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Research Article

SURVEYING THE ABILITY TO INHIBIT XANTHINE OXIDASE ENZYME OF THE EXTRACT OF GREEN AMARANTHUSVIRIDIS L

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ABSTRACT

For a long time, many studies have proven that medicinal plants have potential XO inhibitors, in which Xanthine oxidase (XO) plays an important role in converting purine into uric acid. Overproduction and high accumulation of uric acid in the body cause some serious diseases, especially gout. Therefore, selective XO enzyme inhibitors will be therapeutic agents for some uric acid-related diseases. This study is conducted with the purpose of evaluating the ability of inhibiting XO of extracts from Amaranthus plants at the in vitro level. Plant samples are extracted by soaking with 70% ethanol. The ability to inhibit XO is determined by spectrophotometry based on uric acid formation at 290 nm. The results showed that the Green Amaranthushad an efficiency of extracting ethanol, equivalent to 5.0%. The ability to inhibit XO of the extract is shown by the IC50 value of 243 µg/mL and by a mixed mechanism. The extract has the ability to eliminate DPPH free radicals with a corresponding IC50 value of 207 µg/mL. Qualitative analysis shows that the extract contains XO-inhibiting compounds called flavonoids. Quantitative analysis by colorimetric method with aluminum chloride determined the amount of total flavonoids and flavonols in the ethanol extract of amaranth to be 1.35±0.11 and 0.47±0.03 g/100g of dry substance. In general, the extracts in this study show the ability to inhibit xanthine oxidase enzyme in a mixed way.

Keywords: Gout, Green Amaranthus, Xanthine oxidase, Flavonoids, Urid acid,.

INTRODUCTION

The enzyme xanthine oxidase (XO) plays an important role in the final stage of purine metabolism. It catalyzes the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid. Due to a number of exogenous and endogenous causes, the amount of uric acid in the body will accumulate in high concentrations, causing a number of disorders in the body, including gout. XO inhibitors hinder the biosynthesis of uric acid and reduce uric acid levels in the blood and are widely applied in the treatment of gout.

Allopurinol is an enzyme inhibitor, which is often used to treat gout, but it also causes many side effects such as hepatitis and allergies. Natural products are excellent sources of ingredients that contain compounds capable of treating many diseases. Therefore, active ingredients originating from nature are increasingly receiving research attention because of their safety and effectiveness. Many tests have been conducted on a number of medicinal herbs commonly used by folk to treat gout and some symptoms such as arthritis, inflammation, kidney stones... and have proven their ability to inhibit as well as Identify substances such as phenol, steroids, and flavonoids as the main inhibitory agents.

To find a close and promising source of XO inhibition from nature, the research is conducted on the Amaranth family tree, which is often used by folk to treat gout and some related diseases such as arthritis and inflammation. Studies analyzing the composition of some *Amaranth* plants have isolated a number of biologically active substances such as steroids, saponins, and flavonoids. In particular, flavonoids have a very good antioxidant role, inhibit cancer cell growth and are considered a potential inhibitor of many enzymes such as xanthine oxidase, cyclooxygenase, phosphoinositide 3-kinase and lipooxigenase. The research "Surveying the ability to inhibit xanthine oxidase enzyme of the extract of Green *Amaranthusviridis*L", is conducted to evaluate the ability of ethanol extract to inhibit xanthine oxidase enzyme.

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2. RESEARCH METHODS

2.1. Means and materials

2.1.1. Research time and location

Time: from October 2021 to May 2023.The experiments are conducted at Phat Truong Laboratory, Can Tho University of Medicine and Pharmacy Laboratory.

2.1.2. Materials

Amaranth leaves collected in An Khanh ward, NinhKieu district, Can Tho city. The samples must be picked before 8 to 9 a.m. because during this time the temperature will not be too high to affect the water content in the sample. In addition, at this time there are no UV rays, which will affect the active ingredients in the plant. Keep the medicinal herbs clean, then ish and chop them.

2.1.3. Chemicals

- Extract chemicals: C_2H_5OH 90%
- Qualitative chemicals: KI, I_2, $Pb(OAc)_2$, $FeCl_3$ (Xilong Chemical Co., Ltd;; China).
- Chemicals to investigate the ability to inhibit XO: Xanthine (Sigma; USA); Xanthine oxidase extracted from cow's milk 25 U (Sigma; USA); $Na_2HPO_4.12H_2O$, KH_2PO_4(Xilong Chemical Co., Ltd; China), DMSO (Guangdong Guanghua Sci-Tech Co., Ltd, China)

- Quantitative chemicals for total flavonoids and flavonols: $NaNO_2$; CH_3COONa; NaOH; $AlCl_3$(Xilong Chemical Co., Ltd; China); C_2H_5OH (VnChemsol Co., Ltd; Vietnam); Quercetin (Ministry of Health; Vietnam)
- Chemicals to investigate antioxidant capacity: DPPH (Sigma, USA).

2.1.4. Equipment - Appliances

- Equipment - extract: Electronic scale (Sartorius, Germany); drying oven (Binder, Germany); rotary evaporator (IKA, Germany).
- Equipment and tools for experiments: Analytical balance (Shimadzu, Japan); spectrophotometer (Thermo, USA); pH meter (EUTECH, USA); micropipette (10-100µL, 200µL, 100-1000µL) (Bio-rad, USA).

2.2 Research methods

2.2.1 Extraction method

After collecting the fresh sample, ish it, drain it, weigh the fresh weight, then dry it until the weight remains constant and weigh again to determine the weight of the dry sample. Determine the moisture of samples.Then grind finely into powder and wrap with filter paper to follow the soaking technique with ethanol (70%). Sample/70% ethanol ratio is 1:10 (mg/mL)

After soaking the sample for 3 days (72 hours) at room temperature, the remaining bait or biomass is filtered out, the extracted solution is evaporated under low pressure and at a low temperature of about 55°C to collect the ethanol solvent by rotary evaporation system.After being recovered, the crude extract is dried by using a vacuum dehumidifier, then weighed to calculate yield and stored at 4°C for use in the following experiments.

- Water content in materials

Water content (X) converted to percentage is calculated according to the formula:

$$X = \frac{m_t - m_k}{m_t} \times 100\%$$

In there:

m_K: Mass of samples (minus the massof the sample container) after drying to a constant mass (g).
mt: Mass of fresh samples (minus the mass of sample container) before drying (g).

The general experimental model is presented in Figure 3.1.

Extraction efficiency

$$H\ (\%) = \frac{\text{Dry sample weight - Extract weight}}{\text{Dry sample weight}} \times 100$$

2.2.2. Quality of some compounds contained in the extract

Purpose To check the presence of some compounds: tannins, flavonoids, alkaloids, saponins in the extract

Conduct experiments. Based on the method of Yadav *et al.,* 2014.

Table 2.1. Qualitative testing of some biologically active ingredients in the extract

Compounds	Testing	Observation
Tannin	2 mL extract fluid + 2 mL H_2O + 2-3 drops $FeCl_3$(5%)	Green precipitate
Flavonoid	1 mL extract fluid + 2 mL $Pb(OAc)_4$ (10%)	Yellow precipitate
Alkaloid	1 mL extract fluid + 2-3 drops of Wagner's reagent	Red brown precipitate
Saponin	5 mL extract fluid + 5 mL H_2O + heating	Foam

Evaluation criteria: color and phenomenon of the solution after the reaction. If the reaction appears color or phenomenon corresponding to the results of the tests as Table 2.1, it proves that the extract contains the substance under investigation.

2.2.2. Quantification of total flavonoids in the extract

Experimental purpose: Evaluate total flavonoid and flavonol content in the extract.

Evaluation criteria: Calculated total flavonoid content in 100g of dry substance.

Total flavonoids are determined according to the formula:

$$F_1\ (g/100g\ dry\ samples) = (a_1 \times V/m) \times n_1 \times 10^{-6} \times 100.$$

In which:

a_1: is the quercetin content (µg/mL) determined from the standard line equation

V: is the total volume of extract fluid (mL)

m: is the sample mass (g)

n_1: is the dilution factor

2.2.3. Quantification of flavonols in extracts

The procedure for establishing a quercetin standard line for flavonol quantification is shown in Figure 2.3.

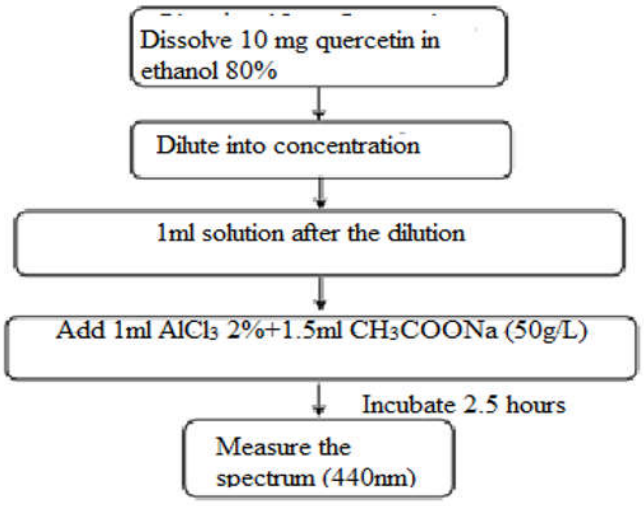


Figure 2.1. The procedure for establishing a quercetin standard line for flavonol quantification

Evaluation criteria: calculated flavonol content in 100g of dry substance. Flavonols are determined according to the formula:

$$F_2 \text{ (g/100g dry samples)} = (a_2 \times V/m) \times n_2 \times 10^{-6} \times 100.$$

In there:

a_2: is the quercetin content (µg/mL) determined from the standard line equation

V: is the total volume of extract fluid (mL)

M: is the sample mass (g)

n_2: is the dilution factor

2.2.4. Survey on the ability to inhibit XO enzyme - Survey substrate concentration

Purpose: Find the optimal substrate concentration range for the experiment to determine the inhibition pattern of the extract.
+ For competitive inhibition
Substrates and inhibitors compete to act on the active center of the enzyme
+ For competitive resistance inhibition
E combines with S to change the spatial structure of the enzyme molecule and create a center that combines with I
This type of inhibition reduces both K_m and V_{max}.

$$\text{Equation} : \frac{1}{V_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

The line has equal inclination and parallels to each other and not to converge at a point.
+ For mixed inhibition type
The inhibitor binds to the free enzyme but also to the ES complex.
+ For non-competitive inhibition
Enzymes can combine with either inhibitors, substrates, or both.

2.2.5. Surveying the antioxidant capacity of ethanol extract by DPPH method

Purpose: Determine the antioxidant capacity of ethanol extract by DPPH method.Evaluation criteria: The ability to inhibit DPPH free radicals is determined through the IC inhibition efficiency (%) calculated according to the following formula:

$$IC\ (\%) = \frac{A_1 - A_2}{A_1} \times 100$$

In which IC: DPPH free radical inhibition efficiency.

A_1: optical density value of the control sample.

A_2: optical density value of real sample.

Construct a standard line to show the correlation between % IC and sample concentration. Based on the standard line equation, determine the IC_{50} value (concentration level capable of eliminating 50% of DPPH).

2.2.6. How to determine IC_{50}:

IC_{50} is a value used to evaluate the strong or weak inhibition ability of the survey sample. IC_{50} is defined as the concentration (mg/mL) of the sample, at which it can inhibit 50% of free radicals, cellular or enzymatic. The higher the activity of the sample is, the lower the IC_{50} value will be.

How to determine IC_{50}:

Conduct a survey on the activity of the sample at many different concentrations.

For samples whose activity varies linearly with concentration,
Draw a straight-line y = ax + b through all points (where y is the inhibition efficiency and x is the concentration).
For samples whose activity does not vary linearly with concentration, approximately, choose two inhibitory concentrations above and below 50% and also draw a straight line y = ax + b to obtain the equation y = ax + b with known coefficients a and b.
From the known equation y = ax + b, substituting y = 50% into the equation will obtain the value x, which is the 50% free radical inhibition concentration (IC_{50}).

2.3 Data processing methods

The data are measured and recorded after each experiment.
Data are statistically analyzed by Minitab 16.0 software and graphed by Microsoft Office Excel software.

3. RESULTS AND DISCUSSION

3.1. Extraction by ethanol solvent

The ethanol extraction process is conducted with fresh samples as the first materials. Samples are chopped and dried at 70°C. Then, soak with ethanol solvent (70%) and a material/solvent ratio of 1/10 (w/v), in dark conditions and at room temperature. After dark incubation for 72 hours, filter the soaking solution through filter paper with a diameter of 13 µm, take the filtrate and discard the residue. The obtained filtrate is removed from the ethanol solvent by a rotary evaporator, then evaporated by a vacuum cleaner and we obtained the extract. The ethanol extract is stored at 4°C for use in the following experiments.

Table 3.1. Results of ethanol extraction

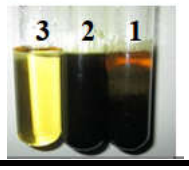
Observation criteria	Green *Amaranthus*
Fresh sample mass (g)	2.300
Water content (%)	87,2
Dry sample mass (g)	300
Raw mass (g)	15,1
Extraction efficiency (%)	5,0

The water content of the Green *Amaranthus*sample is 87.2%, this result is similar to the study of Sharma et al. (2012) with 87.9% of the water content of Green *Amaranthus*. Determining the water content of the leaf sample before performing the extraction helps to know the conditions to determine the appropriate extraction method to obtain the optimal amount of extract. In addition, determining the water content of materials also creates conditions for determining the temperature and drying time of the sample to conduct extraction with higher efficiency. During the sample drying process, high temperatures break down the internal cell structures of the material, facilitating better contact between the solvent and the material, increasing the ability to extract. Besides, the drying process also has the effect of reducing the moisture of the raw materials, helping to increase the ratio of solvent used to the raw materials (Duong Thi Phuong Lien, 2014).

3.2. Quantification of some natural compounds of Green *Amaranthus*

To preliminarily evaluate the chemical composition of the ethanol extract, we conducted a qualitative assessment of the presence of some biologically active compounds such as tannins, alkaloids, flavonoids and saponins. The results are presented in Table 3.2.

Table 3.2. Qualitative results of some compounds extracted from Green *Amaranthus* leaves in ethanol

Compounds	Phenomenon	Green *Amaranthus*
Alkaloid (Wagner reagent)	Brown precipitate	
Flavonoid	Yellow color appearance	
Saponin (Foam reagent)	Foam appearance	
Tannin (Braymer reagent)	Blue and black precipitate	

Notes: +: Positive result; –: Negative result

1: Test sample contains extract and reagents; 2: Test sample contains reagents; 3: Test sample contains extract and water.Many studies have demonstrated that natural compounds extracted from medicinal herbs have XO inhibitory activity and some of them have been included in clinical trials. Therefore, the survey of some natural compounds in medicinal plants is very necessary, making an important contribution to the preliminary assessment for research and application in preparations from medicinal plants. Qualitative analysis results show the presence of a number of biologically active compounds in the studied plant samples. Flavonoids are present in the Green *Amaranthus* plant. All tested samples do not contain alkaloids. Green *Amaranthus*samples contain saponin compounds according to Iqbal *et al.,* (2012) on Green *Amaranthus*leaves, these compounds all have many benefits.

Flavonoids are abundant in foods and drinks of plant origin. These compounds have many biological activities such as anti-cancer, anti-inflammatory, and cardiovascular protection (Li *et al.,* 2013). Many flavonoids are known to be potent antioxidants under in vitro conditions. Besides, flavonoids are also mentioned for their ability to inhibit many enzymes such as cyclooxygenase and lipoxygenase related to inflammation.Tannin is a type of polyphenol compound with high molecular weight and widely distributed in nature. Tannin compounds are found in many plants, helping plants repel harmful insects and regulate plant growth. Tannins are the most commonly found antioxidant compounds in foods and have many important biological activities such as protecting the body from oxidative stress and some degenerative diseases (Ujwala*et al.,* 2012).

3.3. Quantification of total flavonoids and flavonols

Flavonoids are one of the most common phenolic compounds found in plant tissues, along with carotenoids and chlorophyll, flavonoids gives plants colors such as blue, purple, yellow, orange, and red. The flavonoid family includes flavones, flavonols, isoflavonols, anthocyanins, anthocyanidins, proanthocyanidins and catechins (Khoddami *et al.,* 2013).

Flavonoids are potent in vitro antioxidants due to their ability to scavenge free radicals and chelate metals. Besides, many flavonoid groups have been shown to have the ability to inhibit XO enzymes. The quercetin standard line used to quantify total flavonoids is shown in Figure 3.1.

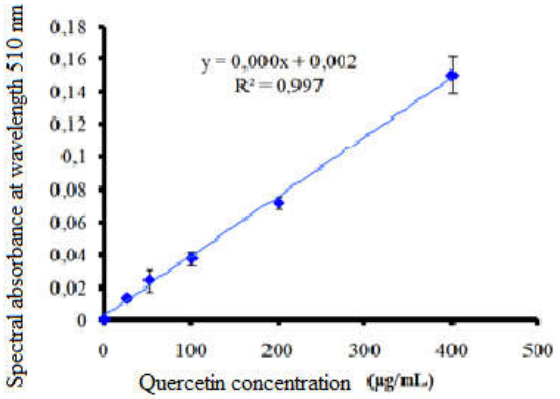


Figure 3.1. Quercetin standard line for total flavonoid quantification

Not all flavonoid groups have the ability to inhibit XO. Nagao et al. (1999) showed that XO inhibitory activity is only present in some groups such as flavones, flavonols, isoflavones, flavanols, in which flavonols, especially some compounds such as kaempferol, quercetin, myricetin, rhamnetin and rutin, are capable of strongest XO inhibitory ability.

The total flavonoid and flavonol content in the plant extract sample is shown in Figure 3.2.

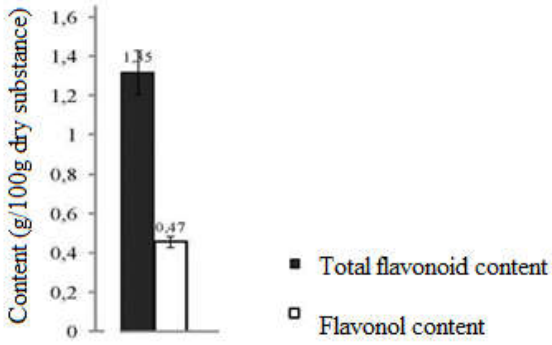


Figure 3.2. Total flavonoid and flavonol content in Green *Amaranthus* sample of extract

Based on the results, the highest total flavonoid and flavonol content is in the ethanol extract of the Green *Amaranthus* plant with total flavonoid and flavonol content of 1.35±0.11 and 0.47±0.02 g/100g dry substance. This result is higher than the results of Borah et al. (2011); The amount of total flavonoids in plant extract samples varies due to many reasons. Firstly, the difference in content is due to the characteristics of the species and genetic differences related to the synthesis and storage of flavonoids in tissues.

3.4. Ability to inhibit XO enzyme of ethanol extract

3.4.1. Inhibition effectiveness of XO enzyme of ethanol extract

Effect of high concentration of ethanol extract of Green *Amaranthu* son the ability to inhibit XO enzyme

The results of investigating the effect of concentration of ethanol extract of Amaranth on the ability to inhibit XO enzyme are presented in Figure 3.3.

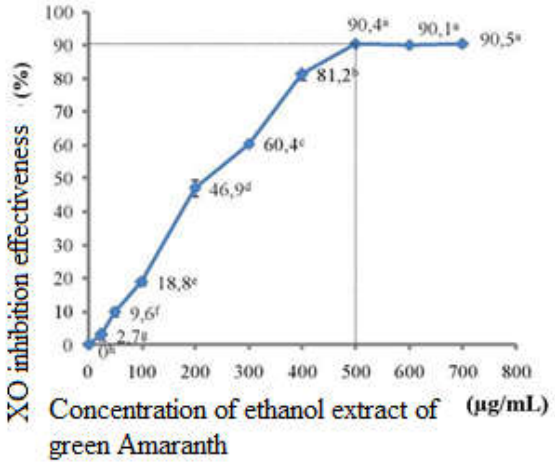


Figure 3.3. Graph shows the ability to inhibit XO enzyme of Green *Amaranthus*ethanol extract

Experimental results show that the ability of Green *Amaranthus*ethanol extract to inhibit XO enzyme is proportional to the extract concentration in the range from 25 to 500 µg/mL. The higher the extract concentration, the stronger the ability to inhibit XO enzyme is and vice versa, the ability to inhibit XO enzyme at high extract concentrations 25; 50; 100; 200; 300; 400; 500 gg/mL is a statistically significant difference ($p<0.05$). Green *Amaranthus* ethanol extract showed the highest inhibitory effect on XO enzyme at a concentration of 500 g/mL with a value of 90.4±0.2%. At the extract concentration of 25 µg/mL, the enzyme is inhibited by 2.7±0.5%. When increasing the concentration of extract from 50 to 100; 200; 300; 400 µg/mL, the enzyme inhibition efficiency also increased, the inhibition efficiency is 9.6±0.5%; 18.8±0.3%; 46.9±0.8%; 60.4±0.2% and 81.2±0.5% respectively. This proves that Green *Amaranthus* ethanol extract has inhibitory activity on XO enzyme. When increasing the extract concentration to 600 and 700 µg/mL, the inhibitory effect is not statistically different from that at the concentration of 500 µg/mL, 90.2±0.2% and 90.5%±0.1% respectively.

The results of quantifying total flavonoids and flavonols in the experiment also explain the ability of Green *Amaranthus* to inhibit XO. Flavonoids are powerful antioxidants and are also used as therapeutic agents for free radical-related diseases. Besides, flavonoids are also an effective inhibitor of many enzymes such as cyclooxygenase, lipooxigenase and especially XO. The IC50 value of the Green *Amaranthus*ethanol extract has an IC_{50} value = 243 µg/mL. Green *Amaranthus*is also quantified for total flavonoids and flavonols with values of 1.35±0.09 and 0.44±0.01 g/100g dry substance, respectively.

3.4.2. Antioxidant capacity of ethanol extract by DPPH method

Oxidation reactions are essential for living organisms, however they also have many harmful effects due to the formation of ROS groups. ROS is one of the intermediate agents that cause a number of diseases and damage to tissues such as the lungs, cardiovascular system, kidneys, liver, digestive system, blood, eyes, skin, muscles, brain and aging process (Shahidi, 1997). ROS are byproducts produced during the body's metabolic processes and immune responses against pathogens, pollution, and cigarette smoke. In biological systems, phenolic compounds such as flavonoids and tannins play a role in eliminating free radicals.

The DPPH method is used to investigate the antioxidant capacity of 4 types of extracts. Under in vitro conditions, DPPH is a stable free radical that accepts electrons or hydrogen radicals. The antioxidant's ability to reduce DPPH is determined by its decrease in absorbance at 517 nm and its color change from purple to yellow. The antioxidant capacity of the extract is also determined by the DPPH method. This method is a simple, fast and stable method commonly used to survey and screen antioxidants (Abalaka *et al.,* 2011).

3.4.3. Effect of concentration of Green *Amaranthus*ethanol extract on antioxidant capacity

The antioxidant capacity of Green *Amaranthus* ethanol extract is shown in Figure 3.4.

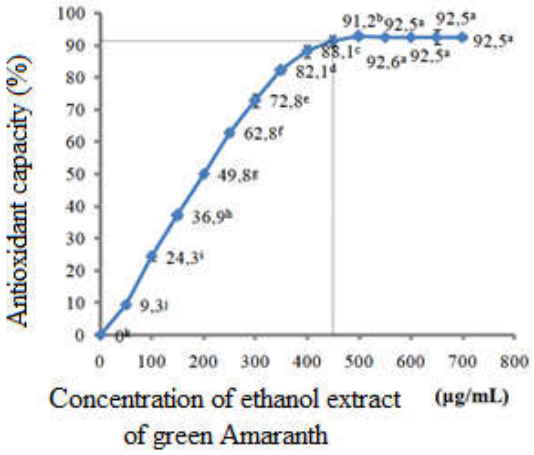


Figure 3.4. Graph shows the antioxidant capacity of Green *Amaranthus*ethanol extract

At concentration levels from 50 to 500 µg/mL, the antioxidant effect of Green *Amaranthus*ethanol extract is statistically different ($p<0.05$). The antioxidant capacity of Green *Amaranthus*ethanol extract is proportional to the concentration of the extract. Specifically, the antioxidant effect of the extract at a concentration of 50 µg/mL is 9.3±0.2%; When continuing to increase the concentration up to 500 µg/mL, the efficiency reached a maximum value of 92.6±0.1%. At concentration extract 550; 600; 650 and 700 µg/mL antioxidant capacity of the extract is not statistically different.

3.4.4. Antioxidant effect of the extract

In order to evaluate the antioxidant effect of ethanol extract, the study determined the IC_{50} value. Vitamin C is a substance with strong activity against free radicals and is used as a standard in many reference documents, so it is used in the study as a positive control substance and a basis for evaluating the antioxidant activity of the extract. The smaller the IC_{50} value is, the lower the concentration that can eliminate 50% of free radicals is, the stronger the antioxidant capacity of the sample under investigation is. From the correlation graph between the investigated concentration and antioxidant performance, proceed to determine the IC_{50} value

The antioxidant IC_{50} values of extracts are shown in Table 3.3.

Table 3.3. Antioxidant IC_{50} values of ethanol and vitamin C extracts

Samples for activity	IC_{50} (µg/mL)
Vitamin C	39
Green *Amaranthus*ethanol extract	207

Inhibiting the XO enzyme not only reduces uric acid levels in the blood, but also reduces the formation of free radicals (Kong et al., 2002). The results of the survey of antioxidant capacity using the DPPH method show that popular and cheap raw materials from 4 *Amaranthus* plants: *Achyranthes aspera*L., Green*Amaranthus*, Alternanthera sessilis, and Gomphrena celosioides Mart have antioxidant ability with IC_{50} value of 187; 207; 625 and 327 µg/mL. Plants are a rich source of antioxidants. These compounds include tocopherol, vitamin C, carotenoid and phenol compounds. Secondary products of photosynthesis can generate free radicals and ROS. Therefore, antioxidant compounds play an important role in plant life. These compounds have in common that the basic molecular structure must have at least one aromatic ring and one hydroxyl group. These compounds include phenol acid, flavonoids, isoflavones, tannins, lignans, coumarine, stilbene, flavone... These compounds have antioxidant properties with different mechanisms (Shahidi, 1997).
The IC_{50} value of the four extracts is many times greater than the IC_{50} of vitamin C, which is 39 µg/mL, proving that the extract has weaker antioxidant capacity than vitamin C. This can be explained by the fact that vitamin C is the commercial substance with high purity, while the ethanol extract still contains many impurities.

The results of studying the antioxidant capacity of the extracts are also compared with previous studies. The IC_{50} value of Green *Amaranthus*is 207 µg/ml, higher than the study by Ahmed et al. (2013), using 80% methanol as the extraction solvent, and the extract has an IC50 value of 83.45 µg/mL.

CONCLUSIONS

Qualitative results showed that the extracts contained flavonoid compounds. The ethanol extract of Green *Amaranthus*has antioxidant properties with an IC_{50} value of 207.

The general conclusion of the research results is that the extracts have the ability to inhibit enzymes and prevent oxidation. This proves that ethanol extract has high potential in further research on xanthine oxidase enzyme inhibitors of medicinal plants closely related to daily life.

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