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RESEARCH

LC–ESI–MS/MS-Based Tentative Phytochemical Profiling and Bioactivity Assessment of *Eucalyptus camaldulensis* Leaf Extract

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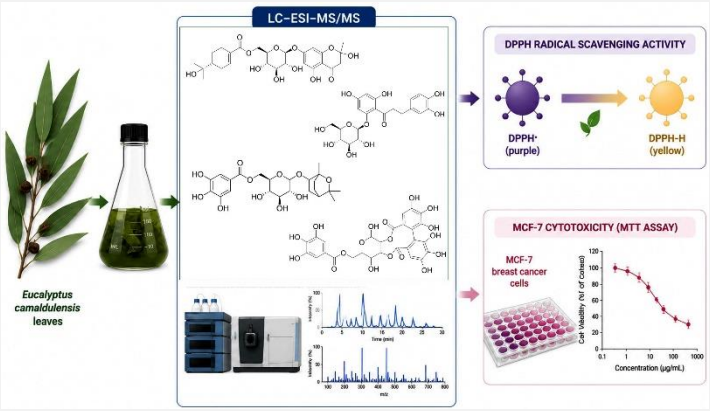
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ABSTRACT

Eucalyptus camaldulensis is a medicinal plant known for its phenolic constituents and antioxidant properties. This study aimed to tentatively characterize the phytochemical profile of its methanolic leaf extract and evaluate its antioxidant activity and effects on MCF-7 breast cancer cell viability. Metabolites were profiled using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS). Antioxidant activity was assessed using the DPPH radical-scavenging assay, and cell viability was evaluated using the MTT assay. A total of 26 metabolites were tentatively annotated, including phenolic acids, flavonoids, flavonoid glycosides, and hydrolyzable tannins, particularly ellagitannin-related derivatives. The estimated DPPH IC_{50} was approximately 135 µg/mL, obtained by linear extrapolation beyond the tested concentration range. The estimated MTT IC_{50} was approximately 183 µg/mL, based on nonlinear regression of concentration–response data. The extract showed antioxidant activity and reduced MCF-7 cell viability at higher concentrations. However, the DPPH IC_{50} should be interpreted cautiously because it was extrapolated beyond the tested range. Further studies involving compound isolation, structural confirmation, and additional cell models are needed to validate the tentative metabolite assignments and investigate the extract's biological effects.



Keywords: *Eucalyptus camaldulensis*, LC–ESI–MS/MS, phenolic compounds, antioxidant activity, cytotoxicity

1. Introduction

Eucalyptus camaldulensis, commonly referred to as the River Red Gum, is one of the most widespread and ecologically adaptable members of the Eucalyptus genus. Native to Australia, where it predominantly grows along riverbanks and floodplains, the species has been introduced into numerous subtropical and temperate regions due to its tolerance to drought, salinity, and variable soil conditions [1,2]. In addition to its ecological contributions to reforestation, erosion control, and carbon sequestration, *E. camaldulensis* has considerable economic value owing to its rapid growth and durable timber [3]. Its longstanding use in traditional medicine across different cultures highlights its medicinal significance and provides a rationale for investigating its properties using contemporary pharmacological approaches [4]. The medicinal relevance of *E. camaldulensis* is associated with its diverse phytochemical composition. Its leaves contain volatile essential oils, often enriched in 1,8-cineole, as well as non-volatile constituents, including phenolic acids, flavonoids, tannins, and formylated phloroglucinol compounds [5–7]. These constituents have been associated with various biological activities, including antimicrobial, antioxidant, anti-inflammatory, and antiproliferative effects [8–10]. Such findings provide a basis for further investigation of the plant's chemical composition and biological properties. However, the presence of diverse phytochemicals alone does not establish which constituents, if any, contribute to a particular biological effect, highlighting the importance of combining chemical profiling with experimental bioactivity assessment.

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Although previous studies have investigated the essential oil composition and total phenolic content of *E. camaldulensis* [11–14], detailed LC–ESI–MS/MS-based characterization of the non-volatile constituents of methanolic leaf extracts from Iranian material, particularly material collected in Khuzestan Province, remains insufficiently documented in the literature cited here. Consequently, the chemical profile of this extract and its relationship to experimentally measured antioxidant activity and effects on cancer-cell viability warrant further investigation. The present study therefore aimed to tentatively characterize the phytochemical constituents of a methanolic leaf extract of *E. camaldulensis* collected in Khuzestan Province, Iran, using LC–ESI–MS/MS, and to assess its antioxidant activity using the DPPH radical-scavenging assay and its effects on MCF-7 breast cancer cell viability using the MTT assay. The metabolite annotations are considered tentative, and the biological assays are intended to provide an initial assessment of the extract rather than establish the activities or mechanisms of individual compounds.

2. Methods

2.1. Plant material

Fresh leaves of *Eucalyptus camaldulensis* were harvested from Abadan, Khuzestan Province, Iran, during February-March 2024, coinciding with the plant's flowering stage a period reported to contain elevated levels of secondary metabolites. Mature, healthy, and pest-free leaves were randomly collected at a height of 1.5–2 m above ground level to minimize environmental variability. The identity of the plant material was confirmed by Dr. Mohammadreza Parishani, Department of Biology, Shahid Chamran University of Ahvaz, Iran. The leaves were shade-dried at ambient temperature to protect heat-sensitive compounds from degradation and stored in airtight containers until further processing.

2.2. Preparation of extract

Dried leaf material was pulverized to a uniform powder and extracted by maceration using methanol as the extraction solvent. Approximately 10 g of the powdered sample was immersed in methanol (sample-to-solvent ratio 1:10, w/v) and kept at room temperature for 24 h with occasional agitation. The mixture was subsequently filtered, and the plant residue was subjected to two additional 24-h extraction cycles under the same conditions to achieve complete extraction. The filtrates obtained from all three maceration steps were combined and then lyophilized using a freeze-dryer to produce a stable, solvent-free dry extract. The extraction yield of the dry methanolic leaf extract was 8.5% (w/w), relative to the initial dry leaf powder. The dried extract was transferred to airtight containers and stored at 4 °C until further analysis.

2.3. Antioxidant activity evaluation

The free radical-scavenging activity of the extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay with slight modifications. Briefly, a 0.1 mM DPPH solution was prepared in methanol. The *E. camaldulensis* extract was dissolved in methanol to prepare solutions at concentrations of 20, 40, 70, 100, and 130 µg/mL. Subsequently, 1 mL of the DPPH solution was mixed with 1 mL of each extract solution. The resulting mixture was vortexed and allowed to stand in the dark at room temperature for 30 minutes. After the incubation period, the absorbance was recorded at 517 nm with a UV–Vis spectrophotometer. Methanol mixed with DPPH solution was used as the control, while methanol served as the blank. The radical scavenging activity was calculated using the following equation:

$$\text{inhibition percent of DPPH radical} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

The IC_{50} was estimated by linear regression of percentage inhibition against extract concentration using the experimental data. As 50% inhibition was not reached within the tested concentration range (20–130 µg/mL), the IC_{50} was estimated by extrapolation beyond the highest tested concentration and should therefore be interpreted cautiously.

2.4. LC–ESI–MS/MS analysis

Comprehensive metabolite profiling of the methanolic extract was conducted using a liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) system consisting of an Alliance 2695 HPLC unit (Waters, USA) coupled to a Micromass Quattro Micro API triple-quadrupole mass spectrometer (Waters, USA). Chromatographic separation was achieved on a Thermo C8 reversed-phase column (150 × 4.6 mm, 5 µm), with the column oven maintained at 35 °C to ensure retention stability and reproducible peak shapes. The mobile phase consisted of solvent A, acetonitrile containing 0.1% formic acid, and solvent B, deionized water with 0.1% formic acid. A linear gradient elution was applied at a flow rate of 0.3 mL/min as follows: 10–50% A (0–8 min), 50–70% A (8–15 min), 70–10% A (15–24 min), followed by column re-equilibration at 10% A (24–26 min). A 10 µL aliquot of each sample was injected under these chromatographic conditions.

Mass spectrometric detection was performed using an electrospray ionization (ESI) source operated in the negative ion mode, which provides efficient ionization of phenolic compounds and other oxygenated secondary metabolites. Nitrogen served as both the desolvation gas and collision gas. The optimized ion source parameters were: capillary temperature, 350 °C; desolvation gas flow, 250 L/h; cone voltage, 25 V; capillary voltage, 4 kV; and collision energy, 30 eV. The triple-quadrupole analyzer was employed in MS and MS/MS acquisition modes to support the tentative characterization of phytochemical constituents based on their molecular ions and fragmentation patterns.

2.5. Cytotoxicity assay on MCF-7 cells

The cytotoxic potential of the methanolic extract of *E. camaldulensis* was assessed on the human breast adenocarcinoma cell line (MCF-7) using the MTT colorimetric assay. The MCF-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. For the experiment, the cells were plated in 96-well culture plates at a density of about 1×10^4 cells per well and incubated overnight to allow proper cell attachment. Following the incubation period, the cells were exposed to various concentrations of *E. camaldulensis* extract prepared in culture medium and incubated for 24 h. The control group was treated with the same volume of the solvent without the extract. After the treatment, MTT reagent was added to each well and the plates were further incubated at 37 °C for 3 h. Subsequently, the formed formazan crystals were dissolved using DMSO, and the absorbance was recorded at 570 nm with a microplate reader. The percentage of cell viability was calculated by comparing the absorbance values of the treated cells with those of the untreated control group.

To assess potential interference of the plant extract with the colorimetric MTT assay, cell-free wells containing the extract and MTT reagent were included. The absorbance of these wells was not appreciably different from the corresponding blank measurements, indicating no detectable substantial background interference under the tested conditions. Therefore, no additional absorbance correction for extract-related interference was applied.

2.6. Statistical analysis

All measurements were performed in three replicates, and the results are presented as mean ± standard deviation (SD). The IC_{50} values were estimated from the concentration–response curves.

3. Results and discussion

3.1. LC–ESI–MS/MS phytochemical profiling of *E. camaldulensis* extract

LC–ESI–MS/MS investigation of the methanolic leaf extract of *E. camaldulensis* led to the tentative characterization of twenty-six metabolites belonging to different categories of phenolic compounds (**Figure 1**). The tentatively characterized constituents, summarized in **Table 1**, included phenolic acids, flavonoids, flavonoid conjugates, and hydrolysable tannins, and were assigned based on their molecular ions, fragmentation behavior, and proposed molecular formulas. The simultaneous occurrence of low-molecular-weight phenolics together with more complex esterified and glycosylated structures reflects the metabolic diversity of the phenylpropanoid pathway in this species. Various flavonoid subclasses, including flavones, flavonols, and flavanones, were also observed in both free and conjugated forms, indicating that glycosylation and related modifications play an important role in metabolite stabilization and accumulation in *E. camaldulensis*. In addition, several tannin-related constituents such as

HHDP-glucose, globulusin A, cypellocarpin B, and other galloyl- or HHDP-substituted glucose derivatives were detected, further demonstrating the strong biosynthetic tendency of this species toward hydrolysable tannin production, a feature commonly associated with members of the Myrtaceae family.

From a chemotaxonomic perspective, the simultaneous abundance of ellagitannins and structurally diverse flavonoids agrees with previously reported phytochemical patterns within the genus Eucalyptus, where these metabolite groups are often considered characteristic markers [15,16]. Nevertheless, comparison with earlier studies indicates that some compounds observed in the present analysis have rarely been reported in *E. camaldulensis*. Among them, irilone, apiforol, and 3-hydroxyphloretin 2′-O-glucoside appear to be uncommon or rarely reported for this species. Their occurrence in the present extract should be considered tentative because authentic standards and NMR data were not available. Further isolation and spectroscopic characterization are therefore required to confirm these assignments.

The results obtained in this study support the potential of *E. camaldulensis* as a source of polyphenolic metabolites, particularly flavonoid derivatives and hydrolyzable tannins, which have also been reported in other Eucalyptus species. Furthermore, the tentative annotation of compounds such as irilone, apiforol, and 3-hydroxyphloretin 2′-O-glucoside provides additional information on the possible phytochemical diversity of the studied extract. However, these assignments require further confirmation using high-resolution mass spectrometry, complementary MS/MS fragmentation analyses, and authentic reference standards. Such investigations may contribute to a more comprehensive understanding of the phytochemical composition and chemotaxonomic characteristics of *E. camaldulensis*.

The metabolite assignments presented in Table 1 should be interpreted as tentative rather than definitive structural identifications. The analysis was performed using a triple-quadrupole mass spectrometer with a single collision energy (30 eV), which limits the extent of structural information available for distinguishing compounds with similar fragmentation patterns, particularly flavonoids and hydrolyzable tannins. In addition, the available data do not provide the high-resolution accurate-mass measurements that can improve confidence in molecular formula assignment. Consequently, the proposed identities are based on the available mass-spectrometric evidence and should be considered putative annotations. Confirmation using high-resolution mass spectrometry, complementary MS/MS fragmentation data acquired at multiple collision energies, and/or authentic reference standards would be required for more confident structural identification.

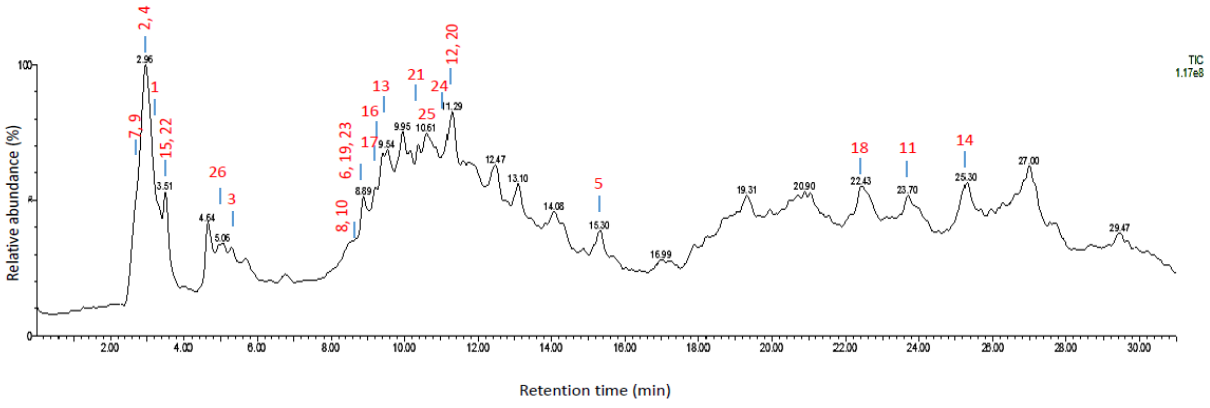


Figure 1. Total ion chromatogram (TIC) of the methanolic leaf extract of *Eucalyptus camaldulensis* obtained by LC–ESI–MS/MS. The red numbers indicate the tentative compound annotations listed in Table 1, according to their retention times.

Table 1. LC–ESI–MS/MS–based tentative characterization of phenolic compounds detected in the methanolic leaf extract of *Eucalyptus camaldulensis*.

No.	Tentative Compound Annotation	Molecular Formula	Retention time (min)	Reported observed m/z $[M-H]^-$	Calculated neutral monoisotopic mass (Da)	Calculated $[M-H]^-$ (m/z)	Δ m/z (reported − calculated)
1	p-Coumaric acid	$C_9H_8O_3$	3.12	163.04	164.047	163.04	0
2	Paeonol	$C_9H_{10}O_3$	2.96	165.08	166.063	165.056	0.024
3	Gallic acid	$C_7H_6O_5$	5.30	168.98	170.022	169.014	-0.034
4	Caffeic acid	$C_9H_8O_4$	2.96	179.03	180.042	179.035	-0.005
5	3,4-Dihydroxyhydrocinnamic acid	$C_9H_{10}O_4$	15.33	180.99	182.058	181.051	-0.061
6	Syringic acid	$C_9H_{10}O_5$	8.88	197.05	198.053	197.046	0.004
7	Pinocembrin	$C_{15}H_{12}O_4$	2.72	255.05	256.074	255.066	-0.016
8	Naringenin	$C_{15}H_{12}O_5$	8.70	271.08	272.068	271.061	0.019
9	Apiforol	$C_{15}H_{14}O_5$	2.70	272.95	274.084	273.077	-0.127
10	Kaempferol	$C_{15}H_{10}O_6$	8.70	285.04	286.048	285.04	0
11	Irilone	$C_{16}H_{10}O_6$	23.67	297.1	298.048	297.04	0.06
12	Ellagic acid	$C_{14}H_6O_8$	11.28	300.97	302.006	300.999	-0.029
13	Gallocatechin	$C_{15}H_{14}O_7$	9.53	305.04	306.074	305.067	-0.027
14	Caffeoyl tartaric acid	$C_{13}H_{12}O_9$	25.29	311.09	312.048	311.041	0.049
15	Glucogallic acid	$C_{13}H_{16}O_{10}$	3.50	331.05	332.074	331.067	-0.017
16	2-O-Coumaroylquinic Acid	$C_{16}H_{18}O_8$	9.39	337.07	338.1	337.093	-0.023
17	Chlorogenic acid	$C_{16}H_{18}O_9$	9.36	353.04	354.095	353.088	-0.048
18	Vitexin	$C_{21}H_{20}O_{10}$	22.45	431.12	432.106	431.098	0.022
19	3-Hydroxyphloretin-2'-O-glucoside	$C_{21}H_{24}O_{11}$	8.87	451.21	452.132	451.125	0.085
20	Kaempferol glucuronide	$C_{21}H_{18}O_{12}$	11.28	461.13	462.08	461.073	0.057
21	Valoneic acid dilactone	$C_{21}H_{10}O_{13}$	10.37	469.02	470.012	469.005	0.015
22	HHDP-glucose	$C_{20}H_{18}O_{14}$	3.50	481.06	482.07	481.062	-0.002
23	Digalloylglucopyranose	$C_{20}H_{20}O_{14}$	8.88	483.07	484.085	483.078	-0.008
24	Globulusin A	$C_{23}H_{32}O_{11}$	11.15	483.19	484.194	483.187	0.003
25	cypellocarpin B	$C_{26}H_{34}O_{12}$	10.59	537.29	538.205	537.198	0.092
26	Galloyl-HHDP-glucopyranose	$C_{27}H_{22}O_{18}$	4.94	633.06	634.081	633.073	-0.013

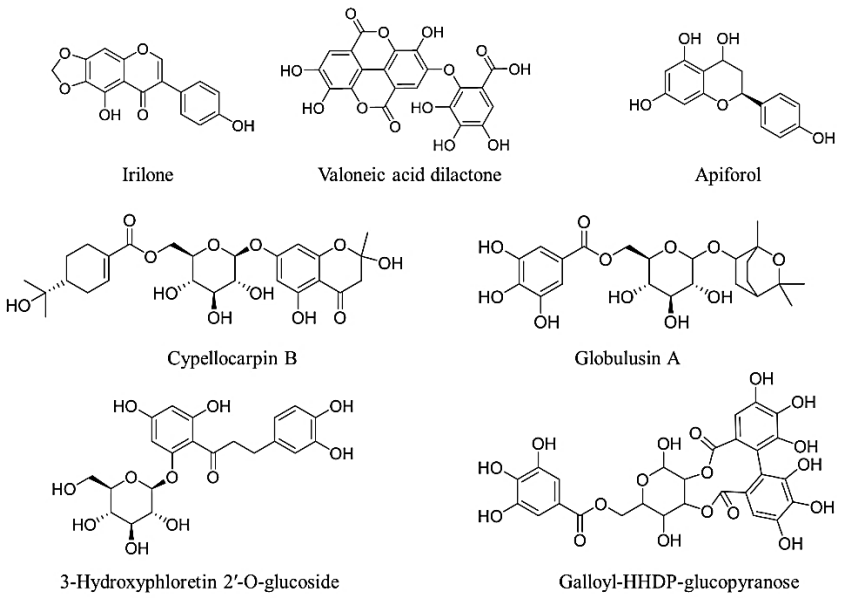


Figure 2. Chemical structures of selected representative phenolic constituents tentatively identified in the methanolic leaf extract of *Eucalyptus camaldulensis* by LC–ESI–MS/MS.

3.2. Antioxidant activity of *E. camaldulensis* extract

The methanolic leaf extract of *E. camaldulensis* demonstrated a gradual enhancement in free-radical scavenging activity as the concentration increased (**Figure 3**). The DPPH radical-scavenging activity increased with increasing extract concentration, ranging from 8.8 ± 0.4% at 20 µg/mL to 48.1 ± 3.5% at 130 µg/mL. Linear regression analysis demonstrated a significant positive concentration-dependent relationship ($R^2 = 0.9780$, $p < 0.001$). One-way ANOVA also revealed a significant difference in radical-scavenging activity among the tested concentrations ($p < 0.001$). The IC_{50} was estimated to be approximately 135 µg/mL by extrapolation, as 50% inhibition was not reached within the tested concentration range.

The antioxidant behavior observed in the assay is likely associated with the abundance of polyphenolic constituents identified in the extract, especially flavonoids, phenolic acids, and hydrolysable tannins, which are recognized for their electron-donating and radical-stabilizing capacities. The continuous increase in scavenging activity with rising concentration indicates that these metabolites collectively contribute to the extract's redox activity. Similar concentration-dependent antioxidant patterns have previously been documented for different Eucalyptus species, including *E. globulus* and *E. camaldulensis*, where DPPH inhibition values commonly increase within the range of 50–200 µg/mL depending on solvent polarity, extraction procedure, and total phenolic content [12,17,18]. In comparison with those reports, the antioxidant performance observed in the present study appears consistent with that generally reported for crude methanolic extracts. The slightly lower maximum inhibition value relative to certain polyphenol-enriched or fractionated samples may indicate that the current extract contains a broader mixture of constituents rather than highly concentrated antioxidant fractions.

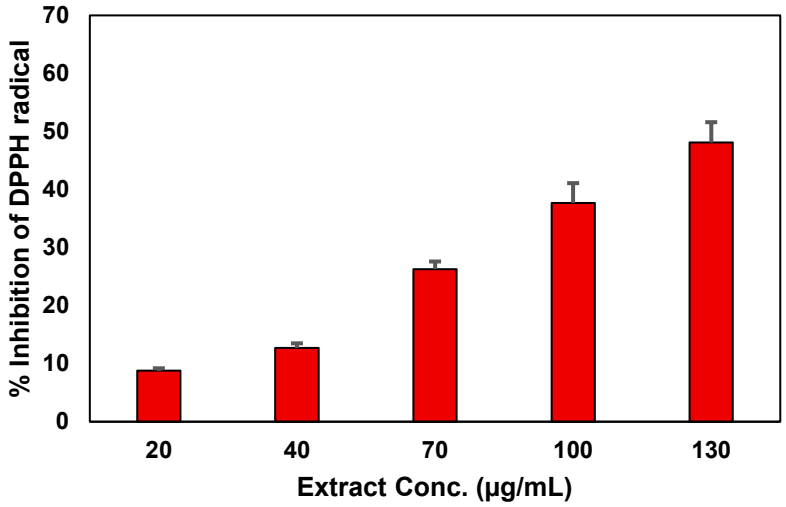


Figure 3. DPPH radical scavenging activity of the methanolic leaf extract of *Eucalyptus camaldulensis* at different concentrations (20–130 µg/mL). The results are expressed as mean ± SD (n = 3).

3.3. Cytotoxicity of *E. camaldulensis* extract

The effects of the methanolic extract obtained from *E. camaldulensis* leaves on MCF-7 human breast cancer cells were evaluated using the MTT assay following 24 h of exposure. As presented in **Figure 4**, treatment with increasing extract concentrations (25–250 µg/mL) resulted in a gradual decline in cell viability. The extract exhibited a concentration-dependent reduction in MCF-7 cell viability, with an estimated 24-h IC_{50} of approximately 183 µg/mL. These findings demonstrate that the crude methanolic extract can reduce MCF-7 cell viability/metabolic activity under the tested experimental conditions, although the observed effect was moderate rather than highly potent.

The reduction in MCF-7 cell viability observed in this study is consistent with previous reports describing moderate biological activity of crude extracts derived from Eucalyptus species. Earlier investigations have commonly demonstrated that non-fractionated Eucalyptus extracts exhibit measurable effects on cancer cell viability, often at relatively high concentrations and with moderate to high IC_{50} values [19–22]. The estimated 24-h IC_{50} of the present extract is therefore within the range generally reported for crude methanolic preparations and may reflect the combined contribution of multiple phenolic metabolites present in the extract.

Considering the antioxidant activity detected in the DPPH assay, the biological effects observed in MCF-7 cells may potentially be related to the complex redox properties of polyphenolic compounds. Although these metabolites can act as free-radical scavengers in chemical systems, some polyphenols may also exhibit pro-oxidant behavior under certain intracellular conditions, potentially influencing cellular redox balance, mitochondrial function, and signaling pathways associated with cell survival and apoptosis [23–25]. However, these mechanisms were not directly investigated in the present study and should therefore be considered possible explanations rather than established mechanisms of action. Importantly, the MTT assay was performed at a single exposure time (24 h), which does not allow the time-dependent effects of the extract to be determined or cytostatic effects to be distinguished from cytotoxic effects. Further investigations using multiple exposure periods (e.g., 24, 48, and 72 h) and complementary assays are needed to clarify the effects of the extract on cell proliferation and survival. Overall, the present findings indicate that the methanolic extract of *E. camaldulensis* produces a measurable, concentration-dependent reduction in MCF-7 cell viability after 24 h of exposure, warranting further investigation.

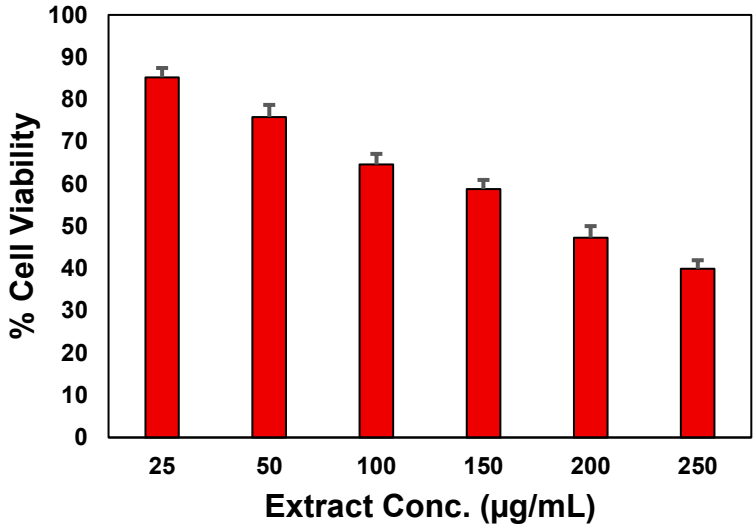


Figure 4. Effect of the methanolic leaf extract of *Eucalyptus camaldulensis* on the viability of MCF-7 cells as determined by the MTT assay. Cells were treated with different extract concentrations (25–250 µg/mL) for 24 h. Data are expressed as mean ± SD (n = 3).

4. Conclusion

The present study provides a combined phytochemical and bioactivity assessment of the methanolic leaf extract of *E. camaldulensis*. LC–ESI–MS/MS analysis enabled the tentative characterization of a broad range of secondary metabolites, including flavonoids, phenolic acids, glycosylated phenolic compounds, and hydrolysable tannin-related constituents, highlighting the chemical diversity of the extract. The extract also exhibited moderate free-radical scavenging activity in the DPPH assay, which may be associated with the presence of polyphenolic constituents.

The extract produced a concentration-dependent reduction in MCF-7 cell viability, indicating moderate cytotoxicity, particularly at higher concentrations. The observed biological effects may reflect the combined contribution of multiple constituents present in the crude extract; however, the specific compounds and cellular mechanisms responsible for these activities were not determined in the present study.

Overall, the findings provide preliminary evidence of the phytochemical diversity and biological activity of the methanolic leaf extract of *E. camaldulensis*. Further studies involving fractionation, purification of individual constituents, comparison with authentic standards, spectroscopic characterization, and mechanistic investigations are warranted to confirm the tentatively characterized metabolites and determine their contributions to the observed biological activities.

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Authors' contributions

Experimental Work and Data Collection: Ahamd Kuti; Conceptualization, Project Design, and Writing Original Draft: Zeinab Mazarei.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be provided upon the author's request.

Statement

Use of Artificial Intelligence: ChatGPT was used solely for language editing, sentence formulation, and improving the clarity and readability of the manuscript. AI tools were not used to generate, analyze, or interpret the experimental data.

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