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IN-VITRO ANTI-LIPOXYGENASE AND PROTEINASE INHIBITORY POTENTIALS OF AQUEOUS LEAF EXTRACT OF *Amaranthus hybridus*L

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ABSTRACT
Inflammation is a reaction of cells or tissues to injury, infections, chemical irritants or allergies usually characterized by heat, swelling, redness and loss of function. The objective of the present work is to study the *in vitro* anti-lipoxygenase and proteinase inhibitory activity of the aqueous leaf extract of *Amaranthus hybridus*. Aqueous extracts of *A. hybridus*was assessed for *in vitro* anti-lipoxygenase and anti-proteinase activity. Qualititive phytochemical screening and estimation of total amounts of phytochemicals present was carried out as well. The extract showed a dose dependent anti-lipoxygenase and proteinase inhibitory action. *A. hybridus* extract at 80 µg/ml displayed significant (P≤0.05) proteinase inhibitory action of 28.861±0.020when compared with the standard drug diclofenac 28.115±0.000. At the different concentrations, the extract displayed significant (P≤0.05) anti-lipoxygenase activity when compared with the standard drug. At 200 µg/ml the extract produced significant anti-lipoxygenase action (52.783±0.002) when compared with the standard drug (79.2481±0.000). Phytochemical screening shows the presence of flavonoid, terpenoids, steroids, Saponins, Coumarins, Anthocyanin, phenols, Alkaloids, fixed oil in the extract. Tannin, glycoside, amino acid, triterpenes, phlobatannin were found absent. Quantitative estimation of phytochemicals revealed that Phenol, flavonoid, alkaloid, steroid and terpeonoid were found present in appreciable amounts in the plant extract with values 3.037±0.000, 2.295±0.002, 2.133±0.019, 2.993 ± 0.000 and 1.548 ± 0.001. In conclusion, results revealed that the leaf extract possess anti-lipoxygenase and anti-proteinase potentials, and this could be due to the differences in the composition and concentration of bioactive compounds.

Keywords: Inflammation, Proteinase, Phytochemical, Lipoxygenase, Amaranthus hybridus.

INTRODUCTION

Inflammation is, for the most part, alluded to as a complex biological reaction of vascular tissues to injurious stimuli. Also, inflammation is connected with soreness, and it necessitates a surge in protein denaturation, a rise in vascular penetrability, and plasma membrane change, among others (Ferrero-Millani, Nelsen, Anderson and Girardin, 2007).

Inflammation is additionally portrayed as the body reacts to inactivate or stave off the attacking stimuli, to subside the pain and set up for tissue rebuild, and the action is quickened by the liberation of chemical mediators from harmed cells or tissues and moving cells (Chandra, Chatterjee, Dey and Bhattacharya, 2012). The movement of leukocytes from the venous frameworks to the site of harm and the liberation of cytokines are known to assume a significant function in the inflammatory feedback (Hollman, 2004). These give rise to the broadening of blood vessels (vasodilation) and the porousness of the capillaries. This will prompt expanded blood outflow to the wounded site (Hollman, 2004). Non-steroidal anti-inflammatory drugs (NSAID) can intercede through different approaches to control inflammatory reactions (Vane, 2000). Although NSAIDs are the most used to control the unpropitious impact related to inflammation, at the same time, anyway research has detailed them to have the diversity of aftereffect (Khan, Saraf, and Saraf, 2017). Long haul utilization of NSAIDs may give rise to gastric disintegration, which can result in stomach ulcers and outrageous cases can prompt hemorrhage bringing about death (Vane, 2000). Because of the different reactions that go along with the utilization of NSAIDs, therapeutic plants have been indicated as a therapy for inflammation.

Some plant strains may fill in as vegetables and at the same time as medicinal plants (Dietrich, Hedwig, and Habegger, 1993). Verdant vegetables are a consequential constituent of food selection in each home (Omotosho, 2013).

Amaranthus hybridus L, (Amaranthaceae) prevalently called "Amaranth or pigweed", is a yearly herbaceous plant, 1-6 feet in height. The leaves are interspersed, petioled, 3 – 6 inches in length, dull green, and unpleasant, shaggy, rhombic with wavy edges. In Nigeria, *A. hybridus* leaves amalgamated with pepper and seasoning are utilized to make soup (Oke, 1983; Mepha, Eboh, and Banigbo, 2007). Leaves are consumed as spinach or green vegetables in Congo (Dhellot et al., 2006). In Mozambique and West Africa, the leaves are simmer and combined with groundnut sauce and masticated as a salad (Oliveria and DeCarvalho, 1975; Martin and Telek, 1979). *A. hybridus* has been utilized customarily for the treatment of liver diseases and spasm of the knee and its purgative, diuretic, and cicatrization properties (Nacoulma 1996); the by-products are utilized especially for stomach hurts, dysentery, and diarrhea.

In spite of the utilization of this plant for such purposes, there is a dearth of information on the in-vitro anti-lipoxygenase and proteinase inhibitory action of *A. hybridus* leaves. This work is accordingly planned for archiving the anti-lipoxygenase and proteinase inhibitory potentials of *A. hybridus* leaf in an offer to deciding its value and appropriateness as the an-inflammatory agent.

MATERIALS AND METHODS

Plant materials

The plant was harvested in crisp condition from Oja-Odan, Ilaro Ogun state Nigeria. The plants was identified and confirmed at the University of Lagos Herbarium, Lagos, Nigeria, and Voucher number LUH: 8236 was allocated. The fresh leaves was altogether flushed and air-dried at room temperature (28°C) and stuffed in a water/air proof sampling sack.

Preparation of plant extracts

The dried samples were pounded, 100g of every sample were taken and submerged in distilled water for 72 hrs (for intensive extraction), after which the mixture was sifted utilizing Whatman's filter paper. The filtrate was concentrated by freeze drying. The crude extract was then set aside at 4ºC. Aliquot of the crude extract was weighed and dissolved in distilled water for use on each day of the study.

Qualitative phytochemical analysis

Phytochemical tests were carried out on the aqueous extracts of the leafy vegetables to ascertain the presence of tannin, saponins, phenols, alkaloids, terpenoids, carbohydrate, flavonoids, cardiac glycosides and phlobatanins, using method described by Sofowora (1993) with minor modifications.

QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS

Determination of coumarin

The method of Makkar et al. (1997) was embraced with slight adjustments. 1ml of the concentrate (0.5g in 1ml ethanol) was added to 0.5 mL of 5N NaOH. The blend was warmed and cooled. 0.75 mL of 5N $(NH_4)_2SO_4$ was added and mixed energetically. 0.25g anhydrous $NaHCO_3$ was in this manner included, blended and moved to the extractor. The mixture was separated for three hours with Pet ether in a water shower at 50 – 55^{0}C. The fluid arrangement was moved to a volumetric flagon and made up to 50 mL with persistent blending. 25 mL was further pipetted and 1% Na_2CO_3 solution added. It was warmed at 85^{0}C for 15 minutes and cooled. 5 mL diazonium solution was added and left to wait 2 hours. Absorbance was read at 540 nm against a blank. Coumarin substance was extrapolated from the standard curve.

Estimation of total phenolic content

The total phenolic content of sample was estimated using the method of Makkar e*t al.* (1997). The aliquots of the extract was taken in a test tube and made up to the volume of 1 ml with distilled water. Then 0.5ml of Folin-

Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially to the test tube. Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. Gallic acid monohydrate was used to plot a standard curve. The linearity obtained was in the range of 1-10 µg/ml. The total phenolic content was extrapolated and expressed as Gallic acid equivalent in mg/g of extract using the standard curve.

Total flavonoid content

Total flavonoid content was estimated utilizing the aluminium chloride colorimetric assay. 1ml of extracts or standard solution of Quercetin (500µg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added. At 6th min, 2 ml of 1M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510nm.

Determination of steroids

0.05g of sample extract was weighed into a 100ml beaker. 20ml of chloroform-methanol (2:1) mixture was added to dissolve the extract upon shaking for 30 minutes on a shaker until the whole mixture until free of steroids. 1ml of the filtrate was pipetted into a 30ml test tube and 5ml of alcoholic KOH was added and shaken thoroughly to obtain a homogenous mixture. The mixture was later placed in a water bath set at 37℃-40℃ for 90 minutes. It was cooled to room temperature and 10ml of petroleum ether added followed by the addition of 5ml distilled water. This was evaporated to dryness on the water bath. 6ml of Liebermann Buchard reagent was added to the residue in dry bottle and absorbance taken at a wavelength of 620nm on a spectronic 21D digital spectrophotometer (Makkar*et al.* 1997).

Determination of saponins

The spectrophotometeric method of Brunner (1984) was used for the analysis of saponins. Briefly, 1g of the finely ground dried sample was weighed into a 250ml beaker and 100ml of isobutyl alcohol was added. The mixture was shaken on a UDY shaker for 5 hours to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No. 1 filter paper into a 100ml beaker containing 20ml of 40% saturated solution of MgCO□. The resulting mixture was again filtered to obtain a clear colourless solution. One milliliter of the colourlessfilterate was pipette into a 50ml volumetric flask and 2ml of 5% FeCl□ solution was added and made up to the marked level with distilled water. This was then allowed to stand for 30 minutes for a blood red colour to develop. 0-10ppm saponins standard was prepared from saponins stock solution. The standard solutions were treated similarly with 2ml of 5% FeCl□ solution as earlier described. The absorbance of the samples as well as standard saponin solutions was read after colour development using a Jenway V6300 spectrophotometer at wavelength of 380nm. Percentage saponin was calculated using the formula:

$$\% \text{ saponin} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 10{,}000}$$

Determination of alkaloids

The quantitative determination of alkaloids was done by distillation and titrimetric methods as described by Henry (1973). Briefly, 2g of finely ground sample was weighed into 100ml beaker and 20mls of 80% absolute alcohol added to give a smooth paste. The mixture was transferred to a 250ml flask and more alcohol added to make up to 1g of magnesium oxide was then added. The mixture was digested in a boiling water bath for an hour and half under a reflux air condenser with occasional shaking. The mixture was filtered while hot through a Buchner funnel. The residue was poured back into the flask and re-digested for another thirty minutes with 50ml alcohol after which the alcohol was evaporated. Distilled water was added to replace the lost alcohol. When all alcohol has evaporated, 3 drops of 10% HCl was added. The whole solution was later transferred into 250ml volumetric flask; 5ml of Zinc acetate solution and 5ml of potassium ferricyanide solution were thoroughly mixed together to give a homogenous mixture. The flask was allowed to stand for a few minutes, filtered through a dry filter paper and 10ml of the filterate was transferred into a separating funnel and the alkaloids present were extracted vigorously by shaking with five successive portions of chloroform. The residue obtained was dissolved in 10ml of hot distilled water and transferred into a Kjeldahl tube with the addition of 0.2g of selenium for digestion to a clear colourless solution. The clear colourless solution was used to determined

Nitrogen using Kjeldahl distillation apparatus the distillate was back titrated with 0.01N HCl and the titre value obtained was used to calculate the % Nitrogen using the formulae:

$$\%N = \frac{Titre\ value \times atomic\ mass\ of\ nitrogen \times Normality\ of\ HCL}{Weight\ of\ sample(mg)} \times 100$$

% Alkaloid = % Nitrogen × 3.26

Where 3.26 is a constant

Determination of total anthocyanins

Total anthocyanin compounds of the sample was estimated using a UV-spectrophotometer by the pH differential method reported by Abu Bakar *et al*. (2009) with slight modifications. Two buffer systems, potassium chloride buffer, pH 1.0 (0.0025 M) and sodium acetate buffer, pH 4.5 (0.4 M) were used. Briefly, 400 µl of extract (3 mg of ground beans in 10 ml absolute methanol) was mixed in 3.6 ml of corresponding buffer solutions and read against a blank at 510 and 700 nm. Absorbance (Ad) was calculated as:

Ad = (A510 - A700) pH1.0 - (A510 - A700) pH4.5

Anthocyanin concentration in the extract was calculated and expressed as cyaniding-3 glycoside (mg g-1) equivalent as $\frac{Ad \times MW \times DF}{(Ma \times 1)} \times 1000$

Where;

Ad is difference in absorbance,

MW is a molecular weight for cyaniding-3-glucoside (449.2),

DF is the dilution factor of the samples,

Ma is the molar absorptivity of cyaniding-3-glucoside (26.900).

Results were expressed as mg of cyaniding-3-glucoside equivalents in 100 g of dried sample.

PROTEINASE INHIBITION ASSAY

The reaction mixture (2 ml) contain 0.06 mg proteinase or trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample/ standard drug, Diclofenac sodium, of different concentration 100600 g/ml. The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein or 4% (w/v) bovine serum albumin was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid or 5% trichloroacetic acid (TCA) is added to terminate the reaction. Cloudy suspension is centrifuged at 3000 rpm for 10 minutes or 2500 rpm for 5 minutes and the absorbance of the supernatant is read at 210 nm or 217 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated using the following equation:

$$\%\ inhibition = \frac{Absorbancecontrol - Absorbancesample}{Absorbancecontrol} \times 100$$

ANTI-LIPOXYGENASE ASSAY

The lipoxygenase activity was assayed according to the method of Tappel (1962) with slight modifications (Wallace and Wheeler, 1975). A total volume of 200 µl assay mixture contained, 160 µl sodium phosphate buffer (100 mM, pH 8.0), 10 µl test extract (50 to 250 µg extracted material in 100 mM Tris buffer pH 7.4) and 20 µl lipoxygenase enzyme. The contents were preincubated for 10 min at 25°C. The reaction was initiated by the addition of 10 µl linoleic acid solution as substrate. The change in absorbance was observed after 6 min at 234 nm. All reactions were performed in triplicates in 96-well microplate reader Spectra Max 190 (Molecular Devices, USA). The positive and negative controls were included in the assay. The percentage inhibition (%) was calculated by the following formula:

$$\%\ inhibition = \frac{Absorbancecontrol - Absorbanceoftest}{Absorbancecontrol} \times 100$$

STATISTICAL ANALYSIS

Data were analysed using software package SPSS with one-way analysis of variance (ANOVA). Post-hoc tests were conducted with the Duncan multiple range test. Data are reported as means ± standard error of mean. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Phytochemical screening

Table 1.0: Qualitative phytoconstituents of the aqueous leaf extract for the three plant material.

Phytochemicals	*A. hybridus*extract
Saponins	+
Tannin	-
Phenol	+
Flavonoids	+
Steroids	+
Terpenoids	+
Coumarins	+
Glycosides	-
Triterpenes	-
Anthocyanin	+
Amino acid	-
Phlobatanin	-
Alkaloids	+
Fixed oil	+

(+)present, (-)absent.

Table 2.0: Quantitative phytochemical analysis of the bioactive compounds

Phytochemicals	*Amaranthus hybridus*
Saponins	0.4385±0.001
Phenol	3.037±0.000
Anthocyanin	0.635±0.002
Alkaloid	2.1325±0.020
Flavonoid	2.2945±0.002
Steroid	2.993±0.000
Terpenoids	1.548±0.001
Coumarins	0.4405±0.005
Glycosides	**NP**

Results are expressed as mean±SEM (n=2)

The result of the qualitative and quantitative phytochemical analysis of secondary metabolites in the aqueous extract of *A. hybridus* was presented in Table 1 and 2. The phytochemical profile in table 1 shows the presence of flavonoid, terpenoids, steroids,Saponins, Coumarins, Anthocyanin, phenols, Alkaloids, fixed oil in the extract. Tannin, glycoside, amino acid, triterpenes, phlobatannin were found absent.

Phenol, flavonoid, alkaloid, steroid and terpeonoid were found present in appreciable amounts in the plant extract with values 3.037±0.000, 2.295±0.002, 2.133±0.019, 2.993 ± 0.000 and 1.548 ± 0.001. The result of the quantitative phytochemical screening of three vegetables is shown in Table 2 The Total Phenolic content was expressed Gallic acid equivalent in mg/g of the extract, Total Flavonoid content was expressed as percentage of Quercetin equivalent per 100g, Total Coumarins was expressed as cyaniding-3 glycoside (mg/g^{-1}) equivalent.

Table 3.0: Proteinase inhibitory activity of aqueous extracts of the three plants.

Concentration(µg/ml)	*A. hybridus*extract	Diclofenac
50	17.465±0.026	24.387±0.017

80	28.861±0.020	28.115±0.000
120	29.713±0.014	30.777±0.047
150	37.870±0.046	49.393±0.021
200	43.451±0.021	54.889±0.051

The extract produced a dose dependent inhibitory action. The percentage inhibition of the extract and standard drug increased with increasing concentration. *A. hybridus* extract displayed significant proteinase inhibitory action of 28.861±0.020 at 80 µg/ml when compared with the standard drug diclofenac 28.115±0.000. However, the extract also produced a significant proteinase inhibitory action at 150 µg/ml and 250 µg/ml respectively but not as much as the standard drug but quite close enough.

Table 4.0: Inhibition of lipoxygenase of aqueous extract of *A. hybridus*

Concentration(µg/ml)	*A. hybridus*extract	Quercetin
50	12.549±0.001	39.5511±0.003
80	33.106±0.000	63.6232±0.001
120	39.551±0.002	69.7267±0.003
150	45.362±0.002	73.1935±0.001
200	52.783±0.002	79.2481±0.000

Results are expressed as %Inhibition of lipoxygenase±SEM (n=3)

A dose dependent lipoxygenase inhibitory action was observed. At the different concentrations, the extract displayed significant anti-lipoxygenase activity when compared with the standard drug. Although the percentage inhibition were not as much as the standard. At 200 µg/ml the extract produced significant anti-lipoxygenase action (52.783±0.002) when compared with the standard (79.2481±0.000).

DISCUSSION

Inflammation is the body common reaction to endogenous factors to help battle attacking pathogens. It is an ordinary reaction to any toxic stimulus that compromises the host and may shift from limited reaction to a summed one up (Medzhitov, 2010). It goes along with cramps, redness swollenness and so forth. The inflammatory response is brought about by the activity of phospholipase A1 which acts on membrane lipids to liberate arachidonic acid. The action of cycloxygenase and lipoxygenase enzymes transforms arachidonic acid to prostaglandins and leukotrienes.

Preliminary phytochemical screening affirms the presence of some secondary metabolites, for example, phenols, alkaloids, flavonoids, glycosides, tannins, and so on. This outcome is as per the exploration work is done by Okoye. (2018).

Neutrophils are known to be a rich wellspring of serine proteinase and are delimitated at lysosomes. It was recently revealed that leukocytes proteinase assumes a significant role in the improvement of tissue harm during inflammatory responses and a huge degree of defense was given by proteinase inhibitors (Leelaprakash and Mohan. 2011; Das and Chatterjee, 1995). Proteinases of leukocytes assume a critical job in the advancement of tissue harm during incendiary procedures. As indicated by Das and Chatterjee, a critical degree of protection was given by proteinase inhibitors. Different latter investigations have demonstrated that numerous flavonoids contributed fundamentally to the antioxidant and anti-inflammatory of numerous plants.

A. hybridus displayed significant antiproteinase action at various concentrations as shown in Table 3. *A. hybridus* demonstrated maximum inhibition of 43.45% at 200µg/ml when contrasted and diclofenac which demonstrated the greatest inhibition (54.89%) at 200µg/ml. The findings of this study demonstrate that *A. hybridus* extract displayed significant antiproteinase action at different concentrations when contrasted with the Diclofenac standard.

As indicated by Gunathilake et al. (2018a), polyphenols, flavonoids, and carotenoids are inherently dominant in verdant vegetables. A dose-dependent proteinase restraint was noted in this study. In numerous past investigations, methanolic extracts of Semecarpus Anacardium bark (Kumar et al., 2013), and an ethanolic concentrate of Wedeliatrilobata (Govindappa et al., 2011) have displayed critical antiproteinase (trypsin) movement in a dose-dependent manner.

Lipoxygenases are the key catalysts in the biosynthesis of leukotrienes. Leukotrienes assume a pivotal role in diverse inflammatory illnesses, for example, joint pain, asthma, malignancy, and unfavorably susceptible ailments (Rackova et al., 2007). Arachidonic acid catabolism assumes a vital part in the sequence of occasions of an incident associated with the process of anti-inflammation (Akinwunmi and Oyedapo, 2015). Lipoxygenase catalyzes deoxygenation of polyunsaturated fatty acids to create cis, trans-conjugated diene hydroperoxides, for example, leukotrienes, which are fundamental mediators in an assortment of inflammatory reactions (Khasawneh et al., 2011). From the outcome exhibited in Table 4, *A. hybridus* indicated 52.78% restraint while standard Quercetin produces 79.25 % hindrance. The most grounded hindrance was acquired at 200µg/ml. Past investigations have demonstrated that a few herbs likewise have high lipoxygenase inhibitory action, for example, Leptadeniapyrotechnica (Khasawneh et al., 2011) and Mahonia aquifolium (Rackova et al., 2007).

These outcomes propose that *A. hybridus* has a conceivably high anti-inflammatory potential, which may be identified with the polyphenol substance of the extract. Past investigations have demonstrated that polyphenols may meddle with the cascade events of arachidonic acid catabolism by repressing lipoxygenase action and, additionally, they may fill in as scroungers of different reactive free radicals which are delivered during the catabolism of arachidonic acid (Trouillas et al., 2003). However, as indicated by Gunathilake et al. (2013b), a large portion of these inflammatory properties are changed by the sort of cooking and the kind of leaves.

CONCLUSION

The present investigation demonstrated promising anti-lipoxygenase and anti-proteinase properties of *A. hybridus* leaves aqueous extracts. Presumably, these activities could be attributed in part to the polyphenolic features of the extract. Therefore, the results encourage the use of *A. hybridus* extracts for medicinal health, functional food, and nutraceuticals applications

CONFLICT OF INTEREST

Authors declare that there are no conflict of interest

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