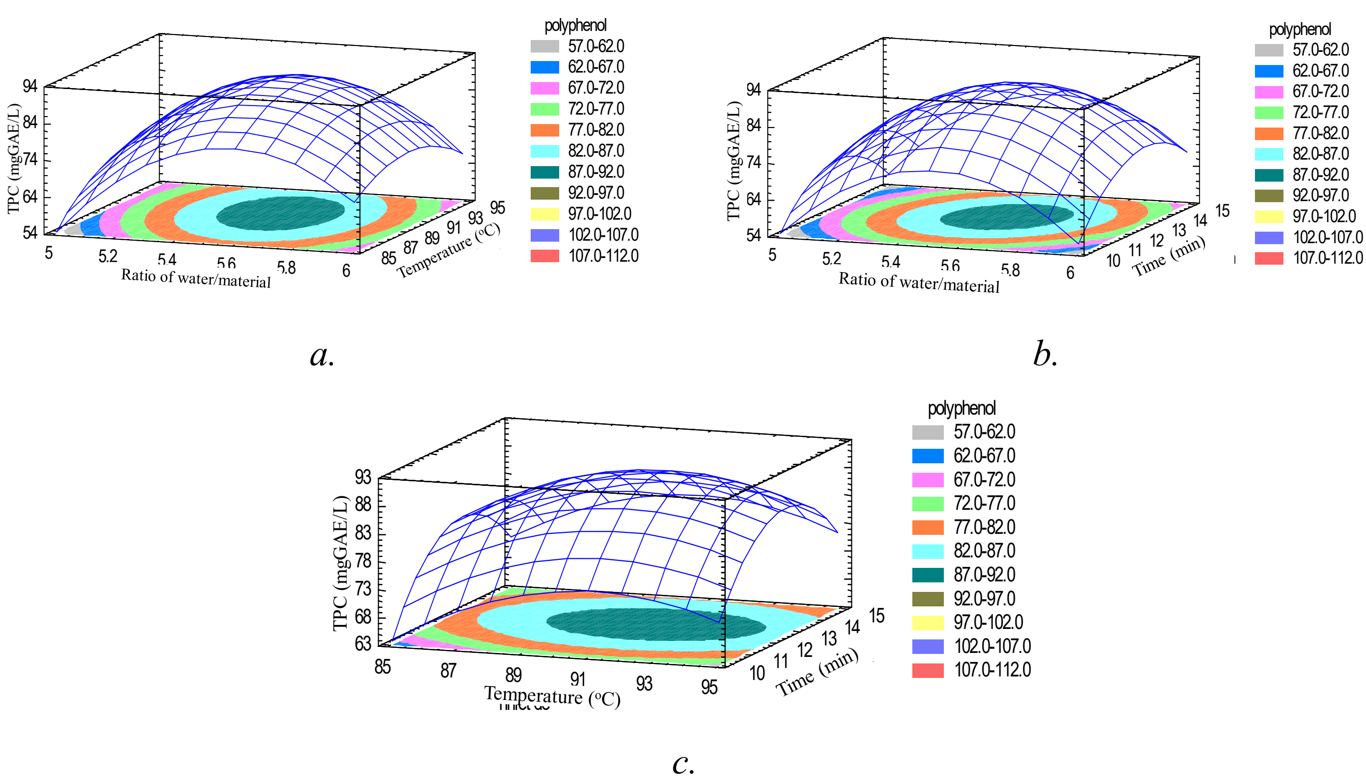


Figure 2. Estimated response surface plots for the effect of extraction variables on TPC (mgGAE/L). (A) At an extraction time of 12.5 min; (B) At an extraction temperature of 90 °C; (C) At a water/material ratio of 5.5:1.

3.2. Optimization of extraction parameters

3.2.1. Optimization of TAC

The optimization of TAC in the extraction process was done by applying a quadratic polynomial equation. The model shows a high significance level and good fit with the experimental data. From the ANOVA, there were eight effects with a P-value less than 0.05, showing that they are significantly different at a 95% confidence level. A comparison of the variability of the current model residuals with the inter-observation variability at repeated settings of the factors was also determined. As the P-value of the lack of fit in the ANOVA table is greater than 0.05 (P-value=0.63), the model fits the observed data at a 95% confidence level. R-squared statistics indicated that the appropriate model explains 96.65% of the anthocyanin variability. The standard error of the estimate shows that the standard deviation of the residual is 0.12. However, there was no significant effect of the interaction between X_2 (temperature) and X_3 (time) on the experimental data. So, this interaction was removed from the model, and a new model (Y_1) was established (Equation 2). This model has high reliability because the lack-of-fit is not significant (F-value=0.48 and P-value=0.75>0.05), indicating that the model fits the spatial influence of the variables on this response with a good prediction (R^2=96.63%) and less standard error of estimation (0.12):

$$Y_1 = -88.72 + 12.38X_1 + 1.49X_2 + 0.61X_3 - 1.66X_1^2 + 0.04X_1X_2 + 0.09X_1X_3 - 0.01X_2^2 - 0.04X_3^2 \quad (2)$$

Where:

Y_1: TAC (mg/L);

X_1, X_2, and X_3: the values of the variables specified in their original units.

Response optimization was performed with the objective of maximizing TAC with the optimal value achieved at 13.72 mg/L under optimal extraction conditions of water/material ratio, extraction temperature, and time of 5.1:1, 90.5 °C, and 12.30 min, respectively.

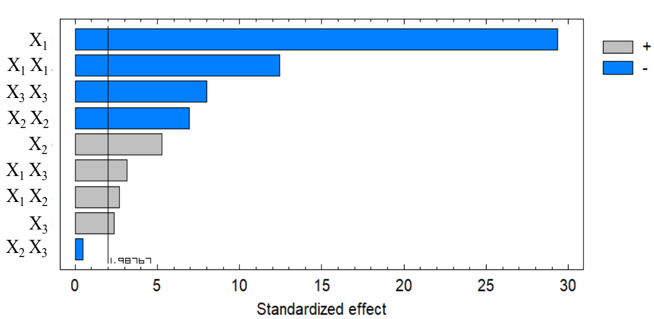
3.2.2. Optimization of total polyphenol

TPC (mg GAE/L) in *P. zeylanica* extract was obtained from 18 experiments (listed in Table 2). The ANOVA showed that the quadratic polynomial model fully represented the experimental data. The coefficient of determination (R^2) for the TPC response is 0.82. The R-squared statistic indicated that the model as fitted explains 82% of the variability in polyphenol. The lack-of-fit test is greater than 0.05 (0.326), and the model appears to be adequate for the observed data at the 95% confidence level. The mean TPC of 18 selected combinations of independent variables ranged from 41.5 mgGAE/L to 93.3 mg GAE/g. However, in the case of TPC, the results of the ANOVA analysis showed that six effects have P-values less than 0.05, indicating that they are significantly different (95% confidence level). The interactions X_1X_2, X_1X_3, and X_2X_3 were not significant (P-value>0.05). Therefore, the regression equation (Equation 3) obtained for the independent and dependent variables TPC (Y_2) is as follows:

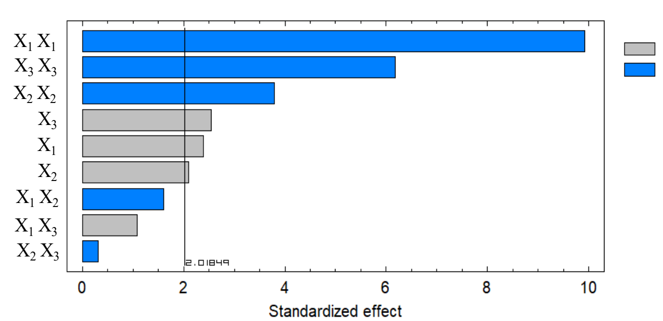
$$Y_2 = -4854.7 + 824.5X_1 + 51.7X_2 + 47.8X_3 - 74.4X_1^2 - 0.3X_2^2 - 1.9X_3^2 \quad (3)$$

Under the optimum conditions of the ratio of water/material (5.54), extraction temperature (90.9 °C), and time (12.9 min), the maximum TPC of 90.15 mgGAE/L could be obtained. The combination of these optimal conditions helped to maximize anthocyanins in the indicated region. The standardized Pareto chart for TAC and TPC is shown in Figure 3.

A Pareto chart is a chart that shows the importance of different variables in a phenomenon. In this study, the Pareto plot showed the influence of three parameters, including solvent/material ratio, temperature, and extraction time, and their interactions on total anthocyanin and total compounds' content. polyphenols. The vertical line in Figure 3 indicates that the effects are statistically significant. The length of each bar scales to the value of the statistic calculated for the associated effect (Thuy et al., 2022b). All bars outside the vertical line are statistically significant at the chosen significance level. This case study has three main parameters with X_1 being the solvent/material ratio, X_2 being the temperature, and X_3 being the time. For TAC (Figure 3A), the values show that all independent linear factors are significant, and the interactions are mostly



a.



b.

Figure 3. Standardized Pareto chart for (A) TAC and (B) TPC.

significant, except for the X_2X_3 interaction (temperature and time, respectively; their effect is meaningless). For TPC (Figure 3B), it is clearly shown that all linear and square interactions are statistically significant except that two-way interactions are non-statistically significant.

3.2.3. Optimize the extraction process and verify the predictive model

The results of the study showed that the extraction conditions for *P. zeylanica* could be optimized to achieve the highest levels of TAC and TPC. RSM was used to determine the optimal X_1, X_2, and X_3 that would maximize the response variables. The results presented above showed that for TAC and TPC, the optimum ratio of water/material is in the range of 5.1:1–5.54:1, and the optimum temperature and time are in the range of 90.5–90.9 °C and 12.3–12.9 min. Therefore, optimizing the two responses TAC and TPC will be more useful when it is necessary to evaluate the impact of multiple variables on these responses. The models of TAC and TPC were properly fit before the optimization of the two responses. Numerical optimization results showed that the desired maximum (0.91) could be obtained using optimal values of the ratio of water/material, extraction temperature, and time were to be 5.38:1, 91 °C, and 12.7 min, respectively. Under these conditions, the optimal TAC and TPC were determined to be 13.55 mg/L and 88.26 mgGAE/L, respectively. The experimental values for the same process conditions for TAC and TPC values were 14.05±0.45 mg/L and 87.95±1.05 mg GAE/L, respectively. The experimental and predicted values are in the range and vary slightly at the level of 5%. Therefore, the regression equations (Y_1 and Y_2) obtained in this study can be used to derive the *P. zeylanica* extract with optimal TAC and TPC.

3.3. Effect of the addition of orange juice to Pouzolzia extract on the quality and antioxidant properties of mixed juice

To improve the nutritional quality and sensory properties of the *Pouzolzia* beverage, the orange juice was fortified as vitamin C after adjusting taste source. Vitamin C is an essential vitamin in the human diet, acting as a natural antioxidant and preventing oxidative stress in the body (Zhao et al., 2018). In this study, the vitamin C content in the ratios of *P. zeylanica* and orange juice was determined and showed significant differences (Table 3).

Table 3. The quality and antioxidant activity of mixed juice at different concentrations of orange juice addition.

Pouzolzia zeylanica: orange juice ratio	Vitamin C (mg/L)	TAC (mg/L)	TPC (mg GAE/L)	DPPH%
90:10	155.93^a±2.3*	12.2^a±0.25	164.43^a±3.5	55.21^a±2.49
85:15	205.6^b±2.6	11.52^b±0.18	202.52^b±4.46	62.17^b±1.58
80:20	255.96^c±2.2	10.84^c±0.21	240.61^c±4.26	67.55^c±2.46
75:25	304.94^d±1.9	10.16^d±0.15	278.70^d±2.14	71.74^d±2.54

*Values are expressed as mean±SD. Means followed by a different letter in columns indicate significant differences at p<0.05.

Immediately after obtaining the finished product, the highest vitamin C content (304.94±2.24 mg/L) was observed in the mixed juice containing 25% orange juice and the lowest (155.90±2.59 mg/L) in the juice containing 10% orange juice. Factors that cause vitamin C degradation may be due to oxygen residues and high-temperature maintenance during fruit juice pasteurization (Herbig et al., 2017; Sapei and Hwa, 2014). TAC and TPC in mixed juice had opposite trends; increasing orange juice caused TPC to increase but TAC to decrease. However, the results of the sensory evaluation showed that, when the extract was added to 20%, the sensory value was still maintained compared to the control sample (100% orange juice). This is understandable since the TAC of mixed juice depends mostly on the *P. zeylanica* used. On the contrary, orange juice is also rich in polyphenols, so the result was an increase in TPC in mixed juice. Enrichment by orange juice also contributed to the increase in AA in mixed *P. zeylanica*-orange juice.

4. Conclusion

In this study, the Box–Behnken response surface design combined with a numerical optimization technique was performed to optimize and study the individual and interaction effects of process variables (i.e., ratio of solvent/material, extraction temperature, and time) for the yield of TAC and TPC from *P. zeylanica* in an aqueous medium. The results showed that the extraction conditions significantly affected the content of biological compounds in the extract. The mixed juice was preferred, and it can be considered an interesting product because of its improved nutritional composition, bioactive compounds, and organoleptic properties. The addition of orange juice can enrich mixed juices with high levels of bioactive substances, thereby improving its health-promoting properties. This research could become important in the pursuit of new raw materials now to produce functional foods and in the design of similar products on an industrial scale in the future. Besides, further research on the combination of *P. zeylanica* extract with other materials rich in other bioactive compounds can be carried out to produce nutrient-rich drinks, especially for the patient or during the recovery period.

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