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Original Article

Kaempferia parviflora Wall. ex Baker against SARS-CoV-2 spike protein: *In silico* and *in vitro* studies

[*Kaempferia parviflora* Wall. ex Baker contra la proteína de la espiga del SARS-CoV-2: Estudios *in silico* e *in vitro*]

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Abstract

Context: SARS-CoV-2 spike (S) protein, governed by its receptor binding domain (RBD), is a key player in mediating viral attachment and fusion into host cells, which leads to infection and transmission of COVID-19. Searching for effective inhibitors against S RBD protein is essential to stop the virus infection.

Aims: To evaluate *Kaempferia parviflora*'s bioactive compounds as inhibitors against SARS-CoV-2 S RBD protein and its complex with human angiotensin-converting enzyme 2 (ACE2) receptor through *in silico*, and to determine the ability of *K. parviflora*'s extract to inhibit the binding of S RBD and ACE2.

Methods: Molecular docking was performed to evaluate the inhibition potentials of *K. parviflora*'s bioactive compounds against S RBD protein and its complex with ACE2. The inhibitory activity of *K. parviflora*'s extract against the binding of S RBD and ACE2 was determined using an *in vitro* inhibition assay.

Results: *K. parviflora*'s compounds had inhibition potentials against S RBD protein in both closed and open states. In the open RBD, these compounds were bound to the key amino acids that were involved in the binding of RBD with ACE2, suggesting their possible roles in preventing the RBD-ACE2 association. *K. parviflora*'s compounds also had strong affinities towards the S RBD-ACE2 complex by interacting with the paired RBD-ACE2 amino acids, which were crucial for the S RBD-ACE2 complex's stability. In addition, *K. parviflora* extract also demonstrated inhibitory activity against the binding of S RBD and ACE2.

Conclusions: This study proposes *K. parviflora* as a promising inhibitor against SARS-CoV-2 S protein for the treatment of COVID-19.

Keywords: angiotensin-converting enzyme 2; bioactive compounds; COVID-19; molecular docking; plant extracts.

Resumen

Contexto: La proteína spike (S) del SARS-CoV-2, gobernada por su dominio de unión al receptor (RBD), es un actor clave en la mediación de la unión y fusión viral en las células huésped, lo que conduce a la infección y transmisión del COVID-19. La búsqueda de inhibidores eficaces contra la proteína S RBD es esencial para detener la infección del virus.

Objetivos: Evaluar *in silico* los compuestos bioactivos de *Kaempferia parviflora* como inhibidores de la proteína S RBD del SARS-CoV-2 y su complejo con el receptor humano de la enzima convertidora de angiotensina 2 (ACE2), y determinar la capacidad del extracto de *K. parviflora* para inhibir la unión de S RBD y ACE2.

Métodos: Se realizó un acoplamiento molecular para evaluar los potenciales de inhibición de los compuestos bioactivos de *K. parviflora* frente a la proteína S RBD y su complejo con ACE2. La actividad inhibitoria del extracto de *K. parviflora* frente a la unión de S RBD y ACE2 se determinó mediante un ensayo de inhibición *in vitro*.

Resultados: Los compuestos de *K. parviflora* presentaron potenciales de inhibición frente a la proteína S RBD tanto en estado cerrado como abierto. En la RBD abierta, estos compuestos se unieron a los aminoácidos clave implicados en la unión de la RBD con ACE2, lo que sugiere su posible papel en la prevención de la asociación RBD-ACE2. Los compuestos de *K. parviflora* también mostraron una gran afinidad hacia el complejo S RBD-ACE2 al interaccionar con los aminoácidos emparejados RBD-ACE2, cruciales para la estabilidad del complejo S RBD-ACE2. Además, el extracto de *K. parviflora* también demostró actividad inhibidora contra la unión de S RBD y ACE2.

Conclusiones: Este estudio propone a *K. parviflora* como un prometedor inhibidor de la proteína S del SARS-CoV-2 para el tratamiento de la COVID-19.

Palabras Clave: acoplamiento molecular; compuestos bioactivos; COVID-19; enzima convertidora de angiotensina 2; extractos vegetales.

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INTRODUCTION

Spike (S) protein, one of the SARS-CoV-2 structural proteins, is highly responsible for the spread and transmission of coronavirus disease-19 (COVID-19). Together with other structural proteins such as envelope, nucleocapsid, and membrane proteins, the S protein forms the SARS-CoV-2 envelope. These proteins share high sequence similarity with SARS-CoV and MERS-CoV counterparts. SARS-CoV-2, like SARS-CoV, uses the same host entry mechanism mediated by its S protein to bind and penetrate host cells (Zhang et al., 2021). Among all the structural proteins, the S protein predominantly contributes to viral attachment, cell membrane fusion, and viral entry.

In homotrimer form, the S protein comprises two key subunits, S1 and S2. The S1 subunit consisted of two functional domains, N-terminal and C-terminal (Shang et al., 2020). The receptor-binding domain (RBD), which is located from amino acid residue 319 to 541 in the C-terminal domain, binds with the human cell receptor, angiotensin-converting enzyme 2 (ACE2), resulting in the cleavage of S protein, allowing the S2 subunit to facilitate the viral fusion into the target cell (Huang et al., 2020). RBD of S protein exists in two distinct conformations, open or closed. In the closed or native state, RBD is inactive and remains in "down" conformation. During infection, RBD undergoes a transition process where its conformation changes from "down" to "up" to initiate the interaction with ACE2 that eventually permits the viral entry (Ray et al., 2021). Identifying or developing therapeutic molecules that can both inhibit this structural change and disrupt the RBD-ACE2 binding is a promising strategy for preventing viral entry and, thus, infection.

Currently, the commonly available antiviral drugs have been repurposed as a strategy in the treatment of COVID-19 infection. The use of the repurposed drugs enables a quick and low-cost therapeutic approach but, so far, they have failed to decrease mortality and reduce hospitalization duration (WHO Solidarity Trial Consortium, 2021). These drugs also exhibit side effects such as gastrointestinal disturbances, diarrhea and abdominal pain, and some of them even affect the nervous system, causing depression, insomnia, and suicidal thoughts (Punekar et al., 2022). In addition, none of these drugs can effectively act via a mechanism that involves blocking the interaction of S protein with ACE2, which has been the primary target in stopping COVID-19 infection (Tsegay et al., 2021). Thus, the search for effective and safe antivirals against SARS-CoV-2 S protein is still highly needed to combat COVID-19 disease. To exploit this, herbs have been targeted as they contain a vast array of pharmacological compounds with promising medicinal properties, especially against infectious diseases.

Kaempferia parviflora Wall. ex Baker, locally known as black ginger, is a medicinal herb with numerous therapeutic properties. Other than having anti-cancer and anti-inflammatory effects, this herb has also been reported to possess antiviral activity. The crude extract of this herb has demonstrated antiviral activity against highly pathogenic avian influenza (H5N1) and HIV-1 (Sookkongwaree et al., 2006; Sornpet et al., 2017). In the case of SARS-CoV-2, bioactive compounds from *K. parviflora* were proven to have inhibitory activities against viral replication proteins, main protease, and RNA-dependent RNA polymerase (RdRp) through *in silico* approach (Supian et al., 2022). Its compounds, 4-hydroxy-6-methoxyflavone and 5-hydroxy-3,7,4′-trimethoxyflavone exhibited inhibition potentials against the main protease, whereas peonidin and 5,3′-dihydroxy-3,7,4′-trimethoxyflavone showed inhibition potentials against RdRp. These compounds had even stronger inhibition effects than repurposing drug hydroxychloroquine. However, the inhibitory effects of *K. parviflora*'s compounds as well as its extract against SARS-CoV-2 S protein, have never been discussed. It is hypothesized that *K. parviflora* inhibits S protein, thus blocking viral entry and infection.

Therefore, in this study, we aimed to explore the potential of *K. parviflora*'s bioactive compounds to interact with SARS-CoV-2 S RBD protein in both closed and open states as well as its conformation with ACE2 receptor through molecular docking in order to prevent the binding of RBD and ACE2, thereby stopping the viral infection. In addition, the ability of *K. parviflora*'s extract to inhibit S RBD protein from binding with ACE2 was also evaluated. The 50% inhibitory concentration (IC_{50}) of this extract was determined. This study offers a promising inhibitor against SARS-CoV-2 S protein as a solution for the management of COVID-19.

MATERIAL AND METHODS

Ligand and receptor preparation

The information on the 20 bioactive compounds from *K. parviflora* was obtained from Yenjai et al. (2004) and Chen et al. (2018). The repurposing drugs, remdesivir and arbidol were used as controls. The compound and drug structures were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) and were ener-

gy-minimized using Open Babel version 2.3. For S protein, closed and open states of RBD were used, as well as its complex with ACE2. The RBD's closed state (7DF3; SARS-CoV-2 S trimer, S-closed at 2.70 Å), RBD's open state (7CAK; SARS-CoV-2 S trimer with three RBD complexed with three H014 Fab at 3.58 Å) and RBD-ACE2 complex (6M0J; SARS-CoV-2 spike RBD bound with ACE2 at 2.45 Å) structures were obtained from RCSB Protein Data Bank (http://www.rcsb.org). Water, hetero atoms, and inhibitors were removed, while hydrogen atoms were added to the protein structures using Discovery Studio Visualizer v21.1.0.20298 (BIOVIA, San Diego, CA, USA).

Molecular docking

Docking simulation was performed using Autodock Vina version 2.5.4. For 7DF3 and 7CAK, RBD was targeted specifically at receptor binding motif (RBM). Grid box was set with an x-, y- and z-sizes of 44 × 62 × 40 and centered at coordinates of x = 184.169, y = 159.360, and z = 218.120 for 7DF3 whereas a box size of 46 × 50 × 50 at center coordinates of x = 251.704, y = 218.851 and z = 141.318 was set for 7CAK. As for 6M0J, a grid box covering the RBD-ACE2 binding interface was set at a size of 38 × 52 × 30 and x-, y- and z-centre coordinates of -37.473, 27.860 and 3.822. The root mean square deviation (RMSD) tolerance was set to 2.0 Å. The docked complexes with the lowest binding energy (kcal/mol) were selected, and their hydrogen and/or hydrophobic interactions were visualized using BIOVIA Discovery Studio Visualizer v21.1.0.20298 (BIOVIA, San Diego, CA, USA) and PyMOL v2.5.4 (Schrodinger, LLC).

Data analysis

The interaction of the ligands and the protein receptors was characterized based on the binding energy estimated by Autodock Vina. The generated output files contain binding poses of ligands along with corresponding binding energies expressed in kcal/mol. Based on these binding energies, the ligands were ranked. The ligand showing the lowest binding energy was selected as the best docking conformation. The interactions of top-ranked compounds with the proteins through hydrophobic and hydrogen bonds were visualized in order to understand the inhibitory mechanism of the compounds.

Plant material and extraction

K. parviflora rhizomes were collected from a glasshouse at the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia (GPS coordinates of 2.98872, 101.70018). Plant identification was conducted by Dr. Mohd Norfaizal Ghazalli (MARDI) and a voucher specimen of *K. parviflora* (MDI 12803) was deposited in MDI Herbarium, MyGenebank™ Complex, MARDI. Before use, the rhizomes were rinsed under running tap water and dried at room temperature. The rhizomes were freeze-dried and ground into powder. Crude extraction was performed using 0.5 g powder samples in 20 mL of methanol in triplicate. Homogenization was carried out for 1 minute prior to mixing by vortexing for 15 minutes. Supernatant and cell debris were separated by centrifugation at 8,900 rpm for 5 min at 4°C. Re-extraction was done for the leftover residues using 10 mL of the same solvent. All collected supernatants were combined and filtered through Whatman® cellulose filter paper 11 µm (Merck, Darmstadt, Germany). The supernatants were then concentrated and dried through a concentrator at room temperature.

In vitro SARS-CoV-2 S RBD-ACE2 inhibition assay

The S RBD-ACE2 inhibition assay was performed using a SARS-CoV-2 inhibitor screening kit (EP-105) (AcroBiosystems, Delaware, United States) according to the manufacturer's instructions. This assay employs a colorimetric ELISA platform where the binding of immobilized SARS-CoV-2 S RBD protein and biotinylated human ACE2 protein was measured. *K. parviflora* extracts were tested for the ability to inhibit the binding of S RBD and ACE2 using different concentrations ranging from 2.5 to 3000 µg/mL. The SARS-CoV-2 inhibitor included in the kit (AcroBiosystems, Delaware, United States) served as a positive control. Measurements were made in triplicate, and the background was subtracted before curve fitting. The IC_{50} value of the extract was calculated from the readings using the non-regression linear model in GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, United States).

Statistical analysis *(in vitro)*

The data of percent inhibition of SARS-CoV-2 S RBD-ACE2 was expressed as mean ± standard error of the means (n = 3). The IC_{50} value was estimated from the dose-response curve using the non-regression linear analysis.

RESULTS

Docking of *K. parviflora* compounds against open and closed states of SARS-CoV-2 S RBD protein

K. parviflora's compounds were docked against closed S protein with binding energies varying from -6.0 to -7.1 kcal/mol. All these compounds were bound to the RBM of closed S protein but not specifically at the key residues that interact with ACE2.

Table 1. Binding energy of *K. parviflora* compounds against closed (PDB ID: 7DF3) and open (PDB ID: 7CAK) states of S proteins as well as S RBD-ACE2 complex (PDB ID: 6M0J).

No.	Compounds name	Binding energy (kcal/mol)		
		7DF3	7CAK	6M0J
1.	Peonidin	-7.1	-6.8	-7.6
2.	4-Hydroxy-6-methoxyflavone	-7.0	-6.9	-7.5
3.	4',5-Dihydroxy-7-methoxyflavone	-7.0	-6.7	-7.6
4.	5-Hydroxy-7-methoxyflavone	-6.5	-7.0	-7.1
5.	5,7-Dimethoxyflavone	-6.5	-6.9	-7.2
6.	5-Hydroxy-3,7-dimethoxyflavone	-6.4	-6.9	-6.5
7.	3,5,7-Trihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)- chromenium	-6.4	-6.4	-6.9
8.	3-Methyl-5,7,3',4'-tetramethoxyflavone	-6.3	-6.5	-7.9
9.	Methoxyflavones	-6.3	-6.9	-7.6
10.	3-Methyl-5,7,4'-trimethoxyflavone	-6.3	-6.3	-7.3
11.	5-Hydroxy-3,7,3',4'-tetramethoxyflavone	-6.2	-6.3	-6.7
12.	5-Hydroxy-7,4'-dimethoxyflavone	-6.2	-6.7	-7.6
13.	5-Hydroxy-7,3',4'-trimethoxyflavone	-6.1	-6.5	-7.7
14.	5-Hydroxy-3,7,4'-trimethoxyflavone	-6.1	-6.2	-6.6
15.	5,3'-Dihydroxy-3,7,4'-trimethoxyflavone	-6.1	-6.1	-6.8
16.	3,5,7,3',4'-Pentamethoxyflavone	-6.1	-6.3	-7.2
17.	5,7,3',4'-Tetramethoxyflavone	-6.0	-6.5	-7.7
18.	3,5,7,4'-Tetramethoxyflavone	-6.0	-6.3	-6.4
19.	5,7,4'-Trimethoxyflavone	-6.0	-6.7	-7.7
20	3,5,7-Trimethoxyflavone	-6.0	-6.8	-6.5
21.	Remdesivir*	-7.0	-6.8	-8.9
22.	Arbidol*	-5.5	-5.9	-6.4

*Reference drugs.

Out of all these compounds, peonidin was the best-docked compound against closed S protein with a binding energy of -7.1 kcal/mol (Table 1). Peonidin formed five hydrogen bonds with Ser469, Glu471, Gln474, Arg457 and Lys458, which were RBM residues (Fig. 1). This compound was also bound to Lys458 through two hydrophobic interactions. In addition to peonidin, the best binding conformations with closed S protein were also demonstrated by 4-hydroxy-6-methoxyflavone and 4',5-dihydroxy-7-methoxy-flavone, both of which had a binding energy of -7.0 kcal/mol. Like peonidin, the compound 4-hydroxy-6-methoxyflavone was bound to the residues within RBM. This compound formed a hydrogen bond with Gln474 and two hydrophobic interactions with Lys458. Meanwhile, the compound 4',5-dihydroxy-7-methoxyflavone interacted with Lys458 through a hydrogen bond. Hydrophobic interactions were also formed between this compound and Lys458 and Pro491. In comparison to the drug remdesivir (-7.0 kcal/mol), peonidin, 4-hydroxy-6-methoxy-flavone, and 4',5-dihydroxy-7-methoxyflavone had comparable inhibition activities against closed S protein to this drug. Remdesivir was docked to Thr345, Arg346, Ser349, and Ala348 through hydrogen bonds as well as to Ala348, Val341, Ala344, and Lys356 through hydrophobic interactions (Fig. 2). Compared to the drug arbidol (-5.5 kcal/mol), these compounds were more potent than this drug in inhibiting closed S protein. Arbidol interacted with Arg457, Gln474, Ser469, and Glu471 through hydrogen bonds and Lys458 through hydrophobic interactions.

K. parviflora's compounds also had inhibition potentials against open S protein by interacting with the important residues of this protein. By having binding energies ranging from -6.1 to -7.0 kcal/mol, these interactions appeared to be comparable to those with closed S protein (Table 1). However, the docking position of these compounds with open S protein differed

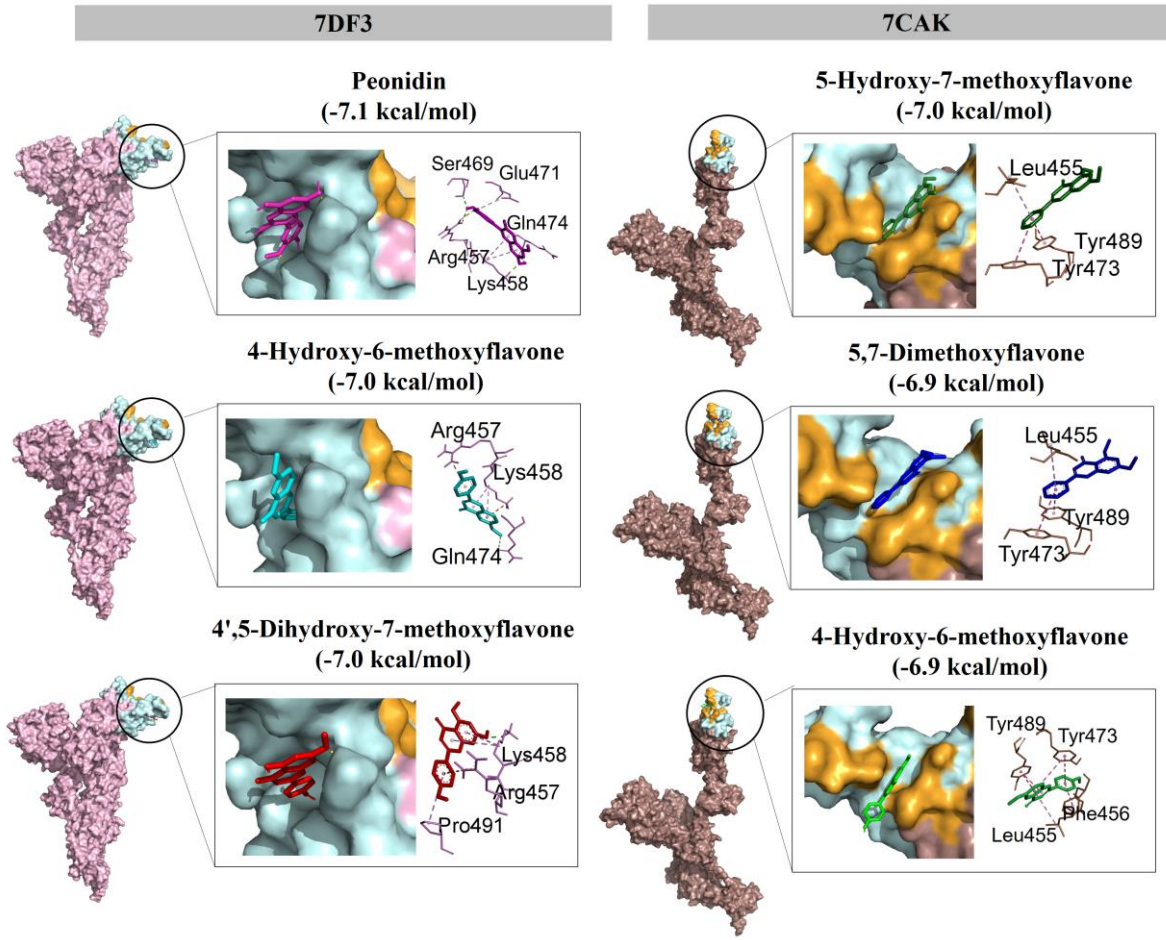

Figure 1. The interaction of the top-ranked *K. parviflora* compounds against closed (PDB ID: 7DF3) and open (PDB ID: 7CAK) states of S protein.

The compounds were represented by sticks, whereas the closed and open proteins were represented by pink and brown surfaces, respectively. The interactions were enlarged in boxes where the RBM of the S protein was highlighted in cyan, whereas ACE2-interacting residues were highlighted in orange. Hydrogen bond and hydrophobic interaction were shown in green and pink dashed lines, respectively.

from that of closed S protein, where these compounds were specifically bound to the ACE2-interacting residues. The compound 5-hydroxy-7-methoxyflavone showed the best binding conformation with open S protein with a binding energy of -7.0 kcal/mol. Hydrophobic interactions bound this compound to Leu455 and Tyr489, which were the residues that interact with ACE2 (Fig. 1). Hydrophobic interaction was also formed with the residue within RBM, Tyr473. The compounds 5,7-dimethoxyflavone, 4-hydroxy-6-methoxyflavone, 5-hydroxy-3,7-dimethoxyflavone, and methoxyflavones shared the same binding energies of -6.9 kcal/mol. The compounds 5,7-dimethoxyflavone and 4-hydroxy-6-methoxyflavone formed hydrophobic interactions with ACE2-interacting residues, Leu455 and Tyr489. Additional hydrophobic interaction was formed between 4-hydroxy-6-methoxyflavone and Phe456. When compared to remdesivir (-6.8 kcal/mol) and arbidol (-5.9 kcal/mol), these compounds were as potent as remdesivir and more potent than arbidol in binding with open S protein. Remdesivir adhered to the RBD interfaces facing ACE2, which was the same region where the compounds were bound (Fig. 2). Remdesivir interacted with RBD's Leu492 and Ser494 through hydrogen bonds. Hydrophobic interactions were also formed with Phe456, Leu455, Tyr473 and Tyr489. Similarly, arbidol was also docked to the ACE2-binding interface of RBD by forming two hydrogen bonds with Tyr489 and Phe490 and hydrophobic interactions with Tyr489 and Phe456. This finding was contrary to those reported by Padhi et al. (2021), who found that arbidol was not bound to this site when docking was performed with RBD alone. This finding indicates that positioning the docking box to the RBD-ACE2 interface has improved the binding of arbidol and S protein.

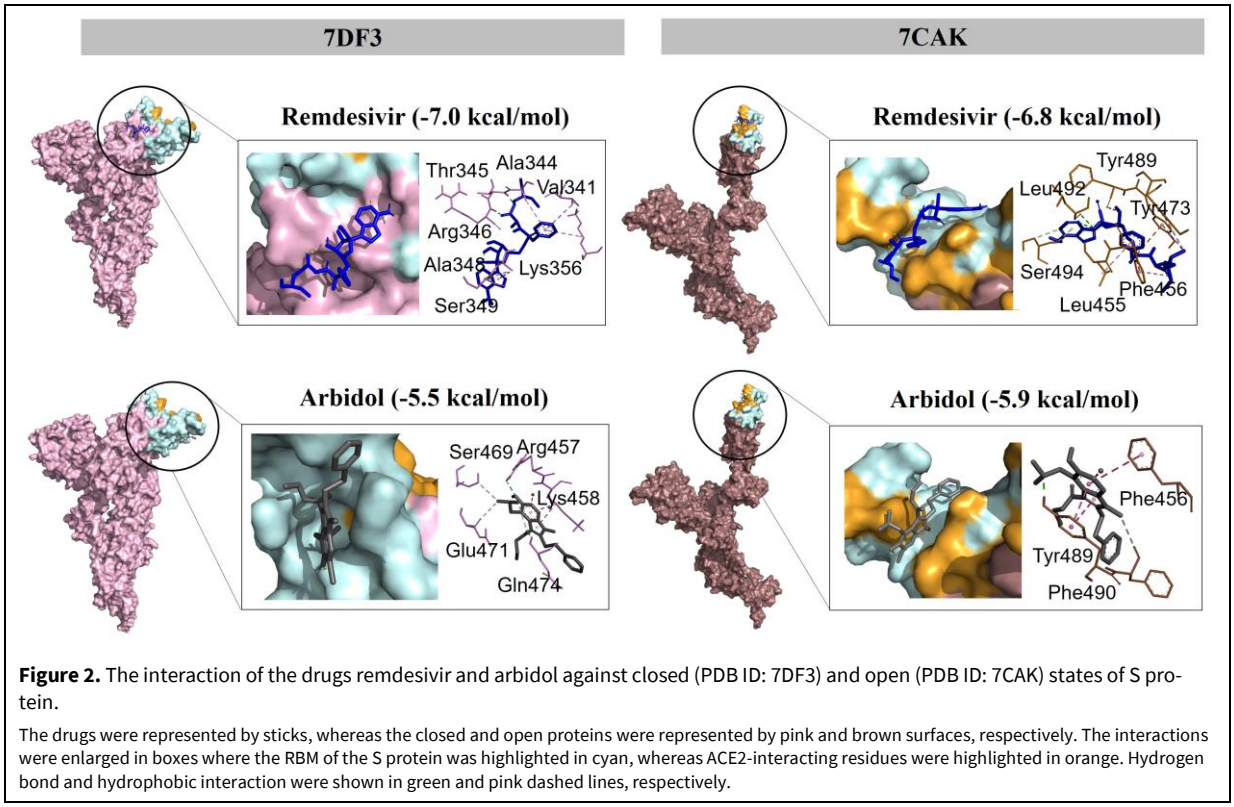

Figure 2. The interaction of the drugs remdesivir and arbidol against closed (PDB ID: 7DF3) and open (PDB ID: 7CAK) states of S protein.

The drugs were represented by sticks, whereas the closed and open proteins were represented by pink and brown surfaces, respectively. The interactions were enlarged in boxes where the RBM of the S protein was highlighted in cyan, whereas ACE2-interacting residues were highlighted in orange. Hydrogen bond and hydrophobic interaction were shown in green and pink dashed lines, respectively.

Docking of *K. parviflora* compounds against S RBD-ACE2 complex

K. parviflora's compounds were found to bind at the S RBD-ACE2 complex with binding energies ranging from -6.4 to -7.9 kcal/mol (Table 1). These compounds appear to interact with this protein complex more strongly than with closed and open S proteins alone, except for 5-hydroxy-3,7-dimethoxyflavone and 3,5,7-trimethoxyflavone. All these compounds were docked at the RBD-ACE2 binding interface (Fig. 3). Among these compounds, 3-methyl-5,7,3',4'-tetramethoxyflavone was the best-docked compound against S RBD-ACE2 complex with a binding energy of -7.9 kcal/mol. This compound was bound to the RBD-ACE2 interface by forming four hydrogen bonds with RBD's residues, Gly496, Ser494, and Arg403. This interaction was strengthened with the formation of five hydrophobic interactions with Tyr453, Arg403, Tyr495, Phe497, and Tyr505 of RBD. This compound also interacted with ACE2's residues, Lys353 and Arg393, through hydrogen bonds. Besides, the compounds 5-hydroxy-7,3',4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone also established strong binding conformations with S RBD-ACE2 complex, with the same binding energies of -7.7 kcal/mol. These compounds formed four hydrogen bonds with RBD's residues Arg403, Gly496, and Ser494. These conformations were tightened through hydrophobic interactions with RBD's residues, Lys417 and Tyr495, which were responsible for its binding with ACE2. In addition, hydrophobic interactions were also formed with Arg403, Phe497, and Tyr505 of RBD. Aside from RBD, 5-hydroxy-7,3',4'-trimethoxyflavone, and 5,7,3',4'-tetramethoxyflavone also interacted with ACE2 at the nucleotide Lys353, which was involved in the RBD-ACE2 binding through hydrogen bonds.

K. parviflora's compounds had higher inhibition potentials than the drug arbidol in binding with the S RBD-ACE2 complex, except for 3,5,7,4'-tetramethoxyflavone. Arbidol exhibited weak binding conformation with this protein complex with binding energy of -6.4 kcal/mol (Fig. 4). This drug formed hydrogen bonds with Arg403 and Tyr505 of RBD, and Asn33, Phe390, His34, Glu37 and Pro389 of ACE2 at the interface of RBD-ACE2 binding, which were in line with those reported by Padhi et al. (2021). However, compared to remdesivir (-8.9 kcal/mol), *K. parviflora*'s compounds were not as potent as this drug. By having lower binding energy than the compounds, remdesivir was docked at the important residues Lys417 and Tyr505 of RBD as well as His34 and Glu37 of ACE2, which were responsible for the RBD-ACE2 binding, through hydrogen bonds. Four more hydrogen bonds were also established between remdesivir and Arg393 of ACE2 and Arg403 and Gln409 of RBD. In addition, ten hydrophobic interactions were also formed with His34, Gln388, and Pro389 of ACE2 as well as Arg403, Lys417, Pro389, Tyr453, Tyr495, and Tyr505 of RBD, which may strengthen the binding of remdesivir to this protein complex.

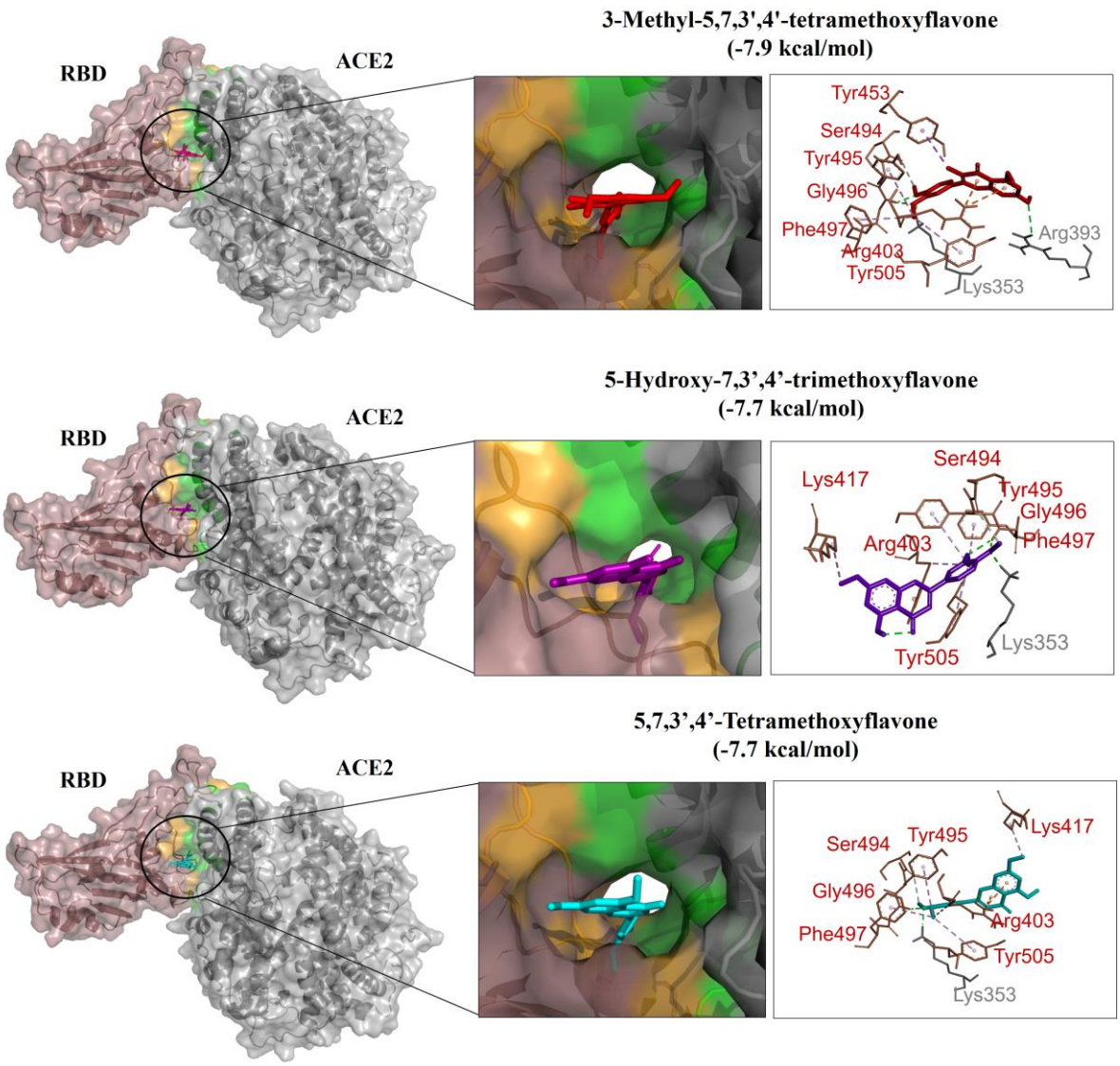

Figure 3. The interaction of the top-ranked *K. parviflora* compounds against S RBD-ACE2 complex (PDB ID: 6M0J).

The compounds were represented by sticks, whereas the S RBD and ACE2 proteins were represented by brown and grey surfaces, respectively. The interactions were enlarged in boxes where ACE2-interacting residues of RBD were highlighted in orange and RBD-interacting residues of ACE2 were highlighted in green. Red letters represented RBD's amino acid residues interacting with the compounds, while grey letters represented ACE2's amino acid residues interacting with the compounds. Hydrogen bond and hydrophobic interaction were shown in green and pink dashed lines, respectively.

In vitro inhibitory activity of *K. parviflora* extract against SARS-CoV-2 S RBD-ACE2

In order to further investigate the effect of *K. parviflora* in inhibiting SARS-CoV-2 S protein, *in vitro* inhibition assay was performed. Different concentrations of *K. parviflora* methanolic extract ranging from 2.5 to 3000 µg/mL were tested for the inhibition of S RBD-ACE2 binding. A maximal inhibition of S RBD-ACE2 binding by the extract was approximately 85% at the concentration of 750 µg/mL (Fig. 5). At higher concentrations than 750 µg/mL, the activity became plateau. The IC_{50} of *K. parviflora* extract was observed at 67.75 ± 1.19 µg/mL.

DISCUSSION

K. parviflora, a therapeutic herb, has been previously used as a folk medicine for managing numerous diseases such as cancer, allergies, and inflammation. However, recently, *K. parviflora* has received much attention as a number of pharmacological research have claimed the valuable benefits of this herb in treating a variety of diseases (Elshamy et al., 2019; Hashiguchi et al., 2022). The therapeutic effects of this herb are mainly contributed by its major constituents, methoxyflavones and its derivatives (Huo et al., 2023; Yoshino et al., 2021). Methoxyflavones are flavonoids that exhibit potent biological activities, including antiviral (Chen et al., 2018). These compounds have

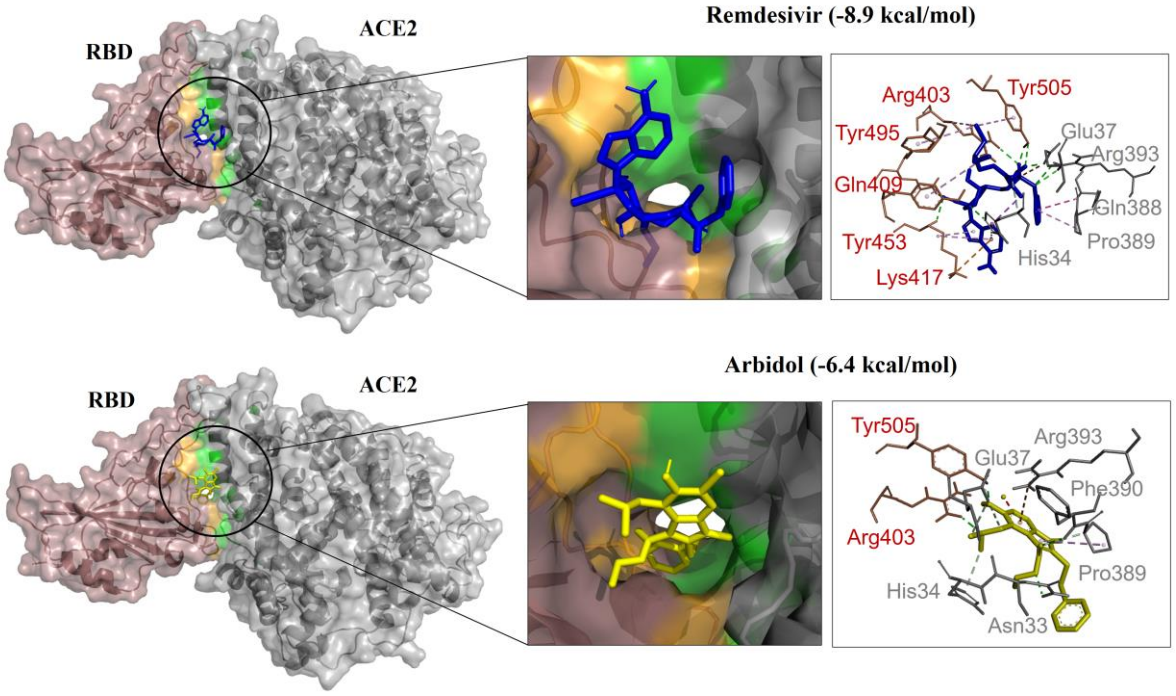

Figure 4. The interaction of the drugs remdesivir and arbidol against S RBD-ACE2 complex (PDB ID: 6M0J).

The drugs were represented by sticks, whereas the S RBD and ACE2 proteins were represented by brown and grey surfaces, respectively. The interactions were enlarged in boxes where ACE2-interacting residues of RBD were highlighted in orange and RBD-interacting residues of ACE2 were highlighted in green. Red letters represented RBD's amino acid residues interacting with the compounds, while grey letters represented ACE2's amino acid residues interacting with the compounds. Hydrogen bond and hydrophobic interaction were shown in green and pink dashed lines, respectively.

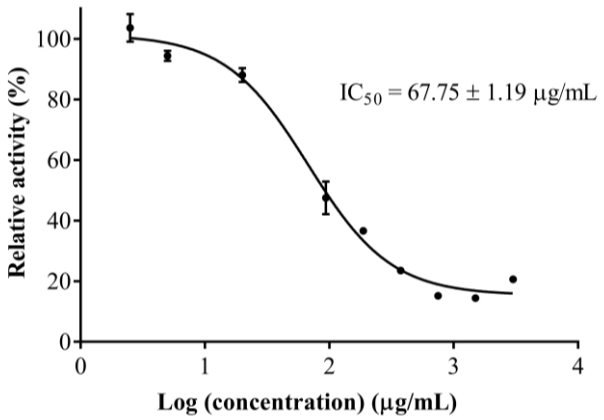

Figure 5. Inhibitory activity of *K. parviflora* extract against the binding of S RBD and ACE2.

The IC_{50} was observed at 67.75 ± 1.19 µg/mL.

been demonstrated to interact with SARS-CoV-2 vital proteins, main protease, and RdRp, which were involved in viral polyprotein proteolysis and viral replication and transcription, respectively, through *in silico* (Supian et al., 2022). Their inhibition effects on these proteins suggest their potential in stopping viral infection. In this study, we further investigated the ability of *K. parviflora*'s compounds to inhibit SARS-CoV-2 by revealing the interaction of these compounds with S protein, which is the first line in the infectious cycle of this virus.

Stopping SARS-CoV-2 infection can be effective by blocking the interaction of the viral S protein and host ACE2 receptor protein. This can be implemented by either directly acting on the viral S protein or the viral S protein bound to the host receptor. To interrogate such potential interactions, molecular docking was performed between *K. parviflora*'s compounds and S

protein bound to ACE2 as well as S protein alone. Interestingly, *K. parviflora*'s compounds exhibited inhibition potentials against both S protein and its complex with ACE2. Moreover, these compounds were bound to close and open states of S protein, suggesting that they may be able to inhibit this protein both before and during the viral infection. In the closed state, *K. parviflora*'s compounds, specifically peonidin, 4-hydroxy-6-methoxyflavone, and 4',5-dihydroxy-7-methoxyflavone were bound to RBM, which is located at the amino acid residues 438 to 508, within RBD. RBM serves as the main functional motif in RBD that creates the interface and makes all the contacts with ACE2 (Jackson et al., 2022; Jawad et al., 2021). However, the interaction of these compounds with RBM has not been formed, particularly with the residues responsible for the binding of S protein with ACE2. It is speculated that the RBD's down conformation prevents this recognition motif from protruding from the interface, preventing it from interacting with these compounds (Jackson et al., 2022). In contrast, in the open state of RBD, *K. parviflora*'s compounds, particularly 5-hydroxy-7-methoxyflavone, 5,7-dimethoxyflavone, and 4-hydroxy-6-methoxyflavone directly interacted with the residues involved with the binding with ACE2. The amino acids Gln493, Tyr505, Gln498, Asn501, Thr500, Asn487, Tyr449, Phe486, Lys417, Phe456, Tyr495, Leu455 and Tyr489 of RBD have been identified as the key residues involved in the tight interaction of RBD with ACE2 (Jawad et al., 2021). The up-confirmation of RBD in the open state exposes this binding site and makes it more accessible, which likely facilitates the interaction with the compounds (Henderson et al., 2020). The interaction of the compounds with these important residues of RBD may suggest their possible roles in preventing the binding of RBD with ACE2.

Despite interacting with the S protein, *K. parviflora*'s compounds have also been found to interact with the S protein bound to ACE2. In terms of binding energy, these compounds appeared to interfere with the S RBD-ACE2 complex more strongly than the S protein alone. The compounds, particularly 3-methyl-5,7,3',4'-tetramethoxyflavone, 5-hydroxy-7,3',4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone had higher affinities with S RBD-ACE2 complex than S RBD alone. These compounds were strongly docked in between RBD and ACE2 binding interfaces in the S RBD-ACE2 complex by establishing more contacts and interactions with RBD residues than ACE2 residues. Their interactions through hydrogen bonds with Gln496 of RBD and Lys353 of ACE2 as well as hydrophobic interaction with Tyr505 of RBD, are of particular interest. The binding of RBD and ACE2 involves the strong pairing between RBD-ACE2 residues, Gln496-Lys353 and Tyr505-Lys353, through hydrogen bonds and hydrophobic interaction, respectively (Jawad et al., 2021). The binding of molecules to the paired residues of the RBD-ACE2 complex has been reported to decrease the stability of the formed protein complex (Basu et al., 2020; Lapaillerie et al., 2022). Thus, the interaction of the aforementioned compounds with these paired residues may possibly affect S RDB-ACE2 conformation stability, which is crucial for virus entry. However, further studies through protein-protein docking are needed to validate the role of these compounds in disrupting the stability of the S RBD-ACE2 complex.

It is intriguing to note that the majority of *K. parviflora*'s compounds had stronger affinities with S RBD protein and its form with ACE2 than the drug arbidol. Arbidol, also known as umifenovir, is an indole derivative with proven antiviral activity against several enveloped and non-enveloped viruses such as influenza, coronavirus, and polio (Blaising et al., 2014; Boriskin et al., 2008). Wang et al. (2020) have discovered that arbidol can efficiently inhibit SARS-CoV-2 infection by targeting S protein to interfere with viral entry to host cells. This work has been supported by Padhi et al. (2021), who revealed that arbidol more efficiently acts on the S RBD-ACE2 complex than RBD alone, which was also in line with our findings. Arbidol binds to the S RBD-ACE2 complex and induces favorable interactions between RBD and ACE2, resulting in an increased affinity between them that leads to structural rigidity in the viral glycoprotein (Padhi et al., 2021). The rigid conformation of the RBD-ACE2 complex restricts the conformational rearrangements associated with membrane fusion required during virus entry (Padhi et al., 2021). In our case, having higher affinities against the S RBD-ACE2 complex than arbidol may suggest *K. parviflora*'s compounds are potent SARS-CoV-2 S protein inhibitors. However, further experiments will be needed to understand the detailed mechanism of action of *K. parviflora*'s compounds on the S RBD-ACE2 complex. Other than arbidol, the top-ranked *K. parviflora* compounds had binding potentials comparable to remdesivir against S RBD protein, implying that these compounds could be as effective as remdesivir. Remdesivir is an RdRp inhibitor that is evidently effective in inhibiting catalytic and NTP-binding residues of SARS-CoV-2 RdRp, resulting in premature termination of RNA synthesis and a decrease in the viral RNA levels (Cao et al., 2020; Eweas et al., 2021). Remarkably, besides RdRp, our study has demonstrated the possible inhibitory action of remdesivir on S RBD protein. Further experiments may be needed to confirm the role of this drug in preventing viral entry and the membrane fusion process.

Based on the *in silico* studies above, *K. parviflora* had strong inhibition potentials towards S protein as well as its complex with ACE2. In addition to this finding, our *in vitro* studies revealed that *K. parviflora* extract was also able to inhibit the binding of S RBD and ACE2 proteins. The inhibition of S RBD-ACE2 binding by this extract was proven in a dose-dependent manner. The maximum inhibition of this extract against the S RBD-ACE2 binding was found at its concentration of 750 µg/mL, which was reported to be safe and have no adverse effects (Saokaew et al., 2017; Yoshino et al., 2019). Considering this, it may be worthwhile to consider *K. parviflora* as a promising candidate for the development of safe and effective SARS-CoV-2 S protein inhibitors.

CONCLUSION

K. parviflora's compounds had inhibition potentials against both closed and open states of S RBD protein. These compounds showed comparable binding energies against the closed and open states of RBD, but interestingly, in the open state, these compounds were bound to key amino acid residues that were involved in the binding of RBD with ACE2, suggesting their possible roles in preventing the binding of RBD and ACE2, thus stopping the viral entry process. *K. parviflora*'s compounds also had strong affinities towards the S RBD-ACE2 complex by interacting with the paired RBD-ACE2 amino acid residues, which were important for the S RDB-ACE2 complex's stability. Furthermore, our study also tested the inhibitory action of *K. parviflora* extract against the binding of S RBD and ACE2 proteins, suggesting that *K. parviflora* could be a potential candidate for the development of SARS-CoV-2 S protein inhibitors for use in the treatment of COVID-19.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Supian S	Ahmad MA	Rozano L	Ab Rahman Z
Concepts or ideas	x			x
Design	x			
Definition of intellectual content	x		x	x
Literature search	x	x		
Experimental studies	x	x		
Data acquisition	x	x	x	
Data analysis	x	x	x	
Statistical analysis	x	x		
Manuscript preparation	x			
Manuscript editing	x		x	x
Manuscript review	x	x	x	x

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