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Phytochemical Screening, Antifungal and Cytotoxic Activities of Trichilia heudelotii Planc (Harm)

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Abstract

This study was designed to evaluate the antifungal and cytotoxic activities as well as phytochemical screening of leaf and stem bark extracts (Aqueous, Acetone and Ethanol) of Trichilia heudelotii Planc Harm. against some human pathogenic fungi using standard methods. The average yield of the extracts ranged from 9.62±0.01 to 10.20±0.01 and 6.39±0.01 to 14.19±0.06 for the leaf and stem bark extracts respectively. Phytochemical screening revealed the presence of alkaloids, tannins, phlobatannins, phenols, anthraquinones, glycosides and flavonoids. Steroids and chalcones were absent in all the extracts. At the concentration of 50mg/ml, the highest zone of inhibition was exhibited by acetone leaf extract against Candida albicans ATCC10231 (17.00±0.58mm) followed by acetone and ethanol stem bark extracts against Trichophyton rubrum and Candida albicans exhibiting zone of inhibition of 16.00±0.00mm respectively. The aqueous leaf extracts showed the least activity of 6.00±0.00 in T. rubrum. The minimum inhibitory concentration (MIC) of all the extracts on the test pathogens ranged between 2.5 and 200mm.The cytotoxic activity of the extracts which was assessed by Brine shrimp lethality bioassay method revealed significant cytotoxic effects with LC_{50} values ranging from 9.01 to 256.17mg/ml. Overall results validated the traditional use of the plant materials in the treatment of skin infections caused by the dermatophytes and other infections caused by the test pathogens.

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Keywords: Antifungal; Cytotoxic; pathogens; Phytochemical; Trichilia heudelotii.

1. Introduction

Medicinal plants have been used to treat various human diseases due to their therapeutic properties. The World Health Organization (WHO) has reported that over 80% of the World's population relies on traditional medicine which is largely plant based, for their primary healthcare needs [1]. The increasing re-emergence of antibiotic resistant pathogens particularly in developing countries is rendering treatment with conventional antibiotics ineffective. This has necessitated the search for new, efficient and cost effective drugs for the control of infectious diseases. Several reports have indicated that medicinal plants constitute a great source of biologically active drugs for the control of pathogenic organisms [2, 3].

Furthermore, fungal infections remain a significant cause of morbidity and mortality despite advances in medicine and emergence of new antifungal agents. Much attention has however, been paid to plant derived antimicrobial and antioxidant compounds, and based on the knowledge that plants have their own defense system [4]. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine shrimp (Artemia salina) lethality assay. This assay is used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds [5, 6, 7].

Trichilia heudelotii Planc Harm. is a species of plant in the family Meliaceae. It is a medium sized tree that grows 12-20m high and up to 40m girth. It is a common medicinal plant in the Western part of Nigeria. It is locally called "Akoko rere" in Nigeria [8] and used in the traditional medicine for the treatment of many microbial infections ranging from gastrointestinal infections to gonorrhea [9].

The evaluation of various plant products according to their therapeutic efficacy leads to the discovery of newer recent drugs for treating various ailments. Therefore, the main objective of the present study was to determine the in vitro antifungal, phytochemical screening and cytotoxic activities of the leaf and stem bark extracts (Aqueous, acetone and ethanol) T. heudelotii on some human pathogenic fungi.

2. Materials and Methods

2.1. Collection, Identification and Extraction of the Extracts

Fresh leaves and stem bark of T. heudelotii Planc Harm. were harvested from uncultivated farmland located in Owo, Ondo State, South-Western Nigeria in May, 2011. The plant materials were then authenticated at the Herbarium of the Department of Botany, University of Lagos and voucher specimen (LVH3617) was deposited at the Department of Forestry and Wood Technology, Federal University of Technology, Akure. The authenticated plant materials were washed and cleaned thoroughly with tap water and then air-dried under shade. The dried samples were then ground into coarse powder with the aid of a mechanical grinder and were stored in clean air- tight containers, and kept in a cool, dry place until required for use.

A 100g portion of the powdered sample was soaked in 300ml of different solvents (acetone, ethanol and water)

for 72hr with intermittent stirring using sterile spatula. The plant extracts were then filtered through Whatman No1. filter paper into bijou bottles and then dried using rotary evaporator at a temperature of 50^0C to yield crude extracts [6]. Different concentrations of the extracts were prepared by diluting 0.10g, 0.20g, 0.30g, 0.40g and 0.50g of the extracts in 100ml of 0.01% Tween-20 to obtain concentrations of 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml and 50mg/ml respectively [10].

2.2. Test microorganisms

The fungi employed in the study were five clinical isolates (Aspergillus flavus, Candida albicans, Candida glabrata, Cryptococcus neoformans and Trichophyton rubrum) and one typed cultures (Candida albicans ATCC 10231) obtained from Federal Medical Center, Owo and Federal Institute of Industrial Research, Oshodi, Lagos State, Nigeria respectively.

2.3. Qualitative phytochemical screening

The extracts of the different plant parts were subjected to qualitative phytochemical analysis for the presence of tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids, glycosides, alkaloids, anthraquinones, chalcones and phenol were carried out on the extracts using standard procedures as described by [11, 12].

2.4. In vitro antimicrobial susceptibility test

The extracts obtained from the test plant were screened against the test organisms by agar well diffusion method [13]. A 25ml aliquot of Sabouraud Dextrose agar (Lab Oratorios Britania, Argentinia) was poured into each Petri plate. When the agar solidified, test organisms were inoculated on the surface of the plates (1 x 10^6 sfu/ml) using a sterile glass spreader, allowed to set and punched with 6mm cork borer. A portion of 50µl of each of the extract concentrations was introduced into the wells. Control wells containing the same volume of 30% Dimethyl sulphoside (DMSO) served as negative control, while Miconazole (100µl) was used as positive control. The tests were carried out in triplicates and plates were incubated at 25^0C for 48h and 72h for the yeast and moulds respectively. The diameters of the zones of inhibition were then measured in millimeters.

2.5. Minimum inhibitory concentration (MIC)

Twofold serial dilutions of the extracts were prepared in Saboraud broth to achieve a decreasing concentrations ranging from the least concentration that produced clear zone of inhibition (10mg/ml to 0.156mg/ml). All tubes including the controls were labeled accordingly. Each dilution was seeded with 1ml of standardized inoculums (1.0×10^6 sfu/ml) incubated at 25^0C for 72hr. A tube containing only seeded broth (i.e. without plant extracts) was used as the positive control while the un-inoculated tube was used as negative control. The lowest concentration of each extract that showed a clear zone of inhibition when compared with the controls was considered as the MIC.

2.6. DPPH free radicals scavenging assay

The DPPH free radical (1,1-diphenyl-2- picrylhydrazyl) scavenging assay was determined using the method described by [2, 14]. A 4mg portion of DPPH was dissolved in methanol to obtain 100µM methanol solution of DPPH. A 3ml portion of the extract concentrations (0.00 to 2.0 mg/ml) was added to 1ml of 100µM methanol solution of DPPH. The mixture was shaken vigorously and incubated in the dark at room temperature for 30min. The absorbance at 517nm was measured against the blank (methanol) and ascorbic acid as positive control using a spectrophotometer. The DPPH radical scavenging activity (%) was then determined by the following equation:

DPPH radical scavenging: Activity (%) = [(Ao −As) /Ao] × 100 where Ao = absorbance of DPPH without sample; As = absorbance of mixture of sample and DPPH. The radical scavenging activity of the samples (Median inhibitory concentration, IC_{50}) value was determined from an equation line obtained by plotting a graph of concentration against percentage inhibition.

2.7. Determination of cytotoxic effect of plant extracts

The brine shrimp (Artemia salina) lethality bioassay was carried out according to the method described by [15]. Brine shrimp eggs were hatched in artificial sea water prepared by dissolving 38g of salt in 1 liter of distilled water, filtered and put in shallow rectangular dish. A plastic divider with several holes of 2mm size was clamped in the dish to make two equal compartments. Brine shrimp eggs were placed in one side of the compartment while the other compartment was illuminated. After 48h of illumination, phototrophic nauplii (Brine shrimp larvae) were collected by using pipette from the lightened side. Samples were then prepared by dissolving 20mg each of the extracts in 2mls of DMSO from where further diluted concentrations of 1000, 100, 10 and 1 ppm were prepared. A 4ml portion of the artificial sea water was added into each test tube and 20 shrimps were transferred into it. This was followed by the addition of 1ml of each of the test extracts and of previously prepared concentrations and maintained under illumination at room temperature. Survivors were counted with the aid of magnifying glass after 24h. The percentage mortality was calculated using Abbot's formula and the LC_{50} was also determined [16, 17].

2.8. Data Analysis

Data were presented as mean±standard error (SE). Significance difference between different groups was tested using two-way analysis of variance (ANOVA) and treatment means were compared with Duncan's New Multiple Range Test (DNMRT) using SSPS window 7 version17.0 software. The significance was determined at the level of $p \leq 0.05$.

3. Results and Discussions

The percentage yields obtained for the plant materials ranged from 9.62±0.01 to 10.20±0.10% and 6.39±0.01 to 14.19±0.01% for the leaf and stem bark extracts respectively (Figure 1). The results revealed that the highest yield of 14.49±0.01 was obtained from using water as solvent while acetone gave the least yield of 6.39±0.01% from the stem bark of the plant. These yields are comparable to those demonstrated in other studies [10]. It also showed that polar solvents have more yields than non-polar one and the abundant metabolites in T. heudelotii are those that easily pass through polar solvents [18].

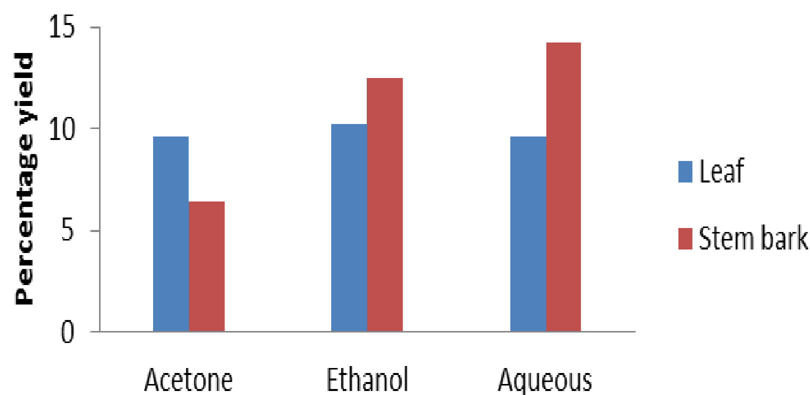

Figure1: Percentage yield of T. heudelotii (leaf and stem) using different solvents.

The phytochemical screening results of the extracts revealed that the leaf and stem bark of T. heudelotii showed the existence of plant constituents such as alkaloids, tannins, phlobatannins, phenols, anthraquinones, glycosides and flavonoids which varied according to the extracting solvents. However, steroids and chalcones were absent in the leaf and stem bark extracts respectively (Table1). The presence of various metabolites in the plant materials could justify its medical use [9]. Most of these compounds are also well known for their large spectrum of pharmacological properties, including antimicrobial (alkaloids and saponins) and antioxidant (tannins) activities [19, 20].

Table1: Qualitative phytochemical property of T. heudelotii extracts.

Phytochemical	Leaf			Stem bark		
	A	E	W	A	E	W
Alk	+++	+++	+	+++	+++	+
Sap	+++	+++	++	+++	+++	++
Tan	++	+	+	++	++	NI
Phlo	+	+	+	+	NI	NI
Phen	+++	+++	++	+++	+++	Ni
Ant	++	++	+	+	++	+
Ter	++	++	NI	+	NI	NI
Card	NI	+	NI	+	+	NI
Ster	NI	NI	NI	+	NI	NI
Glycol	++	++	++	+	++	++
Chal	+	NI	NI	+	Ni	NI
Flav	+	+	NI	NI	NI	NI

Key: A= acetone extract, E= ethanol extracts, W= aqueous extracts, +++ = present in abundance, ++= present in moderate amount, += present in trace amount, NI = completely absent, Alk = alkaloids, Sap=saponins, Tan=Tannins, Phlo=Phlobatannin, Ant=Anthraquinones, Terp=terpenes, Card=Cardenolides, Glyco=Glycolides,

Chalc= Chalcones, Flav=Flavonoids.

The antifungal activity of the T. heudelotii extracts (leaf and stem bark) against the test human pathogens is presented in Tables 3-5. It can be deduced from the results that the activity of the extracts varied according to the extracting solvent and were biologically active against the susceptible organisms. It also revealed that the zones of inhibition (mm) increases with increase in concentration of the plant extracts. At the highest concentration of 50mg/ml, the zone of inhibition recorded for the leaf extract ranged from 6.00±0.00 to 17.00±0.58mm while the stem bark extracts ranged from 10.67±0.00 to 16.00±0.00mm. C. albicans ATCC10231 recorded the highest susceptibility with inhibition zone of 17.00±0.58mm to acetone leaf extract. T. rubrum and C. albicans recorded 16.00±0.00mm zone of inhibition in acetone and ethanol stem bark extracts respectively while the least activity was recorded by the ethanol leaf extract against T. rubrum (6.00±0.00mm). However, the aqueous leaf and stem bark extracts did not show any activity at all the concentrations used. The antifungal activity of the plant extracts against the susceptible pathogens may be due to the presence of the identified metabolites in the plant which may be responsible for the antifungal properties and potency of the extracts. The antifungal properties of the plant extracts were concentration dependent as zone of inhibition increased with increase in concentration of the extract. This is in agreement with previous reports [21, 22]. The minimum inhibitory concentration (MIC) recorded by the plant extracts against the test pathogens ranged from 2.5 to 200mg/ml. T. rubrum recorded the MIC of 2.5 and 5mg/ml in acetone leaf and stem bark extracts, while C. albicans ATCC10231 recorded 5mg/ml in the acetone leaf and ethanol stem bark respectively (Table5). These concentrations indicated significant potency against the respective pathogens.

The percentage lethality of brine shrimp at varying concentrations of the extracts showed lethality in a dose dependent manner [23]. The LC_{50} ranged from 9.87 to 321.50ppm and 9.01 to 256.17ppm in the leaf and stem bark extracts respectively. The acetone extracts of the plant materials exhibited the highest cytotoxic activity while the aqueous extracts showed the least (Table 7). The results of the study indicated that acetone and ethanol extracts exhibited the potential cytotoxic effect in experimental models which supports the claims by traditional medicine practitioners.

Table2: Antifungal activity of acetone extract of T. heudelotii leaf on selected human pathogens.

Conc. (mg/ml)	**10**	**20**	**30**	**40**	**50**	**Myz(100µg/ml)**
Organisms						
A.F	3.67±0.58^{a}	6.33±0.58^{b}	11.67±0.58^{c}	12.00±0.00^{c}	12.00±0.00^{c}	17.33±0.58^{d}
C. A	7.67±0.58^{a}	11.33±0.58^{b}	13.67±0.58^{c}	15.67±0.58^{d}	16.00±0.00^{d}	12.00±0.00^{bc}
C. A ATCC10231	8.33±0.33^{a}	11.67±0.33^{b}	13.67±0.33^{c}	16.67±0.33^{d}	17.00±0.58^{d}	14.00±0.58^{c}
C. N	NI	NI	8.00±0.00^{a}	11.33±0.58^{b}	14.00±0.00^{c}	14.33±0.58^{c}
T. R	6.67±0.58^{a}	9.67±0.58^{b}	12.67±0.58^{c}	15.67±0.58^{d}	16.00±0.00^{d}	10.00±0.00^{b}

Values are Mean±S.E.M (mm), Values followed by different alphabet along the rows are significantly different at $p \leq 0.05$, NI= no inhibition, A.F= Aspergillus flavus, C.A= Candida albicans, C.N= Cryptococcus neoformans,

T. R= Trichophyton rubrum.

Table3: Antimicrobial activity of ethanol extract of T. heudellotii leaf on selected human pathogens.

Conc(mg/ml) Organisms	10	20	30	40	50	Myz(100µg/ml)
A.F	6.67±0.58^{a}	10.67±0.58^{b}	14.33±0.58^{c}	15.00±0.00^{c}	15.00±0.00^{c}	15.33±0.58^{c}
C. N	4.00±0.00^{a}	6.33±0.58^{b}	9.67±0.58^{c}	12.33±0.58^{d}	13.00±0.00^{d}	14.33±0.58^{e}
T. R	NI	NI	NI	4.33±0.00^{a}	6.00±0.00^{b}	10.33±0.33^{c}

Values are Mean±S.E.M (mm), Values followed by different alphabet along the rows are significantly different at $p \leq 0.05$, NI= no inhibition, A.F= Aspergillus flavus, C.N= Cryptococcus neoformans, T. R= Trichophyton rubrum.

Table4: Antimicrobial activity of acetone extract of T. heudelotii stem bark on selected human pathogens.

Conc(mg/ml) Organisms	10	20	30	40	50	Myz(100µg/ml)
C. N	NI	7.33±0.58^{a}	11.00±0.00^{b}	12.67±0.58^{c}	15.67±0.58^{d}	17.33±0.58^{e}
T. R	9.00±0.00^{a}	11.33±0.58^{b}	13.00±0.00^{c}	15.33±0.58^{d}	16.00±0.00^{d}	20.00±0.00^{e}

Values are Mean±S.E.M (mm), Values followed by different alphabet along the rows are significantly different at $p \leq 0.05$, NI= no inhibition, C.N= Cryptococcus neoformans, T. R= Trichophyton rubrum,

Table5: Antimicrobial activity of ethanol extracts of T. heudelotii stem bark on selected human pathogens.

Conc. (mg/ml) Organisms	10	20	30	40	50	Myz(100µg/ml)
A.F	NI	NI	6.00±0.00^{a}	8.33±0.58^{b}	10.67±0.58^{c}	10.00±1.00^{c}
C. A	6.33±0.58^{a}	9.33±0.58^{b}	13.33±0.58^{d}	14.00±0.00^{d}	16.00±0.00^{e}	11.00±0.00^{c}
C. A ATCC10231	6.67±0.33^{a}	9.33±0.33^{b}	13.33±0.33^{d}	14.33±0.88^{de}	15.67±0.33^{e}	13.00±0.58^{c}
T. R	NI	5.67±0.58^{a}	8.00±0.00^{b}	11.00±0.00^{c}	13.00±0.00^{d}	9.00± 0.00^{b}

Values are Mean±S.E.M (mm), Values followed by different alphabet along the rows are significantly different at $p \leq 0.05$, NI= no inhibition, A.F= Aspergillus flavus, C.A= Candida albicans, T. R= Trichophyton rubrum,

Table 6: The MIC of T. heudellotii extracts on the selected pathogens.

Test Organisms	Ethanol Extract		Acetone Extract		Aqueous Extract	
	Leaf	**Bark**	**Leaf**	**Bark**	**leaf**	**Bark**
Aspergilus flavus	5	25	10	NI	NI	NI
Candida albicans	NI	5	5	NI	NI	NI
C. albicansATCC10231	NI	5	5	NI	NI	NI
Candida glabrata	NI	NI	200	NI	NI	NI
Cryptococcus neoformans	7.5	NI	25	12.5	NI	NI
Trichophyton rubrum	40	15	5	2.5	NI	NI

Table7: Percentage mortality of brine shrimps at different concentrations of the extract of T. heudelotii.

		Acetone			Ethanol			Water		
Dose (ppm/ml)	**I.L**	**N.S**	**No. of deaths**	**% mortality**	**N.S**	**No. of deaths**	**% mortality**	**N.S**	**No. of deaths**	**% mortality**
Leaf										
1000	20	0	$20.00{\pm}0.00^{b}$	100	0	$20.00{\pm}0.00^{b}$	100	5	$15.00{\pm}0.10^{a}$	75
100	20	7	$13.00{\pm}0.00^{b}$	65	8	$12.00{\pm}0.01^{b}$	60	11	$9.33{\pm}0.00^{a}$	45
10	20	10	$10.33{\pm}0.01^{b}$	50	12	$8.00{\pm}0.00^{b}$	40	15	$5.00{\pm}100^{a}$	25
1	20	13	$7.00{\pm}0.00^{b}$	35	14	$6.00{\pm}1.00^{b}$	30	17	$3.00{\pm}0.00^{a}$	15
LC_{50}				**9.87**			**72.45**			**321.52**
Stem bark										
1000	20	0	$20.00{\pm}0.00^{b}$	100	0	$20.00{\pm}0.00^{b}$	100	7	$13.00{\pm}0.00^{a}$	65
100	20	5	$15.33{\pm}0.03^{b}$	75	7	$13.33{\pm}0.10^{b}$	65	12	$8.00{\pm}0.11^{a}$	40
10	20	8	$12.33{\pm}0.10^{b}$	60	9	$11.33{\pm}1.00^{b}$	55	16	$4.00{\pm}0.07^{a}$	20
1	20	10	$10.00{\pm}0.01^{b}$	50	12	$8.33{\pm}0.01^{b}$	40	17	$3.33{\pm}1.00^{a}$	15
LC_{50}				**9.01**			**8.82**			**256.17**

Values followed by different superscripts across each row are significantly different at $p \leq 0.05$, IL= initial larva, N.S= number of survivors,

4. Conclusion

The results of this present study provided evidence for the rich phytochemical constituents, antimicrobial and cytotoxic activities of the studied plant extracts. This suggests that further investigations may lead to the use of standardized herbal medicine from T. heudelotii in the treatment of infectious diseases caused by the test

pathogens and could be developed further as anticancer drugs.

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