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Antioxidant and α-Glucosidase inhibitory activity of various solvent fractions from *Amaranthus viridis* L., *Amaranthus tricolor* L., and *Amaranthus spinosus* L.

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ABSTRACT

Amaranthus tricolor L., *Amaranthus spinosus* L., and *Amaranthus viridis* L. are well-known for flavoring food products and traditional medicine. This study investigated the antioxidant and α-glucosidase inhibitory activity of various solvent fractions from the whole plant of *A. tricolor*, *A. spinosus*, and *A. viridis* in Vietnam. The total extract and solvent fractions of petroleum ether (PE), chloroform (CF), ethyl acetate (EA), *n*-butanol (B), and water (W) were tested for antioxidant activity using the DPPH assay and α-glucosidase inhibition. The results showed that the three species of *Amaranthus* had relatively low antioxidant activity, and the total extract of *A. spinosus* L. had the highest antioxidant activity with an IC_{50} value of 324.96 µg/mL. The EA fraction of *A. tricolor* had outstanding α-glucosidase inhibitory activity with an IC_{50} value of 95.94 µg/mL. It is recommended to continue isolation research to find active ingredients with α-glucosidase inhibition potential in the EA extract of *A. tricolor*. Additionally, an *in vivo* assay might be conducted to evaluate the activity and toxicity of this EA fraction.

Keywords: Amaranthus, Antioxidant; α-glucosidase, *Amaranthus tricolor*, Ethyl acetate.

INTRODUCTION

According to the International Diabetes Federation (IDF), the Diabetes Atlas (2021) reports that 10.5% of the adult population (20-79 years) has diabetes. By 2045, IDF projections show that 1 in 8 adults, approximately 783 million, will be living with diabetes, an increase of 46%[1]. Diabetes is a metabolic disorder characterized by increased blood glucose due to absolute or relative insulin deficiency and reduced insulin function. Prolonged hyperglycemia causes disorders of carbohydrate, protein, and lipid metabolism, causing damage to many different organs, especially the heart and blood vessels, kidneys, eyes and nerves causing severe effects crucial to the patient's health[1].

Currently, there are many popular drugs available in the market to maintain stable blood sugar levels in people with diabetes. The major antidiabetic medication classes include biguanides, sulfonyluares, meglitinide, thiazolidinedione, dipeptidyl peptidase 4 inhibitors, sodium-glucose cotransporter (SGLT2) inhibitors and α-glucosidase inhibitors [2]. Acarbose, voglibose and miglitol can inhibit carbohydrate hydrolysis enzymes such as α-amylase and α-glucosidase[2]. Despite the many advances in research and development of modern drugs, the process of finding compounds is expensive, which brings an economic burden to patients' families and society. Moreover, long-term use of these drugs can cause many side

effects. For instance, thiazolidinediones can cause weight gain, hypoglycemia, and increased bad cholesterol in the blood; the sulfonylureas group causes hypoglycemia, tremors, sweating, and dizziness; SGLT2 inhibitors increase the risk of urinary tract infections and fungal infections, and acidosis[2].Therefore, there is a growing trend towards using safe products of natural origin for treatment that are beneficial to human health and have long-term effectiveness.

For centuries, many herbal medicines have been used in the treatment of diabetes such as *Azadirachta indica* A. [3], *Cichorium intybus* L. [4] and *Ginkgo biloba* L. [5]. These medicinal herbs not only have the ability to lower blood glucose but also can reduce complications of diabetes in the kidneys, nerves, retina, hypertension, and hyperlipidemia. Medicinal herbs can be an alternative or supplement to diabetes medications[6]. *Amaranthus* is a typical genus in Vietnam. Many species are common daily and have antidiabetic effects, such as *Amaranthus tricolor* L., *Amaranthus viridis* L., and *Amaranthus spinosus* L. Studies on the antidiabetic effects of species of the *Amaranthus* genus have been published[7-9]. Most of the ability of medicinal herbs to lower blood glucose comes from the following mechanisms: stimulating insulin secretion, enhancing the activity of peroxisome proliferator-activated receptors (PPARs), inhibiting *α*-amylase or *α*-glucosidase enzymes, increasing secretes glucagon-like peptide-1 (GLP-1) agonist, inhibits the formation of advanced glycation products (AGE), scavenges free radicals, is antioxidant (against reactive oxygen or nitrogen species: ROS/RNS), enhances glucose transport by glucose transporter protein type-4and prevents insulin resistance[6].

For those reasons, we wish to carry out the project to achieve the following specific goals: (i) Standardize and obtain the extract, (ii) Identify the extract and fraction with the best antioxidants, and (iii) *α*-glucosidase inhibitory activity.

From there, it provided scientific information about the effects and benefits of *Amaranthus* species that can antioxidants and inhibit *α*-glucosidase as a premise for researching and producing pharmaceuticals capable of treating diabetes in the future. Promoting the local medicinal potential of *Amaranthus* species can enhance the value of this plant in healthcare.

MATERIALS AND METHODS

Collection of plant materials

The whole plant of *A. spinosus* was collected in December 2022 in Ninh Thuan province, Vietnam; *A. viridis* and *A. tricolor* were collected in December 2022 in Ho Chi Minh City, Vietnam.

The collected plants were identified using the *mat*K gene sequencing method [10]. For research, the voucher specimens (accession NTT-DL-023) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Nguyen Tat Thanh University.

Chemicals and reagents

2, 2-diphenyl-1-picrylhydrazyl (DPPH), *α*-glucosidase from *Saccharomyces cerevisiae* acarbose, ρ-nitrophenol glucopyranoside, and ascorbic acid was purchased from Sigma-Aldrich.

Extract preparation

The whole plants were collected and dried at 50 °C in drying oven (UN75, Memmert GmbH & Co. KG, Germany) to obtain a 500 g dry sample, later coarsely powdered in a Willy Mill (JA23852, Japson, India) to 60-mesh size and used for solvent extraction. For sample preparation, 500 g of dried samples were extracted by maceration method with 70% ethanol and concentrated using a rotary evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach) under reduced pressure at 40 °C to yield the samples of total extracts (TE) with the final moisture content less than 20%.

Fractionation

The crude extract was diluted with 250 mL of water, transferred into a separating funnel, shaken, and allowed to settle. Furthermore, 250 mL of petro ether (PE), the least polar solvent, was added and shaken. The content can settle, and the bottom of the separating funnel is opened to remove the aqueous layer. The remaining content in the separating funnel was poured into a clean container for PE fraction. An equal volume of PE was added again, shaken, and separated. The addition continued until after adding PE and shaking, no reasonable quantity of extract appeared to move into the PE portion. A similar cycle was performed for chloroform (CF), ethyl acetate (EA), and *n*-butanol (BU) to get CF, EA, and BU fractions. The remaining portion left after the fractionation is the water (W) fraction, as the crude extract was first dissolved in water [11].

DPPH radical scavenging activity

The DPPH radical scavenging assay was measured as described with minor revisions[12]. The test samples were mixed in MeOH in appropriate ranges to determine half-maximal inhibitory concentration (IC_{50}) values. These samples (100 µL) were added to 100 µL of DPPH solution (0.2 mm in MeOH). After 30 min incubation in the dark at room temperature, absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. All the measurements were carried out in triplicate, and standard deviation was applied.

DPPH scavenging activity (%) = $(A_c - A_t)/A_c \times 100$, where A_t is the absorbance of the test sample and A_c is the absorbance of the control.

All IC_{50} values of tested activities were determined by the logarithm curve (y=aln(x)+b) of the percentage of remaining DPPH radicals against the sample concentration.

α-glucosidase inhibitory activity assay

The inhibition potential of solvent fractions and extracts against *α*-glucosidase was measured to evaluate *in vitro* antidiabetic potential. *α*-glucosidase enzyme inhibitory activity was performed

according to Qaisar et al., (2014) [13]with modifications. The total extracts and solvent fractions were mixed in DMSO in concentration ranges to determineIC_{50} values, controlling DMSO 2.5% in each well. Various sample concentrations were added to phosphate buffer pH 6.8 (40 µL), followed by 40 mL *α*-glucosidase (0,2 U/mL). After 20 min incubation at 37 °C, 40 µL of 4 mM ρ-nitro phenol glucopyranoside was added and incubated for 20 minutes at 37 °C. Terminate the reaction by adding 130 µl of 0.2 M Na_2CO_3 to all wells and measuring absorbance at 405 nm.

α-Glucosidase inhibition (%) = $(A_c - A_t)/A_c \times 100$, where A_c is the absorbance of the control, and A_t is the absorbance of the test sample. The IC_{50} values of all tested activities were determined by the logarithm curve (y=aln(x) +b) of the percentage of remaining *α*-glucosidase against the sample concentration.

Statistical analysis

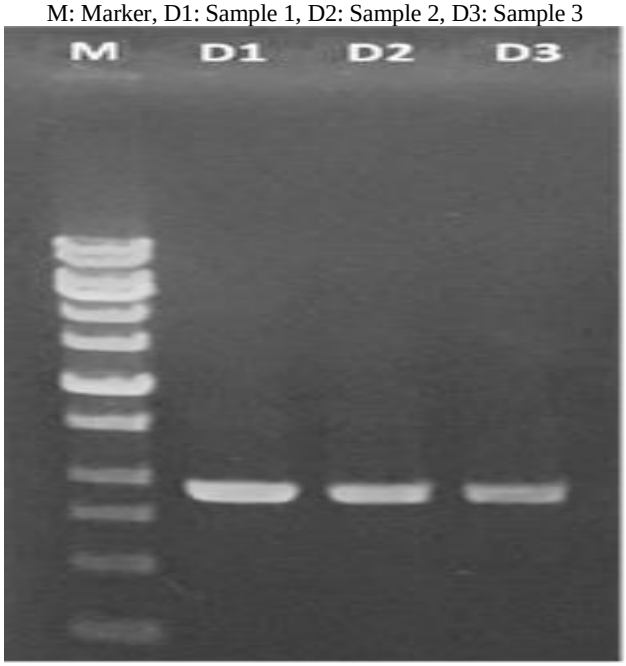
The experimental findings were evaluated for statistical significance using the Fisher's test by *the Microsoft Excel Data Analysis* tool. A probability of 0.05 or less was considered statistically significant.

RESULTS AND DISCUSSION

Identification of *Amaranthus* species by using *mat*K sequences

Samples were identified using the DNA method and *mat*K gene sequencing. The plant DNA was extracted by using a Genomic DNA Purification Kit (Thermo Scientific™), Cat. No. K0512. The polymerase chain reaction (PCR) samples were verified by electrophoresis in 1% agarose gels stained with ethidium bromide. The electrophoresis results of PCR *mat*K are shown in Figure 1.

Figure 1: Electrophoresis results of PCR *mat*K
M: Marker, D1: Sample 1, D2: Sample 2, D3: Sample 3



Results of *mat*K gene sequencing

Sample 1 (809 bp)

CACTATAATAATGAGAAAGATTTCGGCATATACGT
CCAAATCGGTCAATAATATCAGCATCGGATAAATCGGTCC
AGACCGACTTACTAATGGGATGACCTAATCCATTACAAAAT
TTCGCCTTAGCCAACGAGCCAACCAGAGGAATAATTGGAA
CTATGGTATCAAACTTCTTAATAATATTATCTACTAGAAAT
GCATTTTCTAACATTTGACTCCGTATTACTGAAGAATTGAG
TCCCACATTTGAAATAAAACCCATAAAGTCGAGGGAATAG
TTTGATAATTTATTGATATAGATTCTTCTTGGTTGAGACCAC
ACAGAAAAATGACATTGCCAGAAAGCGATAAAGTAATATT
TCCATTTATACATCAGAAAGGATGTCCCTTTTGAAGCCAGA
AGGCATTTTCCTTGATACCGAACATAATGCAGAAAAGGTTC
TTTGAAAAGCCATAGGATAACCCCAAAAACCTTAACTTTGA
CTTTTACTAGATATTTTATCTTTCCGTAAAAATGGATTCGTT
CAAGAAGGGCTCCAAAAGACGTTGATCGTAAATAAGAGGA
TTGCTTGCGTAGAATAACAAAAATGGATTCGTATTCATATA
CAAGAAGATTATATAGGAACAAAAAGAATCTTCGATTCCT
TTTTGAAAAAGTGGAAATGGATTCTTTTGGCCTAATAAGAC
TATTCCAATTACGATACTCGTAAAGAAAGTATCGTAATAAA
TGCAAGGAAGAGGCATCTTTCAACCAATAGCGAAGAGTTT
GAACCAAGATTTCTAGATGGGCAGGGTAAGGTATTAATAT
A

Sample 2 (803 bp)

AGAAAGATTTCGGCATATACGTCCAAATCGGTCAA
TAATATCAGCATCGGATAAATCGGTCCAGACCGACTTACTA
ATGGGATGACCTAATCCATTACAAATTTCGCTTTAGCCAAC
GAGCCAACCAGAGGAATAATTGGAACTATGGTATCAACTT
CTTAATAATATTATCTACTAGAAATGAATTTTCTAACATTT
GACTCCGTATTACTGAAGAATTGAGTCCCACATTTGAAATA
AAACCCATAAAGTCGAGGGAATAGTTTGATAATTGATTGA
TATAGATTCTTCTTGGTTGAGACCACACAGAAAAATGACAT
TGCCAGAAAGCGATAAAGTAATATTTCCATTTATACATCAG
AAAGGATGTCCCTTTTGAAGCCAGAAGGCATTTTCCTTGAT
ACCGAACATAATGCAGAAAAGGTTCTTTGAAAAGCCATAG
GATAACCCCAAAAACCTTAACTTTGACTTTTACTAGATATT
TTAGCTTTCCGTAAAAATGGATTCGTTCAAGAAGGGCTCCA
AAAGACGTTGATCGTAAATAAGAGGATTGCTTGCGTAGAA
TAACAAAAAGGGATTCGTATTCATATACAACAAGATTATAT
AGGAACAAAAAGAATCTTCGATTCCTTTTTGAAAAAGGGG
AAATGGATTCTTTTGGCCTAATAAGACTATTCCAATTACGA
TACTCGGAAAGAAAGTATCGTAATAAATGCAAGGAAGAGG
CATCTTTCAACCAATAGCGAAGAGTTTGAACCAAGATTTCT
AGATGGGCAGGGTAAGGTATTAATATATCTAACA

Sample 3 (794 bp)

CACTATAATAATGAGAAAGATTTCGGCATATACGT
CCAAATCGGTCAATAATATCAGCATCGGATAAATCGGTCC
AGACCGACTTACTAATGGGATGACCTAATCCATTACAAAAT
TTTGCTTTAGCCAACGAGCCAACCAGAGGAATAATTGGAA
CTATGGTATCAAACTTCTTAATAATATTATCTACTATAAAT
GAATTTTCTAACATTTGACTCCGTATTACTGAAGAATTGAG
TCCCACATTTGAAATAAAACCCATAAAGTCGAGGGAATAG
TTTGATAATTGATTGATATAGATTCTTCTTGGTTGAGACCA
CACAGAAAAATGACATTGCCAGAAAGCAATAAAGTAATAT
TTCCATTTATACATCAGAAAGGATGTCCCTTTTGAAGACAG
AAGGCATTTTCCTTGATACCGAACATAATGCAGAAAAGGTT
CTTTGAAAAGCCATAGGATAACCCCAAAAACCTTAACTTTG
ACTTTTACTAGATATTTTAGCTTTCCGTAAAAATGGATTCGT
TCAAGAAGGGCTCCAAAAGACGTTGATCGTAAATAAGAGG
ATTGCTTGCGTAGAATAACAAAAAGGGATTCGTATTCATAT
ACAACAAGATTATATAGGAACAAAAATAATCTTCGATTCCT
TTTTGAAAAAGGGGAAATGGATTCTTTTGGCCGAATAAGA
CTATTCCAATTACGATACTCGTAAAGAAAGTATCGTAATAA
ATGCAAGGAAGAGGCATCTTTCAACCAATAGCGAAGAGTT
TGAACCAAGATTTCTAGATGGGCAGGG

Results of BLAST analysis on GenBank

The *mat*K gene sequence analysis results showed that Sample 1 is *Amaranthus spinosus*, Sample 2 is *Amaranthusviridis*, and Sample 3 is *Amaranthus tricolor* (Table 1).

Table 1: Results of BLAST analysis on GenBank

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Sample 1							
A. spinosus	1495	1495	100%	0	100.00%	2509	MG685171.1
A. spinosus	1495	1495	100%	0	100.00%	150524	NC_065858.1
A. spinosus	1495	1495	100%	0	100.00%	150524	MT526784.1
A. spinosus	1495	1495	100%	0	100.00%	150524	MT526783.1
A. spinosus	1495	1495	100%	0	100.00%	813	KC747161.1
Sample 2							
A. viridis	1483	1483	100%	0	100.00%	898	MK228110.1
A. viridis	1483	1483	100%	0	100.00%	2509	MG685187.1
A. viridis	1483	1483	100%	0	100.00%	913	MG946995.1
A. viridis	1483	1483	100%	0	100.00%	1733	MF159425.1
A. viridis	1483	1483	100%	0	100.00%	893	KX090207.1
Sample 3							
A.tricolor	1467	1467	100%	0	100.00%	2509	MG685180.1
A. tricolor	1467	1467	100%	0	100.00%	1706	MF159454.1
A. tricolor	1467	1467	100%	0	100.00%	1730	MF159453.1
A. tricolor	1467	1467	100%	0	100.00%	150027	KX094399.1
A. tricolor	1467	1467	100%	0	100.00%	893	KX090206.1

Total extract and solvent fractional

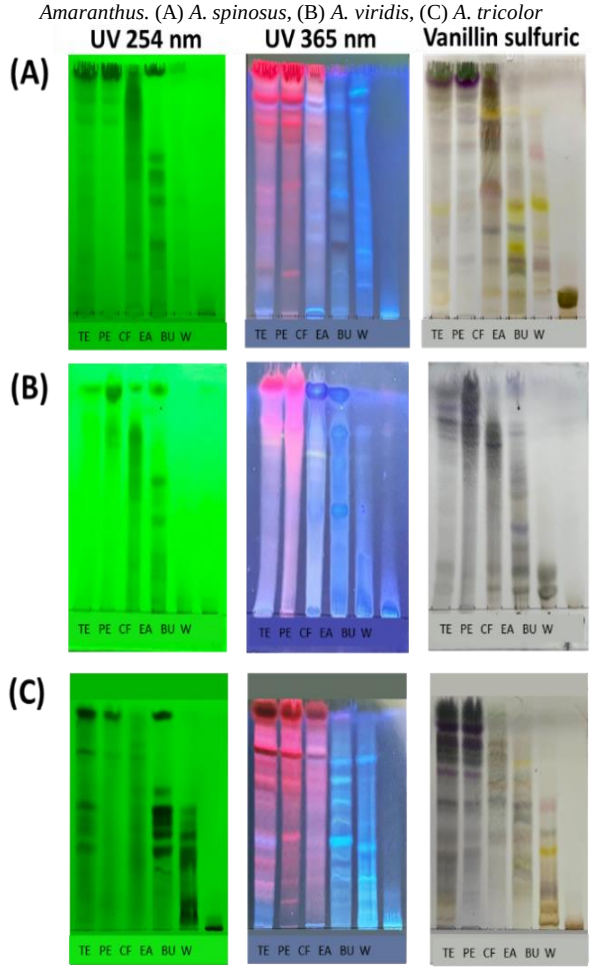
The whole plant powers (500 g) were soaked and evaporated under reduced pressure to obtain TE: *A. spinosus* (48.63 g, moisture 18.24%), *A. viridis* (18, 79 g, moisture 19.98%), *A. tricolor* (53.56 g, moisture 17.8%). Then, take an amount of TE and disperse it into water, then shake and distribute with solvents of increasing polarity, evaporating at reduced pressure to obtain fractionated extracts. The results of extraction and fractionation process are shown in Table 2.

Table 2. The results of extraction and fractionation

Sample		TE	Fractions				
			PE	CF	EA	BU	W
A. spinosus	**Mass (g)**	**38.63**	**18.53**	**1.23**	**3.70**	**4.16**	**4.69**
	Moisture (%)	15.24	10.55	15.31	15.48	13.53	10.95
A. viridis	**Mass (g)**	**14.79**	**7.85**	**1.95**	**2.86**	**8.56**	**0.71**
	Moisture (%)	15.98	11.92	13.94	15.72	9.58	5.15
A. tricolor	**Mass (g)**	**43.56**	**23.62**	**2.18**	**3.77**	**7.11**	**2.63**
	Moisture (%)	15.80	10.19	12.31	14.09	15.38	15.86

The total extracts and solvent fractions were developed on thin-layer chromatography with the solvent system $CHCl_3$-MeOH-H_2O (65:35:10; lower layer) to evaluate the chemical composition preliminarily. The chromatogram results are shown in Figure 2.

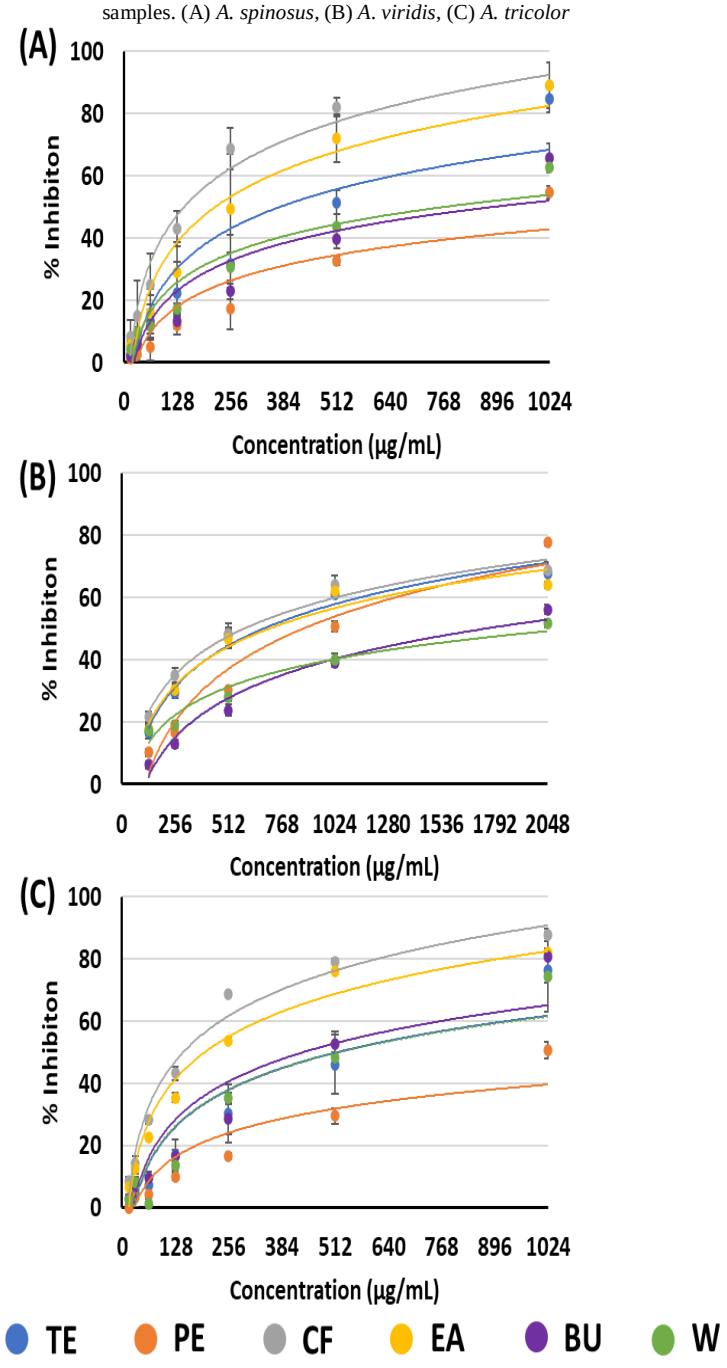
Figure 2: Chromatograms of total extract and fractions of three species of *Amaranthus*. (A) *A. spinosus*, (B) *A. viridis*, (C) *A. tricolor*



Antioxidant activity

The antioxidant activity in percentage (% Inhibition) of the samples is presented in Figure 3. Based on the percentage of inhibition and the tested concentration, a logarithmic nonlinear curve equation of the form y = aln(x) + b was built, and coefficients a and b were evaluated for statistical significance ($p < 0.05$) using Fisher's test. Then, the IC_{50} value (Table 3) is determined by substituting y = 50 into the equation.

Figure 3: The antioxidant activity in percentage (% Inhibition) of the samples. (A) *A. spinosus*, (B) *A. viridis*, (C) *A. tricolor*



Ascorbic acid positive control had IC_{50} = 7.29 µg/mL. The results showed that among the TE samples, *A. spinosus* had the lowest IC_{50} value, and *A. viridis* had the highest IC_{50} value. In the extracts, CF and EA of *A. spinosus* and *A. tricolor* had good antioxidant potential.

Table 3: The IC_{50} value of antioxidant activity

Samples	IC_{50} (µg/mL)		
	A. spinosus	*A. viridis*	*A. tricolor*
TE	324.96	679.84	518.15
PE	1266.37	871.53	2598.66
CF	144.97	588.35	147.22
EA	219.78	709.21	198.84
BU	613.70	1748.39	435.95
W	771.50	2179.42	463.43
Ascorbic acid	7.29		

In *A. spinosus*, the excellent antioxidant activity of CF and EA may be due to compounds such as carotenoids and flavonoids. Besides, the primary plant pigments in *A. spinosus* are amaranthine and iso amaranthine [11], which have better antioxidant capacity than phenolic compounds [12]. In *A. tricolor*, although the CF chromatogram did not have as many diverse spots as EA and BU, it may contain prominent plant pigments such as amaranthine and betacyanin. Both of these compounds have been shown to have antioxidant effects [13]. The results of this study have contributed to supplementing the source of information for previous activities to evaluate antioxidant capacity. In *A. viridis*, previous publications on antioxidant activity mainly used leaves and seeds [14,15], to perform tests, and few studies used the whole *A. viridis* plant to evaluate antioxidant activity. The results of this study contribute to supplementing the source of information for previous activities to evaluate antioxidant capacity.

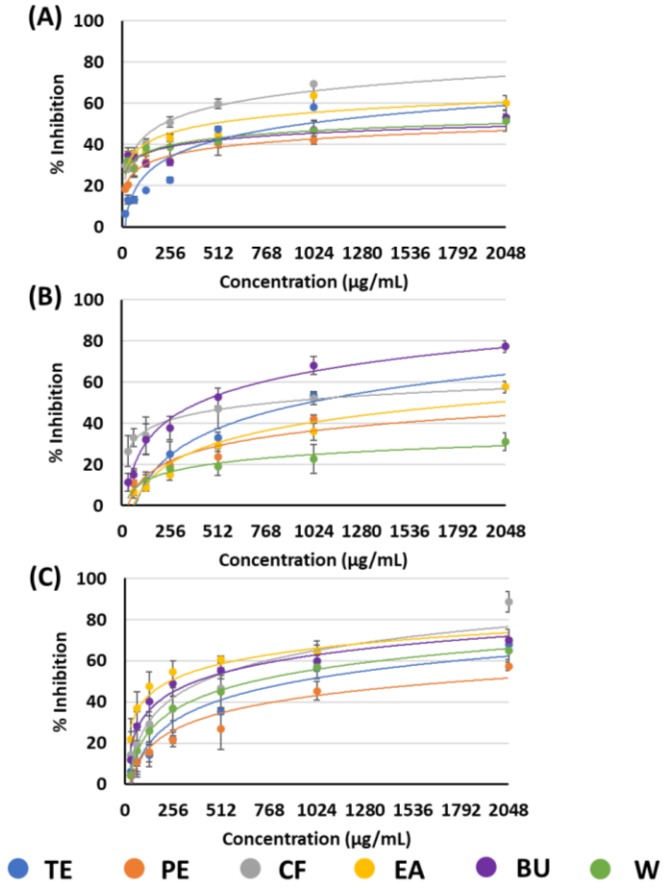
***α*-glucosidase inhibitory activity**

The *α*-glucosidase inhibitory activity (% Inhibition) of test samples is presented in Figure 4. PEof *A. spinosus* and *A. viridis* had a maximum inhibition of 41.95% and 48.11% at a concentration of 1024 µg/mL, and W of *A. viridis* had a maximum inhibition of 31.13% at a concentration of 2048 µg/mL. These solvent fractions had poor solubility andweak *α*-glucosidase inhibitory activity, so the IC_{50} value could not be determined. The remaining solvent fractions had the ability to inhibit *α*-glucosidase by over 50% at the tested concentration range. Evaluate the statistical significance of coefficients a and b in the logarithmic equation y = aln(x) + b ($p < 0.05$) using the Fisher test to determine the regression equation of the test samples. Then, the IC_{50} value (Table 4) was determined by substituting y = 50 into the equation.

Table 4: The IC_{50} value of *α*-glucosidase inhibitory activity

Samples	IC_{50} (µg/mL)		
	A. spinosus	*A. viridis*	*A. tricolor*
TE	865.15	995.67	918.97
PE	-	-	1793.29
CF	239.25	752.96	412.94
EA	364.07	1972.86	95.94
BU	303.04	411.913	378.59
W	4448.83	-	683.38
Acarbose	215.77		

Figure 4: The α-glucosidase inhibitory activityin percentage (% Inhibition) of the samples. A) *A. spinosus*, (B) *A. viridis*, (C) *A. tricolor*



The positive control acarbose had an IC_{50}= 215.77 µg/mL. The results showed that among the TE samples, *A. spinosus* had the lowest IC_{50} value, and *A. viridis* had the highest IC_{50} value. The α-glucosidase enzyme inhibitory activity of the three TE samples was similar, ranging from 865.15 to 995.67 µg/mL. Among the fractions, EA of *A. tricolor* had the best α-glucosidase inhibitory potential (95.94 µg/mL), about 2-fold better than acarbose. This result showed that the EA of *A. tricolor* had the potential to treat diabetes thanks to its ability to inhibit the α-glucosidase enzyme. EA *A. tricolor* was a moderate to strongly polar fraction so the fraction may contain compounds such as flavonoids and phenolic acids. Previous studies have shown that flavonoids and phenolic acids in *A. tricolor* could inhibit the activity of α-glucosidase enzyme [16]. Therefore, the isolation of compounds present in the EA fraction of *A. tricolor* may provide vital information for the development of new treatments for diabetes.

Besides, the good α-glucosidase inhibitory activity of *A. spinosus* CF (239.25 µg/mL) was consistent with the report of Mondal et al. (2015) when they found a new fatty acid with Strong α-glucosidase inhibition – (14E, 18E, 22E, 26E) – methyl nonacosa-14, 18, 22, 26 tetraenoate (IC_{50} = 6.52 mM/mL) and β-sitosterol in the chloroform fraction[17].

CONCLUSIONS

The project has contributed to building documents on the chemical composition and pharmacological effects of antioxidant capacity and α-glucosidase inhibitory activity of three popular *Amaranthus* species in Vietnam.

The chemical composition of the three species was similar, with main groups of active ingredients such as flavonoids, phenolic acids, saponins, and alkaloids. Particularly, *A. tricolor* stands out with its unique anthocyanin active ingredient group. The three species of *Amaranthus* had low antioxidant activity, and the total extract of *A. spinosus* had the highest antioxidant activity with an IC_{50} value of 324.96 µg/mL.The EA fraction of *A. tricolor* had outstanding α-glucosidase inhibitory activity with an IC_{50} value of 95.94 µg/mL.

It is recommended to continue isolation research to find potential active ingredients that inhibit α-glucosidase in the EA fraction of *A. tricolor*.

Conflicts of interest

The authors declare no conflict of interest.

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