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Premna odorata Volatile Oil as a New *Mycobacterium tuberculosis* Growth Inhibitor for the Control of Tuberculosis Disease

Abeer H. Elmaidomy[1*], Hossam M. Hassan[1], Elham Amin[1], Waleed Mohamed[2] and Mona H. Hetta[3]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, 62514, Egypt.
[2]Department of Biochemistry, Kasr El-Eini Teaching Hospital, Cairo University, Egypt.
[3]Department of Pharmacognosy, Faculty of Pharmacy, Fayoum University, 63514, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author MHH designed the work. Authors HMH and EA performed the data collection and GC/MS analysis. Author WM made MeDipro Mycobacterium tuberculosis antigen ELISA technique and PCR analysis. Author AHE wrote the manuscript. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Aims: This study aimed to identify and compare *Premna odorata* Blanco volatile oil (VO) for the first time; isolated from different plant organs (leaves, young stems, and flowers) with evaluating the oil antituberculosis (anti-TB) activity.
Study Design: Experimental design was carried out by using hydrodistillation method, GC/MS analysis and *MeDipro Mycobacterium tuberculosis* (MTB) Antigen ELISA Technique (MMA-ELISA) accompanied by polymerase chain reaction (PCR) analysis.
Place and Duration of Study: This study was carried out at Faculty of Pharmacy, Beni-Suef University, between May to July 2017.
Methodology: *P. odorata* VO was identified using GC/MS analysis, the oil anti-TB activity was evaluated using *in vitro* and *in vivo* MMA-ELISA accompanied by PCR analysis.

**Corresponding author: E-mail: abeerabdelhakium@yahoo.com;*

Results: GC/MS analysis revealed that *P. odorata* VO consisted of monoterpenes, sesquiterpenes, diterpenes and higher alkanes; where monoterpenes and sesquiterpenes were represented the major oils fractions. Trans-caryophyllene (29.403% & 14.638%) and β-phellandrene (22.390% & 11.701%) were the major compounds in the leaves and young stems oils, respectively. While α-pinene (38.160%) was a characteristic component of the flower's oil. MMA-ELISA showed that a dose of 100 µl/ml *in vitro* and 300 µl/ml *in vivo*; the leaves, young stems, and the flowers oils separately had significant anti-TB activities with measured values > 1.5 µg/ml MTB antigen; while the three organs oils in combination 1:1:1 increased the potency of the oils against MTB with measured values < 1.5 µg/ml MTB antigen with PCR negative analysis.
Conclusion: *P. odorata* VO exhibited Anti-TB activity which Anti-TB could be related to the presence of cyclic terpenes (major) and acyclic oxygenated terpenes (minor) compounds.

Keywords: Premna odorata; GC/MS; trans-caryophyllene; β-phellandrene; α-pinene; anti-TB.

1. INTRODUCTION

Despite the availability of efficacious drugs used in treating tuberculosis (TB) infection, TB remained one of the major public health problems in the world. The World Health Organization (WHO) estimated over 8.8 million incident cases of TB in 2010 (140/100 000); of which 3.9 million (62/100 000) were smear-positive [1]. An estimated 1.1 million people died from TB in 2010 among Human Immunodeficiency Virus (HIV) - negative cases of TB with additional 0.35 million deaths among HIV positive cases [1]. The Philippines was ranked ninth among the 22 high TB-burden countries in the world and 3rd among the seven high-TB burden countries in the Western Pacific Region [1]. Although tuberculosis was considered a curable disease, it ranked 6th among the top leading causes of mortality and morbidity in the Philippines [1].

Based on reports from the Department of Health (DOH), there has been significant progress in the implementation of the Directly Observed Short-course Therapy for TB (DOTS) from 1996 to late of 2003 in the country. However, Dr. Woojin Lew, medical officer at WHO-Philippines; said the Philippine government has “a long way to go” in terms of detecting, treating and curing highly infectious TB patients [1]. He further expressed that highly infectious TB patients remain high in the Philippines. Locally, 75 people are dying every day from the disease [1]. The National Tuberculosis Control of the Philippines (NTP) has demonstrated two components of DOTS which are significantly related to improved clinical outcomes. These are government commitment to the program and continuous supply of anti-TB drugs to patients. In a country where drug supply and reliable distribution continue to be a problem and poor sanitation and nutrition, increase the probability of re-infection, adjunctive management for the control of TB can greatly improve its control [1].

P. odorata Blanco (Lamiaceae), popularly known as “alagaw”; synonyms: *Gumira odorata* Blanco, *P. curranii* H. Lam., *P. oblongata* Miq. Var, *P. serratifolia* Blanco and *P. vestita* Schauer; was a tree native to temperate and tropical Asia including the Philippines; where a decoction of the leaves was considered as adiuretic, carminative, febrifuge and used for vaginal irritation, coughs, beri-beri, abdominal pains, and dysentery [2]. It was also one of the seven plants present in a commercialized Philippine herbal preparation called “Pito-Pito” which used in a wide variety of cultural applications as headaches, fever, cough, colds, migraine, asthma, abdominal pains and diarrhea [2].

Traditionally; *P. odorata* leaves decoction was used in the treatment of the endemic tuberculosis disease [2]. However; a recent study showed that the crude methanol extract of the leaves had poor inhibitory activity against MTB [3]. Therefore; our attention was shifted towards the plant VO; as generally VO had long inhibition history against bacteria, viruses, and fungi; and some of these VOs had been proved their anti-TB activities as *Swinglea glutinosa*, *Achyrocline alata* [4], *Thymbra spicata* var. *spicata* [5], *Rhododendron anthopogon* [6], *Cuminum cyminum*, *Eugenia caryophyllata* and *Cinnamomum verum* [7]. Surveying the current literature, no reports could be traced discussing the VO composition of *P. odorata*or proving the oil activity against TB. Consequently, this study was aimed to identifyand compare *P. odorata*VO composition isolate from different plant organs (leaves, young stems, and flowers) with evaluating the oil activity against MTB.

2. MATERIALS AND METHODS

2.1 Chemicals and Kits

All chemicals were of high analytical grade, purchased from Sigma Chemical Co., Ltd (St. Louis, MO, USA). All kits were the products of Biosystems S.A. Costa Brava 30, Barcelona (Spain) and Diasys, Diagnostic systems GmbH, Germany. For conventional PCR targeting TRC4, the primer and red dye Master Mix kit were obtained from Bangalore Genei.

2.2 Plant Materials

The plant materials were collected from The Zoo, Giza, Egypt, May; 2017. It was kindly identified by Dr. Abd El-Halim A. Mohammed, Horticultural Research Institute, Department of Flora and Phytotaxonomy Researchers, Dokki, Cairo, Egypt. A voucher specimen (2016-BuPD 45) was deposited at Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University, Egypt.

2.3 Preparation of the Volatile Oils

The fresh leaves, young stems and flowers (5 kg each) were submitted separately to a hydrodistillation process using Clevenger apparatus for 2 h at temperature 75℃. The oils were collected separately, dried over anhydrous sodium sulfate and stored at 4℃ in air-tight amber glass containers. The yield (V/W %) was calculated based on the fresh weight of the plant material.

2.4 Animals

Albino mice with an average body weight of 25-30 g were obtained from the Animal House; NODCAR; Giza; Egypt. The animals were separated into groups of five, fed with normal commercial pellet diet, given water *ad libitum* and maintained under standard conditions (temperature 24–28℃, relative humidity 55-10% and 12 h light–dark cycle).

2.5 Animal Ethical Statement

This study was approved by the Ethical Committee of Beni-Suef University (2017-Beni-Suef, Egypt, Approval No. 127). All procedures and techniques used in this study were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised1985).

2.6 Acute Toxicity Test

The acute toxicity study was carried out using albino mice as per Organization for Economic Co-operation and Development (OECD) guideline 423 (2001). According to OECD guidelines; acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 h overnight, fasted mice were weighted and divided randomly into three groups each of two mice. As there is no information on a substance to be tested; for animal welfare reasons, OECD recommended using the starting dose of 100 µl/g b w. If mortality was not observed after 24 h, the procedure was repeated for further groups with higher doses such as 200, 300, 400, 500 and 600 µl/g b w.

2.7 GC/MS Analysis

GC/MS spectrometry was used to analyze the VO isolated from the different plant parts. Agilent 6890 gas chromatograph equipped with an Agilent technology 5973 mass selective spectrometric detector using a direct capillary interface and fused silica capillary column PAS-5 MS (30 m × 0.32 mm × 0.25 µm film thicknesses) was used for the analysis. Helium was used as a carrier gas at approximately 1 ml/min, pulsed splitless mode. The solvent delay was 3 min and the injection volume was 1.0 µl. The mass spectrometric detector was operated in electron soft impact ionization mode with ion energy of 70 E V scanning from 50 to 500 m/z. The ion source temperature was 230℃ and the quadrupole temperature was 150℃. The electron multiplier (EM) voltage was maintained at 1250 V above autotune. The instrument was manually tuned using Perfluorotributylamine (PFTBA). The GC temperature program was started at 80℃ then elevated to 280℃ at a rate of 10℃/min and 10 min hold at 280℃, the detector and injector temperature were set at 280 and 250℃, respectively. Wiley and Nist 05 mass spectral database were used for the identification of the separated compounds peaks and certified through comparison with published MS literature data [8].

2.8 *In vitro Medipro Mycobacterium Tuberculosis* Antigen ELISA Technique

In vitro anti-TB activities of the isolated VO were evaluated. ELISA was performedaccording to the manufacturer's instructions [9]. Purified MTB antigen with ESAT-6 and CFP (20 µg/ml) was used as positive control. Five groups were selected: group I, positive control; group II, positive control with leaves oil 1:1; group III, positive control with young stems oil 1:1; group IV, positive control with flowers oil 1:1 and group V, positive control with oils combination (1:1:1) 1:1. Separately, 100 µl of each group was added to individual wells coated with anti-TB antibodies on the ELISA microplate. This step was repeated ten times for each group; separately. The wells were incubated at 37℃ for 60 min then the liquid was aspirated from all wells. A diluted 1x washing solution (280-300 µl) was added to each well. The washing procedure was repeated three times. "Antibody-antigen" conjugate (100 µl) was inserted into each well and incubated at 37℃ for 30 min. The liquid was aspirated from all wells then washed with diluted 1x washing solution three times. The substrate 3, 3', 5, 5'-Tetramethylbenzidine (100 µl) was added to each well, followed by incubation at 37℃ for 10 min. Insert 100 µl of stop solution (1N HCl) to each well to stop the reaction. The optical density (OD) of each well was measured within 30 min of stopping the reaction by a microplate reader (Apollo 11 LB 913) at 450 nm using 650 nm as reference. Calculations of ESAT-6/CFP-10 antigen concentration for different specimens (X) (µg/ml) were carried out using the manufacturer's equation: Y ($OD_{450–650nm}$) = 0.0925X + 0.2791. Safety precautions were taken according to the manufacturer's instructions.

2.9 *In vivo Medipro Mycobacterium Tuberculosis* Antigen ELISA Technique

The effects of *P. odorata* VO on the infected TB mice were evaluated. Thirty normal mice (25-30 g) were selected for this study and divided into six groups each contains five mice as follows:

Group 1: Healthy mice (negative control).

Five groups oftuberculosis-infected mice which infected through injection intravenously with a positive TB solution of purified MTB antigen ESAT-6 and CFP (20 µg/ml) using a dose of 100 µl/d for seven days. Mice had sputum specimens smear positive for acid-fast bacilli (AFB) and/or culture positive for MTB were used in the experiment and classified in groups as follows:

Group 2: Untreated mice (positive control)

Group 3: Treated orally through gavages with leaves oil using a dose of 300 µl/d

Group 4: Treated orally through gavages with young stems oil using a dose of 300 µl/d

Group 5: Treated orally through gavages with flowers oil using a dose of 300 µl/d

Group 6: Treated orally through gavages with (1:1:1) combination of the three oils using a dose of 300 µl/d

After fifteen days, mice were sacrificed and blood samples were collected by puncture the sublingual vein. The blood samples clotting processes were allowed for 10 min prior centrifugation at 3000 rpm for serum separation. The serums were collected and the total MTB antigen level for each sample was separately determined according to *in vitro* MMA-ELISA.

2.10 Statistical Analysis

The data were expressed as mean ± S E (standard error) and statistical analysis was carried out using the one way ANOVA followed by Tukey test. A level of $^{*}P< 0.05$ was considered to be significant. The result was interpreted as positive infection for MTB antigen at a concentration >1.5 µg/ml.

2.11 PCR Analysis

The effect of *P. odorata* VO on the infected TB mice was evaluated qualitatively using PCR analysis. Sputum specimens were taken from *in vivo* MMA-ELISA groups I to VI. PCR analysis was performedaccording to the manufacturer's instructions [10,11]. The specimens were processed and decontaminated by N-acetyl L-Cysteine (NALC) - NaOH method and then centrifuged at 8000 rpm for 15 min. All the collected sediments were aliquot into multiple vials (25 µg nuclear protein/lane) and the remaining were processed for PCR within 24℃ in a freezer until testing. DNA was extracted from specimens. PCR reaction: A single tube nested PCR was performed using the proprietary IS6110 primer sequences targeting MTB. The DNA was amplified with forward and reverse primers of the

outer region of MTB and the first product was amplified with the inner primer in the second amplification. The PCR conditions for the outer sense primers for the first round of amplification: an initial denaturation was performed at 22℃ for 10 min, 94℃ for 5 min, 20 cycles of 94℃ for 30 s, 68℃ for 1 min, 72℃ for 1 min and a final extension of 72℃ for 7 min. The PCR conditions using the inner set of primers for the second amplification consisted of an initial denaturation step at 94℃ for 5 min, 30 cycles of 94℃ for 30 s, 68℃ for 30 s, 72℃ for 30 s and a final extension of 72℃ for 7 min. Samples were amplified using TRC4 Primer-1 (5'-GACAACGACGTGCGCCTACT-3') and TRC4 primer-2 (5'- ACCGAATTAGCGTAGCTCC-3'). The amplification cycles were performed in an automated thermal cycler (MJ Research Corp, Encino, CA, USA). The amplification consisted of 35 cycles of 94℃ for 1 min, 58℃ for 1 min, 72℃ for 1 min and the final primer extension step at 72℃ for 10 min. The detection of amplified PCR products was determined using agarose gel (2%) electrophoresis stained with ethidium bromide (0.5 µg/ml) and subsequently visualized on the 260 nm wavelength UV transilluminator of the gel documentation system (BIO-RAD, Hercules, CA, USA). A result was considered positive for the target when a well-defined DNA band corresponding to the sample was observed along with the controls and molecular weight marker. Samples which have amplified products measuring 123 bp for IS6110 and 173 bp for TRC4 were considered positive. The PCR tests were done three times independently on pulmonary and extra-pulmonary samples. The tests were performed in the same laboratory.

3. RESULTS AND DISCUSSION

3.1 GC/MS Analysis

P. odorata VO isolated from different plant organs (leaves, young stems, and the flowers) were differentiated in their physical properties (Table 1).

GC/MS analysis showed 20, 25 and 20 identified compounds characteristic for the leaves, young stems, and the flowers oils; respectively (Table 2, Figs. 1-3).

According to GC/MS analysis; *P. odorata* VO consisted of monoterpenes, sesquiterpenes, diterpenes and higher alkanes (Table 2); varying in their chemical structures from acyclic to cyclic compounds with qualitative and quantitative variation in each fraction. Sesquiterpenes represented the major oil fraction followed by monoterpenes (Table 3).

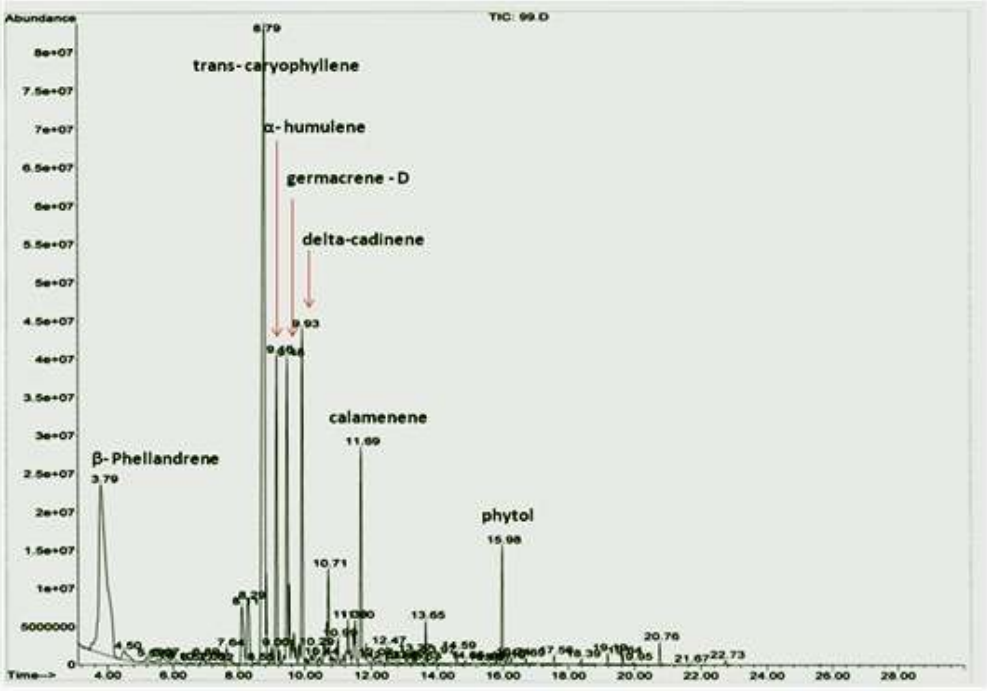

Fig. 1. GC/MS spectrum of *Premna odorata* leaves volatile oil

Table 1. Physical properties and percentage (% V/W) yield of *Premna odorata* volatile oil isolated from leaves, young stems, and flowers, respectively

Physical properties	Leaves oil	Young stems oil	Flowers oil
Color	Greenish yellow	Yellowish white	Dark yellow
Odor	Strong disagreeable	Strong disagreeable	Strong disagreeable
Appearance at room temperature	Faint white turbidity	Semisolid	Clear viscous
Density	Lighter than water	Lighter than water	Lighter than water
% yield (V/W)	0.028	0.011	0.044

Table 2. GC/MS analysis for the identified constituents of *Premna odorata* volatile oil isolated from leaves, young stems, and flowers, respectively

Compound	RRT	M^+	Leaves oil (%)	Young stems oil (%)	Flowers oil (%)
Cyclic monoterpene hydrocarbon					
β-phellandrene	0.432	136	22.390	11.701	nd
α-pinene	0.436	136	nd	nd	38.160*
Acyclic oxygenated monoterpene					
Linalool	0.512	154	0.898	4.384	1.762
Aromatic					
Methyl salicylate	0.659	152	nd	1.242	0.376
Cyclic sesquiterpene hydrocarbon					
α- copaene	0.923	204	2.327	1.068	1.856
Trans-caryophyllene	1	204	29.403*	14.638*	24.488
α-humulene	1.039	204	7.016	3.529	6.701
β-cubebene	1.073	204	nd	3.909	nd
Germacrene-D	1.078	204	9.389	nd	8.496
β-bisabolene	1.099	204	nd	2.549	nd
Delta-cadinene	1.129	204	7.429	4.289	6.518
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	1.150	204	1.665	1.377	0.808
Calamenene	1.166	202	5.103	3.937	3.152
Acyclic sesquiterpeneoxygenated					
Nerolidol	1.170	222	nd	2.161	0.867
Cyclic sesquiterpeneoxygenated					
Caryophyllene oxide	1.218	220	2.868	2.758	1.331
α-cadinol	1.305	222	nd	1.117	0.593
Acyclic sesquiterpene oxygenated					
Farnesol isomer B	1.374	222	nd	2.931	0.318
Acyclic diterpene					
Neophytadiene	1.509	278	0.321	nd	nd
Phytol	1.815	296	2.101	5.133	0.319
Higher alkane					
Eicosane	1.689	282	0.196	1.802	0.031
Heneicosane	1.997	296	0.408	4.086	0.064
Docosane	1.899	310	0.277	6.480	nd
Tetracosane	2.091	338	0.224	2.733	nd
Pentacosane	2.183	352	0.233	nd	0.025
Hexacosane	2.269	366	nd	1.081	nd
Heptacosane	2.361	380	0.388	3.283	0.119
Octacosane	2.464	394	0.031	0.327	nd
Nonacosane	2.586	408	0.132	1.788	0.093
Aromatic ester					
2-propenoic acid 3-(4-methoxyphenyl)- 2-ethylhexyl ester	2.632	290	nd	1.135	nd
% Total identified oil components			92.799	89.438	96.077

*Key: RRT =Relative retention time, M^+ =Molecular ion peak, nd =Not detected, * =major compound*

In *P. odorata* oil; cyclic monoterpenes and sesquiterpenes compounds as α- copaene, trans-caryophyllene, α-humulene, delta-cadinene, caryophyllene oxide and calamenene compounds were common in the leaves, young stems and the flowers oils in different concentrations (Table 2). Germacrene-D was founded only in the leaves and the flowers oils; α-cadinol was in the young stems and the flowers oils only. β -cubebene, β-bisabolene were founded only in the young stems oil; while β-phellandrene was founded in the leaves and the young stems oils. Characteristically; α-pinene was represented only in the flower's oil.

On the other hand, the diterpenes phytol was founded in the leave, young stems and the flowers oils (Table 2) with different concentration 2.1%, 5.1% and 0.3%, respectively. While *P. odorata* VO higher alkanes belonged to heptadecane to tetracosane group (17-54 carbon atoms) which physically solidify at room temperature (Table 2). The highest percentage of this fraction was founded in the young stems oil (21.58%) which affecting its clarity appearance at room temperature (Tables 1, 3).

According to literature, VOs isolated from *Premna* genus consisted of oxygenated and

Table 3. The percentage (%) of identified monoterpenes, sesquiterpenes, diterpenes and higher alkanes constituents in *Premna odorata* volatile oil isolated from leaves, young stems, and flowers, respectively

Class of the compounds	Nature	Leaves oil (%)	Young stems oil (%)	Flowers oil (%)
Monoterpenes	Hydrocarbons	22.390*	11.701	38.160
	Oxygenated	0.898	4.384*	1.762
Sesquiterpenes	Hydrocarbons	62.332*	35.487	52.019
	Oxygenated	2.868	8.776*	3.109
Diterpenes	Hydrocarbons	0.321	nd	nd
	Oxygenated	2.101	5.133*	0.319
Higher alkanes		1.889	21.580*	0.332
Others			2.377*	0.376
% total identified oil compounds		92.799	89.438	96.077

*Key: nd =Not Detected, *=highest percentage identified*

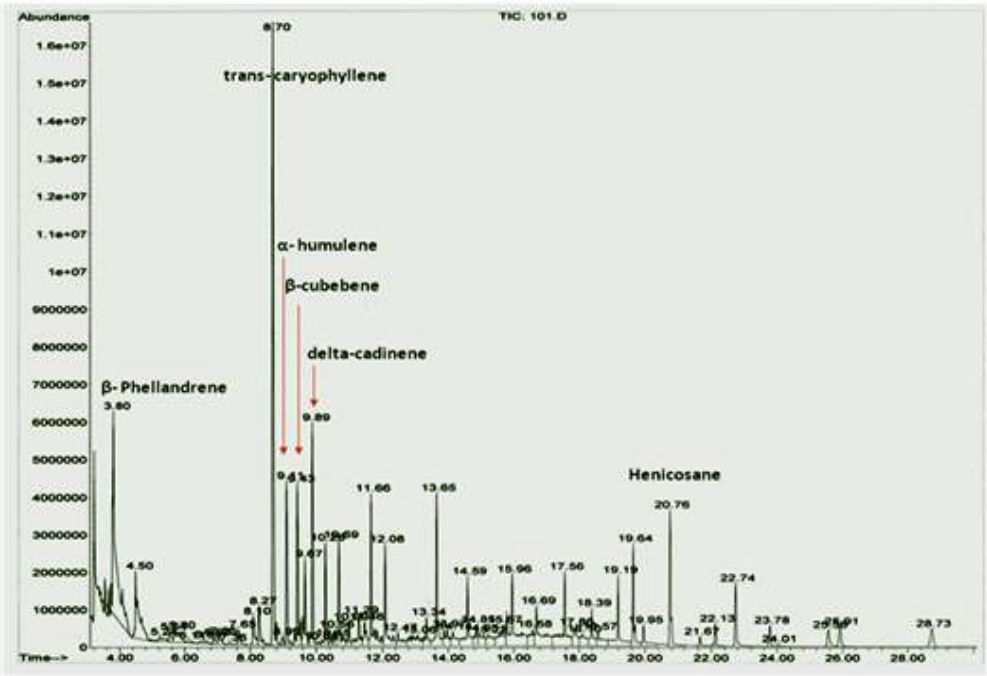

Fig. 2. GC/MS spectrum of *Premna odorata* young stems volatile oil

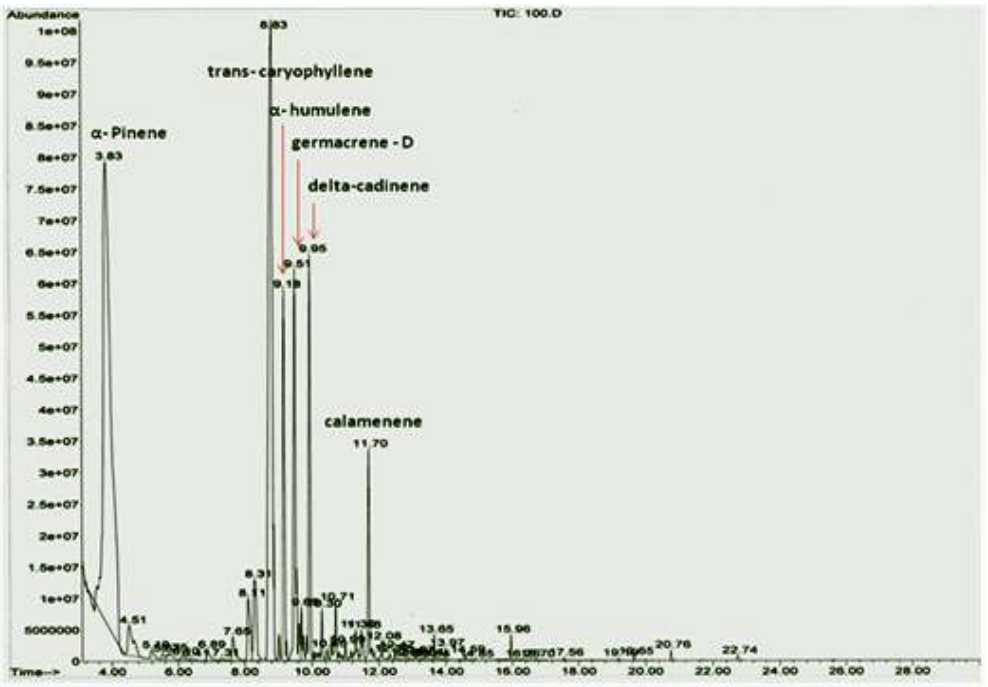

Fig. 3. GC/MS spectrum of *Premna odorata* flowers volatile oil

hydrocarbons compounds related to monoterpenes, sesquiterpenes, and diterpenes classes; while higher alkanes were founded only in *P. integrifolia* in the form of eicosane (0.62%) [12-14]. The chemical profile of *P. odorata* VO in this study turned to have some similarity with those of previous reports isolated VOs from*premna* genus. *P. coriacea*VO isolated from leaves showed to contain similar terpenes compounds as α-copaene (0.68%), caryophyllene (19.27%), bisabolene (4.65%), α-cubebene (0.71%), caryophyllene oxide (1.32%) and phytol (1.42%) [12]. While the chemical composition of the VO isolated from the leaves of *P. integrifolia* showed α- and β- pinene (0.86 and 1.11%, respectively), caryophyllene (0.92%), α-humulene (14.21%), caryophyllene oxide (2.60%), phytol (27.25%) and eicosane (0.62%) which also found in *P. odorata* VO in different quantity manner [13]. While VO isolated from the leaves of *P. angolensis*showed α-pinene (5.00%), linalolol (3.30%), caryophyllene (13.50%), α-humulene (6.40%), cadinene (1.30%), nerlidol (0.70%), α-cadinol (1.00%) and phytol (3.70%) [14]. Also*P. quadrifolia* VO isolated from leaves showed similarity in containing β-phellandrene (1.90%), α-copaene (1.70%), caryophyllene (13.10%), α-humulene (2.90%), germacrene-D (8.90%), β-bisabolene (1.90%), calamenene (0.40%), cadinene (3.30%), caryophyllene oxide (1.30%), and phytol (4.90%) [14].

3.2 Effect of *Premna odorata* Volatile Oil Using *In vitro Medipro Mycobacterium Tuberculosis* Antigen ELISA Technique

According to *in vitro* MMA-ELISA using a dose of 100 µl/ml; *P. odorata* leaves, young stems and flowers VO had a significant reduction in MTB antigen level comparing to the positive control group with measured values > 1.5 µg/ml MTB antigen. While the three organs oil combination 1:1:1 showed a significant reduction in MTB antigen level comparing to positive control with measured value 0.15±0.01 µg/ml (Table 4, Fig. 4).

3.3 Effect of *Premna odorata* Volatile Oil Using *In vivo Medipro Mycobacterium Tuberculosis* Antigen ELISA Technique

Acute toxicity test of the plant VO showed no signs of toxicity up to 1000 µl/g b w. Therefore, we investigated the potential anti-TB effects of the VO at a dose of 1/30 LD50 (300 µl/ml) in TB infected mice.

And in response to TB infection in mice, MTB antigen showed a significant increase in their level comparing to the negative control group (Table 4, Fig. 4). Treatment of the infected mice with *P. odorata* VO leaves, young stems and flowers separately; using a dose of 300 µl/ml showed a significant reduction in MTB antigen level comparing to the positive control group with measured values > 1.5 µg/ml MTB antigen. While treatment the infected mice with the three oils in combination 1:1:1 showed a significant reduction in MTB antigen level comparing to the positive control with measured value 0.97±0.08 µg/mlMTB antigen.

3.4 PCR Analysis

In the sediment of clinical samples taken from groups II, III, IV, and V that were positive by smear microscopy and culture, PCR results were also positive with primer tested. On the other hand, clinical samples taken from groups I, and VI were 100% negative in PCR (Fig. 5).

According to literature; cyclic and acyclic oxygenated terpenes: α-pinene, trans-caryophyllene [5], delta-cadinene [6], caryophyllene oxide [15], α-cadinol [16], calamenene [17], linalool, nerlidol and phytol [18] had anti-TB activities with different MIC values. According to GC/MS; *P. odorata* VO contained these cyclic and acyclic oxygenated terpenes compounds which reflective the *in vitro* and *in vivo* MMA-ELISA results (Table 4, Fig. 4); which showed in a dose of 100 µl/ml *in vitro* and 300 µl/ml *in vivo*; the VO of the leaves, young stems and flowers had a significant reduction in MTB antigen level comparing to the positive control group with a measured values > 1.5 µg/ml MTB antigen.

Table 4. *In vitro* and *in vivo MeDipro Mycobacterium tuberculosis* antigen ELISA technique results measuring the activities of *Premna odorata* volatile oil leaves, young stems, flowers and oil combination, respectively on *Mycobacterium tuberculosis* antigen (µg/ml)

MeDipro	Normal control	Positive control	Leaves oil treatment	Young stems oil treatment	Flowers oil treatment	Oil combination treatment
In vitro	nil	6.26± 0.62	3.01 ± 0.26*	2.35 ± 0.17*	1.83 ± 0.10*	0.15 ± 0.01*
In vivo	0.30±0.037*	14.64±1.16$^{@}$	5.94± 0.46$^{*@}$	2.88 ± 0.16$^{*@}$	1.98 ± 0.12*	0.97 ± 0.08*

Key: nil = not detected,Values represent the mean ± S E (standard error) of ten observations for each group (in vitro), and five mice observations for each group (in vivo), $^{*}P < 0.05$: Statistically significant from thepositive control group (Tukey test), $^{@}P < 0.05$: Statistically significant from thenegative control group (Tukey test)

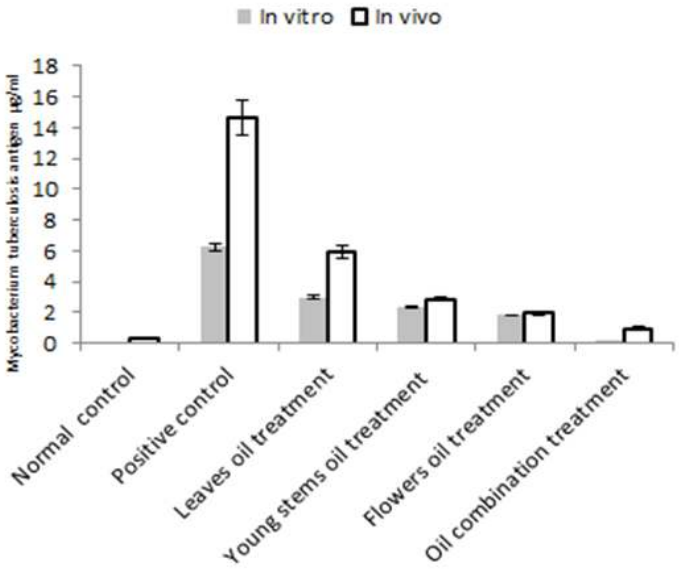

Fig. 4. *In vitro* and *in vivo MeDipro Mycobacterium tuberculosis* Antigen ELISA Technique results calculating as mean ± S E (standard error) testing the antituberculosis activity of *Premna odorata* volatile oils

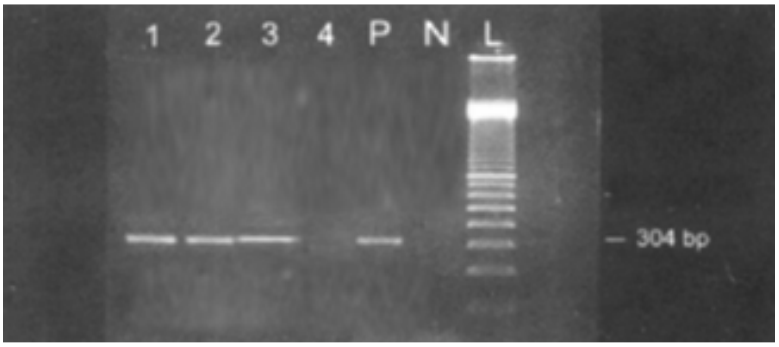

Fig. 5. Gel electrophoresis of PCR products, using primer for TRC4 *Mycobacterium tuberculosis* genes; lanes 1 (*Premna odorata* leaves oil treatment group), 2 (*Premna odorata* young stems oil treatment group), 3 (*Premna odorata* flowers oil treatment group), and positive control group (lane P) were positive for *Mycobacterium tuberculosis* TRC4 DNA (304 bp); lane 4 (*Premna odorata* oils combination 1:1:1 treatment group), and negative control group (lane N) were negative without *Mycobacterium tuberculosis* TRC4 DNA (304 bp); and 100 bp DNA ladder (lane L)

On the other hands; these cyclic and acyclic oxygenated anti-TB terpenes compounds composition differed qualitatively and quantitatively from plant organ to another (Table 2). These terpenes compounds represented 77.191% of the flowers oil composition in the form of monoterpenes, sesquiterpenes, and diterpenes; while the young stems and the leaves contained only 38.417% and 47.802% in the form of sesquiterpenes and diterpenes (Table 2). According to the biological results (Table 4, Fig. 4), these differences influenced the oils activities against MTB where the flower's oil was the most active followed by the stems oil, and the leaves oil (Table 4).

Although; *in vitro* and *in vivo* MMA-ELISA results (Table 4); showed that *P. odorata* VO isolated from the leaves, young stems, and the flowers had anti-TB activities, but these activities were not potent enough separately to bring MTB antigen level to be < 1.5 µg/ml using a dose of 100 µl/ml *in vitro* and 300 µl/ml *in vivo*. However; the oils combination (1:1:1) showed a significant reduction in MTB antigen level with measured values 0.15± 0.01 and 0.97± 0.08 µg/mlMTB antigen; *in vitro* and *in vivo*, respectively (Table 4). This result could be contributed to the combination of *P. odorata* different oils synergized the oils activities of each other through increasing the qualitative and quantitative content of the cyclic and acyclic oxygenated anti-TB terpenes compounds. These results were confirmed using PCR analysis which showed negative results for group VI (the oil combination treated mice group) (Fig. 5).

4. CONCLUSION

P. odorata VO isolated from different plant organs (leaves, young stems, and flowers) consisted of monoterpenes, sesquiterpenes, diterpenes and higher alkanes. Where monoterpenes and sesquiterpenes were represented the major oils fractions. Trans-caryophyllene (29.403% & 14.638%) and β-phellandrene (22.390% & 11.701%) were the major compounds in the leaves and young stems oils, respectively. While α-pinene (38.160%) was a characteristic component of the flower's oil.MMA-ELISA showed that at adose of 100 µl/ml *in vitro* and 300 µl/ ml *in vivo*; leaves, young stems, and the flowers oils separately had significant anti-TB activities with measured values > 1.5 µg/ml MTB antigen; while the three organs oils in combination 1:1:1 increased the potency of the oils against MTB with measured values < 1.5 µg/ml MTB antigen.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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