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Effect of kaffir lime (*Cytrus hystrix*) leaf extract on xanthine oxidase inhibition

Hilyatul Malikhah and Nuniek Herdyastuti *

Department of Chemistry, Faculty of Mathematic and Natural Science, Universittas Negeri Surabaya, Indonesia.

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Abstract

The xanthine oxidase enzyme is an enzyme that catalyzes purine metabolic reactions, the oxidation of hypoxanthine to uric acid through xanthine compounds. High uric acid causes hyperuricemia. One treatment is to inhibit the xanthine oxidase enzyme. It can be conducted with a natural remedy, for example kaffir lime leaf extract. Research showed that kaffir lime leaves contain flavonoids that can inhibit the activity of the xanthine oxidase. This research aimed to examine kaffir lime leaf extract as an inhibitor of xanthine oxidase enzyme activity. Isolation of kaffir lime leaves was conducted by various maceration times of 24, 48, and 72 hours. The method of determining the inhibition value (IC_{50}) of kaffir lime leaf extract to the xanthine oxidase enzyme was UV-Vis spectrophotometer at λ 291,5 nm. Maceration results of kaffir lime leaf extract with phytochemical tests for 48 hours showed that there were phenolic, flavonoids, steroids, alkaloids, tannins, and triterpenoids compounds. FT-IR test showed that there were C=O, C=C aromatic, O-H, and CH-aliphatic groups, which indicated the structure of the flavonoids. Maceration of kaffir lime leaf extract for 48 hours was a weak inhibitor with an IC_{50} value of 285,82 ppm.

Keywords: Flavonoids; Kaffir lime leaf; Uric acid; Xanthine Oxidase

1. Introduction

Hyperuricemia is a disease that affects many Indonesian people. The two causes of hyperuricemia are decreased uric acid excretion in the distal tubule of the kidney's distal tubule and high uric acid levels in the body[1]. Uric acid that exceeds saturated levels in the body causes precipitation and crystallization. The usual uric acid level for men is 3.5-7.0 mg/dl, while the average uric acid level for women is 2.6-6.0 mg/dl [2]. Uric acid comes from the metabolism of purine nucleosides through the purine hypoxanthine, xanthin, and guanine bases assisted by the enzyme xanthine oxidase. The enzyme xanthine oxidase plays a vital role in converting purine bases into uric acid. During the xanthine oxidation reaction to uric acid, oxygen atoms are transferred from the molybdenum to the xanthin. The active decomposition of the molybdenum center is by adding water, as shown in Figure 1 [3]. Increased levels of uric acid due to high purine foods, such as seafood, organ meats, nuts, avocados, cheese, and beans [4]. Reducing uric acid production can treat hyperuricemia by inhibiting the enzyme xanthine oxidase, namely the synthetic drug Allopurinol or Febuxostat [5].

Allopurinol or Febuxostat are the two most used synthetic xanthine oxidase inhibitors in medicine. Febuxostat can inhibit both xanthine oxidase forms (oxidized and reduced) [6]. However, these drugs have side effects such as impaired liver function, skin rashes, nephropathy, hypersensitivity, toxic kidneys, headaches, and digestive tract disorders [7][8]. Thus, natural ingredients such as xanthine oxidase inhibitors that are non-toxic and effective for pharmaceuticals and food are needed.

* Corresponding author: Nuniek Herdyastuti

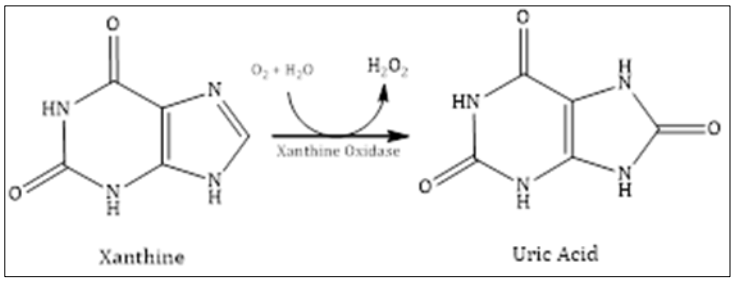

Figure 1 The xanthine reaction to uric acid is catalyzed by the enzyme xanthine oxidase [9]

Recent research reported that flavonoids could function as effective xanthine oxidase inhibitors, such as morin, quercetin, myricetin, kaempferol, and puerarin[10]. Flavonoids are secondary metabolites with a structure similar to xanthine. The charasteristics of flavonoids are having two phenil rings (A and B) and a heterocyclic ring (C) with a hydroxyl group (C-5 and C-7) on one of the phenyl rings. This hydroxyl group contributes to xanthine oxidase inhibitory activity as an electron acceptor for the enzyme xanthine oxidase [11] with a competitive inhibition mechanism [12]. Research showed the phenolic fraction Moringa oleifera lam leaves effectively inhibit uric acid production in rats by regulating serum xanthine oxidase $p<0,01$ at 500 mg/kg and $p<0,05$ at 200 mg/kg[13]. Liu et al. [14] stated the isolation of flavonoids from perilla leaf extract (Frutescens Lamiaceae) showed more potent inhibition (IC_{50} 0.21 μM) than allopurinol (IC_{50} 2.07 μM). Avocado leaves are thought to contain flavonoids with antihyperuricemia activity because these flavonoid can reduce blood uric acid levels by inhibiting xanthine oxidase activity[15]. One of the natural ingredients containing flavonoids is kaffir lime leaves (*Cytrus hystrix*) [16].

Research reveals that kaffir lime leaves contain chemical compounds such as alkaloids, flavonoids, polyphenolics, quinones, monoterpenoids and sesquiterpenoids [17]. Typically, kaffir lime leaves are used as an aromatic, seasoning, and traditional medicine to maintain healthy teeth, gums, and scurvy [18]. Kaffir lime leaf extract is helpful for antioxidant, anti-cancer, and anti-inflammatory activity [19].

Xanthine oxidase enzymes can be isolated from the liver, bacteria, and animal and milk tissues. The research revealed that the enzymes with the best characterization came from the milk of mammals, rats, and chickens. The milk of mammals has xanthine oxidase enzyme activity, for example, cow's milk. Cow's milk fat is the most protein after butyrophilin [20]. Cow's milk fat contains globule membranes where there is a concentrated xanthine oxidase. The activity of cow's milk is known by purification through ammonium sulfate precipitation and affinity chromatography. 1,0 units xanthine oxidase activity enzyme is the formation rate of 1 μM (10^{-6}) uric acid per minute[21]. Xanthine oxidase activity is the number of the products from an oxidation of xanthine substrates [22].

2. Material and methods

2.1. Equipment and Materials

Oven (*Daihan Labtech*), pH meter (*Eutech*), magnetic stirrer (*D-LAB*), thermometer, rotary evaporator (*Buchi R-300*), centrifuge (*Eppendorf 5810*), Magnetic stirrer (*Heidolp MR Hei-Standard*), vortex mixer (*Labnet VX-200*), glass jar, micropipette (*D-LAB*), analytical balance (*Adventurer Ohaus*), waterbath (*Daihan Labtech*), spectrophotometer UV-Vis (*Shimadzu spec. 1800*), spectrophotometer FT-IR (*PerkinElmer*) dan glassware (*Pyrex*).

Kaffir Lime Leaf (*Cytrus hystrix*) obtained from Bojonegoro, East Java. NaOH p.a. (*Merck*), hydrochloric acid (*Merck*), NaCl (*Merck*), xanthine substrate (*Merck*), cow's milk from distributors in Wonocolo, Surabaya. Uric acid standard, Ethanol 96%, aquadest, dimethyl sulfoxida (*Merck*), Allopurinol (*HexaPharm*), phosphate buffer solution pH 7.5, Mg powder (*Sigma Aldrich*), Mayer reagent, $FeCl_3$, chloroform (*Merck*).

2.2. Procedures and Analysis Methods

2.2.1. Kaffir Lime Leaf Extract (Cytrus hystrix)

Kaffir lime (*Citrus hystrix*) leaves were washed, drained and dried at 40°C in oven until the moisture content was below 10%. Then the leaves were mashed and sieved through a 40-mesh sieve. Then, put into a glass jar 100 grams of sieve powder was weighed. Furthermore, 96% ethanol was added with a ratio 1:10[23]. The powder was allowed for 24, 48, and 72 hours. Then, the extract was filtered using a Buchner funnel. Next, the residue was re-macerated with 96%

ethanol. Then, the extract results were evaporated using a rotary evaporator to remove the solvent. The yield of the extract was calculated:

$$\frac{\text{viscous extract weight}}{\text{extracted simplicia weight}}\ x\ 100\%$$

2.2.2. Phytochemical Screening

Identification of flavonoids by adding Hydrochloric acid and Mg powder to the extract, positive results were shown in orange to red. Identifying alkaloids, 1 ml of 2N Hydrochloric acid, 3 ml of the extract solution was added with 6 ml of aquadest. Then the mixture was heated for 2 minutes. The mixture filtered after it was cold. The result of an examination of the filtrate with Mayer's reagent was a white precipitate. Then, saponins were identified by adding the extract to distilled water and shaking vertically for 10 seconds. If stable foam appears for several minutes, it means a positive result. Identify tannins by the reaction between 1 mL of test extract solution and 10% $FeCl_3$. Tannins were indicated by the presence of a dark blue or greenish-black color [24]. —identification of triterpenoids and steroids by dissolving the extract in chloroform. Through the tube wall, 0,5 mL of anhydrous CH_3COOH and 2 mL of concentrated H_2SO_4 were added. Brownish or violet ring at the boundary of the solution indicated a positive result. In comparison, steroids were characterized by the presence of a greenish-blue ring [25]. —phenolic identification by taking 1 mL of extract plus two drops of $FeCl_3$ solution. Phenolic indicated by blue-green or green color[26].

2.2.3. Isolate Xanthine Oxidase from Cow's Milk [27]

250 mL of fresh cow's milk was heated to 30°C. Then, 80 gr of NaCl was added, centrifuged 30 minute at 3000 rpm at 4°C. This supernatant contained crude xanthine oxidase extract.

2.2.4. Xanthine Oxidase Enzyme Activity Test [27].

First, the researcher determined the optimum wavelength. Determination of the optimum wavelength of uric acid standard solution through UV-Vis spectrophotometer test at λ 200-400 nm. The test was showed that 291,5 nm is the optimum standard wavelength of uric acid. These results were used to measure the absorption of the enzyme inhibitory activity. Then the absorbance was measured in standard uric acid solutions with concentrations of 1, 3, 4, 12, and 14 ppm. The correlation curve between concentration and absorbance produced a linear equation. Then, the activity of the xanthine oxidase was tested with 3,9 mL of 0,05 M phosphate buffer solution pH 7,5 plus 2 mL of 0,15 mM substrate solution and preincubated at 35°C for 10 minutes. After that, 0,1 mL of crude xanthine oxidase solution was added and homogenized using vortex mixer. The mixed solution was incubated at 35°C for 30 minutes. The reaction was stopped by adding 1 mL of 1 N hydrochloric acid solution. Then, the absorption was measured at λ 291,5 nm using a UV-Vis spectrophotometer. The absorbance results were used to discover the enzyme concentration. The number of activities was calculated:

$$\text{mU/mL} = \frac{\text{Enzyme concentration (µmol)}}{\text{incubation time (minute)}}\ \text{x}\ \frac{\text{enzyme volume}}{\text{measured total volume}}\ \text{x}\ 1000$$

2.2.5. XanthineOxidase Enzyme Inhibition Test

Xanthine oxidase enzyme inhibition test was conducted in vitro with UV-Vis spectrophotometry at λ 291.5 nm. In addition, the researcher conducted tested Allopurinol and kaffir lime leaf extract (*Cytrus hystrix*). This test was conducted on blank solutions from controls, allopurinol comparators, allopurinol controls, samples, and sample controls. The test sample was kaffir lime leaf extract.

Sample and Allopurinol Test

Allopurinol test was conducted at concentrations of 1, 3, 4, 12, and 14 ppm. In the sample test, the concentration of the extract solution was 5, 25, 50, 75, and 100 ppm. Each 1 mL of solution was put into a test tube. Then 2,9 mL of 0,05 M buffer pH 7,5 and 2 mL of 0,15 mM substrate were added. Furthermore, the mixed solution was preincubated at 35°C for 10 minutes. After that, 0.1 mL of xanthine oxidase enzyme was added and incubated for 30 minutes. After incubation, 1 mL 1 N hydrochloric acid was added, and with a UV-Vis spectrophotometer the absorbance was measured at λ 291,5 nm.

Sample Control and Allopurinol Control Test

Each 1 mL of the test solution was put into a test tube and added 2,9 mL of 0,05 M buffer pH 7,5 and 2 mL of 0,15 mM substrate were. Next, preincubated at 35°C for 10 minutes. Then, 0,1 mL of 0.05 M buffer pH 7.5 was added and

incubated for 30 minutes. After incubation, 1 mL 1 N hydrochloric acid was added, and with a UV-Vis spectrophotometer the absorbance was measured at λ 291, 5 nm.

Blank Solution Test

2 mL of 0, 15 mM xanthine substrate was added to 3,9 mL of 0,05 M buffer solution pH 7,5. Furthermore, preincubated at 35°C for 10 minutes. 0,1 mL of xanthine oxidase enzyme was added and incubated for 30 minutes. After incubation, 1 mL of 1 N HCl was added, and the absorbance was measured at λ 291,5 nm with a UV-Vis spectrophotometer

Blank Solution Control Test

2 mL of 0, 15 mM xanthine substrate was added to 3,9 mL of 0,05 M phosphate buffer solution pH 7,5. Then the solution was preincubated at 35°C for 10 minutes. 0,1 mL of 0.05 M phosphate buffer pH 7.5 was added and incubated for 30 minutes. After incubation, 1 mL of 1 N HCl was added, and the absorbance was measured at λ 291,5 nm with a UV-Vis spectrophotometer.

The inhibitory of xanthine oxidase enzyme was expressed in their presentege of inhibition (I%) [28]=

$$\left[\frac{(A-B)-(C-D)}{(A-B)}\right]$$

- A : blank solution absorbance
- B : blank solution control absorbance
- C : sample absorbance
- D : control sample absorbance

The IC_{50} value was stated in μg/ml of sample concentration as an inhibitor of 50% enzyme activity using the regression equation of the % inhibition vs. concentration curve.

3. Results and discussion

3.1. Kaffir Lime Leaf (Cytrus hystrix) Extract and Phythochemical Screening

The results of the maceration of kaffir lime leaves were a viscous green-brown extract (Figure 2). The yield of kaffir lime leaves maceration for 24, 48, and 72 hours respectively were 21,59; 24,15; dan 22,57%.

Figure 2 Yield of maceration of kaffir lime leaves

The yield of kaffir lime leaf extract (*Cytrus hystrix*) increased in maceration up to 48 hours but decreased after 72 hours. This decrease was due to the extraction process reaching the equilibrium condition so that the optimum point appeared. It resulted in ineffectiveness in obtaining extracted components. This condition was due to the potential loss of compounds in the solution due to evaporation [29]. The yield was related to the bioactive content in kaffir lime leaves. The high yield affected the high content of substances attracted to a raw material [30]. The correct solvent ratio also increased the efficiency of the extraction process [31]. Concentration of 96% ethanol with a solvent ratio of 1:10 was the greatest method for extracting natural products [32]. Kusmartono and Yulianingtyas [33] presented the results of

the optimal conditions for extracting flavonoids from leaves of wuluh starfruit (*Averrhoa bilimbi L.*) which had been macerated for 48 hours. The results of the qualitative phytochemical test of the extract after maceration for 48 hours are shown in Table 1.

Table 1 Phytochemical Test Results of Kaffir lime Leaf Extract on Maceration for 48 Hours

Test	Results	Observations (Indicating Positive Test)
Phenolics	+	The solution was light green, and there was a white precipitate.
Flavonoids	+	The solution turned reddish.
Steroids	+	The solution turned light green after the addition of specific reagents.
Alkaloids	+	A precipitate formed when the Mayer reagent was added.
Saponins	-	No stable foam.
Tannins	+	The solution turned brownish green.
Triterpenoids	+	There was a red-brown color on the surface of the solution.

Phytochemical test results showed that the ethanol extract of kaffir lime leaves contained phenolics, flavonoids, steroids, alkaloids, tannins, and triterpenoids. Ethanol was polar and used as a solvent to attract polar secondary metabolites. The content of bioactive compounds had the potential as an inhibitor for xanthine oxidase belonging to the flavonoids [34]. It was supported by data on the FT-IR test (Figure 3).

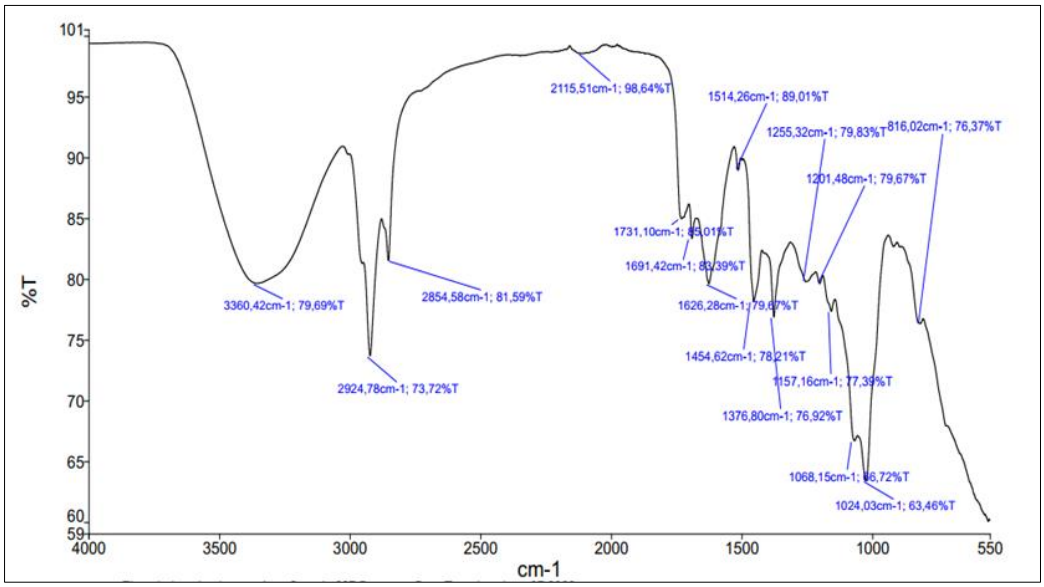

Figure 3 IR Spectra of Kaffir lime Leaf Extract in Maceration for 48 Hours

Figure 3 showed that there was absorption in the form of a wide band at 3360.42 cm^{-1}. It meant that a hydroxy group (O-H) was strengthened by the C-O group at 1300 cm^{-1} -1000 cm^{-1} and a CH-aliphatic group at wave number 2924.78 cm^{-1}. The CH-aldehyde group at wave number 2854.58 cm^{-1}. C=O stretch group with wave number 1731.10 cm^{-1}. C=C aromatic group at wave number 1626.28 cm^{-1} and C-H alkane (CH_3) group at wave number 1376.80 cm^{-1}. The results of the infrared spectrum analysis revealed that the compound had an aromatic group, namely C=O, C=C, O-H, and CH_3. Flavonoids had a carbon skeleton composed of 2 substituted benzene rings connected by a 3-carbon aliphatic chain based on the wave number in the IR result. Gabriella [35] stated that kaffir lime leaf extract contained secondary metabolites, namely flavonoids and citronellal. It was indicated by the bands at 2926.20 cm^{-1}, 2854.75 cm^{-1}, 1732.655 cm^{-1}, 1627.38 cm^{-1} and 1379.01 cm^{-1}. It can be concluded that there were C=C, C=O, CH-aliphatic, CH_2, and CH_3 groups which indicate flavonoid and citronellal compounds. This strengthens the notion that there are flavonoids in kaffir lime leaf extract which function as inhibitors of the xanthine oxidase enzyme. It strengthened the notion that there were flavonoids in kaffir lime leaf extract that function as xanthine oxidase enzyme inhibitors.

3.2. Xanthine Oxidase Enzyme Activity Test

Xanthine oxidase enzyme activity test was conducted at λ 291,5 nm with a UV-Vis spectrophotometer. The absorbance results were converted to uric acid products based on the uric acid standard curve, as shown in Figure 4. So, there was an equation y = 0.0724x - 0.0098.

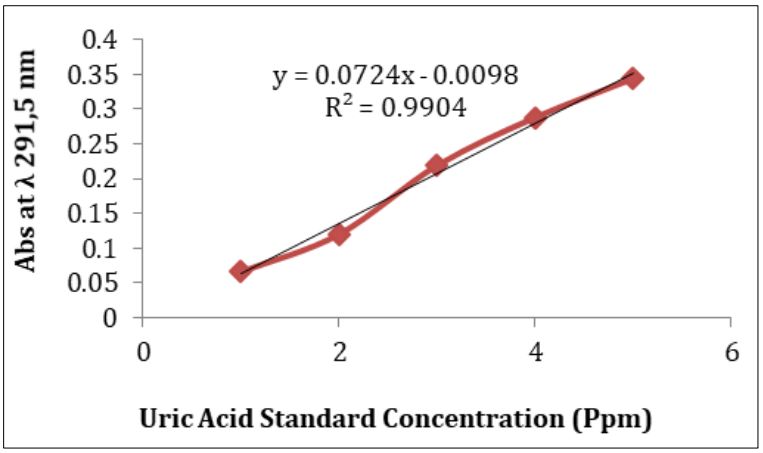

Figure 4 Uric acid standard curve

The xanthine oxidase enzyme activity test showed that the value of xanthine oxidase activity was 20,59 mU/mL. Fajriyah [27] revealed that the value of the XO enzyme activity at 35°C pH 7,5 was 4 mU/mL. Sarwawan [36] reported that the activity of the xanthine oxidase was 0,0006 U/mL at pH 7,5. Meanwhile, Al Fauzi [37] reported that the isolation of xanthine oxidase enzyme from cow's milk at pH 7,5 was 16,84 U/mL. The results are different because of the different types of cow's milk used. 1,0 units xanthine oxidase activity is the formation rate of 1 μM (10^{-6}) uric acid per minute [21].

3.3. Xanthine Oxidase Inhibition Test

The percentage inhibition was calculated by measuring the absorbance of the blank solution, control blank, sample, and control sample. The absorbance measurement of sample control and blank control was used to correct the absorbance result as actual uric acid absorbance. The UV-Visible spectrophotometric test was used because uric acid was a compound with a strong chromophore group. So uric acid could absorb ultraviolet light and visible light [12]. Temperature, pH, and substrate concentration significantly affect enzyme activity. Temperature changes and pH resulted in the structure or charge changes on the residue, which functions in binding the substrate. Substrate concentration also affected the formation rate of uric acid products [12].

The in vitro inhibition test results with a UV-Vis spectrophotometer at λ 291,5 nm and allopurinol as a positive control were shown in Table 2. The IC_{50} value was 8,62 ppm. It meant that allopurinol was able to inhibit 50% of the xanthine oxidase enzyme activity at a concentration of 8,62 ppm. The IC_{50} value of kaffir lime leaf extract (*Cytrus hystrix*) was 285,82 ppm at 5, 25, 50, 75, and 100 ppm (Table 3). It meant that kaffir lime leaf extract affected 50% inhibition of the xanthine oxidase enzyme activity at a concentration of 285,82 ppm. Tables 2 and 3 showed that the high concentration of inhibitors affected the high percentage of inhibition or there was a decrease in the xanthine oxidase enzyme activity.

Table 2 Inhibition of the xanthine oxidase enzyme by Allopurinol

Allopurinol Concentration (ppm)	Inhibition (%)
1	30,92
3	35,31
4	41,55
12	57,68
14	62,78

Table 3 Inhibition of the xanthine oxidase enzyme by kaffir lime leaf extract in maceration for 48 hours

Sample Concentration (ppm)	Inhibition (%)
5	11,69
25	13,50
50	19,32
75	22,54
100	23,49

The results showed that high concentrations of inhibitors (extracts and allopurinol) decreased H_2O_2 absorbance and increased inhibition of xanthine oxidase enzyme activity. Kaffir lime leaf extract had the ability to inhibit xanthine oxidase. It was evidenced by increasing the concentration of the extract, which decreased the absorbance of kaffir lime leaf extract. The low absorbance indicated that the uric acid product was small [12]. The test results showed that kaffir lime leaf extract contained flavonoids. Flavonoids had a structure of two phenyl rings, and a heterocyclic ring with a hydroxyl group on one of the phenyl rings[11] which was similar to the xanthine and allopurinol substrates structure (Figure 5). The hydroxyl group contributed to the inhibitory activity of xanthine oxidase as an electron acceptor. At the same time, The xanthine oxidase enzyme with a competitive inhibition mechanism compared the structural similarities of the enolxanthine with 5,7 dihydroxylflavone (Figure 6).

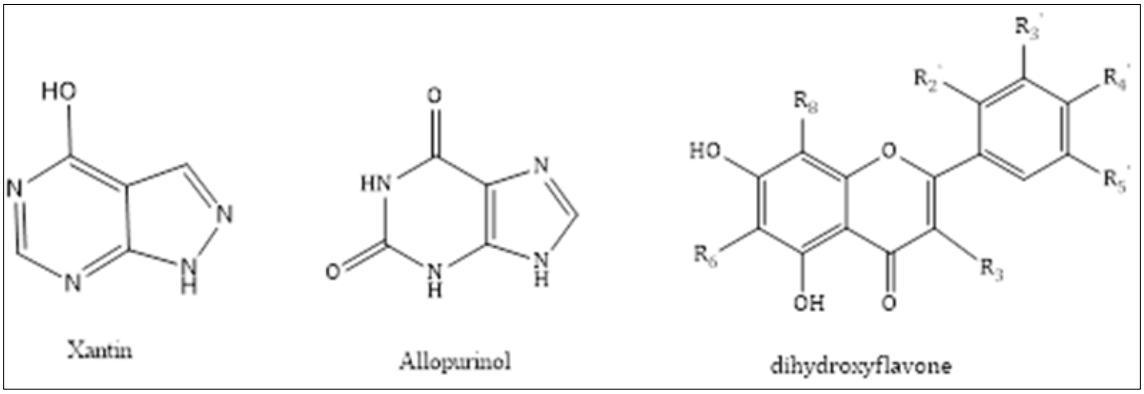

Figure 5 Comparison of structural similarities among tannins, flavonoids and allopurinol [6]

The structural similarity between the xanthine enol and 5,7-dihydroxylflavone forms indicated that the binding site at the allosteric center of xanthine oxidase was similar [12]. Kaffir lime leaf extract was weaker than allopurinol to inhibit xanthine oxidase enzyme activity. It was because natural ingredients had lower inhibitory power than synthetic drugs [38]. Allupurinol is a competitive reversible inhibitor [39] with the molecular formula $C_5H_4N_4O$ [40]. [39]. Allopurinol had a stronger affinity for xanthine oxidase enzymes than xanthin substrates. If the allopurinol inhibitor and xanthin substrate were in the same environment, the xanthine oxidase enzyme reacted more quickly with allupurinol to produce oxypurinol (alloxanthin) [41]. So Allopurinol had a small IC_{50}. The low IC_{50} caused an increase in the inhibitory value of the xanthine oxidase enzyme [42]. The xanthine oxidase enzyme consisted of protein molecules which were flavoprotein enzymes of the oxidoreductase class [43]. Xanthine oxidase enzymes catalyzed purine metabolic reactions, namely the oxidation of hypoxanthine to uric acid through xanthine compounds [22]. The process of xanthine oxidation reactions formed uric acid. The xanthine oxidase enzyme catalyzed the removal of electrons from a substrate using oxygen as its hydrogen or an electron acceptor. Xanthine as a substrate binded to oxygen through the xanthine oxidase enzyme. So uric acid and H_2O_2 were formed [22]. Kaffir lime leaves were classified as weak inhibitors with an IC_{50} value of 100 ppm. IC_{50} was classified as strong if the value was 50-100 ppm, 100-150 ppm was moderate, and more than 150 ppm was weak [44]. Besides inhibitors of the xanthine oxidase enzyme, Sari [45] explained that the flavonoids in kaffir lime leaves had antioxidant activity through free radical scavenging with an IC_{50} value of 187.36 ppm. It also included moderate antioxidants to prevent degenerative diseases. The IC_{50} value of kaffir lime leaf extract was lower than previous studies on avocado leaf extract with an IC_{50} value of 65.55 ppm [15], and papaya seed extract with an IC_{50} value of 101.80 ppm. It was higher than the inhibition value of kaffir lime leaf extract due to differences in the content of flavonoid compounds in each sample [38].

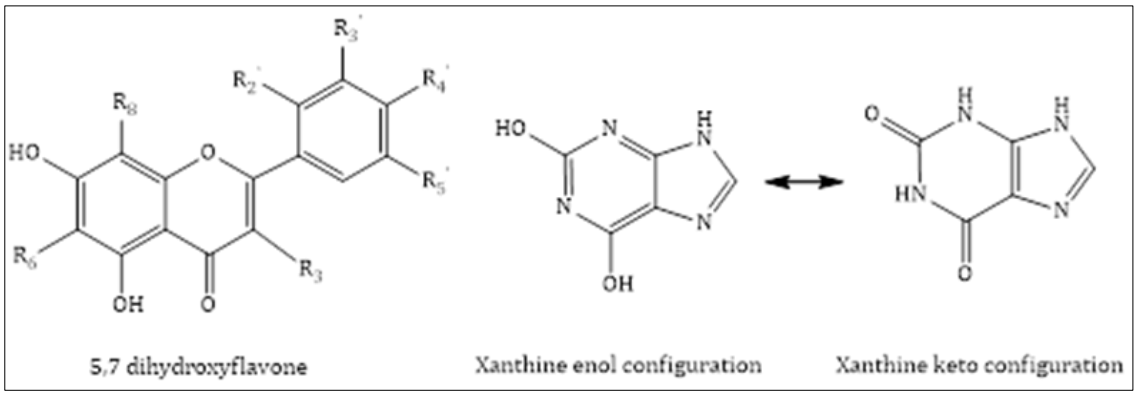

Figure 6 Comparison of structural similarities among xanthine enol and 5,7-dihidroksilflavon [12]

4. Conclusion

Based on the research, it can be concluded that kaffir lime leaf extract macerated for 48 hours is suspected of containing flavonoids and has the ability to inhibit the activity of xanthine oxidase enzyme indicated by IC_{50} value of 285,821 ppm.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared no potential conflicts of interest, financial interest, or personal relationships with respect to the research, authorship, or publication of this article.

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