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OPTIMIZATION OF *Artocarpus altilis* L. EXTRACT FOR XANTHINE OXIDASE INHIBITORY ACTIVITIES USING RESPONSE SURFACE METHODOLOGY (RSM)

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Abstract

Artocarpus altilis is widely used in traditional medicine, especially in Vietnam. Many studies have reported the bioactivities of A. *altilis* in anti-diabetes, while its anti-gout activity has not received much interest. In this study, extraction conditions of A. *altilis* were comprehensively optimized by the response surface methodology (RSM) for the xanthine oxidase inhibitory activity. Furthermore, the effects of the maturity stage on phytochemicals and the xanthine oxidase inhibitory activity of A. *altilis* leaves were assessed. The results indicated that the old leaves were appropriate materials for anti-xanthine oxidase. Under the optimal conditions at ethanol concentration 79.74 %, time extraction 76.68 min, and solid-to-liquid ratio 1:9.56 g:mL, the value of IC_{50} in xanthine oxidase inhibitory activity reached 2.67 µg/mL, nine times less than that of crude extract (24.14 µg/mL). The biological activity of the optimal sample also showed the potentiality of inhibition of α-glucosidase enzyme and antioxidant with IC_{50} values of 64.83 µg/mL and 1.88 µg/mL, respectively.

INTRODUCTION

Gout arthritis is caused by rising uric acid crystals in the soft tissues or joints [1]. Gout is characterized by recurrent attacks of redness, tenderness, pain, hot, and swollen joint [2]. The number of individuals suffering from gout disease has significantly increased in recent years due to modern poor living habits such as fast foods, lack of exercise, and a high prevalence of obesity [3]. In 2015, reports on the prevalence of gout in the world ranged from 0.1% to 10% [3]. Xanthine oxidase inhibitors (XOI) are a crucial component of the supplemental treatment for lowering blood urate levels in gout patients. Allopurinol, a drug used to treat hyperuricemia in gout patients, is the most well-known xanthine oxidase inhibitor [4]. Allopurinol does have certain negative side effects, such as skin rashes, gastrointestinal problems, and hypersensitivity responses. For these reasons, over the years, researchers have searched for alternative therapeutic strategies, particularly those that involve the use of natural products. The current trend is to investigate natural products for gout treatment that contain chemical constituents with potential biological activity.

A. *altilis*, or breadfruit, is one of the traditional medicinal plants in Vietnam; its leaf extraction is popularly used in folk medicines to treat hepatitis, and diabetes [5]. Breadfruit was found to have flavonoids, steroids, and glycosides that were capable of inhibiting xanthine oxidase [6]. A. *altilis* can be considered an anti-xanthine oxidase candidate in gout treatment. However, most research on breadfruit has focused on its antidiabetic or antioxidant activities [7–9]. Rante et al. [10] indicated that ethanol extract from breadfruit leaf has great α-glucosidase enzyme inhibitory activity with IC_{50} of 9.07 µg/mL, compared to one of acarbose of 6.79 µg/mL, while gout disease is often associated with some medical complication, especially diabetes.

In this study, breadfruit leaves in different maturity were compared to the TFC, TPC, and bioactivities to determine the appropriate stages of leaves, which can be used for treating gout. The effects of extraction parameters such as extraction time, solvent concentration, and the ratio of solids

(leaf powder) and liquid (solvent) on the xanthine oxidase inhibitory activity were explored by RSM. The optimal extraction conditions for the highest xanthine oxidase inhibitory activity were also identified. The other bioactivities as anti-inflammatory, antioxidant, and α-glucosidase inhibitory activities that support gout treatment and its complications, were assessed.

MATERIALS AND METHODS

Material and Chemical

Breadfruit leaves were harvested on 06/2022 in Ho Chi Minh, Vietnam. The leaves were washed and dried in the shade until the moisture content bellowed 13% before grinding and storing in a sealed bag for future use. The plant was authenticated by the Department of Ecology and Evolutionary Biology, Ho Chi Minh City University of Science (HCMUS), Vietnam National University.

Chemicals were purchased from commercial suppliers in a pure grade, as follows: aluminum chloride ($AlCl_3$), distilled water, dimethyl sulfoxide (DMSO), diclofenac sodium, ethanol (C_2H_5OH), ferric chloride ($FeCl_3$), methanol (CH_3OH), sodium nitrite ($NaNO_2$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH). The α-glucosidase (100 U/mg), allopurinol, ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), folin-Ciocalteau reagent, quercetin, xanthine oxidase (1 U/mg), xanthine, gallic acid (GA) were provided by Sigma-Aldrich, Singapore.

Preparation of A. *altilis* Extract

A total of 20 g of dried powder was extracted three times with ethanol assisted by an agitator (300-400 rpm). Optimization extraction was performed under controlled conditions such as solvent concentration, extraction time, and solid-to-liquid ratio, described in Table 2. The extracts were filtered by vacuum filtration and removed from the excess solvent by a vacuum rotary evaporator (Buchi, USA).

Qualitative Phytochemical Screening

Phytochemical screening of *Artocarpus altilis* extract was used to determine the presence of bioactive compounds: polyphenols, flavonoids, alkaloids, saponins, carotenoids [11–13].

Total Polyphenol Content (TPC)

The Folin-Ciocalteu colorimetric technique was used to determine the TPC in the extracts [14]. A 40 mL diluted extract at various concentrations was treated with 200 mL of the Folin-Ciocalteau reagent. The mixture was kept for reaction for 5 min at 25 °C before adding 600 µL of Na_2CO_3 20 w/v% and 3160 µL of distilled water. The obtained mixture was homogenized in 30 min by a sonication bath. The absorbance of a mixture was measured at a wavelength of 760 nm by Genesys 10S UV-vis spectrophotometer. Total polyphenol content was determined on mg of gallic acid equivalent per gram of sample (mg GAE/g).

Total Flavonoid Content (TFC)

The total flavonoid content of the extracts was determined using the aluminum chloride technique [15]. A total of 2 mL of distilled water, 0.15 mL of NaNO2 5%, and 0.5 mL of extract dissolved in methanol were mixed. After 5 min for the mixture to react, 0.15 mL of 10% AlCl3 was added. Then, 1.2 mL of distilled water and 1 mL of NaOH 1M were added. The mixture's absorbance was determined at a 425 nm wavelength. TFC was determined on mg of quercetin equivalent per gram of sample (mg GAE/g).

In Vitro Xanthine Oxidase Inhibition Activity Assay

In this study, the XOI activity of the extract was assessed using the method of Rahman et al. [16] with light modification. The assay was carried out on a 96-well plate. 250 µL of extract in DMSO 5%, 175 µL of sodium phosphate buffer (pH 7.5), and 150 µL of enzyme (0.2 mL of xanthine oxidase in phosphate buffer) made up the reaction mixture. The mixture was incubated for 15 min at 37 °C. After that, 300 µL of xanthine (mM) was added to the mixture and settled in 20 min at 37 °C for the reaction before adding 125 µL of 1M HCl to stop the reaction. The absorbance was determined at 290 nm (Genesys 10S UV-Vis Spectrophotometer). The positive control was allopurinol. The assay mixture without a sample was the negative control. XOI activity was calculated as the percentage inhibition of XO, as follows Eq.1:

$$\% \text{ XO inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\% \quad (1)$$

Where: A_{blank}, and A_{sample} are the absorbance at 290 nm of blank and sample.

Albumin Denaturation Assay

In this study, anti-inflammatory activity was measured by inhibiting the albumin denaturation method. Albumin denaturation was carried out using the method of Sangeetha et al. [17]. The reaction mixture (4 mL) consists 0.5 mL of egg albumin, 2.5 mL acetate buffer (pH = 6.4), and 1 mL of different concentrations of extracts. The mixtures were incubated at 37°C for 15 min before heating to 70 °C and held in 5 min. After cooling, the mixture absorbance was measured at 660 nm by Genesys 10S UV-Vis Spectrophotometer. Diclofenac was used as the positive control. The negative control was prepared as reaction mixture without extracts. The percentage inhibition of protein denaturation was determined as follows:

$$\% \text{ Denaturation inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (2)$$

Where $A_{control}$ is the absorbance at 660 nm of negative control; A_{sample} is the absorbance at 660 nm of sample solution.

DPPH Radical-Scavenging Activity

The assay was carried out with slight modifications to the method of Sharma and Bhat [18]. DPPH had a purple color in the methanol solution and gradually changed to a yellow color in reaction to antioxidants. A volume of 180 µL of DPPH in methanol was mixed with 120 µL of sample in methanol at different concentrations. The reaction mixture was homogenized thoroughly by a vortex machine and kept in the dark at 25 ºC for 30 min. Ascorbic acid (vitamin C) and the test sample without extract were used as the positive and negative controls. The absorbance of the mixture was measured at 517 nm using a Genesys 10S UV-Vis Spectrophotometer to calculate the percentage of inhibition as follows:

$$\%\text{DPPH scavening effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (3)$$

Where $A_{control}$ is the absorbance of the negative control (methanol), and A_{sample} is the absorbance of the test solution.

Determination of α-glucosidase Inhibitory Activity

The method of Nair et. al [19], with slight modifications, was used to determine the α-glucosidase inhibitory activity. A volume of 40 µL of sample solution and 20 µL of α -glucosidase enzyme (1 U/mL in phosphate buffer pH 6.8) was added into a well with 100 µL of phosphate buffer. The mixture was then incubated for 5 min at 37 ºC. After that, 40 µL of p-NPG 0.1 mM was added into the reactive mixture and followed by incubation for 30 min at 37ºC. The reaction was stopped by adding 100 µL Na_2CO_3 0.1 M. The absorbance of the mixture was measured at 405 nm using a UV-Vis spectrometer (Genesys 10S UV-Vis Spectrophotometer). Acarbose was used as the positive control, while the mixture without extract was seen as the negative control. The percentage of α-glucosidase inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (4)$$

Where: $A_{control}$ is the absorbance at 405 nm of the negative control; A_{sample} is the absorbance at 405 nm of the sample.

Optimization of Extraction on Xanthine Oxidase Inhibition Activity (Experimental Design)

Response surface methodology (RSM) is an efficient tool for the optimization of bioactive compound extraction as well as bioactivities. In this study, the experiments were carried out following the Central Composite Design (CCD) with 3 factors and 17 experiments because CCD provides high-quality predictions over the entire design space [20]. According to preliminary research, the central point was determined at a solvent concentration of 70% EtOH, extraction time of 60 min, and a solid-to-liquid ratio of 1:10 g:mL. The independent variables included solvent concentration (X_1: 50-90%), extraction time (X_2: 30-90 min), and solid-to-liquid ratio (X_3: 1:5 – 1:15 g:mL). The response variable (Y) was xanthine oxidase inhibitory activity, shown as the percent of inhibition at the concentration of 25 µg/mL. All variables were encoded as -1, 0, and 1 for the factor evaluation of factors (Table 2). The CCD results were generated by Design Expert 12.0. A second-order polynomial regressed equation was established based on the experimental data with the following equation:

$$y = b_0 + \sum_{i=1}^{n} b_i X_i + (\sum_{i=1}^{n} b_{ii} X_i)^2 + \sum_{i<j}^{n} b_{ij} X_i X_j \quad (5)$$

Where: y is the response variables, X_i and X_j are the independent variables, and b_0, b_i, $b_{ii,}$ and b_{ij} present the intercept, linear, quadratic, and interaction constant coefficients, respectively [21].

RESULTS AND DISCUSSION

Phytochemical Screening, TPC, TFC, and XO inhibition activity of A. *altilis* In Different Maturity Levels

The maturity levels of breadfruit significantly affect the bioactive compounds and bioactivities. In this study, three stages of maturity of the breadfruit leaf including were assessed its appearance as fully mature (dark green), young leaf (light green), and old leaf (yellow and fallen off the tree) (Figure 1). The results of the phytochemical screening of breadfruit leaves at three maturity levels are shown in Table 1.

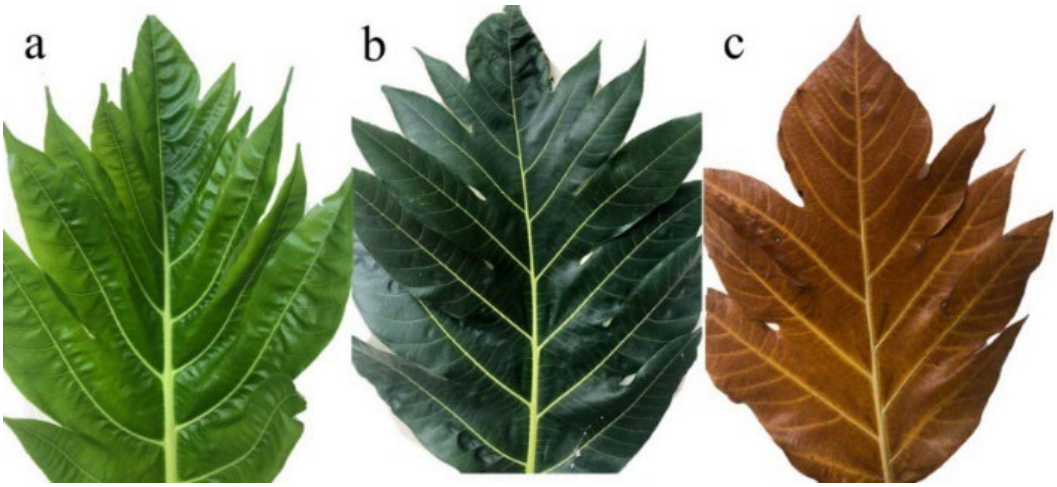

Figure 1. *A. altilis* leaves in three maturity levels, including (a) young leaf, (b) mature leaf, and (c) old leaf.

Table 1. Phytochemical screening results of leaf extract at different maturity levels

Bioactive compounds	The maturity of Leaf Extracts		
	Young	***Mature***	***Old***
Alkaloid	++	++	++
Flavonoid	++	++	++
Tannin	++	+	++
Saponin	+	+	+
Carotenoid	-	-	-
Polyphenol	++	+	++

- Not detected; + Slightly positive reaction; and ++ Strong positive reaction

The results of phytochemical screening of the three stages of the breadfruit leaf were similar. It showed that all extracts have the presence of flavonoid, polyphenol, tannin, alkaloid, and saponin, while there are no traces of carotenoid. These bioactive compounds indicated the potential for diverse biological activity, including xanthine oxidase inhibitory activity [22]. Polyphenols, which are secondary metabolites produced by higher plants, have a variety of biological effects, including antioxidant, anti-inflammatory, anti-carcinogenic, and anti-gout [23]. Flavonoids have the inhibitory activity of various enzymes, such as xanthine oxidase, peroxidase, and nitric oxide synthase, which are involved in producing free radicals, resulting in less oxidative damage to macromolecules [24]. Tannins, the water-soluble polyphenols, have various in vitro bioactivities, the most well-studied of which are antioxidant and antimicrobial properties [25]. Alkaloids show strong biological effects on human organisms such as anti-inflammatory, anticancer and anti-gout [26]. Saponins are natural steroid or triterpene glycosides that have various biological and pharmacological effects, including immunomodulatory, antitumor, anti-inflammatory, molluscicidal, antiviral, antifungal, hypoglycaemic, and hypocholesterolemia [27]. Hence, it can be predicted that all maturity stages of *A. altilis* leaf have an effect on gout and its complication treatment.

The TPC, TFC of the leaf extract at three maturity levels were determined and described in Figure 2. The TPC of three sample extracts ranged from 35 mg GA/g to 154 mg GA/g, while the TFC ranged from 387 mg QUE/g to 487 mg QUE/g. A significant difference in TPC was observed between leaf extracts, with mature leaves showing the highest TPC (154.17 mg GA/g), approximately 2 times higher than young ones. However, young leaf extract was observed to have higher flavonoid content than the mature one because the oxidation of these compounds follows the growing stages of leaves. The xanthine oxidase inhibitory activity of leaf extracts with three different maturity levels was demonstrated in Figure 2b. The results showed that all extracts have strong xanthine oxidase inhibitory activity with an IC_{50} value of 24.06-32.3 µg/mL, compared to allopurinol (1.57 µg/mL). The IC_{50} values of the young and old leaf extracts were almost similar, about 25µ g/mL, respectively, and lower than that of the mature leaf extract, 32.3 g/mL. These results correspond to the difference in total flavonoid content in each maturity stage. Both young and old leaf extracts had the equivalent in the xanthine oxidase inhibitory activity, yet the harvest of the young leaf was more difficult and harmful to the plant's growth, and the young or mature leaves have much mucus and resin. As a result, old breadfruit leaves were appropriated to utilize for anti-gout.

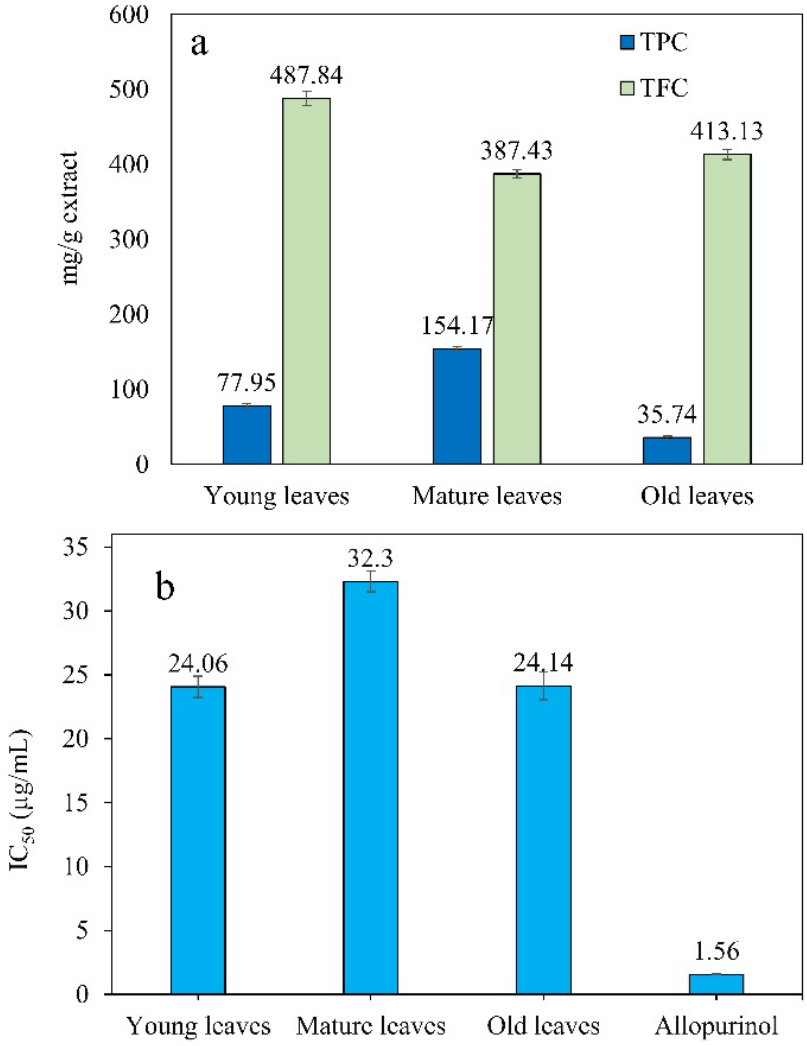

Figure 2. TPC and TFC (a), and XOI activity (b) of A. *altilis* extract in three different maturity levels.

Optimization of A. *altilis* for Xanthine Oxidase Inhibitory Activity By RSM

In this study, the extraction conditions of the old leaves of *A. altilis* were optimized for the xanthine oxidase inhibitory activity via RSM, and the results are shown in Table 2. The experiments at the central point showed the highest percentage of inhibition, and there was repeatability between results at the central point, indicating the value of the optimal point close to the central variables. In contrast, the fifth experiment had the lowest percentage of inhibition (55%) at 50% EtOH, 30 min, and 10 mL/g due to a combination of two disadvantaged extraction conditions.

The coefficient and ANOVA analysis of the model are shown in Table 3 and Table 4, respectively. The effects with *p*-values lower than 0.05 indicated the statistical validity and significance of the models. The correlation between the model and the experimental was evaluated based on the results of the coefficient of determination and the results of variance analysis.

Table 2. Central Composite Design for Three Factors with Response of Inhibition Percentage of the Extracts at 25 µg/mL concentration

No. Unit	Solvent concentration (X_1) (%)	Extraction time (X_2) (min)	Solid to liquid ratio (X_3) (g:mL)	XOI (Y) (%)
1	-1 (50)	-1 (30)	-1 (1:5)	59.66 ± 0.13
2	1 (90)	-1 (30)	-1 (1:5)	63.57 ± 0.85
3	-1 (50)	1 (90)	-1 (1:5)	63.22 ± 1.22
4	1 (90)	1 (90)	-1 (1:5)	69.83 ± 0.11
5	-1 (50)	-1 (30)	1 (1:15)	55.25 ± 1.92
6	1 (90)	-1 (30)	1 (1:15)	60.98 ± 0.56
7	-1 (50)	1 (90)	1 (1:15)	61.28 ± 1.19
8	1 (90)	1 (90)	1 (1:15)	70.31 ± 1.86
9	-1.353(43)	0 (60)	0 (1:10)	59.23 ± 2.11
10	1.353(97)	0 (60)	0 (1:10)	70.20 ± 1.64
11	0 (70)	-1.353 (19)	0 (1:10)	61.91 ± 0.83
12	0 (70)	1.353 (100)	0 (1:10)	71.42 ± 0.62
13	0 (70)	0 (60)	-1.353 (1:3.2)	69.74 ± 0.49
14	0 (70)	0 (60)	1.353 (1:17)	65.84 ± 1.40
15	0 (70)	0 (60)	0 (1:10)	73.22 ± 0.14
16	0 (70)	0 (60)	0 (1:10)	73.34 ± 0.14
17	0 (70)	0 (60)	0 (1:10)	73.12 ± 0.27

Table 3. Coefficient Analysis of the XOI model

Source	Coeff.	Std. Err.	P	Notes
Constant	74.4176	1.16		*
X_1 – Solvent concentration (%)	3.2020	0.5456	0.0006	*
X_2 – Extraction time (min)	3.0149	0.5456	0.0009	*
X_3 – Solid-to-liquid ratio (g:mL)	-1.0997	0.5456	0.0837	
X_1^2	-3.6670	0.6005	0.0005	*
X_2^2	-2.9777	0.6005	0.0016	*
X_3^2	-2.5799	0.6005	0.0036	*
X_1X_2	0.7500	0.7129	0.3277	
X_1X_3	0.5300	0.7129	0.4814	
X_2X_3	0.6925	0.7129	0.3637	

*Indicates significant correlation
Coeff. SC: Coefficient estimate
Std. Err.: Standard Error

Table 4. The regression coefficients and results of ANOVA for XOI response

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	501.95	9	55.77	13.72	0.0011
X_1 - (%)	140.02	1	140.02	34.44	0.0006
X_2 - (min)	124.13	1	124.13	30.53	0.0009
X_3 - (g :mL)	16.52	1	16.52	4.06	0.0837
X_1X_2	4.50	1	4.50	1.11	0.3277
X_1X_3	2.25	1	2.25	0.5528	0.4814
X_2X_3	3.84	1	3.84	0.9437	0.3637
X_1^2	151.60	1	151.60	37.29	0.0005
X_2^2	99.95	1	99.95	24.59	0.0016
X_3^2	75.03	1	75.03	18.46	0.0036
Residual	28.46	7	4.07		
Lack of Fit	28.43	5	5.69	468.68	0.0021
Pure Error	0.0243	2	0.0121		
Cor Total	530.40	16			
R^2	0.9463		**Std. Dev.**	2.02	
Adjusted R^2	0.8774		**Mean**	66.01	
Predicted R_2	0.5953		**C.V. %**	3.05	

df : degree of freedom

The values for the multiple correlation coefficient (R^2=0.9463) and adjusted determination coefficient (R^2 Adj =0.8774) from the ANOVA analysis revealed that 94.63% of the variations could be explained by the explanatory variable and 87.74% of the total variations could be explained by the model. The regression equation with variables is described in the following equation:

$$Y = 74.4176 + 3.2020X_1 + 3.0149X_2 – 3.6670X_1^2 – 2.9777X_2^2 – 2.5799X_3^2 \quad (6)$$

As can be seen in Eq. 6, the appearance of three variables X_1 – solvent concentration, X_2 – extraction time, and X_3 – Solid to liquid ratio proved that these factors influenced the inhibitory activity of the enzyme xanthine oxidase. In addition, the second-order regression coefficients were negative, which indicates the response surface is convex and presents the optimum point. The coefficient of X_1^2 was the largest (3.667) showing that the concentration of extraction solvent has a significant effect on the value of the target function. Thus, solvent concentration was the most important of the three surveyed factors.

The surface response graph can be used to visualize the predicted model equation (Figure 3). The I% value according to the surface response model at three values of each variable was in the range from 57% to 75%. The value of optimal variables around the central point of concentration (70%), extraction time (60 min), and solid-to-liquid ratio (1:10 mL/g). Optimizing the value of inhibitory activity using Design Expert software gave optimal point at solvent concentration 79.74%, extraction time 76.68 min, and solid-to-liquid ratio 1:9.56 mL/g with the predicted value of xanthine oxidase inhibition reached 75.086%.

Three experiments were carried out under optimal conditions to validate the optimal point, and the results are shown in Table 5. The experimental extract at the optimal conditions shows a percentage of inhibition of 75.07% with an error of 0.22%. The error values between the experimental and predicted values were less than 5%, indicating that the model obtained is reliable and meaningful in practice.

Bioactivities of the Optimal Extraction

The biological activity of the optimal sample, such as antioxidant, anti-inflammatory, anti-diabetes, and anti-gout activity, was determined to extend its application capacity (Table 6). The optimal sample of the old leaf of *A. altilis* showed a strong ability in xanthine oxidase inhibitory activity with an IC_{50} value of 2.67 µg/mL, lower 10 times than that of the crude extract. In addition, the antioxidant assay showed an IC_{50} value of 64.83 µg/mL, which was higher than that of the ascorbic acid. The *α*-glucosidase inhibitory activity assay showed very good activity with an IC_{50} value of 1.18 µg/mL, significantly better than acarbose. Clinical evidence suggests that hyperuricemia is a significant risk factor for diabetes in gout patients, and the pathogenesis of acute and chronic pancreatitis can be associated with oxidative stress [28,29]. However, the extract showed low anti-inflammatory activity. From these results, the optimal extracts promise to be applied to treating gout and its comorbidities or complications.

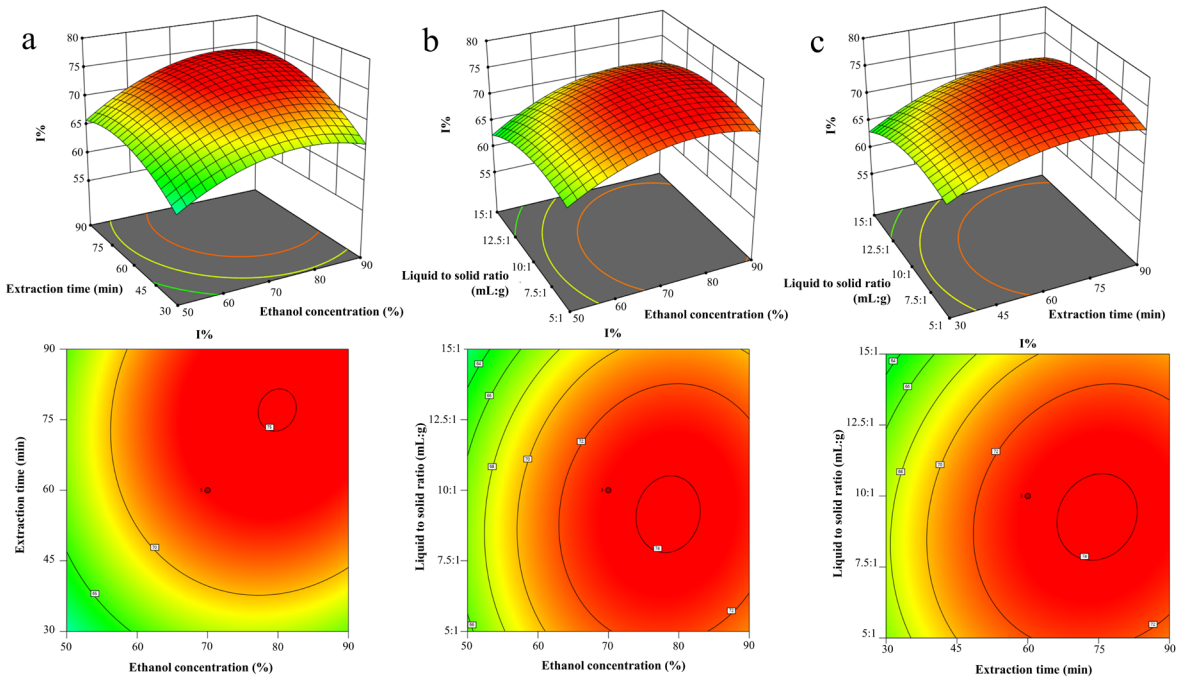

Figure 3. The response surface and contour plot at liquid/solid ratio of 1:10, (a), extraction time of 60 min (b), and EtOH 70% (c).

Table 5. The validation results for XOI responses

Solvent concentration (X_1) - %	Extraction time (X_2) - min	Solid to liquid ratio (X_3) – g:mL	XOI (%)	Error with model (%)
79.74	76.68	1:9.56	74.82± 0.63	0.35
			75.26 ± 0.45	0.23
			75.12 ± 0.61	0.09
		Mean ± SD	75.07 ± 0.22	
		Prediction	75.086	

Table 6. Biological activities of the optimal extract of *Artocarpus altilis*

Bioactivities	Optimal Sample (IC_{50})	Positive control (IC_{50})
XO inhibitory activity	2.67 µg/mL	1.56 µg/mL (Allopurinol)
Anti-inflammatory*	16.18 %*	44.26 %* (Diclofenac)
Antioxidant activity	64.83 µg/mL	2.76 µg/mL (Ascorbic acid)
Anti- α-glucosidase activity	1.88 µg/mL	9.65 µg/mL (Acarbose)

*I% at 12,5 µg/mL

CONCLUSION

In this study, the old breadfruit leaves showed a good xanthine oxidase inhibitory activity with IC_{50} of 24.14 µg/mL. Extraction factors, such as temperature, time, EtOH concentration, and LSR, were proven to exert significant effects on anti-xanthine oxidase activity of *A. altilis* extract. The optimum conditions for xanthine oxidase inhibitory activity of old leaves with IC_{50} of 2.67 µg/mL were observed at ethanol 79.7 % in 76.68 min and liquid to solid ratio of 9.56:1 mL/g. Moreover, the optimal extract also showed high antioxidant activity and α-glucosidase inhibitory activity with an IC_{50} value of 64.83 µg/mL and 1.18 µg/mL, respectively. As result, the old *A. altilis* leaves could be a good candidate for future studies of this plant on gout treatment and its complications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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