



## In Vitro Screening of Plant Extract: Neurotoxic Effects of the “Sierras de Córdoba”, Argentina Plant Extracts

Carlos Alberto Landa<sup>a</sup> and Mónica S. Sanchez<sup>a,b</sup> (\*)

<sup>a</sup>Subsecretaria CEPROCOR, Ministerio de Ciencia y Tecnología, Córdoba, Argentina. <sup>b</sup>Laboratorio de Neurobiología Celular y Molecular, Instituto Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET).

### ABSTRACT

To investigate the neurotoxic effect of some “Sierras de Córdoba” plant extracts, three plants from this place were extracted with ethylic ether, ethanol and milliQ-water. The effects of different extracts on early events in the normal development of neuron were studied using cultured embryonic rat hippocampal cells, which develop in vitro in a stereotypical sequence of events that mimics their development in vivo. In addition, the neuron viability was evaluated using lactate dehydrogenase (LDH) assay. Two ethylic ether, two aqueous extracts from *Thelesperma Megapotamicum* and *Armeniaca Vulgaris* and one ethanolic extract (*Armeniaca Vulgaris*) showed no toxicity against embryonic rat hippocampal cells in culture. The ethanolic extract of *Thelesperma Megapotamicum* showed a slight (but no significative) toxicity against the same neuronal cells. While, the three extract studied of *Aristolochia Stuckertii* Speg, at both doses, showed high toxicity against the rat hippocampal cells in culture. Taken together, our results describes, for the first time, that *Aristolochia Stuckertii* Speg have neurotoxic effects. Further intensive studies are required to clarify how *Aristolochia Stuckertii* Speg produces this effect.

**Keywords:** “Sierras de Córdoba” extract plants; in vitro neurotoxicity; early neuronal development; LDH assay.

---

**Corresponding author:** Sanchez Mónica Silvina, Subsecretaria Ceprococor, Ministerio de Ciencia y Tecnología, Santa María de Punilla (5164). Tel: 54-3541-489650/53. FAX: 54-3541-488181. *E-mail adress:* monicasilvina.sanchez@cba.gov.ar

*Received: February 22, 2010. Accepted: March 10, 2010*



## Introduction

Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infection, cancer and immunological disorders (Newman and Cragg, 2007). About 25% of prescribed drugs in the world originate from plants (Rates, 2001) and over 3000 species of plants have been reported to have anticancer properties (Graham et al., 2000). Argentina is a country with both rich floral biodiversity and cultural diversity. Traditional herbal medicines are important in the health care of most people, and rely heavily on the use of indigenous plants. The use of these medicines was observed to be widespread and prevalent over orthodox medicine. However, the majority of these plants have not yet undergone toxicological studies to investigate their bioactive compound(s) (Goleniowski et al., 2006).

Arising from consideration of the potential environmental triggers of neurodegenerative diseases, we set out to investigate the possible neurotoxic activity in some "Sierras de Córdoba" plants (*Armeniaca Vulgaris*, *Aristolochia Stuckertii* Speg, and *Thelesperma Megapotamicum*).

## Experimental

### Plant Material

Three plants were collected from "Sierras de Córdoba", Argentina. The plant material was identified by the Ceprocor Herbarium, and shade-dried.

### Preparation of Extracts

The dried plant material (50g) was ground into coarse powder and then extracted with ethyl ether, ethanol and milliQ-water. The plant extracts were then filtered and the solvent was evaporated under reduced pressure followed by freeze-drying.

### Cell culture

Dissociated hippocampal pyramidal cells were prepared from embryonic rat brain and cultured as described previously (Sanchez et al., 2008). For cytotoxicity assays, cells were seeded in 24-well plates coated with 1 mg/ml poly-L-lysine (Sigma Chemical Co. St Louis, MO) in minimum essential medium (MEM) containing 10% horse serum. For Morphometric analysis, cells were plated onto glass coverslips, coated with 1 mg/ml poly-lysine (Sigma Chemical Co). After 2 hr, the coverslips were transferred to dishes containing serum-free MEM with N<sub>2</sub>

supplements, 0.1% ovalbumin, and 0.1mM pyruvate.

### Extract exposures

The dried extracts were dissolved in dimethyl sulfoxide (DMSO). Plant extracts were added to the culture medium at concentrations ranging from 0.01 to 0.1  $\mu$ M; while control cultures were treated with DMSO alone.

### Cytotoxicity assays

For the cytotoxicity assays cells were grown in 24-well plates at a density of  $1 \times 10^5$  cells/ml and were treated with each plant extract separately, control cells grown in the same condition. After 24 hr exposure the plated cells were assayed for LDH release assay. The amount of LDH released into the media after exposure to each extract plant was detected using a LDH assay kit (Promega, Madison, WI, USA). The results of LDH release were expressed as percentage of control.

### Immunofluorescence.

Immunocytochemical staining was performed as described (Sanchez et al., 2008). A monoclonal antibody against tyrosinated-tubulin (clone TUB-1A2, mouse IgG; Sigma Chemical Co.) and Alexa 488 were used as primary and secondary antibodies respectively. Rhodamine-phalloidin (Molecular Probes, Eugene, OR) was used to stain filamentous actin (F-actin). Cells were visualized in a conventional confocal (Zeiss Pascal, Carl Zeiss, Inc., Germany) microscope and images processed using Adobe Photoshop, Adobe Systems Inc., San Jose, CA.

### Morphometric analysis of neuronal shape parameters.

Neuronal shape parameters were measured using maximal projection images and the morphometric menu of the confocal microscope. The criteria used for distinguishing pyramidal neurons from nonpyramidal neurons and nonneuronal cells and for determination of pyramidal neuron stage of development were based on the nomenclature first proposed by Dotti et al. (1988). Coverslips were removed 24 hr after plating, and the proportion of neurons in each stage of development (stages 1 to 3) were quantitated for pyramidal neurons in each treatment group.

### Analysis of Neuron Survival

The survival of neurons at 24 hr in each treatment group was determined by comparing the mean number of pyramidal neurons per unit area



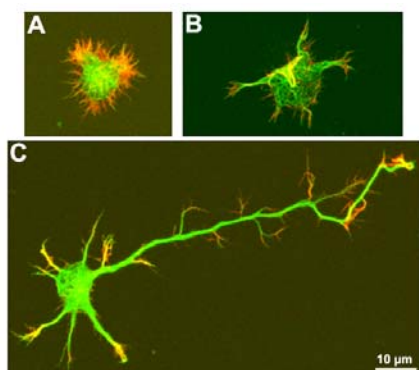
of substrate using methods previously described in detail (Lindsley et al., 2002). Changes in neuronal number over time in culture represent cell death only and are not confounded by proliferation of neurons because less than 1% of the neurons in these cultures divide (Dotti et al., 1988).

### Statistical analyses.

The unpaired two-tailed Student's t-test and one-way ANOVA were used in the statistical analysis when appropriate. Post hoc comparisons of individual groups were performed using the Tukey test. A p-value < 0.05 was considered significant. All results are expressed as mean  $\pm$  SEM for the stated number of observations.

### Results

Hippocampal pyramidal neurons in culture undergo several distinct morphological changes during differentiation that lead to the extension of a single long axon and several short and branched dendrites.

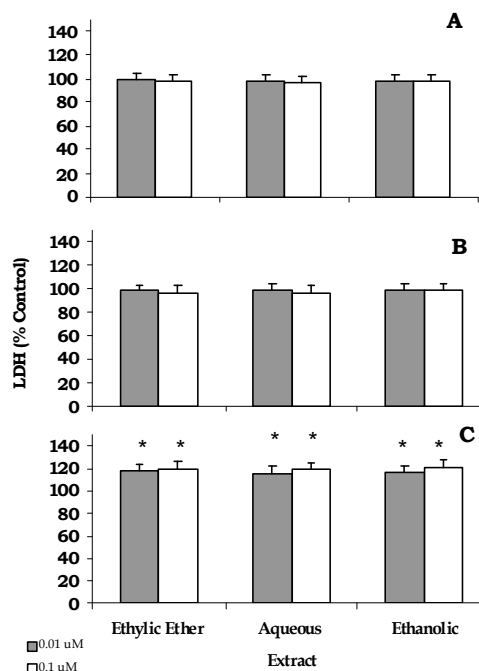


**Figure 1.** Characteristic timing of normal development of hippocampal pyramidal neurons through stages 1 to 3, and corresponding double immunofluorescence images (green: tyrosinated  $\alpha$ -tubulin; red: phalloidin) showing the morphological characteristics of each stage. The cell in (A) has lamellipodia encircling the cell body (stage 1). The cell in (B) has 5 minor processes (stage 2). The cell in (C) has 4 minor processes and an axon (stage 3). The proportion of neurons in each stage of development changes with time as individual neurons progress from stage 1 to stage 3.

The Fig. 1 summarizes the progression of hippocampal neurons developing from stage 1

through stage 3, during the first day in culture. Shortly, after plating there is no discernable polarity, as neurons elaborate lamellipodia (stage 1), and then a symmetrical array of short neurites (minor processes, stage 2). Later on, one of these neurites forms the axon by extending a large and highly dynamic growth cone with labile actin cytoskeleton and abundant dynamic MTs (stage 3) (Bradke and Dotti, 1999); several days later, the remaining minor processes differentiate as dendrites (Dotti et al., 1988). The proportion of neurons with morphological characteristics of each successive stage changes as cells develop processes during the first day in vitro.

Table 1 shows that, as in the control, when neurons were treated with ethyl ether, ethanolic and aqueous extracts from *Thelesperma Megapotamicum* and *Armeniaca Vulgaris* most neurons have reached stage 3 of neuritic development after 24 hr in culture. By contrast, neurons treated with the three extracts studied of *Aristolochia Stuckertii* Speg display a significant inhibition of axon formation, with many of them arrested at stage 2 of neuritic development.



**Figure 2.** Primary rat hippocampal pyramidal neurons were exposed to ethyl ether, ethanol or aqueous extract of *Armeniaca Vulgaris* (A), *Thelesperma Megapotamicum* (B) y *Aristolochia Stuckertii* Speg (C) (0,01 y 0,1  $\mu$ M) for 24 hr. \*



$P < 0.05$  compared to its own corresponding control without any treatment

The Figure 2 shown that the three extracts from *Aristolochia Stuckertii* Speg cause a significant increase of LDH release in cultured hippocampal pyramidal neurons. While the extracts from *Armeniaca Vulgaris* and *Thelesperma Megapotamicum* do not cause cytotoxicity. In addition, cell viability assays revealed that the three extracts of *Aristolochia Stuckertii* Speg, at both studied concentrations, produces a high increase cell death in cultured hippocampal pyramidal neurons. Thus, the number of viable cells quantified in these cultures were significantly lower compared to control ones (data not shown).

### Discussion

Medicinal plants have a long-standing history in many indigenous communities, and continue to provide useful tools for treating diseases in some rural areas.

The majority of people use traditional medications at sometime supporting the presumption of efficacy and safety of the plant materials used in the medicines. Our study describes investigations into the toxic effect of three "Sierras de Córdoba" medicinal plants by screening for neurotoxic activity against hippocampal pyramidal neurons. *Thelesperma megapotamicum* and *Armeniaca Vulgaris* showed no toxicity against neurons, but *Aristolochia Stuckertii* Speg produces an important cytotoxicity against the cell tested.

Although, previous studies indicate that some species of *Aristolochia* can be abortive and nephrotoxic (Pio Correa, 1931; Hu et al., 2004); our results describe, for the first time, that *Aristolochia Stuckertii* Speg have neurotoxic effects.

Since, the etiology of some neurological diseases remains unknown and that recent epidemiological studies have linked exposure to environmental agents with an increased risk of developing the disease, we believe that this study provides an important basis for further investigation into the isolation, characterization and mechanism of neurotoxic compounds from some of the screened "Sierras de Córdoba" plants.

### References

- Bradke F, Dotti CG. (1999). The role of local actin instability in axon formation. *Science* 283:1931–1934.
- Dotti CG, Sullivan CA, Banker GA. (1988) The establishment of polarity by hippocampal neurons in culture. *J Neurosci* 8:1454-1468.
- Goleniowski ME, Bongiovanni GA, Palacio L, Nuñez CO, Cantero JJ. (2006) Medicinal plants from the "Sierra de Comechingones", Argentina. *J Ethnopharmacol.* 107(3):324-341.
- Graham JG, Quinn ML, Fabricant DS, Farnsworth NR. (2000) Plants used against cancer - an extension of the work of Jonathan Hartwell. *J Ethnopharmacol* 73:347–377.
- Hu, S.L., Zhang, H.Q., Chan, K., Mei, Q.X. (2004). Studies on the toxicity of *Aristolochia manshuriensis* (Guanmuton). *Toxicology* 198, 195–201.
- Lindsley TA, Comstock LL, Rising LJ (2002). Morphologic and neurotoxic effects of ethanol vary with timing of exposure in vitro. *Alcohol* 28:197–203.
- Newman DJ, Cragg GM. (2007). Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 70: 461–477.
- Pio Correa, M. (1931). *Diccionario das plantas uteis do Brasil das exóticas cultivadas*, vol. 2.
- Rates SMK. (2001). Plants as source of drugs. *Toxicon* 39:603–613.
- Sanchez M, Gastaldi L, Remedi M, Cáceres A, Landa C. (2008) Rotenone-induced toxicity is mediated by Rho-GTPases in hippocampal neurons. *Toxicol Sci.* 104(2):352-361.



**Table 1.** The table shows the percentage of cells at stage 1, 2, and 3 of neuronal development in control and extract-treated cells. The scoring of stage 1–3 cells was based on their typical morphological features as described (6). The extract was added to the culture medium 2 h after plating. Cultures were fixed and processed for immunofluorescence with a mAb against tyrosinated  $\alpha$ -tubulin and rhodamine phalloidin 24 h after plating. Each value represents the mean  $\pm$  SEM. Values significantly different from those of the control group: (\*)  $p < 0.05$ .

|                                 | Extract   | 0.01 $\mu$ M  | 0.1 $\mu$ M   |
|---------------------------------|-----------|---|---|
| Control<br>(non treated)        |           | Stage 1(%):0.0 $\pm$ 0.0<br>Stage 2(%):4.0 $\pm$ 0.5<br>Stage 3 (%):96.0 $\pm$ 2.8          |   |
| Armeniaca Vulgaris              | Ether     | Stage 1(%):1.0 $\pm$ 0.4<br>Stage 2(%):3.5 $\pm$ 0.6<br>Stage 3(%):95.5 $\pm$ 2.8           | Stage 1(%):1.5 $\pm$ 0.7<br>Stage 2(%):3.5 $\pm$ 0.8<br>Stage 3(%):96.0 $\pm$ 3.5           |
|                                 | Ethanolic | Stage 1(%):1.0 $\pm$ 0.2<br>Stage 2(%):3.0 $\pm$ 0.5<br>Stage 3(%):96.0 $\pm$ 1.4           | Stage 1(%):1.5 $\pm$ 0.7<br>Stage 2(%):3.0 $\pm$ 0.7<br>Stage 3(%):95.5 $\pm$ 2.8           |
|                                 | Aqueous   | Stage 1(%):1.5 $\pm$ 0.4<br>Stage 2(%):4.0 $\pm$ 0.5<br>Stage 3(%):94.5 $\pm$ 1.8           | Stage 1(%):0.5 $\pm$ 0.2<br>Stage 2(%):4.0 $\pm$ 0.8<br>Stage 3(%):95.5 $\pm$ 1.3           |
| Thelesperma<br>Megapotamicum    | Ether     | Stage 1(%):0.5 $\pm$ 0.1<br>Stage 2(%):1.5 $\pm$ 0.8<br>Stage 3(%):98.0 $\pm$ 1.8           | Stage 1(%):0.5 $\pm$ 0.2<br>Stage 2(%):2.0 $\pm$ 1.5<br>Stage 3(%):97.5 $\pm$ 2.8           |
|                                 | Ethanolic | Stage 1(%):1.0 $\pm$ 0.3<br>Stage 2(%):4.5 $\pm$ 1.5<br>Stage 3(%):94.5 $\pm$ 2.8           | Stage 1(%):1.0 $\pm$ 0.5<br>Stage 2(%):4.5 $\pm$ 1.8<br>Stage 3(%):96.0 $\pm$ 1.4           |
|                                 | Aqueous   | Stage 1(%):1.0 $\pm$ 0.4<br>Stage 2(%):3.0 $\pm$ 1.2<br>Stage 3(%):96.0 $\pm$ 3.2           | Stage 1(%):1.0 $\pm$ 0.5<br>Stage 2(%):2.5 $\pm$ 0.7<br>Stage 3(%):96.5 $\pm$ 1.5           |
| Aristolochia Stuckertii<br>Speg | Ether     | Stage 1(%):6.0 $\pm$ 2.1(*)<br>Stage 2(%):23.0 $\pm$ 6.5(*)<br>Stage 3(%):77.0 $\pm$ 4.8(*) | Stage 1(%):8.5 $\pm$ 2.3(*)<br>Stage 2(%):19.5 $\pm$ 3.4(*)<br>Stage 3(%):73.0 $\pm$ 5.1(*) |
|                                 | Ethanolic | Stage 1(%):5.5 $\pm$ 1.4(*)<br>Stage 2(%):20.0 $\pm$ 4.5(*)<br>Stage 3(%):74.5 $\pm$ 6.2(*) | Stage 1(%):5.5 $\pm$ 1.7(*)<br>Stage 2(%):23.5 $\pm$ 3.2(*)<br>Stage 3(%):71.0 $\pm$ 5.6(*) |
|                                 | Aqueous   | Stage 1(%):6.5 $\pm$ 1.5(*)<br>Stage 2(%):16.5 $\pm$ 4.5(*)<br>Stage 3(%):77.0 $\pm$ 5.5(*) | Stage 1(%):8.0 $\pm$ 2.4(*)<br>Stage 2(%):20.0 $\pm$ 5.1(*)<br>Stage 3(%):72.0 $\pm$ 6.3(*) |