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Original Article

Flavonoid glycosides and their putative human metabolites as potential inhibitors of the SARS-CoV-2 main protease (Mpro) and RNA-dependent RNA polymerase (RdRp)

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ABSTRACT

BACKGROUND - Sincethe World Health Organization (WHO) declaredCOVID-19 to be a pandemic infection, importantSARS-CoV-2non-structural proteins (nsp) have been analisedas promising targets in virtual screening approaches. Among these proteins, 3-chymotrypsin-like cysteine protease (3CLpro), also named main protease, and the RNA-dependent RNA polymerase (RdRp), have been identified as the main targets.

OBJECTIVES -Toinvestigate,*in silico*,the main flavonoid glycosides from*Dysphaniaambrosioides*;a medicinal plant found in many regions of the world, along withsome of the putative derivatives of these flavonoid glycosidesin the human organismas potential inhibitors of the SARS-CoV-2 3CLpro and RdRp.

METHODS – Using a molecular docking analysis, the interactions and the binding affinity with SARS-CoV-2 3CLpro and RdRpwere predicted for quercetin-3-*O*-rutinoside (rutin), kaempferol-3-*O*-rutinoside (nicotiflorin) and some of their glucuronide and sulfate derivatives.

FINDINGS - Docking analysis, based on the crystal structure of 3CLpro and RdRp,indicated rutin, nicotiflorin,and their glucuronide derivativesto bethe best inhibitors forboth proteins. Also, theimportance of the hydrogen bond and π-based interactions was evidenced for the presumed active sites.

MAIN CONCLUSIONS - Overall, these results suggest that both flavonoid glycosidesand their putative human metabolites can play a key role as inhibitors of the SARS-CoV-2 3CLpro and RdRp. Obviously, further research is necessary to certifythe docking results reported here, as well as the adequate application ofthese substances. Furthermore, it is necessary to investigate the risks of *D. ambrosioides* as a phytomedicine for use against COVID-19.

Keywords:

COVID-19; rutin; nicotiflorin; glucuronides; *Dysphaniaambrosioides*;phytomedicines

INTRODUCTION

Since the appearance of the first cases, reported in December 2019 in Wuhan, China, the new pandemic disease COVID-19 caused by coronavirus SARS-CoV-2 has

claimedmillions of victims and caused hundreds of deaths around the world. Forthwith, Chinese teams sequenced SARS-CoV-2[1]andits important nonstructural proteins (nsp) were revealed, which included spike proteins,3-chymotrypsin-like cysteine protease (3CLpro), also named main protease (Mpro), papain-like protease (PLpro), and RNA-dependent RNA polymerase (RdRp).[2]The spike proteinsbind the virusto the human receptor –a metallopeptidase named angiotensin- converting enzyme 2 (ACE2), while the 3CLpro and the PLpro providecomponents for packaging new virions from large viral polyproteins translated on host ribosome, andfinally the RdRp replicate the SARS-CoV-2 RNA genome.[2]Many computational reports based on virtual screening have been done on the inhibition of one or more of these SARS-CoV-2 key proteins, mainly 3CLpro and RdRp, most using drugs used against other human diseases or compounds which have beenpreviously described in medicinal plants.[3,4]Fortunately, some of these therapeutic medicinal plants, such as *Dysphaniaambrosioides*(L.) Mosyakin&Clemants (Syn. *Chenopodiumambrosioides* L.),are commonly found in tropical and subtropical regions and are therefore accessible as therapeuticagents.*D. ambrosioides* is popularlyknown in Brazil as“mastruz” or “Erva-de-Santa-Maria” and has been used to treat a number of health problems, such as infections, sinusitis, gastritis, inflammations, and flu.[5,6]From the phytochemical viewpoint, this species is a promising source of flavonoid glycosides.[7]These glycosides present great biological potential, including antioxidant and antiviral activities, and are vital in diets.[8]Since *D.ambrosioides*and several other flavonoid-producing sourcesare used worldwide, the absorption, metabolism and pharmacokinetics of flavonoids havebeen intensively investigated,andglucuronide and sulfates can be highlighted as important metaboliteproducts in this process.[9,10]Thus, in the present study, we screened the main flavonoid glycosides from *D.ambrosioides*along with some of their putative derivatives

in the human organismusing molecular docking, in order to test them as potential inhibitors of the SARS-CoV-2 3CLpro and RdRp.

MATERIALS AND METHODS

Ligand preparation–Initially, the three-dimensional (3D) structures of quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-glucuronide, quercetin-3'-*O*-sulfate, and quercetinwere downloaded from ZINC database (http://zinc15.docking.org/) as SDF files. These files were used as templates to generate, via Marvin Sketch software (https://chemaxon.com/products/marvin),kaempferol-3-*O*-rutinoside (nicotiflorin),kaempferol, and some of their putativemono-glucuronide andsulfatederivatives (Figure 1), based on previous pharmacokinetic studies on the human organism.[9,10]Also, theaflavin, a phenolic compound suggested as potential SARS- CoV- 2RdRpinhibitor,[11]wasdownloaded from ZINC database.Subsequently, all the structureswere subjected togeometry optimization by the semi-empirical method PM7 using MOPAC2016 software (http://openmopac.net/MOPAC2016.html), being the results saved as PDB files.Finally, the ligands were prepared via Autodocktools[12]and saved as PDBQT files.

Protein preparation–The 3D crystal structures of the SARS-CoV-2 3CLpro(PDB ID: 6W63)and RdRp (PDB ID: 6M71)were retrieved from Research Collaboratory for Structural BioinformaticsProtein Data Bank (RCSB PDB) (http://www.rcsb.org) as PDB files. These receptors were prepared via Autodock tools and saved as PDBQT files.

Docking Simulations– The docking simulations were as previously reported,[13] in which the grid box was centered at the ligand X77 in 3CLpro (PDB ID: 6W63)and at the presumed active site[11,14] inthe RdRp (PDB ID: 6M71). The interactions and the binding affinity of the protein-ligand complex were predicted viaa rigid docking process using AutodockVina[15] and viewed withtheDiscovery Studio software.[16] Due to the lack of models with ligands forRdRp in the RCSB PDB, only the 3CLpro was tested for redocking.

RESULTS

Molecular docking with 3CLpro (main protease– Mpro)–Docking analysisrevealed scoring function values for nicotiflorin (-11.2 kcal/mol) and rutin (-10.3 kcal/mol) close to that obtained for the previously described inhibitor X77 (redocking binding free energy = -12.4 kcal/mol, RMSD < 2) when docked with 3CLpro, which suggests the establishment of favorable interactions for theligand-3CLpro complex.

The putative human metabolites from rutin, quercetin-7-*O*-glucuronide (-10.9 kcal/mol) and quercetin-3-*O*-glucuronide (-10.7 kcal/mol) presented better binding free energythan its precursors, while quercetin-3'-*O*-glucuronide (-10.2 kcal/mol) was close to this result. On the other hand, the quercetin sulfates (-9.2 to - 9.5 kcal/mol) presented worse binding free energy than the glucuronides derivatives, irrespectiveof the sulfate'sposition. For quercetin, the binding free energy of -8.3 kcal/mol observed was the worst among the putative human metabolites from rutin. Regarding the putative metabolites from nicotiflorin, the 7-glucuronide derivative (-10.9 kcal/mol) also presented the best scoring function value among the kaempferolglucuronide derivatives, followed by kaempferol-4'-*O*-glucuronide (-9.7 kcal/mol). Surprisingly, the kaempferol-3-*O*-glucuronide (-9.1 kcal/mol) presented binding free energyslightly worse

thankaempferol-3-*O*-sulfate (-9.4 kcal/mol). Similarly toquercetin, kaempferol (-8.1 kcal/mol) presented worstbinding free energythan all its chemical derivatives.

In regards to the observed interactions in the presumed active site of 3CLpro, hydrogen bonds and π-based interactions, such as π-sulfur, π-alkyl, π-π, and π-cation interactions, were dominant in almost all tested compounds (Table 1). The interactions observed for quercetin-7-*O*-glucuronide, the compounds with the best scoring function value in the quercetin-set, were hydrogen bonds with the residues Thr26, Gln189, and Glu166, π-π and π-cation interaction with His41, and π-alkyl interaction with Met49(Figure 2). The second best inhibitor of the quercetin-set, quercetin-3-*O*-glucuronide, presented hydrogen boundswith Thr25, Cys44, Asn142, Ser144, His163, Met165, Glu166, and Arg188, and π-alkyl interaction with Met49 and Met165 (Figure 2). However, rutin, the third best inhibitor of the quercetin-set, also presented some hydrogen bonds, along with π-π and π-cation interactions with His41 and π-alkyl interaction with Met49 (Figure 2). On the other hand, nicotiflorin, the compound with the bestscoring function values in kaempferol-set, presented, besides hydrogen bonds, important π-π and π-cation interactions with His41 and π-alkyl interaction with Met49.Similar interactions were observed forkaempferol-7-O-glucuronide, the second of the kaempferol-set (Figure 2). The main observed interactions between the vegetal flavonoid glycosides andtheir putative human metaboliteswith 3CLpro are summarized in Table 1.

Molecular docking for RNA-dependent RNA polymerase (RdRp)–Docking analysis using RdRprevealed better scoring function values for rutin (-11.7 kcal/mol) andnicotiflorin (-11.3 kcal/mol) than fortheaflavin (-11.2 kcal/mol) (Table 1).

Similarly to the results observed in docking with 3CLpro, the glucuronide derivatives (quercetin = -10.2 to -10.5 kcal/mol; kaempferol = -10.0 to -10.2 kcal/mol) also presented better binding free energy thansulfate derivatives (quercetin = -8.8 to -9.4 kcal/mol; kaempferol = -8.7 to -9.3 kcal/mol), with aglyconesagain presenting the worst binding free energies (quercetin = -8.2 and kaempferol = -7.7 kcal/mol) with RdRp.

Regarding to the observed interactions in the presumed active site of RdRp, hydrogen bonds, π-cation and π-anion interactions were dominant to almost all tested compounds (Table 1). Since this active site includes the motif A (residues 611-626), with the classic divalent cation-binding residue 618, and motif C (residues 753-767), with the catalytic residues 759-761[14],we therefore focused our interpretation on these interactions.The observed interactions for theaflavin were near to that previously described in the literature,[11]and highlightedthe hydrogen bonds observed with Asp623 and Asp760, from motif A and C, respectively, (Figure 3) which is suggestive ofa good approximation of the present model with the previous model by homology. Also, for theaflavin, π-anion interactions were observed with Asp760 and Asp761 from motif C. For rutin, the best of the compounds in this study,among the other interactions, key hydrogen bonds were observed with Tyr619 and Lys621 from motif A, and Asp760, from motif C (Figure 3). Similarly, for nicotiflorin hydrogen bonds were observed with those same residues from motif A and C, along with π-anioninteraction with Glu811(Figure 3). The mainobserved interactions between the flavonoid glycosides and their putative human metaboliteswith RdRp are summarized in Table 1.

DISCUSSION

Rutin and nicotiflorin,as well as other glycoside flavonoids, are present in a large number of therapeutic medicinal plants and are often consumed in the form of herbal

teas.[17]These compounds are vital in dietsandare of great interest due theirantioxidant,anti-inflammatory and antiviral activities and their human metabolites, which include quercetin from rutin hydrolysis.[8,17-20]For this reason,their absorption, metabolism, toxicity, and pharmacokinetics has been intensively investigated.[9,10]Regarding these studies, rutinoside flavonoids, such as rutin and nicotiflorine, aredeglycosylatedprior to being absorbed into the circulation and then conjugated mainly with glucuronate and sulfate, these being the main forms in plasma.[9]Based on this information, it is reasonable to suggest thatboth quercetin and kaempferolglucuronides, demonstrated aboveas promising3CLpro and RdRp inhibitors, could have akey roleagainst these proteins, since the virus is also dominant in plasma. This hypothesis is in accordance with previous *in vivo*antiviral studiesperformed with rutin. These studies indicate thatrutinprotects cells for about 24 h against vesicular stomatitis virus, affords immense viral embarrassment in canine distemper virus, and demonstrates aprofound antiviral effect against avian influenza strain H5N1.[8]Flavonoid glucuronides have also been described as antiviral agents, including quercetin-3-*O*-glucoronide, here described as a potential 3CLpro inhibitor.[21,22]On the other hand, although sulfate products have not showed high binding free energycompared to glucuronides in docking analysis, their antiviral potential should also be considered, since previous works have pointed out their effective anti-HIV and –HSD activities.[23]

Recent autopsy studies have permitted BrazilianCOVID-19 patients to be treated as sufferers ofDissemination Intravascular of Coagulum (DIC), which can cause failure of several organs, mainly the lungs.[24]As a preliminary stage for combating the disease, Low Molecular Weight Heparin (LMWH) has beensuccessfully administered.[25]In addition to its anticoagulant therapeutic effects, LMWH has demonstrated anti-

inflammatory effects, endothelial protection and viral inhibition.[26]Rutin, displayed here as an active agent against 3CLpro and RdRp of SARS-CoV-2, has also proved to have anticoagulant therapeutic effects[27]as well asanti-inflammatory effects and potential protection against acute lung injury (ALI).[28]Intravenous or intranasal administration could be an alternative to oral intake, thus improving its bioavailability.[29,30]*D. ambrosioides* has been used successfully by the riverside population in the Amazon regionfor treating cases of acuterespiratorydistresssyndrome(ARDS) and tuberculosis . These results may also be related to the presence of rutin.[6]

Overall, these results suggest that rutin, nicotiflorin,and their putative human metabolites, mainly glucuronides, but also sulfates, can play a key role as inhibitors of the SARS-CoV-2 3CLpro and RdRp.Such derivatives, which are expected in plasma,are in fact the most likely compounds for targeting these viral proteins, via oral intakeof these flavonoid glycosides. However, at least for rutin,intravenous or intranasal administration can be an alternative for prompt bioavailability. Our results,and much of the reported data,suggestrutin and nicotiflorinas possible alternativesfor combatingthe COVID-19 virus. Rutin could even be considered an alternative to LMWH, given its anticoagulant and anti-inflammatory effects and its potential protection against ALI. Obviously, further research is necessary to corroboratethe docking results reported here, as well as the adequate application ofthese substances. Furthermore, it is necessary to investigate the risks of *D. ambrosioides*as aphytomedicine foruse againstCOVID-19.

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AUTHOR CONTRIBUTIONS

FMAS: Conceptualization, Methodology, Formal analysis, Data Curation, Writing - Original Draft. KPAS:Conceptualization, Investigation. LPMO: Investigation. EVC: Investigation. HHFK: Investigation. MLBP:Conceptualization, Investigation. AQLS:Conceptualization, Investigation, Writing - Original Draft.ADLS:Conceptualization, Methodology, Formal analysis, Data Curation, Writing - Original Draft, Supervision.

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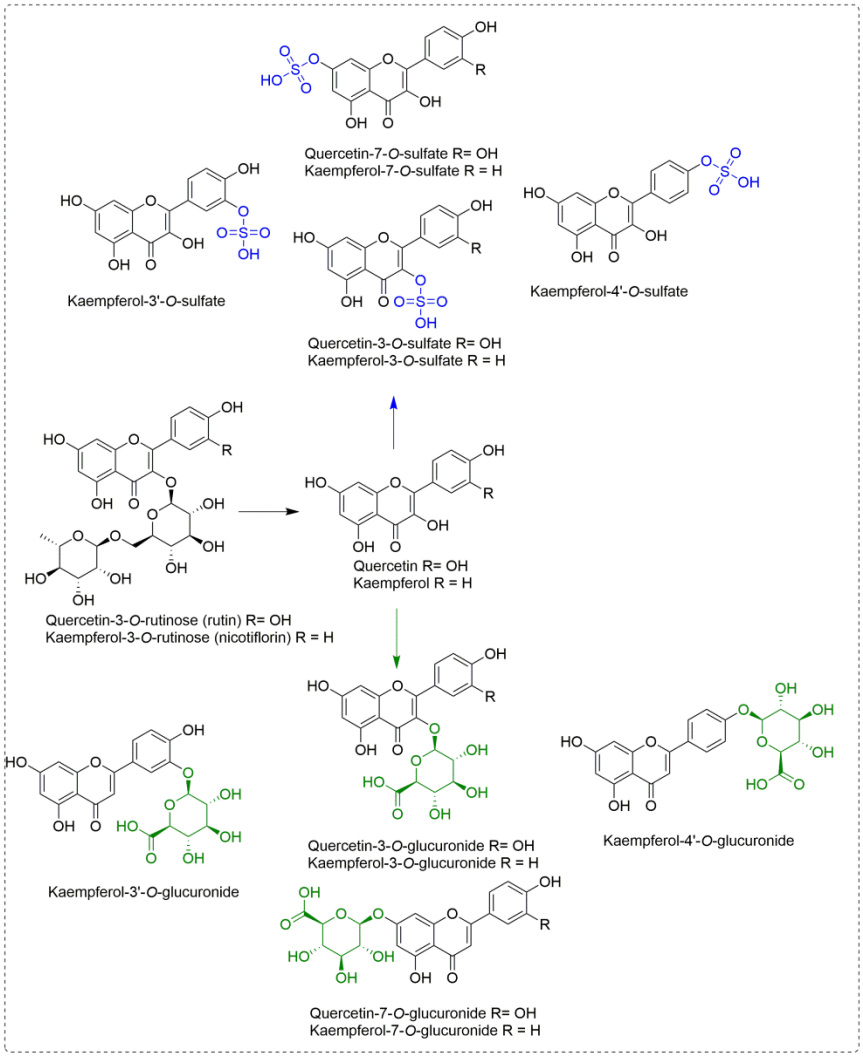

Figure 1. Chemical structures of the main flavonoid glycosides from *D. ambrosioides*, along with some of their putative derivatives in human organism.

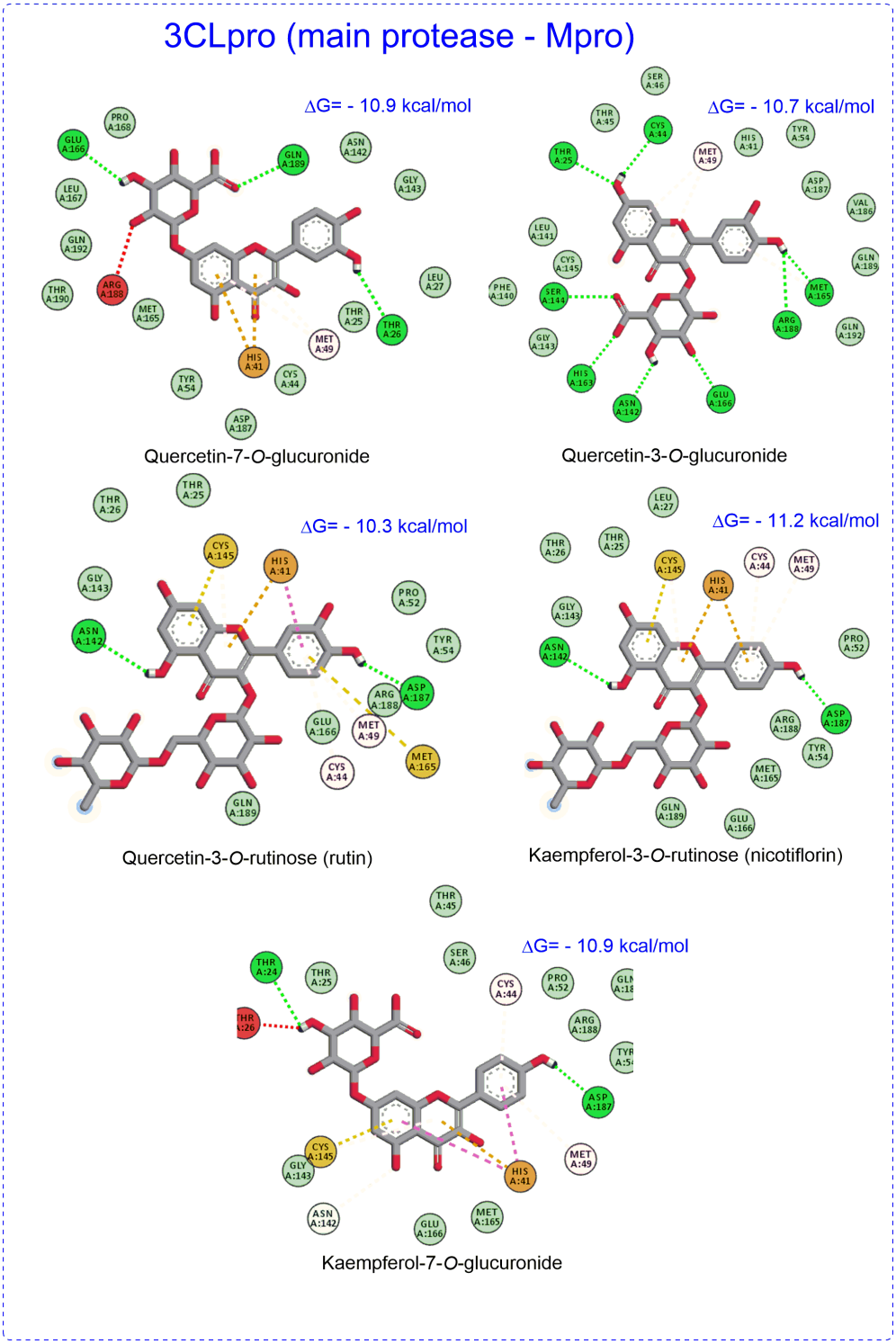

Figure 2. Main interactions observed for the best inhibitors of 3CLpro by docking analysis.

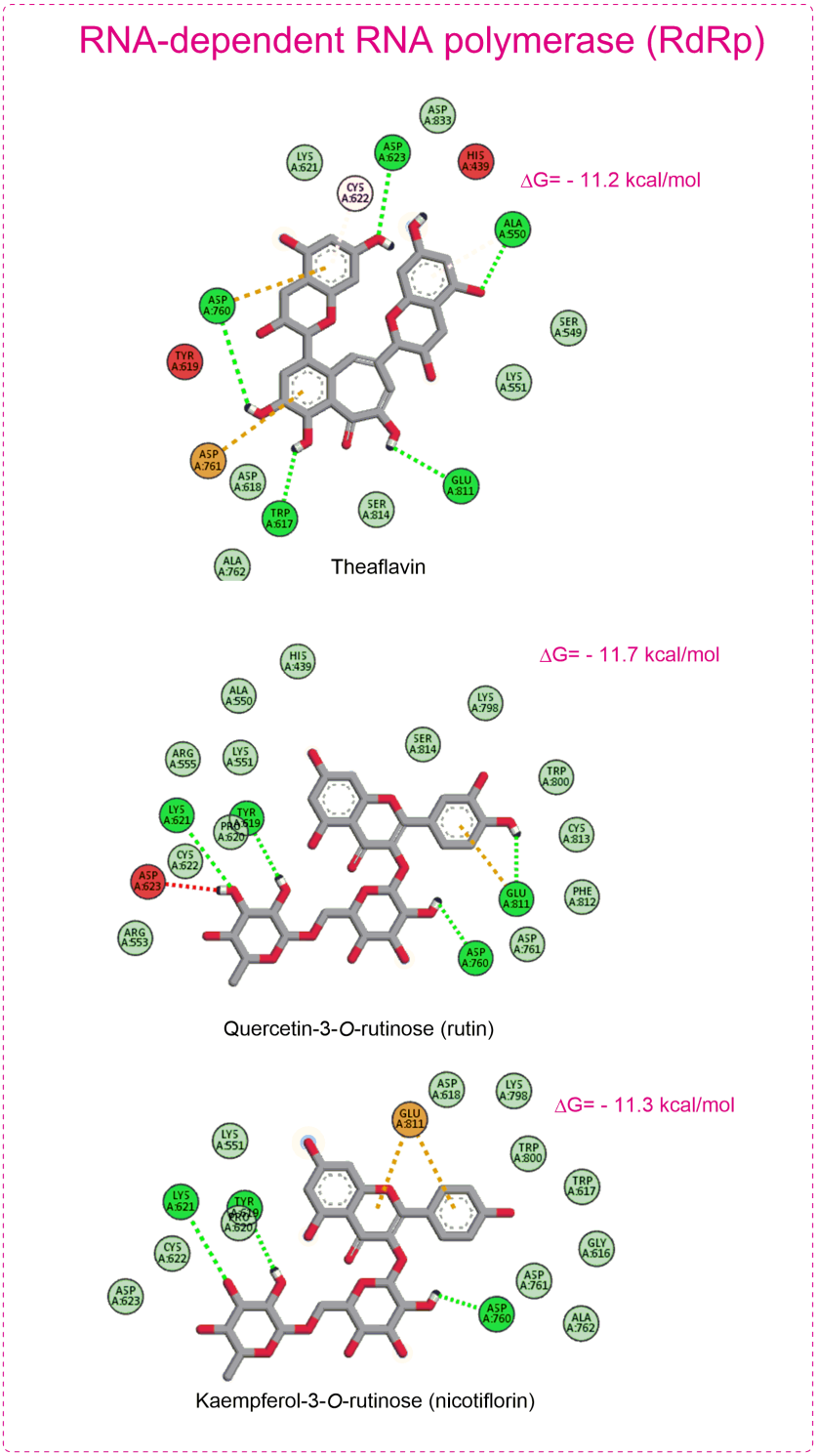

Figure 3. Main interactions observed fortheaflavin, rutin and nicotiflorin-RdRp complex by docking analysis.

Table 1. Docking analysis datafor the main flavonoid glycosides from *D. ambrosioides* along with some of their putative derivatives in human organism

Compounds	Protein (PDB iD)	Bindingenergy (kcal/mol)	Maininteractions
Theaflavin	**RdRp (7M71)**	**-11.2**	**HB (Ala550, Trp617, Aap623, Asp760, Glu811), PA (Asp760, Asp761), PAl (Ala550, Cys622)**
x77	**3CLpro (6W63)**	**-12.4**	**HB (Gly143, Glu166), PS (Met49), PAl (Leu27, His41, Pro168), PAm (Leu 141)**
Quercetin-3-*O*-rutinose (Rutin)	**RdRp (7M71)**	**-11.7**	**HB (Tyr619, Lys621, Asp760, Glu811), PA (Glu811)**
	3CLpro (6W63)	**-10.3**	**HB (Asn142, Asp187), PS (Cys145, Met165), PP (His41), PC (His41), PAl (Cys44, Met49, Cys145)**
Quercetin-7-*O*-glucuronide	**RdRp (7M71)**	**-10.5**	**HB (Arg553, Ser549, Tyr619, Trp800, Glu811, Cys813), PC (ARG555, LYS551)**
	3CLpro (6W63)	**-10.9**	**HB (Thr26, Glu166, Gln189), PP (His41), PC (His41), PAl (Met49)**
Quercetin-3'-*O*-glucuronide	**RdRp (7M71)**	**-10.5**	**HB (Cys622, Asp623, Asp760, Glu811, Cys813, Ser814), PA (Asp618, Asp761)**
	3CLpro (6W63)	**-10.2**	**HB (Gly143, His164), PAl (Met49)**
Quercetin-3-*O*-glucuronide	**RdRp (7M71)**	**-10.2**	**HB (Thr556, Tyr619, Lys621, Thr687, Asn691, Ser759, Asp760), PC (Arg555, Arg553), PA (Asp623), PAl (Lys621)**
	3CLpro (6W63)	**-10.7**	**HB (Thr25, Cys44, Asn142, Ser144, His163, Met165, Glu166, Arg188), PAl (Met49, Met165)**
Quercetin-7-*O*-sulfate	**RdRp (7M71)**	**-9.4**	**HB (Lys545, Lys621, Arg624), PC (Arg555, Arg553), PA (Asp623), PAl (Arg 624)**
	3CLpro (6W63)	**-9.5**	**HB (Thr25, Ser144, Glu166), PS (Cys145), PC (His41), PAl (Met49, Met165)**
Quercetin-3-*O*-sulfate	**RdRp (7M71)**	**-8.8**	**HB (Asp452, Arg553, Thr556, Tyr619), PC (Arg553), PA (Asp623)**
	3CLpro (6W63)	**-9.5**	**HB(Asn142, Thr190), PS (Met165), PSi (Gln189), PAl (Met165)**
Quercetin-3'-*O*-sulfate	**RdRp (7M71)**	**-8.8**	**HB (Ser814), PA (Asp760, Asp761)**
	3CLpro (6W63)	**-9.2**	**HB (Met49, Gly143, Asp187), PSi (Thr25), PP (His41), PC (His41), PAl (Cys44, Met49)**
Quercetin	**RdRp (7M71)**	**-8.2**	**HB (Asp760, Trp800), PA (Asp761)**
	3CLpro (6W63)	**-8.3**	**HB (Glu166), PS (Met49), PC (His41), PAl (Met165)**
Kaempferol-3-*O*-rutinose (Nicotiflorin)	**RdRp (7M71)**	**-11.3**	**HB (Tyr619, Lus621, Asp760), PA (Glu811)**
	3CLpro (6W63)	**-11.2**	**HB (Asn142, Asp187), PP (His41), PS (Cys145), PC (His41), PAl (Cys44, Met49, Cys145)**
Kaempferol-4'-*O*-glucuronide	**RdRp (7M71)**	**-10.2**	**HB (Asp618, Lys621, Asp623, Asp761), PA (Glu811)**
	3CLpro (6W63)	**-9.7**	**HB (Thr24, Cys44), PS (Met49), PP (His41), PAl (Cys44, Met49)**
Kaempferol-3-*O*-glucuronide	**RdRp (7M71)**	**-10.1**	**HB (Tyr619, Asp760, Asp761, Glu811, Cys813)**
	3CLpro (6W63)	**-9.1**	**HB (Asn142, Glu166), PP (His41), PC (His41), PAl (Cys44, Met49)**
Kaempferol-7-*O*-glucuronide	**RdRp (7M71)**	**-10.0**	**HB (Asp760, Trp800), PA (Glu811), PAl (Lys798)**
	3CLpro (6W63)	**-10.9**	**HB (Thr24, Asp187), PP (His41), PS (Cys145), PC (His41), PAl (Cys44, Met49, Cys145)**
Kaempferol-7-*O*-sulfate	**RdRp (7M71)**	**-9.3**	**HB (Lys545, Lys621, Arg624), PC (Arg553, Arg555), PA (Asp623), PAl (Arg624)**
	3CLpro (6W63)	**-9.1**	**HB (Gly143, Glu166), PS (Cys145), PP (His41), PC (His41), PAl (Cys44, Met49, Cys145)**
Kaempferol-4'-*O*-sulfate	**RdRp (7M71)**	**-9.2**	**HB (Arg553, Lys621, Thr687, Asn691), PC (Arg553), PA (Asp623), PS (Cys622), PAl (Lys621)**
	3CLpro (6W63)	**-9.0**	**HB (Leu141, His163), PS (Cys145), PP (His41)**
Kaempferol-3-*O*-sulfate	**RdRp (7M71)**	**-8.7**	**HB (Arg553, Arg555, Thr556, Tyr619, Lys621, Arg624), PC (Arg553), PA (Asp623), PAl (Lys621)**
	3CLpro (6W63)	**-9.4**	**HB (Met49, His41, Gly143, Glu166), PS (Cys44, Met49, Cys145), PP (His41), PC (His41), PAl (Cys145)**
Kaempferol	**RdRp (7M71)**	**-7.7**	**HB (Tyr619, Ser814), PA (Asp760, Asp761)**
	3CLpro (6W63)	**-8.1**	**HB (Met49, Gly143, Asp187), PS (Cys44, Cys145), PP (His41), PC (His41), PAl (Cys145)**

[a]HB – hydrogen bond, PS – π-sulfur, PAl – π-alkyl, PP – π-π, PA – π-anion, PC – π-cation, PSi – π-sigma, PAm – π-amide