# Ganoderma lucidum Methanolic Extraction as a Potent Phytoconstituent: Characterization, in-vitro Antimicrobial and Cytotoxic Activity

**Seyyed Mojtaba Mousavi**
National Taiwan University of Science and Technology
**Seyyed Alireza Hashemi**
University of British Columbia
**Ahmad Gholami** ( Gholami@sums.ac.ir )
Shiraz University of Medical Sciences
**Navid Omidifar**
Shiraz University of Medical Sciences
**Wei-Hung Chiang**
National Taiwan University of Science and Technology
**Vijayakameswara Rao Neralla**
National Taiwan University of Science and Technology
**khadije Yousefi**
Shiraz University of Medical Sciences
**Mansoureh Shokripour**
Shiraz University of Medical Sciences

**Research Article**

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# Abstract

**Background**: *Ganoderma lucidum* has attracted tremendous attention due to its exceptional antimicrobial and anticancer properties that can be delicately tuned by controlling the initial extraction content and concentration. In the present experiment, we detailed the characterization, antimicrobial, and cytotoxic performance of *Ganoderma lucidum* as a potential multi-functional therapeutic agent.

**Methods**: In this study, we used FTIR, XRD, FESEM, EDX, and HPLC techniques to evaluate the samples, which were then followed by disc diffusion and microdilution broth methods to test its antibacterial effects against four Gram-positive and Gram-negative bacterial strains, *Enterococcus faecalis*, *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa*. MTT assay was applied to determine the cytotoxic activity of this natural product against MCF-7 and K562 cancer cell lines.

**Results**: The results revealed that the inhibitory effects of this product had higher antibacterial activity against *E. coli* and *Pseudomonas aeruginosa*. The IC50 values of 0.5 and 0.75 mg/mL were obtained for MCF-7 and K-562 cancer cell lines, which confirmed the higher anticancer activity of the *GLME* against breast cancer cells compared to blood cancer cells.

**Conclusions**: Hence, these data provide pioneer insights into the therapeutic usage of *Ganoderma lucidum* for treating breast and blood cancers. This work is motivated by research studies looking for pharmacological products to address chronic and acute diseases, where further resources and studies are required to explore such products' adverse effects and toxicity.


# 1. Background

Natural products or herbal medicines maybe promising alternatives or supplement for chemotherapy and antibiotic therapy [1, 2]. Herbal medicines have extraordinary properties like high antibacterial, antioxidant, and anticancer activities [3, 4]. For thousands of years, *Ganoderma lucidum* (GL) has been used as a major source of pharmacologically active constituents in Chinese and traditional Japanese medicine. It has piqued the attention of researchers and scientists with its numerous medicinal and pharmacological uses since it contains significant pharmacologically active compounds [5–7]. Proteins, sugars, flavonoids, vitamins, minerals, triterpenes, and polysaccharides are among the biologically active compounds found in extractions of *GL* [8]. The presence of polysaccharides and triterpenoids in *GL's* structure has numerous pharmacological features. Thence, many experiments have been accomplished to investigate the performance of this natural product against different types of cancer like prostate cancer [9], lung cancer [10], colon cancer [11], and cervical cancer [12]. Also, *GL's* other pharmacological characteristics such as anti-inflammatory [13], hypoglycaemic [14], hypocholesterolemic [15], antioxidant activity [16], cardio-protective, hepato-protective, and anti-allergic activity, have been evaluated by researchers [5].

This research was planned and carried out in two parts to determine the characterization of GL methanolic extract (GLME) and investigate this natural product's antibacterial and cytotoxic activity. In

the first part, to enhance *GLME's* properties, sample extraction was done via the standard method. The second portion of this study's antibacterial studies followed the Clinical and Laboratory Standards Institute (CLSI) recommendations. The disc diffusion assay was used to test GLME's antibacterial tolerance against *Staphylococcus aureus*, *E. coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*. The microdilution broth approach was then used to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of this substance, with both bactericidal and bacteriostatic effects defined. In the final section of this investigation, the cytotoxic activity of *GLME* against human breast cancer cells and human blood cancer cells was assessed through MTT assay.

## 2. Materials And Methods

# 2.1. Sample extraction and preparation

Pure dried *GL* was purchased from a traditional market in Shiraz, Iran. The dried *GL* was grounded to form a fine powder, and then its essence was extracted by pouring it with methanol at room temperature. In this regard, 2.5 g of dried *GL* was first blended with 100 mL of methanol and then shook for 24h at 125 rpm. In the next step, the mixture was filtered using filter paper and placed in an oven at 60°C for 12h. After that, 3 mg of the final extracted essence was dissolved in MeOH as the stock solution for biological tests.

# 2.2. Characterization

Different analyses were carried out to test the properties of *GLME*. FTIR spectroscopy with KBr tablets (Bruker model Tensor II) and X-ray diffraction (XRD) (Panalytical model X'Pert Pro, Almelo, Netherlands) was used to explore its crystallinity. The morphology of *GLME* (Tescan model S Max detector, Brno, Czech Republic) was studied using a field emission-scanning electron microscope (FE-SEM, Tescan model Mira III, Brno, Czech Republic) and energy dispersive spectroscopy (EDAX). An Azura HPLC device (Knauer, Berlin, Germany) fitted with a quaternary gradient pump unit and a UV–vis detector (190–700 nm) were used to study the contents of monosaccharides and disaccharides from *GLME*. The detector's wavelength was set to 250 nanometers. The components were separated by a Knauer C18 column (4.6mm 250mm i.d., 5m). Solvents A (acetonitrile) and B (benzene) made up the mobile phase (0.045 percent KH2PO4), where 0.8 mLmin-1 and 20 mL were the injection volume and flow rate, respectively. The mobile process was screened with a 0.45 mm filter and degassed under vacuum until application. The system was run at ambient temperature.

# 2.4. Disc diffusion assay

Briefly, a 0.5 McFarland scale (1.5 $10^8$ colony forming units (CFU)/ml)) bacteria culture was used in this experiment, with an optical density of 590 nm. We applied this bacterial culture to nutrient agar with a sterile swab at this stage. Afterward, the blank discs were placed on the solid surface of nutrient agar while all of the discs were soaked in the *GLME* until saturated. Plates were placed within the incubator at 37ºC for 18 h, and finally, the diameter of inhibition zones was measured [17–19]. A solution containing

20% ethanol in water was used as control group because of probable presence of ethanol in final herbal extract.

## 2.5. Minimum inhibitory concentrations (MICs) assay

Both procedures in this assay were performed according to the Clinical and Laboratory Standards Institute's (CLSI) guidelines for determining *GLME*'s antibacterial susceptibility [20–22]. The 96 well-plate was filled with BHI or liquid medium at a concentration of 90 liters for this experiment. The *GLME* methanolic extraction was then pumped into the culture medium at a concentration of 90 L (with a descending concentration from 1000 g/mL to 7.8 g/mL). In the next step, 10μL of 600 nm OD (0.5 McFarland) microorganisms were transferred into the mentioned wells, which contained the developed samples. The plates were then incubated for 24 hours at 37°C. We used Ampicillin as a standard drug to determine Ganoderma lucidum's antibacterial susceptibility in this method. A solution containing 20% ethanol in water was used as control group because of probable presence of ethanol in final herbal extract. The optical density was estimated at 600 nm in the final step (BioTek, Power WaveXS2). This process was carried out three times.

## 2.6. Minimum bactericidal concentrations (MBCs) assay

The value of MBCs was calculated by culturing the media from nutrient agar wells with no bacterial growth. A solution containing 20% ethanol in water was used as control group because of probable presence of ethanol in final herbal extract. The MBC value is the minor concentration capable of killing 98 percent of microorganisms in culture [23]. This procedure was also done three times.

## 2.7. MTT assay

MCF-7 and K562 cell lines were used to test the cytotoxicity of *GLME*. The positive control was hydrogen peroxide, and the negative control was the culture medium containing 20% ethanol was used as control group because of probable presence of ethanol in final herbal extract. A certain amount of each cell line (10000) was placed in each well containing DMEM culture media and incubated to achieve 85 to 90% confluence. The previous media was then used to substitute 100 μL of *GLME* in a wide variety of concentrations. Then, in each well, 30 mL of MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) stock solution (concentration 4 mg/mL) was transferred and incubated under normal conditions. Purple formazan crystals formed due to the viable cells' mitochondrial function, and we used 100 liters of dimethyl sulfoxide (DMSO) to dissolve these crystals. The plate was shaken in a double orbital manner (for 5 minutes) to dissolve formazan crystals completely. Finally, the optical absorption of the mentioned solution was recorded at 540 nm using an Elisa plate reader (Model 50, Bio-Rad Corp, Hercules, California, USA) [24, 25]. All tests were accomplished in triplicate [26]. The following equation describes the calculations of the cell viability:

$$\%Cellviability = \frac{[OD(cell + compound) - OD(compound)]}{[OD(cell) - OD(Culturemedia)]} * 100$$

## 2.8. Statistical analysis

The statistical interpretations in this analysis were made using the Statistical Package of the Social Sciences (SPSS) 22 software (SPSS Inc., Chicago, IL, USA). The antibacterial and cytotoxic activities of GLME were compared using one-way ANOVA/Tukey experiments. After three trial replications, the significance amount was set at 0.05.

# 3. Results And Discussion

# 3.1. Characterization

With a KBr tablet, FTIR analysis of *GLME* was performed in the 400–4000 $cm^{-1}$ range (Bruker model Tensor II). A narrow band at 2919 $cm^{-1}$, which was allocated to the C-H stretching binding in aliphatic compounds, and 1981 $cm^{-1}$ can confirm the presence of aromatic combination bands, while a peak at 3292 $cm^{-1}$ can be attributed to the stretching bond of the hydroxyl functional groups (-OH), as seen in Fig. 1a. For *GLME*, several transmittance bands in the regions of distinct amide bands suggest the presence of proteins [27]. The stretching frequency of C = O groups is associated with the amide-I band (1634$cm^{-1}$), while the bending vibration of N = H groups is associated with the amide-II band (1538$cm^{-1}$). *GLME* had a critical characteristic band of 1035 $cm^{-1}$, which was close to the stretching of the C-O bond. This banding pattern can also be seen in other Chinese medicines, including *Radix achyranthes, Cordyceps bidentatae*, and *Radix cyathulae* [28]. According to Barker et al. [20], the 893 $cm^{-1}$ band in the fingerprint area of d-glucopyranose is one of the most significant recorded bands. In sugar, this band is a C-H bending vibration. The bending vibration of a saccharide group is represented by the bands at 554$cm^{-1}$ and 529 $cm^{-1}$ [29].

The crystallinity of *GLME* was investigated using XRD, and the resulting pattern is shown in Fig. 1.b. The crystalline plane 002 was assigned to the XRD pattern, which showed an apparent plateau at around 2 = 20°. No peaks existed at higher scattering angles, indicating that the compound was amorphous [30].

FE-SEM images at various scales (i.e., 1 m, 500 nm, and 200 nm) and EDAX analysis of *GLME* are shown in Fig. 2. *GLME* had a rod-shaped structure with particle sizes smaller than 60 nm, as seen in Fig. 2. This natural commodity also contained a lot of carbon, nitrogen, oxygen, magnesium, sulfur, potassium, and calcium, according to EDAX research.

The contents of monosaccharides and disaccharides from *GLME* were calculated using an HPLC method in optimum separation conditions, with acetonitrile-0.045 percent KH2PO4 as the mobile step and a flow rate of 0.8 mLmin-1 at 250 nm as the detector. Polysaccharides from *GLME* were detected by comparing the retention time of each part with standard curves. Monosaccharides and disaccharides, such as lactose, glucose, sucrose, and maltose, were defined in Fig. 3C.

The inhibitory action of *GLME* against selected bacterial strains (i.e., *Staphylococcus aureus, Enterococcus faecalis, E. coli, and Pseudomonas aeruginosa*) was investigated using the microdilution method (Fig. 3a). *GLME* was reported to have antibacterial effects against microorganisms at high

concentrations (1000, 500 g/ml). It can be noted that the antibacterial effects increased in a concentration-dependent manner as the concentration value increased. The obtained results revealed that the inhibitory effects of this product against gram-negative and gram-positive bacterial strains were not similar. According to the presented data in Table 1, it can be understood that this valuable product had higher antibacterial activity against *E. coli* and *Pseudomonas aeruginosa*. The MIC values of the *GLME* were 125 µg/ml and 250 µg/ml for Gram-negative and Gram-positive microorganisms, respectively. The viability of *Staphylococcus aureus* and *Enterococcus faecalis* subjected to *GLME* was 146 percent and 117 percent, respectively, at the most diluted concentration of the experiment (7.8 g/mL), indicating that the extract had some beneficial effects on bacterial growth. *GLME* possesses bactericidal and bacteriostatic effects against Gram-negative and Gram-positive strains, mainly due to polysaccharide components in its structure. Figure 3 (d) shows a view of the disc diffusion method after exposure to four different microorganisms.

A common and acceptable approach for assessing cell viability is the MTT assay. This method can also detect and determine biomaterial toxicity [31–33]. MTT assay can depict the metabolism and mitochondrial activity of cells. This experiment evaluated the viability or proliferation of human breast and blood cancer cells after 24 h of treatment with methanolic extraction of *GLME* (see Fig. 3b). The metabolic performance of cells was changed in a dose-dependent manner by the *GLME*, where the dosage of the sample was varied from 1 to 3000 µg/mL. By increasing the concentration from 1 to 3000 µg/ml, the cell viability percentage was diminished from 108–2.5% for the K-562 blood cancer cell line and 91–6% for the MCF-7 cancerous breast cell line. The IC50 values of 0.5 and 0.75 mg/mL were obtained for MCF-7 and K-562 cancer cell lines, which confirmed the higher anticancer activity of the *GLME* against breast cancer cells compared to blood cancer cells.

Table 1
Performance of the *GLME* against selected microorganisms.

| **Microorganisms** | **Antibacterial tests** | | |
|---|---|---|---|
| | **Zone of inhibition (mm, (Mean ± SD)** | **MIC (µg/ml)** | **MBC (µg/ml)** |
| E.coli | 44 ± 0.09 | 125 | 125 |
| Pseudomonas aeruginosa | 36 ± 0.1 | 125 | > 125 |
| Staphyloccus aureus | 28 ± 0.07 | 250 | 250 |
| Enterococcus faecalis | 34 ± 0.2 | 250 | > 250 |

The mean zones of inhibition in the disc diffusion (DD) method, with a disc diameter of 6 mm, were measured in millimeters. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured mg/mL. The initial doses of both medications were identical (1 mg/mL in 1:1 dilution).

# 4. Conclusions

*GLME* is known to be quite a competent medicinal and nutraceutical agent, owing to its pharmacological, chemical components derived from fruit bodies, mycelium, and spores, which can be health-promoting agents. This work is motivated by research studies looking for pharmacological products to address chronic and acute diseases, where further resources and studies are required to explore such products' adverse effects and toxicity. More clinical studies are needed to validate the efficacy and protection provided by such products. More experiments and clinical research in the future are likely to take place on a wide scale. This research aims to examine the antimicrobial, cytotoxic, and genotoxic activity of the *GLME*. The extract was evaluated for its antibacterial and antifungal efficacy toward different microorganisms by utilizing a microdilution broth system. *GLME* was examined using an approach to assess its cytotoxic activity against breast cancer and blood cancer cells.

# Abbreviations

XRD: X-ray Diffraction

FE-SEM: Field Emission Scanning Electron Microscopy

TEM: Transmission Electron Microscopy

AFM: Atomic Force Microscopy

FTIR: Fourier-Transform Infrared Spectroscopy

UV: Ultraviolet light

HPLC: High-Performance Liquid Chromatography

GL: *Ganoderma lucidum*

GLME: *Ganoderma lucidum* methanolic extract

MIC: Minimum Inhibitory Concentrations

MBC: Minimum Bactericidal Concentrations

EDAX: Energy Dispersive Spectroscopy

CFU: colony forming units

CLSI: Clinical and Laboratory Standards Institute

DMEM: Dulbecco's Modified Eagle Medium

OD: Optical Density

MTT: (3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium)

DMSO: Dimethyl Sulfoxide

# Declarations

### *Ethics approval and consent to participate*

Not applicable

### *Consent for publication*

Not applicable

### *Availability of data and materials*

All data generated or analyzed during this study are included in this published article

### *Competing interests*

The authors declare that they have no competing interests


### *Funding*

There is no funding for this study.


### *Authors' contributions*

SMM and AG developed the idea and structure of the review article. SMM, VRN, SAH and KY wrote the manuscript collecting the materials from databases. MS, AG, W-HC and NO revised and improved the manuscript. AG and NO supervised the manuscript. All the authors have given approval to the final version of the manuscript.


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Not applicable


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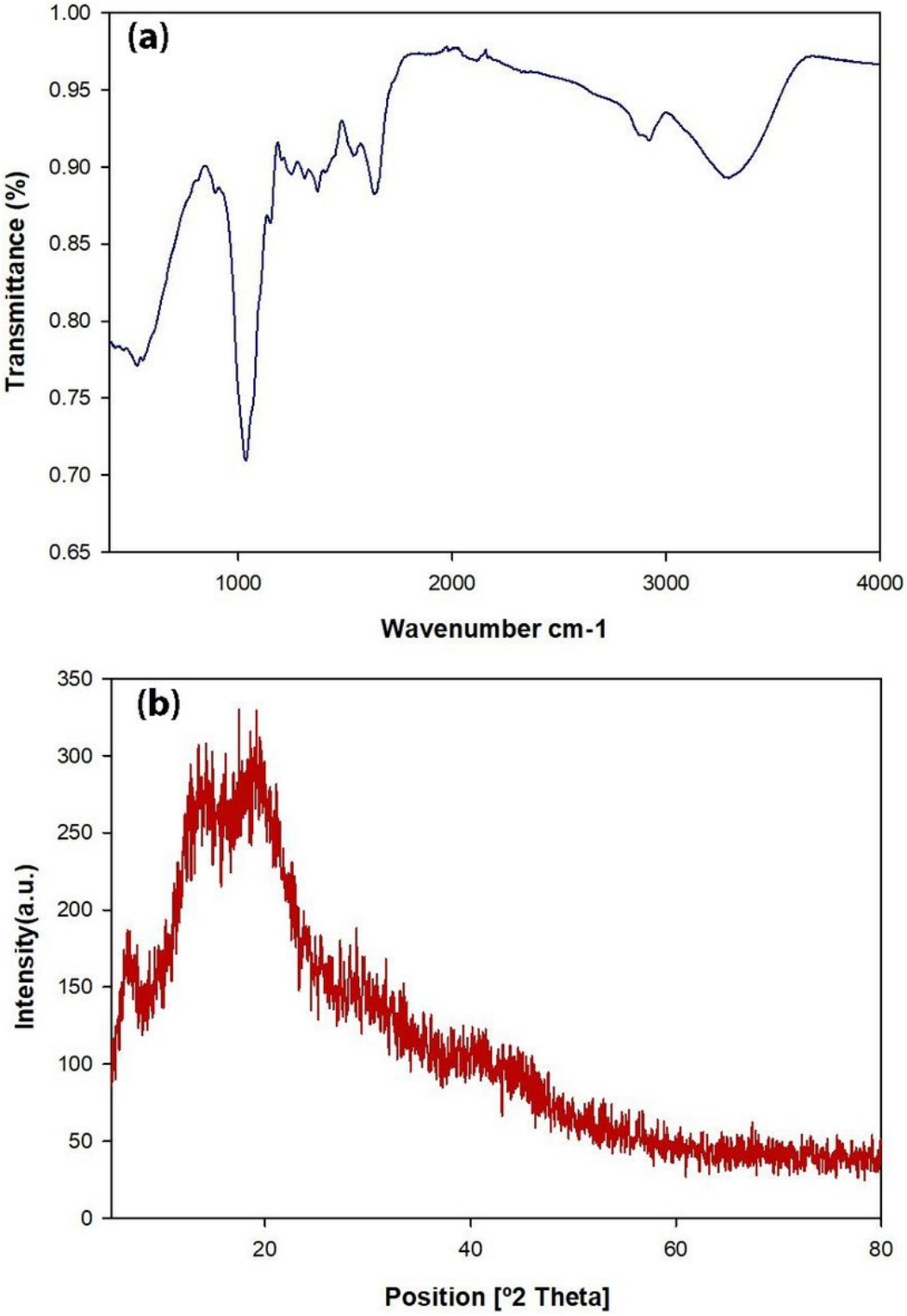
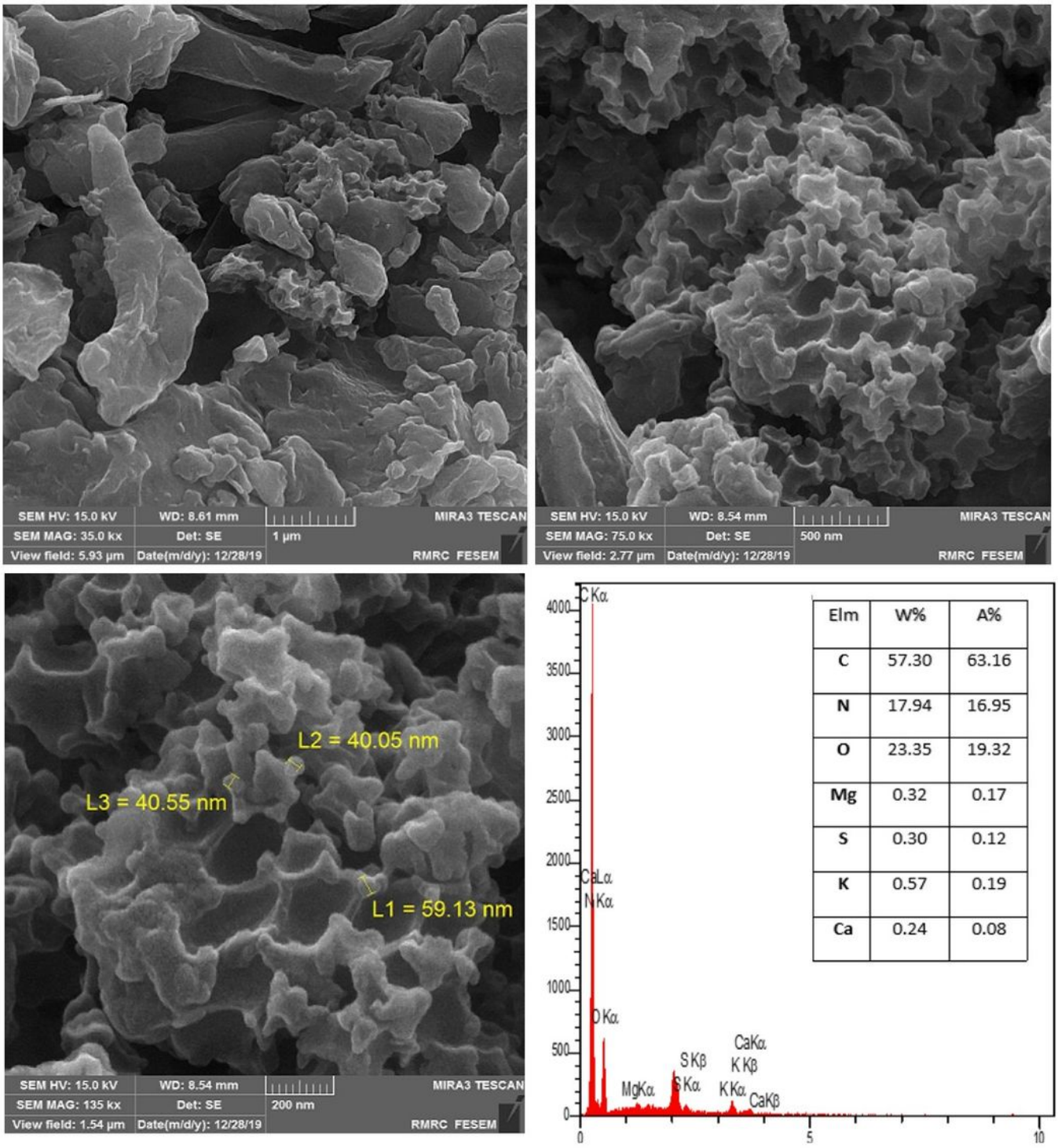
# Figures



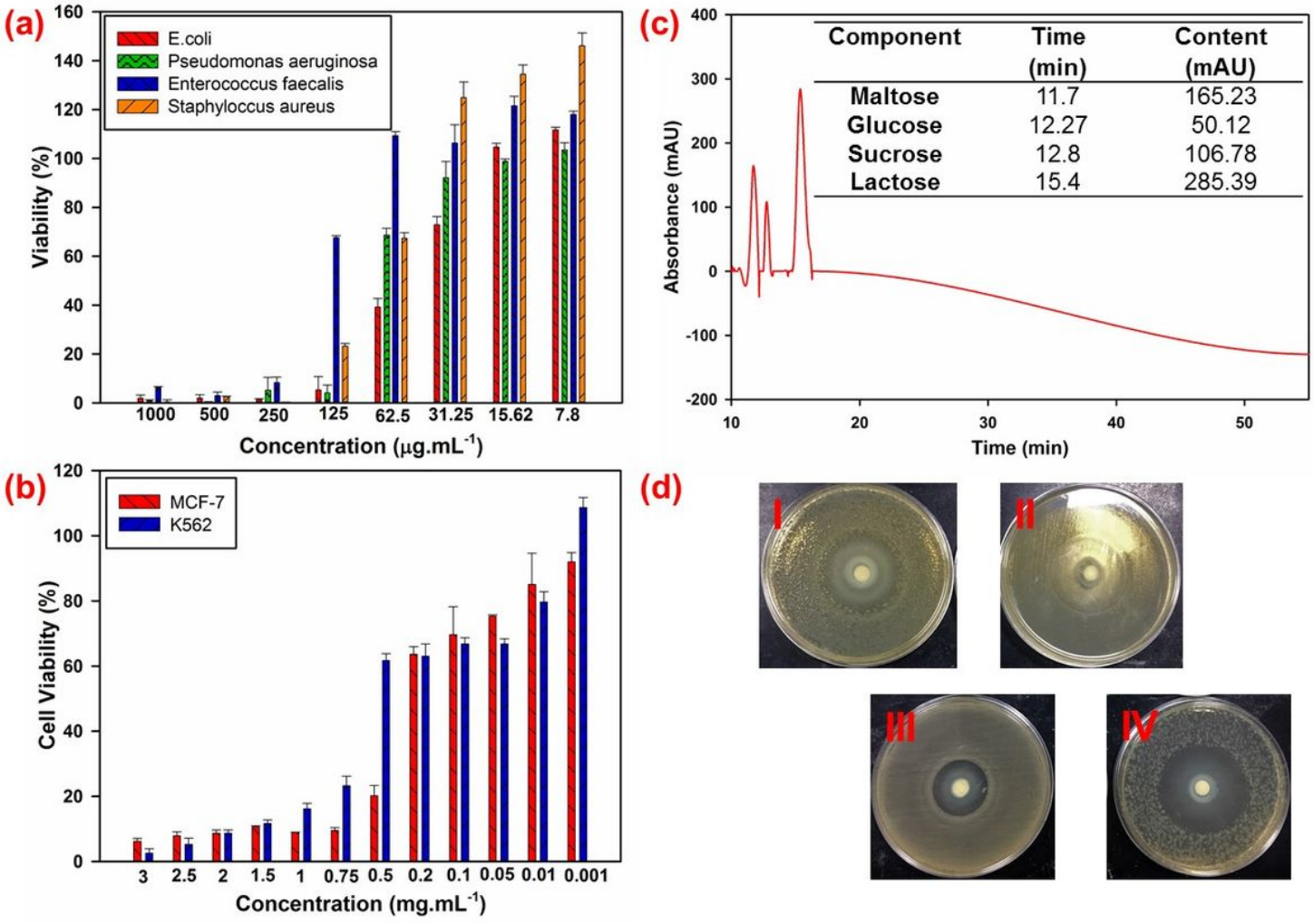

## Figure 1

**a)** FTIR spectrum and b) XRD pattern of *GLME*




## Figure 2

The natural product's FESEM images and EDAX analysis




## Figure 3

(a) Effects of methanolic *GLME* extraction on various microorganism's viability percentages in different concentrations (each bar reflects the mean SD (standard deviation) of three independent tests). b) effects of methanolic *GLME* extraction on human breast and blood cancer cells. *GLME* blocked cell growth in MCF-7 and K-562 cell lines, as shown by the MTT assay, in which cells were treated with various concentrations of *GLME* for 24 hours. The results are interpreted by finding the mean and standard deviation of the three separate experiments. (c) HPLC analysis of the *GLME*. (d) Inhibition zone of *GLME* against (I) *Pseudomonas aeruginosa*, (II) *Enterococcus faecalis*, (III) *Staphylococcus aureus*, and (IV) *Escherichia coli*.