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Original Research Article

Morphology of *Vernonia amygdalina* L. and Study of Its Antioxidant Property, *α*-Amylase and α-Glucosidase Inhibitory Activity, Hepatoprotective Potency and Toxicity

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ABSTRACT

Vernonia amygdalina L., commonly utilized in traditional Vietnamese folk medicine for the management of afflictions including liver diseases, hypertension, diabetes, and elevated cholesterol levels. Despite its extensive employment in local herbal remedies, there exists a scarcity of scientific investigations elucidating its pharmacological and therapeutic potential. This research investigated the antioxidant activity of leaf extracts using a range of solvents, including *n*-hexane, chloroform, ethyl acetate, *n*-butanol, ethanol, and water. The ethyl acetate extract was identified as the most potent antioxidant, exhibiting EC_{50} values of 8.42 and 71.88 μg/mL in ferric reduction and DPPH assays, respectively. The hepatoprotective activity, as measured using the cellular lipid peroxidation method of ethanol leaf extract (IC_{50} = 5.87 μg/mL) is 4.75 times stronger than the trolox. Additionally, the ethanol extract demonstrated significant inhibitory potential against α-amylase and α-glucosidase enzymes (IC_{50} of 480.00 and 72.53 μg/mL, respectively). The acute oral toxicity study conducted on a mouse model indicates a safety margin at the experimental dose D_{max} = 18.44 g extract/kg. Additionally, this study encompassed a leaf microdissection of *V. amygdalina*, elucidating pivotal morphological features. So, it is safe for further studies and producing functional products for disease prevention.

***Keywords*:** antioxidant, hepatoprotective, α-glucosidase, phytochemical screening, *Vernonia amygdalina* L.

Introduction

Plants have been known to exist for thousands of years and have been an essential component of traditional and natural therapeutic frameworks all over the world since ancient times.[1] According to estimates, 80% of people in the third world still primarily obtain their healthcare from medicinal plants, demonstrating the continued usefulness of these plants today.[2] Accordingly, these effects of plants come from biologically active phytochemicals such as: alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides, tannins, volatile oils, etc.[3] These phytochemicals are very diverse, in actions as they may be antioxidants, antivirals, anticancer, antibacterial, antifungal and antiparasitic agents.[4] According to some opinions, the buildup of too many free radicals in the body causes oxidative stress, which causes cellular damage to DNA, lipid, and membranes.[5] It is widely acknowledged that antioxidants play vital role in the body's defence mechanism against free radicals.[6,7]

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Interestingly, many medicinal herbs have been reported to contain large amounts of compounds with antioxidant properties, and have been shown to support and enhance health.[6-8]

Vernonia amygdalina L. (VA) is a perennial plant belonging to the *Asteraceae* family, commonly found in the wild and extensively cultivated in Vietnam (Figure 1). The plant is commonly referred to as "bitter leaf" in English and is known by its indigenous name, "Mat Gau Nam". In Vietnam, this plant has a long history of being used in traditional herbal medicine to treat various conditions, including high liver enzymes, hypertension, diabetes, and elevated blood lipids. The above-ground parts, primarily the stems and leaves, are used for their fever-reducing, laxative, growth-inhibiting, and anti-breast cancer cell properties.[5,9,10] The leaves of *V. amygdalina* contain diversified biochemicals, including saponins, alkaloids, terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, and sesquiterpenes.[11,12] Seven polyphenols in leaf extracts of VA are identified and quantified using HPLC, including 5-*O*-caffeoylquinic acid, luteolin hexoside, 3,4-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, and luteolin dihexoside. Specifically, 3,5-*O*-dicaffeoylquinic acid was determined by Jadwiga Nowak and colleagues in 2022.[13] In a study conducted by Hussen and colleagues (2023), the ability to effectively scavenge free radicals, including DPPH, ABTS, and H_2O_2, was observed for leaf extracts of VA, with respective values of 94.83 μg/mL, 179.8 μg/mL, and 141.6 μg/mL. Additionally, the authors highlighted the diverse phenolic composition's significant role in conferring the antioxidant activity of these extracts.[1] Omede A and collaborators reported in 2018 that the leaf extract of VA exhibited inhibitory properties against 50% of DPPH and NO free radicals, with EC_{50} values of 3.84 ± 1.03 and 71.26 ± 0.48

mg/mL, respectively. Moreover, their research suggested that the extract was non-toxic to shrimp, making it a safe antioxidant source.[14] Julia Joseph and colleagues utilized VA extract to synthesize silver nanoparticles and observed a time and dose-dependent reduction in the viability of MCF-7 cells. This effect was achieved through apoptosis induction and DNA damage, with average EC_{50} values of 67 µg/mL and 6.11 µg/mL after 72 hours of treatment.[15]

The liver is a vital internal organ in the body with multiple crucial functions. Its primary responsibilities include metabolizing nutrients, detoxifying harmful substances, synthesizing bile, producing necessary enzymes, and storing energy in the form of glycogen. However, the liver can be afflicted by various diseases caused by bacteria, viruses, and other factors.[2] Liver diseases are significant global health concern, contributing to approximately 2 million deaths annually worldwide. Approximately one million of these deaths are due to cirrhosis, one million are attributed to viral hepatitis, and hepatocellular carcinoma.[6] Additionally, oxidative stress is increasingly suspected as a contributing factor to the onset and progression of various diseases, including those resulting from alcohol exposure.[16] Therefore, it is imperative to pay close attention to the mechanisms related to drug- and chemical-induced liver damage. Furthermore, the search for effective treatment methods to address liver damage caused by drugs or chemicals is of utmost importance. Hence, researching the antioxidant activity and liver-protective effects of bitter leaf (*V. amygdalina*) aims to develop a new source of antioxidants from herbal medicine and is critically needed.

Materials and Methods

Materials

The *Vernonia amygdalina* plant was harvested in September 2020 in Phong Dien district, Can Tho City (Number: CTU/20-2020). Bitter leaf samples were analyzed for botanical characteristics, anatomy, and compared to the references.[9] To prepare the plant material, 2.0 kg of dried VA leaves were extracted by 96% ethanol. The solvent was then evaporated to obtain 200 grams of ethanol extract. Subsequently, various solvents with increasing polarity were used for fractionation, and the solvents were evaporated under reduced pressure to obtain fractions of *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water. Chemicals used in the study included gallic acid, thiobarbituric acid, Trolox (Calbiochem Ltd. Co.), and several other chemicals.

Preliminary analysis of plant for chemical composition

Preliminary analysis of plant chemical composition involves utilizing specific chemical reactions adjusted to laboratory conditions to detect various compound groups within bitter leaf samples.[12,17]

Alkaloids: Employ Mayer, Dragendoff, and Bouchardat reagents. If the solution becomes cloudier compared to the control tube or if there is the formation of a pale white-gold precipitate (Mayer), orange (Dragendoff), or brown (Bouchardat), it indicates the presence of alkaloids in the solution.

Organic acids: Using sodium carbonate crystals. If there are small gas bubbles evolving from sodium carbonate crystals, it suggests the presence of organic acids in the test sample.

Reducing Compounds: The extracted solution is reacted with Fehling A and Fehling B solutions, followed by heating. If a brick-red precipitate forms at the bottom of the test tube, it indicates the presence of reducing compounds.

Coumarin: React with a 10% KOH solution in alcohol on filter paper. Cover half of the extract spot and inspect under UV light at 365 nm. After a few minutes, if the covered part exhibits weaker fluorescence intensity than the uncovered part, it confirms the presence of coumarin.

Flavonoids using Cyanidin Reaction: The solution exhibits a color ranging from pink to red, suggesting the presence of flavonoids in the sample.

Glycosidic Cyanide Compounds: Employ the Raymon-Marthoud reaction. If a violet color appears, it indicates the presence of cardenolide glycosidic cyanide compounds.

2-Deoxy Sugars: React with a 5 mL xanthydrol reagent, and if the solution turns pink to purple, it suggests the presence of 2-deoxy sugars.

Saponins: The sample forms a stable foam when mixed with 25% alcohol, indicating the presence of saponins.

Tannins: React with a 5% $FeCl_3$ solution; if the solution turns dark green or mossy green, it indicates the presence of polyphenols. React with a gelatin salt solution; if a white flocculent precipitate appears, it confirms the presence of tannins in the test sample.

Polyphenols using Chemical Reactions: The herbal material is placed in a beaker and mixed with 50 mL of 50% ethanol. Shake thoroughly and heat under reflux for about 2 hours. Filter and collect the filtrate.

- React with 5% $FeCl_3$: Add a few drops of 5% $FeCl_3$ to 2 mL of the diluted test solution.
- React with 20% NaOH: Add a few drops of 20% NaOH to 2 mL of the test solution.
- React with 1% $(CH_3COO)_2Pb$: Add a few drops of 1% $(CH_3COO)_2Pb$ to 2 mL of the test solution.

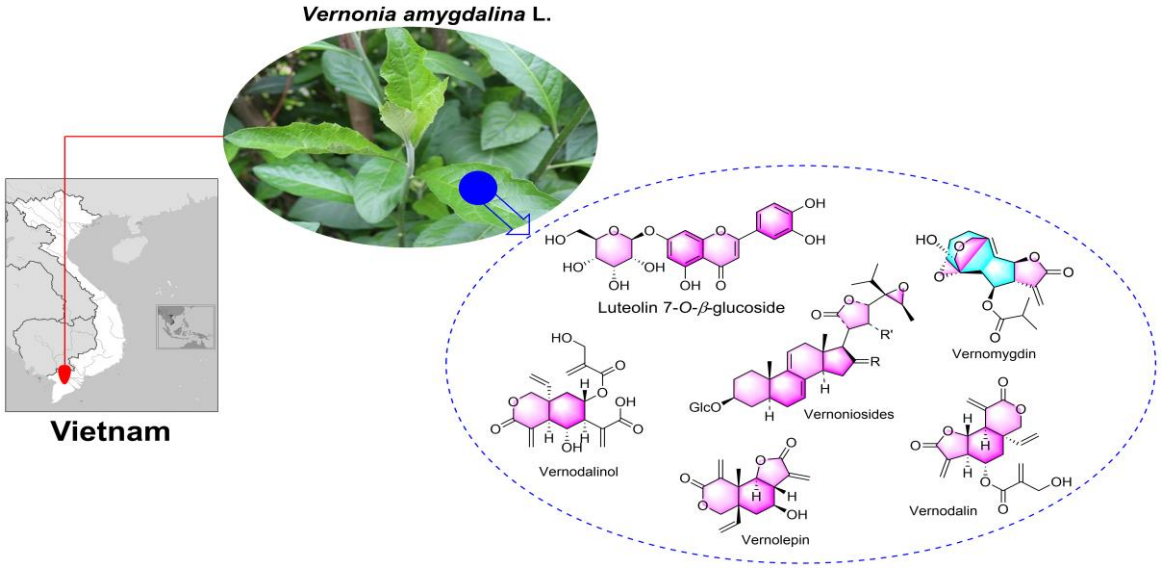

Figure 1: From traditional remedies to modern herbal medicine, the *Vernonia amygdalina* L. is a treasure trove of health-enhancing substances. Explore the chemistry of its foliage, where compounds like alkaloids, flavonoids, and essential oils contribute to its therapeutic properties

Antioxidant activity test based on ferric reduction capacity

The iron-reducing capacity of the ethanol extract of bitter leaf is determined based on its ability to reduce Fe^{3+} in the complex $Fe(CN_6)^{3-}$ to Fe^{2+} in the complex $Fe(CN_6)^{4-}$ in the presence of an antioxidant (ethanol extract). Subsequently, the complex $Fe(CN_6)^{4-}$ continues reacted with Fe^{3+} in $FeCl_3$ to form the $Fe[Fe(CN)_6]$ complex, which exhibited a green color and has the highest absorption at a wavelength of 700 nm.[18] Prepare a full-strength solution with a concentration of 10 mg/mL by weighing 10 mg of the ethanol extract and dissolving it in 1 mL of methanol. Dilute the samples with methanol to various concentrations (in μg/mL). Added 500 μL of phosphate buffer (pH = 6.6) to 5000 μL of the diluted ethanol extract (the negative control is methanol), and 5000 μL of 1% $K_3Fe(CN)_6$. Incubated at 50°C for 20 minutes. Added 5000 μL of 10% CCl_3COOH, centrifuge at 3000 rpm for 10 minutes. Transfer 5000 μL of the upper layer to 5000 μL of distilled water and add 1000 μL of 0.1% $FeCl_3$. Measure the absorbance at a wavelength of 700 nm. Gallic acid is used as a positive control. Repeat the experiment three times. Calculate the results based on the linear equation.

$$\% \text{ RSA} = ([OD_c - OD_t]/ OD_c) \times 100$$

Which,
%RSA: Radical scavenging activity
OD_c: Measured optical density of the control sample.
OD_t: Measured optical density of the test sample.

Investigating the hepatoprotective activity of bitter leaves using the lipid peroxidation method in rat brain cells

The ability to inhibit lipid peroxidation of the research samples was determined by measuring the content of malonyl dialdehyde (MDA), which is a product of the cell membrane lipid peroxidation process. MDA has the capability to react with thiobarbituric acid to form a trimethine complex (pink color) with a maximum absorption peak at λ = 532 nm.[19]

For the experimental samples at different test concentrations, which were mixed with a homologous solution, and then 2 mL of phosphate buffer was added. The reaction mixture was incubated for 15 minutes, and the reaction was stopped with trichloroacetic acid. After centrifugation, the resulting solution was reacted with thiobarbituric acid reagent for 15 minutes. The optical density was measured at a wavelength of 532 nm. Trolox (Calbiochem Ltd. Co.) was used as a reference substance. The experiment was repeated three times.

α-Amylase inhibition assay

The 250 µL of sample solution was combined with an equivalent volume of pH 6.9 buffer solution containing α-amylase (2 U/mL). The resulting mixture was incubated for 15 minutes at 37°C. Subsequently, 250 µL of 1% starch solution was introduced, and the reaction was allowed to proceed for an additional 20 minutes at 37°C. Finaly, 500 µL of 1% 3,5-Dinitrosalicylic acid was added, and the resulting mixture was subjected to boiling for 5 minutes before being allowed to cool. The absorbance of the resulting mixture was measured at 540 nm using Multiplate Reader. Acarbose was used as a standard.

α-Glucosidase inhibition assay

The study followed a protocol similar to previous research.[20] The α-glucosidase enzyme inhibition was conducted in 100 mM sodium phosphate buffer at pH 6.8. A mixture containing 60 µL of sample solution and 50 µL of phosphate buffer containing α-glucosidase solution (0.2 U/mL) was incubated in the wells of a 96-well plate at room temperature for 20 minutes. Subsequently, 50 µL of *p*-nitrophenyl-α-D-glucopyranoside solution, prepared in phosphate buffer, was added to each well, and the wells were further incubated for 10 minutes. The reaction was then terminated by adding 160 µL of 0.2 M Na_2CO_3. The absorbance was measured at 405 nm using Multiplate Reader.

Acute oral toxicity study

Healthy Swiss albino mice, 6 weeks old, with an average weight of 25±2 g, were provided by the Institute of Vaccines and Medical Biologicals, Nha Trang City, Vietnam. The study followed a protocol similar to previous research.[21,22] Prior to the experiments, the experimental mice were subjected to a 12-hour fasting period before administering the maximum allowable dose of the VA extract via oral gavage (dose volume: 20 mL/kg). Monitoring and recording of the number of deceased mice were carried out over a 72-hour period, and observations were continued for a total of 14 days. Surviving mice were subsequently provided with food and maintained under appropriate conditions. The care and handling of the animals were according to the established public health guidelines in Research Center of Ginseng & Material Medical, Ho Chi Minh city, Vietnam.

Statistical analysis

All the measurements were done in triplicate and results are expressed in terms of mean ± standard deviation. The animal experiments adhere to ethical standards, and the animals involved are subsequently cared for and maintained in good health. The study was approved by the Research Center of Ginseng & Material Medical, Ho Chi Minh city, Vietnam.

Results and Discussion

Microdissection of VA leaf

The present works marks the inaugural microscopic dissection of the VA leaf epidermis. This pioneering endeavor yields crucial revelations concerning the internal leaf architecture and morphological attributes of VA. Both the upper and lower epidermal layers manifest an assemblage of irregularly contoured, elongated cells, closely juxtaposed. The midrib exhibits characteristics akin to the leaf petiole (Figure 2). Noteworthy are the presence of protective trichomes on the leaf surface, complemented by glandular trichomes nestled within the leaf axils. Stomata are predominantly localized on the ventral epidermis.

Preliminary analysis chemical components in bitter leaf leaves

This study was conducted to provide a preliminary qualitative assessment for the presence or absence of secondary metabolites in the VA leaf extract. The results showed (Table 1) the presence of compounds such as alkaloids, flavonoids, saponins, various polyphenols, organic acids, and reducing compounds. These results are consistent with the findings reported by Hussen and colleagues in 2023 when assessing VA leaf extracts using ethanol as the solvent.[1] Additionally, the use of polar solvents, such as methanol and ethyl acetate, as highlighted by Norainny Yunitasari in 2022, is known to extract a wider range of plant chemical compounds compared to non-polar solvents.[23]

Antioxidant activity based on iron reducing power (FRAP) of bitter leaf extract

The FRAP (Ferric Reducing Antioxidant Power) assay was employed to measure the antioxidant's reducing potential in the extracts, which reacts with the ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex to produce a blue-colored ferrous tripyridyltriazine (Fe^{2+}–TPTZ). The results presented in Table 2 indicate that extracts from different segments of the bitter leaf all exhibit strong antioxidant activity.

Table 1: Phytochemical analysis of *V. amygdalina* leaf extracts

Compounds	Results
Alkaloid	+
Organic acid	+
Reducing compound	+
Coumarin	-
Flavonoid	+
Glycoside	-
2-Desoxy sugar	-
Saponin	+
Tanin	-
Polyphenol	+

(+): Positive (-): Negative

At a concentration of 13 μg/mL, the ethyl acetate-extracted sample showed the highest antioxidant activity, which is 80.72%, with an IC_{50} value of 8.42 μg/mL. Conversely, at a concentration of 27 μg/mL, the *n*-hexane-extracted sample (EC_{50} = 36.52 μg/mL) showed only 36.74% antioxidant activity, which is less than twice that of the other samples tested.

The antioxidant activity of the ethyl acetate-extracted VA leaf in this study is 25 times higher than the report by Muhammad and colleagues (2023) on VA leaves in Indonesia.[24] This discrepancy may be attributed to differences in climate and soil characteristics in the source regions, as well as variations in the extraction methods. Atangwho and colleagues (2013) pointed out that water and methanol extracts exhibited antioxidant capabilities that could be compared to quercetin, and sometimes even surpass Butylated hydroxytoluene.[25] Okoro (2019) conducted tests on VA leaf extracts and reported that acetone-extracted samples exhibited the highest iron-reducing activity at the highest concentration (400 μg/mL).[26] Ethanol extracts of VA leaves in Nigeria were found to have an iron-reducing capacity of 85 mg Fe(II)/g of extract. The study also revealed that this is a rich source of polyphenolic compounds with TPC and TFC values of 97 mg GAE/g of extract and 65.4 mg GAE/g of extract, respectively.[27] These findings clearly indicate that bitter leaf possesses biologically active compounds providing strong electron/hydrogen donation, making them effective antioxidants. Additionally, previous studies have highlighted the significant role of isolated compounds from this vegetable in its antioxidant activity, such as terpenoids, coumarin compounds, aromatics, unsaturated fatty acids, and fatty acids.[28,29] Based on the observed iron-reducing capacity, it is evident that the various extract segments of bitter leaf demonstrate robust antioxidant activity with EC_{50} values less than 50 μg/mL. This discovery contributes to demonstrating the high value of bitter leaf as a rich source of antioxidants in traditional herbal medicine practices in different countries.

Antioxidant activity based on DPPH assay

The DPPH assay results of VA leaf extracts demonstrated notable antioxidant effects. The scavenging activities of the VA leaf extracts exhibited a concentration-dependent trend. Among all the extracts, the ethyl acetate extract (EC_{50} = 71.88 μg/mL) exhibited significantly stronger antioxidant activity compared to the other extracts. At a concentration of 125 μg/mL, the antioxidant activity of the ethyl acetate extract reached 78.70%. In contrast, the *n*-hexane extract (EC_{50} = 340.71 µg/mL) displayed the lowest antioxidant activity, with only 36.738% at 250 μg/mL.

Hepatoprotective activity of bitter leaves by lipid peroxidation method in rat brain cells

The results obtained in this study indicate that the hepatoprotective activity, assessed through the peroxidation of mouse brain cell lipids, of ethanol extracts from bitter leaf increases with the extract's concentration. Specifically, as the extract concentration rises from 50 μg/mL to 1000 μg/mL, the cellular lipid peroxidation activity also increases from 35.12% to 81.56%. The hepatoprotective activity, as measured using the cellular lipid peroxidation method, of the ethanol-extracted bitter leaf (IC_{50} = 5.87 μg/mL) is significantly stronger than the positive control trolox (IC_{50} = 27.88 μg/mL) by a factor of 4.75 (Figure 3).

Table 2: Antioxidant activity of fractionated VA leaf extracts

Extracts	Iron reduction capacity		DPPH assay	
	%RSA[b]	EC_{50} (μg/mL)	%RSA[e]	EC_{50} (μg/mL)
n-Hexane	36.74	36.52±3.500	36.74	340.71±14.770
Chloroform	74.00	16.10±0.006	68.63	168.22±0.015
Ethyl acetate	80.72[c]	8.42±0.009	78.70[f]	71.88±1.28
n-Butanol	62.42	21.39±0.003	75.26	149.71±6.54
Ethanol	62.68	22.16±4.124	55.89[f]	126.384±6.12
Water	66.86	19.00±4.174	59.52	206.814±0.29
Gallic acid[a]	57.69[d]	10.41±0.070	58.90[g]	4.08±0.010

RSA: Radical scavenging activity; [a]Control. [b]%RSA at 27 μg/mL; [c]RSA at 13 μg/ml; [d]RSA at 12 μg/mL; [e]RSA at 250 μg/mL; [f]RSA at 125 μg/mL ; [g]RSA at 10 μg/mL.

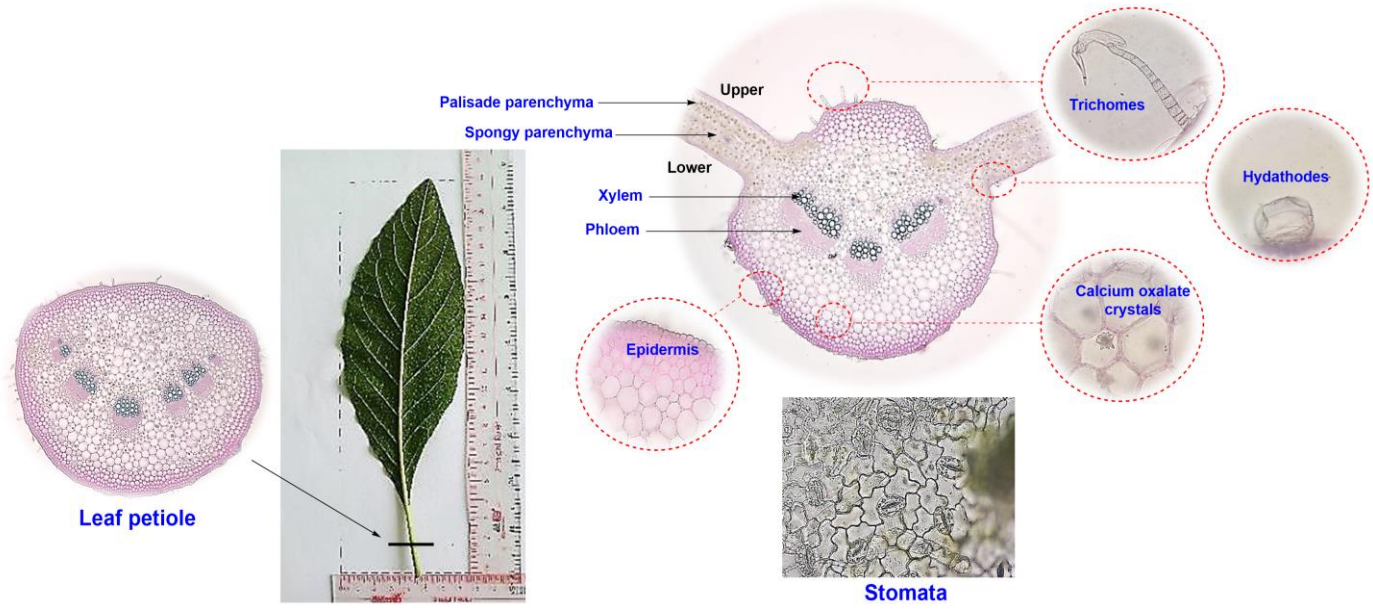

Figure 2: The morphological features of the leaf part and cross-sections of leaf petiole (left) and blades (right) of VA

These findings suggest that bitter leaf exhibits a strong protective effect on the liver. A report by Edet Okon Akpanyung and colleagues (2020) revealed that the use of ethanol as the solvent for *Vernonia amygdalina* extracts significantly increased all antioxidant parameters (SOD, GPx, Catalase, GSH). This report also indicated that these beneficial effects may stem from various phytochemical components, such as polyphenols present in VA extracts, which play a role in stimulating antioxidant enzymes.[30] Michael Okey Enemali and colleagues (2018) demonstrated that water extracts from *V. amygdalina* leaves had the most effective liver-protective effects, even surpassing the standard drug. The research group suggested that this may be due to the fact that *V. amygdalina* extracts contain phenolic compounds that confer potent antioxidant capabilities, safeguarding liver cells.[31]

In summary, extracts from various parts of the bitter leaf plant exhibited strong antioxidant activities with IC_{50} values below 50 μg/mL, with the ethyl acetate extract having an IC_{50} value of 8.4170 μg/mL. Therefore, bitter leaf is a herbal remedy that can be used to supplement the body with antioxidants and potentially contribute to supporting liver function.

In-vitro α-amylase and α-glucosidase inhibitory activity of VA extracts

The inhibitory activity against α-amylase enzyme of ethanol VA leaf extract demonstrate a concentration-dependent increase. At a concentration of 0.1 mg/mL, the inhibition of α-amylase reached 10.37%, escalating to 56.72% at 0.3 mg/mL concentration. The calculated IC_{50} value was determined to be 0.52 mg/mL (Table 3). Notably, the ethanol leaf extract of VA showcases potent inhibition of α-glucosidase enzyme activity. Elevating the concentration of the extract correlates with a proportional increase in the inhibitory activity against α-glucosidase. The inhibitory activity at a concentration of 12.5 µg/mL was measured at 16.61%, rising to 56.94% at a concentration of 100 µg/mL, with an IC_{50} value of 72.53 µg/mL (Figure 4). The IC_{50} value underscores the robust inhibitory efficacy of the ethanol VA leaf extract against α-glucosidase, surpassing acarbose by 1.68-fold. This study sheds light on the promising potential of ethanol VA leaf extract as a candidate for further exploration in the development of antidiabetic drugs with potential therapeutic applications.

Acute oral toxicity study

The acute oral toxicity assessment of ethanol extract from *V. amygdalina* leaves revealed no observable toxicity in the experimental mouse model. The results showed that there was not any toxic sign in mice given orally at the maximum dose (D_{max}) of 18.44 g VA/kg during the 14-day observation period.

Conclusion

The study on *V. amygdalina* highlights its potent antioxidant properties, particularly in the ethyl acetate extract. The ethanol leaf extract demonstrated remarkable hepatoprotective effects and significant inhibition of α-amylase and α-glucosidase enzymes. The acute oral toxicity study suggests the plant's safety for further exploration and product development. Our findings provide valuable insights into the therapeutic potential of *V. amygdalina*, laying the groundwork for future research and preventive health product development.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 3: *In-vitro* α-amylase and α-glucosidase inhibitory activity of ethanol VA leaf extract

Sample	IC_{50} (µg/mL)	
	α-Amylase	α-Glucosidase
Ethanol VA leaf extract	480.00±0.58	72.53±0.33
Acarbose	50.01±0.21	122.20±0.65

Acarbose as control. VA: *Vernonia amygdalina*

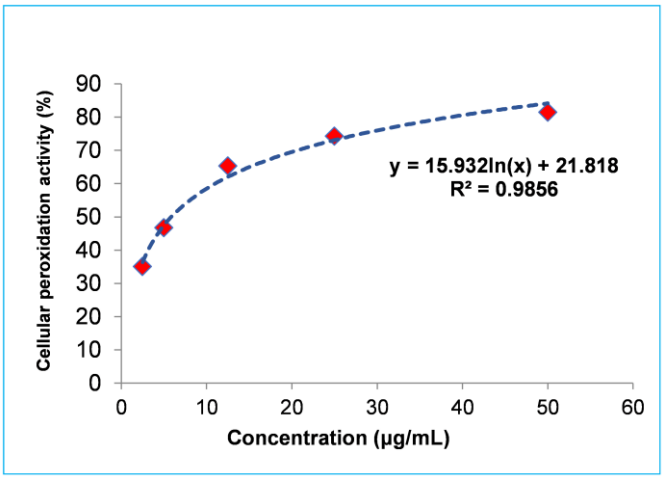

Figure 3: Inhibition activity of bitter ethanol leaf extract of *V. amygdalina* against cellular lipid peroxidation

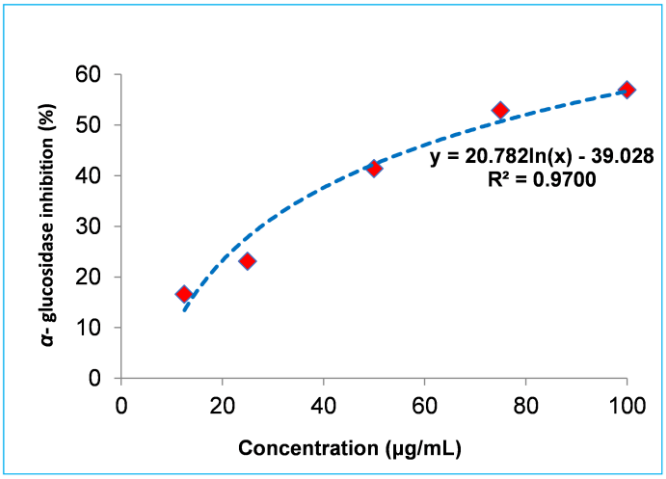

Figure 4: *α*-Glucosidase inhibitory activity of ethanol leaf extract of *V. amygdalina*

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