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In vitro testing and computational analysis of specific phytochemicals with antiviral activities considering their possible applications against COVID-19

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The purpose of this study was to investigate the reservoir of natural products against the SARS-CoV-2 virus and to identify suitable candidates in order to recommend appropriate phytotherapy. Adequately prepared 65 molecules from traditional Chinese medicine with proven antiviral properties were subjected to docking analysis using AutoDock Vina 4 software with the aim to investigate binding affinity and interactions of compounds with Mpro from the SARS-CoV-2 virus. Bioflavonoids and tannins show best docking scores with -9.80 kcal/mol for bioflavonoids and -9.00 kcal/mol for tannins. Bioflavonoids: amentoflavone, agathisfaflavone, robustaflavone, hinokiflavone and rhusflavone were tested for their radical scavenging activity. Partition coefficients were examined by RP-HPLC. Evaluation of drug-likeness properties of investigated bioflavonoids suggested rhusflavone as a molecule with the best ADMET characteristics. Anti-inflammatory activity of rhusflavone was investigated in LPS stimulated RAW264.7 macrophages. Tested bioflavonoids exhibit beneficial effects against inflammation by scavenging free radicals and by suppressing the production of proinflammatory mediators by macrophages. Both predictions of affinity spectra for substances (PASS) and in vitro testing showed promising biological activity of investigated bioflavonoids. A Quantum chemical study was performed in order to calculate the thermodynamic, molecular orbital, and electrostatic potential of selected molecules and to compare their biological and chemical features. Our results highlighted antioxidant, anti-inflammatory and antiviral properties of investigated compounds, emphasizing the significance of bioflavonoid moiety to selected characteristics, which encourage further investigational strategies against COVID-19.

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1. Introduction

The availability of novel drugs and therapeutic approaches for the effective treatment of the emerged coronavirus is an urgent need after the outbreak of the disease caused by the SARS-CoV-2 virus. Since the beginning of the coronavirus spread, extensive research work has been made in order to diagnose, treat and cure the COVID-19 disease. Several countries developed their own preventive measures based on wearing protective masks, avoiding contact with infected persons, daily testing and certain hygiene practice. However, specific antiviral or targeted therapies are still lacking. At present, the main treatment approach for COVID-19 positive patients is supportive care enhanced by the combination of broad-spectrum antibiotics, corticosteroids, oxygen therapy and antivirals (Ali et al., 2020; Ullrich et al., 2020). The global scientific community is involved in comprehensive research with the aim to create appropriate therapy using different approaches such as: drugs repurposing, vaccine development and adjunctive therapies. Since the beginning of the pandemic, several drugs have been subjected to clinical studies including: antiviral drug remdesivir, anti-malarial drugs chloroquine/hydroxychloroquine, anti-rheumatoid arthritis drug tocilizumab and anti-HIV drugs lopinavir/ritonavir (Halim et al., 2021). To date, only remdesivir has been approved to treat COVID-19, but satisfying results to support its use are still lacking (Martinez, 2021).

Vaccine development significantly reduced the number of SARS-CoV-2 infected patients but there is still concern regarding the emergence of new strains (Forni and Mantovani, 2021). In the past year, different computational drug-repurposing strategies have been applied in order to identify appropriate candidates against the SARS-
CoV-2 virus. For example, in the recent study, the binding potential of several compounds was tested on multiple structures of Mpro and a consensus strategy was applied to select the most promising binders. Based on the physiological and pharmacokinetic profile and protein–ligand binding pattern, eleven compounds were identified as good inhibitors of Mpro. After further examination through molecular dynamic simulation, two compounds were retrieved with the highest binding affinities for Mpro and showed the ability to inhibit the replication of SARS-CoV-2 by specifically inhibiting its Mpro enzyme (Halim et al., 2021). Furthermore, another study explored in silico more than 31,000 natural compounds for their capability to bind to S-glycoprotein and block the early stages of the infection. Among these compounds, two (Castanospermine and Karuquinone B) were selected based on their binding affinity and pharmacokinetic profile to be particularly promising in the treatment of respiratory tract viral infections (Al-Sehemi et al., 2020).

Due to the increasing concern about the adverse effects of chemical drugs, the use of natural alternatives to or complementary therapies has received growing attention (Ho et al., 2020). Traditional Chinese medicine provides a huge reservoir of antiviral compounds, from which we can infer advanced therapies and products (Ho et al., 2020; Nguyen et al., 2012; Ryu et al., 2010). Plants can provide a natural, with better side effects profile and cost-effective approaches of drug discovery against many diseases including the COVID-19 disease (Nguyen et al., 2012; Ryu et al., 2010). Hence, there is a vital need to discover novel antivirals that are highly efficacious and cost-effective for the management and control of COVID-19, many natural antiviral products possess synergistic effects with approved antiviral drugs. Considering the COVID-19 pandemic situation our research aims to draw attention to bioactive molecules from natural sources that can serve as potential anti-COVID-19 prophylaxis or as an appropriate scaffold in the further development of suitable drug candidates (Chiong et al., 2016; Park et al., 2012; Zhang et al., 2020).

The significance of plant-derived molecules is well known in terms of nourishing and health-promoting resources. Flavonoids represent one among many phytochemicals which possess different biological properties having a positive influence on human health and thus designated as a functional food (Zhou et al., 2020). To date amentoflavone, agathisflavone, hinokiflavone, volkensiflavone, succedaneflavonan, rhusflavonan and robustaflavonan are recognized for their antiviral properties against HSV, SARS, Dengue virus, HIV, HBV, EBV, Influenza, Parafluinfluenza type 3, Adenovirus type 5, and measles (Li et al., 2019; Coulerie et al., 2013; de Freitas et al., 2020; Lin et al., 1999; Menezes and Campos, 2021).

2. Materials and methods

2.1. General modeling and molecular docking studies

65 molecules that belong to natural products and with proven antiviral properties were selected, and tested using AutoDock Vina 4 software (Kong et al., 2020). Open Babel was employed to transform and generate 3D coordinates of uploaded files. The three-dimensional (3D) structures of selected compounds were drawn using Chemsketch (ACD/Structure Elucidator, version 12.01, http://www.acdlabs.com), MM2 program as a part of Chem Draw Ultra 8.0 were employed for geometry optimization of the molecules. Ligand_prepare.py from MGLTools 1.5.6 was applied in order to convert the ligand files into .pdbqt format with added Gasteiger charge. The protein structures of COVID-19 targets (which imply Structural and Non-structural proteins), were extracted and prepared by MGLTools 1.5.6. In this work, Main protease (Mpro) was selected as target protein. Its structure was imported from Protein Data Bank (PDB) with a code of 6LU7 (Jin et al., 2020).

2.2. Pharmacokinetics and drug-likeness properties of selected bioflavonoid and tannin molecules

Pharmacokinetics, drug-likeness and medicinal chemistry characteristics were calculated for the lead compounds using the SwissADME web tool. The SMILES format of the selected molecules was used as an input file (Daina et al., 2017).

2.3. Chemicals and reagents

Investigated bioflavonoids: amentoflavone (purity >98%), agathisflavone (purity >98%), robustaflavone (purity >98%), hinokiflavone (purity >98%) and rhusflavonan (purity >98%) were obtained from BioCrick and Sigma-Aldrich Co. Following chemicals: 1,1-diphenyl-2-picryl-hydrazyl (DPPH; 95% purity), 2,2-azino-bis(3-ethylbenothiazoline-6-sulfonic acid (ABTS; 98% purity), 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAHP; 97% purity) were purchased from Sigma-Aldrich Co. The mouse macrophage-derived RAW264.7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Applied lipopolysaccharide (LPS) from Escherichia coli O111:B4 was obtained from Sigma-Aldrich Co.

2.4. Partition coefficients estimation

Considering that bioflavonoids represent one of the two most significant classes of molecules in this work amentoflavone, agathisflavone, robustaflavone, hinokiflavone and rhusflavonan were subjected to partition coefficient estimation. Prior to chromatographic analysis samples of selected molecules were dissolved in DMSO (HPLC grade, >99.9%, Alfa Aesar) in the concentration of 2 mg/ml. Chromatographic analysis was carried out using Agilent Technologies 1200 Series HPLC with ZORBAX Eclipse XDB-C18 column (4.6 × 50 mm, 1.8 μm) and Diode Array Detector (DAD). Two mobile phases were used, consisting of water (containing 0.05% acetic acid, A) and acetonitrile (B). The applied flow rate was 1 mL/min. The pH of the mobile phase was maintained on 7 by 0.01 M phosphate buffer (Na2HPO4, KH2PO4, Lach-Ner, p.a.). The column temperature was 26 °C. Injection volume was set at 10 μL. The detection of the compounds was done by a DAD detector 270 nm. The retention of the compounds was measured in triplicate. The capacity factor (k) was calculated using the following equation:

\[ k = \frac{(t_a - t_m)/t_m} \]

where, t_a – retention time of a compound, t_m – dead time (the first disturbance on the chromatogram) logk^' coefficient (Menezes and Campos, 2021) was calculated from the equation:

\[ logk = S \times \psi(MeCN) + logk^0 \]

S – the slope \[ \psi(MeCN) = 0.3 – 0.55v/v, with the step of 0.05 \]

2.5. DFT study of the molecular properties of selected bioflavonoid molecules

DFT (density function theory) represents the quantum mechanical method that is widely applied in the prediction of molecular orbital (MO), thermal and molecular electrostatic potential (MEP) properties (Matin et al., 2020). In order to calculate MEP properties of selected flavonoid compounds Gaussian 09 program was employed and structures of amentoflavone, agathisflavone, robustaflavone, hinokiflavone and rhusflavonan were optimized at B3LYP/6–31 G basis of DFT. Gaussian 3.0 was employed in order to obtain DOS plots. For MEP visualization WebMO demo server was used.
FMO (frontier molecular orbital) energy such as HOMO (highest occupied molecular orbital), LUMO (lowest unoccupied molecular orbital), HOMO-LUMO gap, hardness (\(\eta\)), and softness (\(S\)) were calculated at the same level of theory using the following equations:

\[
Gap = |\text{LUMO} - \text{HOMO}| = n(\text{LUMO} - \text{HOMO})/2; S = 1/n
\]

### 2.6. Antioxidant activity measurements (ABTS, DPPH and crocin bleaching assay)

The applications of ABTS and DPPH solutions are well known in methods for the measurement of the antioxidant capacity of natural products. Both methods are applied according to the previously established method of Di Sotto (Di Sotto et al., 2017). Working solutions of the amentoflavone, agathistaflavone, robustaflavone, hinokiflavone and rhusflavanone were prepared in the following concentrations: 250, 125, 25, 12.5, 2.5, 0.25, 0.125, and 0.025 \(\mu\)g/ml. Prepared DPPH solution (40 \(\mu\)l, 0.1 mM) and the test samples (160 \(\mu\)l) were incubated for 30 min in the dark conditions at room temperature, and then absorbance was measured spectrophotometrically at 515 nm. In the ABTS assay, equal volumes of ABTS (5 mM in PBS) and AAPH (2 mM in PBS) were used, mixed and incubated together for 45 min at 68 °C in order to obtain the ABTS+ radical cation. The sample (20 \(\mu\)l) was added to the radical solution (180 \(\mu\)l), and the plates were incubated for 10 min in the dark room at 37 °C and then measured at 734 nm. The negative controls were a DPPH/ABTS+ ethanol solutions and the positive controls were prepared by mixing a standard (quercetin) and DPPH/ABTS+. The percentage of scavenger activity was calculated as follows:

\[
\text{Scavenging activity} = 100 \times \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}
\]

\(A_{\text{control}}\) is the absorbance of the radical alone and \(A_{\text{sample}}\) is that of radical with the sample. In crocin bleaching assay crocin solution (40 \(\mu\)l; 3.5 mM in PBS 0.1 M, pH 7.4) was used. The sample was mixed with crocin solution, AAPH (10 \(\mu\)l; 0.25 M in PBS), PBS (110 \(\mu\)l) and then incubated at 40 °C in the dark room for 60 min (Di Majo et al., 2008). Measurement of absorbance was carried out spectrophotometrically at 443 nm. The percentage of antioxidant activity was calculated as described in ABTS and DPPH assays. All samples are tested in triplicate.

### 2.7. Nitric oxide (NO) production in RAW264.7 macrophages

RAW264.7 macrophages were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 0.3 mg/ml glutamine, 100 \(\mu\)g/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Cells were plated at a density of 105 cells/ml in 96-well plates and stimulated with 20 ng/mL of LPS for 24 h. The cells were treated with various concentrations of rhusflavanone for 1 h prior to incubation with LPS. Five different concentrations (0.5 \(\mu\)g/ml, 1.5 \(\mu\)g/ml, 2.5 \(\mu\)g/ml, 5 \(\mu\)g/ml and 10 \(\mu\)g/ml) of rhusflavanone were tested. After the incubation period, images of cells were captured under a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany); Supernatants were mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2% phosphoric acid) and was measured at room temperature for 15 min. Accumulation of nitrites was measured spectrophotometrically at 540 nm. From the standard curve generated with NaNO2 nitrite concentrations were obtained.

### 2.8. Statistical analysis

All experiments were performed at least in triplicate. Data were represented as mean ± SD of three independent experiments. \(P\)–values for two groups were determined by two–tailed Student’s t–test. Statistically significant \(P\)–values were labelled as follows: * \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\).

### 3. Results and discussion

#### 3.1. Docking analysis

Main protease (Mpro) which is also named chymotrypsin-like protease was identified as the target protein in the screening of 65 natural products (Table S1) against SARS CoV-2. Diverse chemical structures of natural products were subjected to docking-based virtual screening and ranged according to their docking scores and binding energies. Compounds that show the best docking scores belong to biflavonoids and tannins (Table S2). Among identified biflavonoids specifically highlighted are: amentoflavone (−9.80 kcal/mol), robustaflavone (−9.80 kcal/mol) and agathistaflavone (−9.0 kcal/mol). The docking pose of four selected molecules is presented in Fig. 1 in order to describe the binding properties of biflavonoids: a) amentoflavone b) agathistaflavone and tannins; c) epigallocatechin gallate d) hippomannin A) with Mpro. Docking analysis revealed that amentoflavone interacts with the binding pocket of Mpro. As shown in Fig. 1, the C7 hydroxy group of amentoflavone formed two hydrogen bonds with the nitrogen atom of the imidazole group of His163 and the keto group of Asn142 which are belonging to S1 site of Mpro. Additionally, the C7’ hydroxy group of amentoflavone formed a hydrogen bond with Ser46. In the case of agathistaflavone C5 hydroxyl group forms a hydrogen bond with Asn 142 and C5’ hydroxyl group is bonded via hydrogen bond with -NH group of Glu 166. The hydroxyl group from C4’ atom formed two hydrogen bonds with Asp 187 and Tyr 54. Agathistaflavone possesses lower binding energy and less fitting to S1 pocket of Mpro enzyme. Both biflavonoids consist of two molecules of apigenin, but the main difference is in the coupling of these 2 units (Li et al., 2019). The S1 site of Mpro, consists of the side-chains of His163 and Phe140, and the main-chain atoms of Glu166, Asn142, Gly143 and HisA172. Molecules of epigallocatechin gallate and hippomannin A form hydrogen bonds with these residues thus showing good fitting to S1 pocket. However, docking scores for these molecules are lower compared to selected biflavonoids and their amount for epigallocatechin gallate is −8.90 kcal/mol and for hippomannin A is −9.00 kcal/mol. According to their docking scores, biflavonoids show the best binding affinity to Mpro of SARS-CoV-2 virus and the binding energy (\(\Delta G\)) values of amentoflavone, agathistaflavone, robustaflavone, hinokiflavone and rhusflavanone were calculated to be −9.80 kcal/mol, −9.0 kcal/mol, −9.80 kcal/mol, −9.0 kcal/mol and −8.0 kcal/mol respectively.

#### 3.2. Drug-likeness properties of selected biflavonoids

The biflavonoids represent dimeric structures of corresponding flavonoids which are connected with each other by a C−C or C−O bond. The SwissADME (Daina et al., 2017) is a free web platform for the evaluation of physicochemical properties of investigated compounds. In this work amentoflavone, agathistaflavone, robustaflavone, hinokiflavone and rhusflavanone were studied (Fig. 2). Bioavailability Radars provide a graphical representation of the drug-likeness properties of an orally available compound. The graph is presented as a hexagon (Fig. 3) and vertices represent parameters that define a bioavailable drug. Examined compounds (Fig. 2) show higher polarity, lower solubility and saturation than recommended for an orally available drug. Only rhusflavanone shows better properties compared to the other molecules. Biflavonoids as food-derived bioactive compounds provide promising therapeutic benefits but all five examined molecules show a certain violation of the Lipinski rule of 5 and it is believed to be efficient despite few violations. All examined compounds were not successful in crossing blood-brain barrier so they cannot cause toxic effects at the central nervous system.
3.3. Partition coefficient estimation

The lipophilicity represents an important physicochemical property for the analysis of absorption, distribution, metabolism, and excretion (ADME) properties (Table S3) of different drugs and xenobiotics. Before the implementation of the formulation strategies to improve the solubility of poorly soluble drugs, the lipophilicity of the selected biflavonoids should be evaluated. In this study, we measured the log \( k_0 \) of five selected biflavonoids (Fig. 2.) in the normal internal environment pH buffers and compared these values to the predicted log P values obtained from software Molinspiration (https://www.molinspiration.com). The experimentally obtained partition coefficient values aligned well with the predicted values. The high lipophilicity, with log P values ranging from 4.26 to 5.15 (Table 1), limits their absorption and bioavailability. These findings suggest that an appropriate formulation strategy should be employed in order to enhance the solubility and bioavailability of these valuable molecules (Recharla et al., 2017).

3.4. Assessment of antioxidant capacity

In order to evaluate the antioxidant activity of selected biflavonoids, three different methods: DPPH, ABTS and inhibition of crocin bleaching were performed. In the DPPH analysis, the IC50 (concentration necessary to inhibit the activity of free radicals by 50%) was evaluated and compared to values of quercetin. In relation to the ABTS analysis, selected biflavonoids showed IC50 values several times higher than that of standard quercetin (Table 2). Our results for amentoflavone and agathisflavone are in good agreement with previously published (Di Mayo et al., 2008; Andrade et al., 2018). All examined biflavonoids show mutually similar effects, but are significantly different from quercetin. Quercetin as a molecule with proven antioxidative activity possesses only one flavonoid unit and tested compounds possess two. Under the experimental condition, selected biflavonoids also exhibited antioxidant activity in crocin bleaching assay, being the IC50 values more than 10 fold higher than that of quercetin. It is very well known that many viral infections can cause the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are involved in cell damage and destruction. In the case of respiratory viral infection produced ROS and RNS are involved in the injury of the lung tissue as well as epithelial barrier damage which can facilitate secondary infections. The main enzyme responsible for this reaction is NADPH oxidase isoform 4 (NOX4) (Amatore et al., 2015). By inhibition of NOX4 activity, ROS release is blocked and MAPK phosphorylation is prevented which results in inhibition of nucleoprotein traffic and viral release. Phenolic compounds such as phenolic acids, flavonoids, tannins, and proanthocyanidins are very well known for their antioxidative properties which are the base for specific antiviral, antimicrobial, and antimutagenic effects (Weng et al., 2019; Mahmood et al., 2016; Wang and Liu, 2014). The main process by which biflavonoids exhibit scavenging free radicals is that phenolic hydroxyls of these molecules can react with free radicals in order to complete a chain reaction. The radical scavenging ability of the selected biflavonoids, based on hydrogen and electron transfer, was measured by three different methods. By performing Pearson analysis (Table 3) it has been shown that...
Fig. 2. Chemical structures of examined biflavonoids: a) amentoflavone b) agathisflavone c) rhusflavanone d) robustaflavone e) hinoki flavone.

Fig. 3. The Bioavailability Radars of a) amentoflavone, b) agathisflavone, c) robustaflavone, d) hinoki flavone and e) rhusflavanone. It represents drug-likeness potential of the examined compounds. The pink area refers to the optimal range for each property (Lipophilicity: XLOGP3 should be between \(-0.7\) and \(+5.0\), Size: Mw between 150 and 500 g/mol, Polarity: TPSA between 20 and 130 Å², Solubility: log S not higher than 6, Saturation: fraction of carbons in the sp² hybridization not less than 0.25, and Flexibility: N > 9 rotatable bonds).
measured antioxidant activities are significantly correlated. Albeit DPPH and crocin bleaching measurements are based on the hydrogen transfer for blocking radicals, biflavonoids showed the most potent effects against the DPPH derived radicals with respect to crocin radicals. This difference could be due to the different solubility of tested compounds in used media (aqueous medium for crocin solubilization was applied and ethanol for DPPH).

3.5. Anti-inflammatory potential of rhusflavanone

In order to examine the anti-inflammatory activity of rhusflavanone it was measured rhusflavanone-mediated inhibition of NO production in LPS stimulated macrophage RAW264.7 cell line (Green et al., 1982). Inhibition of NO production was measured using different concentrations of rhusflavanone (0.5 μg/ml, 1.5 μg/ml 2.5 μg/ml, 5 μg/ml and 10 μg/ml). In Fig. 4 it has been shown gradually inhibition of NO production by using five different concentrations of rhusflavanone. Rhusflavanone at 2.5 μg/ml, 5 μg/ml and 10 μg/ml inhibited NO production by 20%, 35%, and 50%, respectively compared to NO production in only LPS treated cells. It has been known that LPS exposure activates NF-kB signaling pathway which is responsible for transcriptional induction of pro-inflammatory cytokines, chemokines and different inflammatory mediators (Green et al., 1982). Additionally, morphological changes in the RAW 264.7 macrophages after treatment with LPS alone were significantly diminished by pretreatment with rhusflavanone in concentrations 2.5 μg/ml, 5 μg/ml and 10 μg/ml (Fig. 4B).

3.6. Prediction of biological properties

Online platform PASS (Prediction of Activity Spectra for Substances; http://www.pharmaexpert.ru/ PASSonline/index.php) was applied in the prediction of biological activities of selected biflavonoids. This software is developed to predict a large number of different biological properties with 90% accuracy. Outcomes are presented as Pa (probability "to be active") and Pi (probability "to be inactive"). Structures of investigated compounds were drawn with ChemDraw 16.0, and then converted into SMILES string which was used to predict their biological potential. In this work PASS platform was used in the prediction of NOS2 (Nitric Oxide Synthase 2) expression inhibition, free radical scavenging, NAD(P)H oxidase inhibition, vasoprotection and antiviral properties estimation. As a part of significant results Pa>Pi and is presented on a scale from 0.00 to 1.00 where in general Pa+Pi<1. Obtained results are presented in Table 4 and clearly indicate high Pa values of NOS2 expression inhibition (0,63–Pa<0,9) for all compounds and the highest value Pa=0,9 for rhusflavanone. NOS2 activation is responsible for multiple inflammatory disorders and it is followed by activation of NF-kB pathway. Significant free radical scavenging ability was noticed for all five biflavonoids (0,58–Pa<0,73) and NAD(P)H oxidase inhibition for four tested molecules was at a remarkable level (0,16–Pa<0,69). All afore-mentioned predicted values are in good agreement with experimentally obtained data (Table 2 and Fig. 4). Investigated biflavonoid compounds show strong vasoprotective effects (0,49–Pa<0,85) which makes them significant resources in the prophylaxis of COVID-19. Clinical outcomes, results from basic science and imaging indicate that COVID-19 represents a vascular disease (Siddiqi et al., 2021). In COVID-19 disease dysregulation of homeostasis disrupts thrombotic balance which causes consequences on the vascular endothelium. Therefore, it is considered that the application of vasoprotective agents has alleviating and preventive effects in the treatment of COVID-19. The antiviral potential of biflavonoids (Table 4) that was determined using PASS software is also in very good correlation with experimentally acquired results published by Lin et al. (Lin et al., 1999). These claims indicate the considerable capability of investigated biflavonoids to serve in prevention, strengthening resilience and response to COVID-19 infection.

3.7. Thermodynamic analysis

After in vitro testing and biological properties estimation thermochemical characteristics of investigated molecules were calculated using Gaussian 09 program. The density functional theory (DFT, B3LYP) method with 6-31 G basis set was applied (Matin et al., 2020) to optimize studied compounds (298.15 K, 1.0 atm) and their structures are shown in Fig. 5. Thermodynamic characteristics of these molecules such as electronic energy (EE), enthalpy, Gibbs free energy (GFE) and dipole moment (DM) are presented in Table 5. If the value of negative energy is greater, the electron is more tightly bound to the nucleus. All five compounds possess similar thermodynamic properties but in the case of rhusflavanone negative electronic energy is slightly higher (~1908.0994 Hartree). Also, it is notable that amentoflavone, agathisflavone, robustaflavone, and hinokiflavone molecules have practically the same molecular formula, only rhusflavanone contains four H atoms more. Gibbs free energy (GFE) indicates spontaneity of a reaction when GFE < 0, and it unites enthalpy and entropy into a single value. It can be concluded from Table 5 that adding H atoms increases GFE, indicating a higher ability of spontaneous binding and interaction with other substrates. The calculated dipole moment (DM) of investigated compounds represents a significant measure of net molecular polarity and binding affinity. The
increased DM values (4.2–8.9 Debye, Table 4) distinctly indicate the higher binding affinity to the receptor during anti-inflammatory action. Among the studied compounds rhusflavanone has been determined as a molecule with the highest thermodynamic properties.

3.8. Molecular orbitals (MO) analysis

The frontier molecular orbitals of the compound are the highest-energy occupied (HOMO) and lowest-energy unoccupied (LUMO) molecular orbitals that are responsible for the chemical reactivity of the molecule. The HOMO and LUMO energy levels of the investigated compounds, obtained from DFT (B3LYP/6–31 G) (Ditchfield et al., 1971), have been shown in Table 6 (Fig. 6). It can be noted that amentoflavone, agathisflavone, robustaflavone, and hinokiflavone molecules possess a higher HOMO-LUMO gap compared to the rhusflavanone. Also, their hardness is increased and softness decreased in comparison with rhusflavanone. These properties can contribute to the chemical and biological characteristics of analyzed compounds.

Table 4
Predicted biological activities using PASS platform for 1) amentoflavone, 2) agathisflavone, 3) robustaflavone, 4) hinokiflavone and 5) rhusflavanone.

| Compound | NOS2 expression inhibitor | Free radical scavenger | NAD(P)H oxidase inhibitor | Vasoprotector | Antiviral (Herpes) | Antiviral (Hepatitis B) |
|----------|---------------------------|------------------------|---------------------------|--------------|-------------------|------------------------|
|          | Pa (Probability to be active) | Pi (Probability to be inactive) | Pa | Pi | Pa | Pi | Pa | Pi | Pa | Pi | Pa | Pi |
| 1        | 0.663 | 0.003 | 0.658 | 0.005 | 0.574 | 0.003 | 0.850 | 0.004 | 0.490 | 0.011 | 0.438 | 0.010 |
| 2        | 0.858 | 0.002 | 0.624 | 0.005 | 0.609 | 0.003 | 0.801 | 0.005 | 0.491 | 0.011 | 0.470 | 0.006 |
| 3        | 0.839 | 0.002 | 0.584 | 0.006 | 0.676 | 0.003 | 0.850 | 0.004 | 0.478 | 0.013 | 0.417 | 0.013 |
| 4        | 0.633 | 0.003 | 0.734 | 0.004 | 0.698 | 0.003 | 0.853 | 0.004 | 0.446 | 0.019 | 0.447 | 0.009 |
| 5        | 0.908 | 0.001 | 0.707 | 0.004 | 0.167 | 0.036 | 0.497 | 0.041 | 0.550 | 0.006 | 0.482 | 0.005 |

Pa=Probability ‘to be active’; Pi = Probability ‘to be inactive’.
3.9. Molecular electrostatic potential analysis

Molecular electrostatic potential (MEP) was calculated to illustrate the molecule’s charge distributions. It can serve in the prediction of reactive sites for the electrophilic and nucleophilic attack as presented in Fig. 7. Red color corresponds to the maximum negative area which is a suitable part of the molecule for the electrophilic attack, the blue color represents the maximum positive area which is the suitable region for the nucleophilic attack, and the green color represents zero potential domain. Additionally, the areas which

![Fig. 5. DFT (631 G basis set) optimized structures of 1) amentoflavone, 2) agathisflavone, 3) robustaflavone, 4) hinokiflavone and 5) rhusflavanone.](image)

| Compound | Ball and stick model | Tube model |
|----------|----------------------|------------|
| 1        | ![Image](image)      | ![Image](image) |
| 2        | ![Image](image)      | ![Image](image) |
| 3        | ![Image](image)      | ![Image](image) |
| 4        | ![Image](image)      | ![Image](image) |
| 5        | ![Image](image)      | ![Image](image) |

Table 5

Molecular formula (MF), electronic energy (EE), enthalpy, Gibbs free energy (GFE) and dipole moment (DM) of 1) amentoflavone, 2) agathisflavone, 3) robustaflavone, 4) hinokiflavone and 5) rhusflavanone.

| Compound | MF      | EE (Hartree) | Enthalpy (Hartree) | GFE (Hartree) | Entropy (cal/mol-K) | DM (Debye) |
|----------|---------|--------------|--------------------|---------------|---------------------|------------|
| 1        | C_{30}H_{18}O_{10} | -1905.8221 | -1905.3672 | -1905.4660 | 207.931 | 4.2038 |
| 2        | C_{30}H_{18}O_{10} | -1905.8256 | -1905.3706 | -1905.4698 | 208.795 | 6.7152 |
| 3        | C_{30}H_{18}O_{10} | -1905.7473 | -1905.2908 | -1905.3889 | 206.345 | 6.0944 |
| 4        | C_{30}H_{18}O_{10} | -1905.7366 | -1905.2801 | -1905.3788 | 207.813 | 8.9097 |
| 5        | C_{30}H_{22}O_{10} | -1908.0994 | -1907.5963 | -1907.6983 | 214.605 | 7.9539 |

Table 6

Energy (eV) of HOMO, LUMO, energy gap, hardness, and softness of 1) amentoflavone, 2) agathisflavone, 3) robustaflavone, 4) hinokiflavone and 5) rhusflavanone.

| Compound | $\varepsilon_{\text{HOMO}}$ | $\varepsilon_{\text{LUMO}}$ | Gap         | Hardness ($\eta$) | Softness ($S$) |
|----------|-----------------------------|----------------------------|-------------|------------------|----------------|
| 1        | -6.452                      | -2.449                     | 8.901       | 4.451            | 0.225          |
| 2        | -6.407                      | -2.434                     | 8.841       | 4.421            | 0.226          |
| 3        | -6.066                      | -2.131                     | 8.197       | 4.099            | 0.244          |
| 4        | -6.100                      | -2.293                     | 3.829       | 4.197            | 0.238          |
| 5        | -5.898                      | -1.490                     | 7.408       | 3.694            | 0.271          |

3.9. Molecular electrostatic potential analysis

Molecular electrostatic potential (MEP) was calculated to illustrate the molecule’s charge distributions. It can serve in the prediction of reactive sites for the electrophilic and nucleophilic attack as presented in Fig. 7. Red color corresponds to the maximum negative area which is a suitable part of the molecule for the electrophilic attack, the blue color represents the maximum positive area which is the suitable region for the nucleophilic attack, and the green color represents zero potential domain. Additionally, the areas which
Fig. 6. DOS Plot and HOMO-LUMO energy gap of a) robustaflavone, b) hinokiflavone and c) rhusflavanone.

Fig. 7. Molecular electrostatic potential (eV) of 1) amentoflavone, 2) agathisflavone, 3) robustaflavone, 4) hinokiflavone and 5) rhusflavanone.
possess negative potential are over electronegative (oxygen atoms), and areas with positive potential are located over hydrogen atoms. MEP plays a significant role in identifying the electrostatic interaction between molecules and can be a useful tool in understanding the electrostatic contribution to the biological activities of tested molecules. The MEP results (Fig. 7) indicate negative red color for keto groups of rhusflavanone (−0.2724) suggesting the maximum possibility of electrophilic attack. Therefore, the maximum positive blue color (+0.1877) for agathistflavone followed by +0.1870 for rhusflavanone express maximum possibilities of nucleophilic attack sites of the investigated compounds. These results help us to understand the biological recognition process and hydrogen bonding interaction with Mpro (Fig. 1) and therefore MEP through visualization of reactive sites enables the understanding process of drug-receptor interaction.

5. Conclusion
Five structurally similar biflavonoids were investigated in this work using molecular docking, quantum chemical study and in vitro testing in order to examine their potential against COVID-19. PASS prediction indicated that studied molecules possess the capability to inhibit NO2 expression, NAD(P)H oxidation expression as well as scavenging of free radicals and antiviral activity, which is in good agreement with in vitro results. In this work, we discussed the free radical scavenging capacity of the biflavonoids in terms of their structures. Presence of hydroxyl functional groups in biflavonoids molecules mediate their antioxidant activities by scavenging free radicals. Related to these findings, DFT based thermodynamic properties were calculated which suggest that the presence of four additional H atoms in rhusflavanone increases GFE and dipole moment which is responsible for higher binding affinity with the target enzyme. ADMET and drug-likeness properties indicated that rhusflavanone shows better characteristics among tested molecules with low toxicity. These results will be helpful in the development of alternative or additional therapeutic/preventive strategies against the SARS-CoV-2 virus.

Author agreement
The authors Jovana Trifunovic Ristovski, Mohammed Mahbubul Matin, Ren Kong, Milica Paut Kusturica and Hao Zhang declare that they have seen and approved the final version of the manuscript being submitted. Authors warrant that the article is the authors’ original work, hasn’t received prior publication and isn’t under consideration for publication elsewhere.

Credit author statement
J.T.R.: Conceptualization, Formal analysis, Writing - original draft, Project administration.: R. K. M.M. and H. Z.: Conceptualization, Software, Experimental and Formal analysis. M.P.K Writing - original draft.

Declaration of Competing Interest
The authors Jovana Trifunovic Ristovski, Mohammed Mahbubul Matin, Ren Kong, Milica Paut Kusturica and Hao Zhang declare they have no conflict of interest.

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Supplementary materials
Supplementary material associated with this article can be found in the online version at doi:10.1101/jj.sajb.2022.02.009.

References
Al-Sehemi, A.G., Oloto, F.A., Dev, S., Pannipara, M., Soliman, M.E., Carradori, S., Mathew, B., 2020. Natural products database screening for the discovery of naturally occurring SARS-cov-2 spike glycoprotein blockers. ChemistrySelect 5 (42), 13399–13317.10.1002/slct.202003349.
Ali, M.J., Hanif, M., Haider, M.A., Ahmed, M.U., Sundas, F.N.U., Hirani, A., Khan, I.A., Anis, K., Karim, A.H., 2020. Treatment options for COVID-19: a review. Front. Med. 7, 480.10.3389/fmed.2020.00480.
Amatore, D., Sgarbanti, R., Aquilano, K., Baldelli, S., Limongi, D., Civitelli, L., et al., 2015. Influenza virus replication in lung epithelial cells depends on redox-sensitive pathways activated by NOX4-derived ROS. Cell. Microbiol. 17 (1), 131–145.10.1111/cmi.12343.
Andrade, A., Machado, K., Machado, K., Figueiredo, D., David, J.M., Islam, M.T., Uddin, S.J., Shilpi, J.A., Costa, J.P., 2018. In vitro antioxidant properties of the biflavonoid agathisflavone. Chem Cent J 12 (1), 75.10.1186/s13065-018-0483-3.
Chiow, K.H., Phoon, M.C., Putti, T., Tan, B.K., Chow, V.T., 2016. Evaluation of antiviral activities of Houttuynia cordata Thumb. extract, quercetin, quercetin and cinanerin on murine coronavirus and dengue virus infection. Asian Pac J Trop Med 9 (1), 1–7.10.1016/j.apjtm.2015.12.002.
Coulier, P., Nour, M., Maciuk, A., Eydoux, C., Guillemot, J.C., Lebouvier, N., et al., 2013. Structure-activity relationship study of biflavonoids on the Dengue virus polymerase DEN-NS5 RdRp. Planta. Med. 79 (14), 1313–1318.10.1055/s-0033-1350772.
Daina, A., Michielin, O., Zoete, V., 2017. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep-UK. 7, 42717.10.1038/srep42717.
de Fraga, C.S., Rocha, M., Sacramento, C.Q., Martorelli, A., Ferreira, A.C., Rocha, N., et al., 2020. Agathisflavone, a Biflavonoid from Anacardium occidentaleoccidentale. Inhibits Influenza Virus Neuraminidase. Curr. Top. Med. Chem. 20 (2), 111–120.10.2174/156802666131950738.
Di Majo, D., Guardia, La, Giammanco, M., La Neve, L., 2008. The antioxidant capacity of red wine in relationship with its polyphenolic constituents. Food. Chem. 111, 45–49.10.1016/j.foodchem.2008.03.037.
Dieck, M.H., Hehr, W.J., Pople, J.A., 1971. Self-consistent molecular orbital methods-9: extended Gaussian-type basis for molecular-orbital studies of organic molecules. J. Chem. Phys. 54, 724.10.1063/1.1674902.
Forni, G., Mantovani, A., et al., 2021. on behalf of the COVID-19 Commission of Accademia Nazionale dei Lincei. COVID-19 vaccines: where we stand and challenges ahead Cell. Death. Differ. 28, 626–639.10.1038/s41418-020-00720-9.
Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. Anal. Biochem. 126, 131–138.10.1016/0003-2697(82)90118-x.
Halim, S.A., Waqas, M., Khan, A., Al-Harrasi, A., 2021. Silico prediction of novel inhibitors of SARS-CoV-2 main protease through structure-based virtual screening and molecular dynamic simulation. Pharmaceuticals 14, 896.10.3390/iph20040896.
Ho, L., Chan, K., Chung, V., Leung, T.H., 2020. Highlights of traditional Chinese medicine frontline expert advice in China the national guidance for COVID-19. Eur. J. Integr. Med. 36, 101116.10.1016/j.eujim.2020.101116.
Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., et al., 2020. Structure of M pro from COVID-19 virus and discovery of its inhibitors. Nature 582, 289–293.10.1038/s41586-020-29648-2.
Kong, R., Yang, G., Xue, R., et al., 2020. COVID-19 Docking Server: a meta server for docking small molecules, peptides and antibodies against potential targets of COVID-19. Bioinformatics.10.1093/bioinformatics/btaa455.
Li, F., Song, X., Su, G., Wang, Y., Wang, Z., Jia, J., et al., 2019. Amentoflavin inhibits HSV-1 and CDV-resistant strain infection by suppressing viral early infection. Viruses 11 (5), 466.10.3390/v11050466.
Lin, Y.M., Flavin, M., Schure, R., Chen, F., Sildwell, R., Barnard, D., et al., 1999. Antiviral Activities of Biflavonoids. Planta. Med. 65 (2), 120–125.10.1055/s-1999-11371.
Mahmood, M.S., Martinez, J.L., Aslam, A., Rafigue, A., Vinet, R., Laradou, C., et al., 2016. Antiviral effects of green tea (Camellia sinensis) against pathogenic viruses in human and animals (a mini-review). Afr J Tradit Complement Altern Med 13 (2), 176–184.10.4314/ajtcam.v13i2.21.
Martinez, M.A., 2021. Lack of effectiveness of repurposed drugs for COVID-19 treat- ment. Front. Immunol. 11, 606688.10.3389/fimmu.2021.635371.
Matin, M.M., Hasan, S., Uzaman, M., Bhuyan, Kibra, S.M., Hossain, E., Rashid, M.H.O., 2020. Synthesis, spectroscopic characterization, molecular docking, and ADMET studies of mannopyranoside esters as antimicrobial agents. J. Mol. Struct. 1222, 12882.10.1016/j.molstruc.2020.128821.
Nguyen, T.T., Woo, H.J., Kang, H.K., Nguyen, V.D., Kim, Y.M., Kim, D.W., et al., 2012. Flav- vonoid-mediated inhibition of SARS coronavirus 3C-like protease expressed in Pichia pastoris. Biotechnol. Lett. 34 (5), 831–838.10.1007/s10529-011-0845-8.
Park, H.R., Yoon, H., Kim, M.K., Lee, S.D., Chong, Y., 2012. Synthesis and antiviral evaluation of 7-O-aryl methylquercetin derivatives against SARS-associated coronavirus (SCV) and hepatitis C virus (HCV). Arch. Pharm. Res. 35 (1), 77–85. https://doi.org/10.1007/s12272-012-0108-9.

Recharla, N., Riaz, M.Z., Ko, S., Park, S.K., 2017. Novel technologies to enhance solubility of food-derived bioactive compounds: a review. J. Funct. Foods. 39, 63–73. https://doi.org/10.1016/j.jff.2017.10.001.

Ryu, Y.B., Jeong, H.J., Kim, J.H., Kim, Y.M., Park, J.Y., Kim, D., et al., 2010. Biflavonoids from Torreya nucifera displaying SARS-CoV 3CL (pro) inhibition. Bioorg. Med. Chem. 18 (22), 7940–7947. https://doi.org/10.1016/j.bmc.2010.09.035.

Siddiqi, H.K., Libby, P., Ridker, P.M., 2021. COVID-19 - A vascular disease. Trends. Cardi-ovasc. Med. 31 (1), 1–5. https://doi.org/10.1016/j.jcmd.2020.10.005.

Ulrich, S., Nitsche, C., 2020. The SARS-CoV-2 main protease as drug target. Bioorg. Med. Chem. Lett. 30, e127377. https://doi.org/10.1016/j.bmcl.2020.127377.

Wang, X.G., Liu, Z.J., 2014. Prevention and treatment of viral respiratory infections by traditional Chinese herbs. In Chinese Med. J. Chinese Med. Assoc. 127 (7), 1344–1350. https://doi.org/10.3760/cma.j.issn.0366-6999.20132029.

Weng, J.R., Lin, C.S., Lai, H.C., Lin, Y.F., Wang, C.Y., Tsai, Y.C., et al., 2019. Antiviral activity of Sambucus Formosana Nakai ethanol extract and related phenolic acid constitu-ents against human coronavirus NL63. Virus Res. 273, 197767. https://doi.org/10.1016/j.virusres.2019.197767.

Zhang, D.H., Wu, K.L., Zhang, X., Deng, S.Q., Pen, B., 2020. In silico screening of Chinese herbal medicines with the potential to directly inhibit 2019 novel coronavirus. J. Integr. Med. 18 (2), 152–158. https://doi.org/10.1016/j.jim.2020.02.005.

Zhou, H., Fang, Y., Xu, T., Ni, W.J., Shen, A.Z., Meng, X.M., 2020. Potential therapeutic targets and promising drugs for combating SARS-CoV-2. Br. J. Pharmacol. 177 (14), 3147–3161. https://doi.org/10.1111/bph.15092.