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Hepatic and Immuno-Remediating Potential of Phenolic Rich Concentrate of *Phyllantus Amarus* (Schum & Thonn) Whole Plant in Acute High Salt Diet Assaulted Animal Model.

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Abstract— Gluttonous habits on high salt diets have been accounted to be deleterious to human ecosystem worldwide, derailing homeostatic threshold, immunological inertia and eventually resulting to hepatic degradation. These influences had prompted many enthusiastic researchers to further investigate on the better/alternative medicinal option to ameliorate the high preponderance of salty-diet related immunological and hepatic derangement, and subsequently complementing the non-readily available but expensive orthodox medicine. Hence, this study thus evaluated the lethal dose and protective potential of Phenolic rich concentrate (PRC) of *Phyllanthus amarus* (Schum and Thonn) whole plant against acute High salt diet (HSD) (8%) assaulted immunological inflammations and hepatic functionality in animal model. The healthy adult male Sprague-Dawley rats n=30 were divided into five groups: Group 1: control, Group 2: High salt diet, Group 3: High salt diet+75mg/kg/bdw, Group 4: High salt diet+100mg/kg/bdw, and Group 5: High salt diet+150mg/kg/bdw of PRC. The results obtained ($p<0.05$) indicated the lethal dose of the concentrate was more than 5000mg/kg/bdw. Moreover, significant excitations were recorded in the level of inflammatory cytokines, interleukin 2(IL-2), interleukin 6(IL-6), interleukin 8(IL-8), Tumor necrosis factor-alpha(TNFα) and serum enzymes, Aspartate transaminase(AST), Alkaline phosphatase(ALP), Lactate dehydrogenase(LDH) and Creatine kinase(CK) of rats fed HSD. Though IL-8 was utterly impaired among others, however, the PRC significantly ameliorated all the compromised markers to near normal level in a dose-dependent manner. The results thus established the connectivity of the plant as used in trado-medicine and also accomplished the efficacy of PRC in remediating HSD induced immunological and hepatic derangements.

Keywords—Inflammations; High salt diet; *Phyllanthus amarus* (Schum and Thonn); Phenol rich concentrate; immunological cytokines; hepatic markers; lethal dose.

I. INTRODUCTION

Immunological subjugation is generally known to be greatly associated with hepatic derangement and other metabolic disorders whose high prevalence has incessantly accounted worldwide in high salty meal related diseases [1]. These are characterized by a significant increment in the level of related inflammatory cytokines, with long-term complications that affect various organs and tissues integrity vis-a-vis; liver, kidneys, heart, endothelial/epithelial tissues, with concomitant degradation of macrophages [2]. Etiologically, readymade meals which are usually preserved by high salt and also generally prefer by present junks accustomed generations, are now being implicated in the aforementioned metabolic lesions and other related pathologies namely; hepatotoxicity, hypertension and immunological derangements [3, 4, 5, 6]. The high salt inflammations thus impaired the homeostatic threshold, triggered by hypertonic electrochemical gradients, and hence compromising hepatic wholeness, infiltrating organs' related markers, and immunological cytokines which are essential in the modulating of immune responses. The overwhelmingly derailed modulators include interleukins, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and serum enzymes; Aspartate transaminase (AST), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH) and Creatine kinase [5, 7]. These cytokines and hepatic but serum based bio-indicators are agitated by compromised neutrophils, monocytes, macrophages, stimulated T, B-lymphocytes and hepatic ribosomes during the pathological state, but are otherwise recuperated by the actions of phytochemicals [8, 9, 10]. More so, significant augmentation in these intercellular cytokines in the interstitium have also been accounted in animal studies with high salt-sensitivity. Fundamentally, plants and animals have multiple types of non-enzymatic phytochemicals; including glutathione, vitamins A, C, E, terpenoids, phenolics, alkaloids, as well as enzymatic biomolecules namely; superoxide dismutase, catalase, and several peroxidases. These are constituents established to

be efficacious against ill-health and its affiliated pathologies [11]. Dietary and exogenous galenicals were noted to exhibit therapeutic abilities on salt-related metabolic lesion, with single or synergetic potentiality.

Phyllanthus amarus ***(Schum and Thonn)(*****figure 1),** belonging to the *Euphorbiaceae* family, commonly known as Stonebreaker, and eyin-olobe by Yoruba people in South-Western, Nigeria, but with more than 600 species [12], is a well-known medicinal plant, traditionally used to manage high salt diet related health challenges in Ogbomosho and the metropolis. Consequently, this study was conducted to ascertain and investigate possible potentials of Phenolic rich concentrate (PRC), though non-existent in the literature, but extracted from *Phyllanthus amarus* **(Schum and Thonn)** whole plant against acute 8% High salt diet (HSD) assaulted immunological inflammations and hepatic related serum enzymes distortion in rats within 8weeks.

Fig 1: Phyllanthus amarus (Schum & Thonn) plant with voucher number 0255

II. RELATED WORK

The therapeutic potentials of the aqueous seed and leaf extract of *Phyllantus amarus* was reported by Adeneye et al., where the accounts revealed dose-dependent anti-lipidemic, anti-obesity and antidiabetic effects in treated mice, by promoting endothelial utilization of glucose [13].

Adomi et al., also evaluated the acute and sub-acute toxicity of the leaf extract of the plant, and a maximum dose of 8000mg/kg/body weight, with significant reduction in plasma ALT and ALP of orally treated experimental rodents were noted, and corresponding minor abnormalities in the liver and renal histopathology [14].

The hypotensive abilities of the aqueous leaf extract of *P. amarus* was also investigated in normotensive male rabbits by Amaechina et al., [15]. Intravenous administration at 5mg to 80mg/kg/body weight doses exhibited a significant fall in systolic, mean diastolic, and arterial values in a dose dependent manner.

In another study by Amonkan et al., two fractions of the aqueous decoction of *P. amarus* were evaluated. The ethanolic fraction was established to exhibit higher hypotensive efficacy than the aqueous fraction. Amonkan et al., also accounted a reasonable composition of alkaloid phytochemical in the ethanolic fraction but not in the aqueous fraction, while the latter has saponin as the active constituents but not in the former. It's of great importance that the aqueous extract signaled the presence of terpenoid (saponin), phenolics (flavonoids and tannins), alkaloids (nitrogenious phytoconstituents) and sterols as the therapeutic agents [16].

Awasthi et al., also evaluated the quantitative and qualitative values of various parts of *P. amarus* and established a great coefficient of terpenoid (saponin), phenolics (flavonoids and tannins), and alkaloids (nitrogenious phytoconstituents) in the aerial part (leaf and stem), while the seeds and roots only showed the presence of terpenoid and phenolics [17].

The anti-hyperlipidemic and anti-adiposity of P. *amarus* aqueous leaf extract was again investigated and accounted by Ezeugwunne et al., in alloxan induced diabetic albino wistar rats. It was established to reduce significantly the serum lipid profile in the diabetic induced rodents [18].

The anti-microbial potency of *P. amarus* on hepatitis B viral (HBV) cell viability was also established by Lee et al., [19]. The investigation accounted the ability of *P. amarus to* suppress the viral mRNA replication and subsequent multiplication of the pathogens in the culture medium.

The phytochemical GC-MS analysis of the *P. amarus* ethanolic leaf extract was also evaluated by Mamza and the following compounds were accounted: heptanoic acid, 13-octadecadiene-1-ol, methyl 14-methyl pentadecanoate, 10-octadecanoate, 3,5-di-t-butylphenol, palmitic acid, 9-hexadecenal, glycerol 1, 3-dipalmitate, and dioctytl ester [20].

Obianime et al., also investigated the possible alterations caused by methanolic leaf extracts of *P. amarus* on some bio-indicators of Guinea pigs (male) and the phytochemical related the presence of cardiac glycosides, alkaloids, favonids and tannins [21]. More so, the extracts exhibited significant and dose dependent decrease in the levels of acid phosphatases, AST, ALT, total cholesterols, kidney related markers vis-à-vis uric acid, urea and total protein of the experimental animals.

Antimicrobial properties of dichloromethane, hexane, ethyl acetate and 70% aqueous methanolic fractions of 95% ethanol leaf extract were also assessed and established to exhibit significant activities against Candida albicans, Staphylococcous aureus and Escherichia coli as reported by Okwute et al. [22].

Yao et al., also reported the folkloric usage of *P. amarus* in the treatment of cardiovascular related disorders and as a diuretic candidate [23]. In addition, an ethanolic fraction of

the plant was also accounted to significantly increase the urinary clearance of water and sodium ion (Na^+), which was attributed to prostaglandins mediated phenomenon in experimental rats.

III. METHODOLOGY

Plant materials

The *Phyllanthus amarus* (Schum & Thonn) whole plant (fig 1) was obtained from traditional healers managing HSD related scourge and also practicing indigenous medicine in Ogbomosho, and attested by Dr. Famuwagun (FUTA) (Federal University of Technology, Akure) and Professor Ogunkunle A., the taxonomist, Department of Applied Biology, LAUTECH (Ladoke Akintola university), Nigeria. The authenticated sample (VN0255) was eventually conserved at the university herbarium for future referencing.

Plant preparation

The whole plant was watched and air-dried at room temperature for 5weeks until a constant weight was obtained, and then pulverised. The resulting powdered sample was immediately extracted as highlighted below and as also depicted in figure 2.

Plant extraction

- **Preparation of Phenol Rich-Concentrate (PRC)**

The extraction proceeded according to the combined accounts described by [24] and [25] with little edification as depicted in figure 2. Powdered sample but dried of the *Phyllantus amarus* (Schum. & Thonn) whole plant (700g) was dissolved in 2000ml of acetone/water/acetic acid (70/28/2 by volume) [26], for 48hours with subsequent shaking at intervals. The dissolution was filtered using Whatman filter paper (150mm), and immediately rotary evaporated under vacuum pressure, and subsequently freeze-dried to obtain the phenolic rich concentrate, which was kept in amber bottles, preserved in the refrigerator for onward administration, evaluation and analysis.

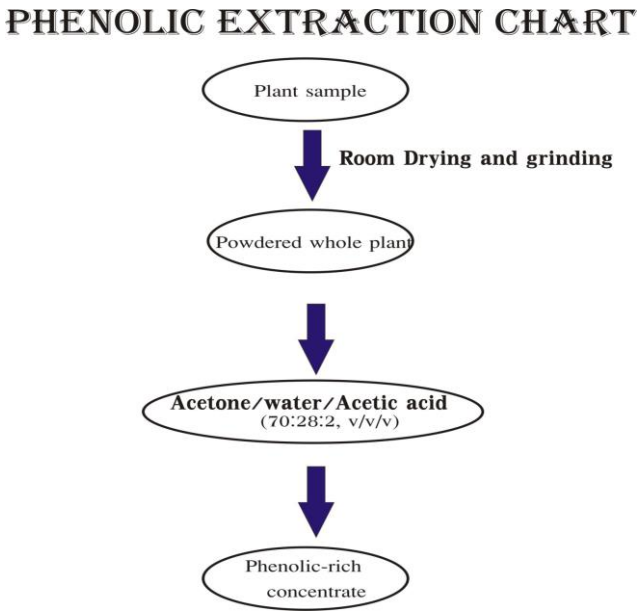

Figure 2: Schematic diagram for phenolic-rich concentrate.

- **Qualitative test for Phenolics**

The phenolic rich extract (4g), was tested by adding 4mls of 5% aqueous ferric chloride, both into a test tube, and the resulting formation of conspicuous blue color indicated rich phenolics [27].

Animal grouping

Thirty Dawley male animals were divided evenly into five groups. Group 1 orally given normal rat chow, while Group 2 received 8% high salt rat chow (HSD). The rats in group 3 received 8% HSD+75mg/kg/body weight of PRC, Group 4: 8% HSD+100mg/kg/body weight of PRC, and Group5: 8% HSD+150mg/kg/body weight of PRC.

All the rats were given drinking water ad-libitum, co-treated in the morning for eight weeks after 2week acclimatization, once daily, and sacrificed by using chloroform as anesthetic after midnight fasting of the last co-treatment, blood collected through cardiac puncture, and the serum decanted immediately after centrifugation. Immunological cytokines and hepatic related serum enzymes assay were then immediately evaluated.

Acute Lethal toxicity quantification of phenol rich concentrates of *Phyllanthus amarus* (Schum & Thonn) whole plant

In this study, the acute lethal toxicity (LD50) of phenol rich concentrate (PRC), was quantified using up and down techniques [26].

Estimation of immunological cytokines

- ✓ **Interleukin 2**

The quantification was done using an Interleukin 2 (IL-2) Immunoassay kit [28]. After the appropriate procedures as stated in the manual, the absorbance of the mixtures in the corresponding wells was taken spectrophotometrically at 450nm.

- **Interleukin-6 (IL-6)**

The RayBio rat IL-6 ELISA is used for the quantitative measurement of IL-6 in the serum [28].

- ✓ **Interleukin-8 (IL-8)**

The quantitative analysis was evaluated using lay down principle by [29]. IL-8 was measured by the enzyme-linked immunosorbent assay fully automated system.

- ✓ **Tumor Necrosis Factor-Alpha (TNF-α)**

The assay employs an antibody for rat TNF-α, coated (immobilized) on a 96-well plate [29]. The TNF-α in the sample bounded to the antibody in the wells, washed, and rat TNF-α antibody was added. After washing away unbound antibody, Tetramethylbenzidine, and other reagent solutions added to the wells for color development. Color produced was measured and proportionate to the amount of TNF-α present in the serum sample at 450nm.

Estimation of serum enzymes

- **Determination of Aspartate transaminase (AST) activity**

The procedure described by Amador and Wacker (1962) and Nwaehujor *et al* (2014) was employed in the determination of the activity of Aspartate transaminase in the serum [30, 31].

- **Determination of Alkaline phosphatase (ALP) activity**

The procedure accounted by Afolabi *et al* (2015) [32] was used for the determination of the activity of alkaline phosphatase (ALP).

- **Determination of Lactate dehydrogenase (LDH) activity**

Lactate dehydrogenase (LDH) metabolically active in the metabolism of glucose and widely abundant in cardiac and skeletal muscles, kidney, liver, and red blood cells. Serum lactate dehydrogenase was detected and quantified according to the method of Fikry (2017) [33] .

- **Determination of Creatine kinase (CK) activity**

This was quantified using Atlas Medical kits. Creatine kinase (CK) catalyzes the reversible reaction of phosphate group release from phosphocreatine in the form of ATP. This phenomenon is coupled to those exhaustively catalysed by hexokinase (HK) and glucose-6-phosphatedehydrogenase (G6P-DH). The coefficient of NADPH produced and measured spectrophotometrically, equates the concentration of creatine kinase (CK).

Statistical Analysis

The data expressed as Mean ± Standard deviation and cross-examined using one-way analysis of variance (ANOVA) at $p < 0.05$ (n=6) and the Statistical Analysis evaluated using Graph Pad Prism.

IV. RESULTS AND DISCUSSION

In this study, the acute lethal toxicity (LD50) of phenol rich concentrate (PRC), was investigated, while table 1 and 2 revealed the effect of high salt diet, significantly inflaming the immunological cytokines; iL-8, iL-6, iL-2, TNF-α and serum enzymes; Aspartate transaminase (AST), Lactate dehydrogenase (LDH), Alkaline phosphatase (ALP) and Creatine kinase (CK), in the rats fed with HSD only (Group 2), when compared against the group fed with normal chow (Group 1). Nevertheless, co-treatment with phenol rich concentrate (PRC) of the *P. amarus* whole plant (Group 3, 4, 5) dose-dependently decreased significantly the level of immunological cytokines (iL-8, iL-6, iL-2, TNF-α) and serum enzymes activities (AST, LDH, ALP, CK) to near level of group fed with normal rat chow (Group 1). It's of interest to note that interleukin 8 which was intensely compromised by HSD, among other immunological parameters studied (iL-6, iL-2 and TNF-α), was however dose dependently ameliorated also to near normal level after co-treatment with PRC.

The acute toxicity of the phenol rich concentrate (PRC) of *P. amarus (Schum &Thonn)* whole plant within 72 hours, was quantified and established to be greater than 5000 mg/kg/bdw upper quartile using up and down method. It was also accounted in this study, that PRC of *Phyllanthus amarus (Schum & Thonn)* whole plant could hence be applied as therapeutic agent against high salt diet assaulted immunological inflammations and hepatic related serum enzymes derangement, for being able to restore the significant aforementioned related markers to near normal level. This account was in agreement with the folkloric use and applications of the plant in the management of HSD affiliated diseases by trado-medicine practitioners in Nigeria and environ. The HSD driven immunological storm (IL2, 6, 8 and TNF-α) accounted, was perhaps significantly actuated by the compromised effects of hypernatremia on white blood cells, macrophages, neutrophils, and that of AST, ALP, LDH, CK in the serum by infiltrated hepatocytes [34]. More precisely, PRC ameliorated the necrotic effect of high salt influx on the intracellular cells by recuperating the denaturing pathology on lymphocytes, monocytes, ribosomes [35] and hepatocytes [36] and hence a good candidate for future altenative against high salt related immunomodulating deficiencies and hepatotoxicity [37]. The findings are also relatively in agreement with the accounts and potency of the *Phyllantus amarus* plant as reported by [38]. The delirious effect of HSD due to the hypertonic and compromised sodium\potasssium Atpase threshold [39], upregulating the serum cytokines and enzymes, also instituted a consistency with the global verdict of the World Health Organization (WHO) (2012) on high salt-diet related diseases and its prevalence [40]. More-over, some enthusiastic researchers had also reported on the pharmacological potencies of *Phyllanthus amarus (Schum & Thonn)* plant, which include di-uretic, anti-hepatitis B-virus, hypoglycemic, and hypocholesterolemic [41, 42, 43]. Others are anti-diabetic, anti- cancerogenic [44], glucose clearance enhancement [45], anti-hypertensive [46] and antihyperlipidemic potentials.

Table 1: Effect of varying doses of phenol rich concentrate (PRC) of Phyllanthus amarus (Schum &Thonn) whole plant on immunological cytokines (ng/ml) of rats fed with high salt diet.

Group	IL8	IL6	IL2	TNF-α
Group1	7.50±1.30	2.32±0.20	1.42±0.10	5.72±1.50
Group2	30.72±3.10	5.21±2.60	3.01±2.10	8.32±3.20
Group3	20.21±3.01	4.42±0.32	2.67±0.11	7.80±0.52
Group4	12.31±0.21	3.69±0.03	2.01±0.50	6.35±0.13
Group5	9.12±0.52	3.05±0.04	1.92±0.02	5.99±0.51

Data were expressed with one way ANOVA using SPSS, as mean ± SD (n= 6) and considered statistically different at $p< 0.05$. The Data with astericks were compared with control group along the same column and are statistically different using Graph pad prism.

Keys:

Group 1- Group administered normal chow diet.
Group 2- High salt diet (HSD) fed group
Group 3- HSD+75mg/kg/bwt of PRE
Group 4- HSD+100mg/kg/bwt of PRE
Group 5- HSD+150mg/kg/bwt of PRE
IL8- interleukin 8, IL6- interleukin 6, IL2- interleukin 2, TNFα- Tumor necrosis factor-alpha.

Table 2: Effect of phenol rich extract (PRE) of Phyllantus amarus whole plant on serum enzymes (U/L) of rats fed with 8% high salt diet.

Group	AST	ALP	LDH	CK
Group1	20.55±0.07	46.21±0.32	240.20±4.01	230.40±6.15
Group2	51.50±0.51	84.32±0.10	452.40±5.22	420.20±8.03
Group3	47.20±1.00	70.12±0.12	378.20±0.07	320.60±0.05
Group4	35.21±0.08	62.12±0.02	300.20±0.02	290.40±0.02
Group5	23.11±0.14	50.12±0.02	258.30±0.06	250.30±0.03

Keys:
AST- Aspartate transaminase
ALP- Alkaline phosphatase
LDH- Lactate dehydrogenase
CK- Creatine kinase
U/L- unit per litre

Withal, the compromising result of IL-8 among other inflammatory cytokines in this study, revealed an immediate and more significant derangement than other immunological affiliates; iL-6, iL-2 and TNF-α, and hence could be recommended as a reliable immuno-inflammatory biomarker in the diagnosis of HSD related immunological diseases. More importantly, other classes of phenolic constituents earlier accounted from the *P. amarus* plant also include flavonoids and tannins , which were established to be beneficial and potentiate delay in the aging process [47].

Possible mode of actions:- In high salt sensitive animals, the influx of sodium ion (Na+) in the interstitial space (hypernatremia) has been accounted to promote hyper-homeostatic barrier, activating sodium\potasssium Atpase threshold, the endothelial modulating enzymes (Angiotensin and Endothelin converting enzymes) [48] and hepatic stellate cells [9], while degrading inotropic functionality, inhibiting coefficient of Nitric oxide [49] and Hydrogen sulfide [50], triggering macrophages, and eventually igniting inflammatory cytokines [51]. All these are perhaps hypothetical/research based mechanisms of high salt diet assaults, hepatic enzymes derangement and immunological storming. Nonetheless, the capability of the hydroxyl functional group of the phenolic rich phytoconstituents from the *P. amarus* whole plant might have potentiated the immunological and hepatic efficacy against HSD assaulted pathologies as accounted in this study. More precisely, the efficacy of the hydroxyl functional group, acting as pro-oxidants' scavenger, can't also be relegated in the latter.

V. Conclusion and Future Scope

The study established a rich concentration of phenolics in the *P. amarus* whole plant. Its lethal dose was also within a maximum of 5000 mg/kg/bdw upper quartile using up and down method, which was partly supported by Adomi et al., [14]. Moreover, the potency of PRC in ameliorating the triggered HSD assaulted immunological cytokines (iL-8, iL-6, iL-2, TNF-α) and hepatic related serum enzymes (AST, LDH, ALP, CK) were unequivocally accounted in co-treated groups when compared to the rodents fed with normal rat chow. Hence, PRC is hereby recommended as therapeutic candidate in the management of high salt diet driven immunological derangements and hepatotoxicity, while the future research direction involves the possible mode of action of the phenolic rich phytoconstituents on high salt diet assaulted immunological and hepatic infringement, and eventual characterization of the active agents in the rich galenicals.

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Author contributions
All authors participated in the study and well intimated about the final manuscript before submission. Specifically, Olorunnisola Sinbad provided the concept and design, Adewale Adetutu engaged in experimental studies and manuscript review, Bamidele Stephen Ajilore sacrificed the experimental rodents and assayed all biomarkers of interest, while Fasan Tope Israel proceeded on the literature search, data acquisition, analysis and manuscript preparation.

Ethical statement
Experimental animals were cared for, following the institutional guidelines of National Institutes of Health guide for use of Laboratory rodents (NIH Publications No. 8023, revised 1978) and European Convention for the Protection of Experimental Animals. Thirty Sprague-Dawley male rats were kept in plastic cages, acclimatized to standard laboratory conditions for two weeks, fed with commercial normal rat chow, liberally supplied with water, while another groups were co-treated for possible efficacy of PRC extracted from the whole plant.

Conflict of interest
We wish to proclaim that there is no counter-opinion or existing conflict in this study.
List of Abbreviation: IL- Interleukins; WHO- World Health Organization**;** PRC-
Phenol rich concentrate, SD- Standard deviation; HSD-High salt diet; TNF- Tumor necrosis factor, Bdw-Bodyweight.

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Legend of Tables and Figure

1. **Table 1:** Effect of varying doses of phenol rich concentrate (PRC) of *Phyllanthus amarus (Schum &Thonn)* whole plant on immunological cytokines (ng/ml) of rats fed with high salt diet.
2. **Table 2:** Effect of phenol rich extract (PRE) of *Phyllantus amarus* whole plant on serum enzymes (U/L) of rats fed with 8% high salt diet.
3. **Figure 1:** *Phyllanthus amarus (Schum & Thonn)* plant with voucher number 0255.
4. **Figure 2:** Schematic diagram for phenolic-rich concentrate.

