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Effects of Essential oil from *Drypetes gossweileri* S. Moore stem barks on Cell Release and DNA Synthesis of *Mycobacterium tuberculosis*

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ABSTRACT

Background: In the recent years, the proliferation of multi-drug-resistant and extensively drug-resistant strain to tuberculosis (TB) suggests that efforts are required to find alternative treatments. The designed study aimed to show the effects of essential oils (EO) from *Drypetes gossweileri* stem barks on *Mycobacterium tuberculosis* cell membrane release and DNA synthesis. Methods: The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined against two clinical isolates (IS53 and IS310) and the reference strain H_{37}Rv ATCC 27294 using microdilution method. The effect of essential oil on cell membrane release of *Mycobacterium tuberculosis* was evaluated by measuring DNA, RNA and proteins release in extracellular medium using NanoDrop 1000 spectrophotometer to show the membrane integrity lose. The effect on DNA was performed by measuring genomic DNA and amplicons of MIRU 04 sequence produced when treated at MICs and MBCs concentrations to put in evidence the inhibitory effect of EO during DNA synthesis. Results: The results revealed that EO from *Drypetes gossweileri* stem barks exhibited strong activity with MIC ranging from 4.88 µg/mL against H_{37}Rv and IS310 to 9.76 µg/mL against IS53. The significant release of DNA, RNA and proteins in extracellular medium were observed for treated cells at MIC and MBC concentrations compare to untreated cells. The most quantified biomolecules were proteins with concentration ranging from 370.9 10^4 ng/µL to 10630.0 10^4 ng/µL released at MIC concentration which increased from 1890.0 10^4 ng/µL to 12000.9 10^4 ng/µL at MBC. The inhibitory effect of DNA synthesis by EOs enhanced lower quantity of DNA for all treated cells at MIC and MBC compare to untreated cells. The results obtained in this study enabled the identification of two cellular targets (cell membrane and DNA) of EO from *D. gossweileri* stem barks on *M. tuberculosis*.

Keywords: Antimycobacterial, Cells release, DNA inhibition, Essential oil.

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INTRODUCTION

Antibiotic resistance remains a considerable problem for tuberculosis (TB) treatment, despite the introduction of new antitubercular drugs into therapy [1]. This situation is very alarming and drug therapy research is an urgent need in the process of management and control of tuberculosis treatment [2]. Therefore, efforts are required to search for new molecules to prevent the spread of the disease. In Africa, applications of traditional knowledge or use of plant extracts in medicinal practices can provide an excellent database for the potential identification of sources new lead compounds with bioactive properties in the field of anti-TB agents. An ethnobotanical survey on anti-TB potential of medicinal plants have been reported and *Drypetes gossweileri* S. Moore is one of the twelve used in two localities of Nkam Division (Littoral region of Cameroon) [3]. *Drypetes gossweileri* S. Moore is a dioic plant of the *Euphorbiaceae* family with an average size of about 30 to 40 meters [4]. Several parts of plants are also used in traditional medicine to treat fever, malaria, intestinal worms and respiratory infections in Central Africa [5]. Glucosinolates are sulfur-containing compounds found in stem barks such as glucosinalbin (4-hydroxybenzyl) and glucotropaeollin (benzyl glucosinolate) [6]. These glucosinolates compounds give isothiocyanate, nitrile and thiocyanate derivatives

through enzymatic pathway depending on pH. Isothiocyanates compounds (ITC) released during extraction of essential oil by hydrodistillation process are the most abundant and reactive compounds [7,8]. Some researchers argued that isothiocyanates derivates play protective role in plant and many studies focus on the medical properties in cancer prevention and food preservatives [9-12]. Furthermore, natural and synthetic isothiocyanates have showed antibacterial activities against *Escherichia coli*, *Clostridium difficile* and antimycobacterial activity against *Mycobacterium tuberculosis* W, $H_{37}R_v$, P and AB strains [3, 13-16]. In addition to that, essential oil from *D. gossweileri* stem barks has several biological activities such as antigerminative effect on *Bacillus* spore, antioxidant, antiinflamatory and antiycobacterial activity against resistant strains [3, 4, 12]. Though high number of studies on the EOs activities have been performed on the investigation of its specific bacterial target (s), no previous studies have yet reported the cellular targets of *D. gossweileri* plant or its causing effects on *Mycobacterium tuberculosis* [17]. Nevertheless, Sieniawka *et al.*, demonstrated that the essential oil from *Mutellina purpurea* L. and its main constituents at MIC might exert morphological changes in the overall *Mycobacterium tuberculosis* $H_{37}Ra$ cell shape and cytoplasm homogeneity [18]. Dwivedi *et al* in 2013 proved that khusenic acid and khusimol two sesquiterpenes of *Vetiveria zizanoides* essential oil showed their antimycobacterial activity by binding to mycobacterial 2Y3P and 1EI1 subunits of DNA gyrase [19]. Based on the results of our previous findings on the antimycobacterial activity of essential oil from *D. gossweileri* stem barks [3], the present investigation was to show its effects on *Mycobacterium tuberculosis* membrane integrity and DNA synthesis.

MATERIALS AND METHODS

Plant material and chemical Analysis of essential oil

Drypetes gossweileri stem barks were identified at Cameroon National Herbarium under the code 25749/SRF/CAM during a previous study carried out by Moni *et al* [3]. The essential oil was obtained by hydrodistillation using Clevenger apparatus and analysed by Gas Chromatography (GC) and Gas Chromatography associated with Mass Spectrometry (GC-MS) as describe by Moni *et al*.

Anti-mycobacterial Activities

Chemical Reagents and Solvents

Glycerol and Tween 80 were purchased from Sigma-Aldrich (France). The Middlebrook 7H9 OADC supplement and Alamar Blue were purchased from Becton Dickinson (USA).

Essential oils and Anti-tuberculosis

Drugs solutions. Stock solutions of essential oils were dissolved in the 7 % tween 80 solution at the concentration of 10 000 µg/mL. Stock solutions were aliquot and kept at -20 °C until use. Standard anti-tuberculosis drugs at 1000 µg/mL: Ofloxacin (OFX) N°GS030093 (TTM e.v, Germany) and Rifampicin (RIF) N°GS030035 (TTM e.v, Germany).

Mycobacterium Strains and Growth Conditions

Three *Mycobacterium tuberculosis* strains were used for this study; the H37Rv ATCC 27294 and two clinical isolates The IS53 and IS310. The sensitivity of IS53 to antibiotic has been provided using Line Probe Assay genotype (Hain Livfescience kits) test at the Laboratory for Tuberculosis Research of the Biotechnology Centre of University of Yaounde I during a previous work meanwhile the sensitivity profile of *M. tuberculosis* IS310 was unknow. These bacterial strains were maintained on slant of both Löwenstein Jensen medium and Löwenstein Jensen medium supplemented with pyruvate (Himedia, India).

Preparation of Inoculum for Biological Assay

From this solid media cultures, a suspension was prepared in Middlebrook 7H9 broth lot N°0000203601 (Himedia, India), containing 0.2 % v/v glycerol, 0.05 % v/v tween 80 (Sigma-Aldrich, France) and 10 % v/v of Middlebrook 7H9 OADC supplement (*oleic acid–albumin–dextrose–catalase*; Becton Dickinson, USA). The suspension was adjusted to turbidity compared to a 0.5 McFarland standard (10^5–10^7 cfu/mL). Prior to antimycobacterial assay, the absence of contamination was confirmed by culturing in the Brain and Heart Infusion (BHI) agar medium [20].

In vitro Anti-mycobacterial Assessment of Essential oils

The geometric serial broth microdilution method was carried out according to the Microplate Alamar Blue Assay (MABA) as described previously by Collins and Franzblau and modified by Jimenez-Arellanes *et al.* [21, 22]. A stock solution was then added to Middlebrook 7H9 broth to reach final samples concentrations ranging from 5 000 µg/mL to 4.88 µg/mL. Serial dilutions were inoculated with mycobacteria inocula (10^6 cells/mL prepared from the Middlebrook 7H9 Broth) to obtain concentration ranging from 2500 µg/mL to 2.44 µg/mL. Each 96 wells microtiter plates were mixed and incubated at 37 °C for 7 days. Rifampicin® and Ofloxacin® at 250 µg/mL to 0.244 µg/mL were used as reference. Upon incubation periods, 20 µL of 0.02 % resazurin salt solution were added to individual wells and the plates reincubated for additional 1 day and checked for colour change. Change in resazurin colour from blue to pink indicated reduction of the indicator and thus bacterial growth.

The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of samples at which the microorganism did not demonstrate growth [23].

The Minimal Bactericidal Concentrations (MBC) of promising essential oils was assessed by sub-culturing MIC test microtiter plates on Middlebrook broth 7H9 medium. The MBC was considered as highest dilution or lowest concentration at which no growth occurred in the medium.

Effects of essential oil from *D. gossweileri* on cell release and DNA synthesis *M. tuberculosis*

Effect on cell release

The leakage of biomolecules was determined in a previous study using spectrophotometric quantification of absorbents materials at 260 nm for nucleic acids and 280 nm for proteins [25]. In this study, the biomolecules release by *M. tuberculosis* H37Rv ATCC 27294, clinical isolate IS53 and IS310 was determined by quantification of cellular materials released in extracellular medium, especially DNA, RNA and proteins using NanoDrop 1000 spectrophotometer (Thermo Scientific) [25]. Essential oil from *D. gossweileri* stem barks at the MIC and MBC concentration was added to the bacterial inoculum (10^7cfu/mL) in sterilized Middlebrook 7H9 supplemented by 10 % of OADC (V/V) and then incubated at 37 °C. After seven days, 100 µL of mycobacterial samples were collected, homogenized and the supernatant was measured using NanoDrop 1000 spectrophotometer. All the measurements were performed in triplicate.

Statistical analysis

The statistical analysis was evaluated using SPSS software (version 20.0). One-way analysis of variance (ANOVA) was performed. The difference in DNA, ARN and proteins

released at MIC and MBC of EO were evaluated using Turkey test. The significant difference was $P < 0.05$ and the data were expressed as mean ± SD.

Effect of EO from *D. gossweileri* on DNA synthesis

The effect on DNA synthesis was performed using DNA inhibition assay evaluated by quantification of total DNA and amplicons of MIRU 04 gene using NanoDrop 1000 Spectrophotometer. After seven days of culture at 37 °C with essential oil from *D. gossweileri* at MIC and MBC concentrations, total DNA was isolated from 50 µL of *Mycobacterium tuberculosis* $H_{37}RV$, IS53 and IS310 using QIAamp DNA Mini kit (Qiagen 51306, Germany). To show the effect of EO on DNA at MIC and MBC concentrations, the genomic DNA extracted were quantified using Nanodrop 1000. The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. When the ratio at 260/230 was appreciably lower than the range of 1.8-2.2, this was indicated the presence of co-purified contaminants [25]. Inhibition effect was detected if the quantity of DNA from treated cells was lower than quantity of untreated cells.

Effect of EO from *D. gossweileri* on MIRU 04 gene

The MIRU 04 gene was amplify by PCR using MIRU 04 forward sequence 5'-GCGCGAGAGCCCGAACTGC-3' (H37302 46-52-87-20/480 Eurofins) and total DNA extracted. To show the effect of EO on MIRU 04 gene, its amplicons were quantified using Nanodrop 1000. When the ratio at 260/230 was appreciably lower than the range of 1.8-2.2, this was indicated the presence of co-purified contaminants [25]. The inhibition effect was detected if the quantity of DNA from treated cells was lower than untreated cells.

RESULTS

Antimycobacterial activity

The analysis of inhibitory activities showed that EO from *D. gossweileri* stem barks is more active against all *M. tuberculosis* strains used for the test with activity at 4.88 µg/mL against $H_{37}Rv$, IS310 and at 9.76 µg/mL against IS53 as present in Table 1. These activities are bactericidal against H37Rv and bacteriostatic against the two isolates.

Table 1: Inhibition parameters of EO from *D. gossweileri* and Ofloxacin against *M. tuberculosis*

Strain	EO		Ofloxacin		Rifampicin	
	Inhibition parameters (µg/mL)					
	MIC	MBC	MIC	MBC	MIC	MBC
H37Rv	4.88	9.76	31.25	62.50	0.48	1.95
IS53	9.76	39.06	62.50	125	0.48	1.95
IS310	4.88	78.12	7.81	7.81	125	250

Legend: MIC = Minimal inhibitory Concentration; MBC = Minimal Bactericidal concentration

Effects of essential oil from *D. gossweileri* on *M. tuberculosis*

Effects of essential oil from *D. gossweileri* on cell release

The cellular targets of EOs from plants in bacteria are scarce and the specific mechanism of action is still not completely understood. The quantities of biomolecules (nucleic acid and proteins) present in extracellular medium are used to identify alterations of the cell membrane permeability. These quantities of biomolecules released by *Mycobacterium tuberculosis* H37Rv, IS53 and IS310 exposed at MIC and MBC of essential oil concentrations are reported in figures 1, 2 and 3 below.

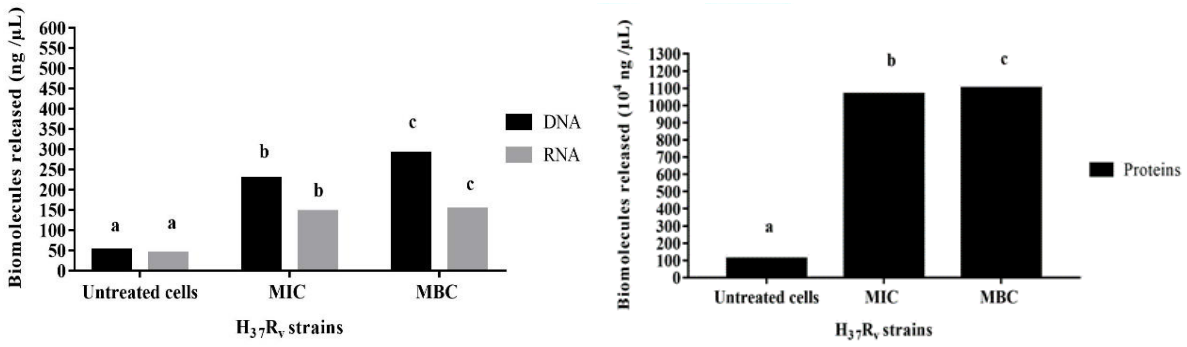

Figure 1: Quantity of biomolecules released by H37Rv treated with *D. gossweileri* EO

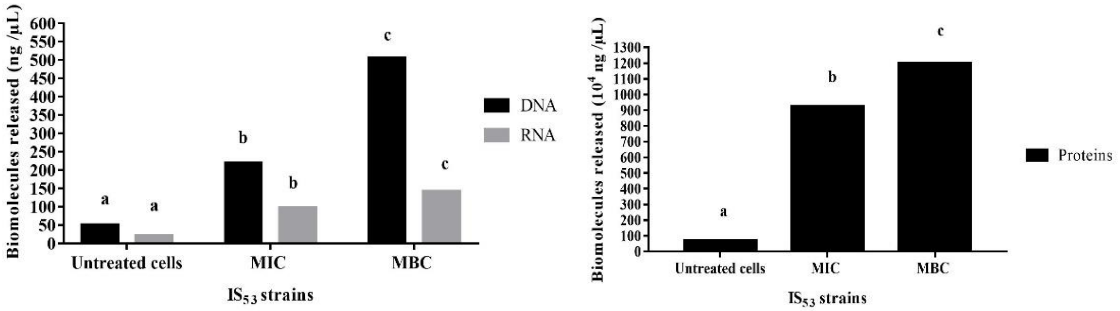

Figure 2: Quantity of biomolecules released by IS53 treated with *D. gossweileri* EO

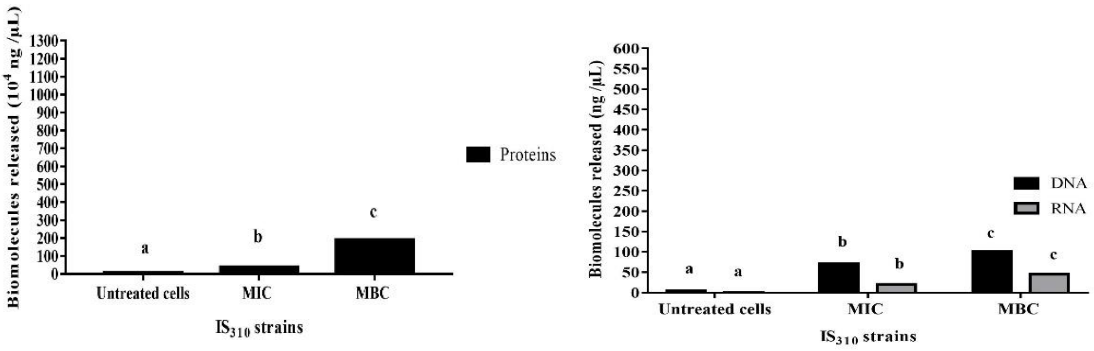

Figure 3: Quantity of biomolecules released by IS310 treated with *D. gossweileri* EO

The results showed that significant quantity of DNA, ARN and proteins was present in extracellular medium for treated cells at MIC concentration which increase at MBC concentration compared to untreated cells (P < 0,05). Proteins are the most released constituents with concentrations ranging from 5 10^4 ± 0.6 ng/µL for IS310 to 107 10^4 ± 2.4 ng/µL for H37Rv in untreated cells and from 37. 9 10^4 ng/µL ± 0.27 for IS310 to 1200.9 10^4 ± 1.20 ng/µL for IS53 in treated cells. Nucleic acids are released in extracellular medium with concentrations ranging from 70 ± 1 ng/µL at MIC in IS310 to 506 ± 2.03 ng/µL at MBC in IS53 for DNA and from 20.2 ± 0.11 ng/µL at MIC in IS310 to 151.6 ± 2.02 ng/µL at MBC in H37Rv for RNA.

Effects of essential oil from *D. gossweileri* on DNA synthesis

Regarding the effect of *D. gossweileri* essential oil on *M. tuberculosis* DNA synthesis, the results are presented by the series of figures 4 to 6. The analysis of these figures showed that the quantities of DNA and MIRU 04 amplicons are low for treated cells than untreated cells. In H37Rv, EO from *D. gossweileri* reduced DNA synthesis at concentration of 2. 2 ng/µL at MIC, 0.2 ng/µL at MBC and reduced the amplification of MIRU gene to 70.0 ng/µL at MIC and 9.80 ng/µL at MBC. In IS53, the concentrations of DNA were ranging from 4. 10 ng/µL at MIC, 0.70 ng/µL at MBC and reduced the amplification of MIRU 04 gene to 20.0 ng/µL at MIC and 4.10 ng/µL at MBC. In IS310, the concentrations of DNA were ranging from 5. 01 ng/µL at MIC, 3.90 ng/µL at MBC and reduced the amplification of MIRU 04 gene to 26.90 ng/µL at MIC and 11.90 ng/µL at MBC.

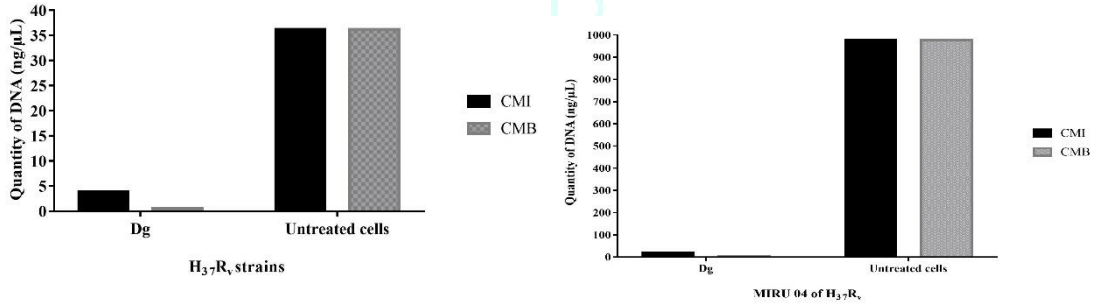

Figure 4: Inhibitory of DNA and MIRU 04 gene synthesis by EO from *D. gossweileri* on H37Rv

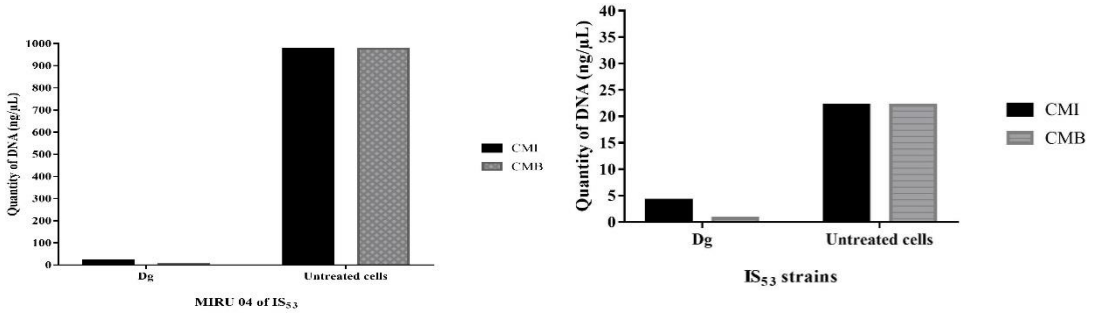

Figure 5: Inhibitory of DNA and MIRU 04 gene synthesis by EO from *D. gossweileri* on IS53

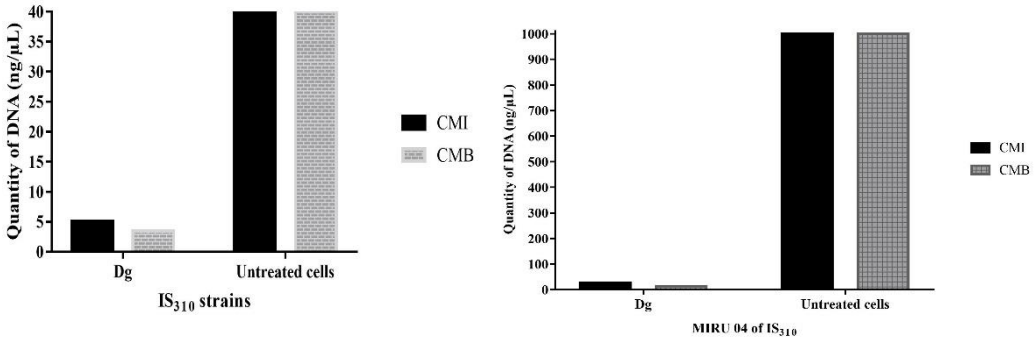

Figure 6: Inhibitory of DNA and MIRU 04 gene synthesis by EO from *D. gossweileri* on IS310

DISCUSSION

Antimycobacterial activity

Moni *et al* showed in a previous study that EO from *D. gossweileri* stem barks presented no activities against multi drug resistant and extensive drug resistant isolates MJ and UJ with MIC > 5000 µg/mL, less active against Isoniazid resistant isolate AC_{79} with MIC of 1250 µg/mL, moderate against Rifampicin resistant isolate AC_{45} with MIC 156, 25 µg/mL. EO from *D. gossweileri* stem barks exhibited antibacterial against four *Bacillus* species and antigerminative against their spore forming bacteria with bacteriostatic activities [5, 7]. The activities are related to synergistic action of all compounds found in essential oil. Voundi, (2017) showed that *D. gossweileri* stem barks essential oil is more active than its lead compound benzyl isothiocyanate against germinative spores of four *Bacillus* species with MIC ranging from 4.88 to 9.72 µg/mL [11, 12]. Although the antibacterial properties of essential oils *D. gossweileri* stem barks and their components have been reviewed against bacteria in the past, it has been suggested that isothiocyanates can covalently cross-links to cellular target like proteins or amino acids causing oxidation reactions [11, 26].

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These results indicated that alterations of cytoplasmic membrane and may be cell wall occurred and leading to the loss of cell constituents such as protein and nucleic acids. In fact, the cytoplasmic membrane has a very important role in the maintenance of cellular homeostasis, as it controls the input and output of intracellular components. DNA, RNA and proteins are usually high in intracellular compartment of cell, their presence in extracellular medium means that membrane permeability has been loosen. Antimycobacterial studies of some essential oils compounds reveal that they caused morphological changes in the overall *Mycobacterium tuberculosis* cell shape and cytoplasm homogeneity resulting in destabilization of cell wall [18]. According to previous studies, due to their hydrophobicity and antibacterial properties, essential oils can disrupt the cell wall membrane causing the permeabilization, loss of ion or molecules and reduce membrane potential [24]. More ever, EOs components can cause structural alterations of the outer envelope with promoting release of cellular biomolecules [27]. Therefore, we concluded that one of the antimycobacterial effect of essential oil from *D. gossweileri* on *M. tubeculosis* was the alteration of membrane and cell wall then, leakage of intracellular absorbent materials which finally resulted in the bacterial death.

The quantities of DNA, RNA and proteins released by IS310 isolate exposed to *D. gossweileri* stem barks EO are lower than those released by H37Rv and IS53 cells. This result reveals that IS310 isolate is more resistant to essential oil action compared to H37Rv and IS53 isolate. In fact, the activity of essential oil against this isolate is equal to those of reference strain H37Rv at MIC value but it lethal concentration is three times more than those of reference strain. More ever, the MIC value of Rifampicin at 125 µg/mL against IS310 is more than 32 µg/mL an indicating parameter of its resistant phenotype [28].

Since the concentrations of DNA produced by normal cells are 36. 20 ng/µL for H37Rv, 22,10 ng/µL for IS53 and 40.0 ng/µL for IS310, the concentrations of DNA in treated cells were ranging from 3.90 ng/µL for H37Rv to 5.10 ng/µL for IS310 at MIC, and reduced the amplification of MIRU 04 gene to 26.90 ng/µL and 70.0 ng/µL at MIC. This inhibition is correlated to the low quantities of DNA in treated cells compare to untreated. Since the concentrations of DNA produced by normal are 36. 20 ng/µL for H37Rv, 22,10 ng/µL for IS53 and 40.0 ng/µL for IS310, the concentrations of DNA in treated cells were ranging from 4. 10 ng/µL at MIC, 0.70 ng/µL at MBC and reduced the amplification of MIRU 04 gene to 20.0 ng/µL at MIC and 4.10 ng/µL at MBC cells. The effect of EO from *D. gossweleiri* on MIRU 04 gene confirmed the hypothesis of DNA inhibition and may be a possible fragmentation action of DNA justified by the absence of MIRU 04 amplicons in treated cells. Our results confirmed that some active constituents in *D. gossweileri* EO interfered directly or indirectly on DNA synthesis. Previous studies explained that isothiocyanates compounds have a very strong antimicrobial effect due to the R-N=C=S group in the molecule which can covalently cross-links to a cellular target [26]. These covalent links on DNA can lead to DNA synthesis inhibition or alterations. Therefore, we concluded that another effect of essential oil from *D. gossweileri* stem barks against *M. tuberculosis* was inhibition of bacterial DNA synthesis. Many studies highlighted the action of EO from plants on DNA inhibition [29, 30, 31]. Xu *et al.* demonstrated that the EO of clove buds at MIC might exert an inhibitory action of DNA replication leading to antibacterial action [27]. Furthermore, Dwivedi *et al* in 2013 showed that khusenic acid and khusimol two sesquiterpenes of *Vetiveria zizanoides* essential oil got their antimycobacterial activity by binding with the Mycobacterial 2Y3P and 1EI1 subunits of DNA gyrase [19].

CONCLUSIONS

The results are important as the effects of essential oil from *D. gossweileri* on *Mycobacterium tuberculosis* was not reported previously. The effects show that essential oil from *D. gossweileri* presents significant antimycobacterial activity against virulent strain H37Rv and two isolates IS53 and IS310 of *M. tuberculosis*. Essential oil from *D. gossweileri* inducactivity is due to action of essential oil on cell

membrane lysis and DNA inhibition. Essential oil from *D. gossweileri* cause alteration significant release of DNA, RNA and protein by *M. tuberculosis* at MIC and MBC.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "conceptualization, M.N.E.D.F. and N.M.A.; methodology, M.N.E.D.F. and A.A.J.P.; validation, N.M.A., A.A.J.P. and M.N.E.D.F; formal analysis, B.D.P.H.; investigation, F.E.G.; writing—original draft preparation, M.N.E.D.F. and B.D.P.V.; supervision, P.B.V.; and E.F-X.

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Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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