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Citrus clementine peels essential oil exhibited anti-SARS-CoV-2 and its modulatory effect against cytokine storm: Evidence from in vitro and in silico studies.

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Abstract

SARS CoV-2 gets over more than four million people all over the world. The challenges for developing vaccines in overwhelming pandemic situations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), developing and screening of unique antiviral agents are peremptorily necessitated. In this study, we aimed to identify the chemical constituents of Citrus clementine peel essential oil (CCPEO) and to investigate its activities as anti-SARS-CoV-2 and anti-inflammatory activities. The chemical profile of CCPEO was identified via Gas chromatography-mass spectrometry analysis (GC/MS). The in-vitro cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the 50% cytotoxic concentration (CC50) of CCPEO was determined. The antiviral effect of citrus clementine extract was determined by plaque reduction assay. A geometry-based molecular docking approach (Patchdock) was performed to create docking modifications that result in good molecular shape complementarity. The antiviral effect of CCPEO was attributed to the downregulation of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) released from Huh7cells, and thus attenuating the SARSCoV-2 infection-associated cytokine storm in severe cases.

Keywords: Citrus clementine; essential oil; SARS CoV-2; IL-6, TNFα; molecular docking

1. Introduction

Coronavirus disease-2019 (Covid19) is considered these days the major risk to human health that is mediated by SARS-CoV-2. The latter is the third new zoonotic coronaviruses (CoVs) to infect humans following the arousal of the Severe Acute Respiratory Syndrome CoV (SARS-CoV) in 2002, the Middle East Respiratory Syndrome CoV (MERS-CoV) in 2012 leading to numerous losses[1]. SARS-CoV-2 was reported to trigger the immune response of the host, causing a cytokine storm, which is implicated in multiorgan disorders. Increasing levels of cytokines in the blood, mainly interleukin (IL-6), are usually found in patients infected with COVID-19, leading to fatalities.[2]

The rate of mortality of SARS-CoV-2 is about 1.78 % all over the world (304 million infected people and 5.4 million deaths) and the rate of mortality in Egypt is about 5.7% (403.000 infection and 22.205 deaths), (WHO 2022) [3].

One of the updated new guidelines for covid-19 treatment by NIH is to control the IL-6 secretion which is particularly important during infection[4], [5].

IL-6 is an important cytokine that has a crucial role in clinical symptoms of viral infection especially COVID-19 and disease severity where its transcription or translation from cells was enhanced by viral products[6] that trigger IL-6 and the secretion of other cytokines causing a dramatic triggering of T cells[7]. The latter and boosting of IL-6 secretion causing cytokine storm which adversely affects COVID-19 patients. Also, the course of various inflammatory diseases are related to high levels of IL-6 expression so in the SARS-COV-2 infection, the patients have pulmonary inflammation and extensive lung damage[8]. In the same way, Many reports explained

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the correlation between the levels of TNF-α and IL-6 and the severity of COVID-19 [4,9,10]. Therefore, the suppression of both TNF-α and IL-6 may be the therapeutic targets for COVID-19 patients and case prognosis[11,12].

Various chemical constituents of essential oils had been reported of possessing a wide range of antiviral activity viz, Adenovirus3, Tobacco Mosaic Virus (TMV), aparapox virus, HSV-1, HSV-2, the Japanese encephalitis virus, influenza viruses; H2N2 and H5N1 [13]. In addition to the ability of some essential oils to fight viral infections, they have anti-inflammatory activity. Many essential oils such as chamomile, eucalyptus, rosemary, lavender, millefolia, have been contributed to the treatment of inflammation [14]. Their activity may be attributed to their antioxidant activities as well as downregulation of various pro-inflammatory genes expression and thus modulation of inflammatory signaling pathways [15].

Citrus genus is flowering plants belonging to the Rutaceae family. Citrus trees are cultivated in many countries mainly China, Brasil, Spain, United states of America and Mexico and they have a great economic value. They are evergreen with different sizes. Citrus fruits are edible and they might be processed into juices, different dishes, and beverages. Many food supplements or functional foods have been prepared due to the beneficial effect of Citrus[16].

Citrus peels act about 45% of the whole fruit weight, unfortunately, it is a byproduct of food waste of citrus processing which causes environmental problems. Citrus fruits are rich in essential oil due to their aromatic containing compounds. Citrus species are containing essential oils in valuable quantities.[17]

Citrus clementina Hort. ex Tan, genus (Rutaceae), as a result of hybridization between mandarin and sweet orange. The CCPEO is rich of essential oils and there is a lack of information about its anti-viral and anti-inflammatory activities[18].

Our investigation aimed to identify the chemical constituents of *Citrus clementine* and to investigate its activities as anti-SARS-CoV-2 and anti-inflammatory activities.

2. Materials and Methods

2.1.Plant materials:

The *Citrus clementine* fruits were purchased from own farm from Belbis, Sharkia governorate, Egypt.

2.2.Extraction:

The fresh *Citrus clementine* (500 g) of fresh were mixed in a blender with 2.5 L of ethanol then the filtrate was collected and 250 ml of water was added to the filtrate then, the filtrate was partitioned with hexane according to the method described by Gouda et al (2021) [19]. The n-hexane extract was evaporated under vacuum using rotatory evaporator at 30°C to yield 7.2 g *Citrus clementine* volatile oil.

2.3.Gas chromatography–mass spectrometry analysis (GC/MS)

GC-MS analysis was done using Agilent GC-MS system [20] which is equipped with 7890B-gas chromatograph and 5977A-mass spectrometer detector at Central Laboratories Network, National Research Centre, Cairo, Egypt. HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 μm film thickness) was used in the GC system. Helium used as the carrier gas at a flow rate of 1.0 ml/min at a split 1:30, injection volume of 1 µl and the following temperature program: 40°C for 1 min; rising at 4°C /min to 150°C and held for 6 min; rising at 4°C/min to 210°C and held for 1 min. The injector and detector were held at 280°C and 220°C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of *m/z* 50-900 and solvent delay 5 minutes. Wiley and NIST Mass spectral Library data were used for identification of peels *Citrus clementine* volatile hexane extract constituents by comparing the spectrum fragmentation pattern with those stored in.

2.4.Anti-SARS-COV-2 activity (Cells and Virus):

Vero-E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel,Switzerland) containing fetal bovine serum (10%) (Lonza), and antibiotic antimycotic mixture (1%) (Lonza). The cells were incubated at 37 ˚C in a humidified atmosphere of 5% CO2. A SARS-COV-2, hCoV-19/Egypt/NRC 03/2020 (Accession Number on GSAID: EPI_ISL_430820) virus was propagated in VERO-E6 cells. The virus was titrated using plaque titration assay.[21]

2.5.Cytotoxicity

To evaluate the *in vitro* cell viability of the CCPEO extract, the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed as previously described [21, 22] with minor modifications. Briefly, cells were seeded in 96-well plates in DMEM supplemented with 10% fetal bovine serum, and 1% antibiotic antimycotic mixture. After 24 hrs of cell preparation cells confluent 90% was checked, the growth medium was aspirated from each well and the cells were washed with 1X phosphate buffered saline (PBS). Different concentrations of CCPEO extract starting from 0.4 µg/ml were two-fold serially diluted in DMEM then added to cultured cells in 96-well plate in triplicate and incubated for 24 hrs post treatment to determine the cytotoxic

concentration 50 (CC_{50}). The medium was then removed and the monolayer of cells was washed with 1X PBS three times before adding MTT solution (20 µL/well of 5 mg/ml stock solution) and incubated at 37 ˚C for 4 h till formulation of formazan crystals. Crystals were dissolved using a volume of 200 µL of dimethyl sulfoxide (DMSO) and the absorbance was measured at λmax 540 nm using an ELISA micro-plate reader. Finally, the percentage of cytotoxicity compared to the untreated cells was determined. The CC_{50} of CCPEO extract was determined from a linear exponential equation[23].

% Cytotoxicity = (Absorbance of cell without treatment – Absorbance of cell with treatment) / Absorbance of cell without treatment X100

2.6. Plaque reduction assay

The antiviral activity of CCPEO extract was determined by plaque reduction assay [24]with minor modifications. Briefly, Vero-E6 cells were seeded in 6-well culture plates (10^5 cells/ml) and incubated overnight at 37 ˚C under 5% CO2 condition. Previously titrated SARS-CoV-2 was diluted to optimal virus dilution, which gave countable plaques, and mixed with the safe concentrations of CCPEO extract. The mixtures of virus and CCPEO extract were incubated for 1 h at room temperature. Growth medium was removed from the 6-well cell culture plates and virus-extract mixtures inoculated in duplicate. After 1 h contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose, 1% antibiotic antimycotic mixture, and 4% bovine serum albumin (BSA) [25] (Sigma, St. Louis, Missouri, USA) were added to the cell monolayer then the plates were incubated at 37 ˚C for 3 days. The cells were fixed using 10% formalin solution for 1 h and the overlayer was removed from each fixed well. Fixed cells were stained using 0.1% crystal violet in distilled water. Untreated virus was included in each plate as a control[23]. Finally, plaques were counted and the percentage reduction in virus count was recorded as follows:

Viral inhibition (%) = (viral count of untreated cells - viral count of the treated cells) / viral count of untreated cells x 100.

2.7. Down regulation of TNF-α and IL-6 released from the Huh7cells by treatment with CCPEO extract:

To investigate whether SARS-COV-2 virus can promote cytokine expression in the human hepatocellular carcinoma (Huh7) cells due to their high susceptibility to SARS-COV2 infection[26], the level of TNF-α and IL-6 were firstly measured in the supernatant of infected Huh7 cells. The levels of TNF-α and IL-6 in the culture supernatant of Huh7 cells were measured using enzyme-linked immunosorbent assay kits. SARS-CoV-2 virus induced the production of TNF-α and IL-6 in the culture supernatant of Huh7 cells in at interval time point according to Ghanim et al (2022)[27].

Then we repeated the experiment with the infected Huh7 cells with the SARS-COV-2 virus in the presence and absence of CCPEO extract. The supernatant of infected cells in two cases were collected at a time ranging from 0–24 hrs and the levels of IL-6 and TNF-α Huh7 were measured using ELISA according to manufacturer instructions.

For further determine of TNF-α and IL-6 levels occurred at the transcriptional level, IL-6 and TNF-α mRNA levels were evaluated by RT-PCR and normalized to β actin and log fold change was calculated. Huh7 cells were infected with SARS-CoV-2 at multiplicity of infection (MOI) 0.1 with and without CCPEO extract at concentration of 0.4 µg/mL.

2.8. PatchDock [28]

It is a geometry-based molecular docking approach. Its purpose is to create docking modifications that result in good molecular shape complementarity. When such changes are applied, they result in extensive interface areas as well as small degrees of steric conflicts. Multiple matching local features of the linked molecules with complementary qualities are assured, resulting in a broad interface. The PatchDock method divides the Connolly dot surface representation [29,30] of the molecules into concave, convex and flat patches. Complementary patches are then matched to produce candidate modifications. Each feasible change is further evaluated using a scoring function that considers both geometric fit and atomic desolvation energy [31]. Finally, to eliminate redundant complexes, the candidate complexes are clustered using RMSD (root mean square deviation).

PatchDock's high efficiency stems mostly from its fast transformational search, which is based on local feature matching rather than brute force scanning of the six-dimensional space [32]. Finally, to eliminate redundant complexes, the candidate complexes are clustered using RMSD (root mean square deviation). It decreases the computational processing time even more by utilizing advanced data structures and spatial pattern recognition algorithms that developed in the field of computer vision, such as geometric hashing and posture clustering, as described in literature [33].

2.9. Statistical analysis

Data were summarized by means ±SD of triplicates and compared by two-way ANOVA followed by Bonferroni multiple comparison test. P-value<0.05 was considered significant.

3. Results and discussion

3.1. Metabolic profiling using GC/MS

The Essential oils of Citrus clementine peel extract cultivated in Egypt were investigated by GC/MS (Figure 1).

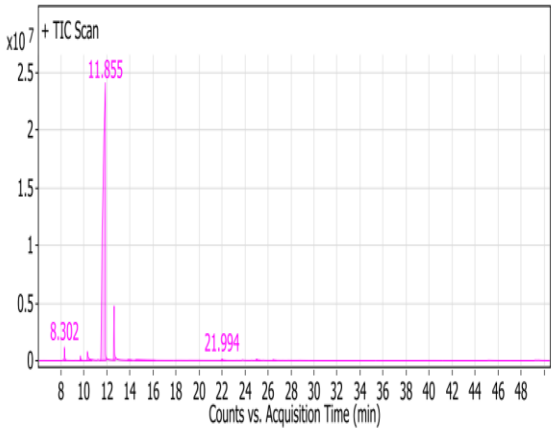

Figure 1 GC/MS profile of metabolites detected in peels Citrus clementine volatile hexane extract.

Ten compounds were detected and identified by comparing the fragmentation pattern with Wiley and NIST mass spectral library data. These identified compounds were mainly one compound limonene (92.27 %) beside minor components acts 7.73 % of the hexane extract including ɤ-terpinene (4%) and β-myrcene (1.3%). in addition to, seven compound of concentration less than 1% are listed in Table 1.

3.2. Anti SARS-CoV-2 and anti-inflammatory activities of Citrus clementine essential oils

Essential oils have shown antiviral effects (*in vitro* and *in vivo* experiments) against Coronavirus infectious bronchitis virus.

The antiviral effects of the essential oils might be attributed to the disintegration of the viral capsid in addition to the expansion of the virus leading to prevention of viral adsorption to the host cells [34]. Essential oils can also hinder the hemagglutinin that allows the virus to invade the host cell. Many essential oils can affect redox signaling pathways and thus inhibit the viral life cycle. [13].

Table 1:GC/MS profile of metabolites detected in peels Citrus clementine volatile hexane extract.

Peak	RT	Name	Formula	Area	Area Sum %
1	8.302	α-Pinene	C10H16	3584586.8	0.92
2	9.686	(1S)-Bicyclo [3.1.1]heptane, 6,6-dimethyl-2-methylene	C10H16	1679019.2	0.43
3	10.298	β-Myrcene	C10H16	5080653.8	1.31
4	11.855	dl-Limonene	C10H16	359106312	92.28
5	12.616	ɤ-Terpinene	C10H16	15581182	4
6	13.852	Camphene	C10H16	407059.79	0.1
7	14.584	Linalyl acetate	C12H20O2	1540746.1	0.4
8	21.994	(3R-trans)-Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)	C15H24	878334.97	0.23
9	25.004	ɤ-Elemene	C15H24	848199.18	0.22
10	26.44	D-Germacrene	C15H24	455057.22	0.12

[a] *P*< 0.05: Statistically significant from control (LSD followed by Dunnett's test).
[b]*P*< 0.05: Statistically significant from acetylsalicylic acid (LSD followed by Dunnett's test).
DME-50, *C. sinaica* defatted extract (50 mg/kg, *p.o*); DME-100, *C. sinaica* defatted extract (100 mg/kg, *p.o*); DME-200, *C. sinaica* defatted extract (200 mg/kg, *p.o*); ASA, Acetylsalicylic acid (150 mg/kg, *p.o*).

Based on the chemical investigation; the essential oil limonene is the most major compound of CCPEO (Table 1). Therefore, the biological activity of Citrus clementine volatile oil is mainly dependent on limonene (92.27%) and might be due to the synergism of limonene with ɤ-terpinene (4%). Accordind to our cytotoxicity and antiviral study, we reported that the CCPEO exhibited a substantial antiviral activity against SARS-COV2 with a CC_{50} and IC_{50} values of (9.03 and 13.1 µl/ml) respectively (Fig. 2). In a previous study, authors reported that the major constituents of the essential oil of 'Laurus nobilus' are 'β-pinene', 'α-pinene', '1,8-cineole' and 'b-ocimene'. The 'Laurus nobilus' oil had been evidenced to exhibit strong antiviral activity against SARS-CoV-1 with a selective index and IC_{50} values of 4.6 and 120 mg/mL, respectively [19]. Forty-eight compounds have been detected in the 'Juniperus *oxycedrus'* essential oil with major constituents as '2.2% δ-cadinene', '2.3% epibicycloses-quiphellandren', '6.7% limonene' and 'α-phellandrene'. They all showed a substantial inhibitory effect versus SARS-CoV-1 coronavirus [36]. Limonene is a common terpene in nature and is widely found in the volatile oils of citrus species. Limonene has been used in food preservation due to its broad-spectrum antimicrobial activity, safety, and low toxicity [37]

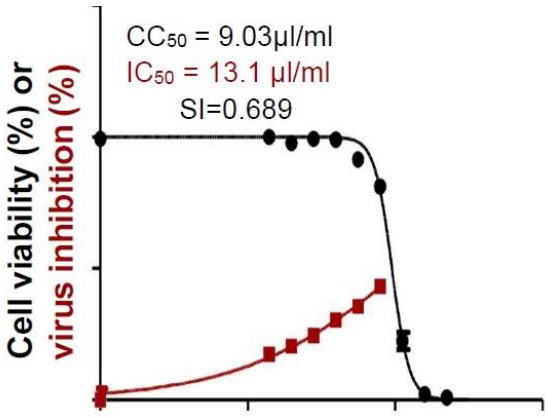

Fig. 2 Citrus clementine peels extract cytotoxicity *and viral inhibition activity on Vero-E6 cell lines.*

3.3. .Down regulation of TNF-α and IL-6 released from Huh7cells by treatment with Citrus clementine peel essential oils extract

CCPEO extract was did not cause any significant changes in 1st two measures but at 8hrs post infection it significantly (p=0.005) and at 16&24hrs post infection CCPEO significantly (p<0.0001) down regulating the levels of TNF-α in the culture supernatant of infected Huh7 cells at interval time points (Fig 3a) while in the assessment of IL-6, the CCPEO extract has significantly (p<0.0001) downregulated the levels of IL-6 in the culture supernatant of infected Huh7 cells at interval time points (Fig 3b) .

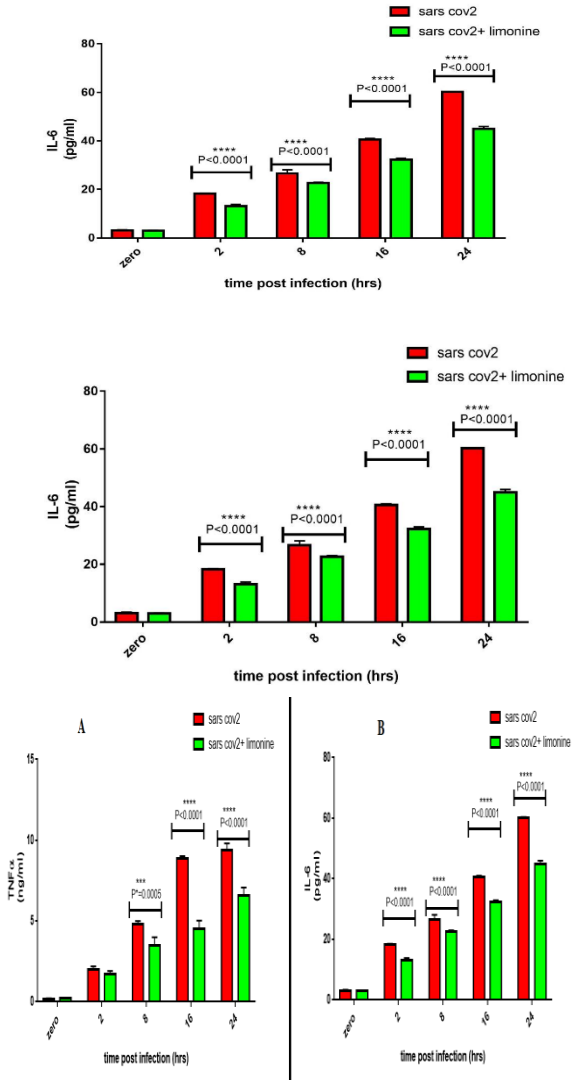

Fig 3. Protein levels of pro-inflammatory cytokines TNFα and IL-6. (A) HUH7 cells treated with CCPEO significantly down regulates TNFα levels at studied time point compared with SARS-COV-2 infected cells. (B) HUH7 cells treated with CCPEO significantly down regulates IL-6 levels at studied time point compared with SARS-COV-2 infected cells.

Treatment with Citrus clementine peel essential oils extract downregulate Transcriptional levels of pro-inflammatory cytokines TNF-α and IL-6 released from Huh7cells: Data explained that SARS-COV-2 strongly induced the transcription level of, TNF-α and IL-6 which was consistent with the release of TNF-α and IL-6 in the supernatants of infected cells. The treated cells with CCPEO extract were significantly down regulating the transcriptional levels of TNF-α by 6.6 folds at 2hrs post infection but did not show any significant changes by the rest of all time points (Fig 6A). On the other hand The IL-6 was significantly down regulated in the treated cells compared to untreated cells after infection with SARS-CoV-2 at at all time points (Fig 6B).

3.4. SARS-CoV-2 Spike protein

SARS-CoV-2 Spike protein, Fig.5, undergoes ten normal mutations. The N-terminal domain (NTD) (residues 27 to 69, 80 to 130, 168 to 172, 187 to 209, 216 to 242, and 263 to 271), NTD′ (residues 44 to 53 and 272 to 293), RBD (residues 334 to 378, 389 to 443, and 503 to 521), the receptor-binding domain (RBD) (residues 403 to 410) and subdomains 1 and 2 (SD1 and SD2) make up the majority of the S1 subunit. Where SD1 (residues 323 to 329 and 529 to 590), SD2 (residues 294 to 322, 591 to 620, 641 to 691, and 692 to 696), A fusion peptide (FP), a fusion peptide proximal region (FPPR), heptad repeats 1 and 2 (HR1 and HR2), a central helix (CH) (residues 985 to 1035), a connector domain (CD) (residues 711 to 716 1072 to 1121), a transmembrane domain (TM), and a cytoplasmic tail (CT) are all found in the S2 subunit. The mutation sites A570D, D614G, P681H, T716I, S982A, and D1118H are in the three structures described in [38].

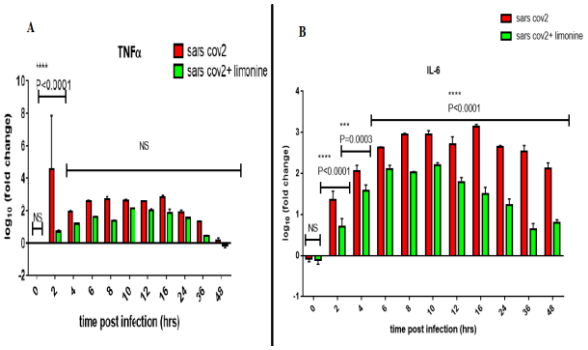

Fig 4. Transcriptional levels of pro-inflammatory cytokines TNFα and IL-6.(A) CCPEO down regulates the log fold change of TNFα induced by SARS- COV-2 infection by 2–3 times at the studied time points. (C) CCPEO down regulates the log fold change of IL-6 induced by SARS-COV-2 infection by 2–3 times at the studied time points.

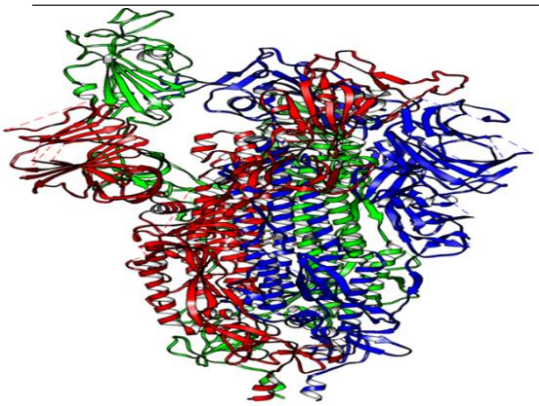

Fig. 5. Spike protein consists of three chains as chain A blue, chain B green, and chain C Red.

The lowest sequence variability of spike protein exists in the S2 subunit (residue range: 816–1141) as mentioned in literature [39]. The HR1, CH, and CD domains (S2 subunit) positioned adjacent to the viral transmembrane formed a huge pocket or a cavity made up of three spike monomers, according to an assessment of less variable sites in spike protein. This reflects the preferred stable complex SARS-CoV-2 spike protein- limonene, Fig.6.

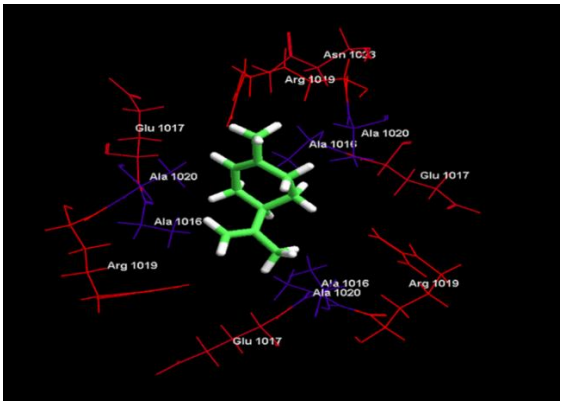

Fig. 6: Central helix of SARS-CoV-2 spike protein bound to limonene

Fig. 6 was mapped by Molegro Molecular Viewer, which uses the Kyte-Doolittle scale [40] to rank the amino acid hydrophobicity, where the blue colour indicates the most hydrophilic, the white colour is equal to 0.0, and the orange-red colour depicted the most hydrophobic. It also demonstrates the amino acids of the spike protein as thin sticks while limonene atoms appeared as thick sticks. The cleavage sequence of 682RRAR685 docks well into the substrate-binding pocket of furin [26]. In the current docking study, limonene binds near to the pocket of furin as shown in Fig.3. limonene binds also near the experimental structure of PDB ID 6VSB [41] also the residues forming the Non-structural protein 16 (nsp16)-nsp10 heterodimer interface can form hydrophobic interaction through cluster D (Leu7042, Met 7045) of nsp16 [41], where Met 7045 form pi-alkyl interaction with limonene, Fig.7.

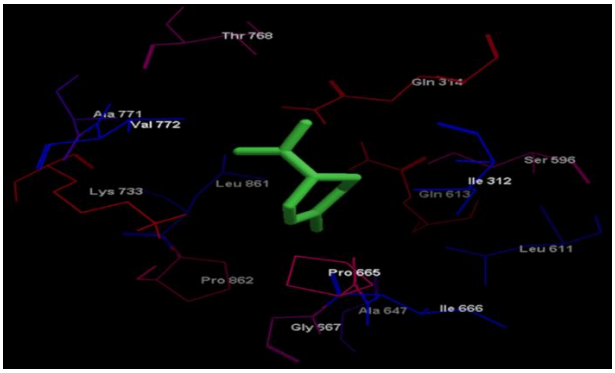

Fig. 7: SD2 and FP regions of SARS-CoV-2 spike protein (7lyo) bound to limonene.

The S-protein supports the viral entrance by attaching to the host cell and also via host-virion membrane union. The attachment of the virus to any host cell depends on the interaction of the S-protein receptor-binding domain (RBD, found in domain S1) with the angiotensin-converting enzyme 2 (ACE2) receptor (Fig. 8). Fusion of the virion to the host cell membrane occurs following the cleavage of the S-protein between the S1 and S2 domains, with an additional cleavage (S20) near the fusion peptide (FP) domain, which is responsible for viral anchoring to the host cell membrane.

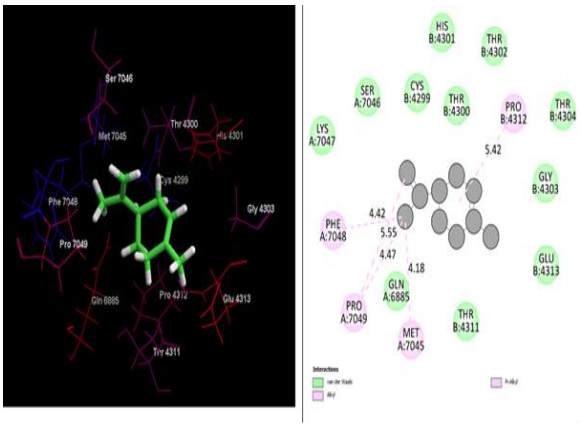

Fig. 8: nsp16 (6wvn) of SARS-CoV-2 bound to limonene.Limonene binds to a different variant of spike protein and to nsp16 (the most conserved region among non-structural proteins of SARS-CoV-2). This conclusion makes limonene a potential lead compound that can target and inhibit SARS-CoV-2.

4. Conclusions

According to our findings, we concluded that the citrus clementine peel essential oil (CCPEO) possessed a potent antiviral effect which is attributed to the binding of limonene to a different variant of spike protein and nsp16 (the most conserved region among non-structural proteins of SARS-CoV-2) and thus inhibits and targets SARS-COV2 infection. Additionally, the CCPEO showed a substantial modulatory effect on cytokine storm in the supernatant of Huh7 cells infected in vitro with SARS-CoV-2. Finally, we recommended this essential oil as a

potentially useful drug to respond to the COVID-19 pandemic after proper testing in vivo and clinical trials.

5. Conflict of interest

Authors declare that there is no conflict of interest.

6. Formatting of funding sources

No source of funding.

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