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The anti-tubercular activity of *Melia azedarach* L. and *Lobelia chinensis* Lour. and their potential as effective anti-*Mycobacterium tuberculosis* candidate agents

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ABSTRACT

Objective: To evaluate the anti-mycobacterial activity of *Melia azedarach* L. (*M. azedarach*) and *Lobelia chinensis* Lour. (*L. chinensis*) extracts against the growth of *Mycobacterium tuberculosis* (*M. tuberculosis*).

Methods: The anti-*M. tuberculosis* activity of *M. azedarach* and *L. chinensis* extracts were evaluated using different indicator methods such as resazurin microtiter assay (REMA) and mycobacteria growth indicator tube (MGIT) 960 system assay. The *M. tuberculosis* was incubated with various concentrations (50–800 μg/mL) of the extracts for 5 days in the REMA, and for 4 weeks in MGIT 960 system assay.

Results: *M. azedarach* and *L. chinensis* extracts showed their anti-*M. tuberculosis* activity by strongly inhibiting the growth of *M. tuberculosis* in a concentration-dependent manner in the REMA and the MGIT 960 system assay. Particularly, the methanol extract of *M. azedarach* and *n*-hexane extract of *L. chinensis* consistently exhibited their effects by effectively inhibiting the growth of *M. tuberculosis* in MGIT 960 system for 4 weeks with a single-treatment, indicating higher anti-*M. tuberculosis* activity than other extracts, and their minimum inhibitory concentrations were measured as 400 μg/mL and 800 μg/mL, respectively.

Conclusions: These results demonstrate that *M. azedarach* and *L. chinensis* extracts not only have unique anti-*M. tuberculosis* activity, but also induce the selective anti-*M. tuberculosis* effects by consistently inhibiting or blocking the growth of *M. tuberculosis* through a new pharmacological action. Therefore, this study suggests the potential of them as effective candidate agents of next-generation for developing a new anti-tuberculosis drug, as well as the advantage for utilizing traditional medicinal plants as one of effective strategies against tuberculosis.

1. Introduction

Recently chronic infectious diseases including tuberculosis, hepatitis, filariasis, leishmaniasis, amoebiasis caused by *Entamoeba histolytica*, as well as acute infectious zoonosis such as malaria, Middle East respiratory syndrome, influenza and Ebola, have been occurred or persisted in various countries, particularly, in less-developed countries globally. Furthermore, the prevalence of tuberculosis has still been causing a serious global challenge of drug-resistant tuberculosis such as extensively drug-resistant tuberculosis (XDR-TB) and multi-drug resistant tuberculosis (MDR-TB) despite various efforts and studies worldwide. *Mycobacterium tuberculosis* (*M. tuberculosis*) is one of the major infectious factors causing the highest mortality through the co-infection with HIV/AIDS as well as the most dangerous infectious bacteria causing resistant strain through fast

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infectivity and the adaptability against environmental variation. Recently, it was estimated that 9.0 million people in the world were confirmed as new tuberculosis cases in 2013, and 1.1 million of them had HIV-positive response. Particularly, tuberculosis cases co-infected with HIV/AIDS showed high-infection rates in the African region compared with other countries, and most of tuberculosis cases in 2013 and 2014 occurred in Asia (56%) and the African region (29%) [1,2]. In these aspects, various studies for the inhibition and/or the treatment of tuberculosis have been carried out in anti-tuberculosis drug discovery/development field. However, the current anti-tuberculosis drugs not only showed the limit that can't inhibit the increase of the tuberculosis patients, but also caused various side effects. Recently, the global pharmaceutical companies for the development of novel/effective anti-tuberculosis drugs are testing the repurposed compounds such as PA-824, SQ109 and linezolid through chemical remodeling of the existing drugs, as well as newly developed compounds such as Q203, TMC207, pyridomycin, and thiophenes in clinical trials [1,3–7]. Nevertheless, the rapid emergence of tuberculosis including MDR-TB and XDR-TB is still inducing serious concerns and problems in the public health field worldwide. Recently, various extracts and/or natural products derived from medicinal plants or traditional oriental medicine were studied or reported for discovering novel anti-*M. tuberculosis* candidate drugs, which are being progressed for developing anti-tuberculosis drugs of more effective/safe next-generation [8–13]. Furthermore, various medicinal plants of oriental medicine have been utilized as traditional resources for treating diseases and/or symptoms such as diabetes, arteriosclerosis, hepatitis, parasite, cancer both "*in vivo*" and "*in vitro*" [14–18].

For these reasons, various studies for developing the effective/safe anti-tuberculosis drugs with novel mechanisms of action, low cytotoxicity, and the least side-effects are urgently needed to block the tuberculosis. In this aspect, this study was carried out to evaluate anti-*M. tuberculosis* effect of *Melia azedarach* L. (*M. azedarach*) and *Lobelia chinensis* Lour. (*L. chinensis*) that are effectively utilized as a medicinal plant in traditional oriental medicine, and to identify the potential of them as candidates for developing novel antitubercular drugs.

2. Materials and methods

2.1. Materials

Various drugs used in this study including rifampicin, isoniazid, resazurin powder and dimethylsulfoxide, were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA), and MGIT™ 960 system indicator 7 mL growth tubes with BACTEC™ MGIT™ 960 supplement kit were purchased from Becton-Dickinson and Company (Sparks, MD, USA). All other chemicals and reagents were purchased from Merck Chemical Co., Ltd. (Darmstadt, Germany) and Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA).

2.2. Preparation and extracts of medicinal plants

The dried roots and barks of *M. azedarach* and dried roots, stem, leaves and flowers of *L. chinensis* were provided by the Oriental Medical Center, Kyung Hee University (Seoul, Republic of Korea) and Bundang Oriental Hospital, Dongguk University (Seoul, Republic of Korea) for this study. The *M. azedarach* and *L. chinensis* were extracted according to solvent extraction system as follows: Five hundred grams of each powdered *M. azedarach* and *L. chinensis* were independently extracted with 4 L of *n*-hexane, chloroform, ethyl acetate, methanol, and distilled water at room temperature for 24 h, respectively. These extracts were filtered using filter paper and a vacuum pump, and then were evaporated under reduced pressure using a rotary evaporator in a vacuum at 45 °C. The extracts were lyophilized after evaporation. All extracts were filtered using a 0.45 μm syringe filter (Roshi Kaisha, Ltd., Tokyo, Japan) and stored at −80 °C until use.

2.3. Preparation of anti-*M. tuberculosis* drugs

The anti-*M. tuberculosis* first-line drugs, isoniazid, was dissolved in sterile distilled water, and rifampicin was dissolved in dimethylsulfoxide, to a concentration of 50 mg/mL according to the manufacturer's instruction. The anti-*M. tuberculosis* first-line drugs were used as reference standard drugs. All compounds were filtered using 0.45 μm membrane syringe filter (Roshi Kaisha, Ltd., Tokyo, Japan) before use and stored at −80 °C deep-freezer until use.

2.4. Preparation and growth conditions of *M. tuberculosis*

M. tuberculosis H37Rv (ATCC 27294) and H37Ra (ATCC 25177) used in this study were purchased from American Type Culture Collection (Manassas, VA, USA). *M. tuberculosis* H37Rv and H37Ra were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase enrichment (Becton–Dickinson and Company, Sparks, MD, USA) and 0.05% (v/v) Tween 80 (Sigma–Aldrich Chemical, St. Louis, MO, USA) to the log phase at 37 °C for 4–5 weeks on shaking incubation of 120 r/min.

2.5. Drug susceptibility testing of *M. tuberculosis*

The *in vitro* anti-*M. tuberculosis* activity of *M. azedarach* and *L. chinensis* extracts was confirmed by resazurin microtiter assay (REMA) using a 96-well micro-plate. Briefly, *M. tuberculosis* was grown in fresh Middlebrook 7H9 broth supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase enrichment and 0.05% (v/v) Tween 80 until the culture reached a turbidity equal to that of 1.0 McFarland standard (3.0×10^8 CFU/mL) at 37 °C. The bacteria were adjusted to a density of 2×10^6 CFU/mL in fresh culture broth. Finally, the bacterial suspensions were inoculated into all wells of a 96-well microtiter plate containing final concentrations (50–200 μg/mL) of *M. azedarach* and *L. chinensis* extracts and anti-tuberculosis first-line drugs (1.25–5.00 μg/mL). *M. tuberculosis* growth controls containing no anti-*M. tuberculosis* first-line drugs and blank controls without inoculation were also included. The 96-well plates, covered with lids, placed in a plastic bag, were incubated at 37 °C for 5 days. After incubation, 20 μL of freshly prepared 0.05% (w/v) resazurin solution was added to all wells, and the plates were re-incubated at 37 °C for 36 h. A change in color from blue to pink indicating bacterial growth was observed after 36 h of incubation. The minimum inhibitory concentration (MIC) was expressed as the lowest concentration of the drug that inhibited *M. tuberculosis* growth or prevented change in color of the resazurin solution from blue to pink based on a REMA.

2.6. Evaluation of drug susceptibility of *M. tuberculosis* by MGIT 960 system assay

For evaluate anti-*M. tuberculosis* effects of *M. azedarach* and *L. chinensis* extracts against the growth of *M. tuberculosis*, the drug susceptibility testing of the strain was further performed using the BACTEC™ MGIT 960 system (Becton, Dickinson and Company, Sparks, MD, USA). In brief, 100 μL of a suspension of *M. tuberculosis* culture, adjusted to 9.6×10^6 CFU/mL, was inoculated in an MGIT growth media tube with BACTEC™ MGIT 960 growth supplement (Becton, Dickinson and Company), which were incubated with different concentrations (25–800 μg/mL) of the extracts and anti-*M. tuberculosis* first-line drugs (10 μg/mL), and *M. tuberculosis* growth controls containing no anti-*M. tuberculosis* first-line drugs were also included. They were incubated into the BACTEC™ MGIT 960 system device for 4 weeks for the accurate determination of *M. tuberculosis* drug-susceptibility.

2.7. Statistical analysis

All results were expressed as mean ± SD of three independent experiments. Statistical analysis of the data was performed using the Student's *t*-test and One-way ANOVA. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Evaluation of anti-*M. tuberculosis* activity of the extracts using REMA

The anti-*M. tuberculosis* activity of *M. azedarach* and *L. chinensis* extracts were evaluated using the REMA. The *M. azedarach* and *L. chinensis* were independently extracted using different solvents at room temperature for 24 h, respectively (Figure 1). After the bacteria were incubated with various concentrations (50–200 μg/mL) of the extracts and anti-*M. tuberculosis* first-line drugs (rifampicin and isoniazid, 1.25–5.00 μg/mL) for 5 days, the growth of *M. tuberculosis* were markedly inhibited in a concentration-dependent manner, and then their viabilities were 0% at a concentration of 100 μg/mL of all extracts (Table 1). The minimal inhibitory concentration (MIC) values of all extracts against the viability of *M. tuberculosis* were measured as 100 μg/mL (Table 2). Furthermore, the extracts obviously demonstrated their *M. tuberculosis*-inhibitory effect through the REMA. Although the superiority and specificity between the extracts regarding anti-*M. tuberculosis* effect were not confirmed in the REMA, the extracts showed similar anti-*M. tuberculosis* activity in both *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv. These results indicate that *M. azedarach* and *L. chinensis* extracts have not only anti-*M. tuberculosis* effects by strongly inhibiting the growth of *M. tuberculosis* but also the potential that can be

Table 1
The inhibitory effects of *M. azedarach* and *L. chinensis* extracts against the growth of *M. tuberculosis* (H37Rv and H37Ra) determined by the REMA.

Medicinal plant	Extracts	Concentrations (μg/mL)					
		H37Rv			H37Ra		
		200	100	50	200	100	50
M. azedarach	*n*-Hexane extract	+	+	–	+	+	–
	Chloroform extract	+	+	+/–	+	+	+/–
	Ethyl acetate	+	+	+/–	+	+	+/–
	Methanol extract	+	+	+/–	+	+	+/–
	H_2O extract	+	+	+/–	+	+	+/–
L. chinensis	*n*-Hexane extract	+	+	+/–	+	+	+/–
	Chloroform extract	+	+	–	+	+	–
	Ethyl acetate	+	+	+/–	+	+	+/–
	Methanol extract	+	+	+/–	+	+	+/–
	H_2O extract	+	+	+/–	+	+	+/–

The *M. tuberculosis* was incubated with different concentrations (50–200 μg/mL) of the extracts at 37 °C for 5 days. The "+" and "–" sign indicates anti-*M. tuberculosis* activity and no activity respectively, and "+/–" sign shows 55%–65% inhibitory rates of the extracts against the growth of *M. tuberculosis*.

Table 2
MICs of *M. azedarach* and *L. chinensis* extracts against the growth of *M. tuberculosis* (H37Rv and H37Ra) determined by different anti-*M. tuberculosis* indicator assays.

Medicinal plants	Extracts	MIC (μg/mL)	
		REMA	MGIT 960 system
M. azedarach	*n*-Hexane extract	100	> 800
	Chloroform extract	100	> 800
	Ethyl acetate	100	> 800
	Methanol extract	100	400
	H_2O extract	100	> 800
L. chinensis	*n*-Hexane extract	100	800
	Chloroform extract	100	> 800
	Ethyl acetate	100	> 800
	Methanol extract	100	> 800
	H_2O extract	100	> 800

M. tuberculosis (H37Rv and H37Ra) were incubated with different concentrations (25–800 μg/mL) of the extracts at 37 °C, and their susceptibility was evaluated by different anti-*M. tuberculosis*-indicator assays. The results of the REMA and the MGIT 960 system assay were determined after 5 days and 28 days of incubation, respectively.

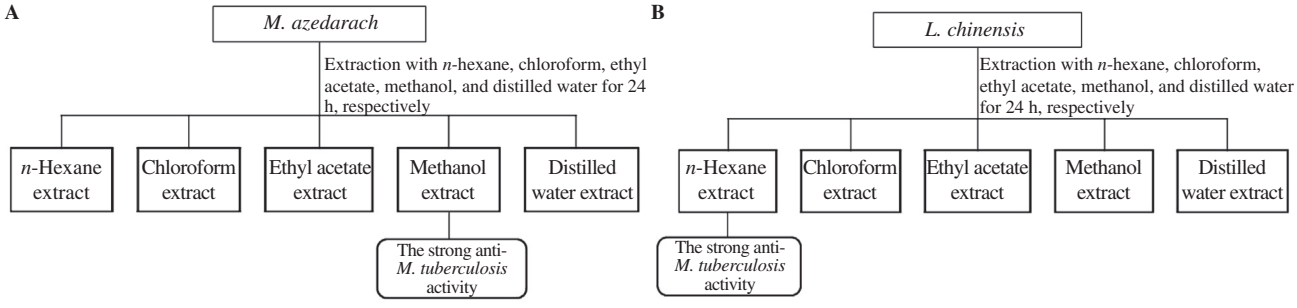

Figure 1. Extraction process of *M. azedarach* (A) and *L. chinensis* (B).

utilized or developed as promising anti-*M. tuberculosis* candidate agents through their new pharmacological activity.

3.2. Anti-*M. tuberculosis* effects of the extracts using the MGIT 960 system assay

The anti-*M. tuberculosis* effects of the extracts were further evaluated using the MGIT 960 system assay. The bacteria were incubated with various concentrations (25–800 μg/mL) of the extracts and anti-*M. tuberculosis* first-line drugs (10 μg/mL) in an MGIT growth media tube of the BACTEC™ MGIT 960 system device for drug susceptibility testing for 4 weeks, and their growth units were markedly inhibited in a concentration-dependent manner (Figure 2). Particularly, the methanol extract of *M. azedarach* and *n*-hexane extract of *L. chinensis* strongly inhibited the growth of *M. tuberculosis* compared with other extracts. When *M. tuberculosis* was incubated with 200 μg/mL of the methanol extract of *M. azedarach*, their growth units were detected at about 8.7 days, whereas 400 μg/mL of the methanol extract strongly inhibited the growth of *M. tuberculosis* compared with other extracts, and the growth unit of *M. tuberculosis* was not detected until 28th day. Furthermore, when *M. tuberculosis* was incubated with 400 μg/mL of the *n*-hexane extract of *L. chinensis*, their growth units were detected at 20.6 days, whereas 800 μg/mL of the *n*-hexane extract strongly inhibited the growth of *M. tuberculosis*, and the growth unit of *M. tuberculosis* was not detected until 28th day. In this MGIT 960 system assay, the methanol extract of *M. azedarach* and the *n*-hexane extract of *L. chinensis* consistently inhibited the growth of *M. tuberculosis* for 4 weeks with a single treatment (400 μg/mL and 800 μg/mL) respectively, and they showed more effectively the selective anti-*M. tuberculosis* activity as well as anti-*M. tuberculosis* action compared with other extracts. In addition, the extracts indicated higher activity against *M. tuberculosis* H37Ra than *M. tuberculosis* H37Rv in the MGIT 960 system assay (Figure 2). Particularly, the differences in anti-*M. tuberculosis* activity of them were obviously confirmed in the MGIT 960 system assay compared with the REMA assay (Table 2). These results demonstrate that the extracts not only induce anti-*M. tuberculosis* activity causing the inactivation of *M. tuberculosis* by effectively or consistently inhibiting the growth of *M. tuberculosis*, but also have unique anti-*M. tuberculosis* properties that inhibit or block the growth of *M. tuberculosis* in a concentration-dependent manner.

4. Discussion

The recent zoonotic diseases such as Middle East respiratory syndrome, avian influenza and Ebola have been occurred in various countries worldwide through diverse infectious pathways, particularly, in Asia, Middle East and Africa. Furthermore, the serious infectious pathogens such as influenza, *M. tuberculosis*, and methicillin-resistant *Staphylococcus aureus* have strongly enhanced their infectivity and viability through resistance or adaptability to drugs as well as environmental variation for the past decades, which induces serious difficulty to treatment and prevention of the disease as well as the development of the effective drug. In particular, these diseases have caused high risk symptoms to young children whose immune function is low and the elderly who have other underlying diseases or complications compared with normal adults. In this aspect, tuberculosis is a disease to cause infection through droplets, which has been causing high mortality and prevalence rate compared with other contagious diseases worldwide. Tuberculosis was one of the leading causes of death among various infectious diseases in 2013 and 2014 globally, particularly, in Africa [1,2]. It is the most dangerous infectious disease causing complications such as bronchiectasis, emphysema, and pneumothorax through chronic symptom. In addition, the rapid increase and appearance of XDR-TB/MDR-TB imply an urgent need to develop anti-tuberculosis drugs of effective/safe next-generation for treating tuberculosis. Until recently, despite various efforts to discover and to develop new anti-tuberculosis drugs, the development of effective/safe anti-tuberculosis drugs is still facing with direct or indirect-difficulty including the cost or time as well as the economic aspect and the profitability of pharmaceuticals. Furthermore, the overuse of antibiotics and antibacterial drugs has rapidly increased the prevalence and incidence rate of resistant-tuberculosis such as XDR-TB and MDR-TB, which has caused more difficulty to treat tuberculosis-patients compared with the past. In addition, the current anti-tuberculosis drugs induce various/serious side effects including hepatotoxicity, ototoxicity, and nephrotoxicity. In this aspect, as one of feasible alternatives for overcoming limitations of the drug's side effects, particularly, including rifampicin, isoniazid, ethambutol and moxifloxacin, various studies for developing anti-tuberculosis drugs were reported from active substances derived from traditional diverse medicinal plants and biological resources [19–23]. However, anti-tuberculosis agents of effective/safe next-generation that can be utilized or used as potential/novel first-line anti-tuberculosis drugs,

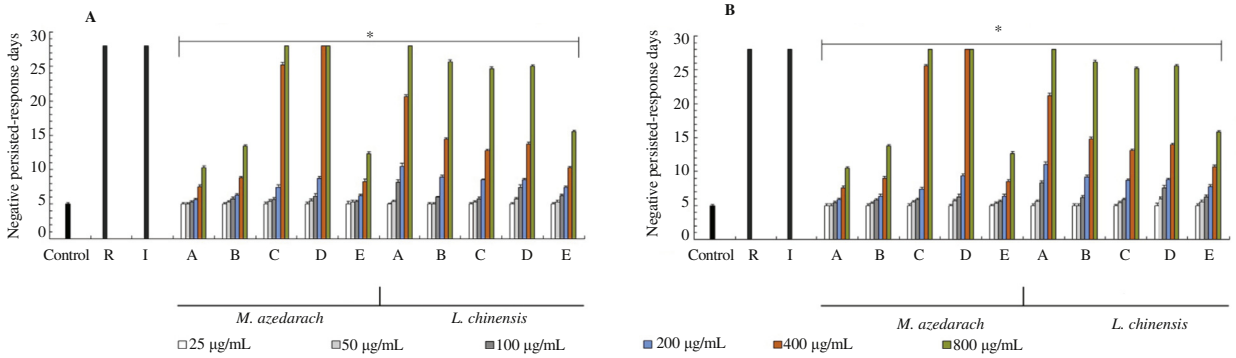

Figure 2. The anti-*M. tuberculosis* activity of *M. azedarach* and *L. chinensis* extracts against the growth of *M. tuberculosis* H37Rv (A) and H37Ra (B). Control: Untreated growth-*M. tuberculosis*; R: Rifampicin 10 μg/mL; I: Isoniazid 10 μg/mL; A: *n*-Hexane extract; B: $CHCl_3$ extract; C: Ethyl acetate extract; D: Methanol extract; E: Distilled water extract. The *M. tuberculosis* (H37Rv and H37Ra) were incubated with different concentrations (25–800 μg/mL) of the extracts in the MGIT 960 system device at 37 °C for 4 weeks, respectively. *: $P < 0.05$ was considered to be statistically significant compared with control.

particularly, the extracts, natural products, and/or semi-synthetic compounds, have not yet been reported in the global pharmaceutical market.

In this perspective, the new pharmacological activity of medicinal plants used in oriental medicine or traditional medicine can provide the advantages of both the safety and efficacy compared with newly developed drugs such as semi-synthetic compounds or biomedicine, which may be used or utilized as one of effective/feasible strategies for developing anti-tuberculosis drugs of effective/safe next-generation. Moreover, the plants selected from the existing medicinal plants through new pharmacological action may increase the potential which can be utilized as a useful/feasible resource for developing new substances in a medical field. For these reasons, this study has been focused on major key points for the discovery of new candidate substances as one of different strategies for developing anti-tuberculosis drugs, which has included crucial factors such as the minimization of side effects and the safety of drugs, as well as the finding of novel pharmacological activity and function of the medicinal plants used in Korean traditional medicine. In these aspects, *M. azedarach* and *L. chinensis* are medicinal plants that are used as biological resources of traditional oriental medicine in Korea, Japan and China as well as various countries and regions globally, which include various bioactive substances. Until recently, pharmacological activity and function of them have been variously reported as follows: (1) the aqueous extracts from *M. azedarach* induce the effect of wound healing by activating the growth of keratinocyte [24]; (2) the pediculicidal activity of ethanol extracts of *M. azedarach* [25]; (3) the leaves and bark extracts of *M. azedarach* through cyclooxygenase-2 and the inducible NO synthase inhibition cause anticancer activity and anti-inflammatory effects [26–28]; (4) the anti-diabetic activity of ethanol extract of *M. azedarach* [29]; (5) the anti-parasitic effect of hexane extract of *M. azedarach* against gastrointestinal nematodes [30]; (6) the antibacterial effect and antifungal activity of the extracts of *M. azedarach* against pathogenic bacterial and fungus strains [31,32]; (7) the methanol extracts and the compounds isolated from *L. chinensis* induce anti-oxidant activity and anti-inflammatory effects through the nuclear factor-κB pathways [33,34]; (8) the anti-viral effects of *L. chinensis* extracts in mouse model infected by herpes simplex virus type 1 [35]; (9) the anticancer effects of the compounds isolated from *L. chinensis* against human lung cancer cells [36,37].

Taken together, these studies show substantial evidence that *M. azedarach* and *L. chinensis* can be used or utilized as therapeutic agents through their unique pharmacological activity and function in both "*in vitro*" and "*in vivo*". However, despite the pharmacological activity or actions of them identified through these studies, their anti-tubercular activity have not yet been reported in both "*in vitro*" and "*in vivo*". For this reason, this study has been begun from hypothesis that *M. azedarach* and *L. chinensis* may strongly inhibit or block and effectively modulate the growth of *M. tuberculosis*. As mentioned above, the results of this study showed novel anti-tubercular activity of *M. azedarach* and *L. chinensis* extracts by effectively inhibiting the growth of *M. tuberculosis* through their novel pharmacological activity and properties. Particularly, anti-*M. tuberculosis* activity and the ability of *M. azedarach* and *L. chinensis* extracts were obviously demonstrated through different *M. tuberculosis* susceptibility-indicator assays such as the REMA and MGIT 960 system assay, and they consistently showed their anti-*M. tuberculosis* activity by strongly inhibiting the growth of *M. tuberculosis* for 4 weeks with a single treatment (400 μg/mL and 800 μg/mL) in MGIT 960 system assay, respectively. In addition, the specificity and differences in anti-*M. tuberculosis* activity of them were obviously confirmed in the MGIT 960 system assay compared with the REMA assay, and the methanol extract of *M. azedarach* more effectively inhibited the growth of *M. tuberculosis* compared with those of *L. chinensis* extracts. These results imply that the intracellular signaling-pathways for replication as well as key-proteins for regulating cell cycle in cytoplasm that accelerate the growth of *M. tuberculosis* and/or functions of the cell wall of *M. tuberculosis* are strongly inhibited or deactivated by the binding of the extracts. Furthermore, the extracts showed higher anti-*M. tuberculosis* activity in *M. tuberculosis* H37Ra than *M. tuberculosis* H37Rv in the MGIT 960 system assay, and it suggests that these results can be associated with differences in pathogenesis and virulence of the two strains or different pathogenic phenotypes between H37Rv and H37Ra.

In conclusion, *M. azedarach* and *L. chinensis* extracts effectively inhibited the growth of *M. tuberculosis* that can cause active tuberculosis in those with weakened immune systems through their novel pharmacological activity or function, and their anti-*M. tuberculosis* activity were obviously demonstrated through different anti-*M. tuberculosis* indicator assays such as the MGIT 960 system and the REMA. These results showed that the effective use of *M. azedarach* and *L. chinensis* extracts can be utilized as potential anti-*M. tuberculosis* agents for consistently inhibiting or blocking tuberculosis causing various complications in clinical fields. Therefore, this study provides significant evidence and the potential that *M. azedarach* and *L. chinensis* extracts can be used or utilized as promising candidate substances for developing novel anti-tubercular drugs of the effective next-generation in the near future through their new pharmacological activity concerning anti-*M. tuberculosis* effect.

Conflict of interest statement

We declare that we have no conflict of interest.

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