Prophylactic and Combination Study on *Phyllanthus amarus* and *Diodia scandens* Bioactive Fractions against Antibiotic Resistant *Staphylococci*

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Abstract: Since centuries ago till date, medicinal plants remain relevant for curing many human infections and diseases due to the many enriched therapeutic and bioactive compounds present in plants. The age-long tradition of drug discovery has always been aided by natural products from plants; which provide the basic elements for drug discovery. This research aims at evaluating the prophylactic effects of the combined extracts of *P. amarus* and *D. scandens* and the bioautography of both plants. Methanolic, n-hexane and ethyl-acetate extracts of the plants were obtained using soxhlet extractor, and the bioactive fractions of both plants were determined using thin layer chromatography. The prophylactic effects of the combined extracts were evaluated on Swiss albino rats infected with antibiotic-resistant coagulase negative *Staphylococcus* strains. The n-hexane yielded more bioactive compounds in *D. scandens* by producing six (6) active bands with retention factor values of 0.07, 0.12, 0.62, 0.68, 0.72 and 0.78, while the cold ethyl-acetate extract yielded more bioactive compounds in *P. amarus* by producing eight (8) active bands with retention factor (R_f) values of 0.096, 0.137, 0.178, 0.260, 0.603, 0.644, 0.767, and 0.838. The experimental mice pre-treated with the combined plant extract showed no sign or symptoms of infection while the uninfected control showed varied signs of infection such as weakness, inflammation, redness of skin and eyes, etc. An elevation of activity or aggression level was also observed only on the pre-treated mice. The least effective dose of 25 mg/kg showed that the prophylactic effects of the combined extracts was more potent than when either plant was used separately. *Phyllanthus amarus* and *D. scandens* possess bioactive compounds which have excellent antibacterial potentials, and the antibacterial efficacy of either plant can be boosted and improved by combining both plants.

Key word: Medicinal plants, bioautography, prophylaxis, combined extracts, antibacterial

INTRODUCTION

Throughout the history of drug discovery and development, natural products have always provided the basic elements for discovery of drugs; which are used for treatment and prevention of infections. Like in many developing countries, new drugs are often not affordable in Nigeria. Approximately 80% of the world's population still relies on traditional medicines as remedies for the treatment of common illnesses (Woo *et al.*, 2012; WHO, 2022). Yuan *et al.* (2016) opined that there is an immense advantage attached to using medicinal plants to cure infections; and despite series of researches that have been conducted, scientists are just beginning to scratch on the surface the great deposit of antimicrobial relevance of the numerous plants in our ecosystem. Medicinal plants, due to their incredible diversity of unique active components and biological activities produce drugs of astounding therapeutic advantages (Sam, 2019). Medicinal plants have been used for centuries as remedies for human diseases because they contain numerous components of therapeutic value (Kavita *et al.*, 2022). In recent years, secondary plant metabolites (phytochemicals) have been extensively investigated as a source of medicinal agents. Phytochemicals with good antimicrobial activity have been reported to be very effective in the treatment of bacterial, fungal, and viral infections (Njeru and Muema, 2020).

The *Phyllanthus* genus belonging to the Euphorbiaceae family was first identified in Central and Southern India in 18th century, but is now found in many countries including Philippines, China, Cuba and Nigeria, among others (Bekoe *et al.*, 2020).

P amarus is locally called Eyinolobe (Yoruba, South-west Nigeria), English (stone breaker or kidney stone plant), Igbo (ngwu) (Ogunmoyole *et al.,* 2020). In a number of countries, the aerial part of *Phyllanthus amarus* is highly valued in traditional medicine for its healing properties. This plant is traditionally used around the world in the treatment of liver ailments and kidney stones. *Phyllanthus amarus* is also known to work as an antifungal, antibacterial and antiviral agent (Ogu *et al.,* 2011). Also, *Diodia scadens* has reportedly been used in curing various ailments such as dysentery, diarrhoea, asthma, convulsion, epilepsy, oedema, gout, swelling and it is said to possess anti-abortifacient, antidotes, antimicrobial, anti-inflammatory properties in Nigeria and other countries (Wada *et al.,* 2022).

Bioautography is a means of target-directed isolation of active molecules on chromatogram. It offers rapid and easy identification of bioactive lead in complex matrices of plant extracts. Thin layer chromatography bioautography is used to isolate and evaluate active components of natural compounds and it is a very useful research tool due to its cost effectiveness and high specificity (Wang *et al.,* 2021). It is an effective and inexpensive technique for the phytochemical analysis of plant extracts to identify bioactive compounds (Hostettmann *et al*., 2015). This study aimed at evaluating the synergistic effect of *D. scandens* and *P. amarus* bioactive fractions as prophylactic agents.

MATERIALS AND METHODS

Collection of test organisms and plant materials: Referenced pure culture antibiotic resistant Coagulase Negative *Staphylococcus* (CoNS 10b: Ojo) and *Staphylococcus aureus* (W241: Ojo) strain were obtained from the Drug Discovery and Infectious Diseases Research Group, Department of Microbiology, Federal University, Oye-Ekiti, Ekiti State, Nigeria. The test strains were cultured and sub-cultured on Mannitol salt agar and nutrient agar respectively with incubation at 35^0C for 24 hours (Ojo *et al*., 2013).

The extracts of whole plant of *Phyllanthus amarus* and *Diodia scandens* were obtained from the Department of Microbiology, Federal University, Oye-Ekiti, Ekiti State, Nigeria.

Determination of Bioactive Fractions using thin layer chromatography (TLC): Thin layer chromatography was performed on a preparative silica gel glass plates (200x200, 60 F254, Merck) to fractionate, active components of the plant extracts. The TLC plates were cut into 9.7x6.6 cm sizes and dried in an oven at 90^0C for 10 minutes. This is to activate the TLC plates by absorbing the moisture content from the plate. The plant extract was reconstituted in chloroform and a 5 µl of extract was spotted at about 1cm apart and away from the bottom of the TLC plates. Thereafter, the plates were placed in ascending direction in a tightly enclosed jar with the different mobile system has developed in (Table 1). The plates were visualized under the iodine fume in an enclosed chamber, the separated spots were marked and the retention factor (R_f) values were calculated (Gujjeti and Mamidala, 2013; Kumar *et al*., 2013; Okwu *et al*., 2015).

Antibacterial Assay of bioactive bands using bioautography: Bioautography as described by Balouiri *et al*. (2016) was done using agar overlay method; inoculums were prepared by suspending the bacteria in Mueller Hinton broth with an approximate concentration of 10^8 cfu/ml just before applying the overlay. The TLC plates were placed in sterile Petri dishes and covered with 4.5ml of seeded molten agar. The agar overlay plates were pre-incubated at room temperature and further incubation for 24 hours at 35^0C. After incubation, the plates were sprayed with 1.0 g/ml of aqueous solution of tetrazolium salt. The sprayed plates were re-incubated for 1h at 35°C, clear zones on the chromatograms indicating inhibition of growth were observed.

Animal Study: Twelve (12) male and female Swiss albino rats, free from contaminating

organisms, weighing 150-165 g were used in the study. They were obtained from the Animal House at Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria. They were grouped into three with four rats per group and a control group, and then kept in a polycarbonated metabolic cage at ambient environmental condition of about 25°C and 50-60% relative humidity. The experimental animals were fed on a standard rodent's diet (growers' mash) consisting of crude protein, fat, calcium, available phosphate, vitamins, crude fibre and constant supply of water. They were also subjected to light and dark cycles of 12 hours respectively and they were allowed to acclimatize for seven days (Eweka and Enogieru, 2011).

Prophylactic treatment: Each mouse in group 1 to group 3 were intubated orally for 4 days after acclimatization, the mouse in group 1 were administered with 0.5 ml of sterile extract of 25 mg/kg dose of ethyl-acetate extract of *Phyllanthus amarus* and *Diodia scandens*. Also the mouse in group 2 were administered with 0.5 ml sterile extract of 50 mg/kg dose of ethyl-acetate extract of *Phyllanthus amarus* and *Diodia scandens* was administered orally into the mouth of the rats. The group 3 which serves as the control were administered with the mixture of 2.5 ml 1% DMSO (Dimethyl sulphoxide), in addition to 15 ml of distilled water for seven days before incubating with the test organisms. Their weights were taken every other day for the 7 days of the experiment (Yao, *et al.*, 2020).

Group A: Infected rats treated with ethyl-acetate extract of *Phyllanthus amarus* and *Diodia scandens* at the concentration of 25 mg/kg (12.5 mg/kg each)

Group B: Infected rats treated with ethyl-acetate extract of *Phyllanthus amarus* and *Diodia scandens* at the concentration of 50 mg/kg (25 mg/kg each)

Group C: Rats not infected but treated with DMSO and distilled water (Control).

Organism challenge test: On the 7th day of the pre-treatment with the administration of the sterile extract, each group was challenged with 0.5 ml of $1x10^8$ cfu in 0.5 McFarland standard of the coagulase negative *Staphylococcus* in Mueller-Hinton broth. The control group was challenged with the test organism after injection with 0.5 ml of the mixture of sterile distilled water and DMSO. The protection offered by the compound was determined by recording the mortality rate of rats in different groups up to 7 days.

RESULTS

The TLC analysis revealed the presence of some bioactive constituents in the plants, *P. amarus* and *D. scandens*. The findings obtained for *P. amarus* indicated that maximum number of spots (8) for cold ethyl acetate extract was observed in the ethyl acetate: n-hexane (80:20) system with 7 active bands having R_f values of 0.09, 0.14, 0.18, 0.26, 0.60, 0.64, 0.77, and 0.84 while for *D. scandens* 5 spots with 5 active bands were observed with R_f values of 0.05, 0.18, 0.43, 0.63, 0.70 in 8:2 system (Table 2). In case of methanol extract of *P. amarus*, maximum number of 5 spots were observed in methanol: ethyl acetate: n-hexane (4: 3: 3) with R_f values of 0.29, 0.47, 0.55, 0.66, and 0.73, whereas *D. scandens* methanol extract of same system has a R_f value of 0.36 (Table 2). The combination of two polar solvents (methanol and ethyl acetate) against non-polar (n-hexane) solvent with R_f value of 0.36 makes it an ideal system. In n-Hexane *Phyllanthus amarus* extract, maximum numbers of spots were seen in methanol: ethyl-acetate: n-Hexane (4: 3: 3) with R_f values of 0.74 and 0.72 (Table 2). The cold ethyl-acetate *D. scandens* extract had a higher number of bands with five R_f values of 0.05, 0.18, 0.43, 0.63 and 0.70 than the soxhlet extract with four bands in the ethyl acetate: n-hexane (8:2) system with R_f values of 0.20, 0.33, 0.43, and 0.30 (Table 2). Though the active bands observed in the cold ethyl-acetate extract was higher than the soxhlet ethyl-acetate, it is therefore evidence that an ideal system was achieved in the soxhlet ethyl acetate with R_f values ranging between 0.30 and 0.33. It was also observed that as the solvent mixture

increased towards the polar solvent from the 7:3 - ethyl acetate: n-hexane mobile solvent system to 8:2 – ethyl acetate: n-hexane mobile system in *D. scandens* study, a more ideal system was achieved, which was seen in the variation of some R_f values of 0.30 and 0.33. This was not observed in *P. amarus* fractions.

The appearance of white area against a purple-red background on the chromatograms denotes inhibition of growth of the microorganism due to the presence of bioactive compounds that inhibits their growth. Actively growing microorganism has the ability to reduce INT (2-(4-iodophenyl)-3-(4-nitropheynl)-5-phenyl-2H-tetrazolium chloride) in the tetrazolium salt to a purple-red color. In the presence of bioactive plant constituents on the chromatograms, the growth of the organism was inhibited. The results obtained showed that the *P. amarus* ethyl-acetate extract which retained at R_f value 0.84 had a 21 mm diameter zone of inhibition against coagulase negative *Staphylococcus*, while the compound which retained at R_f value of 0.84 shows no zone of inhibition against *S. aureus* (Table 3). For methanol extract the compound which retained at R_f value of 0.73 was observed to have a zone size inhibition of 23 mm which is the maximum zone of inhibition against coagulase negative *Staphylococcus*, while the methanol extract compound which retained at the same R_f value of 0.73 against *S. aureus* had no antibacterial activity (Table 3). For n-hexane extract the compound which retained at R_f value of 0.74 shows 21 mm zone of inhibition which is the maximum zone of inhibition against *Staphylococcus aureus*, while the compound which retained at R_f value of 0.74 shows no zone of inhibition against coagulase negative *Staphylococcus* (Table 3).

The prophylactic pre-treatment of the two plant *P. amarus* and *D. scandens* combined are represented in Table 4. The result shows that the synergism of the concentration of 25 mg/kg and 50 mg/kg of the two plants were very effective on the rats. The extracts were not toxic to the rats for the 4 days of pre-treatment with no indication of diarrhoea, no redness of the eyes and skin, no discharge from the eyes, or death. Infected mice in group A treated with 0.5 ml sterile extract of 25 mg/kg ethyl-acetate extract of *P. amarus* and *D. scandens* were aggressive and active from the 1st to the 7th day, while infected rats in group 2 treated with 0.5 ml sterile ethyl-acetate extract of 50 mg/kg of combined *P. amarus* and *D. scandens*, were very aggressive and hyperactive from the 1st to the 7th day. The control group treated with DMSO and distilled water appeared normal.

Table 5 shows protection offered by the combined extract of *P. amarus* and *D. scandens* to the Swiss albino rats being challenge with coagulase negative *Staphylococus*. After 1 hour of the challenged test, rats in group A and B remains hyperactive and normal (healthy) without any observable signs or symptoms and no death recorded, whereas the control group appeared dull (not active).

Table 1: Mobile phase with different solvents ratio for *P. amarus* and *D. scandens*

Solvent system	Composition	Ratio	Type of extract	Ratio	Type of extract
		P. amarus		*D. scandens*	
I	Ethyl acetate: n-Hexane	8: 2	Ethyl acetate	8: 2	Ethyl acetate
II	Methanol: ethyl acetate: n-Hexane	4: 3: 3	Methanol	4: 3: 3	Methanol
III	Methanol: ethyl acetate: n-Hexane	3: 3: 4	n-Hexane	7: 3	n-Hexane

Table 2: Retention factor (R_f) values of *P. amarus* and *Diodia scandens* extracts on TLC solvent system

Phyllanthus amarus					*Diodia scandens*				
Extract	Solvent system	Number of bands	Active bands	R_f of each spots	Extract	Solvent system	Number of bands	Active bands	R_f of each spots
Ethyl acetate (Hot)	E: n-H (8:2)	6	5	0.19,0.65,0.68, 0.72,0.76, 0.84	Ethyl acetate (Hot)	E: n-H (8:2)	4	4	0.20,0.33, 0.43, 0.30
Ethyl acetate (Cold)	E: n-H (8:2)	8	7	0.09,0.14, 0.18,0.26, 0.60,0.64, 0.77,0.84	Ethyl acetate (Cold)	E: n-H (8:2)	5	5	0.05,0.18, 0.43,0.63, 0.70
Methanol (Hot)	M:E: n-H (4:3:3)	5	4	0.29,0.47, 0.55, 0.66, 0.73	Methanol (Hot)	M:E: n-H (4:3:3)	1	1	0.36
Methanol (Cold)	M:E: n-H (4:3:3)	3	2	0.40, 0.60, 0.73	Methanol (Cold)	M:E: n-H (4:3:3)	1	1	0.36
n-Hexane (Hot)	M:E: n-H (3:3:4)	4	3	0.48, 0.57, 0.66, 0.72	n-Hexane (Hot)	E: n-H (7:3)	4	4	0.15,0.23, 0.63, 0.68
n-Hexane (Cold)	M:E: n-H (3:3:4)	4	3	0.52, 0.60, 0.69, 0.74	n-Hexane (Cold)	E: n-H (7:3)	6	6	0.07,0.12, 0.62, 0.68, 0.72,0.78

Key: E-n-H= Ethyl acetate: n-Hexane, M: E: n-H= Methanol: Ethyl acetate: n-Hexane; (C) = Cold, (H) = Hot

Table 3: Antibacterial potential of *Phyllanthus amarus* and *Diodia scandens* extracts on direct bioautographic plates

	Phyllanthus amarus Zones of inhibition (mm)		*Diodia scandens* Zones of inhibition (mm)	
Extracts	CoNS	*S. aureus*	CoNS	*S. aureus*
Ethyl acetate (Hot)	21	0	0	21
Ethyl acetate (Cold)	18	0	20	14
Methanol (Hot)	23	0	28	0
Methanol (Cold)	21	0	29	0
n-Hexane (Hot)	0	19	34	25
n-Hexane (Cold)	0	27	34	4

Key: CoNS – Coagulase Negative *Staphylococcus*; Hot – Soxhlet extraction; cold – cold percolation

Table 4: Response of Swiss Albino Rats to combined dosage administration of *P. amarus* and *D. scandens*

				DAY1								DAY 7						
Groups Mean weight(kg)	T_x	C (mg/kg)	D_o (ml)	Response to dosage								Response to dosage						
				F (n)	Agg (n)	Inf (n)	W (n)	R (n)	D (n)	Di (n)	De (n)	F (n)	Agg (n)	Inf (n)	W (n)	R (n)	D (n)	Di (n)	De (n)
1 (155)	EA1	25	0.5	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0
2 (165)	EA2	100	0.5	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0
Control (190)	DMSO+ Distilled H_2O		0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: Tx- Treatment, D_0- Dosage, C- Concentration, R- Redness of the eye and skin, RD- Response to dosage, Agg- Aggressiveness, INF- Inflammation, D-Discharge in eyes, Di- Key: Tx- Treatment, D_0- Dosage, C- Concentration, R- Redness of the eye and skin, RD- Response to dosage, Agg- Aggressiveness, INF- Inflammation, D-Discharge in eyes, Di-Diarrhoea, D- Death, RD- Response to dosage, EA 1 and EA2- Ethyl acetate 1 and 2, W-Weakness, DMSO+ds H_2O= distilled water

Table 5: Protection offered by the combined extract of *Phyllantus amarus* and *Diodia scandens* on Swiss albino rats

Type	Group	Extract injected per rat	Response to dosage				
			1 hr	24 hrs	3 days	5 days	7 days
Non treated	Control (n=4)	0.5ml DMSO + H2O	W	W	W	W	W
Treated	Gp1 (n=4)	25mg/kg	W	N	N	N	N
Treated	Gp2 (n=4)	50mg/kg	W	N	N	N	N

Key: W= weak N= normal DMSO= Dimethyl sulphoxide

DISCUSSION

The results obtained showed that most of the antibacterial agents detected were present more in extracts of polar solvents (higher in aprotic than protic solvents) than the non-polar solvents as reflected in the number of active bands, which is in accord with the report of Gujjeti and Mamidala (2013), who reported that high amount of 10, 8 and 8 phytochemicals were obtained from methanol, thyl acetate and hexane respectively. Suleiman *et al*. (2010) however, reported more antimicrobials in non-polar than polar solvents. A study by Nguyen *et al.* (2017), who reported nine (9) bioactive compounds from a methanolic extract of *P. amarus*, also supported the trend observed in this current study.

The hexane extracts of *P. amarus* yielded 4 R_f values; with R_f 0.48 as the most polar and 0.74 as the least polar component in the extract. In this study, a R_f of 0.26 and 0.29 was observed in the cold ethyl acetate and hot methanolic extract of *P. amarus* respectively. In corroboration with these findings, Srivastava *et al.* (2015), reported their observation of phyllanthin and hypophyllanthin in *P. amarus* toluene-ethylacetate extract at R_f 0.24 and 0.29 respectively. Ketmongkhonsit *et al.* (2015) also reported phyllanthin as one of the major bioactive compounds in *P. amarus.* The phllanthin of *Phyllanthus* spp has been reported to have many pharmacological effects. Ilangkovan *et al.* (2016) described the inhibitory effect of *P. amarus* and suggested it as a good candidate for immunosuppressive agent.

It was observed in this study that the cold n-hexane extract of *D. scandens* is more efficient than the methanolic and soxhlet extracts; since more active compounds were observed in the n-hexane extract. The polar/non-polar system developed in the *Diodia scandens* study with ethyl acetate/n-hexane solvent (7:3) of the cold n-hexane fractions yielded 6 bands; while the methanolic extract yielded 4 bands on the TLC plate. In addition, in this study four active bands were also observed from the soxhlet n-hexane (7:3) fraction of the ethyl acetate. *D. scandens* has been known to be not only an excellent antibacterial but also as an efficient antifungal agent (Ogu *et al.*, 2011). The presence of phytochemicals of antibacterial activity in *D. scadens* was reaffirmed in this study by the principle of Bioautography (agar-overlay). The TLC-Bioautography of the 5 active spots of cold and soxhlet ethyl acetate extract expressed an antibacterial activity against *S. aureus* and CoNS with zone size diameter ranging from 14 mm to 21 mm. The phytochemicals in *D. scandens* include saponin, tannin, alkaloids and phytin phosphorus (Ojo *et al.*, 2017). Ghosh *et al*. (2022) reiterated the pharmacognositic properties of *P. amarus* which was due to phytochemicals, phyllanthin and hypophyllanthin present in the plant.

In this study, the experimental mice were observed to be protected against the tested pathogen; by showing no presence of any observable symptoms such as diarrhoea, redness of the eyes and skin, discharge from the eyes or death which provides an experimental data to support the notion that *P. amarus* and *D. scandens* are good prophylactic agents; since all the symptoms listed above were observed in the control experiment. The phytochemicals such as saponin, tannin, alkaloids and phytin phosphorus present in *D. scandens*, and

saponin, tannin, alkaloids and phytin phosphorus in *P. amarus* reveals the antibacterial properties of both plants (Ojo *et al.,* 2017). The study of *D. scandens* extract by Wada *et al.* (2022), against *Staphylococci aureus* was reported to have astonishing antibacterial effect against the tested bacterial strains as well as certain fungi strains.

The combination therapy of *P. amarus* and *D. scandens* in this study was observed to yield good positive effects on the tested mice. The administered dose of 25 mg/kg of the combined extract was observed to protect the tested mice against the experimental pathogen. This therapeutic dose was observed to be much lower than the therapeutic dose when *P. amarus* or *D.* scandens was used separately. According to Nwankpa *et al.* (2014), it took 750 mg/kg of *P. amarus* extract to protect the experimented mice against the tested pathogen. The data submitted by Unigwe, *et al*. (2021) recorded a dose of 150 mg/kg as the therapeutic dose which is higher than the dose observed in this current study. An *in vitro* experiment by Okiki *et al.* (2022) who tested *P. amarus* extract against several bacterial strains and fungi showed 100 mg/ml of the extract as the minimum inhibition concentration. Furthermore, Ojo *et al*. (2017) reported an effective dose of 50 mg/kg and 100 mg/kg of *D. scandens and P. amarus* extracts respectively. By comparing data from this current study and previous studies, we observed that the therapeutic dose in this current study is lower than the doses reported when either plant is used separately; it can then be suggested that the prophylactic effects of the combined extracts of these two plants is higher and more effective than when either plant is used separately. In this study, it was also shown that longer time exposure of animals to various doses or concentrations resulted in good and long-term prophylaxis in experimental animals without side effects. The recorded therapeutic dose had no toxic effects on the rats, as no negative behaviour was observed in the experimental mice.

CONCLUSION

Developing new regime of antibiotics has never been more important than now; especially when most current antibiotic drugs seem to be failing. Tapping into the vast antibiotic potentials of *D. scandens* and *P. amarus* involve detecting the specific active components in the plants through thin layer chromatography bioautography. *D. scandens* and *P. amarus* are rich in bioactive compounds that can be harnessed to produce effective antibiotics. The *in vivo* study of the combined extracts in this study confers excellent prophylaxis on the experimented animals against the tested microbes; the antibacterial strength of either plant can be improved by combining both plants. Researchers are continuing to find new ways to develop effective antibiotics to cushion the menace of antibiotic resistance that has plagued our world. It is expected that data from this study and other related studies will help win the fight against antibiotic resistance.

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