Characterization and Efficiency of *Ganoderma lucidum* Biomass as an Antimicrobial and Anticancer Agent

Mohammed Ibrahim Alghonaim,[a] Sulaiman A. Alsalamah,[a] Ahmed Alsolami,[b,*] and Tarek M. Abdel Ghany [c,*]

The microconstituents of *Ganoderma lucidum* biomass (GLB) were evaluated, along with its antimicrobial and anticancer activities. Using gas chromatography-mass spectrometry analysis, 12-octadecadienoic acid (Z,Z)- and *n*-hexadecanoic acid with area% values of 21.0% and 11.0% were recognized in GLB. Uncooked biomass (UCB) and microwave-cooked (CE) biomass of *G. lucidum* caused a significant inhibition of human breast cancer (MCF-7) cell line proliferation in a dose-dependent manner. The inhibition of MCF-7 cell proliferation was 27.22 ± 1.64% using 16 µg/mL of CB while it was 52.29 ± 1.09% using 16 µg/mL of UCB. The cytotoxicity test recorded low IC_{50} (25.63 ± 0.52 µg/mL) of UCE compared to the IC_{50} value (49.99 ± 0.94 µg/mL) of CB. Highest antimicrobial activities were recorded *via* using UCE, compared to CE against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Candida albicans*, and *Aspergillus niger*. The 9,12-octadecadienoic acid (Z,Z)- and *n*-hexadecanoic acid were able to induce both anticancer and antibiotic-resistant properties. A key therapeutic target enzyme for evolving this resistant pathogenic activity on MCF-7 and *K. pneumonia*, was accessed thoroughly *in silico* study. The compounds described, therefore, might provide a great potential for the development of new therapeutics such as anticancer and antimicrobial agents.

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Contact information: a: Department of Biology, College of Science, Imam Mohammad Ibn Saud Islamic University, Riyadh 11623, Saudi Arabia; b: Department of Internal Medicine, College of Medicine, University of Ha'il, Hail 55476, Saudi Arabia; c: Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo 11725, Egypt;
**Corresponding author: tabdelghany.201@azhar.edu.eg; a.alsolami@uoh.edu.sa*

INTRODUCTION

One of the primary causes of death worldwide, and a serious health issue, is cancer. Despite a noticeable rise in early diagnosis methods and advancements in treatment methods, cancer remains one of the most lethal illnesses in the world and presents a significant therapeutic challenge (Qanash *et al*. 2023a). Most governments are dedicated to reducing the harm that cancer poses to human health. The common cause of cancer-related fatalities among women globally is breast cancer (Su *et al*. 2018; Ghasaq *et al*. 2023). Today, the most frequent malignancy in women is breast cancer. Among advanced clinical studies, the major treatments for breast cancer are surgical removal, adjuvant chemotherapy, radiation, and hormone therapy. However, despite these restrictions, certain treatments' efficacies remain unsatisfactory, particularly against triple-negative breast cancer, which does not respond to hormonal or trastuzumab-based therapy, as well as the

rise in drug resistance and undesirable effects of synthetic drugs (Reddy 2011). Prevention of cancer proliferation *via* drug treatment, particularly natural compounds, has become an important health objective. It has become important to find anticancer medications with great effectiveness and minimal side effects. Thus, scientists have been working to identify bioactive components in natural resources. Numerous bioactive compounds, comprising anti-tumor drugs, were recently discovered in a variety of mushrooms (Zhong *et al*. 2019). Polysaccharides, lipids, proteins, ash, alkaloids, glycosides, volatile oils, phenolics, tocopherols, flavonoids, carotenoids, as well as organic acids are among the constituents of mushrooms that play a vital role in biological activities (Aparna *et al*. 2012; Sun *et al*. 2015; Joseph *et al*. 2018; Soliman *et al*. 2022). Asian epidemiological investigators suggested that mushrooms could indeed prevent the breast cancer (Shin *et al*. 2010).

Among mushrooms, *G. lucidum* is one of the most thoroughly studied mushrooms, especially in traditional Chinese medicine and other Asian folk medicine, as a functional food and chemo preventive agent (Oliveira *et al*. 2014). Several studies reported that *G. lucidum* could provide anticancer effects in addition to cancer cell-targeting techniques, such as cell cycle arrest and apoptosis induction (Dai *et al*. 2017; Park 2022), and migration inhibition with more immune improvement (Sun *et al*. 2015). In addition, a recent clinical study demonstrated the effectiveness of *G. lucidum* extracts. The cited study showed that the patient's immune system was improved, and the side effects of chemotherapy and radiotherapy were lessened when using *G. lucidum* extracts along with standard therapies, such as chemotherapy, radiotherapy, and surgery (Zhong *et al*. 2019). The extract from *G. lucidum* has been shown to be cytotoxic to hepatoma, cervical cancer, and lung carcinoma (Ruan *et al*. 2014). Proliferation inhibition of MCF-7 was reported using *G. lucidum* extract (Vidhya and Devara 2011). Another study demonstrated that liver tumor and MCF-7 were inhibited by *G. lucidum* extract (Attoub *et al*. 2013). Lactate dehydrogenase (LDH) is a fascinating potential pharmacological target for the treatment of cancer among glycolysis enzymes. Because LDH is primarily found in cytosol, it can be released into the supernatant during cell damage or lysis. As a result, LDH is frequently used *in vitro* cell culture systems to assess cytotoxicity and cell number. The metabolic changes linked to breast cancer can be investigated using LDH, a useful diagnostic marker for the metabolic syndrome. In the pyruvate-reducing direction, breast cancer cells produce more LDH and express more genes than nearby normal cells (Ghasaq *et al*. 2023). Several indicators were associated with apoptosis of cancer cells (Riedl and Shi 2004). For instance, Feng *et al*. (2017) reported that caspase-3 activities increment is considered as a biomarker of apoptosis as well as a positive indicator for evaluate the efficacy of drugs in treatment of cancer. Moreover caspase-3 stimulates stress encouraged cancer cell proliferation, cellular migration, and angiogenesis of tumor (Zhou *et al*. 2018).

Moreover, several previous studies revealed that *G. lucidum* extract was effective against various bacteria, including *Bacillus cereus*, *B. anthracis*, *B. subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Klebsiella oxytoca*, *K. pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Salmonella typhimurium*, *S. typhi*, and *S. tompson* (Heleno *et al*. 2013; Abdullah *et al*. 2020). Recently *in vitro*, and *in vivo* studies by Soliman *et al*. (2022) demonstrated that *G. lucidum* extract has antibacterial activity particularly Methicillin-resistant *Staphylococcus aureus*. Various studies indicated that texture and chemical constituents might be changed as a result of cooking process, such as microwaving, pressure-cooking, griddling, frying, and steaming (Sun *et al*. 2014; Yuan *et al*. 2022) to evaluate the nutritional value but not for pharmacological value. The authors' research with other published papers will contribute to over knowledge about the

right way of management of cancer and multidrug resistance microorganisms by *G. lucidum* extract, as well as its utilization as an adjunct to prevent the chemotherapy effects.

G. lucidum is a vital pharmaceutical mushroom that has been applied in Oriental medicine for several years. As declared above and according to published papers, numerous secondary metabolites have been extracted and separated from *G. lucidum.* These compounds have various chemical formulas and possess several biological activities. The selection of suitable conditions for extraction of these metabolites is essential in order to determine their chemical contents, activities, and extracted quantities. Therefore, the current study aimed to assess the influence of cooked sample *via* microwave heating on the chemical constituents of *G. lucidum* extract and study its activity against human breast cancer cell line (MCF-7 cells) and pathogenic microorganisms to compare its activity with uncooked *G. lucidum* extract. In addition, the molecular docking interaction among the main detected compounds 9,12-octadecadienoic acid (z,z) and *n*-hexadecanoic acid on the extract with MCF-7 cells and *Klebsiella pneumonia* proteins was investigated.

EXPERIMENTAL

Materials

Preparation of Ganoderma lucidum extract sample

The *G. lucidum* product in fresh form was obtained from Hypermarket in Egypt.

The moisture content of the fresh fruiting bodies of the sample was calculated on a wet basis (72.24 ± 2.5 %) utilizing the oven at 80 °C for 36 h. The obtained sample was divided into two parts. The first part (50 g) was extracted with 200 mL of mixed solvents containing methanol, chloroform, and distilled water, (2:2:1 v/v/v); then the upper part of solvent containing the extract was removed, solvent was removed *via* rotary evaporator (40 °C), and the obtained extract was concentrated and weighted (Uncooked extract). The second part (50 g) was cooked without additional water by placing it on a glass plate and cooked using a home microwave oven for 1.5 min at 900 W pending tender. This was followed by extraction (Cooked extract), as mentioned in the first part. For further studies, the extracts were stored at -10 °C (Hamad *et al*. 2022).

GC-MS assay of G. lucidum constituents

The constituents of uncooked extract (UCE) and cooked extract (CE) were dissolved in dimethyl sulfoxide (DMSO) and were analyzed by gas chromatograph interface united with a mass spectrometer (GC-MS) model (Thermo-Scientific, Waltham, MA, USA; MS (ISQ Single Quadrupole Mass Spectrometer)). One mL/min of high quality, 99.99% helium was used as the carrier gas at a constant flow rate, while a split ratio of 1:100 was used to inject 1 µL of the extract into the gas chromatograph (GC), using the capillary column, which has dimensions of 30 m × 0.32 mm × 0.25 µm (TR5MS). The ion-source temperature was 280 °C, whereas the injector temperature was kept at 250 °C. The oven was set at 110 °C for 2 min and then increased gradually by 10 °C/min up to 200 °C/min, and one more increase by 5 °C/min to be extended to 280 °C/min, and finally held at 280 °C for 9 min. The mass spectra were recorded at 70 eV, and the chromatography procedure took 20 min. According to the average peak area of each discovered constituent to the total areas, the level percentage of each detected constituent was estimated. As previously mentioned, the GC-MS spectra were compared to the

database made available by the National Institute of Standard and Technology (NIST) (https://www.nist.gov/) (Abdelghany *et al.* 2021; Al-Rajhi *et al*. 2022b).

Cytotoxicity and microscopic studies of cooked and uncooked G. lucidum extract against MCF-7 cell line propagation

Human breast cancer cell line (MCF-7 cells) obtained from the American Type Culture Collection (ATCC, Rockville, MD) were tested to see how the cells' proliferation could be inhibited by CE and UCE samples of *G. lucidum*. The grown tested cells (5×10^4) in Dulbecco's modified Eagle's medium (DMEM), which contained L-glutamine (1%) and gentamycin (50 g/L), were kept in culture under specific conditions (37 °C, a humid environment, and 5% of CO_2) for 24 h. The growth medium containing different concentrations (2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL) of the examined extracts was added to the wells; then the plates were incubated at certain conditions (37 °C, a humid environment, and 5% CO_2). Through a microplate reader model SunRise (Tecan, Inc., Lyon, France), the viability of the cells was determined, using the MTT colorimetric method at 570 nm. The following formula was employed to determine the cytotoxicity percent (CY%):

$$\text{CY}\ (\%) = 100 - [1 - \frac{\text{Optical density of wells treated with the tested extract}}{\text{Optical density of the untreated cells (Control)}} \times 100] \quad (1)$$

To determine the MCF-7 survival curve following treatment, the relationship between living cells and extract concentration was plotted. The quantity required to have toxic effects on 50% of whole cells was known as the 50% inhibitory concentration (IC_{50}). Using GraphPad Prism software (San Diego, CA, USA), the IC_{50} was calculated from the graphic plots of the quantity response curve for each concentration. An inverted microscope (CKX41; Olympus, Tokyo, Japan) and digital camera were used to take pictures of the treated and untreated MCF-7 under the microscope (Abdelghany *et al*. 2023). Vinblastine sulfate (1 mM) was utilized as a drug control in cell culture wells. The DMSO as a negative control was applied also against cancer cells.

Assessment of lactate dehydrogenase (LDH) in MCF-7 cell line

A 24-well cell culture plate was cultured with 1.5×10^5 cells per well and incubated for 12 h. Then, some cultured wells were exposed to different concentrations of CE and UCE of *G. lucidum*, while other wells were unexposed. All plates were incubated for two days. The collected supernatant was then added to a black 96-well culture plate (200 µL per well). Based on the manufacturer's instructions, an LDH cytotoxicity test kit was used to assess LDH liberation. The absorbance in each well was measured utilizing an enzyme-linked immunoassay device at a wavelength of 450 nm (Zaqout *et al*. 2012).

Assay of caspase-3 in MCF-7 cell line

Based on the manufacturer's instructions, caspase-3 was detected *via* colorimetric method using Caspase substrate Set Plus (BioVision, Milpitas, CA, USA) (Sharma *et al*. 2014). The MCF-7 cells at level 2 M 10^6/mL were incubated for one day followed by treatment with the different concentrations of UCE and CE of *G. lucidum.* Then, the treated and untreated MCF-7 cells were collected and centrifuged at 1000 rpm for 5 min to obtain cells pellet. Buffer was added to the cells pellet for cells lyses, and then kept in ice for 10 min. The lysate cells were centrifuged for 2 min at 5000 rpm. Then 50 µL of supernatant containing lysate cells were transmitted to a microplate. Each created reaction mixture

needed 50 µL of 2x reaction buffer involved 10 Mm of D-1,4-dithiothreitol DTT. Five µL of caspase substrate (*p*-nitroaniline) was added to each well at the last step, and then the specimen was incubated at 37 °C for 2 h. The peptide cleavage by the caspase released the chromophore *p*-nitroaniline. At 405 nm wavelength, the absorbance of developed color was measured *via* reader of FLUOstar Omega microplate (BMG Labtech, Mannheim, Germany). Caspase enzymatic potential level was proportionately directly to the color reaction. The caspase-3 colorimetric assay kit was obtained from Abnova, Germany.

Antimicrobial activity cooked and uncooked G. lucidum extract

The tested microorganisms in the current study were bacteria and fungi, including *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* (ATCC13883), *Candida albicans* ATCC 10231, and *Aspergillus niger*. The disc diffusion technique was used to evaluate the antimicrobial activity of CE and UCE of *G. lucidum*. After being propagated into BHI agar, the microorganisms were left to grow for 24 h at 37 °C (for bacteria) and for 3 days at 30 °C (for fungi). The cellular suspension (McFarland 0.5, 1.5 x 10^8 UFC/mL) was then grown on Muller-Hinton agar plates and incubated at 37 °C for 24 h. Gentamicin (5 µL, 100 g/mL) was added to the discs' surface as control. The disc was loaded with DMSO, then dried and used as a negative control in disc diffusion technique. Zones of halo inhibition (mm) were used to express the results of antimicrobial activity (Al-Rajhi *et al*. 2022a).

Molecular docking study

Three D structures of breast cancer cell line and *Klebsiella pneumonia* were obtained from the online database Protein Data Bank (http://www.rcsb.org/pdb) with an access code PDB: 2LWL and 5ZGN, respectively. Two D structures of 9,12-octadecadienoic acid (z,z) and n-hexadecanoic acid were drawn using ChemDraw Ultra 15.0 and saved as MDL files (.sdf) for MOE. Hardware was applied for calculation, molecule modeling, and docking molecule personal computer (Dell Core i7 processor 1.99 GHz, 16 GB memory with Windows 10, 64-bit operating system). The software was applied, using Molecular Operating Environment (MOE 2019). The Department of Chemistry, Faculty of Mathematics and Natural Science at Padjadjaran University was granted the license for MOE 2019.0102 (Chemical Computing Group www.chemcomp.com) to create protein and ligand structures. While applying for, creating, and simulating the process, docking was the program MOE dock. Program Alpha Site Finder was used for predicting the position and binding pocket volume of the site of the ligand's interaction with macromolecules. Preparation of target proteins are detailed below:

1. The co-ligand and water molecules from the protein's active site were eliminated.
2. The hydrogen atoms were added with their typical shape to the structure.
3. The dummy sites served as the binding pocket by generating the active binding sites created by the MOE site finder.
4. The obtained pocket was saved as MOE to predict the ligand-protein.

Preparation of ligands

Through using the MMFF94 force field, the native ligands were reduced to their lowest energy state. Following 3D protonation and the rectification procedure, the final form was produced. The ligands were inserted into the site, using the triangle matcher approach after the general docking scenario was run for 100 ns on the stiff receptor atoms.

The GBVI/WSA dG procedures for rescoring were used, along with the London dG as a scoring function. The binding free energy (S kcal/mol) and hydrogen bonds between substances and protein-containing amino acids with lengths under 3.5 A were used to rank the top five poses. Additionally, the results pose-with-pose in the co-crystal ligand position and before and after amendment, respectively, was compared using the RMSD and RMSD-refine fields (Qanash *et al*. 2022; Yahya *et al*. 2022).

Statistical analysis

All measurements were performed in triplicate to calculate the ± standard deviation (SD) and variance *via* SPSS ver. 22.0 software (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Detected Constituents of *G. lucidum* Extract *via* GC-MS

The CE and UCE of *G. lucidum* were subjected to GC-MS analysis and various biological activities (Fig. 1). Several compounds were detected with different retention time and area (%) (Tables 1 and 2, Figs. 2A and 2B) in CE and UCE of *G. lucidum*. The 9,12-octadecadienoic acid (Z,Z)- and *n*-hexadecanoic acid represented the greatest detected compounds with 20.99% and 10.97 %, respectively, while moderate area (%) was recognized for linoleic acid ethyl ester (7.01%) and propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)- (4.67%) in UCE of *G. lucidum*. Moreover, cholestan-3-ol, 2-methylene-, (3á,5à)- and docosanoic acid, 1,2,3-propanetriyl ester were detected with the lowest area (0.92%) (Table 1). The GC-MS analysis showed differences in the numbers and area % of the recognized compounds of CE compared to UCE of *G. lucidum*. There were less numbers of the detected compounds in the CE than UCE of *G. lucidum* (Table 2), where several compounds, such as betulin, á-sitosterol, neoergosterone, glycidyl (Z)-9-heptadecenoate, and 2-bromotetradecanoic acid among others, were detected in UCE of *G. lucidum*. In contrast, some compounds were not affected by the coking process, such as octadecanoic acid and n-hexadecanoic acid. At the same time, some compounds were not detected in CE but appeared in UCE of *G. lucidum*; these probably were generated *via* transformation of original compound. The most detected constituents exhibited various biological activities similar to other studies. For example, n-hexadecanoic acid exhibited anti-inflammatory activity *via* inhibition of phospholipase A2, an anti-inflammatory enzyme (Aparna 2012). Moreover, n-hexadecanoic acid revealed anticancer potential against HCT-116 cells with excellent IC_{50} value (0.8 µg/mL) (Lokesh and Kannabiran 2017). Tian *et al.* (2018) reported that antioxidant activities were associated with the natural extracts, containing (Z,Z)-9,12-octadecadienoic acid (linoleic acid). Kusumah *et al.* (2020) documented the antibacterial activities of linoleic acid and α-linolenic acid against *B. subtilis* and *S. aureus*. As previously reported, several fatty acids could possess antifungal and antibacterial properties (Agoramoorthy *et al.* 2007); furthermore, fatty acids could regulate the immune responses by acting directly on T cells. The present results indicated the presence of n-hexadecanoic acid and 9,12-octadecadienoic acid (Z,Z)- in the extract of *G. lucidum* with high area %. According to Ohiri and Bassey (2016), n-hexadecanoic acid and 9,12-octadecadienoic acid (Z,Z)- represent the predominant constituents of *G. lucidum* that were detected via GC/MS analysis. Therefore these compounds were selected for molecular docking studies.

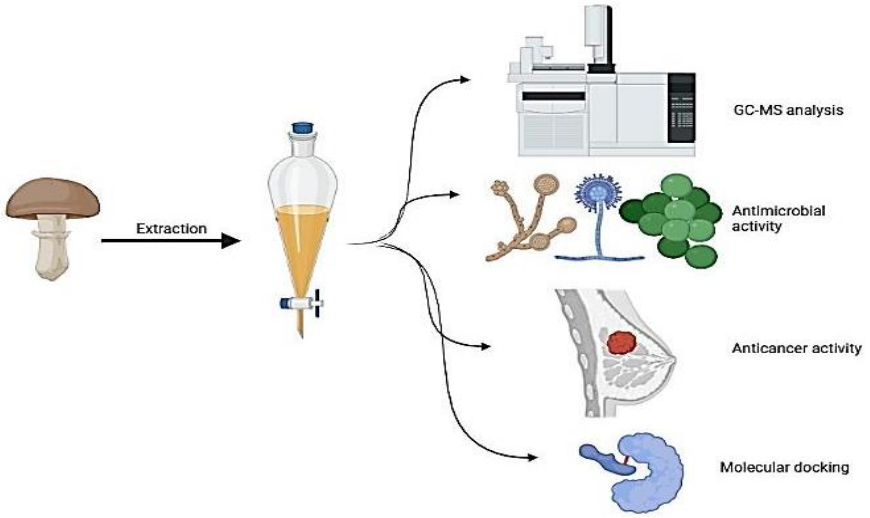
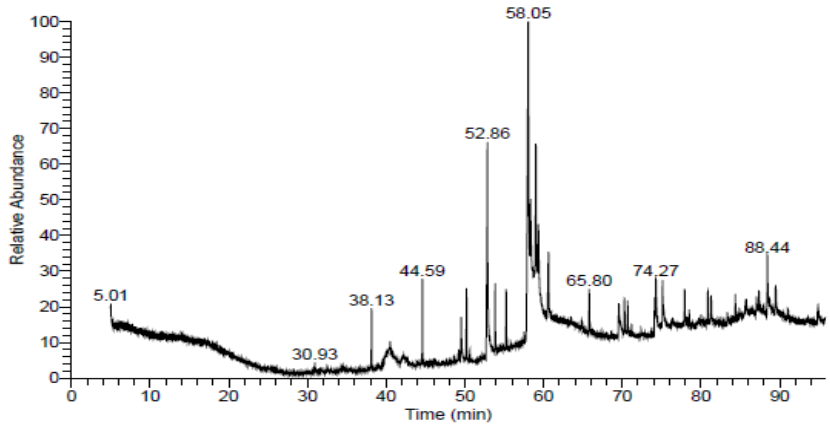


Fig. 1. Extraction method and various applicable tests to detect the constituents, antimicrobial activity, anticancer activity, and molecular docking of *G. lucidum.* This Image was designed *via* BioRender.com.



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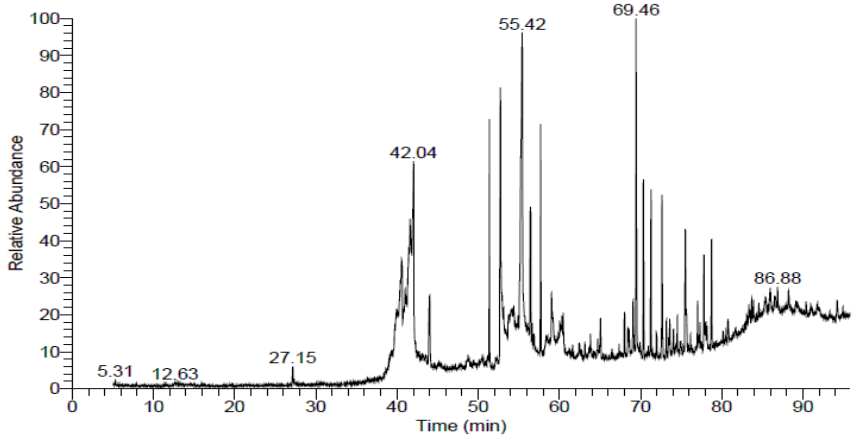


B

Fig. 2. GC-MS chromatogram analysis of UCE (A) and CE (B) samples of *G. lucidum*

Table 1. Detected Compounds of UCE of *G. lucidum* Assessed by GC-MS

Compound	R.T.*	Area%	M.F.*	M.W.*
Cycloheptasiloxane, tetradecamethyl	38. 13	2.75	$C_{14}H_{42}O_7Si_7$	518
Cyclooctasiloxane, hexadecamethyl	44. 59	3.77	$C_{16}H_{48}O_8Si_8$	592
Pentadecanoic acid	49. 52	2.51	$C_{15}H_{30}O_2$	242
Cyclononasiloxane, octadecamethyl	50. 21	3.50	$C_{18}H_{54}O_9Si_9$	666
n-Hexadecanoic acid	52. 86	10.97	$C16H_{32}O_2$	256
Hexadecanoic acid, ethyl ester	53. 84	3.03	$C_{18}H_{36}O_2$	284
Cyclodecasiloxane, eicosamethyl	55. 24	2.98	$C_{20}H_{60}O_{10}Si_{10}$	740
9,12-Octadecadienoic acid (Z,Z)-	58. 04	20.99	$C_{18}H_{32}O_2$	280
Oleic Acid	58. 34	3.57	$C_{18}H_{34}O_2$	282
Linoleic acid ethyl ester	59. 01	7.01	$C_{20}H_{36}O_2$	308
Octadecanoic acid	59. 31	3.86	$C_{18}H_{36}O_2$	284
Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	60. 61	4.67	$C_{27}H_{42}O_4$	430
Glycine, N-[(3à,5á)-24-oxo-3-[(trimeth ylsilyl)oxy]cholan-24-yl]-, methyl ester	65. 79	2.26	$C_{30}H_{53}NO_4Si$	519
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	69. 56	1.90	$C_{35}H_{68}O_5$	568
3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	70. 67	1.75	$C_{28}H_{25}NO_7$	487
9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	74. 09	1.52	$C_{21}H_{38}O_4$	354
Buta-20,22-dienolide, 3,14-dihydroxy-, (3á,5á)-	74. 28	2.04	$C_{24}H_{34}O_4$	386
Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	75. 12	2.34	$C_{21}H_{42}O_4$	358
Cholestan-3-ol, 2-methylene-, (3á,5à)-	78. 48	0.92	$C_{28}H_{48}O$	400
Neoergosterone	80. 86	1.69	$C_{27}H_{38}O$	378
1-Acetylcur-16-en-20-ol	81. 27	1.31	$C_{21}H_{26}N_2O_2$	338
2-Bromotetradecanoic acid	84. 37	1.20	$C_{14}H_{27}BrO_2$	306
Glycidyl (Z)-9-Heptadecenoate	85. 74	1.43	$C_{20}H_{36}O_3$	324
Docosanoic acid, 1,2,3-propanetriyl ester	87. 05	0.92	$C_{69}H_{134}O_6$	1058
Vitamin A palmitate	87. 37	1.04	$C_{36}H_{60}O_2$	524
á-Sitosterol	88. 44	3.27	$C_{29}H_{50}O$	414
Betulin	94. 86	1.40	$C_{30}H_{50}O_2$	442

Retention time (R.T.); molecular formula (M.F.), molecular weight (M.W.), the same is true for Table 2

Table 2. Detected Compounds of CE of *G. lucidum* Assessed by GC-MS

	R.T.*	Area%	M.F.*	M.W.*
n-Hexadecanoic acid	40. 61	2.25	$C16H_{32}O_2$	256
Hexadecanoic acid, ethyl ester	44. 01	2.79	$C_{18}H_{36}O_2$	284
Octadecanoic acid	55. 42	15.39	$C_{18}H_{36}O_2$	284
Methyl stearate	57.69	6.08	$C_{19}H_{38}O_2$	298
Glycidyl palmitate	60.48	0.63	$C_{19}H_{36}O_3$	312
Pseudojervine	65.07	1.36	$C_{33}H_{49}NO_8$	587
Hexadecanoic acid, 3-[(trimethylsilyl)oxy]propyl ester	68.02	1.24	$C_{22}H_{46}O_3Si$	386
2-Hydroxy-3-(stearoyloxy)propyl stearate	68.45	0.81	$C_{39}H_{76}O_5$	624
6,8-Di-C-á-Glucosylluteolin	69.07	1.65	$C_{27}H_{30}O_{16}$	610
Phthalic acid, bis(2-ethylhexyl) ester	69.46	8.76	$C_{24}H_{38}O_4$	390
1,2-Benzenedicarboxylic acid	70.36	4.41	$C_{24}H_{38}O_4$	390
1,2-Cyclohexanedicarboxylic acid, di-(2-ethylhexyl) ester	71.28	4.30	$C_{24}H_{44}O_4$	396
2-(4-Chlorophenyl)-4,5-di(methoxycarbonyl)-3,6-hexanopyridine	72.64	4.3	$C_{21}H_{22}ClNO_4$	387
2,2-Dimethyl-3-oxa-5à-cholestane	73.17	1.03	$C_{28}H_{50}O$	402
p-Menthane-1,2,3-triol	73.59	1.11	$C_{10}H_{20}O_3$	188
Glucobrassicin	74.05	0.75	$C_{16}H_{20}N_2O_9S_2$	448
Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	74.51	0.88	$C_{27}H_{58}O_4Si_2$	502
1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	75.51	4.15	$C_{24}H_{38}O_4$	390
(Z,Z)-1,3-Dioctadecenoyl glycerol	75. 62	1.36	$C_{21}H_{24}N_2O_3$	352
Oleanan-2-ol	77.03	1.32	$C_{30}H_{52}O$	428
2-Monooleoylglycerol trimethylsilyl ether	77.32	0.84	$C_{27}H_{56}O_4Si_2$	500
3-O-(Trimethylsilyl)-5,7,3',4'-tetra-o-methylquercetin	77. 81	2.49	$C_{22}H_{26}O_7Si$	430
Cholesta-3,5-diene	78.74	2.77	$C_{27}H_{44}$	368

Inhibitory activity of cooked and uncooked G. lucidum extract against MCF-7 cancer cell line with morphological deviations

The MCF-7 cell line when exposed to different concentrations of CE and UCE of *G. lucidum* showed increment inhibition (%) of cell proliferation in a dose-dependent manner (Fig. 3). However, UCE exhibited more cytotoxicity against MCF-7 cell line than CE, where UCE at 2 µg/mL showed a significant inhibition (3.71 ± 0.16%) of cell proliferation, but at the same concentration of UCE, no cytotoxicity was reported. At 16 µg/mL, the inhibition of cells using CE and UCE was 27.22 ± 1.64% and 52.29 ± 1.09%, respectively. However, at high concentration of 512 µg/mL, the cell inhibition was approximately similar with slight differences for cells treated with UCE (87.56 ± 0.39%) and CE (88.66 ± 0.31%). Vinblastine sulfate, a positive drug of cancer treatment was used to evaluate the potential application of the extracts against MCF-7 cell line. IC_{50} was 25.63 ± 0.52 µg/mL for UCE and 49.99 ± 0.94 µg/mL for CE of *G. lucidum* extract, compared to the IC_{50} (6.87 ± 0.43 µg/mL) of vinblastine sulfate. Different extracts of mushrooms exhibited anticancer activities against MCF-7 cell line and other cancer cells with different activities. Kolniak-Ostek *et al.* (2022) has shown the potential cytotoxicity toward MCF-7 cell line of *G. lucidum* extract with IC_{50} 209.6 µg/mL.

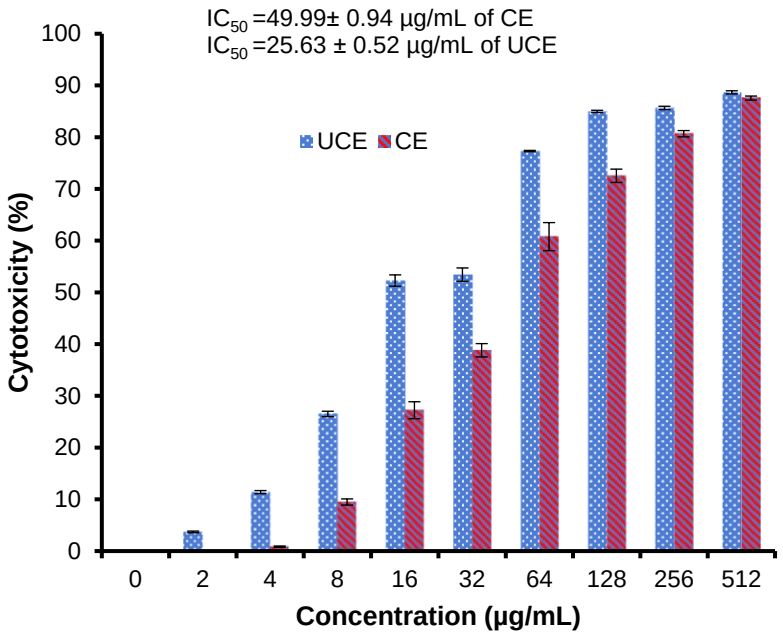


Fig. 3. Anticancer activities of CE and UCE samples against MCF-7 cell line

Cytotoxic effects of *G. lucidum* metabolites were reported against different cancer cell lines including breast, liver, lung, and, colon, due to the immunomodulatory properties of these metabolites (Cör Andrejč et al. 2022). However, other investigations are still required to further explain the immunomodulatory effect mechanisms in addition to the anticancer activities of *G. lucidum*. Recently, Kolniak-Ostek *et al.* (2022) tested the cytotoxicity of *G. lucidum* extract against tested cancer cell lines and normal cells. They demonstrated the extract exhibited cytotoxic effects on tumor cells and did not displayed toxicity against normal cells, which reveals a particular antitumor potential. From results of Sharmila *et al.* (2021), they demonstrated the successful application of *G. Lucidum* extract as anticancer agent because exhibited greater inhibition and mild activity towards breast cancer cell line and normal cells, respectively. A previous study reported the anticancer activity of *Flammulina velutipes* extract toward MCF-7 and MDA-MB-231 with different IC_{50} (17.7 to 38.36 µg/mL and 114.5 to184.2 µg/mL, respectively) values (Ukaegbua *et al*. 2018). The extracted polysaccharides of *Agaricus bisporus* displayed a strong block of MCF-7 cell growth but showed negligible cytotoxicity against colon, prostate, and gastric cancer (Jeong *et al*. 2012). Appropriately, colon and MCF-7 cell lines proliferation were affected by *Pleurotus ostreatus* extract with marked changes in the cell morphology, accompanied with the elongation in shape of MCF-7 cells (Jedinak and Sliva 2008; Mishra *et al*. 2022). In addition, the inhibitory mechanism of *G. lucidum* extract on MCF-7 cell lines included apoptosis induction, vitality reducing, and controlling key signaling molecules (Suarez-Arroyo *et al*. 2016). In the current findings, the differences among the activities of CE and UCE may be due to loss or transformation of active ingredients to other inactive ingredients because of cooking process. These observations were recorded in previous studies, but the antioxidant activity was investigated. Barros *et al*. (2007) claimed that the cooking of mushrooms using heat could damage the phenolics constructions, followed by declining their contents with a great antioxidant action into other phenolic constituents or altering phenolic constituents with little antioxidant action.

Sun *et al.* (2014) also documented the low antioxidant activity that might be due to cooking process as not only reducing the phenolic compounds, but also altering the type and relative quantity of phenolics. The DMSO as a negative control did not exhibit toxicity against cancer cells**.**

MCF-7 cell line lactate dehydrogenase

According to several reports, the release amount of lactate dehydrogenase (LDH) represents one of the biomarkers to indicate the cancer cell destruction and apoptosis (Rose *et al*. 1993; Lai *et al*. 2008; Van Wilpe *et al.* 2020). In the current study, releasing LDH from MCF-7 cell line confirmed that UCE exerted more effect than CE (Fig. 4). Therefore, the quantity of the released LDH was high in cells exposed to UCE at different concentrations than its quantity when exposed to CE. The released amount of LDH was 16 and 9 Unit/mL at 256 µg/mL, and 13 and 7 Unit/mL at 512 µg/mL of UCE and CE, respectively.

Untreated cells released a higher quantity of LDH than treated; these cells indicated that of the tested extracts either UCE or CE affect the number of tumor cells. Several studies demonstrated that various tumors reveal high LDH expression (Buchakjian and Kornbluth 2010; Levine *et al*. 2010; Feng *et al*. 2018), which was attributed to bio-characteristics of malignant cells. These studies supported the authors' explanation.

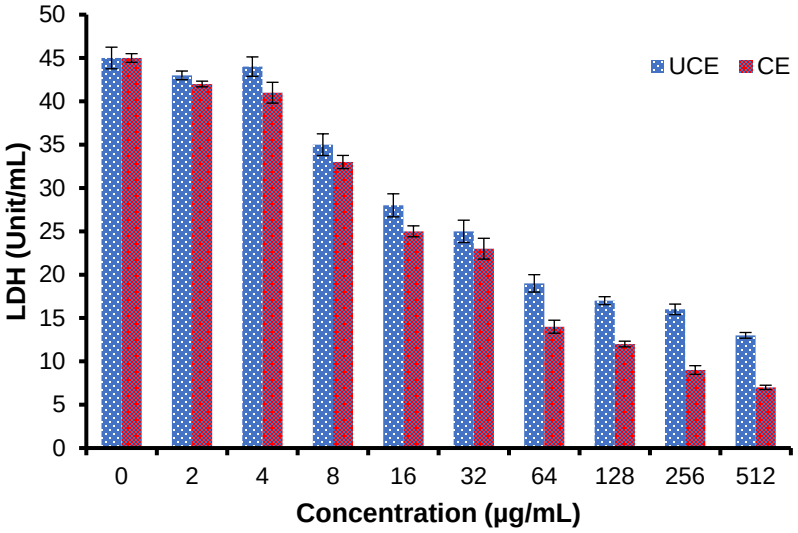


Fig. 4. MCF-7 cell line lactate dehydrogenase exposed to different concentrations of UCE and CE of *G. lucidum*

Activity of caspase-3 in exposed MCF-7 cells to UCE and CE of G. lucidum

Zhou *et al*. (2018) reported that caspase-3 is a main mediator of apoptosis stimulated during exposure of the cells to cytotoxic compounds, immunotherapy, or radiotherapy. It is often utilized as an indicator for the effectiveness of cancer therapy. In the current study, caspase-3 assay showed that the activity of caspase-3 increased in MCF-7 cells exposed to the different concentrations of UCE and CE of *G. lucidum* in a dose-dependent manner (Fig. 5). At all applied concentrations, the activity of caspase-3 was highest using UCE compared to the activity using the same concentration of CE, confirming the efficacy of UCE against MCF-7 cells. However, a brisk increment was

observed in the activity of caspase-3 with increasing the concentration of the extracts, but exposure to 256 µg/mL of UCE and CE of *G. lucidum* displayed the major rise in the activity of caspase-3. Then, a slight increment in the activity was observed at 512 µg/mL. The results from this study were in line with other reports (Buchakjian and Kornbluth 2010), where in the other report the extract of *Teucrium mascatense* exhibited activity against MCF-7 cells. Caspase pathways are appropriate candidate objectives to find the drugs specific for cancer treatment as mentioned previously (Riedl *et al*. 2004). Generally, these findings support the findings that *G. lucidum* extract contains bioactive compounds that can cause death of MCF-7 cells *via* caspase-dependent apoptosis.

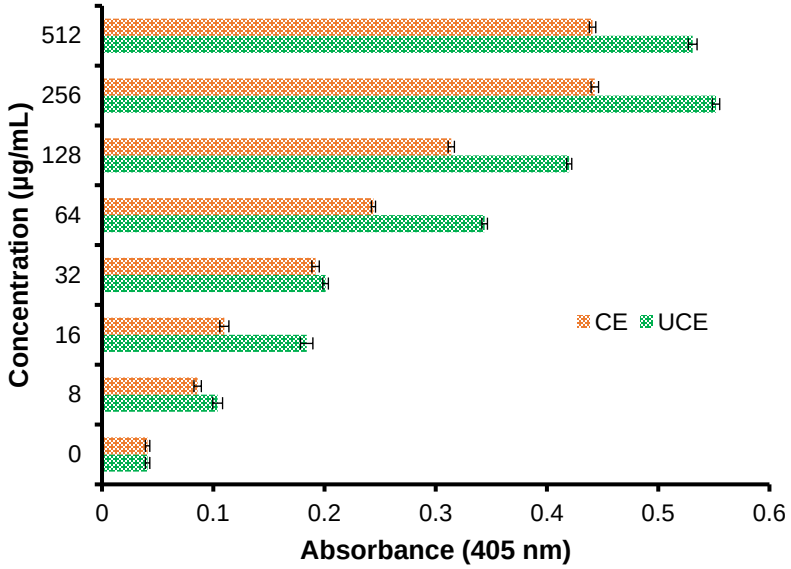


Fig. 5. Effect of UCE and CE of *G. lucidum* on the activity of Caspase-3 in MCF-7 cells

Antimicrobial activity of cooked and uncooked G. lucidum extract

Different species of bacteria and fungi were subjected to the CE and UCE of *G. lucidum* to evaluate the antimicrobial activity. It was clear that UCE provided antibacterial and antifungal activities more than CE against all tested microorganisms with different degree of inhibition zones (Fig. 6). *Bacillus subtilis* was the most sensitive bacteria with inhibition zone of 21 mm and 15 mm, while *K. pneumonia* was the most resistant bacteria with inhibition zones of 17 mm and 13 mm to UCE and CE, respectively. Promising anticandidal activity was recorded through using UCE and CE extracts against *C. albicans* with 20 mm and 17 mm inhibition zones, respectively. In contrast, both extracts exhibited less inhibitory action against *A. niger* with 8 mm and 6 mm inhibition zones, respectively. All obtained antimicrobial activities were compared utilizing standards of antibiotic and antifungal compounds (Fig. 7). As discussed in the anticancer activity of CE and UCE of *G. lucidum*, differences in the antimicrobial activity may be dependent on the components of the extract that were affected by the cooking procedures. Recently, Yuan *et al.* (2022) contributed to the number of detected volatile flavor compounds (54, 61, 53, 63, and 49 compounds) and the type of cooking methods (raw, steamed, boiled, microwaved, and fried) of *Clitocybe squamulosa* samples (Zhou *et al*. 2018). In the current study, the disc loaded with DMSO did not indicate any antimicrobial activity.

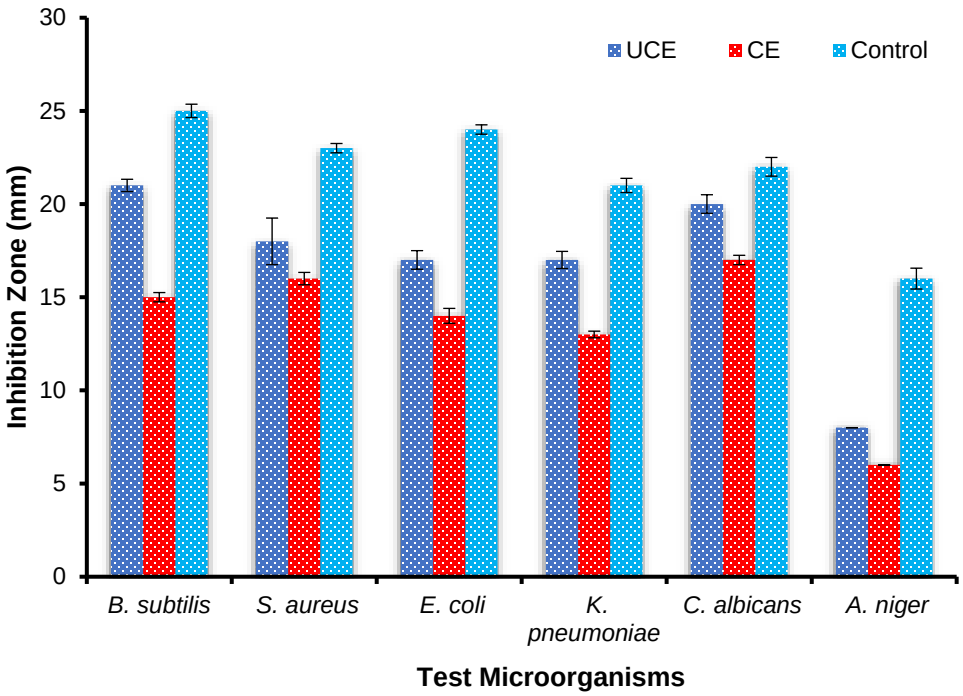


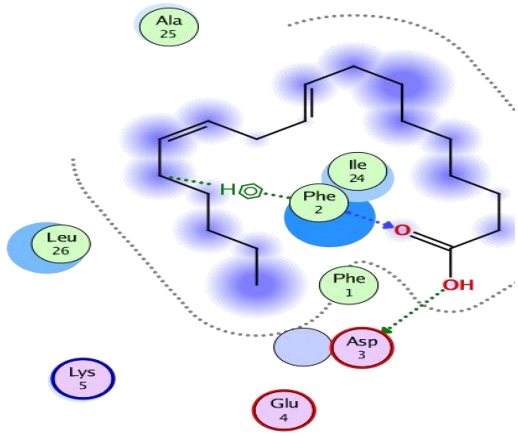
Fig. 6. Antimicrobial activities of CE and UCE *G. lucidum* extracts

Molecular docking study of 9,12-octadecadienoic acid (Z,Z) and n-hexadecanoic acid with MCF-7 cancer cell line 2LWL and K. pneumonia 5ZGN

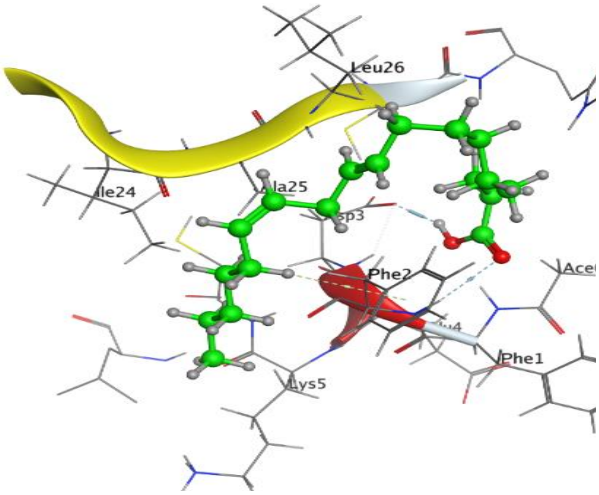
The 9,12-octadecadienoic acid (Z,Z) and n-hexadecanoic acid were investigated to evaluate the binding affinities of MCF-7 cell line (2LWL) and *K. pneumonia* (5ZGN). This study observed that docking the compounds under study had higher binding scores against active sites of 5ZGN (-7.09431 kcal/mol and -6.65358 kcal/mol respectively) than that of 2LWL (-5.95503 kcal/mol and-5.68074 kcal/mol). 9,12-Octadecadienoic acid (Z,Z) was bound to the binding cavity of breast cancer (2LWL) with the residues (ASP-3 and PHE-2) *via* O-1, O-48, and C-32, respectively. Meanwhile, interacting with *K. pneumonia* (5ZGN) by (GLY-107 and PHE-144) *via* O-48 and C-19, respectively was documented. In contrast, n-hexadecanoic acid formed two hydrogen donor interactions against 2LWL with the residues of ASP-3 *via* O-1 and C-4. The interaction between n-hexadecanoic acid and the active site bound of 5ZN also revealed the presence of three acceptor hydrogen atoms between GLN-106, GLY-107, and TRP-108 amino acid residues and O-46 atom in the ligand. Several hydrogen bonds that existed between the identified proteins and the selected compounds were detected (Tables 3 and 4). In addition, the interaction of 9,12-octadecadienoic acid (Z,Z) and n-hexadecanoic acid with 2LWL and 5ZGN proteins was reported (Tables 5 and 6). The compounds docked to adopt their best-fitted postures (Figs. 7 and 8).

Some studies compared between biological activities of detected active constituents *via* molecular docking, for example Gupte *et al.* (2018) studied the molecular docking of four compounds including ganoderal A, ganoderol A, ganoderol B, and ganoderic acid Y as a natural constituents of *G. lucidum* with Lanosterol 14 α-demethylase enzyme as well as the inhibitory action of ganomycin1 and 2 on HIV 1 protease and Tyrosinase. The docking findings reflected the inhibitory potential of these constituents, demonstrating the probability that they can be utilized as potent drugs in the future. In another study, the

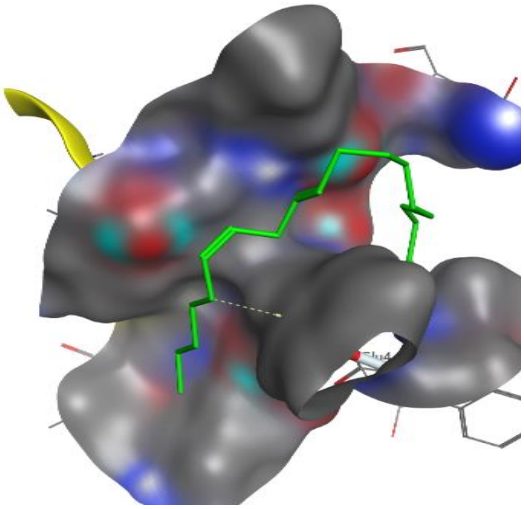
docking interaction tool was performed to compare between interaction of ellagic acid and chlorogenic acid with the crystal structures of pathogenic yeasts including *C. albicans* (4YDE) and *G. candidum* (4ZZT). The obtained results indicated that chlorogenic acid was more active with a docking score of −7.84379 kcal/mol, than ellagic acid with a docking score of −6.18615 kcal/mol (Alsalamah *et al.* 2023). Recently, *in silico* studies on interaction of *P. ostreatus* active constituents with MCF-7 cancer cell line was documented (Mishra *et al*. 2022). Several scientific reports about docking scores were described in various reports studied, confirming the effectiveness of natural constituents from plants and fungi to repress the proliferation of different cancer cells as well as the inhibition of human pathogenic microbes (Al-Rajhi *et al*. 2022c,d,e; Al-Rajhi *et al*. 2023; Qanash *et al*. 2023b).



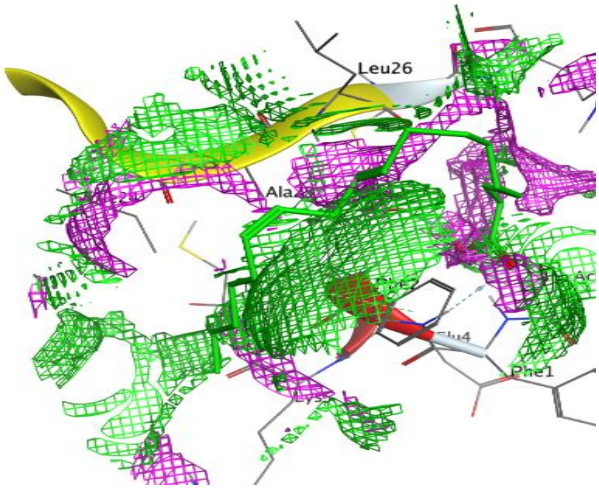
The interaction between 9,12-octadecadienoic acid (Z,Z) and active sites of 2LWL protein



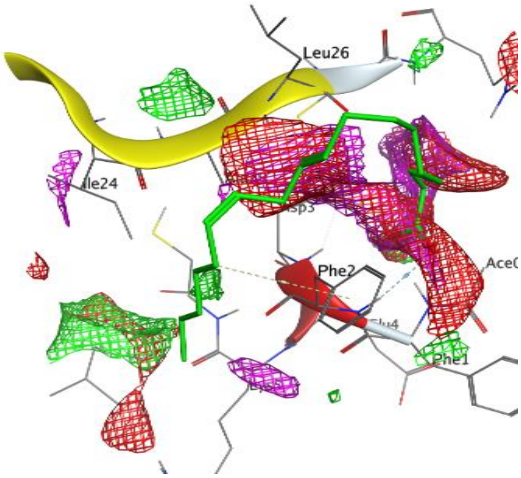
The most likely binding conformation of 9,12-octadecadienoic acid (Z,Z) and the corresponding intermolecular interactions are identified



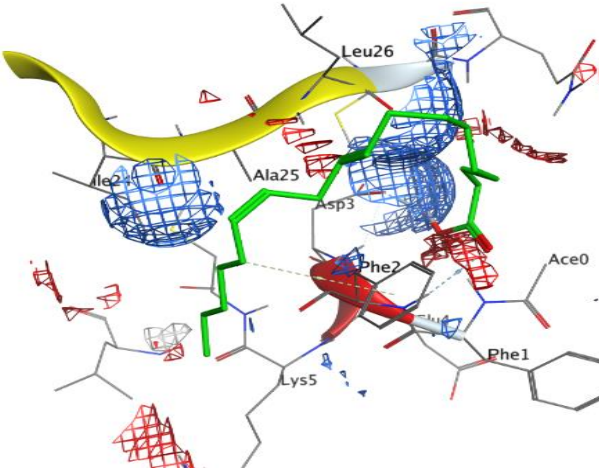
Molecular surface of 9,12-octadecadienoic acid (Z,Z) with 2LWL



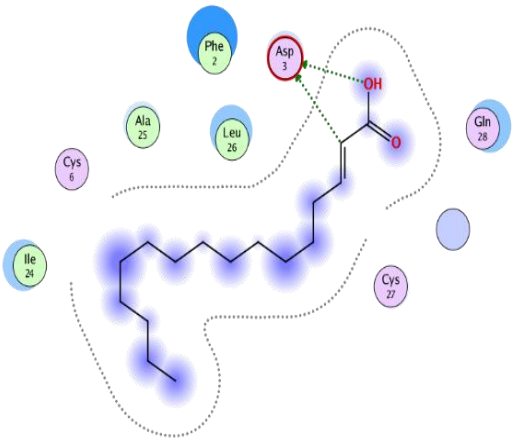
The contact preference of 9,12-octadecadienoic acid (Z,Z) with 2LWL



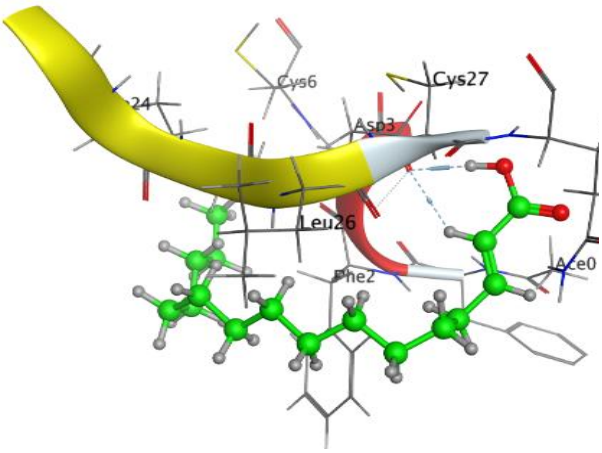
Interaction potential of 9,12-octadecadienoic acid (Z,Z) with 2LWL



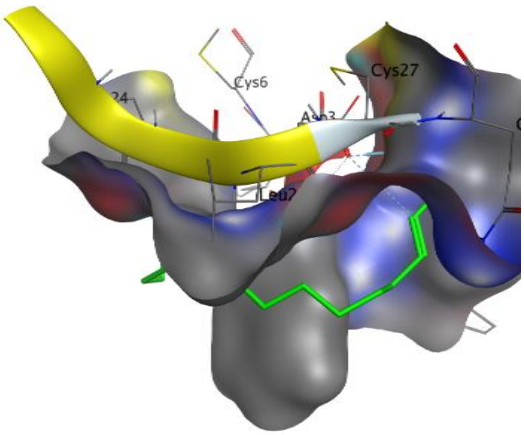
Electrostatic map of 9,12-octadecadienoic acid (Z,Z) with 2LWL



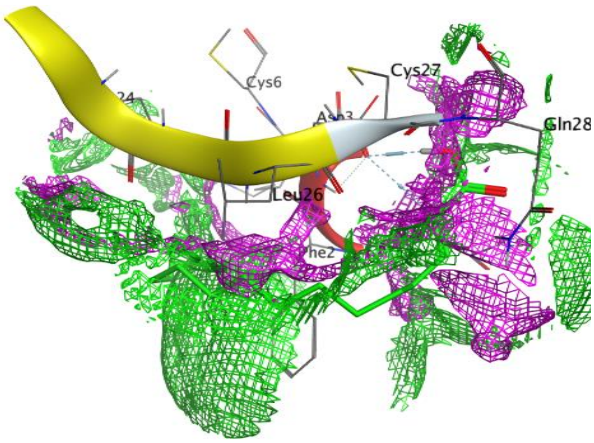
The interaction among n-hexadecanoic acid and active sites of 2LWL protein



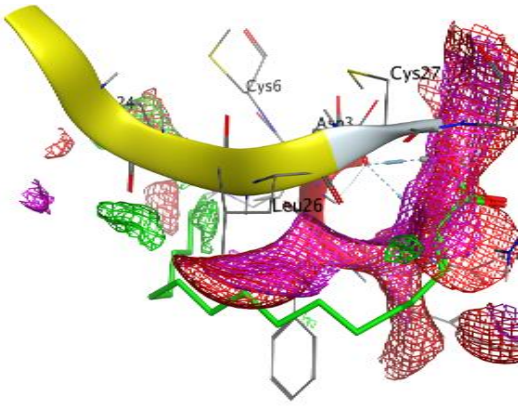
The most likely binding conformation of n-hexadecanoic acid and the corresponding intermolecular interactions are identified



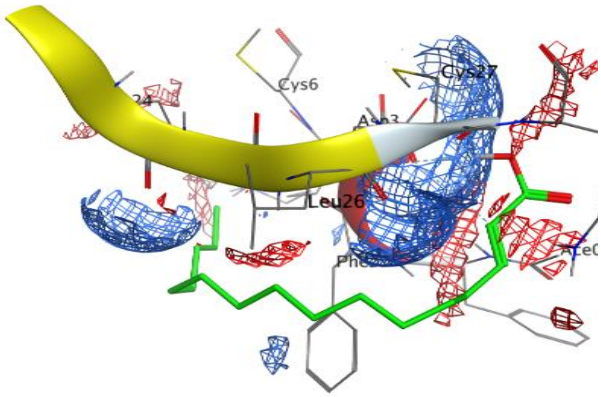
Molecular surface of n-hexadecanoic acid with 2LWL



The contact preference of n-hexadecanoic acid with 2LWL

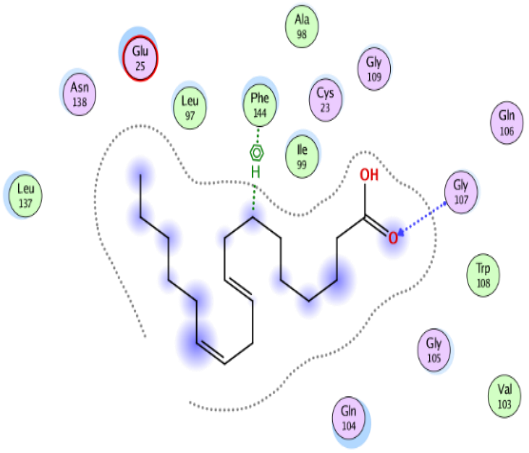


Interaction potential of n-hexadecanoic acid with 2LWL

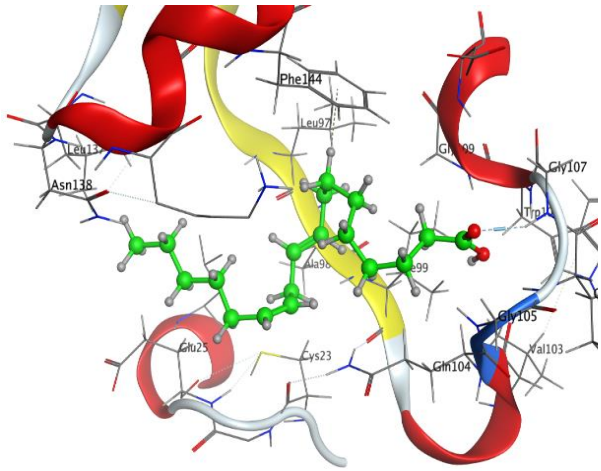


Electrostatic map of n-hexadecanoic acid with 2LWL

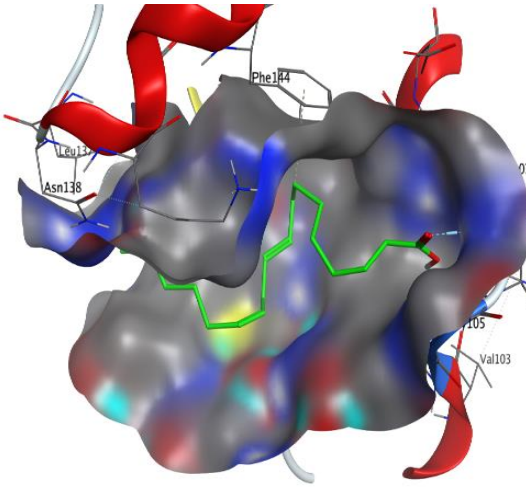
Fig. 7. Molecular docking processes of 9,12-octadecadienoic acid (Z,Z) and n-hexadecanoic acid with 2LWL protein



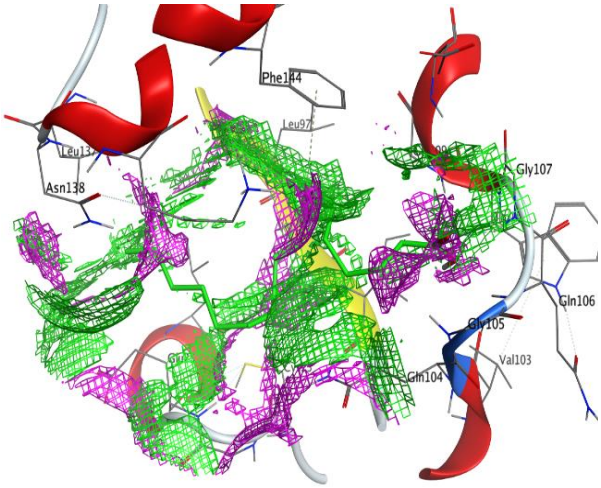
The interaction among 9,12-octadecadienoic acid (Z,Z) and active sites of 5ZGN protein



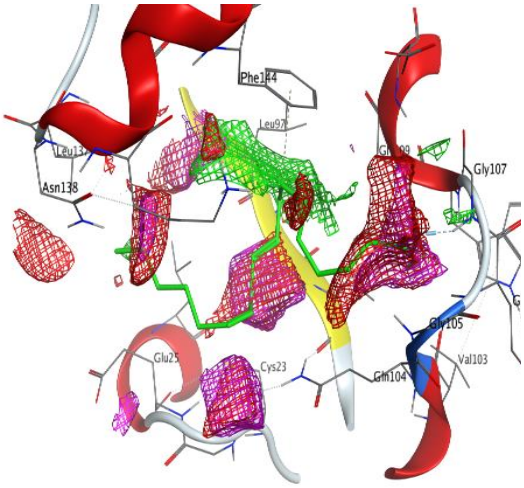
The most likely binding conformation of 9,12-octadecadienoic acid (Z,Z) and the corresponding intermolecular interactions are identified



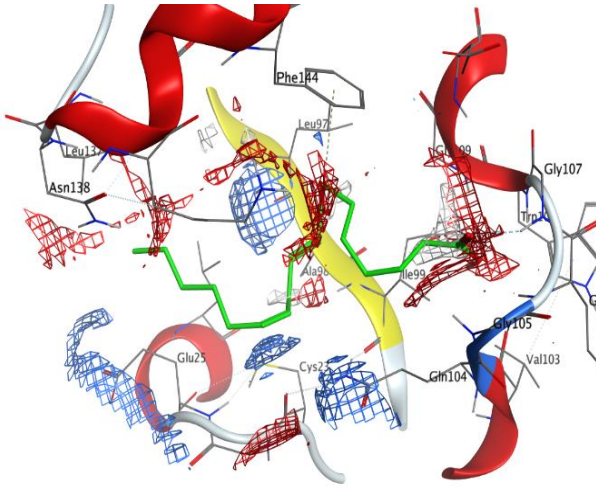
Molecular surface of 9,12-octadecadienoic acid (Z,Z) with 5ZGN



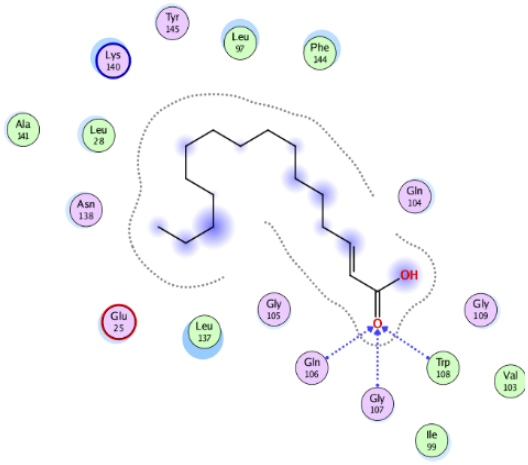
The contact preference of 9,12-octadecadienoic acid (Z,Z) with 5ZGN



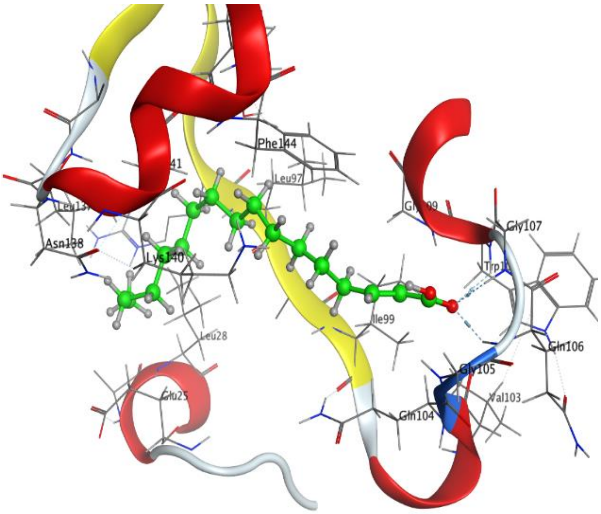
Interaction potential of 9,12-octadecadienoic acid (Z,Z) with 5ZGN



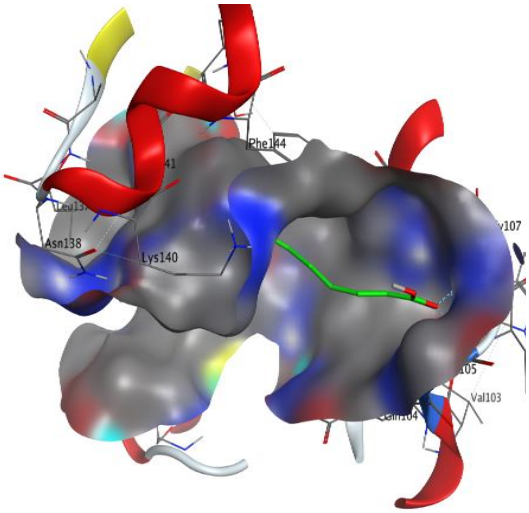
Electrostatic map of 9,12-octadecadienoic acid (Z,Z) with 5ZGN



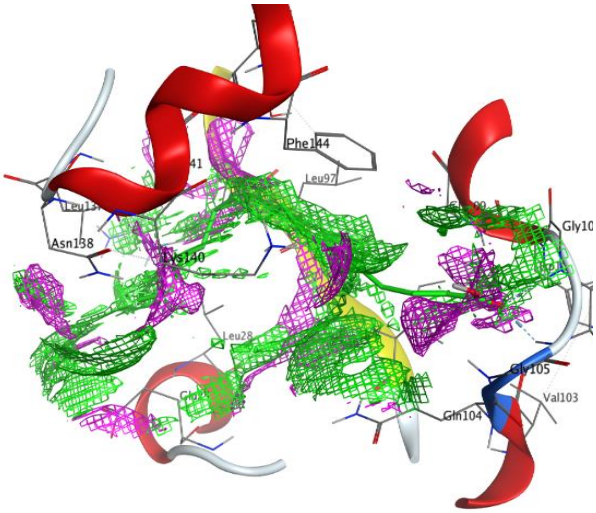
The interaction among n-hexadecanoic acid and active sites of 5ZGN protein



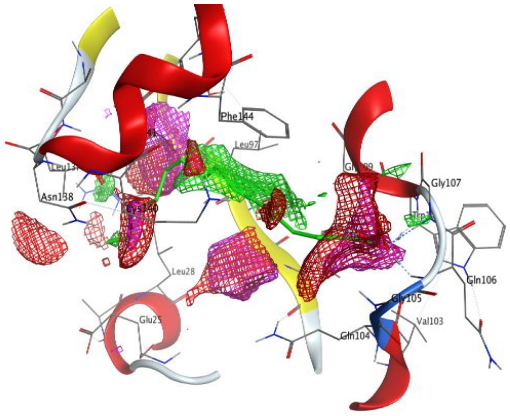
The most likely binding conformation of n-hexadecanoic acid and the corresponding intermolecular interactions are identified



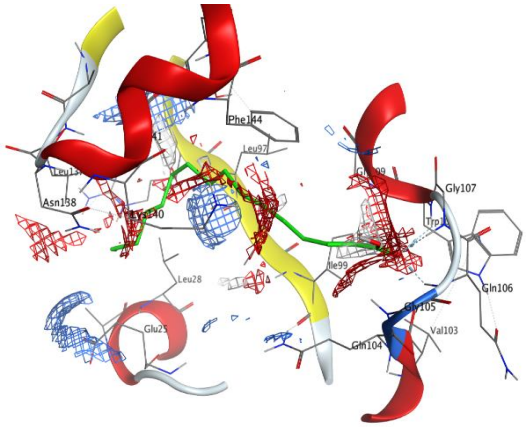
Molecular surface of n-hexadecanoic acid with 5ZGN



Contact preference of n-hexadecanoic acid with 5ZGN



Interaction potential of n-hexadecanoic acid with 5ZGN



Electrostatic map of n-hexadecanoic acid with 5ZGN

Fig. 8. Molecular docking processes of 9,12-octadecadienoic acid (Z, Z) and n-hexadecanoic acid with 5ZGN protein

Table 3. Docking Score and Energies of 9,12-Octadecadienoic Acid (z,z) and n-Hexadecanoic Acid with 2LWL Receptors

Mol	rseq	mseq	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
9,12-Octadecadienoic acid (z,z)	1	1	-5.95503	2.749589	-15.9656	-31.0542	-8.08373	-31.6015	-5.95503
9,12-Octadecadienoic acid (z,z)	1	1	-5.9068	1.710867	-18.121	-46.9759	-7.70645	-28.0728	-5.9068
9,12-Octadecadienoic acid (z,z)	1	1	-5.83955	2.645402	-19.5809	-48.3545	-8.04807	-31.7419	-5.83955
9,12-Octadecadienoic acid (z,z)	1	1	-5.55384	2.193975	-20.3393	-32.1463	-7.6603	-25.7304	-5.55384
9,12-Octadecadienoic acid (z,z)	1	1	-5.46903	1.447283	-17.7714	-24.159	-7.70341	-22.4325	-5.46903
n-Hexadecanoic acid	1	2	-5.68074	2.049251	-12.3094	-54.0689	-8.62062	-29.466	-5.68074
n-Hexadecanoic acid	1	2	-5.54615	1.417887	-13.4786	-45.3928	-8.90371	-25.8478	-5.54615
n-Hexadecanoic acid	1	2	-5.49714	4.062923	-13.0606	-36.5464	-8.79817	-27.1568	-5.49714
n-Hexadecanoic acid	1	2	-5.47295	3.888174	-13.6179	-46.2524	-8.54579	-26.8438	-5.47295
n-Hexadecanoic acid	1	2	-5.46346	1.173401	-14.4088	-52.4235	-8.49557	-29.9713	-5.46346

Table 4. Docking Score and Energies of 9,12-Octadecadienoic Acid (Z,Z) and n-Hexadecanoic Acid with 5ZGN Receptors

Mol	rseq	mseq	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	E_score2
9,12-Octadecadienoic acid (Z,Z)	1	1	-7.09431	1.9222269	-11.7365	-68.0562	-9.21079	-36.4883	-7.09431
9,12-Octadecadienoic acid (Z,Z)	1	1	-6.96591	0.92460543	-13.2403	-82.1339	-9.18789	-37.7229	-6.96591
9,12-Octadecadienoic acid (Z,Z)	1	1	-6.60378	1.549034	-11.4106	-71.2026	-9.17939	-36.7804	-6.60378
9,12-Octadecadienoic acid (Z,Z)	1	1	-6.59155	1.4171782	-15.0103	-76.9719	-10.6056	-30.4551	-6.59155
9,12-Octadecadienoic acid (Z,Z)	1	1	-6.56856	0.93718886	-17.1544	-77.8059	-9.11086	-32.9545	-6.56856
n-Hexadecanoic acid	1	2	-6.65358	1.3210641	-7.43884	-64.979	-9.14334	-32.3135	-6.65358
n-Hexadecanoic acid	1	2	-6.62392	1.4558727	-1.29912	-78.5707	-10.3738	-34.412	-6.62392
n-Hexadecanoic acid	1	2	-6.57175	1.556402	-9.16978	-78.2708	-9.45118	-35.8296	-6.57175
n-Hexadecanoic acid	1	2	-6.41443	2.1907792	-7.54893	-67.1569	-9.19692	-31.9479	-6.41443
n-Hexadecanoic acid	1	2	-6.39599	1.0914258	-11.3795	-70.0204	-11.4927	-30.7879	-6.39599

Table 5. 9,12-Octadecadienoic Acid (Z,Z) and n-Hexadecanoic Acid Interactions with 2LWL Protein

Mol	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
9,12-Octadecadienoic acid (Z,Z)	O 1	OD1 ASP 3 (A)	H-donor	2.71	-5.6
	O 48	N PHE 2 (A)	H-acceptor	3.48	-0.8
	C 32	6-ring PHE 2 (A)	H-Pi	4.51	-0.6
n-Hexadecanoic acid	O 1	OD1 ASP 3 (A)	H-donor	2.92	-7.0
	C 4	OD1 ASP 3 (A)	H-donor	3.35	-0.6

Table 6. 9,12-Octadecadienoic Acid (Z,Z) and n-Hexadecanoic Acid Interactions with 5ZGN Protein

Mol	Ligand	Receptor	Interaction	Distance	E (kcal/mol)
9,12-Octadecadienoic acid (Z,Z)	O 48	N GLY 107 (C)	H-acceptor	2.99	-3.3
	C 19	6-ring PHE 144 (C)	H-Pi	3.99	-0.5
n-Hexadecanoic acid	O 46	N GLN 106 (C)	H-acceptor	3.03	-2.1
	O 46	N GLY 107 (C)	H-acceptor	2.94	-0.7
	O 46	N TRP 108 (C)	H-acceptor	3.04	-0.6

CONCLUSIONS

1. Various constituents with different biological activities were detected in the *Ganoderma lucidum* biomass (GLB).
2. Increment in the inhibition (%) of MCF-7 cell line proliferation was recorded, using CE and UCE of GLB; however, UCE showed higher cytotoxicity than CE.
3. The released quantity of LDH from treated MCF-7 cell line was more than the cells exposed to CE. Moreover, it might be concluded that *G. lucidum* extract, particularly UCE, might inhibit the proliferation and development of MCF-7 cells *via* stimulation of apoptosis by caspase-dependent pathways.
4. The tested microorganisms were more affected by UCE compared to CE.
5. MOE 2019.0102 is applicable to predict the best conformer of ligand structure and investigate the binding free energies of these inhibitors inside the target receptor. In this study, the docked 9,12-octadecadienoic acid (Z,Z) and n-hexadecanoic acid structural activities *in silico* with breast cancer cell line (2LWL) and *K. pneumonia* (5ZGN) drug target enzyme were reported.

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