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Full Length Research Paper

Effect of Crude Ethanolic Leaf Extract of *Phyllanthus amarus* and Highly Active Antiretroviral Therapy (Tenofovir-Lamivudine Combination) in Wistar Rats

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ABSTRACT: The effect of the ethanolic crude leaf extract and fractions of *Phyllanthus amarus* on the deleterious effects of the prolonged administration of highly active anti-retroviral therapy (HAART) (Tenofovir-Lamivudine) in Wistar rats was investigated. Qualitative phytochemical screening of the crude leaf extract and fractions showed the presence of tannins, saponins, alkaloids, flavonoids, terpenoids, and phenols. Quantitatively, alkaloids (3.73±0.10, 1.70±0.10 for crude extract and fraction I respectively); tannins (9.08±0.07, 4.17±0.07 for crude extract and fraction I respectively); phenols (7.41±0.07, 2.09±0.07 and 4.96±0.44 for crude extract, fraction I and fraction II respectively); flavonoids (11.87±0.56, 4.58±0.24 and 3.17±0.07 for crude extract, fraction I and fraction II respectively); saponins (5.99±0.19, 2.02±0.20 and 3.01±0.14 for crude extract, fraction I and fraction II respectively) and terpenoids (1.27±0.10 for crude extract only) were estimated. Invitro antioxidants status of the crude leaf extract and fractions was determined using DPPH, FRAP and TBARS with the ethanolic crude leaf extract showing more of the antioxidant potentials than its fractions. The administration ethanolic crude leaf extract and fractions of *Phyllanthus amarus* and HAART which lasted for 21 days showed that animals that received 300mg/kg body weight of HAART showed a significant increase ($P<0.05$) in the levels of AST, ALT, total bilirubin, urea, creatinine levels. However, co-treatment of these animals with ethanolic crude leaf extract, fraction 1 and fraction 2 (100, 200mg/kg body weight respectively) of *Phyllanthus amarus* revealed a significant decrease ($P<0.05$) in the levels of AST, ALT, total bilirubin, urea and creatinine levels with a corresponding significant increase ($P<0.05$) in total protein and albumin levels. The results from this research showed that the ethanolic crude leaf extract and fractions of *Phyllanthus amarus* has expressed its ameliorative potential by reversing the deleterious effects of prolonged HAART administration on the experimental animals.

Keywords: *Phyllanthus amarus*, ameliorative, ethanolic crude leaf extract and fractions, highly active anti-retroviral therapy (HAART), antioxidant

INTRODUCTION

Plant materials are used in traditional medical practices and have remained useful sources of new drugs (O'Brien, 2004). Although orthodox medical practice is generally acceptable, alternative healthcare is still relied on all over the world. In the developing countries of the world traditional herbal medicine is often used side by side western medicine with herbal medicine taking the upper hand when that of the western is beyond reach (Busia, 2005). In more recent years, with considerable research it has been found that many plants do indeed have medicinal

values (Sofowora, 1993). Man is able to obtain from plants a wondrous assortment of industrial chemicals. Plants based natural constituents can be derived from any part of plant such as the barks, leaves, roots, flowers, fruits, and seeds. Any part of the plant may contain bioactive components (Prashant *et al*., 2011). Today, liver damage is one of the common ailments in the world resulting in several mild and serious metabolic disorders that could lead to death (Raheim *et al.,* 2013). Being a versatile organ concerned with regulation of internal chemical environment, damage inflicted on it by hepatotoxic agent may pose a grave consequence (Sreedevi *et al.,* 2009).

Free radicals are species (atoms, molecules, ions) that have one or more unpaired electrons in their outer orbital that make them very unstable and quite reactive with other molecules by pairing up their electron(s) to generate a more stable compound (Salwa *et al.,* 2011). Reactive oxygen species (ROS) or free radicals, formed during physiological and pathological conditions in the body are extremely reactive and react with proteins, lipids, carbohydrates and nucleic acids (Carmen *et al.,* 2012). Free radicals are derived from oxygen, nitrogen and sulphur to form reactive oxygen species, reactive nitrogen species and reactive sulphur species respectively. The nitrogen derived free radicals include nitric oxide (NO), peroxy nitrite anion (ONOO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3); the thiol derived free radicals include sulphite (SO_3^{2-}), disulfide S oxide (DSSO), sulfenic acid (RSOH) and sulfenyl (RS.) radicals (Lu *et al*., 2010). When the body is overloaded with free radicals and cannot be gradually destroyed, their accumulation in the body generates a phenomenon called oxidative stress (Carmen *et al.,* 2012).

Antioxidants are exogenous or endogenous compounds which either prevent or delay the generation of toxic oxidants or intercept those that are already generated to inactivate them, thereby blocking the chain of propagation reaction by these oxidants (Halliwell, 2010). They can also initiate repair processes (e.g damaged DNA repaired by sulphaoxide reductase) which remove damaged macromolecules to prevent their accumulation that may further hinder cellular process and viability. They have the ability to protect human body cells from the damages caused by unstable free radicals (highly reactive chemicals that play part in generating oxidative stress in biological system) by stabilizing them via electron donation (Lu *et al.,* 2010).

HIV belongs to the retrovirus family of viruses. It affects the immune system of infected persons by destroying T-lymphocytes cells, which the body relies upon to fight infection (NASCOP, 2002). When HIV enters the body, it infects T helper cells which display the $CD4^+$ receptor binding sites. The virus commandiers the genetic material of the host cell, instructing it to replicate and produce new viral particles which break free from the host, destroying the cell in the process and which then move on to infect and destroy other new uninfected lymphocytes (WHO and UNAIDS, 2015). There are two distinct serotypes of HIV viruses: type 1 and type 2. The HIV-1 is the primary cause of AIDS worldwide while, HIV-2 is found predominantly in West Africa and its vertical transmission develops more slowly and milder compared to HIV-1 (Sanders *et al*., 2007). AIDS is the late stage of HIV infection, a condition characterized by destruction of $CD4^+$ T cells which help the body fight diseases (NASCOP, 2002). The syndrome was first identified in 1981 among homosexual men and intravenous drug users in New York and California and after its detection evidence of an AIDS epidemic grew shortly after among heterosexual men, women, and children in sub-Saharan Africa (CDC, 2009). Although initial infection with HIV can result in flu-like symptoms, infected persons typically can show no symptoms for many years but as HIV replicate in the body, infected persons begin to show signs and symptoms such as shingles, tuberculosis, oral or vaginal thrush, herpes simplex virus, and Kaposi sarcoma (WHO, 2009) which are a reflection of a weakened immune system or loss of the body's ability to fight infection.

Description of *Phyllanthus amarus*

Phyllantus amarus is a small tropical herb that belongs to the family Euphorbiaceae. The plant grows up to a height of 10-60cm. It is an annual and widespread throughout the tropics and subtropics. It is found in sandy regions as a weed in cultivated and wastelands. The plant is a common tropical weed that grows well in moist, shady and sunny places (Nanden-Amattaram, 1998). Some common names of *Phyllanthus amarus* in North, Central and South America are black catnip, carry-me seed, egg woman, hurricane weed, quinine creole, quinine weed, seed-under-leaf, stone breaker among others (Morton, 1981). The Spanish called it 'Chancapiedra' meaning the 'stone breaker'. The plants are monoecious or monogamous. It has smooth cylindrical stem (1.5-2mm) thick and deciduous horizontal branch lets (4-12cm) long and 0.5cm thick, with 15 to 30 leaves. The leaves are simple, alternate or opposite and leathery, and borne on petioles 0.3 to 0.5 mm long. The flowers are very small and diclinous, often in clusters borne in greenish cup-shaped structures with glands. The fruit is a three-lobed capsule containing six seeds and extends from the cup with a long stalk pendant about 1-2mm (Lewis and Elvin-Lewis, 1977). The seeds are triangular (like an orange segment), light brown, 1mm long, and with 5-ribsback and the seeds are hurled away when the fruits burst open (Morton, 1981).

Objectives of the study

The objectives of this study is to qualitatively and quantitatively determine the presence of phytochemical

components in the ethanolic crude leaf extract and fractions of *Phyllanthus amarus*, to determine the *in vitro* antioxidant status of the crude leaf extract and fractions of *Phyllanthus amarus*, to determine the effect of ethanolic crude leaf extract and fractions of *Phyllanthus amarus* and highly active anti-retroviral therapy (HAART) on Wistar rats.

MATERIALS AND METHODS

The study area lies on geographic location between latitude 7° N, 22° North and longitude 9°E, 3° 0" E; East Google earth, (2016). The leaf of *Phyllanthus amarus* was harvested from Yandev Gboko Local Government Area of Benue state, Nigeria. The plant was authenticated by a botanist in the Department of plant science, School of Life Sciences, Moddibo Adama University of Technology, Yola. The leaf was washed thoroughly with tap water and rinsed with distilled water.

Preparation of leaf extracts

The method of Alade and Irobi (1993) was used in the preparation of ethanolic crude leaf extract of *Phyllanthus amarus.* The leaf was air dried at room temperature and was blended into powder using electronic blender. The powder was weighed using an electronic weighing balance and was soaked in 80% ethanol. The mixture was agitated and kept under room temperature for 48h. The extract was filtered using Cheese cloth and filtered again using Whatman No. 9 filter paper. The filtrate was concentrated by evaporating to dryness using an Electronic water bath at a regulated temperature of 37-45°C.

Fractionation of the ethanolic crude leaf extract of *Phyllanthus amarus*

The fractionation method described by Abbot and Andrews (1970) was used to separate the extract into its fractions.

Principle

The principle is based on differential affinities (strength of adhesion) of the various components of the extract towards the stationary and mobile phases resulting in the differential separation of the components. Affinity is in turn dictated by two properties of the molecule which are adsorption and solubility.

Procedure

Fractionation of the ethanolic crude leaf extract of *Phyllanthus amarus* was carried out with silica gel column chromatography to separate the extract into its fractions using the wet packing silica gel (150g) as the stationary phase and varying solvent combination of increasing polarity as the mobile phase. In the setting up the column chromatography, the lower part of the glass column was stocked with glass wool with the aid of glass rod. The slurry prepared by mixing 200g of silica gel and 350ml of hexane was poured down carefully into the column with the aid of the glass rod. The tap of the glass column was left open to allow free flow of solvent into a beaker placed directly below the column tap. The solvent was then be drained freely to ensure better packing. At the end of the packing process, the tap was closed and the column was closed and the column was allowed to stand for 24h without disturbance for it to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain to the silica meniscus. About 6g of the sample was adsorbed with 22.0g of silica gel in 100ml of ethanol (74:12:8) and then dry on a hot plate. The column tap was then opened to allow the eluent to flow at rate of 40 drops per minute. Elution of the extract was done with solvent system of gradually increasing polarity using hexane, ethanol, ethylacetate and acetone. The ratios of solvent combinations were sequentially used in the elution process at varying ratio of each different solvent and sample. A measured volume (500ml) of each solvent combination was collected gradually with a 10ml syringe and sprayed uniformly by the sides of the glass into the column each time.

Qualitative screening of phytochemical constituents of ethanolic crude leaf extract and fractions of *Phyllanthus amarus*

Tests for flavonoids

One millilitre of each ethanolic crude leaf extract and fractions was added 1ml of 10% lead acetate solution. The formation of a yellow precipitate indicated the presence of flavonoids (Sofowora, 1993).

Test for tannins

Each ethanolic crude leaf extract and fractions (2ml) were mixed with 2ml of distilled water and few drops of $FeCl_3$ solution were added. The formation of a green precipitate was an indication for the presence of tannins (Trease and Evans, 1989).

Test for steroids

A red colour produced in the lower chloroform layer when 2ml each of crude ethanolic leaf extract and fractions was

dissolved in 2ml of chloroform together with 2ml concentrated tetraoxosulphate (VI) acid added indicates the presence of steroids (Harborne, 1973).

Test for saponins

For saponins, 5ml each of ethanolic crude leaf extract and fractions was shaken vigorously with 5ml of distilled water in a test tube and warmed. The formation of stable foam was an indication for the presence of Saponins (Sofowora, 1993).

Test for terpenoids (Salkowski Test)

Two millilitres each of ethanolic crude leaf extract and fractions was dissolved in 2ml of chloroform and evaporated to dryness. Then 2ml of concentrated sulphuric acid was added and heated for about 2min. A greyish colour observed indicated the presence of terpenoids (Harborne, 1973).

Test for cardiac glycosides (Keller-Killani Test)

One hundred milligrammes each of the ethanolic crude leaf extract and fractions was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was under-layed with 1ml of concentrated H_2SO_4. A brown ring observed at the interface indicated the presence of de-oxy sugar; characteristic of cardenolides (Ngbede *et al.,* 2008).

Test for alkaloids

Three millilitres each of ethanolic leaf extract and fractions was stirred with 3ml of 1% HCl on a steam bath. Mayer's and Wagner's reagents were added to the mixture. Turbidity of the resulting precipitate was an indication for the presence of alkaloids (Trease and Evans, 1989).

Test for phenols

Two millilitres each of ethanolic crude leaf extract and fractions were shaken with equal volume of ferric chloride in a test tube. A deep bluish-green solution observed indicated the presence of phenols (Ngbede *et al.,* 2008).

Quantitative estimation of phytochemical constituents presents in ethanolic crude leaf extract and fractions of *Phyllanthus amarus*

Estimation of total phenols

The total phenolics content was estimated using the Folin-Ciocalteau procedure described by Siddhuraju and Becker (2003). About 1g of leaf extract was used and made up to 1ml, with distilled water. Then 0.5ml of diluted Folins-phenols reagent (1:1 ratio with water) and 2.5ml of (20%) sodium carbonate was added. The mixture was shaken and incubated for 40min at room temperature for the development of colour. Distilled water was used as blank. Absorbance was measured at 760nm using Thermo Fisher double beam spectrophotometer. Gallic acid was used as standard and the result was expressed as microgramme of gallic acid equivalents per gramme dry mass of extract (µg GAE/gDM).

Estimation of total flavonoids

The total flavonoids content was estimated using the procedure described by Zhishen *et al* (1999). 1ml of plant extract was diluted with 200µl, of distilled water separately followed by the addition of 0.150ml of (5%) sodium nitrite solution. The mixture was incubated for 5 min and then 0.150ml of (10%) aluminum chloride solution was added and allowed to stand for 6 min. Then 2ml of (4%) sodium hydroxide solution was added and made up to 5ml with distilled water. The mixture was well shaken and allowed to stand at room temperature for 15min. The absorbance was read at 510nm against the blank. Appearance of pink colour showed the presence of flavonoids content. The total flavonoids content was expressed as rutin equivalent mg/g extract on a dry weight basis using standard curve.

Estimation of total alkaloids

Total of 200ml, of 20% acetic acid was added to 1g of ethanolic crude leaf extract and fractions taken in separate 250ml, beaker and covered to stand for 4h. The mixture solution was filtered and the volume of the filtrate was reduced to one quarter using water bath. To the sample concentrated ammonium hydroxide was added drop-wise until a precipitate was formed. The solution was allowed to settle and the precipitate was collected by filtration and weighed (Obadoni and Ochuko, 2001). The percentage of total alkaloids content was calculated as:

$$\text{Percentage of total alkaloids (\%)} = \frac{\text{Weight of residue x 100}}{\text{Weight of sample that was taken}}$$

Estimation of total tannins content

Tannins content of the ethanolic crude leaf extract and fractions was estimated by the method of Siddhuraju and Manian (2007). A total of 0.5ml the extracts were taken in test tube separately and treated with 100mg of polyvinyl polypyrrolidone and 0.5ml of distilled water.

The solution was incubated at 4°C for 4h. Then the sample was centrifuged at 5000rpm for 5min and 0.02ml of the supernatant was taken. This supernatant only contains simple phenolics free of tannins (tannins were precipitated along with the polyvinyl polyprrolidone). The phenolics content of the supernatant was measured at 725nm and expressed as the content of free phenolics on a dry matter basis. From the above result, the tannins content of the extract was calculated as follows: Tannins (mg GAE/g) = Total phenolics (mg GAE/g) – Freephenolics (mg GAE/g).

Estimation of total saponins content

Estimation of total saponins content was determined by the method described by Makkar *et al.* (2007) based on vanillin–sulphuric acid colorimetric reaction with some modification about 0.05ml of plant extracts was added with 0.25ml of distilled water. To this mixture, 0.25ml of vanillin reagent (800mg of vanillin in 10ml of 99.5% ethanol) was added. Then 2.5ml of 72% sulphuric acid was added and mixed well. This solution was kept in a water bath at 60°C for 10min. After 10min, it was allowed to cool in ice cold water and the absorbance was read at 544nm. The value was expressed as diosgenin equivalent (mg DE/g extract derived from a standard curve.

Estimation of total terpenoids content

Two grammes of extract was weighed and soaked in 50ml of 95% ethanol for 24 h. The extract was filtered and filtrate was extracted with petroleum ether (60-80%) and concentrated to dryness. The dried ether extract was treated as total terpenoids (Ferguson, 1956).

Determination of DPPH radical scavenging activity

The antioxidant activity of the plant extract was estimated using the DPPH radical scavenging method described by Sutharsingh *et al.,* 2011. DPPH solution (0.004 % w/v) was prepared in 95% ethanol. A stock solution of ethanolic extract, extract fractions and standard ascorbic acid were prepared in the concentration of 10mg/100ml. From stock solution, 2ml, 4ml, 6ml, 8ml and 10ml of this solution were taken in five test tubes respectively. With same solvent made the final volume of each test tube up to 10ml whose concentration was 0.02mg/ml, 0.04mg/ml, 0.06mg/ml, 0.08µg/ml and 0.1mg/ml respectively. Two mililitres of freshly prepared DPPH solution (0.004 % w/v) was added in each of the test tubes. The reaction mixture was incubated in the dark for 15 min and thereafter the optical density was recorded at 523nm against the blank.

The capability of scavenging DPPH radical was calculated using the following equation:

$$\text{Percentage DPPH activity} = \frac{\text{Absorbance of Control - Absorbance of Test} \times 100}{\text{Absorbance of Control}}$$

Determination of ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay (FRAP) was used to estimate the reducing capacity of leaf extracts, according to the method of Benzie and Strain, (1996). In ferric reducing antioxidant power assay, 1ml of test sample of ethanolic extract in different concentration were mixed with 1ml of 0.2M sodium phosphate buffer (pH 6.6) and 1ml of 1% potassium ferricyanide in separate test tubes. The reaction mixtures were incubated in a temperature-controlled water bath at 50°C for 20min followed by addition of 1ml of 10% trichloroacetic acid. The mixtures were then centrifuged for 10 min at room temperature. The supernatant obtained (1ml) was added with 1ml of deionized water and 0.2ml of 0.1$FeCl_3$ solution. The blank was prepared in the same manner as the samples except that 1% potassium ferricyanide was replaced by distilled water. The absorbance of the reaction mixture was measured at 700nm. The reducing power was expressed as an increase in absorbance of the sample after blank subtraction.

Determination of thiobarbituric acid reactive substances (TBARS method)

The standard method as described by Sutharsingh *et al.,* 2011 was used.The mixture containing 4mg of the extract in 4ml of 99.5% ethanol (final concentration 0.02%). Exactly 4.1ml of linoleic acid in 99% ethanol, 8ml of 0.05M phosphate buffer (pH 7.0) and 3.9ml water was added to test tube and incubator at 40°C in the dark separately. To 2ml of this mixture, 2ml of 20% trichloroacetic acid and 2ml of 0.67% TBA solution was added. This mixture placed in water bath (100ºC) for 15 min and after cooling at room temperature, then centrifuged at 3000rpm for 30min. Antioxidant activity was based on the absorbance at 532nm. The percentage of antioxidant activity was calculated by following formula:

$$\text{Percentage TBARS activity} = \frac{\text{Absorbance of Control - Absorbance of Test} \times 100}{\text{Absorbance of Control}}$$

Forty albino wistar strain rats weighing between 100±10g were used for this study. The rats were obtained from Department of Biochemistry, Faculty of Sciences Federal

University of Agriculture Makurdi. The animals were allowed for two weeks to acclimatize, the rats weighed and housed in wooden cages with wooden bottom and wire-mesh top (North Kent Co. Ltd) under well ventilated room with a temperature and relative humidity of 29±2ºC and 70% respectively. The animals were exposed to 12h light-dark cycle and handled according to standard protocol. The animals were fed *ad libitum* with standard laboratory chaw throughout the period of the study.

Lamivudine and tenofovir disoproxil fumarate

Lamivudine and tenofovir disoproxil fumarate was obtained from the Federal Medical Centre Makurdi (FMC) Pharmacy and was used to managed human immunodeficiency virus (HIV) and hepatitis B virus (HBV), it decreases the patient's total burden of hepatitis and HIV, maintaining function of the immune system and prevents opportunistic infections that could lead to death. The antiretroviral drugs include deoxythymidine, zidovudine, stavudine, didanosine, zalcaitabine, abacavir, it is commonly known as highly active antiretroviral therapy. Lamivudine and Tenofovir disoproxil fumarate works by binding directly to reverse transcriptase and prevent RNA conversion to DNA and prevent immunodeficiency virus from replicating. In this study, this drug combination (Lamivudine – Tenofovir) was administered orally to reference control group of rats in a dose of 300 mg/kg body weight suspended in distilled water.

Experimental design

Forty Wistar strain rats divided into eight groups of five animals each was used for normal and biochemical indices analysis. Group I - VIII were treated according to the schedule in (Table 1). The administration was done once daily by orogastric intubation for a period of twenty-one days.

Collection of blood samples for analysis

The experimental animals were anaesthetised with chloroform and whole blood was collected from the animals by cardiac puncture using a sterile syringe. The blood sample was put into clean sterile sample bottles and allowed to clot before centrifugation. Sera collected were kept in the refrigerator at 4°C.

Determination of aspartate aminotransferase (AST) activity

AST activity was determined by the method described by Reitman and Frankel, (1957).

Principle

In this reaction L-Aspartate and α-Ketoglutarate react in the presence of AST in the sample to yield oxaloacetate and L-glutamate. The reaction is monitored by measurement of the decrease in the absorbance of NADH at 340nm. The rate of reduction in absorbance is proportional to AST activity in the sample.

Procedure

One millilitre (1ml) of reagent added to test tubes, 0.05 ml of sample was added to the sample test tube and none to the blank. It was incubated at room temperature for 20min, mixed immediately and first absorbance of test was read exactly at 1min and thereafter at 30, 60, 90 and 120 seconds at 340 nm.
The mean change in absorbance per minute was determined and the test results were calculated.
Serum AST activity (IU/L) = A /min × F.
A = Change in absorbance per minute
F = 3376 (Based on the millimolar extinction coefficient of NADH at 340nm).

Determination of aspartate aminotransferase (ALT) activity

AST activity was determined as described by Reitman and Frankel, (1957).

Principle

In this reaction, L-alanine and α-ketoglutarate react in the presence ALT in the sample to yield Pyruvate and L-glutamate. The reaction is monitored by measurement of the decrease in absorbance at 340nm. The rate of reduction is proportional to ALT activity in the sample.

Procedure

One millilitre (1ml) of reagent added to all required test tubes, 0.05ml of the sample was added to the test sample tube test and none to the blank. It was incubated at room temperature for 20min, it was mixed immediately and first absorbance of test was read at exactly 1 minute and thereafter at 30, 60, 90 and 120sec at 340nm. The mean change in absorbance per minute was determined and test results were calculated.

Serum ALT activity (IU/L) = A/min × F.
A = Change in absorbance per minute
F = 3376 (Based on the millimolar extinction coefficient of NADH at 340nm).

Table 1: Experimental design and treatment schedule for determination of the effect of the ethanolic crude leaf extract and fractions of *Phyllanthus amarus* and highly active anti- retroviral therapy (HAART) on rats:

Groups	Number of animals	Type of Treatment
I	5	Distilled water: Normal /control
II	5	Tenofovir – Lamivudine 300mg/kg body weight
III	5	Crude leaf extract of Phyllanthus amarus 100mg/kg body weight and Tenofovir – Lamivudine 300mg/kg body weight
IV	5	Crude leaf extract of Phyllanthus amarus 200mg/kg body weight and Tenofovir – Lamivudine 300mg/kg body weight
V	5	Fraction 1 100mg/kg body weight and Tenofovir – Lamivudine 300mg/kg body weight
VI	5	Fraction 1 200mg/kg body weight and Tenofovir – Lamivudine 300mg/kg body weight
VII	5	Fraction 2 100mg/kg body weight and Tenofovir – Lamivudine 300mg/kg body weight
VIII	5	Fraction 2 200mg/kg body weight and Tenofovir – Lamivudine 300mg/kg body weight

Determination of alkaline phosphatase (ALP) activity

Serum activity of alkaline phosphatase (ALP) was determined by the method described by Haussament, (1977).

Principle

$$\text{P-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{phosphate} + \text{p-nitrophenol}$$

Alkaline phosphatase in a sample hydrolyses P-nitrophenyl phosphate into P - nitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405 nm at 37°C due to liberation of P-nitrophenol is proportional to the alkaline phosphatase activity.

Procedure

Reagent (1ml) containing diethanolamine buffer, magnesium chloride and substrate (P-nitrophenylphosphate) was added into a clean test tube and incubated at 37°C followed by the addition of 0.02ml of sample. This was mixed thoroughly and immediately absorbance of test was read exactly at 30, 60, 90 and 120sec at 405nm against the reference blank (distilled water). The mean change in absorbance per minute was determined and the test results were calculated.

ALP activity was calculated using the following formulae:

Serum Alkaline phosphatase Activity (IU/L) = A/min x F.

A = Change in absorbance per minute

F = 2713 (calculated on the basis of molar extinction coefficient for P-nitrophenol and ratio of total assay to sample volume)

Determination of serum total bilirubin concentration

The serum total and direct bilirubin concentrations were determined by the method described by Dangerfield and Finlayson, (1953).

Principle

Bilirubin is estimated by reacting it with diazotised sulfanilic acid obtained from sodium nitrite and sulfanilic acid solutions. Bilirubin when reacted with diazotised sulfanilic acid forms a pink coloured azo-compound that is measured at 546nm. The unconjugated or free bilirubin takes longer time to react and requires caffeine as an accelerator. The indirect bilirubin is calculated from the difference between the total and direct bilirubin.

Procedure

For direct bilirubin, 0.05ml of sample was pipette into both the standard and test tubes in which 1.0ml of normal saline was added and followed by the addition of 0.1ml of 2-bilirubin solution. Also, 1.0ml of normal saline was added to the test tubes and followed by the addition of 0.1ml of working reagent. The reaction mixture was incubated at room temperature for 3 min and absorbance was read at 546nm against sample blank.

Serum Bilirubin (mg/dl) = (Absorbance of sample – Absorbance of sample blank) x F

F =26.312 (Molar extinction coefficient of diazotised sulfanilic acid).

Determination of total protein

Total protein was determined according to the method described by Fine, (1935).

Principle

The method is based on the knowledge that cupric ions in an alkaline medium, interact with protein peptide bond resulting in the formation of purple colour copper- protein complex which was read at 540nm.

Procedure

Biuret reagent (2.5ml) was added to all the required test

tubes (sample blank, standard and test sample). 0.05ml of the sample was added to the test sample tubes and 0.05ml of the standard reagent was added to the standard test tube. It was mixed well and allowed to stand at room temperature for 10min. The absorbance of the test sample and standard were read at 540nm against sample blank. The concentration of the sample was calculated using the formula.

$$\text{Total protein concentration} = \frac{\text{Absorbance of sample x conc.of standard}}{\text{Absorbance of standard}}$$

Determination of albumin

This was determined using Randox Assay Kit (Bromocresol Green) based on the method described by Spencer and Price, (1977).

Principle

Albumin measurement is based on its quantitative binding to the indicator, bromocresol green, BCG (3, 3, 5, 5,-tetrabromo-M-cresolsulphonapthalein). The albumin – BCG-complex formed absorbed at 578nm and its colour intensity is directly proportional to the concentration of albumin in the sample.

Procedure

Three clean test tubes labelled standard, sample and reagent blank were set. Into standard test tube, 0.1ml of standard was put, 0.01ml of sample into sample test tube, 0.1ml of distilled water into reagent blank test tube. Into each of these tubes 1ml of BCG reagent was added, mixed and incubated for 5min at 37°C. Absorbance of standard and sample was read at 578 nm, against reagent blank.

$$\text{Albumin conc. (g/dl)} = \frac{\text{Absorbance of sample x conc. of standard}}{\text{Absorbance of standard}}$$

Determination of serum creatinine concentration

Creatinine concentration was determined according to method described by Folin and Wu, (1919).

Principle

Creatinine present in the serum reacts with alkaline picrate to form a coloured complex. The rate of formation of coloured complex is directly proportional to creatinine concentration. This rate of reaction (intensity of orange colour produced) is measured colorimetrically at 510nm and is compared with that of the standard.

$$\text{Creatinine} + \text{Picric acid} + \text{NaOH} \longrightarrow \text{Creatinine picrate.}$$

Procedure

Working reagent (1ml) containing picric acid and sodium hydroxide was added into sample and standard test tubes containing 0.1ml of test sample and 0.1ml of standard solution respectively. The content in each test tube was mixed and after 20sec, the absorbance A1 of the standard and A1 of sample was read at 510nm. Exactly 80sec later, absorbance A2 of standard and A2 of sample were read at 510nm against distilled water blank.

$$\text{Creatinine concentration (mg/dl)} = \frac{\text{A2 – A1 (sample) x Concentration of standard}}{\text{A2 – A1 (standard)}}$$

Determination of serum urea concentration

This was determined using the method described by Fawcett and Scout, (1960).

Principle

Urease breaks down urea into ammonia and carbon dioxide. In alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicarboxyindophenol, a coloured compound. The reaction is catalysed by sodium nitroprusside. The intensity of colour produced is measured at 578nm.

$$H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{Phenol} \longrightarrow \text{Indophenol (blue compound).}$$

Procedure

Reagent (1 ml) containing sodium nitropusside and urease was added into three clean test tubes labelled as sample, standard and reagent blank containing 0.01ml sample, 0.01 ml standard reagent and 0.01ml distilled water respectively. The content in each of the test tube were mixed and incubated at room temperature for 10min. The absorbance of the test sample and standard were read against reagent blank at 578nm.The serum urea concentration was calculated using the formula below;

$$\text{Urea concentration (mg/dl)} = \frac{\text{Absorbance of sample x conc. of standard.}}{\text{Absorbance of standard}}$$

Table 2: Phytochemical components detected in the ethanolic crude leaf extract and fractions of *Phyllanthus amarus.*

Phytochemical components	Crude leaf extract	Fraction 1	Fraction 2
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	+	+	_
Alkaloids	+	+	_
Phenols	+	+	+
Terpenoids	+	_	_
Steroids	_	_	_
Cardiac glycosides	_	_	_

Key: + = Presence of phytochemical constituent.
- = Absence of phytochemical constituent

Qualitative Phytochemical Constituents of Ethanolic Crude Leaf Extract and Fractions of *Phyllanthus amarus*

The column fractionation of ethanolic crude leaf extract of *Phyllanthus amarus* yielded two fractions (Fraction 1 and 2). Phytochemical constituents detected in ethanolic crude leaf extract, fraction 1 and fraction 2 of extracts of *Phyllanthus amarus* is shown in (Table 2).

RESULTS AND DISCUSSION

Qualitative detection of phytochemicals in *Phylanthus amarus* showed the presence of saponins, tannins, phenols, terpernoids, alkaloids and flavonoids in the crude ethanolic leaf extract of Phyllanthus amarus. The fraction 1 showed the presence of all the phytochemicals detected in the ethanolic crude leaf extract with the exception of terpernoids while fraction 2 saponins, flavonoids and phenols were the only phytochemical constituents detected.

Quantitatively, flavonoids, phenols, tannins, saponins, alkaloids and terpernoids were found in varying concentrations in the ethanolic crude leaf extract and fractions of *Phylllanthus amarus.* This variation may be however, attributed to the difference in polarity of the solvents and molecular size of compounds present in the plant extracts (Ali *et al*., 2011). Plant phenolics and flavonoids constitute major groups of phytochemicals acting as primary *in vitro* antioxidants or free radical scavengers (El-Sayed *et al*., 2012). Therefore, (Tables 3, 4, 5, and 6) showed the scavenging activities of free radicals in a concentration dependent manner exhibited by DPPH Scavenging activity, FRAP and TBARS assay of ethanolic crude extract and fractions of *Phyllanthus amarus,* which signified antioxidant activities. The results of DPPH was expressed in terms of percentage inhibition and half maximum inhibitory concentration (IC_{50}) crude leaf extract, fractions 1 and fraction 2 of *Phyllanthus amarus* and L-ascorbic acid were found to be 35.65mg/ml, 38.38mg/ml, 41.95mg/ml and 23.78mg/ml respectively. The low IC_{50} value (23.78mg/ml) of the L-ascorbic acid suggests that it is a better free radical scavenger compared to the crude extract, fraction 1 and fraction 2 of the extracts. However, among the extracts the crude extract suggests a better free radical scavenger based on its lowest IC_{50} value (35.65mg/ml). This showed that the extract has reducing activity against free radicals.

The result of FRAP assay was expressed in terms of percentage inhibition and half maximum inhibitory concentration (IC_{50}) crude leaf extract, fraction 1 and fraction 2 of *Phyllanthus amarus* and L-ascorbic acid were found to be 29.25mg/ml, 23.78mg/ml, 38.68mg/ml and 26.25mg/ml respectively. The low IC_{50} value (23.73mg/ml) of the fraction 1 suggests it is a better reducing antioxidant power compared to the crude extract, fraction 2 and L-ascorbic acid. This showed that fraction 1 has better antioxidant reducing power than that of L-ascorbic acid, crude extract and fraction 2. TBARS assay showed that ethanolic crude leaf extract, fractions 1 and 2 of *Phyllanthus amarus* and L-ascorbic acid were found to be 29.34mg/ml, 31.25mg/ml, 41.61mg/ml and 19.16mg/ml respectively. L-ascorbic acid suggests a better antioxidant activity compared to the crude leaf extract, fraction 1 and fraction 2 of *Phyllanthus amarus*. However, among the extracts the crude extract suggests a better antioxidant activity base on its lowest IC_{50} value (29.34mg/ml) as compared to fractions 1 and 2. The antioxidant activities revealed by DPPH, TBARS and FRAP could be attributed to the presence of some phytochemicals in the leaf extracts of *Phyllanthus amarus*.

The administration of Tenofovir-Lamivudine drug combination (highly active antiretroviral therapy (HAART) to the rats resulted in a significant elevation ($P<0.05$) of ALT, AST and also decreased levels of total protein and albumin in rats. These observations were in agreement with previous reports that serum ALT, AST and bilirubin levels in rats after Tenofovir-Lamivudine drug combination administration were significantly elevated and with a corresponding decrease in total protein,

Table 3: Concentrations of some phytochemical constituents in the ethanolic crude leaf extract and fractions of *Phyllanthus amarus* (g/100g).

Phytochemical constituent	Crude leaf extract	Fraction 1	Fraction 2
Total flavonoids	11.87±0.56	4.58±0.24	3.17±0.07
Total saponins	5.99±0.19	2.02±0.20	3.01±0.14
Total tannins	9.08±0.07	4.17±0.07	_
Total alkaloids	3.73±0.10	1.70±0.10	_
Total terpenoids	1.27±0.10	_	_
Total phenols	7.41±0.07	2.09±0.07	4.96±0.44

Values are Mean ± Standard deviation for three determinations

Table 4: DPPH Radical scavenging activity of ethanolic crude leaf extract and fractions of *Phyllanthus amarus.*

Concentrations mg/ml	Ascorbic acid % Inhibition	Crude leaf extract % Inhibition	Fraction 1 %Inhibition	Fraction 2 %Inhibition
20	0.14±0.10	0.11±0.06	0.13±0.05	0.19±0.08
40	0.20±0.09	0.0.15±0.07	0.15±0.03	0.13±0.05
60	0.25±0.12	0.18±0.11	0.17±0.10	0.15±0.08
80	0.30±0.13	0.20±0.11	0.20±0.08	0.18±0.10
100	0.31±0.10	0.23±0.09	0.23±0.11	0.22±0.16
IC_{50} (mg/ml)	35.65	38.38	41.95	23.75

Key: Values are expressed as Mean ± Standard error of mean for three determinations.
IC_{50} = Half maximal inhibitory concentration; is the concentration causing 50% Inhibition of the desired activity.
F 1 = Fraction 1; F 2 = Fraction 2

Table 5: Ferric reducing antioxidant power (FRAP) assay of ethanolic crude leaf extract and fractions of *Phyllanthus* amarus.

Concentration (mg/ml)	Ascorbic acid %Inhibition	Crude leaf extract %Inhibition	Fraction 1 %Inhibition	Fraction 2 %Inhibition
20	0.13±0.05	0.11±0.02	0.10±0.01	0.08±0.04
40	0.22±0.01	0.39±0.06	0.12±0.02	0.16±0.03
60	0.26±0.03	0.24±0.02	0.16±0.02	0.09±0.01
80	0.32±0.07	0.28±0.01	0.24±0.03	0.16±0.03
100	0.27±0.02	0.34±0.03	0.26±0.03	0.20±0.01
IC_{50} (mg/ml)	26.25	29.31	23.78	35.68

Key: Values are expressed as Mean± Standard error of Mean for three determinations.
IC_{50}=Half maximal inhibitory concentration; is the concentration causing 50% inhibition of the desired activity.
F1= Fraction 1; F2= Fraction 2

Table 6: Thiobarbituric acid reactive substances (TBARS) of ethanolic crude leaf extract and fractions of *Phyllanthus amarus.*

Concentration (mg/ml)	Ascorbic acid %Inhibition	Crude leaf extract %Inhibition	Fraction 1 %Inhibition	Fraction 2 %Inhibition
20	0.20±0.06	0.15±0.08	0.11±0.07	0.10±0.09
40	0.32±0.12	0.18±0.06	0.13±0.10	0.12±0.03
60	0.33±0.17	0.21±0.13	0.16±0.05	0.13±0.10
80	0.40±0.12	0.25±0.17	0.20±0.10	0.17±0.07
100	0.42±0.14	0.28±0.10	0.24±0.06	0.20±0.01
IC_{50} (mg/ml)	19.16	29.34	31.20	41.60

Key: Values are expressed as Mean ± Standard error of mean for three determinations
IC_{50} = Half maximal inhibitory concentration; is concentration causing 50% inhibition of the desired activity.
F 1 = Fraction; F 2 = Fraction 2.

albumin (Gnanasekaran *et al*., 2015; Akram *et al*., 2012; Ama *et al*., 2013; Muhammad *et al.,* 2013).

Normal liver functions are demonstrated by the balanced activities of serum marker enzymes AST, ALT, ALP (Singh *et al.,* 2011). Liver toxicity caused by prolonged administration of highly active antiretroviral therapy can be inflicted through several mechanisms. The pathogenesis often remains enigmatic.

Table 7: Effects of ethanolic crude leaf extract and fractions of *Phyllanthus amarus* and Tenofovir-Lamivudine drug combination on liver markers enzymes in Wistar rats.

Groups	Treatment	AST(iu/l)	ALT (iu/l)	ALP (iu/l)
I	Normal/Control	85.00±3.48	27.80±1.07	73.32±0.51
II	TL: 300mg/kg	96.40±0.81[a]	48.20±3.84[a]	72.28±0.98
III	CE:100mg+TL300mg/kg	89.00±3.39[a]	37.60±2.01[a]	73.40±1.11[b]
IV	CE:200mg+TL300mg/kg	91.00±1.82[a]	43.20±1.36[a]	73.68±0.59[b]
V	F1:100mg+TL300mg/kg	86.70±3.43[a]	30.20±0.97[a]	73.20±1.44[b]
VI	F1:200mg+TL300mg/kg	88.20±3.09[a]	33.60±5.64[a]	73.42±1.77[b]
VII	F2:100mg+TL300mg/kg	87.70±2.89[a]	34.60±2.20[a]	73.34±1.06[b]
VIII	F2:200mg+TL300mg/kg	89.60±3.05[a]	36.20±1.69[a]	73.46±0.58[b]

Key: Values are expressed as Mean ± Standard error of mean (n = 5).
a= significantly higher (P<0.05) compared to normal/control in the same column.
b = Values fall within the same range with the normal/control in the column at (P<0.05).
F1 = Fraction 1; F2 = Fraction 2; TL = Tenofovir – Lamivudine drug combination;
CE = Crude extract

Table 8: Effects of ethanolic crude leaf extract and fractions of *Phyllanthus amarus* and Tenofovir-Lamivudine drug combination on some biochemical parameters in Wistar rats.

Groups	Treatment	Total protein (g/l)	Albumin (g/l)
I	Normal/Control	48.00±1.48	29.40±1.17
II	TL: 300mg/kg	46.40±1.03[b]	28.00±1.52[b]
III	CE:100mg+TL300mg/kg	48.60±1.54[c]	29.40±0.68[c]
IV	CE:200mg+TL300mg/kg	49.80±1.91[a]	31.80±2.44[a]
V	F1:100mg+TL300mg/kg	48.33±0.48[c]	29.60±0.51[c]
VI	F1:200mg+TL300mg/kg	49.50±2.97[a]	30.80±1.43[a]
VII	F2:100mg+TL300mg/kg	48.20±2.35[c]	30.00±3.19[c]
VIII	F2: 200mg+TL300mg/kg	48.80±0.37[c]	39.40±1.40[a]

Key: Values are expressed as Mean ± Standard error of Mean (n = 5).
a = significantly higher (P<0.05) compared to normal in the same column.
b = significantly lower (P<0.05) compared to normal in the same column.
c = Values fall within the same range with the normal in the same column at (P<0.05).
F1 = Fraction 1; F2 = Fraction 2; TL = Tenofovir – Lamivudine drug combination; CE = Crude extract.

Five categories are proposed: direct mitochondrial inhibition, direct cell stress, hypersensitivity reactions, immune reconstitution in the presence of viral hepatitis co-infection, and disturbances of lipid/sugar metabolism and steatosis (Núñez, 2010). Treatment with both the ethanolic crude leaf extract and fractions of *P. amarus* and HAART at different concentrations significantly decreased level of liver marker enzymes (Table 7). The reduction in the levels of these enzymes after the administration of the crude extract and fractions of this plant signifies recovery through increased in protective factors and decreased in the aggressive factors; couple with natural immune system activity. This is in agreement with research carried out by Gnanasekaran *et al*., (2015) reported on the ameliorative effect of aqueous-ethanolic extract of *Calpurnia aurea* seed on the effects of HAART prolonged intake on the liver.

The results suggested that the possible mechanism of this activity might be due to free radical-scavenging and antioxidant activity of the leaf extracts of *Phyllanthus amarus*. HAART may induce (i) oxidative stress (ii) decrease in free radical scavenging protection, or (iii) a failure to restore oxidative damage. Therefore, free radical-scavenging is the most significant way to protect the liver against hepatotoxicity caused by HAART administration. Tannins have strong antioxidant properties and have the ability to chelate metal ions such as Fe^{2+}, unlike primary antioxidants they donate hydrogen atom or electron (Maytin *et al.,* 1999). The prevention of membrane lipid peroxidation by tannins can act via the inhibition of cyclooxygenase (Zhang *et al.,* 2004). On the other hand, the significant elevation ($P<0.05$) of total bilirubin levels in the HAART-treated rats correlates with the findings of Aluko *et al.,* (2013). Possibly, elevated bilirubin levels may be associated with reduced hepatocyte uptake of bilirubin, impaired conjugation of bilirubin (Dominic *et al*., 2012; Khan *et al*., 2012). However, administration of the crude extracts and fractions significantly reversed the effect of HAART on the liver by reducing bilirubin, total protein and albumin levels to a near normal which can be compared to the works of Babalola *et al*., (2011), Aluko *et al.,* (2013) and

Table 9: Effects of ethanolic crude leaf extract and fractions of *Phyllanthus amarus* and Tenofovir-Lamivudine drug combination on serum creatinine, urea and total bilirubin levels of Wistar rats.

Groups	Treatment	Total Bilirubin (µmol/l)	Creatinine (mmol/l)	Urea (mmol/l)
I	Normal/Control	6.28±1.07	17.52±1.63	11.83±0.68
II	TL:300mg/kg	17.94±1.64[a]	53.40±2.71[a]	14.74±0.20[a]
III	CE:100mg+TL 300mg/kg	7.30±0.16[ab]	28.10±1.37[ab]	12.96±0.24[ab]
IV	CE: 200mg+ TL 300mg/kg	10.22±0.56[ab]	43.24±1.28[ab]	13.38±0.28[ab]
V	F1: 100mg+ TL 300mg/kg	8.34±0.47[ab]	30.30±2.31[ab]	12.40±0.26[bc]
VI	F1: 200mg+ TL 300mg/kg	10.54±0.70[ab]	46.38±1.01[ab]	13.12±0.19[ab]
VII	F2 : 100mg+ TL 300mg/kg	8.94±0.21[ab]	20.00±1.09[ab]	11.91±0.32[bc]
VIII	F2 : 200mg+ TL 300mg/kg	10.92±0.38[ab]	24.20±2.10[ab]	12.980.36[ab]

Key: Values are expressed as Mean ±Standard error of Mean (n = 5)
a = significantly higher (P<0.05) compared to normal in the same column.
b = significantly lower (P<0.05) compared to Tenofovir – Lamivudine drug combination.
c = Values fall within the same range with the normal/control in the same column at (P<0.05).
F1= Fraction 1; F2 = Fraction 2; TL = Tenofovir – Lamivudine drug combination; CE = Crude extract.

Jude *et al*., (2016).

Similarly, administration of HAART caused nephrotoxicity as indicated by significant elevation (P<0.05) of urea and creatinine levels in the serum of the experimental animals (Table 8). These results are in agreement with earlier findings by Yacout *et al*., (2012). From this study, it is evident that significant elevation in serum urea and creatinine levels can be attributed to damaged nephron structural integrity (Khan and Siddique, 2012). It was obvious that administration of HAART caused marked impairment in renal function with significantly raised levels of creatinine and urea concentrations (Table 9). Serum urea and creatinine levels elevations might have resulted from remarkable leakage due to hypercellularity of the glomeruli and degradation. Treatment with *Phyllanthus amarus* ethanolic crude leaf extract and fractions at both high and low doses (200mg/kg and 100mg/kg body weight) has affected the nephrotoxic effect of HAART positively by significantly lowering the levels of urea and creatinine.

Conclusion

In conclusion, this study has demonstrated that the ethanolic crude leaf extract and fractions of *Phyllanthus amarus* is a good source of phytochemicals such as flavonoids, terpenoids, alkaloids, tannins and phenols. The antioxidative potentials of these phytochemicals might have contributed significantly to its ameliorative effect on the deleterious effects of prolonged HAART administration on the experimental animals. The ameliorative potential of *Phyllanthus amarus* is depicted by the reversal and/or improvement of the hepatotoxic/nephrotoxic effect of HAART administration.

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