



Study of the Antimycobacterial and Anti-Biofilm Activity of the Essential Oils of *Duguetia confinis* [Engl & Diels (Chatrou)] and of *Vetiveria zizanioides* (L.)

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Received: October 23, 2025

Published: November 24, 2025

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Abstract

The aim of this study was to evaluate the antimycobacterial and antibiofilm potential of the essential oils of the bark *Duguetia confinis* [(Engl & Diels) Chatrou] and the rhizomes *Vetiveria zizanioides* (L). The essential oils were extracted by hydrodistillation and tested on the strains of mycobacteria, namely *Mycobacterium tuberculosis* H37Rv with ATCC (American Type Cells Cultures) code 2794 and a clinical isolate. The technique used for antimycobacterial tests is that of microdilution in liquid media for the determination of MIC and CMB inhibition parameters on planktonic cells. Then, the biofilm was induced by liquid culture on a polystyrene support. Finally, the biofilm inhibition parameters were determined in liquid media (CMIB and CMEB) and a comparison of the activity of planktonic cell inhibition parameters and biofilm inhibition parameters was made. The essential oils were obtained with a yield of 0.03% for *Duguetia confinis* and 0.26% for *Vetiveria zizanioides*. The minimum inhibitory concentrations (MIC) were 156.25 µg/mL for *V. zizanioides* on both the reference strain and clinical isolate, and 1250 µg/mL for *D. confinis* on both, showing bactericidal activity. The activity of the recorded biofilm inhibition parameters shows that Both *D. confinis* and *V. zizanioides* showed notable biofilm inhibition, with *D. confinis* at 156.25 µg/mL on the clinical isolate with a MIBC of 156.25 µg/mL on the clinical isolate. The activity of these EOs confirms the use of these plants for the treatment of respiratory tract diseases in traditional medicine and the results of the activity of *D. confinis* provide data missing in the literature review. These results show the potential antimycobacterial of the EOs tested and what could be a promising alternative solution for the treatment of tuberculosis.

Keywords: *Vetiveria zizanioides*; *Duguetia confinis*; Essential Oils; Antimycobacterial Activity; Anti Biofilm Activity

Introduction

Tuberculosis is an endemo-epidemic disease that is transmitted by airborne aerosols from a sick person to a healthy person. It generally attacks the lungs and is caused by infection with bacilli of the *Mycobacterium tuberculosis* complex. This complex mainly

comprises *Mycobacterium tuberculosis*, *M. bovis* and *M. africanum*, as well as other species rarely found in humans [1]. A WHO estimate for 2022 shows that approximately 10.6 million people worldwide, 2.5 million in Africa, and 44,000 in Cameroon respectively, had contracted tuberculosis. The number of deaths that same year

was 1.1 million worldwide, 310,000 in Africa and 7,400 in Cameroon [2]. In Cameroon, the disease doubled between 2002 and 2006, then stabilised at around 25,000 cases per year [3]. This increase led to a rise in the incidence rate from 77/100,000 in 2000 to 91/100,000 in 2004 out of a population of 18 million [3]. However, in 2020, the number of cases recorded was 9.9 million cases worldwide, 2.5 million in Africa and 45,000 cases in Cameroon. According to experts, in 2021, 1.5 million deaths were due to tuberculosis worldwide, 501,000 in Africa and 12,000 in Cameroon. In this context, it is important to highlight the case of drug-resistant tuberculosis, which represents a limitation in the treatment of the disease. Indeed, on a global scale, the WHO reported in 2021 that around 450,000 people were suffering from multidrug-resistant tuberculosis (MDR/RR-TB), 77,000 in Africa and 810 in Cameroon [4].

This phenomenon of resistance to anti-tuberculosis drugs is associated with the formation of biofilms. Three main hypotheses have been put forward to explain the mechanisms of biofilm resistance to antibiotics. The first is based on the notion of a physical barrier, which would explain the slow and incomplete penetration of certain antibiotics. The second hypothesis is linked to the specific environment of the biofilm, where the deepest zones, rich in acid residues and poor in oxygen and nutrients, could hinder the action of the antibiotic. Finally, the last hypothesis is based on the phenotypic changes observed in certain biofilms, in which the constituent micro-organisms could present more resistant forms [5]. *Mycobacterium tuberculosis* can develop a biofilm that makes treatment for the disease difficult. The *Mycobacterium tuberculosis* biofilm plays an important role in the process of breakage necrosis and cavity formation in lung tissue [6]. Bacterial biofilms are generally defined as aggregates of bacterial cells attached to a surface and embedded in a polymeric matrix [7]. These cells have the ability to adhere to new surfaces and reform a biofilm. It has been suggested that the importance of studying biofilms in this disease is due to the process of breakage necrosis and cavity formation in lung tissue, a site in which *M. tuberculosis* could form a biofilm [8,9]. Other experiments have shown a decrease in the activity of anti-tuberculosis drugs against tuberculosis biofilms [10,11]. These findings have prompted interest in the mechanisms of biofilm formation as a potential target for new TB therapies. Following

the appearance of biofilms, researchers are studying the benefits that could be derived from the use of medicinal plants and are exploring molecules to disrupt or prevent the polysaccharide matrix formation or target quorum sensing signals [12]. A medicinal plant is a plant used for its therapeutic properties [13]. Over the course of evolution, plants have developed substances (referred to here as active principles) with different functions, which may be a means of defence against parasites or other aggressors (micro-organisms).

In view of the spread of the resistance phenomenon and the limited number of antibiotics being developed, the discovery of new antibacterial agents has become more than essential. The use of essential oils (EO) is a highly promising alternative in the field of aromatherapy. Several clinical studies have confirmed the effectiveness of essential oil-based phytomedicines such as GelMyrteol®, a medicine used in German medicine to treat bronchitis and coughs [14]. Several plants in Cameroon are used to treat respiratory tract infections. The choice of *V. zizanioides* EO is based on its chemical composition rich in sesquiterpenes; compounds that have shown strong antibacterial activities according to the work of [15-21] and the choice of *D. confinis* based on its use in traditional medicine for the treatment of respiratory tract infections. The aim of this study was to evaluate the antimycobacterial activity and determine the effect of essential oils (EOs) on *M. tuberculosis* biofilms.

Materials and Methods

Collection and identification of the species studied

The bark of *Duguetia confinis* collected in the southern region in the locality of Bengbis with GPS coordinates (Latitude, 3° 26' 20.62 "N; Longitude 12° 26' 53.99 "E) and the roots of *Vetiveria zizanioides* collected in the Far North region in the locality of Mokolo with GPS coordinates (Latitude, 10° 44' 24.00 "N; Longitude 13° 48' 0.0 "E). Identification was made at the National Herbarium of Cameroon by comparison with the specimen identified under number 29365/HNC for *Duguetia confinis* and the 1st sample of *Vetiveria zizanioides* identified under number 67515/HNC.

Extraction of essential oils from *D. confinis* and *V. zizanioides*

The essential oils were extracted by hydrodistillation using "'Clevenger"-type equipment', dried with anhydrous sodium sulphate and stored at 4°C before biological testing [22].

Assessment of antimycobacterial activity

Bacterial strains

This study used an "ATCC: American Type Culture Collection" reference strain, namely: *Mycobacterium tuberculosis* H37Rv. This strain was offered by the tuberculosis and pharmacology research laboratory of the Nkolbisson Biotechnology Centre and kept by the Sangmélina Reference Hospital; and a clinical isolate from the Sangmélina hospital sample bank.

Culture media and reference substance

Lowenstein Jensen medium; Middlebrook 7H9 liquid medium supplemented with glycerol (2%); tween 20 (0.05% V/V), ketoconazole (antifungal drug) and a mixture of OADC (Oleic Acid Dextrose Catalase; nutrient supplement for the growth of mycobacteria) + PANTA (Polymixin B, Nalidixic acid, Trimethoprim, Azlocylline; a cocktail of antibiotics that inhibits the growth of germs other than mycobacteria) at 10% were used. Rifampicin® was used as the reference anti-tuberculosis drug and Ofloxacin® as the second-generation anti-tuberculosis drug.

Preparation of bacterial inocula

Pure *M. tuberculosis* colonies were picked with a plastic loop from Lowenstein Jensen agar slants and plated in MbK 7H9 broth supplemented with glycerol (2%), tween 80 (0.05%, v/v) and OADC + PANTA (10%) and inoculated for 7 days at 37°C. The optical density (OD) was read at 625 nm and ranged from 0.08 to 0.1, allowing the concentrated bacterial inoculum to be standardised at 10⁶ CFU/mL. Determination of inhibition parameters using the liquid microdilution method. Antimycobacterial activity was determined using the microdilution method [23]. Each well of a 96-well microplate received a volume of 100 µL of 7H9+10% OADC and PANTA medium supplemented with 2% glycerol and 0.05% v/v tween 80. Next, 100 µL of EO solutions were introduced into the first three wells of line 1 (test wells A, B and C) and from these wells, successive dilutions were made in a geometric progression of reason 2 in the direction from 1 to 12 up to the eleventh well; the concentration range varied from 5000 µg/mL to 4.88 µg/mL. Finally, a volume of 100 µL of mycobacterial inoculum was introduced into each well. Thus, the final volume is 200 µL in each well, with the concentration range varying from 2500 µg/mL to 2.44 µg/mL. All tests were run in triplicate. In a fourth column after the triplicate, a negative control test for EO activity is used, with wells containing 7H9 culture medium and HE at decreasing concentrations

from 2500 µg/mL to 2.44 µg/mL. Rifampicin® and Ofloxacin® were taken as reference antibiotics and tested using the same protocol as for EHs, but with previously prepared stock solutions of Ofloxacin® and Rifampicin® varying the final concentrations from 250 µg/mL (for wells in row 1) to 0.244 µg/mL (for wells in row 11). The twelfth row of the microplate is used as a positive control for mycobacterial growth not exposed to EHs. The microplate was sealed with its lid, covered with parafilm and incubated at 37°C for 7 days. After incubation, mycobacterial growth was detected by adding 20 µL of Blue Alamar solution to the wells of two of the three test columns. The wells of the third column were used to determine the BMC. The set was reincubated at 37°C for 1 day. The change in coloration of the medium from blue to pink indicates growth.

The MIC is defined as the lowest extract concentration for which there is no visible mycobacterial growth [23].

For the determination of MBCs, 50 µL of the wells of the third test column with concentrations greater than or equal to the MIC are subcultured in 150 µL of 7H9 medium contained in the wells of a microplate and incubated at 37°C for 7 days, then mycobacterial growth is demonstrated by adding 50 µL of a Blue Alamar solution to each well of the three test columns. The set was reincubated at 37°C for one day. The BMC is defined as the lowest concentration of EHs for which no visible germ growth is observed [23]. The experiment was performed in triplicate.

The BMC/MIC ratio was used to determine the bacteriological profile of the essential oils. The bactericidal effect will be observed if the CMB/CMI ratio is less than 4, bacteriostatic if CMB/CMI is between 4 and 16, and tolerant with respect to the microorganism present, if the CMB/CMI ratio is greater than 16 [24].

Evaluation of anti-biofilm activity

The anti-biofilm activity of essential oils was determined using the method of [25]. Firstly, a 96-well microplate was filled with 200 µL of inoculum containing a bacterial load of CFU/mL, then incubated at 37°C for 5 weeks. After incubation, floating microbial cells were removed from the wells by aspiration using a sterile syringe and the wells were then rinsed three times with phosphate-buffered saline (PBS). At the same time, concentrations of each EO were prepared in distilled water plus 7% Tween 80 and diluted in

Middlebrook 7H9 + PANTA + 10% OADC liquid culture medium to give final HE concentrations ranging from 5 to 0.002 mg/mL, the microplate wells were filled with 100 µL of 10% Middlebrook 7H9 + PANTA + OADC liquid culture medium containing each HE concentration, except for the wells in the 12th row, which were filled with 100 µL of culture medium and are considered controls. The microplate was then re-incubated under the same conditions. After re-incubation, biofilm growth was revealed by adding 20 µL of Blue Alamar. The CMIB was determined as the lowest concentration of the test product (essential oil or standard) for which the change in Blue Alamar colouration is not observed with the naked eye [26]. The experiment continued in the same microplates used for the determination of CMIBs. In the same day after reading the CMIBs results, the culture medium was removed from the microplate by aspiration using a syringe and using a micropipette, 100 µl of 0.4% crystal violet was added to each well for a period of 15 min to stain the biofilms. The wells were then rinsed four times with distilled water to remove the unbound crystal violet, and the microplates were left to dry for 2 h at 37°C. At the end, 100 mL of 95% (v/v) ethanol was added to each well, and the plate was shaken for 10 min to release the stain from the biofilms [27]. BMECs were determined as the concentration of test substance that resulted in biofilm destruction.

Results and Discussion

Results

The results obtained are reported in Table I; from this table it can be seen that the minimum inhibitory concentrations of the

essential oils varied from 1250 µg/mL (for *Duguetia confinis*) to 156.25 µg/mL (for *Vetiveria zizanioides*) on the reference strain and clinical isolate respectively. *D. confinis* EO showed the lowest activity on the clinical isolate and on the reference strain H37Rv with MICs equal to 1250 µg/mL, while *V. zizanioides* EO's activity remains unclassified due to undetermined MBC with MICs of 156.25 µg/mL. The clinical isolate tested compared with the reference strain showed moderate activity to Ofloxacin® and was resistant to Rifampicin®, a first-line anti-tuberculosis drug, with an MIC of 312.5 µg/mL compared with the sensitive reference strain H37Rv with an MIC of 4.88.

The parameters were used to calculate the MBC/MIC ratio, which indicates bactericidal or bacteriostatic action, or to determine bacterial 'tolerance'. *D. confinis* EO was bactericidal on the H37Rv strain and the clinical isolate, while *V. zizanioides* EO showed bacteriotolerant activity on the reference strain and the clinical isolate, as the MBC was not determined. These activities are nevertheless lower than those of the reference antituberculosis drugs, with the exception of the clinical isolate whose MIC for *V. zizanioides* EO is the same as that for ofloxacin®, namely 156.25 µg/µL.

The results presented in Table II show the biofilm inhibition parameters (CMIBs and CMEBs) achieved by the microdilution method; The table shows that the minimum biofilm inhibitory concentrations of the essential oils vary from 156.25 µg/mL (for *Vetiveria zizanioides* EO) to 2500 µg/mL (for *Duguetia confinis* EO) for the reference strain and from 156.25 µg/mL (for *Duguetia confinis* EO)

Table I: MBC and MIC inhibition parameters; MBC/MIC ratio and activity of test substances.

		MIC (µg/ml)	MBC (µg/ml)	Rapport MBC/MIC	Activity of test substance
Isolate	<i>V. zizanioides</i>	156,25	∅	∅	Not determined
	<i>D. confinis</i>	1250	1250	1	Bactericidal
	Rifampicin	312,5	625	2	Bactericidal
	Ofloxacin	156,25	312,5	2	Bactericidal
Reference strain H37Rv	<i>V. zizanioides</i>	156,25	∅	∅	Not determined
	<i>D. confinis</i>	1250	1250	1	Bactericidal
	Rifampicin	4,88	19,53	4	Bactericidal
	Ofloxacin	78,125	78,125	1	Bactericidal

to 312.5 µg/mL (for *Vetiveria zizanioides* EO) for the clinical isolate. *V. zizanioides* EO had the lowest activity on the biofilm of the clinical isolate with a MIC of 312.5 µg/mL and the highest activity was recorded for *Duguetia confinis* EO with a MIC of 156.25 µg/mL; on the biofilm of strain H37Rv, *D. confinis* EO had the highest activity with a MIC of 156.25 µg/mL. *Confinis* EO was the least active with a MIC of 2500 µg/mL and *Vetiveria zizanioides* EO was the most active with a MIC of 156.25 µg/mL. The search for CMIBs enabled us to qualify the activities of the EOs and the CMIB/CMEB ratio to give their efficacy. These results show that all the HEs have anti-biofilm properties. We found that the biofilm from the clinical isolate was sensitive to Ofloxacin® but resistant to Rifampicin® with CMIBs of

19.53 µg/mL and 1250 µg/mL respectively; while on the biofilm of the reference strain, Rifampicine® and Ofloxacin® have moderate activity with CMIBs of 312.5 µg/mL and 156.25 µg/mL respectively. These parameters were used to calculate the CMEB/CMIB ratio, indicating bactericidal or bacteriostatic action, or to determine bacterial “tolerance”. The CMIB and CMEB values calculated showed that *V. zizanioides* EO has bactericidal activity (CMEB/CMIB ratio ≤ 4) and bacteriostatic activity for *D. confinis* EO (CMEB/CMIB ratio ≥ 4) on the biofilm of the clinical isolate. On the reference strain H37rv, *V. zizanioides* EO has bacteriostatic activity and *D. confinis* EO is bactericidal. As for the anti-tuberculosis drugs studied, we found bactericidal activity (the ratio of CMEB/CMIB ≤ 4).

Table II: Biofilm inhibition parameters (CMIBs and CMEBs); CMEB/CMIB ratio and activities of test substances.

		CMIB (µg/mL)	CMEB (µg/mL)	MBC/MIC ratio	Activity of test substances
Reference strain H37Rv	<i>V. zizanioides</i>	156,25	1250	8	Bacteriostatic
	<i>D. confinis</i>	2500	2500	1	Bactericidal
	Rifampicin	312,5	625	2	Bactrricidal
	Ofloxacin	156,25	625	4	Bactericidal
Isolate	<i>V. zizanioides</i>	312,5	1250	4	Bactericidal
	<i>D. confinis</i>	156,25	1250	8	Bacteriostatic
	Rifampicin	1250	1250	1	Bactericidal
	Ofloxacin	19,53	156,25	4	Bactericidal

Discussion

Evaluation of the antimycobacterial activity showed that the EOs used in this study exhibited antimycobacterial activities with MICs ranging from 156.25 to 1250 µg/mL. According to the classification of antibacterial activities, an EO has strong activity if its MIC < to 100 µg/mL; medium or moderate if its MIC is between 1000 µg/mL > MIC > 100 µg/mL and weak if its MIC > 1000 µg/mL [28]. Of the EOs tested, that of *V. zizanioides* showed average activity with MICs of 156.25 µg/mL on all the mycobacteria tested and that of *D. confinis* showed low activity with MICs of 1250 µg/mL on all the mycobacteria tested. This result justifies their use in traditional medicine for the treatment of respiratory tract infections. According to [29], the ability of a plant to inhibit microbial growth is influenced by its chemical composition. If these EOs have an effect on the growth of *M. tuberculosis*, it is because they contain antimicrobial compounds such as monoterpenes, hydrocarbons and oxygenated sesquiterpenes. This composition is dominated by

the presence of bicyclogermacrene, humulene epoxide II, spathulenol, germacrene D, caryophyllene oxide, viridiflorene, α-pinene, β-caryophyllene, and β-pinene for *D. confinis* [30]; the oxygenated sesquiterpenes (Khusimol, (E)-Isovalencenol, α-Vetivone) for *V. zizanioides* [16]. Terpenes act against mycobacteria, through the action of trans-β-caryophyllene, p-cymene, α-phellandrene and α-pinene [31]. Their elucidated mechanism of action on mycobacteria shows morphological deformations caused as a result of increased membrane permeability [32]. These are active on various pathogenic microorganisms with an MIC of 31.25 to 500 µg/mL [16]. The deleterious effect on the structure and function of the microbial membrane and cell wall has generally been used to explain the antimicrobial action of essential oils and their components, particularly monoterpenoids. It has been shown that monoterpenes are capable of interacting with phospholipid membranes, functioning as interstitial impurities in the ordered structure of the lipid bilayer [33] due to their lipophilic nature; the prefer-

ence of terpenes for microbial membrane structures can then be described [34]. This hypothesis demonstrates the potential use of terpenes and phenylpropanes as antimycobacterial agents, since the cell wall of mycobacteria is highly lipophilic due to the presence of mycolic acids. The lipophilicity of the main components of essential oils allows them to interact easily with the mycobacterial cell wall, resulting in changes to cell permeability and microbial death. This difference in the activity of these two EOs is thought to be due to their chemical composition: in *V. zizanioides* EO, the main compounds are oxygenated sesquiterpenes, whereas in *D. confinis* EO, in addition to oxygenated sesquiterpenes, there are monoterpenes and hydrocarbons. In addition, the strong activity is mainly due to the inhibitory action of khusimol and khusenic acid. Khusimol and khusenic acid, two sesquiterpenes in the essential oil of *Vetiveria zizanioides*, have been shown to inhibit susceptible and resistant strains of *M. smegmentis* and *M. tuberculosis* by binding to both DNA gyrase subunits [14]. *V. zizanioides* essential oil obtained by hydrodistillation showed potent anti-tuberculosis activity at a minimum concentration of 500 µg/mL [20]. Lemongrass, geranium and vetiver essential oils reduce the expression of IL-6 and ISG 54 after infection with the Ross River virus when applied as a co-treatment [16]. Based on work by [19], *Vetiveria zizanioides* root extracts showed antimycobacterial activity against *M. tuberculosis* at a dose of 500 µg/ml. Anti-tuberculosis activity was shown in the hexane fraction at a very low concentration (50 µg/ml). The effect would remain active in the long term even after elimination of the oil. EOs from the *Duguetia* genus have demonstrated activity against *Staphylococcus aureus* and *Candida guilliermondii* with inhibition diameters of 12mm [35]. The pharmacological effects of essential oils of the *Duguetia* species are anti-inflammatory, antinociceptive, antibacterial, antifungal, antioxidant, anti-trypanosomal, cytotoxic and also antitumour properties [29]. The results obtained in our study are in line with other reports indicating that, due to their hydrophobicity, HEs probably act on the lipids in the wall of mycobacteria or mitochondria, functionally altering these structures by promoting increased permeability to proton [33]. The OEs showed significant activity on mycobacterial biofilms, with CMIBs ranging from 156.25 to 2500 µg/mL. Overall, the HE from *D. confinis* showed the best activity on the resistant clinical isolate, due to its predominant composition of Bicyclogermacrene, humulene epoxide II, germacrene D and caryophyllene oxide, α-pinene, β-caryophyllene, and β-pinene; compounds that have

better anti-biofilm properties [17]. This result shows that EOs are effective in the fight against biofilms. They are complex mixtures of compounds with biological activities attributed in most cases to their main chemical constituents. However, certain constituents in low concentrations can interact with each other to have synergistic effects, thereby increasing the activity of EOs, or antagonistic effects, thereby reducing their activity [36]. The work of [37] refers to the fact that *in vitro* biofilms of *M. tuberculosis* are more persistent against antibiotics than their unicellular planktonic counterparts, hence the tests with essential oils. According to the work of [38], essential oils have the ability to destroy biofilms by altering the quorum sensing phenomenon. In [39] reported that Antibiotic resistance through phenomena such as biofilm formation, for example, is linked to a microbial cell-to-cell communication system known as quorum sensing. In this respect, bioactive molecules derived from plants, in particular terpenoids, flavonoids, etc. and essential oils, have numerous anti-quorum sensing mechanisms via inhibition of the release of autoinducers, sequestration of QS-mediated molecules and deregulation of QS gene expression; bearing in mind that in biofilms, cells live in synergy, these mechanisms are fatal to them. However, the clinical applications of these molecules are not fully covered, which limits their use against infectious diseases. Ojha, *et al.* have shown that in non-motile mycobacteria, shorter-chain mycolic acids play an important role in the development of biofilm structure [40]. They also proposed that these shorter mycolic acids could form a hydrophobic extracellular matrix [9]. Burt's work in 2004 showed that essential oils may have a greater capacity to penetrate this matrix, and have the capacity to disrupt the structure of the extracellular matrix of biofilms, thus weakening their integrity and making bacterial cells more vulnerable [41,42]. These reasons could explain why *Duguetia confinis* EO is more active on the biofilm of the isolate than on free bacteria and why *Vetiveria zizanioides* EO is more active on the biofilm of the reference strain H37Rv.

Conclusion

The results of this study highlight the inhibitory effect of *Vetiveria zizanioides* and *Duguetia confinis* essential oils on the ATCC code *Mycobacterium tuberculosis* H37Rv strain and on the clinical isolate from the Sangmélina hospital sample bank. In the light of the results obtained, we can deduce that Cameroon's flora may constitute a significant reserve of interesting plant species whose active prin-

ciples may be used in several fields. Future studies will develop plant-based medicine to combat tuberculosis, reduce treatment duration, and address resistance by identifying active ingredients, testing components, combining oils, and testing on *Mycobacterium tuberculosis* complex strains and other microorganisms an improved plant-based traditional medicine to combat tuberculosis, reduce the duration of treatment and resistance, firstly by identifying the active ingredients responsible for antimycobacterial activity using high-performance analytical techniques and testing the different components of these essential oils separately, then to combine these essential oils and test them on strains of mycobacteria from the *Mycobacterium tuberculosis* complex and extend the tests to other micro-organisms. However, it would be necessary to carry out research under *in vivo* conditions to assess the probable toxicity and pharmacological effects.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The Tuberculosis and Pharmacology Research Laboratory of the Nkolbisson Biotechnology Centre, the Laboratory of the Sangmelima Reference Hospital and the Bacteriology Laboratory of the Yaoundé University Hospital.

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