

## Anti-dormant mycobacterial activity of *Melissa officinalis* L. (Lemon balm).

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### Abstract

*Mycobacterium tuberculosis* is one of the most common causes of morbidity and mortality among adults who have tested HIV-positive or those living in poverty. One of the major reasons for the need of extended chemotherapeutic regimens and widespread epidemicity of tuberculosis is that the causative agent, *M. tuberculosis*, has the ability to be in a dormant state. Therefore, there is an urgent need for new lead compounds that are effective against *M. tuberculosis* in both its active and dormant states. In this study, we investigated the anti-mycobacterial activity of the total ethanol extract and fractions of *Melissa officinalis* L. (Lemon balm) under both actively growing and dormancy inducing conditions. As a result, the total extract of *M. officinalis* exhibited antimicrobial activity against both active and dormant states of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG with Minimum Inhibitory Concentration (MIC) values ranging from 12.5 µg/mL to 50 µg/mL. Among the fractions, the *n*-hexane fraction showed the most ideal activity against active and dormant states of *M. smegmatis* (MIC = 6.25 µg/mL). In addition, the *n*-hexane fraction also exhibited antimicrobial activity against actively growing *M. bovis* BCG (MIC = 12.5 µg/mL), while moderate activity (MIC = 50 µg/mL) was observed against the dormant state of *M. bovis* BCG.

### Key words

Lamiaceae, *Melissa officinalis*, Tuberculosis, Dormant state, Antimicrobial activity.

### 1. Introduction

Human beings have been battling with microbes historically, particularly bacteria, which have been causing considerable morbidity and mortality globally. Tuberculosis is among the important infectious diseases mainly caused by *Mycobacterium tuberculosis*. In 2019, tuberculosis accounted for an estimated 10 million new cases and 1.2 million deaths [1]. Mycobacteria have developed a genetically programmed process for adapting to the host response against infection. Among the host responses against infection is the arrest of replication of mycobacterial bacilli by nutrient starvation, hypoxia, and other stresses in the infected region called granuloma [2–4]. It is now generally accepted that a minimum of 6 months of tuberculosis treatment is required owing to the difficulty of eradicating non-replicating persistent *M. tuberculosis*. Therefore, many current approaches are seeking to discover antibacterial agents targeting *M. tuberculosis* in its active and dormant state. Recently, we established a screening system to search for substances that exhibit activity against dormant *Mycobacterium* spp., by using hypoxic culture conditions [5, 6].

*Melissa officinalis* L. (Lemon balm) belongs to the Lamiaceae family. *Melissa officinalis* L is a perennial herb, up to 1 m high, growing in the Mediterranean region, western Asia, southwestern Siberia, and northern Africa. Parts mostly used are dried leaves; which often present flowering tops [7]. Phytochemical studies carried out with *M. officinalis* L. have demonstrated the occurrence of many classes of constituents, including polyphenolic compounds (rosmarinic acid, caffeic

acid and protocatechuic acid), essential oils (citral), monoterpenoid aldehydes, sesquiterpenes, flavonoids (luteolin), and tannins [7]. In addition, *M. officinalis* has been traditionally used for different medical purposes such as antispasmodic, hepatic protector, carminative, diaphoretic, a tonic, surgical dressing for wounds, sedative-hypnotic strengthening the memory, and for relieving stress induced by headache. In modern pharmacology, *M. officinalis* exhibits efficacy in the management of mild to moderate Alzheimer's, against migraine and rheumatism, as well as exhibits antitumor and antioxidant activities [8, 9]. In addition, *M. officinalis* also has scientific reported uses, and can be used as an antibacterial, antifungal, antiviral, antihistaminic [9-11], and antilipidemic [12].

In this study, we evaluated the anti-dormant mycobacterial activity of the total ethanol extract of *M. officinalis*, which was not reported previously, in order to explore new seeds for anti-tuberculosis drugs. In addition, as a part of further purification, we also evaluated the activity of some fractions that were prepared by partitioning with different solvents.

### 2. Materials and methods

#### 2.1. Plant material

Leaves and stems of *M. officinalis* were collected from plants cultivated in the botanical garden of SEKEM Company, Cairo, Egypt. Authentication of the plant was established by Dr. Saber Hendawy, the director of SEKEM botanical garden. A voucher specimen (Mn-Ph-Cog-014) was deposited in the herbarium of

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## 2.2. Extraction and fractionation

The air-dried, powdered leaves and stems (500 g) of *M. officinalis* were extracted by maceration with 95% ethanol, and then the solvent was evaporated under reduced pressure to obtain a total ethanol extract (5.8 g). This was partitioned into a water/EtOAc mixture. The active EtOAc soluble fraction (3.0 g) was further partitioned into an *n*-hexane/90% aqueous MeOH mixture. As a result, the soluble fractions of *n*-hexane and 90% aqueous MeOH were obtained as 1.2 g and 0.8 g, respectively.

## 2.3. Anti-mycobacterial assay

*M. smegmatis* mc<sup>2</sup>155 and *M. bovis* BCG Pasteur were grown in the Middlebrook 7H9 broth containing 10% oleic albumin dextrose catalase (OADC, both BD, Franklin, NJ, USA), 0.5% glycerol, and 0.05% Tween 80. The MIC values of the test compounds against *M. smegmatis* and *M. bovis* BCG under actively growing aerobic conditions and dormancy inducing hypoxic conditions were determined using the established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Merck KGaA, Darmstadt, Germany) assay [5]. In the case of the aerobic conditions, mid-log phase bacilli [*M. smegmatis* ( $1 \times 10^4$  CFU/0.1 mL) or *M. bovis* BCG ( $1 \times 10^5$  CFU/0.1 mL)] were inoculated into a 96-well plate, and then, serially diluted samples were added to the 96-well plate. The bacteria were then incubated at 37°C for 36 h (for *M. smegmatis*) or for 7 days (for *M. bovis* BCG). In the case of the hypoxic conditions, the mycobacterial bacilli were grown in Middlebrook 7H9 broth at 37°C under a nitrogen atmosphere containing 0.2% oxygen until the optical density reached 0.8 at 600 nm. Subsequently, the bacilli were inoculated to the 96-well plate at the same density under aerobic conditions and incubated at 37°C under a nitrogen atmosphere containing 0.2% oxygen for either 96 h (for *M. smegmatis*) or 14 days (for *M. bovis* BCG). After the initial incubation, 50 µL of MTT solution (0.5 mg/mL) was added to each well and further incubation was completed at 37°C for an additional 12 h under aerobic or hypoxic conditions. The optical density at 560 nm was measured to determine the MIC value.

## 3. Results and Discussion

The aqueous decoctions of *M. officinalis* leaves are known as a functional beverage with antioxidant, antitumor, and antimicrobial effects [13]. It has been also reported that different extracts and aqueous decoctions of lemon balm exhibited noteworthy inhibitory actions against a variety of pathogenic microorganisms such as *Staphylococcus aureus*, *Bacillus* spp., *Sarcina lutea*, *Micrococcus flavus*, *Listeria monocytogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Trichoderma viride*, *Aspergillus* spp., and *Penicillium* spp. [13-16]. However, the antimicrobial activity of the extract against *Mycobacterium* spp., especially against dormant state bacilli, has not been investigated.

On the other hand, hypoxia is known to be a major factor inducing a nonreplicating persistence of tubercle bacilli. Wayne L. G. *et al.* proved that oxygen depletion triggered the dormancy response, such as isoniazid resistance in mycobacterial bacilli [17, 18]. Based on these observations, we established a screening system to search substances that exhibit anti-

microbial activity against dormant mycobacteria. Indeed, the MIC values of isoniazid against *M. smegmatis* and *M. bovis* BCG were observed at 2.5 and 0.03 µg/mL, respectively, under aerobic conditions. The MIC values against *M. smegmatis* and *M. bovis* BCG shifted to 25 µg/mL and more than 100 µg/mL, respectively, under 0.2% oxygen of hypoxic conditions (Table 1). In this study, we found that the total ethanol extract from *M. officinalis* exhibited anti-microbial activity against *M. smegmatis* under both actively growing aerobic and dormancy-inducing hypoxic conditions with MIC values of 12.5 µg/mL. The total extract also showed the activity against *M. bovis* BCG under both aerobic and hypoxic conditions with MIC values of 25 and 50 µg/mL, respectively. The EtOAc fraction partitioned from the total extract exhibited similar activity to that of the total extract, whereas the specific activity of the EtOAc fraction did not increase for unknown reasons. In addition, as a part of further purification, the active EtOAc soluble fraction was further partitioned into an *n*-hexane/90% aqueous MeOH mixture, and the activity of the fraction was evaluated. As shown in Table 1, the *n*-hexane fraction exhibited the most ideal activity against the active and dormant states of *M. smegmatis* (MIC = 6.25 µg/mL). The *n*-hexane fraction also exhibited antimicrobial activity against actively growing *M. bovis* BCG (MIC = 12.5 µg/mL), while moderate activity (MIC = 50 µg/mL) was observed against dormant state of *M. bovis* BCG. On the other hand, 90% MeOH aq fraction was active against *M. smegmatis* and actively growing *M. bovis* BCG, whereas no effect was observed on the dormant state of *M. bovis* BCG (MIC ≥ 100 µg/mL). These results suggested that further purification of *n*-hexane fraction might provide new seeds for anti-tuberculosis drugs that are effective against active and dormant states of mycobacterial bacilli.

**Table 1:** Anti-mycobacterial activity of total ethanol extract and fractions.

Samples	MIC (µg/mL)			
	<i>M. smegmatis</i>		<i>M. bovis</i> BCG	
	Aerobic	Hypoxic	Aerobic	Hypoxic
<b>Total ethanol extract</b>	12.5	12.5	25	50
<b>Ethyl acetate fraction</b>	25	25	25	100
<b><i>n</i>-hexane fraction</b>	6.25	6.25	12.5	50
<b>90% MeOH fraction</b>	12.5	12.5	50	> 100
<b>Isoniazid</b>	2.5	25	0.03	> 100

## 4. Conclusion

The present study provided the new finding that the total ethanol extract prepared from the leave and the stem of *M. officinalis* L. (Lemon balm) exhibits anti-dormant mycobacterial activity against both species of *M. smegmatis* and *M. bovis* BCG. In addition, we also found that *n*-hexane fraction showed ideal inhibitory activity against both species. Although further studies, phytochemical analysis and purification of the fraction,

are necessary, *n*-hexane fraction from total extract of *M. officinalis* L. is expected to contain medicinal potential for a new anti-tuberculosis drug.

## 5. Conflict of Interest

The authors declare no conflict of interest.

## 6. Acknowledgments

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