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Pre-Clinical Assessment of Phyllanthus Amarus Leaf Fraction on Arsenate Treated Hepato-Renal and Pancreatic Cells

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ABSTRACT

With increasing anthropogenic activities, man is faced with carcinogenic substances like arsenate, which according to the Agency for Toxic Substances and Diseases Registry, is top on the list of twenty most toxic substances of public health concern. Therefore, this research investigated the effects of P. amarus leaf fraction on arsenate toxicity on key cell lines, with focus on addressing the challenge of arsenic poison in food and the environment. Various assays such as free radical scavenging assays and in-vitro cell lines assays on Human hepatic (HepG2), Kidney epithelial (LLCPK1), fibroblast and clonal pancreatic cells were employed using crude and pure fractions of the leaf. Data obtained were subjected to analytical tools such as SPSS, and Mnova software. From five solvent extracts screened, ethyl acetate extract had strongest antioxidant activity against in-vitro radicals. In-vivo LD_{50} of sub-acute toxicity on Wistar rats is 707.11mg/Kg. The cell line study revealed that 10µg/mL and above of crude leaf fraction protected pancreatic cells from cytotoxic effect of sodium arsenate (about 33% cells are viable against 50mg/mL arsenate), while on normal cells, 1.0-100µg/mL of the fraction showed improved cell viability. The fraction is less toxic on kidney epithelial cells and no effect on kidney fibroblast compared to Doxorubicin drug. Moreso, the fraction activated Pregnane X Receptor (PXR) which is responsible for CYPs mRNA genes expression for enzymes involve in xenobiotic metabolism, in concentration-dependent manner. Pure compounds isolated also showed same activation on PXR. In Conclusion, P. amarus leaf fraction act as ameliorative agent against arsenic toxicity as demonstrated through rejuvenation of cell growth due to its polyphenols, known for their membrane stabilizing activity by inhibiting ROS. It also acts as molecular ligand that activates PXR receptor. Although, it contains niranthin and cubebin dimethyl ether with same inhibitory potential as Ketoconazole which has been verified to have side effects.

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KEYWORDS: Arsenic; Cell line; In-vitro assay; Phyllanthus amarus; Pregnane X receptor

1.0 INTRODUCTION

Arsenic recently was ranked first on list of 20 hazardous substances by the Agency for Toxic Substances and Diseases Registry (John, et al. 2019). A known human carcinogen (referred to as 'king of poison') with acute toxic effect of 0.002mg/L, currently poisoning tens of millions of people worldwide (Liu, et al. 2009). Arsenic is mainly transported in the environment by water as arsenate (WHO, 2000). It is ubiquitous; exist in organic and inorganic forms, of which the inorganic form is highly toxic. Although, arsenic is used for production of insecticides, wood preservatives, feed additives, etc, these are part of the anthropogenic sources of

environmental pollution beside the natural sources (Yong-Chul, et al. 2016). Several physico-chemical techniques have been employed in a bid to solve arsenic pollution in water of which the best is iron based sorbent at pH of 6-8.5 (Sylvester and Arun, 2015). However, there is need to address its toxicity in human health with better alternatives from natural plant sources as drugs like metformin, diazepam, etc used for liver diseases have side effects. P. amarus is one of the choicest traditional herbs for the treatment of several liver diseases (Mohammed and Idris, 2018).

2.0 MATERIALS AND METHODS

Collection and Preparation of Plant Extract

The leaves were collected from Mkar community in Gboko Local Government area, Benue state, Nigeria, and were identified at the Department of Plant Biology, Bayero University, Kano with herbarium accession number BUKHAN 0278. The extraction was by maceration method for 72hours. 40g/400mL of distilled water, chloroform, acetone, hexane, methanol and ethyl acetate were prepared. Each crude extract of 20g/250mL in percentage ratio of 100% ethyl acetate, 100% chloroform, Ethyl acetate-chloroform (1:1), Chloroform-methanol (1:1) was used for extraction using silica gel chromatography. Eluents were collected for thin layer chromatography (TLC). LD_{50} was by the method of (Lorke, 1983).

Determination of Toxic Concentration of Sodium Arsenate

Pancreatic cells (BRIN-BD11) were incubated with graded concentrations of sodium arsenate (0–100 mg/mL). Lactate Dehydrogenase (LDH) release was expressed as a percentage of LDH released from control incubated in the absence of sodium arsenate. Dotted line in Figure 1 indicates the selected concentration which is 50mg/mL.

Anti-cytotoxicity and Cell Viability Assessment of P. amarus on Pancreatic (BRIN-BD11) and Kidney epithelial (LLCPK1) cell lines

This was assessed using CytoTox-96 non-radioactive cytotoxicity assay kit to measure the amount of LDH from damaged cells and (3-(4,5-Dimthylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assays for cell viability as described by Van de Loosdrecht et al. (1994). For Kidney epithelial cell line, the assessment of anti-cytotoxic activity of the plant was compared with Doxorubicin drug.

Assessment of Modulatory Activity of P. amarus on CYP3A4-PXR Microsome

CYP inhibition assays were performed using Vivid® CYP-450 kits according to instructions provided in the manual. Ketoconazole was used as positive control for CYP3A4 inhibition. IC_{50} were obtained from concentration-response curves generated by plotting log-concentration vs. percent inhibition. The effects of crude and pure compounds of P. amarus leaf sample on PXR activity were compared with a standard drug (Rifampicin) while Ketoconazole was used as inhibitory drug for CYP (CYP3A4).

Isolation and Characterization of Pure Compounds from Leaf Fraction of P. amarus.

Isolation of pure compounds from 100% ethyl acetate fraction was achieved by using several chromatographic techniques described by Bae et al. (2019).

3.0 STATISTICAL ANALYSIS OF DATA

Data were expressed as mean ± SD of triplicate assays analyzed using one-way Analysis of Variance on Statistical Package for Social Sciences (version 20). The IC_{50} were determined by log probit analysis after performing Duncan Test. GraphPad Prism 8 software was used for plotting bar charts while Mnova software was used to access NMR spectra. Values at $P<0.05$ were regarded as significant compared to appropriate standards.

4.0 RESULTS

Free radical scavenging assays were employed on five sample fractions and their IC50 determined and the ethyl acetate fractions showing the most promising activity as shown in Table 1A

Determination of LD_{50} of 100% Ethyl Acetate Fraction (E100) of P. amarus Leaf

Acute toxicity of 100% ethyl acetate fraction was measured at dose range of 100 to 5000mg/Kg.bw for 24hrs. Exposure for 10days, death was observed for dose of 1000mg/kg and above. Thus, LD_{50} of sub-acute toxicity is 707.11mg/Kg.

Effect of P. amarus Leaf Fraction (E100) on Pancreatic and Kidney Cell Lines

In Figures 2, 3 and 4 illustrated the effect of P. amarus Leaf on sodium arsenate-induced pancreatic cell toxicity. About 33% cells were viable in 50mg/mL sodium arsenate administered. The cytotoxic effect of the fraction on kidney epithelial cells and fibroblast was compared with Doxorubicin and the IC_{50} calculated as shown in Table 1B.

Isolation of Phytocompounds from Ethyl Acetate Fraction of P. amarus Leaf.

Total of six pure isolates of lignans class of phytochemicals (phyllanthin, niranthin, cubebin dimethyl ether, 5-demethroxyniranthin, hypophyllanthin, and isolintetralin) were obtained from 100% ethyl acetate fraction of the P. amarus given in Fig. 5.

Modulatory Effect of P. amarus Leaf on CYP3A4 via Pregnane X Receptor System

Luciferase reporter gene assay was employed to determine the increase in transcriptional activity of PXR. Results shows the fraction activated PXR in a concentration-dependent manner as illustrated in Fig. 6. It elicited strongest activity (>6 fold) at 20µg/mL.

Inducive Effect of Pure Compounds from Fraction of P. amarus Leaf on PX Receptor

The pure isolates were individually tested for their effect on PXR system and each showed strong activation in varying folds at highest concentration (10mg/ml) which is significantly similar to that of the standard drug (Rifampicin). See Fig 7. The IC_{50} for each compound was also compared to the standard inhibitory drug (Ketoconazole) in Table 2. Hypophyllantin and phyllantin has the least inhibitory effect on CYP3A4 gene expression.

5.0 DISCUSSION

Live is threatened by environmental toxicants from anthropogenic activities which may not be completely avoided. Hence, we are compared to seek protective remedies to their effects. The case with arsenic is a difficult one to handle because of its vase usefulness. To clean the environment of its pollution, several physical-chemical methods have been employed but to address its threat to physiology of human life is an aspect we must look into. To do this with less cost, we have to research into the nature's vegetations with vase medical potentials, a science known as herbology. Herbology is an important area in health sciences due to its enormous applications in formulation of drugs, foods, cosmetics, chemicals and supplements; all attributed to the rich heritage of phytonutrients/phytochemicals that exhibits pharmacophoric ability on bioreceptors to modulate, induce or inhibit biological effects.

Anti-cytotoxic and Cell Viability Potentials of Leaf Fraction of P. amarus on Cell lines Impregnated with Sodium Arsenate

The overall objective of this research is to examine the in-vitro effect of P. amarus leaf on key cell lines impregnated with arsenic toxicity to have a broad view of its potency in therapeutics with focus on addressing the challenge of arsenic poison. And from the cell line study, 10 to 1000µg/mL of fraction demonstrated protective effect on sodium arsenate-induced lactate dehydrogenase (LDH) release from pancreatic cells. 33% of the cells were viable against 50mg/mL sodium arsenate treated with 1000µg/mL of the fraction. On normal cells, 1.0-100µg/mL of the fraction showed improved cell viability.

In research, IC_{50} measurement provides quantitative information regarding specific dosage of drugs and their effects. The IC_{50} of P. amarus was higher to that of Doxorubicin on kidney epithelial cells and fibroblast and according to studies, the higher the IC_{50}, the lower the potency to cause toxicity; thus P. amarus is less toxic on kidney epithelial cells and has no cytotoxic effect on the kidney fibroblast. It can elicit protective effect against cytotoxicity and could also enhance in-vitro cell proliferation.

Modulatory Effect of Leaf Fraction of P. amarus on CYP3A4 via PX Receptor

It becomes interesting to know if it could act as molecular ligand with inducive effects on cytochrome gene expression. According to Sprouse and Breeman (2016), there are two major cytochrome isoforms (CYP3A4 and CYP2C9) in human hepatic cell line (HepG2) via activation of PXR. These two PXR-activated CYPs are used in more than 70% of all recognized drug-drug interaction studies. The effect of ligands as inhibitors or inducers may lead to increased drug toxicity due to decreased metabolic clearance or loss of drug efficacy due to increase metabolic clearance. Results showed that the leaf fraction activated PXR in a concentration-dependent manner. The IC_{50} of the fraction is <0.21µg/mL and that of the standard drug (Ketoconazole) is 0.008 ±0.001µM.

Effect of Pure Compounds from Leaf Fraction of P. amarus on Pregnane X Receptor

The pure compounds were individually tested for their effect on PXR and each showed strong activation at highest concentration (10mg/mL) which is significantly similar to that of the standard drug (Rifampicin). At 1.1µg/mL, Phyllantin has the best activation effect (2.775fold), followed by niranthin (2.427-fold) compared to Rifampicin (1.845fold). According to Shmizu, (2010) and Pius et al. (2017), the ability of a drug to cause more than a two-fold increase in PXR activation can significantly induce expression of CYP mRNA gene for synthesis of xenobiotic metabolizing enzymes and/or transporters, which is a causative factor for herb-drug interaction (HDI). And from the result, three (phyllanthin, niranthin, isolintetralin) out of the six isolates exhibited above 2.5fold increase at 3.3µg/mL while the crude extract was as high as 6-fold/20µg/mL. This implies that both the crude extract and the pure compounds significantly modulate the transcriptional activity of PXR signaling and may likely induce CYP3A4 mRNA gene expression; inferably phyllanthin and niranthin for the synthesis of enzymes for arsenic detoxification. This agrees with the findings of Pius et al. (2017).

More so, the IC_{50} for each compound revealed that, hypophyllantin and phyllantin has the least inhibitory effect on CYP3A4 gene expression, this suggest that, metabolic clearance activity of the CYPs may not be hampered to a level

that could lead to drug toxicity or itself exciting cytotoxicity toward HepG2 cells. However, niranthin and cubebin dimethyl ether have same inhibition with Ketoconazole. This demand careful use of the extract and particularly these isolates in terms of dose and duration because of their possible potential for adverse reactions including hepatotoxicity (US-FDA, 2017).

Nevertheless, this work could not particularly ascertain whether the level of activation of the PXR could lead to expression of the CYP3A4 mRNA genes. This can be truly verified by using real time PCR (RT-PCR) and further enzyme assays as suggested by Islam et al. (2021). In conclusion, ethyl acetate fraction of P. amarus leaf contains phytomolecules that act as molecular ligand and as antioxidants scavenging free radicals or boosting endogenous antioxidant system.

Ethical Approval

Not applicable.

6.0 CONCLUSION

In conclusion, ethyl acetate leaf fraction of Phyllanthus amarus has ameliorative and therapeutic effects on arsenate toxicity.

7.0 RECOMMENDATIONS

The plant extract has some active compounds that could be used as a model for drug design against toxicity elicited by injecting arsenic through drinking water or any other possible ways. Therefore, it can be recommended as diet in form of tea or vegetable especially by people living in smelt areas and places noted for high arsenic poison. However, it contains Niranthin and Cubebin dimethyl ether with same inhibitory potential as Ketoconazole which has been verified to have side effects.

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Table 1A Inhibitory Concentration of Fractions of P. amarus Leaf on Different Radicals

(µg/mL) Sample Fraction	DPPH	ABTS	NO^-	FRAP	OH^-
Ethyl Acetate	22.08±0.04[b]	31.69±0.37[b]	54.29±0.07[b]	50.21±0.07[b]	60.39±0.10[b]
Ethyl-chloroform	141.39±0.04[a]	125.64±0.49[a]	36.72±0.03[a]	73.56±0.16[a]	91.54±0.16[a]
Chloroform	118.85±0.13[c]	252.70±0.36[c]	53.98±0.10[b]	122.24±0.14[c]	90.30±0.31[e]
Chloro-methanol	136.21±0.04[d]	128.88±0.49[d]	52.70±0.40[c]	115.48±0.14[f]	90.58±0.30[e]
Methanol	130.88±0.04[e]	135.18±0.08[e]	50.05±0.80[d]	71.10±0.01[e]	92.86±0.11[c]
Vitamin C (standard)	55.41±0.21[f]	111.70±0.18[f]	42.12±0.10[f]	66.72±0.22[g]	74.36±0.16[d]

Values are expressed as mean ±SD (n=3). Values in the same column having different superscripts differ significantly at P<0.05.

Table 1B. Cytotoxic Effect of Fraction of P. amarus Leaf on Kidney Cell Lines

SAMPLE	LLCPK1	VERO
Inhibitory Concentration (µg/mL)		
P. amarus	47.0±0.02	NC
Doxorubicin	1.8±0.04	5.0±0.01

Values are expressed in Mean ± SE (n=3). **Key:** LLCPK1-Kidney epithelial cells; VERO- Kidney fibroblast; NC- No cytotoxic effect

Table 2. IC_{50} of Pure Compounds from Fraction of P. amarus Leaf on CYP3A4 Gene

S/N	Sample Code	Name of Compound	IC50µg/mL
1	BA-1-53-8-Pa	Phyllantin	1.1±0.05
2	BA-1-53-1-Pa	Niranthin	0.03±0.02
3	BA-1-64-2-Pa	Cubebin dimethyl ether	0.05±0.01
4	BA-1-56-3-Pa	Isolintetralin	0.39±0.08

5	BA-1-59-1-Pa	5-demethroxyniranthin	0.12±0.03	
6	BA-1-59-3-Pa	Hypophyllanthin		3.6±0.40
7	Standard Drug(μM)	Ketoconazole	0.01±0.01	

Values are expressed in Mean± SD (n=3)

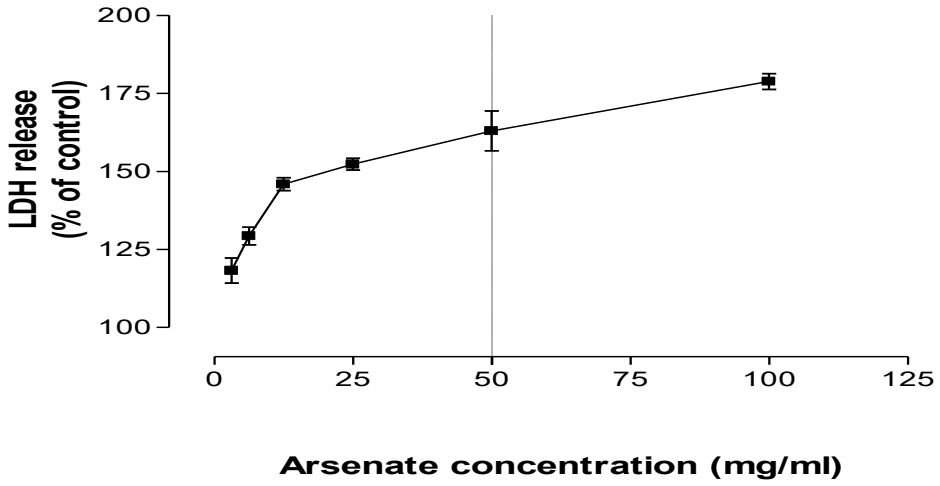

Fig.1 Effective Toxic Concentration of Sodium Arsenate on Clonal Pancreatic Cell Line

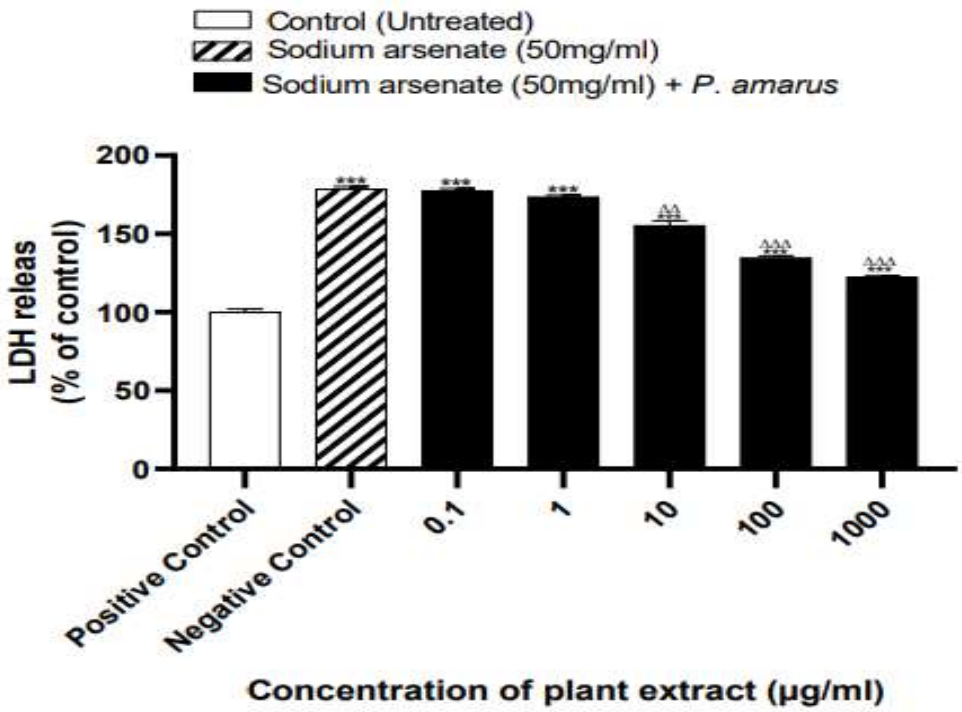

Fig. 2. Effect of Ethyl Acetate Fraction of Phyllanthus amarus Leaf on Sodium Arsenate-induced LDH release from Clonal Pancreatic Cells (BRIN-BD11) by non-radioactive Cytotox-96 LDH assay.

Values are mean ±SEM with n = 4. ***P<0.001 compared with untreated cells. ΔΔΔP<0.001, ΔΔP<0.01 compared with cell treated with sodium arsenate (50mg/ml) alone.

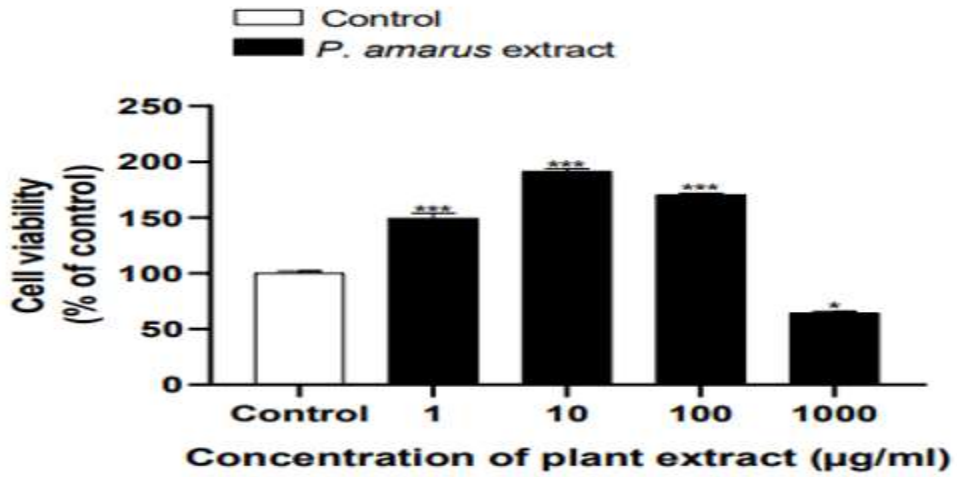

Fig. 3. Effect of Ethyl Acetate Fraction of Phyllanthus amarus Leaf on viability of Pancreatic Cells (BRIN-BD11) by MTT assay.

Values are mean ±SEM with n = 4. ***P<0.001 compared with control incubation using media alone.

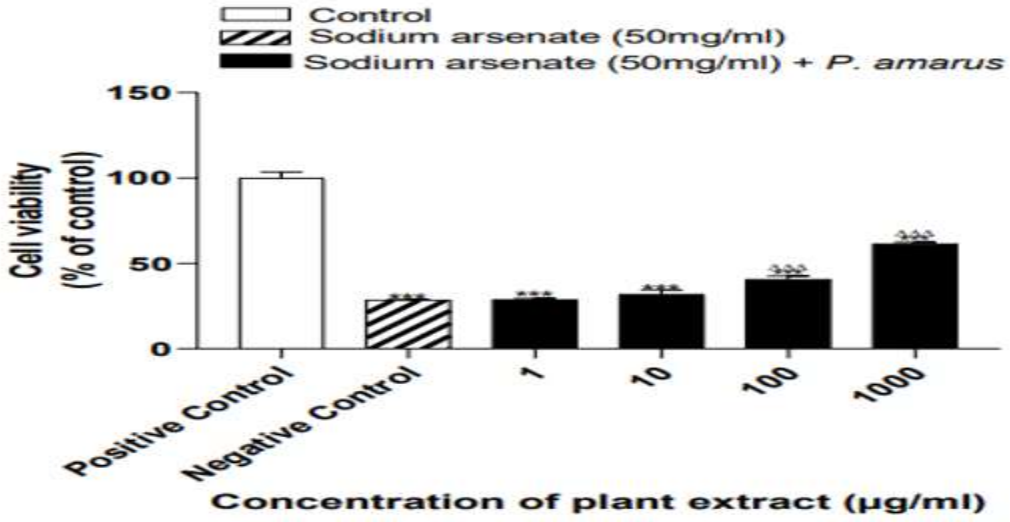

Fig. 4. Effect of Ethyl Acetate Fraction of Phyllanthus amarus Leaf on Arsenate-induced Cell Death in Pancreatic Cells (BRIN-BD11) by MTT assay.

Values are mean ±SEM with n = 4. ***P<0.001 compared with control incubation using media alone. $^{\Delta\Delta\Delta}$P<0.001 compared with incubation performed with sodium arsenate alone.

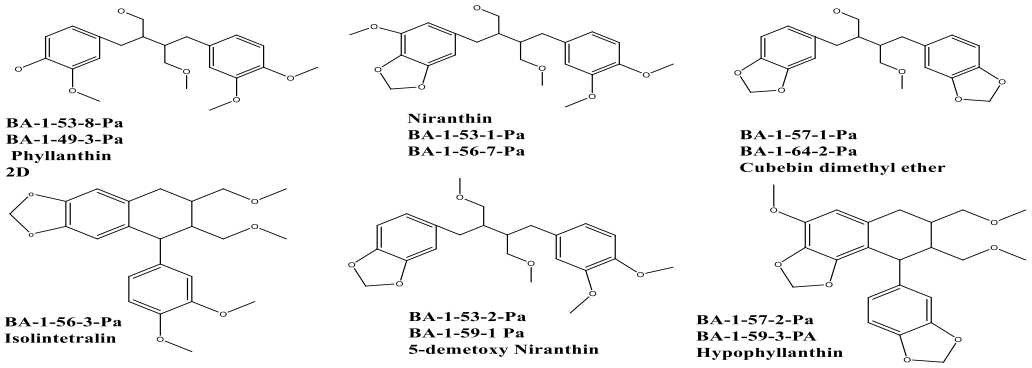

Fig. 5. Structures of Pure Compounds Isolated from 100% Ethyl Acetate Fraction of P. amarus Leaf by NMR Technique.

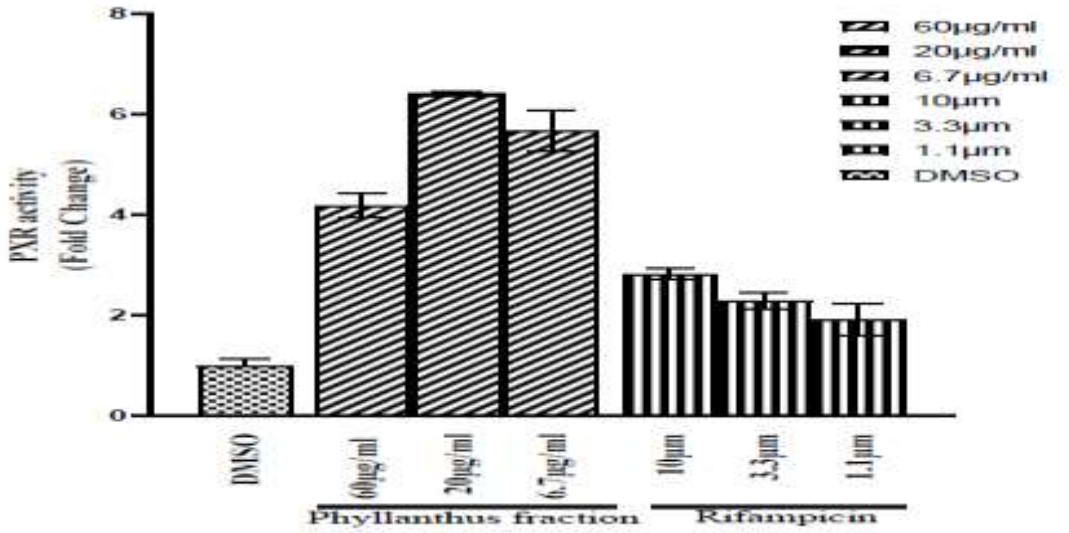

Fig.6. Effect of P. amarus and Rifampicin on PXR Activity

The data was represented as Mean±SE (n=3). Statistical significance is shown compared to DMSO as control at P<0.05.

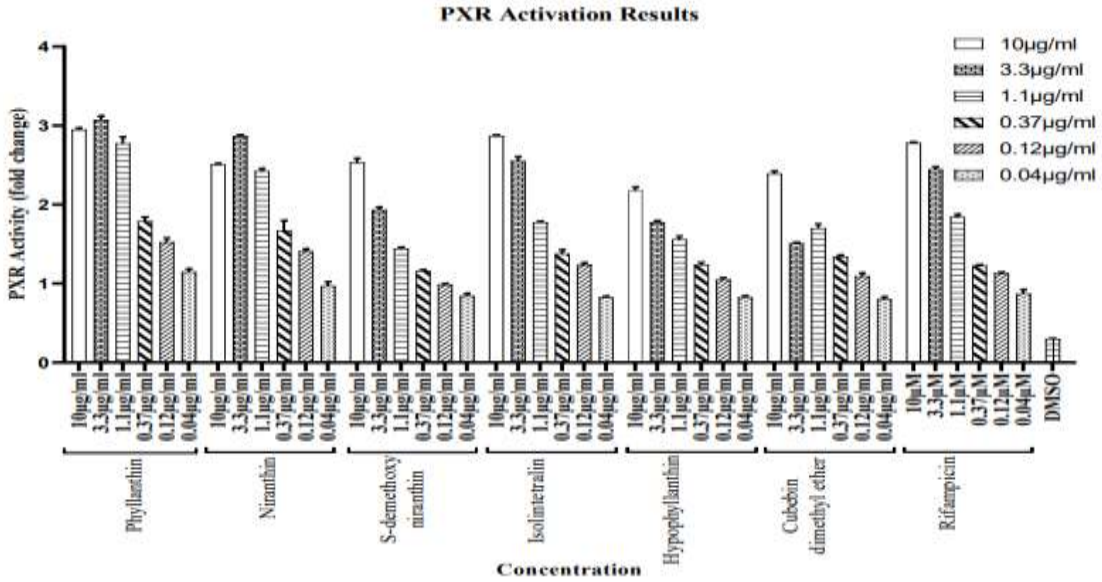

Fig 7. Effect of Pure Compounds Isolated from Ethyl Acetate Fraction of P. amarus Leaf on Pregnane X Receptor Activity.

Abbreviation:

CYPs- Cytochrome P450
HepG2- Hepatocellular carcinoma
LD_{50} – Lethal dose
LDH-Lactate Dehydrogenase
LLCPK1- Kidney cell line for study of kidney function and transport
mRNA- messenger Ribonucleic acid
MTT - (3-(4,5-Dimthylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PXR- Pregnane X Receptor
ROS- Reactive oxygen species
RT-PCR- Real time-Polymerase Chain Reaction

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