

## Research Article

# Identification of Phytochemicals by GC-MS, and Antifungal Efficacy of the Ethanolic Extract of the Plant *Eucalyptus globulus*

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## Abstract

**Background:** *Eucalyptus globulus* has many uses in traditional medicine, but the chemical composition and antifungal properties of its ethanolic leaf extract have not been sufficiently researched. **Objective:** To identifying the main constituents of the extract through GC-MS analysis and evaluating its in vitro antifungal properties against two pathogenic fungi. **Methodology:** The leaves of *Eucalyptus globulus* were collected in Abu Al-Khasib city in Basrah province, Iraq. Ethanolic leaf extract was obtained through microwave-assisted extraction. It was analyzed by GC-MS and assayed against *Candida albicans* and *Aspergillus niger* (concentrations ranging from 10 to 100 mg/mL) using agar well diffusion test, followed by determining the MIC and MFC concentrations by broth microdilution. **Results:** The extraction yield was  $14.6 \pm 0.8\%$  (w/w). Thirty-one chemical compounds were tentatively identified through GC-MS and consisted mainly of oxygenated monoterpenoids (eucalyptol, thymol, pinocarvone), sesquiterpene alcohols (carotol,  $\alpha$ -santalol), phenolic (homovanillic acid), fatty acids (palmitic acid), phytol, and phytosterols. The antifungal activity showed dose dependence and statistical significance ( $p < 0.05$ ); maximum inhibition zones were  $20.7 \pm 0.6$  mm and  $18.0 \pm 0.5$  mm for *Candida albicans* and *Aspergillus niger*, respectively, with 100 mg/mL of extract; while the activity started with 20 and 30 mg/mL, respectively; the solvent had no effect on inhibition zone. The MIC and MFC values were 20 and 40 mg/mL, and 40 and 80 mg/mL, respectively, for *Candida albicans* and *Aspergillus niger* with MFC/MIC ratio being 2, which indicated fungicidal activity. **Conclusion:** The current study offers a combined evaluation of the chemistry and antifungal properties of the microwave-assisted ethanolic leaf extract of *E. globulus*.

## 1. Introduction

*Eucalyptus globulus* Labill. (Myrtaceae) (*E. globulus*) is a widely-used species in traditional medicine and pharmacy applications. Biological properties of eucalyptus are determined by the presence of essential oils, phenolics, flavonoids, tannins, terpenoids and other secondary metabolites, which ratios may depend on genotype, plant part, location, environmental conditions, and extraction techniques. *Eucalyptus* essential oil is characterized by high content of 1,8-cineole (eucalyptol) along with other components such as  $\alpha$ -pinene, limonene, p-cymene and several sesquiterpenes [1]. Of special interest are leaves since they contain both volatile and non-volatile constituents. Essential oil, usually extracted via hydrodistillation or steam distillation and representing a combination of volatile substances, contrasts with the ethanolic extract, which contains a larger variety of polar and semi-polar substances including phenolic acids, flavonoids, tannins, and less volatile

terpenoids. Hence, the chemical profile of an ethanolic extract should not be necessarily considered similar to the one of the essential oil. Differences in chemical profiles between *Eucalyptus* species can determine their antibacterial, antifungal and antiviral activities [2].

For chemical characterization of volatile and semi-volatile phytochemicals gas chromatography–mass spectrometry (GC–MS) is usually used. While gas chromatography allows separating compounds on the basis of volatility and interaction with the column, mass spectrometry provides characteristic spectra, which can be compared with the spectra available in the libraries and standards in order to identify compounds and estimate their relative abundance. Library matching cannot serve as the only source for identifying compounds; however, it can be combined with retention indices, retention times, and if possible, the authentic standards. In case of *E. globulus*, the correlation between chemical composition of the oil and its antimicrobial activity against food-spoiling microorganisms illustrates the necessity of chemical characterization in explaining biological effects [3]. Apart from identifying terpenoids, GC–MS analysis can also reveal fatty acids, esters, alcohols, terpenes, phenolic derivatives, and others present in plant extracts. Nonetheless, non-volatile or heat-labile compounds may require derivatization or complementary liquid chromatography–mass spectrometry. Chemical characterization of methanolic extract of *E. globulus* leaves revealed the importance of chemical profiling when estimating biological activity and toxicity [4]. More advanced approaches include GC–MS, UPLC–MS/MS, and molecular docking that allows predicting potential active compounds in eucalyptus leaves. Such predictions are valuable for generating hypotheses but cannot replace experimental verification [5].

Possible mechanisms of antifungal action of eucalyptus preparations include several complementary pathways. Terpenoid and other aromatic compounds can damage fungi cell membrane, increase its permeability, disrupt proton gradient, and affect respiration and enzymes' functioning. Phenolic compounds can increase the level of oxidative stress and inhibit functioning of essential proteins. Consequently, the activity of an essential oil depends on the main constituent, its concentration, chemical interactions, species and conditions of experiments. In the review dedicated to the essential oils, the disruption of membrane is mentioned as the common antimicrobial mechanism while the role of composition and interactions are highlighted [6]. Several experiments provided evidence of possible interactions between constituents. *E. globulus* essential oil and 1,8-cineole inhibited growth of storage fungi such as *Aspergillus flavus* and *A. parasiticus*, and in some experiments, the complete oil was more effective, which suggests that minor constituents could increase response [7]. Eucalyptus oil can also show activity against *Candida albicans*, other filamentous fungi, and *Candida tropicalis*, while the vapor exposure demonstrates higher activity in comparison with the direct contact under certain conditions [8]. Experiments with different species of eucalyptus demonstrate that the antifungal effect is associated with chemical composition of particular species and not with the volume of the oil [9]. Evaluation of antifungal activity requires rigorous methodology. The disk-diffusion measurements can be influenced by the diffusion of compounds through the agar, whereas minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are more informative parameters if inoculum density, solvent concentration, incubation conditions, and controls are controlled. An ethanol control is necessary because the solvent influences fungi growth, and a well-known antifungal drug must be used as a positive control. Reproducibility of plant-derived antimicrobial studies is also determined by extraction and assay methods [10]. Thus, standardized susceptibility testing methods recommend inoculum density, incubation conditions, reading time, and endpoints [11].

While the essential oil of *E. globulus* is extensively studied, the ethanolic extract represents a unique chemical preparation that contains phenolic and semi-polar compounds, which are not present in volatile fraction. Moreover, its composition and activity can be influenced by altitude, climate, and place of collecting, since the altitude-related differences in *E. globulus* oils have been correlated with their chemical composition and antimicrobial activity [12]. Therefore, the investigation of the extract should not be combined with the data about the essential oil. Plants extracts are examined as potential complementary material for treatment of pathogenic and food-spoilage fungi in view of growing interest in natural preservatives [13]. Natural origin does not mean safety, efficacy, or appropriateness for therapeutic use. Reviews of eucalyptus preparations highlight the importance of evaluation of dose, toxicity, bioavailability, interactions and preparation-specific variation [14]. Moreover, the entire mixture may exhibit more or less activity in comparison with separate components due to the synergistic and antagonistic interactions [15]. The current gap in research is in the lack of correlation between GC–MS analysis and antifungal activity of ethanolic *E. globulus* extracts. This study is aimed to identify the major phytochemicals in the extract by GC–MS, and investigate its *in vitro* antifungal activity with appropriate controls.

## 2. Materials and methods

### 2.1. Collection of plant materials

In April 2025, fresh leaves of *E. globulus* Labill. (globe eucalyptus) were collected from the gardens of Abu Al-Khasib in Basrah, Iraq. Identification of the species was performed by a faculty member at the University of Basrah, College of Science. The leaves were cleaned to remove dust and other materials, sliced into small parts, air-dried until constant weight was achieved at room temperature, and ground into fine powder using an electric mill. The powder was kept in an airtight bottle at 4°C until extracted.

### 2.2. Microwave-Assisted Extraction of Eucalyptus Leaf

Microwave-assisted extraction (MAE) technique was used to obtain ethanolic extract of the leaves. To be more precise, 5 g of Eucalyptus leaves powder was transferred to a round-bottom conical flask, and 100 mL of 99% ethanol was added to it. After mixing and letting it stay for 20–30 min, MAE was performed on the mixture. Extraction was performed using a microwave-assisted system at 50°C for 5 min. Then, the extract was allowed to cool down to room temperature for 10–20 min and centrifuged at 3000 rpm for 15 min. Finally, the extract was separated from solid matter using Whatman filter paper and collected in a clean and airtight container. It was kept at 4°C until further analysis. Hameed et al.'s protocol was followed in the process, any changes made were mentioned [16]. Extraction yield was calculated gravimetrically after evaporation of the solvent and expressed in percentage relative to the dry weight of the plant, using the following formula:

$$\text{Yield (\%)} = \frac{(\text{Weight of dried extract})}{(\text{Weight of dry plant powder})} \times 100$$

### 2.3. GC–MS Analysis of the Ethanolic Leaf Extract

The ethanolic leaf extract of the plant was subjected to GC–MS for phytochemical analysis. The GC analysis was performed by the use of Agilent 7890B Gas Chromatograph fitted with Agilent 5977A Mass Selective Detector (MSD, USA). The analysis was controlled via MassHunter workstation software. Before injection, the extract was filtered using a syringe filter. Separation was accomplished using a 5% phenyl methyl siloxane capillary column. Oven temperature started from 40°C and increased to 50–280°C at the rate of 10°C per minute. Inlet temperature was maintained at 290°C for four minutes after the solvent delay time. Helium was used as the carrier gas with a flow rate of 1.0 mL min<sup>-1</sup>, while the inlet pressure was kept at 6.0799 psi. The analysis was performed by using pulsed splitless injection. Mass spectrometric analysis was performed in the range of 35 to 650 m/z [17]. Tentative identification of the identified compounds was performed by comparing the obtained mass spectra of each compound with NIST library. Identified compounds together with their relative abundance values were determined from GC-MS analysis and NIST library matching results. Since NIST matching gives only tentative identifications of compounds, the assignment of the compounds needs to be supported by retention index, authentic standard or other analytical methods if available.

### 2.4. Antifungal Activity Assay

Antifungal activity of the ethanolic extract of *E. globulus* leaf was investigated against *Candida albicans* (*C. albicans*) and *Aspergillus niger* (*A. niger*). Isolates were grown on Sabouraud Dextrose Agar (SDA). For *C. albicans*, incubation was performed at 35°C for 24–48 h, while for *A. niger*, incubation was performed at 28–30°C for 3–5 days until there was adequate growth [18]. Fungal suspension was prepared in sterile saline and standardized to approximately 0.5 McFarland standard.

#### 2.4.1. Agar-Well Diffusion Assay

SDA plates were streaked with the fungal suspension. Sterile wells of diameter 6 mm were punched in the agar plates. Ethanol extracts were added to the wells at the following concentrations: 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/mL. The negative control was prepared by using only the solvent, while the positive control was prepared by using a suitable standard antifungal drug (voriconazole). The plates with *C. albicans* isolates were incubated at 35°C for 24–48 h, and plates with *A. niger* isolates were incubated at 28–30°C for 3–5 days [19]. Diameter of the inhibition zone was measured in millimeters. This procedure was carried out in three replicates and results expressed as mean ± standard deviation.

#### 2.4.2. Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined by broth microdilution technique. The ethanol extracts were prepared in similar concentrations used in the agar-well diffusion (10–100 mg/mL). They were mixed with the selected broth medium and inoculated with the standardized fungal suspension. Broth microdilution assay was carried out using 96-well microplates. There was a growth control comprising broth and fungal inoculum but no extract. A sterility control comprising broth but not fungal inoculum was used. There was also a solvent control and a positive control that consisted of a standard antifungal drug. Fungal growth was assessed spectrophotometrically after incubation at appropriate conditions for each fungal isolate. MIC was the lowest extract concentration that resulted in complete inhibition of fungal growth compared to the growth control. If absorbance measurements were used, the percent inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \frac{[A_{\text{control}} - A_{\text{sample}}]}{A_{\text{control}}} \times 100$$

Where,  $A_{\text{control}}$  represents the absorbance of the growth control, while  $A_{\text{sample}}$  is the absorbance of the treated extract sample after adjusting for the absorbance of the extract blank.

#### 2.4.3. Identification of the Minimum Fungicidal Concentration

In order to identify the minimum fungicidal concentration (MFC), subculturing from the MIC well and from those wells where there was no growth was carried out on SDA plates without any extract. These plates were incubated for (24–48) h at 35°C for *C. albicans* and for (3–5) days at (28–30)°C for *A. niger*. MFC was considered as the lowest concentration of extract resulting in the absence of fungal growth after subculture [20]. Values of MIC and MFC were expressed in mg/mL for each fungal strain.

### 2.5. Statistical Analysis

The experiments were performed in triplicates. The results were subjected to statistical analysis using one-way analysis of variance (ANOVA) and appropriate post hoc test. Statistical significance of differences was set at  $p < 0.05$ . Antifungal susceptibility testing procedures followed the recommendations of CLSI M27 for yeasts and CLSI M38 for filamentous fungi with appropriate modifications to the use of plant extract.

## 3. Results

### 3.1. Physical Properties and Extraction Yield of the Ethanolic Extract

Microwave-assisted extraction (MAE) of the dried ground leaves of *E. globulus* Labill. using 99% ethanol resulted in a dark-green, viscous ethanolic extract with an aromatic smell. Extraction of 5 g of dried ground leaf powder in 100 mL of ethanol for 5 minutes at 50°C. Thus, the MAE method can be used as an efficient way of obtaining the extract from the eucalyptus leaves. The yield of the ethanolic extract obtained was 14.6±0.8% (w/w).

### 3.2. Phytochemical Composition by GC–MS Analysis of the Ethanolic Extract

GC–MS analysis of the ethanolic extract of the *E. globulus* leaves revealed a diverse chemical composition of the sample. In total, 31 different phytochemical compounds were tentatively identified based on their similarity to those of known compounds from the NIST library of mass spectra with RTs varying between 9.267 and 34.952 minutes Table 1 & Figure 1. These compounds belonged to several categories of phytochemicals, mainly to monoterpenes and oxygenated monoterpenoids, sesquiterpenes and sesquiterpene alcohols, phenolics and phenylpropanoids, fatty acids, fatty alcohols, and fatty-acid esters as well as sterols and triterpenoids; there were also some nitrogenous compounds and others.

**Table 1:** Phytochemical compounds in the ethanolic extract of *E. globulus* by GC–MS

| No. | RT     | Chemical formula                                            | Name compound                               |
|-----|--------|-------------------------------------------------------------|---------------------------------------------|
| 1   | 10.372 | C <sub>10</sub> H <sub>18</sub> O                           | Eucalyptol                                  |
| 2   | 11.154 | C <sub>5</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub> | Thymine derivatization                      |
| 3   | 11.454 | C <sub>10</sub> H <sub>16</sub> O                           | alpha.-Campholenal                          |
| 4   | 11.875 | C <sub>10</sub> H <sub>18</sub> O                           | Fenchol                                     |
| 5   | 12.72  | C <sub>10</sub> H <sub>14</sub> O                           | Pinocarpone                                 |
| 6   | 13.25  | C <sub>10</sub> H <sub>16</sub> O                           | Myrtenol                                    |
| 7   | 13.49  | C <sub>8</sub> H <sub>8</sub> O                             | -Benzofuran, 2,3-dihydro                    |
| 8   | 13.755 | C <sub>17</sub> H <sub>21</sub> NO <sub>4</sub>             | Isoborneol 4-nitrobenzoate                  |
| 9   | 13.938 | C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>              | Myrtenyl angelate                           |
| 10  | 14.639 | C <sub>10</sub> H <sub>14</sub> O                           | p-Cymen-7-ol                                |
| 11  | 14.756 | C <sub>10</sub> H <sub>14</sub> O                           | Thymol                                      |
| 12  | 16.326 | C <sub>17</sub> H <sub>20</sub> O <sub>2</sub>              | Bisphenol C                                 |
| 13  | 16.642 | C <sub>10</sub> H <sub>16</sub> O <sub>3</sub>              | Pinonic acid                                |
| 14  | 16.701 | C <sub>15</sub> H <sub>26</sub> O                           | Carotol                                     |
| 15  | 16.78  | C <sub>15</sub> H <sub>24</sub>                             | Aromandendrene                              |
| 16  | 18.598 | C <sub>15</sub> H <sub>26</sub> O                           | Globulol                                    |
| 17  | 18.477 | C <sub>16</sub> H <sub>34</sub> O                           | Hexadecanol-1                               |
| 18  | 19.109 | C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>               | Epi-Inositol                                |
| 19  | 19.214 | C <sub>9</sub> H <sub>10</sub> O <sub>4</sub>               | Homovanillic acid                           |
| 20  | 19.947 | C <sub>15</sub> H <sub>24</sub> O                           | alpha.-Santalol                             |
| 21  | 20.716 | C <sub>15</sub> H <sub>32</sub> O                           | n-Pentadecano                               |
| 22  | 21.282 | C <sub>18</sub> H <sub>36</sub> O                           | -Pentadecanone, 6,10,14-trimethyl-2         |
| 23  | 22.414 | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>              | n-Hexadecanoic acid                         |
| 24  | 24.496 | C <sub>12</sub> H <sub>25</sub> NO                          | Dodecanamide                                |
| 25  | 25.197 | C <sub>20</sub> H <sub>40</sub> O                           | Phytol                                      |
| 26  | 26.126 | C <sub>18</sub> H <sub>35</sub> NO                          | -(Octadecenamide, Z-9)                      |
| 27  | 26.269 | C <sub>9</sub> H <sub>19</sub> NO                           | Nonanamide                                  |
| 28  | 28.151 | C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>              | Bornanediol-2,3                             |
| 29  | 29.862 | C <sub>29</sub> H <sub>50</sub> O <sub>4</sub>              | alpha.-Tocospiro A.                         |
| 30  | 34.373 | C <sub>29</sub> H <sub>46</sub> O                           | Stigmasta-3,5-dien-7-one                    |
| 31  | 34.952 | C <sub>32</sub> H <sub>54</sub> O <sub>2</sub>              | -(Cyclolanostan-3-ol, acetate, 3.beta-9,19) |

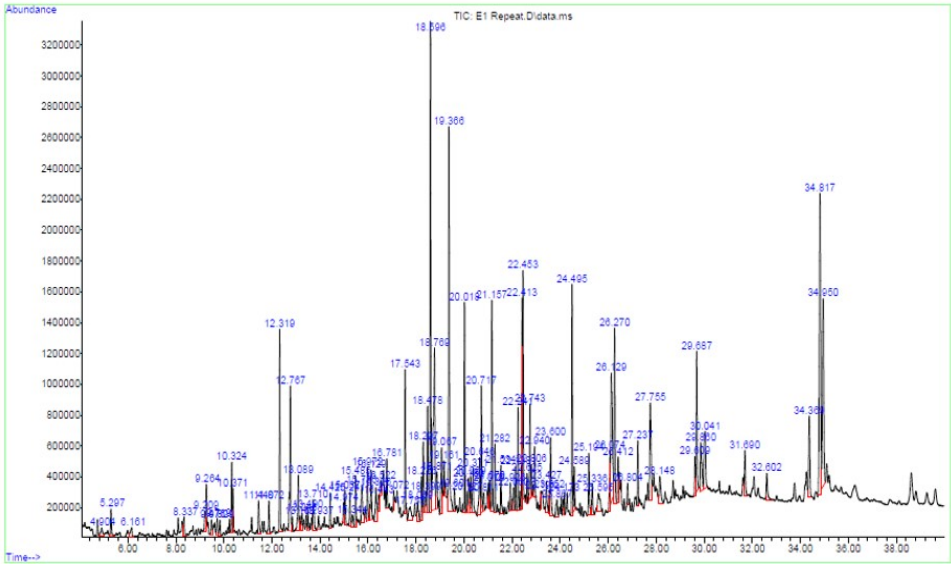


**Figure 1:** Total ion chromatogram of ethanolic leaf extract of *E. globulus*

### 3.3. In vitro Antifungal Activity of Ethanolic Extract

#### 3.3.1. Agar-well diffusion technique

The antimicrobial activity of the ethanolic extract of leaves of *E. globulus* against *C. albicans* (yeast) and *A. niger* (filamentous fungi) at ten concentrations (10–100 mg/mL) has been determined using agar-well diffusion technique. The extract showed a clear dose dependent inhibitory activity against both the test fungi as manifested from increase in diameter of the inhibition zone as the concentration of the extract increases Table 2. There were no zones of inhibition around the negative control wells (solvents) implying that the solvent used for extraction did not play any role in the antifungal activity at the volume used. Voriconazole (25  $\mu$ g/mL), used as a positive control, produced zones of inhibition of  $22.3 \pm 0.6$  mm against *C. albicans* and  $18.7 \pm 0.6$  mm against *A. niger*. The extract at 100 mg/mL concentration produced zones of inhibition of  $20.7 \pm 0.6$  mm against *C. albicans* and  $18.0 \pm 0.5$  mm against *A. niger*. Inhibition started at concentrations of 20 and 30 mg/mL respectively. There was statistically significant concentration dependency ( $p < 0.05$ ) in which concentrations  $> 50$  mg/mL were more potent than lower concentrations. Overall, the extract exhibited broad spectrum antifungal activity with *C. albicans* being more sensitive than *A. niger*.

**Table 2:** Diameter of inhibition zones (mm) produced by the ethanolic extract of *E. globulus* against *C. albicans* and *A. niger* (mean  $\pm$  SD, n = 3)

| Concentration (mg/mL)                            | Inhibition zones (mm) |                 |
|--------------------------------------------------|-----------------------|-----------------|
|                                                  | <i>C. albicans</i>    | <i>A. niger</i> |
| 10                                               | $0.0 \pm 0.0$         | $0.0 \pm 0.0$   |
| 20                                               | $7.3 \pm 0.6$         | $0.0 \pm 0.0$   |
| 30                                               | $9.0 \pm 0.5$         | $7.7 \pm 0.6$   |
| 40                                               | $10.7 \pm 0.6$        | $9.3 \pm 0.6$   |
| 50                                               | $12.3 \pm 0.6$        | $10.7 \pm 0.6$  |
| 60                                               | $14.0 \pm 0.5$        | $12.0 \pm 0.5$  |
| 70                                               | $15.7 \pm 0.6$        | $13.3 \pm 0.6$  |
| 80                                               | $17.3 \pm 0.6$        | $15.0 \pm 0.5$  |
| 90                                               | $19.0 \pm 0.5$        | $16.3 \pm 0.6$  |
| 100                                              | $20.7 \pm 0.6$        | $18.0 \pm 0.5$  |
| Negative control (ethanol)                       | $0.0 \pm 0.0$         | $0.0 \pm 0.0$   |
| Positive control<br>(voriconazole 25 $\mu$ g/mL) | $22.3 \pm 0.6$        | $18.7 \pm 0.6$  |

#### 3.3.2. Determination of Minimum inhibitory concentration (MIC)

MIC results obtained through broth microdilution technique are given in Table 3. Ethanol extract had a complete inhibitory effect on growth of *C. albicans* at a minimum concentration of 20 mg/mL while its MIC value against *A. niger* was 40 mg/mL. Control wells had expected growth of the fungi while sterility control wells had no growth and solvent control wells had no inhibition. Therefore, the assay was confirmed. Percentage of inhibition calculated using absorbance measurements of the samples correlated well with visually defined endpoints and demonstrated  $> 90\%$  of inhibition at the obtained MICs of both fungi.

#### 3.3.3. Determination of Minimum fungicidal concentration (MFC)

Inoculation of clear wells at MIC concentrations on the Sabouraud dextrose agar without extract provided MFC values of 40 mg/mL for *C. albicans* and 80 mg/mL for *A. niger* Table 3. Ratio between MFC and MIC was equal to 2 in the case of *C. albicans* and 2 in the case of *A. niger*. Since MFC/MIC ratio  $\leq 4$  is considered a good indicator of a fungicidal effect of an antifungal agent, ethanol extract of *E. globulus* can be referred to as fungicidal against tested fungi.

**Table 3:** MIC and MFC values (mg/mL) of the ethanolic extract of *E. globulus* against the test fungi

| Fungal isolate     | MIC (mg/mL) | MFC (mg/mL) | MFC/MIC ratio |
|--------------------|-------------|-------------|---------------|
| <i>C. albicans</i> | 20          | 40          | 2             |
| <i>A. niger</i>    | 40          | 80          | 2             |

## 4. Discussion

The current study combines MAE, GC-MS profiling and standardized in vitro susceptibility testing to characterize the ethanolic leaf extract of Iraqi *E. globulus* and evaluate its antifungal properties. The high extraction yield of  $14.6 \pm 0.8\%$  obtained in only 5 minutes at  $50^\circ\text{C}$  suggests that MAE is a evident and solvent-saving method to isolate bioactive metabolites from eucalyptus foliage. The optimization of MAE of eucalyptus leaves showed that short times of irradiation favor maximum phenolic extraction with minimal thermal decomposition, a plausible reason behind the chemical richness observed in the present extract [21]. According to GC-MS, 31 different compounds were tentatively identified belonging to monoterpenoids, sesquiterpenoids, phenolics, fatty-acid derivatives and phytosterols, indicating that the ethanolic extract has a completely different chemical profile in comparison with the essential oil obtained by steam distillation, which is mainly composed of 1,8-cineole. For example, the Algerian *E. globulus* essential oil has been found to contain 94.03% 1,8-cineole [22], while the current extract contains eucalyptol in combination with thymol, globulol, carotol,  $\alpha$ -santalol, and homovanillic acid. This semipolar mixture of components corresponds to the solvent selectivity of ethanol and the known chemocomposition of eucalyptus leaves rich in

phenolics, flavonoids and tannins in addition to volatile terpenes [23]. It is important to note the detection of globulol and aromadendrene, since these sesquiterpenes are the chemotaxonomic markers of the genus *Eucalyptus* and possess antimicrobial properties.

The extract demonstrated dose-dependent inhibitory effect on *C. albicans* and *A. niger* with inhibition zones of  $20.7 \pm 0.6$  mm and  $18.0 \pm 0.5$  mm, respectively, at the concentration of 100 mg/mL. These results are lower than those for pure essential oils but biologically significant for a crude semipolar extract tested in an agar medium, in which diffusion of lipophilic components is naturally limited. The antifungal activity of *E. globulus* oil against food spoilage fungi was previously shown to strongly depend on the format of the assay, with the vapor-phase exposure being usually more active than direct contact [24], hence the use of well diffusion design in the present study might be expected to underestimate the real activity of volatile fraction. The greater susceptibility of *C. albicans* compared with *A. niger* is consistent with previous reports of increased sensitivity of yeasts to eucalyptus oil, whereas filamentous molds were shown to be more resistant to its action due to their chitin-rich, melanized cell wall and slower growth rate [25].

The MIC values (20 mg/mL for *C. albicans*; 40 mg/mL for *A. niger*) and MFC/MIC ratio of 2 indicate a fungicidal rather than fungistatic mechanism of the action. Similarly to whole *E. globulus* oil, this extract was reported to be more active against storage *Aspergillus* species than isolated 1,8-cineole [26], suggesting that minor components of the extract (thymol, pinocarvone, myrtenol and the sesquiterpene alcohols) act additively or synergistically with the major monoterpene. Synergy between minor components is one of the known features of complex essential oil mixtures, as the membrane disruption, proton gradient dissipation and enzymes inhibition act as the common mechanism of antifungal activity of the majority of these mixtures [27]. Several components identified in the extract have individually described antifungal mechanisms that make the reported results more plausible. For example, thymol interferes with ergosterol biosynthesis and impairs the integrity of the membrane of pathogenic fungi [28], while palmitic acid (n-hexadecanoic acid) inhibits virulence factor expression in *Candida* species [29]. The diterpene alcohol phytol possesses the broad spectrum of antimicrobial and antibiofilm activities [30], while the phenolic compound (homovanillic acid) can contribute by the pro-oxidative and enzyme inhibitory mechanisms. Hence, the co-existence of several antimicrobial components in a single mixture enables a multitarget chemical attack that cannot be provided by single compounds, making less probable the emergence of resistance.

Regarding methodology, the inclusion of solvent, growth, sterility and positive controls, triplicate testing and use of CLSI standardized procedures ensure the internal validity of the susceptibility test results. Agar diffusion measurements were shown to be affected by the polarity of a compound and composition of agar medium, and dilution methods were recommended to supplement them [31], which is implemented in the present combined well diffusion and microdilution design. Still, the tentative nature of library-based GC-MS identification should be noted, and it is necessary to confirm the results with retention index determination and using authentic standards or complement the analysis with LC-MS of nonvolatile components, as recommended for multiplatform approaches [10]. The chemical and biological variability of eucalyptus preparations depending on the geographical origin was previously shown, and altitude alone has been shown to have significant influence on the composition and antifungal properties of *E. globulus* oil [32]. The described extract, obtained from trees growing in the hot, humid, low altitude environment of Abu Al-Khasib (Basrah, Iraq), represents another chemotype of eucalyptus with unique chemical composition that cannot be extrapolated data from other geographical regions. From practical point of view, the fungicidal activity of this extract against yeast and mold of clinical and food spoilage importance makes it a candidate natural preservative or alternative antifungal agent in the context of growing interest in plant-derived antimicrobials [33]. However, natural origin does not equal safety, and the toxicological testing of a methanolic *E. globulus* leaf extract demonstrates the kind of dose-response and toxicity testing that should be carried out before any consideration of its therapeutic application [34]. Finally, biological activity of the essential oil-type extracts depends on their composition, dose and interaction between components [35], therefore bioassay-guided fractionation and in vivo studies are essential subsequent steps.

## 5. Conclusion

The current study offers a combined evaluation of the chemistry and antifungal properties of the microwave-assisted ethanolic leaf extract of *E. globulus*, native to Basrah, Iraq. The microwave-assisted extraction (MAE) technique was fast and effective in producing  $14.6 \pm 0.8\%$  of a complex chemically active extract, which was analyzed using gas chromatography-mass spectrometry (GC-MS) and found to comprise 31 components. The most dominant among them were identified. The extracts showed high concentration-dependent fungicidal action towards *C. albicans* (MIC 20 mg/mL; MFC 40 mg/mL) and *A. niger* (MIC 40 mg/mL; MFC 80 mg/mL) fungi, *C. albicans* being more sensitive to the effect of the tested extract.

## Article Information

**Data Availability:** The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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