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Screening of Secondary Metabolites in *Artemisia annua* as Potential Inhibitors of Coronavirus Proteases by *in silico* Approaches

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Abstract: To date, no specific drug has been proven to treat COVID-19. It encourages people to use medicinal plants to treat or protect themselves against these diseases. *Artemisia annua* is one of the promising plants that have already been used in coronary disease. However, the antiviral compounds present in this plant remain poorly known. In this study, we aimed to identify some of these molecules by *in silico* approach. During the screening, 102 secondary metabolites of *Artemisia annua* were selected and the two viral proteins 3CLpro and PLpro of *SARS-CoV2* were selected as targets. Then, a preliminary analysis was performed to determine the inhibition capacity of these phytoligands for the two viral proteins. Then, the phytoligands with stronger interaction energy with these target proteins were selected and their physicochemical properties and ADMET profile were analyzed. Consequently, 13 molecules of *Artemisia annua*, namely *Apigenin*, *Axillarin*, *Crysoeriol*, *8-Hydroxygalangin*, *Isorhamnetin*, *Kaempferol*, *Luteolin*, *Luteolin-7-methyleter*, *Quercetagetin-3-4-dimethyleter*, *Quercetagetin-3-4-dimethyleter*, *Quercetin-3-methyleter*, *Quercetin, Rhamnetin*, and *Tamarixetin* can inhibit the two proteases of *SARS CoV2*. They also have a good physicochemical profile and an ADMET property in the human. These molecules may be compounds promoting an antiviral treatment in *Artemisia annua*. To complete these results, in vitro tests are necessary.

Keywords: *SARS-Cov2*, 3CLpro, PLpro, *Artemisia Annua*, Secondary Metabolites, Docking, ADMET

1. Introduction

Since the SARS or Severe Acute Respiratory Syndrome outbreak in 2003, *coronavirus* (CoV) diseases have become a permanent threat to humans [1]. The current COVID-19 pandemic's, the first of this millennium, caused by the *SARS-CoV2* virus has created a public health emergency worldwide [2, 3]. However, no specific therapy or clinically approved drugs are available, making it difficult to manage the patients and control the associated pandemic [4].

Several attempts have been made in the search for therapies [2]. All chemotherapeutic agents, which have been shown to be effective, have had limited success in humans [5]. Indeed, various antiviral molecules such as *Remdesivir, Lopinavir and Ritonavir* have been tested and found to have an inhibitory effect in vitro [6, 7]. However, the clinical response of these antiviral molecules is not very encouraging [8].

Thus, the characterization of new drug candidates to overcome the human losses caused by the pandemic and to cope with future emergence of *CoV* strains remains a global concern. We need an active drug or combination of drugs that work. Remdesivir has raised hopes. It is an antiviral that works by attacking an enzyme that a virus needs to replicate inside our cells. Some experts believe that *Remdesivir*, *Lopinavir, Ritonavir* have a clear, significant positive effect in reducing recovery time and can therefore block this virus. This can be a miracle solution [9]. In this context, traditional medicine (herbal medicine) has gained importance and is seen as a potential source of new drugs for COVID-19 [10]. Indeed, the use of medicinal plants is an ideal alternative to treat COVID-19. Moreover, plants are considered to be sources of chemical structures (or active ingredients) essential for the development of future drugs [11].

After the discovery of *Artemisinin*, used as an antimalarial [12], *Artemisia annua*, a plant of great therapeutic importance [13], has been the subject of extensive research leading to the identification of active compounds as anti-tumour [12], immunomodulatory, anti-inflammatory [12], antibacterial [12] and even antiviral [14]. The antiviral action of *Artemisia annua* has been demonstrated in the infection of Human immunodeficiency virus- HIV-1,2 [15], *Herpes Simplex Virus* - HSV-1,2 [16], Bovine *Pestivirus*–BVDV [17], *Hepatitis B Virus* - HBV [18] and *SARS-CoV1* during 2002 *CoV* epidemic [3]. Given its efficacy in the treatment of *SARS-CoV*, *Artemisia annua* could be used against SARS-CoV2. The latter is structurally related to SARS-CoV1 [19].

The *CoV* genome, a single-stranded RNA of positive polarity (RNA+), will be translated into two large polyproteins (pp1a and pp1b), which are the source of the sixteen non-structural proteins, and four or five structural proteins (S, N, E, M and sometimes H) during virus replication [20]. The replication cycle of *CoV* consists of several steps, namely attachment followed by penetration, decapsidation, early translation of non-structural proteins as well as autolytic cleavage of polypeptides, viral replication and transcription, translation of structural and accessory proteins, viral assembly and budding [21]. These different steps may be promising therapeutic targets for the control of *SARS-CoV2* [22].

Early translation will generate the non-structural proteins of *SARS-CoV2* responsible for autolytic cleavage of pp1a and pp1b. The two endopeptidases which are responsible for this early translation, are 3CLpro (*chymotrypsin-like protease*) and PLpro *or Papaine-like protease* [23]. By blocking the activities of these two proteases, the synthesis of mature non-structural proteins such as RNA-dependent RNA polymerase (RdRp) and helicase that are essential for *CoV* transcription and replication as well as structural proteins are compromised [24]. In addition to its role in cleaving viral polyproteins, *PLpro* is also a deubiquitinating enzyme that can dampen the host antiviral response by hijacking the ubiquitin system [25, 26]. Besides, these two proteases do not have closely related homologues in vertebrates, particularly in humans [22]. This makes these two proteases (*3CLpro* and *PLpro*) interesting targets for new drug design against *SARS-CoV2* [4].

The present study, based on *in silico* approach, will provide a means of large-scale investigation to identify promising molecule(s) against *CoV* diseases such as COVID-19 using medicinal plants like *Artemisia annua*. This approach involves both molecular docking and pharmacological studies of the molecules.

2. Materials and Methods

2.1. Selection and Preparation of Ligand Files

A literature review identified 102 secondary metabolites from *Artemisia annua* (Supplementary Data S1), belonging to the family of coumarins (7), flavonoids (46), monoterpenes (8); diterpenes (2), triterpenes (7), sesquiterpenes (20), alkaloids (2), benzenoids (4), heterocyclic oxygen (2), steroids (2), a phenolic acid and an alkaloid [27, 28]. Six antiviral molecules *Lopinavir* [29], *Ritonavir* [29], *Darunavir* [30], *Rupintrivir* [31], *Nelfinavir* [32] and *Boceprevir* [33], used by other researchers as protease inhibitors were selected as references ligands in this study [31, 34-36].

The 3D structures file of these secondary metabolites and reference ligands were downloaded in .sdf format from the PubChem[1] database [37].

2.2. Preparation of Receptors (Macromolecules)

The 3D structures of 3CLpro and PLpro of *SARS-CoV2* retrieved from the PDB database in pdb format contain two A and B chains, forming a homodimer [38] and four asymmetric A, B, C, D chains, forming a homotetramer [39]. The A-chains of both structures were used in the preparation of the macromolecule. The water molecules and ligands, which were still attached, were removed in BIOVIA-DS and the receptors were stored in.pdb format.

2.3. Study of the Active Site of Macromolecules (3CLpro and PLpro)

The active site study defined the key residues involved in the receptor-ligand interaction and analyzed the X, Y and Z coordinates of the gate box during molecular docking [40]. The active site region of 3CLpro and PLpro is predicted using the COACH_D[2] [41].

1: https://pubchem.ncbi.nlm.nih.gov/
2: https://yanglab.nankai.edu.cn/COACH-D/

2.4. Molecular Docking

Molecular docking was performed with AutoDock Vina 4.2.1 [42] using the graphical interface of the PyRx program version 0.8 [43]. In this program, using the Open Babel tool [44], the .sdf files of the phytoligands were energetically minimized using mmff94 [45] and then converted to a .pdbqt format file. The rotational bonds of these phytoligands have been established to be free [42]. The interaction between macromolecules and phytoligands takes place in the center of an Auto Grid box: x = -13.76Å, y = 14.26Å, z = 69.56Å for 3CLpro and x = 27.29Å, y = 68.27Å, z = 7.67Å for PLpro. The Lamarckian genetic algorithm or LGA [42] was used to analyze the docking poses and the resulting binding energies were reported in Kcal/mol [42]. The best molecular docking results, with the lowest binding energy, obtained for each compound were visualized as a pdbqt file using the BIOVIA-DS visualization tool.

Two rounds of docking were performed: the first round with the six references ligands and the second round with the phytoligands. The phytoligand-protease complexes with the lowest interaction energy [42], compared to the docking of references ligands with these proteases, in this study (energy lower or equal to that of the reference ligands) were selected as the most plausible interaction mode. In addition, docking was considered optimal if the phytoligands are well anchored in the enzyme catalytic sites and interact correctly with the key residues in the proteolytic activity of the protease [46], in particular the cystein catalytic dyad (*Cys* and *His*) or triad (*Cys*, *His* and Asn) in the active site.

2.5. Physicochemical Property Prediction, Drug Similarity and Pharmacokinetic Analysis (ADMET)

The physicochemical properties and drug similarities as well as the pharmacokinetic properties analysis of Absorption, Distribution, Metabolism, Excretion (*ADME)* and Toxicity of the selected phytoligands with the best binding energies to *SARS-CoV2* proteases (*3CLPro* and *PLpro*) after molecular docking were examined using the online servers SwissADME[3] [46] and pkSCM[4] [47] respectively. These servers used 2D format file named canonical SMILES [48]. The canonical SMILES file for each preselected phytoligands was downloaded from PubChem database.

The estimation of these different properties was done in three phases: (i) prediction of drug similarity with *Pfizer*'s Lipinski [49], *GlaxoSmithKline*'s Veber [50] and *Boehringer Ingelheim*'s Muegge [51] rules; (ii) prediction of pharmacokinetic [47, 52] as well as (iii) toxicities of phytoligands with AMES predictions for mutagenicity [53], hERG-I/II (*human Ether-a-go-go Related Gene*) for cardiac potassium channel inhibition [53] and hepatotoxicity [54].

3: https://www.swissadme.ch

4: http://biosig.unimelb.edu.au/pkcsm/prediction

3. Results

3.1. Structure and Active Site Analysis of 3CLpro and PLpro

The 3D structures showed of 3CLpro, contain three domains DI, DII and DIII (Figure 1A). The active site of CoV/3CLpro is located in the center of the cleft between DI and DII and divided into five pockets S1', S1, S2, S3 and S4. Amino acids at positions 24-25, 27, 41, 49, 54, 140-145, 163-168, 172 and 187-192 are predicted from COACH-D as active site residues. The catalytic site of *SARS-CoV2*/3CLpro is a dyadic site and the residues responsible for this activity are Cys^{145} and His^{41} (Figure 1A).

In contrast, the visualization of the 3D structure of SARS-Cov2/PLpro shows the conservation of four domains, namely the ubiquitin-like domain (UBL), the thumb domain, the palm domain, and the finger domain. The active site is located at the interface of the thumb and palm subdomains (Figure 1B). Amino acids at positions 106-109, 111-112, 116, 162-166, 208, 245-248, 264, 266-273, 286 and 301-302 are predicted as active site residues. The catalytic site of PLpro, which is a triadic site responsible for enzymatic activities, is all found in the S1 pocket and the oxanyon inside of S1' pocket (Figure 1B).

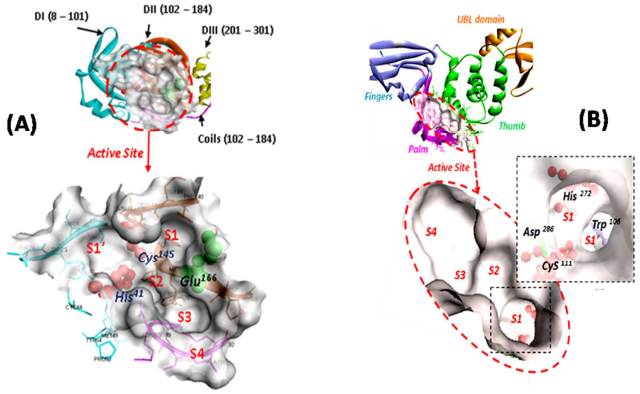

Figure 1. *3D structure and active site presentation of 3CLPRO SARS-cov2 (pdb ID = 6lu7, chain A) and PLpro SARS-COV2 (pdb ID = 6wuu): (A) the different domains in 3CLpro are colored in different color: DI (cyan), DII (chestnut), DIII (yellow). The two dyad site residues (HIS^{41} and CYS^{145}) are presented in the form of red balls while the oxanyon glu^{166} is presented in the form of green balls. (B) The 4 domains are colored in different colors: UBL (Beige), Thumb (Green), Palm (violet) and Fingers (gray). The residues of the triad site are presented in sticks Green (Asp^{286}), red (Cys^{111}, His^{272}) and Blue (oxanyon Trp^{106}). The surface of accessibility to cavity solvents is presented in gray.*

3.2. SARS-CoV2/3CLpro and SARS-CoV2/PLpro Inhibiting Compounds

3.2.1. Molecular Docking of Reference Ligands with SARS-CoV2/3CLpro and SARS-CoV2/PLpro

The six references ligands used in this study are all well anchored in the active site of 3CLpro and PLpro which interact with hydrogen bonds, hydrophobic bonds and Van Der Waals interactions (Table 1 - Figure 2). The binding energy between these references ligands and these *SARS-*

CoV2 proteases ranges from -8.3 kcal/mol to -6.8 kcal/mol (3CLpro) and -7.8 kcal/mol to -6.7 kcal/mol (PLpro).

Table 1. *Interaction of synthetic ligands with 3CLPRO and plpro of SARS-COV2.*

N°	*References ligands*	*Energy (kcal/mol)*	
		3CLpro	**PLpro**
1	*Nelfinavir*	-8.3	-7.6
2	*Darunavir*	-8.2	-7.1
3	*Ritonavir*	-8.2	-6.7
4	*Lopinavir*	-7.9	-7.3
5	*Rupintrivir*	-7.9	-7.8
6	*Boceprevir*	-6.8	-7

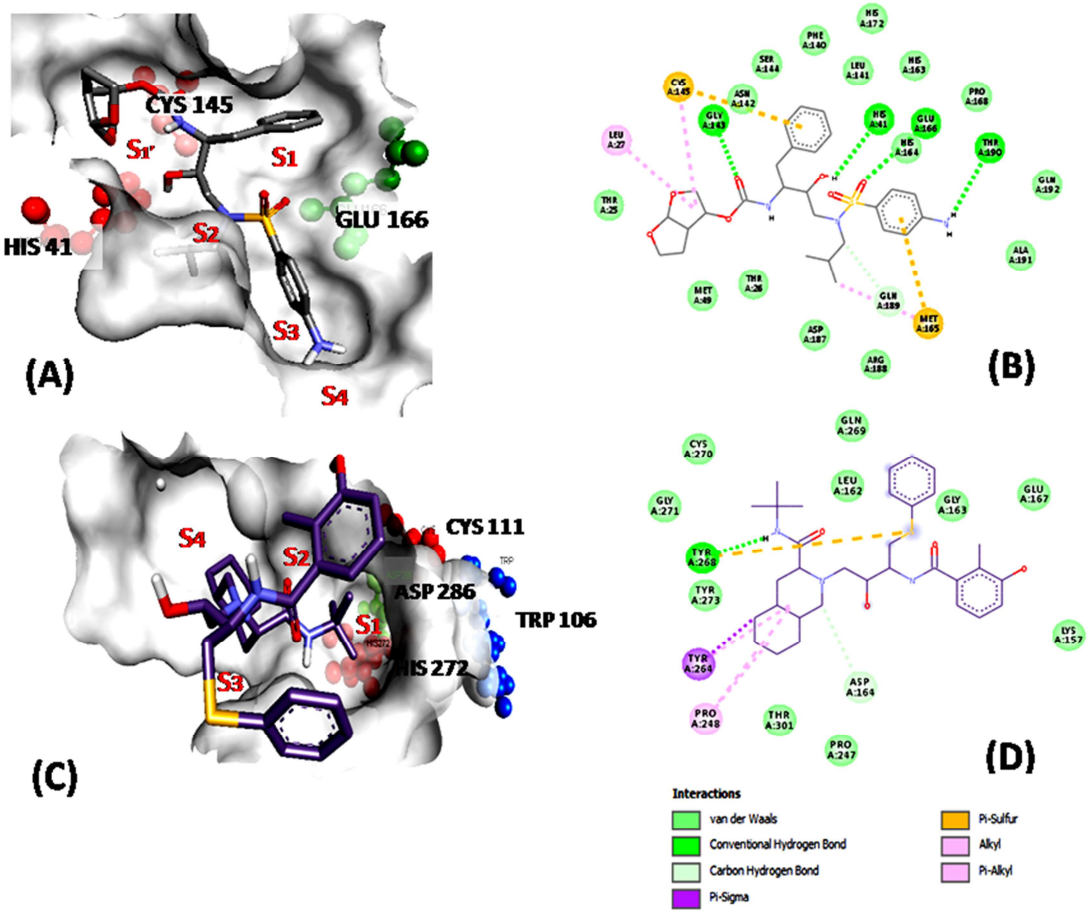

Figure 2. *3D and 2D visualization of reference ligands with the active site of SARS-COV2/3CLPRO (pdb ID = 6lu7, chain A) and of SARS-COV2/PLPRO (pdb ID= 6wuu chain A). (A, B): 3D and 2D interaction of Darunavir CID = 213039 with SAR-SCov2/3CLpro. (C, D) 3D and 2D interaction of Nelfinavir (CID = 64143) with SARSCoV2/ PLpro.*

3.2.2. SARS-CoV2/3CLpro and SARS-CoV2/PLpro Inhibiting Phytoligands

On the 102 phytoligands from Artemisia annua analyzed, 62 molecules have interaction energy of less than -6.8 kcal/mol (table 2) and can anchor properly in the active site by creating hydrogen bonds, hydrophobic bonds and Van der Waals interactions with the residues of the *SARS-CoV2*/3CLpro catalytic site (Supplementary Data S2). Among the 62, some phytoligands such as *Rutin* (-8.8 kcal/mol), *Astragalin* (-8.7kcal/mol), *Isoquercitrin* (-8.6kcal/mol), *Quercetagetin-3,4,6,7-tetramethylether* (-8.6kcal/mol), *Kaempferol-6-methoxy-3-O-β-O-glucoside* (-8,6kcal/mol), *Quercimetrin* (-8.6kcal/mol) and *Cinarin* (-8.4 kcal/mol) have a binding energy higher than the highest binding energy of six references ligands used in this study. They are included in the flavonoid class (Supplementary data S1, table 2). During molecular docking, these phytoligands occupy the pockets of the catalytic site in close direct interaction between the His^{41} (S1') and Cys^{145} (S1) dyad residues, either with the chromen-4 forming A and C rings and these hydroxides such as *Apigenin* and *Kaempferol* (Figure 3A, B) or with the 2-phenyl ring and these hydroxides such as *Rhamnetin*, and *Tamarixetin* (Figure 3C, D). *Apigenin* occupies the three pockets (S1', S1 and S2) of the active site and interacts with the two residues of the dyad site (His^{41} and Cys^{145}) and with Glu^{166} oxygen through pi-alkyl, pi-sulfur links and hydrogen bonding respectively (Figure 3A).

In the case of *SARS-CoV2*/PLpro, of the 102 secondary metabolites of *Artemisia annua* selected in this study, 51 phytoligands have interaction energy of less than -6.7 kcal/mol (table 2). Among them, *Rutin* (-8 kcal/mol), *Dihydro-Artemisinin B* (-8 kcal/mol), Astragalin (-7.9 kcal/mol), *Friedelin* (-7.9 kcal/mol), *Taraxasterone* (-7.9 kcal/mol) and *Mearnsetin* (-7.9 kcal/mol) show the highest interaction energy compared to the reference ligands used. All of which are belong to the flavonoid, triterpene and sesquiterpene classes (Supplementary Data S1). Visualization of the anchoring mode of these phytoligands in the *SARS-CoV2*/PLpro active site shows that they are all well positioned either in the S2/S3/S4 pockets and interact with the residues forming the pockets by non-covalent bonds (electrostatic effects, π-effects, Van der Waals forces and hydrophobic effects) visible on the 2D representations (Figure 4 A, B, C, D and supplementary data S2). In contrast, none of these references ligands as well as the phytoligands manages to anchor in the S1/S1' pockets.

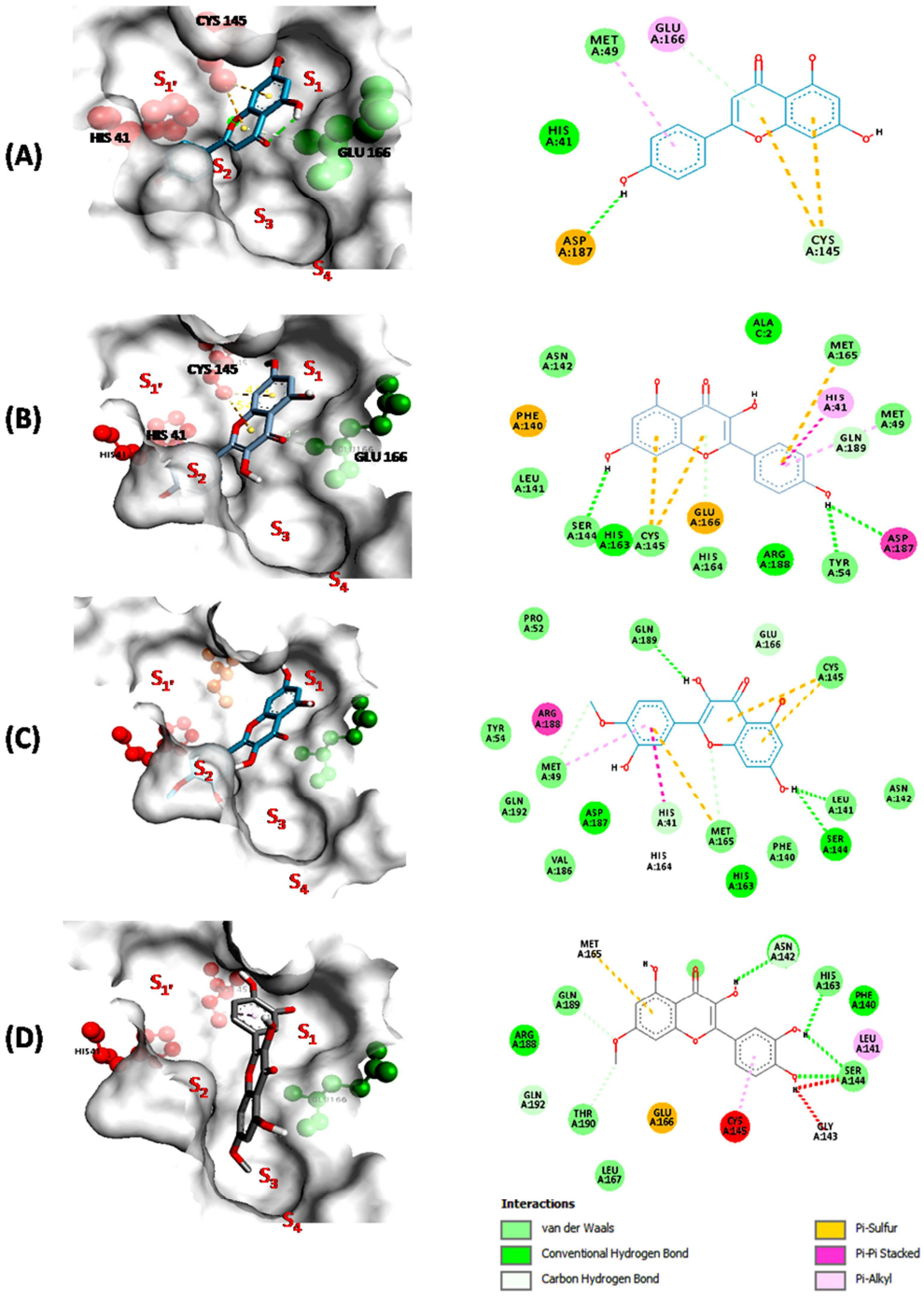

Figure 3. *3D and 2D interaction of Artemisia phytoligands with SARS-COV2/3CLPRO (pdb ID = 6lu7, chain A)- (A) apigenin CID =5280443; (B) kaempferol CID =5280863; (C) tamarixetin CID =21633679 (D) rhamnetin CID =5281691). The dyad and oxanyon site residues are represented in the form of red/green ball and the surface of the active site is colored gray.*

In total, 50 phytoligands, can anchor both *SARS-CoV2*/PLpro (binding energy less than -6.7 kcal/mol) and *SARS-CoV2*/3CLpro (less than -6.8 kcal/mol) proteases. Furthermore, with the exception of *Dihydro-Artemisinin B* and *Taraxasterone*, the binding energies recorded with *SARS-CoV2*/3CLpro are higher than with SARS-CoV2/PLpro. So, these 63/102 phytoligands with binding energies greater than or equal to -6.8 kcal/mol (SARS-CoV2/3CLpro) or -6.7 kcal/mol (*SARS-CoV2*/PLpro) could have the capacity to anchor one or both *SARS-CoV2* proteases (table 2).

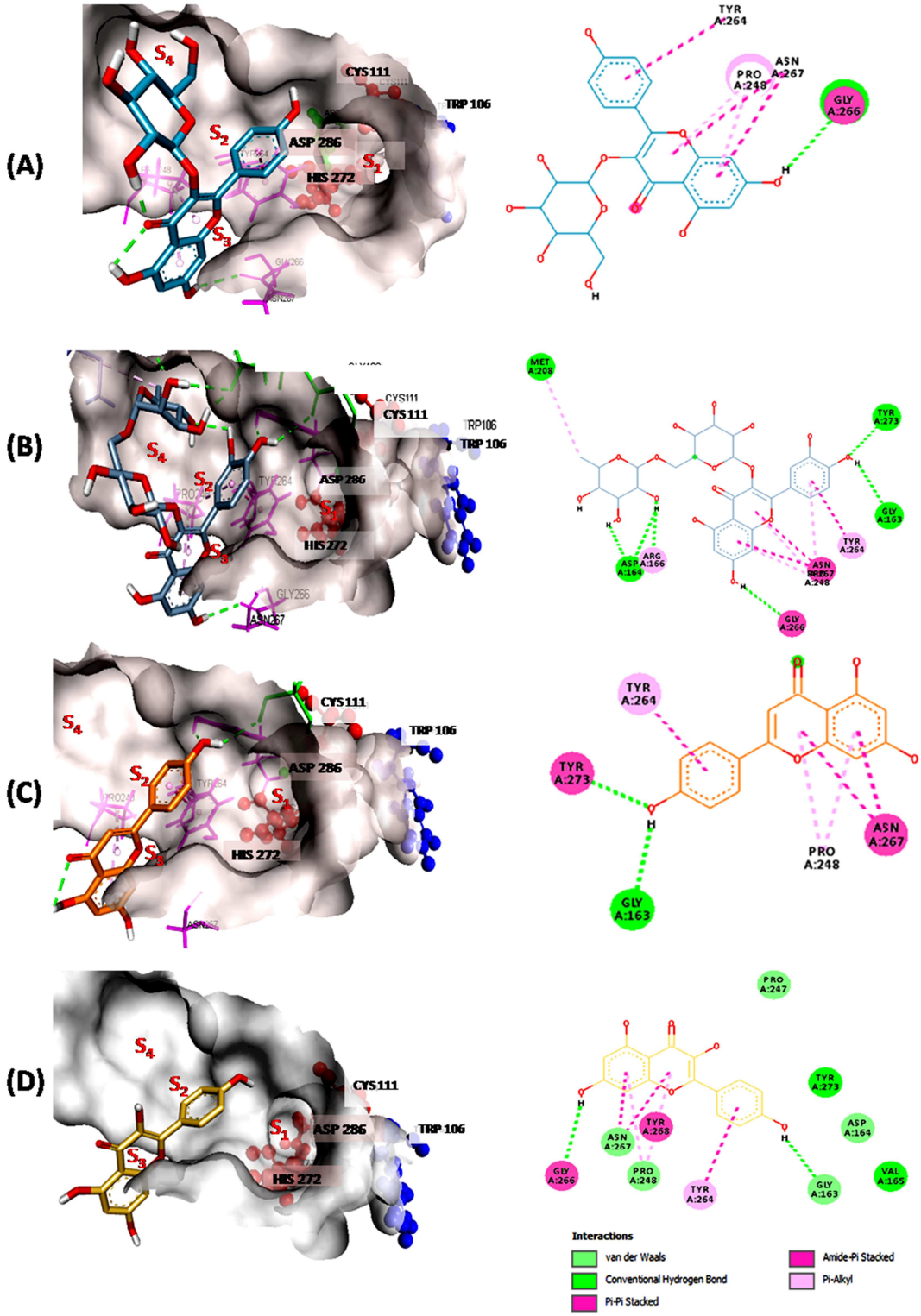

Figure 4. *3D and 2D interaction of Artemisia phytoligands with SARS-COV2/PLPRO (pdb ID = 6wuu, chain A) (A) Astragalin CID =5282102; (B) rutin CID =5280805; (C) apigenin CID =5280443; (D) kaempferol CID =5280863. The residues of the triad site and the oxanyon are represented in the form of red/blue ball and the surface of the active site is colored gray.*

Table 2. *Docking energy (-Kcal/mol) of 63 phytoligands with 3CLpro and PLpro.*

N°	Ligands names	Docking energy (kcal/mol)	
		3CLpro	PLpro
1	Rutin	-8,8	-8
2	Astragalin	-8,7	-7,9
3	Isoquercitrin	-8,6	-7,8
4	Kaempferol-6-methoxy-3-O-beta-D-glucoside	-8,6	-7,7
5	Quercetagetin-3-4- 6-7-tetramethyl ether	-8,6	-7,2
6	Cinarin	-8,4	-6,7
7	Cirsimaritin	-8,2	-6,8
8	Friedelin	-8,2	-7,9
9	α-amyrenon	-8	-7,3
10	Quercimeritrin	-8	-7,7
11	Dihydro Artemisinin B	-7,9	-8
12	Aurantiamide acetate	-7,9	-7,5
13	Cinaroside	-7,9	-7,7
14	α-amyrin	-7,8	-7,3
15	Apigenin	-7,7	-7
16	Kaempferol	-7,7	-7
17	Acacetin	-7,6	-6,8
18	Chlorogenic acid	-7,6	-7,2
19	Oleanolic acid	-7,6	-6,8
20	Patuletin-3 glucoside	-7,6	-7,8
21	Quercetagetin-4-methyl ether	-7,6	-6,9
22	Tamarixetin	-7,6	-7,3
23	Caftaric acid	-7,5	
24	Taraxasterone	-7,5	-7,9
25	Cirsiliol	-7,4	-6,8
26	Laricitrin	-7,4	-6,9
27	Luteolin	-7,4	-7
28	Mearnsetin	-7,4	-7,9
29	Scopolin	-7,4	
30	Taraxerol acetate	-7,4	-7,1
31	Artemetin	-7,3	
32	Axillarin	-7,3	-6,8
33	Chrysoeriol	-7,3	-7
34	Luteolin-7-methylether	-7,3	-6,9
35	Patuletin	-7,3	-6,9
36	Quercetagetin-3-methyl ether	-7,3	-6,9
37	Quercetin	-7,3	-7
38	Rhamnetin	-7,3	-6,9
39	Arcapillin	-7,2	-6,8
40	Chrysosplenol C	-7,2	-6,8
41	8-Hydroxygalangin	-7,2	
42	IsoKaempferide	-7,2	-7
43	Quercetagetin-3-4-dimethyl ether	-7,2	
44	Quercetin-3-methylether	-7,2	-6,9
45	Benzyl isovalerate	-7,1	-6,7
46	Chrysoplenetin	-7,1	-6,7
47	Chrysosplenol D	-7,1	-6,7
48	Eupatin	-7,1	
49	Isorhamnetin	-7,1	-7
50	Stigmasterol	-7,1	-7
51	Zeatin dihydroriboside	-7,1	
52	5-hydroxy- 3-4-6-7-tetramethoxyflavone	-7	
53	Arteanuin H	-7	-6,8
54	Cirsilineol	-7	-6,8
55	Pachypodol	-7	-6,9
56	Syringetin	-7	-6,9
57	Arteanuin C	-6,9	
58	Artemisinin	-6,9	-7
59	Beta-sitosterol	-6,7	
60	Gossypetin- 3,8-dimethylether	-6,7	
61	Mikanin	-6,7	
62	Penduletin	-6,7	-6,7
63	3-Hydroxy Deoxy Dihydro Artemisinin		-7,5

3.3. Analysis of Physicochemical Properties, Drug-like Properties, Pharmacokinetics and Toxicity

All predictions of physicochemical properties, drug-like properties, pharmacokinetics and toxicity were performed with the online servers SwissADME and pkSCM.

On the 63 phytoligands that met our criteria and are predicted to be *SARS-COV2* protease inhibitors, 38 of them responded favorably to the rules of: Lipinski [MW≤ 500; XLOGP≤ 4, 15; H-bond acc≤10 and H.bond donn ≤ 5], Veber [TPSA≤140 and Num.rotable bond≤10] and Muegge [200≤MW≤600; -2≤XLOGP≤5; TPSA≤150; Num.ring≤7; Num.carbon>4; Num.heteroatom>1; Num.rotable bond≤15; H-bond acc≤10 and H.bond ≤ 5]. In other words, these 38 phytoligands all possess the drug-like properties predicted by these three rules (Supplementary Data S3).

Among these 38 phytoligands that passed the drug-like properties, two phytoligands (*Scopolin* and *Quercetagetin-4-methyl ether*) were analyzed *in silico* as poorly absorbed in the intestine (Low: absorption value ≤ 0.3), with the online tool pkSCM. In other words, all 36 phytoligands have acceptable intestinal absorption profiles who these predicted $CaCo_2$ permeability are between high value 0.99 to moderate value 0.67 (Supplementary Data S3).

Continuing with the second step (Distribution), 16 phytoligands failed the prediction test. Among these 16 phytoligands, one (*Syringetin*) is a P-gp substrate, three (*Tetraflavone*, *Artemetin* and *Arteannuin_H*) are Pgp-I inhibitors, one (*IsoKaempferide*) has a VDss less than -0.1 (VDss<-0.1) and the remaining 11 (*Arcapillin*, *Chrysosplenetin*, *Chrysospenol_C*, *Chrysospenol_D*, *Circilineol*, *Eupatin*, *Gossypetin-3,8-dimethylether*, *Mikanin*, *Pachypodol, Penduletin and Quercetagetin-3-4-6-7-tetramethyl_ether*) are P-glycoprotein I/II or P-gp I/II inhibitors. None of them have the properties to cross the blood-brain barrier or BBB (logBB<-1). On this second step of prediction of ADME properties, the 20 phytoligands that have acceptable distribution profiles in the organism; is neither P-gp substrate, nor P-gp-I/II inhibitor. They also have a VDss>-0.1 (Supplementary Data S3).

Table 3. *ADMET property of 13 phytoligands from Artemisia annua selectionned in this study.*

N°	Name	Absorption				Distribution	
		HIA	P-gp substrate	P-gp I inhibitor	P-gp II inhibitor	VDss (human)	BBB permeant
1	Apigenin	93.25	No	No	No	0.822	-0.734
2	Axillarin	82.844	No	No	No	0.346	-1.522
3	Chrysoeriol	82.844	No	No	No	0.741	0.741
4	8-Hydroxygalangin	71.859	No	No	No	0.788	-1.213
5	Isorhamnetin	76.014	No	No	No	1.123	-1.135
6	Kaempferol	74.29	No	No	No	1.274	-0.939
7	Luteolin	81.13	No	No	No	1.153	-0.907
8	Luteolin-7-methylether	84.881	No	No	No	0.278	-1.254
9	Quercetagetin-3-4-dimethyl ether	67.831	No	No	No	0.251	-1.582
10	Quercetin-3-methylether	76.069	No	No	No	0.217	-1.16
11	Quercetin	77.207	No	No	No	1.559	-1.098
12	Rhamnetin	80.214	No	No	No	0.419	-1.345
13	Tamarixetin	73.005	No	No	No	1.089	-1.161

The results of ADME prediction at the third (metabolism) and fourth (excretion) step of ADME prediction showed that five phytoligands (*Acacetin*, *Circiliol*, *Circimaritin*, *Arteanuin_C* and *Artemisinin*) are substrates of CYP3A4 and can be degraded (metabolism) during their serum transport (distribution). The remaining 15 are not inhibitors of CYP2D6 or interfere with the action of other molecules. Also, these 15 phytoligands can be easily eliminated renally (Excretion) as they are not substrates of renal OCT2 (Supplementary Data S4).

The prediction of the AMES test or carcinogenicity test, proved the absence of mutagenic potential with the exception of *3-Hydroxy Deoxy Dihydro artemisinin* and *Benzyl isovalerate* was predicted to possess hepatoxicity. Also, all the remaining 13 phytoligands are not predicted to be hERGII inhibitors, a potassium channels gene. Inhibition of this gene lead the prolongation of action potential duration and increase QT interval measured on an electrocardiogram.

At the end of the *in silico* analyses, all the 13 phytoligands (Figure 5) belong to the flavonoid class and are predicted to be good inhibitors of the two *SARS-CoV2* proteases (PLpro and 3CLpro), have good pharmacophysical, pharmacokinetic (ADME) properties and are not carcinogenic or hepatoxic. In other words, they are not toxic to humans.

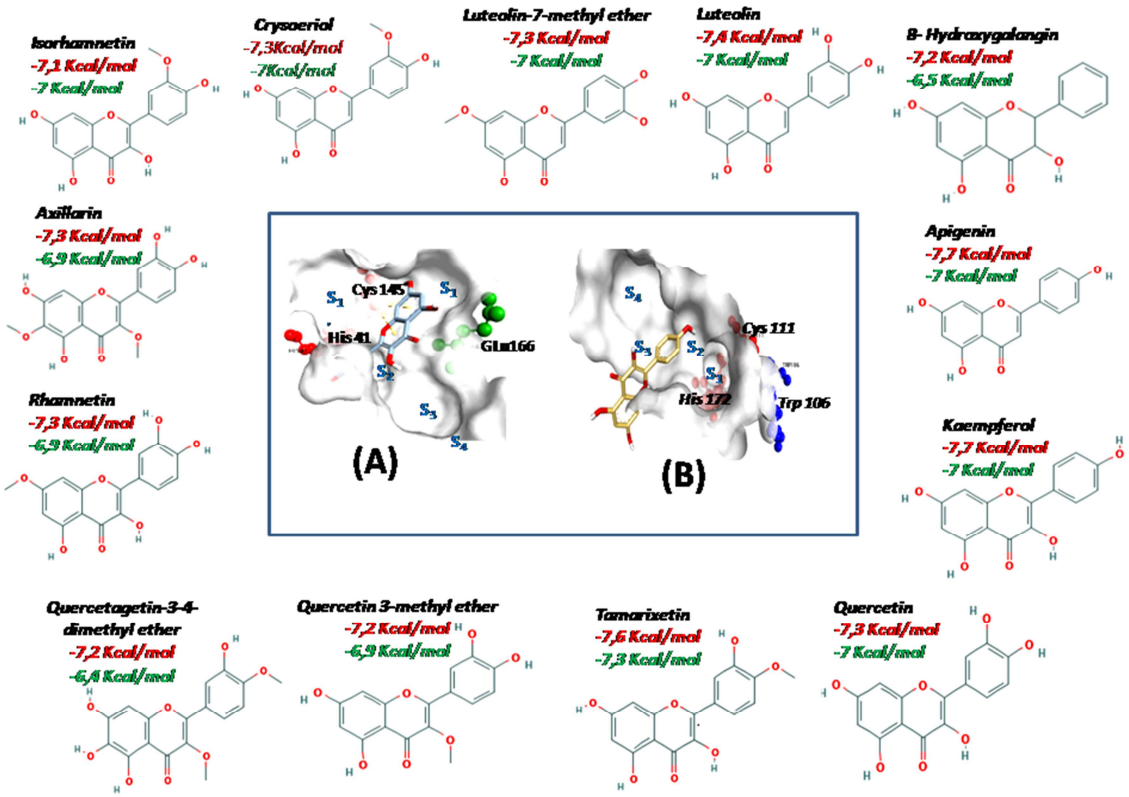

Figure 5. *Secondary metabolites of Artemisia annua predicted as inhibitors of 3CLPRO (A: red value) and PLPRO (B: green value) of SARS-COV2 and having an acceptable ADMET property.*

4. Discussion

The cyclical epidemic of CoV since 2003, resulting in human deaths, except HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKI1 responsible for human *cold flu* or *common cold* [20, 55], suggests to us the idea of having a universal antiviral drug. In order to provide candidate molecules for a future drug directed against *CoV*, in particular *SARS-CoV2*, we screened bioactive compounds from *Artemisia annua* that may have pharmacological activities, particularly against these cystein proteases *3CLpro* and *PLpro* of *SARS-CoV2* [56].

Indeed, *PLpro* cleaves its own polypeptide pp1a/b, after glycine at position P1: Leu4-(Asp^{P3}/Arg^{P3})-Gly^{P2}-Gly^{P1}↓(Ala$^{P'1}$/Lys$^{P'1}$)-(Val$^{P'2}$/Pro$^{P'2}$/Iso$^{P'2}$), which subsequently lead to the release of three non-structural proteins nsp1, nsp2 and nsp3 [26, 57]. Due to the de-ubiquitination and de-ISGylation activities of SARS-COV2 of PLpro, it plays an important role in countering the innate immune response of its host during CoV infection [58]. Also, this protease involved in the inhibition of the production of cytokines and chemokines that are responsible for the activation of the host's innate immune response against a viral infection [59]. *For 3CLpro,* it also cleaves its own pp1a/b polypeptide after glutamine (Gln^{P1}) which follows leucin (Leu ^{P2}), before an amino acid serine (Ser), alanine (Ala) or glycine (Gly): X4-X^{P3}-Leu^{P2}-Gln^{P1}↓ Ser$^{P'1}$/ Ala $^{P'1/}$ Gly $^{P'1}$-X$^{P'2}$-X$^{P'3}$-X$^{P'4}$ [60]. Glutamine (Gln), at the P1 position of the substrate, is an amino acid with a relatively long side chain leading to a polar amide group with hydrogen donor/acceptor potential [61]. The protease *3CLpro* cleaves this polypeptide pp1a/b between the non-structural proteins nsp4 to nsp16 [25, 26]. All these nsp, generated after the processed phase, are used by the *CoV* during its life cycle and the inhibition of this phase leads to the inhibition of this *CoV* life cycle.

To accelerate the design of new drugs, in particular against SARS-CoV2 responsible for the COVID-19 pandemic, computer-assisted or *in silico* screening methods are an effective approach used by the pharmaceutical industries [62-64], especially for the hundreds of essential phytoligands in *Artemisia annua*. However, the specification of its efficacy and safety for humans is a real challenge. Indeed, *in vivo* studies with different preliminary steps are necessary in order to evaluate the efficacy and toxicity of a plant [65]. Thus, the implementation of *in silico* methods is an important step before performing *in vitro* and *in vivo* studies [65]. This approach has been used to modeling the interaction of phytoligands with macromolecules (such as the viral proteases SARS-CoV2/PLpro and SARS-CoV2/3CLpro), predicting physicochemical "druglikeness" parameters as well as pharmacokinetic parameters (ADMET) that give them a potential chance in the way of drug discovery [66]. Two *in silico* methods, involving molecular docking and pharmacological study of 102 selected phytoligands (secondary metabolites of Artemisia annua) of Artemisia annua were used in this study (Supplementary data S1).

The molecular docking results of 102 phytoligands were

compared with six molecules: *Lopinavir* [29], *Ritonavir* [29], *Darunavir* [30], *Rupintrivir* [31], *Nelfinavir* [32] and *Boceprevir* [33], which we used as references ligands (Table 1). These six ligands are already proven by other researchers to be anti-proteases of CoV or other viruses [29-33]. This molecular docking consists of predicting the 3D and 2D structures of the complexes formed between the 102 phytoligands and the proteases *SARS-CoV2*/3CLpro and *SARS-CoV2*/PLpro as well as the binding energy scores between the two molecules in kcal/mol. The computational algorithms used (Autodock Vina) randomly generate a large number of possible orientations to find the "best way to insert" a phytoligand into the CoV protease at the active site [42]. The key principle is that the optimal spatial conformation between phytoligands and proteases is characterized by the lowest energy in kcal/mol [67].

After molecular docking, 63 selected phytoligands from *Artemisia annua* were shown to fit exactly into the pocket containing the catalytic sites of the proteases *SARS-CoV2*/3CLpro *and SARS-CoV2*/PLpro (Supplementary Data S2). These phytoligands form different arrangements of interaction (H bond, Alkyl-alkyl/π -alkyl, π-sulphide bond, π-π hydrophobic, Van Der Vaals) with the key amino acids forming the pocket (Figures 3 and 4). Thus, the binding energies generated (-8.8 to -6.7 kcal/mol), some of which (such as *Rutin*:-8.8 Kcal/mol and *Astragalin*: -8.7 Kcal/mol (table 2) are higher than the references ligands in this paper (-8.8Kcal/mol to -6.7Kcal/mol). The binding energy (Kcal/mol) generated for each phytoligand is used to compare and study their affinities. Thus, the higher affinity of the phytoligand for the receptor (the proteases), more the accessibility of the substrate (polypeptide pp1a/b) to the active sites of this receptor is restricted, leading to its subsequent inhibition. Hence, these 63 phytoligands that prevent or interfere with the ability of *SARS-CoV2*/3CLpro and *SARS-CoV2*/PLpro to properly perform their protease functions [4, 68] could be chosen as a potential drug for further studies [69]. The anti-protease activity on SARS-CoV1 of most of these compounds has already been reported by other authors [70] including *Quercetin* and *Luteolin* on 3CLpro [71, 72], *Circilineol, Kaempferol, Rhamnetin, and Oleanolic acid* on PLpro [73].

On the 63 phytoligands, 42 are flavonoids. In addition, they are secondary metabolites found in large numbers of vegetables, seeds, fruits, beverages such as red wine and tea [74, 75]. Also, these flavonoids are dominant secondary metabolites in medicinal plants in angiosperm botanical families such as Artemisia annua [76, 77]. In particular, flavonoids in whole plant preparations of *Artemisia annua*, have already been identified as secondary metabolites responsible for synergistic [78-81], in the treatment of rodent malaria [78, 79], and in the treatment of artesunate-resistant malaria patients [80]. And some scientific reviews have even suggested that *artemisinin* and its semi-synthetic analogues become more effective in treating parasitic diseases (such as malaria) and cancer if administered simultaneously with flavonoids [28]. In addition, a recent study revealed that *Artemisia annua* extracts had specific activity against *SARS-CoV2* variants by in vitro assays and confirmed that the inhibition of viral replication was not associated with "*artemisinin*" [82]. However, the whole plants of *Artemisia annua*, in addition to these flavonoids, also contain other secondary metabolites in the groups of coumarin, triterpene, sesquiterpene, alkaloid, benzenoid, steroid, phenolic acid and peptide alkaloid [12, 83]. Some molecules in these groups also fulfilled anti-protease functions and could also be chosen as potential drugs for further studies [69].

These results demonstrate that phytoligands contained in *Artemisia annua*, can interact synergistically to inhibit *SARS-CoV2*/PLpro and *SARS-CoV2*/3CLpro and other key viral life cycle proteins such as viral polymerases [70, 76, 84], act as an immunomodulator and anti-inflammatory [85] and also act as an antioxidant [28]. However, whole plant preparations contain hundreds of secondary molecules that may cause macromolecular malfunctions in the body [70]. In order to avoid these possible malfunctions, laboratory tests on cell culture *in vitro* and/or on animals *in vivo* are necessary to determine the physicochemical characteristics and pharmacokinetic properties of these ligands but these tests are limited by time, ethical considerations and financial burden [86]. To overcome these limitations and to accelerate the processes of drug discovery and development today, the use of *in silico* screening methods is essential, especially for phytoligands in medicinal plants. As with *in vitro* and *in vivo* methods, the main objective of the drug discovery and development process, by first going through *in silico* screening, is to find a molecule or molecules with both good pharmacodynamic properties and good pharmacokinetic properties [87]. The prediction of these properties was performed with the online tool SwissADME [52] and pkSCM [88].

Filters for predicting pharmacodynamics properties in SwissADME include lipophilicity and solubility as well as *drug-likeness* [52]. These properties are grouped into the *Pfizer* Lipinski [49], *GlaxoSmithKline* Veber [50] and *Boehringer Ingelheim* Muegge [51] rules. Analysis of the results revealed that 38 of the 63 phytoligands with higher docking scores with the *SARS-CoV2* proteases did not violate any of these rules. These 38 phytoligands selected have acceptable physicochemical and "*drug-like*" characteristics (Supplementary Data S3). In addition, they do not present problems of oral bioavailability [89], which are verified by the *in silico* predictions of Caco-2 permeability (high> 0.90) and of the VDss value superior to -0.1 (Supplementary Data S4).

After the four phases of *in silico* ADME analysis: Absorption, Distribution, Metabolism and Excretion [90], 15 phytoligands passed the analytical filters. Indeed, these 15 phytoligands also have an intestinal adsorption capacity of more than 70%. During migration to the target cells, they are not metabolized by the major blood cytochromes P450 (CYP3A4 and CYP2D6). Secondly, they are neither substrates nor inhibitors of P-glycoproteins (P-gp) of the major membrane transport molecules [91]. In addition, all 15 phytoligands are able to cross the cell membrane (lipophilicity characteristics) to reach their

potential targets in the cell cytoplasm. With regard to excretion, the analysis of the results (Supplementary Data S4) shows that none of these 15 phytoligands filtered in the ADME assay are substrates of OCT2 and cannot affect the functioning of this ionic transporter, hence the possibility of their elimination by renal route [92].

The prediction of toxicity by AMES test showed that among the 15 phytoligands that have an acceptable ADME property, *3-Hydroxy Deoxy Dihydro artemisinin*, and benzyl isovalerate are predicted to have AMES toxicity (Supplementary DataS4). With regard to cardiotoxicity, hepatotoxicity, and skin sensitization, none of the remaining 13 phytoligands is likely to be associated with them (Figure 5). These molecules cannot influence the action of the liver in elimination or reabsorption, hence the possibility also of hepatic elimination, nor can they cause a fatal ventricular tachyarrhythmia called torsade de pointes or TdP linked tohERG I/II inhibition [93-95].

The 13 phytoligands released in this study (*Apigenin*, *Axillarin*, *Crysoeriol*, *8-Hydroxygalangin*, *Isorhamnetin*, *Kaempferol*, *Luteolin*, *Luteolin-7-methylether*, *Quercetagetin-3-4-dimethyl ether*, *Quercetin 3-methyl ether*, *Quercetin*, *Rhamnetin* and *Tamarixetin*) all belong to the group of flavonoids that are already cited by other researchers in other medicinal plants and some of them have also been shown to have antiviral activity, in particular, *Apigenin*, *Luteolin*, *Quercetin* and *Kaempferol*, by inhibiting the proteolytic activity of *SARS-CoV/*3CLpro [96].

In addition to these antiviral roles, these flavonoids are also known for their anti-inflammatory activity. Upon infection, *SARS-CoV2* leads to an increase in the secretion of pro-inflammatory cytokines such as IL-1β, IFN-γ, IP-10, MCP-1, IL-4 and IL-10 subsequently leading to the cytokine storm associated with the severity of COVID-19 [97]. As most flavonoids, *Apigenin*, and *Luteolin* have already been shown to be an anti-inflammatory, the addition of these compounds to treatment may reduce mortality due to respiratory distress [98]. Indeed, various flavonoids are currently known to have antiviral and anti-inflammatory roles [99]. For this reason, phytonutrients and/or nutraceuticals (P/N) have become a trend in recent years to boost immune health [98].

Other than viral anti-proteolysis, anti-inflammatory and immunostimulatory, these flavonoids, in significant amounts in *Artemisia annua*, exert various biological roles. It has been shown that flavonoids such as *Apigenin*, *Quercetin* and *Kaempferol* can increase intracellular glutathione levels via induction of gamma-glutamyl cysteine synthetase transcription [100] and act as antioxidants [101].

The herbal remedy is usually prepared in the form of a beverage such as tea obtained either by infusion (soaking or immersion in hot water) or decoction (boiling with water). Infusion or decoction allows the extraction of several molecules that can play a synergistic role in the treatment of the disease [102].

Indeed, the use of several molecules can target several diseases simultaneously or direct the effect against a single disease and treat it more effectively [103]. This is the case with *Artemisinin* derivatives such as *Artesunate*. Metabolic and pharmacokinetic studies show that *Artesunate* has a short half-life (1 to 2 hours) in the blood when taken orally [28]. But the infusion or decoction of *Artemisia annua* can improve the bioavailability of *Artesunate*, allowing it to exert its effect in the long term [104].

5. Conclusion

In conclusion, the present study identified, using an *in silico* approach that 13 phytoligands (*Apigenin*, *Axillarin*, *Crysoeriol*, *8-Hydroxygalangin*, *Isorhamnetin, Kaempferol, Luteolin, Luteolin-7-methylether, Quercetagetin-3-4-dimethyl ether, Quercetin 3-methyl ether, Quercetin, Rhamnetin, and Tamarixetin*) of *Artemisia annua* likely to inhibit *SARS-CoV2* by acting on the proteases 3CLpro and PLpro. Studies of the physicochemical properties, drug-like properties and pharmacokinetics of these molecules have shown that they have good absorption and permeability when administered orally in humans. Some properties of these molecules (bioavailability, toxicity) do not allow them to act alone. But their simultaneous presence in *Artemisia annua* tea causes a synergism resulting in beneficial effects during treatment. In this study, we only used the *in silico* approach to identify relevant molecules in *Artemisia annua* capable of inhibiting *SARS-COV2* proteases. It is therefore recommended to move on to a next phase of drug development, which consists of an *in vitro* and *in vivo* evaluation of the antiviral capacity of these 13 proposed phytoligands, to develop new drugs against *SARS-CoV2*.

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