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Determination of Non-Toxic and Subtoxic Concentrations of Potential Antiviral Natural Anthraquinones

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***SUMMARY*. Anthraquinones-rich extracts of *Heterophyllaea pustulata* Hook f. *(Rubiaceae)* exhibited *in vitro* antiviral activity against Herpes Simplex Virus Type I, from which several anthraquinones (AQs) were isolated and identified. The Maximum Non-Cytotoxic Concentration (MNCC), the subtoxic concentration (SubTC), and the CC50 of each AQ were determined on a mammalian eukaryotic cell line (Vero cells) by means of Neutral Red uptake assay; the cytopathic effect was simultaneously evaluated by optical microscopy. The range of concentrations where each AQ did not exhibit cytotoxicity was established, which is limited by the MNCC: rubiadin 1-methyl ether, damnacanthol and pustuline were found to be markedly less cytotoxic. To the remaining AQs, we could estimate a SubTC (about 10 μg/mL) that assures 80 % cellular viability. Therefore, we determined a concentration range which could be used to evaluate the antiviral effect of each AQ since it ensures the viability of the host cell.**

INTRODUCTION

In search of new structures with potential biological activity, it is essential to evaluate by means of toxicity tests the security in its use. Among the wide variety of bioassays currently available for this purpose, the *in vitro* tests with cellular cultures (cytotoxicity assays) are largely employed [1]. They allow a rapid assessment of a high number of compounds by using small amounts of substance; they are also inexpensive and raise no ethical objections [2].

Cytotoxicity studies are particularly useful for monitoring changes at cellular structure and/or metabolic functions that could be altered by a compound. The damages produced can be detected early as morphologic changes in cells (cytopathic effects) by optical microscopy, and measured as a decrease in the cellular proliferation and/or survival by means of colorimetric assays such the Neutral Red uptake test (NR) [2-4], widely used to evaluate the cytotoxicity of microbial toxins, food additives and pharmaceuticals [5,6]. A mammalian eukaryotic cell line such as Vero cell constitutes a good option for these kinds of assays, since the combination of NR and visual morphologic tests and this cellular line allows determining the non-cytotoxic and/or subtoxic concentration range [7], which can be used to evaluate the *in vitro* antiviral effect of a compound [8].

Previously, we demonstrated that the extracts obtained from aerial parts of *Heterophyllaea pustulata* Hook f. *(Rubiaceae)* enriched in AQs exhibit *in vitro* antiviral activity against HSV-I [9]. The chemical investigation of these bioactive extracts showed that they contain 9,10-anthraquinone aglycones (AQs): soranjidiol (**1**), soranjidiol 1-methyl ether (**2**), rubiadin (**3**), rubiadin 1-methyl ether (**4**), damnacanthal (**5**), damnacanthol (**6**), 2-hydroxy-3-methyl anthraquinone (**7**), heterophylline (**8**), pustuline (**9**) and 5,5'-bisoranjidiol (**10**) (Fig. 1) [9,10]. These preliminary results have encouraged us to assess the potential biological effects of each AQ iso-

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lated from this plant. Nevertheless, prior to the antiviral tests, it is necessary to evaluate the cytotoxicity of these compounds on host cells. Therefore, in this work the *in vitro* non-cytotoxic and subtoxic concentrations of each AQ were determined, as a necessary step prior to studying their antiviral effects in a concentrations range with a safety margin. In addition, bearing in mind that some AQs isolated from *H. pustulata* increased the superoxide anion production in human leukocytes [11], the influence of this factor on the viability of Vero cells was analyzed.

MATERIALS AND METHODS

Samples

Only nine of the ten AQs isolated and purified from aerial parts of *H. pustulata* were assayed (Fig. 1) because 2-hydroxy-3-methyl anthraquinone (**7**) was obtained in trace amounts. Each AQ was identified by comparing their spectral data (UV-Vis, MS and RMN) with those reported [9,10]. The vegetal material used to obtain the AQs analyzed was collected in La Almona (Jujuy province, Argentina), and a voucher specimen was deposited at Museo Botánico de Córdoba (U.N.C.) as M.E. Lázzaro s/n, CORD 305.

Reagents

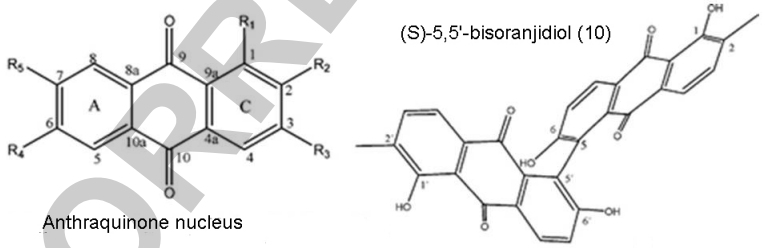
The following reagents were used: Eagle's minimum essential medium (EMEM) (Gibco), Fetal calf serum (FCS) (Natocor), L-glutamine (Calbiochem), gentamicine (Klonal), dimethyl sufoxide (DMSO) (Tetrahedron), Neutral Red (NR) (Gibco), Phosphate buffer saline (PBS), Nitroblue Tetrazolium (NBT, Sigma), sodium dodecyl sulfate (Sigma). Chloroform ($CHCl_3$) was distilled before use.

Cells

African green monkey kidney cells (*Cercopithecus aethiops,* Vero 76 ATCC CRL-587) were used. They were grown and kept alive under humid atmosphere with 5 % CO_2 at 37 °C. EMEM supplemented with 10 % FCS, 1 % L-glutamine and gentamicin (50 µg/mL) was used as growth medium, whereas EMEM plus 2 % FCS containing the same formulation as described above and 1 % DMSO was used as maintenance medium (MM).

In Vitro Toxicity Test

The cytopathic effect produced by each AQ on the morphology of Vero cells was observed by optical microscopy [12]. From a stock solution of each AQ (1 mg/mL in PBS with 1 % DMSO as co-solvent), 15 consecutive dilutions were prepared, within a range of 1 to 30-50 µg/mL according to AQ solubility. Each dilution was inoculated in duplicate on a confluent cell monolayer (2.5 ± 0.6 x 10^5 cells/mL, 48 h incubation), including cell controls (CC) that contain only MM. The cells were incubated at 37 °C during 72 h, and the development of cellular alterations such as rounding, membrane retraction, cell de-



	AQs	R_1	R_2	R_3	R_4	R_5
1	soranjidiol	OH	CH_3	H	OH	H
2	soranjidiol 1-methyl ether	OCH_3	CH_3	H	OH	H
3	rubiadin	OH	CH_3	OH	H	H
4	rubiadin 1-methyl ether	OCH_3	CH_3	OH	H	H
5	damnacanthal	OCH_3	COH	OH	H	H
6	damnacanthol	OCH_3	CH_2-OH	OH	H	H
7	2-hydroxy-3-methyl anthraquinone		OH	CH_3		
8	heterophylline	OH	CH_3	H	OH	OCH_3
9	pustuline	H	OH	OCH_3	H	CH_3

Figure 1. Natural anthraquinones obtained from *Heterophyllaea pustulata*.

tachment and the presence of granules in the cytoplasm was daily observed [13].

The cellular viability depending on the concentration of each AQ was measured by means of the uptake NR assay. Each dilution was inoculated in triplicate on a confluent monolayer of cells (2.5 ± 0.6 x 10^5 cells/mL), according to the methodology described by Borenfreund & Puerner [12]. The absorbance of the NR extracted after 48 h of incubation at 37 °C was measured at 540 nm on a microplate reader (BioTek ELx800). The percentage of cellular viability was calculated by comparison with CC (100 % viability). The concentration of the compound that reduces the viable cells to 50 % (CC_{50}) was determined by regression (R^2> 0.9) from the plot of cellular viability percentage vs. AQ concentration. Maximum Non-Cytotoxic Concentration (MNCC) was defined as the maximal sample concentration showing more than 90 % viable cells and exerting no cytotoxic effect as detected by microscopic monitoring [14].

Determination of $O_2\cdot^-$ production

To evaluate the intracellular generation of $O_2\cdot^-$, the NBT bioassay adapted to a confluent monolayer of cells attached to a multiwell plate was used [15]. In this test, the yellow-colored NBT is absorbed by cells and reduced to water-insoluble Blue Formazan by the action of $O_2\cdot^-$ intracellular, in the presence and absence (basal situation) of each AQ as a trigger of the oxidative burst. The NBT was dissolved in PBS (0.1 % DMSO) at a concentration of 1 mg/mL; Vero cells grown in 24-well plate for 48 h were used (2.5 ± 0.6 x 10^5 cells/mL). Each AQ was tested in duplicate at two concentrations: 10 µg/mL and CC_{50}, following the methodology described by Choi *et al.* [16]. The absorbance of intracellular Blue Formazan was measured on a microplate reader (BioTek ELx800) at 570 nm. The $O_2\cdot^-$ production in the presence of oxidant (AQ) was expressed as an increase in absorbance compared to the basal situation.

AQ incorporation assay in Vero cells

This assay constitutes a spectrophotometric determination of intracellular content of AQs needed to stimulate $O_2\cdot^-$ production [11] and other biologic effects. Compound 1 was chosen to study the incorporation of AQs in Vero cells for two reasons: this AQ is the predominant compound in the aerial parts of *H. pustulata* and significant differences are not expected in the accumulating capacity of each AQ, since AQs tested have similar partition coefficients [17]. Thus, 1 mL of 1 in MM (2.46 x 10^{-4} M) was incubated in duplicate with 1 mL of suspended Vero cells (10^7 cells/mL) in MM at 37 °C during 30 min. Control samples (in duplicate) were processed without AQ and under the same working conditions. After incubation, samples were centrifuged at 1500g for 25 min. The supernatant (S1) was discarded. The pellet was washed twice with MM. After that, 2 mL of 2 % sodium dodecyl sulfate was added with the aim of disrupting the membranes and liberating the intracellular AQ. After centrifugation, the supernatant (S2) was partitioned with $CHCl_3$ and the absorbance of the organic phase (S3) was measured at 268 nm. The concentration of **1** in S3 was obtained from a calibration curve ($CHCl_3$), previously performed by using a concentration range of 7.5 x 10^{-5} – 2.9 x 10^{-7} M, thus obtaining an $\varepsilon_{(268\ nm)}$ = 34789.8. A Hewlett–Packard 8452A, diode array spectrophotometer was used.

Data analysis

MNCC, SubTC and CC_{50} values were graphically obtained from the dose-response curves, which have a non-lineal regression analysis (Sigmoidal Origin, R^2 > 0.9). The values were expressed as (mean ± standard error) from three independent experiments. Thus, for each concentration, 6 replicates were carried out to determine cytopathic effect, 9 replicates to quantify cell viability, 6 replicates to evaluate the intracellular $O_2\cdot^-$ production and 6 replicates to establish of intracellular content of AQs. The *t*-test (Origin) was used to assess the degree of statistical difference of MNCC, CC_{50} and the SubTC values; differences between means were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

By analyzing the plots of cellular viability percentage (% CV) *vs.* AQ concentration (Figs. 2-4), we determined the concentration range where cellular viability was equal to or higher than 90 %. Whereas **4**, **6** and **9** were notable (Fig. 2) for showing MNCC (90 % CV) values within 16 and 22 µg/mL range (Table 1), this concentration was not higher than 10 µg/mL for the other AQs (Figs. 3 and 4). Thus, the MNCC for **2**, **8** and **10** (Fig. 3) was near 10 µg/mL, and approximately 6 µg/mL for **1**, **3** and **5** (Fig. 4).

Similar results were obtained by analyzing the CC_{50} of each AQ (Table 1), which established that **4** and **6** were less cytotoxic, followed, in increasing order of cytotoxicity, by **9**,

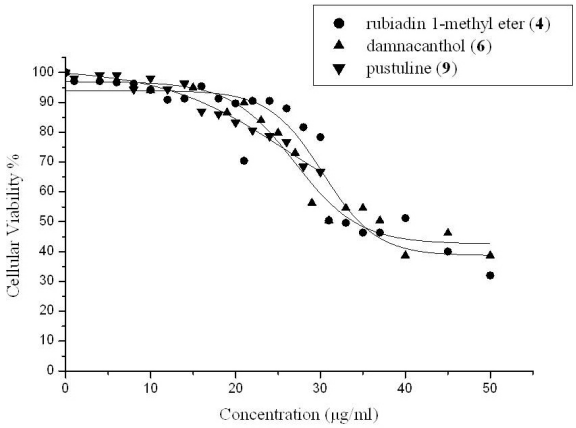


Figure 2. Cytotoxicity of rubiadin 1-methyl ether (**4**), damnacanthol (**6**) and pustuline (**9**) on Vero cells (cellular viability percentages *vs.* concentrations).

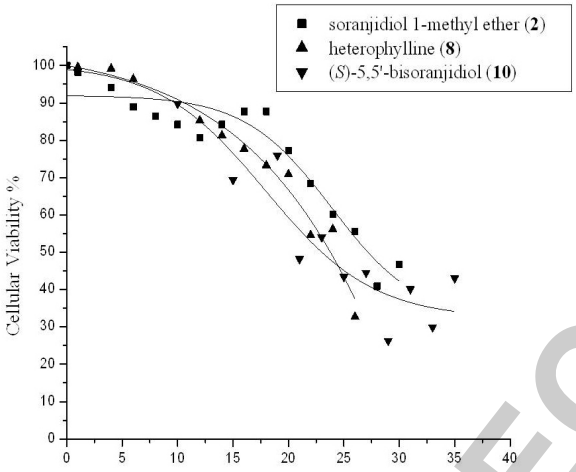


Figure 3. Cytotoxicity of soranjidiol 1-methyl ether (**2**), heterophylline (**8**) and (S)-5,5'-bisoranjidiol (**10**) Vero cells (cellular viability percentages *vs.* concentrations).

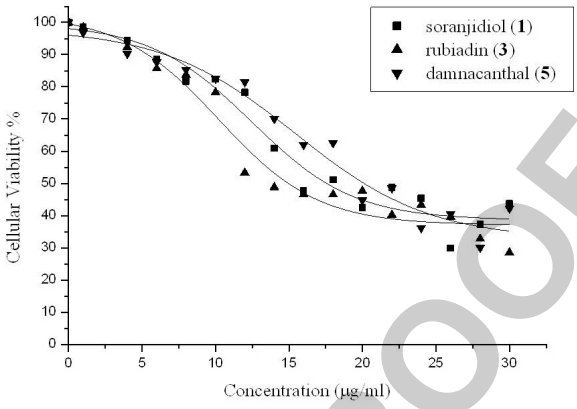


Figure 4. Cytotoxicity of soranjidiol (**1**), rubiadin (**3**) and damnacanthal (**5**) on Vero cells (cellular viability percentages *vs.* concentrations).

2, **8**, **10**, **5**, **1**, **3**. The CC_{50} of **9** was not estimated because concentrations higher than 30 µg/mL could not be tested due to solubility problems; however, its MNCC was determined. In addition, a subtoxic concentration (SubTC) was estimated for all AQs tested, defined as the concentration that causes 10-20 % cellular death [18] and produces slight morphologic changes observed by microscopy (less than 20 % of swollen and rounded cells, with cytoplasmic inclusions, slight vacuolization, and the nuclear membrane remaining intact). The statistical treatment of MNCC, CC_{50} and SubTC is shown in Table 1.

Comparing the cytotoxic activity of compounds that differ in the substituent at position-1 of AQ nucleus: soranjidiol (**1**) with soranjidiol 1-methyl ether (**2**) (Fig. 5) and rubiadin (**3**) with rubiadin 1-methyl ether (**4**) (Fig. 6), we can ob-

AQs	MNCC [a] (µg/mL)	CC_{50} [b] (µg/mL)	SubTC [c] (µg/mL)
rubiadin 1-methyl ether (**4**)	22.2 ± 0.4	34.4 ± 0.2	26.8 ± 0.1
damnacanthol (**6**)	19.6 ± 0.2	33.7 ± 0.2	23.9 ± 0.1
pustuline (**9**)	16.1 ± 0.3	nc	22.3 ± 0.1
soranjidiol 1-methyl ether (**2**)	10.5 ± 0.3	27.1 ± 0.2	18.4 ± 0.1
heterophylline (**8**)	9.7 ± 0.2	23.69 ± 0.04	15.64 ± 0.04
(S)- 5,5'-bisoranjidiol (**10**)	9.5 ± 0.2	22.7 ± 0.1	13.9 ± 0.2
damnacanthal (**5**)	6.7 ± 0.2	20.1 ± 0.1	11.2 ± 0.1
soranjidiol (**1**)	6.3 ± 0.2	17.5 ± 0.2	9.9 ± 0.1
rubiadin (**3**)	5.3 ± 0.3	14.9 ± 0.2	8.2 ± 0.1

Table 1. Cytotoxic Concentration to 50 % (CC_{50}), Maximum Non-Cytotoxic Concentration (MNCC) and Subtoxic Concentration (SubTC) for each AQ tested on Vero cells. nc: not calculated. Results given as mean ± SD, n=3. Differences between means were considered significant at $p < 0.05$. [a] The values are significantly different ($p < 0.05$), except **8** respect to **10**. [b] The values are significantly different ($p < 0.05$), except **4** respect to **6**. [c] The values are significantly different ($p < 0.05$).

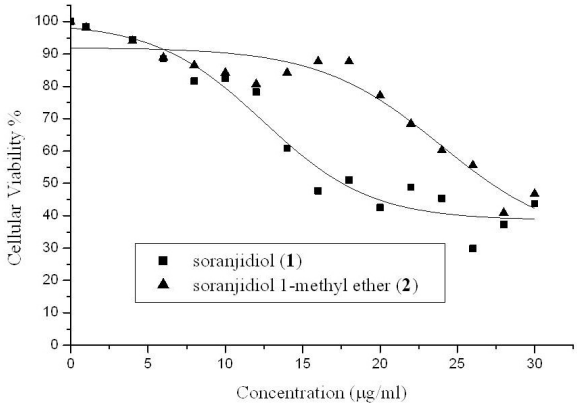


Figure 5. Cytotoxicity of soranjidiol (**1**) and soranjidiol 1-methyl ether (**2**) on Vero cells (cellular viability percentages *vs.* concentrations).

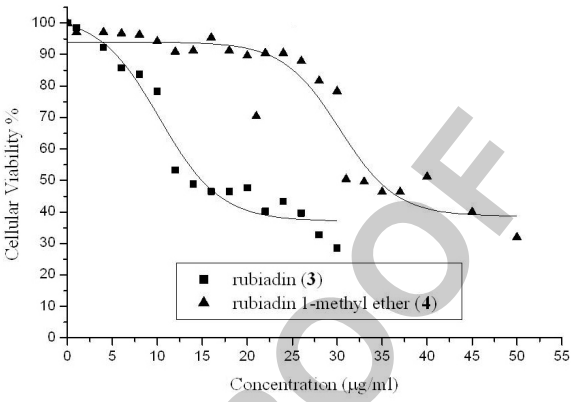


Figure 6. Cytotoxicity of rubiadin (**3**) and rubiadin 1-methyl ether (**4**) (cellular viability percentages *vs.* concentrations).

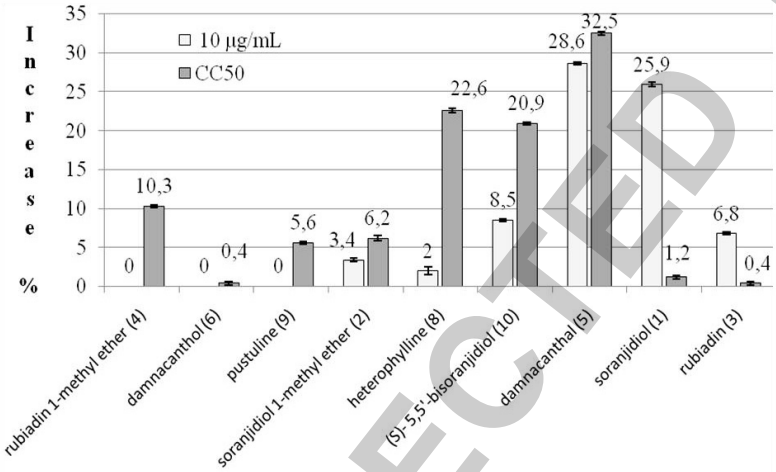


Figure 7. NBT assay. Increase in percentage of $O2^{*-}$ in Vero cells with respect to basal situation, produced by two different concentrations of every AQ.

serve that the presence of a methoxyl group ($-OCH_3$) instead of a hydroxyl group (–OH) in position-1 confers a low cytotoxicity to the AQ, reflected in an increase in the values of CC_{50} and MNCC for the methoxylated derivatives (Table 1).

In previous studies we demonstrated that some of the AQs tested had the ability to increase the $O_2{\cdot}^-$ production in human leukocytes [11]. Considering that this effect could generate cytotoxic activity of AQs on Vero cells, the ability of each AQ to produce this reactive species of oxygen (ROS) and its relation to the cytotoxic effect were evaluated. To this aim, the NBT reduction bioassay was carried out under aerobic conditions and each AQ was assessed at 10 µg/mL, representing a non-cytotoxic or subtoxic concentration dependent on the compound. We observed that **4**, **6** and **9** did not increase the production of $O_2{\cdot}^-$ at 10 µg/mL (Fig. 7), which is a non-cytotoxic concentration for these AQs since it assures approximately 95 % CV (Fig. 2) without evidencing any cytopathic effect. For the other AQs, this amount corresponds to a subtoxic value, which increased the generation of $O_2{\cdot}^-$ (Fig. 7). It was noted that those compounds that produced a large increase in the $O_2{\cdot}^-$ generation at 10 µg/mL (**1** and **5**, Fig. 7) exhibited a low CV % (about 80 %, Fig. 4) with increased cell damage. However, those AQs that at same concentration showed a low increase in the $O_2{\cdot}^-$ production (**2**, **8**, **10** and **3**), revealed a high CV % (between 86 and 90 %, Figures 2 and 3), except for **3**. In general, we might conclude that an increased production of $O_2{\cdot}^-$ causes a great cytopathic effect as observed by microscopy, with a concomitant decrease in cellular viability. Compound **3** is excepted from this behavior, producing a significant cytopathic effect at 10 µg/mL, which results in a significant decrease in CV (30 %, Fig. 4), despite having a low production of $O_2{\cdot}^-$ (Fig. 7). We may there-

fore assume the presence of another mechanism in the cytotoxicity of this compound. In addition, when each AQ was tested at their CC_{50}, the increase in $O_2^{\cdot -}$ production was not the same in all AQs. Therefore, the intracellular increase of this ROS would not be the sole responsible for the loss of cellular viability at CC_{50}.

Since we are measuring the intracellular production of $O_2^{\cdot -}$, we have established that **1** enters Vero cells, 29 ± 3 % with respect to the initial concentration after 30 min incubation. Although only a single AQ was tested, no significant differences are expected in the rate of incorporation for the other AQs since they all have similar partition coefficients [19].

CONCLUSIONS

This work allowed us to establish the concentration range where each AQ exhibits low or no cytotoxic effect. These concentrations were therefore used in another study for testing *in vitro* antibacterial properties [19]. In addition, many of the AQs assayed in this work, especially damnacanthal, and rubiadin 1-methyl ether, have shown to exhibit *in vitro* antimicrobial effects [20-21], without evaluating the potential cytotoxicity on a mammalian eukaryotic cell line at the concentrations tested. Therefore, the results obtained in this study can be used as a parameter to compare cytotoxicity in relation to the antimicrobial effects reported in the literature.

From the nine AQs tested, we identified three derivatives: **4**, **6** and **9**, with low or no cytotoxicity (95 ± 5 % VC) in a concentration range limited by the MNCC (Table 1). The estimation of the subtoxic concentrations for the other AQs (**1**, **2**, **3**, **5**, **8** and **10**) allowed us to consider that a concentration of about 10 µg/mL could be used to test other biological activities, since this concentration ensured in our experiments more than 80 % CV (Table 1).

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REFERENCES

1. Fernandes, M.J.B., C. Limas, M.H. Rossi, E. Gonçales & I.C. Simoni (2005) *Braz. J. Microbiol.* **36**: 338-41.
2. Harbell, J.W., S.W. Koontz, R.W. Lewis, D. Lovell & D. Acosta (1997) *Food Chem. Toxicol.* **35**: 79-126.
3. Fotakis, G. & J.A.Timbrell (2006) *Toxicol. Let.* **160**: 171-7.
4. Carbonell, G.V., A.F. Alfieri, A.A. Alfieri, M.C. Vodotto, C.E. Levy, A.L.C. Darini *et al.* (1997) *Braz. J. Med. Biol. Res.* **30**: 1291-8.
5. Greer, C.N. & P.E. Shewen (1986) *Vet. Microbiol.* **12**: 33-42.
6. Zuang, V. (2001) *Altern. Lab. Anim.* **29**: 575-99.
7. Valdivieso-García, A., R.C. Clarke, K. Rahn, A. Durette, D.L. MacLeod & C.L. Gyles (1993) *Appl. Environm. Microbiol.* **59**: 1981-3.
8. Govorkova, E.A., G. Murt, B. Meignier, C. Taisne & R.G. Webster (1996) *J. Virol.* **70**: 5519-24.
9. Núñez Montoya, S.C., A.M. Agnese, C. Pérez, I.N. Tiraboschi & J.L. Cabrera (2003) *Phytomedicine* **10**: 569-74.
10. Núñez Montoya, S.C., A.M. Agnese & J.L.Cabrera (2006) *J. Nat. Prod.* **69**: 801-3.
11. Núñez Montoya, S.C., L.R. Comini, M. Sarmiento, C. Becerra, I. Albesa, G.A. Argüello *et al.* (2005) *J. Photochem. Photobiol. B: Biol.* **78**: 77-83.
12. Borenfreund, E. & J.A. Puerner (1985) *Toxicol. Lett.* **24**: 119-24.
13. Ooi, L.S.M., H. Wang, Ch. Luk & V.E.C. Ooi (2004) *J. Ethnopharmacol.* **94**: 117-22.
14. Liu, A., S. Shu, H. Qin, S.M.Y. Lee, Y. Wang & G. Du (2009) *Planta Med.* **75**: 337-9.
15. Rook, G.A.W., J. Steele, S. Umar & H.M. Dockrell (1985) *J. Inmonolog. Met.* **82**: 161-7.
16. Choi, H.S., J.W. Kim, Y. Cha & Ch. Kim (2006) *J. Immunoassay Immunochem.* **27**: 31-44.
17. Comini, L.R., S.C. Núñez Montoya, G.A. Argüello & J.L. Cabrera (2006) *Lat. Am. J. Pharm.* **25**: 252-5.
18. Cholewa, M., G.J. Legge, H. Weigold, G. Holan & C.J. Birch (1994) *Life Sci.* **54**: 1607-12.
19. Comini, L.R., S.C. Núñez Montoya, P.L. Páez, G.A. Argüello, I. Albesa & J.L. Cabrera (2011) *J. Photochem. Photobiol. B: Biol.* **102**: 108-14.
20. Ali, A.M., N.H. Ismail, M.M. Mackeen, L.S. Yazan, S.M. Mohamed, A.S.H. Ho *et al.* (2000) *Pharm. Biol.* **38**: 298-301.
21. Koumaglo, K., M. Gbeassor, O. Nikabu, C. de Souza & W. Werner (1992) *Planta Med.* **58**: 533-4.