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Anti-mycobacterium activity and Bioassay guided fractionation of the leave extract of *Ximenia* Americana L.

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***Abstract*- This work was aimed at providing preliminary findings that unraveled the promising potential of *X. americana* for the management of *Mycobacterium bovis*. The study tried to assess the anti-mycobacterial activity of *Ximenia americana* using the leave extract after extracting with methanol for 48 hours and the extract subjected to serial solvent partitioning with hexane, chloroform and n-butanol. The various solvent fractions obtained from the partitioning were screened against *Mycobacterium bovis* and the result showed hexane, chloroform and n-butanol extracts to inhibit the growth of the bacteria with a minimum concentration (MIC) of 3200 µg/ml, 1800 µg/ml and 3500 µg/ml respectively. The activity of the chloroform extract was found to be higher than the hexane and n-butanol as such Bio-assay guided fractionation of the chloroform extract was further carried out using column chromatography and nine fractions were obtained. The fourth and Fifth fractions showed strongest activity at MICs of 130 µg/ml and 140 µg/ml. The mixture of these two most active components gave an improved minimum inhibitory concentration of 78 µg/ml fraction after preparative thin layer chromatography.**

***Index Terms*— Tuberculosis, Bioassay, solvent partition, Ximenia Americana**

I. Introduction

Mankind, throughout his existence, depended on plants mainly for food, shelter, clothing, fragrances and flavour. During the course of his interaction with plants for these purposes, man was able to identify plants that have physiological effects on his system. This knowledge was passed from one generation to another. The information accumulated over time made plants to form the basis of sophisticated traditional medicine system that have been in existence for centuries [1]. In Nigeria, the use of traditional medicine is still practiced to treat and manage disease conditions despite the introduction of modern medicine [2]. The absence of synthetic drugs for the treatment of some disease conditions and the emergence of some new strains of bacteria in the society today could be an incentive for the use

of herbal medicine in most communities in Nigeria. This draw-backs have shifted the attention of people from the use of synthetic drugs to the use of natural therapies (herbal medicine), which are found to be effective in treating some disease conditions. Millions of new and relapse cases of tuberculosis is reported every year [3]. Tuberculosis is an infectious disease caused by mycobacterium tuberculosis. The bacterium has of recent developed resistance to first and secondary anti-tubercular drug and efforts to treat and/or cure tuberculosis have been relatively unsuccessful [4]. However, many plants extracts have been reported to show anti-mycobacterial activity. Recently, Sandoval-Montemayor [4] reported that hexane extract of the fruit peels of *Citrus aurantiifolia* showed activity against *Mycobacterium tuberculosis*.

Ximenia americana have been used for the treatment and management of respiratory diseases among the natives of Mushere people in Central Nigeria. *Ximenia americana* is a tropical plant which belongs to the family Olacaceae, commonly found widely in the tropical and temperate regions of the world [5]. The plant is a scrambling spiny shrub or small tree that grow to the height of about 6 m. Traditionally, the extract of the plant is used in the treatment of skin infections, ulcer, leprosy, malaria and *Trypanosoma congolense* infections [6,]. The anti- inflammatory, anti-neoplastic anti-tripanosomal, anti-rheumatic, antioxidant, analgesic, moluscicide and pesticidal, antifungal, anti-cancer, and antimicrobial activity of the plant have been reported[6, 7, 8]. The compounds, 3-Methyl-1-oxoiso chroman-8-Carboxylic acid and Ergosta- 4, 6, 8, 22-tetraen-3-one, have been isolated from *X. americana* [7]. Tuberculosis (TB) is a long-term disease that has been debilitating man and if left untreated, the disease can be life threatening. It is a disease that can be treated, however resistant strains are on the increase as such the search for the discovery of new anti-tuberculosis compounds with improved efficacy and potency demands concerted efforts. this informs research for a new drug that can substitute the old drug. Indigenous knowledge about the native use of plants to treat illnesses could be harnessed for the discovery of new drugs. *X. americana* is claimed to be used for the management of tuberculosis, however there is no documented scientific evidence to support such claim. The aim of this work is to determine the anti mycobacterium activity of the plant and to used bioassay guided fractionation to isolate the active substance in the leave extract.

II. Sampling of Plant Material

The plant was collected from Mushere in Bokkos LGA of Plateau State, Nigeria in October 2010 and was identified by an Ethnobotanist, Department of Traditional Medicine and Research (TMR/TM), National Institute for Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria and the specimens were deposited at the same department.

III. Preparation of Extract

The shade dried powder leaves were extracted with methanol for 48 hours. The extract was obtained by filtration on a Whatman No1 filter paper and the solvent was recovered on rotary evaporator at reduce temperature. The crude methanol extract was subjected to serial solvent partitioning with hexane, chloroform and n-butanol in this order. by dissolving 60g of the extract in 100ml of methanol and water added to make up 80% aqueous solution and then extracted with the solvents . The solution was partition with hexane, chloroform and n-butanol in a separatory funnel each and the process repeated four times until no much colour was observed. The extracts obtained from the solvent partition were dried at low temperature on a rotary evaporator and each extract screened against *Mycobacterium bovis*, a strain of tuberculosis bacteria. The chloroform extracts with the best minimum inhibitory concentration (MIC) was further subjected to bio-assay guided fractionation. The bio-assay guided fractionation was carried out on silica gel(60-80 mesh) in hexane packed column chromatography. by dissolving the crude chloroform extract (10g) in chloroform, and this was adsorbed on 30g of silica gel (60-80 mesh) and allowed to dry at room temperature. The dried adsorbed powder was introduced on the column and it was eluded with hexane, and subsequently with hexane containing increasing concentrations of chloroform. Fractions were collected and monitored by thin layer chromatography (TLC) to ensure single component in each fraction and fractions with similar TLC profiles were combined and tested for activity. Fractions F4 and F5 were combined and purified using preparative thin layer chromatography (PTLC) using methanol/chloroform (9:1) as the solvent mixture. Spray reagent (paramolybdic acid/ethanol) was used to monitor the band of separation. The purity of the compound was ascertained on TLC plates using three difference solvent systems.

IV. Anti-mycobacterium Test

The activity of the plant extract was tested on *Mycobacterium bovis. Mycobacterium bovis strain* was collected in stock solution and sub-cultured in Middlebrook 7H9 broth base supplemented with glycerol. About 1.5 g of Middlebrook 7H9 broth base was suspended in 250 ml of distilled water in a amber bottle (500 ml) followed by addition of 1 ml of glycerol. The mixture was heated and later autoclaved at 120 °C for 25 minutes. The mixture was left to cool to 31 °C, before separately being inoculated with *M. bovis*. The *M.bovis* was incubated at 37°C and optimal growth of the bacteria cultures was observed after 5 days [9].The two fold micro broth dilution methods was used to determine the MIC values of extracts and bio-assay guided fraction against *M.bovis* strains by adopting the method according to Eloff [10]. *M.bovis* was prepared from five days grown cultures in middlebrook 7H9 broth base containing dimethyl sulphoxide (DMSO) and the turbidity was adjusted to approximately 1.2 x108 CFU/ml. The concentrations of stock solution of all test extracts and fraction were all known before serial dilutions. The extracts were serially diluted two folds with a broth base containing DMSO. The serial dilution was performed by addition of 50 µl of extracts and fractions into the first well which had 50 µl of broth base, and thereafter mixed well and transferred 50 µl of the first well sample-broth base mixture to next and subsequent wells of each row. The remaining 50 µl of the mixture was discarded from the last well (well 12) of the row. This was followed by the separate inoculation of 50 µl of mycobacteria cultures in each well, to complete a twofold broth micro-dilution. A duplicate of two additional wells were used as growth controls, where no drugs were added as negative control, and while a row with inoculums and control drugs were used as positive control [11]. The inoculated plates were incubated at 37°C for 7 days. The minimum inhibitory

concentration (MIC) values of each extracts and fraction were read at the concentrations where no growth inhibition was noted. Positive control used in this study was rifampicin.

V. Results and Discussions

Results of extraction and anti-mycobacteria evaluation of the fractions obtained from solvent partition of the methanol extract of the leaves of *X. americana* showed that hexane extracted 5g of the active component, chloroform 12g while n-butanol extracted 22g. Fractions of hexane, chloroform and n-butanol from the methanol extract showed that the fractions inhibited the growth of *M.bovis* with MIC 3200 µg/ml, 1800 µg/ml and 3500 µg/ml respectively. This result revealed that the fractions inhibited the growth of the test bacteria but with varying potency and this seems to agree with the activities of the plant extract on other tested bacteria and microorganism [5, 7]. The chloroform fraction exhibited stronger activity compared to other two extracts as such it was therefore selected for the pursuance of bioassay-guided isolation of its active constituent(s) [12]. The results of the anti-mycobacterium screening of the chloroform fractions from the bioassay-guided column chromatography indicated that nine fractions could be isolated [Table I]. The fractions are coded F1 – F9 with F4 and F5 showing strongest activity and F9 being non-detectable. Despite all fractions showed activity with the exception of F9, the fraction with the strongest activity constitute a very small percentage of the isolates (Table I) The MICs of 130 and 140 µg/ml for F4 and F5 respectively suggest these fractions to be the most active metabolites that are soluble in the solvent mixture that eluded F4 and F5. All other fractions with MICS >200 µg/ml showed less activity. The purified combined fractions of F4 and F5 gave three fractions after elution. Of the three fractions, only one fraction gave a good yield and so was screened against the *M. bovis.* The result of the test showed the extract to inhibit the bacteria with a MIC of 78µg/ml. This technique seems to improved the activity of the extract by not less than 60%. All tested fractions exhibited less anti-mycobacterial activity than the positive control used, rifampicin (Table I). This work provides preliminary findings that unraveled the promising potential of *X. americana* for the management of *Mycobacterium bovis*. The chemical and structural reinvestigation of the plant extract of *X. americana* may be the way out of the search for a promising tuberculosis management. It also supports the claimed uses of the *X. americana* in the traditional system of managing tuberculosis and other respiratory infectious diseases caused by *M.tuberculosis.*

TABLE I. Column chromatographic Fractions of chloroform extract

Fractions	F1	F2	F3	F4	F5	F6	F7	F8	F9	Rifampicin
Quantity extracted (mg)	800	460	200	400	600	1800	1200	1500	100	-
Percent Yield (%)	8.0	4.6	2.0	4.0	6.0	18	12	15	1.0	-
MIC (µg/mL)	2000	350	220	130	140	250	750	2800	ND	0.2

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