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Effect of naringenin, hesperetin and their glycosides forms on the replication of the 17D strain of yellow fever virus

(Efecto de la naringenina, hesperetina y sus formas glicosidadas sobre la replicación de la cepa 17D del virus de la fiebre amarilla)

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Abstract (english)

Infections produced by flaviviruses are threats to public health worldwide. The absence of effective drugs treatment makes imperative the quest for efficient antivirals. Using a plaque inhibition assay we studied the antiviral action of the non-cytotoxic flavanones naringenin, hesperetin, and their glycoside forms on the replication of the 17D strain of yellow fever virus (YFV17D). Using plaque assay the glycosylated forms did not show antiviral effect at the highest concentration used, naringenin and hesperetin reduced the plaque size and the infectious titer up to 85.51% and 100% respectively at the maximum concentration employed. The effective dose (ED_{50}) of naringenin (0.0013 μM), calculated by plaque assay, was approximately tenfold lower than hesperetin (ED_{50} 0.01 μM), and neither showed any virucidal effect. Evaluation of kinetic entrance of YFV to cell showed that after 2h of infection 80% of the virus was already into the cell, but in the presence of naringenin or hesperetin, this entrance were inhibited to 48.58% and 55.2% respectively ($p \leq 0.05$). Study of molecular parameters of naringenin and hesperetin demonstrated that naringenin Log P = -1.56 is ten times higher than hesperetin (Log P = -2.56), and therefore less hydrophilic and probably more effective in passing through membranes, which could be related to its ability to interfere with the viral replication beside the entrance level

Keywords (english)

Flavanones, Flavivirus, Antiviral, Yellow Fever Virus 17D

Resumen (español)

Las infecciones producidas por flavivirus representan una amenaza para la salud pública a nivel mundial. La ausencia de fármacos eficaces para su tratamiento hace imperativa la búsqueda de antivirales efectivos. Empleando ensayos de inhibición de placa se estudió la acción antiviral de las flavanonas no citotóxicas naringenina, hesperetina, y sus formas glicosidadas sobre la replicación de la cepa 17D del virus de la fiebre amarilla (YFV17D). Las formas glicosidadas no mostraron efecto antiviral con la máxima concentración. La naringenina y hesperetina fueron capaces de reducir el tamaño y número de placas virales en un 80% y 100% respectivamente. La dosis efectiva (ED_{50}) de la naringenina (0.0013 μM) fue aproximadamente diez veces menor que la hesperetina (ED_{50} 0.01 μM) y ninguna mostró efecto virucida. Después de 2h de infección el 80% del virus ha entrado a la célula pero en presencia de naringenina o hesperetina dicho proceso es inhibido en un 48,58% y 55,2% respectivamente ($p \leq 0,05$). El estudio de parámetros moleculares de ambas flavanonas demostró que el Log P=-1.56 de la naringenina es diez veces mayor que el de hesperetina (Log P=-2,56) indicando que es menos hidrofílico y probablemente más eficaz pasando a través de membranas, lo que podría estar relacionado con su capacidad de interferir en la replicación viral a niveles distintos de la entrada.

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Palabras clave (español)

Flavanonas, Flavivirus, Antiviral, Virus fiebre amarilla 17D

Introduction

The family Flaviviridae includes four genera: Flavivirus, Hepacivirus, Pegivirus and Pestivirus characterized by being small-enveloped single-stranded RNA viruses (1-5). Flaviviruses like Dengue (DENV), Yellow Fever (YFV) and Japanese encephalitis (JEV), and also the Hepacivirus Hepatitis C (HCV) are threats to public health worldwide (6-8). YFV, the prototype of the Flavivirus, causes a severe febrile disease with haemorrhage, multi-organ failure and shock with a high mortality (up to 50% of cases) (9). It is a zoonotic agent that, despite the availability of a safe and efficient vaccine, continues to be reintroduced into human population from wild animal reservoirs, causing outbreaks in endemic regions of South America and Africa (10). The lack of antiviral compounds targeting flaviviruses represents a significant problem in the development of strategies for treating their infections (11-13). The vacunal strain 17D of YFV has been used previously in the study of the antiviral effect of a series of compounds (14, 15). Flavanones are a type of flavonoid found in citric fruits as orange and grapefruit (16); previous studies with the glycosylates flavanones naringin and hesperidin, have shown an inhibitory effect on in vitro rotavirus infections (17) but not DENV type-2 (DENV-2) (18). However, their aglycone form inhibited Sindbis virus (SINV) (19), Chikungunya virus (20) and HCV replication (21). Studies in a number of animal models have shown that diets supplemented with grapefruit juice, orange juice (22), naringenin or hesperidin (23) result in reduced triglyceride (24) and cholesterol (25) plasma levels. Naringenin inhibited hepatocyte apoB (26) and HCV (21, 27) secretion via interaction with cholesterol and lipids pathway. Naringenin has already been pharmacologically evaluated as a potential antioxidant (28), nephroprotective (29) and hepatoprotective (30).

Understanding the relation between structure and reactivity or biological activity has been a major goal in Quantitative Structure–Activity Relationship (QSAR) studies. The identification of structural motifs or descriptors that related to activity and the topological arrangement used to define a pharmacophore by substructure searching. A number of molecular parameters have been calculated using computational methods to identify the ones which may be related to activity (31). Among this parameters are molecular volume, surface area, shape indices, refractivity, dipole moments, polarizability, global and local hardness (32, 33), hydration energy, octanol-water partition coefficient (log P) (32), and quantum mechanical descriptors such as: electron density maps (33-35), frontier orbital HOMO-LUMO, population analysis Mülliken (36) and Fukui functions (33). These parameters used in correlations with active and inactive compounds against a determined biological target, to obtain a QSAR equation.

The present study shows the antiviral action of naringenin and hesperetin on YFV17D replication, and studies a number of molecular parameters using electronic structure calculations at semi-empirical level PM3 in order to understand the possible relationship to the biological activity.

Materials and methods

Cells, virus and compounds. Vero cells (ATTC® CCL-81™) were cultured in growth medium [Minimum Essential Medium (MEM), 8% inactive Fetal Bovine Serum (FBS), 1% glutamine, 1% non-essentials amino acids and 1% gentamicine] and maintained at 37°C in a 5% CO2 atmosphere. Lyophilized YFV17D stock suspended in 1 mL of MEM. Naringenin, hesperetin and their glycoside forms naringin and hesperidin (*Sigma-Aldrich Chemicals*) were dissolved in 1 mL of maintenance medium [MEM, 1% inactive Fetal Bovine Serum (FBS), 1% glutamine, 1% non-essentials amino acids and 1% gentamicine] at 10% dimethylsulphoxide (37) to obtain a final concentration of 1 mg/mL. MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide, *Sigma Chemical Company*) was used for cytotoxic assay.

Cytotoxic assay. A modified colorimetric assay was performed to determine the effect of naringenin, hesperetin, and their glycosides forms in Vero cells (19, 38). The following criteria were employed to determine the type of effect of these compounds over cells: cellular viability between 80 and 120% was considered innocuous, lower than 80% cytotoxic and higher than 120% proliferative (19). Vero cells 100 µL ($4x10^5$ cell/mL) were grown to confluence in a 96-well plate during 24h and then incubated with 100µL twofold serial dilutions of each compound in maintenance medium. All compounds were assayed at

25 μg/mL-0,005 μg/mL concentration range, but presented in μM: hesperidin (0.003 $\mu M - 0.041\ \mu M$), hesperetin (0.005 $\mu M - 0.083\ \mu M$), naringin (0.003 μM $- 0.043$ μM), and naringenin (0.006 $\mu M - 0.092\ \mu M$), mock-treated cells were included in each plate. After 24, 48, 72, 96 and 120 h, supernatants were removed and 10 μL of MTT (1 μg/mL) was added and incubated for 3 h, followed by the addition of 100 μL of lysis buffer (20% SDS, 50% N,N-dimethylformamide). Plates read at 570 nm after 18 h of incubation in the same conditions. Experiments were performed in quadruplicate and the cellular viability was calculated as follow:

% Cellular viability =100 – [(A – B)/ A] x 100, with A= Optical Density (OD) of mock treated cells (corresponding to cells incubated with medium), and B= OD treated cells.

Flavanones effects on viral replication. To evaluate the compounds effect on YFV replication, a modified plaque inhibition assay was carried out (39). 1mL of Vero cells (2,5x105 cel/mL) were grown to confluence in 24 well plates and maintained at 37 ºC in a 5% CO2 atmosphere. After 24 h media was removed and 120 μL of YFV17D (approximately 40 UFP/well) and 50 μL of compounds at the same range of concentrations used in the cytotoxic assay were added. After 2 hours of incubation at 37 ºC, 5% CO2, 500 μL of the overlay gel (50% MEM 2X; 1% FBS, 1% glutamine; 0.1% gentamicine; 0.075% NaHCO3; 0.6% carboximethyl cellulose) was added in each well and incubated during 5 days. Untreated cells were set as cellular and control virus. Cells were stained for 20 min, prior removal of the medium, with Naphtol Blue Black [0.05 g Naphtol Black Blue (*Sigma-Aldrich Chemical*) 0.68 g sodium acetate; 3 mL glacial acetic acid 99.8% and 47 mL of bidestiladed water], washed three times with running water and the viral plaques counted. All the experiments were performed in triplicate and the inhibition of plaque formation by flavanones was calculated as follow:

% Inhibition = [(A-B)/A] x 100, with A = number of average plaques in control, and B = number of average plaques in treated cells.

The inhibition data were plotted as dose-effect curves, from which the 50% effective doses (ED_{50}) were obtained by regression curve (19).

Inhibition mechanisms of naringenin and hesperetin. To determine if the inhibitory effect of flavanones on YFV17D infections is at cellular or viral level three conditions were tested. All essays were performed in triplicate for at least two independent experiments and the plaque numbers averaged: ***a)*** *Virus entrance effect*: Monolayers of Vero cells were seeded on 24 wells plates and infected with 120 μL of YFV17D (approximately 40 UFP/well) at 37 ºC in presence and absence of hesperetin and naringenin at their ED_{50} (50 μL of each one). After 0, 1, 2 and 3 hours post infection (hpi), extracellular virus were inactivated using 500 μl of acid glycine (8 g of NaCl, 0.38 g of KCl, 0.1 g of MgCl2 6H2O, 0.1 g of CaCl2 · 2H2O and 7.5 g of glycine, pH adjusted to 3 with HCl) (39) during 1 min at room temperature. Monolayers were washed with PBS (Phosphate Buffer Saline, pH 7), overlaid and incubated during 5 more days and stained as before. The number of plaques formed on cells without acid glycine treatment was considered as 100% (PBS control). ***b)*** *Virucidal effect*: Monolayers of Vero cells were seeded on 24 wells plates and infected with 120 μL of YFV17D (approximately 40 UFP/well) which was previously incubated with 50 μl hesperetin or naringenin at their ED_{50} during 1 h at 37 ºC; mocked treated cells and viral control were set for each condition. After 2 h of incubation extracellular viruses were eliminated by acid treatment with glycine, added overlay gel followed by incubation of cells monolayers and stained as previously described. ***c)*** *Antiviral effect*: Monolayers of Vero cells were seeded on 24 wells plates and infected with 120 μL of YFV17D (approximately 40 UFP/well) during 2 h at 37 ºC. Cells were treated with acid glycine and incubated with 50 μl hesperetin or naringenin (ED_{50}). After 1, 3 and 6 hours of incubation, monolayers were washed with PBS to remove the compounds. Infected monolayers were overlaid, incubated during 5 days and stained as previously described.

Computational methods and models. Molecular dynamics simulations were performed to obtain energetically accessible conformations of the flavanones naringenin, hesperetin and their glycosides naringin and hesperidin. The MD simulations were carried out for 5 ps at 300 K in vacuum using MM+ force field implemented in Hyperchem 7.5 (36). Selected low energy conformations were further optimized employing semi-empirical model Hamiltonian PM3 and these conformations were employed to calculate a number of molecular parameters including frontier orbital energies, HOMO-LUMO gap, hydration energy, log P, refractivity, polarizability, surface area, and electrostatic potential maps.

Statistical analysis. Kruskal-Wallis test were used for statistically analysis.

Results

Cytotoxicity evaluation of Flavanones over Vero cells. The effects of naringenin, hesperetin and their aglycones naringin and hesperidin on Vero cells were determined using the MTT colorimetric assay. The four compounds were innocuous in the range of assayed concentration. Figure 1 shows that the percentage of cellular viability obtained after 24h of incubation were between 80 and 120% (33) and same results were obtained at 48, 72, 96 and 120 h (data not showed), so the same concentrations were used in the following experiments.

Flavanones effect on YFV17D replication. Flavanones effect on yellow fever virus infection was determined by a modified plaque inhibition assay. In the range of concentrations evaluated, the glycosylated flavanones did not reduce the number of viral plaques obtained after infection with YFV17D in Vero cells (figure 2a). However, the aglycone naringenin and hesperetin inhibited replications up to

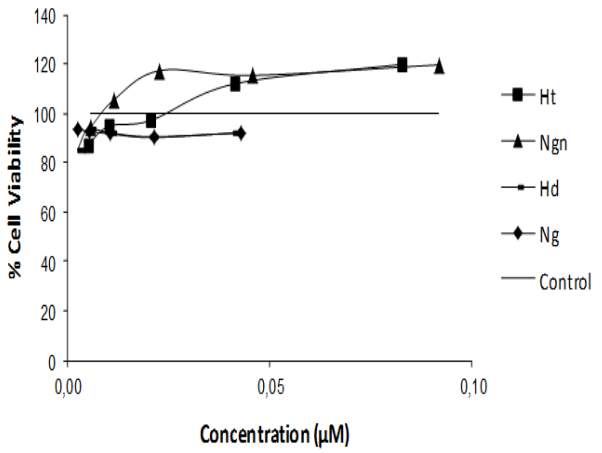

Figure 1. Viability of Vero cells in presence of hesperidin (Hd), hesperetin (Ht), naringin (Ng) and naringenin (Ngn) after 24 h of incubation

85.51% (±1.25) and 100% (±0) respectively at the highest concentration employed (0.092 µM for naringenin and 0.083 µM for hesperetin, p = 0.003). At the lowest concentration used, hesperetin (0.003 µM, 46.38% (±6.11)) was almost two times less effective than naringenin (0.004 µM, 85.51% (±6.64)) .

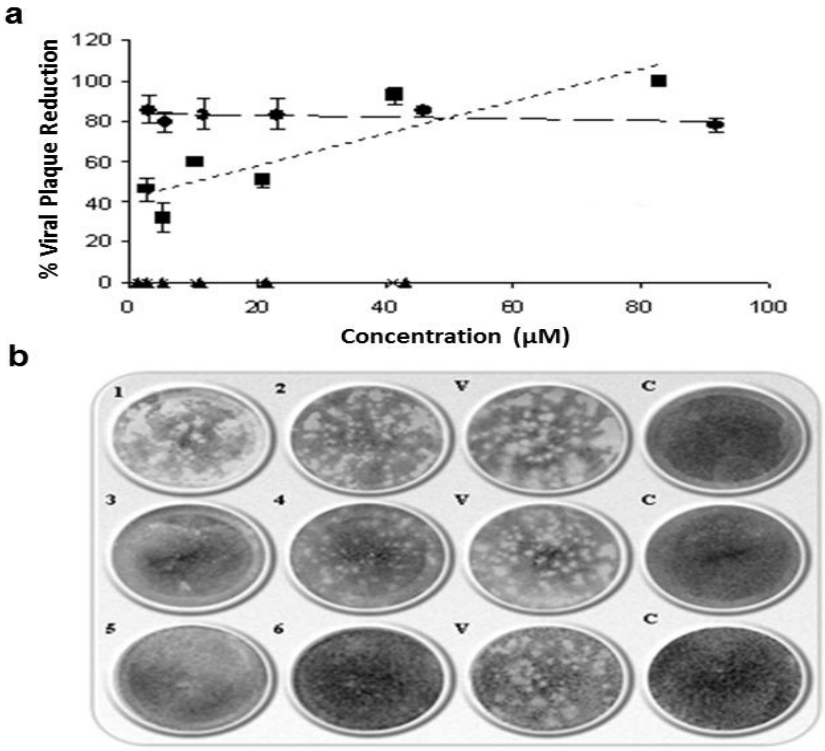

Figure 2. 2a. Effect of different concentrations of hesperetin (■ Ht), naringenin (▲ Ngn), hesperidin (- Hd) and naringin (♦ Ng) on the infectivity of YFV17D measured by a plaque inhibition assay. **p*= 0.003. **2b.** Plaque inhibition assay: Vero cells monolayers were infected with YFV17D and treated with (1, 2) naringin at 0.043 µM and 0.001 µM; (3, 4) hesperetin at 0.083 µM and 0.003 µM; (5, 6) naringenin at 0.092 µM and 0.004 µM. V and C corresponds to viral and cellular controls.

Hesperetin induced a dose-dependent inhibition of infectious titer from 40 to 100% over the concentration range used. The lack of effect of glycoside naringin on virus replication was shown on figure 2b, where the number of plaques obtained at the highest and lowest concentration was similar to the number of plaques in the viral control well. Similar results were obtained with hesperidin (data not showed). Naringenin and hesperetin were able to reduce the number and size of the viral plaques (figure 2b) and their ED_{50} was 0.0013 µM and 0.01 µM respectively.

Inhibition mechanism of naringenin and hesperetin. To evaluate if the inhibitory mechanism of flavanones on YFV17D infections in Vero cells is virucidal or antiviral (acting during or after viral entrance) naringenin and hesperetin, at their ED_{50} (0.0013 µM and 0.01 µM respectively) were: a) added during viral entrance into the cell, b) incubated with the virus before infecting cells and c) added to the cells after virus entrance. During viral entrance assay (a) 80% of the virus has entered the cell after 2 h of infection, but in presence of naringenin and hesperetin this entrance was reduced at 48.58% and 55.2% respectively ($p \leq 0.05$). However, after 3 hpi, the entrance reduction to cell was similar but only naringenin had a statistically effect significance (p = 0.0463) (figure 3). The infection reduction obtained in the virucidal assay (b) with both flavanones was 31% and not 50% as expected, because the experiments were carried out with their ED50 (figure 4a) but the results were not statically significant (p = 0.1717) and there were no changes on plaque morphology (figure 4b) as seen on the experiments where the compounds were present throughout the assay (figure 2b). When naringenin and hesperetin were added after virus entrance (c) there were no changes in the amount of viral plaques obtained with or without flavanone, but both compounds were capable of reduce the plaque size and the effect were proportional to the time of incubation (data not showed).

Molecular modeling studies. To understand the conformational flexibility of the molecules under study and rationalize the activity results obtained in terms of the molecular parameters that may be involved in such activity, molecular modeling of the four structures were carried out. The two glycosides forms did not have any effect on YFV replication, thus, it was important to relate which molecular properties are changed compared to the aglycone forms. Observation of the electronic properties of the glycosides and aglycones revealed important

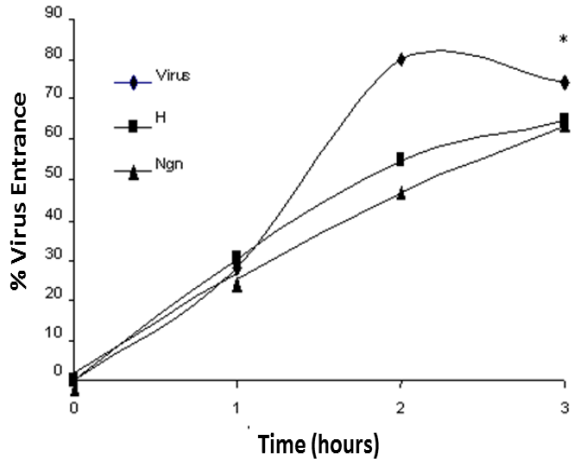

Figure 3. Kinetics of YFV17D entry into Vero cells (♦) or in presence of hesperetin (■) or naringenin (▲) * $p < 0.05$. Results shown as a percentage of plaque formation when compared with controls in which acid glycine buffer substituted for PBS.

differences in the electron density maps. In the aglycone the electron density distributed in rings A and B and the hydroxyl groups with some density on the oxygen atoms in ring C (figure 5a), while in the glycosides, the electron density shifted to the glycoside part; this could be a reason for the lack of viral replication inhibition. Molecular area and volume were also changed; this can be of importance if the

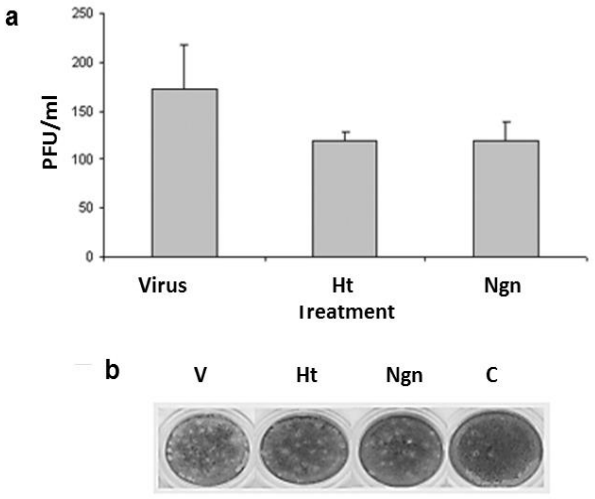

Figure 4. 4a. Virucidal effect of hesperetin and naringenin on YFV17D infections. **4b.** Morpholgy of viral plaque after treatment with hesperetin (Ht) and naringenin (Ngn). V corresponds to cells incubated only with virus (virus control) and C corresponds to cells incubated only with medium (cell control).

steric factor is involved on the entrance of the viral particle to the cell.

The lower energy conformations of the aglycones are close to planar with regards to rings A, C while ring B is almost perpendicular to A-C. In the glycosides, the sugar part rotated out of the plane defined by A-C rings. Frontier orbitals do not show significant changes from flavanones to their glycosides. In the case of naringenin, the HOMO is mostly in ring A, and LUMO in ring A, and C10-C4 bond and carbonyl moiety in ring C. The HOMO and LUMO orbitals of hesperetin were localized in rings A and B (figure 5b).

For the respective glycosides, naringin HOMO and LUMO orbital remains in the same rings with no contribution from the glycoside. However, in hesperidin HOMO is mostly in ring B while LUMO orbital is in the A-C system. HOMO-LUMO energies and HOMO-LUMO energy gaps are similar in all compounds (Table 1). The log P parameter, which related to a partition coefficient octanol/water, may be of importance for biological activity. Log P is negative for flavanones and their glycosides are more negative. The hydration energy is also negative for all compounds with higher values for the glycosides. Dipole moments are similar for naringenin and hesperetin. Lower values are found for the naringin and even lower for hesperidin. Polarizability is also similar in the flavanones and significantly bigger for their glycoside counterparts.

Discussion

The flavanones naringenin and hesperetin are ubiquitous plant secondary metabolites present in citric fruits that had demonstrated to possess anti-oxidant, anti-inflammatory and anti-carcinogenic properties (28, 40). It has also been reported that both flavanones reduce the activity and the expression of the microsomal triglyceride transfer protein and the acyl-coenzyme A cholesterol acyltransferase 2, which affect the Apo B secretion (26) that is important for HCV budding from human hepatoma cell line (26, 41). Naringenin had demonstrated inhibition of virion assembly (42) and more recently docking studies of HCV nonstructural protein (NS2) protease as targets showed that naringenin is potential inhibitors of the virus replications (43).

In our studies, none of the flavanones is citotoxic to Vero cells as has been described for others cells types (44, 45). Unlike the aglycones naringenin and hesperetin, the glycoside forms (naringin and hesperidin) did not inhibit the YFV17D replication, implying that the presence of the rutinoside moiety prevents the antiviral activity. Naringenin was approximately tenfold more effective than hesperetin inhibiting viral replication as shown by their ED50 (0.0013 ?M naringenin and 0.01 ?M hesperetin). A similar effect was observed in infections with other viruses, like the neurovirulent strain (NSV) of SINV, where glycosides forms has no inhibitory activity being naringenin the most effective inhibitor of the viral replication (19). In addition, our results indicate that in YFV17D infections both flavanones are able to reduce the number and size of viral plaque, therefore we evaluated if their effect was antiviral or virucidal and at which stage of the viral replication.

During viral entrance, naringenin and hesperetin inhibited the process, being naringenin the

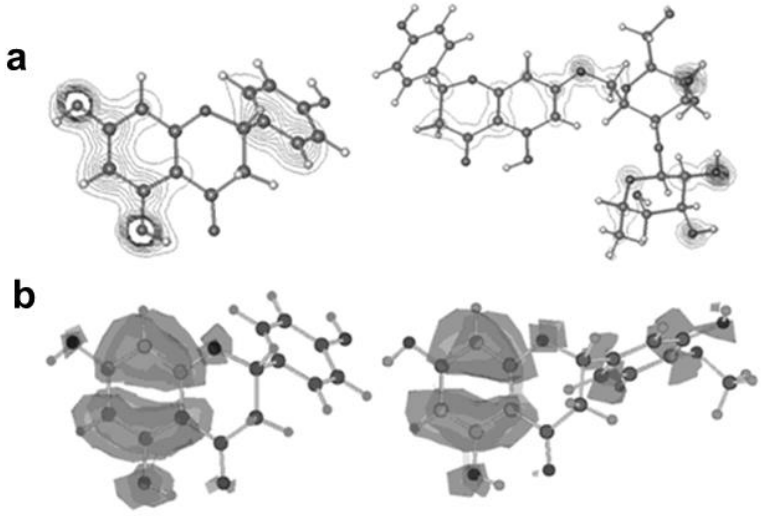

Figure 5. 5a. Charge density maps of naringenin (left) and its glycoside naringin (rigth). **5b.** HOMO of flavanones: naringenin (left) and hesperetin (right).

Table 1. Molecular parameters for flavanones and their glycosides

	Ehid kcal/mol	logP	refract ^{A3}	μ (Debye)	polariz A^3	Ehomo ev	Elumo ev	ΔEhomo-lumo ev
Naringenin	-23.25	-1.56	78.26	1.974	27.46	-9.26411	-0.58076	-8.68335
Naringin	-37.73	-3.73	83.81	1.663	55.5	-9.12609	-0.48687	-8.64003
Hesperetin	-22.75	-2.56	84.63	1.833	29.93	-9.28328	-0.65512	-8.62816
Hesperidin	-40.34	-4.45	147.93	0.4034	56.14	-8.89885	-0.57856	-8.32029

most effective (55% of inhibition, $p < 0.05$). Inhibition of viral entry by flavonoids has been reported for baicalein and epigallocatechin-3-gallate in HIV (46) and HCV (47) infections respectively. The reduction of viral entrance up to 3 hours (~60%) taken together with plaque size drecrease obtained when flavonoids were added after virus entrance, suggest two different inhibition mechanism as it has been reported for other flavonoids in HCV *in vitro* infection (45, 48).

Several compounds within a series of derivatives of thiazoles targeting the envelope protein were shown to have potent activity against YFV in a cell-based assay (49) not naringenin or hesperetin showed stadistically effect on this YFV17D glycoprotein E. Natural products demonstrate some antiviral activity against YFV. The venom component sPLA2s from the rattlesnake *Crotalus durissus terrificus* showed to be highly active against YFV and DENV in Vero Cell E6, although this activity was primarily restricted to pretreatment/inactivation of the virus (50) contrary to the effect reported for us with flavanones.

Both flavanones did not inhibit significantly viral replication when virus and compounds were incubate together prior infecting cells. On the other hand, when compounds were added after viral entrance, naringenin and hesperetin reduced the plaque size in a time-depended manner, indicating that both aglycones possess antiviral activity over YFV17D. It has been reported that morphology changes on viral plaque are present when an aspartic acid is substituted by glycine at 360 position on E glycoprotein of YFV17D (51). Also is known that naringenin is capable of regulate RNA levels into the cell (52, 53). However, in DENV-2 infections it has been reported that naringenin is capable of reduce the foci unit formation and also decrease the viral RNA only when added directly to the virus suspension before infecting cells, but have not antiviral activity when added to cells before or after viral entrance (54). These results might suggest that the mechanism employed by naringenin to inhibit viral replication would be different between flaviviruses.

Analysis of shared and unshared features in the two flavanones can give some insight of the molecular properties related to the biological activity. Naringenin is ten times more effective than hesperetin inhibiting YFV17D replication, has a smaller molecular volume, lower refractivity and slightly higher dipole moment. Also, the Log P = -1.56 obtained with naringenin is ten times higher than hesperetin (Log P = -2.56) (Table 1) indicating that it is less hydrophilic and probably more effective passing through membranes. Another important difference is the localization of the HOMO orbital. In the case of naringenin, this orbital localized, with contributions of atoms in ring A and oxygen 1 in ring C. For hesperetin however, the HOMO is more spread with contributions of atoms in ring A, ring B and oxygen 1 in ring C (figure 5). The localization of the HOMO therefore appears to be important and possibly related to electron-donor interactions in the mechanism of action of the drug on the virus replication but further studies needed in a homologous sufficiently large series of compounds to better understand this relationship.

Flavivirus life cycle occurs in a membranous system where virions binds to cellular receptor localized on the surface membrane of host cells and are internalized by endocytosis, followed by viral replication and assembly. The last replication steps occur in the endoplasmic reticulum and Golgi network (56, 57) so compounds that could interact with membranes would be capable of inhibit or modify viral replication at different levels. Recently it has been reported that phenothiazines, a small tricyclic compounds that resemble the flavonoid main structure, is able to inhibit HCV entry increasing the fluidity of cellular membranes by intercalating into cholesterol-rich domains which also increase the energy barrier needed for virus-host fusion (58). Also, using a CHIKV replicon system the expression of viral RNA levels were reduce in presence of naringenin (20). In the same study, authors screened several compounds, using a different replicon system, and reported that naringenin was capable of inhibit the viral entrance of Semliki Forest virus. The molecular parameters of naringenin and hesperetin make them

good candidates as antivirals compounds due to its solubility and size could easily diffuse through membranes and would be able to access to cytoplasm or any other cellular compartment.

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