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Research Article

# THE LEAVE EXTRACTS OF *HEERA INSIGNIS* (DEL) INHIBITS RESISTANCE BACTERIA, FUNGI AND *MYCOBACTERIUM BOVIS*

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**ABSTRACT**

The hexane (HE), dichloromethane (DCM), ethylacetate (EA) and methanol (ME) extracts of the leaves of *Heeria insignis.* A plant with wide ethnomedicinal application in Northern Nigeria, was evaluated for anti-tuberculosis and antimicrobial activities against the following clinical isolates; *Bacillus subtilis, Enterobacter Sp,Staphylococcus aureus, Shigelladysenteriae, Methicillin resistant Staphylococcus aureus (MRSA), Vancomycin resistant enterococci (VRE), Streptococcus feacalis, Pseudomonas aeruginosa, Proteus rettgeris*, *Candida albicans, Candida Pseudotropicalis, Candida stellatoidea and mycobacterium bovis.* The result of the zone of inhibition (ZI) of the test extracts on the microorganism ranges from 20 to 34 mm against all test organism with the exception of *P.aeruginosa*, *S. feacalis* and *C. albicans*. Minimum inhibitory concentration (MIC) determination reveals that a low concentration of 5mg/mL of EA and DCM inhibited the growth of all test organism with the exception of *VRE* which was inhibited at 10 mg/mL. These extracts also showed a bactericidal and fungicidal effect at 10 mg/mL. While the rest of the extracts showed MIC, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) ranging from 10 to 40 mg/mL. The anti-TB evaluation reveals that the DCM extract was the most active with MIC of 0.625 mg/mL.The result of this investigation clearly shows the potential of the plant in the search for anti-TB and antimicrobial agents from nature.

**Keywords**: *Heeria insignis*, resistance bacteria, Fungi, Antituberculosis.

## INTRODUCTION

Tuberculosis, also called TB, is currently a major health problem especially in the developing Countries. It has been classified as one of the neglected tropical diseases (NTD), the emergence and wide spread of TB is mainly due to multidrug-resistant forms of TB strains[1]. Long regime of treatment of effective drugs and treatment involves drug combination therapy. Global efforts are underway to eradicate TB using new drugs with new modes of action, higher activity and fewer side effects in combination with vaccines. For this reason, unexplored new sources need to be examined, to explore the possibilities of developing drugs from these new sources.

*Heeria insignis* which belong to the *Anacardiaceae* family is a shrub that may grow to a tree 6.5 m high, it is an indigenous African shrub found extensively in the southern Savanna from Senegal to Niger and Nigeria[2]. In northern Nigeria, this plant species is used widely in the treatment of diarrhoea, venereal disease, tapeworm and hookworm, schistosomiasis, kidney trouble and in the treatment of tuberculosis[3].

In the current investigation, we report our findings on the antimicrobial and anti-tuberculosis activity of the methanol, ethyl acetate, dichloromethane and hexane extracts of the leaves of *Heeria insignis* against pathogenic bacteria, fungi and *Mycobacterium bovis*.

## MATERIALS AND METHODS

### Plant material

The plant material was collected fresh from Zaria, Nigeria in September, 2013. Taxonomical identification was done at the Herbarium of the Biological Sciences Department, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen with number 014 was deposited in Herbarium. The plant was air-dried under shade, segregated and pulverized by mechanical pounding using wooden mortar and pestle. The pulverized plant material was stored away from moisture until needed for extraction.

### Extraction

The pulverized leaves of *Heera insignis* (500 g) was carefully weighed and macerated with 95% methanol for one week. It was then decanted and filtered, the process was repeated three times for exhaustive extraction. The three sets of extracts were combined on confirmation to be the same by TLC analysis. The combined methanol extract was then partitioned with hexane, dichloromethane and ethyl acetate. The solvent fractions were then concentrated in vacuum at 40˚C using rotary evaporator and later subjected to air drying to give dried crude extracts.

### Phytochemical screening

The hexane, dichloromethane, ethyl acetate and the methanol extracts of the plant, were subjected to phytochemical screening using standard techniques[4]. The metabolites tested for include, carbohydrates, tannins, saponin, flavonoids, anthraquinones, cardiac glycosides, steroids, terpenes and alkaloids.

### Antimicrobial studies

The antimicrobial activities of the HE, DCM, EA and ME extracts and standard drugs (Ciprofloxacin, Sparfloxacin and Fluoconazole) were determined using microbial strains and fungi obtained from the Department of Medical Microbiology, Ahmadu Bello University

Teaching Hospital Zaria, Nigeria (ABUTH); *Shigella dysenteriae, Salmonella typhi, Corynebacterium ulcerans, Klebsiella pneumoniae, Staphylococcus aureus, Methicillin resistant staphyllococcus aureus* (MRSA)*, Proteus mirabilis, Streptococcus pneumoniae, Candida tropicalis,Candida krusei* and *Candida albicans*.

The agar-in-well diffusion method of Preeti *et al* (2014)[5] was used to determine the antimicrobial activity of the test extracts. Briefly; pure cultures of the bacterial organisms were inoculated on to Mueller Hinton Agar (MERCK) and incubated for 24h at 38 ºC. About 5 discrete colonies were asceptically transferred using sterile wire loops into tubes containing sterile normal saline (0.85% NaCl) and were adjusted to a turbidity of 0.5 MacFarland Standard. The suspensions were then inoculated on the surface of sterile Mueller – Hinton Agar plates using sterile cotton swabs. A sterile 6 mm diameter cork borer was used to make holes (wells) into the set of inoculated Mueller-Hinton Agar. The wells were filled with different concentration of the test extracts. The plates were incubated for 24h at 38 ºC, while the fungi were incubated at 34ºC for 48h. All the tests were performed in triplicate and the antibacterial activities were determined as mean diameters of inhibition zone (mm) produced by the test extracts/drugs.

**Minimum Inhibitory Concentration (MIC)**

The minimum inhibition concentrations (MIC) were determined for the extracts using micro broth dilution method in accordance withVollekova *et al* (2001)[6]. Serial dilution of the least concentration of the extracts that showed activity were prepared using test tubes containing 9 mL of double strength nutrient broth (OXOID). The test tubes were inoculated with the suspension of the standardized inocula and incubated at 38 ºC for 18h. Minimum inhibition concentrations (MIC) were recorded as the lowest concentrations of the compounds showing no visible growth (turbidity) in the broth.

**Minimum Bactericidal Concentration (MBC/MFC)**

The minimum bactericidal and minimum fungicidal concentration were determined by aseptically inoculating aliquots of culture, from the minimum inhibition concentration (MIC) tubes that showed no growth, on sterile nutrient agar (OXOID) plates and incubated at 38ºC for bacteria and 34ºC for fungi for 48h. The MBC/MFCs were recorded as the lowest concentration of extracts showing no bacterial growth at all.

**Tuberculosis**

The anti-tuberculosis activity of the extracts was carried out using the method describe by Oladosu *et al* (2014)[7].

**Preparation of Extract**

About 100 mg of each extract was transferred into a sterile bottle, then dissolve with 0.5 mL dimethylsulphoxide (DMSO) and 0.5 mL distill water. The extracts were further diluted (1:10) in 7H9 Middlebrook broth to give 10 mg/mL concentration.

**Preparation of *Mycobacterium bovis* (BCG)**

*Mycobacterium bovis* (500 µL) freshly prepared stock was inoculated into 50 mL of sterile Middlebrook 7H9/ADC broth medium and incubated at 30ºC for 5 to 7 days. The optical density of resulting culture was measured using a uv-spectrophotometer. The optical density (OD) of resulting culture determined at 650 nm was approximately 0.2 which is equivalent to $10^9$cfu/mL.

**Anti-TB assay**

The micro broth dilution in sterile 96 microwell plate method as describe by Oladosu *et al* (2014)[7] was employed for the determination of anti-TB activity of the extracts. Into each well of 96 microwell plate was transferred 50 µL of sterile 7H9 broth starting from well 2 to 12. To each of the first well was added 100 µL of 10% DMSO, 100 µL of 25 µg/mL solution of rifampicin (control drug, prepared by dissolving 250 mg of rifampicin powder in 10 mLDMSOand diluted 1: 1000 by dispensing 25 µL of rifampicin in 25 mL 7H9 Middlebrook broth) and 100 µL of each plant extract. Using a multichannel pipettor 50 µL was carefully removed from well 1 to 2, mixed thoroughly and the process continued to well 11 from which 50 µL was withdrawn and discarded. The wells were inoculated with 50 µL of diluted BCG culture and incubated at 30ºC for a period of seven days. The results were confirmed by staining the wells with tetrazolium dye after the incubating period. The reduction of tetrazolium salt from colourless to brightly coloured derivative in the wells is an indication of sample inactivity but if the dye remains colourless that confirms the activity of the sample. The last well where there was no colour change is regarded as the minimum inhibitory concentration (MIC) of the sample.

**RESULTS AND DISCUSSION**

Recently there has been considerable interest in the use of plant material as an alternative method to control pathogenic microorganism[8] and many components of plants products have been shown to be specially targeted against resistant pathogenic bacteria[9]. The emergence of multidrug resistant strain of many pathogens is a serious threat and makes chemotherapy more difficult. Moreover, the current cost of most of the chemotherapeutic agents is unbearable to the public especially in developing countries[10].Therefore attempts must be made towards the development of effective natural, non-toxic drug for treatment. The present work was done to explore the antimicrobial and antituberculosis property of *Heeria insignis*, a medicinal plant used in Nigeria for various purposes including skin diseases, diarrhoea and tuberculosis. Preliminary phytochemical study on the leaves of *H. insignis* revealed the presence of Carbohydrate, Glycosides, Cardiac glycoside, Saponins, tannins flavonoids, alkaloids, Steroids and Triterpenes (Table 1)**.** These result are similar to a large extent to that reported by others[11,12,13], except that we found alkaloids present in our findings but Agunu *et al* (2011)[11] reported that alkaloids were absent.

The result of the zone of inhibition (ZI) of the hexane extract shows ZI ranging from 20 to 22 mm, the DCM (26-31 mm), ME ( 20 and 26 mm) while EA ranges from 20 to 34 mm against all test organism with the exception of *P. aeruginosa*, S. *feacalis* and *C. albicans*. Minimum inhibitory concentration (MIC) result reveals that a low concentration of 10 mg/mL of HE and ME inhibited the growth of all test organism, also, a concentration of 5 mg/mL of EA and DCM inhibited the growth of all test organism with the exception of *VRE*. The DCM and EA extracts did not only inhibit the growth of the test microbes, but were found to be bactericidal and fungicidal at 10 mg/mL. While the rest of the extract showed MIC, MBC and MFC ranging from 10 to 40 mg/mL. The ethyl acetate extract exhibited the highest activity against *B. subtilis* with ZI of 34 mm, MIC of 5mg/mL, MBC/MFCof 10 mg/mL. This activity against *B. subtilis* was comparable with the standard antibiotic Sparfloxacine (34 mm) and even more active than Ciprofloxacine (31 mm) as shown in Table 2. Anti-TB evaluation (Table 5) showed that the DCM fraction had the highest activity against *Mycobacterium bovis* with MIC of 0.625 mg/mL, followed by ethyl acetate extract (1.25 mg/mL), hexane and methanol extracts did not show activity. Our investigation, clearly demonstrated the potential of *H. insignis* in the search for new anti-TB and antimicrobial agents from nature.

**Table 1. Phytochemical screening of the extracts of *Heeria Insignis***

| Metabolites | Hex | DCM | EA | MeOH |
|---|---|---|---|---|
| Carbohydrate | - | + | + | + |
| Cardiac glycoside | + | + | + | + |
| Tannins | - | + | + | + |
| Saponins | - | + | - | + |
| Flavonoids | - | + | + | + |
| Anthraquinones | - | - | - | - |
| Steroids | + | + | - | + |
| Triterpenes | + | + | - | + |
| Glycosides | + | + | + | + |
| Alkaloids | - | + | + | + |

**Key:** + = present, - = absent, HE = hexane extract, DCM = dichloromethane extracts, EA = Ethyl acetate extracts, ME = Methanol extracts

**Table 2. Determination of Zones of Inhibition of test extracts and drugs**

| TEST ORGANISMS | DCM | EA | ME | HEX | CPX | SPX | FCZ |
|---|---|---|---|---|---|---|---|
| ***MRSA*** | 27 | 31 | 25 | 22 | 35 | 35 | - |
| ***VRE*** | 26 | 30 | 24 | 20 | - | 35 | - |
| ***S. aureus*** | 20 | 20 | 20 | 20 | 37 | 41 | - |
| ***S. feacalis*** | - | - | - | - | 34 | 37 | - |
| ***B. subtilis*** | 31 | 34 | 26 | 21 | 31 | 34 | - |
| ***P. aeruginosa*** | - | - | - | - | - | 32 | - |
| ***Enterobacter sp*** | 27 | 33 | 24 | 21 | 34 | 35 | - |
| ***P. rettgeris*** | - | - | - | - | 35 | 37 | - |
| ***S. dysenteriae*** | 30 | 32 | 26 | 22 | 39 | 40 | - |
| ***C. Stellatoidea*** | 28 | 32 | 24 | 20 | - | - | 37 |
| ***C. pseudotropicalis*** | 29 | 33 | 25 | 21 | - | - | 32 |
| ***C. albicans*** | - | - | - | | - | - | 36 |

**Key**: HE = Hexane extract, DCM= Dichloromethane extracts, EA = Ethyl acetate extracts, ME = Methanol extracts, CPX=ciprofloxacine, SPX=sparfloxacine, FCZ = fluconazole, - = no activity

**Table 3. Results of Minimum Inhibitory Concentration (MIC)**

| Test Organisms | DCM | EA | ME | HEX |
|---|---|---|---|---|
| ***MRSE*** | 5 | 5 | 10 | 10 |
| ***VRE*** | 10 | 5 | 10 | 10 |
| ***S. aureus*** | 5 | 5 | 10 | 10 |
| ***B. subtilis*** | 5 | 5 | 10 | 10 |
| ***Enterobacter sp*** | 5 | 5 | 10 | 10 |
| ***S. dysenteries*** | 5 | 5 | 10 | 10 |
| ***C. stellatoidea*** | 5 | 5 | 10 | 10 |
| ***C. pseudotropicalis*** | 5 | 5 | 10 | 10 |

**Key:** HE = Hexane extract, DCM= Dichloromethane extracts, EA = Ethyl acetate extracts, ME = Methanol extract

**Table 4. Results of Minimum Bactericidal /Fungicidal Concentration (MBC/MFC)**

| Test Organisms | DCM | EA | ME | HEX |
|---|---|---|---|---|
| ***MRSA*** | 20 | 10 | 20 | 20 |
| ***VRE*** | 20 | 10 | 20 | 40 |
| ***S. aureus*** | 10 | 10 | 20 | 20 |
| ***B. subtilis*** | 10 | 10 | 20 | 20 |
| ***Enterobacter sp*** | 20 | 10 | 20 | 20 |
| ***S. dysenteries*** | 10 | 10 | 20 | 20 |
| ***C. stellatoidea*** | 10 | 10 | 20 | 40 |
| ***C. pseudotropicalis*** | 10 | 10 | 20 | 20 |

**Key:** HE = Hexane extract, DCM= Dichloromethane extracts, EA = Ethyl acetate extracts, ME = Methanol extract

**Table 5: Results of Antituberculosis screening against *Mycobacterium bovis***

| Extract Concentration (mg/mL) | HE | DCM | EA | ME | Rf |
|---|---|---|---|---|---|
| 5 | NA | + | + | NA | + |
| 2.5 | NA | + | + | NA | + |
| 1.25 | NA | + | + | NA | + |
| 0.675 | NA | + | NA | NA | + |
| 0.3125 | NA | NA | NA | NA | + |

**Key** : NA= not active; + = active; Rf = rifampicin Hex= Hexane; Dcm= Dichloromethane; EA= ethyl acetate; ME= Methanol

## CONCLUSION

The extracts of *Heeria insignis* were found to inhibit *Mycobacterium bovis* and most of the clinical isolated microorganism and fungi. The broad spectrum exhibited by the extracts from this plant seems to justifie the traditional uses of the plant in folk medicine.

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