# INNOCOUS USE OF AQUEOUS EXTRACT OBTAINED FROM THE HEARTWOOD OF *Enterolobium cyclocarpum* (Jacq.) Griseb.

[1]Rosa E. Martinez Muñoz, [1]David Raya Gonzalez, [3]Marco Cajero Juarez, [1]Martin Calderon Raya, [1]Rosa E del Rio, [*1]Mauro Manuel Martinez Pacheco.

[1]Instituto de Investigaciones Quimico Biologicas, [2]Facultad de Ingenieria en Tecnologia de la Madera, [3]Facultad de Medicina Veterinaria. Universidad Michoacana de San Nicolas. Ed. B3; Cd. Universitaria; Francisco J. Mujica s/n; Col. Felicitas del Rio; Morelia, Michoacan; Mexico; C.P. 58060. e-mail: mpacheco@umich.mx

## Summary

The median inhibitory and median lethal concentrations ($IC_{50}$/$LC_{50}$) of an aqueous extract obtained from the heartwood of *E. cyclocarpum* were determined in organisms of different trophic levels. The $LC_{50}$ for the crustacean *Artemia salina*, the termite *Incisitermes marginipennis* (Latreille) and the Wistar rat were 1.3, 2 mg/ml and > 2000 mg/$kg_{ind}$, respectively. In the microbial isolates the $IC_{50}$ was > 1 mg. In the cellular tumor line MCF-7 the $IC_{50}$ was 1.3 mg. In comparing the observed $IC_{50}$/$LC_{50}$ with permissible levels of toxicity, it is concluded that the extract is not toxic for these organisms and infer us that nor for human being.

**Key words**. $IC_{50}$/$LC_{50}$, crustacean, termite, rat, bacteria, fungus, oomycete, MCF-7 cells.

## Introduction

The deterioration of dry wood caused by boring insects produces millions of dollars in economic losses annually; additionally it drastically and sensibly affects the wooden cultural patrimony of towns. The phytochemical research of wood with natural durability and resistance to biodeterioration could potentially be the method to find new molecules with biocide activity to control wood boring insects. Heartwood, prized in the fabrication of furniture due to its resistance to biodeterioration, is obtained from *E. cyclocarpum*. It is a species of agroforestral use that is distributed from the central slopes of the Pacific Ocean and Gulf of Mexico to the North of Brazil. A protecting effect of aqueous extract from heartwood of *E. cyclocarpums* on pine wood was reported and to it was attributed an antifeedant action and repellant property against the dry wood termite *I. marginipennis* [1]. Considering it as a potential candidate to preserve perishable dry wood and it is required to know the toxicity it could cause.

## Material and methods

**Biological material**. Eggs of *A. salina* [Salt Creek Co™] and synthetic sea water Red Sea Salt™ [Red Sea Fish Pharm] were obtained in the local market. Specimens of *I. marginipennis* were collected from a termite colony situated at 10º 59' 22''N, 101º 16' 31''W. The Wistar™ rats were obtained from the university animal breeding center. The MCF-7 tumor cell line of mammalian gland epithelium was obtained from the American Type Culture Collection. The enterobacteria were maintained and propagated on BAB (Blood Agar Base) at 37 ºC for 24 h. Wild isolates of the fungus *Candida albicans* and of the oomycete *Phytophthora cinnamomi* were grown and maintained on Sabouraud medium and potato dextrose agar (PDA), respectively. *E. cyclocarpum* (voucher 10277) was collected from a site at 9º14'N, 101º28'W and identified by botanist Javier Madrigal, M.S. This species is not at risk of extinction and was handled in accordance with the official natural resources standards [2].

**Obtaining the aqueous extracts**. A decoction was obtained with 100 g of plant powder and 500 ml distilled and deionized water, it was filtered and the solid was eliminated (3X). The final suspension was concentrated by lyophilization. The amount of aqueous extract for the median inhibitory and median lethal concentrations ($IC_{50}$/$LC_{50}$) determinations was taken as Total Phenol Equivalent. The powder contained 0.2626 mg Total Phenol Equivalent to *p*-coumaric acid/ml (mgTFE/ml) [3].

***A. salina* assay**. It was performed in accordance with the Meyer method [4]. Phototrophic nauplii (instar I and II) were collected from sea water with a light source (1000-4000 lux) at 25 ºC for 24 h. 10 – 15 nauplii (100 µl) were placed in wells of an ELISA plate and 100 µl of the aqueous extract was added (0.1, 1, 10 µg). Berberine sulfate was used as a positive control. The nauplii were incubated at ambient temperature for 24 h and the dead and live nauplii were counted. 100 µl of methanol were added to annihilate the surviving nauplii. Survivability was determined by the ratio of live nauplii to total nauplii (10), the criterion was: a $LC_{50}$ > 20 µg extract/ml was not toxic [5].

**Termite *I. marginipennis* assay**. In a non-selection of food assay, the food substrates were discs of filter paper impregnated with the aqueous extract (0.2626. 0.1313, 0.026 and 0.0026 µg/ml), without aqueous extract, and a single dose of 842 mg/ml of boron salts (BS) [0.7% $Na_2B_4O_7 \cdot 10H_2O$ and 0.5% $H_2BO_3$] as a positive control. 25 nymph termites were placed on each disc and were fed for eight weeks in a dark room at ambient temperature and relative humidity of 11 – 15 %, each week the dead termites and consumption of food substrate were monitored. The median lethal time ($TL_{50}$) for BS was 15 days.

**Wistar rat assay**. The acute toxicity test was determined according to the "limit test" which utilizes a single dose 2000 $mg/kg_{individual}$ [6]. Five rats (234 ± 42 g of 6 – 10 months of age) per group for each treatment. The aqueous extract was administered with an oro-gastric tube, the positive control was quercitin (1, 10, 100 and 1000 mg/kg of weight), water was administered to the negative control group. Dead animals were recorded after seven days.

**Microorganism assay**. Filter paper discs measuring 7 mm in diameter were impregnated with 6 µl aqueous extract (0.2626, 0.1313, 0.026, 0.0026 µg/ml) and were placed in triplicate within solid growth medium previously inoculated with the microorganisms. ($1x10^6$ cells/ml).

The bacterial cultures were incubated at 37 ºC for 72 h, the fungal and oomycetal cultures at 25 ºC for 72 and 192 h, respectively. The positive controls were: cefotaxime, 10 µg (bacteria); 4-(1H-1,3-benzodiazol-2-il)-1,3-thiazole, 6 µg (*C. albicans*) and mefenoxam, 6 µg (*P. cinnamomi*), maximum halo of inhibition was 20 ± 3 mm. The empirical expression, $HI = DT - DP$ was utilized to determine growth inhibition (mm). Where: $HI$ = halo of inhibition of the extract, $DT$ = total diameter of inhibition, $DP$ = diameter of the paper.

**Cell line assay**. MCF-7 cell lines and 3T3 rat fibroblasts were routinely maintained in modified DMEM medium. 2000-3500 cells were placed in wells of an ELISA plate, they were incubated with logarithmic concentrations of the aqueous extract and of the positive control, 5-fluorouracil (5-FU). Cell viability was determined at 24 and 48 h post-treatment, by the reduction of yellow tetrazolium to purple formazan in living cells. It was considered that $IC_{50}$ >100 µg are not toxic [7].

**Leaching assay**. Discs of pine wood 4.5 cm in diameter and 1 mm thick were impregnated with the aqueous extract (0.2626 mg/ml of TFE) for 3 h. The impregnated discs were placed in deionized water for five days. In each procedure, the preserved and treated discs were dried to constant weight to determine by weigh difference the retained solids and the leachate was concentrated by lyophilization.

**Analysis of results**. For each organism, the concentration that inhibits its growth or that annihilates 50 % of the population ($IC_{50}/LC_{50}$) in an interval of 24 to 168 h post-treatment was calculated by means of a dose-response curve. A completely randomized experimental design was done. The data was analyzed by ANOVA and *t*-Student tests with a significance of $p < 0.05$.

## Results

**Leaching of the aqueous extract from the pine wood discs**. The pine wood discs retained 43.7 mg of the aqueous extract (100 % retention), they were subjected to an extreme condition with deionized water for five days, up to 62.92 % of the extract was leached and the wood retained 37.08 %. Toxicological assays were done with the leachate.

**Toxicity of the aqueous extract in animals**. The median lethal concentration of the aqueous extract on *A. salina* was determined to be 1.3 mg/ml and the effect of berberine sulfate was lethal at 0.01 mg/ml within 5 min, see Table 1. The aqueous extract exhibited an anti-termite effect against *I. marginipennis* dependant on the time and concentration. The $LC_{50}$ was 2 mg/ml and the effective median lethal time ($TL_{50}$) was 5 weeks (Table 1). In the Winstar rat, the acute toxicity caused by the aqueous extract was null, bristling of hair and a diminishing of motor activity was observed, signs of which disappeared after three hours of feeding with the extract (Table 1). The quercitin induced an acute toxicity with 200 mg/kg ($LC_{50}$) causing 50% of death in the rat population in four days ($TL_{50}$).

**Microorganism sensibility**. No growth inhibitory effect caused by the aqueous extract was observed in the six bacterial isolates (Gram positive and Gram negative bacteria) exposed to 1 mg. However, in *K. pneumoniae* the $IC_{50}$ was 300 µg for up to six hours. No toxic growth effect was observed in the fungus *C. albicans* neither in the oomycete *P. cinnamomi* (Table 1).

*Table 1. The median inhibitory and median lethal concentrations ($IC_{50}/LC_{50}$) of the aqueous extract obtained from the heartwood of* E. cyclocarpum *on animals, microorganism and cell lines test.*

| Organism | Species/Cell | *$CL_{50}/CI_{50}$ (mg/ml) | Time |
|---|---|---|---|
| Crustacean | *A. salina* | *1.3 mg/ml | 24 h |
| Xylophagous insect | *I. marginipennis* | *2 mg/ml | 5 weeks |
| Rodent | *Wistar rats* | > *2000 mg/kg | > 168 h |
| Bacteria | *P. auriginosa, P. mirabilis, P. vulgaris, S. aureus, S. epidermidis, S. saprophyticus* | >1 mg/ml | >6 -- |
| Fungus | *C. albicans* | >1 mg/ml | >120 |
| Oomycete | *P. cinnamomi* | >1 mg/ml | >120 |
| Non-tumor cell line | Fibroblasts | 0.0017 mg/ml | 72 |
| Tumor cell line | MCF-7 | 0.0013 mg/ml | 72 |

**Citotoxic effect**. Toxic effect on MCF-7 tumor cell line and rat 3T3 fibroblasts. The $IC_{50}$ of the aqueous extract was 1.3 mg/ml for the MCF-7 cell line and for the 3T3 fibroblasts it was 1.70 mg/ml, both were sensitive at 2 to 5 µg/ml of 5-FU after three days post-treatment (Table 1).

## Discussion

The median inhibitory concentration and median lethal concentration ($IC_{50}/LC_{50}$) were determined for an aqueous extract obtained from the heartwood *E. cyclocarpum* on organism of different trophic levels, since there are numerous examples of venomous plants or vegetal components that in low doses cause diverse toxic effects that include death. It was observed that in the target organism (termite) a $LC_{50}$ and $TL_{50}$ superior to the control was required, strengthening the observation that the aqueous extract contains deterrent substances [1].

In the *A. salina* biological toxicity indicator, for its credibility and reliability, a $LC_{50}$ was observed to be superior to the toxicity criteria [8]. The Wistar rat, a reasonably analogous model for humans, was utilized to assay the acute toxicity of substances of interest, in which the $LC_{50}$ was determined in accordance with the IACUC guidelines [6]. We accept the probability that the ingested solid substance of more than 2,000 $mg/kg_{ind}$ may exert an acute oral toxicity [9].

The use of rodents to estimate the median lethal dose/median lethal concentration ($LD_{50}/LC_{50}$), presents a low correlation between the observed effect in the rodent and the human. ($R^2$ = 0.61 and 0.65 for rat and mouse) [10]. There is also a low prediction of the effects of acute toxicity in for humans (43 %) and a formal validation is required by modern standards to establish a relevance for humans when $LD_{50}/LC_{50}$ or any other protocol for estimating the acute toxicity is used [11,12]. Therefore, the determination of the toxicity of the extract was extended utilizing other biological models [13].

We note that the aqueous extract had a bactericidal effect only for *K. pneumoniae* and had no antimicrobial effect for other bacteria or *C. albicans* or *P. infestans*. It was observed that in cancerous MCF-7 and non cancerous 3T3 cells more than 100 µg/ml of plant extract was required in relation to the cytotoxic activity considered dangerous, the $IC_{50}$ was higher than the indicator suggesting that the aqueous extract has no cytotoxic components [5,14].

The water leached the aqueous extract, however, the results of this study suggest that leached solids are not factors that acute toxicity. Therefore, since the obtained aqueous extract does not cause acute toxicity nor cytotoxicity in the organisms exposed to it, and due to the possibility of utilizing the solid residues generated in the manufacturing of furniture made from the heartwood of *E. cyclocarpum*, we believe there is a potential application of the aqueous extract to protect processed woods.

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