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Docking-Based Evidence for the Potential of ImmunoDefender: A Novel Formulated Essential Oil Blend Incorporating Synergistic Antiviral Bioactive Compounds as Promising Mpro Inhibitors Against SARS-CoV-2

Ayoub Ksouri,^{a,b} Anis Klouz,^c Balkiss Bouhaouala Zahar,^{a,b,c} Fathi Moussa,^d and Mounir Bezzarga^a

^a Université Tunis El-Manar, Campus Universitaire Farhat Hached B.P. n° 94 - ROMMANA 1068 Tunis, Tunisie.

^b Laboratoire des Biomolécules, Venins et Applications Théranostiques, Institut Pasteur de Tunis, 13 Place Pasteur, BP74, Tunis Belvedere- University of Tunis El Manar, Tunisia

^c Faculté de Médecine de Tunis, 15 rue Djebel Lakhdhar, La Rabta, 1007-Université Tunis El Manar, Tunisia

^d Institute of Physical Chemistry, CNRS - UMR 8000. University Paris - Saclay. Rue Noetzlin, 91190 Gif-sur-Yvette. France.

* Correspondence:

Abstract: Essential oils have demonstrated antiviral activity, but their toxicity can hinder their use as therapeutic agents. Recently, some essential oil components have been used within safe levels of acceptable daily intake limits without causing toxicity. The "ImmunoDefender," a novel antiviral compound made from a well-known mixture of essential oils, is considered highly effective in treating SARS-CoV-2 infections. The components and doses were chosen based on existing information about their structure and toxicity. Blocking the Main Protease (Mpro) of SARS-CoV-2 with high affinity and capacity is critical for inhibiting the virus's pathogenesis and transmission. *In-silico* studies were conducted to examine the molecular interactions between the main essential oil components in "ImmunoDefender" and SARS-CoV-2 Mpro. The screening results showed that six key components of ImmunoDefender formed stable complexes with Mpro via its active catalytic site with binding energies ranging from -8.75 to -10.30 kcal/mol, respectively for Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, Syzyginin B, Procyanidin C1, and Tenuifolin. Furthermore, three essential oil bioactive inhibitors, Cinnamtannin B1, Cinnamtannin B2, and Pavetannin C, have a significant ability to bind to the allosteric site of the main protease with binding energies of -11.12, -10.74, and -10.79 kcal/mol. These results suggest that these essential oil bioactive compounds may play a role in preventing the attachment of the translated polyprotein to Mpro, inhibiting the virus's pathogenesis and transmission. These components also had drug-like characteristics similar to approved and effective drugs, suggesting further pre-clinical and clinical studies are needed to confirm the generated *in-silico* outcomes.

Keywords: ImmunoDefender; Essential oils (EO); Bioactive molecules; antiviral; SARS-CoV-2; Main-Protease; active & allosteric sites

1. Introduction.

The COVID-19 pandemic has caused global human health threats, resulting in a large number of infections, severe forms of the disease, long-term health consequences, and high mortality rates, especially among older and vulnerable populations [1, 2]. As an obligate intracellular pathogen, the SARS-CoV-2 virus requires entry into host cells to multiply, and the crucial role of the non-structural protein nsp5 or Main protease in the virus's life cycle has been identified [3, 4, 5]. Since SARS-CoV-2 Mpro has no human homolog, it is considered an ideal target for antiviral treatments [6, 8].

The development of new drugs against COVID-19 is a time-consuming and expensive process, and natural sources have been considered a crucial therapeutic approach. Recent studies have suggested that herbal extract substances and essential oils possess antiviral, anti-inflammatory, and immunomodulatory properties [9, 22, 23, 24].



Additionally, using a blend of essential oils can provide a broader range of therapeutic benefits, as each oil may have unique properties that can target different aspects of the condition being treated. By using a mixture of 10 essential oils, we can potentially achieve a more well-rounded and effective treatment approach compared to using just a few oils.

Furthermore, the use of multiple essential oils in a blend is a common practice in aromatherapy and has been shown to be safe and effective. Studies have shown that aromatherapy can have beneficial effects on a range of conditions [5,6,7].

This study investigates the potential of a mixture essential oil treatment approach for COVID-19, exploring the interaction of essential oil components with viral key protein targets inside of cells. Essential oils contain a complex mixture of chemical compounds that can act synergistically with each other to enhance their overall efficacy. This synergy is a well-known phenomenon in aromatherapy and is one of the reasons why blends of essential oils are often used to achieve specific therapeutic effects [49].

The suggested EO components possess antiviral, anti-inflammatory, and immune-stimulating properties and actions, making them ideal candidates for the development of a natural antiviral compound. A mixture of bioactive compounds consisting of small, efficient, and tolerable amounts of essential oils (EOs) is combined with a natural organic excipient to enhance absorption. Sesame oil is a widely utilized natural and non-aqueous excipient in pharmaceutical formulations. Its primary function is to serve as a vehicle for lipophilic drugs and active ingredients.

The major components of each essential oil have been identified, and chemical data and information (molecular properties and drug-like qualities) have been collected and tested for their ability to inhibit the crucial SARS-CoV-2 non-structural protein target Mpro. This study's findings could potentially contribute to the development of a natural and effective treatment approach for COVID-19.

2. Material & Methods

2.1. Essential oils quantification

The objective of this study was to determine the appropriate doses of essential oils and active constituents used in an antiviral herbal medicinal extract. To ensure the accuracy, safety, and efficacy of our study, we followed a rigorous methodology.

We selected a potent source of antiviral herbal medicinal extract, which comprised of ten pure essential oil substances. The essential oils used were Spearmint (*Mentha spicata*), Menthol (crystal), Water mint (*Mentha aquatica*), Cloves (*Syzygium aromaticum*), Peppermint (*Mentha piperita*), Pitch mint (*Mentha poulegatum*), Eucalyptus (*Eucalyptus*), Cinnamon (*Cinnamomum zeylenicum*), Cajeput (*Melaleuca cajuputii*), Camphor (*Cinnamomum camphora*), and Sesame (*Sesama*).

The quality of the essential oils used in our study was of paramount importance. We took great care to ensure that our selection of essential oils was based on the rates content of active compounds, as specified by the European pharmacopea, in order to achieve the highest level of purity and potency. Additionally, the source and extraction method of the essential oils were given careful consideration, ensuring that the integrity and quality of the oils were maintained.

To determine the appropriate doses of essential oils and active constituents, we took into account toxicity data and carefully considered the required molecular inhibition action while avoiding exceeding toxicity thresholds. We also provided a rationale for the selection of doses, citing previous studies or literature that supported their use. Furthermore, we used an inverse problem of mathematical expectation to determine the appropriate weighting for each compound based on their kinetic effects of temporarily irreversible inhibition of the key enzymes in the replication of the SARS-CoV-2 virus.

Our rigorous adherence to these high standards of selection and dosing ensured the accuracy, safety, and efficacy of the essential oils and active constituents used in our antiviral herbal medicinal extract. This methodology not only allowed us to achieve reli-

ble and reproducible results in our research, but also increased the reliability and validity of our findings.

We considered a dose of one gram of bioactive antiviral substances from essential oils of ten genera of aromatic plants. To determine the appropriate weighting for each compound, we used an inverse problem of mathematical expectation. We assigned weights to each compound based on the kinetic effects of temporarily irreversible inhibition of the key enzymes in the replication of the SARS-CoV-2 virus. We ensured that the accurate ingredient amount combination covers the molecular inhibition action without exceeding toxicity thresholds.

Table 1 and Table S1 in the Supplementary data (Appendix A) provide a well-described description of the immunoDefender formulation. We hope that these additions will provide a better understanding of our study and ensure that the results are replicable, accurate, safe, and effective.

Table 1. ImmunoDefender" EO list and detailed acceptable daily intake (ADI).

| Plants | Essential Oil (EO) | Acceptable daily intake (mg/kg body weight / day) | References |
|----------------------------------|--------------------|--|------------|
| Mentha spicata | Spearmint EO | 40 | [27] |
| Mentha | Menthol (crystals) | 4 | [28] |
| Mentha aquatica | Watermint EO | 5 | [29] |
| Syzygium aromaticum | Cloves EO | 2.5 | [30] |
| Mentha piperita | peppermint EO | 200 | [31] |
| Mentha poulegatum | Pennyroyal EO | 2.3 | [32] |
| Eucalyptus globulus | Eucalyptus EO | 4.28 | [33] |
| Cannula | Cinnamon EO | 0.1 | [34] |
| Melaleuca leucadendron cajuputii | Cajeput EO | 0.17 | [35] |
| Cinnamomum camphora | Camphor | 50 | [36] |
| Sesama | Sesame Oil | 15000 | [37] |

2.2. Data source: Protein and ligands molecule files preparation

In this study, a dataset of bioactive phytochemicals was obtained from the PubChem database [10], this database contains a wealth of chemical structures, bioactivity, health & safety, and spectra data. The 3D structures were retrieved in the ".sdf" format and converted to ".mol2" format using the OpenBabel web tool [11]. The X-Ray Crystal Structure of the SARS-CoV-2 Main Protease (3C-like protease) was retrieved from the RCSB Protein Data Bank (PDB) [12] with the code (PDB ID: 6LU7) [13]. This 3D structure was selected primarily based on its high resolution of 2.16 Å. Since the Main protease is functional only in homodimer form, two chains A & B or a dimeric form were modeled, starting from the 6LU7 monomer. After removing the N3 inhibitor from the active catalytic pocket of the Mpro, a refinement step was performed using the RELAX application binary executable from the Rosetta package [15]. The protein and ligand structure files were prepared in ".pdbqt" format as follows: hydrogen atoms were added using the PDB2PQR software [16] to the query input file (.pdb for receptor & .mol2 for ligands) with a pH of 7.4. The output ".pqr" file was converted to ".pdb" and then to the ".pdbqt" file using the MGLTools software [17].

2.3. Receptor-ligand Docking process

In order to conduct a thorough analysis of molecular interactions, a comparative docking approach was utilized, employing AutoDock Vina [18] for blind docking and SwissDock for driven docking. SwissDock [19], which utilizes the EADock DSS docking software [20], generates a large number of binding modes (BMs) and calculates their CHARMM energies on a grid. The most favorable BMs are ranked based on the FACTS implicit solvation model, and then clustered to obtain the most favorable clusters. This process allows for accurate docking assays to be conducted in just a few minutes. For the

driven docking approach, active site residues of the Main Protease were identified using literature and checked with the molecular visualization system PyMOL. The grid box around these active sites was then set to the active site residues with a spacing of 1 angstrom.

2.4. Drug-likeness prediction for the major EO bioactive compound

In addition to the molecular docking study, an analysis of the drug-likeness score was conducted using Molsoft software [29]. This analysis considered several key molecular properties, including molecular weight, number of hydrogen bond donors and acceptors, octanol/water partition coefficient, water solubility, molecular polar surface area and volume, pKa of the most basic/acidic group, and the Blood-Brain Barrier score. The drug-likeness chemical fingerprints scores were then projected onto a large-scale data template standard graph of training approved drug sets, as well as non-drug compounds as a negative control. To ensure the accuracy of the data, the approved drug sets were sourced from the World Development Indicators (WDI) database. This comprehensive approach enabled a thorough assessment of the drug-likeness potential of the compounds under investigation.

3. Results

The objective of our study was to investigate the potential inhibitory effects of ImmunoDefender essential oil components on the SARS-CoV-2 viral particle's non-structural protein Mpro. To achieve this, we performed molecular docking simulations of Mpro with various ligands, including major components of the essential oil, ensuring that each ingredient was accurately dosed within safe limits of toxicity and acceptable daily intake (ADI). In determining appropriate doses, we carefully considered toxicity data and the required molecular inhibition action, while citing previous studies or literature supporting their use. We also employed a mathematical approach to assign appropriate weights to each compound based on their kinetic effects on the crucial enzymes implicated in the replication of SARS-CoV-2 (Mpro) and the acceptable daily intake of each compound (Mathematical matrix data is available upon request from the corresponding author) . This analysis enabled us to pinpoint several bioactive compounds that displayed robust binding affinity to Mpro and exhibited significant inhibitory effects on the key enzymes. These findings suggest the potential utility of these compounds in the development of novel therapeutic options for treating COVID-19. The estimated ΔG (kcal/mol), with which ligand binds to the active catalytic pocket of the targeted protein also to the allosteric sites mainly involved in the interaction with the viral polyprotein, was the principal output of the docking carried out using AutoDock Vina as a driven approach and SwissDock as a blind approach for molecular interaction studies. The docking outputs of the ligands were summarized in Table 2 and Table 4 for the Mpro target protein. For docking data generated via AutoDock Vina software (driven docking based on grid complexes around the active catalytic pockets sites), complexes with the best conformations of Mpro binding energy were not relevant compared to the results generated using Swissdock software (Blind docking against the whole targeted protein structures Mpro) (data details are well illustrated in Table S2 : Appendix A. Supplementary data. Complex outputs of Swissdock were visualized in ChemeraX software [21], and the 3D interaction of the protein-ligand complex is shown in (Figure 1). The proposed essential oil (EO) mixture of ImmunoDefender product has been found to contain 24 bioactive molecule ligands (see Table S1 : Appendix A. Supplementary data) . In-silico interaction simulations with the target protein Mpor have demonstrated the presence of a significant number of bioactive ligands that interact with high affinity with the proteins. Among these ligands, Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, Syzyginin B, Procyanidin C1, and Tenuifolin have been shown to interact with remarkable stability and high affinity with the target protein. These ligands are capable of ensuring stable complex conformations via their active catalytic pocket sites, with a free binding energy of -9.56, -9.40, -10.30, -10.10, -9.05 and -8.75 (kcal/mol), respectively.

Furthermore, it is noteworthy that nirmatrelvir, the bioactive molecule present in the Paxlovid brand of Pfizer, was tested using the same computational approach of docking. A value of -9.24 was obtained, indicating that the ligands in ImmunoDefender product's EO mixture have a higher affinity and stability compared to nirmatrelvir. This finding highlights the potential of ImmunoDefender product's EO mixture in providing therapeutic benefits through its interaction with target proteins.

Intriguingly, the homodimeric structure of the target protein revealed a significant free binding energy in its concave region. Specifically, Cinnamtannin B1, Cinnamtannin B2, and Pavetannin C1 exhibited free binding energies of -11.12, -10.74, and -10.79 (kcal/mol), respectively, within this region. Further analysis showed that these ligands established several hydrogen-bond interactions with specific amino acids of the Mpro group, including Glu14, Asn95, Lys97, Lys100, Pro122, and Asp155 for Cinnamtannin B1, Glu14, Gly15, Ala70, Gly71, Ser121, and Tyr154 for Cinnamtannin B2, and Glu14, Met17, Gly71, Lys97, and Asn119 for Pavetannin C1. This indicates that these ligands possess high affinity and stability towards the target protein, and can form stable complex conformations via their active catalytic pocket sites, making them promising candidates for further investigation.

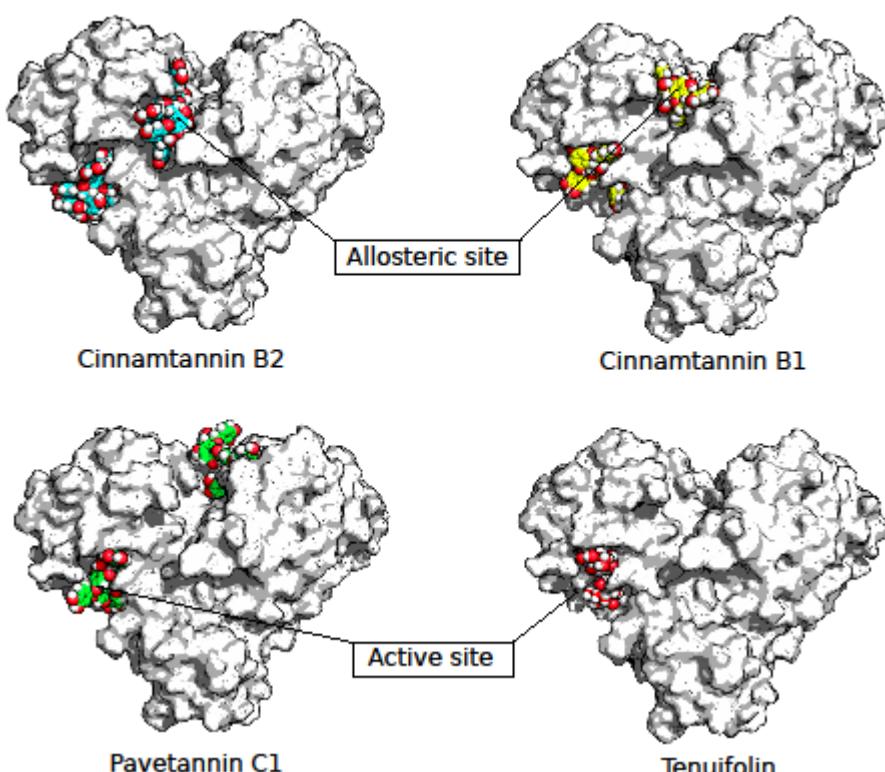


Figure 1. Inhibition of SARS-CoV-2 Main Protease Active and Allosteric Sites by Bioactive Ligands. The SARS-CoV-2 main protease (Mpro) is depicted as a gray surface, while Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, and Tenuifolin are shown (colored by element) as yellow, blue, green, and red spheres, respectively, interacting with both active and allosteric sites.

Figure 1 clearly displays the Mpro-ligand complexes, which were visualized using the PyMOL molecular visualizer software [14]. Notably, several ligands, including Tenuifolin and Procyanidin C1, exhibited remarkable potential in blocking Mpro enzymes, as outlined in Table 2.

Table 2. Docking molecular simulation of the main bioactive compounds of "ImmunoDefender" EO mixture against the Sars-Cov2 Mpro .

| No | Ligands | Ligands Topo-logical Polar Surface Area (Å ²) | Binding affinity (kcal/mol) “Active site” | Binding affinity (kcal/mol) “Allosteric site” |
|----|-----------------|---|--|--|
| 1 | Cinnamtannin B1 | 320 | -9.56 | -11.12 |
| 2 | Cinnamtannin B2 | 431 | -9.40 | -10.74 |
| 3 | Pavetannin C1 | 431 | -10.30 | -10.79 |
| 4 | Syzyginin B | 349 | -10.10 | - |
| 5 | Procyanidin C1 | 331 | -9.05 | - |
| 6 | Tenuifolin | 214 | -8.75 | - |
| 7 | Nirmatrelvir | 131 | -9.24 | - |

Of particular interest, the ligands displayed an exceptionally strong affinity for the Mpro enzyme, as evidenced by the formation of several hydrogen bonds (non-covalent interactions) with crucial amino acid residues in the concave region of the Mpro homodimeric form. This critical interaction region is depicted in detail in Figure 3.

The main bioactive molecules present in the proposed EO mixture of ImmunoDefender experimental products were carefully selected for further analysis. In order to predict their molecular properties and drug-likeness features, an overall drug-likeness score was computed for each compound based on several molecular properties values. As a result, Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, and Tenuifolin were found to have drug-likeness scores of 0.75, 0.79, 0.79, and 0.70, respectively (as shown in Table 3). Figure 2 illustrates how these score values conform to commercialized drug active molecules with a high level of tolerance and efficiency.

Table 3. Molecular Properties and Drug-likeness value of the EO mixture (ImmunoDefender product) main active compounds.:

| Active compounds | Molecular formula | Molecular weight (kDa) | Number of HBA | Number of HBD | Mol-LogP | LogS (mol es/L) | MolPSA (Å ²) | MolVol (Å ³) | pKa | BBB Score | Number of stereo centers | Drug-likeness model score |
|--|-------------------|------------------------|---------------|---------------|----------|-----------------|--------------------------|--------------------------|------------|-----------|--------------------------|---------------------------|
| Pa-vetan- nin C1 | C60H48O24 | 1152.25 | 24 | 19 | 3.46 | -3.04 | 344.95 | 1039.90 | <0. / 9.52 | 0 | 11 | 0.79 |
| Tenui- folin | C36H56O12 | 680.38 | 12 | 8 | 1.02 | -1.30 | 168.92 | 737.65 | <0. / 5.17 | 0.34 | 15 | 0.70 |
| Cin- nam- tannin B1 | C45H36 O18 | 864.19 | 18 | 14 | 2.40 | -2.60 | 257.26 | 782.13 | <0. / 9.52 | 0 | 8 | 0.75 |
| Cin- nam- tannin B2 | C60H48O24 | 1152.25 | 14 | 19 | 3.12 | -3.06 | 345.71 | 1039.96 | <0. / 9.52 | 0 | 11 | 0.77 |

Table 4. Table illustrating the main bioactive compounds of “ImmunoDefender” EO mixture making H-bound interaction with a group of amino acids.

| Residue | AA | Distance H-A | Distance D-A | Donor angle | Donor Atom | Acceptor Atom |
|---------|-----|--------------|--------------|-------------|------------|---------------|
| 14A | GLU | 2.18 | 3.07 | 150.97 | 28 [O2] | 311 [O-] |
| 14A | GLU | 1.74 | 2.69 | 163.65 | 20 [O2] | 306 [O2] |
| 95A | ASN | 3.53 | 3.94 | 106.70 | 1568 [Nam] | 24 [O2] |
| 95A | ASN | 2.51 | 3.10 | 118.88 | 24 [O2] | 1569 [O2] |
| 97A | LYS | 2.40 | 3.13 | 131.54 | 18 [O3] | 6275 [O2] |
| 97A | LYS | 2.37 | 3.31 | 150.71 | 1598 [N3+] | 63 [O2] |

| | | | | | | |
|------|-----|------|------|--------|-------------------|-----------|
| 100A | LYS | 3.22 | 3.75 | 115.47 | 6322 [Nam] | 22 [O2] |
| 122A | PRO | 2.91 | 3.86 | 162.78 | 30 [O2] | 1996 [O2] |
| 155A | ASP | 3.13 | 3.82 | 129.57 | 16 [O2] | 7160 [O-] |
| | | | | | -Cinnamtannin B2- | |
| 14A | GLU | 2.04 | 2.80 | 132.84 | 9387 [O2] | 4889 [O2] |
| 15A | GLY | 2.00 | 2.83 | 141.70 | 9395 [O2] | 4904 [O2] |
| 70A | ALA | 2.11 | 3.04 | 159.71 | 9389 [O3] | 5762 [O2] |
| 71A | GLY | 2.65 | 3.20 | 114.54 | 5769 [Nam] | 9381 [O2] |
| 71A | GLY | 3.19 | 3.64 | 110.53 | 9371 [O3] | 5772 [O2] |
| 121A | SER | 3.38 | 3.89 | 115.24 | 6570 [O3] | 9449 [O2] |
| 122A | PRO | 2.86 | 3.58 | 131.51 | 9403 [O2] | 6579 [O2] |
| 154A | TYR | 2.74 | 3.15 | 105.73 | 9401 [O2] | 2354 [O2] |
| 154A | TYR | 2.86 | 3.81 | 165.39 | 9385 [O2] | 2354 [O2] |
| | | | | | -Pavetannin C1- | |
| 14A | GLU | 1.90 | 2.82 | 156.43 | 9409 [O2] | 212 [O-] |
| 14A | GLU | 1.87 | 2.80 | 159.59 | 9385 [O3] | 207 [O2] |
| 17A | MET | 2.13 | 2.77 | 122.36 | 9397 [O2] | 240 [O2] |
| 17A | MET | 3.17 | 3.48 | 100.00 | 237 [Nam] | 9387 [O2] |
| 17A | MET | 2.07 | 2.82 | 131.63 | 9387 [O2] | 240 [O2] |
| 71A | GLY | 2.29 | 3.04 | 130.92 | 1087 [Nam] | 9397 [O2] |
| 97A | LYS | 3.14 | 3.87 | 128.25 | 1499 [N3+] | 9367 [O2] |
| 119A | ASN | 2.57 | 3.19 | 121.41 | 9403 [O2] | 1865 [O2] |

Interestingly, it was observed that Cysteine 145 (Cys145) is the main amino acid responsible for making polar, hydrophobic, and hydrogen bound contacts in the Mpro catalytic pocket between chains, within a distance of 4 ångström. This observation is illustrated in Figure 4. The presence of these interactions further highlights the potential of the selected bioactive molecules to effectively block the Mpro enzyme activity.

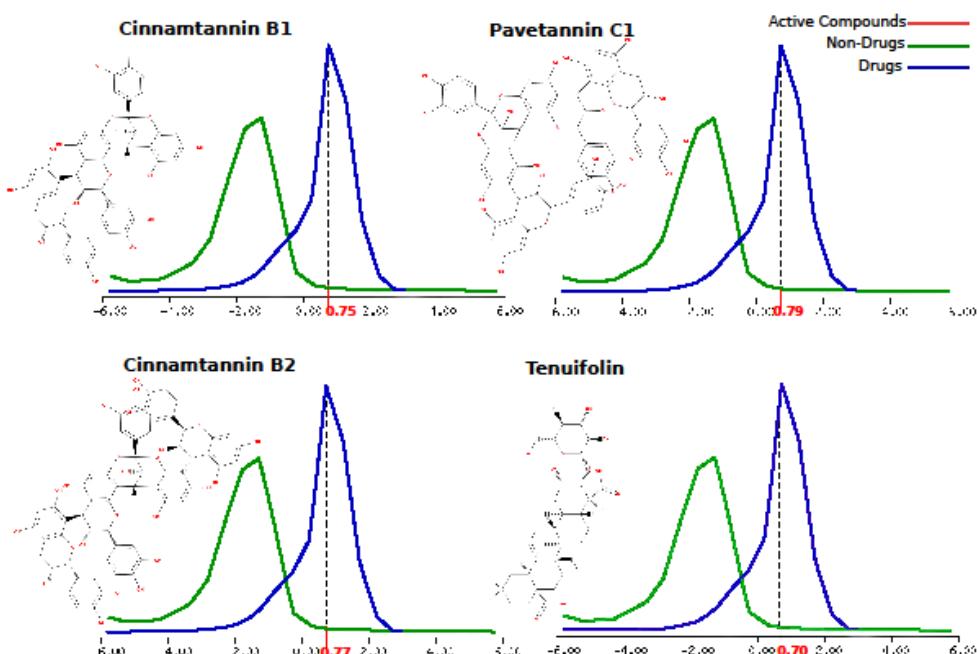


Figure 2. Chemical Structures and Drug-Likeness Scores of Main ImmunoDefender Bioactive Compounds. The 2D chemical structures of Main ImmunoDefender bioactive compounds are illustrated, along with their respective drug-likeness scores projected on a drug and non-drug graph template graphs. The values described on the x-axis are the Drug-Likeness Scores of the bioactive ligands in question provided by Molsoft software.

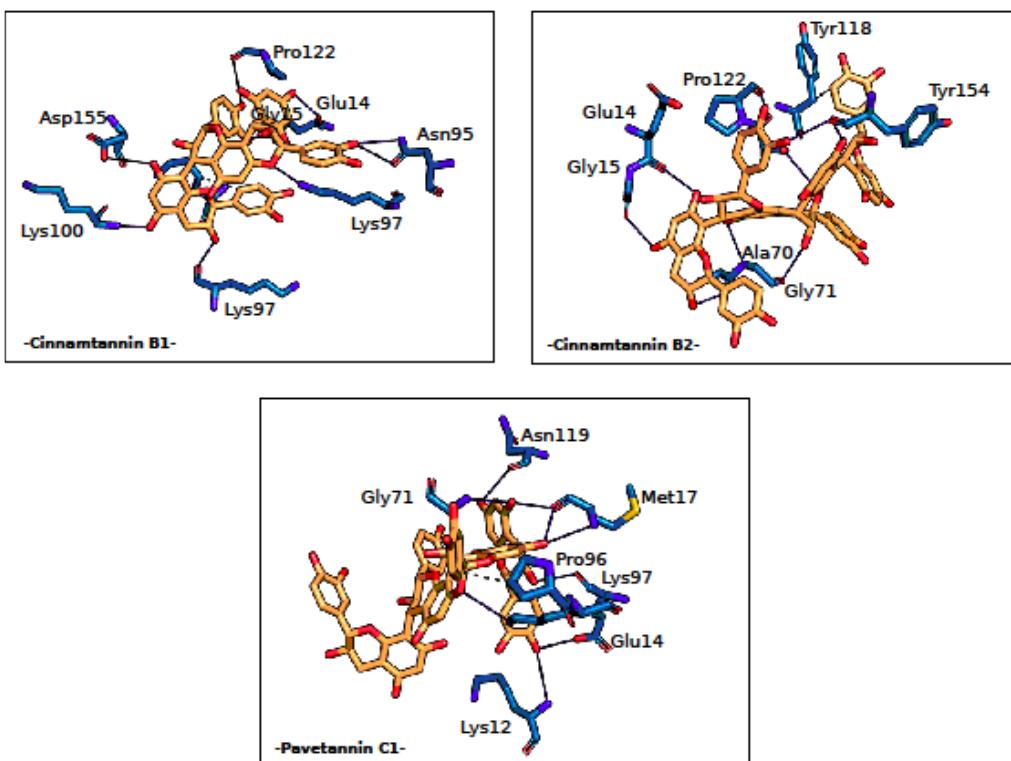


Figure 3. Non-Covalent Interactions of ImmunoDefender Bioactive Ligands with Allosteric Sites in SARS-CoV-2 Main Protease. ImmunoDefender bioactive ligands form non-covalent interactions, including hydrogen bonding, with crucial amino acid residues that are involved in the formation of allosteric sites in the SARS-CoV-2 main protease.

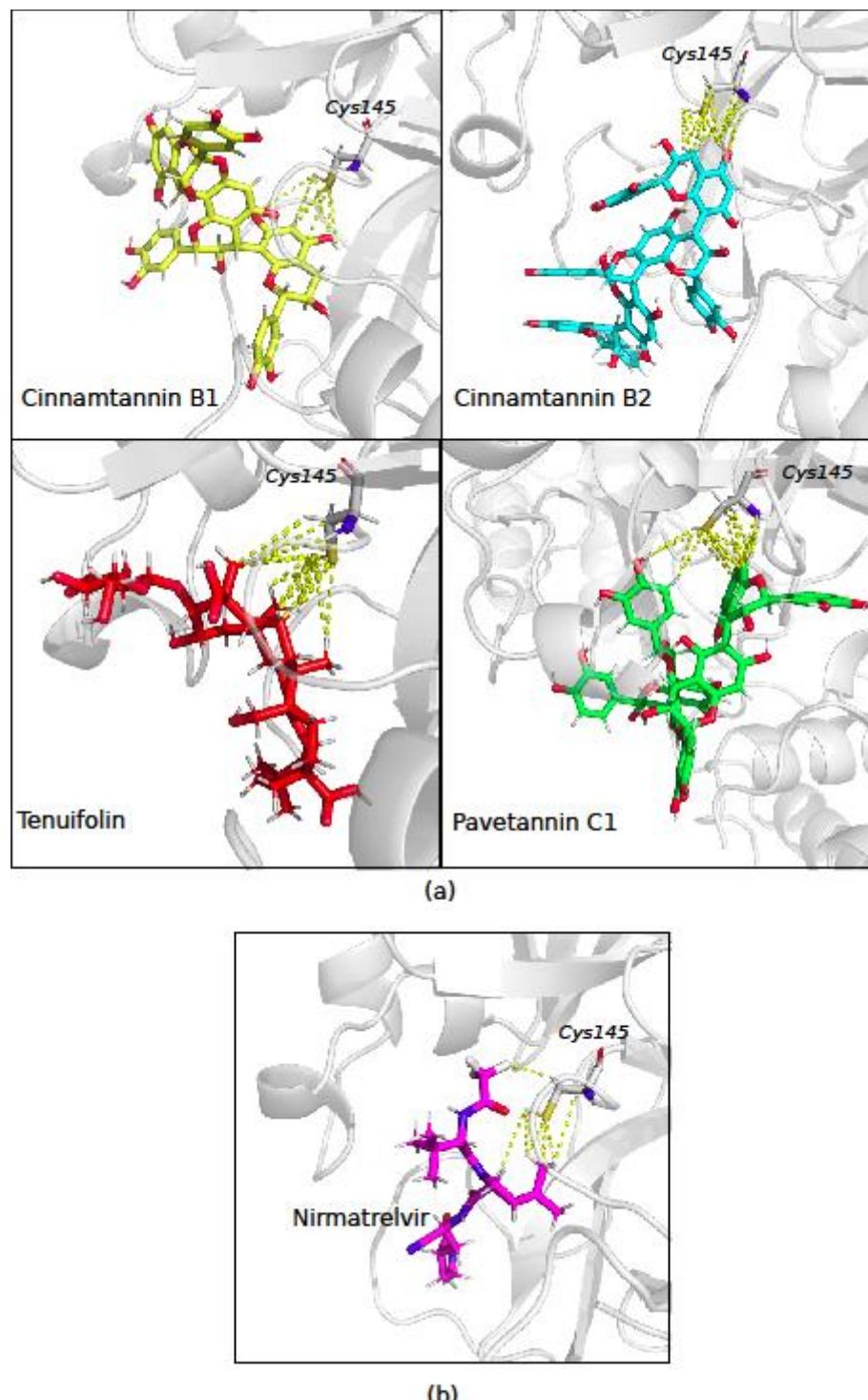


Figure 4. Catalytic Residue Cys145 Interactions with Main ImmunoDefender Ligands and Paxlovid Drug. The catalytic residue Cys145, which makes polar, hydrophobic, and hydrogen bound contacts in the Mpro catalytic pocket, is highlighted as a transparent cartoon. Main ImmunoDefender ligands and Nirmatrelvir, a component of the anti-COVID-19 drug Paxlovid, are shown as sticks, and their interactions with Cys145 are illustrated as yellow dashed lines. (a) Interaction of Mpro Cys145 with Main ImmunoDefender ligands. (b) Interaction of Mpro Cys145 with Nirmatrelvir of Paxlovid.

4. Discussion

The COVID-19 pandemic has highlighted the urgent need for rapid and effective health responses against viral outbreaks. However, the traditional development and marketing of antiviral products have always been a lengthy and challenging process, taking several years or even decades. In this context, the search for alternative approach-

es to combat viral infections has gained considerable attention, and herbal medicinal products based on essential oils have emerged as a promising option.

Several studies have shown that essential oils derived from certain plants contain bioactive compounds with antiviral properties [22,23,24,40]. For example, eugenol, the main bioactive ligand found in clove essential oil, has been shown to exhibit significant antiviral activity against certain pathogenic viruses [23]. Similarly, Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, and Tenuifolin, which are bioactive compounds found in cinnamon essential oil, have also been reported to have antiviral effects [24,40].

Tisserand and Young (2013) explain that combining different essential oils can lead to a synergistic effect, where the combined action of the oils produces a greater result than the sum of their individual actions. This phenomenon is well-known in aromatherapy and is one reason why blends of essential oils are often used to achieve specific therapeutic effects. Similarly, Edwards (2000) highlights the importance of synergy in the use of essential oil blends for therapeutic purposes [47,48]. Based on this understanding of the potential benefits of essential oil blends, our aim was to develop an effective antiviral product using a carefully selected combination of essential oils. By blending oils with known antiviral properties, we sought to create a synergistic effect that would enhance their individual actions and provide a more powerful antiviral effect. Through our research and experimentation, we hope to contribute to the growing body of evidence supporting the use of essential oil blends for therapeutic purposes. Our preliminary study aimed to develop a safe and effective antiviral product based on a blend of essential oils. We identified bioactive molecules with antiviral properties and a long history of safe use in humans. These were carefully formulated to create a potent blend of essential oils that can be safely consumed at appropriate doses. We also used advanced computational techniques to evaluate the efficacy of our product and ensure it targets the virus without affecting human catalytic pathways. Our study represents an important step forward in developing safe and effective antiviral products based on essential oils.

Our results demonstrated that the selected bioactive molecules interacted strongly with the Mpro target enzymes, affirming the stability of the target-ligand complex and providing a better association constant [26]. These findings suggest that the electrostatic interactions dominate the non-covalent interaction in the cited ligands, making them efficient in targeting Mpro.

The current COVID-19 pandemic has resulted in numerous research studies aimed at finding a cure or a way to manage the disease. Among the potential targets for therapy is the non-structural protein Mpro of SARS-CoV-2, which has been identified as an effective target for COVID-19 therapy [38]. In computational molecular simulation approaches, the selection of the best Mpro 3D structure is crucial to ensure the reliability of the results. Previous studies on the Mpro enzyme have used the monomer structure of PDB: 6LU7 to simulate ligand-enzyme interaction [39,40,41]. However, in the present study, the homodimeric structure (PDB: 1Q2W) was considered, as it was found to be a better choice for modeling candidate ligand efficiency.

Blocking the Mpro active catalytic site and preventing the attachment of the translated polyprotein from its allosteric site is the goal of this study. Several ligands were found to bind effectively to the target protein, demonstrating the blocking and inhibiting capacity of Mpro despite the low doses of EO fraction used for the proposed EO mixture formulation. The computational approach used in this study sheds light on how low doses of associated ligands can have a strong effect on blocking the Mpro catalytic active and allosteric sites.

Furthermore, recent studies have shown that the Mpro enzyme also interacts with several host cell proteins, highlighting its importance in the pathogenesis of SARS-CoV-2 [45]. In this context, targeting Mpro via allosteric inhibition offers a promising strategy to disrupt its interactions with host cell proteins and limit the pathogenicity of the virus.

In addition to its potential as a therapeutic target, Mpro has also been studied as a diagnostic target for COVID-19. Several research studies have reported the development of Mpro-based diagnostic assays, which show promising results in terms of sensitivity

and specificity [46,47]. The availability of sensitive and reliable diagnostic assays targeting Mpro can greatly contribute to the early detection and control of COVID-19 outbreaks.

The alternative approach of targeting Mpro via allosteric inhibition offers a new avenue for drug discovery and can potentially lead to the development of more effective antiviral agents. The development of Mpro-based diagnostic assays can also greatly aid in the early detection and control of COVID-19 outbreaks. Further research is needed to optimize the targeting of Mpro and to develop safe and effective drugs and diagnostic tools to combat this global health crisis.

The use of essential oils in aromatherapy is a popular approach for achieving specific therapeutic effects. This is because essential oils contain a complex mixture of chemical compounds that work synergistically to enhance their overall efficacy. Although certain constituents of essential oils may show good binding energy with a target, it is important to consider the potential benefits of using a blend of oils, as each oil may have unique properties that can target different aspects of a condition. Using a mixture of 10 essential oils can provide a more well-rounded and effective treatment approach compared to using only a few oils, and this practice has been shown to be safe and effective. In the case of targeting the main protease, 24 ingredients from the 10 essential oils were tested to identify the most effective bioactive molecules against Mpro. Only six components consistently displayed a strong interaction with Mpro, and their selection was based on a comprehensive evaluation of their overall contribution to the inhibition of Mpro activity. It is worth noting that the composition of essential oils is determined by a non-linear mathematical model, resulting in a complex interplay between their individual components. Therefore, the resulting composition of a blend of oils is not a simple sum of their individual components but a dynamic interplay between them.

The proposed ImmunoDefender bioactive ligands demonstrate a high affinity for both the active and allosteric sites of the Mpro enzyme, which is a significant finding that suggests their potential as inhibitors. To further contextualize these results, a similar molecular docking study was conducted by Sourav Das et al. in 2020, in which 33 molecules, including four anti-viral drugs (ritonavir, hydroxychloroquine, penciclovir, and lopinavir), were tested to identify possible inhibitors of the SARS-CoV-2 main protease [45]. Although the results showed that these drugs bind within the Mpro active site with varying free binding energy values, their affinities were notably lower than those of the ImmunoDefender bioactive molecules.

Moreover, the proposed bioactive compounds, particularly Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, and Tenuifolin, exhibit excellent drug-likeness scores, indicating their potential effectiveness and tolerability, particularly in terms of the Blood-Brain Barrier (BBB). These findings further support the potential use of ImmunoDefender EO mixture as a therapeutic option for COVID-19 treatment. However, it is important to note that further experimental studies, such as in vitro and in vivo studies, are necessary to confirm the efficacy of these ligands in blocking the Mpro enzyme and inhibiting viral replication. Such studies may include cellular assays, animal studies, and clinical trials, which will provide more comprehensive information on the safety and efficacy of the ImmunoDefender EO mixture as a potential treatment for COVID-19.

The study analyzed the molecular interactions and binding intensity of Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, Syzyginin B, and Tenuifolin. The free binding energy scores indicated strong molecular interactions between the ligands and the target protein, which were further supported by the presence of intense hydrogen and hydrophobic bonds.

In addition, the study used Molsoft's chemical fingerprints to predict the overall drug-likeness score of the selected compounds. The drug-likeness score is a crucial parameter that helps to assess the suitability of a compound for drug development. The results showed that the selected compounds had good drug-likeness scores, indicating that they possess ideal pharmacological properties such as efficacy and tolerability.

The findings of this study are consistent with previous research that has highlighted the potential pharmacological benefits of Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, Syzyginin B, and Tenuifolin. For example, Cinnamtannin B1 has been shown to possess potent antioxidant and antitumor activities, while Tenuifolin has been reported to have neuroprotective and anti-inflammatory effects.

In addition, we further evaluated the drug-likeness properties of four compounds in the mixture, namely Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, and Tenuifolin. These compounds showed drug-likeness scores ranging from 0.70 to 0.79, indicating their molecular properties are similar to those of commercialized drug active molecules with high tolerance and efficiency. The potential of these compounds as drug candidates was also supported by our molecular docking studies, which demonstrated their strong molecular interactions and binding intensity with the target receptor. Therefore, the identified compounds in the proposed EO mixture of ImmunoDefender may have potential as lead compounds in drug development.

Overall, the results of this study suggest that the selected compounds have the potential to serve as lead compounds for the development of novel drugs with improved pharmacological properties. Further experimental studies, such as *in vitro* and *in vivo* assays, are necessary to validate the findings of this study and to confirm the potential of these compounds as drug candidates.

Antiretroviral drugs have shown promise in treating COVID-19 by inhibiting the viral protease, a key enzyme necessary for viral replication. Pfizer's Nirmatrelvir/Ritonavir, sold under the brand name Paxlovid, is one such orally administered antiviral inhibitor that has been developed to target COVID-19.

The mechanism of action of Nirmatrelvir/Ritonavir is based on inhibiting the catalytic cysteine (Cys145) of the coronavirus protease, which is essential for viral replication. Structural analysis has revealed that the Cys145 of the Mpro catalytic site interacts with the "ImmunoDefender" bioactive compounds, making it a crucial amino acid residue within a distance of 4 Ångströms. This highlights the potential efficacy of the "ImmunoDefender" product as a treatment for COVID-19, reducing the risk of complications associated with the disease.

It is important to note that the bioactive molecules of ImmunoDefender have a strong binding energy, indicating a high affinity for the viral protease enzyme. This suggests that ImmunoDefender could potentially be a highly effective treatment for COVID-19. By targeting the crucial Cys145 amino acid residue of the viral protease, ImmunoDefender could inhibit viral replication and reduce the severity of the disease.

5. Conclusion

In conclusion, our study highlights the potential of natural products in drug discovery for COVID-19 therapies. By screening phytoconstituents from well-established plant extracts, we identified the most potent bioactive molecules that bind to the Mpro protein of SARS-CoV-2.

It is important to note that while our study identified Cinnamtannin B1, Cinnamtannin B2, Pavetannin C1, Syzyginin B, Procyanidin C1, and Tenuifolin as the most potent compounds, the therapeutic effect of the proposed EO mixture may not solely rely on these individual constituents. Essential oils contain complex mixtures of chemical compounds that may act synergistically with each other, enhancing their overall efficacy. Therefore, it is possible that the therapeutic effect of our proposed EO mixture may be attributed to the synergistic interactions between its various chemical constituents. Further studies are needed to fully elucidate the therapeutic potential of the proposed EO mixture for COVID-19. Nonetheless, our study provides a promising starting point for the development of new and effective natural product-based therapies for COVID-19.

Conflicts of interest: The authors declare no conflict of Interest.

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