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In vitro Antimycobacterial Screening of *Ficus sycomorus* Extracts on Susceptible Strain of *Mycobacterium tuberculosis*

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAS designed the study. Authors MAS, KA and SM performed the statistical analysis. Authors MAS, MMA and DAA wrote the protocol. Authors MAS and KM wrote the first draft of the manuscript. Authors MMA, DAA and MSA managed the analyses of the study. Authors EN, AI, PM and IM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the Anti-mycobacterial activity of *Ficus sycomorus* extracts by *in vitro* screening against susceptible strain of *Mycobacterium tuberculosis to* standard TB drugs.
Study Design: Hospital/University based cross sectional study.
Place and Duration of Study: National Tuberculosis and Leprosy training center Zaria, Department of Biochemistry, Ahmadu Bello university Zaria. March 2015 to February 2017.
Methodology: The anti-mycobacterial activity of *Ficus sycomorus* (stem bark, root bark, leaves and

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fruits) was studied *in vitro* using standard Nitrate Reductase Assay techniques against susceptible strain of *Mycobacterium tuberculosis.* Phytochemical analysis of the n-hexane fruit extract was done using standard test methods. Partial fractionation of the n-hexane fruit extract was done using Thin layer and column chromatography assay.

Results: n-hexane fruit extract showed activity against the tested *Mycobacterium tuberculosis* (*M-Tb)* strain. This activity was observed between 100-400 µg/mL against the susceptible strain to standard TB drugs. The crude n-hexane, leaves, root bark and stem bark extracts lacked activity against the susceptible *M-Tb* strain. The methanol and aqueous extracts of fruit, leaves, stem bark and root bark also lacked activity against the tested susceptible *M-Tb* strain. The n-hexane fruit extract (the most active extract) was then partially purified using thin layer chromatography and Column chromatography where four fractions A-D was obtained, where A lacked activity but B, C and D was measured for their Minimum inhibitory concentration (MIC) as 6.25 µg/mL, 25 µg/mL and 100 µg/mL respectively, therefore fraction B had the lowest minimum inhibitory concentration against the tested *M-Tb* strain as 6.25 µg/mL. The crude n-hexane fruit extract also revealed phytochemicals of which saponins and alkaloid had the highest percentage content, (16.67±0.04%) and (6.00±0.02%) respectively

Conclusion: Therefore, these findings indicate that if *Ficus sycomorus* is properly explored and standardized it could be used as herbal drug against *Mycobacterium tuberculosis*.

Keywords: Nitrate reductase assay; Ficus sycomorus; Mycobacterium tuberculosis; susceptible strain.

1. INTRODUCTION

Tuberculosis (TB) is a chronic infectious airborne disease caused by the tubercle bacillus *M. tuberculosis* [1]. TB infections are characterized by the growth of rod-shaped bundles of the *M. tuberculosis* bacteria which in susceptible animals, including humans produce microscopic "tubercles" consisting of chronic granulomas, some with caseous necrosis. Lung tissue is frequently infected, but other parts of the body can be involved [2]. In 2016, 6.3 million new TB cases have occurred with an incidence of 10.4 million incidences along with 1.7 million deaths, which represent an inversion in the global down trend [3]. Nigeria has a high incidence rate of TB (322 cases/100000 population in 2016) it ranked third among the 10 countries that account for 76% of the total gap between TB incidence and reported cases [3]. Although one-third of the world's population is infected by *M. tuberculosis*, only approximately 5-10% of the infected population who are HIV uninfected will develop TB at some stage in their life [3]. Factors that contribute to the development of tuberculosis disease are complex and not completely understood but suppression of cell-mediated immunity plays a key role [4].

Tuberculosis is mostly asymptomatic and is aggravated when the immunity is compromise which arises due to conditions like malnutrition, diabetes, malignancy, and HIV/AIDS. A major problem for the control of TB is the requirement of drug regimens for six to nine months. These lengthy regimens lead to non-compliance with therapy, relapse and development of drug resistance. In order to shorten the duration of therapy, novel drugs that are active against *Mycobacterium tuberculosis*, which act through mechanisms different from those employed by the existing frontline and secondary anti-TB drugs are urgently needed. The use of herbs and other alternative therapies for the treatment of Tuberculosis is on the increase.

Phytochemicals are chemical compounds formed during the plants normal metabolic processes, as such the therapeutic advantage conferred by these plant based products have surpassed the chemical counter parts owing to their lesser side effect and more potent therapeutic effect. Natural products continue to play the most significant role in the drug discovery and development process [5]. Hence it is a demanding need to study the various pharmacologically valuable aspects of these medicinal plants and one of which is *Ficus sycomorus* in treating human diseases. The anti Mycobacterium activity can be detected by the conventional method such as Nitrate Reductase Assay by incorporating the various dilutions of the test antibiotic compound into Lowenstein Jensen (LJ) Medium and inoculating a known amount of test organisms.

Ficus sycomorus known as Baure in Hausa is a large, semi-deciduous spreading savannah tree, up to 21 (max. 46) m, occasionally buttressed which is found almost round the subtropics of Africa [6]. All parts of the plant are used in

medicine. The dried fruits are taken orally by adult human beings in Venda (Cyprus) for the management of tuberculosis [7], it has also been reported for the treatment of snake bites, jaundice, chest pains, dysentery, cool, coughs and throat infections [8]. Roots extracts are also recommended for cough related cases including tuberculosis, cold, and other related cases [9]. Stem-bark, extracts are reported to contain pharmacologically active substances such as gallic tannins, saponins, reducing sugars, alkaloids and flavone aglycones, and was relatively safe in rats with LD_{50} of 720 mg/kg, causing no hematological, hepatic and renal toxicities [10,11]. Therefore the current study is carried to study the anti mycobacterial activity of the n-hexene extract of *Ficus sycomorus.*

2. MATERIALS AND METHODS

2.1 Collection and Processing of Plant Material

Fresh leaves, Root back, Stem back and Fruit of *Ficus sycomorus* were obtained from Saye village of Zaria Local Government Area, Kaduna State, Nigeria. The sample was authenticated at the herbarium of the Department of Biological Sciences, Ahmadu Bello University Zaria. The dried leaves, root back, stem back and fruit were pulverized to powder and 100 g of each were extracted in 500 ml of Distilled water, methanol, and n-hexane respectively by maceration within 48 hrs. The solution of extract was gently evaporated to dryness in a water bath at 40°C. The extract was stored in a refrigerator at 4°C until when required.

2.2 Microbial Strain for Anti *Mycobacterium tuberculosis* Activity

ATCC 27294 (American culture type collection) standard strains of *Mycobacterium tuberculosis* susceptible to both isoniazid and Rifampicin were obtained from National Tuberculosis and Leprosy Training center saye Zaria.

2.3 Susceptibility Testing of Extracts against *Mycobacterium tuberculosis*

2.3.1 Nitrate reductase assay method

Nitrate reductase assay (NRA) was performed as described by Golyshevskaia et al. [12] and Angeby et al. [13] for anti-mycobacterial activity. The critical concentrations of 0.2 µg/mL for Isoniazid (INH), 40 µg/mL for rifampicin and different extracts concentrations (400, 200, 100, 50, 25, 12.5, 6.25 µg /mL) of each test extract sample of *Ficus sycomorus* were used. The Lowenstein Jensen (LJ) media and potassium nitrate (KNO_3 1 mg/mL) were added to the media, and growth of *M. tuberculosis* strains (in the form of pink color) was observed as growth. For each sample test of a concentration, three control bottles were prepared for inoculation of *M. tuberculosis* strain and all the bottles were inspissated.

For each concentration of the extract, 0.2 mL of inoculum suspension was inoculated into the bottles containing LJ medium with potassium nitrate. Similarly, 0.2 mL of inoculum suspension was also inoculated into the standard anti-tubercular drugs, while 0.2 mL of the 1:10 inoculum was inoculated into drug free media which served as growth controls. Falcon tubes in triplicate were utilized for each test sample extracts, anti-tubercular drugs and control bottles. After which all inoculum were incubated at 37°C for a maximum of 21 days.

2.4 *In-vitro* Assay to Show Antimycobacterial Activity

The method used was the macro-tube dilution method described by Adeniyi et al. [14]. The fractions were serially diluted from the solutions of 10000 µg/mL to obtained varying concentrations (400, 200, 100, 50, 25, 12.5 and 6.25 µg/ml). The concentrations were incorporated into LJ media base and 6 ml each was dispensed into falcon tubes, then inspissated at 85°C for 45 mins and inoculated with 0.2 ml each with the standardized mycobacterium strain into the various test tubes containing varying concentrations. Another set of three test tubes containing only LJ media base were used as positive controls. Also test tubes containing standard anti-tuberculosis drugs and test organisms were used as negative controls. All the tested tubes and control tubes were then incubated at 37°C for a maximum of 21 days.

Griss reagent (HCl, 2% sulphanilamide and 1% n-1-napthyl-ethylenediamine dihydrochloride, in the ratio 1:2:2) were used as an indicator of growth, that was added to one drug-free control bottle after 7, 14 or 21 days of incubation. When the color of control bottle changed to pink, then bottles with drugs were then tested with this reagent to indicate activity.

2.5 Statistical Analysis

Analysis was performed using one way ANOVA.

3. RESULTS

The results of the anti mycobacterial activity by Nitrate Reductase assay is presented in the following tables against ATCC 27294 which is susceptible standard strain of *Mycobacterium tuberculosis.*

Table 1 showed the results of the anti-mycobacterial screening at different concentrations (400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL) of the aqueous, methanol and n-hexane crude extracts against the susceptible standard *Mycobacterium tuberculosis* strain. Crude n-hexane Fruit extract was found to be the active at a concentration of 100 µg/mL. No activity was observed in the n-hexane Leave, stem bark and root bark extracts respectively when compared to the control because there was conversion of nitrate to nitrite which was an indicator by color change to pink, likewise the aqueous and methanol extracts were found to have no activity against the susceptible mycobacterium strain. Two potent first line antibiotics against tuberculosis (rifampicin 40 µg/mL and isoniazid 0.2 µg/mL) were used as standard drugs against the tested organisms and a free antibiotic drug control.

Table 2 showed the quantitative phytochemical analysis of n-hexane *Ficus sycomorus* fruit extracts. Tannins, Flavonoids Saponins, Phenols, and Alkaloid, were all present. The highest percentage was obtained in saponins (16.67±0.04) while the lowest percentages were obtained in phenols (0.18±0.10).

The n-hexane fruit extract was subjected to a partial purification using thin layer chromatography and column chromatography where four fractions A-D were obtained. A lacked activity then B, C and D were screened to determine their minimum inhibitory concentration (MIC).

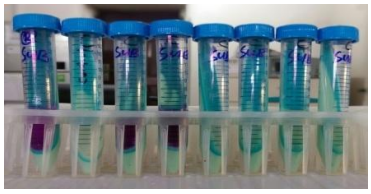
Table 3 showed Minimum Inhibitory Concentration (MIC) of column chromatographic fractions of *Ficus sycomorus* n-Hexane fruit extract. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of test samples that resulted in a no color change on the LJ slant, which signifies complete inhibition of growth in the LJ media. All the fractions exhibited low MIC against the tested strain. Fraction B exhibited the lowest MIC at concentration of 6.25 µg/mL (Fig. 1), while fraction C exhibited MIC at concentration of 25 µg/mL (Fig. 2), and lastly, Fraction D exhibited MIC at 100 µg/mL (Fig. 3).

4. DISCUSSION

The result of the investigation were very encouraging that all the Mycobacteria exposed to the B fraction of *Ficus sycomorus* fruit extract at a lowest concentration of 6.25 µg/ml had Mycobacterial activity (Table 3 & Fig. 1), as such these result is in concordance with the result obtained from Arnold & Galamain [15] in the use of *Ficus sycomorus* dried fruit for the management of tuberculosis among the people of Venda (Cyprus) and Mousa et al. [16] in the use of *Ficus sycomorus* fruit as Antibacterial and antifungal activities. Therefore these could be due to more viability of active compounds in the n-hexane *Ficus sycomorus* fruit extract with more of non polar extracting properties which had strong affinity to the bacilli. Because one the virulence properties of *M. tuberculosis* is the waxy nature of their cell wall which has high lipid content that includes mycolic acids and a trehalose-mycolic acid component called cord factor [1], Therefore compounds of non-polar nature will have more activity towards the mycobacteria cell wall.

Fraction B

Fig. 1



Fraction C

Fig. 2

Fraction D

Fig. 3

Figs. 1,2,3. MIC of Chromatographic fraction B, C and D against the standard susceptible *Mycobacterium tuberculosis* strain

Fraction B exhibited the lowest MIC at concentration of 6.25 µg/mL (Fig. 1), while fraction C exhibited MIC at concentration of 25 µg/mL (Fig. 2), and lastly, Fraction D exhibited MIC at 100 µg/mL (Fig. 3).
Key: D-CONC (Drug control), M-CONC (Media control), SUB (Susceptible)

Table 1. Anti-mycobacterial activity of aqueous, methanol and n-hexane extract of *Ficus sycomorus* against susceptible strain of Standard *Mycobacterium tuberculosis*

CONC (µg/mL)	Aqueous				Methanol				N-hexane			
	Leaves	Stem	Root	Fruit	Leaves	Stem	Root	Fruit	Leaves	Stem	Root	Fruit
400	–	–	–	–	–	–	–	–	–	–	–	+
200	–	–	–	–	–	–	–	–	–	–	–	+
100	–	–	–	–	–	–	–	–	–	–	–	+
50	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–
12.5	–	–	–	–	–	–	–	–	–	–	–	–
6.25	–	–	–	–	–	–	–	–	–	–	–	–
Negative control												
RIF 40	+											
INH 0.2	+											

Control without Drug (positive control) = Pass

Key: + = Active (Growth absent), – = Not active (Growth present), CONC (Concentration), RIF (Rifampicin), INH (Isoniazid)

Table 2. Quantitative phytochemical analysis of crude n-hexane *Ficus sycomorus* fruit extract

Phytochemical	% Quantitative value
Tannins	4.16±0.03
Flavonoids	4.00±0.04
Saponins	16.67±0.04
Phenols	0.18±0.03
Alkaloids	6.00±0.02

Mean ± SD of Triplicate Determinations

Table 3. Minimum Inhibitory Concentration (MIC) of column chromatographic fractions of n-hexane fruit extract against the susceptible standard *Mycobacterium tuberculosis* strain

CONC (µg/mL)	B	C	D
200	+	+	+
100	+	+	+
50	+	+	–
25	+	+	–
12.5	+	–	–
6.25	+	–	–
Negative control			
RIF 40	+		
INH 0.2	+		

Control without Drug (Positive control)

Key: + = Active (Growth absent), – = Not active (Growth present), CONC (Concentration), RIF (Rifampicin), INH (Isoniazid)

Aqueous and methanol extracts lacked activity (Table 1) against *M. tuberculosis* which may be as a result of non availability of active compounds in the extracts due to their polar extracting properties that is against the reported by Morayi, [17] for the management of tuberculosis.

Ficus sycomurus fruit extracts have phytochemicals apart from its high nutritive value [18]. The phytochemicals evaluated ware flavonoids, Tannins, Alkaloids, Saponins and Phenols Table 2, which might have also contributed to the activity among others, as established in several studies such as Sandabe. [10], Sandabe [11], Ladda et al. [19], Mousa et al. [16], reported the use of phytochemicals for active killing of Micro organisms. The extract has high content of Saponins, Alkaloid and Flavanoid which is in agreement with the report of Okoronkwo et al. [18]; as such it might have been responsible for the activity.

The activity could either be through inhibition of cell wall, nucleic acid and enzymatic synthesis etc, which may help in protection against chronic diseases [20], as postulated by the following studies, Saponins could be responsible for the mycobacterial activity because saponins are thought to form complexes with bile and/or cholesterol, therefore preventing absorption of cholesterol by the small intestine of the gastrointestinal tract as such cholesterol will be unavailable for the formation of mycobacterial cell wall [21]. Saponins also help to fight infection and microbial inversion due the fact that they are glucosides, which includes steroid saponins and triterpenoid saponins which also can form complex with proteins to reduce protein digestibility [21].

Jha et al. [22] reported six quinazoline alkaloid from *Justicia adhatoda* having significant antimycobacterial activity, and in silicon analysis confirmed that these alkaloids inhibit β-ketoacyl-acyl-carrier protein synthase III (FabH), an enzyme involved in the initial step of fatty acid

biosynthesis, leading to poor cell wall development and survival of bacilli [22]. Sharma, et al. [23] reported that a flavanoid epigallocate-chin gallate/epigallocatechin-3-gallate directly inhibits fatty acid synthase systems I and II which is the fourth step of the fatty acid elongation cycle is carried out by an enoyl-acyl carrier protein reductase (InhA in *M. tuberculosis*) which catalyses an NADH-dependent reduction of the trans-2-enoyl fatty acyl chain to the saturated fatty acyl chain, by interacting with the residues near the NADH binding site.

Reports from literature show that long-chain unsaturated fatty acids such as oleic and linoleic acid extracted from n-hexane are selective inhibitors of the enoyl-acyl carrier protein reductase, which is a potent inhibitor of cell wall fatty acid synthesis [24].

A number of studies have explored a wide range of natural products with strong activity against *M. tuberculosis* of which include, *Ricinus communis Lin* [19], *Alliu sativum*, *Zingiber officinale* [25], *Adhatoda vasica* [26] and [27], *Acalypha indica* and *Allium cepa* [28] are among the few reported to have anti-mycobacterium activity.

5. CONCLUSION

The anti mycobacterial activity showed that n-hexane *Ficus sycomorus* fruit extract has the potential to cure tuberculosis and is a promise for future therapeutic interventions, which may be probably be due to the phytochemical constituents present in the plant and could be a function of either the individual or the additive effects of the phytochemical constituents. All these findings justify the claim made in the indigenous system of medicine *Ficus sycomorus* use for the treatment of tuberculosis.

Further detailed in-vivo screening and bio activity studies need be carried out using crude solvent extracts as well as further purified constituents to comprehend their role in anti-tuberculosis activity and also pure purification to strain the active compound that confers this activity so as to develop suitable short time regiment drugs that can effectively kill Mycobacteria with lesser toxicity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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