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Evaluation of *In-Vitro* Anti-Tuberculosis Activity of *Tetrapleura tetraptera* Crude and Fractions on Multidrug Resistant *Mycobacterium tuberculosis*

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Abstract

The management, control and elimination of tuberculosis (TB) have been difficult with the advent of HIV and cases of multidrug resistant (MDR-TB) tuberculosis. The cases of multidrug resistance to rifampicin and isoniazid pose greater challenges on first line and second line drugs to eliminate TB. The study is aimed at establishing anti-tuberculosis activity of *Tetrapleura tetraptera* against *Mycobacterium tuberculosis* and MDR-TB and the phytochemical present. The leaves of *Tetrapleura tetraptera* were collected, weighed, dried and pulverized to powder. The pulverized leaves of *Tetrapleura tetraptera* were subjected to 70% methanol extraction and screened for phytochemical. The crude extract was further purified into fractions using silica gel and thin layer chromatography techniques. *M. tuberculosis* and MDR-TB were obtained from positive acid fast bacilli sputa of TB patients and confirmed using GeneXpert to differentiate genotypic drug susceptible *M. tuberculosis* and MDR-TB. The sputa were digested using sodium hydroxide-cysteine technique and cultured in Middlebrook 7H9. The crude extract and fractions were screened for anti-tuberculosis activity using tetrazolium microtitre plate assay. The results showed that *Tetrapleura tetraptera crude* had activities against *M. tuberculosis* at 7.4 ± 0 mg/ml and 27.5 ± 0 mg/ml for MDR-TB. One of the fractions inhibited the growth of *M. tuberculosis* at 0.24 ± 0 mg/ml and MDR-TB at 0.89 ± 0 mg/ml. The phytochemical screened includes tannins, alkaloids, saponins, fla-

vonoids, phenols and resins. *T. tetraptra* possesses anti-tuberculosis potential at low concentration on MDR-TB and can be a lead compound in drug development for the treatment of tuberculosis and multidrug resistant tuberculosis.

Keywords

Anti-Tuberculosis, *Tetrapleura tetraptera*, Tetrazolium Assay, Tuberculosis, Phyto-Chemical, MDR-TB

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, a bacterium that infects a one third of the world population with 2 billion people infected and 1.8 million deaths in 2016 [1] [2]. According to WHO [2] estimates that between year 2000 and 2020 nearly one billion people will become newly infected with, 200 million people will get sick and another 35 million will die from tuberculosis. The advent of HIV/AIDS pandemic in the 1980s, struck a blow to this optimism and there has been a global resurgence of TB [3]-[9]. MDR tuberculosis is among the most worrisome element of the pandemic of antibiotic resistance because TB patients fail treatments which have high risk of death [10] [11] [12]. In 2016, the WHO estimated that 4.1% of new cases and 20% of the previously treated cases of TB were MDR-TB [2]. There were also approximately 190,000 deaths from MDR TB. More than half of these patients were in India, China and the Russia Federation. Among patients with infectious (smear positive) pulmonary disease, MDR TB was seen in 35% of newly detected cases, and in a massive 76% of previously treated patients [12]. There are high burden drug resistant TB countries including India and South Africa. Today MDR TB spreads unchecked in most of the world. It is fueled by poverty at the individual and family levels, limiting access to effective treatment and at the regional and national levels where under resourced governments lacks the capacity to tackle this disease [13]. After the introduction of rifampicin, no worthwhile antituberculosis drug with new mechanisms of action has been developed in over thirty years [11]. Globally, there is a challenge to develop new drugs especially for the MDR TB that is ravaging the pandemic of antibiotic resistance because TB patients fail treatments which have a high risk of death [4]. The available TB drugs used especially for the MDR TB have varied degrees of toxicity on the users. The plants are diverse in nature and source of future drugs lead [14] [15]. Anochie *et al.* [15] reported that some Nigerian medicinal plants claimed by the traditional medicinal practitioners to cure TB were found not active against *M. tuberculosis*, the plants include *Crinum glaucum*, *Treculis aficana*, *Erthrina mildaedi*, *Fucus thonningis* and *Xylopis aethiopica.* Nigerian plants have been reported to possess antitubercular activities. Ethnobotanical surveys on Nigerian medicinal plants have been reportedly carried in the different Nigeria geo-political zones. Ibekwe *et al.* [16] reported the findings of the efficacy of some Nigerian plants used by tradi-

tional medicine practitioners in the management of tuberculosis. Others reported the potentials of Nigerian plants in the treatment of tuberculosis in the Western part of Nigeria [17], Middle belt areas of Nigeria [3], Adamawa State of Nigeria [18]. Some medicinal plants indigenous in Ethiopia including *Pterolobium stellatum*, *Vernonia amygdalina*, *Allium ursium* and *Dodonaea angulstifolia* have been reported to possess anti-mycobacterial activity [13] [19]. *Tetrapleura tetraptera* is a woody plant growing in the deciduous and fringing forest. The plant can grow up to 80 ft high and over 4 ft girth with sharp buttresses. The foliage is dark and bi-pinnate leaves with thin bark. The leaves of *T. tetraptera* are commonly used as flavor because of the caramel-like smell [20]. The medicinal potentials have been explored by the locals in West Africa. The Binis use the seeds to improve taste of soup. The fruits are used as medicinal for children in Yorubaland of Nigeria. The common names include Twi by the Ashanti in Ghana [20], Ighirehimi in Esan (Edo) [21]. It is commonly used in soups for nursing mothers to prevent post-partum contractions [22]. The soft parts of the fruit and bark are known to contain sugars, tannins, traces of saponin and amino acids [22]. Many works have been done on the plants of *T. tetraptera* [22] [23] [24] [25] [26] but little had been reported on the anti-mycobacterial activity of the leaves of *T. tetraptera* in the literature. The study is aimed to investigate the anti-mycobacterial activity and phytochemical screening of the leaves of *T. tetraptera.*

2. Materials and Methods

2.1. Collection and Authentication of the Plants

The *T. tetraptera* plant materials were collected from the wild in forest reserve in Ihievbe Town in Owan East of Edo State of Nigeria. The leaves and seeds were identified and authenticated at the Herbarium Unit of National Institute for Pharmaceutical Research and Development with a voucher number NIPRD/H/6808. The leaves were washed thoroughly in tap water and with distilled water and air dried at room temperature to constant weight. The plant materials were sorted out and dry milled into the powder form, sieved and kept safe until needed in the Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development.

2.2. Preparation of Extracts and Fractionation

Two hundred gram of the pulverized powder was extracted by maceration with 70% aqueous methanol for 72 hours at 25˚C on rotary shaker at 1000 rpm [27]. The extracts were then filtered and concentrated aseptically under reduced pressure using rotary evaporator to remove methanol and some water.

2.3. Preparation of Purified Fractions

The crude methanol extract of the plant was purified using column chromatography and thin layer chromatographer (TLC). Glass column was packed with silcal gel which then adsorbed the crude extract. It was then packed onto

the column layer. The mobile phase consisted of three solvents, hexane, ethyl-acetate and methanol mixed in different proportions 100, 80, 60, 40, and 20 mL of methanol followed by 20, 40, 60, 80 and 100 mL ethyl-acetate. The various proportions of the solvents were pushed through the bed. Ten fractions were obtained. The fractions were pooled together by thin layer chromatography according to fingerprint (Table 1). This reduced the number of the fractions to six. Fractions A, B and C were used for anti-tubercular activity. The three fractions produced minute fraction and therefore not used in anti-mycobacterial assay.

2.4. Analysis for the Phytochemical Components

The plant extracts were screened for the presence of biological active components. The phyto-chemical tested for included alkaloids, anthraquinones, catachol, flavonoids, phenols, saponin, steroids, tri-terpenoids, and tannins using the standard methods. The standard techniques adopted were for screening the qualitative analysis of the phyto-chemical components. [14] [28].

2.5. Isolation and Identification of *M. tuberculosis* and MDR Tuberculosis Isolates from Patient Sputum

The sputa obtained from DOTS center of National Institute for Pharmaceutical Research and Development (NIPRD) were screened for acid fast bacilli (AFB) using Ziehl-Neelsen technique [29] [30] [31]. The positive AFB sputa were

Table 1. TLC spots on TLC plate of *Tetrapleura tetraptera* fractions.

Spots on TLC plate	Rf values
1	Rf1 = 0.79
	Rf2 = 0.89
2	Rf1 = 0.26
	Rf2 = 0.42
	Rf3 = 0.58
	Rf4 = 0.66
	Rf5 = 0.77
	Rf6 = 0.91
3	Rf1 = 0.77
	Rf2 = 0.91
4	Rf1 = 0.40
	Rf2 = 0.77
	Rf3 = 0.91
5	Rf1 = 0.17
	Rf2 = 0.32
6	Rf1 = 0

analysed with GeneXpert [29] to confirm drug susceptible and multi-drug-resistant tuberculosis. These sputa positive for drug susceptible and resistant TB were digested and decontaminated with NaOH-cysteine technique [32] [33] and were cultured in Middlebrook 7H9 (Sigma) for seven days with daily observation for turbidity and monitor for AFB with Ziehl-Neelsen technique.

2.6. Determination of Minimum Inhibitory Concentration

Two milligram each of the extract was dissolved in 1.0 mL dimethyl-sulphoxide (DMSO) to aid dissolution and made up with sterile distilled water to give 5.0 mL volume and filtered with syringe fixed membrane filters (0.45 µm). Two fold dilutions of the crude extract and fractions were made to obtain the desired starting concentrations mg per ml of the both crude and fractions to be used. 50 µl of sterile Middle Brook 7H9 broth was dispensed onto the 96 labeled micro well plates excluding the first row each of the eight rows. 100 µl of diluted extract was dispensed onto the first row of the 96 micro well plates. Two fold dilutions were carried out using 50 µl broth of each of the 1stwell and dispensed onto the 2nd well and continue to the 9th. The 10th well contained 100 µl of the extract concentration (extract sterility), 11th well contained 100 µl of media (media sterility control) and the 12th well contained the organism viability control. The procedure was repeated in triplicate. All inoculated plates were incubated at 37˚C for 5 days. At the end 5th day 25 µl of tetrazolium salt was added to wells to determine color change indicative of growth and not activity, while no color change showed further incubation for 48 hours. At end 48 hours 25 µl tetrazolium was further dispensed into controlled wells for activity by change in color. The absence of color change showed inhibition of *M. tuberculosis* (activity) and presence of color showed no inhibition or growth of *M tuberculosis*. The highest dilution that inhibits the growth of the *Mycobacterium* specie is the minimum inhibitory concentration of the plant extract. The above procedure was carried out for the different plants and on the different test M*ycobacterium* species. The above procedure was adopted from their works [34] [35].

2.7. Minimum Bactericidal Concentration

The results from the MIC plates were used for the MBC determination in the wells that did not show growth by means of no color change, 100 µl each was inoculated into sterile Middle Brook 7H10 agar and incubated at 37˚C for 7 days and count the number of colonies and compared with the initial growth per ml (10^5 cfu/ml). The minimum bactericidal concentration was computed if the growth was less than 0.1%. The procedure was adopted from his work [36].

2.8. Statistical Analysis

The statistical used included basic tool and graphical representation to analyze the data generated and use of SSPS version 22 software to analyze the data.

3. Results

3.1. Phytochemical Screening

Table 2 depicts the phytochemical composition of methanol extract of *T. tetraptera*. The qualitative test carried out on the *T. tetraptera* showed the presence of alkaloid, tannins, flavonoids and other phytochemical metabolites.

3.2. Anti-Tubercular Assay

Table 3 depicts the susceptibility profile of crude and fraction extracts of *T. tetraptera* against drug susceptible *M. tuberculosis*. The crude extract inhibited the growth of *M. tuberculosis* at the concentration of 7.4 ± 0 mg/ml (MIC) and 14.8 ± 0 mg/ml (MBC). The fractions A and B inhibited the growth of *M. tuberculosis* at concentrations of 0.25 ± 0 (MIC) and 0.50 ± 0 mg/ml (MBC). The fraction A extract had activity at concentrations of 0.11 ± 0 mg/ml (MIC) and 0.22 ± 0 mg/ml (MBC). The rifampicin had activity at 0.002 ± 0 mg/ml. The susceptible profile of *M. tuberculosis* as summarized in Figure 1.

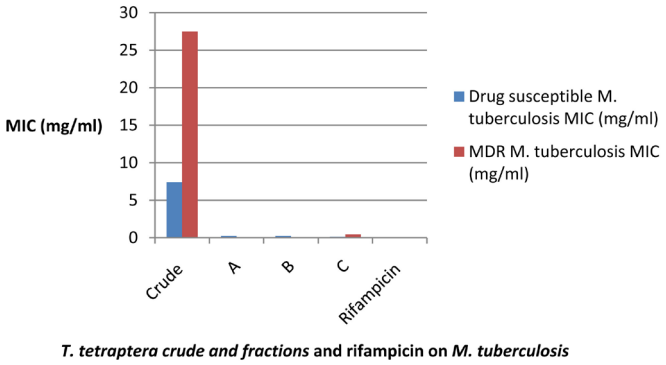

Figure 1. MIC of crude and fractions of *T. tetraptera* and Rifampicin against *M. tuberculosis*.

Table 2. Phytochemical compositions of methanol crude extract *T. tetraptera* screened.

Parameters	*Tetrapleura tetraptera*
Tannins	+
Alkaloids	+
Saponins	++
Flavonoids	+
Phenols	+
Terpenes	-
Steroids	-
Glycosides	-
Resins	+
Anthraquinones	-

Table 4 depicts the susceptibility profile of crude and fraction extracts of *T. tetraptera* against MDR-TB isolates. The crude extract had activity against MDR-TB isolates at the concentration of 27.5 ± 0 mg/ml (MIC). The fraction C had activity against MDR-TB isolates at concentrations of 0.87 ± 0 mg/ml (MIC) and 1.78 ± 0 mg/ml (MBC). The fractions A and B extracts and rifampicin had no activity against MDR-TB isolates which was indicated with growths of MDR-TB isolates in the presence of different concentrations of fractions and rifampicin (0.002 mg). The susceptible pattern of *T. tetraptera* extracts and rifampicin summarized in Figure 2.

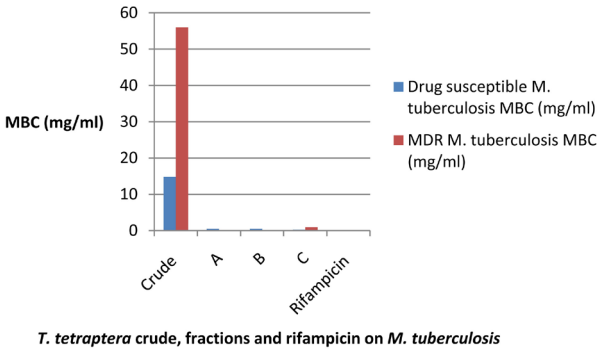

Figure 2. MBC of crude and fractions of *T.tetraptera* and rifampicin against *M. tuberculosis*.

Table 3. Anti-TB activity of crude extract and fractions *T. tetraptera* against drug susceptible *M. tuberculosis*.

T. tetraptera	Initial (mg)	WELLS									MIC	MBC
		1	2	3	4	5	6	7	8	9		
Crude	236.8	-	-	-	-	-	-	+	+	+	7.41	4.8
A	16	-	-	-	-	-	-	+	+	+	0.25	0.50
B	16	-	-	-	-	-	-	+	+	+	0.25	0.50
C	7.04	-	-	-	-	-	-	-	+	+	0.11	0.22
Rifamp	0.002	-	-	-	-	-	-	-	-	-	(0.002)	-

Keys: (-) Activity, (+) No Activity, (A, B, C) fractions, (Rif) Rifampicin, MIC Minimum inhibitory concentration. MBC Minimum bactericidal concentration.

Table 4. Anti-TB activity of crude extract and fractions of *T. tetraptera* against multidrug-resistant tuberculosis.

T. tetraptera	Initial (mg)	WELLS									MIC	MBC
		1	2	3	4	5	6	7	8	9		
Crude	110	-	-	-	+	+	+	+	+	+	27.5	ND
A	8	+	+	+	+	+	+	+	+	+		R
B	8	+	+	+	+	+	+	+	+	+		R
C	7.04	-	-	-	-	-	+	+	+	+	0.891	1.78
Rif	0.002	+	+	+	+	+	+	+	+	+	(0.002)	R

Keys: (-) Activity, (+) No Activity, (R) Resistant (A, B, C) fractions, (Rif) Rifampicin ND Not Done, MIC Minimum inhibitory concentration. MBC Minimum bactericidal concentration.

4. Discussion

There have been ethno-botanic survey reports on the potentials of Nigerian medicinal plants used for the treatment of tuberculosis [15] [16] [17] [27]. Others reported on other plants from other countries for the management of TB in Ethiopian and South Africa [31] [37] [38] [39]. Akintola *et al.* [40] reported the anti-tubercular activity of *Crinum jagus* against *M. tuberculosis*. Similarly, Oladosu *et al.* [41] reported the activity of bioactive compounds from fruits extract of *Acacia nilotica* against *M. tuberculosis*. The information of activity of *T. tetraptera* on *M. tuberculosis* was found to be scanty and not documented.

The findings of this study revealed the presence of phytochemicals including tannins, alkaloids, saponins, flavonoids, phenols and resins with absence of terpenes, steroids, glycosides, and anthraquinones in *T. tetraptra*. The findings of this study agree the results of other researchers on phytochemical screening of *T. tetraptera* plant [23] [42]-[47]. The findings of this study also differed from report of Ebama [48] the presence of glycosides in the leaves of *T.* tetraptera. The presence of phytochemical secondary metabolites is responsible for the antimicrobial activity of the medicinal plants [49] [50]. Copp [51] reported presence of steroids, alkaloids; terpenes are associated with anti-mycobacterial activities. *T. tetraptera* methanol extracts contain saponnins, tannins, alkaloids, flavonoids. *T. tetraptera* does not have volatile oil and resins and may not have antioxidant potential. Abdel-Hamid *et al.* [39] reported the presence of glycosides, phenol, volatile oil, and resin that enhances immunity against pathology induced free radical generation.

T. tetraptera crude extract has anti-tubercular activity against susceptible *M. tuberculosis* at low concentration but differed with other researchers who reported lower concentrations of activity with different plants against *M. tuberculosis* [3] [16] [52] [53]. Adebiyi *et al.* [54] reported activity of *Guiera senegalensis* at 40 mg/ml against *M. tuberculosis*. Endale *et al.* [19] reported MICs range of 250 to 12.5 mg/ml for *Allium ursinum* and *Dodonaea anguistfolia* respectively. Kahaliw *et al.* [55] reported that *Pterolobium stellatum* had MIC of 0.039 mg/ml. The findings in this study, the MIC of *T. tetraptera* on *M. tuberculosis* was 7.4 mg/ml which was found lower in concentrations when compared with other reports. The methanol fractions of *T. tetraptera* showed much lower concentrations of 0.25 and 0.11 mg/ml (MICs) that inhibited the growth of *M. tuberculosis.* This study also revealed that the crude of *T. tetraptera* at the concentration of 27.5 mg/ml (MIC) had activity against MDR-tuberculosis isolates. The methanol fraction of *T. tetraptera* inhibited the growth of MDR-tuberculosis isolates at the concentration of 0.89 mg/ml (MIC), while the concentration of 0.002 mg/ml of rifampicin concentration was found to be resistant to MDR-TB. There was no information on the anti-tuberculosis activity of *Tetrapleura tetraptera* on *M. tuberculosis* and MDR-TB.

5. Conclusion

The *T. tetraptera* inhibited the growth of genetically analyzed drug susceptible

M. tuberculosis at low concentrations. *T. tetraptera* is indicating a potential drug lead and the ability to further inhibition of MDR-TB that shows the diversity of the plant as it possesses anti-tuberculosis activity. *T. tetraptera* is a potent anti-tuberculosis drug against drug susceptible and MDR-TB.

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Conflicts of Interest

There is no conflict of interest such that all authors cited in the study were acknowledged.

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