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Full Length Research Paper

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

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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.

Keywords: Plant extracts, Test organisms, antimycobacterial activity, BMM, MIC,

List of 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

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

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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 meninges, 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 (Mariita *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. lamiifolum* (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. lamiifolum* 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. lamiifolum* and *M. africana* against *M. tuberculosis* (H37Rv) and *M. bovis* (SB 1176).

MATERIALS AND METHOD

Plant materials

Plant material collection

Literature survey on ethnomedicinal uses of plants indicated that *Clausena. antisata*, *Ocimum. lamiifolum* 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.lamiifolum* were obtained from local market in Addis Ababa in February 2011in the same week of above plants collection. Plants were authenticated by botanist in Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University. Voucher specimens were deposited in the mini-herbarium of ALIPB.

Plants material preparation and extraction

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; Mariita *et al*., 2010; Emeruwa, 1982; Trease and Evans, 1996). Briefly, 75grams each of the powdered material of *O. lamiifolum* 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

Figure 1. Oven dry of MeOH extract and Electrical shaking of solvent-powder mixture

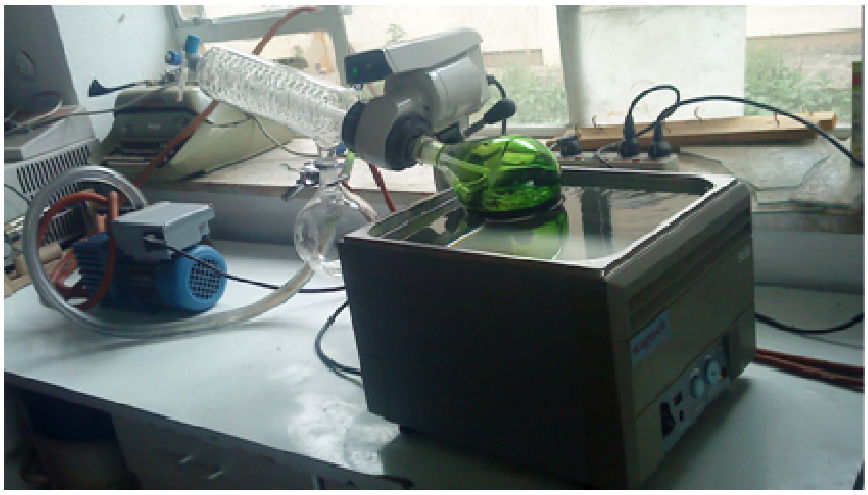

Figure 2. MeOH extract concentration using rotary evaporator

subjecting to electrical shaker for 4hrs (Figure 1) and standing at ambient temperature (25–30^{0}C) for remaining 44 hours, 6hours 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^{o}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; Mariita *et al*., 2010; Emeruwa, 1982 ; Trease 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

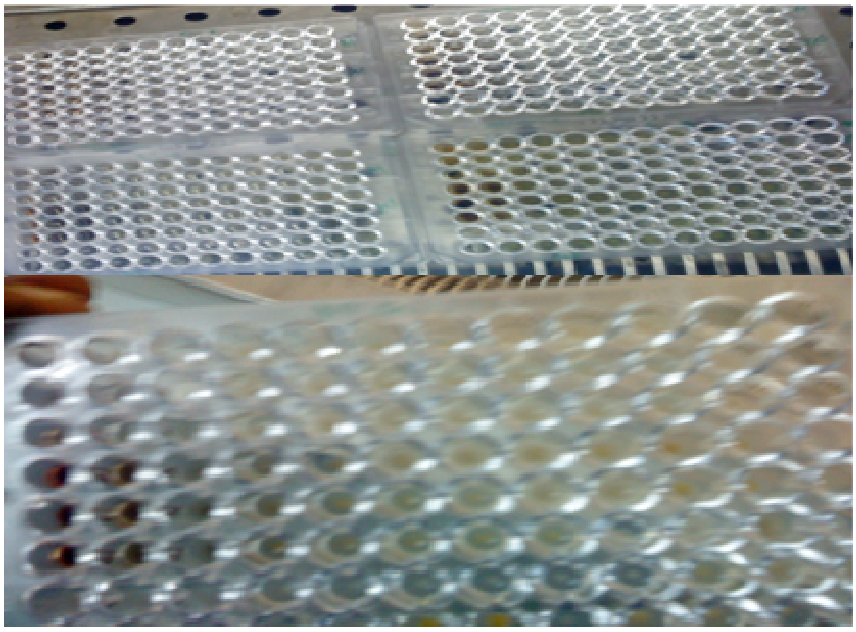

Figure 3. Broth microdiltution test

strain and *M. bovis* (SB 1176) arrested at deep freez were obtained from TB laboratory of Animal Health and Zoonotic Diseases Research Unit at Aklilu Lemma institute of Pathobiology. The test organisms were revived from deep freeze(-70^{0}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^{0}C for 3-4weeks 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 3.0x10^8 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). Brifely, 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 µL7H9 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^{0}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.

Table 1. Plant extracts with their corresponding yields and test organism

Plant species	Part of plant Used	Used powder in grams	Percentage Yield	
			MeOH	Aqueous
C. anisata	Leaf	50	2.2%	7.11%
O. lamiifolum	Leaf	75	3.7%	10.29%
M. africana	Leaf	75	5.3%	2.25%

Table 2. Mycobacterial Growth and inhibition with respect to test extracts

Plant species	*Mycobacterium tuberculosis*		*M. bovis*	
	MeOH	Aqueous	MeOH	Aqueous
C. anisata	+	+	—	—
O. lamiifolum	+	—	+	+
M. africana	—	—	—	—
Positive control (RIF)	—		—	
Negative control	+		+	
Solvent control	+		+	
Sterile control	no growth at all		No growth at all	

Key MeOH=MeOH, + (no visible inhibition observed at all), — (inhibition observed) Negative control (Media and bacteria without any extract), Solvent control (2% of dimtylsulfoxide (DMSO)), sterile control (enriched media only)

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. lamiifolium* (10.29) and MeOH of *C. anisata* (2.2) (Table1). During aqueous extraction the foam formation when shacking was observed in *M. africana.* The yield percentage was relatively higher for aqueous extracts of *C. anisata and O. lamiifolum* than MeOH extract. MeOH extract of *Myrsine africana* better yielded than aqueous one (Table1) 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. lamiifolum* 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. lamiifolum* 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

Table 3. Minimum inhibitory concentration of extracts and respective test organisms

Plant species	MIC (µg/ml) against *M. tuberculosis*		MIC(µg/ml) against *M. bovis*	
	MeOH	Aqueous	MeOH	Aqueous
Clausena anisata	NA	NA	1600	800
O. lamiifolum	NA	400	NA	NA
M. africana	800	400	800	800
Positive control(RIF)	0.125		0.5	

Key. MIC (minimum inhibitory condition), NA(not active), RIF= rifampicin

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 (Table1). 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 Mkhombo (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-postive 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(Ngadjui *et al*., 1989; Ito *et al*., 2000), group of t erpenoids, 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 extracts of *O. lamiifolum* and *M. africa*na 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 benzoquinoes 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 africa na* 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/m 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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