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Xanthine Oxidase Inhibitor Activity of Dichloromethane Fraction, Ethyl Acetate Fraction of Ethanol Extract of Nephelium Lappaceum L. Leaves

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Abstract

The prevalence of hyperuricemia increases from year to year in various countries. In Italy, increased from 85.4/1,000 to 119.3/1,000 in 2005-2009. In Ireland from 2006-2014, it increased from 19.7% to 25.0%. Allopurinol, as a commonly used drug, causes various side effects, so it needs an alternative treatment based on natural ingredients that have higher safety. This study aims to determine the activity of ethanol extract, dichloromethane fraction, and ethyl acetate fraction Nephelium lappaceum L. leaves in inhibiting xanthine oxidase and identification of flavonoid compounds. The xanthine oxidase testing was carried out using UV-Vis spectrophotometry (λ 295 nm) with allopurinol as a positive control. Flavonoid identification was carried out using TLC (cellulose as stationary phase and HOAc 30% as mobile phase) and spectral observations with NaOH 2M, NaOAc, NaOAc/H3BO3, AlCl3, AlCl3/HCl. The results showed that ethanol extract, dichloromethane fraction, and ethyl acetate fraction of N. lappaceum leaves had activity as xanthine oxidase inhibitors, with the highest activity in ethyl acetate fraction (IC50 17.506 µg/mL), then ethanol extract (IC50 31,148 µg/mL), and dichloromethane fraction (IC50 41,737 µg/mL) with allopurinol (IC50 3,582 µg/mL). The identification found that ethyl acetate fraction of leaves N. lappaceum was a flavonol flavonoid and had an Rf value of 0.14, which was the same as quercetin.

Keywords: Nephelium lappaceum L. leaves; Hyperuricemia; Xanthine oxidase; Inhibitory activity; Flavonoids

Introduction

Hyperuricemia is a condition that increases serum uric acid levels above normal values, in men more than 7 mg/dl and in women more than 6 mg/dl. In various countries, the prevalence of hyperuricemia continues to increase from year to year. In Italy, the prevalence of hyperuricemia increased from 85.4 per 1,000 population in 2005 to 119.3 per 1,000 population in 2009. The incidence increased with increasing age and four times higher in men [1]. In Ireland from 2006 to 2014, the prevalence of hyperuricemia increased from 19.7% to 25.0% in men and from 20.5% to 24.1% in women [2]. Based on research conducted in the Tenganan Pegringsingan Village of Karangasem, Bali, in 51 men and 49 women in the age range of 13 years to 69 years, the incidence of hyperuricemia was found at 28%, namely 21% in men and 7% in women [3]. The prevalence of hyperuricemia in Depok City, West Java, is 18.6% and is more common in men [4]. Prolonged hyperuricemia can cause gout. Gout occurs due to the deposition of monosodium urate crystals in tissues or due to the supersaturation of uric acid in the extracellular fluid [5]. The synthetic drug commonly used to treat gout is allopurinol. But allopurinol has several side effects, which can cause peripheral neuritis, spinal cord depression, sometimes aplastic anemia, liver toxicity, intestinal nephritis, and can also cause cataracts because allopurinol can be bound to the eyepiece [6]. Allopurinol works by inhibiting xanthine oxidase in converting hypoxanthine to xanthine and xanthine to uric acid. Xanthine oxidase is a very versatile flavoprotein enzyme. In xanthine oxidase, purine hydroxylation is catalyzed, especially the conversion of xanthine to uric acid, which is more responsible for several diseases such as gout, kidney

disease, and stone formation urinary system [7]. Natural xanthine oxidase inhibitors with a higher safety level are readily available and affordable prices can be an alternative treatment. One of the plants that can be utilized is N. lappaceum leaves, widely available in Indonesia. N. lappaceum leaves contain secondary metabolites, namely flavonoids, saponins, and tannins with isolation compounds, namely flavonoid flavonol compounds which are thought to have xanthine oxidase inhibitors [8,9]. According to Cos et al. (1998), flavonoids can inhibit xanthine oxidase and flavonoid flavone, and flavonol groups have higher inhibitory power than other flavonoid groups because the position of the hydroxyl group is easier to capture electrons from the active side of xanthine oxidase. The ability of flavonoids to inhibit xanthine oxidase activity is through competitive inhibition mechanisms and interactions with enzymes in the side groups [10]. N. lappaceum leaves have been investigated in previous studies in various activities, including antioxidants, antidiabetic, and antibacterial [11,12]. However, there has never been a study of xanthine oxidase inhibitors from N. lappaceum leaves. Based on the above explanation, the authors want to conduct a study on the activity of xanthine oxidase inhibitors from ethanol extract, dichloromethane fraction, and ethyl acetate fraction of N. lappaceum leaves and identify their flavonoid compounds.

Research Procedure

This section covers the detailed research procedure applied in this research.

Extracts Preparation and Hydrolysis

The extraction of N. lappaceum leaves was carried out using the maceration method for 2 × 24 hours. Powder of N. lappaceum leaves weighed as much as 500 g. The solvents used were 70% ethanol with a ratio of 1:10 on the first day and 1: 4 on the second day [13]. The first and second macerates were evaporated using a rotary evaporator 30-40ºC and then evaporated with a water bath to obtain thick extracts [14,15]. The extract and the yield obtained were calculated. 50-gram ethanol extract of N. lappaceum leaves was hydrolyzed with methanol: HCL 2 N (1: 1) as much as 250 ml, then refluxed at 1000C for 1 hour [16].

Making dichloromethane fraction and ethyl acetate fraction

The ethanol extract, which has been hydrolyzed, is then ± 100 ml of distilled water so that the ethanol extract can be dispersed in aquabides to facilitate the distribution of compounds based on the solubility that occurs during fractionation. Fractionation was carried out by using 250 ml dichloromethane solvent (divided into 3 shuffles) in a separating funnel and followed by fractionation using 250 ml ethyl acetate solvent (divided into 3 shakes) [17, 18].

Identification of flavonoids by thin layer chromatography (TLC)

Quercetin, ethanol extract of N. lappaceum leaves, dichloromethane fraction, and ethyl acetate fraction were weighed 5 mg then dissolved with p.a ethanol as much as 5 ml. Each sample and comparison quercetin was then bottled on the cellulose plate and eluted with 30% glacial acetic acid. Spots were observed under 366 nm UV light before and after ammonia evaporation and were observed after spraying the sitroborate reagent and heated in a 105 0C oven for 5 minutes, recording the results [19].

Making xanthine solution 100 µg / ml

Pure xanthine is weighed as much as 100 mg, then dissolved with a few drops of 0.01 N NaOH, then added phosphate buffer to 100 ml so that the concentration of 1000 µg / ml is obtained. From xanthine 1000 µg / ml solution, 1 ml was taken, then added phosphate buffer to 10 ml so that a concentration of 100 µg / ml was obtained.

Making 0.1 U / ml xanthine oxidase solution

Xanthine oxidase is made at a concentration of 0.1-0.2 U / ml in fresh conditions (Anonim, 1994). 0.1 ml of xanthine oxidase was dissolved in phosphate buffer pH 7.5 to 3.5 ml volume so that the concentration of 0.1 U / ml was obtained.

Making test solutions

(Allopurinol, ethanol extract, dichloromethane fraction, and ethyl acetate fraction)
The mother liquor is made using each sample weighed as much as 10 mg then added DMSO as much as 5 drops and added aquabidestilata to a volume of 10 ml (1000 µg / ml). For allopurinol, the mother liquor is diluted so that a concentration of 2.5 µg / ml is produced; 5 µg / ml; 7.5 µg / ml; and 10 µg / ml. Whereas for ethanol extract samples, dichloromethane fraction, and ethyl acetate fraction, concentrations of 10 µg / ml, 13 µg / ml, 17 µg / ml, and 20 µg / ml were made.

Determination of xanthine oxidase activity

The 450 µg / ml (xanthine) substrate (450 µl) was added with a mixture of 450 µl xanthine oxidase 0.1 U / ml and 424 µl phosphate buffer pH 7.5. The speed of uric acid formation from xanthine was observed in spectrophotometry at a wavelength (λ) 295 nm from the 0th minute to the 5th minute at 25ºC. The data obtained is in the form of a rate (Δ A295 / minute). The method follows with several changes [20].

Determination of inhibition of xanthine oxidase activity

The 450 µg / ml (xanthine) substrate (450 µl) was added with a mixture of 450 µl xanthine oxidase 0.1 U / ml and 424 µl phosphate

buffer pH 7.5. Then added allopurinol 200 µl at a concentration of 2.5 µg / ml; 5 µg / ml; 7.5 µg / ml; and 10 µg / ml. In the same way, it was also determined the inhibition of xanthine oxidase activity by 200 µl of the test solution namely ethanol extract, dichloromethane fraction, and ethyl acetate fraction at concentrations of 10 µg / ml, 13 µg / ml, 17 µg / ml, and 20 µg / ml. The rate of uric acid formation was observed in spectrophotometry at wavelength (λ) 295 nm from 0 minutes to 5 minutes at 25ºC. The data obtained is in the form of a rate (Δ A295 / minute). The method follows with several changes (Figure 1).

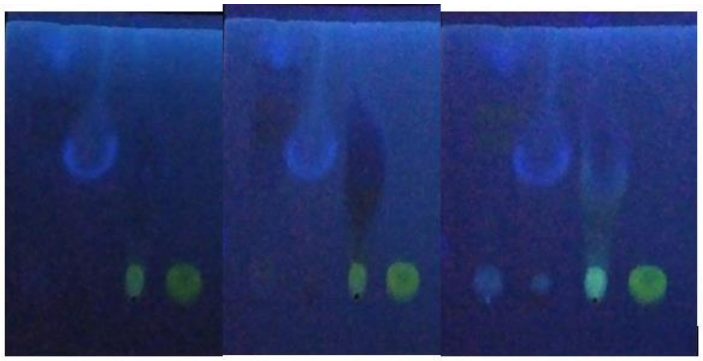

Figure 1: *TLC plate profile with cellulose stationary phase and 30% glacial acetic acid mobile phase seen on UV light 366 nm, A: before ammonia is steamed, B: after ammonia is evaporated, C: After being sprayed with sitroborate. Information: E = ethanol extract, DCM = dichloromethane fraction, EA = ethyl acetate fraction, K = quercetin.*

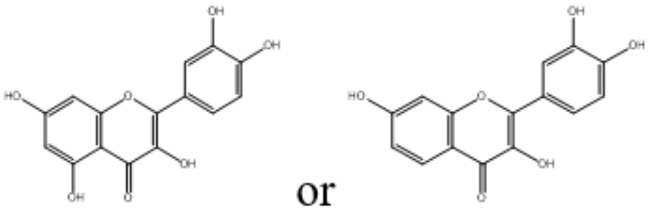

Figure 2: *Possible structure of flavonoids: (1)2-(3,4-dihidroksifenil)-3,5,7-trihiroksi-4H-chromen-4-one or (2) 2-(3,4-dihidroksifenil)-3,7-dihiroksi-4H-chromen-4-one.*

Characterization flavonoid by using spectrophotometri UV-Vis

As much as 0.1 mg of ethyl acetate fraction was dissolved with 10 ml methanol pa, then the spectrum was measured using UV-Vis spectrophotometry at a wavelength of 200-400 nm with methanol pa as blank, then NaOH, NaOAc, NaOAc / H3BO3, AlCl3 were added. And AlCl3 / HCl. The types of flavonoids are determined and changes in the spectrum caused by various shear reactants are interpreted (Diniatik, 2016).

Data Analysis

a. Identification of flavonoids

Rf is clculated by formula (21):

$$Rf = \frac{distance\ of\ sample}{distance\ of\ eluen}$$

b. Activity of xanthine oxidase inhibitors

The xanthine oxidase activity is calculated by the formula (Markham, 1988):

$$\text{Activity (unit/ml enzim)} = \frac{(\text{rate x } 1.324)}{(12{,}2 \text{ x } 0{,}450)}$$

Information:

1,324 = total mixture volume (ml)
12.2 = uric acid extention coefficient (mM)
0.450 = volume of enzyme used (ml)
Rate = absorbance / minute

For xanthine oxidase activity after addition of the test solution or sample, the total volume of the mixture becomes 1.524 ml.

Then% inhibition was calculated using xanthine oxidase activity data using the formula: % inhibisi

$$= \frac{activity\ without\ sample - sample\ activity}{activity\ without\ sample}\ x\ 100\%$$

IC50 values indicate the number of inhibitors needed to achieve a 50% inhibition of the enzyme. The IC50 value was determined by linear regression analysis where the x axis showed the sample concentration and y axis indicating% inhibition. From the equation y = a + bx the IC50 value can be calculated using the following formula (Putri, 2016):

$$IC50 = (50\text{-}a) / b$$

Results and Discussion

The extraction process of N. lappaceum leaves was carried out by the maceration method using ethanol solvent. Ethanol has volatile properties, is cheap, easy to obtain, and relatively safe. 70% ethanol solvent was chosen because 70% of ethanol can attract polar, semipolar, and non-polar compounds. Maceration is the simplest extraction method because it is easy to do, inexpensive, and does not require sophisticated equipment (Diniatik, 2016). From the maceration process, we obtained 130 grams of the thick extract with a 26% ration. Hydrolysis is carried out to separate aglycones and glycons. Fractionation is intended to separate the mixture of chemical components contained in the extract using two solvents that do not mix. The chemical components present in plant extracts will dissolve into solvents that are in accordance with the level of polarity possessed by the compound. From the results of the interpretation of the colour of the spots, the possibility of flavonoids contained in the ethanol extract, namely chalkon and isoflavone, in the dichloromethane fraction, namely isoflavones, and in the ethyl acetate fraction, there may be flavonol, khalkon, and flavone. Whereas on the KLT plate, after being sprayed with sitroborate, the quercetin spots and ethyl acetate fraction produced

a greenish-yellow glow under the light of 366 nm, which was more intense than before being sprayed with the sitroborat. This condition shows the possibility that ethyl acetate fraction contains quercetin flavonoids, which are supported by Rf values. The Rf value of ethanol extract is 0.14; 0.725 and 0.95; the dichloromethane fraction is 0.95 and 0.125, whereas in ethyl acetate fraction is 0.14; 0.412; and 0.487; with comparison of quercetin Rf 0.14. Spots 1 on the ethyl acetate fraction have the same Rf value as quercetin and produce a yellow luminescence after being sprayed with sitroborate, thus proving that the ethyl acetate fraction of N. lappaceum leaves contain quercetin flavonoids. According to ethanol extract, n-hexane extract, and ethyl acetate extract of N. lappaceum leaves contain quercetin flavonoids around 1.83-9.59 g quercetin equivalents / 100 g with quercetin content highest on ethyl aseta extract [21-23].

Xanthine Oxidase Inhibitor Activity Test

This study aims to determine the ability of ethanol extract, dichloromethane fraction, and ethyl acetate fraction of N. lappaceum leaves and compared with allopurinol in inhibiting xanthine oxidase enzyme activity in vitro. The principle of xanthine oxidase reaction, xanthine, has linear reaction kinetics for 6 minutes.

Table 1: *Extract.*

Powder	Extract	Randemen	Characteristics		
			Form	Colour	Odor
500 g	130 g	26%	Thick	Green	Unique

Table 2: *Results of fractionation of ethanol extract of N. lappaceum.*

Ethanolic extract weight	Fractions	Weight (g)	Randemen (%)	Characteristics		
				Form	Form	Form
50 g	Dichloromethane	0.2	0.4	Thick	Green	Unique
	Ethyl Acetate	0.42	0.84	Thick	Chocolate	Unique

Table 3: *Test results for xanthine oxidase activity.*

Sample	Concentration(µg/ml)	Activity (unit/mg solid)
Placebo	0	0.001447
Ethanol extract	10	0.001249
	13	0.001194
	17	0.001083
	20	0.000999
Dichloromethane fraction	10	0.001415
	13	0.001360
	17	0.001276
	20	0.001193
Ethyl acetate fraction	10	0.000971
	13	0.000860
	17	0.000749
	20	0.000638
Allopurinol	2,5	0.000777
	5.0	0.000666
	7.5	0.000527
	10	0.000471

Table 4: *% inhibition value of ethanol extract, dichloromethane fraction, ethyl acetate fraction N. lappaceum leaves, and allopurinol.*

Sample	Concentration(µg/ml)	Inhibition (%)
Placebo	-	0
Ethanol extract	10	13.67
	13	17.50
	17	25.18
	20	30.93

Dichloromethane fraction	10	2.16
	13	5.99
	17	11.75
	20	17.50
Ethyl acetate fraction	10	32.85
	13	40.52
	17	48.20
	20	55.87
Allopurinol	2.5	46.28
	5.0	53.95
	7.5	63.54
	10.0	67.38

Table 5: *IC50 values of allopurinol, ethanol extract, dichloromethane fraction, and ethyl acetate fraction of N. lappaceum leaves*.

Sample	**Linear Regression Equations**	**IC_{50} (µg/ml)**	**Rata-rata IC_{50} (µg/ml) ± SD**
Ethanol extract	y = 1.5877x – 0.5521	31.853	31.148 ± 0,997
	y = 1.9184x – 8.3912	30.443	
Dichloromethane fraction	y = 1.4554x – 11.037	41.949	41.737 ± 0,300
	y = 1.5877x – 15.9	41.524	
Ethyl acetate fraction	y = 2.2492x + 12.546	18.359	17.506 ± 1,206
	y = 2.2492x + 8.7092	16.653	
Allopurinol	y = 2.6091x + 40.529	3.630	3.582 ± 0,067
	y = 3.223x + 38.61	3.533	

Table 6: *UV-Vis spectrum data from ethyl acetate fraction before and after addition of shear reagents*.

Solvent	**Wavelength(nm)**		**Sift**		**Conclusion**
	Pita I	**Pita II**	**Pita I**	**Pita II**	
Ethyl acetate + methanol	372	255	-	-	Flavonol (3-OH bebas)
Methanol + NaOH	431	281	+59	+26	4´-OH
Methanol + NaOAc	383	269	+11	+14	7-OH
Methanol + NaOAc/H_3BO_3	389	263	+17	+8	o-diOH at cincin B
Methanol + $AlCl_3$	428	266	+56	+11	Maybe 3-OH (with or without 5-OH)
Methanol + $AlCl_3$/HCl	431	266	+59	+11	-

The velocity of uric acid formation has 295 nm with a linear curve from minutes 0-3 and ramps in the 4th minute and the xanthine oxidase activity value is 0.001447 units/ml enzyme. The decrease in xanthine oxidase activity is caused by the addition and increase in the test material concentration, where the higher the concentration, the greater the decrease in xanthine oxidase activity produced (Tables 1-3). The % inhibition value in table shows that the greater the concentration of the test material, the greater the percentage of inhibition so that the xanthine oxidase activity decreases. Based on the% inhibition value, the IC50 value was then determined for each test material. The IC50 value is obtained from a linear regression equation between the concentration of the test material (x-axis) and the percentage of inhibition of xanthine oxidase activity by the test material (y-axis). The smaller the IC50 value indicates, the better the inhibition of xanthine oxidase activity. The test for inhibition of xanthine oxidase activity showed that the ethanol extract of N. lappaceum leaves, dichloromethane fraction, and ethyl acetate fraction had xanthine oxidase inhibitor activity (Table 4). The IC50 values in sequence are ethyl acetate fractions with IC50 value 17.506 µg / ml; ethanol extract with IC50 value 31,148 µg / ml; and dichloromethane fraction with IC50 value 41.737 µg / ml. Allopurinol, as a positive control, had an IC50 value of 3,582 µg / ml. The IC50 value of allopurinol produced was not much different from the IC50 value of allopurinol carried out by which was 3.16 µg / ml. The ethyl acetate fraction has the best IC50 value, but it is still fragile compared to IC50 allopurinol. Ethanol extract and dichloromethane fraction are thought to contain compounds other than flavonoids to have a large

IC50 value. Flavonoids can inhibit xanthine oxidase caused by the presence of hydroxyl groups on C-5 or C-7 atoms and the double bond between C-2 and C-3, which allows an addition reaction (oxidase by xanthine oxidase), so that ring B becomes co-planar against rings A and C. The ability of flavonoids to inhibit xanthine oxidase activity is through competitive inhibition mechanisms and interactions with enzymes in the side groups. The results showed that quercetin flavonoids had considerable xanthine oxidase inhibition activity with inhibitory values of 0.44 µM, compared to luteolin of 0.96 µM, and kaempferol of 0.67 µM. So plants containing quercetin have xanthine oxidase inhibitors and have the potential to treat gout. According to in addition to flavonoid compounds, polyphenols and saponins can also inhibit the action of the xanthine oxidase enzyme. Based on data analysis using one way ANOVA, the significance value was 0,000 <0,05, which means there were significant IC50 differences between allopurinol, ethanol extract, dichloromethane fraction, and ethyl acetate fraction from N. lappaceum leaves. So it can be concluded that the variation of solvent has a significant effect on IC50 values.

Identification of Flavonoids with UV-Vis Spectrophotometer

The structure of flavonoids contains a conjugated aromatic system so that it shows strong absorption bands in the UV spectrum region and visible spectrum [15]. The spectrum determination was carried out on ethyl acetate fraction as the most active fraction using a UV-Vis spectrophotometer with the addition of 2 M NaOH, NaOAc, NaOAc / H3BO3, AlCl3, and AlCl3 / HCl. Based on the results, ethyl acetate footage in methanol has a wavelength in band I of 372 nm and band II of 255 nm, indicating the presence of flavonol. According to Markham (Markham, 1998), flavonoid flavonols (free 3-OH) have wavelengths in the band I range between 350-385 nm and band II between 250-280 nm. Based on previous research conducted by isolate compounds found in N. lappaceum leaves are flavonoid compounds, namely flavonol. The UV-Vis spectrum data from ethyl acetate before and after added shear reagent can be seen in (Table 6). The addition of NaOH 2 M to ethyl acetate solution in methanol, causing a right-handed batochrome shift of 85 nm without a decrease in absorption strength, showed a hydroxyl group at 4´-OH position. The addition of a base of sodium hydroxide which is a strong base, can ionize many hydroxy groups in the flavonoide nucleus which show phenolic compounds with batochromic shift characteristics. The NaOAc shear reagents added shifted the position of the maximum absorbance in the sample by 11 nm in the I band and 14 nm in the II band. This shift occurs because of ionization in the most acidic hydroxyl groups of flavonoids, which in flavonoids are owned by 7-OH groups and oxygenation in C6 or C8. Shear reagent NaOAc / H3BO3 is used to detect the o-diOH (ortho-dihydroxy) group, which reacts by bridging the two hydroxyl groups in the o-diOH group. The addition of NaOAc / H3BO3 shear reagents to the sample caused a spectrum shift of 17 nm in the I band and 8 nm in the II band, which showed o-diOH in ring B. The addition of the AlCl3 shear reactor caused a shift in 56 nm in the I band and 11 nm in the II band. The increase in the spectra's intensity is because there is still glucose in the sample, which can increase the intensity. This condition shows that there are 3-OH with or without 5-OH.

While the addition of AlCl3 / HCl, there was a shift in the spectrum of the I band of 59 nm and the band II of 11 nm. The addition of AlCl3 and AlCl3 / HCl can form acid-resistant complexes between conflicting hydroxyl and ketone groups and form acid-resistant complexes with ortho-dihydroxy groups, these reagents can be used to detect both groups. So the AlCl3 spectrum is the sum of the effects of all complexes on the spectrum, while the spectrum of AlCl3 and HCl is only the influence of the hydroxy-keto complex. Based on the results of UV spectra, it is possible to predict the possibility of flavonoid structures found in the ethyl acetate fraction of N. lappaceum leaves, namely:

Conclusion

The possibility of flavonoids contained in ethanol extracts is chalkon and isoflavone, in dichloromethane fractions namely isoflavones. In contrast, in ethyl acetate fractions namely chalcone, flavone, flavonols containing 3-OH are free and have or do not have 5-OH free (sometimes derived from dihydroflavonol) and has an Rf value of 0.14 which is the same as quercetin.Ethanol extract, dichloromethane fraction, and ethyl acetate fraction from N. lappaceum leaves have activities as xanthine oxidase inhibitors. The highest IC50 value was found in ethyl acetate fraction with IC50 value 17.506 µg / ml; ethanol extract has an IC50 value of 31,148 µg / ml; while the dichloromethane fraction had the smallest IC50 value of 41.737 µg / ml with positive control of allopurinol, which produced an IC50 value of 3.582 µg / ml. The peak of the UV-Vis spectrum ethyl acetate fraction is at 372 nm (band I) and 255 nm (band II) shows the presence of flavonol (3-OH is free). Possible structures found in ethyl acetate fractions are 2- (3, 4-dihydroxyphenyl) -3, 5, 7-trihiroksi-4H-chromen-4-one or 2- (3, 4-dihydroxyphenyl) -3, 7-dihroxy -4H-chromen-4-one.

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