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Research Article

A comparative study of antioxidant potentials and phenolic acid and flavonoid profiles of *Amaranthus spinosus* leaf, stem and root extracts

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Abstract
Oxidative stress has been identified as a contributing factor in the onset of several illnesses and disorders. This has led to the adoption of multiple studies in recent years aimed at improving clinical outcomes by increasing antioxidant levels. The plant studied, *Amaranthus spinosus*, has been used in tribal cultures worldwide for its ethnomedicinal properties. Here, the present investigation characterises the phenolic acids and flavonoids in the methanolic extracts of *A. spinosus* from the leaf (ASLE), stem (ASSE), and root (ASRE) using High-performance liquid chromatography (HPLC). Additionally, a comparative evaluation of their antioxidant activity was conducted both *in vitro* and *in vivo*. ASLE exhibited high levels of rutin (12.93±0.06 µg/mg) and syringic acid (8.84±0.09 µg/mg), while ASSE was rich in gallic acid (3.39±0.03 µg/mg) and protocatechuic acid (2.05±0.004 µg/mg). *In vitro* antioxidant assays demonstrated comparable radical scavenging activities among the extracts, despite differences in phytochemical profiles. Also, *in vivo* experiments on thioacetamide-induced oxidative stress in mice (*Mus musculus*) demonstrated considerable antioxidant capabilities among all extracts. Administration of extracts restored catalase and SOD enzyme activity, elevated glutathione levels, and decreased lipid peroxidation in liver homogenates, indicating their therapeutic value. Therefore, the presence of important phenolic acids (e.g., syringic and chlorogenic acids) and flavonoids (e.g., rutin and quercetin) might explain the observed effectiveness. However, there were no significant variations in *in vivo* antioxidant activity amongst the extracts. This study highlights the therapeutic potential of *A. spinosus*, which supports its traditional use in combating oxidative stress. However, further research is necessary to identify the specific phytochemical combinations responsible for its antioxidant properties and their underlying mechanisms.

Keywords: Antioxidant, Flavonoids, Oxidative stress, Phenolic acid, Plant extracts

INTRODUCTION

Oxidative stress is the primary causative element behind the onset and advancement of several conditions, such as cancer, diabetes mellitus, cardiovascular illnesses, neurodegenerative disorders, and inflammatory diseases, among others (Arika *et al.*, 2019). The condition is caused by an overabundance of unbound oxygen and nitrogen species or an insufficient regulation of these species within the cell. Molecules containing free radicals, such as oxygen and nitrogen species, are inherently unstable. These compounds exist in the environment (exogenous) and are also synthesized within the body (endogenous) through normal aerobic metabolic processes (Bhat *et al.*, 2015). Various sources produce free radicals, including cigarette smoke, ozone exposure, ionising radiation, and certain medications. Endogenous sources of free radicals encompass the

electron transfer chain of mitochondria, the xanthine oxidase pathway, and many disease conditions (Moriasi *et al*., 2020). The human body possesses a complex antioxidant system consisting of enzymatic and nonenzymatic components, which work together to maintain a stable equilibrium between prooxidants and antioxidants. This system is crucial for maintaining an individual's overall well-being (Arika *et al*., 2019). Propyl gallate (PG), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) are commonly used as synthetic antioxidants to mitigate oxidative stress. However, their usage is associated with adverse effects, including liver damage and cancer (Ndhlala *et al*., 2010). They have limited availability, high cost, and lack of stability, which reduces their popularity. Hence, there is a demand for alternative, safer, and more effective antioxidants (Moriasi *et al*., 2020).

Plants, such as medicinal herbs, have been used for thousands of years for therapeutic purposes. *Amaranthus spinosus*, a plant with antioxidant properties, has a long history of use in conventional medical practices and may have potential as a herbal remedy (Gossell-Williams *et al*., 2006; Hardy, 2021; Pattanayak *et al*., 2016). Different parts of the plant have been utilised for their medicinal properties, including cooling, laxative, diuretic, stomachic, antipyretic, and other characteristics, by many cultural and ethnic groups in India (Chandrashekhar, 2019; Tanmoy *et al*., 2014). The plant has been utilized in Ayurveda for the management of bronchitis, blood disorders, leprosy, and piles, as well as for its antipyretic, laxative, and diuretic qualities (Kumar *et al*., 2014). It also has nutritional and therapeutic potential due to its phytoconstituents, which include betalains, flavonoids, and minerals (Ganjare and Raut, 2019). Various researchers have investigated the pharmacological actions of *A. spinosus*, which may include antidiabetic, antitumor, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepato-protective, spermatogenic, antifertility, antimalarial, and antioxidant properties (R. P. Kumar *et al*., 2014). It has a variety of phytochemicals that contribute to its ethnopharmacological characteristics. Phenols and flavonoids are plant-derived biologically active polyphenolic compounds with multiple possible health advantages mainly due to their antioxidant properties. They possess a wide range of molecular properties and can act as antioxidants due to their specific structural arrangements and propensity to donate hydrogen. Their actions have been examined for their utilization in medical sciences, and their levels in urine and blood have been measured for metabolic investigations (Stalikas, 2010).

Thioacetamide (TAA) is a hepatotoxicant that causes liver damage by imitating oxidative stress. The toxicity of TAA arises from its activation through a mechanism including mixed-function oxidases, mainly CYP2E1 and FAD monooxygenases. Physiological stimulation of TAA produces reactive metabolites, including free radicals from thioacetamide-S-oxide and reactive oxygen species (ROS) formed as intermediates (Chilakapati *et al*., 2007). Reactive metabolites can chemically bind to large biological molecules, causing oxidative damage. ROS generation caused by TAA treatment leads to lipid peroxidation, depletion of glutathione, and a decrease in SH-thiol groups (Okuyama *et al*., 2003; Pallottini *et al*., 2006). The prevalence of lifestyle disorders, such as obesity and type 2 diabetes, is currently rising due to the excessive intake of fried and processed foods. This leads to oxidative stress in the body, which is the primary cause of most diseases, particularly those related to lifestyle. Hence, it is imperative to comprehend the antioxidant capacity of plants such as *A. spinosus* to manage these diseases effectively.

Despite the well-established medicinal properties of *A. spinosus*, there is a notable lack of thorough research examining the phenolic acid and flavonoid content in various parts of the plant, such as the leaf, stem, and root, and their potential antioxidant effects. There has been limited data integration on the entire range of phytochemicals and their antioxidant capacities in the plant. These results are based on previous studies that have concentrated on analyzing specific plant constituents or particular types of phytochemicals (Gandhi *et al*., 2021). Moreover, there is a lack of agreement *in-vivo* and *in-vitro* assessments of antioxidant levels for various plant components. The present study aimed to compare and analyse the phenolic and flavonoid compounds present in the methanolic extracts of the leaves, stems, and roots of *Amaranthus spinosus*, and to assess the *in vitro* radical scavenging capacities of these individual plant parts. Additionally, the *in vivo* antioxidant capabilities of these extracts were evaluated using a Thioacetamide (TAA) induced mice (*Mus musculus)* model.

MATERIALS AND METHODS

Plant collection

Specimen of *A. spinosus* were collected from Midnapore block, District Pashchim Medinipur (22.4257° N, 87.3199° E), West Bengal, India, from December to February, (2019-20,2021-22 and 2022-23).

Extract preparation

The collected plants were washed thoroughly under running water to eliminate any dirt attached to the leaves. The leaves, stems, and roots were separated after being shade-dried for seven days. They were then chopped into smaller pieces and crushed into a powder using a grinder. 5gm of dried and powdered leaf, stem, and roots were taken separately in 50ml falcon tubes, and 80%-20% methanol-water solution was added to each falcon tube containing the powdered, dried plant

parts. The mixture was kept on a rocker (Tarsons, Rockymax) for 72 Hours. After 72 hours, the solutions were filtered through filter paper, and the filtrates were poured onto Petri dishes (this was done to increase the surface area for faster evaporation) and kept in an incubator at 37°C until all the methanol had evaporated. The Petri dishes were then transferred to the freeze dryer to further dry the extract under low temperatures and vacuum. The dried crude extracts were then scraped off the Petri dish and weighed. The percentage yield of each extract was determined using the following formula:

$$Percentage\ yield(\%) = \frac{Weight\ of\ extract(g)}{Weight\ of\ ground\ dry\ plant(g)} \quad \ldots\ldots(1)$$

Qualitative estimation of total phenolic acids and flavonoids

For the qualitative and quantitative assessment of phytochemicals, extracts were prepared in methanol at a concentration of 1 mg/mL.

Test for flavonoids and phenols

The alkaline reagent test was performed to detect the presence of flavonoids. Here, five drops of 5% NaOH were combined with 0.5 mL of extract to produce a yellow colour. After that, the solution was supplemented with a few drops of 2M HCl. The cessation of the yellow coloration verified the presence of flavonoids (Gul *et al*., 2017).

The presence of phenols was determined using the ferric chloride test. For this test 10% $FeCl_3$ solution was added after 2 ml of plant extract and 2 ml of distilled water were combined. An appearance of a blue-black colour was indicative of the presence of phenol (Kumar Shah and Yadav, 2015).

Quantitative estimation of total phenolic acids and flavonoids

Estimation of total phenolic content (TPC)

Quantitative estimation of Phenolic acids was done spectrophotometrically with the Folin-Ciocalteau reagent. To 100µl of extract (1mg/ml) or standard dissolved in methanol, 1.9 ml of distilled water was added. In this solution, 1 ml Folin-Ciocalteau (1N) and 20% Na_2CO_3 were added. This mixture was incubated at room temperature in the dark for 40 minutes. After 40 minutes, absorbance was recorded at 725 nm in a spectrophotometer (Muanda *et al*., 2011). A calibration curve was generated using Gallic acid at various doses, including 100-1000 µg/ml. Total phenolic content was expressed in mg/g of Gallic acid equivalents.

Estimation of total flavonoid content (TFC)

The total flavonoid content of the extracts was determined by the method described by Zaiter *et al.* (2016). 50 µl of extract (1mg/ml) or standard dissolved in methanol was added to the test tube containing 0.95 ml of distilled water. 75 µl of 5% sodium nitrite ($NaNO_2$) was added to the test tube and incubated for 5 minutes at room temperature. After 5 minutes, 75 µl of 10% aluminium chloride ($AlCl_3$) was added to the test tube, and the mixture was incubated for 6 minutes at room temperature. After 6 minutes, 500 µl of 1N NaOH was added to the test tube. Finally, 2.5 ml of distilled water is added to the test tube and mixed. Absorbance is measured at 510 nm. A calibration curve was generated using quercetin at various doses, including 10, 20, 40, 60, 80, and 100 µg/ml. The total flavonoid content of the plant extract was estimated by extrapolating from the standard curve. Total flavonoids were expressed as mg of quercetin equivalent per gram of dry matter (mg quercetin/g dry matter).

High-Performance liquid chromatography (HPLC) of the plant extract

HPLC separation was accomplished using a DIONEX Ultimate 3000 (Thermo Scientific, USA) fitted with a Quarternary pump LPG 3400SD and a photodiode array (PDA) detector. The separation was performed in an Acclaim 120 C-18 column (250mm x 4.6mm, 5µm) with a C-18 guard column with a 20µl loop. The gradient elution was 90 % solvent B and 10% solvent A, and the flow rate was settled from 1mL/min to 0.7 mL/min in 27 min, from 10 to 40 % solvent A with flow rate 0.7 mL/min for 23 min, 40% solvent A and 60% B with flow rate 0.7 mL/min primarily for 2 min and then flow rate altered from 0.7 to 0.3 mL/min in 65min, from 40 to 44% solvent A with flow rate 0.3 to 0.7mL/min in 70 min, 44% solvent A with flow rate 0.7 to 1mL/min for 10 min duration, solvent A changed from 44% to 58 % with flow rate 1mL/min for 5 min, 58 to 70% solvent A in 98 min at constant flow rate 1 mL/min. Then, 10% A was injected in 101 min and equilibrated for 4 min for the next injection.

Identification of phenolic acids and flavonoids

Phenolic acids and flavonoids were identified using an external standard method. Reference standards protocatechuic acid, gentisic acid, chlorogenic acid, p-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid, gallic acid and ellagic acid, catechin, rutin, myricetin, quercetin, naringin, apigenin and kaempferol were used for identification. Standard curves were performed for the quantitation of each component. The detection wavelengths used were 260 nm, 280 nm, and 310 nm.

Experimental animal maintenance

Swiss Albino mice (*Mus musculus)* weighing 20 ± 2 g were obtained from Saha Enterprise, Kolkata. Animals were housed in an air-conditioned room (22 ± 2 °C)

under a 12-hour light-dark cycle. The animals underwent a 7-day acclimatisation period prior to the commencement of the experiment. They were fed a typical pellet meal often used in research settings and received filtered drinking water for the entire experiment.

Animal ethical compliance

The research was conducted in compliance with the Animal Ethics Committee of Vidyasagar University (VU/IAEC/CPCSEA/2/6/2022).

Experimental model

Animals were administered TAA three times a week for 8 weeks at a dose of 150 mg/kg body weight intraperitoneally (Wallace *et al*., 2015). The purchased animals were divided into 11 groups (Groups I to XI) (n = 6), as listed in Table I. Animals from Groups VI, VII, VIII, IX, X, and XI were first administered TAA at 150 mg/kg body weight, three times a week, for 8 weeks. After 8 weeks of TAA treatment, Groups VII, VIII, and IX were given oral dosages of leaf, stem, and root Aqueous-methanolic extracts of *A. spinosus* at 250 mg/kg body weight, respectively, for 15 days, and the animals from Group X received 0.5% Carboxymethylcellulose (CMC) of the same volume as the extracts while Group XI received normal food and water. Group I was the Control Group (N), Groups II(L), III(S), and IV(R) received oral doses of Aqueous-methanolic Leaf (ASLE), stem (ASSE) and root (ASRE) extracts of *A. spinosus* at 250 mg/kg body weight respectively for 15 days. Animals from group V(C) were given 0.5% CMC orally for 15 days at the same volume as the extracts. All oral dosages were administered using a 24G gavage with a 1 mL Luer-lock syringe. The volume of extract dissolved in 0.5% CMC solution was kept under 0.4 mL, and the oral dosages were administered under fasting conditions (McConnell *et al*., 2010). CMC was used as a carrier molecule to help the methanolic extract dissolve completely in water.

Animals of groups I and VI were sacrificed by cervical dislocation one day after the 8-week TAA treatment. Subjects of Groups VII to XI were administered with their respective oral doses for 15 days after 8 weeks of TAA treatment. They were then sacrificed by cervical dislocation one day after the oral dosage schedule ended. Blood was collected by heart puncture for liver function tests and the FRAP assay, and tissue was collect-

Table 1. Details of animal groups for the experimental design

Group	No. of animals taken	Description
Group I : Control Group (N)	6	Control with normal rodent food
Group II : Leaf Extract Group (L)	6	Mice with normal rodent food and were given 15 days oral dose of ASLE dissolved in 0.5% CMC at 250 mg/kg body weight
Group III : Stem Extract Group (S)	6	Mice with normal rodent food and were given 15 days oral dose of ASSE dissolved in 0.5% CMC at 250 mg/kg body weight.
Group IV : Root Extract Group (R)	6	Mice with normal rodent food and were given 15 days oral dose of ASRE dissolved in 0.5% CMC at 250 mg/kg body weight
Group V : Vehicle group (C)	6	Mice with normal rodent food and 0.5% CMC were given to them during treatment in CMC Suspension.
Group VI : Thioacetamide Group (T)	6	Mice were given IP injections of thioacetamide 150mg/kg body weight 3 times a week for 8 weeks.
Group VII : Treated with leaf extract (TL)	6	Mice were given IP injections of thioacetamide 150mg/kg body weight 3 times a week for 8 weeks and were followed by 15 days oral dose of ASLE dissolved in 0.5% CMC at 250 mg/kg body weight
Group VIII : Treated with stem extract (TS)	6	Mice were given IP injections of thioacetamide 150mg/kg body weight 3 times a week for 8 weeks and were followed by 15 days oral dose of ASSE dissolved in 0.5% CMC at 250 mg/kg body weight
Group IX : Treated with root (TR)	6	Mice were given IP injections of thioacetamide 150mg/kg body weight 3 times a week for 8 weeks and were followed by 15 days oral dose of ASRE dissolved in 0.5% CMC at 250 mg/kg body weight
Group X : Treated with Vehicle control (TC)	6	Mice were given IP injections of thioacetamide 150mg/kg body weight 3 times a week for 8 weeks, followed by 15 days oral dose of 0.5% CMC.
Group XI : Treatment withdrawn (TW)	6	Mice were given IP injections of thioacetamide 150mg/kg body weight 3 times a week for 8 weeks, followed by normal rodent food and water ad libitum.

ed for other in vivo antioxidant activity assays.

***In vitro* antioxidant assay**

For the *in vitro* assessment of the antioxidant capacity of the extracts, the extracts were prepared in methanol at a concentration of 1 mg/ml. Extracts were serially diluted in 0, 200, 400, 600, 800, and 1000 µg/ml. The minimum concentration of plant extracts and the standards used was 200 µg/ml, and the maximum concentration was 1000 µg/ml.

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay

DPPH Radical Scavenging capacity of antioxidants was determined and expressed as Trolox Equivalent Antioxidant Capacity (TEAC) by a method described by Gulcin and Alwasel (2023). 0.5 ml extract solution was added to 3 ml of 0.1mM DPPH in a methanol, vortexed and incubated at room temperature in the dark for 30 minutes. After 30 minutes, absorbance was recorded at 517 nm in a UV spectrophotometer. The reaction mixture that does not contain a sample or standard served as a control. The scavenging effect of the extracts on the DPPH radical was determined by calculating their antioxidant capacity in terms of Trolox equivalent using a calibration curve. A calibration curve was prepared using Trolox, following the same procedure using concentrations of Trolox at 0 to 1000 µg/ml in methanol. The radical scavenging capacity was calculated with the help of the following Equation (2)

$$Radical\ Scavenging\ Activity\ (RSA)\% = \frac{A_c - A_s}{A_c} \times 100 \quad \text{.....(2)}$$

A_c is the absorbance of the Blank solution, A_s is the absorbance of the sample or standard at 517 nm.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

The ABTS Radical Scavenging capacity of antioxidants was determined and expressed as Trolox Equivalent Antioxidant Capacity (TEAC) using a method described by Chaves *et al.* (2020). A solution containing ABTS (7mM) and potassium persulfate (4.95mM) in equal volumes was prepared and incubated in darkness at room temperature for 16 hours. This was done to release $ABTS^{\cdot+}$ radical, through the oxidation of ABTS by potassium persulfate. After 1 hour, the mixture was diluted with methanol until it reached absorbance values of 1 to 1.5 at 734 nm. Then, 3.9 ml of ABTS dilution was mixed with 0.1 ml of extract or standard solution. This mixture was kept at rest for 1 minute, and then absorbance was noted at 734 nm. A reaction mixture without a sample or standard was considered blank. A calibration curve was prepared using Trolox, following the same procedure using concentrations of Trolox at 0 to 1000 µg/ml in methanol. The radical scavenging capacity was calculated as in the DPPH assay with the help of Equation 2.

Hydrogen peroxide (H_2O_2) scavenging assay

The hydrogen peroxide scavenging capability of the extracts was determined by the method described by Kumaran and Karunakaran (2007). A 40mM H_2O_2 solution was prepared in phosphate buffer saline(PBS) (pH 7.4). 0.1 ml aliquots of extract dilutions were transferred to test tubes, volumes were made up to 0.4 ml with PBS, and 0.6 ml of H_2O_2 solution (40 mM) was added and incubated at room temperature for 10 minutes. The absorbance of H_2O_2 was noted at 230 nm after 10 minutes of reaction. The percentage of H_2O_2 scavenging activity was calculated using the following equation (3). A graph of % H_2O_2 scavenging activity versus concentration of extracts was made. A solution devoid of plant extract was considered as a control.

$$\%\ H_2O_2\ Scavenged = \frac{A_c - A_s}{A_s} \times 100 \quad \text{.....(3)}$$

A_c is the absorbance of the blank solution, and As is the absorbance of the sample or standard at 230 nm.

Nitric oxide scavenging assay

The nitric oxide scavenging capacity was determined using the method described by Patel et al. (2010). Two millilitres of 10 mM Nitroprusside were added to 0.5 millilitres of PBS, and 0.5 millilitres of sample or standard was added to the reaction mixture. This solution was incubated at 25 °C for 150 minutes. After incubation, 0.5 ml of this mixture was taken and added to 1 ml sulfanilic acid, and it was allowed to stand for 5 minutes. Then, 1 ml of Napthyl Ethylene diamine was added to this mixture and was incubated at 25°C for 30 minutes. The concentration of nitrite was evaluated by noting absorbance at 546 nm. The amount of nitric oxide radical inhibition was calculated using Equation 4.

$$\%\ \text{inhibition of}\ Nitric\ oxide\ radical = \frac{A_c - A_s}{A_s} \times 100 \quad \text{.....(4)}$$

A_c is the absorbance of the blank solution, and As is the absorbance of the sample or standard at 546 nm.

Superoxide radical scavenging assay

Superoxide radical scavenging assay was determined using the method described by Alam (Alam *et al.*, 2013). To 0.5 ml of NBT (0.3mM) and 0.5 ml of NADH (0.12mM), 1 ml of extract dilutions or standard were added, and to this, 0.5 ml of Tris-HCl buffer was added. In this reaction mixture, 0.5 ml PMS was added to initiate the reaction and was incubated at 25°C for 5 minutes. The absorbance was measured at 560 nm against blank. In blank, instead of extracting, only solvent was added, which, in this case, was methanol. The superoxide radical scavenging activity was determined using Equation 5.

$$Superoxide\ Radical\ Scavenging\ activity\ \% = \frac{A_c - A_s}{A_s} \times 100 \quad \ldots\ldots\ldots(5)$$

A_c is the absorbance of the Blank solution, A_s is the absorbance of sample or standard at 560 nm.

Calculation of IC50 values

The inhibitory concentration (IC50) is a quantitative measure that represents the quantity of a certain inhibitory substance necessary to interfere with a biological process or biological component by fifty per cent *in vitro* (Gomis-Tena, 2020). The IC50 Values of *the in vitro* antioxidant assays discussed have been calculated using the linear slope-intercept equation for each antioxidant versus the percentage scavenging capacity of that antioxidant.

***In vivo* antioxidant assay**

For the *in vivo* antioxidant assay, blood was collected in a microcentrifuge tube containing EDTA. The isolated tissue from the animal was weighed and then homogenised in 0.1 M Tris-HCl Buffer (pH 7.5) at a ratio of 1:9 to create a 10% tissue homogenate. Protein concentration was estimated using Lowry's method for supernatants obtained from animals of all groups, and an equal amount of protein was used in all assays (Lowry *et al*., 1951). The collected blood was kept undisturbed at 4°C for 1 hour. After 1 hour, the whole blood was centrifuged at 5000 RPM for 10 minutes at 4°C. The straw-coloured fluid, collected after centrifugation, was stored at 4°C for the FRAP assay.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP test was performed according to the method of Benzie and Strain to measure the ferric reduction activity of plasma from various groups (Benzie and Strain, 1999). The absorbance of the reaction mixtures was measured at 593 nm against the blank, and the difference in absorbance between the initial and 4-minute readings was calculated. A solution with everything except the sample was taken as blank. A standard curve of ascorbic acid was used parallel to the experiment every time the assay was done. FRAP Value (µM) was measured with the help of Equation (6).

$$FRAP\ Value(\mu M) = \frac{0\ to\ 4\min\ \Delta A_{593}\ test\ sample}{0\ to\ 4\min\ \Delta A_{593}\ standard} \times FRAP\ Value\ of\ standard\ (\mu M) \quad \ldots\ldots\ldots\ (6)$$

Estimation of reduced glutathione (GSH)

The concentration of reduced glutathione was estimated in liver tissue from animals in each group using the procedure established by Ellman (Ellman, 1959; Hosseini *et al*., 2024; Moron *et al*., 1979). A tissue homogenate with 1 mg of protein was prepared, and an equal volume of 5% TCA in 1 mM EDTA was added. The mixture was then allowed to stand for 5 minutes at room temperature. Then, the solution was centrifuged for 10 minutes at 2000 rpm. The supernatant was collected, and 100µL of it was added to 2 ml of Ellman's reagent. The intensity of the yellow colour developed was measured in a UV Spectrophotometer at 412 nm against blank. The blank solution contained everything except the sample. A standard curve of GSH was made using GSH solutions with concentrations ranging from 0 to 25 µM.

Catalase assay

For estimation of Catalase activity in liver homogenate, a methodology described by Hugo Aebi in 1984 was used, which took into account the incorporation of 1 % Triton-X 100 and ethanol (0.01ml/ml of supernatant) in the steps of tissue homogenate preparation as suggested by Cohen and team earlier in 1970 (Aebi, 1984; Cohen *et al*., 1970). 2 ml of 50 mM sodium phosphate buffer (pH 7) was mixed with 1 ml 30 mM H_2O_2 and tissue homogenate with 1 mg protein was added to this solution. The change in absorbance was recorded from 0 to 60 seconds at 240 nm using a UV Spectrophotometer. The blank solution contained everything except the sample. The enzyme activity was assessed by measuring the mM of H2O2 degraded per minute, using a 43.6 M cm-1 concentration. One unit of activity (U) was defined as 1 mmol of H_2O_2 degraded per minute and was expressed as units per milligram of protein (Alam *et al*., 2013).

Lipid peroxidation assay

Estimation of lipid peroxidation was done following Okhawa's procedure. Tissue homogenate containing 1 mg of protein was added to a mixture of 200 µl of 8.1 % SDS, 1.5 ml of 20% Acetic acid, and 1.5 ml of 0.8% Thiobarbituric acid (TBA). The mixture volume of the solution was adjusted to 4 ml. Then it was incubated in a water bath at 95°C for 30 minutes. After incubation, the mixture was cooled under tap water. Then, 1 ml of distilled water was added, and 5 ml of 15:1 n-butanol and pyridine were added. Then, the mixture was centrifuged at 4000 RPM for 10 minutes. The absorbance of the organic layer was taken at 532 nm using a UV spectrophotometer (Ohkawa *et al*., 1979). The assay, commonly called the TBARS assay, measures the quantity of Malondialdehyde (MDA)-TBA adduct in the reaction mixture at a wavelength of 532 nm. A standard curve was drawn using concentrations of Tetraethoxypropane (TEP), which gives the same amount of equivalent moles of MDA in the reaction mixture on hydrolysis in the range of 0 to 60 µM (Fauziah *et al*., 2018).

Superoxide dismutase (SOD) activity

SOD activity was assessed using a modified protocol established by Kakkar in 1984, based on the methodology initially described by Nishikimi (Nishikimi *et al*., 1972; Kakkar *et al*., 1984). 1.2 ml of 0.052 mM sodium phosphate buffer, 0.1 ml PMS, and 0.3 ml NBT were added to two different test tubes. 0.2 ml of distilled wa-

ter was added to one test tube, and tissue homogenate containing 1 mg protein was added to the other. A second test tube containing all components, including tissue homogenate, except for PMS, was designated as the control. 0.2 ml of 780 µM NADH was added to all three test tubes and incubated at 30°C for 90 seconds. The reaction was stopped by the addition of 1 ml of Acetic acid. Then, 4 ml of n-butanol was added, and the mixture was shaken and allowed to stand for 10 minutes. The mixture was centrifuged at 4000 rpm for 10 minutes at 4°C. The absorbance of the organic layer obtained from the last step of this assay was recorded at 560 nm in a UV spectrophotometer. Percentage inhibition was calculated according to Equation 7, which is given below. The enzyme concentration required for 50% inhibition of chromogen production was considered one enzyme activity unit(U).

$$Percentage\ inhibition = \frac{\Delta A_{Control} - \Delta A_{Sample}}{\Delta A_{Control}} \times 100 \quad \text{.....(7)}$$

RESULTS

Amount of extracts obtained

Crude extraction was performed on several components of *A. spinosus* using an extraction solvent containing 80% methanol in water. From 5 g of powdered leaf material, 311.2 mg of extract yielded 6.22%. Similarly, the stem extract yielded 201.3 mg (4.02%) from 5 g of powdered material, while the root extract yielded 258.7 mg (5.17%) from the same quantity. These data show that the leaf had the maximum extraction efficiency, followed by the root and then the stem.

Qualitative estimation of total phenolic acids and flavonoids

The initial qualitative phytochemical analysis of methanol extracts from all three plant parts suggested the presence of both phenolic acids and flavonoids.

Quantitative estimation of total phenolic acids and flavonoids

The Total Phenolic Content (TPC) of ASLE, ASSE, and ASRE were calculated to be 26.54, 20.83, and 25.74 mg/g of gallic acid equivalent (GAE), respectively while the total flavonoid content (TFC) of ASLE, ASSE, and ASRE were calculated to be 9.635, 9.11, and 10.176 mg/g of quercetin equivalent (Table 2).

High-performance liquid chromatography (HPLC) of the plant extract

The HPLC analysis of aqueous methanolic extract (80% methanol) of *Amaranthus spinosus* leaf, stem, and root indicated various phenolic acids and flavonoids with different retention times. The detailed concentration of the flavonoids and phenolic acids found in leaf, stem, and root extracts (ASLE, ASSE, and ASRE) are given in Tables 3 and 4, respectively. ASLE and ASRE contained particular flavonoids such as Rutin and Quercetin, whereas Caffeic acid, a phenolic acid, was only detected in ASLE and ASRE. Narinigin and p-Hydroxy benzoic acid are specific flavonoid and phenolic acids only present in ASLE and ASSE. In addition to the flavonoids mentioned above, Catechin, Myricetin, Naringenin, Apigenin, and Kaempferol were also found in all the extracts. Two phenolic acids, salicylic acid and ellagic acid, were found only in ASRE and ASSE. All the information presented above can be seen in Figs. 3 and 4.

As observed in Table 3, the flavonoid rutin was found at a concentration of 12.932±0.06376 µg/mg extract in ASLE, which is the highest among all the flavonoids identified in the extracts of all three plant parts. In ASRE, Rutin was found at 5.805 ± 0.0106 µg/mg but was present in a negligible concentration, 0.035 ± 0.0035 µg/mg, in the stem extract. Among other flavonoids, quercetin was present in the second-highest concentration in the leaf extract (6.840±0.162 µg/mg). In the stem extract, quercetin was found at a concentration of 1.889 ± 0.0045 µg/mg. However, in the root extract, this flavonoid was found at a concentration of 0.699 ± 0.0104 µg/mg (Table 3). Other flavonoids found in high concentrations were Catechin and Naringin, both present in the stem extract, at concentrations of 6.123±0.0062 µg/mg and 7.213±0.0047 µg/mg, respectively. Among these two flavonoids, catechin was found in comparatively, more minor concentrations than the other two extracts. Naringin was not observed in the Root extract but was found at a concentration of 0.345±0.0103 µg/mg in the leaf extract. Apigenin was absent in the Stem extract and was found in inconsequential amounts in ASRE. Other flavonoids, such as myricetin and naringenin, were found in small concentrations in all three extracts. Apart from these flavonoids, kaempferol was found in almost similar concentrations in all three extracts (Table 3).

Table 2. Total Phenolic acids and flavonoids in Leaf (ASLE), stem (ASSE) and root extracts (ASRE)

	Phenolic Acid (mg/g GAE)	Flavonoid (mg/g QE)
Leaf extract	26.543 ± 0.953	9.635 ± 0.397
Stem extract	20.832 ± 1.04	9.109 ± 0.364
Root extract	25.744 ± 0.672	10.176 ± 0.355

Three phenolic acids were found at significant concentrations in the leaf extract: Syringic acid, p-coumaric acid, and Chlorogenic acid at concentrations of 8.836±0.0942 µg/mg, 4.461 ± 0.0128µg/mg, and 2.999±0.007 µg/mg respectively (Fig. 2; Table 5). The concentration of Syringic acid was much lower in Root extract (1.396±0.0087 µg/mg) and very low in stem extract (0.547±0.0056 µg/mg). The concentration of p-Coumaric acid in the root extract was 0.786±0.0062 µg/mg, and its concentration in the stem extract was found to be 0.404±0.0058 µg/mg, both of which were significantly low when compared to the leaf extract. Phenolic acids that were higher in other extracts were gallic acid and Protocatechuic acid in ASSE, which were found at concentrations of 3.394 ± 0.0274 µg/mg and 2.051 ± 0.0043 µg/mg, respectively (Fig. 2; Table 5). p-Hydroxybenzoic acid was completely absent in ASSE and present in very low concentrations in the other two extracts. On the other hand, Caffeic acid was found in leaf and root extracts in insignificant amounts (0.303 ± 0.00473 µg/mg and 0.117 ± 0.0055 µg/mg, respectively), while being absent in the stem extract (Table 4). Salicylic acid and ellagic acid were detected in minimal amounts exclusively in the extracts derived from the Root and Stem. Other phenolic acids, such as ferulic acid, vanillic acid, and sinapic acid, were found in insignificant concentrations in all three extracts (Table 4). Details of peak height, area, and retention time (RT) of all phenolic acids and flavonoids are specified in Tables 5, 6, and 7.

In vitro antioxidant assay

The study evaluated the IC50 values of three plant extracts, ASLE, ASSE, and ASRE, for their ability to inhibit DPPH and ABTS radicals. The IC50 values were significantly higher than those of Trolox in DPPH and ABTS radical inhibition, with all three extracts showing effective DPPH and ABTS radical scavenging activity (RSA). However, differences in RSAs among the three plant extracts were statistically insignificant. This suggests that the radical scavenging activity (RSA) of ASLE, ASSE, and ASRE was lower than that of Trolox for scavenging DPPH and ABTS. However, they still exhibited considerable RSA individually, as seen in Table 8, Fig. 6, and Fig. 7.

In terms of H_2O_2 scavenging, the IC50 values were 883.5 ± 34.26 µg/mL for ASLE, 865.6 ± 18.98 µg/mL for ASSE, and 806.4 ± 14.55 µg/mL for ASRE. The standard used for assessing H2O2 scavenging was ascorbic acid, which had an IC50 value of 633.5 ± 6.37 µg/ml. However, all three extracts were significantly less than the standard used but still showed effective H2O2 scavenging capability (Table 8; Fig. 8). The IC50 Values for the NO scavenging capacity of scorbic acid were found to be 644.3 ± 3.206 µg/ml. ASLE, ASSE, and ASRE yielded IC50 values of 1186 ± 135.8 µg/ml, 1091 ± 107.2 µg/ml, and 935.5 ± 45.06 µg/ml, respectively. The values of ASLE and ASSE were significantly higher than the IC50 values of Ascorbic acid, as confirmed by ANOVA followed by Tukey's test at $p < 0.05$. However, it was observed that the IC50 of ASRE for NO scavenging showed a slight, yet significant, similarity with the IC50 value of Ascorbic acid (Table 8; Fig. 9). Ascorbic acid's superoxide radical scavenging capacity was determined by its IC50 value, which was 564.8±6.225 µg/ml. Compared to the IC50 values of ASLE, ASSE, and ASRE, the difference in the IC50 values of their corresponding standards, Ascorbic acid, was statistically significant (Table 8; Fig. 10). However, all three extracts showed effective scavenging activity against H2O2, NO, and superoxide radicals.

In vivo antioxidant assay

The FRAP assay results, as shown in Fig. 11, showed a sharp drop in serum antioxidant potential in Group VI (T), in which oxidative stress was induced in the liver when compared to animals of Group I. Animals of Group XI (TW) and Group X(TC) showed no significant difference when compared to the antioxidant capacity

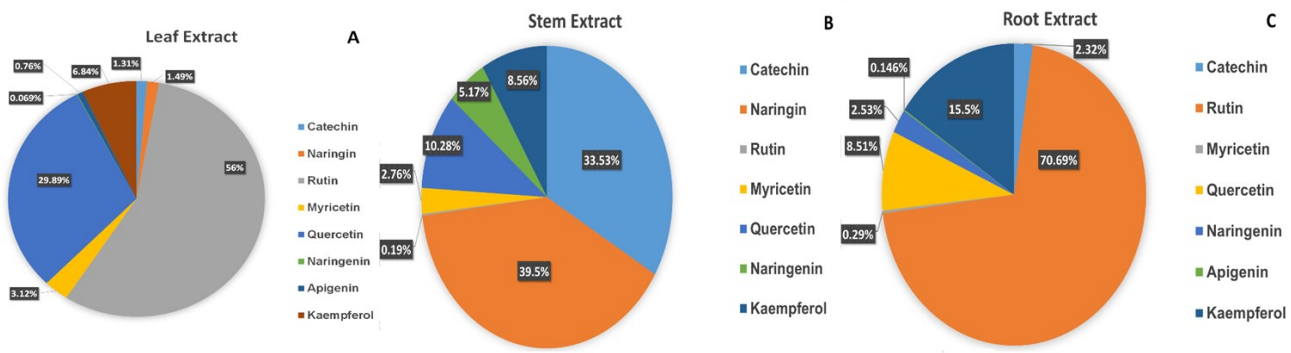

Fig. 1. *Percentage composition of various flavonoids in the preparations of the leaf (A), stem (B), and root (C);Rutin present in high concentrations in leaf and root extracts, but in very minor quantities in stem extracts. The stem extract contains Catechin and Naringin in a similar proportion. Additionally, leaf extract contains a substantial amount of Quercetin, another flavonoid. Rutin, quercetin, catechin, and myricetin are the common flavonoids present in all three extracts.*

Table 3. Flavonoid compounds identified from leaf extract (ASLE) stem extract (ASSE) and root extract (ARSE)

Flavonoids	Leaf extract µg/mg	Root extract µg/mg	Stem Extract µg/mg
	Mean± SEM	Mean ± SEM	Mean ± SEM
Catechin	0.299 ± 0.007	0.190 ± 0.0035	6.123 ± 0.0062
Naringin	0.345 ± 0.0103	-	7.213 ± 0.0047
Rutin	12.932 ± 0.06376	5.805 ± 0.0106	0.035 ± 0.0035
Myricetin	0.713 ± 0.01965	0.024 ± 0.0059	0.504 ± 0.0064
Quercetin	6.840 ± 0.162	0.699 ± 0.0104	1.879 ± 0.0045
Naringenin	0.016 ± 0.0033	0.208 ± 0.0064	0.945 ± 0.0044
Apigenin	0.174 ± 0.0071	0.012 ± 0.0032	-
Kaempferol	1.564 ± 0.0069	1.273 ± 0.0114	1.563 ± 0.0046

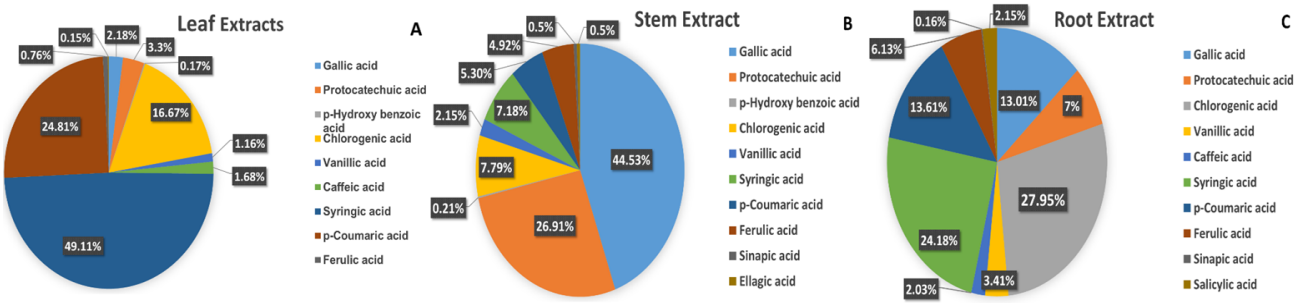

Fig. 2. *Percentage composition of different phenolic acids in Leaf (A), Stem (B), and Root (C) extracts. Syringic acid is found in high proportions in Leaf extract, Chlorogenic acid in Root extracts, and Gallic acid in stem extract. The leaf extract and stem extract also have protocatechuic acid in the second highest proportions, and root extract has syringic acid in the second highest proportion. Gallic acid, Protocatechuic acid, syringic acid, Chlorogenic acid, Vanillic acid,p-coumaric acid, and ferulic acid are the common phenolic acids found in all three extracts.*

of animals of group VI(T). Animals of groups VII, VIII, and IX, which received ASLE, ASSE, and ASRE for 15 days, showed significant elevation in antioxidant potential compared to group VI animals. On the other hand, there was no significant difference in the antioxidant potentials between animals of Group I(N) and groups VII, VIII, and IX. The antioxidant potential of the plant extracts in animals of Groups II, III, and IV, which received ASLE, ASRE, and ASRE, respectively, increased after 15 days of oral dosage. Furthermore, when the antioxidant potential of animals of Group XI (TW) and Group X(TC) were compared, the difference was insignificant, and Group XI showed prominent changes in antioxidant capacity when compared to that of Groups VII, VIII, and IX.

In the case of catalase and SOD activities (Fig.s 12 and 13), enzyme activity in Group VI(T) was significantly decreased when compared to animals of Group I(N). Animals of Group XI (TW) and Group X (TC) showed no significant difference in catalase and SOD activities compared to those of Group VI (T). Animals of groups VII(TL), VIII(TS), and IX(TR), which received ASLE, ASSE, and ASRE for 15 days after inducing oxidative stress, showed significant recovery in catalase and SOD activities in liver tissue when compared to that of group VI animals and showed no noticeable change when compared to group Group I animals. The catalase and SOD activities in liver tissue of animals of Groups II, III, IV, and V did not show significant differences with tissue from animals of Groups I. Furthermore, when the activities of these enzymes in the liver of animals from Group XI (TW) and Group X (TC) were compared, the difference was insignificant. However, Group XI showed prominent changes in catalase and SOD activities compared to those of Groups VII, VIII, and IX.

A comparison of GSH concentration in the liver tissue of control animals (Group I) and TAA-treated animals (Group VI) indicated a decrease in GSH concentration in animals of Group VI. Again, the concentration of GSH increased considerably in Groups VII (TL), VIII (TS), and IX (TR) compared to Group VI (T), while Group X (TC) showed a small and insignificant increase from Group VI. GSH concentration did not vary considerably within Groups VI and XI, as well as within Groups X and XI. Additionally, the GSH concentration in the liver tissue of animals in Groups II, III, IV, and V did not show a significant difference compared to tissue from animals in Groups I and XI. However, a prominent variation in GSH concentration was observed in Groups VII, VIII, and IX when compared to Groups II, III, IV, and V (Fig. 14).

Lipid peroxidase activity vastly increased in TAA-treated animals (Group VI) compared to control animals

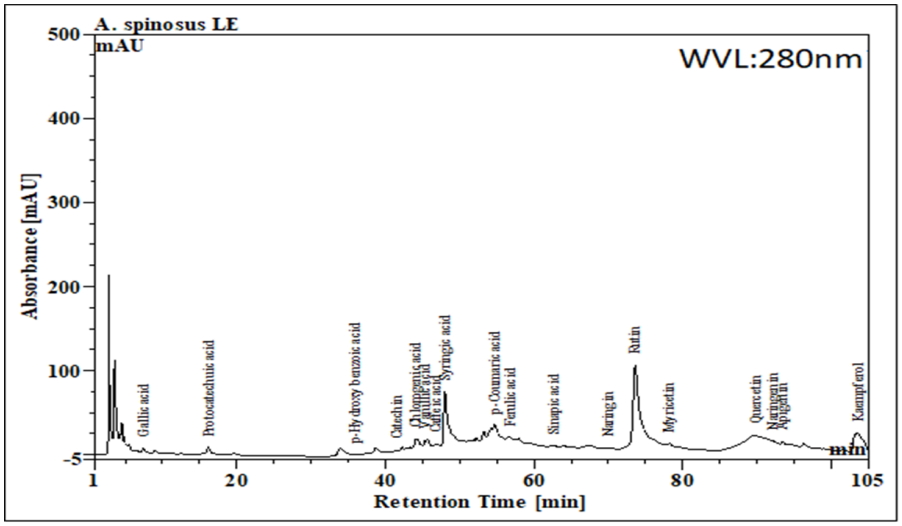

Fig. 3. *Chromatograph from HPLC analysis of ASLE. Flavonoid with highest concentration found in ASLE was Rutin, and the phenolic acid with highest concentration was Syringic acid.*

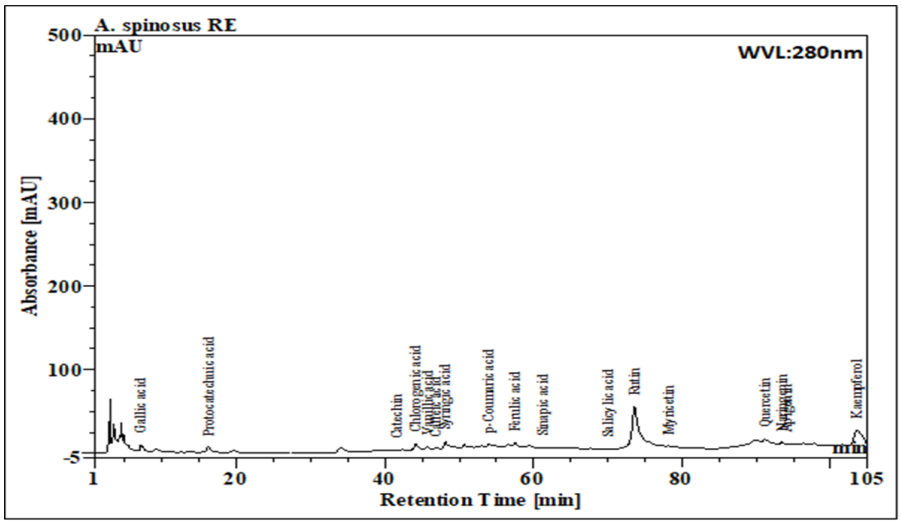

Fig. 4. *Chromatograph from HPLC analysis of ASRE. Flavonoid with highest concentration found in ASRE was Rutin, and the phenolic acid with highest concentration was Chlorogenic acid.*

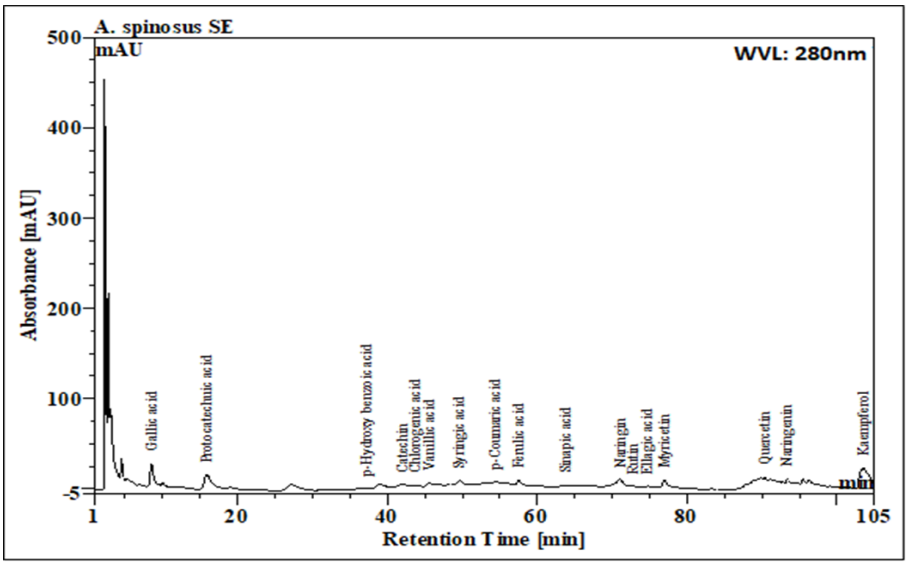

Fig. 5. *Chromatograph from HPLC analysis of ASSE. Flavonoid with highest concentration found in ASSE was Naringin, and the phenolic acid with highest concentration was Gallic acid.*

Table 4. Phenolic acids identified from leaf extract (ASLE), stem extract (ASSE) and root extract (ARSE)

Phenolic acid	Leaf extract (µg /mg)	Root extract (µg /mg)	Stem Extract (µg /mg)
	Mean± SEM	Mean± SEM	Mean± SEM
Gallic acid	0.393±0.0206	0.751±0.00754	3.394±0.0274
Protocatechuic acid	0.594 ±0.0038	0.426±0.0077	2.051±0.0043
p-Hydroxy benzoic acid	0.030±0.0026	-	0.016±0.0035
Chlorogenic acid	2.999±0.007	1.614±0.0108	0.594±0.00353
Vanillic acid	0.209±0.0075	0.197±0.011	0.164±0.00473
Caffeic acid	0.303±0.00473	0.117±0.0055	-
Syringic acid	8.836±0.0942	1.396±0.0087	0.547±0.00561
p-Coumaric acid	4.464±0.0128	0.786±0.0062	0.404±0.0058
Ferulic acid	0.137±0.00416	0.354±0.0062	0.375±0.00384
Sinapic acid	0.027±0.0045	0.009±0.0015	0.038±0.0046
Salicylic acid	-	0.12±0.0067	-
Ellagic acid	-	-	0.038±0.0041

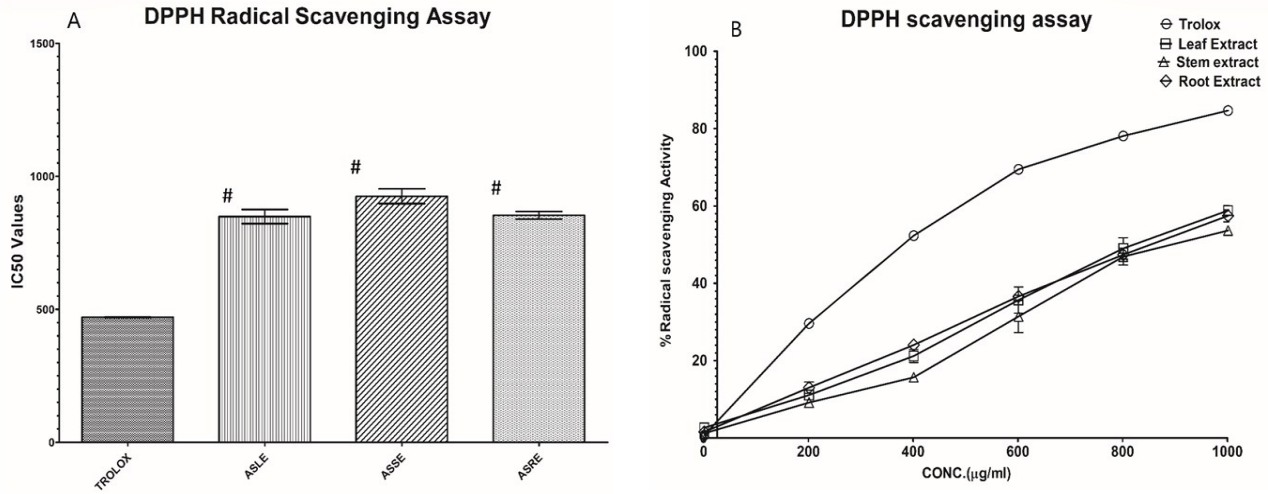

Fig. 6. *(A) Comparative IC50 of DPPH Radical Scavenging Capacities of ASLE, ASSE, and ASRE. (B) Comparative % Radical Scavenging Activities of ASLE, ASSE and ASRE. The IC50 values of ASLE, ASSE, and ASRE are significantly higher than those of the IC50 values of Trolox. "#" denotes significant difference in IC50 with the standard Trolox. A Higher IC50 value signifies that a greater quantity of the antioxidant is required to neutralise 50% of free radicals or oxidative species, indicating reduced efficacy or decreased antioxidant activity.*
The data suggests that all three extracts possess antioxidant properties but are not as potent as Trolox.

(Group I). Again, the enzyme activity decreased considerably in Groups VII (TL), VIII (TS), and IX (TR) compared to Group VI (T). Additionally, Groups X (TC) and XI (TW) exhibited a significant decrease in lipid peroxidation compared to Group VI. The difference in lipid peroxidase activity within groups X and XI was not substantial. However, liver homogenate from Groups VII, VIII, and IX had a prominently reduced lipid peroxidase activity compared to that of Group XI. Additionally, lipid peroxidase activity in the liver tissue of animals in Groups II, III, IV, and V did not show significant differences compared to liver tissue from animals in Group I (Fig. 15).
All the changes described above were significant at $p < 0.05$, according to ANOVA followed by Tukey's multiple comparison test.

DISCUSSION

This study examines the antioxidant capacity and phytochemical content of methanolic extracts derived from different parts of *Amaranthus spinosus*. A comprehensive approach was undertaken to enhance the understanding of how bioactive chemicals are distributed throughout the plant's structure and their potential as antioxidants. This enhances the present study's comprehension of the therapeutic applications of this plant. The HPLC analysis of the plant revealed that rutin was detected in the leaf and root extracts at the most significant concentration among all flavonoids. In contrast, stem extracts contained naringin and catechin. In all three extracts, kaempferol was present in equivalent concentrations. The most dominating phenolic acid in

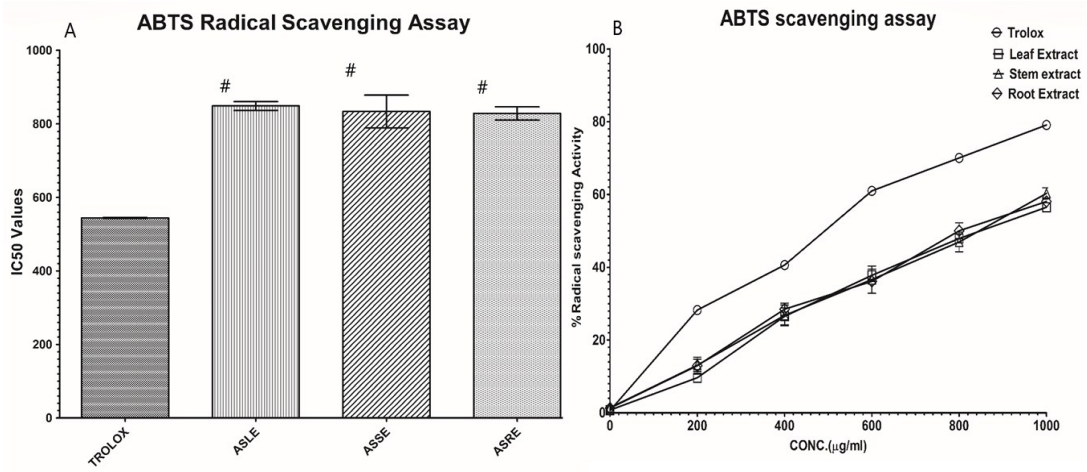

Fig. 7.*(A) Comparative IC50 of ABTS Radical Scavenging Capacities of ASLE, ASSE, and ASRE. (B) Comparative % ABTS Radical Scavenging Activities of ASLE, ASSE, and ASRE. "#" denotes significant difference in IC50 with the standard Trolox. A higher IC50 value indicates that a greater quantity of the antioxidant is necessary to neutralize 50% of free radicals or oxidative species, which suggests a decrease in antioxidant activity or efficacy. This implies that all three extracts possess antioxidant properties; however, they are not as potent as Trolox.*

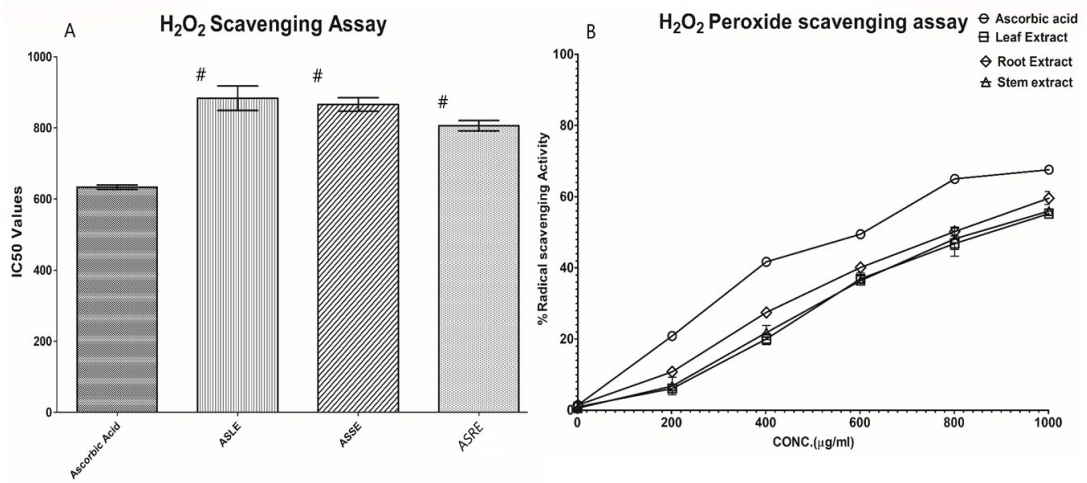

Fig. 8. *(A) Comparative IC50 of H_2O_2 Scavenging Capacities of ASLE, ASSE and ASRE (B) Comparative H_2O_2 Scavenging Activities of ASLE, ASSE and ASRE. "#" denotes a significant difference in IC50 with the standard ascorbic acid. A Higher IC50 value signifies that a greater quantity of the antioxidant is required to neutralise 50% of free radicals or oxidative species, indicating reduced efficacy or decreased antioxidant activity. The results show that all three extracts have antioxidant capacities but are less effective than ascorbic acid.*

leaf extract was syringic acid, followed by p-coumaric acid and chlorogenic acid. Gallic acid and protocatechuic acid were the highest in stem extract and syringic and chlorogenic acids were dominant in root extracts. The leaf (ASLE), stem (ASSE), and root (ASRE) extracts demonstrated considerable antioxidant activity owing to their varied phenolic acid and flavonoid compositions. These findings are consistent with prior research emphasising the therapeutic benefits of *A. spinosus* and its use in traditional medicine to treat oxidative stress-related disorders Abir and Ahmad, 2021). Several species of Amaranthus, such as *A. caudatus*, *A. caudatus*, *A. hypochondriacus*, and *A. tricolor*, have been extensively studied for their antioxidant activities. Sarker *et al*. (2022) found that *Amaranthus tricolour* leaf extracts had substantial radical scavenging activity, which was attributed to considerable quantities of flavonoids such as rutin and quercetin, as well as phenolic acids such as ferulic acid and caffeine. Similarly, this investigation found rutin and quercetin to be important contributors to the antioxidant capacity of *A. spinosus*, with the leaf extract (ASLE) having the highest concentration (Fig. 1, Table 3).

Amaranthus caudatus has been proven to have potent antioxidant activity in both its seeds and leaves. Its leaf extract is found to be high in rutin and kaempferol, chemicals that are also found in *A. spinosus*. However, seeds of this species of genus Amaranthus have a higher concentration of betacyanins, which contributes to their antioxidant qualities (Martinez-Lopez *et al*.,

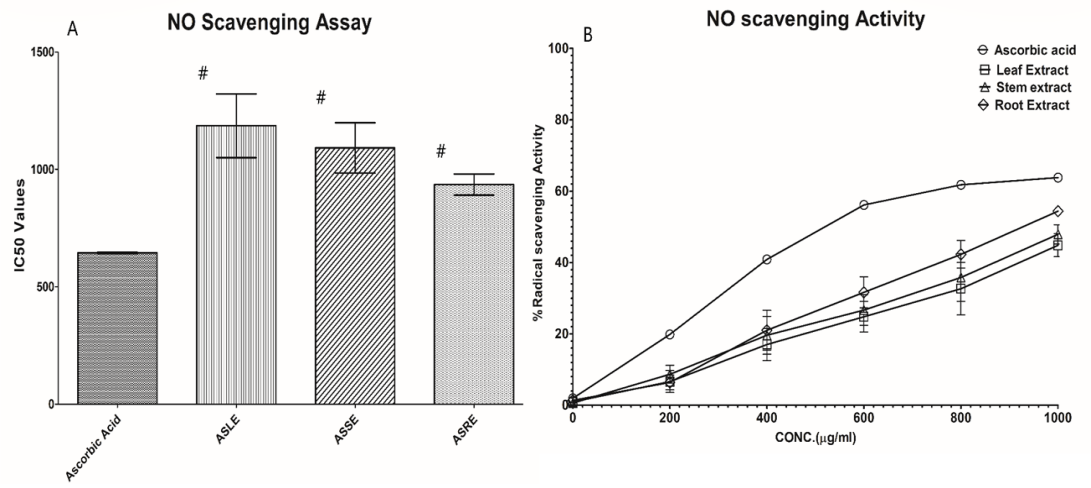

Fig. 9. *(A) Comparative IC50 of NO Scavenging Capacities of ASLE, ASSE and ASRE. "#" denotes significant difference in IC50 with the standard Trolox. (B) Comparative NO Scavenging Activities of ASLE, ASSE, and ASRE. "#" denotes significant difference in IC50 with the standard ascorbic acid. A higher IC50 value indicates that a greater quantity of the antioxidant is necessary to neutralize 50% of free radicals or oxidative species, which suggests a decrease in antioxidant activity or efficacy. This implies that all three extracts possess antioxidant properties; however, they are not as potent as ascorbic acid.*

Table 5. Flavonoids and phenolic acid peak specifications found in leaf extract (ASLE)

Peak ID	Retention time (minutes)	Height(mAU)	Area (mAU*mins)	Concentration (µg /mg)
Gallic acid	7.43	4.849	2.029	0.397
Protocatechuic acid	16.17	8.592	6.368	0.601
p-Hydroxy benzoic acid	35.94	0.817	0.621	0.034
Catechin	41.60	0.661	0.210	0.297
Chlorogenic acid	44.13	11.284	8.715	3.113
Vanillic acid	45.29	8.994	2.196	0.210
Caffeic acid	46.80	2.658	1.328	0.296
Syringic acid	48.01	62.863	44.879	8.827
p-Coumaric acid	54.70	18.213	20.377	4.461
Ferulic acid	56.79	3.029	1.031	0.129
Sinapic acid	62.66	0.768	0.562	0.025
Naringin	70.03	0.871	0.518	0.345
Rutin	73.61	100.144	131.133	12.806
Myricetin	78.19	6.612	9.784	0.701
Quercetin	89.82	17.419	88.042	6.840
Naringenin	92.10	0.120	0.078	0.010
Apigenin	93.45	3.004	1.189	0.171
Kaempferol	103.57	22.023	30.620	1.568

2020). This demonstrates a different phytochemical profile in *A. caudatus* when compared to *A. spinosus,* which lacks betacyanins but compensates with greater concentrations of phenolic acids such as syringic acid and p-coumaric acid in leaf (Fig. 2, Table 4), both of which have previously demonstrated to be high in therapeutic potential (Abazari *et al.,* 2021; Adeyi *et al*., 2023). The antioxidant potential of Amaranthus hypochondriacus is attribut to its high levels of gallic acid, ferulic acid, and catechin (Sarker and Oba, 2019). *A. hypochondriacus* has a more diversified variety of phenolic acids than *A. spinosus*, yet it shares common components like catechin, which was discovered largely in *A. spinosus'* stem extract (ASSE). The consistency in catechin concentration between species supports a conserved antioxidant activity throughout the genus.

Table 6. Flavonoids and phenolic acid peak specifications found in stem extract (ASSE)

Peak ID	Retention time (minutes)	Height(mAU)	Area(mAU*mins)	Concentration(µg /mg)
Gallic acid	8.50	26.754	17.511	3.425
Protocatechuic acid	15.90	16.519	21.811	2.059
p-Hydroxy benzoic acid	37.25	0.249	0.179	0.01
Catechin	42.08	2.874	4.335	6.122
Chlorogenic acid	43.68	1.495	1.676	0.599
Vanillic acid	45.53	2.657	1.696	0.162
Syringic acid	49.64	3.928	2.769	0.545
p-Coumaric acid	54.42	1.744	1.849	0.405
Ferulic acid	57.53	5.673	2.985	0.373
Sinapic acid	63.70	0.708	0.869	0.038
Naringin	71.03	8.449	10.818	7.212
Rutin	72.86	0.509	0.350	0.034
Ellagic acid	74.74	0.926	0.779	0.037
Myricetin	77.01	8.810	7.035	0.504
Quercetin	90.41	11.637	24.283	1.887
Naringenin	93.38	9.007	7.691	0.944
Kaempferol	103.59	22.192	30.505	1.563

Table 7. Flavonoids and phenolic acids peak specifications found in Root extract (ASRE)

Peak ID	Retention time (minutes)	Height(mAU)	Area(mAU*mins)	Concentration(µg / mg)
Gallic acid	7.12	7.692	3.836	0.750
Protocatechuic acid	16.19	6.535	4.542	0.429
Catechin	41.56	0.384	0.138	0.196
Chlorogenic acid	44.15	7.833	4.510	1.611
Vanillic acid	45.70	3.291	2.012	0.192
Caffeic acid	46.84	0.977	0.528	0.118
Syringic acid	48.16	8.415	7.102	1.397
p-Coumaric acid	53.98	4.550	3.647	0.798
Ferulic acid	57.52	5.477	2.851	0.356
Sinapic acid	61.36	0.267	0.132	0.006
Salicylic acid	70.00	0.222	0.099	0.122
Rutin	73.60	48.758	59.398	5.801
Myricetin	78.15	0.643	0.301	0.022
Quercetin	91.15	7.979	9.001	0.699
Naringenin	93.45	3.166	1.695	0.208
Apigenin	94.03	0.221	0.046	0.007
Kaempferol	103.60	19.331	24.793	1.270

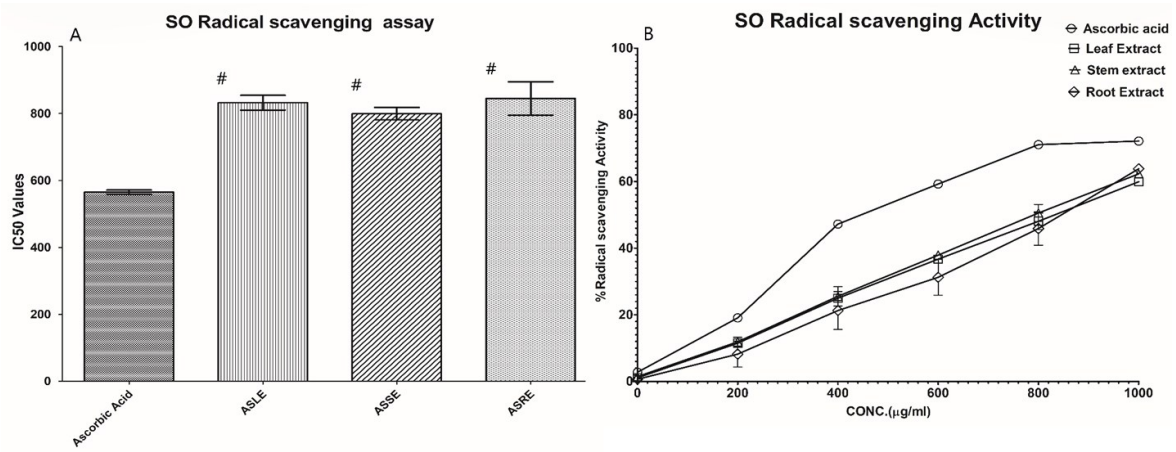

Fig. 10. *(A) Comparative IC_{50} of Superoxide Radical Scavenging Capacities of ASLE, ASSE, and ASRE. "#" denotes significant difference in IC_{50} with the standard Trolox. (B) Comparative Superoxide Radical Scavenging Activities of ASLE, ASSE, and ASRE. "#" denotes a significant difference in IC_{50} with the standard ascorbic acid. A higher IC50 value indicates that a larger amount of the antioxidant is necessary to neutralize 50% of free radicals or oxidative species, signifying diminished efficacy or lower antioxidant activity. All three extracts possess antioxidant capabilities, but less efficient than ascorbic acid.*

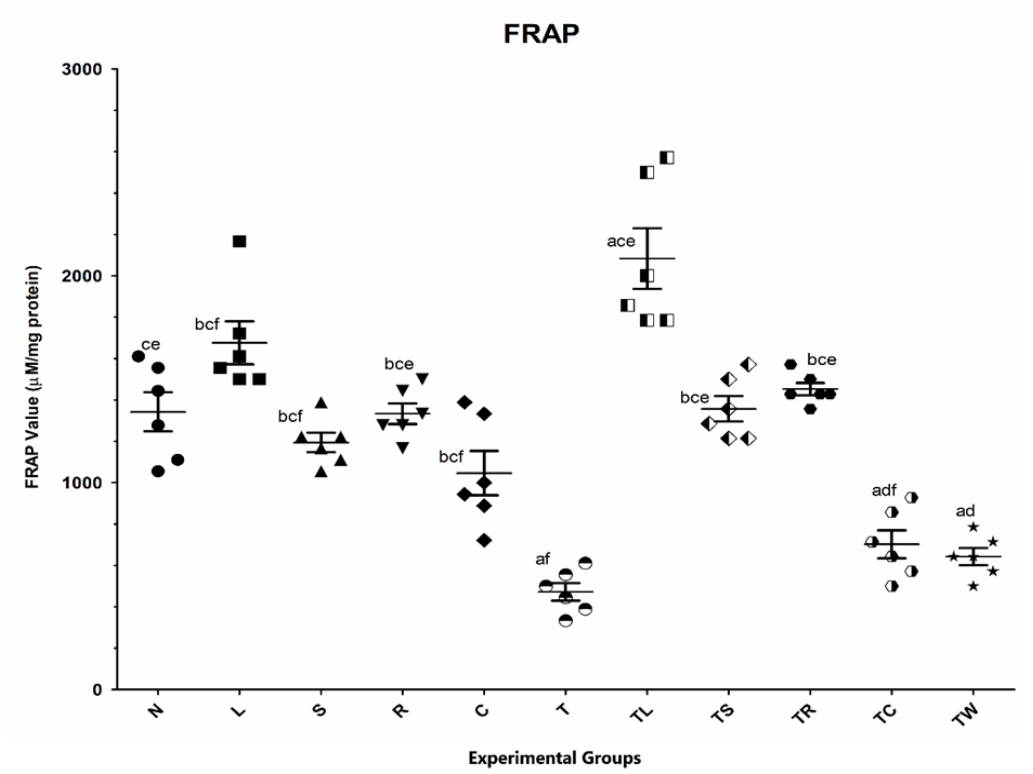

Fig. 11. *FRAP Values of serums of animals from 11 experimental groups indicated that ASLE had higher in-vivo FRAP capacity when compared to ASSE and ASRE. Fibrosis in the liver caused a prominent drop in in-vivo FRAP value, which did not increase significantly in animals where TAA was withdrawn (TW), or only vehicle (CMC) (TC) was administered but increased significantly in animals administered with the three extracts, ASLE(TL), ASSE(TS), ASRE(TR). (N: Control group, L: Animals orally dosed with ASLE, S: Animals orally dosed with ASSE, R: Animals orally dosed with ASRE, C: Animals orally dosed with CMC, T: Animals treated with TAA, TL: TAA Animals orally dosed with ASLE, TS: TAA Animals orally dosed with ASSE, TR: TAA Animals orally dosed with ASRE, TC: TAA Animals orally dosed with CMC, TW: Animals treated with TAA then treatment was withdrawn with no oral dosage).*

*Value representations are in Mean±SEM; (n=6) *p<0.05;a: statistically significant with Group N (Control);b: statistically not significant with Group N (Control); c: statistically significant with Group T (Thioacetamide Treated);d: statistically not significant with Group T (Thioacetamide Treated); e: statistically significant with TW (Thioacetamide Treated and withdrawn); f: statistically not significant with TW (Thioacetamide Treated and withdrawn)*

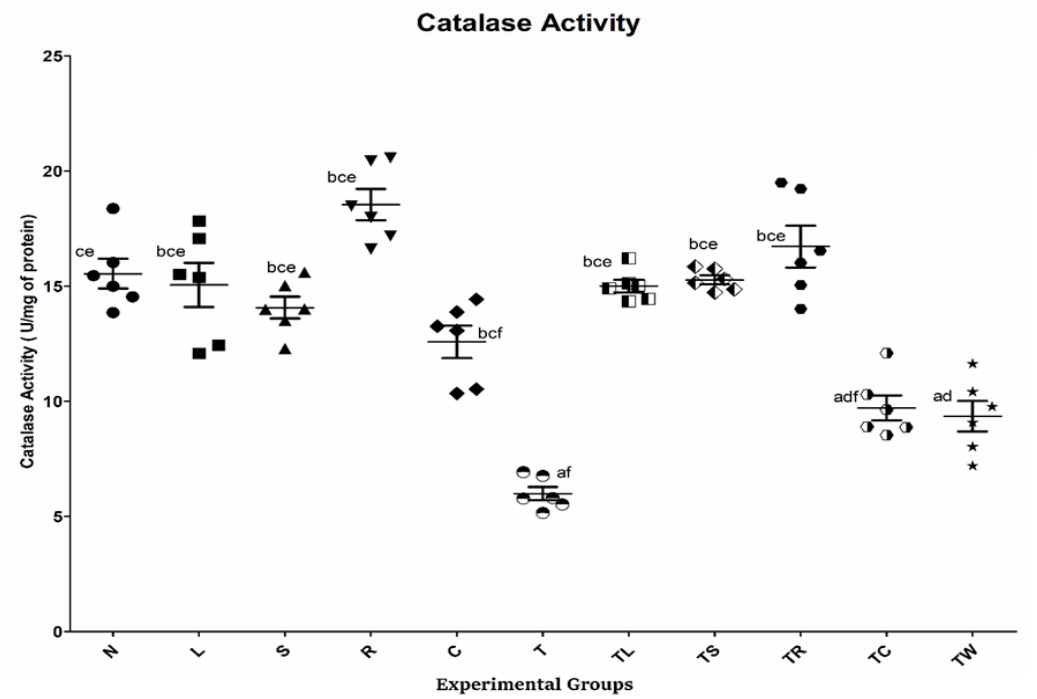

Fig. 12. *Catalase activity in liver homogenates from animals of 11 experimental groups indicated that all three extracts had almost the same in-vivo antioxidant potential by the breakdown of H_2O_2. Higher catalase activity means the cell or tissue has a greater capacity to break down H_2O_2, hence protecting against oxidative damage. Fibrosis in the liver caused a prominent drop in in-vivo Catalase activity, which did not increase significantly in animals where TAA was withdrawn (TW), or only vehicle (CMC) (TC) was administered but increased significantly in animals administered with the three extracts, ASLE(TL), ASSE(TS), ASRE(TR). (N: Control group, L: Animals orally dosed with ASLE, S: Animals orally dosed with ASSE, R: Animals orally dosed with ASRE, C: Animals orally dosed with CMC, T: Animals treated with TAA, TL: TAA Animals orally dosed with ASLE, TS: TAA Animals orally dosed with ASSE, TR: TAA Animals orally dosed with ASRE, TC: TAA Animals orally dosed with CMC, TW: Animals treated with TAA then treatment was withdrawn with no oral dosage);Value representations are in Mean±SEM; (n=6) *p<0.0*
a: statistically significant with Group N (Control);b: statistically not significant with Group N (Control);c: statistically significant with Group T (Thioacetamide Treated);d: statistically not significant with Group T (Thioacetamide Treated);e: statistically significant with TW (Thioacetamide Treated and withdrawn); f: statistically not significant with TW (Thioacetamide Treated and withdrawn)

Table 8. IC50 values Leaf extract (ASLE) Stem extract (ASSE) and Root extract (ARSE) in different *in-vitro* antioxidant assays

Sl. No.	Antioxidant assay	Standard		ASLE	ASSE	ASRE
		Trolox	Ascorbic acid			
		Mean± SEM (µg/ml)	Mean± SEM (µg/ml)	Mean± SEM (µg/ml)	Mean± SEM (µg/ml)	Mean± SEM (µg/ml)
1.	DPPH radical scavenging assay	470.2±1.355	-	848.7±26.32	925.1±27.9	854±14.07
2.	ABTS radical scavenging assay	544.2±1.119	-	848.7±12.27	833.5±44.8	828.5±17.9
3.	H2O2 scavenging assay	-	633.5±6.37	883.5±34.26	865.6±18.98	806.4±14.55
4.	NO scavenging assay	-	644.3±3.206	1186±135.8	1091±107.2	935.5±45.06
5.	Superoxide scavenging assay	-	564.8±6.225	832.1±22.02	799.1±18.68	844.9±50.01

Table 9: *In vivo* antioxidant assays of liver procured from different experimental groups

Sl. No.	Experimental Groups	Antioxidant Assays				
		FRAP	Catalase	R-GSH	Lipid peroxi-dase	SOD
		Mean ± SEM (µM/mg protein)	Mean± SEM (U/mg of protein)	Mean± SEM (µM/mg protein)	Mean± SEM (µM/mg protein)	Mean± SEM (U/mg protein)
1.	N (Group I)	1425.93±158.22	15.89±1.32	95.49±2.39	5.02±0.89	1.37±0.03
2.	L (Group II)	2814.82±227.56	15.25±1.56	118.82±1.89	3.83±0.32	1.52±0.02
3.	S(Group III)	1166.67±32.08	14.37±0.63	102.35±1.23	7.80±0.41	1.5±0.03
4.	R (Group IV)	1314.82±97.99	18.75±0.99	109.41±2.23	5.18±0.41	1.48±0.09
5.	C (Group V)	1000±200.31	12.94±1.22	94.71±3.11	8.99±0.73	1.32±0.03
6.	T (Group VI)	388.89±32.08	6.16±0.38	47.45±2.93	39.71±4.13	0.37±0.03
7.	TL (Group VII)	2047.62±227.13	10.74±1.48	159.22±4.48	7.72±0.38	1.28±0.02
8.	TS (Group VIII)	1359.52±84.55	13.91±1.89	99.8±2.55	9.67±0.63	1.26±0.01
9.	TR (Group IX)	1428.57±41.244	16.59±1.50	146.08±3.21	5.82±0.73	1.19± 0.02
10.	TC (Group X)	666.67±103.78	9.49±0.51	63.53±1.89	24.98±2.22	0.69± 0.08
11.	TW (Group XI)	642.86±41.24	9.13±0.98	58.63±1.19	22.96±0.87	0.73±0.02

FRAP: Ferric Reducing Antioxidant Power;SOD: Superoxide Dismutase;R-GSH: Reduced glutathione;U/mg of protein Catalase: One unit of activity (U) was defined as 1 mmol of H_2O_2 degraded per minute and was expressed as units per milligram of protein;U/mg protein SOD: The enzyme concentration required for 50% inhibition of chromogen production

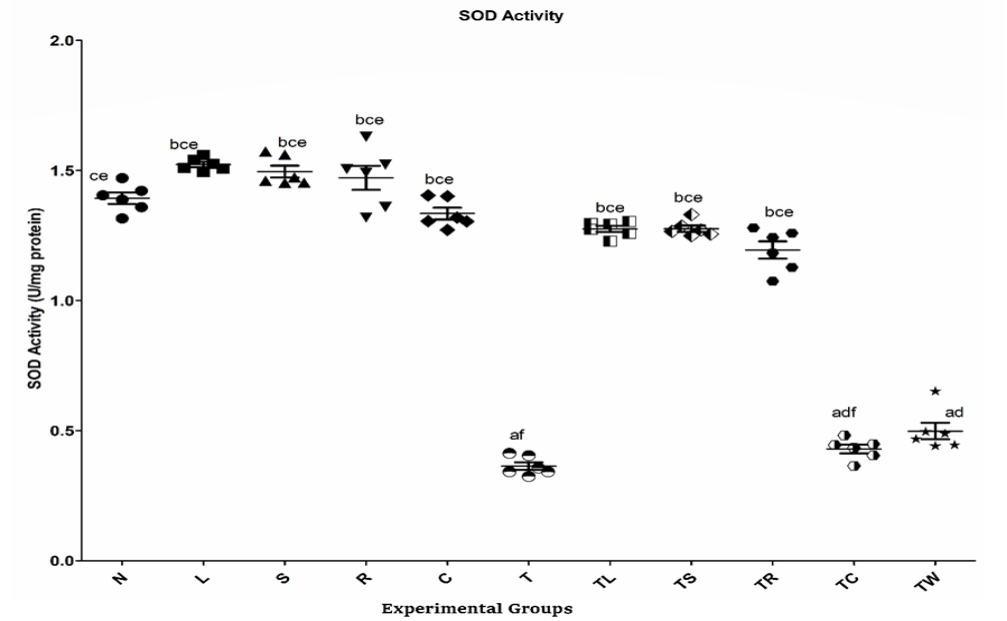

Fig. 13. *Superoxide dismutase activity in liver homogenates from animals of 11 experimental groups indicated that all three extracts had almost the same in-vivo SO radical scavenging potential. Elevated SOD (Superoxide Dismutase) activity in a tissue sample signifies an enhanced ability of the tissue to neutralise superoxide radicals (O2•-), which are detrimental reactive oxygen species. It signifies that the tissue possesses a more robust antioxidant defense mechanism, crucial for safeguarding cells against oxidative damage and preserving cellular health. Fibrosis in the liver caused a prominent drop in in-vivo SO radical scavenging potential, which did not increase significantly in animals where TAA (TW) was withdrawn, or only vehicle (CMC) (TC) was administered but increased significantly in animals administered with the three extracts, ASLE(TL), ASSE(TS), ASRE(TR). (N: Control group, L: Animals orally dosed with ASLE, S: Animals orally dosed with ASSE, R: Animals orally dosed with ASRE, C: Animals orally dosed with CMC, T: Animals treated with TAA, TL: TAA Animals orally dosed with ASLE, TS: TAA Animals orally dosed with ASSE, TR: TAA Animals orally dosed with ASRE, TC: TAA Animals orally dosed with CMC, TW: Animals treated with TAA then treatment was withdrawn with no oral dosage);Value representations are in Mean±SEM; (n=6) *p<0.05;a: statistically significant with Group N (Control);b: statistically not significant with Group N (Control);c: statistically significant with Group T (Thioacetamide Treated);d: statistically not significant with Group T (Thioacetamide Treated);e: statistically significant with TW (Thioacetamide Treated and withdrawn); f: statistically not significant with TW (Thioacetamide Treated and withdrawn)*

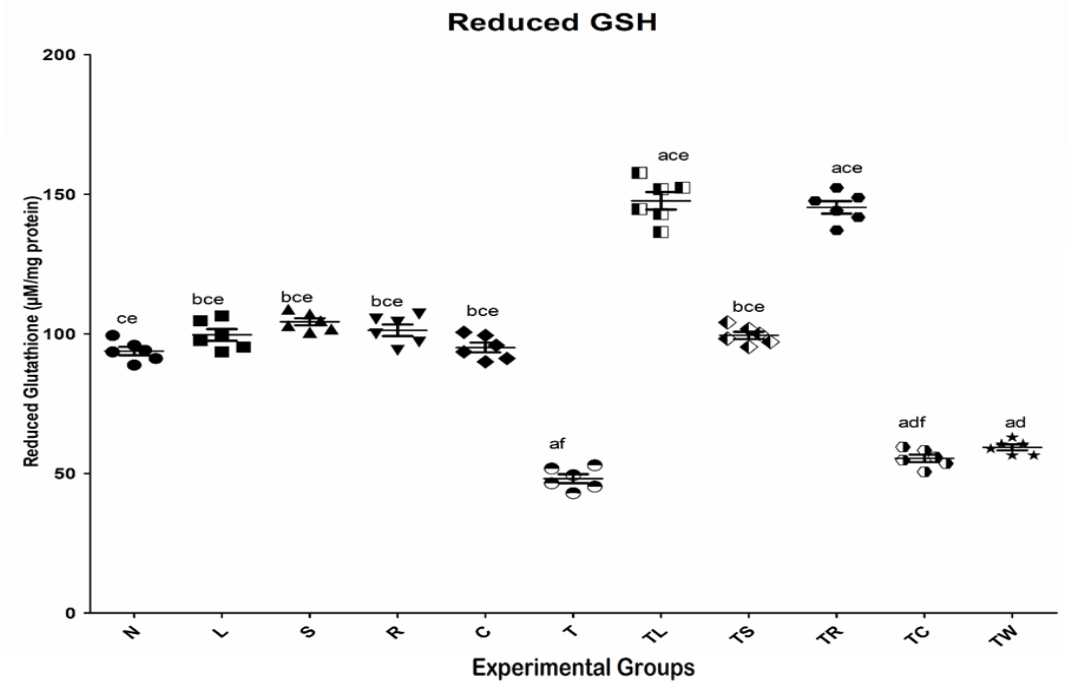

Fig. 14. *The concentration of reduced glutathione (GSH) in liver homogenates from animals across 11 experimental groups demonstrated that all three extracts exhibited nearly identical in-vivo antioxidant capabilities. An increase in GSH levels indicates enhanced antioxidant defense mechanisms in the tissue, contributing to the mitigation of oxidative stress and the safeguarding of cellular integrity. Hepatic fibrosis resulted in a severe reduction of in-vivo GSH, which did not exhibit a significant rise in animals from whom TAA (TW) was withdrawn or those supplied only vehicle (CMC) (TC); nevertheless, a significant increase was observed in animals treated with the three extracts, ASLE(TL), ASSE(TS), and ASRE(TR). (N: Control group, L: Animals orally dosed with ASLE, S: Animals orally dosed with ASSE, R: Animals orally dosed with ASRE, C: Animals orally dosed with CMC, T: Animals treated with TAA, TL: TAA Animals orally dosed with ASLE, TS: TAA Animals orally dosed with ASSE, TR: TAA Animals orally dosed with ASRE, TC: TAA Animals orally dosed with CMC, TW: Animals treated with TAA then treatment was withdrawn with no oral dosage);Value representations are in Mean±SEM; (n=6) *p<0.05;a: statistically significant with Group N (Control);b: statistically not significant with Group N (Control);c: statistically significant with Group T (Thioacetamide Treated);d: statistically not significant with Group T (Thioacetamide Treated);e: statistically significant with TW (Thioacetamide Treated and withdrawn);f: statistically not significant with TW (Thioacetamide Treated and withdrawn)*

In this study, *A. spinosus* has shown some unique phytochemical profiles. It shares many of its bioactive components with other Amaranthus species; however, its antioxidant profile is differentiated by the dominance of syringic acid in the leaf extract and the presence of salicylic acid in the root extract. Syringic acid has been shown to possess neuroprotective and anti-inflammatory properties, while salicylic acid is well-known for its analgesic and anti-inflammatory effects Ekinci *et al*., 2011; Adeyi *et al*., 2023). These findings suggest that *A. spinosus* may possess novel therapeutic benefits that are not typically associated with other Amaranthus species. The IC50 values, which represent the concentration required to inhibit 50% of free radical activity, provide critical insight into the antioxidant efficacy of the extracts. In this study, the IC50 values for DPPH and ABTS radical scavenging activity revealed that all three extracts of *Amaranthus spinosus* (ASLE, ASSE, and ASRE) exhibited effective antioxidant activities, although their efficacy was lower than that of synthetic antioxidants such as Trolox and ascorbic acid. The root extract (ASRE) demonstrated slightly better IC50 values for nitric oxide (NO) and superoxide radical scavenging compared to the leaf and stem extracts, suggesting that its unique phytochemical composition, including salicylic acid, contributes to its superior antioxidant performance(Fig.s 6-10).

The IC50 values for all the radical scavenging capacities (DPPH, ABTS, NO, H2O2 and Super oxide), in *A. spinosus* extracts are comparable to those reported in other Amaranthus species. For instance, in *A. tricolor*, methanolic leaf extracts showed IC50 values ranging from 400–600 µg/ml, with rutin and quercetin being the major contributors to its activity (Sarker and Oba, 2019). Similarly, *A. caudatus* leaf extracts exhibited DPPH IC50 values which can be attributed to their rich flavonoid and phenolic acid profiles(Karamać *et al*., 2019). These comparable IC50 values reinforce the role of rutin, quercetin, and chlorogenic acid as key antioxidants across the genus Amaranthus. The IC50 values in this investigation for ABTS scavenging are consistent with those found by Sarker and Oba (2020)

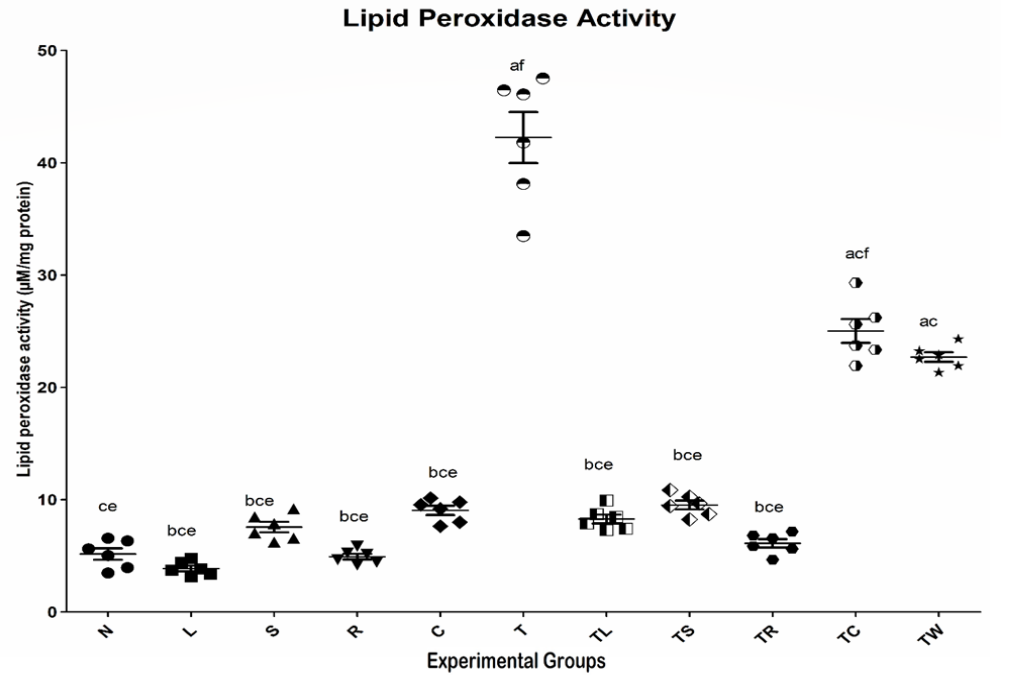

Fig.15. *Lipid Peroxidase (LPO) activity in liver homogenates from animals across 11 experimental groups demonstrated that all three extracts exhibited nearly identical in-vivo antioxidant efficacy. An increase in LPO activity suggests heightened antioxidant stress, whereas a decrease in LPO activity reflects enhanced defense mechanisms within the tissue, contributing to the mitigation of oxidative stress and the safeguarding of cellular integrity. Fibrosis in the liver resulted in a marked increase in in-vivo lipid peroxidation (LPO) activity, which dramatically decreased in animals from which TAA (TW) was withdrawn or those received only the vehicle (CMC) (TC), but not as markedly as in the groups treated with ASLE (TL), ASSE (TS), and ASRE (TR). (N: Control group, L: Animals orally dosed with ASLE, S: Animals orally dosed with ASSE, R: Animals orally dosed with ASRE, C: Animals orally dosed with CMC, T: Animals treated with TAA, TL: TAA Animals orally dosed with ASLE, TS: TAA Animals orally dosed with ASSE, TR: TAA Animals orally dosed with ASRE, TC: TAA Animals orally dosed with CMC, TW: Animals treated with TAA then treatment was withdrawn with no oral dosage);Value representations are in Mean±SEM; (n=6) *p<0.05;a: statistically significant with Group N (Control); b: statistically not significant with Group N (Control);c: statistically significant with Group T (Thioacetamide Treated);d: statistically not significant with Group T (Thioacetamide Treated);e: statistically significant with TW (Thioacetamide Treated and withdrawn); f: statistically not significant with TW (Thioacetamide Treated and withdrawn)*

in a study involving *Amaranthus hypochondriacus* leaf extract. The slightly diminished potency of *A. spinosus* extracts compared to synthetic antioxidants may be attributed to the synergistic but milder effects of natural phytochemicals versus the strength of pure, isolated substances such as Trolox and ascorbic acid. The root extract (ASRE) exhibited the most significant NO scavenging activity (IC50 ~935 µg/mL), compared to the leaf and stem extracts. This observation was consistent with the presence of salicylic acid, a chemical known for successfully neutralising reactive nitrogen species. These data show that phytochemicals, such as salicylic acid, in the root extract boost its NO scavenging activity.

It was found that the IC50 values for superoxide scavenging (ASLE: ~1120 µg/mL, ASSE: ~1150 µg/mL, ASRE: ~1080 µg/mL) were greater than those for ascorbic acid (IC50: ~565 µg/mL). These findings are consistent with previous research on *Amaranthus caudatus*, which showed superoxide scavenging IC50 values in a similar range(Bang *et al*., 2021). Similar actions across species demonstrate the conserved significance of flavonoids, such as catechin and quercetin, in superoxide neutralisation, which were found in all *A. spinosus* preparations. The IC50 values for H2O2 scavenging in *A. spinosus* (ASLE: ~883 µg/mL, ASSE: ~865 µg/mL, ASRE: ~806 µg/mL) were slightly greater than those of ascorbic acid (~633 µg/mL). These findings are in agreement with those of *Amaranthus tricolour*, which had IC50 values for H2O2 scavenging in a similar range(Sarker and Oba, 2020). ASRE's higher efficacy may be attributed to its combination of rutin, quercetin, and phenolic acids such as gallic acid, caffeic acid, and protocatechuic acid, which have been shown to decompose hydrogen peroxide into water and oxygen, acting as antioxidants by scavenging peroxide radicals (Kut *et al*., 2024)

The in-vivo experiments demonstrated that *A. spinosus* extracts had strong therapeutic potential. The TAA-induced oxidative stress paradigm resulted in decreased antioxidant enzyme activity (SOD and catalase), lower GSH levels, and increased lipid peroxida-

tion. This demonstrated the effective development of oxidative stress. However, oral treatment of ASLE, ASSE, and ASRE effectively restored these effects by restoring antioxidant defences and decreasing lipid peroxidation in liver tissues. These findings are similar to previous research, which shows that phytochemical-rich extracts reduce oxidative damage in animal models Park *et al*., 2020).Following the withdrawal of TAA, animals treated with ASLE, ASSE, and ASRE exhibited a significant alteration in antioxidant activity compared to the treated (T) animals and animals from group XI (TW). The results indicated that the liver's capacity for self-regeneration in TW mice was less effective compared to mice administered any of the three extracts. As a result, these extracts aided in the process of liver regeneration. Additionally, the mice in the vehicle group (TC) exhibited no significant difference in antioxidant activity compared to the TW group. This indicated that the lack of extract molecules in the system resulted in the liver of these animals operating in a manner comparable to that of TW animals.

The hepatoprotective effects of the extracts may primarily be due to their flavonoid concentration. In several in vivo experiments, quercetin and rutin have been showno increase hepatic antioxidant enzyme activity and prevent lipid peroxidation (Ahmed *et al*., 2022). Furthermore, syringic acid and chlorogenic acid, present in ASLE and ASRE, have been shown to preserve liver function by lowering ROS and inflammatory mediators (Adeyi *et al*., 2023; Miao and Xiang, 2020; Shi *et al*., 2013). Rutin and quercetin have been found to possess high antioxidant, antidiabetic, anti-inflammatory, anti-allergic, and antitumor properties (Ghorbani, 2017; Vollmannová *et al*., 2024; Koval'skii *et al*., 2014; Patel and Patel, 2019; Prasad and Prasad, 2019; Yang *et al*., 2008) and quercetin is also known to have cardioprotective and hepatoprotective properties (Ogut *et al*., 2022; Yang *et al*., 2020). Notably, despite differences in phytochemical profiles, the antioxidant activity of ASLE, ASSE, and ASRE in liver homogenates of treated mice was similar. This showed that the extracts have a shared mechanism of action, which most likely involved modulating oxidative stress pathways and increasing endogenous antioxidant enzyme levels. Gallic acid, protocatechuic acid, naringin, and catechin are powerful phenolic acids and flavonoids that exhibit strong anti-inflammatory, anticancer, antioxidant, antidiabetic, and anti-microbial properties. According to Kahkeshani *et al*. (2019), they possess a defensive effect against cardiovascular, gastrointestinal, neurophysiological, and metabolic disorders. This suggests that the stem extract may have potential benefits for the issues mentioned above. Conversely, the root extract contains chemicals comparable to those found in the leaf extract, except one of its components, salicylic acid. Salicylic acid is a widely recognised pharmacological compound that serves as an analgesic, antioxidant, and anti-inflammatory agent (Randjelović *et al*., 2015; Das *et al*., 2022). This discovery provides evidence for indigenous populations' utilization of the plant's root to alleviate pain.

The findings of this study align with those of previous studies, which reported significant antioxidant activity in various species of Amaranthus, primarily driven by theirhenolic and flavonoid content (Sarker *et al*., 2024). However, the present study extends this knowledge by comparing the antioxidant potentials of different plant parts and identifying the unique phytochemical profiles of each. Additionally, the in vivo data highlight the role of these extracts in restoring antioxidant defences in a TAA-induced liver damage model, which has not been previously reported for *A. spinosus*. Further investigation is warranted to fully comprehend the therapeutic potential and safety profile of *A. spinosus*, owing to its significant antioxidant properties. This finding can simplify the development of novel antioxidant therapies derived from this conventional medicinal plant, which would assist in managing and preventing diseases induced by oxidative stress. Integrating traditional Indigenous knowledge with modern scientific approaches underscores the significance of ethnobotanical resources in advancing medical research and enhancing health outcomes.

Conclusion

The present study shows that the ASLE, ASSE, and ASRE of *A. spinosus* exhibit distinct phytochemical profiles and antioxidant activities. The ASLE had the most phenolic and flavonoid content. *In vitro* studies demonstrated that all extracts had substantial radical scavenging capacities for DPPH, ABTS, nitric oxide, and hydrogen peroxide, with ASRE having the best nitric oxide scavenging activity. Furthermore, *in vivo* study verified the antioxidant activity of the extracts by significantly restoring endogenous antioxidant defences (catalase, SOD, and glutathione) and lowering lipid peroxidation in TAA-damaged liver. The presence of salicylic acid in ASRE, along with its antioxidant activity, confirms the novelty of this study. This investigation suggests that the root extract of *A. spinosus* or its individual components may be a potential candidate for developing targeted antioxidant therapy against oxidative stress-related hepatic diseases.

Conflict of interest

The authors declare that they have no conflict of interest.

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