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Materials Today: Proceedings

journal homepage: www.elsevier.com/locate/matpr

Antimycobacterial activity and phytochemical properties of *Eucalyptus camaldulensis* (eucalyptus) extracted by deep eutectic solvents

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ARTICLE INFO

Article history:

Available online xxxxx

Keywords:

Antimycobacterial
Eucalyptus camaldulensis
 Medicinal plant
 Broth microdilution
 Phytochemical content

ABSTRACT

The tuberculosis (TB) is one of the world's top causes of illness and death, it is a major public health problem. *Eucalyptus eucalyptus camaldulensis* has been traditionally used against many diseases, such as respiratory disease infections, including pulmonary (TB), The investigate the antimycobacterial activity of extracts by different deep eutectic solvents against multidrug-resistant (MDR) *Mycobacterium tuberculosis* compared with aqueous traditional solvents and the correlation between the phytochemical composition and antimycobacterial activity, The deep eutectic solvents (DESs) and standard water solvents were used to extract the samples. Agar disc diffusion experiment was used to investigate the antimycobacterial activity of extracts against multidrug-resistant (MDR) *Mycobacterium tuberculosis*. The minimal inhibitory was determined spectrophotometrically at 570 nm and followed measured the bactericidal concentrations. Phytochemical analysis was used in the study to investigate the total phenolic and flavonoid concentrations (TPC and TFC), the demonstrated, extraction by DESs is more a beneficial procedure than traditional solvent extraction of camaldulensis extracts, which have remarkable effectiveness against tuberculosis activities. Therefore, the use of DES should be considered for further investigation.

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Selection and peer-review under responsibility of the scientific committee of the XII th Biennial National Conference of Physics Academy of North East (PANE 2021).

1. Introduction

Tuberculosis (TB) is a serious worldwide health threat because the multidrug-resistant (MDR) activity [5]. TB is endemic in all countries worldwide and causes more deaths in developing countries than other diseases [11]. TB mainly affects the respiratory system but can also cause extensive lesions in the reproductive, digestive, sensory and skeletal systems [20]. The existing TB medications include adverse effects such as headache, hepatotoxicity, skin rashes, and gastrointestinal symptoms [21]. Therefore, developing a new approach for TB with new mode of actions, low toxicity and higher efficacy is urgently needed.

Eucalyptus camaldulensis (eucalyptus) belongs to the Myrtaceae family, is native to Southeast Asia and Australia and also grows in the Middle East and North Africa [5,25]. *E. camaldulensis* leaves have been used against many diseases, such as pulmonary TB, and used in folk medicine to reduce many clinical respiratory signs, including cough and nasal sinus infections [15,19]. All parts of this plant have been investigated against several bacterial and viral pathogens with great biological activities [14,19]. In addition, *E. camaldulensis* is a rich source of essential oils and has received much interest from several industries, such as medicine, cosmetics and food preservatives [24,26]. 1,8-Cinole is a major bioactive essential oil compound in *E. camaldulensis*; this compound has different biological activities and is related to agriculture and genetic factors [13]. Furthermore, phytochemicals, such as flavonoids and phenolics, in *E. camaldulensis* have antioxidant and antimicrobial against various bacterial strains [13,24].

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<https://doi.org/10.1016/j.matpr.2022.06.017>

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Selection and peer-review under responsibility of the scientific committee of the XII th Biennial National Conference of Physics Academy of North East (PANE 2021).

Deep eutectic solvents (DESs) consist of two or three components and were first reported in 2003 [12]. These solvents have low toxicity and are non-inflammable, biodegradable, low cost; these characterisations may overcome the limitations of traditional solvents [1,23]. DESs of many varieties have been created by combining substances such as sugars, organic acids, and amino acids [12]. DESs have become a new green extraction solvent for scientific research and possess selective and efficient performance compared with conventional organic solvents for extracting bioactive compounds from medicinal plants [3,6,8].

Multidrug resistance to isoniazid, rifampicin, pyrazinamide and ethambutol has increased; hence, the search for novel anti-TB drugs is an important task [17,22]. This current study for the first time was conducted to determine the antimycobacterial activity for *E. camaldulensis* extracted through used various types from deep eutectic solvents (DESs) depended on natural renewable products as well as the total phenol and flavonoid content were also determined compared with water extract.

2. Materials and methods

2.1. Chemicals and plants material

L(+) lactic acid (85%) was obtained from Chem-lab NV (Belgium). Glycerol ($\geq 99\%$) was purchased from Panreac (Spain). Choline chloride (98%) and xylitol (98%) were purchased from Xi'an Geeke Biotech (China). Middlebrook 7H10 agar, Middlebrook 7H9 broth medium, glucose anhydrous ($\geq 99\%$) and D(-) fructose ($\geq 99\%$) were obtained from HiMedia (India). Whole *E. camaldulensis* plants were collected from Ramidi City, Al Anbar Governorate and authenticated at the Centre of Desert Studies of Al Anbar University, Iraq.

2.2. DES preparation

Methods for preparing DESs were based on previous research [31]. The mixture was placed in a sealed flask with constant heating and stirred until the liquid turned colourless and homogeneous. Table 1 lists the synthesized DESs.

2.3. Extraction procedure

The extraction procedure used in this research was modified somewhat from that of a prior one [4]. The steps were conducted for one hour, we heated and stirred the extract in a sealed glass container at 40 °C. Dry plant powder 20 mg and 1 mL from various DESs were mixed in equal parts. The sample was centrifuged at 9000 rpm for 10 min. Prior to use and analysis, the suspension was filtered using a 0.45 m nylon membrane. The extraction process for each extract was performed in triplicate.

2.3.1. MDR – *Mycobacterium tuberculosis* strain

The clinical isolate was obtained from the Clinic of Respiratory and Thoracic Diseases in Al-Ramadi City, Iraq. The isolate was verified by GeneXpert assay (Cepheid, CA, USA); cultured on Löwen-

Table 1
Type of DESs used in this study.

DES Code	Full Name	M.R*
DES1: LGH	Lactic acid, Glucose and Water	5:1
DES2: Tailor	Glycerol, Xylitol and D(-)-Fructose	3:3:3
DES3: ChGI	Choline Chloride: Glycerol	1:2
DES4: ChXI	Choline Chloride: Xylitol	1:1

* M.R = molar ratio.

stein-Jensen medium and subcultured on Middlebrook 7H10 agar or Middlebrook 7H9 broth with supplement materials are 10% (v/v) oleic acid–albumin–dextrose–catalase (HiMedia, India) and albumin–dextrose–catalase (HiMedia, India).

2.4. Determination of inhibitory zone by agar disk diffusion assay

The method was adopted from a previously study [28] with slight modifications. 200 mg/ml of stock solution from each extracts were prepared and diluted for different series concentrations (100, 50, 25 mg/ml). A filter paper (6 mm) was sterilized and impregnated with 20 μ L for each concentrations. Rifampicin 50 μ g/ml was used a positive control. Middlebrook 7H10 agar plates were seeded by 100 mL bacterial suspension. Prepared filter paper carefully transferred onto the cultured agar plates. The inoculated plates with parafilm sealed were incubated at 37 °C for three day. Recorded the result by measured the diameters of inhibition zone and this assay was performed in triplicate.

2.5. Determination of minimal inhibitory concentrations MIC and minimal bacterial concentrations MBC

Spectrophotometric method through using culture dilution tubes was performed based on [10] with minor modification. Briefly, the inoculum was cultured, and the suspension density was adjusted to 0.5 McFarland standards. Extraction extracts were diluted six times each in Middlebrook 7H9 broth to provide a range of concentrations of 6.25–200 mg/ml for testing. Additionally, 100 μ L for all except one of the culture tubes had bacterial inoculum introduced, and this was the control growth tube. The control tube contained Middlebrook 7H9 broth and bacterial strain suspensions. All test tubes were incubated at 37 °C for 96 h. Afterward, The extract's MIC was calculated by comparing it to a control growth tube through visual reading and determining the lowest concentration at which all microbial growth was inhibited. Optical density (OD) was determined at 570 nm, and MIC was measured as the 50% growth inhibition compared with the control tube growth. The test was performed in triplicate. The tubes that showed bacterial inhibition were transferred to Middlebrook MH10 agar and then incubated at 37 °C for 72 h. MBC was measured on plates as the lowest concentration of plants sample extracts that showed no viable cells.

2.6. Phytochemical analysis

The TPCs of the plant extracts obtained by DESs were determined by the Folin-Ciocalteu method [16]. The TFCs of the plant extracts were determined by aluminium chloride method according to a recent study with slight modification [27]. The results were expressed on Gallic acid and rutin standard curve. The final results gained were expressed in equivalent per 100 g dry mass.

2.7. Statistical analysis

This study used SPSS version 17.0 and Pearson's correlation analysis to investigate the correlation between phytochemical content in plants and anti-TB action. The significance level was $P < 0.05$.

3. Result and discussion

The inhibitory effects of four DES extracts (i.e. DES1–4) and water extract against MDR *M. tuberculosis* as determined by agar disc diffusion assay are presented in Table 2.

Table 2
Diameter of inhibitory zone (DIZ) of plant crude extracts against MDR – *Mycobacterium tuberculosis*.

Type of DESs	Extract concentration of <i>E.camaldulensis</i>			
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml
DES1: LGH	11.33 ± 0.57	8.66 ± 1.15	7.33 ± 0.57	NA
DES2: Tailor	13.00 ± 1.00	9.33 ± 0.57	8.33 ± 0.57	NA
DES3: ChGI	10.33 ± 0.57	8.33 ± 1.52	NA	NA
DES4: ChXI	12.33 ± 1.52	9.33 ± 0.57	NA	NA
Water	8.66 ± 1.15	NA	NA	NA
Rif 50 µg	13.33 ± 1.52			

Notes: NA = not active. The values were expressed as the mean perform in triplicates.

The 200 mg/mL DES2 displayed the largest inhibition activity (DIZ = 13.00 ± 1.73 mm), followed by DES4, DES1 and DES3 when compared with the water extract. At 100 mg/mL concentration, DES2 and DES4 had equally higher activities compared with the other extracts. In comparison, the 50 mg/mL *E. camaldulensis* extracts in all solvents showed no inhibition activity against MDR *M. tuberculosis*, except DES2 and DES1 (DIZ = 8.33 ± 0.57, 7.33 ± 0.57 mm, respectively).

All the extracts were not active at 25 mg/mL concentration. In comparison, the positive control rifampicin showed inhibition activity against MDR *M. tuberculosis* with DIZ = 13.33 ± 1.52 mm at this concentration.

The MICs and MBCs of the *E. camaldulensis* extracts are shown in Table 3. DES2 extract showed the lowest MIC (12.5 mg/mL) and MBC (50 mg/mL). DES3 and DES4 exhibited equal MICs (25 mg/mL) but different MBCs (not active and 100 mg/mL, respectively). DES1 showed the highest MIC (50 mg/mL) and was not active in terms of MBC. By contrast, the water extract did not exhibit any activity in both parameters. This result is in line with the previous studies, in which *E. globulus* and *E. camaldulensis* have antibacterial activity against different pathogens and *M. tuberculosis* [13,18,25]. The antimycobacterial activities of *E. camaldulensis* extracts obtained by different kinds of DESs against MDR *M. tuberculosis* had not been previously investigated prior to the current study.

Table 4 shows the TPCs and TFCs of the *E. camaldulensis* extracts obtained by various DESs. The highest TPC value (1.430 mg/100 g dry weight [DW]) was obtained in DES2, followed by DES3 (1.35 mg/100 g DW), DES1 (1.260 mg/100 g DW) and DES4 (0.923 mg/100 g DW). The TPCs of the DES extracts were higher than that of the aqueous extract (0.716 mg GAE/100 g DW). The present study showed that TFC ranged from 0.0077 mg RE/g to 0.0026 mg RE/g. The highest TFC value was found in DES2 (0.0077 mg RE/g), followed by DES1, DES4, aqueous extract and DES3. The extraction efficiencies of the DESs for phytochemical contents compared with aqueous solvent are displayed in Fig. 1a and b. Antimicrobial action in *E. camaldulensis* extracts is due to the presence of different phytochemicals contents such as alkaloids, flavonoids, polyphenols, tannins, carbohydrates, saponins, terpenoids, carbohydrates, and steroids [7,18,29].

The reasons for the different results between the present study and previous literature are the differences in environmental or cli-

Table 3
The antimycobacterial activity of *E. camaldulensis* extracts.

Type of DESs	MIC (mg/mL)	MBC (mg/mL)
DES1: LGH	50 mg	NA
DES2: Tailor	12.5 mg	50 mg
DES3: ChGI	25 mg	NA
DES4: ChXI	25 mg	100 mg
Water	NA	NA

Notes: NA = not active. The values were expressed as the mean perform in triplicates.

Table 4
The phytochemical analysis of *E. camaldulensis* extracts.

Type of DESs	TPC (mg GAE/100 g DW)	TFC (mg RE/100 g DW)
DES1: LGH	1.260 ± 0.135	0.0055 ± 0.0004
DES2: Tailor	1.430 ± 0.017	0.0077 ± 0.0010
DES3: ChGI	1.35 ± 0.200	0.0026 ± 0.0006
DES4: ChXI	0.923 ± 0.015	0.0036 ± 0.0003
Water	0.716 ± 0.020	0.0033 ± 0.0008

Notes: NA = not active. The values were expressed as the mean perform in triplicates.

mate conditions, geographical location and place of plant collection, which subsequently lead to differences in phytochemical contents. The extraction efficiency was evaluated based on the levels of phytochemical contents, DESs had a greater extraction efficiency than standard aqueous solvents. Probably, the higher extraction efficiency of the DESs depends mostly on their higher polarity properties compared with that of water, which leads to a higher solubilising capacity [2]. Additionally, the results suggested that DES2 showed a higher potential for phenol and flavonoid content extraction than all the other DESs tested. The current results reflected the higher interaction of triple DES with phytochemicals than double DES, which ultimately leads to a better response against microorganisms [30]. Furthermore, the importance of double DES composition, such as those in DES1, DES3 and DES4, compared with conventional solvents should not be overlooked [9,31].

The correlation between the phytochemical composition and antimycobacterial activity of *E. camaldulensis* extracts was determined using correlation analysis. Table 5 shows that the anti-TB activity of DES2 showed a highly strong positive correlation with both phytochemical contents with $r^2 = 1.000$.

The TPC and TFC of DES4 displayed strong positive correlations with antimycobacterial activity with $r^2 = 0.982$ and $r^2 = 0.994$, respectively. Moreover, The TPC and TFC of DES3 and DES1 extracts showed positive correlations with antimycobacterial activity. By contrast, the antimycobacterial activity of the extract obtained by water extraction showed moderate correlation with TPC and TFC with $r^2 = 0.770$ and $r^2 = 0.693$, respectively.

4. Conclusion

The *E. camaldulensis* extracts obtained by different DESs had improved inhibition activity against MDR *M. tuberculosis* than the extract obtained by classical water extraction because of the increment in TPC and TFC contents. The findings showed that at 200 mg/mL concentration, DES2 displayed the largest inhibition activity (diameter of inhibition zone = 13.00 ± 1.73 mm), followed by DES4, DES1 and DES3. DES2 exhibited the best antimycobacterial activity against MDR *M. tuberculosis* with MIC and MBC values of 12.5 and 50 mg/mL, respectively. The highest TPC and PFC were found in DES2 extract (1.430 mg GAE/100 g and 0.0077 mg RE/100 g, respectively). These extracts may be refined and stan-

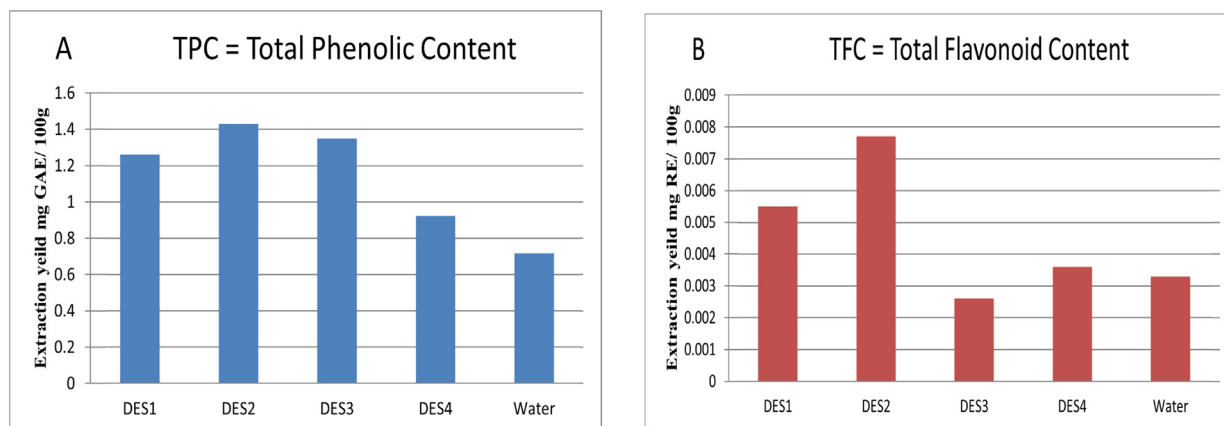


Fig. 1. Extraction efficiency of DESs compared with water solvents. (a) TPC and (b) TFC.

Table 5

Correlation coefficients of each analysis.

Correlation	DES1	DES2	DES3	DES4	Water
TPC versus DIZ	0.896	1.000**	0.866	0.982	0.693
Sig. (2-tailed)	0.293	0.000	0.333	0.121	0.512
TFC versus DIZ	0.866	1.000**	0.924	0.994	0.770
Sig. (2-tailed)	0.333	0.000	0.249	0.069	0.440

** Correlation is significant at the 0.01 level (2-tailed).

andardised to be used as an alternative or complementary medicine. Moreover, further studies should be carried out to isolate bioactive compounds, which could be potential anti-TB drug leads.

CRedit authorship contribution statement

Ali Sami Dheyab: Conceptualization, Methodology, Software. **Abdul Jabbar Khaleel Ibrahim:** Data curation. **Ekremah Kheun Aljumily:** . **Mohamed Khalid AlOmar:** Visualization. **Mohd Fad-zelly Abu Bakar:** Software. **Siti Fatimah Sabran:** Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.matpr.2022.06.017>.

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