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Evaluation of Phytochemical, Antimicrobial and Toxicity Studies of Ethanolic Stem Extract of *Phyllanthus Amarus* Thonn and Schum (Euphorbiacea)

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Abstract:
This study investigated the phytochemical composition, antimicrobial activities and acute toxicity studies of ethanolic stem extract of Phyllanthus amarus. The Phytochemical analysis revealed the presence of saponins, tannins, flavonoids, terpenoids, alkaloids, cardiac glycosides, anthroquinones, steroids and carbohydrates. Antimicrobial sensitivity test using eleven human pathogenic microorganisms across various concentrations (200-1600mg/ml) showed that P. amarus has bactericidal effect on five pathogens namely; Klebsiella pneumonia, salmonella typhii, Shigella dysentrea, Streptococcus pyogene, and Candida albicans all at 100 mg/ml except Streptococcus pyogene which is 200 mg/ml. The extract also demonstrated significant inhibitory effect on Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhii, Candida albicans and Klebsiella pneumonia with Salmonella typhii having the highest zone of inhibition of 20 mm which is even higher than standard drug (Tetracycline) with only 18 mm while the least zone of inhibition at the same concentration (1600 mg/ml) is 12 mm for Streptococcus pyogene. The result of acute toxicity of CEE of P.amarus on the wistar albino rats shows that the LD_{50} is calculated to be 1720 mg/kg using arithematic method of Karbar 1931 as modified by Aliu and Nwude1982 which translates that the plant is safe for human consumption.

Keywords: *Phyllanthus amarus, phytochemical screening, antimicrobial activity, acute toxicity, ethanol extract*

1. Introduction

The prevalence of multi-drug resistant pathogens has continue to threaten the clinical efficacy of many existing antibiotic drugs leading to extensive investigation of medicinal plants for potential antimicrobial activity in recent years (Oyewole *et al*., 2013). Some plants have been identified to contain medicinal constituents which have potentially significant therapeutic applications against human pathogens including bacteria, fungi and viruses (Okigbo and Omodamiro, 2006, Oyewole *et al*., 2013).

Plants with their complex chemical storehouse of biodynamic compounds serve as plant defence mechanisms against inversion by microorganisms and insects and can provide valuable sources of natural antibacterial agents (Abel and Busia, 2005, Roosita *et al*., 2008). The active principles isolated from plants appear to be one of the important alternatives, when compared to many sub-standard orthodox synthetic medicines, because of their less or no side effects and better bio-availability (Scazzochio *et al*., 2001, Joshi *et al*., 2011).

Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very rich botanical wealth and a large number of diversity types of plants grow in different parts of the country. Nigeria is rich in all the three levels of biodiversity, namely species diversity, genetic diversity and habitat diversity. In Nigeria thousands of species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times. Herbal medicine is still the mainstay of about 75-80% of the whole population, and the major part of traditional therapy involves the use of plant extract and their active constituents (Akerele, 1993).

Antimicrobials of plants origin have an extremely large therapeutical potential. They are effective in the treatment of infectious diseases, while simultaneously alleviating many of the side effects that are often connected with synthetic antimicrobials (Iwu *et al*., 1999, Mamza *et al*., 2013).

Medicinal plants have the richest bioresource of drugs of traditional systems of medicine, modern medicine, neutraceuticals, intermediates and chemical entities for synthetic drugs (Harmmer *et al*., 1999, Joshi *et al*., 2011, Mamza *et al*., 2013). The phytochemicals have made significant contribution in maintaining human health. The significant of drugs derived from plants cannot be over emphasized with the recent trend of high percentage of resistance of microorganisms to the present day antibiotics (Ibekwe *et al*., 2000, Oluwafemi and Debiri, 2008).

Phyllanthus amarus (Thonn and Schum) is one of the important medicinal plants belonging to Euphorbiacea commonly known as "stone breaker". The plant is locally called gyeron tsuntsaye (Hausa), eyin olobe (Yoruba), ngwu (Igbo) and shaffa pitu (Marghi) all in Nigerian Languages (Mamza *et al*., 2013, Mamza *et al*., 2012). *P. amarus* is a herb common to central and southern India and can grow to 30-60cm height. It is found in forest areas, arid lands, savannah areas, leached or exhausted soil in many countries including China, India, Nigeria, Cuba and the Philipines among others (Mamza *et al*., 2012, Bharatiya, 1992, Akah and Nwambie, 1994, Burkill, 1994).

Phyllanthus amarus has a long history of usage by people, because of its rich medicinal values. It has been reported to posses potent anti-inflammatory (Mahat and Patil, 2007), antihepatotoxic (Kumar *et al*., 2007), antispasmodic, antiviral, antiamnesic (Joshi, 2007), antilithic, analgesic, hypotensive, dieuretic, antimutagenic and hypoglycemic properties (Kiran *et al*., 2011, Thyagarayan *et al*., 1998). This plant is used in the treatment of diarrhoea in the traditional medicine system in the tropical countries and it is routinely prescribed as an antidiarrhoeal and antimicrobial drug in Nigerian traditional medicine (Mamza *et al*., 2014, Mamza *et al*., 2013, Oluwafemi and Debiri, 2008, Sen and Batra, 2012). *P amarus* has also been reported to offer good treatment for leprosy, hiccup, peptic ulcer, asthma, good laxative, typhoid fever, kidney stones, jaundice, malaria, dysentery, gonorrhea, astringent, detoxifier, antiviral, antifungal among others (Akinjogula *et al*.,2010, Idowu *et al*., 2009, Sharma,2011, Ahmad and Beg, 2001, Mamza *et al*., 2013). Previous findings have revealed that extracts from different parts of *P.amarus* demonstrated antioxidant, anti-inflammatory, hypocholestrolemic, anti-carcinogenic and anti-HIV potential (Adeneye *et al*., 2006, Clardy and Walsh, 2004, Ogueke *et al*., 2007, Harikumar and Kuttan, 2007, Oyewole *et al*., 2013).

Despite the fact that a number of Laboratory and Clinical studies have been conducted on the therapeutic efficacy of this plant there is no reported toxicity investigation on it especially *P.amarus* growing in Nigeria. We therefore intend to determine the major phytochemicals present in this plant as well as evaluating its antimicrobial efficacy. Bio-safety of this plant species growing in Nigeria will also be ascertained since plant composition of the same species may vary in different geographical location.

2. Materials And Methods

2.1. Collection of Plant Material and Identification

The stem of *P. amarus* used for this study were freshly collected from Suleimanti in Jere Local Government Area of Borno State Nigeria in August 2011. The plant material was identified and authenticated taxonomically by Prof. S.S. Sanusi at the Department of Biological Sciences, University of Maiduguri. A voucher specimen (1360) of the collected sample was deposited in the Postgraduate Research Laboratory, Department of Chemistry, University of Maiduguri for future reference.

2.2. Preparation and Extraction of Plant Material

The stem of *P. amarus* were washed three times with tap water and rinsed with distilled water to removed dirt and shade dried. The dried materials were powdered and extracted exhaustively with petroleum ether to remove the fatty materials and the marc was further extracted with 99% ethanol using soxhlet method. The crude extract obtained was concentrated to dryness between 40^{o}c to 45^{o}c using water bath and extract coded CEE-crude ethanolic extract (CEE:yield 29.23%,w/w) and this were used for further studies.

2.3. Phytochemical Screening

The ethanol extract (CEE) was screened for phytochemical constituents using standard procedures of analysis (Harborn, 1993; Sofowora, 2008 and Trease and Evans, 2000).

2.4. Test Microorganisms

A total of eleven microorganisms were used in this study: four Gram negative bacteria (*Escherichia coli, Salmonella typhii, Klebsiella pneumonia and Shigella dysentriae*), four Gram positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Corynbacterium spp and Bacillus cereus*) and three fungal strains (*Candida albicans, Penicillium spp and Aspergillus niger*). These organisms are clinical isolates obtained from the Department of Medical Microbiology, University of Maiduguri Teaching Hospital (UMTH), Maiduguri, Nigeria. The microorganisms were obtained as pure cultures on agar plates after biochemical test. The bacteria were confirmed for their identity using biochemical tests with 24hrs broth culture (Bello, 2000). The fungi were identified using the germ tube tests with lactophenol cotton blue stain (Cheesbrough, 2004). The standard susceptibility antibiotic disc used was tetracycline (2.5×10^{5} mg/disc) which was prepared in the Laboratory from 250µg tetracycline capsule.

3. Antimicrobial Investigation

Antimicrobial sensitivity testing was carried out on the various concentrations of the extract as well as tetracycline, a standard antibiotic using the broth dilution assay procedure (Nostro *et al*., 2000) and their zones of inhibition measured. A cloudy solution (turbidity) indicated the presence of the pathogen in each solution. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract was determined for sensitive pathogens following standard method (Cheesbrough, 2004).

4. Experimental Animals

Sixty wistar strain albino rats of both sexes (average weight 120g) were used for these studies. They were obtained from the National Research Institute Vom, Plateau state Nigeria. The rats were kept in well ventilated plastic cages under standard conditions of temperature (25±2^{o}C) and light approximately 12/12 hr (light/dark cycle), humidity 65±5% in the Department of Physiology Pharmacology and Biochemistry Laboratory, Faculty of Veterinary Medicine, University of Maiduguri, Nigeria for two weeks to acclimatized before the commencement of the experiment.

5. Acute Toxicity Analysis

The acute toxicity (LD_{50}) values of the CEE *P. amarus* were determined using conventional procedure as described by Karbar 1931 as modified by Aliu and Nwade (1982). In this study, two different routes of administration were considered, that is oral and intraperitoneol. The rats were sorted into six groups (A, B, C, D, E and F) of five rats each. Rats in group A served as the control and were administered with normal saline (2ml/kg), while groups B, C, D, E and F were administered intraperitoneolly with graded doses (200mg/kg, 400mg/kg, 800mg/kg,1600mg/kg and 3200mg/kg) of the ethanolic extract of *p. amarus* respectively. The rats were allowed free access to food and water *ad libitum* and they were observed for 24hr for clinical signs of toxicity or death. Postmortem findings were also carried out. For the oral route, the same protocol was observed. The LD_{50} with 95% confidence for crude ethanol extract for both *i.p* and *po* were calculated and recorded using the relation:

$LD_{50} = LD_{100} - (Md*Dd)/n$

Where Md is the mean death of rats and Dd is dose difference and n is the number of rats in each group.

6. Results

The preliminary phytochemical screening of the CEE of *P. amarus* leaves is shown in Table 1. This reveals the presence of flavonoids, tannins, saponins, cardiac glycosides, terpenoids, alkaloids, anthraquinones and steroids while phylobatannins are absent.

S/No	Phytochemicals	Test	Result	Observation
1	Alkaloids	(i) Dragendorff's Test	+	Orange-red
		(ii)Mayer's test	-	No ppt formed
2	Cardiac	(i) Keller-Killiani's Test	+	Greenish color
	Glycosides	(ii) Liebermann-Burchard Test	+	Bluish green
		(iii)Salkowski Test	+	Reddish brown
3	Flavonoids	(i) Ferric chloride Test	+	Bluish green
		(ii)Lead Ethanoate Test	+	Buff color ppt.
		(iii) Shinada's Test	+	Light pink color
		(iv) Sodium Hydroxide Test	-	Yellow color
4	Anthroquinones	(i) Free anthroquinone Test	-	No color Formed
		(ii) Combined anthroquinone Test	+	Violet color Formed
5	Tannins	Ferric chloride Test	+	Deep blue black
6	Saponins	Froth Test	+	Foam formed
7	Phlobatanins	Hydrochloric acid Test	-	No color change
8	Terpenenoids	General Test	+	ppt. formed
9	Steroids	Liebermann-Burchard Test	+	Bluish green
10	Carbohydrates	(i) General Test (Molisch's Test)	+	purple color
		(ii)Monosaccharides(Barfoed's Test)	+	Brick red ppt.
		(iii)Free Reducing sugar (Fehling test)	+	Deep Brick red
		(iv)Combined Reducing Sugar	+	Deep brick red
		(v)Ketoses (Resorcinol or Selivanoff's Test)	+	Deep rose color

Table 1: Phytochemical Screening of Crude Ethanolic Stem Extract (CEE) of P. amarus

Key:

+ =Present

- = Absent

The results of antimicrobial sensitivity tests of CEE *P. amarus* stem against eleven human pathogens compared with tetracycline (standard drug) is shown in Table 2. It shows the susceptibility of Gram positive and Gram negative organisms and a fungal strains *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumonia,Shigella dysentiea, Salmonella typhii and Candida albicans* respectively. The other pathogens; *Escharihia coli, Corynebacterium spp., Bacillus cereus, Arspergillus niger* and *Penicillium spp.* were not inhibited by the extract at all. Tetracycline antibiotics inhibited all the eight pathogens except for the three which was not tested.

Tested pathogens	**Zones of inhibition (mm)**				
	Concentration of CEE (mg/ml)				**Tetracycline (standard)**
	1600	**800**	**400**	**200 (mm)**	**(250mg)***
Salmonella typhii (-)	20	14	10	9	18
Escherichia coli (-)	R	R	R	R	25
Shigella dysentriae (-)	18	15	12	10	18
Klebsiella pneumonia (-)	15	12	11	10	19
Staphylococcus aureus (+)	12	10	8	R	25
Streptococcus pyogene (+)	18	14	10	9	25
Corynebacteria species (+)	R	R	R	R	20
Bacillus cereus (+)	R	R	R	R	NT
Aspergillus niger (FF)	R	R	R	R	NT
Candida albicans (Y)	15	13	11	10	24
Penicillium species (FF)	R	R	R	R	NT

Table 2: In-vitro Antimicrobial Sensitivity Test of CEE of P. amarus Stem on Eleven Human Pathogens Compared with Tetracycline

Key:
CEE = Crude Ethanolic Extract
R = Resistant (i.e. not sensitive)
+ = Gram +ve
- = Gram –ve
* = standard drug (2.5×10^5µg/disc)
Y = Yeast
FF = Filamentous fungus
NT = Not tested

The result of the minimum inhibitory concentration (MIC) assay is presented in Table 3. It revealed the concentrations of the extract which can inhibit the growth of the bacteria or fungal species (bacteriostatic and fungistatic concentrations). *Klebseilla pnemoniae, Shigella dysentriea, Salmonella typhii, Streptococcus pyogene* and *Candida albicans* all had the MIC of 50 mg/ml except *Streptococcus pyogene* which is at 100 mg/ml.

Pathogens	**Extract Concentration (mg/ml)**				
	12.5	25	50	100	200
Klebsiella pneumoniae	+	+	β	-	-
Shigella dysentriea	+	+	β	-	-
Streptococcus pyogene	+	+	+	β	-
Candida albicans	+	+	β	-	-
Salmonella typhii	+	+	β	-	-

Table 3: Minimum Inhibitory Concentration (MIC) Values for Human Pathogens Isolates Against Ethanolic Extract of Phyllanthus amarus Stem

Key:
+ = Resistant (growth of bacteria or turbidity)
- = Concentrations showing no turbidity (inhibition of bacterial growth)
β = Least concentration showing no turbidity (MIC)

The result of the minimum bactericidal/fungicidal concentration assay is shown in Table 4. *Klebseilla pneumonia, Shigella dysentriea* and *Candida albicans* had moderate bactericidal/fungicidal effect with MBC values of 100mg/ml while *Streptococcus pyogene* had low bactericidal effect with MBC value of 200mg/ml.

Pathogens	Extract Concentration (mg/ml)				
	12.5	25	50	100	200
Klebsiella pneumoniae	+	+	+	β	-
Shigella dysentriea	+	+	+	β	-
Streptococcus pyogene	+	+	+	+	β
Candida albicans	+	+	+	β	-

Table 4: Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) Values for Human Pathogens Isolates Against Ethanolic Extract of Phyllanthus amarus Stem

Key:
+ = Resistance (growth of bacteria)
- =bactericidal Concentrations
β =Minimum Bactericidal concentration (MBC)

The results for acute toxicity test (LD_{50}) of ethanolic stem extract of *P. amarus* both *i.p* and *p.o* are presented in Tables 5 and 6 respectively.

Group (n)	Extract/dose (mg/kg)	Number of death	Percentage mortality (%)
A	**-ve** Control	0	0
B	200	0	0
C	400	0	0
D	800	1	20
E	1600	3	60
F	3200	4	80

Table 5: Acute Toxicity in Rats given Ethanolic Stem Extract i.p. at different Doses

Key:
-ve control=Rats given normal saline orally
n= number of rats in each group (5)

Group (n)	Extract/dose (mg/kg)	Number of death	Percentage mortality (%)
A	**-ve** Control	0	0
B	200	0	0
C	400	0	0
D	800	0	0
E	1600	0	0
F	3200	0	0

Table 6: Acute toxicity in Rats given ethanolic Stem Extract orally at different Doses

Key:
-ve control=Rats given normal saline orally
n= number of rats in each group (5)

7. Discussion

Results obtained in this study showed that CEE of *Phyllanthus amarus* contain at least eight important bioactive components of medicinal plants namely saponins, alkaloids, tannins, flavonoids, cardiac glycosides, terpenoids, anthroquinones and steroids. The study also reveals the presence of carbohydrates (reducing sugars and combined sugars) (Table 1).

The extract also showed antimicrobial effect as it exhibited bactericidal and inhibitory activities against human pathogens with zones of inhibition of comparable to that of tetracycline (standard drug). This antimicrobial effect could be attributed to the bioactive phytochemicals present in the plant. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives. These secondary metabolites exert antimicrobial activity through different mechanisms. Tannins form irreversible complexes with proline rich protein in bacteria, resulting in the inhibition of cell protein synthesis. Flavonoids disrupt microbial cell wall by forming complex with extracellular soluble proteins in the bacteria (Oluwosulu and Ibrahim 2006; Mamza *et al*., 2012; Mamza *et al*., 2014). Saponins posses membrane permeabilizing properties inhibits histamine release *in vitro* and leads to vacuolization and disintegration of teguments. Alkaloids produce analgesic, inflammatory and

adaptogenic effects which help to develop resistance against diseases and endurance against stress (Gupta, 1994; Sodipo *et al*., 2012). Cardiac glycosides are known to have laxative, diuretic and antiseptic properties (Robinson, 1969, Frantisek, 1991). Carbohydrates in this extract occupy an important position in metabolism, so the method for their detection is useful in phytochemistry. Carbohydrate have no therapeutic actions, but they possibly increase the effectiveness of the biologically active principles in the plant, thus most therapeutic principles isolated from plants occur in combination with sugar as glycosides (Iwu,1984). The combined effects (synergistic effect) of these phytochemicals are not unconnected to the broad spectrum activity exhibited by this extract. The current investigation confirms previous records that the plant has antimicrobial properties on certain bacterial species (Gill, 1992, Oyewole *et al.,* 2013).

The result of acute toxicity of CEE of *P.amarus* on the wistar albino rats shows that the LD_{50} is calculated to be 1720mg/kg (*i.p*) using arithmetic method of Karbar 1931 as modified by Aliu and Nwude1982. According to Clark and Clark, 1977, any substance whose *intraperitoneal* LD_{50} in rats, falls between 50 and 500mg/kg is regarded as toxic and between 500 and 1000mg/kg is moderately toxic. But any substance whose LD_{50} value is more than 1000mg/kg is considered as non toxic. Therefore, considering the LD_{50} values obtained in this study (1720mg/kg), it clearly shows that this plant is not toxic. This result agrees with the previous result of several researchers (Odetola and Akojenu, 2000, Adeneye, 2006, Akinjogula *et al.,* 2010) that when the LD_{50} value is more than 1000mg/kg it is considered to be non-toxic. The oral route of administration of the extract also confirms that the plant is safe since there is no mortality rate recorded.

8. Conclusion

The phytochemical analysis has revealed the presence of cardiac glycosides, flavonoids, tannins, saponins, alkaloids, anthroquinones, terpenoids and steroids, some of which have associated with antibacterial properties. The result of the *in vitro* antibacterial studies shows that the extract possesses antibacterial and antifungal properties. This is evident from the results of susceptibility test assay, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC). From this there is validity for the folkoric usage of the plant in the treatment of ailment caused by these pathogenic microorganisms. This suggests the treatment of bacterial and fungal infections.

From the toxicity study, it also shows that the use of *P. amarus* is not toxic. We hereby conclude that the use of *P.amarus* as herbal remedy for the treatment of various ailments is safe.

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