Scholars Journal of Applied Medical Sciences
Abbreviated Key Title: Sch J App Med Sci
ISSN 2347-954X (Print) | ISSN 2320-6691 (Online)
Journal homepage: https://saspublishers.com

OPEN ACCESS

Pharmaceutical Technology

Hepatoprotective and in Vivo Antioxidant Enzyme Activity of Phenol Rich Leaf Extract of *Amaranthus spinosus*

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DOI: 10.36347/sjams.2022.v10i06.018 | **Received:** 18.05.2022 | **Accepted:** 11.06.2022 | **Published:** 23.06.2022

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Abstract **Original Research Article**

This study was designed to evaluate the total phenol content, in vivo and antioxidant activity and hepatoprotective activity of methanol leaf extract o*f Amaranthus spinosus.* 15 wistar albino rats having an average mean weight of 104.64 kg was divided into five groups consisting of three animals each group. Group A served as normal control, group B served as negative control, group C served as positive control while group D and E served as test control administered with different doses of *Amaranthus spinosus*. Hepatotoxicity was induced using CCL_4 injected interperitoneally for groups 2-5. The experimental period lasted for seven days after which the animals were sacrificed and their blood collected for analysis. The phenol content was estimated using Folin-Ciocalteu reagent method. The in-vivo antioxidant screening as also estimated determining the concentration in serum of SOD, CAT and MDA and the liver function test was evaluated by also determining the concentration in serum of ALT, AST and ALP spectrophotometrically. The results obtained were presented as mean ± SEM. There was significant increase in the SOD activities of treated groups compared to the negative control group. Result showed a significant ($p<0.05$) increase in the catalase activity of the groups administered 400 and 200 mg/kg of the extract compared to the negative control group. Also there was a significant ($p<0.05$) increase of MDA level in the untreated group (negative control) compared with the normal control. Interestingly, other groups treated with the leaf extract at different doses reflected a significant ($p<0.05$) decrease in the level of MDA with respect to the untreated group. For hepatoprotective activity, the groups treated with different doses of *A. spinosus* leaf extract were observed to have a very significant ($p<0.05$) reduction in the levels of these enzymes bringing their values closer to that of the normal control. However, the ALP level of the negative group was not significantly different ($p>0.05$) from the normal control group. Therefore, the study suggests that *Amaranthus spinosus* has a hepatoprotective activity and free radical scavenging properties.

Keywords: Phenolic compounds, *Amaranthus spinosus*, Liver, Antioxidant, Medicinal plants.

INTRODUCTION

Plants have been one of the important sources of medicines since the down of human civilization and still remain one of the major sources of drugs in modern as well as in traditional systems of medicine. (Pan *et al*., 2013) speculated that to date approximately 80% of antimicrobial, cardiovascular, immune suppressive, and anticancer drugs are of plant origin. The use of herbs has been a long tradition apart from the conventional medicine. It is increasingly becoming a mainstay as enhancements in the analysis, quality control along with advances in pre-clinical research has proven that these herbs are of therapeutic benefit in disease management and control (Tanmoy *et al*., 2014). In these contemporary times, research has been channeled towards exploration of medicinal plants scientifically.

Free radicals have been implicated in the development of a number of disorders, including cancer, neurodegeneration and inflammation, giving rise to studies of antioxidants for the prevention and treatment of diseases (Baba and Malik, 2015). Most of the drugs that are currently available on the market for the treatment of various serious diseases have limited potential because they are expensive and produce detectable side effects. Therefore, it necessary to find effective treatments for various disorders excluding the above mentioned limitations of the marketed drugs. Fruits, vegetables, and herbal plants have been shown to be rich sources of chemicals with the potential to

prevent incurable diseases (Al-Matani*et al*., 2015). Potential phytochemicals from plants for therapeutic purposes have received more attention in recent decades. This is because numerous phytochemicals have been shown to have antioxidant properties and lessen the risk of a variety of diseases, particularly those linked to oxidative stress including cardiovascular disease and cancer. Antioxidants are substances that significantly delay or inhibit oxidation of an oxidizable substrate when present at low concentrations in comparison with those of the substrate (Halliwell and Gutteridge, 1999; Kapadiya*et al*., 2016; Rakesh *et al*., 2010). Endogenous antioxidants are synthesized within the system of living organisms and repair free radical damage internally by initiating cell regeneration while exogenous antioxidants which are derived from sources outside the living systems such as diets (Jaouad and Torsten, 2010) stimulate cell repair externally.

An extensive report by Tanmoy *et al*. (2014) revealed that "The juice of *A. spinosus* is used to prevent swelling around stomach while the leaves are boiled without salt and consumed for 2-3 days to cure jaundice''. ''The plant is consumed as a vegetable for its high concentration of antioxidant components (Tanmoy *et al*., 2014) and high nutritive values due to presence of fiber, proteins and high concentration of essential amino acids, especially lysine''. The root is used as an expectorant; lessens the menstrual flow, useful in leucorrhoea and leprosy. The seed is used as a poultice for broken bones. *Amaranthus spinosus* is used as ''febrifuge, antipyretic, laxative and diuretic. Besides its culinary value, it is used to repute for treat digestible, bronchitis, appetizer, biliousness, galactagogue, haematinic, stomachic, nausea, flatulence, anorexia, blood diseases, burning sensation, leucorrhoea, leprosy and piles. Phytochemical investigations prove its importance as valuable medicinal plant. It is known as rich source of alkaloids, flavonoids, glycosides, phenolic acids, steroids, amino acids, terpenoids, lipids, saponin, betalain, b-sitosterol, stigmasterol, linoleic acid, rutin, catechuic tannins and carotenoids'' (Kumar *et al*., 2014).There has been undeniable evidence of interest when it comes to finding natural antioxidants from plant materials. As shown in recent years, natural antioxidants discovered in plants have attracted some interest due to their widely acclaimed nutritional and therapeutic values. This study focused on antioxidant enzyme activity of phenolic rich methanolic leaf extract of *Amaranthus spinosus* and its effect on liver enzyme markers.

MATERIALS AND METHOD

Reagents and Chemicals

All chemicals, equipments and solvents used were of good analytical grades.

Sample Collection and Preparation

The leaves of *A. spinosus* were collected from Azuiyi, of Ikwuano in the state of Abia, Nigeria, and were authenticated at the herbarium, Department of Botany, Michael Okpara University of Agriculture Umudike**.** The sample collected was washed with clean water to removed dirt and air dried for 2weeks at room temperature. It was then grinded to powder using pulverizing machine.

Extraction Method

Extraction was done using Maceration (soaking) method for 3 days using methanol as a polar solvent.

Determination of Total Phenolic Content (TPC)

Colorimetric protocol (Ahmed *et al*., 2014) was used to determine total phenolic content of methanol extract of *Amaranthus spinosus* leaf. In a test tube, 40 µL (1 mg per 1 mL of butanol) of the plant extract (or standard gallic acid solution), 3.16 mL distilled water and 200 µL Folin-Ciocalteu reagent were put and mixed by shaking gently. After an incubation of 8 min, 600µL sodium carbonate solutions was added and mixed. The mixture was incubated at 40 °C for 30 min before recording its absorbance in a spectrophotometer at 765 nm against a blank. The blank contained 40 µL methanols in place of sample. Gallic acid was used as a standard. Its calibration curve was drawn and the total phenolic content was expressed as micrograms per milliliter of gallic acid equivalents (µg/mL of GAE).

Experimental Animals

A total of fifteen (15) healthy Wister albino rats of both sexes were used for the study. The rats were between the 5-10 weeks old. The animals were kept in a well aerated laboratory cages in the biochemistry animal house and were allowed to acclimatize to the environment for a period of two weeks before the commencement of the experiment. The animals were maintained on a standard animal feed and drinking water during the acclimatizing process.

Experimental design

The rats were weighed and a mean weight was obtained. They were then divided into 5 groups. Group 1 as Normal Control, Group II as Negative control, Group III as positive control, Group IV as Test control & Group Vas Test control.

Group II-V was induced with 25% Carbon tetrachloride (CCL_4) (0.5ml/kg /body weight) interperitoneally to induce liver toxicity.

Group I (Normal control): was fed with feed and water only
Group II (Negative control): was induced but was not treated.
Group III (positive control): was administered500mg/kg of Silymarin orally.
Group IV (Test control): animals were administered 400mg/kg of *Amaranthus spinosus* extract orally.

Group V (**Test control**): animals were administered 200mg/kg of *Amaranthus spinosus* extract orally.

The plant extract was prepared using distilled water and Tween 80 given orally to the animals against CCl4 induced liver damage. Animals were sacrificed 24hours after last administration. Blood was taken from the heart and centrifugated using a centrifuge to separate the hemolyte for analysis.

LIVER ENZYME FUNCTION TEST

Determination of Alkaline Phosphate (ALP)

The serum alkaline phosphate was determined by the method described by Englehardt 1970.

Principle

The principle of this method is based on the reaction of alkaline phosphate and a colorless substrate of phenolphthalein mono phosphate giving rise to phosphoric acid phenolphthalein which at an alkaline pH values, turn pink that can be determined spectrophotometerically.

P-nitrophenyl phosphate + H_2O —-ALP--PO_4^{2-} + P-nitrophenol (pink at pH=9.8)

Calculation: The alkaline phosphatese activity was calculated as follows:

$$\text{Activity of ALP} = \frac{\text{Absorbance of sample}}{\text{Observance of standard}} \times 3300$$

Determination of Alanine Aminotransferase (ALT)

The serum Alanine aminotransferase was determined by the methods described by Schimidt *et al.* 1963.

Principle

Alanine aminotransferase (ALT) is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-ditrophenylhydrazine. The color intensity is measured against the blank at 540nm.

Determination of Aspartrate Aminotransferase (AST)

The serum Alanine Aminotransferase was determined by the method described by Schimidt *et al*. 1963.

Principle

Aspartrate aminotransferase (AST) is measured by monitoring the concentration of oxaloacetate hydrozone formed with 2,4-dinitrophenylhydrazine. The color intensity is measured against the blank at 546 nanometer.

Method

The blank and the sample test tubes were set up in duplicates. A volume, 0.1ml of serum was pipetted into the sample tubes and 0.5ml of reagent one (1) was pipetted into both sample and blank tubes. The solutions were thoroughly mixed and incubated for exactly 30 minutes at the 37^{o}C and pH 7.4. 0.5ml of reagents (2) two containing 2, 4- dinitrophenyhydrazine was added into all the test tubes followed by 0.1ml of sample into the blank tubes. The tubes were mixed thoroughly and incubated for exactly 25 minutes at 25^{o}C and 5.0ml of sodium hydroxide solution was then added to each test tube and mixed. The absorbance was read against the blank after 5 minutes at 546nm for 14 days.

ANTIOXIDANT ANALYSIS

Determination of catalyst activity (CAT)

Principle in the UV range hydrogen peroxide shows continuous absorption of without decreasing wave length. The reaction is based on the reaction of enzymes which methanol In the presence of optimal concentration of hydrogen peroxide. The formaldehyde produced is measured choloremetrically with 4- amino-3-hydrazine-5-mercapto-1, 2, 4-triazole as the chromogen. The decomposition of hydrogen peroxide can be followed directly by the decrease in the extinction at 750nm. The difference in extinction (AE750) per unit time is a measure of catalyst activity. Procedure: The catalase activity was determined according to UV assay method of Aebi (1994). The catalase activity was calculated using the equation.

$$K = \frac{2.3}{\triangle t} \times \log \frac{E1}{E2}$$

E1= Absorbance after 18 seconds
E2= Absorbance after 180 seconds.
$\triangle$t= change in time.

Determination of Superoxide Dismustase (SOD)

PRINCIPLE

The ability of superoxide to inhibit auto oxidation of adrenaline is the basis of the SOD assay. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-redopheny l)-3(-4 –nitrophenol)-5-phenyoltetra zolium chloride (INI) to form a red formazen dye. The superoxide dismutase activity is then measured by the degree of inhibition of the reaction.

Procedure

Superoxide dismutase activity was determined by Epinephrine method of payoric *et al*, (2003) 0.1ml of serum was added to 2.5ml of 0.5ml of phosphate buffer (Ph 7.3).0.3ml of aderaline solution (0.59%) was added and the absorbance was read at 750nm using spectrophotometer for 15 seconds , 30 seconds and 1 minute.

Calculation

$$\text{Increase in absorbance for substrate} = \frac{A_3 - A_0}{3}$$

Where A0 = aborbance after 15 seconds

A_3 = absorbance after 90 seconds

% inhibition = 100 - [increase in absorbance substrate] / [Increase in absorbance of blank]

Estimation of Lipid Peroxidation

Lipid peroxidation was assessed by measuring malondialdye (MDA) formation, using the method of Okhawa, *et al*., (1979). Malondiadehyde (MDA), the end product of lipid peroxidation, is a good marker of free radical mediated damage and oxidative stress.

Principle

This method consists in the reaction of MDA thiobarbituric acid (TBA) in acidic conditions and at a higher temperature (90-100oc) to form a pink MDA-(TBA) 2 complex, which can be qualified spectrophotometrically at 530nm. In the procedure, 0.5ml of 20% TCA was added to 0.5ml of the tissue homogenate, then there was an additional of 1ml of 0.67% TBA. The mixture was incubated for 1000c for 15minutes in a water bath, cooled and then added 4ml of *n*butanol and centrifuged at 3000rpm for 15 minutes. The absorbance of the clear pink supernatant was then read against a blank at 532nm spectrophotometrically. The concentration of MDA is expressed in *nmol/g* of the tissue.

RESULT AND DISCUSSION

Table-1: Liver Enzyme Function Test

GROUPS	AST	ALT	ALP
GROUP 1	10.00^{a}± 0.00	10.10^{c}± 0.10	3.02^{a}± 0.02
GROUP 2	76.25^{d}± 0.05	20.05^{d}± 0.05	3.01^{a}± 0.01
GROUP 3	91.00^{e}± 0.00	7.02^{b}± 0.02	4.03^{b}± 0.02
GROUP 4	21.15^{c}± 0.15	7.01^{b}± 0.01	7.02^{c}± 0.01
GROUP 5	15.05^{b}± 0.05	5.01^{a}± 0.01	8.02^{d}± 0.01

The result was represented as mean ± SEM, values with different super script varies significantly (P<0.05).

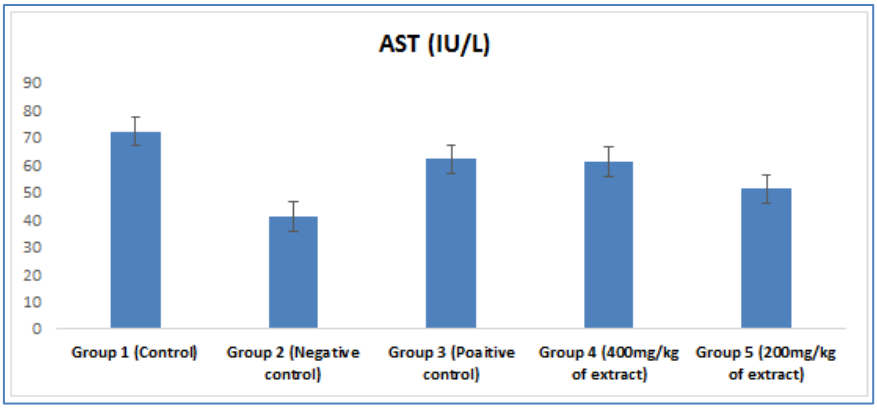

Fig-I: AST Chart

Result indicated AST concentrations was also significantly (p<0.05) higher in Positive and negative control when compared to normal control, 400mg/kg and 200mg/kg. 400mg/kg was significantly (p<0.05) higher when compared to 200mg/kg.

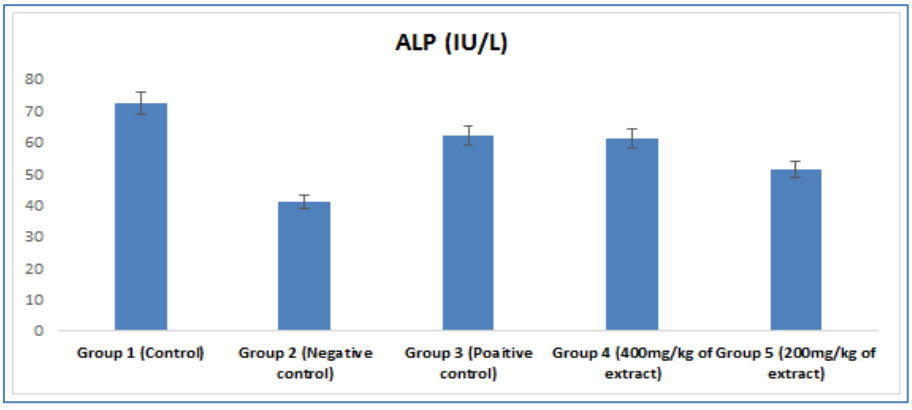

Fig-II: ALP Chart

Result revealed that ALP concentrations was also significantly (P<0.05) higher in Test group treated with 200mg/kg than group treated with 400mg/kg when compared to normal control, Negative control and Positive control group.

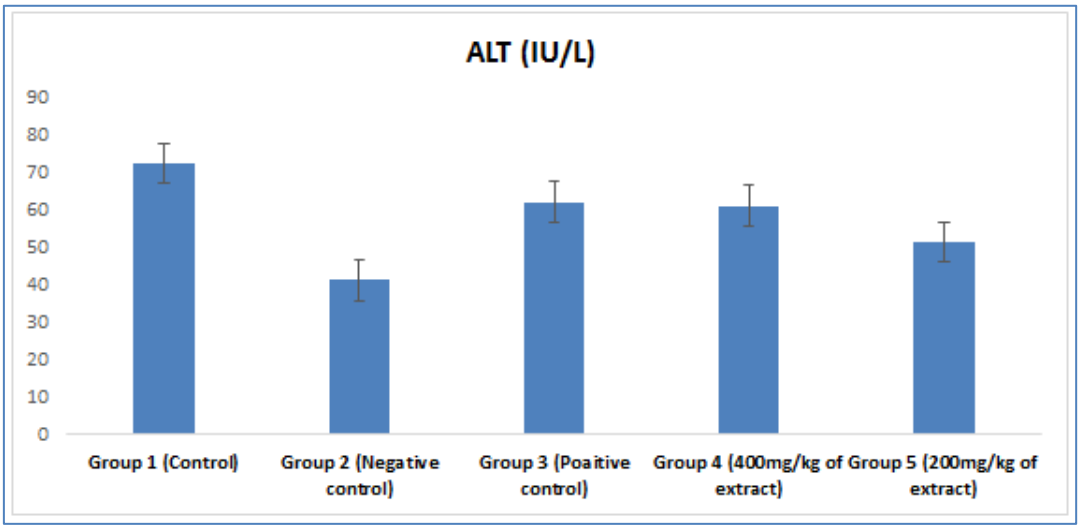

Fig-III: ALT Chart

ALT result showed that the Negative control group was significantly higher (P<0.05) when compared to the normal, positive control, and the test groups. However, result revealed that groups treated with 400mg/kg were not significantly (P>0.05) different from the positive control group but ALT concentrations in Group III-V were significantly lower (P< 0.05) when compared to the Normal control group.

Table-2: In-Vivo Antioxidant Test

GROUPS	CAT	SOD	MDA
GROUP 1	72.41^{e}± 0.01	60.25^{a}± 0.01	10.25^{a}± 0.00
GROUP 2	41.18^{b}± 0.01	72.42^{b}± 0.01	18.14^{e}± 0.00
GROUP 3	62.11^{d}± 0.01	94.31^{d} ± 0.01	14.05^{b}± 0.01
GROUP 4	61.11^{c}± 0.01	95.31^{e}± 0.01	15.88^{c}± 0.01
GROUP 5	51.24^{b}± 0.01	90.24^{c}± 0.01	15.94^{d}± 0.01

The result was represented as mean ± SEM, values with different super script varies significantly (P<0.05)

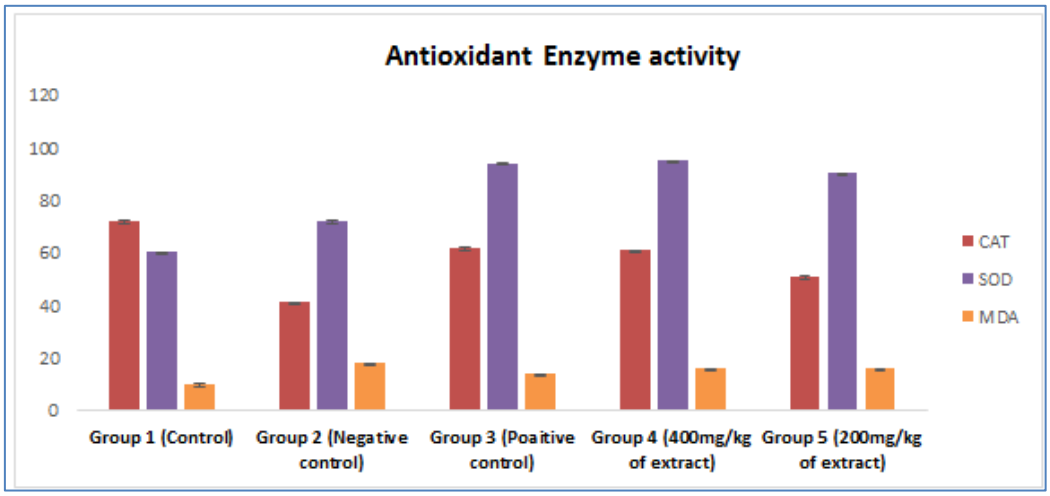

Fig-IV: CAT, SOD & MDA Chart

CAT Results revealed that the Negative control group was significantly (P<0.05) lower on comparison to other groups while the Normal control were significantly (P<0.05) higher than all other groups. However, SOD result revealed that all groups were significantly (P<0.05) different from each other having the normal control group to be significantly lower (P<0.05) on comparison to other groups. Lastly, the

MDA concentration results showed that all groups also significantly different (P<0.05) from each other having the negative control group to be significantly (P<0.05) higher than all groups.

Table-3: Total Phenolic Content

Concentration of extract used	Absorbance	Concentration of samples (mg/ml)
Methanol extract		
0mg/ml	0.021	0.020161
10mg/ml	0.167	2.96371
20mg/ml	0.189	3.407258
30mg/ml	0.239	4.415323
40mg/ml	0.322	6.08871

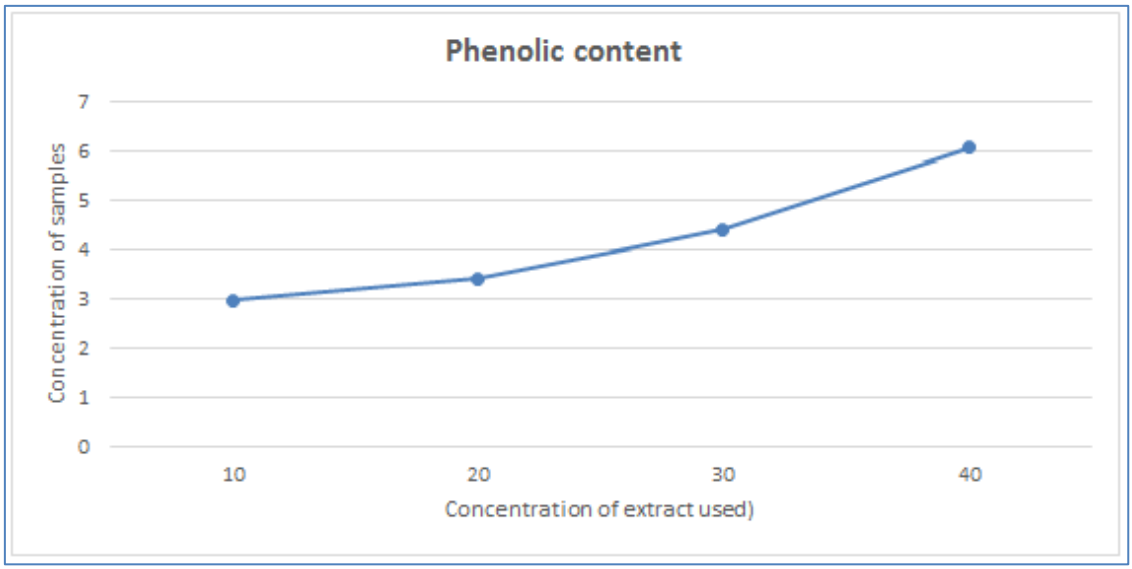

Fig-V: Total Phenolic Content Chart

DISCUSSION

Plants have good scavenging ability because of the phenolic constituents contained in them which has propelled a lot of studies focus on the pharmacological potentials of these phenolic compounds which makes them a potential antioxidant (Kumar *et al*., 2010). The result of the present study has revealed that methanolic leaf extract of *A.spinosus* contains some moderate amount of phenol in a concentration-dependent manner which is in line with study of Amabye (2015). Another report revealed that the methanolic root extract of *Amaranthus spinosus* contains a higher amount of phenols supporting the claims that the plant contains phenols (Barku *et al*., 2013).

Reactive species are highly active molecules, some of which may be direct oxidants and others of which contain oxygen or oxygen-like electron - withdrawing components created within the cell during cellular metabolism or physiological circumstances. These species are beneficial and plays a crucial role in plants ranging from growth and development function but these species upon shift in equilibrium state can be harmful and dangerous (Das &Roychoudhury, 2014). ‘’Plants have developed efficient antioxidant machinery having two arms, (i) enzymatic components like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR); (ii) non-enzymatic antioxidants like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline. These two components work hand in hand to scavenge ROS’’ (Das &Roychoudhury, 2014).

In a bid to fight against oxidation, SOD plays a role of converting free radicals to water and H2O2 only (Luo *et al*., 2019). From the result of this study, there was significant increase in the SOD activities of treated groups compared to the negative control group. CAT serves as a main H2O2 scavenging enzyme (Luo *et al*., 2019), a decrease in CAT is reported to be correlated with the carcinogen-initiated emergence of the malignant phenotype in mouse keratinocytes (Nishawa *et al*., 2002). Result showed a significant (p<0.05) increase in the catalase activity of the groups administered 400 and 200 mg/kg of the extract compared to the negative control group. ‘’Malondialdehyde is an endogenous genotoxic product of enzymatic and reactive oxygen species-induced lipid peroxidation. MDA level is widely used as a marker of lipid peroxidation in states of elevated oxidative stress’’ (Madubuike *et al*., 2015). Increased lipid peroxidation

in the blood and organs can lead to various degenerating diseases. There was a significant ($p<0.05$) increase of MDA level in the untreated group (negative control) compared with the normal control. Interestingly, other groups treated with the leaf extract at different doses reflected a significant ($p<0.05$) decrease in the level of MDA with respect to the untreated group. The decreased level of MDA shows that *A. spinosus* leaf extract can improve the pathological condition of lipid peroxidation.

Liver function parameters are used to detect the presence of liver disease or potential harm to the liver includes the serum level of the enzymes AST, ALT and ALP. Usually, any kind of liver injury can cause a rise in ALT, and the release of ALT and AST from the cytosol occurs when there is injury to hepatocytes, especially in membrane damage (Chikwendu *et al*., 2015). The results of this study showed that there was a significant ($p<0.05$) increase in the levels of AST, and ALT in the negative control group compared to the values of the normal control group. But the groups treated with different doses of *A. spinosus* leaf extract were observed to have a very significant ($p<0.05$) reduction in the levels of these enzymes bringing their values closer to that of the normal control. However, the ALP level of the negative group were not significantly different ($p>0.05$) from the normal control group nevertheless, there was an increase in the ALP level of the rats following the administration of the standard drug and leaf extract of *A. spinosus* which was in disagreement with the study of Rjeibi *et al.* (2016). The increase in the ALP level of the rats may suggest that the plant may be quite toxic at some concentrations.

CONCLUSION

This study has demonstrated antioxidant and hepaprotective activity of methanolic extract of *A.spinosus* in protecting the body from free radicals. This effect may be attributed to the high phenolic content of the plant extract which is present in the plant. The result from the study also suggests that the leaf extract of *A. spinosus* may promote liver function parameters, maintain normal kidney function indices but maybe toxic at higher concentrations.

ACKNOWLEDGEMENT

The researchers want to acknowledge TETfund and the Management of Federal Polytechnic Nekede for their continuous support towards staff development in research.

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