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Antigout Activity of The *Spatholobus littoralis* Hassk. Extract Fractions Against Xanthine Oxidase: Its Metabolite Profile and Inhibition Kinetics

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ABSTRACT

The *Spatholobus littoralis* Hassk. (*S. littoralis* Hassk.) is a native plant in Indonesia and has been widely used in traditional Dayaknese medicine for non-communicable degenerative diseases. One of these illnesses, known as gout, is caused by excessive uric acid in the blood, which is the catalytic byproduct of a xanthine oxidase (XO) enzyme. In this work, we investigated the inhibition kinetics of XO and identified bioactive compounds from the stem extract fractions of *S. littoralis* Hassk. using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Fractionation was carried out to obtain *n*-hexane, dichloromethane, and 1-butanol fractions from the water and 70% ethanol extracts. Fraction of 1-butanol from 70% ethanol and water extract displayed potent inhibitors of XO with IC_{50} value 116.91±3.51 and 137.15±5.00 mg/L, respectively. Lineweaver-Burk plot analysis showed that the 1-butanol fraction from the two extracts inhibited XO competitively. The 1-butanol fraction from the two extracts has been further identified as a bioactive fraction. The majority of the compounds in the two active fractions were phenolics. These findings revealed that the 1-butanol fraction from the two extracts is promising as an antigout treatment in the future.

1. Introduction

The bajakah tampala is also referred to as the *Spatholobus littoralis* Hassk. (*S. littoralis* Hassk.). This plant belongs to the Leguminosae family and originates from Southeast Asia; in Indonesia, it can be found on the islands of Kalimantan and Sumatra peatland forests (Ridder-Numan 1998). The Dayak culture has traditionally used this plant as local knowledge to alleviate aches and diarrhea, lessen body lumps, and lower uric acid levels. Flavonoids, tannins, phenolics, and saponins have all been scientifically confirmed to be present in the phytochemical composition of this species (Fitriani *et al.* 2020).

A buildup of monosodium urate crystals in periarticular tissue, joints, and bones causes inflammatory joint disease (gout) (Pacher *et al.* 2006; Ayoub *et al.* 2021). Hyperuricemia, which occurs when the serum uric acid content is higher than average for men and women above 7.0 mg/dl and 6.5 mg/dl, is another cause of gout (Kang and Johnson 2020). Gout increases the risk of catching SARS-Cov-2 (severe acute respiratory syndrome-coronavirus-2) (The Ministry of Home Affairs Working Team for 2020 COVID-19 Task Force Support 2020). If left untreated, this illness could progress into chronic gout, resulting in kidney failure and a stroke (Recommendations of the Indonesian Rheumatology Association 2018).

Xanthine oxidase (XO) is a crucial enzyme that catalyzes the production of reactive oxygen species (ROS) simultaneously with the conversion of xanthine to uric acid and hypoxanthine to xanthine (Pacher *et al.* 2006; Voet and Voet 2010). The sort of inhibitory kinetics induced by XO as a target and therapeutic candidate compound can explain how this affinity is developed and whether it is transitory (competitive and uncompetitive inhibition) or permanent (non-competitive inhibition) (Iswantini *et al.* 2014; Zhao *et al.* 2020). Herbal plants containing flavonoid extracts as XO inhibitors have been widely studied,

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including *Sida rhombifolia* L. (Iswantini *et al.* 2014), *Apium graveolens* L. (Iswantini *et al.* 2012), *Saraca thaipingensis* (Argulla and Chichioco-Hernandez 2014), *Rhodiola crenulate* (Hung-Chu *et al.* 2014), *Toona sinensis* (Yuk *et al.* 2018), *Sonchus arvensis* (Trivadila *et al.* 2020), *Chrysanthemum morifolium* Ramat (Peng *et al.* 2020), *Filipendula ulmaria* (Gainche *et al.* 2021).

In the present study, we investigated the inhibition kinetics of XO from the stem extract fractions of *S. littoralis* Hassk. through Lineweaver-Burk plot analysis. Bioactive compounds from the two active fractions were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Water extract and 70% ethanol extract were fractionated to produce *n*-hexane, dichloromethane, and 1-butanol fractions.

2. Materials and Methods

2.1. Materials

Potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany), dipotassium hydrogen phosphate (K_2HPO_4) (Merck, Germany), *n*-hexane (Merck, Germany), dichloromethane (DCM) (Merck, Germany), 1-butanol (Merck, Germany), methanol (Merck, Germany), ethanol (Merck, Germany), HCl (Merck, Germany), *Artemia salina* L. (SandersTM Great Salt Lake Artemia Cysts), seawater, distilled water, xanthine X0626-5G (Sigma Aldrich, USA), Allopurinol A8003-5G (Sigma Aldrich, USA), and the xanthine oxidase X1875-25 UN type Bovine Milk Grade I (Sigma Aldrich, USA).

2.2. Plant Collection

The bajakah tampala sample was collected from Muara Teweh, North Barito Regency, Central Kalimantan Province, and was determined by a botanist in the National Research and Innovation Agency at Cibinong, West Java, Indonesia. The Tropical Biopharmaca Research Center, IPB University, Indonesia received a voucher specimen of the sample and filed it there with the reference number BMK0490102021.

2.3. Sample Preparation and Fractionation

The stems of the bajakah tampala were washed and sundried. Then, it was ground and sieved to obtain 40 mesh simplicia powder. The fractionation method followed the method reported by Trivadila *et al.* (2020) and has been slightly modified. Water and 70% ethanol extracts (30 g) were separately dissolved in 90% methanol. The mass ratio of extract:90% methanol was 1:8, then sonicated for 30 minutes. Methanol and *n*-hexane were used in a 1:1 (v/v) ratio inside a separating funnel to carry out the fraction in *n*-hexane solvent. After shaking the separating funnel, the *n*-hexane fraction was separated and concentrated, while the 90% methanol fraction was diluted to 50% methanol to increase its polarity, then fractionated with DCM solvent in a ratio of 1:1. It was done to separate and concentrate the DCM fraction. The 50% methanol solvent was then removed using a rotary evaporator until the remaining solvent was water. The water fraction was then fractionated with 1-butanol at a ratio of 1:1. The 1-butanol fraction was also separated and concentrated. The yield of each fraction is calculated by the formula (1):

$$\text{Fraction yield (\%)} = \frac{\text{fraction weight}}{\text{fraction weight}} \times 100\% \quad (1)$$

2.4. Determination of Toxicity by using the Brine Shrimp Lethality Test (BSLT)

This method followed the method reported by Meyer *et al.* 1982. The initial stage of the toxicity assay was the hatching of *Artemia salina* Leach (*A. salina* L.) eggs. The eggs (50 mg) were weighed and put into an Erlenmeyer containing filtered seawater. The Erlenmeyer was also equipped with an aerator and incubated for 48 hours under lighting until the larvae hatched entirely. The hatched larvae were used in the toxicity assay. Furthermore, ten larvae were inserted through a micropipette into a 24-well plate filled with seawater and fractions of water, and 70% ethanol extracts. The concentration series were 1,000, 500, 100, and 50 ppm, each in a final volume of two ml in triplicates. After 24 hours, the results were determined for the dead larvae. Applying IBM SPSS Statistics version 21 application, the lethal concentration 50 (LC_{50}) value was calculated by visualizing the mortality rate using probit analysis at a 95% confidence level.

2.5. *In Vitro* Xanthine Oxidase Inhibition Assay and IC_{50} Determination

The XO inhibitory was performed at previously determined optimal settings. The fractions (1 ml) were put into a test tube with various concentrations based on the toxicity value. First, potassium

phosphate buffer (1.9 ml, 50 mM) was added at the optimum pH. Then, 1 ml xanthine at the optimum concentration was mixed. The reaction was initiated by adding 0.1 U/ml XO (0.1 ml) and maintained for 45 minutes at the optimal temperature. The process was halted by adding 0.58 M HCl (1 ml). The absorbance of the latter was then examined using a UV-Vis spectrophotometer at a maximum wavelength (270.0 nm) to determine the unreacted xanthine remaining in the sample. We used allopurinol as the positive control. The formulas (2 and 3) will express XO activity and inhibition capacity (%). This assay has been slightly modified from the method of Iswantini *et al.* (2014).

$$\text{XO activity} = \frac{\text{reacted xanthine (mM)}}{\text{enzyme volume (L)} \times \text{incubation time (minute)}} \times 100\% \quad (2)$$

$$\text{Inhibition capacity} = \frac{\text{XO activity control} - \text{XO activity sample}}{\text{XO activity control}} \times 100\% \quad (3)$$

The inhibitory concentration 50 (IC_{50}) was estimated by entering 50 as the y value in the regression equation to obtain the x value as the sample's IC_{50}.

2.6. *In Vitro* Kinetics of Xanthine Oxidase Assay

The concentration of the two active fractions was selected to determine the inhibition kinetics of XO based on the IC_{50} and LC_{50} values. The range of xanthine concentration was 0.10 to 1.60 mM (0.10 mM interval). Following the formulas (4 and 5), the data were interpreted into the Lineweaver-Burk plot analysis to produce the inhibition constant (K_I), Michaelis-Menten constant (K_m), and the maximum reaction rate (V_{max}).

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \frac{1}{[S]} = \frac{1}{V_{max}} \quad (4)$$

$$K_m' = K_m \left(1 + \frac{[I]}{K_I}\right) \quad (5)$$

Where:
V_0 = reaction rate
V_{max} = maximum reaction rate
K_m = michaelis–menten constant when without the extract addition
K_m' = Michaelis–Menten constant after extract addition
$[S]$ = substrate concentration
$[I]$ = inhibitor concentration
K_I = inhibition constant

2.7. Putative Identification of Compounds by LC-MS/MS

LC-MS/MS was used to conduct putative identification of compounds from the two active fractions. Chromatographic separations were achieved on the Accucore C_{18} column of 100 mm × 2.1 mm, 1.5 µm particle size. The chromatographic conditions were as follows: mobile phase A (water + 0.1% formic acid); mobile phase B (acetonitrile + 0.1% formic acid); flow rate of 0.2 ml/min; column temperature of 30°C; and sample injection volume of 5.0 µL. The gradient profile was optimized as the following: 0-1 min, 5% B; 1-25 min, 5-95% B; 25-28 min, 95%B; 28-30 min, 5%B.

The electrospray source of the MS was operated in negative mode, and the parameters were: 3.8 kV spray voltage, 320°C capillary temperature, 15 ml/min flow rate of sheath gas, and 3 ml/min flow rate of auxiliary gas. The collision energy was adjusted to 18, 35, and 53 eV for MS/MS experiments. Mass spectra were recorded in range m/z 100-1,500 range.

UHPLC-Q-Orbitrap HRMS data were processed using Compound Discoverer 2.2 with an in-house database collected from genus *Spatholobus* information to identify the compounds putatively. The compounds were identified using retention time and fragmentation patterns, compared to data from other research journals; online databases, such as Human Metabolome Database (HMDB), ChemSpider, and PubChem.

2.8. Statistical Analysis

Data were presented as mean±standard deviation (SD) (n = 3), and a one-way analysis of variance (ANOVA) was used to examine them before the Tukey test at a 95% confidence level. A significant difference between samples was indicated by a p-value ≤0.05.

3. Results

3.1. The Fraction Yields and Toxicity

The highest yield came from the 1-butanol fraction of the 70% ethanol extract (8.20%) and 1-butanol

fraction of the water extract (3.78%), followed by the DCM fraction and the *n*-hexane fraction on each extract (Figure 1). The results of the toxicity assay of each extract are shown in Figure 2. The fractions of 70% ethanol extract have a lower LC_{50} than those of water extract. However, the 1-butanol fraction of 70% ethanol extract gave the lowest LC_{50} (528.57 mg/L) among all fractions, followed by the DCM fraction and the *n*-hexane fraction.

3.2. IC_{50} of Active Fractions Against XO Activity

The IC_{50} value of 1-butanol fraction from the 70% ethanol extract (116.91±3.51 mg/L) and water extract (137.15±5.00 mg/L) resulted in the lowest value, meaning the two active fractions. The inhibitory activity increased with the increasing polarity of the fraction (Figure 3). This outcome is consistent with the LC_{50} values, which we can observe in Figure 2.

3.3. Inhibition Kinetics of Active Fractions Toward XO Activity

We determined the concentrations of the two active fractions in the inhibitory kinetic assay based on the IC_{50} and LC_{50} values. The IC_{50} value for 1-butanol fraction of 70% ethanol extract was 116.91 ±3.51 mg/L, while for 1-butanol the fraction of water extract was 137.15±5.00 mg/L (Table 1). Thereby we used values below the LC_{50} for both fractions. The LC_{50} values for 1-butanol fraction of 70% ethanol extract and 1-butanol fraction of water extract were 528.57±40.71 mg/L and 675.47±47.98 mg/L, respectively. Therefore, the concentration of the two active fractions we used in the inhibition kinetics test was 200 mg/L. The level of XO activity was determined in the free of inhibitors and the presence of inhibitors expressed in mM/min at various substrate (xanthine) concentrations ranging from 0.10 to 1.60 mM for the inhibition kinetics test. The Lineweaver-Burk plot analysis was carried out to investigate the mechanism of extracts' and fractions' inhibition of XO activity (Figure 4).

Figure 4 shows that the Vmax for the two active fractions remained relatively unchanged, while the Km increased. The highest α value was given by the 1-butanol fraction of 70% ethanol extract (2.36); and the value of inhibition constant (KI) for a 1-butanol fraction of 70% ethanol extract was obtained as the least (147.44 mg/L). Based on these results, the identified inhibition kinetics for the active fractions is competitive.

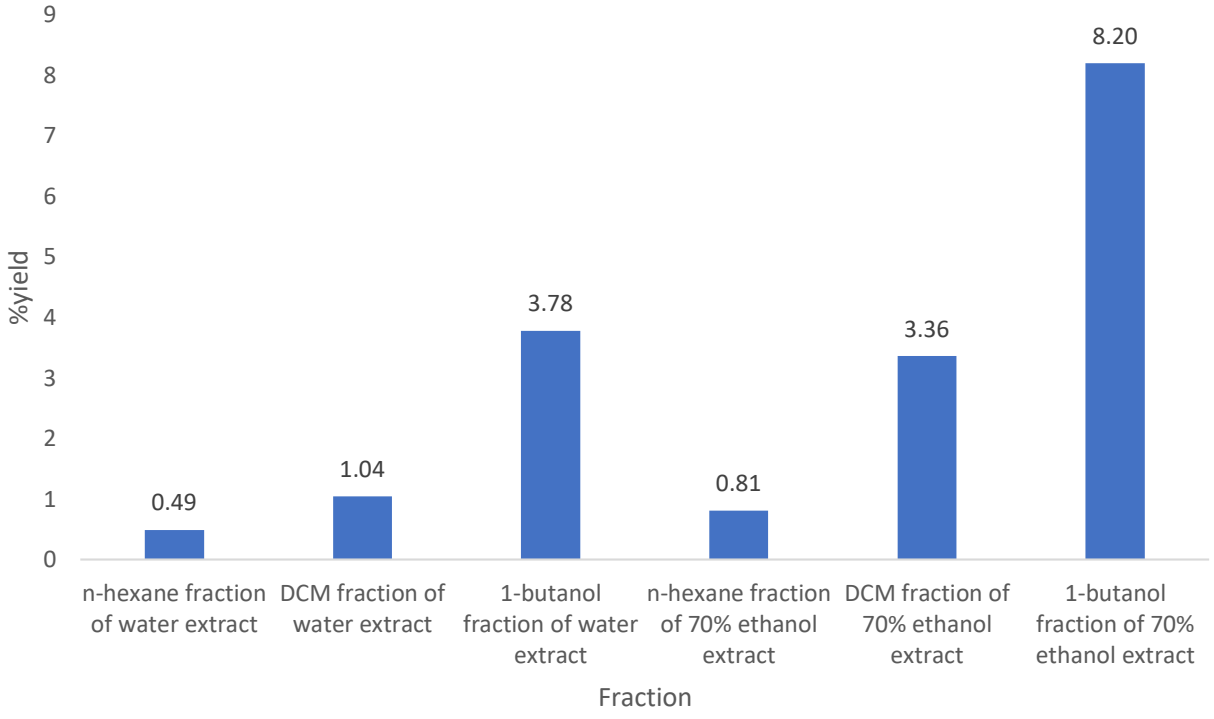

Figure 1. The yield of the fractions of *S. littoralis* Hassk. stem

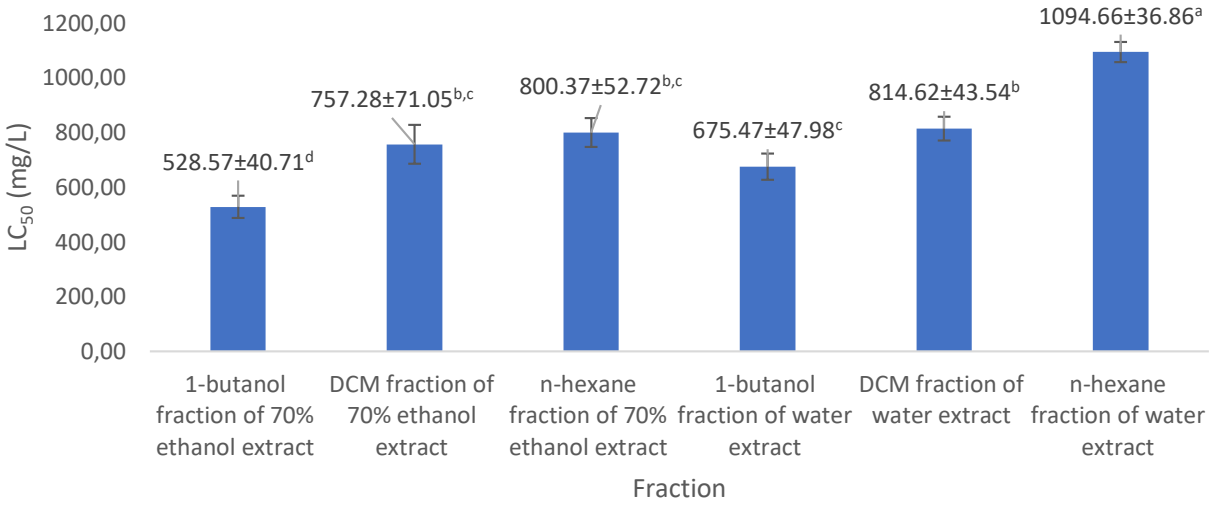

Figure 2. The LC_{50} value of the fractions. For each sample, values with different letters showed significant differences in p-value≤0.05 based on one-way ANOVA and continued with Tukey's test (n = 3). Results are sorted in ascending order: a>b>c>d

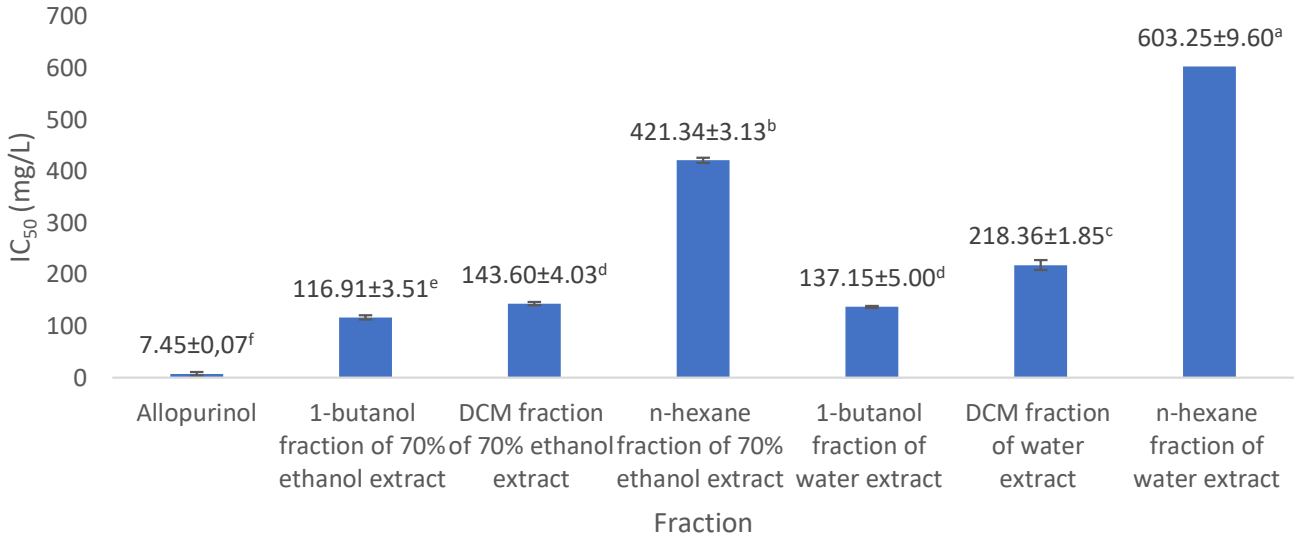

Figure 3. The IC_{50} value of the fractions. For each sample, values with different letters showed significant differences in p-value≤0.05 based on one-way ANOVA and continued with Tukey's test (n = 3). Results are sorted in ascending order: a>b>c>d>e>f

Table 1. IC_{50}, LC_{50}, and inhibition kinetics parameters of the two active fractions of the *S. littoralis* Hassk. stem 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/min)	K_m (mM)	α	K_I (mg/L)	
No extract	-	-	-	0.0144	0.7320	-		-
1-butanol fraction of 70% ethanol extract	116.91±3.51	528.57±40.71	200	0.0143	1.7248	2.36	147.44	Competitive
1-butanol fraction of water extract	137.15±5.00	675.47±47.98	200	0.0145	1.6088	2.20	166.95	Competitive

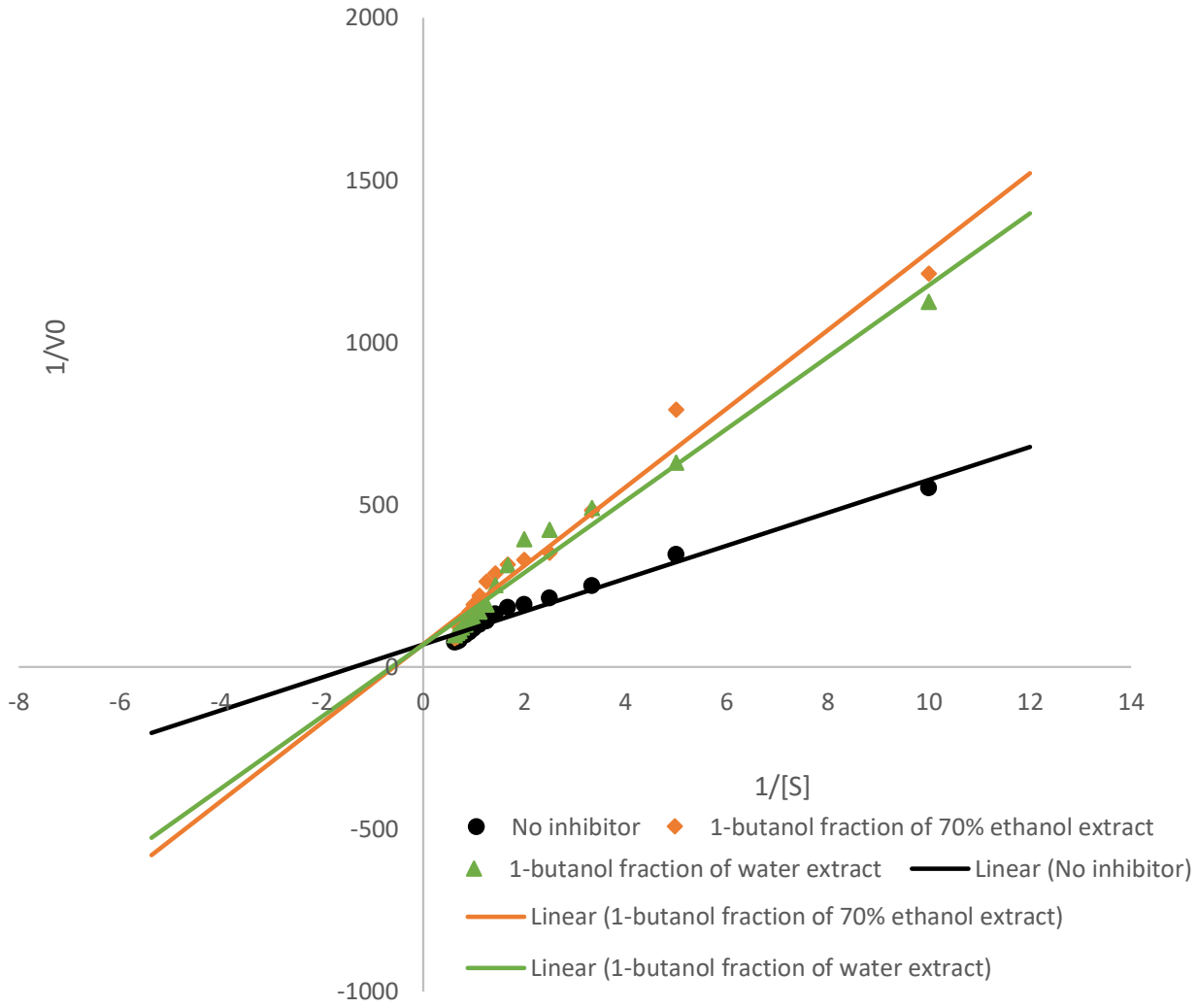

Figure 4. Lineweaver-Burk plots of the 1-butanol fraction of water extract and 1-butanol fraction of 70% ethanol extract

3.4. Putative Compounds in Active Fractions by LC-MS/MS

To putatively identify the bioactive compounds in the two active fractions with the least IC_{50} values (1-butanol fraction of water extract and 1-butanol fraction of 70% ethanol extract) were examined by LC-MS/MS. The three highest peaks in the 1-butanol fraction of 70% ethanol extract were formononetin, piscidic acid, and (15Z)-9,12,13-trihydroxy-15-octadecenoic acid (Figure 5A). Meanwhile, in 1-butanol fraction of the water extract consisted of piscidic acid, methylmalonic acid, and DL-malic acid (Figure 5B). These compounds belong to the subclass of phenolic acids, flavonoids (isoflavones), and fatty acids, respectively.

The 1-butanol fraction of 70% ethanol extract contained 39 compounds, whereas the 1-butanol fraction of the water extract had 23 compounds, according to the compound profile (Supplementary 1 and 2). Phenolics, including flavonoids, dominated the two active fractions. The 1-butanol fraction of the 70% ethanol extract contained 51.30% of the total phenolic chemicals, compared to 47.83% for the 1-butanol fraction of the water extract (Figure 6A and B). We used the method of the percentage of the class composition contained in both fractions, as can be observed in Supplementary 3. Analysis of the Venn diagram (Figure 7), which was processed based on the database of compounds in the two active fractions, explained that there were 22 compounds exclusively in 1-butanol fraction of 70% ethanol extract, six compounds exclusively in 1-butanol fraction of water extract, and 17 compounds that were in both factions (Table 2).

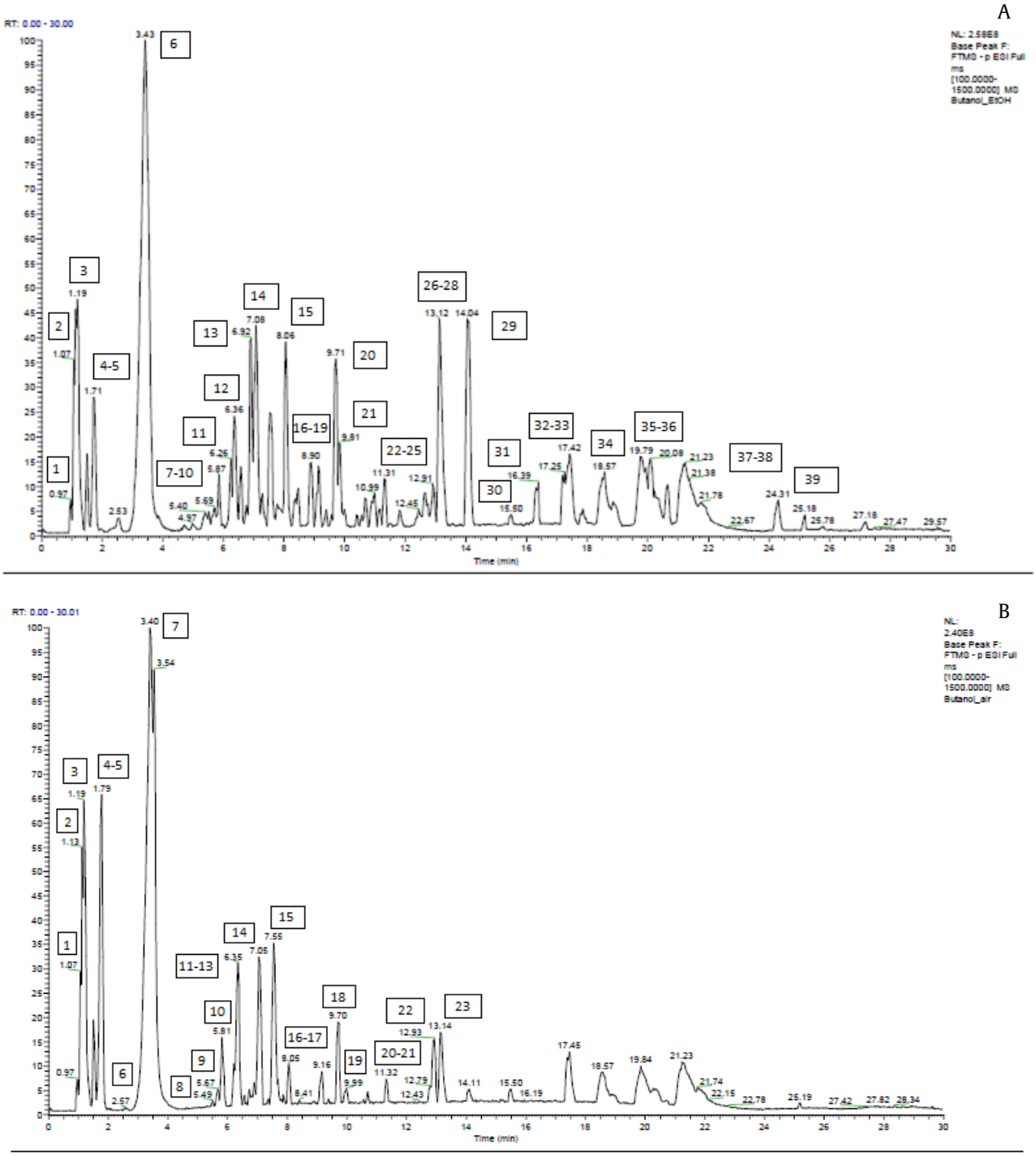

Figure 5. (A) Chromatogram of 1-butanol fraction of 70% ethanol extract, (B) chromatogram of 1-butanol fraction of water extract

4. Discussion

Water and 70% ethanol extract were fractionated with nonpolar, semipolar, and polar solvents. The principle of this fractionation is the separation of chemical components between two immiscible solvent phases. Chemical components will separate into two phases according to their level of polarity, which refers to the "like-dissolve-like" rule (Zhuang *et al.* 2021). The *n*-hexane solvent was used to extract the nonpolar compounds, and the DCM and 1-butanol solvents extracted semipolar compounds

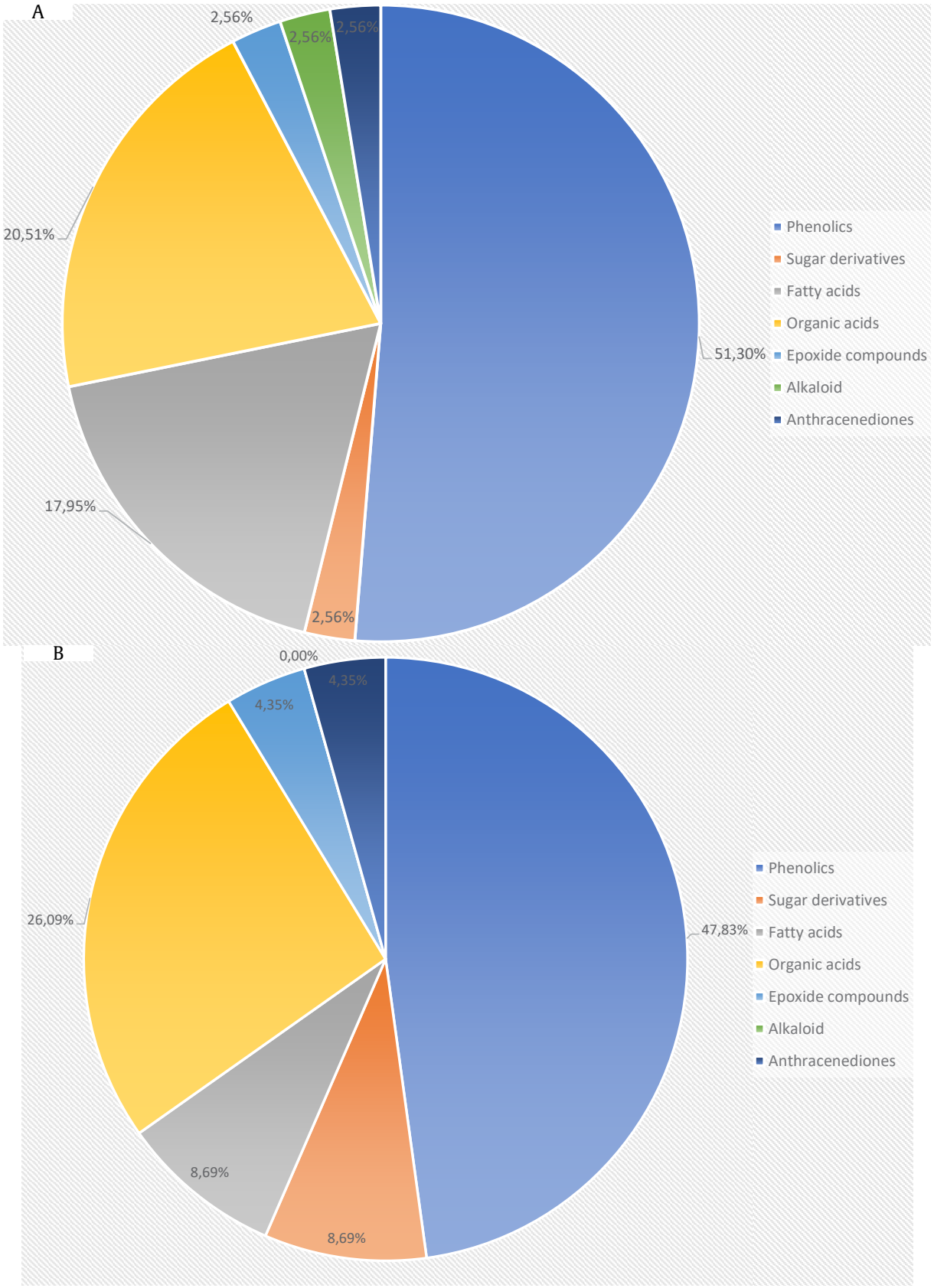

Figure 6. (A) Class composition in 1-butanol fraction of 70% ethanol extract, (B) class composition in 1-butanol fraction of water extract

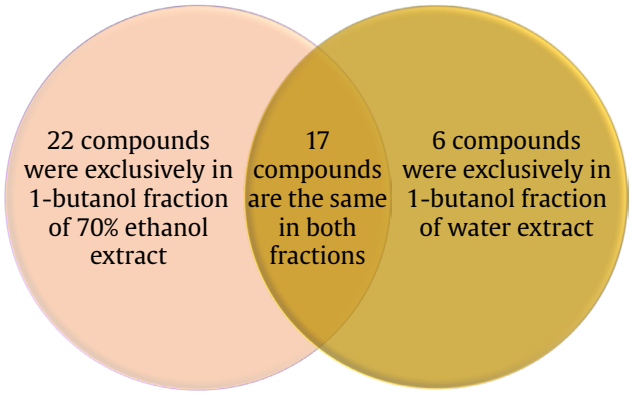

Figure 7. Venn diagram for compounds in the two active fractions

Table 2. List of 17 compounds that are the same in both fractions

Compound
D-(-)-Quinic acid
D-(+)-Galactose
DL-Malic acid
Methylmalonic acid
Piscidic acid
Gentisic acid
Diphenol glucuronide
Mitoxantrone
Salicylic acid
N-feruloylglycine
(2E)-3-(3,4-Dimethoxyphenyl) crylic acid
5-Methoxysalicylic acid (Isovanilic acid)
Etoglucid
8-Hydroxyhexadecanedioic acid
2-[3,8-Dihydroxy-8-(hydroxymethyl)-3-methyl-2-oxodecahydro-5-azulenyl]-2-propanyl hexopyranoside (Daidzein)
(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid
Formononetin

from the extracts. Based on yield fractions, the more polar the solvent, the higher the fraction yield obtained, and semipolar compounds can be extracted more easily in the water and 70% ethanol extracts. Nonpolar compounds are triterpenoids, steroids, and alkaloids. Meanwhile, semipolar compounds are flavonoids, phenolics (tannins, quinones), and saponins (Harborne 1987; Vrancheva *et al.* 2021).

The LC_{50} value represents the concentration of toxic compounds that can generate up to 50% organism mortality. *A. salina* L. larvae are susceptible to changes in their surroundings and chemical contamination (Rasyid *et al.* 2020). In this study, we applied the BSLT method using *A. salina* L. larvae as the organism. The result on the lethality of each fraction on brine shrimps is with LC_{50} values (Figure 2) below 1,000 mg/L, but only the *n*-hexane fraction of water extract which had LC_{50} above 1,000 mg/L. Figure 2 also indicates that the more polar the fraction, the higher the toxicity level. Meyer's toxicity scale classed only *n*-hexane fraction of water extract as non-toxic fraction (LC_{50} above 1,000 mg/L). Therefore, bioactive compounds are represented in more polar fractions. The LC_{50} of each fraction determines the concentration in the XO inhibition assay, considering that in the drug formula, it will be safer if it is used below its LC_{50} thereby preventing overdose (Sulaksono and Syamsudin 2012; Waghulde *et al.* 2019; Handayani *et al.* 2022). The compounds could be absorbed into the larvae's digestive tract. Following the absorption process, the compounds are dispersed throughout the larvae body, where they cause metabolic problems. Metabolic diseases have immediate impacts that can be seen just within 24 hours, causing 50% death of the larvae. The concentration and combined effects of several compounds in the fractions were strongly correlated with the death rate of larvae (Sami *et al.* 2019).

The IC_{50} value of the 1-butanol fraction of 70% ethanol extract (IC_{50} 116.91±3.51 mg/L) and the 1-butanol fraction of water extract (IC_{50} 137.15±5.00 mg/L) was obtained as the smallest among the other fractions, but the IC_{50} value was greater than allopurinol (positive control), or the activity value was smaller than allopurinol. The IC_{50} value of the 1-butanol fraction of 70% ethanol extract decreased compared to the IC_{50} of 70% ethanol extract (IC_{50} 224.14±8.62 mg/L). Likewise, the IC_{50} value of 1-butanol fraction of water extract also decreased compared to water extract (IC_{50} 348.83±4.85 mg/L) (Rahminiwati *et al.* 2023). This fractionation proves an increase in the inhibition of XO activity. The IC_{50} value of these two active fractions was still above the IC_{50} of the herbs *Sida rhombifolia* (IC^{50} 91.15±5.74 mg/L) (Iswantini *et al.* 2014), *Saraca thaipingensis* (IC_{50} 0.033 mg/ml) (Argulla and Chichioco-Hernandez 2014), *Rhodiola crenulate* (IC_{50} 24.24±1.80 M) (Hung-Chu *et al.* 2014), *Toona sinensis* (IC_{50} 78.4 M) (Yuk *et al.* 2018). However, the IC_{50} value of the two active fractions is below the IC_{50} of *Sonchus arvensis* (IC_{50} 119.02 mg/L) (Trivadila *et al.* 2020).

The highest α value and the lowest inhibition constant (K_i) value for 1-butanol fraction of 70% ethanol extract means that the inhibition is the strongest and the binding affinity of the enzyme-

inhibitor exceeds the binding affinity of the enzyme-substrate (Liu *et al.* 2020a). Throughout the competitive inhibition mechanism, the inhibitor compound's structure is similar to the substrate, so it can attach to the same enzyme's active site in competition with the substrate. Another condition that indicates the occurrence of competitive inhibition kinetics is the increase in the value of Km but the value of Vmax tends to remain (Nelson and Cox 2017; Robinson 2015; Pathak *et al.* 2020; Fadillah *et al.* 2022). The α values of the two active fractions increased from the extracts of 70% ethanol and water, respectively, going from 2.10 to 2.36 and 2.06 to 2.20. The K_I values of the two active fractions decreased: for the 1-butanol fraction of 70% ethanol extract and 70% ethanol extract, they moved from 272.68 mg/L to 147.44 mg/L, and for the 1-butanol fraction of water extract and water extract, they decreased from 378.44 mg/L to 166.95 mg/L (Rahminiwati *et al.* 2023). These also prove that fractionation makes the inhibition kinetics more competitive.

The competitive inhibition kinetics data of the two active fractions was confirmed by the abundant presence of phenolic compounds. The highest peak on the chromatogram of LC-MS/MS, also indicating the presence of phenolics and the similarity of 17 compounds between the two fractions (Table 2) strongly suggest that these compounds function as competitive inhibitors of XO. These findings are consistent with Sianipar *et al.* (2022) study that flavonoids predominated in 25 Indonesian medicinal plants used as XO inhibitors. This identification's outcomes are also in line with the earlier phytochemical screening (more flavonoid compounds were contained in 70% ethanol extract than water extract) (Rahminiwati *et al.* 2023). Typical flavonoid compounds in Leguminosae family are isoflavones (Harborne 1987; Veitch 2013), proven in the analysis of bioactive compounds in the *S. littoralis* Hassk. plant containing formononetin as one of the most compound compositions in 1-butanol fraction of 70% ethanol extract, in addition to daidzein. In both fractions, glycitein is present in the 1-butanol fraction of water extract. Phenolics such as phenolic acids and flavonoids can bind to the XO active site and interacts with the amino acid residues of XO through van der Waals forces, hydrophobic interactions, and hydrogen bonds (Ayyappan and Nampoothiri 2020; Liu *et al.* 2020b).

In conclusion, the results obtained in this work indicate that the fractionation can improve the inhibition kinetics from the water extract and 70% ethanol extract. In addition, the two active fractions: 1-butanol fraction of water extract and 1-butanol fraction of 70% ethanol extract of *S. littoralis* Hassk. are rich in phenolics. The phenolics are thought can competitively inhibit XO activity.

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Supplementary Materials

Supplementary 1. Secondary metabolites contained in 1-butanol fraction of 70% ethanol extract

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
1	0.97	0.17812	-4.79	192.06247	191.05519	173.04451; 155.03418; 131.03392; 111.04386; 101.02326; 85.02835	$C_7H_{12}O_6$	D-(-)-Quinic acid	Organic acids
2	1.07	0.67753	-5.45	180.06241	179.05443	163.06021; 113.02326; 89.02325; 71.01268; 59.01273	$C_6H_{12}O_6$	D-(+)-Galactose	Sugar derivatives
3	1.19	2.44440	-8.61	134.02037	133.01315	115.00253; 87.00761; 71.01269	$C_4H_6O_5$	DL-Malic acid	Organic acids
4	1.55	0.15481	-10.04	116.00979	115.00252	98.02357; 71.01270; 67.56075	$C_4H_4O_4$	Fumaric acid	Organic acids
5	1.71	1.44075	-10.08	118.02542	117.01817	99.00755; 73.02832	$C_4H_6O_4$	Methylmalonic acid	Organic acids
6	3.41	14.56923	-2.21	256.05774	255.5046	193.05013; 165.05521; 135.04442; 119.04952; 107.04927	$C_{11}H_{12}O_7$	Piscidic acid	Phenolic acids (Phenolics)
7	4.97	0.38774	-6.24	154.02565	153.01837	109.02839; 84.00808; 59.51074	$C_7H_6O_4$	Gentisic acid	Phenolic acids (Phenolics)
8	5.40	0.24456	-0.13	286.06883	285.06149	232.50786; 179.07074; 163.03906; 152.01048; 108.02054	$C_{12}H_{14}O_8$	Diphenol glucuronide	Phenolics
9	5.69	0.28573	-3.72	444.19923	443.19196	299.38110; 118.27349; 113.02322; 101.02323; 71.01268; 59.01274	$C_{22}H_{28}N_4O_6$	Mitoxantrone	Anthracenediones
10	5.87	0.37792	-7.54	138.03065	137.02338	93.03340; 65.01323	$C_7H_6O_3$	Salicylic acid	Phenolic acids (Phenolics)
11	6.26	0.48722	-0.68	290.07884	289.07156	245.08154; 203.07034; 179.03416; 165.01825; 161.05961; 123.04404; 109.02837	$C_{15}H_{14}O_6$	Catechin	(Flavan-3-ol) Flavonoids
12	6.36	1.41039	-1.99	240.06291	239.05563	194.4051; 179.03396; 177.05486; 149.05972	$C_{11}H_{12}O_6$	2-(3-carboxypropionyl)-6-hydroxy-cyclohexa-2.4-diene carboxylic acid	Organic acids

Supplementary 1. Continued

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
13	6.92	1.22196	-9.13	122.03567	121.02839	109.58405; 92.02530; 75.39960	$C_7H_6O_2$	4-Hydroxybenzaldehyde	Phenolics
14	7.08	2.52640	-1.75	251.07893	250.07166	206.08029; 132.02910; 88.03923	$C_{12}H_{13}NO_5$	N-feruloylglycine	Organic acids
15	8.06	1.57920	-3.29	208.07287	207.0656	163.07552; 135.04399; 122.03617	$C_{11}H_{12}O_4$	(2E)-3-(3,4-Dimethoxyphenyl) acrylic acid	Phenolic acids (Phenolics)
16	8.27	0.17267	-6.31	152.04638	151.03911	136.01553; 121.02850; 107.04912; 91.02913; 81.04485	$C_8H_8O_3$	Vanillin	Phenolics
17	8.47	0.28280	-5.5	168.04133	167.03406	152.01053; 148.04788; 135.00763; 123.00739; 108.02054; 97.03942; 71.01246	$C_8H_8O_4$	5-Methoxysalicylic acid (Isovanilic acid)	Phenolic acids (Phenolics)
18	8.90	0.28669	-1.93	233.06836	232.06108	188.07065; 186.93271; 175.94037; 160.07617; 144.95819; 134.06009; 117.03352; 114.01859; 70. 02870	$C_{12}H_{11}NO_4$	Casimiroin	Quinolines (Alkaloid)
19	9.25	0.64524	-0.63	262.14147	261.1341	252.74991; 187.09669; 176.03433; 125.09607	$C_{12}H_{22}O_6$	Etoglucid	Epoxide compounds
20	9.71	1.84078	-4.26	188.10406	187.09676	143.10672; 125.09607; 115.91956	$C_9H_{16}O_4$	Azelaic acid	Organic acids
21	9.81	0.60013	-0.68	302.20912	301.20164	283.19128; 265.18094; 227.45804; 201.11235; 183.10184	$C_{16}H_{30}O_5$	8-Hydroxyhexadecanedioic acid	Fatty acids
22	10.99	0.16824	-1.09	254.05763	253.0503	225.05492; 208.05226; 196.05215; 181.06499; 169.06447; 148.92700; 133.02829; 112.98575; 93.04716; 69.81467; 54.65988	$C_{15}H_{10}O_4$	2-[3,8-Dihydroxy-8-(hydroxymethyl)-3-methyl-2-oxodecahydro-5-azulenyl]-2-propanyl hexopyranoside (Daidzein)	Isoflavone (Flavonoids)

Supplementary 1. Continued

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
23	11.31	0.49130	0.23	286.0478	285.04053	272.0674; 243.02957; 228.78293; 215.03409; 201.01898; 183.91209; 140.02333; 135.04424; 121.02834; 91.01741; 68.94187	$C_{15}H_{10}O_6$	Luteolin	Flavone (Flavonoids)
24	12.45	0.16322	-0.93	272.06822	271.06097	270.04868; 227.07370; 177.01831; 151.00272; 119.04908; 107.01272; 93.03339; 69.05736; 63.02300	$C_{15}H_{12}O_5$	Naringenin	Flavanone (Flavonoids)
25	12.91	0.44743	-0.73	270.05263	269.04535	239.92270; 225.05682; 197.06056; 181.06477; 151.00262; 135.04396; 119.04913; 117.03366; 83.01237; 68.90342	$C_{15}H_{10}O_5$	Apigenin	Flavone (Flavonoids)
26	13.12	3.49199	-1.21	330.24022	329.23294	255.38583; 229.14388; 211.13329; 193.12473; 183.13829; 171.10170; 137.09601; 127.11177; 99,08027	$C_{18}H_{34}O_5$	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	Fatty acids
27	13.20	0.11737	-0.86	302.07878	301.0715	286.04800; 242.05663; 229.14384; 215.03334; 196.00127; 177.01842; 151.00264; 134.03624; 107.01273; 75.69307; 67.75343	$C_{16}H_{14}O_6$	3',5,7-Trihydroxy-4'-methoxyflavanone (Hesperetin)	Flavanone (Flavonoids)

Supplementary 1. Continued

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
28	13.32	0.30866	-1.29	300.063	299.05576	284.03229; 283.02383; 256.03644; 240.04305; 223.40569; 200.04709; 177.01788; 149.05960; 148.01529; 108.02058	$C_{16}H_{12}O_6$	3,5,7-trihydroxy-4'-methoxyflavone (Kaempferide)	Flavonols (Flavonoids)
29	14.04	3.24540	-1.36	268.07319	267.06592	252.04236; 251.03436; 224.04620; 196.05147; 135.00717; 132.02056; 70.42388; 63.93503	$C_{16}H_{12}O_4$	Formononetin	Isoflavones (Flavonoids)
30	15.50	0.11753	-0.89	312.22978	311.22256	264.82047; 255.09013; 179.05550; 168.52384; 119.03384; 113.02336; 101.02325; 89.02323; 71.01269; 59.01273	$C_{18}H_{32}O_4$	(10E,12Z)-9-Hydroperoxy-10,12-octadecadienoic acid	Fatty acids
31	16.39	0.23257	-1.00	284.06819	283.06094	268.03735; 240.04272; 223.03937; 211.03943; 141.39938	$C_{16}H_{12}O_5$	5,6-Dihydroxy-7-methoxyflavone (Negletein)	Flavone (Flavonoids)
32	17.25	0.11694	-0.52	310.21425	309.20694	291.19571; 273.18634; 229.97525; 211.13383; 201.11273; 185.11752; 174.95499; 171.10167; 137.09599	$C_{18}H_{30}O_4$	(9Z,11E,13S,15Z)-13-Hydroperoxy-9,11,15-octadecatrienoic acid	Fatty acids
33	17.42	0.97610	-1.49	340.13057	339.12335	286.11780; 266.79657; 206.59961; 187.11203; 151.00266	$C_{20}H_{20}O_5$	(-)-8-Prenylnaringenin	Flavanone (Flavonoids)
34	18.57	0.49398	-0.62	294.21931	293.21204	275.20117; 231.21095; 183.10252; 171.10165	$C_{18}H_{30}O_3$	(9Z)-11-{3-[(2Z)-2-Penten-1-yl]-2-oxiranyl}-9-undecenoic acid	Fatty acids

Supplementary 1. Continued

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
35	19.79	1.58303	-0.91	326.19127	325.18402	275.21072; 265.38651; 183.01123	$C_{18}H_{30}O_3S$	4-Dodecylbenzenesulfonic acid	Organic acids
36	20.08	0.87672	-0.96	408.19328	407.18591	394.00562; 377.70285; 339.19876; 271.17047; 243.13847; 151.03906	$C_{25}H_{28}O_5$	6,8-Diprenylnaringenin	Flavanone (Flavonoids)
37	21.78	0.21787	-1.17	410.20885	409.20157	391.19244; 353.98651; 326.18918; 289.13522; 246.15869; 151.03908	$C_{25}H_{30}O_5$	1-(2,4-Dihydroxyphenyl)-3-[2-[(2E)-3,7-dimethyl-2,6-octadien-1-yl]-3,4-dihydroxyphenyl]-1-propanone	Chalcones (Flavonoids)
38	22.67	0.14120	-0.72	294.21928	293.21204	249.22310; 220.14763; 196.19997; 185.11726; 167,10614; 9695892	$C_{18}H_{30}O_3$	(9Z,11E,13S,15Z)-13-Hydroxy-9,11,15-octadecatrienoic acid	Fatty acids
39	24.31	0.67995	-0.76	272.23494	271.22766	253.21716; 225.22182; 133.34665; 81.55684	$C_{16}H_{32}O_3$	16-Hydroxyhexadecanoic acid	Fatty acids

Supplementary 2. Secondary metabolites contained in 1-butanol fraction of water extract

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
1	1.07	0.6409	-1.57	342.1157	341.1084	179.05516; 89.02325; 71.01270; 59.01273	$C_{12}H_{22}O_{11}$	α,α-Trehalose	Sugar derivatives
2	1.13	0.7830	-5.45	180.0624	179.05443	163.06021; 113.02326; 89.02325; 71.01268; 59.01273	$C_6H_{12}O_6$	D-(+)-Galactose	Sugar derivatives
3	1.19	4.8498	-8.39	134.0204	133.01315	115.00253; 87.00761; 71.01269	$C_4H_6O_5$	DL-Malic acid	Organic acids
4	1.52	0.9855	-4.79	192.06247	191.05519	173.04451; 155.03418; 131.03392; 111.04386; 101.02326; 85.02835	$C_7H_{12}O_6$	D-(-)-Quinic acid	Organic acids
5	1.79	4.9457	-9.82	118.0255	117.01815	108.02873; 99.00761; 73.02834; 57.16619	$C_4H_6O_4$	Methylmalonic acid	Organic acids

Supplementary 2. Continued

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
6	2.57	0.1209	-2.38	219.1102	218.10287	184.82854; 172.97586; 146.08134; 126.94264; 88.03923	$C_9H_{17}NO_5$	D-pantothenic acid	Organic acids
7	3.40	23.3155	-1.85	256.0577	255.5046	193.05013; 165.05521; 135.04442; 119.04952; 107.04927	$C_{11}H_{12}O_7$	Piscidic acid	Phenolic acids (Phenolics)
8	5.49	0.1029	-3.44	444.1992	443.19196	299.38110; 118.27349; 113.02322; 101.02323; 71.01268; 59.01274	$C_{22}H_{28}N_4O_6$	Mitoxantrone	Anthracenediones
9	5.67	0.2424	-6.04	154.0257	153.01837	109.02839; 84.00808; 59.51074	$C_7H_6O_4$	Gentisic acid	Phenolic acids (Phenolics)
10	5.81	0.3623	-5.41	168.0413	167.03406	152.01053; 148.04788; 135.00763; 123.00739; 108.02054; 97.03942; 71.01246	$C_8H_8O_4$	5-Methoxysalicylic acid (Isovanilic acid)	Phenolic acids (Phenolics)
11	6.35	2.6959	-1.99	240.0629	239.05563	194.4051; 179.03396; 177.05486; 149.05972	$C_{11}H_{12}O_6$	2-(3-carboxypropionyl)-6-hydroxy-cyclohexa-2,4-diene carboxylic acid	Organic acids
12	6.61	0.1492	-2.81	212.0315	211.02422	196.00056; 167.03377; 152.01053; 138.96245; 123.04406; 108.02058	$C_9H_8O_6$	5-carboxyvanillic acid	Phenolic acids (Phenolics)
13	6.84	0.2621	-0.34	286.0688	285.06149	232.50786; 179.07074; 163.03906; 152.01048; 108.02054	$C_{12}H_{14}O_8$	Diphenol glucuronide	Phenolics
14	7.05	2.8804	-1.51	251.0789	250.07166	206.08029; 132.02910; 88.03923	$C_{12}H_{13}NO_5$	N-feruloylglycine	Organic acids
15	7.55	3.0875	-3.44	208.0729	207.0656	163.07552; 135.04399; 122.03617	$C_{11}H_{12}O_4$	(2E)-3-(3,4-Dimethoxyphenyl) acrylic acid	Phenolic acids (Phenolics)
16	8.05	0.1935	-0.52	432.1054	431.09814	381.06042; 341.06653; 311.05585; 283.06082; 269.04465; 239.07059; 161.02391; 117.03296; 101.02299; 89.02320	$C_{21}H_{20}O_{10}$	Apigetrin (Apigenin 7-O-beta-D-glucoside)	Flavone glucoside (Flavonoids)

Supplementary 2. Continued

Peak number	Retention time (minute)	% Area	Mass error (ppm)	Calculated molecular weight (g/mol)	[M-H]- (m/z)	Fragment ions (m/z)	Molecular formula	Proposed compound	
								Compound	Class
17	9.16	0.4324	-0.6	262.1415	261.1341	252.74991; 187.09669; 176.03433; 125.09607	$C_{12}H_{22}O_6$	Etoglucid	Epoxide compounds
18	9.70	0.5944	-7.54	138.0307	137.02338	93.03340; 65.01323	$C_7H_6O_3$	Salicylic acid	Phenolic acids (Phenolics)
19	9.99	0.1508	-0.85	254.0576	253.0503	225.05492; 208.05226; 196.05215; 181.06499; 169.06447; 148.92700; 133.02829; 112.98575; 93.04716; 69.81467; 54.65988	$C_{15}H_{10}O_4$	2-[3,8-Dihydroxy-8-(hydroxymethyl)-3-methyl-2-oxodecahydro-5-azulenyl]-2-propanyl hexopyranoside (Daidzein)	Isoflavone (Flavonoids)
20	11.32	0.1553	-0.68	302.2091	301.20164	283.19128; 265.18094; 227.45804; 201.11235; 183.10184	$C_{16}H_{30}O_5$	8-Hydroxyhexadecanedioic acid	Fatty acids
21	12.43	0.0960	-0.57	284.0683	283.06104	268.03720; 240.04237; 224.04646; 212.04779; 174.95461; 145.06088; 127.77939; 97.15845; 85.93065; 55.12834	$C_{16}H_{12}O_5$	Glycitein	Isoflavone (Flavonoids)
22	12.93	0.3316	-1.27	330.2402	329.23294	255.38583; 229.14388; 211.13329; 193.12473; 183.13829; 171.10170; 137.09601; 127.11177; 99.08027	$C_{18}H_{34}O_5$	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	Fatty acids
23	13.14	0.5510	-0.9	268.0732	267.06592	252.04236; 251.03436; 224.04620; 196.05147; 135.00717; 132.02056; 70.42388; 63.93503	$C_{16}H_{12}O_4$	Formononetin	Isoflavones (Flavonoids)

Supplementary 3. Secondary metabolites contained in 1-butanol fraction of 70% ethanol extract and 1-butanol fraction of water extract and percentage of the class composition

Class	Sub class	Compound	1-butanol fraction of 70% ethanol extract			1-butanol fraction of water extract		
			Presence	Quantity	Percentage	Presence	Quantity	Percentage
Phenolics	Phenolic acids	Piscidic acid	√	1		√	1	
		Gentisic acid	√	1		√	1	
		Salicylic acid	√	1		√	1	
		(2E)-3-(3,4-Dimethoxyphenyl) acrylic acid	√	1		√	1	
		5-Methoxysalicylic acid (Isovanilic acid) 5-carboxyvanillic acid	√	1		√	1	
	Phenolic acids	Catechin	√	1				
		2-[3,8-Dihydroxy-8-(hydroxymethyl)-3-methyl-2-oxodecahydro-5-azulenyl]-2-propanyl hexopyranoside (Daidzein)	√	1		√	1	
		Luteolin	√	1				
		Naringenin	√	1				
		Apigenin	√	1				
		3',5,7-Trihydroxy-4'-methoxyflavanone (Hesperetin)	√	1				
		3,5,7-trihydroxy-4'-methoxyflavone (Kaempferide)	√	1				
		Formononetin	√	1		√	1	
		5,6-Dihydroxy-7-methoxyflavone (Negletein)	√	1				
		(-)-8-Prenylnaringenin	√	1				
		6,8-Diprenylnaringenin	√	1				
		1-(2,4-Dihydroxyphenyl)-3-[2-[(2E)-3,7-dimethyl-2,6-octadien-1-yl]-3,4-dihydroxyphenyl]-1-propanone	√	1				
		Apigetrin (Apigenin 7-O-beta-D-glucoside)				√	1	
		Glycitein				√	1 1	
	Other phenolics	Diphenol glucuronide	√	1		√		
		4-Hydroxybenzaldehyde	√	1				
		Vanillin	√	1				
			sub total	20	51.30%	sub total	11	47.83%
Sugar derivatives		D-(+)-Galactose	√	1		√	1	
		α,α-Trehalose				√	1	
			sub total	1	2.56%	sub total	2	8.69%

Supplementary 3. Continued

Class	Sub class	Compound	1-butanol fraction of 70% ethanol extract			1-butanol fraction of water extract		
			Presence	Quantity	Percentage	Presence	Quantity	Percentage
Fatty acids		8-Hydroxyhexadecanedioic acid	√	1		√	1	
		(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	√	1		√	1	
		(10E,12Z)-9-Hydroperoxy-10,12-octadecadienoic acid	√	1				
		(9Z,11E,13S,15Z)-13-Hydroperoxy-9,11,15-octadecatrienoic acid	√	1		√	1	
		(9Z)-11-{3-[(2Z)-2-Penten-1-yl]-2-oxiranyl}-9-undecenoic acid	√	1				
		(9Z,11E,13S,15Z)-13-Hydroxy-9,11,15-octadecatrienoic acid	√	1				
		16-Hydroxyhexadecanoic acid	√	1				
			sub total	7	17.95%	sub total	2	8.69%
Organic acids		D-(-)-Quinic acid	√	1		√	1	
		DL-Malic acid	√	1		√	1	
		Fumaric acid	√	1				
		Methylmalonic acid	√	1		√	1	
		2-(3-carboxypropionyl)-6-hydroxy-cyclohexa-2.4-diene carboxylic acid	√	1			1	
		N-feruloylglycine	√	1		√		
		Azelaic acid	√	1				
		4-Dodecylbenzenesulfonic acid	√	1				
		D-pantothenic acid				√	1	
		2-(3-carboxypropionyl)-6-hydroxy-cyclohexa-2,4-diene carboxylic acid				√	1	
			sub total	8	20.51%	sub total	6	26.09%
Epoxide compounds		Etoglucid	√	1		√	1	
			sub total	1	2.56%	sub total	1	4.35%
Alkaloid	Quinolines	Casimiroin	√	1		√	1	
			sub total	1	2.56%	sub total	1	0.00%
Anthracenediones		Mitoxantrone	√	1		√	1	
			sub total	1	2.56%	sub total	1	4.35%
			Total	39	100.00%	Total	23	100.00%