Antimycobacterial Activities of Selected Ethiopian Traditional Medicinal plants used for treatment of symptoms of Tuberculosis

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Abstract

Tuberculosis (TB) is serious infectious diseases affecting many people across the world particularly sub-Saharan Africans. Ethiopia is ranked 7th among TB burden shouldering countries in the world. Conventional chemotherapeutic control approach has faced serious, flourishing drug resistance strains. Traditional herbal remedies have endeavored to supplement or replace ineffective drugs. This study determined the antimycobacterial activity of selected Ethiopian medicinal plants traditionally used to treat TB. Leaf of Ocimum lamiifolum, Clausena antisata and Myrsine africana were collected, air dried and extracted with distilled water and absolute methanol (MeOH). The crude aqueous and MeOH crude extracts of the plants were tested against Mycobacterium tuberculosis H37Rv strain and M. bovis(SB 1176). Broth micro-dilution method (BMM) was used to determine the anti-mycobacterial activities and minimum inhibitory concentration of the plant extracts. MeOH and aqueous crude extracts of O. lamiifolum, C. antisata and M. africana have demonstrated promising activity against at least one species of two Mycobacterium species. Both MeOH and aqueous crude extracts of M. africana were active against both species. Antimycobacterial activity was documented within inclusive MIC range of 400-1600µg/m for the extracts of three plant species. The plant extracts have anti-mycobacterial activities pin pointing scientific ground for ethnomedicinal use of the plants against TB. This finding could serve as baseline information for further antimycobacterial agent study of these plants. Future studies ought to assess the exact chemicals involved and identify, if any toxicity. There will also be way to encourage the traditional use of the plant against TB after further research.

Kew Words: Plant extracts, Test organisms, antimycobacterial activity, BMM, MIC.

Introduction

Tuberculosis (TB) is one of the leading infectious disease and health burden in the world (Dye et al., 1999). It has been estimated that one third of the world’s population is infected with TB, with more than nine million new cases diagnosed and approximately two million people killed annually (WHO, 2002). TB still remains the most prevailing cause of morbidity and mortality in the developing countries particularly sub-Saharan Africa more than any part in the world (Walzl et at., 2008). According to the WHO (2009) report on the epidemiological burden of TB, Ethiopia was ranked 7th among the 22 countries in the world with a high TB burden.

Pulmonary TB features (Cough, fever, sweats, weight loss and haemoptysis) and extra-pulmonary lymphnode swelling (lymphadenitis) are leads that used in identifying diseases symptomatically. Apart from lung and lymphnode the disease can occur in any part of the body, including the mienenges, bone and kidneys that land marks disseminated/miliary TB (Fitzgerald and Haas, 2005). Conventional control approach has strived for half a century predominantly focusing on chemotherapy. For the purpose, a number of efficacious agents originally intended for TB treatment were introduced to the market starting in late 1940s and halted with introducing rifampicin in the 1960s (Schraufnagel, 1999). These agents had reasonable efficacy when introduced. The use or often misuse of drugs over the years has led to flourishing drug resistant strains (Nachega and Chaisson, 2003). The emerging and re-emerging global deadly drug resistant strains (multidrug resistance (MDRTB) and extensive drug resistance (XDR-TB)) coupled with
significant drug hepatotoxicity and lengthy therapy paved
the irony road toward global TB therapeutic crisis (WHO,
2010; Dye et al., 1999; Mann et al., 2007; Amin et al.,
2009).

Paralleling or even preceding the modern anti-
tuberculosis treatment endeavors, traditional herbal
remedies have been practical. Ethnomedicinal plants are
main constituents in the traditional treatment of TB and
related diseases globally (Mann et al., 2007). Several
plants are locally reported to treat TB in parts of Africa
(Marita et al., 2010; Mann et al., 2009; Green et al.,
2010). Ethiopian ethnomedicinal system has documented
use of C. anisata (Yineger and Yewhalaw, 2007), O.
lamiiifolium (Mesfin et al., 2005; Mesfin 2009; Getahun
1976) and M. africana (Desisa, 2000; Yineger and
Yewhalaw, 2007) against respiratory problems like cough
and TB.

Different regimens for treating TB have faced different
level of resistance. Combination of drugs and original
regimens are extremely difficult to follow. Despite, World
Health Organization recommendation of directly observed
treatment (DOTS) to standardize the treatment, the TB
treatment crisis is still a great worry. As a result,
evaluating antimycobacterial activity of traditional
medicinal plants is very dominant event in many parts of
the world in search of new efficacious agent. Long served
Ethiopian traditional medicinal plants against TB and
respiratory ailments are either not evaluated yet for
antimycobacterial activities or inaccessible to scientific
community. Screening for antimycobacterial activities of
C. anisata, O. lamiifolium and M. africana against M.
tuberculosis and M. bovis (SB 1176) was not yet
evidenced in the pool of scientific data base. This study
attempted to determine antimycobacterial activities of
Ethiopian ethnomedicinal plants namely C. anisata, O.
lamiifolium and M. africana against M. tuberculosis
(H37Rv) and M. bovis (SB 1176).

Materials and method

Literature survey on ethnomedicinal uses of plants
indicated that Clausena. antisata, Ocimum. lamiifolium
and Myrsine africana are part of Ethiopian traditional
medicinal system to treat respiratory problems like cough
and TB. The fresh leaves of C. anisata and M. africana
were collected from Menagesha-Suba forest and the
fresh leaves of O.lamiifolium were obtained from local
market in Addis Ababa in February 2011in the same
week of above plants collection. Plants were
authenticated by botanist in Akililu Lemma Institute of
Pathobiology (ALIPB); Addis Ababa University. Voucher
specimens were deposited in the mini-herbarium of
ALIPB.

Leaves attached with small branches were washed
thoroughly in distilled water. The washed plant materials
were dried at room temperature allowing continuous
ventilation and turning parts up and down to prevent
fungal development. The air-dried leaves were broken off
the branches and pulverized in to powder using wooden
pistol and mortar. The powder was sieved to separate
from unbreakable plant parts.

The preparation and cold maceration was performed
following the combination of procedures previously
described by different researchers (Asres et al., 2001;
Sato et al., 2008; Marita et al., 2010; Emeruwa, 1982;
Trezas and Evans, 1996). Briefly, 75grams each of the
powdered material of O. lamiiifolium and M. africana
were macerated in 375ml (1:5 w/v) of absolute MeOH and
distilled water whereas 50grams of C. anisata in 250 ml
of absolute MeOH (99.9%) and distilled water contained
in a 500 ml sterile conical flask and covered with cotton
wool plug and wrapped with aluminum foil. Extraction
was allowed to proceed exhaustively for 48hrs by
subjecting to electrical shaker for 4hrs (Figure.1) and
standing at ambient temperature (25–30°C) for remaining
44 hours, 4hours interval manual shaking at day hours
were also applied. This process was repeated for three
times while taking out the extract (juice) by filtering using
clean muslin cloth. The three successive filtrates in
separate flasks pooled together and again filtered using a
Whatman No. 1 filter paper to avoid fibrous portion of
plant completely.

The MeOH extracts of three plants were subjected to
partial concentration using a rotary evaporator (Laborota
4000, SN 090816862, Germany) attached to a vacuum
pump and set in a water bath at 40°C (Figure.2). The
partially concentrated extracts contained in petri-dish
were placed in oven at 40°C to dry completely (Figure.1).
The aqueous extract of plants were allowed to
lyophilization to obtain fine crude extract (Asres et al.,
2001; Sato et al., 2008; Marita et al., 2010; Emeruwa,
1982 ; Trezeas and Evans, 1996). The powder (dry
residues) of both MeOH and aqueous extracts were
weighed and the yield percentages were estimated
according to Parekh and Chanda (2007) as:

(Dry weight recovered / Dry material weight) × 100.

The stock solutions of the extracts were prepared one
day before use in 4% dimethyl sulphoxide (DMSO) at
a concentration of 32 mg/ml. Prior to the bioassay, working
solutions of the extracts were prepared by diluting the
stock solutions in middlebrook 7H9 medium to at a
concentration of 3.2mg/ml. The working solutions were
sterilized by filtration using a cellulose membrane of
0.22µm pore size as indicated by Mohamad et al., (2011).

In Vitro Antimycobacterial activity test

Test organisms and inoculums standardization

The test organisms, Mycobacterium tuberculosis H37Rv
strain and M. bovis (SB 1176) arrested at deep freeze
were obtained from TB laboratory of Animal Health and
Zoonotic Diseases Research Unit at Akilu Lemma institute of Pathobiology. The test organisms were revived from deep freeze(-70°C) in middlebrook 7H9 supplemented with 10% OADC and 2% glycerol and then subcultured in middlebrook 7H9 medium containing 2% of glycerol and enriched with 10% OADC. These were incubated at 37°C for 3-4 weeks until lag-phase was reached. Prior to test, the preparation of standard suspension was carried out according to recommendation of NCCLS (1997). Briefly, the test organisms were diluted by adding the middlebrook 7H9 broth to obtain just as equal concentration of mycobacterium in McFarland standard. The 1.0McFarland standard which contains approximately $3.0 \times 10^8$ of bacteria suspension was used as standard and the suspension standardization was carried out by visually comparison following procedures recommended by NCCLS (1997). Before test the standardized organism was diluted in middlebrook 7H9 media in 1:25 ratio.

**In vitro evaluation of antimycobacterial activity**

Antimycobacterial activity of plants were test by Microbroth dilution method (BMM) previously described by Mann *et al.*, (2008c) with minor modification of some measurement were made by parts of method in Mohamad *et al.*, (2011). Briefly, the susceptibility test was performed using the broth microdilution method (BMM) in 96 well microtitre plates (Figure.3). All 96-wells of sterile plates were filled 100 μL 7H9 medium supplemented with...
10% OADC and 2% of glycerol. 100μL of 3200μg/ml plant extract solutions were added in column 1 in triplicates, after thorough mixing 100 μL mixture to next column and then in the respective rows by using multichannel pipette. The process was continued by double dilution until the final 12th column of every row from A-C MeOH extract, D-F aqueous extract of the same plant. Finally, the excess 100μg was discarded from the 12th column. Each well in their respective row had concentration range of 1.56μg/ml - 3200μg/ml. 100μL rifampicin solution containing 32μg/ml of active ingredient and media without extract were used as positive and negative control respectively. 2% of Dimethy sulfoxide (DMSO) and media only also were included as solvent and sterile control respectively. Finally 100μL of mycobacterium species were inoculated to every well except for sterile control wells. The plates were then sealed with parafilm and incubated at 37°C. The test of every plant extract was performed in triplicates. The presence of activity was determined by careful visual reading. The reading was carried out against black ground; any well observed as cloudy for visual reading against non-white back ground, counted as positive growth and absence of cloudiness as growth inhibition.

**Determination of Minimum inhibitory concentration (MIC)**

The broth dilution test in micro-dilution (microtitre) plate with a capacity of 300μl was used to test in the determination of Minimum Inhibitory Concentration (MIC) for natural products as well for commercial pharmaceutical product. Minimum inhibitory concentration was defined in both cases as the lowest concentration or highest dilution that exhibits no visible growth by visual reading of microtiter plate wells (Mann et al., 2008c; Yeung et al., 2009). MIC interpreted as a lowest concentration in the well that prevented visible growth or cloudiness.

**Quality assurance**

All aspects of procedures were performed in safety cabinet. For laboratory activities like culturing, media preparation and sterilization (filtration) of extracts were carried out following recommended standard and manufacturer’s procedure. Measuring concentration and dilution were maintained at standard recommended level. Media purity was checked by 24hrs incubation before actual test.

**Results**

Highest and lowest percentage yield were obtained from aqueous extract of *O. laumifolium* (10.29) and MeOH of *C. anisata* (2.2) (Table1). During aqueous extraction the foam formation when shaking was observed in *M.*
The yield percentage was relatively higher for aqueous extracts of *C. anisata* and *O. lamiifolium* than MeOH extract. MeOH extract of *Myrsine africana* better yielded than aqueous one (Table 1) MeOH.

The MeOH and aqueous extracts of *C. anisata* showed promising growth inhibition on *M. bovis* (SB 1176), but no growth inhibition against *M. tuberculosis* H37Rv. Both MeOH and aqueous crude extracts of *M. africana* showed inhibitory activity against both mycobacteria. Only aqueous crude extract of *O. lamiifolium* prevented growth of *M. tuberculosis* in test wells (Table 2).

The minimum inhibitory concentration of extracts with antimycobacterial activities against both mycobacteria fell in inclusive range of 400µg/ml-1600 µg/ml (Table 3). The higher activities or lowest MIC was obtained in aqueous extracts of *O. lamiifolium* and *M. africana* against *M. tuberculosis*. The lower activity or highest MIC was obtained in MeOH of *C. anisata* against *M. bovis* (SB1176). 800µg/ml MIC was obtained from *M. africana* in both solvents against *M. bovis* (SB1176) and in MeOH against *M. tuberculosis*; from *C. anisata* in aqueous extract against *M. bovis* (SB1176).

**Discussion**

Three plants have shown variation in percentage yield of aqueous and MeOH extracts (Table 1). The highest yield (10.29 %) was obtained from aqueous extraction of *O. lamiifolium* whereas lowest (2.2%) was obtained from MeOH extracts of *C. anisata*. Comparable yield percentage of *C. anisata* was reported by Mkhmbo (2010).

The MeOH and aqueous, crude extracts of *C. anisata* showed promising growth inhibition on *M. bovis* species (Table2). Previously, reports confirmed that alcoholic extract of stem bark of *C. anisata* was active against Gram-positive and Gram-negative bacteria (Chakraborty et al., 1995); such extracts were also tested using microbroth dilution method against infective *Candida* species and exhibited a strong antifungal activity (Hamza et al., 2006). Anti-insect activity of volatile oil of the plant...
was proved against grasshopper species, and its repellency against some tick and mosquito species (Okunade, 1987). The previously documented biological activities against different organisms, imply that the potential use of *C. anisata* as a chemotherapeutic source against different infections including deadly TB. The argument can sufficiently be built up by chemical constituents identified from the plant like coumarins, limonoids (Ngedjui et al., 1989; Ito et al., 2000), group of terpenoids, sesquiterpenoids (Adesina and Ette, 1982; Lakshmi et al., 1984; Hutchings et al., 1996; Ojewole, 2002). Some studies proved antimycobacterial activity of these chemicals in other plants (Maneerat et al., 2008; Wube et al., 2005; Asres et al., 2001).

The minimum inhibitory concentration of extracts having antimycobacterial activities against both test organisms fell in inclusive range of 400µg/ml-1600 µg/ml (Table 3). This was comparable to MIC of 400 µg/ml-1600 µg/ml obtained somewhere in Malaysia from crude extract of other plants against *M. tuberculosis* H37Rv (Mohamad et al., 2010). Lowest MIC, 400µg/ml (higher activity) was obtained by aqueous extract of *O. lamiifolum* and *M. africana* against *M. tuberculosis* whereas high MIC, 1600µg/ml (lower activity cut off) was observed in MeOH extract of *C. anisata* against *M. bovis* (SB1176). Growing evidences suggest that minimum inhibitory concentration of the crude extracts may or may not be indicative for success full identification of active compound. This is because either an extract with a relatively low MIC (high activity) may contain large quantities of only very few moderately active major constituents or moderately active crude materials could lead to minor compounds with high activity (Cantrell et al., 1999).

Only aqueous crude extract of *O. lamiifolum* prevented growth of *M. tuberculosis* in the test plate wells which could be due to the fact that active biological constituents are more soluble in aqueous solvent than in MeOH. Absence of activity against *M. bovis* (SB1176), in this context, could be due to either a chemical uptake differences or previously acquired resistance against chemical analogs in under use drugs to *M. bovis* (SB1176) since *M. tuberculosis* H37Rv strain has been counted as virtually susceptible to all drugs at market. The information on past biological activity was more concentrated on analgesic, anti-inflammatory, antipyretic aspects (Debella et al., 2003; Hakkim et al., 2008). Few reports are available on biologically active constituents against insects and nematodes (Deshpande and Tipnis, 1997; Chaterje et al., 1982). No clear scientific report obtained from literature survey on antimicrobial and antifungal activities. However, the plant contains valuable essential oils, phenolic compounds (Debella et al., 2003) alkaloids, flavonoids, terpenoids - steroids, saponins and tannins (Mukazayire et al., 2010) which have confirmed antimycobacterial activities in other plants (Maneerat et al., 2008; Wube et al., 2005; Asres et al., 2001; Mann et al., 2008; Suksamrarn et al., 2004).

Both MeOH and aqueous crude extracts of *M. africana* showed inhibitory activity against both mycobacteria (Table 2) in the concentration range of 1.56µg/ml - 3200µg/ml. This shows that *M. africana* has got antimycobacterially active constituents that are readily soluble in both aqueous and MeOH solvents. Previously, the plant was tested against human and veterinary infectious agents and have shown different level of activities against bacteria, fungi and helminthes (Habtamu et al., 2010; Kang et al., 2007; Ahmad et al., 2011). The plant constitutes different chemicals such as benzoquinones and its derivatives (Manguro et al., 2003; Gathuma et al., 2004), group of flavonoids (Zou, 2009; Kang et al., 2007) and triterpenoids (Lavaud et al., 1994), which have been reported to have antimycobacterial activity from some other plants (Maneerat et al., 2008; Wube et al., 2005; Asres et al., 2001; Mann et al., 2008; Suksamrarn et al., 2004).

MeOH and aqueous crude extract of *C.anisata* and *O.lamiifolum* against *M. tuberculosis*, and that of aqueous extract of *O.lamiifolum* against *M. bovis* showed no observable inhibitory activity even at 3200µg/ml, upper marginal concentration. The reason behind such inactivity may be related to the presence of constituents that are insoluble in used solvent and/or due to insensitive test organisms. Positive control RIF showed MIC average of 0.125µg/ml against *M.tuberculosis* and 0.5µg/ml *M. bovis* this is comparable to that of Mann et al., (2008c), however very larger than Suksamrarn et al., (2004) report. This may be due to relatively susceptible strain was used by earlier report. DMSO showed no inhibition at 2% control against test organisms.

The antimycobacterial activity obtained in this study may not be an exact attribute of chemical constituents mentioned in this text; it could either be due to an attribute of these chemicals or a combination of these chemicals with others that are not yet known to be isolated and identified. In the current study, effort was made to show the presence of antimycobacterial activity in the three plants and this could supplement other ethnomedicinal studies in the search for potent remedies against the deadly disease.

Conclusions and recommendations

*Ocimum lamiifolum*, *Clausena antisata* and *Myrsine africana* have promising antimycobacterial activity. For the first time, MeOH and aqueous crude extracts were found to be active within inclusive MIC range of 400-1600µg/ml against at least one test organism. This finding pointed out the scientific ground for ethnomedicinal use of the plants against TB.

This finding can be used as baseline information in further antimycobacterial activity studies of these plants. Future studies are recommended to prove the exact
chemicals responsible for observed activity and to identify toxicity, if any. To conclude, there should be way to encourage the traditional use of the plants in combination with conventional ones to control tuberculosis after further research on them.

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Abbreviations

BMM= Microbroth dilution method
DMSO = Dimethyl Sulphoxide
DOT=directly observed treatment
MDR= Multidrug resistance
MeOH= Methanol
MIC= minimum inhibitory concentration
NCCLS= National Committee for Clinical Laboratory Standards
OADC= Middlebrooke enrichment (oleic acid, Albumin, Dextrose, Catalase )
TB= tuberculosis
XDR= extensive drug resistance

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