Anti-Sindbis Activity of Flavanones Hesperetin and Naringenin

Adriana Paredes,[a] Maríaelena Alzuru, Jeannette Mendez,[b] and Morella Rodríguez-Ortega*,[a]

[a] *Biomedical Institute, Venezuelan Central University; PO BOX 4043, Caracas 1010A, Venezuela: and* [b] *Laboratory of Natural Products, Venezuelan Central University; PO BOX 4043, Caracas 1010A, Venezuela.*

Received January 10, 2002; accepted October 1, 2002

The effect of hesperetin, naringenin and its glycoside form on the Sindbis neurovirulent strain (NSV) replication *in vitro* was studied. All flavanones tested were not cytotoxic on Baby Hamster cells 21 clone 15 (BHK-21). Antiviral effect was evaluated by a colorimetric assay using MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide) and by plaque reduction assay. Hesperetin and naringenin had inhibitory activity on NSV infection. The 50% inhibitory doses ($ID_{50\%}$) of both compounds were 20.5 and 14.9 μg/ml respectively, as established by plaque assay. However their glycosides, hesperidin and naringin did not have inhibitory activity. Implying that the presence of rutinose moiety of flavanones blocks the antiviral effect. Oxygenation on the 3′ positions at the B rings on the hesperetin skeleton decrease the anti viral activity at 25 μg/ml.

Key words flavanone, hesperetin, naringenin, antiviral, sindbis virus

Sindbis virus is a positive single stranded RNA virus of the Togaviridae family. Wild type and a neurovirulent strain, NSV, have provided a valuable model system for studying alphavirus induced encephalomyelitis in mice.[1—3] In recent years, there has been a growing interest in search for antiviral substances with high efficacy, low toxicity and minor side effects, one approach is the search for viral inhibitors of plant origin. Flavanones, also called citrus flavonoids, possess many biological activities,[4] previous studies have shown its antiviral activity, hesperetin was found to inhibit the replication of herpes simplex virus type 1, poliovirus type 1, parainfluenza virus type 3 and influenza.[5,6] Hesperidin and Naringin had inhibitory activity on rotavirus infection.[7] Here, we report the effects of hesperetin, naringenin and its glycosides on BHK-21 clone 15 cells, as well as their inhibitory effect on the replication of a neuro-adapted strain of Sindbis (NSV).

MATERIALS AND METHODS

Chemicals The four flavanones studied were hesperidin, naringin and its aglycones hesperetin and naringenin, purchased from Sigma Chemical, St. Louis, Mo, U.S.A. A stock solution of each compound was prepared by dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C.

Cells and Virus BHK-21 clone 15 cells were kindly provided by Dr. Guadalupe Guzmán (Virology Department of the Pedro Kourí Institute, Havana, Cuba). The neurovirulent strain (NSV) of Sindbis virus was donated by Dr. Dianne Griffin (Medical School, John Hopkins University, Baltimore, U.S.A.). BHK-21 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% inactivated fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamicine (growth medium). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Virus stock NSV was grown in BHK-21 cells with maintenance medium (MEM, 1% FBS, 2 mM glutamine and 50 μg/ml gentamicine) for 24 h. Viral titer was obtained by plaque assay.[8]

Cytotoxic Assay The effect of the compounds over cell viability was determined by a modified MTT assay.[9] Briefly, cells were adjusted to 4×10^4 cells/100 μl in growth medium and seeded in a 96-well plate. Cells were stabilized for 24 h and then incubated with twofold serial dilutions of each compound from 25—0.09 μg/ml in maintenance medium. After 24 h 10 μl (10 mg/ml) of MTT were added to each well; it was metabolized for 3 h, and 100 μl of the lysis buffer (20% SDS, 50% *N*,*N*-dimethylformamide) was added. Plates were read at 570 nm after 18 h.

Antiviral Activity The effect of these compounds over the mammalian cells was assayed in the same conditions as the cytotoxic assay. BHK cells were infected with NSV at a multiplicity of infection (moi) of 0.1 pfu/cell. The infections were carried out in the presence or absence of different concentrations of compound. After 24 h, the viability of the cells was determined by MTT assay as described before. In addition, plaque reduction assay were carried out.[8] Briefly, BHK-21 clone 15 at a final concentration of 2×10^5 cell was seeded in 24-wells plates and stabilized for 24 h. Viral suspension at moi 0.02 and serial dilutions of the compounds were incubated for 1 h at 37 °C, then 50 μl of each solution were added to the cells. After the 3 h of infection, cells were overlaid with 3% carboxymethylcellulose; after 24 h cells were fixed and stained with naphthol blue black, and the numbers of plaques were counted. All experiments were performed in triplicate, and each experiment was reproduced a minimum of three times. The antiviral activity of the compounds was determinate by the plaque reduction as follows:

$$\%PR = (\text{viral control} - \text{treated cells})/\text{viral control} \cdot 100$$

The inhibition data were plotted as dose-effect curves, from which the 50% inhibitory doses ($ID_{50\%}$) were obtained. Differences on the inhibitory effect between the compounds assayed were analyzed through the Student's *t* test. A $p<0.05$ value was defined as statistically significant.

RESULTS AND DISCUSSION

The four flavanones assayed over BHK-21 clone 15 were innocuous at all concentration evaluated. The flavanone glycosides did not inhibit the NSV replication by neither of the two assays employed; However, their agliconas, hesperetin and naringenin could block viral replication; showing an $ID_{50\%}$ of 15.9 and 14.5 μg/ml respectively by MTT assay and $ID_{50\%}$ of 20.5 and 14.9 μg/ml by plaque reduction assay

* To whom correspondence should be addressed. e-mail: mrodri@telcel.net.ve

Table 1. Activity of Flavanones on Sindbis Virus Infected Cells

Flavanones	Molecular weight	Substituents				$RF_{50\%}MTT$ (μg/ml)	$RF_{50\%}PA$ (μg/ml)
		R5	R7	R3′	R4′		
Hesperetin	302.3	OH	OH	OH	OCH_3	15.9	20.5
Hesperidin	610.6	OH	Rut	OH	OCH_3	Inactive	Inactive
Naringenin	272.3	OH	OH	—	OH	14.5	14.9
Naringin	580.5	OH	Rut	—	OH	Inactive	Inactive

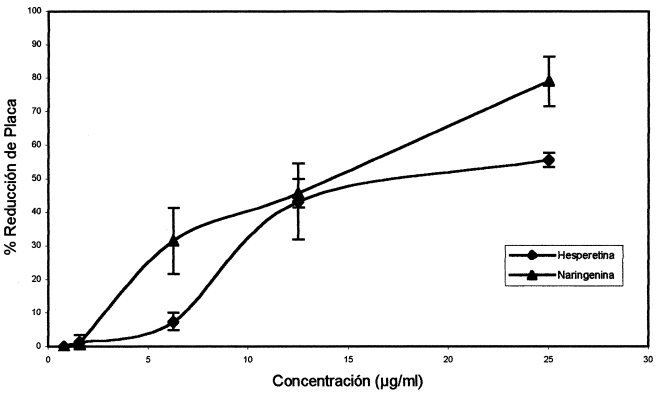

Fig. 1. Plaque Reduction Assay

Plaques were counted at each concentration and the results represented as plaque formation unit.

(Table 1). These flavonones were capable of causing a concentration–dependent decrease in plaque formation. Being, naringenin the most effective, reaching up to 80% of viral replication inhibition at 25 μg/ml; in contrast, hesperetin only inhibited the 50% at the same concentration; this differences was statistically significant ($p<0.01$). (Fig. 1). The oxigenation on the 3′ position at the B ring on the hesperetin skeleton, could be responsible for decrease the antiviral activity at this concentration (Fig. 1). Similar results were obtained using these compounds against *Helicobacter pylori*.[10] It is important to notice that the presence of the rutinoside moiety, inhibit the antiviral activity in both flavavones used. However previous report showed that this moiety is the active site against Rotavirus infection *in vitro*.[7]

Acknowledgements This work was supported by CONICIT project N° G-97000627. We thank to Miguel Marzal for the statistical analysis.

REFERENCES

1) Griffin D. E., "The Togaviridae and Flaviviridae Family," ed. by Schlesinger S., Schlesinger J. M., Plenum, New York 1986, pp. 201—210.
2) Griffin D. E., *Neurosc. Biobehav. Review*, **22**, 721—723 (1998).
3) Tucker P. C., Lee S. H., Bui N., Martinie D., Griffin D. E., *J. Virol.*, **71**, 6106—6112 (1997).
4) Pathak D., Pathak K., Singla A. K., *Fitoterapia*, **LXII**, 371—389 (1991).
5) Kim H. K., Jeon W. K., Ko B. S., *Planta Med.*, **67**, 548—549 (2001).
6) Kaul T. N., Middleton E., Jr., Ogra P. L., *J. Med. Virol.*, **15**, 71—79 (1985).
7) Bae E.-A., Han M. J., Lee M., Kim D.-H., *Biol. Pharm. Bull.*, **23**, 1122—1124 (2000).
8) Kurokawa M., Nagasaka K., Hirabayashi T., Uyama S., Sato H., Kageyama T., Kadota S., Ohyama H., Hozumi T., Namba T., Shiraki K., *Antiviral Research*, **27**, 19—37 (1995).
9) Shearman M. S., Ian Ragan C., Iversen L. L., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 1470—1474 (1994).
10) Bae E.-A., Han M. J., Kim D.-H., *Planta Med.*, **65**, 442—443 (1999).