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Evaluation of the toxicity of *Uncaria tomentosa* by bioassays in vitro

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Abstract

Aqueous extracts of *Uncaria tomentosa* (Willdenow ex Roemer and Schultes) DC. (Rubiaceae) ('Uña de gato'), were analyzed for the presence of toxic compounds in Chinese hamster ovary cells (CHO) and bacterial cells (*Photobacterium phosphoreum*). Toxicity was evaluated by four systems: Neutral red assay (NR), total protein content (KB), tetrazolium assay (MTT) and Microtox test. The extracts of *U. tomentosa* did not show toxicity in vitro at the concentrations tested. Testing in vitro could be a valuable tool for evaluating toxicity of medicinal plants. © 1997 Elsevier Science Ireland Ltd.

Keywords: Toxicity; *Uncaria tomentosa*; Cell culture; Luminescence

1. Introduction

The traditional use of plants for treating various illnesses is widespread. Herbs have been used as medicinals throughout history. Today herbal and health food remedies, recommended for the treatment of a variety of diseases, are experiencing increased popularity. Hundreds of natural products are available to the consumer, although some could be potentially toxic when ingested in overdoses or in combination with other medications.

Uncaria tomentosa (Willdenow ex Roemer and Schultes) DC. (Rubiaceae), popularly known as 'Uña de gato' is one of the plants used in the Amazon basin. Although it is consumed mainly for its highly anti-inflammatory properties (Aquino et al., 1991), it is increasingly consumed in developing countries for its effects as a stimulant.

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Previous studies of this plant have mainly focused on the structure, extraction and metabolic determination of the active principles (Senatore et al., 1989; Aquino et al., 1990). Aquino et al. (1989) carried out pharmacological studies on different compounds of *U. tomentosa*. Rizzi et al. (1993) investigated the antimutagenic effect of extracts of this plant. However its toxic effects have not been studied.

This paper shows the results of a study on potential toxic effects in vitro of aqueous extracts of *U. tomentosa* barks, testing different concentrations of this plant on mammalian and bacterial cells.

2. Methodology

2.1. Preparation of extracts

U. tomentosa barks were obtained from the lianas used by the ethnic groups in northern of Perú (Duke and Vasquez, 1994). The voucher specimen of the plants used in the present study was kept for record in the herbarium of the San Pablo-CEU University (USP), Madrid, Spain (voucher no. 196797). They were collected in Tarapoto (Department of San Martin, Peru) and identified by J. Albán, Professor in the Department of Ethnobotany of the Universidad Nacional Mayor de San Marcos (Lima, Peru). Aqueous extracts of *U. tomentosa* barks were prepared at a concentration of 500 mg/ml; the mixture was infused for 7–10 min in boiling distilled water and then centrifugated (1500 rpm, 15 min). The extracts were then added to the culture medium at seven concentrations (10, 20, 30, 40, 50, 75 and 100 mg/ml).

2.2. Cells

Chinese hamster ovary (CHO) cells were obtained from Flow Laboratories (McLean, VA). For each experiment, cultures were seeded from frozen stocks and maintained in Ham F-12 medium (Flow Lab.) supplemented with 10% fetal calf serum and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37°C. Three independent cultures were used for each concentration.

2.3. Cytotoxicity assays

Cells were harvested and diluted to a concentration of 2×10^4 cells/well in medium. The cell suspension was shaken and then 200 μl aliquots were added to each well of 96-well tissue culture plates, incubated overnight at 37°C. The next day, the medium was replaced by 200 μl aliquots of the solution extracts and after a 24 h exposure period, the toxic endpoints were determined.

2.3.1. Neutral red (NR) assay

The Borenfreund and Puerner (1985) method of measuring the inhibition of cell growth, which uses the vital dye neutral red, absorbed only by living cells, was employed. After a 24 h period, the test substance was replaced by 200 μl of neutral red solution (50 μg/ml) and after 3 h of in situ incubation, plates were rinsed with warmed phosphate buffered saline (PBS). Cells were destained with 200 μl of destaining solution (glacial acetic acid–96% ethanol–water; 1:50:49 v/v). The absorbance of the solution in each well was measured at 540 nm with a Microplate Reader (EL 312c).

2.3.2. Total protein content assay (KB)

The method used was that proposed by Knox et al. (1986) and consists of cell protein stain with kenacid blue. Cells were stained for 30 min with a 200 μl/well of the dye solution (0.4 g of kenacid blue in 250 ml of ethanol and 630 ml of water). Immediately before use, 12 ml of glacial acetic acid were added to each 88 ml of stain and the solution was filtered. Cells were destained twice with 200 μl of destaining solution (96% ethanol–glacial acetic acid–water; 2:1:17 v/v), the final destain was removed and 200 µl/well of desorbing solution (1 M potassium acetate in 70% ethanol) was added. The absorbance of each well was determined at 620 nm using a Microplate Reader (EL 312 c).

2.3.3. Tetrazolium (MTT) assay

It is based on inhibition of the reduction of soluble yellow MTT tetrazolium salt by chemical injury to a blue insoluble MTT formazan product of mitochondrial succinic dehydrogenase, accord-

ing to Borenfreund et al. (1988). After the 24 h exposure period, cells were rinsed with 200 μl of Ca-, Mg-free Earle medium balanced salt solution. Then 100 μl of prewarmed phenol red-free modified Eagle medium were added to each well. Cells were stained for 4 h at 37°C with 10 μl/well of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; 5 mg/ml in PBS). At the end of this period, 100 μl/well of acidified isopropanol were added to solubilize the purple formazan crystals produced. The absorbance of each well was measured at 570 nm with a Microplate Reader (EL312c).

Each experiment (NR, KB and MMT assays) was repeated at least three times and the average absorbance at each dose level was calculated and plotted as the percentage absorbance of control against dose.

2.3.4. Microtox assay

This assay measures the decrease in light output of a luminiscent bacterium, *Photobacterium phosphoreum*, as an indicator of toxicity in the bacterium (Bulich et al., 1990). The effect of test substances on bacterial metabolism can be determined indirectly by measuring luminescence (Bruner et al., 1991). The control value of light emission from the bacteria was photometrically determined immediately before the addition of test substance solution. Bacteria were then incubated with the test substance solutions and the light output was measured at 5 and 15 min. The ratio of light lost to light remaining (γ) was calculated and graphs of log γ against log test substance concentration plotted. The concentration that gave a γ value of 1.0 (50% reduction in light output) was calculated using least squares fit linear regression analysis. Three independent assays were carried out. The Microtox assay system was supplied by Microbics Corporation (CA).

2.4. Statistical analysis

A bidirectional analysis of variance (ANOVA) with three replicates and two variation factors (concentrations of the extracts and cytotoxicity test) was carried out, using the Pearson correlation test to determine differences in methods of measuring cytotoxicity. In addition, we carried out a Student's t-test of concentrations.

Table 1
Percentage absorbance (mean ± S.E.M.) in three cytotoxicity assays

Concentration (mg/ml)	NR	KB	MTT
10	88.00 ± 4.35	92.00 ± 2.67	91.00 ± 3.46
20	87.00 ± 1.45	90.00 ± 2.89	90.00 ± 2.30
30	80.00 ± 0.58	87.00 ± 2.40	87.33 ± 1.76
40	76.00 ± 1.73	78.33 ± 2.60	76.66 ± 1.76
50	67.33 ± 1.76	72.66 ± 1.85	74.33 ± 2.60
75	66.00 ± 2.08	69.33 ± 1.76	69.66 ± 0.88
100	66.33 ± 2.33	68.00 ± 1.53	68.00 ± 3.05

NR, neutral red assay; KB, total protein assay; MTT, tetrazolium assay.

3. Results

Results of cytotoxicity are shown in Tables 1 and 2. Statistical analyses appear in Tables 3–5.

Table 1 represents the results of cytotoxicity for 24 h as measured by the three endpoint systems (NR, KB and MTT assays). Results are expressed as the percentage of absorbance of cells incubated with different doses of aqueous extracts against control. Cytotoxicity was similar in all the systems, independent of the toxic endpoints employed (Table 3). The correlation between data of the three cytotoxicity systems was excellent ($P < 0.01$, Table 4). As shown in Table 1, the extracts of *U. tomentosa* did not show cytotoxicity at the concentrations tested. However, there were signifi-

Table 2
Microtox data (Cr/γ)

Concentration (mg/ml)	5 min	15 min
10	0.26 ± 0.03	0.19 ± 0.08
20	0.35 ± 0.02	0.36 ± 0.01
30	0.45 ± 0.02	0.43 ± 0.02
40	0.65 ± 0.02	0.58 ± 0.02
50	1.05 ± 0.31	1.04 ± 0.29
75	2.25 ± 0.29	2.07 ± 0.26
100	2.53 ± 0.11	2.46 ± 0.39

Tabular values represent the mean ± S.E.M.

Table 3
Statistical analysis of variance (ANOVA)

Variable	df	F
Doses	6	50.09*
Cytotoxicity assay	2	3.04 NS
Interaction	12	0.64 NS

Data expressed in F values.
* $P<0.01$; NS, not significant.

cant differences ($P < 0.01$) of cytotoxicity among doses under conditions used in the three test systems (ANOVA, Table 3). The highest concentrations analyzed (50, 75 and 100 mg/ml) showed a similar cytotoxic effect. There were no significant differences of cytotoxicity between 10 and 20 mg/ml (Table 5). Similar results were obtained by Microtox assay at 5 and 15 min (Table 2). The concentrations tested did not show a toxic effect on bacterial cells in the established conditions.

4. Discussion and conclusions

The most significant finding from our results was that *U. tomentosa* extracts did not exert a toxic effect on the battery of bioassays used. It is well-known that other herbal medicines can have adverse effects, in which case it is convenient to evaluate the benefit-risk profile. Thus, different compounds of *U. tomentosa* have been investigated. Senatore et al. (1989) isolated betasitosterol, stigmasterol and campesterol in steroidal fractions of this plant. Aquino et al. (1989, 1990, 1991) reinvestigated the *U. tomentosa* barks, elucidating the structures of some triterpenes and quinovic acid glycosides by spectral and chemical studies. There are pharmacological studies of these compounds. In the same line, Aquino et al. (1989) carried out a series of antiviral tests with all isolated glycosides. Furthermore, Senatore et al. (1989) and Aquino et al. (1991) demonstrated anti-inflammatory activity of *U. tomentosa* extracts.

Table 4
Correlation analysis of cytotoxicity assays

Variables	df	r
NR-KB	19	0.87*
NR-MTT	19	0.88*
KB-MTT	19	0.84*

* $P<0.01$.

Table 5
Student's t-test of concentrations tested

Variables (mg/ml)	df	t
10–20	16	0.54 NS
10–30	16	2.29*
20–30	16	2.21*
20–40	16	7.36**
30–40	16	4.33**
40–50	16	3.00**
50–75	16	1.72 NS
50–100	16	2.07 NS
75–100	16	0.56 NS

* $P<0.05$; ** $P<0.01$.

However, the toxic effects have been poorly studied. Our results demostrated a non-toxic effect in bacterial cells (*Photobacterium phosphoreum*) using the Microtox assay (Table 2). These results agree with those of Rizzi et al. (1993), who reported mutagenic and antimutagenic activity of extracts and chromatographic fractions of *U. tomentosa* barks. These extracts were not able to induce mutagenesis in different strains of *Salmonella typhimurium*.

Our data also provided evidence for the non-cytotoxicity of *U. tomentosa* extracts. Little information is available on their potential cytotoxic effects. We used one Chinese hamster cell line (CHO), widely employed in cytotoxicity tests because of its several advantages (Bradley et al., 1981). Several dilutions of the extracts showed different inhibition of cell growth (Table 1) but the values obtained were higher than 65% of cell growth. Thus, cytotoxic activity was not considered significant. Cytotoxicity was also similar in all the systems (Tables 1 and 4), independent of the toxic endpoints employed, supporting the basal cytotoxicity concept, which implies that most cell lines should have a similar response to toxicants when toxicity is measured by various endpoints, corresponding to inhibition or destruc-

tion of basal functions and structures (Ekwall, 1995).

Tests in vitro used in this paper are reliable short-term assays that can be performed easily and seem to predict cytotoxicity (Bulich et al., 1990; Ekwall et al., 1991; Santa Maria et al., 1996). Our results indicate that they are valuable tools for evaluating the toxicity of natural products widely used in popular medicine. We can conclude that *U. tomentosa* shows no toxic effects in the concentrations tested.

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References

Aquino, R., De Simeone, F., Pizza, C., Conti, C., Stein, M.L., 1989. Plant metabolites stucture and in vitro antiviral activity of quinovic acid glycosides from *Uncaria tomentosa* and *Guettarda platypoda*. Journal of Natural Products 52, 679–685.

Aquino, R., De Simeone, F., Vincieri, F.F., Pizza, C., Gacs-Baiz, E., 1990. New polyhydroxilated triterpenes from *Uncaria tomentosa*. Journal of Natural Products 53, 559–564.

Aquino, R., De Feo, V., De Simeone, F., Pizza, C., Cirino, G., 1991. Plant metabolites. New compounds and antiinflamatory activity of *Uncaria tomentosa*. Journal of Natural Products 54, 453–459.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24, 119–124.

Borenfreund, E., Babich, H., Martin-Alguacil, N., 1988. Comparison of two in vitro cytotoxicity assays: The neutral red (NR) and tetrazolium (MTT) tests. Toxicology in Vitro 2, 1–6.

Bradley, M.D., Bhu Yan, M.C., Francis, R., Langebach, A., Peterson, A., Huberman, E., 1981. Mutagenesis by chemical agents in Chinese hamster cells: A report of the GenTox Program. Mutation Research 87, 81–142.

Bruner, L.H., Kain, D.J., Roberts, D.A., Parker, R.D., 1991. Evaluation of seven in vitro alternatives for ocular safety testing. Fundamental and Applied Toxicology 17, 136–149.

Bulich, A.A., Tung, K.T., Scheibner, G., 1990. The luminiscent bacteria toxicity test: Its potential as an in vitro alternative. Journal of Bioluminescence and Chemiluminescence 5, 71–77.

J.A. Duke, R. Vasquez, Amazonian Ethnobotanical Dictionary, CRC Press, Boca Raton, FL, 1994.

Ekwall, B., Bondesson, I., Hellberg, S., Högberg, S., Romert, L., Stenberg, K., Walum, E., 1991. Validation in vitro cytotoxicity test-past and present strategies. Alternative to Laboratory Animals 19, 226–233.

B. Ekwall, in: A. Goldberg, L. Zutphen (Eds.), The Basal Cytotoxicity Concept. Alternative Methods in Toxicology, Lieber, New York, 1995.

Knox, P., Uphill, P.F., Fry, J.R., Bendford, D.J., Balls, M., 1986. The Frame multicentre project on in vitro cytotoxicity. Food Chemical Toxicology 24, 457–463.

Rizzi, R., Re, F., Bianchi, A., De Feo, V., De Simeone, A., Bianchi, L., Stivala, L.A., 1993. Mutagenic and antimutagenic activities of Uncaria tomentosa and its extracts. Journal of Ethnopharmacology 38, 63–77.

Santa Maria, A., Pozuelo, J.M., Lopez, A., Sanz, F., 1996. Toxicity of potential irritants in mammalian cells in vitro. Ecotoxicology and Environmental Safety 34, 56–58.

Senatore, A., Cataldo, A., Iaccarino, F.P., Elberti, M.G., 1989. Phytochemical and biological study of *Uncaria tomentosa*. Bolletino Società Italiana Biologia Sperimentale 65, 517–520.