Isolation of an Antibacterial Principle from *Terminalia ivorensis* chev. Stem bark

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ABSTRACT

Background: Widespread resistance to current antibiotic therapies has necessitated the search for new anti-infectives from natural and synthetic sources. Ethnobotanical information indicated the use of *Terminalia ivorensis* in the treatment of wounds, syphilis and inflammation.

Objectives: This study was undertaken to investigate the antibacterial properties of the *Terminalia ivorensis* extract and isolate the bioactive compound from the extract.

Methods: Sensitivity of four nosocomial microorganisms (*Bacillus substilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus)* to the acetone extract of *T. Ivorensis* was investigated by agar cup diffusion method. Bioguided solvent–solvent fractionation was carried out on the extract. The most active fraction was subjected to open column chromatography to obtain a bioactive compound. The compound was characterized by application of spectroscopic techniques (ESI-MS, HR-MS, 1D and 2D NMR).

Results: Four microorganisms were sensitive to the whole extract. Column separation led to the isolation of 2-(3,5-dihydroxyphenyl)-benzofuran-5,6-diol as an active principle in the extract. MIC values obtained for the bioactive compound and the whole acetone extract were 1.25mg/ml and 12.5mg/ml respectively.

Conclusion: This study suggests that there is a scientific basis for the application of *T. ivorensis* extract in the treatment of infections. Further studies on its *in vivo* activities and the toxicity profile are required before application in humans.

Key words: *Terminalia ivorensis,* nosocomial microorganisms, 2-(3,5-dihydroxyphenyl)-benzofuran-5,6-diol.

L'isolement d'un principe antibactérien de Terminalia ivorensis chev. L'écorce de la tige

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RÉSUMÉ

Contexte: La résistance répandue aux traitements antibiotiques actuels ont nécessité la recherche de nouveaux anti-infectieux provenant de sources naturelles et synthétiques. Informations Ethnobotanical indiqué l'utilisation de Terminalia ivorensis dans le traitement des plaies, de la syphilis et l'inflammation.

Objectifs: Cette étude a été entreprise pour étudier les propriétés antibactériennes de l'extrait de Terminalia ivorensis et isoler le composé bioactif de l'extrait.

Méthodes: La sensibilité de quatre micro-organismes nosocomiales (Bacillus substilis, Escherichia coli, Pseudomonas aeruginosa et Staphylococcus aureus) à l'extrait acétonique de T. ivorensis a été étudiée par la méthode de diffusion sur gélose de coupe. Bioguided fractionnement par solvant-solvant a été réalisé sur l'extrait. La fraction la plus active a été soumis à une Chromatographie sur colonne ouvrir pour obtenir un composé bioactif. Le composé a été caractérisé par l'application de techniques spectroscopiques (ESI-MS, RS-MS, RMN 1D et 2D).

Résultats: Quatre micro-organismes sont sensibles à l'extrait entier. Colonne de séparation conduit à l'isolement de la 2-(3,5-dihydroxyphényl) -benzofuran-5,6-diol en tant que principe actif dans l'extrait. Les valeurs de CMI obtenues pour le composé bioactif et l'ensemble extrait d'acétone étaient de 1,25 mg / ml et 12,5 mg / ml, respectivement.

Conclusion: Cette étude suggère qu'il existe une base scientifique pour l'application de l'extrait de T. dans le traitement des infections. D'autres études sur ses activités in vivo et le profil de toxicité sont nécessaires avant l'application chez l'homme.

Mots clés: Terminalia ivorensis, micro-organismes nosocomiales, 2 (3,5-dihydroxyphényl) -benzofuran 5,6-diol.

INTRODUCTION

Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth. The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs.[1]

The genus *Terminalia* is highly versatile in ethnobotanical applications. Our group reported the antisickling potential of *Terminalia catappa*[2] while Yang *et al.*[3] reported its anticancer potential. Although several African species of the *Terminalia* genus have a long history of successful application in local therapies,[4-6] only a few have been studied beyond the crude extract level. *Terminalia ivorensis* is one of the species reputed for several applications locally with paucity of research that links their biological activity with isolated bioactive compounds.

T. ivorensis is a common plant that is mostly distributed between Guinea, Cameroon, Cote d'Ivoire and Nigeria. The plant has several ethnomedicinal uses. In Ghana, the bark is used for the treatment of wounds, sores and cuts. It is also used in the treatment of rheumatism and syphilis among the Jukuns. *T. ivorensis* was reported to be effective against *T. brucei rhodensiense* parasite, one of the causative organisms of trypanosomiasis.[7,8] Current studies involved a bioguided investigation of the antibacterial activities of *T. ivorensis* which led to the isolation of a bioactive compound.

METHODS

Nuclear Magnetic Resonance (NMR) data were obtained on a Bruker 500 MHZ model using Deuterated methanol as solvent and TMS as Internal standard. Electrospray Ionization Mass Spec (ESI-MS) and High Resolution Mass Spec (HRMS) data were obtained from Agilent Mass Spectrophotometer.

(All Spectral data were obtained from the Facilities of Indian Institute of Integrative Medicine, Jammu, India).

Plant material

Fresh leaves of *Terminalia ivorensis* was collected from the Botanical Garden of the University of Ibadan. The Curator of the Botanical Garden identified the sample and Voucher specimen was deposited at the Department. The leaves were air dried, milled into fine powders and stored in closed glass bottles in the dark cupboard until use.

Extraction

The dried powdered plant material of *T. Ivorensis* (~500g) was extracted with 5 litres of acetone by cold maceration for 72 hours with intermittent shaking. The extract was filtered with Whatman No. 1 filter paper and concentrated on a Rotary evaporator to minimum volume at 45^{0}C. The extract was finally dried under a stream of cool air and the weight of the extract was determined. The quantity of material extracted from *Terminalia ivorensis* was 9 %.

Antimicrobial activity of acetone extract

The microbial cultures used in the determination of the microbial sensitivity and minimum inhibitory concentration (MIC) test were isolates obtained from the Department of Pharmaceutical Microbiology, University of Ibadan. They are: *Bacillus substilis, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa*. Experiment was carried out in duplicate.

The susceptibility testing of the bacteria to the plant extract was performed using Agar cup diffusion method. 0.05mls (a drop) of each actively growing culture of the four organisms were introduced into four sterile plates. Molten agar that was cooled to 45^{0}C was added to the sterile plates, mixed thoroughly with the organism and allowed to set. A cork borer was used to bore a hole in each plate and two drops of the solution of the extract was introduced into the hole, which was allowed to diffuse and then incubated at 37^{0}C for 24 hours after which the zone of inhibition was measured around each hole containing the extract at different concentrations. Gentamicin (40mg/ml) was used as positive control.

Solvent-solvent fractionation of dried plant extract

Thirty grams of the dried acetone extract was subjected to solvent-solvent fractionation according to the method described by Suffness *et al*[9] with some modifications. This involved a partition of the extract suspended in 80 % aqueous methanol into three fractions: hexane, ethyl acetate and Aqueous methanol fractions. The three fractions were tested for antimicrobial activity at 15mg/ml by measuring the zone of inhibition against *E. coli* to identify the most active fraction.

Chromatographic separation of ethyl acetate fraction

Acetone extract (2.5g) was subjected to open column chromatographic separation and eluted with a gradient solvent system of hexane and ethyl acetate. 400ml each of 80/20, 70/30, 60/40, 200ml of; 50/50, 40/60, 30/70,

20/80, 10/90, 100% of ethyl acetate and 50/50 acetone-ethyl acetate. The column was then washed with 100% acetone.

A total of 78 fractions of 30mls each were collected. Combinations into 7 major fractions were made based on TLC fingerprint developed in chloroform: ethyl acetate (5:5) mobile system. F4 which was the most active was obtained as a single spot for spectroscopic analysis. The antibacterial activity of the fractions was carried out using the method described below (MIC).

Minimum Inhibitory Concentration (MIC) determination

The MIC values for the column fractions and the crude plant extracts were determined by serial agar plate dilution method. Five serial two fold dilutions for each fraction were made as follows: 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml and 0.625mg/ml. The ethyl acetate fraction and whole (or acetone) extract were also serially diluted: 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.5625mg/ml. Each bottle containing 18ml of nutrient agar was melted and cooled to 45-50˚C and 2ml of each of the concentration or dilution was added. The bottle was mixed properly and poured in a Petri dish. The agar was then allowed to set firmly. The bacterium (*E.coli*) was then streaked accordingly and the plates incubated at 37˚C for 24 hours from which the MIC values were determined.

Spectroscopic analysis of isolated compound

Spectral data (ESI-MS, HRMS, 1D and 2D NMR on a Bruker 500 MHz model) data were obtained for the compound and the structure of the isolated compound elucidated.

Statistical analysis

The experiments were done in duplicate. The results are given as mean +standard deviation (SD). One- way Analysis of Variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant at $p < 0.05$ (GraphPad Prism 5 was used).

RESULTS

Antimicrobial activity of acetone extract

Activity of the whole extract presented in Table 1 showed that the four nosocomial organisms are sensitive to the extract. *E.coli* and *S. aureus* had the highest susceptibility (15.625mg/ml). The Gram-positive organism, *B. substilis* exhibited the least sensitivity to the extract, with its growth only being inhibited at a concentration of 125mg/ml.

Table 1: Antimicrobial activity of acetone extract of *T. ivorensis*

Concentration	*S. aureus*	*E. coli*	*B. substilis*	*P. aeruginosa*
125mg/ml	1.2±0.05	1.2±0.05	1.1±0.018	1.0±0.062
62.5mg/ml	0.9±0.03	1.0±0.044	-	-
31.25mg/ml	0.9±0.04	1.1±0.05	-	-
15.625mg/ml	0.9±0.06	1.0±0.012	-	-
6.25mg/ml	-	-	-	-
Gentamicin (40mg/ml)	3.0±0.08	1.7±0.064	2.0±0.057	-

Key (-): No inhibition

Average zone of inhibition of the crude extract of *Terminalia ivorensis* bark against four bacteria species using different concentrations of extract. Zone of inhibition is expressed as the diameter in cm. Values represent means and Standard deviations of zones of inhibition.

Antibacterial activity of solvent-solvent fractions

From the results shown in Figure 1, the hexane fraction had the least activity, while the Water/methanol fraction had medium activity. Ethyl acetate had the highest zone of inhibition which is significantly higher than the other fractions when compared by Tukey's post test in a One-way ANOVA analysis.

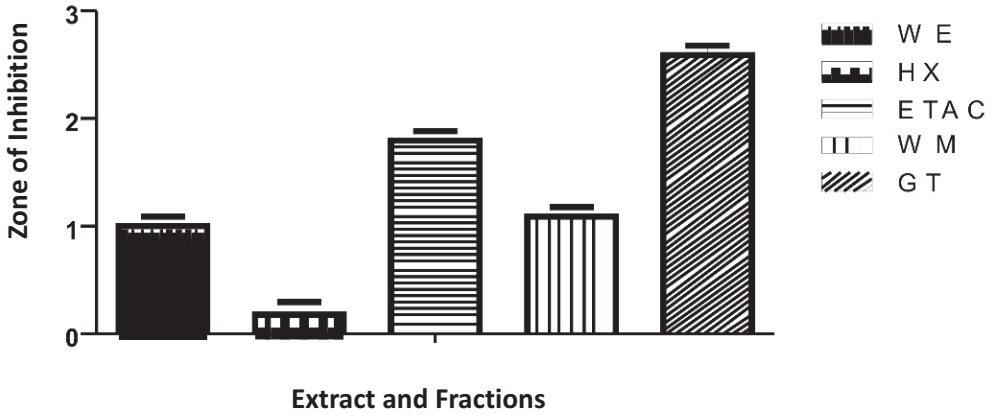


Figure 1. Zone of inhibition of the solvent –solvent fractionation of *T. ivorensis. WE represents the whole extract; HX is the hexane fraction; ETAC is the ethyl acetate fraction; WM is the water-methanol fraction; GT is Gentamycin (positive control).*

Activity of column fractions.

The result of the antibacterial activity of column fractions against *E. coli* is shown in Figure 2.

Fraction F4 had the highest activity with the lowest MIC value at 1.25mg/ml. Its MIC value is significantly lower than the other fractions. Fractions 6 and 7 had medium activity but the activity is significant when compared with the other inactive fractions by One-way ANOVA post analysis Tukey's comparison.

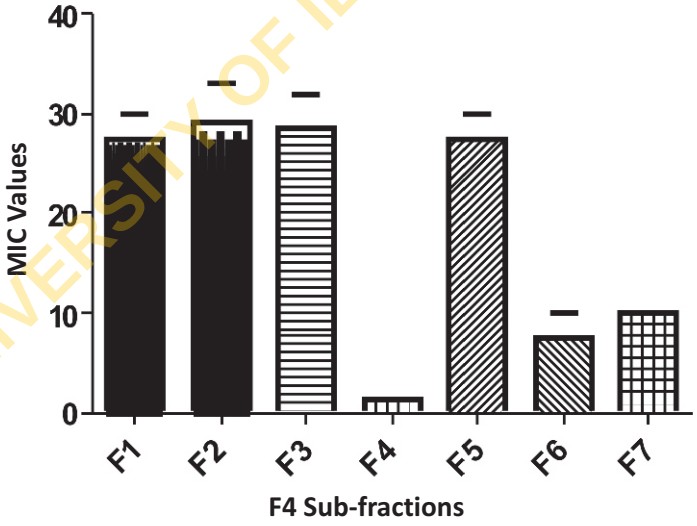


Figure 2. MIC of various fractions from Column Chromatography. Column fractions of ethyl acetate fraction with different MIC values. The lowest bar (F4) showed the highest activity with the lowest MIC value.

Structural elucidation of isolated compound

ESI- MS indicated the isolated compound have *m/z* 259 $(M+H)^+$ corresponding to m/z 258; while HRMS m/z 259.0607$(M+H)^+$ (calculated 259.0601 for $C_{14}H_{11}O_5^+$) confirmed the molecular formulae to be $C_{14}H_{10}O_5$.

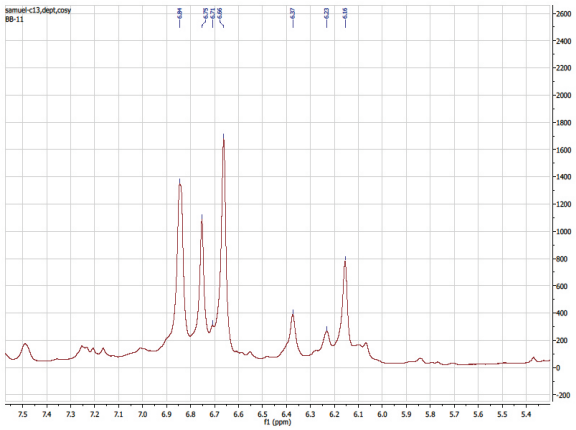


Figure 3: ^{1}H NMR Spectrum of F4

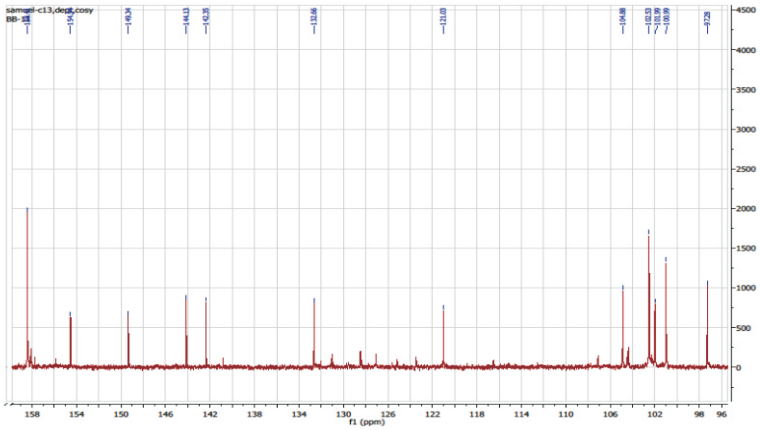
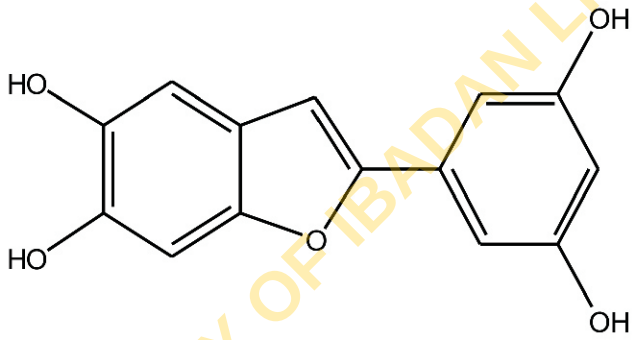


Figure 4: ^{13}C NMR Spectrum of F4

The ^{1}H and ^{13}C spectra data in Figures 3 and 4 [expanded downfield region] showed signals above 5.5ppm and 95ppm respectively indicating a compound that is essentially aromatic with no aliphatic linkages or methoxy substitutions. Correlation of the NMR, IR and MS data with literature confirm the compound to be 2-(3,5-dihydroxyphenyl)benzofuran-5,6-diol.[10,11]



2-(3,5-dihydroxyphenyl) benzofuran-5,6-diol

DISCUSSION

It is known that *Staphylococcus aureus* is the most frequently encountered microorganism in wounds.[12] From the susceptibility profile shown in Table 1, *S. aureus* and *E.coli* had the best sensitivity to the extract with zones of inhibition observed at 15.625mg/ml. These are commonly associated with human infections. Ethnobotanical information indicated treatment of infections as a major application of *Terminalia ivorensis*. The observed sensitivity of *S. aureus* to the extract possibly obviates the local application.[13]

Bioactivity screening of solvent- solvent partitioning indicated that the ethyl acetate fraction had the highest activity. Other workers investigating the activity of *T. ivorensis* extract against *T. brucei rhodensiense* also found ethyl acetate as the most active fraction.[8] There is possibly a link in the bioactive compound responsible for the anti-trypanosome studies they carried out and the current study. The observed independent activities of ethyl acetate fraction against virulent microorganisms suggest that even if non-purified fractions of the plant is to be used due to the commonly observed synergistic effect in bioactive extracts; ethyl acetate fraction is possibly a valuable option.[14]

The most polar fraction is often rich in sugars and high molecule weight polyphenols and a few glycosides. Eloff[15] observed they often have low antimicrobial activity. The medium polarity fraction is often associated with aglycones of several classes of compounds such as the flavonoids, terpenoids, saponins, among others which are associated with high biological activity.

2-(3,5-dihydroxyphenyl)benzofuran-5,6-diol which was identified as a major active compound in this extract is in a group of the compounds referred as phytoalexins. Phytoalexins are known to protect plants against invasion by microbes.[16] They are reported to have strong antimicrobial and antioxidant activities.[17] Preliminary findings by our group also indicated very significant

antioxidant activities for this extract. These characteristics possibly explain the rationale for the multipurpose application of this plant in local therapies. Since it is a known fact that oxidative stress is the baseline for a number of diseases such as cancer, ulcer and inflammatory diseases; a compound in a class that has significant antioxidant and antibacterial activities is potentially valuable in the treatment/management of several ailments. The structure of the isolated compound also makes it amenable for Structural Activity Relationship studies through modifications for optimization of activity and toxicity profile.

Our results suggest there is a basis for the application of this extract in diseases mediated through the four nosocomial microorganisms used in the current study.

Further studies to establish the *in-vivo* activity and full toxicity profile for this extract or fractions will be valuable before the consideration of developing this product as a phyto-drug.

CONCLUSION

Terminalia genus is a potential source of effective therapies for the treatment of infectious diseases. The whole extract of *T. Ivorensis* was active against all screened organisms (significantly against *E. coli* and *S. aureus*).

The MIC values for 2-(3,5dihydroxyphenyl)benzofuran-5,6-diol, ethyl acetate fraction and whole extract against *E. coli* were 1.25mg/ml, 3.125mg/ml and 12.5mg/ml respectively.

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