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Phytochemical Analysis and Antibacterial Activity of *Trema orientalis* (Ulmaceae) Stem Bark Extracts on Respiratory Tract Bacteria

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ABSTRACT

Respiratory tract infection causing micro-organisms are becoming increasingly resistant to common antibiotics. This negative health trend has rekindled interest in the search for plant constituents as sources of antibacterial agents that can be used for the treatment of respiratory tract infectious diseases. The objective of this study is to carry out phytochemical analysis and antibacterial activity of *Trema orientalis* stem bark extracts on respiratory tract bacteria.
Phytochemical screening and thin layer chromatography (TLC) were done on *T. orientalis* stem bark extracts obtained by successive extraction with n-hexane, chloroform, ethyl acetate, methanol and water. Also, the antibacterial activity of the extracts were carried out using clinical isolate of *Pseudomonas fluorescens, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, and Proteus mirabilis*. The most active chloroform extract was further screened by TLC bioautography using the test microorganisms.
The phytochemical screening of the stem bark extracts revealed the presence of flavonoids, steroids, terpenoids, saponins and tannins. The chloroform extract inhibited the growth of all the test organisms especially *E. coli* and *K. pneumonia*. The minimum inhibitory concentration (MIC) for *E. coli* and *K. pneumonia* were 0.4 mg/mL and 1.6 mg/mL respectively; while the minimum bactericidal concentration (MBC) was 0.8 mg/mL for *E. coli* and 12.8 mg/mL for *K. pneumonia*. This study has justified the traditional use of this plant for the treatment of respiratory tract infections.

Keywords: Phytochemical analysis, antibacterial activity, TLC bioautography, *Trema orientalis*

Introduction

Since ancient times, people explored nature, particularly plants in search of new drugs. This has resulted in the use of large number of medicinal plants with curative properties to treat various diseases.[1] The use of medicinal plants as a source of relief from illness can be traced back to over five millennia to the written documents of the early civilization in China, India and the north east.[2] Medicinal plants are relied upon by 80% of the world's population and a number of plants have been documented for their biological and antimicrobial properties.[3]

Trema orientalis (Linn.) Blume enjoys wide spread distribution within savanna vegetation belts of sub Saharan Africa. Each specie grows to become a moderately tall tree with acceptable merchantable girth.[4] The stem bark of *T. orientalis* contains Octacosanoic acid, 1-octacosanyl acetate, simiarenone, simiarenol, episimiarenol, and a new triterpene alcohol, trematol.[5] The leaves contain tannins, saponins, flavanoids, triterpenoid (simiarenol, simiarenone, trematol).[6] The tree has got various traditional medicinal uses in a wide range of cultures. The leaves and the bark are used to treat coughs, sore throats, asthma, bronchitis, gonorrhoea, yellow fever, toothache and as an antidote to general poisoning.[7] The bark is used for dysentery, also used as an inhalant for chest diseases and as a vermifuge. Stems and twigs are used for coughs and other respiratory ailments, fevers, toothache and venereal diseases. Pods and seeds are used for tired muscles and aching bones.[8] The fruits and flowers are used to prepare infusion that are administered to children as a therapy for bronchitis, pneumonia and pleurisy.[8] In African folk medicine, it is used to treat many diseases like dysentery, hypertension.[9]

According to World Health Organization (WHO), over 13 million people die each year from infectious and parasitic diseases: one in two deaths in some developing countries.[10] In Nigeria, lower respiratory tract infections constituted the second leading cause of death in all age brackets in 2002, a year in which tuberculosis was the seventh leading cause of death, accounting for 4% of all deaths.[11] The present study describes the antibacterial activity of the bark extracts of *T. orientalis* against five different bacterial strains viz., *Klebsiella pneumoniae*, *Pseudomonas flourescens*, *Proteus mirabilis*, *Staphylococcus aureus*, and *Escherichia coli*.

It is in view of this that this study was carried out to determine the antibacterial activity of the stem bark of *T. orientalis* on clinical isolates from the respiratory tract and then carry out thin layer chromatography bioautography to determine the bioactive spot.

Materials and Methods

Plant source and Collection

The stem bark of *Trema orientalis* was collected along Ijegun road, Iba Local Government, Lagos state and was authenticated at the Herbarium of the Department of Botany and Microbiology, Faculty of Science,

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University of Lagos, Akoka, Lagos State where a voucher specimen was deposited with voucher number LUH 6870.

Preparation of plant extract

The stem bark of the plant was dried, size reduced to small bits and ground into coarse powder using an electric grinder. 500 g of the resulting powder was weighed and transferred into two wide mouthed bottles in equal proportion and was successively extracted using 1 L each of five different solvents in the order of increasing polarities n-hexane, chloroform, ethyl acetate, methanol and water. Each mixture was allowed to stand for 48 h and then filtered using a filter bag. All the organic solvent extracts were concentrated using a rotary evaporator while the aqueous extract was freeze dried.

Phytochemical screening

The phytochemical screening of the extract was carried out using the Harborne, 1984 procedures with slight modifications.[12]

Microbial strains and Standardization of isolates

All microbial strains used in the study are clinical strains, and was kindly provided by the Department of Microbiology, Lagos University Teaching Hospital. They are Gram positive: - *Staphylococcus aureus*; Gram negative: - *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas fluorescence*.

All the isolates were grown on pre-dried Mueller Hinton agar from their various stock cultures and incubated at 37ºC for 24 h. They were then subcultured in a sterile nutrient broth and the turbidity was adjusted to 0.5 McFarland standards (1.5 x 10^8 cfu/mL). Mueller-Hinton agar was then used for antimicrobial assay. All the broth cultures were incubated at 37°C for 24 h.

Antibacterial activity

Antibacterial activity of *T. orientalis* extracts was evaluated by means of agar-well diffusion assay technique with some modifications. Twenty-five millilitres (25 mL) of the molten agar (45°C) in each of sample bottles were poured into sterile petri dishes. The working cell suspensions were prepared, and 1 mL was seeded into the bottle of Mueller-Hinton agar and was adequately mixed before pour-plating. The plates were allowed to set aseptically, 12 mm diameter wells were bored in the agar with a sterile cork borer. The extracts obtained were dissolved in 10% Dimethyl Sulphoxide (DMSO) to yield the final working concentrations of 200, 100 and 50 mg/mL. Thus, 100 µL of the working samples were placed into the wells and the plates were allowed to stand on the bench for four hours for diffusion to take place before growth of assay organisms after which the plates were incubated lid-up at 37°C for 24 h.[13]

10% Dimethyl Sulphoxide (DMSO) were used as negative control while the standard antibiotic, levofloxacin at concentrations 50 µg/mL, 25 µg/mL and 12.5 µg/mL was used as positive control.

Antibacterial activity was evaluated by measuring the diameter of circular inhibition zones around the well with the aid of a zone reader. A positive result was defined as an inhibition zone (halo size) of 9 mm or more appearing around the holes, indicating presence of antibacterial substance in the extracts tested.

Determination of the minimum inhibitory concentration (MIC)

Agar dilution (solid dilution) technique was used for the determination of the minimum inhibitory concentration of the *T. orientalis* extracts. Ten sample concentrations were also used for the minimum inhibitory concentration determination. The extracts were incorporated from a stock concentration into the sterile molten Mueller Hinton Agars (MHA) of which the volumes were calibrated in sample bottles so as to arrive at final volumes and final working concentrations of the extracts in the agars.

The medicated agars were poured and allowed to set. The surfaces were dried in the sterile biosafety cabinet before the inoculation of assay organisms was done. The lowest concentration of the extract in the agar that inhibited the bacteria in the assay was recorded for the minimum inhibitory concentration (MIC) of that extract and against that particular test organism.[13]

Thin-Layer Chromatography-Direct Bioautography (TLC-DB)

The method of direct bioautography described by Choma and Jesionek, 2015 was used with slight modifications.[14] Developed TLC plates were covered with sterile molten soft agar gel seeded with the vulnerable microorganisms using sterile spatula for spreading after which the plates were incubated at 37ºC. The organisms grew directly on the plates excluding the spots of the active fractions of the extract. Visualisation was done by spraying with tetrazolium salt. Creamy spots called inhibition zones appeared against a pink background of living bacteria, confirmed the presence of antimicrobial agents.

Results and Discussion

The phytochemical constituents present in the stem bark extract were terpenoids, saponin, bound sugars, tannins, cardiac glycoside, flavonoids and free anthraquinones (Tables 1); this result is consistent with the result obtained by Adinortey *et al.*, 2013. [6]

The antibacterial assay of the stem bark showed activity on both gram positive and very strong activity on some gram-negative bacteria.

It is noteworthy that the water extract being the solvent used traditionally for extraction of medicinal plants; inhibited the growth of two out of all the tested bacteria with zones inhibition of 9 mm for *S. aureus*, 10 mm for *K. pneumoniae* at 100 mg/mL and 13 mm for both bacteria at 200 mg/mL.

The chloroform extract of the stem bark was active against all the test bacteria.at all concentrations with zones of inhibition ranging from 15 mm- 30 mm for *P. flourescens*, 16 mm- 35 mm for *S. aureus*, 12 mm - 25 mm for *P. mirabili*, 12 mm- 24 mm for *K. pneumoniae* and 10 mm - 20 mm for *E. coli* (Table 2). Levofloxacin was used as standard drug on the test bacteria; it had activity against all the tests bacteria except *P. fluorescence*. The results of our study is in contrast to previous study by Chowdhury and Islam, 2004 on the antimicrobial activity of ethyl acetate, n-hexane and methanolic extracts of the root of *T. orientalis*

Table 1: Phytochemical Constituents of *Trema orientalis* stem bark extracts.

Phytoconstituents	**Stem bark Extracts**				
	n Hexane	**Chloroform**	**Ethyl acetate**	**Methanol**	**Aqueous**
Tannins	+	+	+	+	+
Saponins	-	+	+	+	+
Reducing sugars	+	+	+	+	+
Free anthraquinone	-	+	+	-	-
Bound anthraquinone	-	-	-	-	-
Cardiac glycosides	-	+	+	+	+
Flavonoids	+	+	+	+	+
Alkaloids	_	-	-	-	-
Terpenoids	+	+	+	+	+
Bound Sugars	+	+	+	+	+

Key: + = present; - = absent

Table 2: Zones of inhibition of *T. orientalis* stem bark extracts on the test bacteria.

Test bacteria	n-Hexane			Chloroform			Ethyl acetate			Methanol			Water			Levofloxacin		
	Conc. (mg/mL)															Conc. (µg/mL)		
	Zones of inhibition (mm)																	
	200	100	50	200	100	50	200	100	50	200	100	50	200	100	50	50	25	12.5
P. flourescens	-	-	-	30 ± 0.50	21 ± 0.24	15 ± 1.10	15 ± 1.02	11 ± 1.01	11 ± 1.05	13 ± 1.01	12 ± 1.00	-	-	-	-	-	-	-
S. aureus	-	-	-	35 ± 0.44	25 ± 1.02	16 ± 1.10	20 ± 0.51	-	-	15 ± 0.25	13 ± 0.54	11 ± 1.06	13 ± 1.02	9 ± 1.01	-	37 ± 1.10	33 ± 0.50	28 ±1.14
P. mirabilis	13 ± 0.5	-	-	25 ± 1.10	17 ± 0.50	12 ± 1.30	14 ± 1.01	11 ± 1.05	-	13 ± 1.05	-	-	-	-	-	16 ± 0.20	12 ± 1.13	-
K. pneumoniae	6 ± 1.01	-	-	24 ± 1.61	14 ± 0.40	12 ± 0.21	20 ± 0.52	12 ± 1.04	-	17 ± 1.03	-	-	13 ± 1.05	10 ± 1.01	-	26 ± 0.30	25 ± 0.25	23 ± 0.42
E. coli	13 ± 1.0	-	-	20 ± 0.82	14 ± 1.11	10 ± 1.00	-	-	-	-	-	-	-	-	-	30 ± 1.11	28 ± 0.23	27 ± 1.10

Values are expressed as mean ± SEM

Table 3: Minimum inhibitory concentrations (mg/mL) of *T. orientalis* stem bark chloroform extract against test bacteria.

Test bacteria	Conc. (mg/mL)									
	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2
P. flourescens	+	+	+	+	+	+	+	+	-	-
S. aureus	+	+	+	+	+	+	+	-	-	-
P. mirabilis	+	+	+	+	+	+	+	-	-	-
K. pneumoniae	+	+	+	+	-	-	-	-	-	-
E. coli	+	+	-	-	-	-	-	-	-	-

Key: + Growth - No Growth

Table 4: Minimum bactericidal concentrations (mg/mL) of *T. orientalis* stem bark chloroform extracts against test bacteria.

Test bacteria	Conc. (mg/mL)									
	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2
P. flourescens	+	+	+	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	+	+	+	+	+
P. mirabilis	+	+	+	+	+	+	+	+	+	+
K. pneumoniae	+	+	+	+	+	+	+	-	-	-
E. coli	+	+	+	-	-	-	-	-	-	-

Key: + Growth - No Growth

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that showed no activity against *Klebsiella sp, P. aeruginosa, B. subtilis* and *S. aureus* even at a dose of 500 mg/disc; only methanolic extract showed significant activity against *E. coli*.[15] However, the results of our study are similar to that obtained by Jayashree *et al*., 2012 with significant activity of water, methanol, ethanol and acetone extracts of *T. orientalis* bark against *Klebsiella spp, Pseudomonas spp, Bacillus subtilis* and *Staphylococcus. aureus*.[16]

The MIC of the chloroform extract was determined for all the test bacteria; the MIC results ranged from 0.8- 25.6 mg/ mL. The MIC results for the chloroform bark extract of *T. orientalis* on the bacteria were as follows *E. coli* (0.4 mg/mL), *K. pneumoniae* (1.6 mg/mL), *S. aureus* and *P. mirabilis* (12.8 mg/ mL) while *P. flourescens* had the highest MIC 25.6 mg/ mL (Table 3). The low MIC values of the extract on *E. coli* and *K. pneumoniae* are indicative of a very good activity.[17]

The Minimum bactericidal concentration MBC of the chloroform extract showed significant activity for *K. pneumoniae* at 12.8 mg/mL and 0.8 mg/mL for *E. coli* for the bark of *T. orientalis* (Table 4).

The type of antimicrobial activity exhibited by an antimicrobial agent may be determined as either static or cidal by calculating the MBC to MIC ratios. MBC to MIC ratios greater than 4 indicates a bacteriostatic action while ratios less than 4 indicate a bactericidal activity.[18] The MBC to MIC ratios of chloroform bark extract of *T. orientalis* on *E.coli* was less than 4 indicating the extract had a cidal effect on the organism while it ratio for *K. pneumoniae* was greater than 4 indicating that the extract had a bacteriostatic effect on the organism (Tables 3 and 4).

The TLC of the chloroform extract of *T. orientalis* bark using mobile phase: Chloroform/ Ethyl acetate (7: 3 v/v) separated into five spots labelled a – e with retention factor (R_f) values were (a) 0.11 (b) 0.30 (c) 0.61 (d) 0.82 (e) 0.91. On spraying with Vanillin-sulphuric acid reagent spot c stained purplish pink confirming the presence of terpenoids compound which was detected in the phytochemical screening of the extracts in Table 1.

TLC bioautographic studies carried out showed that the chloroform extract from the bark of *T. orientalis* had a bactericidal effect on two of the test bacteria used, which are *E. coli* and *K. pneumoniae*. This produced a white background against the pink background when sprayed with tetrazolium salt showing that the bacteria present in that zone had been inhibited while life bacteria still existed in the pink zone (Figures 1 and 2).[19]

The zone of inhibition in the plate C and D of the bioautogram indicated that it is the terpenoids compound in spot c on the TLC plates A and D that is responsible for the antibacterial activity on *E. coli* and *K. pneumoniae* (Figures 1 and 2).

It was also observed that the TLC spot that exhibited this bactericidal activity against *E. coli* and *K. pneumoniae* had the same Rf values indicating that the same constituent was most likely responsible for the activity and suspected to be a terpenoids compound from the purplish pink colour produced on TLC using vanillin sulphuric acid as spray reagent.

Conclusion

The phytochemical analysis and antibacterial activity of *T. orientalis* stem bark was carried out and all its extracts studied possessed activity against one or more of the test bacteria, but the chloroform extract of stem bark exhibited the highest activity against all the test bacteria. The likely active constituent of the chloroform extract responsible for the antibacterial activity was identified as terpenoid compound. Also, the water extract inhibited the growth of two out of all the tested bacteria; *S. aureus* and *K. pneumoniae*.

This study has justified the traditional use of this plant for the treatment of respiratory tract infections whose causative agents are some of the bacteria used in this study and *T. orientalis* stem bark may therefore be considered as a potential source of bioactive compounds with antibacterial activity.

Conflict of interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

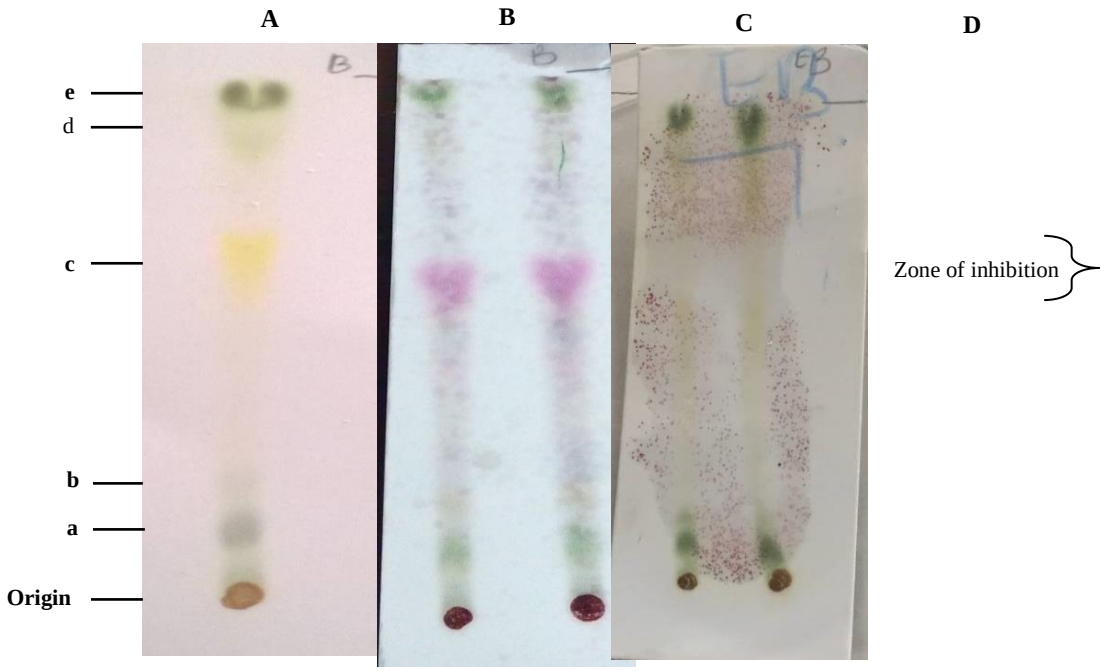

Figure 1: Chromatogram and Bioautogram of the chloroform extract of *T. orientalis* stem bark against *E. coli*. Chromatogram A and B and Bioautogram C and D for the chloroform extract of *T. orientalis* stem bark against *E. coli*. Plate A indicates daylight view while B indicates spots visualised when sprayed with Vanillin / sulphuric acid reagent. Zone of inhibition is marked on plate D and observed as clear spots against a pink background for *E. coli*. Rf values of spots labelled a-e include: (a) 0.11 (b) 0.30 (c) 0.61 (d) 0.82 (e) 0.91. Mobile phase: Chloroform/ Ethyl acetate (7: 3 v/v).

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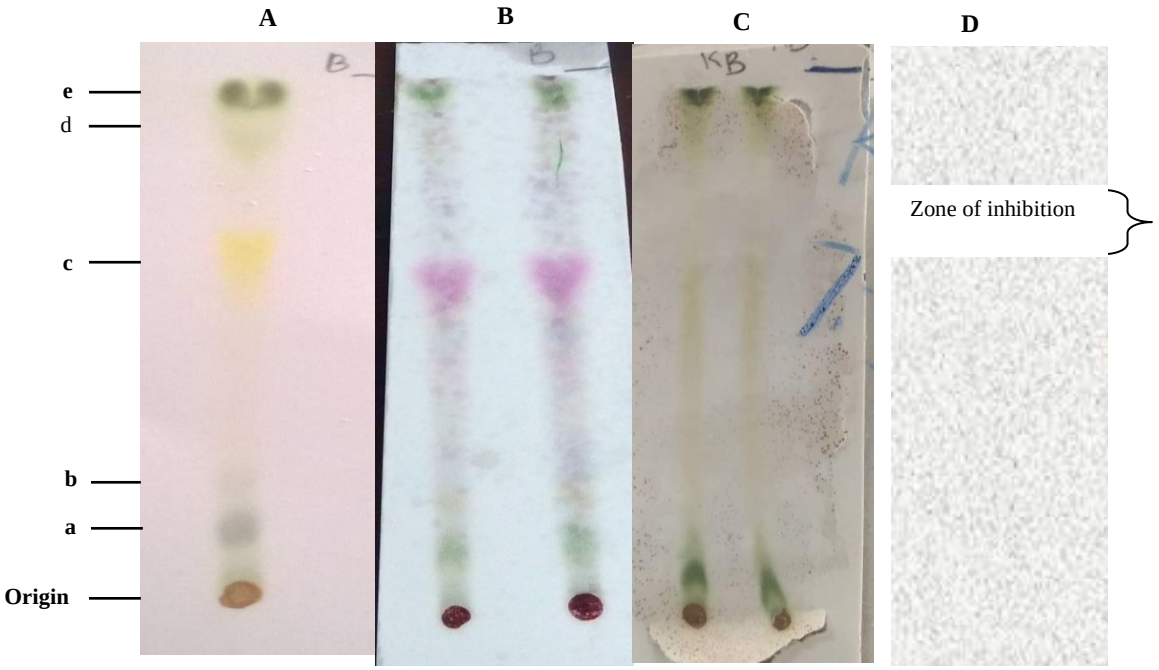

Figure 2: Chromatogram and Bioautogram of the chloroform extract of *T. orientalis* stem bark against *K. pneumoniae*. Chromatogram A and B and Bioautogram C and D for the chloroform extract of *T. orientalis* stem bark against *K. pneumoniae*. Plate A indicates daylight view while B indicates spots visualised when sprayed with Vanillin / sulphuric acid reagent. Zone of inhibition is marked on plate D and observed as clear spots against a pink background for *Kleb. pneumoniae*. Rf values of spots labelled a-e include: (a) 0.11 (b) 0.30 (c) 0.61 (d) 0.82 (e) 0.91. Mobile phase: Chloroform/ Ethyl acetate (7: 3 v/v)

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