

## Article

# Molecular Interaction Studies and Phytochemical Characterization of *Mentha pulegium* L. Constituents with Multiple Biological Utilities as Antioxidant, Antimicrobial, Anticancer and Anti-Hemolytic Agents

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**Abstract:** Multiple biological functions of *Mentha pulegium* extract were evaluated in the current work. Phytochemical components of the *M. pulegium* extract were detected by Gas Chromatography-Mass Spectrometry (GC-MS) and High-performance liquid chromatography (HPLC). Moreover, *M. pulegium* extract was estimated for antioxidant potential by 2,2-Diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging, antimicrobial activity by well diffusion, and anticoagulant activity via prothrombin time (PT) and activated partial thromboplastin time (APTT). GC-MS analysis detected compounds including cholesterol margarate, stigmast-5-en-3-ol, 19-nor-4-androstenediol, androstan-17-one, pulegone-1,2-epoxide, isochiapin B, dotriacontane, hexadecanoic acid and neophytadiene. Chrysoeriol (15.36 µg/mL) was followed by kaempferol (11.14 µg/mL) and 7-OH flavone (10.14 µg/mL), catechin (4.11 µg/mL), hisperdin (3.05 µg/mL), and luteolin (2.36 µg/mL) were detected by HPLC as flavonoids, in addition to ferulic (13.19 µg/mL), cinnamic (12.69 µg/mL), caffeic (11.45 µg/mL), pyrogallol (9.36 µg/mL), *p*-coumaric (5.06 µg/mL) and salicylic (4.17 µg/mL) as phenolics. Antioxidant activity was detected with IC<sub>50</sub> 18 µg/mL, hemolysis inhibition was recorded as 79.8% at 1000 µg/mL, and PT and APTT were at 21.5 s and 49.5 s, respectively, at 50 µg/mL of *M. pulegium* extract. The acute toxicity of *M. pulegium* extract was recorded against PC3 (IC<sub>50</sub> 97.99 µg/mL) and MCF7 (IC<sub>50</sub> 80.21 µg/mL). Antimicrobial activity of *M. pulegium* extract was documented against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, but not against black fungus *Mucor circinelloides*. Molecular docking was applied using MOE (Molecular Operating Environment) to explain the biological activity of neophytadiene, luteolin, chrysoeriol and kaempferol. These compounds could be suitable for the development of novel pharmacological agents for treatment of cancer and bacterial infections.

**Keywords:** *Mentha pulegium*; anticancer; anticoagulant; antimicrobial; antioxidant

## 1. Introduction

At the present time, with the spread and outbreak of diseases, and the resistance of pathogens to drugs, scientists in the medical and pharmaceutical fields have increased their search for safe alternative sources that can solve these problems [1]. Plants are one of the best sources of herbal preparations because they contain different and diverse phytoconstituents. Despite many biologically active compounds of plant origin having been discovered, there are many that so far remain undiscovered. There are many medicinal plants that have been traditionally used without any scientific basis, requiring further study for the conversion from traditional use to scientific validity [2,3].

*Mentha* (commonly named pennyroyal) of the family *Lamiaceae* contains 61 species with approximately 100 varieties which are disseminated across temperate regions including North America, Africa, Asia, Europe, and Australia [4]. The family *Lamiaceae* is considered a rich source of flavonoids, particularly flavones such as apigenin, luteolin and 6-hydroxyflavone [4]. According to Marzouk et al. [5] the genus *Mentha* is represented in the flora of Egypt by two species, namely *Mentha longifolia* and *M. pulegium* L. Numerous uses have been recorded for the *M. pulegium* plant [6] including for relief of colds, coughs, headaches, kidney problems, liver complaints, and gallbladder ailments; in addition, it has been applied in drink and food additives. Despite these therapeutic and nutritional uses, *M. pulegium* is well-known for its poisonousness [4]. Additionally, some reports have indicated the presence of certain toxic compounds in *M. pulegium*, although Caputo et al. [7] suggested that the soil conditions may influence the expression of toxic compounds such as pulegone and its derivatives. Numerous biological activities have previously been reported for *M. pulegium* extract as well as its essential oils, and occasionally the activity has differed based on cultivation conditions. Previous studies reported a number of biological activities including antimicrobial activity against many microorganisms [8,9], decremental inflammation with oral mucosa treatment, anti-hemolytic, and anticancer activities [10] of *Mentha*.

Recently, Alharbi et al. [11] demonstrated the bactericidal potential of *M. pulegium* extract towards four gram-positive and five gram-negative bacteria; also, the development of *Aspergillus niger*, *A. flavus* and *Candida albicans* was inhibited by *M. pulegium* extract. Baali et al. [9] suggested food and pharmaceutical applications of Algerian *M. pulegium* as a preservative, antimicrobial, and antioxidant agent. Moreover, the anthelmintic properties of *M. pulegium* extract were recently documented [12]. Some scientific papers have focused on the application of *M. pulegium* essential oils [13,14] in biological activities including antiviral and antifungal activities. Bektašević et al. [15] observed excellent antioxidant activity in addition to moderate cholinesterase inhibition using essential oils, and therefore recommended their use in Alzheimer disease. The most serious diseases worldwide in the last decade have been cancers, which in 2020 led to about 9.9 million deaths [16]. Promising sources of phenolic and flavonoid contents in plants can play a main role in cancer treatment and treatment of other diseases [17,18]. The inhibitory effect of plant flavonoids on the spread of cancer cells through apoptosis induction has been detected [19,20]. The existence in *M. pulegium* of several phenolic acids was reported, including syringic acid and ferulic acid, as well as several compounds of flavonoid including isorhamnetin-3-*O*-glucoside and kaempferol-3-*O*-rutinoside; these compounds reflected high antioxidant activity [12]. Positive correlations among the phenolic contents of certain *Mentha* species (*M. rotundifolia* and *M. pulegium*) and their biological activities, namely antimicrobial and antioxidant activities, were documented by Alharbi et al. [11].

A computer simulation technique such as molecular dynamic simulation (MDS) can be utilized [20] to screen and estimate the physical movements of molecules and atoms. This allows searchers to measure flexibility, rigidity, and secondary construction prediction

in terms of gain or loss during the simulation time. Recently, the silico protocol was applied to select the most suitable structures for biological observation of active compounds; this protocol was dependent on the correlation among biological activity values of the input structures and the target proteins in pathogenic organisms, cancer cells, etc. [20–25]. The present investigation was designed to explore the phytochemical characterization of *M. pulegium* extract by GC-MS and HPLC, studying different biological applications comprising antitumor, antimicrobial, anti-hemolytic and anticoagulant activities.

## 2. Results and Discussion

### 2.1. GC-MS and HPLC Analysis of *M. pulegium* Extract

Research into the pharmaceutical value of medicinal plants depends on the detection and identification of phyto-constituents and their chemical structures, and understanding their pharmacodynamic action when used for the treatment of any illness. In the current study, extract of *M. pulegium* was chemically evaluated using GC-MS and HPLC analysis (Figure 1) for performing various biological activities. The GC-MS analysis of *M. pulegium* (Figure 2) revealed the existence of 38 compounds (Table 1) and, as indicated by chromatogram (Figure 2), the most abundant component by peak area was shown to be hexadecanoic acid (20.88%), followed by methyl 10-ketostearate (10.84%), cis-13-Octadecenoic acid (6.84%), stearic acid (4.87%), oxiranoctanoic acid 3-octyl-methyl ester (4.72%), methyl hexadecanoate (4.56%), elaidic acid, methyl ester (4.17%), and 1,2-benzenedicarboxylic acid (3.60%). Neophytadiene (diterpenoid) was identified as a constituent of *M. pulegium* extract (Table 1); this constituent has been found to include multiple functionalized agents according to earlier reports [26,27], including anti-inflammatory, analgesic, antipyretic, antioxidant, and antimicrobial. Among the *M. pulegium* extract constituents, phytosterols including cholesterol margarate, stigmast-5-en-3-ol, 19-nor-4-androstenediol, and androstan-17-one (Table 1), can play an important biological role in protection against various cancers of the stomach, breast, prostate, lung, liver, and ovaries, as well as regulation of sugar uptake in diabetics, as mentioned by previous researchers [28–30]. Raju et al. [31] documented the safe properties (non-carcinogenic, non-poisonous, and non-mutagenic) of these phytosterols, and the anti-hypercholesterolemic qualities of stigmast-5-en-3-ol have also been reported [32]. Furthermore, the obtained results showed the presence of other compounds in *M. pulegium* extract, namely pulegone-1,2-epoxide (oxygenated terpene), which has vasorelaxant activity according to Lima et al. [33]. The non-hemolytic, non-toxic, and non-irritant qualities of pulegone-1,2-epoxide have been recognized [34], therefore its application for medicinal purposes was validated. Other important ingredients of *M. pulegium* extract (Table 1), namely isochiapin B (sesquiterpene lactone) and dotriacontane, have been identified as antioxidants [35] and bacteriocidal agents [36], respectively. From the GC-MS, it is obvious that *M. pulegium* extract is rich in biologically active constituents. Different and various flavonoids and phenolic acids within *M. pulegium* extract were recognized by HPLC analysis (Table 2 and Figures 3 and 4). Among the detected flavonoids, chrysoeriol was the most abundant (15.36 µg/mL), followed by kaempferol (11.14 µg/mL), and 7-OH flavone (10.14 µg/mL) (Table 2), while luteolin, hesperidin, and catechin were detected in the extract with low concentrations of 2.36, 3.05, and 4.11 µg/mL, respectively. From a scientific study [37] of thirteen *Mentha* species, the detected flavonoid levels varied within the species, particularly in the incidence of hesperidin, luteolin, and kaempferol, which ranged from 0.73 to 109.39 µg/g, 1.84 to 31.03 µg/g, and 1.30 to 33.68 µg/g, respectively; in contrast, levels of phenolic acids did not significantly differ. Six phenolic acids were identified in the *M. pulegium* extract, namely ferulic, cinnamic, caffeic, pyrogallol, *p*-coumaric, and salicylic with concentrations of 13.19, 12.69, 11.45, 9.36, 5.06 and 4.17 µg/mL, respectively. Similar observations were recorded in a previous study [38], where caffeic acid represented the main compound (31.48 mg/g) in *M. pulegium*. Different flavonoids and phenolic acids were detected in *M. pulegium*, depending on the origin, as mentioned previously in the literature. Caffeic (as phenolic acid), acacetin 5-O- $\alpha$ -L-rhamnopyranosyl(1-2)-O- $\alpha$ -L-rhamnopyranoside, 7-O- $\alpha$ -rutinosides of apigenin and

luteolin, vicenin, and 5-hydroxy-6,7,3',4'-tetramethoxyflavone as flavonoids were detected in *M. pulegium* of Egyptian origin [39]. Caffeic, vanillic, and ferulic as phenolic acids, in addition to apigenin, luteolin, naringenin, and catechin as flavonoids, were detected in *M. pulegium* of Greek origin [40]. 4-Hydroxy benzoic, caffeic, *p*-coumaric, chlorogenic, and rosmarinic acids as phenolic acid, in addition to luteolin, diosmin, and kaempferol, were detected in *M. pulegium* of Algerian origin [41]. Hesperidin was recorded in *M. pulegium* at low concentrations compared with the other detected flavonoids (Table 2), was although it has been recorded in various levels in other species, for example 0.21 mg/g in *M. piperita* and 11.83 mg/g in *M. spicata* [42]. The main reason for the traditional therapeutic potential of *Mentha* spp. may be due to the existence of these flavonoids and phenolics that provide different health benefits. In a recent evaluation [43], multiple biological functions including anticancer, antioxidant, antimicrobial and anti-inflammatory properties of chrysoeriol and luteolin were documented. Moreover, Wei et al. [44] demonstrated in vivo activity of chrysoeriol against human lung carcinoma development. Cancer-preventive action without distracting normal cells and anti-inflammatory activities of kaempferol were recorded by Sharma et al. [45], in vivo and in vitro. Recently, Abbou et al. [46] identified different compounds, namely *p*-coumaric acid, gallic acid, naringenin, quercetin, and ferulic acid, in the extract of *M. pulegium* L aerial parts, which could be supportive in the regulation of oxidative stress leading to Alzheimer's disease and diabetes.

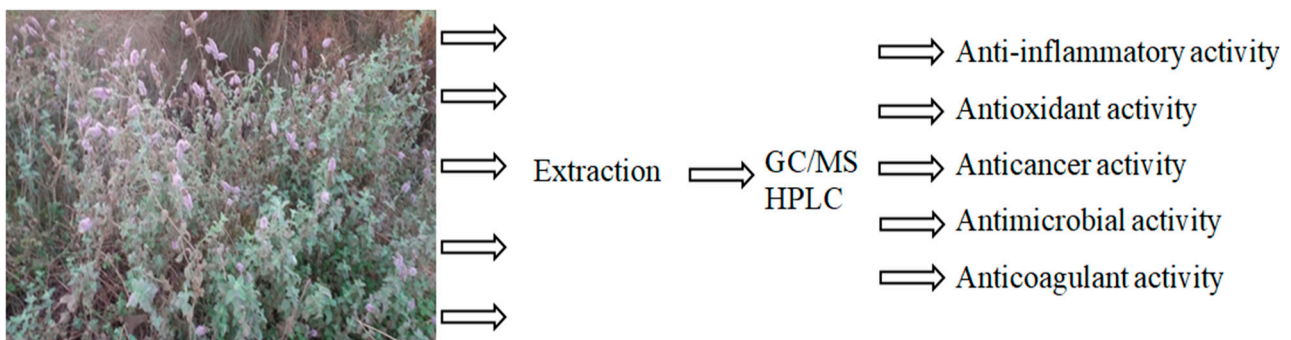


Figure 1. Diagram illustrating *M. pulegium* plants with the analysis methods and biological activities.

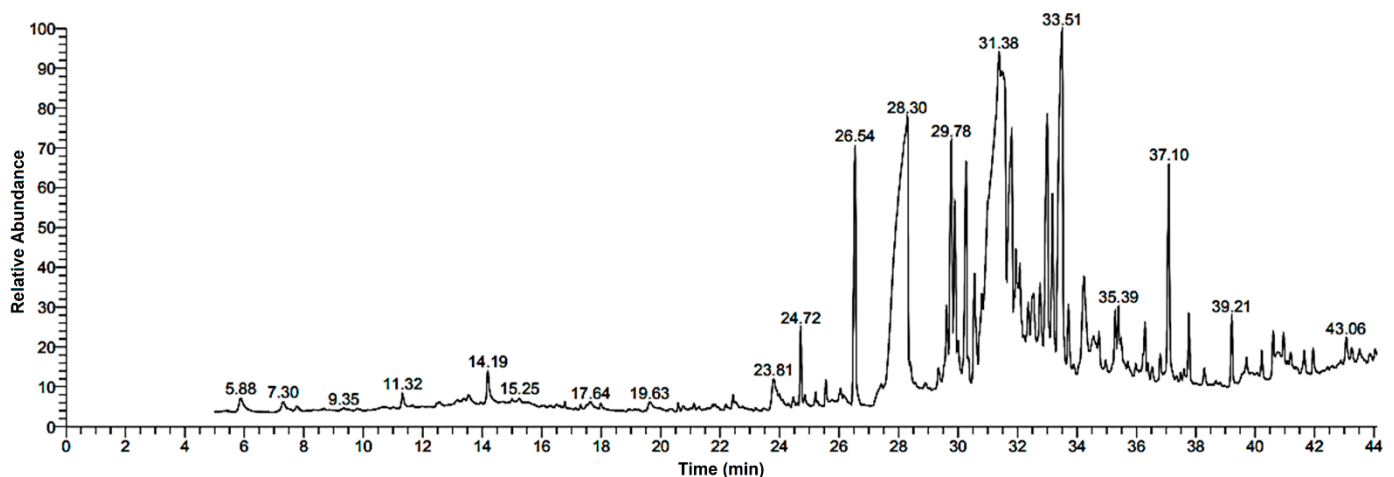


Figure 2. GC-MS chromatogram analysis of *M. pulegium* extract.

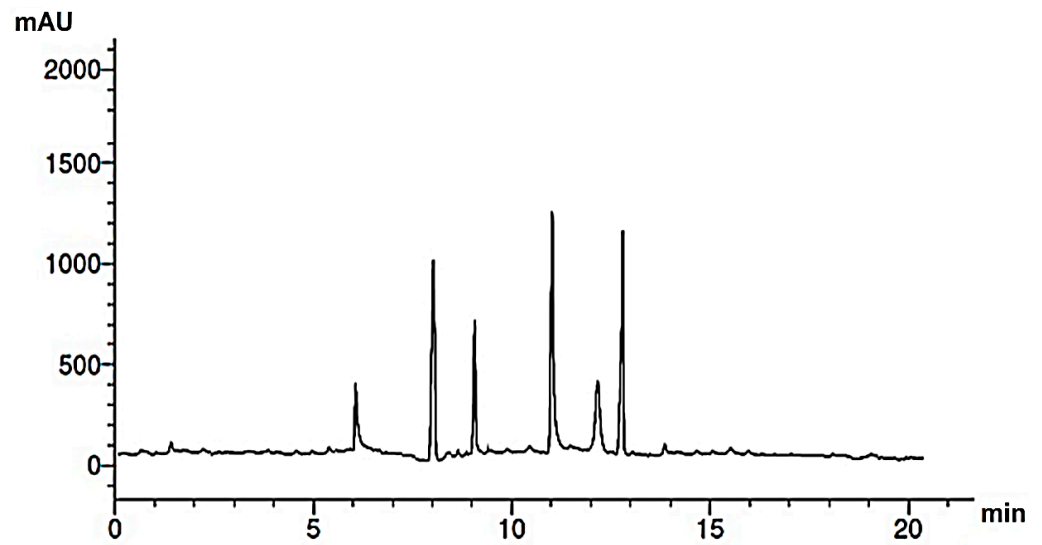


Figure 3. HPLC chromatogram of phenolic acids of *M. pulegium* extract.

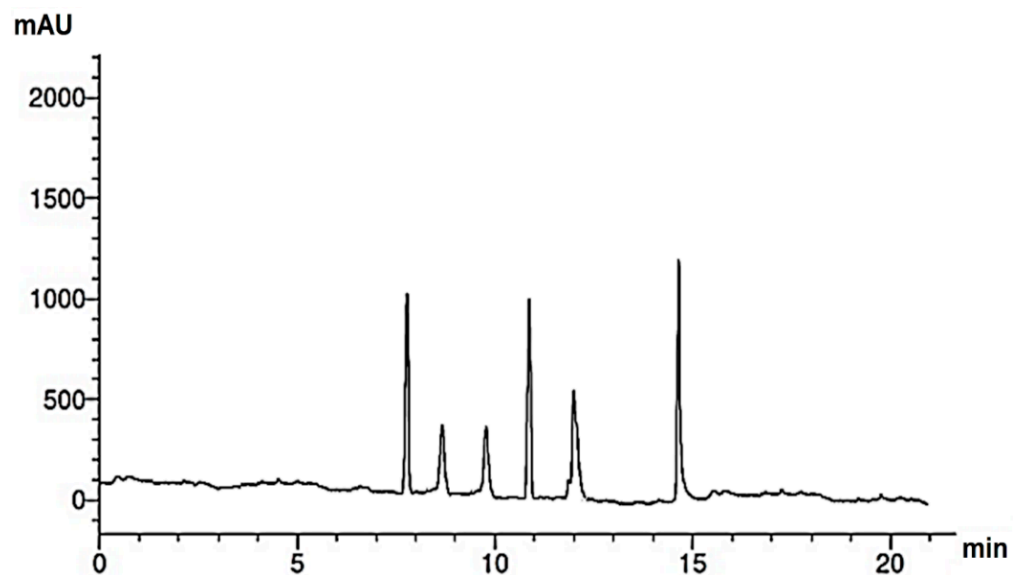


Figure 4. HPLC chromatogram of flavonoids of *M. pulegium* extract.

Table 1. Phyto-constituents of *M. pulegium* extract identified by GC-MS.

| Phyto-Constituent                         | RT *  | Area % | Molecular Formula                              | Molecular Weight |
|---|-------|--------|--|------------------|
| Pulegone-1,2-epoxide (oxygenated terpene) | 14.18 | 0.52   | C <sub>10</sub> H <sub>14</sub> O <sub>2</sub> | 166              |
| Methyl tridecanoate                       | 23.80 | 0.69   | C <sub>14</sub> H <sub>28</sub> O <sub>2</sub> | 228              |
| Neophytadiene (Diterpenoid)               | 24.72 | 1.07   | C <sub>20</sub> H <sub>38</sub>                | 278              |
| 2-cis-9-Octadecenyl oxyethano             | 25.56 | 0.36   | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub> | 312              |
| Methyl hexadecanoate                      | 26.54 | 4.56   | C <sub>17</sub> H <sub>34</sub> O <sub>2</sub> | 270              |
| Hexadecanoic acid                         | 28.30 | 20.88  | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 256              |
| 2,3-Dihydroxypropyl palmi                 | 29.34 | 0.32   | C <sub>19</sub> H <sub>38</sub> O <sub>4</sub> | 330              |

Table 1. Cont.

| Phyto-Constituent   | RT *  | Area % | Molecular Formula   | Molecular Weight |
|---|-------|--------|---|------------------|
| Linolelaidic acid, methyl ester                               | 29.62 | 0.87   | C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>                | 294              |
| Elaidic acid, methyl ester                                    | 29.78 | 4.17   | C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>                | 296              |
| cis-11-Octadecenoic acid, methyl ester                        | 29.90 | 2.80   | C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>                | 296              |
| Isochiapin B  | 30.01 | 0.32   | C <sub>19</sub> H <sub>22</sub> O <sub>6</sub>                | 346              |
| Octadecanoic acid, methyl ester                               | 30.27 | 3.94   | C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>                | 298              |
| Methyl (7E,10E)-7,10-octadecadienoate                         | 30.55 | 2.06   | C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>                | 294              |
| Ethyl linoleate   | 30.80 | 0.65   | C <sub>21</sub> H <sub>38</sub> O <sub>2</sub>                | 322              |
| Oleic acid  | 30.99 | 0.79   | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>                | 282              |
| 6-Octadecenoic acid   | 31.39 | 3.58   | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>                | 282              |
| cis-13-Octadecenoic acid                                      | 31.57 | 6.84   | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>                | 282              |
| Stearic acid  | 31.81 | 4.87   | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>                | 284              |
| 19-nor-4-androstenediol (Phytosterol)                         | 31.94 | 1.39   | C <sub>18</sub> H <sub>28</sub> O <sub>2</sub>                | 276              |
| Androstan-17-one (Phytosterol)                                | 32.08 | 1.20   | C <sub>21</sub> H <sub>34</sub> O <sub>2</sub>                | 318              |
| cis-13-Eicosenoic acid  | 32.36 | 0.52   | C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>                | 310              |
| Oxiraneoctanoic acid, 3-octyl-methyl ester                    | 33.00 | 4.72   | C <sub>19</sub> H <sub>36</sub> O <sub>3</sub>                | 312              |
| Methyl 10-ketostearate  | 33.50 | 10.84  | C <sub>19</sub> H <sub>36</sub> O <sub>3</sub>                | 312              |
| Arachidic acid methyl ester                                   | 33.71 | 1.00   | C <sub>21</sub> H <sub>42</sub> O <sub>2</sub>                | 326              |
| 7,7,8,8,9,9,10,10,10-Nonafluorodecane-1,2-Diol                | 34.24 | 2.10   | C <sub>10</sub> H <sub>13</sub> F <sub>9</sub> O <sub>2</sub> | 336              |
| Dasycarpidan-1-methanol, acetate (ester)                      | 34.56 | 0.75   | C <sub>20</sub> H <sub>26</sub> N <sub>2</sub> O <sub>2</sub> | 326              |
| 6,7-Dimethoxy-1,2-Dimethyl-1,2,3,4-Tetrahydro-5-isoquinolinol | 35.39 | 0.95   | C <sub>13</sub> H <sub>19</sub> NO <sub>3</sub>               | 237              |
| Hahnfett  | 35.48 | 0.34   | ND **   | ND **            |
| 2,3-Dihydroxypropyl elaidate                                  | 35.29 | 0.79   | C <sub>21</sub> H <sub>40</sub> O <sub>4</sub>                | 356              |
| Dotriacontane   | 36.29 | 0.84   | C <sub>32</sub> H <sub>66</sub>                               | 450              |
| Di-2-Benzothiazole Disulfane                                  | 36.80 | 0.36   | C <sub>14</sub> H <sub>8</sub> N <sub>2</sub> S <sub>4</sub>  | 332              |
| 1,2-Benzenedicarboxylic acid                                  | 37.09 | 3.60   | C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>                | 390              |
| Heptacosane   | 37.78 | 0.96   | C <sub>27</sub> H <sub>56</sub>                               | 380              |
| Docosanoic acid, 1,2,3-propanetriylester                      | 38.29 | 0.31   | C <sub>69</sub> H <sub>134</sub> O <sub>6</sub>               | 1058             |
| Docosanoic acid, methylester                                  | 39.71 | 0.49   | C <sub>23</sub> H <sub>46</sub> O <sub>2</sub>                | 354              |
| Cholesterol margarate(Phytosterol)                            | 41.65 | 0.42   | C <sub>44</sub> H <sub>78</sub> O <sub>2</sub>                | 638              |
| 6,8-DI-C-á-Glucosylluteolin                                   | 41.95 | 0.39   | C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>               | 610              |
| Stigmast-5-EN-3-OL (Phytosterol)                              | 43.06 | 0.48   | C <sub>28</sub> H <sub>44</sub> O <sub>4</sub>                | 444              |

\* RT, retention time; \*\* ND, not detected.



**Table 2.** Flavonoids and phenolic acids contained in *M. pulegium* extract, identified by HPLC.

| Flavonoids |              |                       | Phenolic Acids |                         |                       |
|------------|--------------|-----------------------|----------------|-------------------------|-----------------------|
| RT *       | Compound     | Concentration (µg/mL) | RT *           | Compound                | Concentration (µg/mL) |
| 7.8        | Kaempferol   | 11.14                 | 6              | <i>p</i> -Coumaric acid | 5.06                  |
| 8.7        | Luteolin     | 2.36                  | 8              | Caffeic acid            | 11.45                 |
| 9.8        | Hesperidin   | 3.05                  | 9              | Pyrogallol              | 9.36                  |
| 11         | 7-OH flavone | 10.14                 | 11             | Ferulic acid            | 13.19                 |
| 12         | Catechin     | 4.11                  | 12             | Salicylic acid          | 4.17                  |
| 15         | Chrysoeriol  | 15.36                 | 13             | Cinnamic acid           | 12.69                 |

\* RT, retention time.

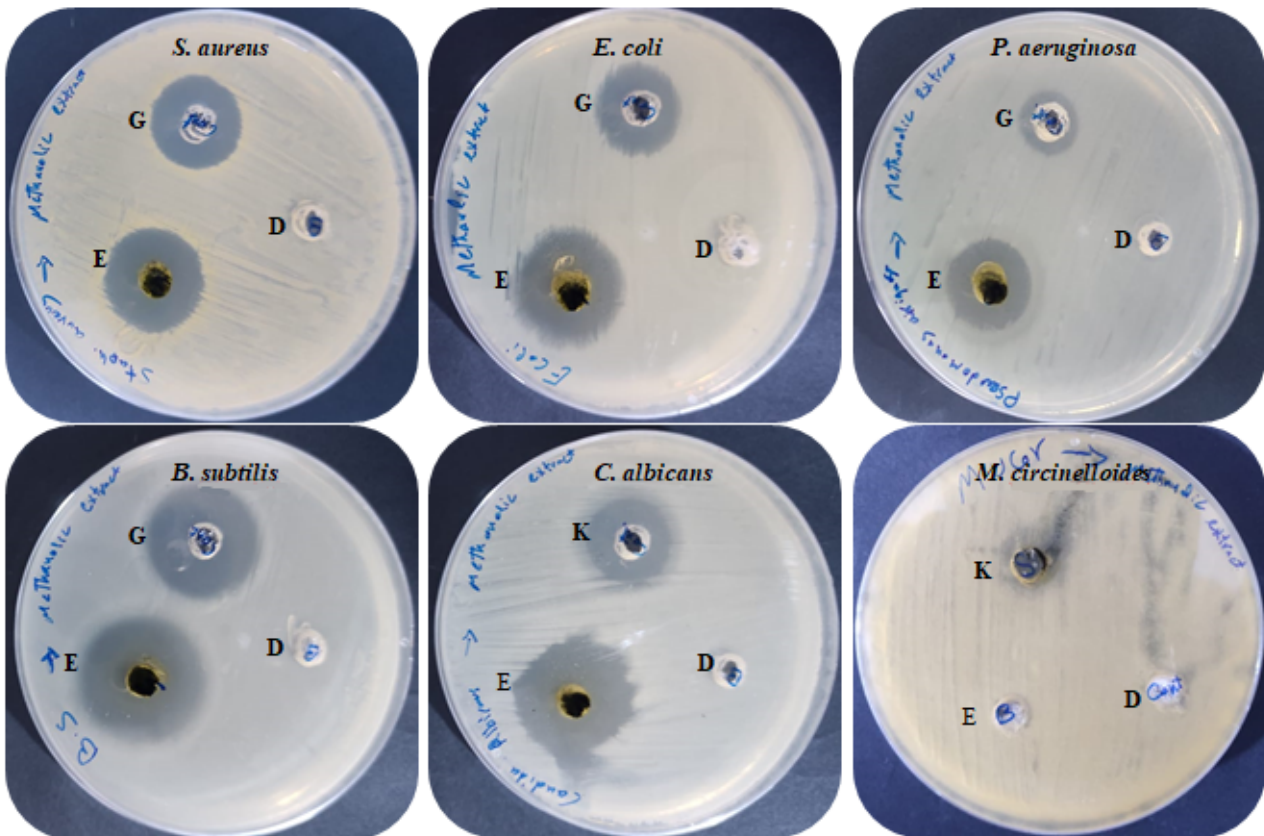
## 2.2. Antimicrobial Potential of *M. pulegium* Extract

Antimicrobial activities of *M. pulegium* extract were assessed against four bacteria, one unicellular fungus, and one black mold as presented (Table 3 and Figure 5). The findings indicated that all the tested bacteria and *C. albicans* were sensitive to *M. pulegium* extract. Growth of *B. subtilis* (IZ, 27 mm) was more affected by *M. pulegium* extract than other bacteria, namely *E. coli* (IZ, 26 mm), *S. aureus* (IZ, 19 mm) and *P. aeruginosa* (IZ, 19 mm). Surprisingly, the extract was more effective on the tested organisms than the positive control (Gentamycin antibiotic). However, *C. albicans* was inhibited where the IZ was 25 mm, while *Mucor circinelloides* growth was not inhibited. These results may be due to differences in cell wall composition or genes responsible for extract resistance. According to a recent study [47], *M. pulegium* extract exhibited antibacterial potential against *S. aureus* only, and therefore it was applied for wound healing. Previous reports have indicated the antimicrobial activity of *M. pulegium* extract towards many bacteria (including five gram-positive and five gram-negative types) and fungi including six fungal species [7]. Numerous studies have shown that *M. pulegium* essential oil exerts antimicrobial activities on bacteria and fungi. A study by Aimad et al. [48] indicated that *M. pulegium* oil completely inhibited *Aspergillus niger* growth, and inhibited *B. subtilis* where the IZ was 25 mm. The antibacterial activity is perhaps attributable to the occurrence of natural ingredients of *M. pulegium* extract including neophytadiene, isochiapin B, and other constituents mentioned in Table 3. The current finding is in good agreement with the results of Ceyhan-Güvensen and Keskin [49], who reported the antimicrobial activity of *M. pulegium* extract and explained this activity by the presence of high neophytadiene content.

**Table 3.** Antimicrobial activity of *M. pulegium* extract.

| Tested Microorganisms  |                          | Inhibition Zone (mm) |           |               |
|------------------------|--------------------------|----------------------|-----------|---------------|
|                        |                          | Extract (100 µL)     | Control * | MIC *** µg/mL |
| Gram-positive bacteria | <i>S. aureus</i>         | 23                   | 21        | 62.50         |
|                        | <i>B. subtilis</i>       | 27                   | 25        | 45.25         |
| Gram-negative bacteria | <i>E. coli</i>           | 26                   | 20        | 33.60         |
|                        | <i>P. aeruginosa</i>     | 19                   | 14        | 15.62         |
| Fungi                  | <i>C. albicans</i>       | 25                   | 22        | 65.20         |
|                        | <i>M. circinelloides</i> | 0.0                  | 11        | ND **         |

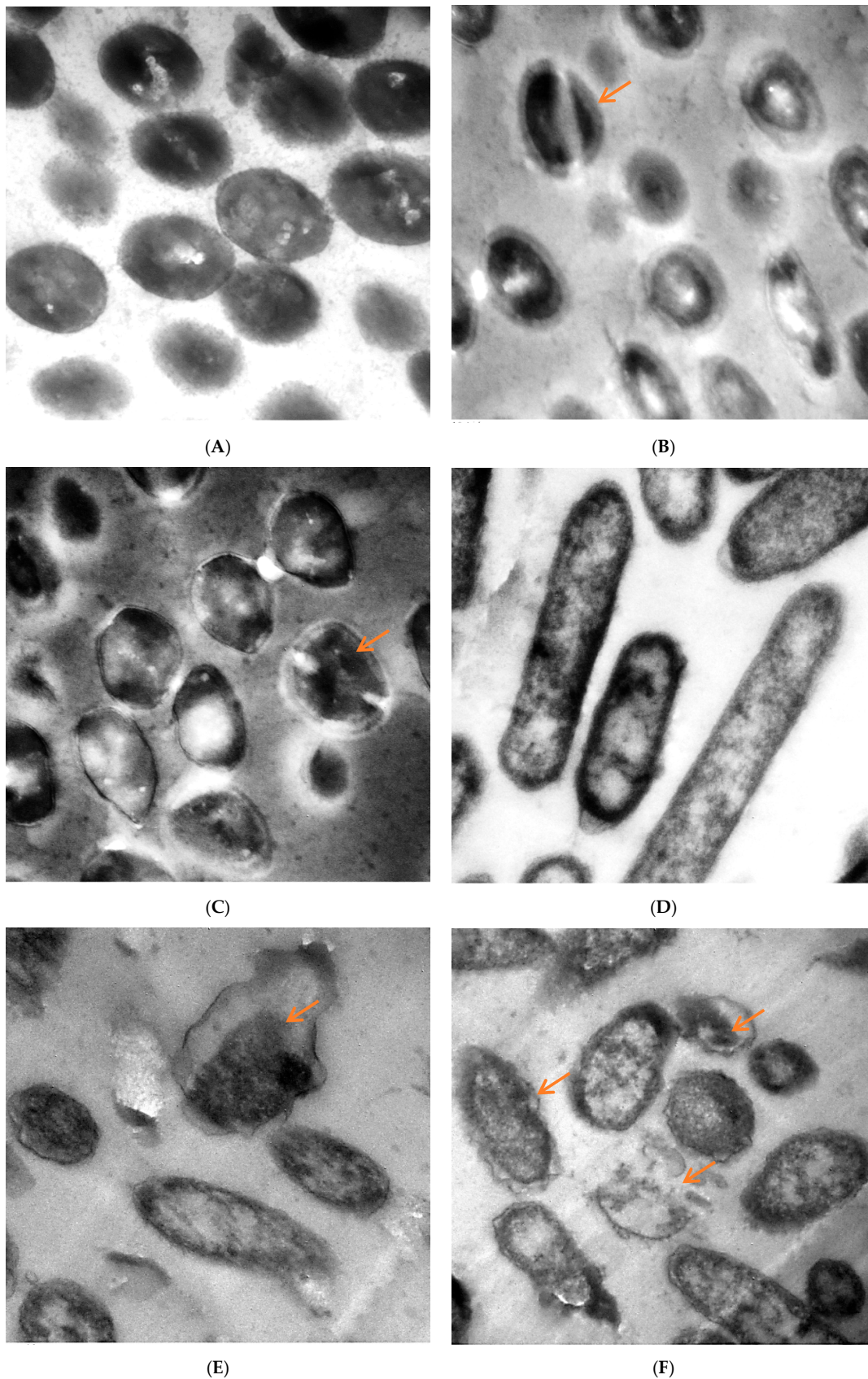
\* Ketoconazole, or \* Gentamycin as antifungal or antibiotic, respectively. ND \*\*, not detected. \*\*\* Minimum inhibitory concentration.



**Figure 5.** Antimicrobial activities of *M. pulegium* extract against different bacteria and fungi: (E) *M. pulegium* extract; (D) methanolic loaded disc, (G) Gentamycin, (K) Ketoconazole.

TEM of untreated and treated *S. aureus* and *P. aeruginosa* with *M. pulegium* extract are shown in Figure 6. The untreated cells of *S. aureus* had a distinct structure with cocci shape and intact cellular walls, and showed no signs of damage or morphological change (Figure 6A). Some deformations were observed in *S. aureus* treated by *M. pulegium* extract (Figure 6B,C), including partially digested cell wall and exfoliated fragments from the surface of bacterial cell, exposition of the patches in the cytoplasmic membrane, lack of cell wall or its detachment from the membrane, thickening of the outer membrane, and abnormal septa with the presence of ghost cells. Toxic effects of the *M. pulegium* extract on the structure and function of the membrane have generally been used to describe the antimicrobial action of the extract and its components. Untreated *P. aeruginosa* cells revealed a normal rod-shaped structure with an intact outer membrane and the cytoplasmic membrane near the cell wall, and the intracellular content was well-maintained. The periplasmic area was narrow and homogenous (Figure 6D). Several alterations in cell shape were detected after treatment with *M. pulegium* extract, and the periplasmic space indicated separation of the cell wall from the plasma membrane. The internal structures were disordered, and the components of the bacterial cell membrane were distorted and distributed from their original form. Draining of the intracellular contents was seen at particular sites within the cells, mostly at the septal and polar regions, due to breaches of the cell membrane. There were numerous cells without membranes and null cells of *P. aeruginosa* (Figure 6E,F). Approximately the same alteration was recorded in *P. aeruginosa*, leading to cell death [50].





**Figure 6.** TEM micrographs of (A) untreated *S. aureus*, (B) *S. aureus* treated at 62.50 µg/mL and (C) at 100 µg/mL, (D) untreated *P. aeruginosa*, (E) *P. aeruginosa* treated with *M. pulegium* extract at 15.62 µg/mL and (F) at 30 µg/mL. Scale Bar = 100 nm, 5000×.

### 2.3. Antioxidant Activity of *M. pulegium* Extract

The extract of *M. pulegium* showed excellent antioxidant activity according to the DPPH scavenging method. DPPH scavenging (%) increased with the increment of concentration, and reached 88.1% at 1000 µg/mL (Table 4). Surprisingly, the IC<sub>50</sub> of *M. pulegium* extract was 18 µg/mL, compared with IC<sub>50</sub> of 15.0 µg/mL for ascorbic acid. The current results can therefore confirm the antioxidant activity of this extract. The current study suggested that the main contributors to the antioxidant potential are associated with phenolic and flavonoid contents. Strong antioxidant activity of *M. pulegium* extract has been documented via numerous techniques including DPPH, trolox equivalent antioxidant capacity, ferric reducing antioxidant power, and oxygen radical absorbance capacity [47]. Many studies have reported relationships between phenolic content and antioxidative activity in plants [10,11]. However, the IC<sub>50</sub> of the *M. pulegium* essential oil was 7.7 mg/mL [48] less than the current result (IC<sub>50</sub>, 18 µg/mL) for *M. pulegium* extract, although Aimad et al. [48] confirmed their obtained result using essential oil while our study dealt with the raw extract. Aimad et al. [48] suggested that the antioxidant activity of *M. pulegium* extract obtained by total antioxidant capacity and DPPH techniques may be due to the occurrence of pulegone.

**Table 4.** Antioxidant activity of *M. pulegium* extract.

| Concentration (µg/mL)                          | O.D * Mean | DPPH Scavenging (%) | SD ** | SE *** |
|--|------------|---------------------|-------|--------|
| 1000   | 0.172      | 88.1                | 0.003 | 0.001  |
| 500  | 0.215      | 85.1                | 0.004 | 0.001  |
| 250  | 0.307      | 78.7                | 0.002 | 0.001  |
| 125  | 0.419      | 70.9                | 0.003 | 0.001  |
| 62.5   | 0.529      | 63.3                | 0.003 | 0.001  |
| 31.25  | 0.653      | 54.7                | 0.005 | 0.001  |
| 15.62  | 0.762      | 47.1                | 0.006 | 0.002  |
| IC <sub>50</sub> of <i>M. pulegium</i> extract |            | 18 µg/mL            |       |        |
| IC <sub>50</sub> of ascorbic acid              |            | 15.0 µg/mL          |       |        |

\* OD, optical density; \*\* SD, standard deviation; \*\*\* SE, standard error.

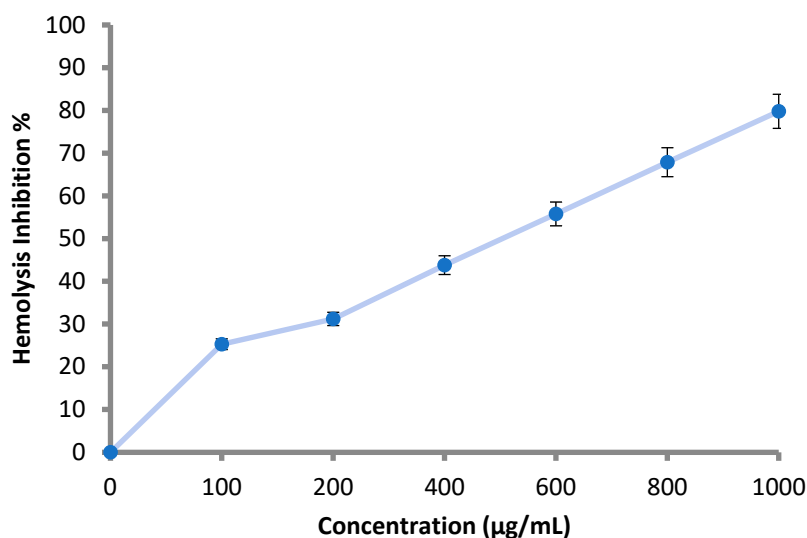
### 2.4. Hemolysis Inhibition by *M. pulegium* Extract

According to the obtained results, all the applied concentrations (100–1000 µg/mL) of the *M. pulegium* extract inhibited the lysis of the erythrocyte membrane as shown (Table 5 and Figure 7). Hemolysis inhibition increased with increment of the concentration, and reached 79.8% at 1000 µg/mL (Table 5). The result was compared using 200 µg/mL of indomethacin, where hemolysis inhibition was 91.0%. Hemolysis inhibition reflects the anti-inflammatory activity of *M. pulegium* extract, and the obtained results are in accordance with previous studies demonstrating that *M. pulegium* exhibited anti-inflammatory activities [41,51]. There are similarities between the membranes of red blood cells and lysosomal membrane contents; lysis of lysosomes occurs during inflammation. When the red blood cells are exposed to hypotonic conditions, cell membrane lysis is caused, leading to hemolysis in addition to haemoglobin oxidation. Prevention of hypo-tonicity leading to lysis of the red blood cell membrane that was performed to observe anti-inflammatory activity. The obtained results suggest a potential anti-inflammatory action that is probably due to the presence of pulegone-1,2-epoxide in the extract of the *M. pulegium*. This was noted in the GC-MS analysis, and it was previously demonstrated that pulegone can inhibit inflammation mechanisms [51,52]. Recently, Baali et al. [47] mentioned the promising activity of *M. pulegium* extract as a wound healing agent and documented a scientific rationale that confirmed the traditional application of this plant.

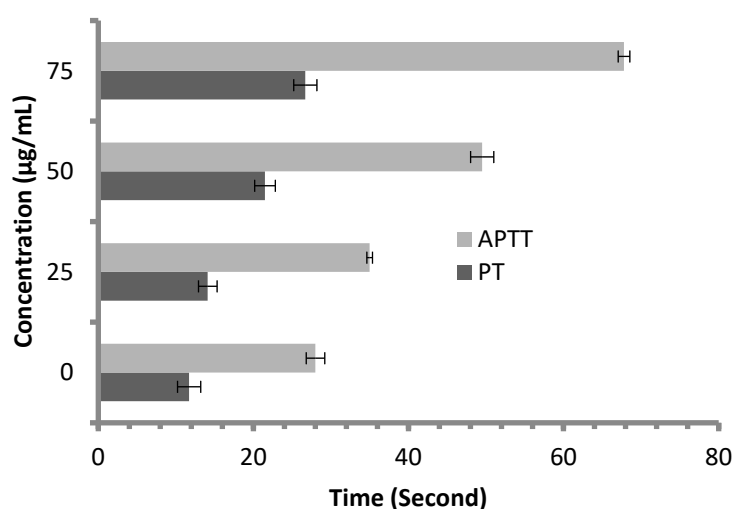
**Table 5.** Effect of *M. pulegium* extract on hemolysis inhibition.

| Concentration ( $\mu\text{g/mL}$ )   | Hypotonic O.D * | Hemolysis Inhibition % | SD ** | SE *** |
|--------------------------------------|-----------------|------------------------|-------|--------|
| Control                              | 1.038           | 0                      | 0.003 | 0.001  |
| 1000                                 | 0.301           | 79.8                   | 0.009 | 0.003  |
| 800                                  | 0.417           | 67.9                   | 0.005 | 0.002  |
| 600                                  | 0.527           | 55.8                   | 0.008 | 0.003  |
| 400                                  | 0.641           | 43.8                   | 0.002 | 0.001  |
| 200                                  | 0.766           | 31.2                   | 0.004 | 0.001  |
| 100                                  | 0.822           | 25.3                   | 0.008 | 0.002  |
| Indomethacin at 200 $\mu\text{g/mL}$ | 0.130           | 91.0                   | 0.005 | 0.002  |

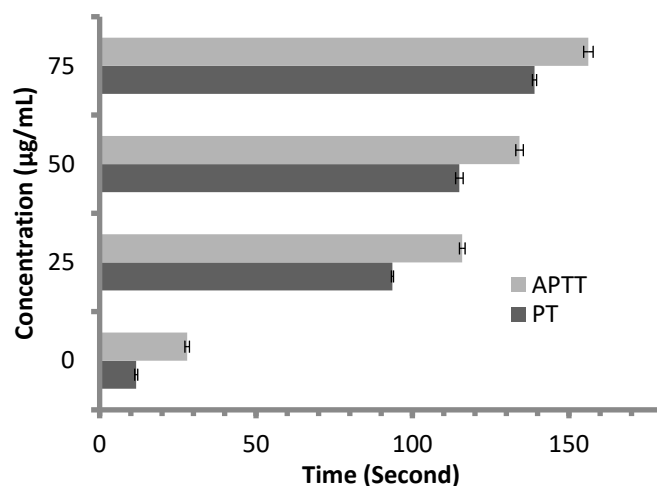
\* OD, optical density; \*\* SD, standard deviation; \*\*\* SE, standard error.

**Figure 7.** Hemolysis inhibition (%) at different concentration of *M. pulegium* extract 3.5. Anticoagulant Activity of *M. pulegium* Extract.

Anticoagulant activity of *M. pulegium* extract was evaluated using APTT assay, measuring the activity of all coagulation factors in the intrinsic pathway, and PT assay, measuring the activity of the extrinsic pathway. PT and APTT results increased with incremental concentrations of the *M. pulegium* extract (Figure 8). *M. pulegium* extract showed PT and APTT of 21.5 s and 49.5 s, respectively (Figure 8), while heparin as a positive control showed PT and APTT as 115 s and 134.2 s, respectively, at 50  $\mu\text{g/mL}$  (Figure 9). At 75  $\mu\text{g/mL}$  of *M. pulegium* extract, PT and APTT were at 26.7 s and 67.8 s, respectively. Recently Leite et al. [53] studied the anticoagulant activity of numerous plants, among which *Mentha crispa* (APTT = 51.25) showed greatest anticoagulant potential. Ku and Bae [54] suggested that the inhibition of intrinsic and common pathways was associated with the prolongation of APTT, while the inhibition of the extrinsic coagulation pathway was associated with the prolongation of PT. The anticoagulant activities exhibited by *M. pulegium* extract may be due to the presence of natural constituents as observed by GC-MS analysis in the current study. The obtained results proved that the *M. pulegium* extract was effective as an anticoagulation agent.



**Figure 8.** Anticoagulant activity of *M. pulegium* at different concentrations, represented by PT and APTT.



**Figure 9.** Anticoagulant activity of heparin at different concentrations, represented by PT and APTT.

### 2.5. Anticancer Activity of *M. pulegium* Extract

Cytotoxic activity of *M. pulegium* against two cancer cell lines (PC3 and MCF7) was assessed by MTT in vitro. The obtained results showed a dose-dependent decline in cancer cell line proliferation after exposure to *M. pulegium* extract. The highest cytotoxic activity was 97.17% and 96.71% against PC3 and MCF7, respectively, at 500 µg/mL (Table 6). PC3 was more resistant than MCF7 at low concentrations of *M. pulegium* extract, where acute toxicity was 0.60 and 10.34% for PC3 and MCF7, respectively, at 31.25 µg/mL. Toxicity was 28.88 and 48.46% for PC3 and MCF7, respectively, at 62.5 µg/mL. A similar study was conducted previously [55], where the anticancer potential of *M. pulegium* was documented against the MCF7 breast cancer cell line with acute toxicity of 88.547% at 500 µg/mL. However, the anticancer activity was recorded against the two tested cells, but different values of IC<sub>50</sub> (97.99 and 80.21 µg/mL for PC3 and MCF7, respectively) were recorded according to the type of cell (Table 6) or plant origin, as reported in other literature. Low IC<sub>50</sub> (59 and 48.86 µg/mL against MCF7 and A549, respectively) of *M. pulegium* L extract was recorded in another study [56]. Numerous authors have mentioned that the anti-proliferative and apoptotic activities of *M. pulegium* L extract, as well as those of other plants, may be due to the presence of flavanones, particularly hesperidin [10,20]. Cell death mechanism caused by the *M. pulegium* L extract was observed by cell morphology assay, where the acute toxicity was accompanied by a morphological change of PC3 and MCF7 (Figure 10), particularly at high concentrations. Cell shrinkage was observed with exposure

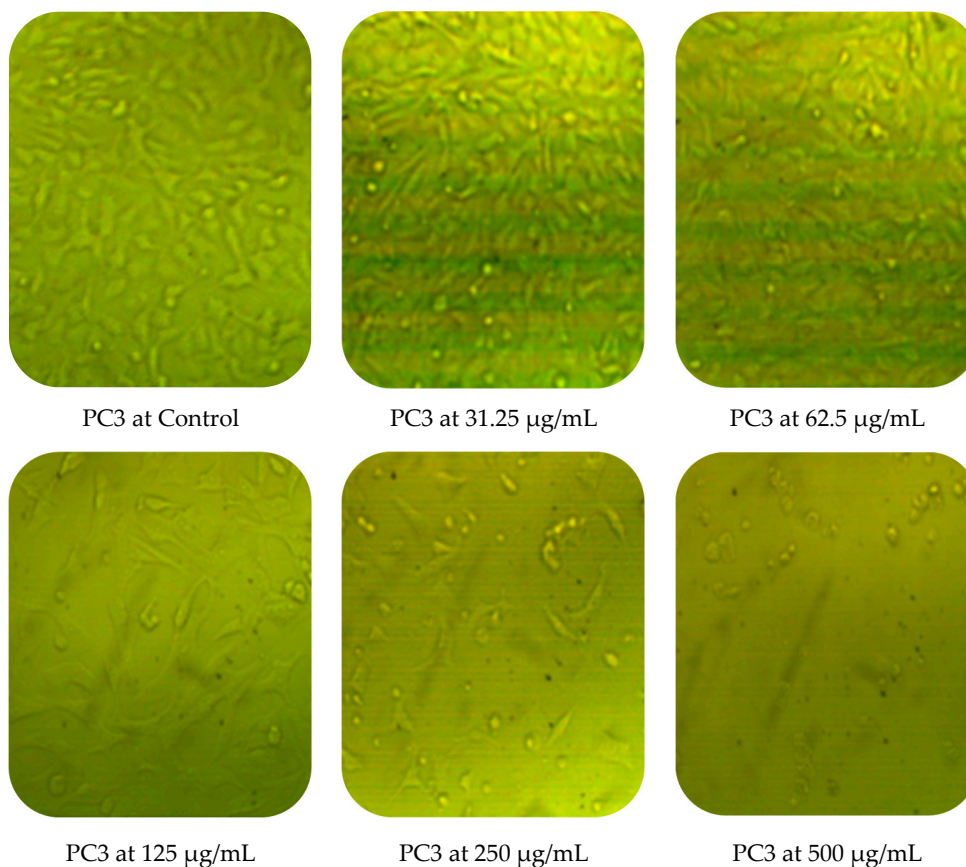


to 125  $\mu\text{g}/\text{mL}$ , in addition to the appearance of round cells and membrane breakdown with cell disruption at 250 and 500  $\mu\text{g}/\text{mL}$ , possibly underlining a genotoxic interference, although this postulation requires further study.

**Table 6.** Cytotoxicity of *M. pulegium* L extract against PC3 and MCF7 at different concentrations.

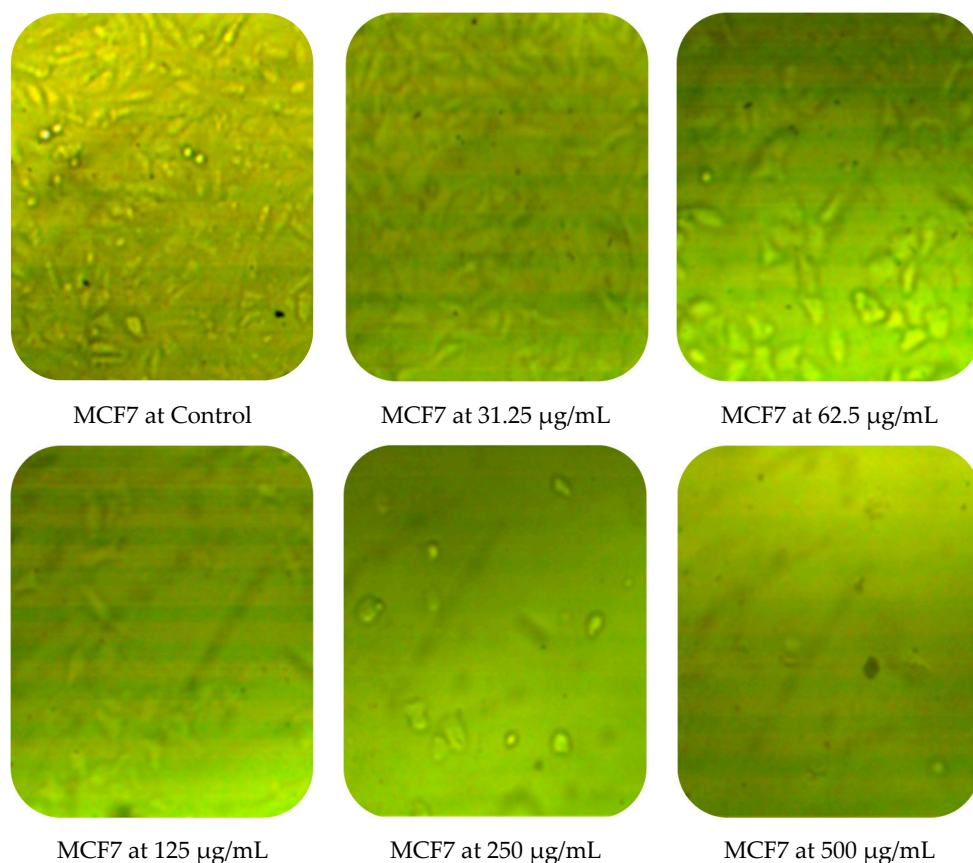
| Concentration<br>$\mu\text{g}/\text{mL}$ | PC3        |       |                               |            | MCF7       |       |                               |            |
|--|------------|-------|-------------------------------|------------|------------|-------|-------------------------------|------------|
|  | Mean O.D * | SE ** | Viability %                   | Toxicity % | Mean O.D * | SE ** | Viability %                   | Toxicity % |
| Control                                  | 0.554      | 0.010 | 100                           | 0.0        | 0.476      | 0.01  | 100                           | 0.0        |
| 31.25                                    | 0.55       | 0.007 | 99.40                         | 0.60       | 0.43       | 0.005 | 89.57                         | 10.43      |
| 62.5                                     | 0.39       | 0.008 | 71.12                         | 28.88      | 0.25       | 0.016 | 51.547                        | 48.46      |
| 125                                      | 0.18       | 0.007 | 32.31                         | 67.69      | 0.11       | 0.010 | 23.53                         | 76.47      |
| 250                                      | 0.11       | 0.005 | 19.19                         | 80.81      | 0.05       | 0.007 | 10.85                         | 89.15      |
| 500                                      | 0.06       | 0.001 | 2.83                          | 97.17      | 0.02       | 0.001 | 3.29                          | 96.71      |
| 1000                                     | 0.02       | 0.001 | 3.13                          | 96.87      | 0.02       | 0.001 | 3.71                          | 96.29      |
| IC <sub>50</sub>                         |            |       | 97.99 $\mu\text{g}/\text{mL}$ |            |            |       | 80.21 $\mu\text{g}/\text{mL}$ |            |

\* OD, optical density; \*\* SE, standard error.



**Figure 10.** Cont.



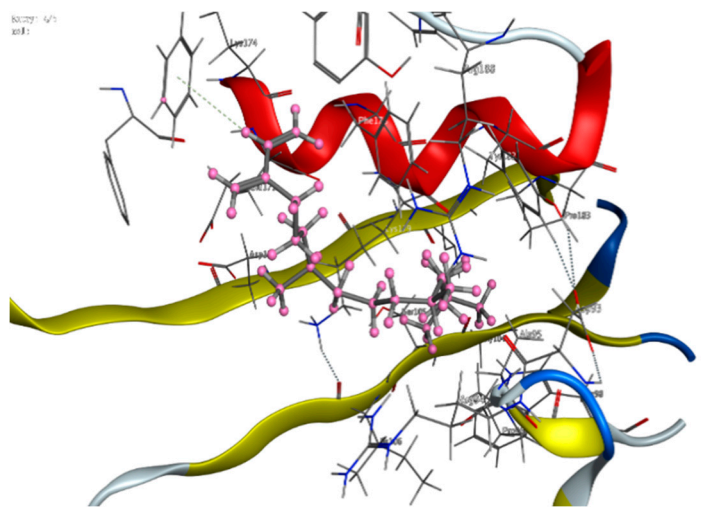
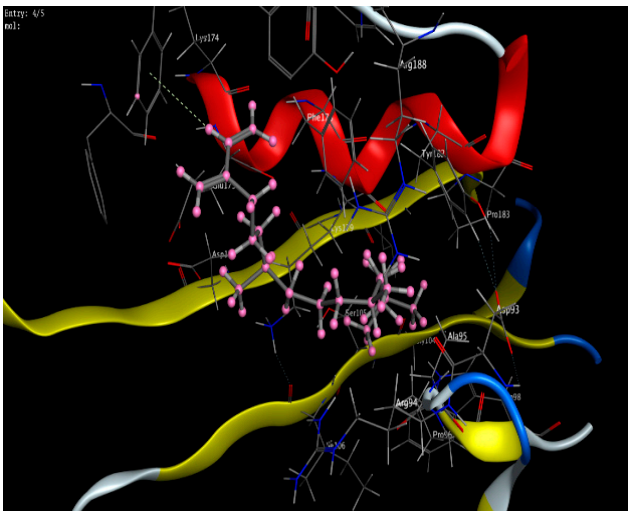


**Figure 10.** Effects of different concentrations of *M. pulegium* extract on morphological changes of PC3 and MCF7.

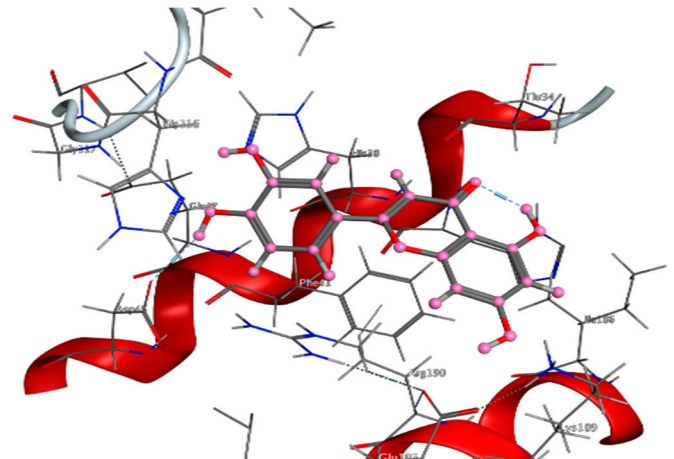
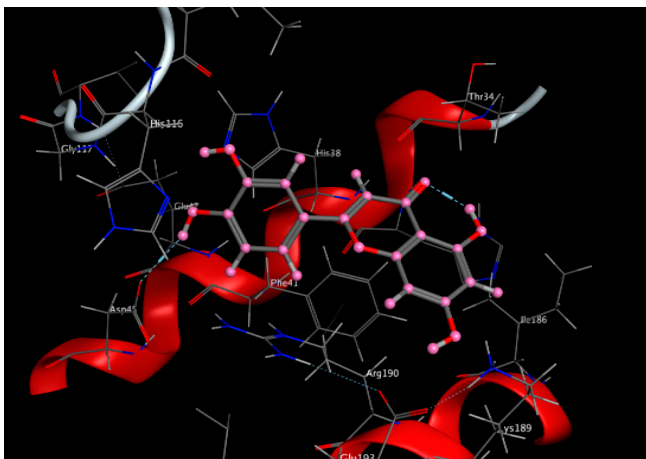
### 2.6. Molecular Modeling: Docking Study

As described in the “Method” section, the current docking protocol was authenticated for all target receptors. Neophytadiene, luteolin, chrysoeriol, and kaempferol were analyzed for binding affinity with *P. aeruginosa* (PDB = 7BCZ), crystal structure of *E. coli* DNA (PDB = 7C7N), crystal structure of human prostate (PDB = 3QUM), and the crystal structure of the breast-cancer-associated protein (PDB = 1JNX) (Figure 11). The amino acid residue interactions of different proteins with active compounds are presented in Figure 12. Molecular modeling calculation was carried out to investigate the binding free energies of this inhibitor inside the target receptor, as mentioned previously [23].

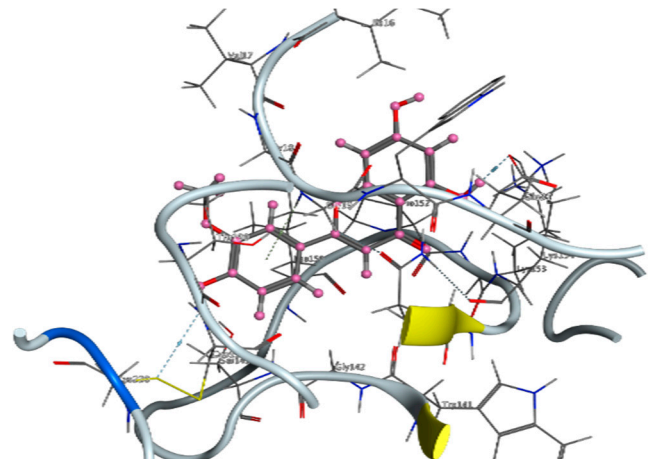
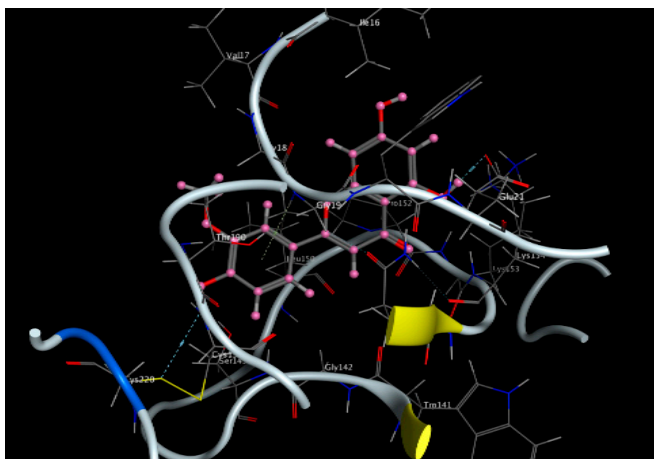
Docking of neophytadiene with active receptor sites of (7BCZ) indicated the occurrence of a hydrogen bond among C1 atoms in the ligand with aromatic ring PHE191 amino acids residue at a distance of 4.37 °A. Also, the interaction between luteolin and the active site binding of *E. coli* DNA (7C7N) revealed the presence of a hydrogen donor atom between the O28 atom in the ligand and ASP 45 amino acid residue, at a distance of 3.26 °A. The docked chrysoeriol and kaempferol were superimposed on the crystal structure of human prostate (PDB = 3QUM) and the crystal structure of the breast-cancer-associated protein (PDB = 1JNX), with RMSD values (1.0946, 1.7265) and (1.7518, 1.3456), respectively, and binding free energies of ( $-6.3350 \text{ kcal}\cdot\text{mol}^{-1}$ ,  $-5.0644 \text{ kcal}\cdot\text{mol}^{-1}$ ) and ( $-6.3593 \text{ kcal}\cdot\text{mol}^{-1}$ ,  $-5.2916 \text{ kcal}\cdot\text{mol}^{-1}$ ), respectively. The total obtained results of docking scores and energies of compounds are presented in Table 7. The interactions between almost all atoms in the compounds and amino acid residues of enzymes are shown in Table 8. The effectiveness of the compounds tested in the current study was evaluated on the basis of docking scores, as described previously [24,25] for other compounds, namely deguelin and its derivatives.



Structures of 7BCZ protein showing the binding sites with neophytadiene

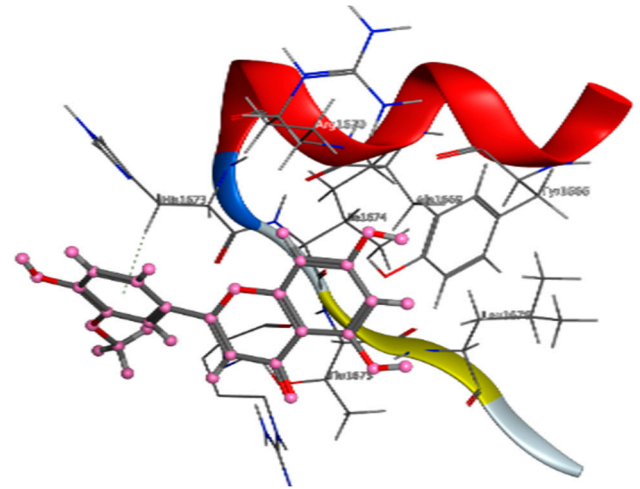
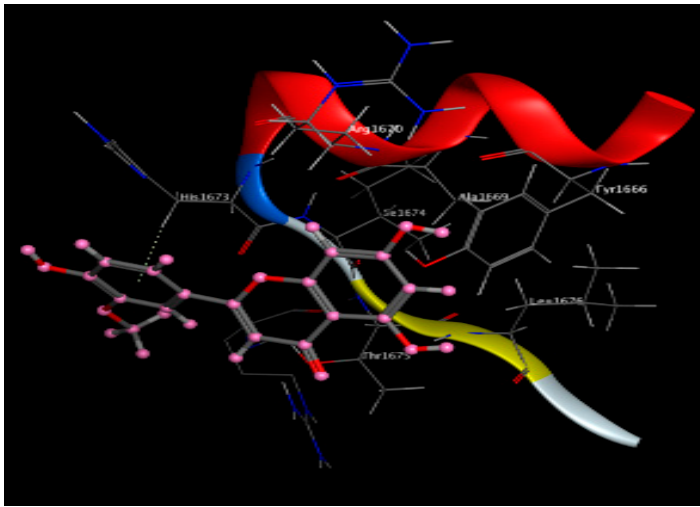


Structures of 7C7N protein showing the binding sites with luteolin

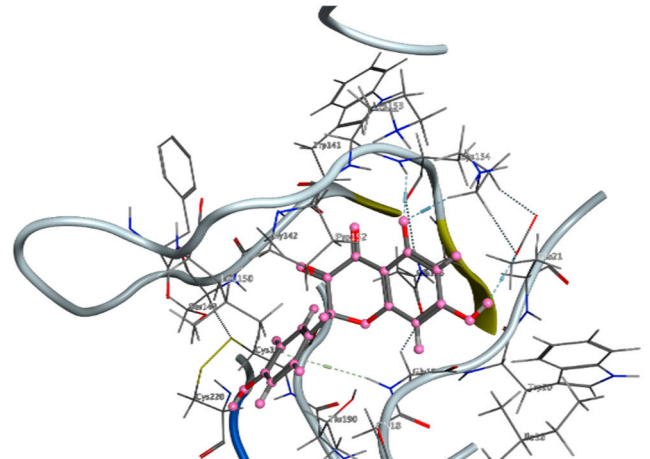
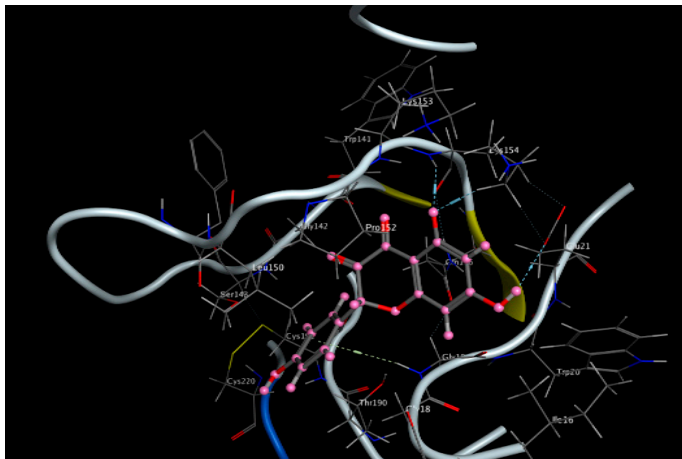


Structures of 3QUM protein showing the binding sites with chrysoeriol

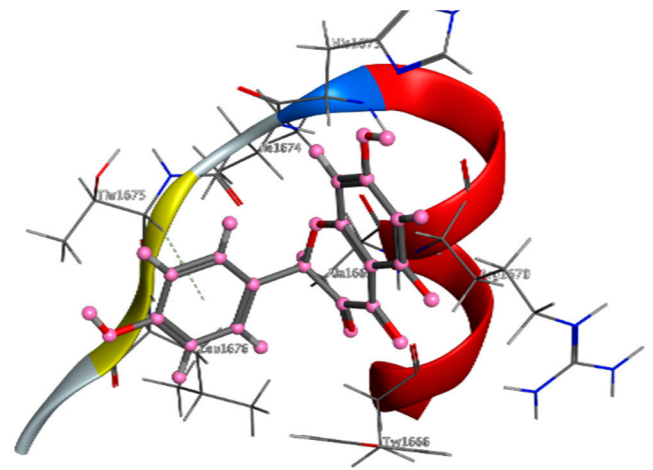
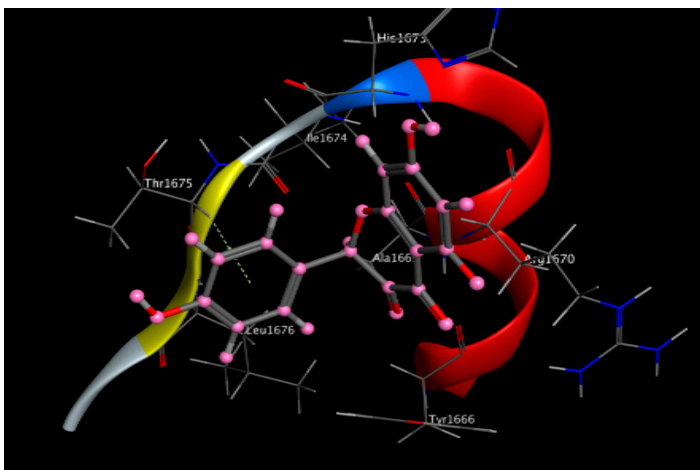
Figure 11. Cont.



Structures of 1JNX protein showing the binding sites with chrysoeriol



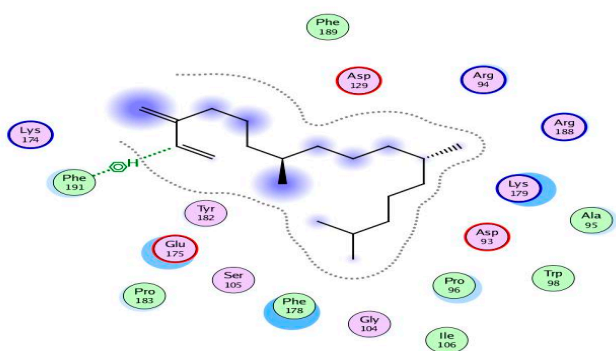
Structures of 3QUM protein showing the binding sites with kaempferol



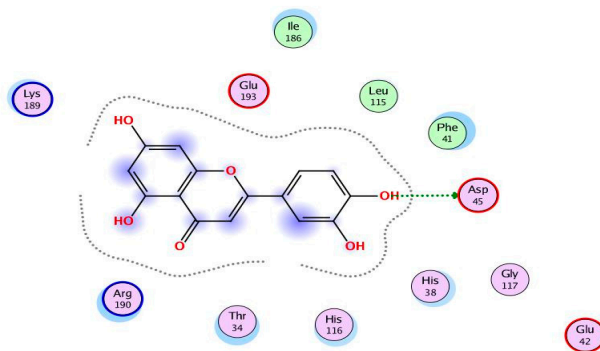
Structures of 1JNX protein showing the binding sites with kaempferol

**Figure 11.** Structures of different proteins showing their respective binding sites for active compounds.

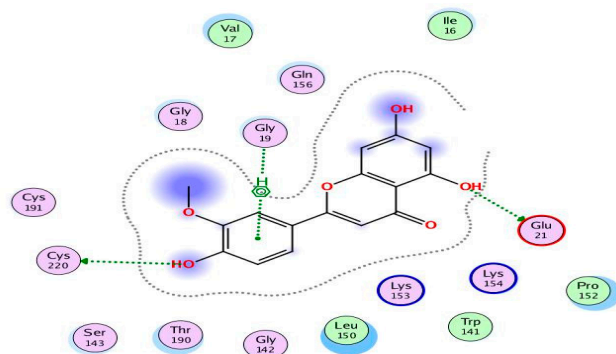




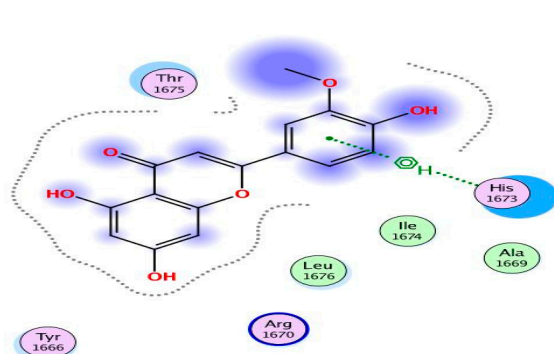
Amino acid residues interaction of 7CBCZ with neophytadiene



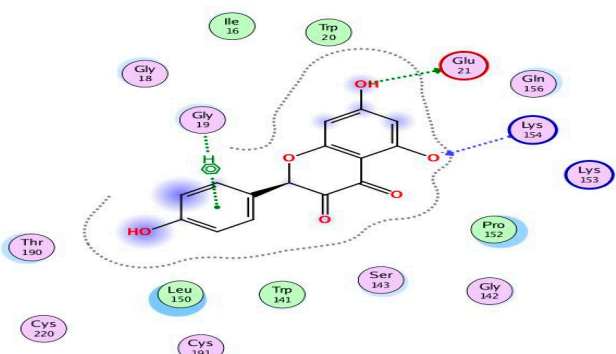
Amino acid residues interaction of 7C7N with luteolin



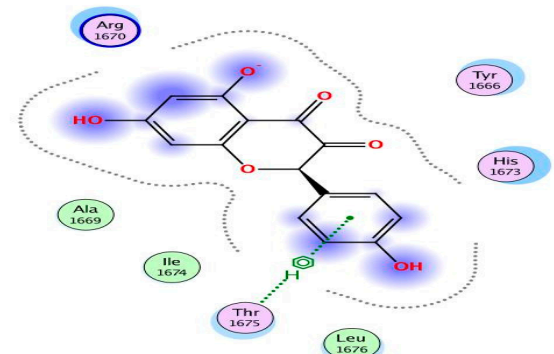
Amino acid residues interaction of 3QUM with chrysoeriol



Amino acid residues interaction of 1JNX with chrysoeriol



Amino acid residues interaction of 3QUM with kaempferol



Amino acid residues interaction of 1JNX with kaempferol

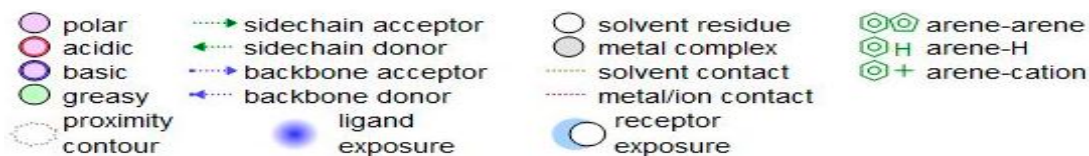


Figure 12. Amino acid residue interactions of different proteins with active compounds, and the representative key of the types of interaction between active compounds and proteins.

**Table 7.** Docking scores and energies of compounds with 7BCZ, 7C7N, 3QUM, and 1JNX proteins.

| Compound      | Receptor | mseq | S       | rmsd_refne | E_conf   | E_place  | E_score1 | E_refne  | E_score2 |
|---------------|----------|------|---------|------------|----------|----------|----------|----------|----------|
| Neophytadiene | 7BCZ     | 1    | -7.1580 | 1.2822     | 24.5600  | -57.8312 | -7.8518  | -31.7035 | -7.1580  |
| Neophytadiene | 7BCZ     | 1    | -6.9125 | 2.3982     | 13.1121  | -49.8856 | -8.0566  | -31.8298 | -6.9125  |
| Neophytadiene | 7BCZ     | 1    | -6.8013 | 1.3756     | 26.6025  | -54.2040 | -7.6831  | -27.2223 | -6.8013  |
| Neophytadiene | 7BCZ     | 1    | -6.7640 | 1.1550     | 86.2092  | -72.2128 | -8.5950  | -31.8384 | -6.7640  |
| Neophytadiene | 7BCZ     | 1    | -6.6269 | 1.8035     | 10.0713  | -39.6643 | -7.4382  | -32.7957 | -6.6269  |
| Luteolin      | 7C7N     | 1    | -5.2683 | 1.2104     | -30.1027 | -53.0515 | -10.7811 | -21.5450 | -5.2683  |
| Luteolin      | 7C7N     | 1    | -5.2098 | 1.0321     | -33.5362 | -58.5419 | -10.8163 | -25.1026 | -5.2098  |
| Luteolin      | 7C7N     | 1    | -5.1764 | 2.0993     | -31.8830 | -50.5262 | -9.9086  | -23.7861 | -5.1764  |
| Luteolin      | 7C7N     | 1    | -5.0736 | 1.1214     | -34.9514 | -74.7281 | -10.4863 | -24.8905 | -5.0736  |
| Luteolin      | 7C7N     | 1    | -5.0535 | 0.6345     | -30.7855 | -62.0186 | -9.5565  | -20.9379 | -5.0535  |
| Chrysoeriol   | 3QUM     | 1    | -6.3350 | 1.0946     | -15.0487 | -75.6312 | -11.4227 | -36.8350 | -6.3350  |
| Chrysoeriol   | 3QUM     | 1    | -6.2655 | 1.7381     | -20.6640 | -84.1280 | -11.5780 | -37.1195 | -6.2655  |
| Chrysoeriol   | 3QUM     | 1    | -6.2171 | 0.7234     | -14.4679 | -76.0750 | -11.4356 | -33.1380 | -6.2171  |
| Chrysoeriol   | 3QUM     | 1    | -6.1692 | 2.1441     | -15.4071 | -78.4542 | -11.5705 | -36.1058 | -6.1692  |
| Chrysoeriol   | 3QUM     | 1    | -6.1571 | 1.1044     | -15.2604 | -84.8887 | -11.8802 | -35.8331 | -6.1571  |
| Chrysoeriol   | 1JNX     | 1    | -5.0644 | 1.7265     | -17.5303 | -62.1961 | -9.9634  | -25.0239 | -5.0644  |
| Chrysoeriol   | 1JNX     | 1    | -5.0101 | 0.9227     | -15.7642 | -42.2704 | -9.1471  | -24.0765 | -5.0101  |
| Chrysoeriol   | 1JNX     | 1    | -4.9971 | 1.6559     | -14.5476 | -43.9988 | -9.2024  | -23.3186 | -4.9971  |
| Chrysoeriol   | 1JNX     | 1    | -4.9616 | 1.4387     | -16.8284 | -44.6490 | -9.6921  | -23.2991 | -4.9616  |
| Chrysoeriol   | 1JNX     | 1    | -4.8836 | 2.0835     | -17.2031 | -52.3723 | -9.3292  | -24.7783 | -4.8836  |
| Kaempferol    | 3QUM     | 1    | -6.3593 | 1.7518     | -54.3337 | -63.8852 | -12.2329 | -35.5105 | -6.3593  |
| Kaempferol    | 3QUM     | 1    | -6.2456 | 1.7957     | -53.7207 | -84.7006 | -11.2066 | -33.4084 | -6.2456  |
| Kaempferol    | 3QUM     | 1    | -5.9016 | 1.4734     | -50.9818 | -65.7556 | -11.2285 | -27.7261 | -5.9016  |
| Kaempferol    | 3QUM     | 1    | -5.8823 | 1.1136     | -48.9402 | -68.5376 | -11.2814 | -31.7357 | -5.8823  |
| Kaempferol    | 3QUM     | 1    | -5.8293 | 1.7911     | -50.1359 | -67.1079 | -11.2273 | -31.8993 | -5.8293  |
| Kaempferol    | 1JNX     | 1    | -5.2916 | 3.4082     | -57.8566 | -54.0164 | -7.9325  | -25.2956 | -5.2916  |
| Kaempferol    | 1JNX     | 1    | -5.2264 | 1.3456     | -58.4060 | -23.5095 | -7.0560  | -24.2946 | -5.2264  |
| Kaempferol    | 1JNX     | 1    | -4.9339 | 2.1388     | -58.0519 | -35.5016 | -6.9689  | -23.3973 | -4.9339  |
| Kaempferol    | 1JNX     | 1    | -4.9124 | 2.8442     | -58.3635 | -31.1941 | -7.3013  | -22.9019 | -4.9124  |
| Kaempferol    | 1JNX     | 1    | -4.8548 | 1.5679     | -59.0462 | -43.3097 | -8.0880  | -23.1065 | -4.8548  |

Where S = final score, which is the score of the last stage that was not set to none. rmsd = root mean square deviation of the pose, in Å, from the original ligand. This field was exist if the site definition was identical to the definition of ligand. rmsd\_refine = root mean square deviation between the pose before refinement and the pose after refinement. E\_conf = energy of the conformer. If there was a refinement stage, this is the energy calculated at the end of the refinement. Note that for force field refinement, by default, this energy was calculated with the solvation option set to Born. E\_place = score from the placement stage. E\_score 1, E\_score 2 = scores from rescoring stages 1 and 2. E\_refine = score from the refinement stage, calculated to be the sum of the van der Waals electrostatic and solvation energies, under the generalized Born solvation model (GB/VI).



**Table 8.** Interaction of active compounds with proteins.

| Neophytadiene Interaction with 7BCZ Protein |                    |             |          |              |
|---|--------------------|-------------|----------|--------------|
| Neophytadiene                               | Receptor           | Interaction | Distance | E (kcal/mol) |
| C 1   | 6-ring PHE 191 (A) | Pi-H        | 4.73     | −0.5         |
| Luteolin interaction with 7C7N protein      |                    |             |          |              |
| Luteolin                                    | Receptor           | Interaction | Distance | E (kcal/mol) |
| O 28  | OD2 ASP 45 (A)     | H-donor     | 3.26     | −1.7         |
| Chrysoeriol interaction with 3QUM protein   |                    |             |          |              |
| Chrysoeriol                                 | Receptor           | Interaction | Distance | E (kcal/mol) |
| O 23  | SG CYS 220 (P)     | H-donor     | 4.29     | −1.0         |
| O 26  | OE2 GLU 21 (P)     | H-donor     | 2.85     | −2.0         |
| 6-ring                                      | N GLY 19 (P)       | Pi-H        | 4.40     | −0.6         |
| Chrysoeriol interaction with 1JNX protein   |                    |             |          |              |
| Chrysoeriol                                 | Receptor           | Interaction | Distance | E (kcal/mol) |
| 6-ring                                      | CB HIS 1673 (X)    | Pi-H        | 3.65     | −0.5         |
| Kaempferol Interaction with 3QUM protein    |                    |             |          |              |
| Kaempferol                                  | Receptor           | Interaction | Distance | E (kcal/mol) |
| O 28  | OE2 GLU 21 (P)     | H-donor     | 3.26     | −0.9         |
| O 27  | N LYS 154 (P)      | H-acceptor  | 3.01     | −3.1         |
| O 27  | CE LYS 154 (P)     | H-acceptor  | 3.18     | −2.6         |
| 6-ring                                      | N GLY 19 (P)       | Pi-H        | 4.38     | −1.5         |
| Kaempferol interaction with 1JNX protein    |                    |             |          |              |
| Kaempferol                                  | Receptor           | Interaction | Distance | E (kcal/mol) |
| 6-ring                                      | CA THR 1675 (X)    | Pi-H        | 4.09     | −1.1         |

### 3. Material and Methods

#### 3.1. Collected Plant Samples and Process of Extraction

Leaves, stems, and flowers of *M. pulegium* were collected from Menofia Governorate throughout August 2021. The collected plant parts were washed with running tap water followed by sterile water to eliminate any dust, then dried and ground by electric mixer. Then, the obtained dried plants (140 g) were ground using the electric mixer into a fine powder, homogenized, then macerated in a stoppered container with 500 mL of 85% methanol for a period of 7 days. Then, for conventional extraction, the extract was processed in a sonicator at 40 °C for 60 min. This extract was filtered, then concentrated in a vacuum at 40 °C for 30 min using Rota vapor, to provide 7.0 g crude extract. Chemical analysis and biological activity analyses were performed on the extract.

#### 3.2. Analysis of *M. pulegium* Extract by Gas Chromatography-Mass Spectrometry (GC-MS)

GC (THERMO Scientific Corp., Waltham, MA, USA)-MS (ISQ Single Quadrupole Mass Spectrometer) was used for *M. pulegium* extract analysis. Chromatographic conditions included film thickness of the capillary column TR-5MS of 30 m × 0.32 mm × 0.25 µm. Temperature cycling to 60 °C was used for the initial analysis, incrementing to 240 °C, followed by a gradual increase to the maximum temperature 290 °C by 30 °C/min, remaining isothermally constant up to 2 min. The protected temperature for injector was 250 °C and for MS transfer was 260 °C. One mL/min of high purity helium at constant flow was applied as carrier. One µL extract of *M. pulegium* was injected using the Auto sampler AS1300 linked to GC in the split manner. At a range of *m/z* 40–1000 achieved by electron energy with 70 eV application, the spectra of the electron ionization mass were

collected in full scan mode. By calculation of mass spectra and retention time (RT) of the detected contents, these were identified by comparison with the existing evidence in the mass spectral library at the National Institute of Standards and Technology [1].

### 3.3. High-Performance Liquid Chromatography (HPLC) for Flavonoid and Phenolic Contents Determination

The analysis of flavonoid and phenolic contents of the extract was performed using HPLC. The chromatographic conditions included HPLC-(Agilent 1100) (Agilent, Santa Clara, CA, USA), equipped with two LC pumps, a UV/Vis detector, 125 mm × 4.60 mm, with 5 µm particle size of the C18 column. Twenty µL of the extract was injected in HPLC using methanol and 1/25 of acetic acid/water (60:40 v/v) as a mobile solvent for separation of phenolic acids. In case of flavonoids separation, the used mobile solvent with an isocratic flow rate of 1.0 mL min<sup>-1</sup> was methanol/water in a 50:50 ratio v/v, adjusted using phosphoric acid to obtain pH 2.8. The obtained peaks of the separated flavonoid and phenolic content were compared with the different standard stock solutions of pure phenolic and flavonoids in methanol extract, using the RT (retention time) and UV-Vis spectra [57].

### 3.4. Antimicrobial Potential of *M. pulegium* Extract

Study of the antimicrobial potential of *M. pulegium* extract was carried out using well diffusion assay against bacteria including four bacterial species (*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*), as well as yeast (*Candida albicans*), and filamentous black mold (*Mucor circinelloides*). Ain Shams University Hospitals, Egypt were the source of the tested bacteria, while Assiut University Mycological Centre provided the tested fungi. A hole (6 mm) was cut aseptically into agar using a sterile cork borer; extract solution (100 µL) was poured into the hole. Next, the plates were inoculated, then kept for 30 min in the refrigerator for proper diffusion of the extract, followed by incubation at 28 °C for fungi and 37 °C for bacteria and yeast. The diameter of the apparent inhibition zone (IZ) surrounding the wells was measured (mm) [58]. Gentamycin and ketoconazole were used as antibiotic and antifungal, respectively, as positive control. Dimethyl sulfoxide (DMSO) as a solvent of plant extract was applied as control. MIC was detected by microdilution protocol by making serial dilutions of 1 mL of *M. pulegium* extract (dissolved in DMSO) in broth, using a new pipette for each subsequent dilution step. The antimicrobial and broth solutions were dispensed into the plastic microdilution trays. The inoculum was prepared by making saline suspension of bacterial or yeast colonies ( $2 \times 10^8$  colony-forming units/mL) from 20 h old, cultivated agar plates; 100 µL from the relevant suspension was inoculated into each well. Then the inoculated macro-dilution tubes were incubated ( $35 \pm 2$  °C) for 18 h (specific for bacteria) or for 24 h (specific for yeast) The MIC of *M. pulegium* extract was detected by measuring OD at 600 nm, and compared with growth without treatment as control [18].

### 3.5. Antioxidant Activity of *M. pulegium* Extract

The antioxidant activity of the extract was observed using a 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test. Different concentrations of the dried methanolic extract of *M. pulegium* were dissolved in DMSO. Then the different concentrations (15–1000 µg/mL) were prepared by dilution, and 2 mL of 0.1 mM prepared DPPH in methanol as solvent solution was added to each of the diluted extracts, then mixed by vortex, followed by keeping under shade for 1 h. The addition of 2 mL of the DPPH solution to 1 mL of methanol was utilized as a negative control [59]. The mean of three absorbance readings of the plant extract and negative control at 514 nm was recorded, then the activity of DPPH radical scavenging (%) was determined by the following formulation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Control sample absorbance} - \text{Extract sample absorbance}}{\text{Control sample absorbance}} \times 100$$

Inhibitory concentration 50% (IC<sub>50</sub>) of the extract was determined.

Ascorbic acid at different amounts from 5 to 80 µg/mL was used to determine antioxidant activity as a positive control, as mentioned in the description of the antioxidant activity of the extract.

### 3.6. Anti-Hemolytic Activity Evaluation of *M. pulegium* Extract In Vitro

For the erythrocyte preparation, 3 mL of fresh whole blood was collected from one of the current research authors, in heparin blood collection tubes. The collected red blood cells from the blood sample obtained via centrifugation at 3000 rpm for 10 min were dissolved using normal saline solution (*v/v* supernatant), then reconstituted up to 40% *v/v* suspension by sodium phosphate (10 mM) as isotonic buffer solution adjusted to pH 7.4. Successive dilutions of plant extract in DMSO (from 100 to 1000 µg/mL) were added to 5 mL of distilled water to obtain the hypotonic solution.

Suspension of erythrocyte (0.1 mL) was added to the prepared hypotonic solution, followed by gentle mixing and incubation (37 °C) for 60 min. For the next incubation period it was centrifuged at 1200 × *g* for 5 min, and the collected supernatant was subjected to assessment of its haemoglobin content via spectrophotometer (Milton Roy) at 540 nm. Indomethacin at 200 µg/mL was used as a positive control. Haemolysis inhibition (%) caused by the plant extract was calculated by the equation:

$$\text{Haemolysis inhibition (\%)} = 1 - \frac{\text{AD2} - \text{AD1}}{\text{AD3} - \text{AD1}} \times 100$$

where AD1 is the absorbance of the treated sample in the isotonic solution, AD2 is the absorbance of the treated sample in the hypotonic solution, AD3 is absorbance of the control sample in the hypotonic solution. Hemolysis % was assessed with regard to hemolysis in distilled water (100%) [60].

### 3.7. Coagulate Activity Evaluation of *M. pulegium* Extract In Vitro

The dried methanolic extract of *M. pulegium* was dissolved in dimethyl sulfoxide (DMSO) then evaluated as an anticoagulant agent using prothrombin time (PT) and activated partial thromboplastin time (APTT) as a classical coagulant test. Nine parts of blood collected from an author of the current research were added to one part of 3.2% sodium citrate, followed by centrifugation (5000 × *g* rpm) for 10 min, and the collected supernatant was utilized for carrying out the PT and APTT tests. Citrated normal human plasma was combined with different dilutions of the extract and incubated for 3 min at 37 °C. The reagents of the PT and APTT were incubated at 37 °C for 3 min before carrying out the tests, then 0.10 mL of the APTT reagent was added to each mixture of plasma and extract, then incubated at 37 °C for 5 min, then 0.10 mL of 0.025 mol/L CaCl<sub>2</sub> (pre-incubated at 37 °C for 3 min) was added, and the clotting time was recorded. Mixtures of citrated normal human plasma with different dilutions of the extract were amended with 0.20 mL PT reagent, then clotting times were recorded to assess PT. These procedures were carried out using heparin as control [57].

### 3.8. Antitumor Assay and Morphological Characteristics of the Treated Cancer Cells

Cytotoxicity of the extract against MCF7 and PC3 cells was performed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The solution of MTT was prepared in phosphate buffered saline (PBS) as 5 mg/mL. The cells (1 × 10<sup>5</sup> cells/mL) were inoculated in the 96-well tissue culture plate, followed by incubation at 37 °C for 24 h to develop a complete monolayer sheet. Then, the growth medium was decanted from the 96-well micro titer plates after confluent sheets of cells were formed, and the cell monolayer was washed twice with wash media. Six-fold dilutions of the extract were made in Roswell Park Memorial Institute (RPMI) 1640 medium with serum (2%) as maintenance medium. Each dilution (0.1 mL) was tested in different wells, leaving three wells as control receiving only maintenance medium, then incubated at 37 °C and scanned for any physical signs of

toxicity. To each well, 20  $\mu$ L MTT solution was added, and the MTT was mixed into the media. It was placed on a shaking desk for 5 min at around 150 rpm, then incubated at 37 °C under 5% CO<sub>2</sub> for 1–5 h to permit the metabolization of MTT. The resulting MTT metabolic product was re-suspended in 200  $\mu$ L of DMSO, followed by shaking at 150 rpm for 5 min to mix it into the solvent. The optical density (correlated directly with cell numbers) was measured at 560 nm with subtract background at 620 nm. Cell lines in growth medium without plant extract were studied as a control [59].

### 3.9. Molecular Docking

A molecular modeling study was undertaken using the Molecular Operating Environment (MOE) [61], a drug discovery software platform that integrates visualization, modeling and simulations, as well as methodology development, into one package. Molecular modeling studies were performed using Molecular Operating Environment (MOE, 2019) software on Dell Core i7 processor with 1.9 GH, 16 GB memory, and a Windows 10, 64-bit operating system. Energy minimizations were performed using MOE, with a RMSD (root mean square deviation) gradient of 0.05 kcal/mol and MMFF94X force field, and partial charges were automatically calculated. The X-ray crystallographic structures were obtained from the Protein Data Bank (PDB) ([www.rcsb.org/pdb](http://www.rcsb.org/pdb) accessed on 19 July 2022); the PDB files were (7BCZ), (7C7N), (3QUM) and (1JNX).

The target proteins were prepared for docking by:

1. Removing the water molecules and co-ligand from the active site of the protein.
2. Addition of hydrogen atoms to the structure, with standard geometry.
3. Using the MOE site finder to generate the active binding sites, to create the dummy sites as the binding pocket.
4. Saving the obtained pocket was saved in MOE, to be used for predicting the ligand and protein.

Moreover, the native ligands were minimized to their lowest energy using the MMFF94 force field. Then, the final form was obtained after 3D protonation and the correction process. The general docking scenario was run to 100<sub>ns</sub> on the rigid receptor atoms, and the ligands were placed in the site using the triangle matcher method. London dG was utilized as a scoring function and the GBVI/WSA dG methods were used for rescoring. The five best poses were ranked by their binding free energy (S, kcal/mol), and the lengths of hydrogen bonds between the compounds and amino acids in the protein did not exceed 3.5 Å. RMSD and RMSD-refine fields were used for pose-with-pose comparison of the results in the co-crystal ligand position before and after amendment, respectively.

### 3.10. Statistical Analysis

The current experiments were realized in replicate, and results were therefore calculated as  $\pm$  standard deviation (SD) or standard error (SE) means. GraphPad Prism<sup>®</sup> (version 5.0, San Diego, CA, USA) software was applied to obtain graphs for the IC<sub>50</sub> values of DPPH radical scavenging activity.

## 4. Conclusions

The obtained findings highlight the phytochemical and pharmaceutical application of *M. pulegium* extracts. In the current study, the extract was rich in several active components that showed biological activity including anticancer, antimicrobial, antioxidant, and as anti-hemolytic activity. These data suggest that this plant extract is a potential candidate for further experiments towards its use as an alternative drug. The MOE molecular modeling environment was used to study inhibitor activity for (7BCZ), (7C7N), (3QUM) and (1JNX). It was found that the energy scores of the molecular docking study were in good agreement with the experimental results. In conclusion, the antibacterial and antifungal activities of *M. pulegium* extract obtained from plants growing in Egypt were more effective than traditional antibiotics against the microorganisms studied.

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## Abbreviations

|          |  |
|----------|--|
| HPLC     | High-performance liquid chromatography                         |
| GC-MS    | Gas Chromatography-Mass Spectrometry                           |
| RT       | Retention Time   |
| MIC      | Minimum inhibitory concentration                               |
| DPPH     | 2-Diphenyl-1-picryl-hydrazyl-hydrate                           |
| PT       | Prothrombin time   |
| APTT     | Activated partial thromboplastin time                          |
| IZ       | Inhibition zone  |
| DMSO     | Dimethyl sulfoxide   |
| TEM      | Transmission Electron Microscopy                               |
| SD       | Standard deviation   |
| SE       | Standard error   |
| AD1      | Absorbance of treated sample in the isotonic solution          |
| AD2      | Absorbance of treated sample in the hypotonic solution         |
| PBS      | phosphate buffered saline                                      |
| AD3      | Absorbance of control treated sample in the hypotonic solution |
| O.D      | Optical density  |
| RPMI     | Roswell Park Memorial Institute                                |
| MCF7     | Michigan Cancer Foundation-7                                   |
| PC3      | Prostate cancer cell   |
| MOE      | Molecular Operating Environment                                |
| RMSD     | Root mean square deviation                                     |
| GBVI/WSA | Generalized-Born Volume Integral/Weighted Surface area         |

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