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Article

Comparison of bioassays using the anostracan crustaceans *Artemia salina* and *Thamnocephalus platyurus* for plant extract toxicity screening

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RESUMO: "Comparação de bioensaios com os crustáceos *Artemia salina* e *Thamnocephalus platyurus* para abordagem de extratos de plantas com toxicidade". Três bioensaios de letalidade com o crustáceo de água salgada *Artemia salina* Leach, Artemiidae, (teste convencional em microplaca de 96 poós Artoxkit microbiotest M) e o crustáceo de água doce *Thamnocephalus platyurus* Packard, Thamnocephalidae (Thamnotoxkit microbiotest F), foram comparados utilizando extratos de dez espécies de plantas da Guatemala. Foi previamente observado que cinco delas possuem atividade anti-*Artemia: Solanum americanum* Mill., Solanaceae, *Gliricidia sepium* (Jacq.) Kunth ex Walp., Fabaceae, *Neurolaena lobata* (L.) Cass., Asteraceae, *Petiveria alliacea* L., Phytolaccaceae e *Ocimum campechianum* Mill., Lamiaceae. As outras cinco espécies, *Curatella americana* L., Dilleniaceae, *Prunus barba*ta Koehne, Rosaceae, *Quercus crispifolia* Trel., Fagaceae, *Rhizophora mangle* L., Rhizophoraceae e *Smilax domingensis* Willd., Smilacaceae, não. Todas as plantas sem atividade anti-*Artemia* não tiveram nenhum efeito letal em ambos os ensaios com *A. salina*. Para as plantas com atividade anti-*Artemia* o M Artoxkit não foi sensível a *G. sepium* e teste convencional de *Artemia* não foi sensível a *S. americanum*, *G. sepium* e *N. loba*ta. Todos os extratos vegetais, exceto o de *C. americana*, apresentaram um efeito letal sobre *T. platyurus* e a concentração letal média (CL50) para este organismo em todos os casos foram substancialmente inferiores aos da espécie de teste de água salgada. Este estudo revelou que *T. platyurus* é teste promissor para uma investigação aprofundada na seleção de extratos de plantas com potenciais propriedades medicinais.

Unitermos: Plantas medicinais, toxicidade, *Artemia salina, Thamnocephalus platyurus,* Artoxkit M, Thamnotoxkit F.

ABSTRACT: Three lethality bioassays, using the salt-water crustacean *Artemia salina* Leach, Artemiidae, (conventional 96 microwell plate test and the Artoxkit M microbiotest) and the freshwater crustacean *Thamnocephalus platyurus* Packard, Thamnocephalidae, (Thamnotoxkit F microbiotest), were compared using extracts of ten Guatemalan plant species. It was previously observed that five of them have anti-*Artemia* activity. These were: *Solanum americanum* Mill., Solanaceae, *Gliricidia sepium* (Jacq.) Kunth ex Walp., Fabaceae, *Neurolaena lobata* (L.) Cass., Asteraceae, *Petiveria alliacea* L., Phytolaccaceae, and *Ocimum campechianum* Mill., Lamiaceae. The five others: *Curatella americana* L., Dilleniaceae, *Prunus barbata* Koehne, Rosaceae, *Quercus crispifolia* Trel., Fagaceae, *Rhizophora mangle* L., Rhizophoraceae, and *Smilax domingensis* Willd., Smilacaceae, do not. All plants without anti-*Artemia* activity had no lethal effects in both assays with *A. salina.* For the plants with anti-*Artemia* activity the Artoxkit M was not sensitive to *G. sepium* and the conventional *Artemia* test was not sensitive to *S. americanum, G. sepium* and *N. lobata*. All the plant extracts, except for that of *C. americana*, had lethal effects on *T. platyurus* and the lethal median concentration (LC50) levels for this organism were in all cases substantially lower than those of the salt-water test species. This study revealed that *T. platyurus* is a promising test species worth further in depth investigation for toxicity screening of plant extracts with potential medicinal properties.

Keywords: Medicinal plants extracts, toxicity screening, *Artemia salina, Thamnocephalus platyurus,* Artoxkit M, Thamnotoxkit F.

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INTRODUCTION

The brine shrimp, *Artemia salina* Leach, Anostraca: Artemiidae, (Figure 1), has been used for several decades as a test organism (Michael et al., 1956) in natural products research (Meyer et al., 1982) and to assess the effect of chemicals in aquatic environments (Persoone & Wells, 1987). The *Artemia* assay is used routinely in laboratories around the world for prescreening of plant extracts with potential medicinal properties (e.g., antimicrobial or antiparasitic), for bioguided fractionation of bioactive constituents of plant extracts and for cytotoxic effects detection (Trotter et al., 1983; Anderson et al., 1991; Beloz, 1992; Solis et al., 1993; Sam, 1993; Zani et al., 1995; Adoum et al., 1997; Awachie & Ugwu, 1997; Chávez et al., 1997; Cáceres et al., 1998; Moshi et al., 2004; Oliva et al., 2007).

The major reason why this salt-water anostracan crustacean is used widely for toxicity testing of plant extracts is due to the commercial availability of dormant eggs (cysts), which are harvested in huge amounts in salt lakes and pans. The larvae hatched from the cysts are used worldwide in aquaculture and in aquariology as live food for juvenile fish. Dormant brine shrimp eggs remain viable for many years and are therefore a suitable biological source for rapid, simple and inexpensive bioassays. A drawback of this bioassay, however, is that the saline medium decreases the solubility and bioavailability of some substances, thus limiting the detection of possible bioactive plant constituents.

In recent years, the controlled production of dormant stages of a number of aquatic test organisms has been achieved and stock culture and maintenance-free microbiotests have been developed (see the Cyst-based Toxicity Tests series of publications in www.microbiotests.be). These assays are available commercially as Toxkits (Persoone, 1991). One of these microbiotests, the Thamnotoxkit F with the fairy shrimp, *Thamnocephalus platyurus* Packard, Anostraca: Thamnocephalidae, (Figure 2), is particularly interesting for toxicity testing and screening of potential medicinal plants. *T. platyurus* is a freshwater relative of the brine shrimp and is very sensitive to a number of chemicals and environmental pollutants. A variety of research papers on environmental toxicity assessment using this organism have already been published, several of which are listed on the website www.microbiotests.be.

In the present study we compared the response of three bioassays to extracts of ten Guatemalan plant species used in traditional medicine: the *Artemia* test normally used for prescreening and toxicity testing of plant extracts (Solis et al., 1993; Cáceres et al., 1998), the Artoxkit M and the Thamnotoxkit F microbiotests. To our knowledge, this is the first attempt to compare the effects of plant extracts on *A. salina* with those on its freshwater relative *T. platyurus*.

Figure 1. Recently hatched nauplius of *A. salina*, about 300 µm long (photograph courtesy of Microbiotests, Inc.).

Figure 2. Instar I nauplius of *T. platyurus* (around 300 µm long) (photograph courtesy of Microbiotests, Inc.).

MATERIAL AND METHODS

Plant material and extracts

Plants were collected in several Guatemalan locations. The specimens were authenticated by Ana María Ortiz (C.F.E.H. Herbarium), Juan José Castillo (Faculty of Agronomy, San Carlos University of Guatemala), Elfriede Pöll (UVAL Hebarium) and Mario Véliz (BIGU Herbarium). The specimens are stored in Farmaya's Ethnobotanical Herbarium (C.F.E.H), in Guatemala City, with the voucher numbers indicated in Tables 1 and 2.

Plant material was shade-dried and extracted with 95% ethanol and concentrated to dryness by rotary evaporator. The essential oil of *Ocimum campechianum* Mill., Lamiaceae., was obtained by Neocleavenger hydrodistillation (BHMA, 1996). All material assayed (from now on referred to as extracts) was provided by the Department of Cytohistology, Faculty of Chemical Sciences and Pharmacy, San Carlos University of Guatemala.

The plant species selected for the present study are used in Guatemala for medicinal purposes and their activities against *A. salina* have been previously

Table 1. Guatemalan plant species used, reported to have anti-*Artemia* activity.

Species (part used)	Reference	Collection site	Herbarium and voucher number
Solanum americanum Mill., Solanaceae (leaf)	Cáceres et al. (1998)	Samayac	FARMAYA's Ethnobotanical Herbarium (C.F.E.H), 401
Gliricidia sepium (Jacq.) Kunth., Fabaceae (bark)	Berger et al.(1998)	Samayac	C.F.E.H, 339
Neurolaena lobata R.Br., Asteraceae (leaf)	Berger et al.(1998)	Samayac	C.F.E.H, 160
Petiveria alliacea L., Phytolaccaceae (root)	Berger et al.(1998)	Samayac	C.F.E.H, 367
Ocimum campechianum Mill., Lamiaceae (leaf, essential oil)	Cruz (2001)	Samayac	C.F.E.H, 1049

Table 2. Guatemalan plant species used, reported to be inactive against *A. salina.*

Species (part used)	Reference	Collection site	Herbarium and voucher number
Curatella americana L., Dilleniaceae (leaf)	Cáceres et al. (2001)	Biosphere Reserve Sierra de las Minas (RBSM)	C.F.E.H., 1130
Prunus barbata Koehne, Rosaceae (leaf)	Cáceres et al. (2001)	RBSM	C.F.E.H, 755
Quercus crispifolia Trel., Fagaceae (leaf)	Cáceres et al. (2001)	RBSM	C.F.E.H, 754
Rhizophora mangle L., Rhizophoraceae (bark)	Previously tested as negative at our laboratory	Chiquimulilla	C.F.E.H, 323
Smilax domingensis Willd., Smilacaceae (rhizome)	Cáceres et al. (1998)	Samayac	C.F.E.H, 662

determined by this Department. The 5 species found to have anti-*Artemia* activity are listed in Table 1.

Cáceres (1999) describes botanical aspects, habitat, history, agriculture, medicinal uses, other popular uses, pharmacology, chemical composition, pharmacognosy, toxicology and therapeutic indications of these five plants.

The other five species reported to be inactive against *A. salin*a are listed in Table 2. Some of their medicinal uses are mentioned in the respective references.

Twenty mg of each extract concentrate were dissolved in 10 mL of the standard seawater or freshwater medium for *A. salina* or *T. platyurus* respectively. In the case of *O. campechianum*, 2 mg of the essential oil were dissolved in 40 µL dimethyl sulfoxide (DMSO) and 1.9 mL medium. DMSO is not toxic to any of the test organisms in the concentration used.

Conventional microwell *Artemia* test

The brine shrimp cysts of *A. salina* for the conventional *Artemia* test (*Artemia* test) were bought in a pet fish shop in Guatemala City.

The bioassay with *A. salina* was originally described by Michael et al. (1956). The procedure was adapted to natural product research by Meyer et al. (1982), and the conventional microwell bioassay methodology used in this investigation followed the method described in Solis et al. (1993) and Cáceres et al. (1998).

Artoxkit M

The Artoxkit M kits used were obtained from Microbiotests, Inc., Mariakerke-Gent, Belgium. This microbiotest was developed and standardized in the Laboratory for Biological Research on Aquatic Pollution at the University of Ghent in Belgium (Vanhaecke & Persoone, 1981, 1984; Van Steertegem & Persoone, 1993).

The chemical compounds methodology indicated in the Standard Operational Procedure (SOP) of the Artoxkit M was followed (Artoxkit M, 2003).

The Artoxkit M microbiotest differs from the *Artemia* test on several points, which are mentioned below. The kit contains all the materials necessary to perform up to six tests, including *Artemia* cysts of a standard reference stock. A 24-microwell plate with 1 mL of sample per well is used, instead of a 96-microwell plate with 200 µL per well. The hatching of the cysts is carried out by exposure to a fluorescent light source of 3000-4000 lux for 30 h at 25 °C and newly hatched nauplii are used since they molt rapidly to older stages. The nauplii are transferred from the hatching medium to a rinsing well containing the test solution (this avoids dilution from transferring hatching medium to the test sample). Then, ten nauplii are exposed in triplicate to each concentration of the test sample in the remaining wells and the dead larvae are counted at the end of the test. Methanol killing is not necessary since the exact number of organisms is known. The incubation is carried out in the dark at 25 °C with results read at 24 h and, if no effect has been observed, at 48 h. A range finding test is performed

with 1:10 dilutions of the stock solution, followed by the preparation of a logarithmic set of five dilutions between the dilutions showing 0 and 100% mortality in the range finding test to determine a precise median lethal concentration (LC50) and a 95% confidence interval.

Thamnotoxkit F

The Thamnotoxkit F kits used were obtained from Microbiotests, Inc., Mariakerke-Gent, Belgium.

Freshly hatched larvae of the freshwater fairy shrimp *T. platyurus* are used as test organisms in the Thamnotoxkit F microbiotest. The test is performed according to the chemical compounds procedure indicated in the SOP of this assay (Thamnotoxkit, 2003). The procedure is very similar to the SOP of the Artoxkit M, except that standard freshwater is used and the hatching takes place under constant exposure to a fluorescent light source (3000-4000 lux), for a 20-22 h period.

In all bioassays used here, a negative control was carried out exposing the test organisms to standard dilution water (fresh or sea water). If mortality exceeded 10% the assay should be repeated. With the Artoxkit M and Thamnotoxkit F, a test with the reference toxicant potassium dichromate was carried out for quality assurance and the LC50's obtained were within the range indicated in the specification sheets provided with each kit.

LC50 estimation

A computerized program was used to estimate the LC50's and the 95% confidence intervals (US EPA, 1985). Extracts with LC50's higher than 1000 µg/mL were considered not to have biological activity (Berger et al., 1998).

RESULTS AND DISCUSSION

The LC50's and 95% confidence intervals for the three bioassays on the extracts of the ten plants are presented in Table 3.

For the *Artemia* tests, all the extracts known to have no anti-*Artemia* activity did not induce lethal effects either in the Artoxkit M or in the *Artemia* test. The Artoxkit M was not sensitive (even after 48 h of exposure) to *G. sepium* while the *Artemia* test was not sensitive (after 24 h) to *S. americanum, G. sepium* and *N. lobata*. Prolongation of the exposure time to 48 h (in the Artoxkit M) instead of 24 h (as in the *Artemia* test) resulted in detectable LC50 values for *S. americanum* and *N. lobata.*

All extracts induced lethal effects on the Thamnotoxkit F crustaceans except for *C. americana.* LC50's were in all cases substantially lower for the Thamnotoxkit F assays than for the *Artemia* tests, except for *O. campechianum* where the difference, between the Thamnotoxkit F and the Artoxkit M data, was slight.

Although three of the five extracts known to have activity against *A. salina* had no effect on the *Artemia* test, four did on the Artoxkit M. We cannot explain why *G. sepium* had no activity in either of the *A. salina* tests. This may possibly be due to natural variations in the concentration of bioactive components in the original plant material, or perhaps due to storage conditions. As an example of the first possibility, in Cáceres et al. (1998) N. lobata showed no activity against *A. salina* but was active

Table 3. LC50's and 95% confidence intervals (CI) of plant extracts, tested with the *Artemia* test, Artoxkit M and Thamnotoxkit F.

	LC50 in µg/mL (and 95% CI)		
Plant species	*Artemia* test 24h	Artoxkit M 24 h [and 48 h[a]]	Thamnotoxkit F 24 h
	With previously demonstrated anti-*Artemia* activity		
S. americanum	>1000	>1000 [565 (380-845)]	219 (173-281)
O. campechianum	328 (260-393)	114 (86-166)	95 (68-132)
P. alliacea	228 (157-271)	481 (367-669)	12 (9-14)
G. sepium	>1000	>1000	492 (399-587)
N. lobata	>1000	>1000 [902 (794-1032)]	251 (225-274)
	Without previously demonstrated anti-*Artemia* activity		
C. americana	>1000	> 1000	>1000
R. mangle	>1000	> 1000	84 (63-110)
P. barbata	>1000	> 1000	405 (344-486)
Q. crispifolia	>1000	> 1000	201 (177-226)
S. domingensis	>1000	> 1000	70 (52-92)

[a]This is analogous to the *Daphnia magna* acute assay (ISO 6341), for which the exposure period is also 24 h, but it can be (and has been) prolonged to 48 h. This was done only in cases where no toxic response was observed after 24 h. If no other value is indicated, the LC50 was still >1000 µg/mL after 48 h.

against several pathogens. Berger et al. (1998) reported that this plant had both anti-*Artemia* (LC50=495.1 µg/mL±42.1) and anti-trypanosome activity. However, the extracts without previously reported anti-*Artemia* activity showed no anti-*Artemia* activity in the present study.

The results of the present study clearly show that the bioassay with *T. platyurus* detects bioactive components that *A. salina* may not. In fact, nine of the plant species used here, including three of those previously reported not to have anti-*Artemia* activity (*R. mangle, Q. crispifolia* and *S. domingensis*) have proven to have biocide properties against one or more human pathogens (bacteria, fungi, trypanosomes and leishmanias) and mosquito larvae (Cáceres et al., 1991; Cáceres et al., 1998; Berger et al., 1998; Cáceres, 1999; Cáceres et al., 2001). *C. americana,* which did not have lethal effects against either crustacean, showed antibacterial activity (Cáceres et al., 2001). Although *P. barbata* had activity against *T. platyurus*, no antipathogen activity has been reported (Cáceres et al., 2001). Based on these results, however, it could be worthwhile to determine if it causes other pharmacological effects (*e.g.*, anti-inflammatory and antiaterogenic effects, hypoglycemia, analgesia, diuresis, hepatoprotection, and effects on the central nervous system). Morales et al. (2001) and Morales Cifuentes et al. (2001) have reported effects of *G. sepium, N. lobata* and *P. alliaceae* extracts on the central nervous system of Swiss albino mice.

Bioguided fractionation of plant extracts using *T. platyurus* could be of interest in future studies. However, "Very often the chemical complexity of the crude or partially purified extract seems to be essential for the bioavailability of the active constituents… It is often found that, when individual constituents are isolated from the plant extract there is loss of specific bio-activity." (Bhattacharya, 2009). Therefore, when initial bioactivity screening is done, the use of complex whole plant extracts is more suitable and significant than studying fractions.

The toxicity of pharmaceuticals has been determined with many test organisms, including *T. platyurus* and *A. salina*. Nałęcz-Jawecki & Persoone (2006) present toxicity information for several pharmaceuticals using *T. platyurus*. From the data published by these authors and those reported by Calleja et al. (1994) on assays with *A. salina,* it also appears that *T. platyurus* is more sensitive to paracetamol, caffeine and theophylline.

Finally, it is worth mentioning not only that *A. salina* is still regularly used for cyanotoxin detection and screening of biologically active substances from blue green algae (*e.g.*, Mian et al., 2003), but that Törökné (1999) reported better results using *T. platyurus* for the same purpose. Marsalek & Blaha (2003) reported that, out of nineteen bioassay species, *T. platyurus* was the most sensitive for cyanotoxin detection.

Our findings therefore show that *T. platyurus* is more sensitive than A. salina for detecting toxic effects of plant extracts. Because of this, we suggest that the acute bioassay with the freshwater fairy shrimp is worth pursuing as an interesting substitute for the brine shrimp test in bioactive natural products research. A second study is currently being carried out to test a larger set of plant extract samples in order to confirm the present trend.

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