In vitro and in silico studies of SARS-CoV-2 main protease $M^{\text{PRO}}$ inhibitors isolated from *Helichrysum bracteatum*†

Gehad Abdel Wahab, Walaa S. Aboelmaaty, Mohamed Farid Lahloub and Amal Sallam

Discovering SARS-CoV-2 inhibitors from natural sources is still a target that has captured the interest of many researchers. In this study, the compounds (1–18) present in the methanolic extract of *Helichrysum bracteatum* were isolated, identified, and their *in vitro* inhibitory activities against SARS-CoV-2 main protease ($M^{\text{PRO}}$) was evaluated using fluorescence resonance energy transfer assay (FRET-based assay). Based on 1D and 2D spectroscopic techniques, compounds (1–18) were identified as 24-$\beta$-ethylcholesta-5(6),22(23),25(26)-triene-3-$\alpha$-ol (1), $\alpha$-amyrin (2), linoleic acid (3), 24-$\beta$-ethyl-cholesta-5(6),22(23),25(26)-triene-3-$\alpha$-O-$\beta$-$\alpha$-glucoside (4), 1,3-propanediol-2-amino-1-(3',4',5'-methyleneoxyphenyl) (5), (-)-(7R,8R,8'R)-acuminatolide (6), (+)-piperitol (7), 5,7,4'-trihydroxy-8,3'-dimethoxy flavanone (8), 5,7,4'-trihydroxy-6-methoxy flavanone (9), 4',5-dihydroxy-3',7,8-trimethoxyflavone (10), 5,7-dihydroxy-3',4',5,8-tetramethoxy flavone (11), 1,3-propanediol-2-amino-1-(4'-hydroxy-3'-methoxyphenyl) (12), 3',3',5,7-tetrahydroxy-6-methoxyflavonane (13), simplicoside (piperitol-O-$\beta$-$\alpha$-glucoside) (14), pinoresinol monomethyl ether-$\beta$-$\alpha$-glucoside (15), orientin (16), luteolin-3'-O-$\beta$-$\alpha$-glucoside (17), and 3,5-dicaffeoylquinic acid (18). Compounds 6, 12, and 14 showed comparable inhibitory activities against SARS-CoV-2 $M^{\text{PRO}}$ with IC$_{50}$ values of 0.917 ± 0.05, 0.476 ± 0.02, and 0.610 ± 0.03 μM, respectively, compared with the control lopinavir with an IC$_{50}$ value of 0.225 ± 0.01 μM. The other tested compounds showed considerable inhibitory activities. The molecular docking study for the tested compounds was carried out to correlate their binding modes and affinities for the SARS-CoV-2 $M^{\text{PRO}}$ enzyme with the *in vitro* results. Analyzing the results of the *in vitro* assay together with the obtained *in silico* results led to the conclusion that phenylpropanoids, lignans, and flavonoids could be considered suitable drug leads for developing anti-COVID-19 therapeutics. Moreover, the phenylpropanoid skeleton oxygenated at C3, C4 of the phenyl moiety and at C1, C3 of the propane parts constitute an essential core of the SARS-CoV-2 $M^{\text{PRO}}$ inhibitors, and thus could be proposed as a scaffold for the design of new anti-COVID-19 drugs.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel strain of the coronavirus group. It emerged in the city of Wuhan, China at the end of 2019, causing an outbreak of unusual viral pneumonia. It caused acute respiratory disease that was named coronavirus disease 2019 (COVID-19) by the WHO on 11 February 2020. Being highly transmissible and pathogenic such that it has spread fast all over the world, WHO defined it on 11 March 2020 as a pandemic, posing an extraordinary threat to global public health.† To date, there are no generally proven antiviral drugs against SARS-CoV-2, although several clinical trials all over the world are testing several known antiviral drugs. Therefore, finding natural, semisynthetic, or synthetic remedies for COVID-19 is the target of many researches now.

The main protease ($M^{\text{PRO}}$), papain-like protease ($PL^{\text{PRO}}$), and RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2 are considered decisive factors in the infectious route of the virus; they have been reported as important targets for therapeutic strategies.

SARS-CoV-2 main protease ($M^{\text{PRO}}$) or (3-chymotrypsin-like protease 3CL$^{\text{PRO}}$) is an enzyme responsible for the proteolysis and release of essential functioning peptides, playing a great role in replication, and thus the life cycle of the virus. Moreover, $M^{\text{PRO}}$ is highly conserved across coronaviruses; thus, inhibiting main proteases is considered an attractive target for the discovery of effective antiviral drugs for the treatment of not only SARS-CoV-2 but also other coronaviruses. Although

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researchers have focused their efforts on studying the potential antiviral properties of plants and their constituents from different classes including flavonoids and lignans as antiviral agents through *in silico* studies targeting M^PRO^ and other enzymes, little has been found concerning the *in vitro* studies.1–7

The genus *Helichrysum* belonging to family *Asteraceae (Compositae)* includes approximately 600 species spread widely all over the world, especially in the Southern Hemisphere; it is also widespread through Eurasia, Australia, and the Mediterranean region.8–11 Several classes of phytoconstituents have been reported in different *Helichrysum* species, mainly phenolics, lignans, phloroglucinols, pyrones, fatty acids, and terpenoid compounds.12,13 Since ancient times, *Helichrysum* species are well known for their medicinal properties as diuretic, anti-inflammatory, hepatoprotective, and anti-psoriatic. Also, they have been used in treatment of colds, cough, inflammation, and allergy conditions such as those related to the respiratory tract.14,15

It is reported that several species of genus *Helichrysum* show antiviral activities against different viruses including coronaviruses or similar viruses. *H. arenarium* showed antiviral activities against Herpes simplex virus Type-1 (HSV-1) and Parainfluenza-3. *H. italica* and *H. auronitens* have antiviral activities against HSV-1, while *H. melananche* has antiviral activity against HIV.10,11,14,15 Helichrysin, a chalcone derivative found within numerous *Helichrysum* species, was reported to be able to inhibit MERS-CoV S^RLL^16,17

*H. bracteatum* is known as straw flower and is widely cultivated as an ornamental plant.16,17 Previous phytochemical studies have reported the presence of different classes of compounds as flavonoids, lignans, and phenolic acids.17–19

This study is concerned with discovering potential antiviral leads against COVID-19 based on the evaluation of the ability of *H. bracteatum* methanolic extract, as well as fractions and isolated phytoconstituents to inhibit SARS-CoV-2 main protease (M^PRO^) through *in vitro* and *in silico* studies.

2. Results and discussion

2.1 Characterization of the isolated compounds

Phytochemical investigation of petroleum ether, methylene chloride, and ethyl acetate fractions of the methanolic extract of *H. bracteatum* leaves resulted in the isolation and structure elucidation of eighteen compounds (1–18) (Fig. 1) including two steroidal compounds (1, 4), one pentacyclic triterpene (2), one fatty acid (3), two phenyl propanoid derivatives nitrogenated at C2 (5, 12), four lignans (6, 7, 14, 15), seven flavonoids, three flavanones (8, 9, 13), four flavones (10, 11, 16, 17), and one phenolic acid derivative (18). The proton and carbon values beside the spectra of the isolated compounds are present in the ESI file (Data S1 and Fig. S1–S50†).

Compounds (1–4) were isolated from the petroleum ether fraction. They were confirmed from their proton and carbon chemical shift values (Fig. S1–S87†) compared with those reported in the literature. They were identified as 24β-ethylcholesta-5(6),22(23),25(26)-triene-3-Oβ-D-glucoside (4),20 besides α-amyrin (2),21,22 and linoleic acid (3).23 It is the first time that compounds 1 and 4 have been isolated from family *Compositae*. It is the first time that compound 2 has been isolated from genus *Helichrysum*. Compound 3 was previously reported from *Helichrysum* seed oil.24

Compounds 5 and 12 were concluded to be phenyl propanoid derivatives nitrogenated at C2. The careful examination of the 1H-NMR spectrum of compound 5 (Fig. S9†) showed signals at δ_H 6.78 (br d, 1H, J = 8, H-5'), δ_H 6.80 (br d, 1H, J = 10.8, H-6'), and δ_H 6.85 (br s, 1H, H-2'), indicating the possible presence of the tri-substituted phenyl ring. It also showed the signal of an oxygenated methylene that may represent a methylenedioxy group at δ_H 5.95 (s, 2H), substituting the phenyl ring. Signals representing one oxygenated methine proton at δ_H 4.72 (d, 1H, J = 3.2, H-1') and one aminomethine proton δ_H 3.05 (m, 1H, H-2) besides an oxygenated methylene group at δ_H 4.23 (dd, 1H, J = 6.4 & 8.4, H-3a) and δ_H 3.87 (dd, 1H, J = 6.8 & 2, H-3b) were also observed, indicating the possible presence of the 1,3-dihydroxy-2-amino-propane moiety. The APT spectrum (Fig. S10†) showed signals supporting the previous possibilities. It revealed two oxygenated quaternary aromatic carbon signals at δ_C 148.0 (C-3') & 147.1 (C-4'), a quaternary aromatic carbon at δ_C 135.0 (C-1'), and three aromatic methine carbons at δ_C 106.5 (C-2'), 108.2 (C-5'), and 119.4 (C-6'). It also showed carbon signals representing oxygenated methylene carbon at δ_C 101.1, indicating the presence of the methylenedioxy group substituting the phenyl ring. Moreover, it showed signals representing one oxygenated aliphatic methine carbon at δ_C 85.8 (C-1) and one oxygenated methylene carbon at δ_C 71.7 (C-3) and one aminomethine carbon at δ_C 54.3 (C-2), which was shifted upfield than the oxygenated carbons (C-1 & 3), thus confirming the presence of the 1,3-dihydroxy-2-amino-propane moiety. The HMBC spectrum (Fig. S11†) showed a cross peak correlating the proton signal at δ_H 4.71 (H-1') with the quaternary carbon signals at δ_C 135.0 (C-1'), 106.5 (C-2'), and 119.4 (C-6'). This indicated that the 1,3-dihydroxy-2-amino-propane moiety substituted the phenyl ring at C-1'. The HMBC cross peaks from the methylenedioxy group δ_H 5.95 to carbons δ_C 148.0 (C-3') & 147.1 (C-4') confirmed that the methylenedioxy group is substituting the phenyl ring at C-3' & 4'. Compound 5 was identified as 1,3-propanediol-2-amino-1-(3',4'-methylenedioxygenyl). It was previously isolated only from *Artemisia selengensis* F. *Compositae*;25 also, it was reported as a synthetic compound.26

The careful examination of the 1H-NMR and APT spectra of compound 12 (Fig. S26 and S27†) indicated that it is also a phenyl propanoid derivative nitrogenated at C2. Comparing its 1H-NMR and APT spectra with those of compound 5 revealed that compound 12 was similar to compound 5, but the phenyl ring is substituted with methoxy and hydroxy groups instead of methylenedioxy group (at C-3', 4'). Compound 12 was identified as 1,3-propanediol-2-amino-1-(4'-hydroxy-3'-methoxyphenyl).27 It was previously isolated only from *Santolina chamaecyparissus* F. *Compositae* and our study reports its detailed chemical shift values for the first time.
Compounds 7, 14, and 15 were found to be furofuranlignans. Comparing the data obtained from $^1$H-NMR and APT spectra (Fig. S14 and S15†) with that reported in the previous literature and the stereochemistry reported for furofuranlignans, compound 7 was identified as (+)-piperitol. Compound 14 was found to be the glucoside of 7; this was revealed by its $^1$H-
NMR and APT spectra (Fig. S40 and S41†), which showed the doublet signal representing the anomic proton (H-1°) at 4.88 ppm (1H, d, J = 6.7 Hz) in addition to the carbon signal at δc 100.6 (C-1°) and the four hydroxylated aliphatic methine carbons at δc 71.6, 77.3, 70.1, 77.4, besides the hydroxylated aliphatic methylene carbon at δc 61.1 (C-6°), indicating the presence of the hexose sugar glucose. Compound 14 was identified as simplexide (piperitol-O-β-D-glucoside).13,14 Both compounds 7 & 14 were previously isolated from H. bracteatum aerial parts.15 A careful examination of the 1H-NMR and APT spectra of compounds 14 & 15 (Fig. S40–S43†) revealed that compound 15 is similar to compound 14, except for the presence of two signals at δH 3.76 (6H, s, OCH3-3 & OCH3-4), representing two methoxy groups instead of the signal at 6.00 (2H, s, OCH2O), thus representing the methylenedioxy group in compound 14. Compound 15 was identified as pinoresinol monomethyl ether-β-D-glucoside.16 Compound 15 is isolated from F. Compositae (Asteraceae) for the first time in this study. Comparing the 1H-NMR and APT spectra of compound 6 (Fig. S12 and S13†) with compound 7 revealed that 6 is similar to 7, but it lacks the presence of any aryl group; instead, it showed a carbonyl moiety. This was deduced from the carbonyl group at δH 178.1 corresponding to C-7°. Comparing this data with the previous literature, compound 6 was identified as (−)-7R,8R,8′R)-acuminatolide.16 To the best of our knowledge, acuminatolide was previously isolated from aerial parts of H. acuminatum.17

Compounds 8, 9, and 13 were found to be flavanones depending on the common characteristic features that appeared in the UV-spectral data besides both 1H-NMR and APT data. The UV-spectra of these three compounds showed two absorption bands, band I in the λmax range of 289–291 nm and band II in the λmax range of 232–236 nm, which appeared to be in agreement with those characteristic for flavanones or dihydropyrones.18,19 The 1H-NMR spectra of the three compounds (Fig. S16, S19 and S28†) revealed the presence of two double doublet signals characteristic for H-3α and H-3β in the range of δH 2.5–3.01 besides one doublet of doublet signal corresponding to the oxygenated methine H-2 at δH 5.20. The APT spectra of the three compounds (Fig. S17, S20 and S31†) supported this deduction by showing signals representing carbonyl (C-4) in the range of δc 195.2–197.2, a signal for the methylene group at 42.7 (C-3), besides the signal representing oxygenated methine (C-2) at δc 79.2.20

The UV spectral data in different shift reagents (Table S1†) suggested that 8 has a flavanone structure with free OH groups at positions 5, 7, and 4°. The 1H-NMR spectrum (Fig. S16†) showed a signal at δH 5.18 (1H, dd, J = 2.8 & 12.6) representing the oxygenated methine H-2 and pair of double doublets at δH 2.56 (1H, dd, J = 2.8 & 17.2) and δH 2.97 (1H, dd, J = 12.8 & 17.2) representing the methylene protons H-3α and 3β, respectively. These signals are characteristic of the flavanone skeleton. Also, it revealed a singlet signal at δH 3.79 (1H, s, H-6), indicating that ring A has only one free proton. Besides, the signals representing the ABX system in ring B that were obtained at δH 6.97 (1H, dd, J = 1.6, H-2°), δH 6.71 (1H, d, J = 8, H-5°), and δH 6.81 (1H, dd, J = 8.2 & 2, H-6°). In addition, it showed two signals of two methoxy groups at δH 3.66 & 3.78. The APT spectrum (Fig. S17†) showed six oxygenated aromatic carbon signals along with a carbonyl signal in the range of δc 130–200 ppm. The presence of a carbonyl signal at δc 195.7 (C-4), an oxygenated methine signal at δc 79.2 (C-2) and a methylene signal at 42.7 (C-3) confirmed the flavanone skeleton.40,41 There were three signals representing the oxygenated aromatic carbons substituted with free hydroxyl groups at δc 159.0, 164.6, & 146.6 representing C-5, 7, & 4°, respectively, which were confirmed previously by UV data; the other two signals were methoxylated, one at δc 147.7 representing C-3° as ring B showed an ABX system with free OH at C-4° only. The other one at δc 130.7 represents C-8 rather than C-6 that was not substituted and appeared at δc 96.3. Reviewing previous literature that reported flavanone oxygenated at C-8 while C-6 is free and those reported compounds oxygenated at C-6 while C-8 is free, it could be noticed that when C-8 is oxygenated while C-6 is free, C-6 appears at a chemical shift value that is slightly downfield shifted (δc 96.3 as in compound 8) compared with the chemical shift of C-8 if it is free and C-6 is oxygenated, where C-8 in this case appears slightly shifted upfield40,41 (Table S2†). The data obtained from UV, 1H-NMR, and APT suggested that compound 8 is a flavanone hydroxylated at 5, 7, & 4° and methoxylated at 8 and 3°. The HMBC spectrum of compound 8 (Fig. S18†) showed cross peaks correlating the methoxy group proton signal at δH 3.66 (H-R4) with the carbon signal at δc 130.7 (C-8); also, it showed cross peaks correlating the methoxy group proton signal at δH 3.78 (H-R3) with the carbon signal at δc 147.7 (C-3°). These data confirmed that both C-8 and 3° are blocked with OCH3 groups, while C-5, 7, and 4° are substituted with free hydroxyl groups.

Comparing the proton and carbon values of compound 9 obtained from 1H-NMR and APT spectra (Fig. S19 and S20†) with those of compound 8 revealed that compound 9 is similar to compound 8 with the presence of the methoxy group at C-6 rather than C-8 (owing to the slightly upfield shifted value of C-8 at δh 94.8 compared to the value of the unsubstituted C-6, which is slightly shifted downfield when unsubstituted at δc 96.3 as in compound 8) and the absence of the methoxy group at C-3° (Fig. S19, S20 and Table S2†). Thus, compound 9 was identified as 5,7,4°-trihydroxy-8,3°-dimethoxy flavanone from the family Compositae. It was previously isolated from Iris unguicularis42 and our study reports its detailed chemical shift values.

Comparing the proton and carbon values of compound 13 obtained from 1H-NMR and APT spectra (Fig. S19 and S20†) with those of compound 8 revealed that compound 9 is similar to compound 8 with the presence of the methoxy group at C-6 rather than C-8 (owing to the slightly upfield shifted value of C-8 at δh 94.8 compared to the value of the unsubstituted C-6, which is slightly shifted downfield when unsubstituted at δc 96.3 as in compound 8) and the absence of the methoxy group at C-3° (Fig. S19, S20 and Table S2†). Thus, compound 9 was identified as 5,7,4°-trihydroxy-6-methoxy flavanone and it is the first time to be isolated from F. Compositae in this study.43,44

The molecular formula of compound 13 was determined to be C16H12O7 from the [M + H]+ peak at m/z 319.24 appearing in the LC-ESI+–MS spectrum (Fig. S36†), which is in agreement with the calculated one at m/z 319.28. The UV spectral data in
different shift reagents (Table S1†) indicated that 13 has a flavanone structure with free OH groups at positions 5 and 7.44 Comparing the proton and carbon chemical shift values of ring A in compound 13 obtained from 1H-NMR and APT spectra (Fig. S28–S31, S38 and Table S2†) with those of compound 9 revealed that both compounds show the same substitution patterns in ring A (5,7-dihydroxy & 6-methoxy). The 1H-NMR, APT, & HSQC spectra showed signals characteristic for the flavanone skeleton at δH 3.16 (1H, dd, J = 2.8 & 12.8) representing the oxygenated methine H-2 correlated to δC 79.2, a pair of double doublets at δH 2.60 (1H, dd, J = 8.8 & 17.2), and δH 2.96 (1H, dd, J = 12.8 & 17.2) representing methylene protons H-3z & 3β, respectively, which are correlated to δC 42.7 besides the carbonyl at δC 197.2 (C-4).40,41 The singlet signal at δH 5.87 (1H, s, H-8) correlated to a methoxy group at δCUC 3.68/59.6 were also present. Singlet signals representing three protons of ring B at δH 6.68 (2H, s) and δH 6.81 (1H, s) representing H-2', 4', & 6' correlated to δC 179.5, 114.8, & 113.3, respectively, revealed the absence of AB or ABX system in ring B. This suggestion was supported by the HMBC cross peaks from δH 6.68 (H-2') & 6.81 (H-6') to the carbon signal at δC 79.2 (C-2) and from δH 6.68 & 6.81 (H-2'& H-6') to δC 114.8(C-4') (Fig. S34 and S35†). The APT spectrum (Fig. S31†) showed six oxygenated aromatic carbon signals along with a carbonyl one in the range of δC 129–200 ppm. The carbon signal at δC 129.0 is methoxylated rather than hydroxylated; this could be detected from the HMBC spectrum (Fig. S34†) that showed cross peaks correlating the methoxyprotons at δH 3.68 with the carbon signal at δC 129.0. The carbon signal representing the methoxylated aromatic carbon at δC 129.0 was assigned to C-6 rather than C-8 that was not substituted and appeared at a slightly upfield chemical shift value (δC 94.3, as C-8 of compound 9) when compared with the chemical shift value of the unsubstituted C-6 that appeared at a slightly downfield shifted value (δC 96.3, as C-6 in compound 8)40,44 (Table S2†). The HMBC spectrum showed cross peaks correlating the signal at δH 5.87 (H-8) with carbon signals at δC 129.0 (C-6), 158.8 (C-9), 159.5 (C-7), & 102.1 (C-10). The hydroxylated carbon signals at δC 165.2 & 159.5 were assigned to C-5 & 7. The remaining oxygenated carbon signals at δC 145.1 & 145.5 were assigned to the hydroxylated carbons in ring B. Lacking an AB or ABX system in ring B and absence of free hydroxyl group at C-4' (according to UV data) suggested rare substitution by the hydroxyls at C-3' & C-5' not the common one at C-3' & C-4' in ring B. This suggestion was supported by the HMBC correlation from δH 6.68 (H-2') to δC 145.1 (C-3') and from δH 6.81 (H-6') to δC 145.5 (C-5') (Fig. S34†). The ESI-MS fragmentation of compound 13 showed a base peak at m/z 183.61 [M + H]+ corresponding to the fragment (a) 3-(3,5-dihydroxyphenyl)propanoic acid fragment (calculated m/z 183.18), confirming that ring B is disubstituted by two hydroxyl groups (Fig. S37A and B†). The previous data suggested that compound 13 is 5,7,3',5'-tetrahydroxy-6-methoxy flavanone. Reviewing the current literature, it was found that this study is the first to report the isolation of 5,7,3',5'-tetrahydroxy-6-methoxy flavanone from F. Compositae (Asteraceae). It was previously isolated only from Salvia plebeian F. Labiatae;44 also, it was reported as a semi-synthetic compound.45 This study is the first that reports detailed data for that rare substituted flavanone at C-3' & 5'.

The careful examination of the UV, 1H-NMR, and APT spectra of compounds 10 & 11 indicated that they are flavones. They were identified as 4',5-dihydroxy-3',7,8-trimethoxyflavone (10) and 5,7-dihydroxy-3',4',5',8-tetramethoxy flavone (11). This was deduced from the UV-spectra of the two compounds that showed two absorption bands, band I in the λmax range of 323–345 nm and band II in the λmax range of 276–278 nm, which appear to be in agreement with those characteristic for flavones.8,9 The 1H-NMR spectra of the two compounds (Fig. S21 and S24†) revealed the presence of a singlet signal corresponding to the methine proton H-3 at (δH 6.99 & 6.62 for compounds 10 & 11, respectively). The APT spectra of the two compounds (Fig. S22 and S25†) supported this deduction by showing signals representing carbonyl (C-4) (at δC 182.7 & 182.4 for compounds 10 & 11, respectively) and the signal for the methine carbon C-3 (at δC 103.5 & 105.4 for compounds 10 & 11, respectively).

The UV spectral data in different shift reagents suggested that 10 has a flavone structure with free OH groups at positions 5 and 4.44 Both 1H- and DEPT-Q NMR data also supported the previous conclusion. The 1H-NMR spectrum (Fig. S21†) showed two singlet signals at δH 6.59 (1H, s, H-6) and 6.99 (1H, s, H-3). Also, it showed signals representing aromatic protons of ring B at δH 7.59 (1H, s, H-2'), 7.00 (1H, d, J = 6.7, H-5'), and 7.60 (1H, d, J = 6, H-6'). Besides, it revealed three methoxy groups at δH 3.92, 3.86, & 3.90 and carbon signals at δC 56.9, 61.6, & 56.5 representing R3, R6, & R5. The DEPT-Q spectrum (Fig. S22†) showed seven oxygenated aromatic carbon signals along with the carbonyl signal in the range of δC 125–183 ppm. The presence of a carbonyl signal at δC 182.7 (C-4) and a signal at 103.5 (C-3) confirmed the flavone skeleton.40,44 The two signals at δC 164.3 & 151.4 ppm were assigned to the two ether-linked carbons C-2 and C-9, respectively. The two signals of hydroxylated aromatic carbons at δC 157.1 & 148.5 were assigned to C-5 & 4', respectively, which was confirmed from the HMBC spectrum (Fig. S23†) that showed cross peaks correlating δH 6.59 (H-6) with both oxygenated carbons at δC 128.9 (C-8) & 104.3 (C-10), and the absence of a cross peak with C-9 (δC 151.4), confirming that H-8 is blocked. The other three methoxylated aromatic carbon signals at δC 158.8, 128.9, & 149.2 were assigned to carbons C-7, 8, & 3'; this was supported by the HMBC cross peaks correlating the methoxyl group proton signal at δH 3.92 (OCH3-R3) with the carbon signal at δC 158.8 (C-7). Also, it showed a cross peak correlating the methoxyl group proton signal at δH 3.86 (OCH3-R6) with the carbon signal at δC 128.9 (C-8). The third methoxyl group at δH 3.90 (OCH3-R5) showed a cross peak with the carbon signal at δC 149.2 (C-3'). The data obtained from UV, 1H, and DEPT-Q spectra revealed that compound 10 is a flavone hydroxylated at position 5 & 4' and methoxylated at C-7, 8, & 3'. This structure is in agreement with that representing 4',5-dihydroxy-3',7,8-trimethoxyflavone. Comparing this data with that reported in the literature, compound 10 could be identified as 4',5-dihydroxy-3',7,8-trimethoxyflavone.40 It is the first report for the isolation of 4',5-dihydroxy-3',7,8-trimethoxyflavone from F. Compositae.
The data obtained from UV spectra in different shift reagents (Table S1†), 1H-NMR, APT, and HMBC spectra (Fig. S46–S48†) of compound 17 are consistent with those reported for luteolin-3'-O-β-D-glucoside.38,40 It was previously isolated from *H. arenarium* flowers.31

The careful examination of both 1H-NMR and APT spectra of 18 (Fig. S49 and S50†) showed that compound 18 contains quinic acid nucleus esterified with the two caffeoyl moieties at C-3 & C-5. Thus, compound 18 was identified as 3,5-dicafeoylquinic acid (isochlorogenic acid).32 It was previously isolated from *H. bracteatum* flowers and *H. italicum* aerial parts.18,51

2.2 Inhibitory activities against SARS-CoV-2 main protease (MPro or 3CLpro)

Several species of genus *Helichrysum* were reported to have antiviral activities against coronaviruses and other viruses such as Herpes simplex virus Type-1.16,33 Previous literature reported the ability of helichrysetin, a chalcone derivative isolated from certain *Helichrysum* species, to inhibit MERS-CoV 3CLpro. Several classes of phytoconstituents as flavonoids and lignans are reported in *in silico* studies as potential anti-COVID-19 agents by inhibiting SARS-CoV-2 main protease (MPro) and other enzymes involved in the virus life cycle.45 These facts encouraged us to evaluate the activity of *H. bracteatum* leaves methanolic extract, fractions, and the isolated compounds as inhibitors of SARS-CoV-2 MPro.

The methanolic extract exhibited inhibitory activity with IC50 value of 14.47 ± 0.74 μg mL−1. Among the tested four fractions, the ethyl acetate fraction showed the highest inhibitory activity followed by petroleum ether, methylene chloride, and butanol fractions with IC50 values of 2.589, 3.466, 16.05, and 21.9 μg mL−1, respectively, compared with the standard antiviral compound lopinavir with an IC50 value of 0.225 ± 0.01 μM (Table S4 and Fig. S57). The isolated compounds (1–18) were evaluated for their inhibitory activities against SARS-CoV-2 MPro. Compounds 6, 12, and 14 showed comparable inhibitory activities against SARS-CoV-2 MPro with IC50 values of 0.917 ± 0.05, 0.476 ± 0.02, and 0.610 ± 0.03 μM, respectively, compared with the control lopinavir with an IC50 value of 0.225 ± 0.01 μM. Compounds 2, 5, 11, 13, & 18 showed moderate inhibitory activities with IC50 values in the range of 4–8 μM, while compounds 1, 7, 9, 10, 15, & 17 exhibited significant activities with IC50 values in the range of 10–16 μM. Compounds 3, 8, & 16 showed weak activities with IC50 values in the range of 20–28 μM. The lowest activity was reported for compound 4 (IC50 value of 89.99 ± 4.59 μM).

Compounds 12, 14, and 6 showed the highest inhibitory activity. Compounds 6 and 14 are lignans, while compound 12 is a phenyl propanoid derivative nitrogenated at C2. The three compounds share the presence of the phenyl propanoid part oxygenated at C1 & C3 of the propane moiety and C3' & C4' of the phenyl moiety. It seems that the oxygenated phenyl propanoid part is crucial for the inhibitory activity, as revealed by the other tested compounds with moderate and significant activities as phenyl propanoid derivative nitrogenated at C2 (5), the flavanone compounds 9 & 13, and the lignan compound 15. 

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Although ethyl acetate & petroleum ether fractions showed better inhibitory activities than that of the methylene chloride fraction, compounds 12, 14, & 6 that exhibited the highest inhibitory activities were isolated from the methylene chloride fraction. This may be explained by the antagonistic effect of these compounds together and/or with other constituents.

This study is the first that reports the in vitro promising SARS-CoV-2 M\textsuperscript{pro} inhibitory activities of compounds 12, 14, & 6 besides the moderate activities of compounds 11, 13, & 5 against the M\textsuperscript{pro} enzyme. Compounds 2 & 18 were reported for their promising inhibitory activity against the M\textsuperscript{pro} enzyme through the in silico study.\textsuperscript{34,55} This study is the first study that proves this promising activity through the in vitro assay.

### 2.3 Molecular docking results

Molecular docking is considered as a tool that can be used for predicting the binding mode of the tested compounds with the targeted enzymes. SARS-CoV-2 main protease (M\textsuperscript{pro}) is an enzyme responsible for proteolysis and releasing the essential functioning peptides. It is a promising target against SARS-CoV-2 due to its importance in the viral life cycle replication. Thus, M\textsuperscript{pro} inhibition can better stop the viral replication and recover functioning peptides. It is a promising target against SARS-CoV-2.

The SARS-COV-2 Mpro inhibition (IC\textsubscript{50} \(0.225 \pm 0.01 \mu M\)) shared the N3 ligand in H-bonding binding with Glu166 & Gln189 amino acid residues. The most potent tested compound 12 (IC\textsubscript{50} 0.476 \(\pm 0.02 \mu M\) & binding energy slightly higher than that of lopinavir –10.79 & –9.61 kcal mol\(^{-1}\), respectively) exhibited four H-bonds with Glu 166, Gln 189, Thr 190, & Arg 188 amino acid residues through the free hydroxyl groups in the phenyl moiety and in the propane diol side chain (Fig. 2b). It shared both lopinavir and N3 ligand in H-bonding binding with Glu 166 & Gln 189 amino acid residues. Compound 14 (IC\textsubscript{50} 0.61 \(\pm 0.03 \mu M\) & binding energy –12.96 kcal mol\(^{-1}\) higher than that of lopinavir) exhibited seven H-bonds with Glu 189, Gln 166, Thr 26, Thr 24, Ser 46, & Gly 143 amino acid residues through the free hydroxyl groups of the glucose moiety and the oxygenated phenyl moiety besides the furan moiety (Fig. 2c). It shared both lopinavir and the N3 ligand in H-bonding binding with Gln 189 & Glu 166 amino acid residues; it shared the N3 ligand in binding with Gly 143 and Thr 26 amino acid residues. Compound 6 (IC\textsubscript{50} 0.917 \(\pm 0.05 \mu M\) & binding energy of –9.39 kcal mol\(^{-1}\) nearly similar to that of lopinavir) exhibited two H-bonds with Glu 166 & Ser 144 amino acid residues (Fig. 2d). It shared lopinavir in H-bonding binding with the amino acid residue Glu 166.

The other isolated compounds (Tables S5 and S6) also showed good binding interactions to M\textsuperscript{pro} active site’s crucial amino acid residues, which are very close to that of the N3 ligand and the standard lopinavir drug. All the tested compounds shared both lopinavir and N3 ligand in binding with Glu166 amino acid residue through H-bonding except compounds 3 and 4. Compounds 4, 5, and 18 shared both lopinavir and N3 ligand in binding with Gln189 amino acid residue. The results of the docking scores and different interactions with amino acids of the protein pocket (two-dimensional visualization) are shown in Tables S5 and S6.†

### Table 1

The SARS-COV-2 M\textsuperscript{pro} inhibition (IC\textsubscript{50} \(\mu M\)), docking scores of the isolated compounds [1–18], and the standard compound (lopinavir)

| Compound (code) | In vitro SARS-COV-2 M\textsuperscript{pro} IC\textsubscript{50} \(\mu M\) (kcal mol\(^{-1}\)) (docking score) | Compound (code) | In vitro SARS-COV-2 M\textsuperscript{pro} IC\textsubscript{50} \(\mu M\) (kcal mol\(^{-1}\)) (docking score) |
|----------------|------------------------------------------------------------------------------------------------|----------------|------------------------------------------------------------------------------------------------|
| Lopinavir\textsuperscript{a} | 0.225 ± 0.01 | 10 | 12.83 ± 0.65 | 14 | 0.61 ± 0.03 | Total 14 compounds were tested with IC\textsubscript{50} values ranging from 0.225 to 0.61 \(\mu M\), with the highest activity observed at 0.225 \(\mu M\). The results show that the tested compounds have potential inhibitory activity against the SARS-CoV-2 main protease. The docking scores and IC\textsubscript{50} values provide a basis for further in vitro and in vivo studies.

\textsuperscript{a} The standard used Lopinavir.
The three-dimensional visualization of the docking results and the binding pocket surface mapping were also shown to simulate ligand binding to the M₃₉₀ active pocket (Table S7†). Analyzing the docking results of all the tested compounds, compared to the ligand inhibitor N3 of M₃₉₀ and lopinavir, represented a good idea about their binding modes and affinities. However, the tested compounds showed variable binding strengths, as discussed above.

2.4 Structure activity relationship study

Observing the structure activity relationships of the isolated compounds depending on their binding affinities and binding modes to the M₃₉₀ pocket and comparing it with their in vitro inhibitory activity results against the M₃₉₀ enzyme compared to lopinavir can give an insight into the characteristic features for the compounds that can be considered as leads for designing...
anti-COVID-19 drugs. In this study, generally, compounds 12, 14, and 6 achieved nearly the same inhibitory activities against the M\textsuperscript{pro} enzyme compared with lopinavir; they shared the presence of the phenyl propanoid part oxygenated at C1 & C3 of the propane moiety and C3’ & C4’ of the phenyl moiety, which seemed to be potential sources for developing anti-COVID-19 drugs (Table S5\textsuperscript{†}).

Compounds 5 and 12, the two phenyl propanoid derivatives nitrogenated at C2 and shared both N3 ligand and lopinavir in binding with both Gln189 and Glu166 amino acid residues, showed different \textit{in vitro} inhibitory activities. This may be attributed to the free hydroxyl group located at C-4\textsuperscript{′} in compound 12 that increased its binding affinities compared to 5, where this free phenolic hydroxyl binds by two hydrogen bonds with Thr190 & Arg188 amino acid residues, while the methylenedioxy group in compound 5 binds by only one hydrogen bond with Gln192 (Tables S5 and S6\textsuperscript{†}). This may explain the results of the \textit{in vitro} inhibitory activity assay where compound 12 showed higher inhibitory activity than 5 (IC\textsubscript{50} value of 0.476 ± 0.02 and 8.532 ± 0.43 \textmu M, respectively) (Fig. S51\textsuperscript{†}).

A careful study of the two lignans 7 and its glucoside 14 revealed that the glucose moiety of compound 14 increased the binding affinities of this compound compared to its aglycone 7 as the glucose moiety in compound 14 shared the N3 ligand and the standard lopinavir in binding by two H-bonds with both Gln189 and Glu166 amino acid residues; in addition, it shared the N3 ligand only in binding by two H-bonds with Gly143 amino acid residue, while the aglycone 7 shared the N3 inhibitor ligand and lopinavir in binding with only Glu 166 amino acid residue (Tables S5 and S6\textsuperscript{†}). This is in agreement with the results of the \textit{in vitro} inhibitory activity assay where compound 14 showed an IC\textsubscript{50} value of 0.61 ± 0.03 and compound 7 showed an IC\textsubscript{50} value of 16.31 ± 0.83 (Tables S5 and S6\textsuperscript{†}) (Fig. S53\textsuperscript{†}).

Comparing the binding affinities of the two lignans 14 and 15, the methylenedioxy group in 14 increased its binding affinities compared with compound 15 that showed two methoxy groups at C-3 & 4 instead of the methylenedioxy group in compound 14. The methylenedioxy group in compound 14 is bound by the hydrogen bond with two amino acids (Thr 24 & Ser 46), while one of the two methoxy groups in compound 15 is bound by only one hydrogen bond with Thr 26. This matches with the results of the inhibitory activity assay where compound 14 showed an IC\textsubscript{50} value of 0.61 ± 0.03 \textmu M and compound 15 showed an IC\textsubscript{50} value of 11.46 ± 0.58 \textmu M (Tables S5 and S6\textsuperscript{†}) (Fig. S54\textsuperscript{†}).

In the steroidal compound 4, the glucose moiety decreased the activity of this compound compared to its aglycone 1, where compound 4 showed an IC\textsubscript{50} value of 89.99 ± 4.59 \textmu M, while 1 showed an IC\textsubscript{50} value of 12.51 ± 0.64 \textmu M. This revealed that the blocking of the hydroxyl group at the C-3 of sterol decreased the inhibitory activity against M\textsuperscript{pro} (3CL\textsuperscript{pro}) (Tables S5 and S6\textsuperscript{†}) (Fig. S52\textsuperscript{†}).

3. Experimental

3.1 Reagents and apparatus

A UV-visible spectrophotometer (Shimadzu 1601 PC, model TCC-240A, Japan) was employed. ESI-HPLC-Mass, TSQ Quantum Access MAX triple stage quadrupole mass spectrometer equipped with an electrospray ionization (ESI) was operated in the positive ionization mode, Thermo Scientific, New York, USA and Accela U-HPLC system using Accela 1250 quaternary pump and Accela open autosampler (operated at 25 °C) New York, USA. Nuclear Magnetic Resonance spectra (\textit{H}-NMR, \textit{APT, DEPT-Q, HMBC, and HSQC}) using TMS as an internal standard were recorded on a Bruker AV-400 spectrometer at 400 MHz for \textit{H} and 100 MHz for \textit{13C} NMR. Compounds were dissolved in CDCl\textsubscript{3}, CD\textsubscript{3}OD, or DMSO-\textit{d6}. Chemical shifts were given in ppm with a TMS as an internal standard. Column chromatography was performed on silica gel 60 (Merck, Germany) and thin-layer chromatography on pre-coated silica gel 60 GF\textsubscript{254} (20 × 20 cm × 0.2 mm thick) on an aluminum sheet (Merck, Germany), RP-C\textsubscript{18} (Merk, Germany), and Sephadex LH 20 (Pharmacia, USA). Spots were visualized by exposure to vanillin sulfuric spraying reagent.

For the FRET-based activity assay, 3CL Protease (SARS-CoV-2) Assay Kit (Catalog #79955-1) was used to measure the main protease activity. The kit comes in a convenient 96-well format with purified main protease, fluorogenic substrate, and main protease assay buffer for 100 enzyme reactions. Also, lopinavir was included as a positive control. In addition, microtiter plate-reading fluorimeter was used to measure the fluorescence intensity.

(6042 Cornerstone Court West, Ste. B) San Diego CA 92121, Email: info@bpsbioscience.com).

3.2 Preparation of the plant material

The aerial parts of \textit{H. bracteatum} ornamental plant were collected from El-Orman Garden, Cairo, Egypt in June 2019. The plant identity was confirmed by Associate Prof. Dr Mahmoud Makram Qassem, Department of Vegetables & Floriculture, Faculty of Agriculture, Mansoura University, Egypt. Voucher specimens were coded as Hb-1-2019 and kept in Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Egypt.

3.3 Extraction and isolation

The leaves of the plant were separated, air dried in shade, and then powdered. The dried powdered leaves (2.5 kg) were extracted with 70% hydro-alcoholic methanol (6 × 10 L) by maceration. The collected methanol extracts were evaporated under reduced pressure to give (444 g) of a dark green viscous residue. The dried methanolic extract was dissolved in CDCl\textsubscript{3}, C D\textsubscript{3}OD, or DMSO-\textit{d6} (300 mL). The solvent, in each case, was evaporated to dryness under reduced pressure giving petroleum ether extract (115 g), methylene chloride (10 × 500 mL), and \textit{butanol (5× 300 mL). The solvent, in each case, was evaporated to dryness under reduced pressure giving petroleum ether extract (115 g), methylene chloride extract (150 g), ethyl acetate extract (19 g), and \textit{butanol (17 g). The petroleum ether extract (70 g) was subjected to normal silica gel column chromatography, eluted with petroleum ether : ethyl acetate : methanol (100 : 0) till (0 : 100), and then ethyl acetate : methanol (100 : 0) till (0 : 100) to give
two groups, namely, group 1 and group 2. Group 1, eluted with petroleum ether : ethyl acetate (90 : 10), when left for crystallization, precipitated a white powder (compound 1, 14 mg), while the supernatant was collected, dried, and then re-chromatographed over normal silica gel column using petroleum ether : ethyl acetate (100 : 0) till (0 : 100) to give two subgroups 1A and 1B; subgroup 1A (fractions 28–43), eluted with petroleum ether : ethyl acetate (98 : 2), was also re-chromatographed over a normal silica gel column using petroleum ether : ethyl acetate (100 : 0) till (0 : 100) and yielded sub-fractions (41–49), eluted with petroleum ether : ethyl acetate (88 : 12), which produced compound 2, 5 mg, while subgroup 1B (fractions 81–103) was further re-chromatographed over normal silica gel column using petroleum ether : methylene chloride (100 : 0) till (0 : 100), yielded sub-fractions (103–109), eluted with methylene chloride (100%), produced compound 3, 5 mg. Group 2, eluted with petroleum ether : ethyl acetate (20 : 80), was re-chromatographed over a normal silica gel column using methylene chloride : methanol (100 : 0 till 0 : 100), sub-fractions (35–49), eluted with methylene chloride : methanol (96 : 4), when left for crystallization, white substance precipitated (compound 4, 86 mg).

Methylene chloride extract (120 g) was subjected to normal silica gel column chromatography, elution was carried out using petroleum ether : ethyl acetate (100 : 0) till (0 : 100) then ethyl acetate-methanol (100 : 0) till (0 : 100) to give seven groups 1–7. Group 1 (fractions 81–97), eluted with petroleum ether : ethyl acetate (88 : 12), was re-chromatographed over a normal silica gel column using petroleum ether : ethyl acetate (94 : 6) to yield sub-fractions (33–39), when left for crystallization, precipitated a white substance (compound 5; 13 mg). Group 2 (fractions 139–155), eluted with petroleum ether : ethyl acetate (79 : 21), was re-chromatographed over normal silica gel column using petroleum ether : methylene chloride (60 : 40 till 0 : 100) for elution; two subgroups were obtained, namely, 2A and 2B. Subgroup 2A (fractions 65–80), eluted with petroleum ether : methylene chloride (95 : 5), when left for crystallization, precipitated a white needle substance (compound 6; 7 mg). Subgroup 2B (fractions 121–125), eluted with methylene chloride (100%), was re-chromatographed over normal silica gel column using petroleum ether : methylene chloride (50 : 50 till 0 : 100) for elution giving fractions (30–52), eluted with petroleum ether : methylene chloride (10 : 90), when left for crystallization, a white substance was precipitated (compound 7; 1 g). Group 3 (fractions 168–175), eluted with petroleum ether : ethyl acetate (76 : 24), was re-chromatographed over normal silica gel column using methylene chloride : methanol (99 : 1) using isocratic elution, yielded two subgroups 3A and 3B. Subgroup 3A (fraction 9) was re-chromatographed over normal silica gel preparative TLC using methylene chloride : methanol (98 : 2), when left for crystallization, precipitated a yellowish white substance (compound 8; 7 mg). Subgroup 3B (fractions 50–80), when left for crystallization, precipitated a yellowish white substance (compound 9; 33 mg). Group 4 (fractions 180–200), eluted with petroleum ether : ethyl acetate (73 : 27), when left for crystallization, precipitated a yellow substance (compound 10; 20 mg). Group 5 (fractions 208–230), eluted with petroleum ether : ethyl acetate (70 : 30), was re-chromatographed over normal silica gel column using methylene chloride : methanol (100 : 0 till 98 : 2) to elute fractions (36–39), when left for crystallization, precipitated yellow substance (compound 11; 6 mg). Group 6 (fractions 243–276), eluted with petroleum ether : ethyl acetate (67 : 33), was re-chromatographed over normal silica gel column using petroleum ether : methylene chloride (5 : 95 till 0 : 100) then methylene chloride : methanol (100 : 0 till 0 : 100) yielded two subgroups 6A and 6B. Subgroup 6A (fractions 60–75), eluted with methylene chloride (100%), when left for crystallization, precipitated colorless needles (compound 12; 10 mg), while subgroup 6B (fractions 119–131), eluted with methylene chloride : methanol (98 : 2), when left for crystallization, a yellow powder precipitated (compound 13, 6 mg). Group 7 (fractions 360–375), eluted with ethyl acetate : methanol (95 : 5), was re-chromatographed over a normal silica gel column using methylene chloride : methanol (100 : 0 till 0 : 100) and produced two subgroups 7A and 7B. Subgroup 7A (fractions 31–41), eluted with methylene chloride : methanol (96 : 4), when left for crystallization, a white powder precipitated (compound 14, 1.5 g). Subgroup 7B (fractions 42–50), also eluted with methylene chloride : methanol (96 : 4), was re-chromatographed over normal silica gel preparative TLC using methylene chloride : methanol (90 : 10), when left for crystallization, a white substance precipitated (compound 15, 5 mg).

The ethyl acetate extract (15 g) was subjected to normal silica gel column chromatography, eluting with ethyl acetate : methanol (100 : 0) till (0 : 100) to give two groups: group 1 and group 2. Group 1 (fractions 26–39), eluted with ethyl acetate; methanol (95 : 5), when left for crystallization, precipitated a yellow substance (compound 16, 65 mg). Group 2 (fractions 40–45), also eluted with ethyl acetate : methanol (95 : 5), was re-chromatographed over a Sephadex LH 20 column using methanol (100%), yielded two subgroups 2A and 2B. Subgroup 2A (fractions 28–37), which was re-chromatographed over normal silica gel column using methylene chloride : methanol (100 : 0 till 0 : 100) to give sub-fractions (50–100), eluted with methylene chloride : methanol (85 : 15), when left for crystallization, precipitated a yellow powder (compound 17, 5 mg). Subgroup 2B (fractions 43–70) was re-chromatographed over reversed silica gel column RP-C<sub>18</sub> using water : methanol (100 : 0 till 0 : 100), yielded fractions (17–23), eluted with water : methanol (95 : 5), when left for crystallization, a yellow substance was precipitated (compound 18, 7 mg).

3.4 FRET-based activity assay (Fluorescence Resonance Energy Transfer assay)

The inhibitory activity against the SARS-CoV-2 main protease (M<sub>prO</sub> or 3CL<sub>prO</sub>) assay was carried out based on the FRET-based activity assay. The principle of the assay depends on the C-terminal of the peptide substrate being linked to a fluorophore (Edans) and the N-terminal has a fluorescence quencher (Dabecyl) that quenches the fluorescence signal of Edans. Thus, the peptide substrate exhibits low fluorescence because the fluorescence intensity of Edans in the C-terminal is quenched by the Dabecyl in the N-terminal of the substrate.
When the M^pro hydrolyzes the substrate, it yields two fragments: non-fluorescent Dabyl fragment and highly fluorescent Edans fragment. Consequently, an increase in the fluorescence signal proportional to the protease activity occurs. Main protease (M^pro) inhibitor causes the inhibition of fluorescent fragment release, and thus decrease intensity of the fluorescence signal. Fluorescence intensity is measured with a fluorescence microtitr plate reader capable of reading excitation/emission = 360/460 nm.\(^5\)

3.5 Molecular modelling simulation study

The binding affinities of the isolated compounds 1–18 to SARS-CoV-2 main protease (M^pro) pocket amino acids residues were predicted by carrying out a docking experiment for them and comparing their results with the co-crystallized ligand (N3 inhibitor)\(^6\) and the standard lopinavir. Lopinavir was previously reported for its promising inhibitory activity against SARS-COV-2 main protease (M^pro) inhibitor through in silico computational study\(^7\) and it was also reported for inhibiting SARS-CoV-2 replication in vitro study.\(^8\)

3.5.1 Ligand and protein preparation. The structures of compounds (1–18) that was docked in their neutral forms were built using the builder of Molecular Operating Environment (MOE) version 2009.10 (Chemical Computing Group Inc. software. https://www.chemcomp.com). Ligands (the structures that will be docked) were prepared for docking by minimizing their energy to get the most stable conformers of ligands and they were imported and saved in the form of MDB file to be ready for docking into the M^pro active site. For protein preparation, the crystal structure was protonated, hydrogen atoms were added, an automatic correction to check for any errors in the atom’s connection and the type was applied, and the fixation of the potential of the receptor and its atoms were done. Site Finder was used for the selection of the same active site of the co-crystallized inhibitor in the protease structure using all default items and dummy atoms of the pocket were created. The program specifications were adjusted so that the docking site was specified as dummy atoms, triangle matcher as the placement methodology, and London dG as the scoring methodology for the selection of the best 19 poses from 380 different poses for each tested compound. The isolated compounds were docked into the binding site of the M^pro enzyme; PDB code ID 6LU7 was used as the target enzyme.

3.5.2 Molecular modeling visualization. All the visualization of the docking files was done using MOE software. After the completion of the docking processes, the obtained poses were studied. The best compounds are those showing the more negative docking scores that refer to the better capability of a compound to dock with the target and make more desirable ligand–enzyme interactions.\(^4\)

4. Conclusion

The methanolic extract, petroleum ether, methylene chloride, and ethyl acetate fractions of H. bracteatum leaves besides eighteen isolated and identified compounds (1–18) were in vitro evaluated for their inhibitory activities against SARS-CoV-2 main protease (M^pro) using fluorescence resonance energy transfer assay (FRET-based assay). The tested isolated compounds (1–18) included phenylpropanoid derivatives nitrogenated at C-2, lignans, and rare flavonoids. The methanolic extract and fractions exhibited promising inhibitory activities. Compounds 6, 12, and 14 showed comparable inhibitory activities against SARS-COV-2 M^pro with IC\(_{50}\) values of 0.917 ± 0.05, 0.476 ± 0.02, and 0.610 ± 0.03 \(\mu M\), respectively, compared with the control lopinavir with an IC\(_{50}\) value of 0.225 ± 0.01 \(\mu M\). The other tested compounds showed significant inhibitory activities. Thus, the methanolic extract of H. bracteatum leaves and the isolated phenylpropanoid derivatives, lignans, followed by flavonoids, could be considered promising natural SARS-COV-2 M^pro inhibitors. The molecular docking study for the tested compounds was carried out to correlate their binding modes and affinities for M^pro enzyme with the in vitro results. Combining the results of the in vitro and in silico studies led us to suggest the structural basis of potential inhibitors targeting SARS-COV-2 M^pro. It could be concluded that phenylpropanoids skeleton oxygenated at C3, C4 of the phenyl moiety and at C1, C3 of the propane part is the essential fragment. Consequently, an increase in the fluorescence signal proportional to the protease activity occurs. Main protease (M^pro) inhibitor causes the inhibition of fluorescent fragment release, and thus decrease intensity of the fluorescence signal. Fluorescence intensity is measured with a fluorescence microtitr plate reader capable of reading excitation/emission = 360/460 nm.\(^5\)

Conflicts of interest

We declare that we have no conflict of interest.

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