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Original Article

Optimization of Xanthine Oxidase Activity, Phytochemical Screening, Toxicity Assay, and Antigout Activity of *Spatholobus littoralis* Hassk. Extract

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ABSTRACT

Introduction: A medicinal plant from Central Kalimantan, *Spatholobus littoralis* Hassk., with a local name bajakah tampala, has long been used to treat gout or uric acid disease. This study aimed to develop the optimum conditions of xanthine oxidase (XO) activity, phytochemical screening, and to obtain the antigout activity of *S. littoralis* Hassk. **Methods:** Before performing the phytochemical screening, we performed a simplicia maceration with water and 70% ethanol solvents. The development of the optimum conditions for XO was carried out using the Response Surface Methodology (RSM) combined Box-Behnken Design (BBD). Toxicity assay (LC_{50}) on both extracts was assessed by BSLT (Brine Shrimp Lethality Test). The two extracts were then evaluated for antigout activity. **Results:** The yield of 70% ethanol extract (11.24%) was higher than the aqueous extract (7.29%). The phytochemical screening on the simplicia and the two extracts positively contained steroids, flavonoids, saponins, and tannins. The optimum conditions of XO activity were temperature of 20 ℃, pH of 8.0, and xanthine concentration of 1.40 mM. The toxicity of the 70% ethanol extract (LC_{50} 662.25 mg/L) was stronger than the aqueous extract (LC_{50} 774.68 mg/L). Moreover, the 70% ethanol extract (IC_{50} 224.14±8.62 mg/L) showed higher XO inhibition than the water extract (IC_{50} 348.83±4.85 mg/L). Lineweaver-Burk plot analysis showed that 70% ethanol and water extracts inhibited XO competitively with the inhibition constant (K_i) of 272.68 mg/L and 378.44 mg/L, respectively. **Conclusion:** This preliminary study can be a reference that *S. littoralis* Hassk. is potentially an antigout.

Key words: Box-Behnken Design, Gout, Lineweaver-Burk plot, *Spatholobus littoralis* Hassk, Xanthine Oxidase.

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INTRODUCTION

The primary enzyme responsible for gout is a xanthine oxidase (XO).[1] Gout or the formation of uric acid crystals in the joints is led on by this enzyme activity.[2] The endogenous and exogenous nucleotides in the human body are metabolized into nucleosides and then to purine bases. Guanine from guanosine and hypoxanthine from inosine, generated from adenosine-by-adenosine deaminase, gives comparable breakdown products. Guanine deaminase converts the guanine to xanthine, which is ultimately converted to uric acid by XO. Hypoxanthine is converted by the XO into xanthine and then uric acid.[3] This activity also coincides with the production of reactive oxygen species (ROS).[4]

The term "gout" itself is derived from the Latin word "gutta," which means "to drip," and in the thirteenth century, it was thought that poison drops that fell on the joints produced gout.[5] The disease primarily suffers people in Middle Ages men and their postmenopausal stages women.[6] If left untreated, this illness can intensify to chronic gout, even leading to kidney failure and a stroke.[7] The U.S. has reported that gout patients worldwide are rising annually.[8] Gout prevalence reaches 4.49% in Asian nations, for instance, Taiwan.[9] The in Indonesia, the prevalence of gout is in the range of 1.6-13.6 per 100.000 people.[10]

The type of inhibition kinetics generated can describe how XO as a target is inhibited and how therapeutic candidate molecules bind to it, forming an affinity that may be either temporary (competitive and uncompetitive inhibition) or permanent (non-competitive inhibition).[11,12] Due to the presence of secondary metabolites, substances with the ability to inhibit XO, some medicinal herbs can be mainly used to treat gout.[13] The five Indonesian medicinal herbs, *Phaleria macrocarpa, Ruellia tuberosa, Sida rhombifolia, Cyperus rotundus,* and *Syzygium polyanthum,* exhibit competitive inhibition kinetics against XO.[14]

Hasskarl discovered bajakah tampala in 1842; its scientific name is *Spatholobus littoralis* Hassk. (*S. littoralis* Hassk.). Twenty-nine species in the genus *Spatholobus* are distributed throughout Southeast Asia. Consequently, it spreads from West India to South China, the Philippines, and West Malaysia. *Spatholobus* was expanded throughout in the Malay Peninsula, Borneo (Kalimantan Island), and southeast Sumatra.[15,16] Traditional medicines, including bajakah tampala, are frequently used to treat degenerative diseases.[17] According to Iskandar and Warsidah (2020),[18] the ethanol extract also apparently exhibits antioxidant activity, with an IC_{50} of 8.25 g/mL. Ayuchecaria *et al.* (2020)[19] revealed that the bajakah tampala stem extract's total phenolic content is 12.32 mg GAE/g.

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In this current research work, we investigated the antigout activity of *S. littoralis* Hassk. extract. We applied the water extract and 70% ethanol extract of *S. littoralis* Hassk. stem. Response Surface Methodology (RSM) is a useful technique for optimizing XO activity. The experimental design chosen in the RSM is the Box-Behnken Design (BBD). BBD has the advantage of not containing any combination factors at the cube's vertices (high/low points), resulting in fewer tests.[20,21] Optimization of XO activity based on three combination factors: pH, temperature, and xanthine concentration. After obtaining these optimum conditions, we analyzed the inhibitory capacity of the water extract and 70% ethanol extract toward XO activity and their inhibition kinetics through Lineweaver-Burk plot analysis. We also evaluated the phytochemical screening and toxicity assay of both extracts.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were HCl (Merck, Germany), H_2SO_4 2M, anhydrous CH_3COOH (Merck, Germany), magnesium powder (Merck, Germany), amyl alcohol (Merck, Germany), 10% $FeCl_3$, 1N NaOH, Meyer's reagent, Dragendorf's reagent, Wagner's reagent, *Artemia salina* L. (Sanders™ Great Salt Lake Artemia Cysts), seawater, distilled water, potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany), dipotassium hydrogen phosphate (K_2HPO_4) (Merck, Germany), ethanol (Merck, Germany). UV-Vis spectrophotometer U-2800 (Hitachi, Tokyo, Japan) was used for determining and recording absorbances in the inhibition assay. Xanthine X0626-5G, the enzyme xanthine oxidase X1875-25 UN type Bovine Milk Grade I, and allopurinol A8003-5G were purchased from Sigma Aldrich, USA.

Plant collection and sample preparation

The species *S. littoralis* Hassk. was collected in Muara Teweh, North Barito Regency, Central Kalimantan Province, and was identified by a botanist at the National Research and Innovation Agency at Cibinong, West Java, Indonesia. The Tropical Biopharmaca Research Center, IPB University, Indonesia, has deposited a voucher specimen of the sample (number BMK0490102021). The stem parts of the plant were cleaned, sundried, and ground to give 40 mesh-size powder to obtain the simplicia.

Determination of moisture content[22]

The porcelain cup was dried for 30 minutes in a 105 °C oven, then placed in a desiccator for 30 minutes of cooling before weighing. Next, the simplicia (3 g) was placed into the known-weight porcelain cup and dried in a 105 °C oven for 3 hours. Finally, the porcelain cup was cooled in a desiccator for 30 minutes and weighed. The procedure was carried out repeatedly until a constant weight was obtained by a difference of less than 1 mg. Moisture content determination was carried out in triplicates. The moisture content was calculated using the following formula (1):

$$Moisture\ content\ (\%) = \frac{W1-W2}{W1} \times 100\% \quad (1)$$

Abbreviations: W_1= sample weight before drying (g); W_2 = sample weight after drying (g).

Extraction[23]

The simplicia powder (100 g) was macerated with water, and 70% ethanol, at room temperature with a simplicia and solvent ratio of 1:10. The sample and solvent were stirred and allowed to stand for 24 hours at room temperature, then filtered. Next, the filtrate was separated, and the process was repeated three times. Finally, the filtrate was concentrated using a rotary evaporator at 40–60 °C. The extracts were weighed, and the yield was calculated using the following formula (2):

$$Extract\ yield\ (\%) = \frac{a}{b\ (1-\frac{c}{100})} \times 100\% \quad (2)$$

Abbreviations: a = extract weight (g); b = sample weight (g); c = moisture content (%).

Phytochemical screening[24]

A phytochemical assay was carried out to correlate the presence of secondary metabolites from the simplicia and the extracts with the inhibition of XO. This analysis used the Harborne method to identify alkaloids, triterpenoids, steroids, flavonoids, saponins, tannins, and quinones.

Toxicity assay[25]

The toxicity assay was carried out using the BSLT (Brine Shrimp Lethality Test) method. Hatching of *A. salina* eggs was the first stage of the toxicity test. The eggs (50 mg) were weighed before being placed in an Erlenmeyer with sufficient filtered seawater. The Erlenmeyer was equipped with an aerator and incubated under light sources for 48 hours until all eggs hatched. Then, the toxicity test was performed on the hatched larvae. A 24-well plate containing seawater and extracts (water extract, 70% ethanol extract) was also added with 10 larvae per well. In a total volume of 2 mL with three replications, the concentration series were 1000, 500, 100, and 50 ppm. After 24 hours, the dead larvae were counted. Using the IBM SPSS Statistics version 21 application, the lethal concentration 50 (LC_{50}) was calculated by visualizing the mortality rate using probit analysis at a 95% confidence level.

Determination of the maximum wavelength (λ_{max}) of xanthine (Modification from Desma 2017)[26]

Xanthine solution (1.0 mM) was prepared in potassium phosphate buffer (pH 7.5, 10 mL, 50 mM). The maximum wavelength was determined in the range of 200–400 nm. The determined wavelength was used as a reference in the XO inhibitory assay. Measurements were made using a U-2800 double-beam spectrophotometer (Hitachi, Tokyo, Japan).

Optimization of XO activity (Modification from Trivadila *et al*. 2020)[27]

The experiment condition for XO activity determination was optimized at 20–30 °C temperature, 6.0–8.0 pH, and 0.70–1.40 mM xanthine concentration. RSM with BBD was used to optimize XO activity. This method was performed by entering a combination of factors of independent variables in the statistical software Minitab.v.14 English. Afterward, the experiment was carried out by determining three factors and 2-replicate. Thirty combinations generated the optimized condition. Then, the optimized results were verified by a one-sample *t*-test based on the value of the reacted xanthine concentration from the Minitab optimization results.

In Vitro xanthine oxidase inhibition assay and IC_{50} determination (Modification from Iswantini *et al*. 2014)[11]

The XO inhibitory was performed under previously determined optimum conditions. Various extract (1 mL) concentrations were added to the test tube depending on the toxicity value. At the optimum pH, 1.9 mL of potassium phosphate buffer (50 mM) was added. The optimum concentration of xanthine in 1 mL was then blended. The

reaction was initiated by slowly adding 0.1 U/mL XO (0.1 mL), and it was then incubated for 45 minutes at the optimum temperature. The process was stopped by adding 0.58 M HCl (1 mL). In order to determine how much of the unreacted xanthine was still present in the test sample, the mixture's absorbance was then measured using a UV-Vis spectrophotometer at its maximum wavelength. The results were compared to a commercial antigout drug (allopurinol) to determine the inhibitory capacity of each extract. The formulas (3 and 4) were employed to express XO activity and inhibitory capacity (%):

$$XO\ activity = \frac{reacted\ xanthine\ (mM)}{Enzyme\ volume\ (L) \times Incubation\ time\ (minute)} \quad (3)$$

$$Inhibition\ capacity\ (\%) = \frac{XO\ activity\ control - XO\ activity\ sample}{XO\ activity\ control}\ x\ 100\% \quad (4)$$

By entering 50 as the *y* value in the regression equation to obtain the *x* value as the inhibition concentration 50 (IC_{50}) of the sample, the inhibition concentration 50 (IC_{50}) was determined.

In Vitro inhibition kinetics of xanthine oxidase assay (Modification from Iswantini *et al.* 2014)[11]

The extract concentrations were determined based on the IC_{50} and LC_{50} values to evaluate the XO inhibition kinetics. The xanthine concentration ranged from 0.10 to 1.60 mM. (0.10 mM interval). The data were interpreted into the Lineweaver-Burk plot analysis to obtain the maximum reaction rate (V_{max}), Michaelis-Menten constant (K_m), and constant of inhibition (K_I) following the formulas (5 and 6):

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (5)$$

$$K_m' = K_m \left(1 + \frac{[I]}{K_I}\right) \quad (6)$$

Abbreviations: V_0 = reaction rate; V_{max} = maximum reaction rate; K_m = Michaelis–Menten constant when without the addition of extract; $K_{m'}$ = Michaelis–Menten constant when adding extract; [*S*] = substrate concentration; [*I*] = inhibitor concentration; K_I = inhibition constant.

Statistical analysis

Data were presented as the mean of standard deviation (SD) (n = 3), and one-way analysis of variance (ANOVA) was used to examine them well before the Tukey test at a 95% confidence level. The P-value<0.05 indicates that the differences between samples are significant.

RESULTS AND DISCUSSION

Moisture content

In this study, the cleaned, dried (Figure 1), ground to 40-mesh powder of stem pieces of bajakah tampala was used (Figure 2). The simplicia powder was brown and had a distinctive aroma. Simplicia drying was intended to avoid microbial attack. In addition, the grinding of samples was intended to facilitate solvent diffusion into the plant cell walls to optimize extraction. Meanwhile, sieving aims to homogenize the size of the simplicia powder.

The moisture content of the simplicia was determined by the gravimetric method. Moisture content determination is useful for determining how to store the simplicia and avoid damage due to microbial activity (fungi and bacteria). In addition, the moisture content also acts as a correction factor for calculating the extract yield. The moisture content

Figure 1: The cleaned and dried stems.

Figure 2: Simplicia.

in this study was 5.81% ± 0.0827. The simplicia can be stored relatively long, depending on the findings for further use at the extraction stage, following the requirements of the Indonesian Food and Drug Authority (2014), [28] where the simplicia moisture content should be less than 10%. Microbial development may be slowed down in this optimum condition.

Extract yields

Maceration was used to extract secondary metabolites such as antigout. The mechanism of maceration is the process of solvent diffusion into the plant cell wall to extract the plant's compounds. The advantage of maceration is that the equipment used is simple, has low energy consumption, and is relatively inexpensive. The maceration method, on the other hand, requires a longer extraction time.[29,30]

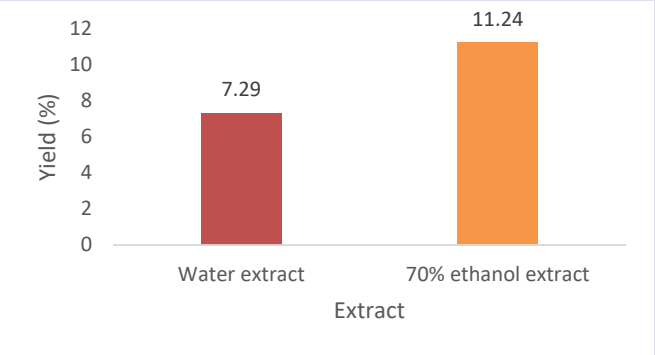

Figure 3: The yield of water extract and 70% ethanol extract of *S. littoralis* Hassk. Stem.

Table 1: Phytochemical compositions of simplicia, water extract, and 70% ethanol extract from *S. littoralis* Hassk. stem.

Phytochemical constituents	Assay results		
	Simplicia	Water extract	70% ethanol extract
Alkaloids	-	-	-
Triterpenoids	-	-	-
Steroids	+	+	+
Flavonoids	++	++	+++
Saponins	+++	+++	+++
Tannins	+++	+++	+++
Quinones	-	-	-

Remarks: the sign (+) indicates the level of color intensity and (-) indicates secondary metabolites are not present in the simplicia and in the extract.

The use of water and ethanol solvents in this study refers to the regulation of the Indonesian Food and Drug Authority (2012)[23] regarding the reference to Herbal Preparations (*Acuan Sediaan Herbal*). People use water to prepare traditional drink ingredients. Meanwhile, ethanol is generally considered safe for human consumption and a solvent for natural food and medicine ingredients.[31]

Water is a polar solvent with the highest polarity (1.000). On the other hand, ethanol is a semipolar solvent with a polarity of 0.654.[32] The yield of 70% ethanol extract was higher (11.24%) than the water extract (7.29%) (Figure 3). It indicates semipolar compounds can be extracted more easily in 70% ethanol than in water extract. The "*like-dissolve-like*" rule states that chemical components will separate into two phases based on their level of polarity.[33] Meanwhile, semipolar compounds are flavonoids, phenolic compounds (tannins, quinones), and saponins.[24]

Phytochemical composition

The phytochemical assay is a preliminary test to determine the secondary metabolite content that can inhibit XO in simplicia and extracts. Table 1 showed that the simplicia and extracts (water and 70% ethanol) were positive for flavonoids, tannins, saponins, and steroids. The flavonoids in the 70% ethanol extract were higher than in the water extract. Saputera *et al.* (2019)[34] showed a negative result for alkaloids in 70% ethanol extract of the same species collected in Pulang Pisau Regency, Central Kalimantan and this result is the same as this research. In addition, the 96% ethanol extract from Lamandau Regency, Central Kalimantan, also displayed negative results for alkaloids.[35] However, in the Ayuchecaria *et al.* (2020)[19] study, the ethanol extract showed a negative result for saponins, which is different from our findings. Meanwhile, Iskandar and Warsidah (2020)[18] stated that the ethanol extract of the same species from Palangkaraya, Central Kalimantan was positive for alkaloids. This difference may occur due to internal factors, such as gene regulation and external environmental factors (the growing site, the presence of light, temperature, moisture content, salt content, and soil type).[36] The quantity of secondary metabolites in plants is also affected by the harvest time and the post-harvest handlings.[37]

Toxicity

The BSLT method was used to determine the toxicity of *S. littoralis* Hassk. extracts. This method is a simple and direct bioassay for measuring the toxicity of natural products. The percentage of *A. salina* larvae mortality was used to determine the probit value. The obtained probit value is then plotted against the concentration log in a graph to determine the LC_{50} value. According to Meyer's criteria, an extract is considered very toxic if its LC_{50} value is less than 30 mg/L, toxic if its LC_{50} value is between 30 and 1,000 mg/L, and not toxic if its LC_{50} value is greater than 1,000 mg/L.[25]

Figure 4 displays the LC_{50} of each extract and the negative control (without extract). The LC_{50} for the 70% ethanol extract (662.25 mg/L) has a lower value than the water extract (774.68 mg/L), indicating that the 70% ethanol extract is more toxic than the water extract. contains more secondary metabolites and is more toxic to *A. salina* L. The high toxicity of 70% ethanol extract suggests that it contains a higher concentration of secondary metabolite components.[38] These results are consistent with phytochemical screening that 70% ethanol extract has a higher content of flavonoids. After the secondary metabolite components are absorbed, they are distributed throughout the larvae body, going to cause metabolic disorders. The mortality rate of larvae was closely related to the concentration and synergistic effect of several compounds in the extract.[39] The LC_{50} of each extract determines the concentration in the XO inhibition assay because it is safe and secure in the drug formula when employed below its LC_{50}.[40]

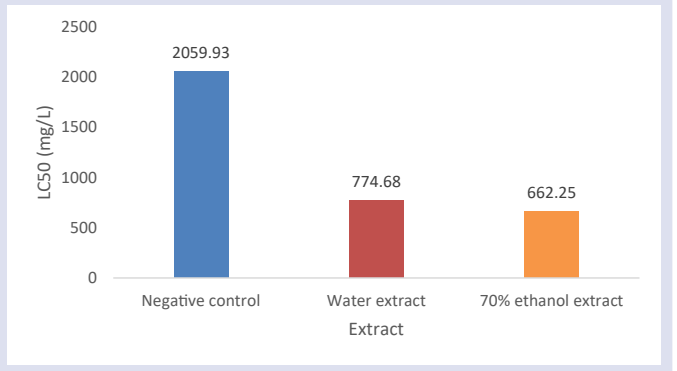

Figure 4: The LC_{50} of negative control; water extract and 70% ethanol extract of *S. littoralis* Hassk. stem as compared to the control.

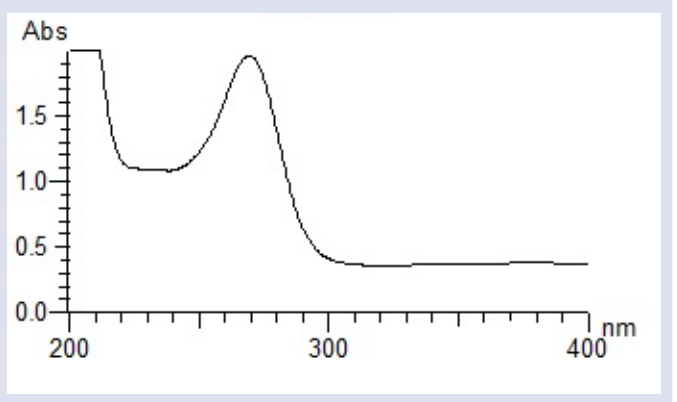

Figure 5: Maximum wavelength (λ_{max}) of xanthine at 270.0 nm.

The maximum wavelength (λ_{max}) of xanthine

Determination of the maximum wavelength was carried out before the xanthine oxidase inhibition assay. Measurement of the absorption of a solution at the maximum wavelength can reduce errors or have high accuracy in determining a compound by spectrophotometry. This is because measurements at the wavelength (λ) with maximum absorption will increase the sensitivity of the analysis. Ultraviolet (UV) measurements were carried out in the wavelength range of 190–400 nm because the compounds to be measured are colorless.[41,42] The maximum wavelength (λ_{max}) of xanthine was 270.0 nm (Figure 5). A similar result was reported by Kim *et al.* (2020)[43] and Desma (2017).[26]

Optimum conditions for XO activity

The response surface methodology (RSM) applied to obtain optimum conditions for xanthine oxidase activity is the Box-Behnken Design (BBD). Variables examined in response: pH (6.0-8.0), temperature (20-30 °C), and xanthine concentration (0.70-1.40 mM). These variables are included in the main factors that affect enzyme activity.[44,45] Meanwhile, the selection of ranges for each variable was based on research conducted by Iswantini *et al.* (2014),[11] Rahmatullah (2019),[46] and Trivadila *et al.* (2020).[27] Table 2 describes the results of the optimization conditions for determining XO activity with reacted xanthine as a response.

Analysis of Variance (ANOVA) is a method used to determine the optimized XO activity from each response, such as pH, temperature, and xanthine concentration.[47] Based on the ANOVA (Table 3) of various pHs, temperatures, and xanthine concentrations in the linear regression model, the P-value was 0.000. It indicates that the three variables have a significant effect ($P<0.05$) on the response (concentration of the reacted xanthine). Meanwhile, in the squared regression model, temperature and temperature had no significant effect, with a P-value of 0.832. In addition, for 2-way interaction, the interaction between pH and temperature; the interaction of pH and xanthine concentration had a significant effect with P-values of 0.040 and 0.001 respectively; while the interaction of temperature and xanthine concentration had no significant effect with a P-value of 0.904. Data on lack of fit obtained a P-value of 0.000, which means that the model used is suitable to be applied in determining optimum conditions. It is also supported by the R-sq value of 95.22% which states that the variables analyzed affect the resulting response.

The rate of a chemical reaction increases, as the temperature increases, increasing the kinetic energy of the system. However, if the temperature of the system exceeds its optimum conditions, the enzymes will be denatured. The concentration of the substrate is the following variable. Most of the enzyme molecules (E) are free at relatively low substrate concentrations. The number of enzyme molecules involved in the production of the enzyme-substrate complex (ES) increases as the substrate concentration (S) rises. When the substrate concentration exceeds a certain point, almost all of the enzyme molecules are occupied by the substrate (assuming the concentration of the enzyme remains constant). At this condition, the enzyme becomes saturated with the substrate. If the substrate concentration continues to increase and greatly exceeds the amount of enzyme, a steady state is reached where the reaction rate does not increase any further. Any addition of substrate can no longer increase the activity of the enzyme and the reaction behaves as zero order.[48]

Table 2: Optimizing conditions for XO activity experiment using Box-Behnken design.

Running order	pH	Temperature (°C)	[Xanthine] (mM)	Absorbance	[Unreacted xanthine] (mM)	[Reacted xanthine] (mM)
1	6.0	25	0.70	0.735	0.43	0.27
2	6.0	25	0.70	0.745	0.44	0.26
3	6.0	20	1.05	0.826	0.53	0.52
4	6.0	20	1.05	0.827	0.53	0.52
5	6.0	30	1.05	1.022	0.75	0.30
6	6.0	30	1.05	1.032	0.76	0.29
7	6.0	25	1.40	1.048	0.78	0.62
8	6.0	25	1.40	1.055	0.79	0.61
9	7.0	20	0.70	0.777	0.19	0.51
10	7.0	20	0.70	0.765	0.18	0.52
11	7.0	30	0.70	0.911	0.36	0.34
12	7.0	30	0.70	0.927	0.38	0.32
13	7.0	25	1.05	1.070	0.56	0.49
14	7.0	25	1.05	1.075	0.57	0.48
15	7.0	25	1.05	1.083	0.58	0.47
16	7.0	25	1.05	1.074	0.57	0.48
17	7.0	25	1.05	1.068	0.56	0.49
18	7.0	25	1.05	1.073	0.56	0.49
19	7.0	20	1.40	1.018	0.49	0.91
20	7.0	20	1.40	1.045	0.53	0.87
21	7.0	30	1.40	1.171	0.69	0.71
22	7.0	30	1.40	1.162	0.68	0.72
23	8.0	25	0.70	1.036	0.35	0.35
24	8.0	25	0.70	1.032	0.35	0.35
25	8.0	20	1.05	1.059	0.37	0.68
26	8.0	20	1.05	1.052	0.37	0.68
27	8.0	30	1.05	1.514	0.77	0.28
28	8.0	30	1.05	1.529	0.78	0.27
29	8.0	25	1.40	1.060	0.38	1.02
30	8.0	25	1.40	1.064	0.34	1.02

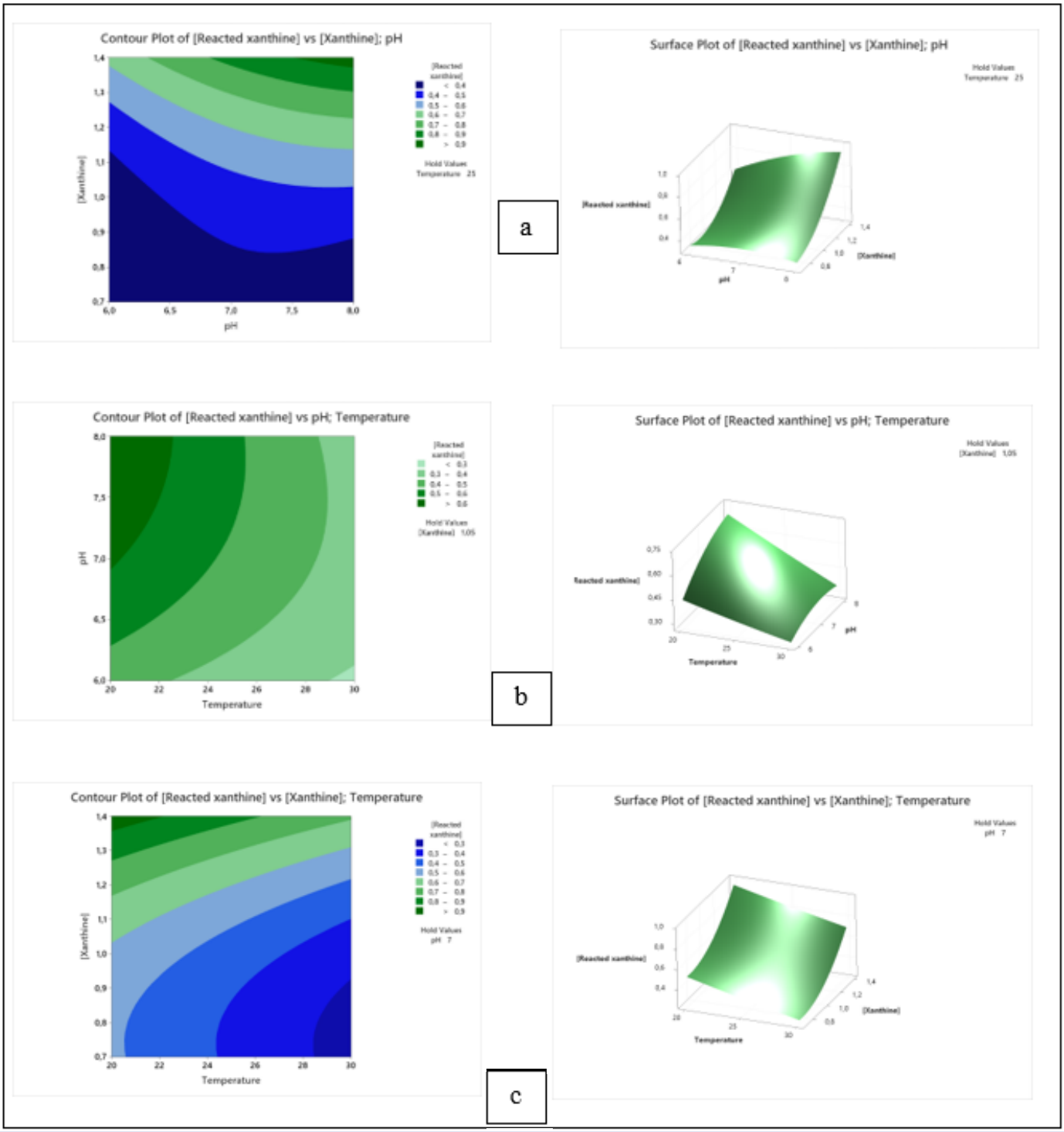

Figure 6: **Contour plot and surface plot describe the relationship between (a) pH and xanthine concentration, (b) temperature and pH, and (c) temperature and xanthine concentration to reacted xanthine concentration.**

Table 3: ANOVA results for the optimum conditions of XO activity.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	1.34090	0.148989	44.22	0.000
Linear	3	1.13635	0.378783	112.43	0.000
pH	1	0.09923	0.099225	29.45	0.000
Temperature	1	0.24503	0.245025	72.73	0.000
[Xanthine]	1	0.79210	0.792100	235.10	0.000
Square	3	0.13710	0.045699	13.56	0.000
pH*pH	1	0.01523	0.015232	4.52	0.046
Temperature * Temperature	1	0.00016	0.000155	0.05	0.832
[Xanthine]*[Xanthine]	1	0.11462	0.114617	34.02	0.000
2-Way Interaction	3	0.06745	0.022483	6.67	0.003
pH*Temperature	1	0.01620	0.016200	4.81	0.040
pH*[Xanthine]	1	0.05120	0.051200	15.20	0.001
Suhu*[Xanthine]	1	0.00005	0.000050	0.01	0.904
Error	20	0.06738	0.003369		
Lack-of-Fit	3	0.06575	0.021917	228.11	0.000
Pure Error	17	0.00163	0.000096		
Total	29	1.40828			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,0580445	95.22%	93.06%	87.78%

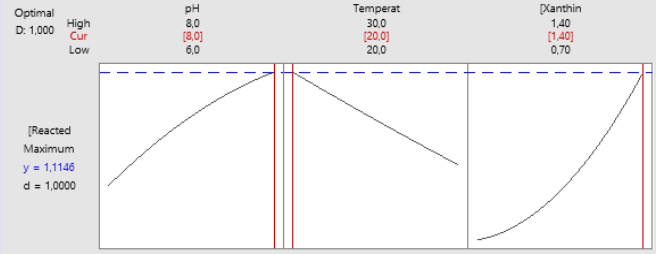

Figure 7: Response optimizer (RO) for XO activity at optimum conditions at 20 °C, pH 8.0, and xanthine concentration of 1.40 mM.

The contour plot and surface plot analysis in Figure 6 shows the relationship between the 3 variables and the response (reacted xanthine concentration). Both of these plots produce XO activity at optimum conditions. The darkest green color indicates the maximum response, while the darkest blue color shows the minimum response. Figure 6 shows that the maximum response will be obtained in the pH range of 7.5–8.0; the temperature of 20 °C; and the xanthine concentration of 1.40 mM. These results are also following the optimization produced by the Minitab software through response optimizer (RO).

The RO analysis explained that the optimum activity conditions of XO were reached at pH 8.0, the temperature of 20 °C, and the xanthine concentration of 1.40 mM (Figure 7). The desirability value of the RO shows that it reaches 1.0. The higher the desirability value (up to 1.0), the higher the suitability of the variable combinations to achieve the response.[49] The expected value of the Minitab reacted xanthine (optimized result) was 1.1146 (Table 4). When tested based on the optimum conditions, the average value of xanthine concentration was reached at 1.1067. When H_0; $\mu = 1.1067$; H_1: $\mu \neq 1.1067$; then the P-value of the one-sample *t*-test is 0.423 or greater than 0.05. It means that H_0 is accepted (not significantly different), and the verification follows the expected value (Table 5).

The research data for optimizing XO activity in the study also did not differ significantly, as shown in Table 6. The difference in the optimum conditions for XO activity was caused by measurement time, environmental conditions, and differences in laboratory equipment.[11] Furthermore, differences in the enzyme type used will impact XO, typically isolated from mammalian milk, which is influenced by the animal's sex, season, and diet.[50] Jadhao *et al.* (2018)[51] reported that the optimum temperature and pH obtained from cow's milk were 25 °C and 7.4. Meanwhile, the optimum pH of XO obtained from buffalo milk is 8.0.[51]

Inhibitory capacity and IC_{50} of extracts toward XO activity

Allopurinol from Sigma and commercial allopurinol were used as positive controls. Figure 8 represents the effect of increasing the concentration of allopurinol on XO. The analysis results explained that allopurinol exhibited a much stronger inhibitory power than water extracts and 70% ethanol. The IC_{50} of allopurinol was 7.45±0.07 mg/L, while the commercial allopurinol was 2.69 mg/L. Previous studies have shown varying IC_{50}, as in Trivadila *et al.* (2020)[27] at 9.58 ppm; Rodriguez *et al.* (2021)[52] at 3.61 mg/L; Nile and Park (2013)[53] at 10.5 ppm; and Ernis (2016)[54] at 3.12 ppm. Meanwhile, the IC_{50} of the commercial allopurinol obtained by Trivadila *et al.* (2020)[27] is 1.76 mg/L. The difference in IC_{50} is due to differences in the optimum conditions for XO activity, the type of commercial allopurinol used, and the instrumentation in the laboratory.[11]

The IC_{50} of 70% ethanol extract is 224.14±8.62 mg/L. This value is lower than the water extract (348.83±4.85 mg/L) and this condition indicates that 70% ethanol extract is more active than water extract. The inhibitory activity is increasing with the increasing polarity of the fractions (Figure 5). This result is in line with the LC_{50} result obtained previously. Semipolar compounds are assumed to be present in the 70% ethanol extract. XO inhibitors are phenolic compounds such as phenolic acids and flavonoids.[55] The total phenolic content of the ethanol extract of bajakah tampala stem reported by Ayuchecaria *et al.* 2020[19] is 12.33 mgGAE/g. Meanwhile, according to Kurnianto *et al.* (2020),[56] the total phenolic content is 14.952 mg/100g GAE and the flavonoid content of the ethanol extract of the stem is 79.739 mg/100g QE. The pharmacological effects of these compounds not only reduce uric acid concentrations but also as anti-inflammatory and suppress the formation of free radicals such as superoxide anion and hydrogen peroxide.[57,58]

Inhibition kinetics of extract toward XO activity

The concentration of the extract and the most active fraction were determined in determining the kinetics of inhibition of XO based on IC_{50} and LC_{50} values (Table 6). The inhibition kinetics parameters calculated were V_{max}, K_m, α, and K_I. V_{max} indicates the maximum rate when the enzyme is saturated with the substrate, whereas K_m is the Michaelis-Menten constant which represents the concentration of the substrate when it is half of the V_{max} value. K_m is also expressed as the

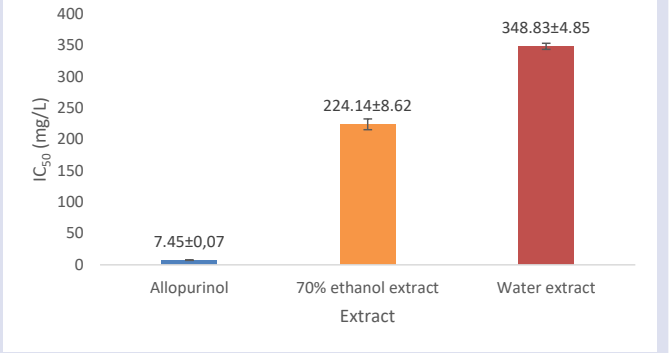

Figure 8: The IC_{50} of water extract and 70% ethanol extract.

Table 4: Response optimization (reacted xanthine).

Parameters						
Response	Goal	Lower	Target	Upper	Weight	Importance
[Reacted xanthine]	Maximum	0.26	1.02		1	1
Solution						
Solution	pH	Temperature	[Xanthine]	[Reacted xanthine] Fit	Composite Desirability	
1	8	20	1.40	1.1146	1	

Table 5: One-sample t-test results to verify optimization conditions.

Descriptive statistics				
N	Mean	StDev	SE Mean	95% CI for μ
3	1.1067	0.00577	0.0033	(1.09232; 1.12101)
μ: population mean of [reacted xanthine based on the lab]				
Test				
Null hypothesis	H_0: μ = 1.1067			
Alternative hypothesis	H_1: μ ≠ 1.1067			
T-Value	P-Value			
-1.00	0.423			

Table 6: Data from several studies on the optimization of XO activity.

Variables	Data from several studies on the optimization of XO activity				
	Iswantini and Darusman (2003)	Iswantini et al. (2014)	Rahmatullah (2019)	Trivadila et al. (2020)	This research (2023)
pH	7.5	7.5	7.5	7.4	8.0
Temperature (°C)	20	30	20	20	20
Xanthine concentration (mM)	0.70	1.00	1.40	1.40	1.40

Table 7: IC_{50}, LC_{50}, and inhibition kinetics parameters of The *S. littoralis* Hassk. stem extracts on XO activity.

Extract	IC_{50} (mg/L)	LC_{50} (mg/L)	Concentration of inhibition kinetics (mg/L)	Parameters of inhibition kinetics				Type of inhibition kinetics
				V_{max} (mM/minute)	K_m (mM)	α	K_I (mg/L)	
No extract	-	2059.928	-	0.0144	0.7320	-		-
70% ethanol extract	224.14±8.62	662.253	300	0.0146	1.5372	2.10	272.68	Competitive
Water extract	348.83±4.85	774.682	400	0.0147	1.5056	2.06	378.44	Competitive

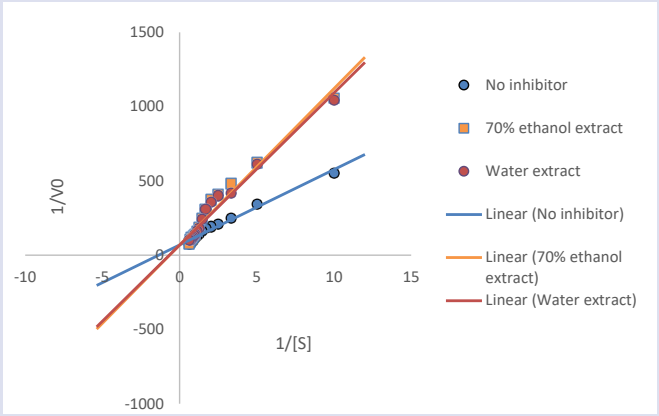

Figure 9: Lineweaver-Burk plots of water extract and 70% ethanol extract.

affinity of the enzyme for the substrate. The ratio of the value of Km in the condition of adding the inhibitor and the Km in the condition without the inhibitor will reach the value of α. This value is also the affinity of the inhibitor for XO. The greater the value of α, the higher the competitive inhibition that occurs.[45,59]

The concentrations of inhibition kinetics on the water and 70% ethanol extracts were 400 and 300 mg/L, respectively. During the inhibition kinetics assay, the activity rate of XO was measured both in the absence and presence of the inhibitor expressed in mM/min at various substrate (xanthine) concentrations ranging from 0.10 to 1.60 mM. In addition, a Lineweaver-Burk plot investigation was carried out to establish the type of inhibition kinetics from extracts toward XO activity (Figure 6).

The V_{max} and K_m values without inhibitors were 0.0144 mM/min and 0.7320 mM. This condition means that XO will reach half of its maximum rate at a substrate concentration of 0.7320 mM with a maximum rate of 0.0144 mM/min. Table 6 shows that V_{max} values for extracts remained relatively unchanged, while K_m values increased. The highest α value was obtained in the 70% ethanol extract (2.36), while the lowest was in the water extract (2.06). The value of α which is greater than 1 indicates a strong inhibitory power. The value of the inhibition constant (K_I) for the 70% ethanol extract was obtained as the smallest (272.68 mg/L), which means that the inhibition is stronger and the binding affinity of the inhibitor-enzyme exceeds the binding affinity for the enzyme-substrate.[45] The type of inhibition kinetics for the extracts obtained is competitive inhibition kinetics. This is based on an increase in the value of K_m but a relatively constant V_{max}.[60] The Lineweaver-Burk plot (Figure 9) shows a decreasing x-intercept due to the increasing K_m value. However, in the competitive inhibition mechanism, the value of V_{max} tends to remain constant, resulting in no change in the y-intercept in the Lineweaver-Burk plot.

In the mechanism of competitive inhibition, the structure of the inhibitor compound resembles that of the substrate so that it

competes with the substrate in binding to the active site of the enzyme. Competitive inhibitors and substrates have the same affinity for occupying the active site of the enzyme so that when the concentration of the inhibitor exceeds the concentration of the substrate, it will form an enzyme-inhibitor complex (EI), and no product is formed.[45,59,61]

In the world of pharmacokinetics, the selected drug candidates are competitive inhibitor types because they are easily absorbed in the body's metabolism (bioavailability) and reduce the effects of toxicity in the body.[62] In general, flavonoids can be absorbed by the small intestine around 0-60% of the dose and the elimination half-life ranges from 2-28 hours.[63] Flavonoids that lead to competitive inhibitors of XO are isoflavones (genistein), flavonols (kaempferol), flavones (luteolin, apigenin), flavanones (eriodictyol), and flavanols (catechins).[14] These flavonoid compounds are thought to be contained in the water and 70% ethanol extracts from the stem of *S. littoralis* Hassk.

CONCLUSIONS

The Box-Behnken Design was successfully employed to optimize XO activity. Water and 70% ethanol extracts from the stem of *S. littoralis* Hassk. competitively inhibit xanthine oxidase activity. The yield, phytochemical screening, and toxicity correspond with these findings. However, further fractionation of the extracts is required to increase their inhibitory activity.

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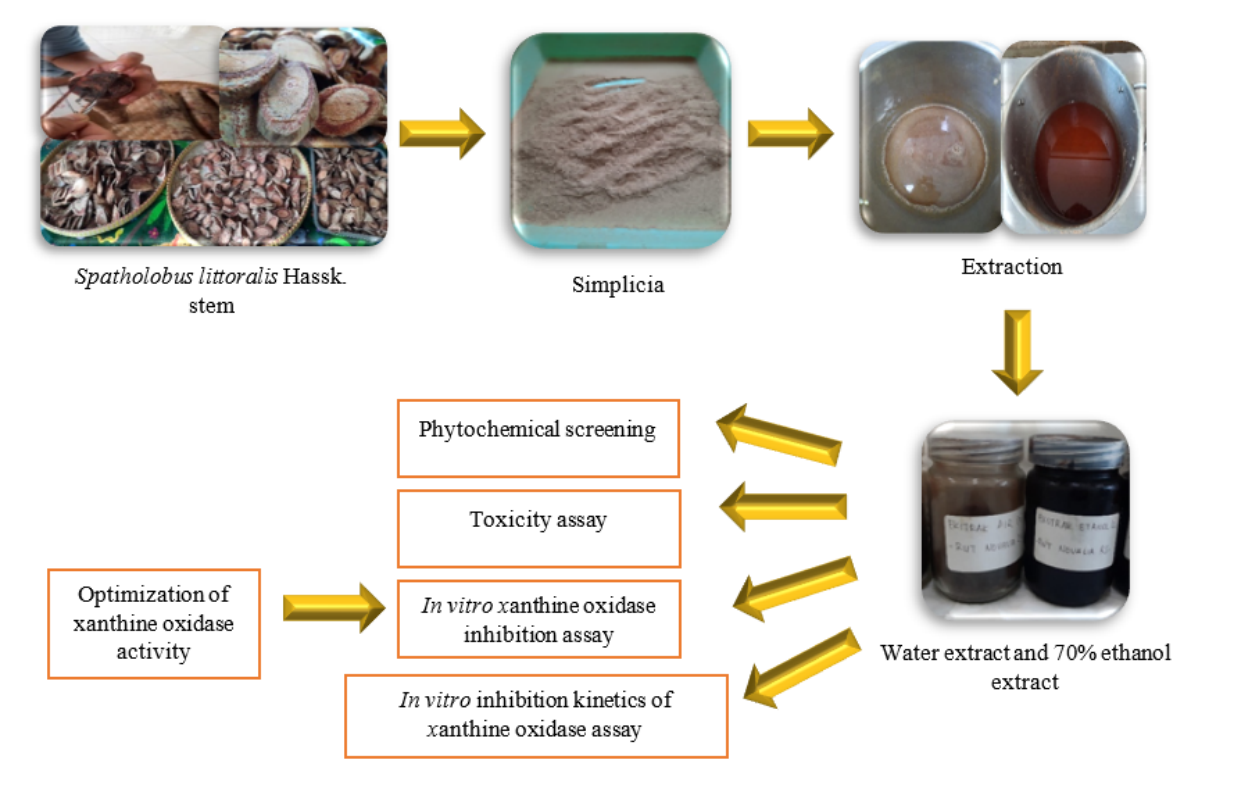
GRAPHICAL ABSTRACT



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