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Phytochemical screening and antimicrobial evaluation of the methanol extract and fractions of the leaves of *Piper umbellatum* Linn (Piperaeceae)

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Abstract

Piper umbellatum is widely distributed tropical plant species. The plant has been reported to possess an array activity including anti-inflammatory, antioxidant, antifungal, analgesic and skin protection. It is consumed as a vegetable and used ethnomedicinally in the treatment of stomach pain in Edo state, Nigeria. The antimicrobial property of the plant is being advocated. This present study aims at evaluating the phytochemistry of the methanol extract of *P. umbellatum* including analytical thin layer chromatography of the extract. This study evaluated the antimicrobial activity of the methanol extract of *P. umbellatum* as well as the *n*-butanol; *n*-hexane and chloroform fractions of the extract obtained by partitioning the methanol extract using clinical isolates in agar dilution technique. This study also went further to determine the minimum inhibitory concentration (MIC) of the methanol extract and the n-hexane fraction of the extract. Phytochemical screening of the dry leaves indicated the presence of carbohydrates, cardiac glycosides, saponins, tannins and alkaloids. TLC revealed that a non-polar solvent system is preferable for isolation. The methanol extract, n-hexane fraction and n-butanol fractions inhibited all the test microorganisms at the doses used. The MIC for both the methanol extract and n-hexane fraction were found to be <25mg/ml. This study therefore confirms the antimicrobial activity of *Piper umbellatum*.

Keywords: *Piper umbellatum*; Methanol extract; Antimicrobial activity; Phytochemistry; Chromatography

INTRODUCTION

The potential of the Nigerian flora as a veritable source of pharmaceutical and other therapeutic materials have been emphasised and herbs have usually served as a repository of healing materials (Gbile and Adesina, 1986). The observed physiological and pharmacological activities of medicinal plants depend on the presence of active constituents mostly the secondary metabolic products. (Sofowara 1993).

Piper umbellatum Linn is a tropical plant species widely distributed in Mexico, America, South America, Africa and West indies (Carles and Roersch, 2010). It is consumed as a vegetable and used ethnomedicinally in the treatment of stomach pain in parts of Edo State, Nigeria (Mensah, *et al.*, 2008). Other documented activities

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include Antioxidant activity (Sen *et al.*, 2002), Anti-fungal activity (Tabopda *et al.*, 2008); Anti-inflammatory, analgesic and skin protection (Carles and Rosersch 2010). Antibacterial activity studies are being advocated (Carles and Roersch, 2010). Phytochemical studies on *P. umbellatum* indicate the presence of Alkaloid, Saponin, and Tannin (Mensah *et al.*, 2008). Chemical Studies on the plant resulted in the isolation of three pure compounds (Tabopda *et al.*, 2008).

This present study aims at evaluating the phytochemistry of the methanol extract of *P. Umbellatum* including analytical thin layer chromatography of the extract. This study also evaluated the antimicrobial activity of the methanol extract of *P. umbellatum* as well as the n-Butanol; n-Hexane and Chloroform fractions of the extract obtained by partitioning the methanol extract against clinical isolates of *E. coli*, *S. aureus*, *P. aeruginosa* and *Candida albican.* This study also went further to determine the minimum inhibitory concentration (MIC) of the Methanol extract and the n-Hexane fraction of the extract. All antimicrobial tests were done by agar dilution method.

EXPERIMENTAL

Plant materials and extraction. Fresh leaves of *Piper umbellatum* were collected in Ogwa, Esan West Local government area of Edo State.The plant was identified by Dr. B. A. Ayinde (Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin city, Nigeria). The leaves were air dried at room temperature for two weeks and milled into a powder. 350g of the powder was cold macerated in methanol for 48 hours and there after filtered severally through a sieve. The filtrate was concentrated and dried under reduced pressure using Rotatory evaporator with a yield of 15.94% w/w (55.78g).

Partitioning was done with the aid of separating funnel using sequentially 60ml of n-hexane, chloroform and n-butanol respectively. The different fractions were dried under reduced pressure using a rotatory evaporator after which they were stored in a fridge at 4^{o}C until used for experiment.

Analytical thin layer chromatography was used to determine an appropriate elute solvent (or solvent system). The solvent/solvent systems used were: Chloroform (100%), chloroform: dichloromethane (1:1), chloroform: methanol (1:1), chloroform: hexane (1:1) and methanol (100%). The volume of solvent (or solvent system) used was between 6-10 ml in all cases. The chromatogram was allowed to develop. The Rf values were thereafter recoded.

Phytochemical screening was carried out as described by Evans (1989) for carbohydrates, tannins, saponins, alkaloids, and glycosides.

Preparation of stock solutions.

i. Extract and fractions. The methanol extract and n-hexane fraction stock solutions of 200mg/ml each were obtained by dissolving 2g of each in 10% Tween 80. The stock of chloroform and n-butanol fractions of 500mg/ml were prepared by dissolving 0.5g each in lml of 10% Tween 80.

ii. Ciprofloxacin stock of was prepared according to EUCAST (2000) Formula:

i.e. weight of powder(mg) =

$$\frac{\text{Conc (µg/ml) X Volume of solvent (ml)}}{\text{Potency of powder (µg/ml)}}$$

Potency of ciprofloxacin powder = 980ug/mg

iii. Fluconazole stock solution of 5,000 mg/ml was prepared by dissolving 50 mg fluconazole in 10ml of 10% Tween 80.

Test organisms. The experiment described in this study was performed using clinical isolates of *Escherichia coli, Psuedomonas aeruginosa, Staphylococus aureus* and *Candida albicans* obtained from the

microbiology Department of the University of Benin Teaching Hospital (UBTH), Benin City.

Media. All media were prepared form dehydrated commercial products and were made up strictly according to the manufacturer's instructions. They were sterilized by autoclaving at 121^0c for 15 minutes, unless otherwise stated. All Agar media after sterilization were allowed to cool to about 47^oC before dispensing into appropriate sterile dishes.

Preparation of inocula. Four sterile plates were used to collect the test organisms *Escherichia. coli*, *Psuedomonas aeruginosa*, *Staphylococus aureus* and *Candida albicans* and labelled accordingly.
Bacteria inoculum was prepared by suspending the colonies of the organism in 5ml nutrient broth and incubated aerobically at 37^0c for 3 hours. Inoculum of *Candida albicans* was prepared by suspending 5 colonies in sabouraud broth and incubated aerobically at room temperature for 24 hours.

Screening for antimicrobial activity (agar dilution method). 9ml of sterile molten agar cooled to about 40^0 C were aseptically seeded with l ml of the stock solutions of the extract and its various fractions. This yielded test concentrations of 200 mg/ml for the methanol extract (ME) and Hexane fraction (HF) and 50mg/ml for the Chloroform fraction (CF) and n-Butanol fraction (BF). Ciprofloxacin and fluconazole stock solutions were diluted to concentrations of 100ug/ml before seeding the agar to yield test concentrations of 10ug/ml each. The seeded agar were aseptically poured into sterile Petri-dishes and allowed to solidify at room temperature. A drug free plate (containing lml of 10% tween 80 + 9ml of molten agar) was included to serve as control (EUCAST 2000).

About 5 ml of each inoculum was spotted and incubated on each plate. Plates were incubated aerobically at 37^0c for 18-24 hours for bacteria and at room temperature for 72 hours for *Candida albicans*. The plates were checked for growth after incubation. (Lunagtongkum *et al.*, 2007).

Determination of minimum inhibitory concentration (MIC). The agar dilution method was employed for the determination for the MIC for methanol extract (ME), hexane fraction (HF) and reference antimicrobial agents (ciprofloxacin and fluconazole). 9ml of sterile molten agar cooled to about 40^oC was aseptically seeded with 1ml each of ME, HF and the reference antimicrobial agents at three different concentrations respectively. The seeded agar were aseptically poured into sterile Petri dishes and allowed to solidify at room temperature. A test agent free plate was included to serve as control. About 5ul of each inoculum was spot inoculated on each plate.

Table 1: Phytochemical screening of *Piper umbellatum* leaves

TEST	*RESULT*
Carbohydrates: Soluble carbohydrates	+
Reducing sugars	+
Glycosides: Cardiac glycosides	+
Cyanogenetic glycosides	-
Anthraquinone glycosides	-
Saponins	+
Tannins: True tannins	+
Psuedotannins	-
Phlobatannins	+
Alkaloids	+

+ = present,- = absent

Table 2: Analytical TLC result of methanol extract of *Piper umbellatum* L.

Solvent system	*No. of spots*	*Rf value*	*Colour of spot under sunlight*	*Colour of spot under 366nm UV light*
i. Chloroform (100%)	7	0.02	Green	Grey
		0.1	Green	Grey
		0.3	Green	Grey
		0.5	Yellow	Red
		0.6	Green	Grey
		0.8	Green	Grey
		0.9	Green	Grey
ii. Methanol (100%)	4	0.4	Green	Faint red
		0.5	Green	Grey
		0.6	Faint green	Faint red
		0.7	Faint green	Faint red
iii. Methanol: chloroform (1:1)	2	0.7	Green	Faint red+Grey
		0.8	Green	Grey
iv. Chloroform: hexane (1:1)	7	0.02	Green	Grey
		0.03	Green	Grey
		0.04	Green	Grey
		0.08	Green	Faint red
		0.1	Faint green	Faint red
		0.2	Faint green	Faint red
		0.3	Faint green	Faint red
v. Chloroform: Dichloromethane (1:1)	6	0.01	Yellow	Grey
		0.2	Green	Grey
		0.3	Yellow+Green	Red+Green
		0.5	Green	Grey
		0.6	Faint green	Faint red
		0.8	Faint green	Faint red

Table 3: Antimicrobial activity of methanol extract of *Piper umbellatum*, fractions and reference antimicrobial agents

Test organism	*Source*	*Control*	*ME (200mg/ml)*	*HF (200mg/ml)*	*CF (50mg/ml)*	*BF (50mg/ml)*	*CIP (10ug/ml)*	*FLU (10ug/ml)*
EC	Urine	+++	-	-	+++	-	-	NT
SA	Ear	+++	-	-	+++	-	-	NT
PA	Eye	+++	-	-	+++	-	-	NT
CA	HVS	+++	-	-	+	-	NT	-

Key: +=slight growth, +++ =heavy growth, - = no growth, NT= Not tested: EC=*Eschericia coli*, PA= *Pseudomonas aeruginosa*, SA=*Staphylococcus aureus*, CA=*Candida albicans;* HVS=High vaginal swab; ME=Methanol extract, CF=Chloroform fraction, HF=n-Hexane fraction, BF=n-Butanol fraction, CIP=Ciprofloxacin, FLU=Fluconazole.

Table 4: Results of MIC determination of methanol extract of *Piper umbellatum*, n-Hexane fraction and reference antimicrobial agents.

Test organism	*Control*	*ME(mg/ml)*			*HF(mg/ml)*			*CIP(ug/ml)*			*FLU(ug/ml)*		
		100	*50*	*25*	*100*	*50*	*25*	*5*	*2.5*	*1.25*	*5*	*2.5*	*1.25*
EC	+++	-	-	-	-	-	-	-	+	++		NT	
SA	+++	-	-	-	-	-	-	-	-	+		NT	
PA	+++	-	-	-	-	-	-	-	-	-		NT	
CA	+++	-	-	-	-	-	-		NT		-	-	++

+ =slight growth, ++ =moderate growth, +++ = heavy growth

Plates were incubated at 37^{o}C for 18-24hr for bacteria and at room temperature for 72hr for *Candida albicans*. Plates were observed for growth after incubation.

RESULTS AND DISCUSSION

The weight of the dried methanolic extract was 55.78g, while the dried fractions weighed 6.62g, 4.56g and 2.34g for the n-Hexane, Chloroform and n-butanol respectively.

The percentage yield of the methanol extract was found to be 15.3% while the yields of the various fractions were found to be 6.62g (33.1%), 4.56g (22.8%) and 2.34g (11.7%) for the n-Hexane, chloroform and n-butanol fractions of the methanolic crude extract.

Thin layer chromatography (TLC) is a major separation and analytical tool usually preferred for its speed, sensitivity and resolution. Analytical TLC of the methanol crude extract using different solvent systems indicates that the constituents of the extract have more polar constituents owing to the low Rf values obtained with non-polar solvent systems (i, iv); with polar solvent systems and semi-polar solvent systems (ii , iii). The spots migrated more than they did with systems i and iv. The number of spots also reduced suggesting a possible overlapping of the components of the extract. High Rf valves of 0.7, 0.8 and 0.9 with non-polar solvent systems also indicate the presence of non-polar components with non-polar characteristics. (or at best compounds with amphoteric properties).

The number of spots identified from the different solvent systems indicates that a non-polar system will be preferable for resolution and isolation because the spots are more separated out and methanol extract of *Piper umbellatum* indicates the presence of reducing sugars, cardiac glycosides, saponins, Tannins and alkaloids, saponins have been reported to have anti-microbial activity (Mahoto *et al.,* 1999). Other secondary plant metabolites such as alkaloids, tannins and sterols have also been shown to have anti-microbial activity (Parekh, *et al.,* 2005, Banso and Adeyemo, 2007). Studies have been conducted to establish the antimicrobial activity of medicinal plants (Habsah *et al.,* 2000), (Sudhakar *et al.,* 2006). In this present study, it was observed that clinical isolates of *E. coli, Staph aureus, P. aeruginosa* and *Candida albicans* were tested against the methanol extract of *Piper umbellatum* as well as its n-butanol and n-hexane fractions which exhibited total inhibition of the clinical isolates utilized in this study: The chloroform fraction however, did not show any activity at inhibiting the organisms used. This may be as a result of n-Hexane extracting most non-polar active constituents before chloroform was used to partition the extract.

The n-butanol fraction exhibited 10% activity at inhibiting all the test organisms. This indicates that the polar fraction of the extract has a good amount of anti-microbial secondary plant metabolite. This can also be buttressed because the n-butanol fraction was the last portion to have been obtained by partitioning, it also had the lowest yield and was effective at inhibiting the test organisms even at one-quarter the concentration of the extract and its n-Hexane fraction (i.e. 50mg/ml).

The reference drugs used showed activity in line with already documented spectrum of activity (Goodman and Gilman, 2001) ciprofloxacin inhibited all the bacteria species (i.e *E. coli, P. aeruginosa, S. aureus*) at a concentration of 10mg/ml. Fuconazole a known systemic anti-fungal agent inhibited *Candida albicans* in this test at a concentration of 10mg/ml.

Dilution methods are used to determine the minimum inhibiting concentrations (MICs) of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing (EUCAST 2000). The MIC assay was carried out to

determine the lowest concentrations of the methanol extract and n-Hexane fraction that will inhibit the test organisms. It was observed that for both samples there was total inhibition of the test organisms at the test concentrations used of 100mg/ml, 50mg/ml and 25mg/ml. The MIC for both samples was found to be less than 25mg/ml.

The MIC of ciprofloxacin was found to vary among the test organisms as follows *E. coli* (5ug/ml) *S. aureus* (2.5ug/ml) and *P. aeruginosa* (<1.25ug/ml) fluconazole was also found to have an MIC of 2.5ug/ml for *Candida albicans* in this study.

This study in conclusion has established the anti-microbial activity of leaves *Piper umbellatum* and confirmed its ethno medicinal use as an antibiotic. This study also established that the antimicrobial constituents of the leaves can be found in both the polar and non-polar fractions of the plant extract.

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