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RESEARCH ARTICLE

MODULATORY EFFECTS OF HYDROALCOHOLIC EXTRACT OF Phyllanthus amarus ON PURINERGIC ENZYMES IN ALBINO RAT TISSUES AND ITS ANTIOXIDANT POTENTIALS.

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Abstract

Phyllanthus amarus is a medicinal plant with immense benefits. Several experimental investigations have explored and establish its phytochemical constituents. This study was carried out to evaluate the antioxidant potentials and inhibitory effects of the hydro-alcoholic extract of the leaves on two major enzymes involved in the hydrolysis of extracellular ATP- Nucleoside triphosphate diphosphohydrolase (NTPDases) and 5'-nucleotidase in rat tissues using in-vitro models. The extract shows a dose dependent scavenging abilities against free radicals such as 2,2 diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), hydroxyl radical, superoxide radical, and nitric oxide(NO) radicals. The ferric reducing power, Vitamin C content and total phenol content also increases as the concentration increases. The extract of *Phyllanthus amarus* showed significant inhibition ($P< 0.05$) of NTPDase and 5'-nucleotidase enzymes in rat tissues with increasing concentrations. Research has shown that targeting these purinergic enzymes could be a novel target for cancer immunotherapy and have been projected to be valuable tools in clinical therapeutics such as in the treatment of chronic pain, immune system diseases and cancer, as they would prolong the physiological effects of extracellular nucleotides or simultaneously administered nucleotide analogues. The hydro- alcoholic extract of *Phyllanthus amarus* with its high radical scavenging ability and inhibitory effects on the purinergic enzymes could be explored in the treatment of chronic pain, cancer and immune system related diseases.

Introduction:-

Purinergic enzymes are enzymes involved in purinergic signalling. Adenosine triphosphate, in addition to its cellular function in metabolism, also serves as an extracellular signaling molecule (Abbracchio, 2009). It is released by exocytosis or via transporters to the extracellular space. Examples of purinergic enzymes include the Ecto-nucleoside Triphosphate diphosphohydrolases (NTPDases) which dephosphorylates ATP via ADP to AMP and 5-nucleotidase (5'- NT) which catalyses the hydrolysis of AMP to adenosine. Extracellular ATP inhibits the growth of

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a variety of human tumours, including prostate, breast, colon, liver, ovarian, colorectal, oesophageal and melanoma cancer cells, partly by mediating apoptotic cancer cell death (Stagg et al., 2011).

Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants.This imbalance leads to damage of important bio-molecules and cells, with potential impact on the whole organism (Durackova, 2010). Oxidative damage accumulates during life cycle and has been implicated in diseases, aging and age dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders such as Lou Gehrig's disease, Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple sclerosis, and other chronic conditions (Rahman, 2003).

An antioxidant refers to any molecule capable of stabilizing or deactivating free radicals so they are unable to oxidize cells (Eboh, 2014). Antioxidant is one of the major essential ingredients of today therapy. They can be endogenously obtained or obtained exogenously as part of a diet.

Phyllanthus amarus (Schum&Thonn) is a plant of the family *Euphorbiaceae* and has about approximately 800 species which are found in tropical and subtropical countries of the world (Mazumder*et al.,* 2006). The name '*Phyllanthus*' means "leaf and flower" and named so because its flower, fruit and leaf appears fused (Kumar *et al.,* 2011). *Phyllanthus amarus* is a branching annual herb which is 30-60 cm high and have slender, leaf-bearing branchlets, distichous leaves which are subsessile elliptic-oblong, obtuse, rounded base. Flowers are yellowish, whitish or greenish. Fruits are depressed-globose like smooth capsules present underneath the branches and seeds are trigonous, pale brown with longitudinal parallel ribs on the back (Itoro*et al.,* 2013). *Phyllanthus amarus* have numerous phytocompounds such as alkaloids, flavonoids, tannins, lignins, polyphenolic compounds and tetracyclic triterpenoids, and as such, has served as lead for several experimental explorations. The objective of this research is to evaluate the antioxidant potentials and inhibitory effects of the hydro-alcoholic extract of the leaves *Phyllanthus amarus* on purinergic enzymes which have been projected to be valuable tools in clinical therapeutics using *in-vitro* models.

Materials and Methods:-

Collection of samples;

Fresh samples of *Phyllanthus amarus* leaves were collected from Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria. The plants were identified and authenticated at the Herbarium of the Department of Plant Science, Ekiti State University, Ado- Ekiti. Voucher specimens with reference number UHAE 2015/15 was deposited in the herbarium for reference purposes. The fresh leaves were air-dried at room temperature until it was completely dry, pulverized using an electrical blender and obtained powdery form were stored until further use.

Preparation of ethanolic extract:

100g of the powdery form was dissolved in 500ml of 70% ethanol for 48hours. The crude extract was filtered with Whatman filter paper and evaporated under pressure at 60°C using a rotary evaporator.

Preparation of tissues:

The tissues were all removed from decapitated adult Wistar rats. The tissues were quickly removed, placed on ice and homogenized in cold 50mM Tris-HCl pH 7.4. The homogenate was centrifuged at 4,000x*g* for 10minutes to yield the low-speed supernatant fraction that was used for the assays.

Nucleoside triphosphate diphosphohydrolase (NTPdase) assay:

NTPDase activity was determined in a reaction medium as described by Battastini*et al.* (1991) on three tissues. 200µL of the plant sample was added to the reaction mixture and pre-incubated for 10minutes at 37^0C. The reaction was initiated by the addition of ATP, and was terminated using 5% TCA + 10mM $HgCl_2$. The absorbance was read at 650nm.

5'-Nucleotidase assay:

5'-Nucleotidase activity was determined by the method described by Heyman*et al.* (1984). 200µL of the plant sample was added to the reaction mixture and pre-incubated for 10minutes at 37^0C. The reaction was then initiated by the addition of AMP and then incubated for another 30minutes at 37^0C. They reaction was terminated by adding 5% TCA + 10mM $HgCl_2$. Addition of distilled water, Ammonium Molybdate and Vitamin C gave a characteristic

blue colouration whose absorbance was read at 650nm. Lower absorbance readings indicated low enzymatic activities.

Determination of Ferric Reducing Property of the Extracts:
The reducing property of the extracts was determined by (Warokar et al., 2010). 0.25ml of the extracts was mixed with 0.25ml of 200mml of sodium phosphate buffer pH 6.6 and 0.25ml of 1 % potassium ferro cyanide (KFC). The mixture was incubated at 50^{O}C for 20mins, there after 0.25ml of 10% trichloro acetate (TCA) was also added and centrifuge at 2000 rpm for 10mins, 1ml of the supernatants were mixed with 1ml of distilled water and 0.1% iron (iii) chloride (FeCl3) and the absorbance was measured at 700nm.

DPPH Scavenging Assay of the Extracts:
The free radical scavenging ability of the extracts against 1,1-diphenyl-2-picryhydrazyl (DPPH) using the method of Gyamfi et al., 1999. 1ml of the extracts were mixed with 1ml of 0.4mm methanolic solution of the DPPH, the mixture was left in the dark for 30mins before measuring the absorbance at 570nm. The scavenging ability of the extract was calculated as.
DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/ (Abs control) ×100
Where Abs control is the absorbance of DPPH radicals + methanol; Abs sample is the absorbance of DPPH radical + extract or standard

Hydrogen peroxide scavenging assay:
The modified method of Hazra et al., 2008 was employed for the hydrogen peroxide scavenging assays of the extracts. The stock solution contained 4mM hydrogen peroxide prepared in 0.1M phosphate buffer (pH 7.4). A volume of 0.6 ml of the solution was added to 2 ml of the extract and standard (200 – 1000 µg/ml) and incubated for 15 min at room temperature. The absorbance was read at 230 nm and the percentage inhibition of hydrogen peroxide was calculated as:
H_2O_2 scavenging activity (%) = [(Abs control – Abs sample)] / (Abs control) ×100 Where Abs control is the absorbance of H_2O_2 radicals; Abs sample is the absorbance of H_2O_2 radical + extract or standard.

Determination of Nitric oxide (NO) Radical Scavenging Ability of the Extracts:
Sodium Nitropruside in an aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Greiss reagent. Scavenging of NO compete with oxygen, reading to reduce production of NO. Briefly 5mm sodium nitropruside in phosphate saline was mixed with the extracts before incubation at 25^{O}C for 15mins. Thereafter, the reaction mixture was added to Greiss reagent before measuring the absorbance at 546nm, relative to the absorbance of standard solution of potassium nitrate treated in the same way with Greiss reagent (Bao et al., 2005). The percentage nitric oxide inhibition by the extracts was calculated using the following equation.
NO scavenging activity (%) = [(Abs control – Abs sample)]/ (Abs control) ×100 Where Abs control is the absorbance of NO radicals; Abs sample is the absorbance of NO radical + extract or standard.

Superoxide Anion Scavenging Activity Assay of the Extracts:
The superoxide anion radicals were produced in 2ml of phosphate buffer (100mm), pH 7.4 with78µm β-nicotinamide adenosine dinucleotide (NADH) 50µm nitro blue tetrazolium chloride (NBT) and test samples at different concentrations. The reaction mixture was kept for incubation at room temperature for 15mins. It was then added with 5-methyl phenaziniummethosulphate (PMS) (10µm) to initiate the reaction and incubated for 5mins at room temperature. The colour reaction between superoxide anion radical and NBT was read at 560nm. Gallic acid was used as a positive control agent for comparative analysis. The reaction mixture without sample is used as control and without PMS is used as blank (Selvakumar et al., 2011) .The percentage inhibition of superoxide anion radical was calculated as.

Superoxide anion radical scavenging activity (%) = [(Abs control – Abs sample)] / (Abs control) ×100
Where Abs control is the absorbance of superoxide anion radicals; Abs sample is the absorbance of superoxide anion radical + extract or standard.

Hydroxyl (OH) Radical Scavenging Ability of the Extracts:
The ability of the extracts to prevent Fe^{2+}/ H_2O_2 induced decomposition of deoxyribose will be carried out using the method of(Halliwell and Gutteridge , 1999). Freshly prepared extracts (0-100µl) added to a reaction mixture containing 120µl, 20mml deoxribose, 400µl, 500µm, FeSO4 and volume were been made to 800µl with distilled water. The reaction mixture was incubated at 37oc for 30min and the reaction was stop by the addition of 0.5ml of 2.8% trichloro acetate (TCA) this was followed by the addition of 0.4ml of 0.6% thiobabithoric acid solution (TBA). The tubes were subsequently incubated in boiling water for 20mins. The absorbance was measured at 523nm in spectrophotometer.

Determination of Total Phenol in the Extracts:
The method of (Wolfe *et al*, 2003) was used to determine the total phenolic content. The reaction mixture contained 2.5 ml of 10 % (v/v) Folin- Ciocalteu reagent, 2 ml of 7.5 % (w/v) of sodium carbonate and 0.5 ml (1 mg/ml) of the extract. The mixture was mixed together and incubated at 40oC for 30 min after which the absorbance was measured at 765nm. Gallic acid was used as standard. The total phenolic content was calculated from the equation obtained from the calibration curve of gallic acid and expressed as mg/g of dry extract.

Analysis of Data:-
The results were expressed as Mean ± SD of 2 or 3 replicates and were analysed by analysis of variance (ANOVA) followed by Duncan's multiple range test using Statistical Package for the Social Sciences (SPSS) 17. Differences between groups were considered significant when $p < 0.05$. Graphs were drawn using GraphPad Prism.

Results:-

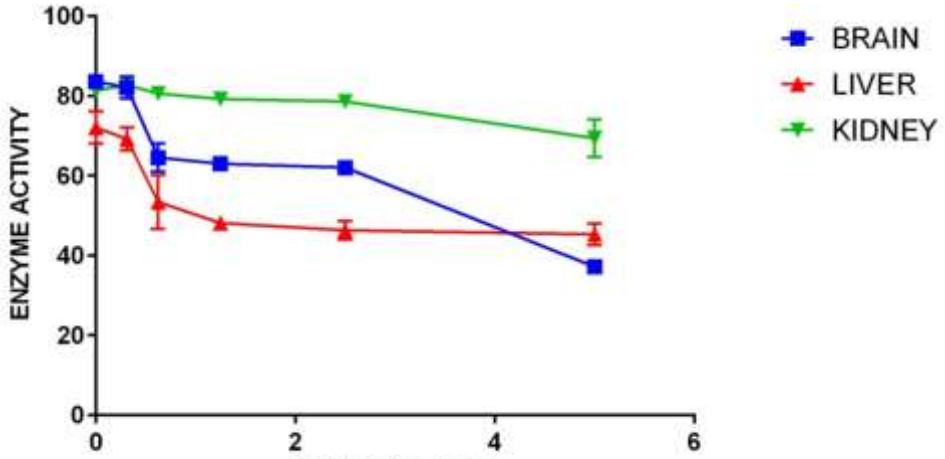

Figure 1: Inhibitory Effect of PhyllanthusAmaruson Nucleoside Triphosphate Diphosphohydrolase (NTPDase) In Rat Tissues

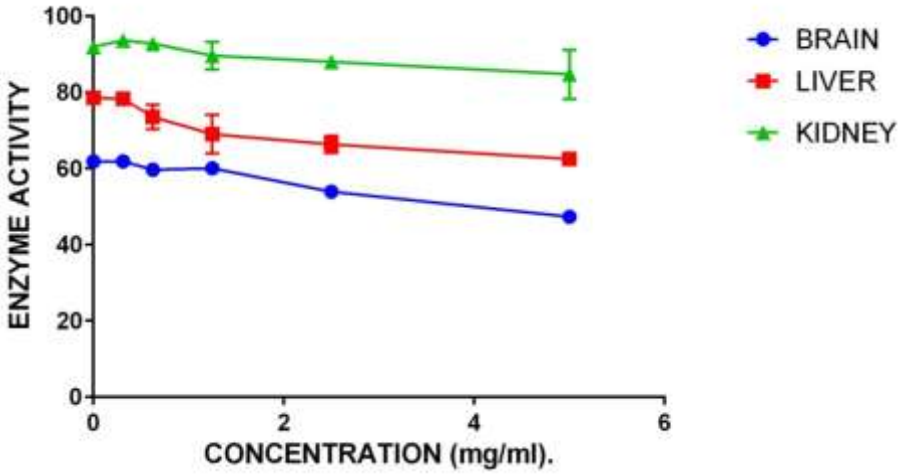

Figure 2: Inhibitory Effect of *Phyllanthus amarus* on *5'-Nucleotidase* In Rat Tissues *in Rat Tissues*

Table 1:-Phenol and Vitamin C concentration of the Ethanolic Extracts *Phyllanthus amarus*

Concentration (mg/ml)	**Vitamin C (mg/g)**	**Phenol (mg/g)**
1	0.84±0.00	1.24±0.05
2	1.80±0.08	1.67±0.05
3	4.90±0.01	2.22±0.10

Values represent Mean ± SD of triplicate readings in mg/g.

Table 2:-Ferric Reducing Properties of Ethanolic Extract of *Phyllanthus amarus*

Concentration (mg/ml)	**Standard Vitamin C (mg/g)**	**Ethanolic extract (mg/g)**
1	23.78	2.49±0.06
2	47.56	2.74±0.03
3	71.33	3.33±0.01

Values represent Mean ± SD of triplicate readings of the *Ferric Reducing Properties of Ethanolic Extract of Phyllanthus amarus*

Table 3:-DPPH Radical Scavenging ability of Ethanolic Extract of *Phyllanthus amarus*

Concentration (mg/ml)	**Standard Trolox (%)**	**Ethanolic extract (%)**
1	78.96	61.92±1.42
2	89.98	75.45±0.71
3	92.99	82.50±0.71

Values represent Mean ± SD of triplicate readings of the *DPPH Radical Scavenging ability of Phyllanthus amarus* in %

Table 4:-Hydrogen Peroxide Scavenging ability of Ethanolic Extract of *Phyllanthus amarus*

Concentration (mg/ml)	**Standard Trolox (%)**	**Ethanolic extract (%)**
1	64.29	16.67±1.12
2	73.81	66.67±0.71
3	90.48	88.10±3.37

Values represent Mean ± SD of triplicate readings of *Hydrogen Peroxide Scavenging ability of Phyllanthus amarus* in %

Table 5:-Nitric Oxide Scavenging ability of Ethanolic Extract of *Phyllanthus amarus*

Concentration (mg/ml)	**Standard Trolox (%)**	**Ethanolic extract (%)**
1	54.62	40.80±2.87
2	74.10	47.80±2.84
3	80.52	69.10±2.37

Values represent Mean ± SD of triplicate readings of *Nitric OxideScavenging ability of Phyllanthus amarus* in %

Table 6:-Superoxide Radical Scavenging ability of Ethanolic Extract of *Phyllanthus amarus*

Concentration (mg/ml)	**Standard Gallic (%)**	**Ethanolic extract (%)**
1	72.41	39.20±1.28
2	81.76	57.80±0.64
3	91.38	82.00±1.37

Values represent Mean ± SD of triplicate readings of *superoxide radical Scavenging ability of Phyllanthus amarus* in %

Table 7:-Hydroxyl Radical Scavenging ability of Ethanolic Extract of *Phyllanthus amarus*

Concentration (mg/ml)	**Standard Trolox (%)**	**Ethanolic extract (%)**
1	65.71	14.30±0.00
2	82.86	35.71±2.02
3	88.57	37.14±2.12

Values represent Mean ± SD of triplicate readings of *Hydroxyl Radical Scavenging ability of Phyllanthus amarus* in %

Discussion:-

Adenosine is a potent immunosuppressive metabolite, and it is often found elevated in the extracellular tumour microenvironment (Allard *et al,* 2017). Targeting adenosine-generating enzymes or their plasma-bound receptors has emerged as a new means of cancer treatment (Zitvogel*et al*., 2011). Two of these enzymes are Ectonucleoside triphosphate diphosphohydrolase-1, also known as CD39 (Sévigny*et al.,* 1997) and 5'-nucleotidase (5'-NT), also known as CD73 (Misumi*et al.,* 1990). Nucleoside triphosphate diphosphohydrolases also known as CD39 are involved in the hydrolysis of ATP and ADP resulting in the formation of nucleoside monophosphates and orthophosphate. NTPDase inhibitors therefore are currently target for various cardiovascular diseases and some types of cancer (Ashraf *et al.,* 2011). 5-nucleotidase (5'-NT) catalyse the hydrolysis of AMP to adenosine.

Recent studies confirm that CD73 promotes invasion, migration and adhesion of human breast cancer cells. The strong expression of CD73 has been suggested as a clinical diagnostic aid in the differential diagnosis of thyroid tumours. Also, expression of E-NTPDase1 (CD39) by vascular endothelium or by tumour cells accelerates tumour growth and spread of rat glioma, mouse colorectal cancer, and mouse melanoma (Braganhol*et al.,* 2009).

All these point to one possible avenue for intervention when it comes to cancer treatment. Available evidence in several experimental tumour models clearly shows that reducing the adenosine concentration halts tumour progression and prevents metastasis (Stagg*et al.,* 2011). Administration of CD39 and CD73 inhibitors will achieve this purpose by maintaining ATP levels and reducing Adenosine concentrations (Beavis *et al.,* 2012).

Ethanolic extract of *Phyllanthus amarus* showed a significant inhibitory trend for these enzymes, with the enzyme activities decreasing with increasing concentration in the tissues of albino rats (Figure1 and figure 2).
One of the *in vitro* antioxidant parameters used for testing the potency of plants is their ability to scavenge free radicals. The mechanism involves the deprotonation of the unstable 2,2-diphenyl-1-picryl hydazyl (DPPH) radicals turning it to stable diamagnetic molecule which is visually noticeable as a discoloration from purple to golden yellow. Interestingly, Ethanolic extract of *Phyllanthus amarus* demonstrated marked radical scavenging activity in a concentration dependent manner against DPPH radical, Hydrogen peroxide radical, Nitric oxide radical, superoxide radical and hydroxyl radical towards the standard used in this study.

Conclusion:-

This research work has shown that inhibitors of Ectonucleoside triphosphate diphosphohydrolase and 5'-nucleotidase can be found in plants. Although further research works has to be done in the identification and isolation of these compounds, this *in-vitro* analysis will serve as a background for further exploration as this could pave way for the creation of non-invasive cancer treatments.

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