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Antimycobacterial Activity of *Rosmarinus officinalis* (Rosemary) Extracted by Deep Eutectic Solvents

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Abstract: Tuberculosis (TB) is a massive problem for public health and is the leading cause of illness and death worldwide. Rosemary (*Rosmarinus officinalis*) is used traditionally to treat many diseases, such as infections of the lungs including pulmonary TB. *R. officinalis* was collected from Al Anbar Governorate, Iraq, and was extracted with deep eutectic solvents (DESs) of many different kinds and with conventional water solvent. The antimycobacterial activities of the *R. officinalis* extracts were tested against multidrug-resistant (MDR) *Mycobacterium tuberculosis* by agar disc diffusion assay. Minimum inhibitory concentrations were measured spectrophotometrically at 570 nm. Then, a time-kill assay and cell membrane integrity analysis were conducted to investigate the effects of the most active extracts on cell growth. The *in vitro* cytotoxicity of the most active extracts was evaluated against Rat Embryonic Fibroblasts (REF) cell line by MTT assay. Liquid chromatography-mass spectrometry (LC-MS) was conducted to analyze the chemical components of the most active extracts. At 200 mg/mL concentration, a significant inhibition activity was seen in DES2: Tailor (DIZ = 17.33 ± 1.15 mm), followed by DES3: ChGl, DES1: LGH and DES4: ChXl. The best result was DES2: Tailor, which had a MIC of 3.12 mg/mL and an MBC of 12.5 mg/mL. The DES2 extract exhibited a high drop in the number of colonies over time, killing more than 80 colonies. The main phytochemical compounds of the *R. officinalis* extract were camphene, camphenilol, α-pinene, limonene, apigenin, camphor, carnosol, linalool and myrcene. *R. officinalis* extracts obtained by DESs have shown evident power in treating tuberculosis, and extraction by DES is a greener procedure than the methods involving conventional extraction solvents. As a result, additional research into the application of DES should be considered.

Keywords: *Rosmarinus officinalis*; medicinal plant; DESs extract; antimycobacterial; broth microdilution; LC-Mass

Citation: Dheyab, A.S.; Kanaan, M.Q.; Hussein, N.A.; AlOmar, M.K.; Sabran, S.F.; Abu Bakar, M.F. Antimycobacterial Activity of *Rosmarinus officinalis* (Rosemary) Extracted by Deep Eutectic Solvents. *Separations* **2022**, *9*, 271. https://doi.org/10.3390/separations9100271

Academic Editor: Grzegorz Boczkaj

Received: 22 July 2022
Accepted: 30 August 2022
Published: 29 September 2022

1. Introduction

Tuberculosis (TB) is a major global health issue due to drug resistance, which is referred to as multi-drug resistance (MDR) [1]. Tuberculosis is widespread globally and kills more people in impoverished nations than other illnesses [2]. Although tuberculosis is primarily a lung infection, it may also cause severe damage to the reproductive, gastrointestinal, nervous, and bone-skeletal systems [3]. Headache, liver damage, rashes, and stomach problems are some of the possible adverse effects of existing tuberculosis medications [4]. As a result, creating a novel therapy for tuberculosis with a novel method of action, minimal toxicity, and increased effectiveness is critical. Indigenous peoples in different regions have used medicinal plants, which have significantly treated human and animal ailments [5]. In recent years, many modern medicines have been made from the parts of plants that have been taken out and studied for their ethnopharmacological uses [6]. Civilizations

have used medicinal plants for a long time to help people and animals feel better when sick. They are now being studied more often due to their potential advantages and fewer adverse effects compared with pharmaceutical medications. They may also be used with other therapies to expedite therapeutic success [7]. Because people worry about the harmful effects of synthetic chemicals in food, "clean label goods" must be made. As such, people are becoming more interested in alternatives to synthetic additives, including natural extracts, because of: (a) synergy with other methods of preservation; (b) perceived safety; and (c) their unique antioxidant, antidiabetic, antimutagenic, antioxygenic and antibacterial capabilities [8]. Natural products have become more important in drug research when bioactive chemicals are used as therapeutic agents, raw materials for drugs, or models for new biologically active molecules. [9]. Known botanically as *Rosmarinus officinalis* L., this Lamiaceae shrub has tall stems, white or blue flowers, and dark green leaves. This plant, which most people call rosemary, comes from the Mediterranean region. Fresh or dried rosemary leaves can be used as a spice or herbal tea [10–12]. The primary active ingredients in rosemary extract are phenolic diterpenes, triterpenes, and phenolic acids have significant medicinal properties, including antioxidant, anti-inflammatory, and antimicrobial properties [10,11]. They possess several unique therapeutic qualities, including hypoglycaemic, antiatherogenic, antihypertensive, hypocholesterolaemia, anti-inflammatory, anti-depressive, hepatoprotective, anti-proliferative and antimicrobial capabilities. Rosemary extract may help patients suffering from asthma, cataracts, renal colic, peptic ulcers and physical and mental tiredness [13]. Instead of using traditional solvents, deep eutectic solvents (DES) are more efficient and more environmentally friendly than organic solvents such as hexane, acetone and chloroform; moreover, the use of these solvents is limited by green chemistry concepts [14]. Green solvents (biocompatible, biodegradable, and comfortable to use) have been suggested to replace dangerous organic solvents [15]. These solvents are made up of a halide salt or other type of hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD). These complexes are made with well-known stoichiometric ratios, so they can be called new components. DESs have been used in various applications in recent years, including metal processing, purification and biodiesel synthesis [16]. They have also been used to remove environmental pollutants, separate azeotropes, or isolate and fractionate chemicals [17,18]. Additionally, this sort of solvent may establish hydrogen bonds with certain chemicals, such as phenolic compounds, thereby boosting their solubility [19]. Due to these reasons, phenolic compounds are increasingly extracted with DES. DES is a suitable solvent for separating and stabilizing these biomolecules [20]. The microbialtoxicity for deep eutectic solvents differ depending on their individual components, such as phosphonium-based DESs, and organic acid-based DESs had a significant inhibitory effect against various bacterial strains [21,22]. Conversely, other kinds of deep eutectic solvents such as amino and sugar based DESs do not have inhibitory bacterial growth as a result of their individual components; these may be sources of energy, the absorption of which occurs in bacteria by simple or facilitated diffusion [22,23]. There is multi-drug resistance among TB pathogens; hence, the quest for innovative anti-TB medicines is critical [24]. The present study aims to look into the antimycobacterial activity and the phytochemical composition of *R. officinalis* extracts prepared using different DESs. The extract obtained with DES was compared with the extract obtained using water (herein called water extract). For the first time, it was found how well *R. officinalis* DES extracts kill MDR-*Mycobacterium tuberculosis*.

2. Materials and Methods

2.1. Chemicals and Reagents

All the chemical materials and reagents presented in Table 1.

Table 1. Chemical materials and reagents used in this study.

Names	%	Supplier Company	Originated
L(+) lactic acid	85	Chem-lab NV	Belgium
Choline chloride	98%	Xi'an Geekee Biotech	China
Glycerol	≥99%	Panreac	Spain
Xylitol	98%	Xi'an Geekee Biotech	China
Glucose anhydrous	99%	HiMedia	India
D(-) fructose	99%	HiMedia	India
Middlebrook 7H10 agar		HiMedia	India
Middlebrook 7H9 broth medium		HiMedia	India
Gallic acid	99%	Sigma-Aldrich Chemicals	USA
Rutin	99%	Sigma-Aldrich Chemicals	USA

2.2. Plant Materials

Whole *R. officinalis* plants were collected in Ramadi City and authenticated at Al Anbar University's Centre of Desert Studies in Iraq. After it was gathered, it was washed and dried in a hot oven at 40 °C for 48 h. The dried sample was ground into a powder and put through a sieve with a 2 mm mesh.

2.3. DES Preparation

DESs were made using a previously explained method [22]. A flask with a tight seal was used to hold the mixed material and was heated continuously while being stirred until it became colourless and homogeneous. Table 2 has a list of the synthesized DESs.

Table 2. This study used the following types of DESs.

DES Types	Full Name	Molar Ratio
DES1: LGH	Lactic acid, glucose and water	5:1
DES2: Tailor	Glycerol, xylitol and D-(-)-fructose	3:3:3
DES3: ChGl	Choline chloride: glycerol	1:2
DES4: ChXl	Choline chloride: xylitol	1:1

2.4. Extraction Procedure

Extraction was performed based on the method used in a previous study with some modifications [25]. For the extraction process, 20 mg of plant powder that had been dried and 1 mL of DES were combined and mixed, then heated and stirred in a closed glass bottle at 40 °C for one hour. A 9000 rpm centrifuge was used to spin down the sample for a full ten minutes. Before it was used and analysed, the suspension was purified by a nylon membrane with a thickness of 0.45 m. The extractions were performed in triplicate.

2.5. Antimycobacterial Activity

2.5.1. MDR-*M. tuberculosis* Bacterial Strain

The clinical isolate was provided from the TB centre in Al-Ramadi City, Iraq, and the identity of the isolate was confirmed by GeneXpert assay (Cepheid, Sunnyvale, CA, USA). For culturing it was grown on Löwenstein-Jensen medium, and for subculturing on Middlebrook 7H10 agar containing supplemented material (HiMedia, Maharashtra, India) or on Middlebrook 7H9 broth also containing supplemented material (HiMedia, Maharashtra, India).

2.5.2. Disk Diffusion Assay for Determining the Inhibitory Zone (DIZ)

The applied technique was based on a previous study [26]. Stock solutions from each extract (200 mg/mL) were prepared and diluted to obtain different concentrations (25, 50 and 100 mg/mL). Sterilized Whatman filter paper (6 mm) was used, and 20 µL of each concentration was applied to it. As a positive control, 50 µg/mL of rifampicin was used. Middlebrook 7H10 agar plates were inoculated using 100 mL of a bacterial suspension., The prepared filter paper was carefully put on the cultured agar plates. The parafilm was used to seal the plates that had been inoculated, and they were kept at 37 °C for three days. DIZ was measured to record the results, and this test was performed in triplicate.

2.5.3. Determination of Minimal Inhibitory Concentrations (MICs)

Based on this, a spectrophotometric approach was used with culture dilution tubes [27]. The inoculum was grown, and the density of the suspension was changed to meet 0.5 McFarland standards. Six serial twofold dilutions of the extracts were done in Middlebrook 7H9 broth to obtain the different testing concentrations in the range of 6.25–200 mg/mL. Additionally, 100 µL of bacterial inoculum was added to all culture tubes except for the control growth tube. Bacterial strain suspensions only were placed in the control tube. For 96 h, all of the test tubes were kept at 37 °C. Afterwards, MIC was determined as the lowest extract concentration with total microbial growth inhibition through visual reading compared to the control growth tube. Optical density (OD) was measured at 570 nm, and the minimum inhibitory concentration (MIC) was calculated as the growth inhibition at 50% compared to control tube growth. The experiment was carried out three times for accuracy.

2.5.4. Determination of Minimal Bacterial Concentrations (MBCs)

The MBC was measured by streak agar plate assay. The tubes that showed no growth were tested and then cultured in Middlebrook MH10 agar and incubated at 37 °C for three days. The MBC concentration was the lowest in samples that failed to grow cell viability on plates.

2.6. Time-Kill Assay

A method described in a previous study was used [28]. The strongest crude results were used with different MIC volumes (1, 2, and 3) to find variations in the killing. Standard and positive controls were included in the assay using cultures without extract samples and rifampicin. MDR-*M. tuberculosis* with a density of about 1.0×10^5 CFU/mL was grown in 250 mL shake flasks with 10 mL of Middlebrook 7H9 broth medium and an adequate amount of crude extract. The flasks were incubated by the shaking incubator and were set to 150 revolutions per minute and 37 °C. It was decided to harvest the aliquots at 0, 24, 32, 48, 56, 72, 80 and 96 h serial dilutions in Middlebrook 7H9 broth culture, prepared after inoculation to identify viable cell counts by the drop spread plate, as previously explained by [29]. Drying time was allowed for the dilution samples (10 µL) to be placed at the appropriate distance on Middlebrook 7H10 agar plates. After 72 h of incubation at 37 °C, the total colony counts were calculated. Every experiment was repeated three times. The mean log (CFU/mL) was calculated as follows:

$$\text{CFU/mL} = \frac{\text{No of individual colonies} \times \text{dilution factor } (10^n)}{0.1 \text{ mL (volume plated)}}$$

2.7. Cell Wall Integrity

The integrity of the cellular membrane was assessed by monitoring the release of cell components into the cell suspension at 280 nm. A method recently described by [30] was used with minor changes. MDR-*M. tuberculosis* colonies were scraped from Middlebrook 7H10 plates and inoculated into Middlebrook 7H9 broth (10 mL). The bacterial suspension was homogenized using a vortex mixer (Dragon lab, DLAB, Beijing, China, MX-S), and the turbidity was adjusted to meet the 0.5 McFarland standard. This resulted in a suspension

containing approximately 1.5×10^8 CFU/mL. The adjusted MDR-*M. tuberculosis* suspensions were treated with different multiples of the selected crude extracts' MICs (1, 2 and 3). The test included cultures without any extracted material, rifampicin as a standard, and a positive control. The flasks were then incubated at 37 °C for 8 h with shaking at 150 rpm. A spectrophotometer was used to measure the absorbance of cell supernatant at 280 nm (Spectra Max 250, Molecular Devices, Hampton, VA, USA). Afterwards, centrifugation of 1 mL aliquots of the bacterial suspensions was performed at 6000 rpm for 10 min. The amount of chemical leakage from the cytoplasm at 280 nm was given as the ratio of the values found in treated cells to those found in control cells that had not been treated.

2.8. In Vitro Cytotoxicity Assay

2.8.1. Cell Lines and Cell Cultures

We used normal Rat Embryonic Fibroblast (REF) cell lines in this study. They were generously provided by the Biotechnology Research Center at Al Nahrain University in Baghdad, which is located in Iraq. Cells were cultured in RPMI-1640 medium (EroClone, Milan, Italy) and supplemented with 10% foetal bovine serum (Biowest, LubioScience GmbH, Zürich, Germany, South America origin) and 1% penicillin-streptomycin (Gibco, South Cleveland, Atlanta, GA, USA). The cell lines were grown as a monolayer in a humidified atmosphere at 37 °C with 5% CO_2.

2.8.2. Cytotoxicity Assay

The cytotoxic assay was carried out according to [31,32] with some modifications. The *in vitro* cytotoxic effects of the extracts were evaluated using the MTT assay. MTT powder 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Macklin, Shanghai, China) was dissolved in PBS to prepare the MTT solution (5 mg/mL). Before exposing cells to extracts and guaranteeing cell adherence, 7×10^3 cells were seeded in each well and incubated overnight in a 96-well plate. Various concentrations from minimally effective inhibitory concentrations (12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL) of the plant extracts in addition to the control vehicle (CV) were added to the cells in triplicate wells. After 24 h of incubation, the growth medium was aspirated from the plate, and all wells were washed with PBS. Into each well, 20 µL of serum-free media was added to a similar volume of MTT solution (5 mg/mL) per well before incubation for 3 h longer in the dark at 37 °C. Fifty microliters of dimethyl sulfoxide (DMSO) were then added with shaking for 10 min to dissolve the MTT. A microplate reader (Expert Plus reader; Hitech GmbH, Eugendorf, Austria) was then used to measure absorbance at 620 nm. The formula below was used to measure the percentage of viability from raw absorbance data, as follows:

$$\text{Viability } \% = \frac{\text{A test}}{\text{A control}} \times 100$$

where A represents absorbance. GraphPad prism software was utilized to plot the viability curve and determine the growth inhibitory concentration that decreases 50% of viability (GI_{50}) from the same curve.

2.9. Phytochemical Analysis

2.9.1. Total Phenolic and Flavonoid Contents. [TPC and TFC]

The Folin–Ciocalteu method was used to determine the TPCs [33]. The aluminium chloride method was used to measure the TFCs [34]. Standard curves for gallic acid and rutin were used to determine the final results per 100 g of dry mass.

2.9.2. Identification of Bioactive Constituents Using Liquid Chromatography-Mass Spectroscopy

The chromatography analysis was performed according to a recent studies [35,36], in which 50 µL of the extract was injected into an Agilent Zorbax Eclipse XDB-C18 column (2.1 × 150 mm × 3.5 µm) and held at 35 °C with a constant flow rate of 0.5 mL/min

and a total LC run time of 20 min. The separation by HPLC was performed with mobile phases containing 0.3% (*v*/*v*) formic acid in water (solvent A) and 0.3% (*v*/*v*) formic acid in acetonitrile. The mobile phase gradually increased from 5–100% in 15 min. The eluent was monitored by Shimadzu LC-MS 8030 with electrospray ion mass spectrometer (ESI-MS) under positive ion mode and scanned from 100 to 1000 *m*/*z*. The sample was injected into the mass detector using a Shimadzu CBM-20A system controller, LC-30AD pump and SIL-30AC autosampler. The nebulizer pressure was 45 psi. Drying gas high purity nitrogen (99.999%) was used with a flow rate of 10 L/min. The capillary temperature was 350 °C with a cooler; a CTO-30 column oven was used.

2.10. Statistical Analysis

All experiments were done three times, and the data were expressed as the mean of triplicates ± standard error.

3. Results

Figure 1 shows how the agar disc diffusion assay showed the inhibitory effects of four DES extracts and the water extract on MDR-*M. tuberculosis* from growing.

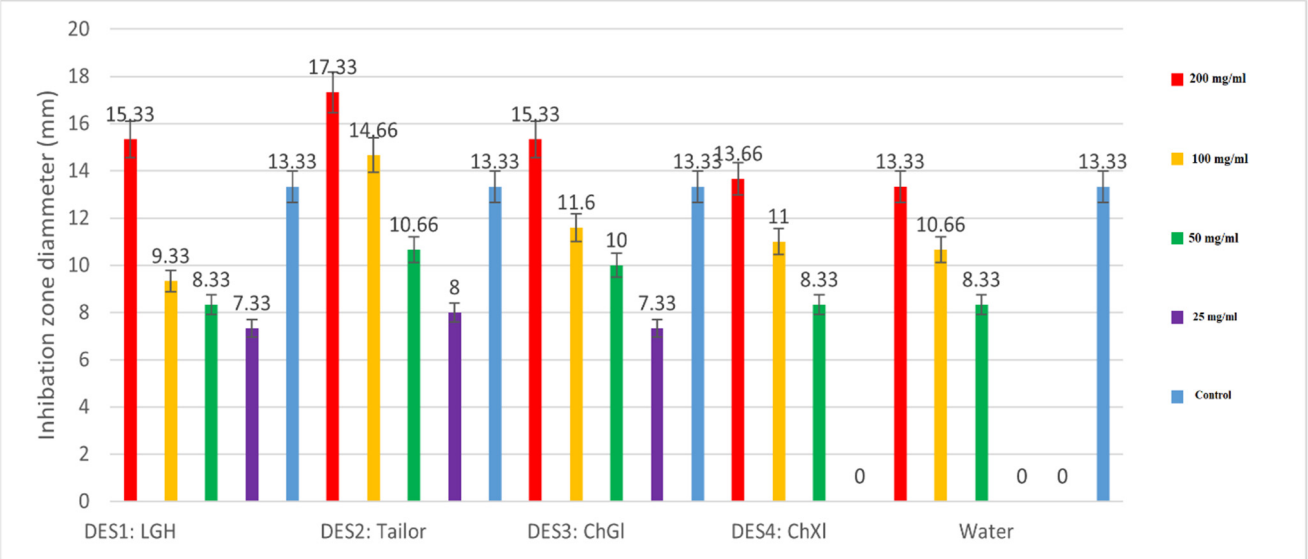


Figure 1. Results of DIZ assay for various DESs plants extracts. The values were presented as the mean ± SD performed in triplicate.

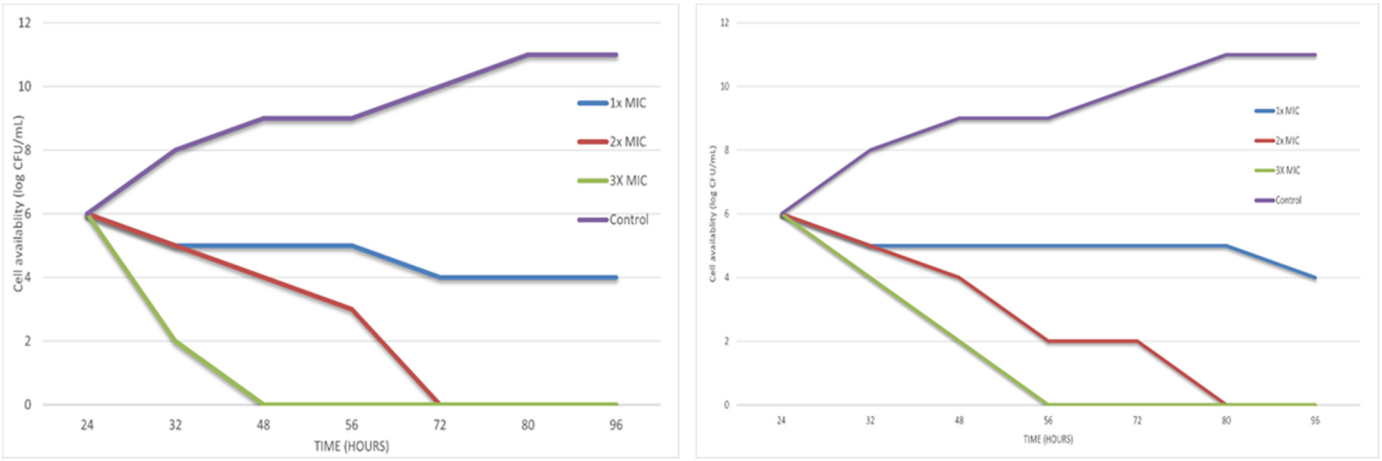
Compared with the water extract, the DES2: Tailor extract at 200 mg/mL concentration showed the highest DIZ activity result (17.33 ± 1.15 mm), followed by the DES1, DES3, which had identical results, and then DES4. At 100 mg/mL, the DES2: Tailor extract showed higher activity (DIZ = 14.66 ± 2.08 mm) than the other extracts. DES3 and DES4 had similar results, and DES1 had the lowest result. Meanwhile, the 50 mg/mL concentration of plant extracts did not show good DIZ activity (low inhibition), except for DES2: Tailor and DES3: ChGl (DIZ = 10.66 ± 0.57, 10.0 ± 1.73 mm, respectively). In comparison, all the extracts were not active (except DES2; DIZ = 8.00 ± 1.00 mm) at 25 mg/mL concentration. The positive control rifampicin showed DIZ activity at 50 µg concentration.

The MICs and MBCs antimycobacterial activity of the *R. officinalis* extracts are illustrated in Table 3. The MIC for DES2: Tailor extract was 3.12 mg/mL, and the MBC was 12.5 mg/mL. DES3: ChGl extract exhibited MIC and MBC values (6.25 and 25 mg/mL, respectively). In addition, DES1 showed intermediate results in MIC and MBC values (12.5 and 100 mg/mL, respectively). DES4: ChXl had the highest MIC, and MBC was not active. By contrast, the water extract showed a very high MIC and did not exhibit any activity in MBC parameters.

Table 3. The MIC and MBC activity of *R. officinalis* extracts.

Type of DESs	MIC (mg/mL)	MBC (mg/mL)
DES1: LGH	12.5 mg	100 mg
DES2: Tailor	3.12 mg	12.5 mg
DES3: ChGl	6.25 mg	25 mg
DES4: ChXl	25 mg	NA
Water	50 mg	NA

For both DES2: Tailor and DES3: CHGl extracts of *R. officinalis*, Figure 2 shows that the MDR-*M. tuberculosis* growth was not suppressed by the selected crude extracts at 1X MIC just 24 h after the commencement of the treatment. However, slight reproliferation (7.5–8.73 log CFU/mL) occurred after 32 h because the 1X MIC concentration of selected crude extracts was insufficient to sustain the bacteriostatic effect. Figure 2A shows an exciting result that exhibits the total bactericidal effect at 2X MIC of DES2: Tailor extracts for *R. officinalis* after 72 h of treatment. Figure 2B shows that for the 2X MIC of DES3: CHGl extracts of *R. officinalis*, the total bactericidal effect was obtained at 80 h of treatment; RIF exhibited a bactericidal effect after 72 h of treatment. The treatment with MDR-*M. tuberculosis* with the concentration of 3X MIC showed a fantastic result. Figure 2B shows sharp decreases in the log CFU/mL bacterial count (99.9%) after 56 h incubation with DES3: ChGl extract. By contrast, DES2: Tailor extracts of *R. officinalis* showed an early reduction of over 99.9% of the bacterial population after 48 h of incubation.



(**A**) (**B**)

Figure 2. Time-kill curves of the extracts (**A**) DES2: Tailor *R. officinalis* and (**B**) DES3:CHGl extracts of *R. officinalis* against MDR-*M. tuberculosis*.

Figure 3 shows the ratios of intracellular compounds' absorbance at 280 nm (OD280) released by MDR-*M. tuberculosis.* Cells were treated for 8 h with increasing concentrations of both DES2: Tailor and DES3: CHGl extracts of *R. officinalis* and RIF as a positive control compared with those released by untreated MDR-*M. tuberculosis*. The release of the cellular constituents' absorbance at 280 nm when the cells were treated with DES2: Tailor of *R. officinalis* at the concentrations of 1×, 2× and 3× MIC increased by 4.4, 3.7 and 4.5 times, respectively, compared with the positive control. By comparison, DES3: CHGl extracts of *R. officinalis* at the concentrations of 1×, 2× and 3× MIC increased by 3.8, 3.4 and 3.9 times, respectively, compared with the positive control.

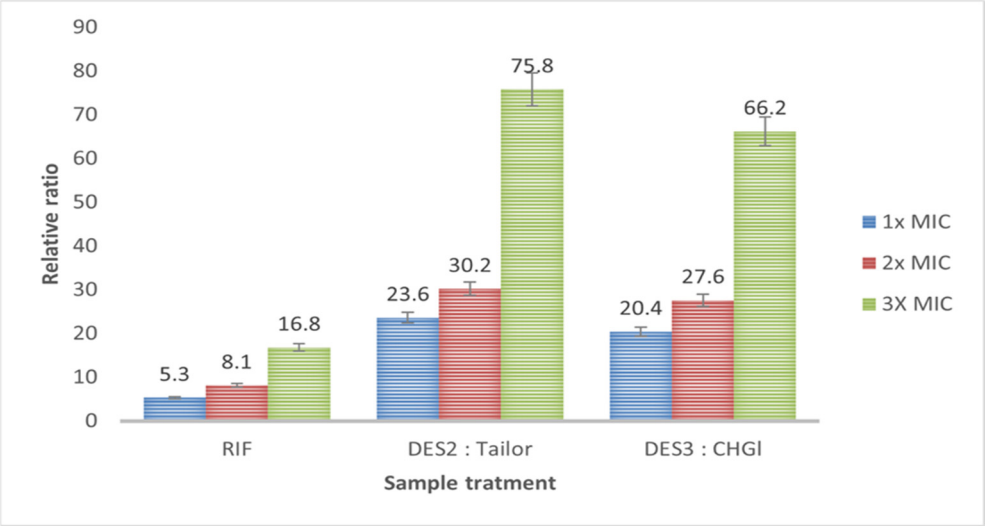
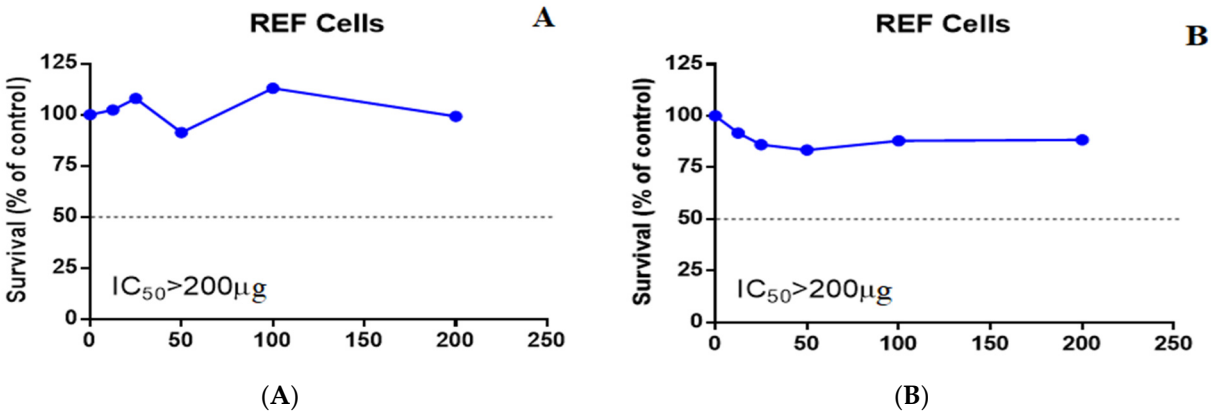


Figure 3. The effect of DES2: Tailor and DES3: CHGl extracts of *R. officinalis* and RIF on the release of cell constituents at 280 nm from MDR-*Mycobacterium tuberculosis*.

Figure 4A,B reveals that the *in vitro* cytotoxicity of the *R. officinalis* extracted by DES2: Tailor and DES3: CHGl were not toxic to the REF cell line. The survival rates at the highest concentration (200 µg/mL) for both extracts were (99.2% and 88.4%), respectively. This effect was not significantly different compared with control cells.



(**A**) (**B**)

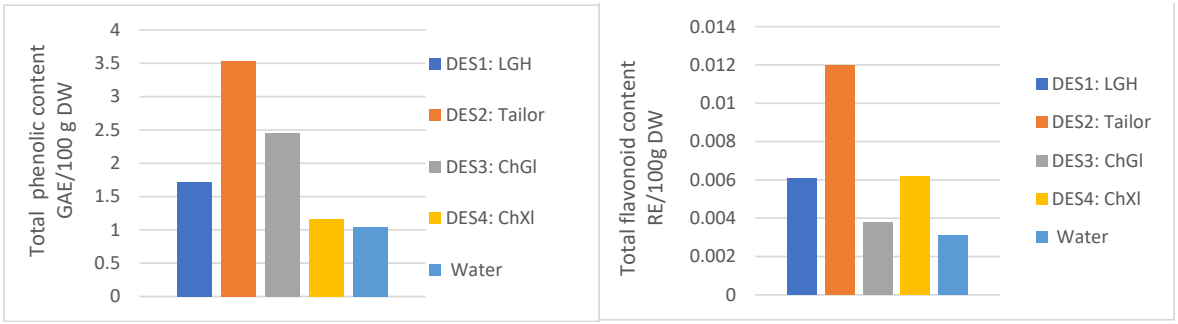
Figure 4. *In vitro* cytotoxicity of the *R. officinalis* extracted on REF cell line (**A**): DES2: Tailor, (**B**): DES3: CHGl.

Table 4 shows the total phenolic and flavonoid contents of the *R. officinalis* extracts. TPC with the highest value (3.530 mg/100 g dry weight [DW]) was found in DES2: Tailor, followed by DES3: ChGl (2.450 mg/100 g DW), DES1: LGH (1.720 mg/100 g DW) and DES4: ChXl (1.160 mg/100 g DW). The total phenolic contents of the *R. officinalis* extracts were higher than the water extract (1.050 mg GAE/100 g DW). The current study showed that the total flavonoid contents ranged from 0.012 mg RE/g to 0.0062 mg RE/g. The highest TFC at DES2: Tailor extract (0.012 mg RE/g), followed by DES1: LGH, DES4: ChXl, aqueous extract and DES3: ChGl. Figure 5A,B depicts the relative efficacy of the DESs for extracting phytochemicals compared to the water solvent.

Table 4. Phytochemical contents of *Rosmarinus officinalis* extracts.

Type of DESs	TPC mg GAE/100 g DW	TFC mg RE/100 g DW
DES1: LGH	1.720 ± 0.096	0.0061 ± 0.0003
DES2: Tailor	3.530 ± 0.251	0.012 ± 0.0017
DES3: ChGl	2.450 ± 0.200	0.0038 ± 0.001
DES4: ChXl	1.160 ± 0.076	0.0062 ± 0.0009
Water	1.050 ± 0.132	0.0031 ± 0.0016

The values are expressed as the mean ± SD performed in triplicates.



(**a**) (**b**)

Figure 5. The extraction efficiency of DESs compared with water solvents. (**a**) TPC, (**b**) TFC.

Both DES2: Tailor and DES3: CHGl extracts of *R. officinalis* were analysed and profiled by LC-MS/MS analysis in order to characterise the chemical constituents in *R. officinalis* qualitatively. To our knowledge, this is the first validated method for detecting active compounds in *R. officinalis* whole plant extracts using these kinds of DESs through LC-MS/MS analysis. The base peak chromatogram is depicted in Figures 6 and 7. Compounds were characterized by their retention times, the absorption spectrum in the UV-vis region, the mass spectrum obtained by MS-ESI and the fragmentation profile; these properties were compared with those mentioned in previous reports [31]. The results obtained from the LC-MS/MS analysis allowed the tentative assignment of many chemical constituents, and the same observations were shown by [37]. The compounds identified are listed in Tables 5 and 6.

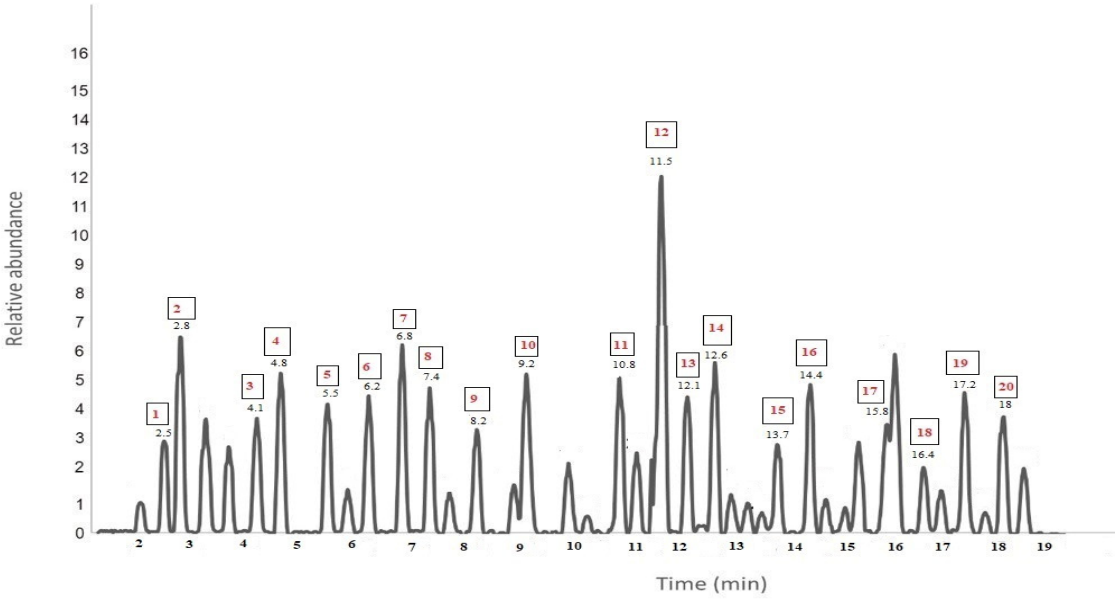


Figure 6. LC-MS chromatogram from *R. officinalis* extracts obtained by using DES2: Tailor.

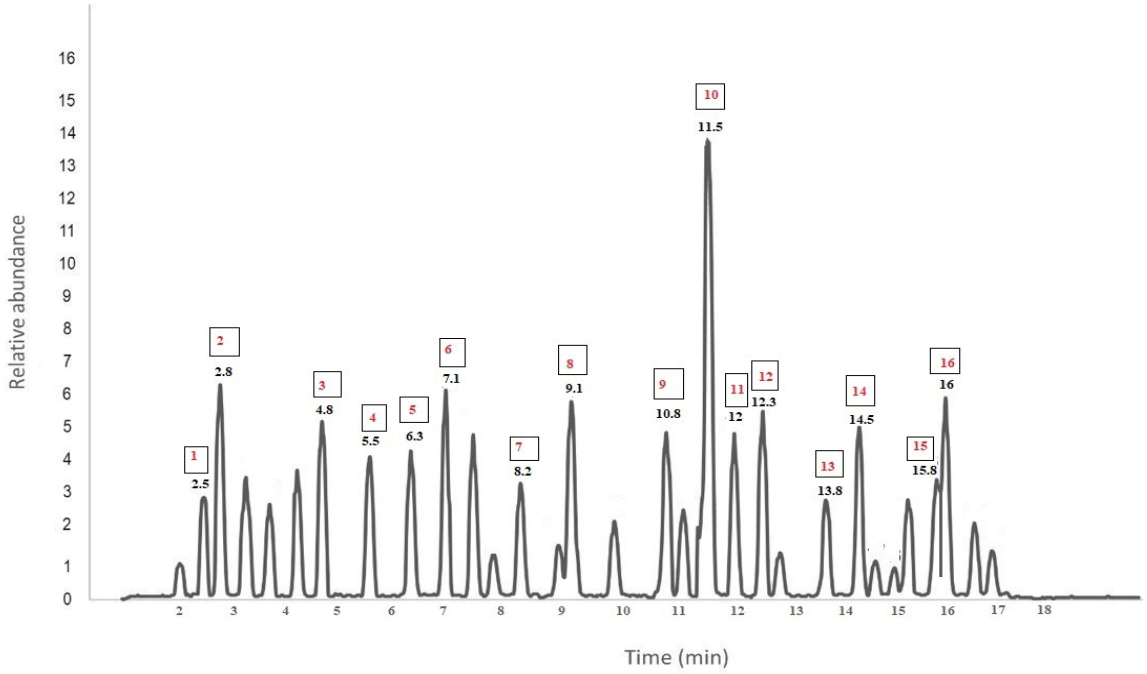


Figure 7. LC-MS chromatogram from *R. officinalis* extracts obtained by using DES3: CHGl.

Table 5. Tentative identification of chemical constituents in the *R. officinalis* extract was obtained using DES2: Tailor.

No	Chemical Compound	R.T (min)	%	Molecular Weight (g/mol)	Molecular Formula
1	Rosmarinic acid	2.5	1.6	360.3	$C_{18}H_{16}O_8$
2	Camphene	2.8	10.3	136.23	$C_{10}H_{16}$
3	Thymol	4.1	1.6	150.22	$C_{10}H_{14}O$
4	Camphenilol	4.8	5.3	140.22	$C_9H_{16}O$
5	Diosmin	5.5	1.6	608.5	$C_{28}H_{32}O_{15}$
6	Cirsimaritin	6.2	1.7	314.29	$C_{17}H_{14}O_6$
7	α-pinen	6.8	9.6	136.23	$C_{10}H_{16}$
8	Betulinic acid	7.4	1.7	456.7	$C_{30}H_{48}O_3$
9	Oleanolic acid	8.2	1.5	456.7	$C_{30}H_{48}O_3$
10	Limonene	9.2	3.4	136.23	$C_{10}H_{16}$
11	Apigenin	10.8	4.8	270.24	$C_{15}H_{10}O_5$
12	Camphor	11.5	11.2	152.23	$C_{10}H_{16}O$
13	Carnosol	12.1	4.2	330.4	$C_{20}H_{20}O_4$
14	Linalool	12.6	8.2	154.24	$C_{10}H_{18}O$
15	Carnosic acid	13.7	1.5	332.4	$C_{20}H_{28}O_4$
16	Myrecne	14.4	6.3	136.23	$C_{10}H_{16}$
17	Luteolin 3-O-beta-D-glucuronide	15.8	1.6	462.4	$C_{21}H_{18}O_{12}$
18	Limonene	16.4	7.5	136.23	$C_{10}H_{16}$
19	Rosmanol	17.2	3.1	346.4	$C_{20}H_{26}O_5$
20	Ursolic acid	18	2.9	456.7	$C_{30}H_{48}O_3$

Table 6. Tentative identification of chemical constituents in the *R. officinalis* extract was obtained by using DES3: CHGl.

No	Chemical Compound	R.T (min)	%	Molecular Weight (g/mol)	Molecular Formula
1	Cirsimaritin	2.5	0.7	314.29	$C_{17}H_{14}O_6$
2	α-pinen	2.8	11	136.23	$C_{10}H_{16}$
3	Carnosol	4.8	6.9	330.4	$C_{20}H_{20}O_4$
4	Luteolin 3-O-beta-D-glucuronide	5.5	1.5	462.4	$C_{21}H_{18}O_{12}$
5	Rosmadial	6.3	1.5	334.4	$C_{20}H_{24}O_5$
6	Linalool	7.1	9.6	154.24	$C_{10}H_{18}O$
7	Hesperidin	8.2	1.1	610.6	$C_{28}H_{34}O_{15}$
8	Limonene	9.1	8.4	136.23	$C_{10}H_{16}$
9	Oleanolic acid	10.8	6.2	456.7	$C_{10}H_{18}O$
10	Camphor	11.5	12.2	152.23	$C_{10}H_{16}O$
11	Ursolic acid	12	6	456.7	$C_{30}H_{48}O_3$
12	Camphene	12.3	9.1	136.23	$C_{10}H_{16}$
13	Methyl carnosate	13.8	0.8	346.5	$C_{21}H_{30}O_4$
14	Myrecne	14.5	8.1	136.23	$C_{10}H_{16}$
15	Rosmarinic acid	15.8	1.1	360.3	$C_{18}H_{16}O_8$
16	Camphenilol	16	7.5	140.22	$C_9H_{16}O$

4. Discussion

All of the powerful eutectic solvents used in this study were tested against MDR-*Mycobacterial tuberculosis* on their own to see if they were toxic to the test organism, which would affect the assay results. The solvents were not toxic to the test organism. All the extracts showed different anti-TB activity in a lab setting against MDR-*M. tuberculosis*. Based on the values of their DIZ, MIC, and MBC results, it appears that these sections could have the most bioactive parts. Researchers have found that plant extracts with varying degrees of inhibition contain active phytochemicals in reviews of natural products with anti-tuberculosis effects [1,38,39].

The relative anti-TB activity of different solvents shows that DES2: Tailor was the most effective, followed by DES3: ChGl and DES1: LGH. This indicates that the bioactive components against *M. tuberculosis* were possibly extracted in more significant quantities using the triple solvents. These results were similar to other anti-TB and natural product analyses [40,41]. In extraction, the polarity of the components of DES mixtures is also a significant part of the modulation mixture. The polarity of DES changes based on its composition and is thought to be linked to the molecular structure of HBD [14,42]. Based on the results of this study, most of the active ingredients may be of a type called "lipophilic". These results were supported by the fact that the polar aqueous solvent did not have a great effect.

On the other hand, the outer membrane of mycobacteria is lipid-bounded, and since most molecules in these polar aqueous solvents were predicted to have hydrophilic groups, the solvents were effectively inaccessible. Because the cell wall of *M. tuberculosis* has a double layer with an inner electron-dense layer and an outer electron-transparent layer of peptidoglycan, it is known that hydrophilic molecules cannot get through the outer membrane [43]. Our results were similar to those from other studies, which found that *R. officinalis* had antibacterial effects [44]. This study is the first to examine the anti- TB activities of *R. officinalis* extract by various DESs against multidrug-resistant *Mycobacterium tuberculosis.* Rifampicin was chosen as the control drug because of its high potency against tuberculosis and high specificity for *Mycobacterium tuberculosis* (DIZ = 13.33 1.52 mm at 50 mg concentration). This controlled drug has been used to confirm that the assay techniques were correct.

We looked into the most active DES2: Tailor and DES3: CHGl extracts of *R. officinalis* in order to learn more about how these partitions kill bacteria at the MIC values. We did this by measuring how many mycobacteria were killed at different times during their exposures,

as the mycobacterial population is only exposed to the highest drug concentrations that can be reached in living organisms for a concise time [45]. The *in vitro* endpoint assay method used to figure out MIC and MBC could not show what happened during an infection. Therefore, in order to highlight the susceptibility results, the rate at which the mycobacterial growth was stopped was also measured. Also, according to the National Committee for Clinical Laboratory Standards (NCCLS), antimicrobial susceptibility tests should include a time factor because the killing rate is more clinically significant than the degree of killing [46]. The killing rate of antimicrobials concerning exposure time during the first infection with *M. tuberculosis* is an essential measure of therapeutic efficacy to prevent resistance from developing [47]. In this study, the untreated control mycobacterial cells grew in lag, log, and death phases, in that order. This is how Mycobacterium species typically grow when put into a new medium [48]. The high and fast death rates for both DES2: Tailor and DES3: CHGl extracts of *R. officinalis* means that these extracts might have active agents that kill bacteria well. To stop TB from returning and to lower the risk of resistance, it would be good to have anti-TB drugs that kill any remaining live bacilli [49]. Our study revealed that both DES2: Tailor and DES3: CHGl extracts of *R. officinalis* shoot significantly reduced 99.9% of the viable cells count of MDR-*M. tuberculosis* compared with the control. A compound can be regarded as an antitubercular agent when it reduces $\geq$90% of viable cell counts in the test medium compared with the untreated control. This result substantiated the susceptibility of MDR-*M. tuberculosis* to both DES2: Tailor and DES3: CHGl extracts of *R. officinalis*.

The cell wall of mycobacteria is an integral part of how the cells grow and is a critical factor in how dangerous they are. It also acts as a static barrier against many drugs used to treat TB and biological stresses [50]. A cell's metabolism can be hurt by even small changes or breaks in the structure of the cell membranes. This can eventually lead to the death of the cell [51]. Leaking amino acids, proteins, nucleic acids, and enzymes are signs that the membrane has been damaged or that the cell has died [52]. The crude extract treatments that were examined in this study showed signs of damage to the cell membrane, which caused more cell membrane leakage than in the untreated control. In the past, some studies of natural compounds showed that they could kill Mycobacterium by targeting their membranes. Green tea extract was used to obtain epigallocatechin gallate, a plant-based chemical [53]. Because of structural changes in lipomannan and lipoarabinomannan, it was found that the antimicrobial agent was particularly effective against M. smegmatis due to its vulnerability in maintaining its cell wall integrity [54].

Because *in vitro* toxicity tests can support *in vivo* assays, they are of great scientific importance. There will be a reduction in the number of animals used due to the use of *in vitro* assays in screening for potentially toxic compounds. Toxicity tests for essential oils are necessary for them to be used. The toxicity of many herbal compounds makes them unsuitable for clinical use, even though they have biological activity [55]. Most DESs benefit from ChCl's qualifications, and choline is the preferred cellular raw material for membrane synthesis phospholipids [56]. As a result, ChCl has been categorized as a relatively safe salt, but the DESs cytotoxic profiles obtained thus far do not share the ChCl negligible cytotoxic label. Glycerol, like triglycerides and phospholipids, is a precursor [57]. Cellular glycerol turnover is increased in normal cell lines because glycerol can be used for gluconeogenesis [58]. In light of the previous, the IC50 values show that these carbohydrate-based eutectics are likely to have higher cellular tolerance than previously thought. In order to reduce their cytotoxicity, biomaterials appear to be an important asset. Organic acids, on the other hand, should be used with caution because they can amplify the harmful effects of DESs, as previously reported.

Further *in vitro* and *in vivo* studies may be possible at concentrations below 1000 g/mL toxicity [59]. According to a separate study, both the essential oil and the constituents of *R. officinalis* have potent antioxidant properties. This last point may help to support the claim of low toxicity [60]. The tested extracts possessed low toxicity, which made them good candidates for the green extraction of *R. officinalis*. The *in vitro* cytotoxicity of the

R. officinalis extracts obtained by different DESs against the REF cell line had not been previously investigated.

It has been reported that Lamioideae plants contain a wide range of compounds, including terpenes, flavonoids, and phenolic compounds such as di- and triterpenes and essential oils [61]. Antibacterial, antiviral, antioxidant, and anti-inflammatory properties of phenolic acids such as rosmarinic acid are found in the Lamiaceae family of plants. [7]. DESs were better at extracting phytochemicals than traditional aqueous solvents, based on the number of phytochemicals they contained. Most likely, the higher extraction efficiency of DESs was mostly since they are more polar than water, which gives them a higher solubilizing capacity [62].

The results also showed that DES2 had a higher chance of extracting phenol and flavonoid content than any of the other DESs tested. The current results show that triple-DES interacts with phytochemicals more than double-DES, which leads to a better response against microorganisms [41]. Also, it is important not to forget how vital double DES compositions like those in DES1, DES3, and DES4 are compared to common solvents [63].

Many phytochemicals from the *R. officinalis* extracts were found to be volatile and semi-volatile organic compounds with lipophilic properties, as was the case with the various DES methods used to extract them. It is important to remember that the results presented in this study were constrained by the materials used and by the LC-MS methods, thus the number of identified compounds and their identities cannot be taken as absolute for each partition [64]. Previous studies have demonstrated that flavonoids and phenolic acids possess high biological and pharmacological activities [65]. In the LC chromatogram, the peak at retention time (RT) of 2.8 min was assigned to the presence of camphene and α-pinene, as reported by previous studies [37]. In fact, carnosol showed potent anti-inflammatory activity [13,66]. In this study, camphene and α-pinen, natural essential oils, were the main compounds obtained in the *R. officinalis* extract. A previous study also reported the presence of camphene in other *R. officinalis* species [67]. As reviewed by [7], camphene and α-pinen showed potent antibacterial activity [68,69].

Moreover, Bjapai et al. demonstrated that α-pinene has antimicrobial activity against *S. aureus*, being effectively able to disorganize the cell membrane and therefore promote the lysis [70]. Hence, in the present study, we speculated that the antimycobacterial effects of *R. officinalis* might be due to the presence of camphene and α-pinene at high concentrations in the extract. To exert inhibitory effects on *M. tuberculosis*, the lipophilic nature of the compounds in these plant partitions was an essential property of the bioactive constituents. The discovery of these compounds confirmed the higher anti-TB activity of non-polar solvent partitions, as previously discussed. It is ideal if the active ingredients can be isolated and studied in detail. Our collaborators currently use a lengthy chemical-biological approach to isolate and elucidate these compounds. Nevertheless, their isolation and elucidation should be made much easier with the recent availability of several modern, sophisticated hyphenated separation and spectroscopic techniques. Once these active components are isolated, additional assays could be conducted to determine their mechanism of action on tubercle cells.

5. Conclusions

In recent decades, manufacturers of foods, cosmetics, and pharmaceuticals have increased their demand for medicinal plants. The importance of conducting such research lies then not only in the chemical characterization of the plants but also in the possibility of linking their chemical contents with specific functional properties. The extracts of *R. officinalis* by different kinds of DESs demonstrated antimycobacterial activity, possibly through mycobacterial cell wall damage. However, the active compounds identified in this study need to be isolated and purified. The results above conclude that the antimycobacterial action of the *R. officinalis* bioactive substances is due to their components working together.

Author Contributions: Conceptualization, A.S.D.; Data curation, A.S.D.; Formal analysis, M.Q.K.; Investigation, A.S.D.; Methodology, A.S.D.; Resources, N.A.H.; Supervision, M.K.A., M.F.A.B. and S.F.S.; Writing—original draft, A.S.D.; Writing—review & editing, M.K.A., M.F.A.B. and S.F.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors extend their appreciation to the Directorate of Health of Al-Alanbar (DOH), Iraq, for supporting this work.

Conflicts of Interest: The authors declare no conflict of interest.

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