PANAMJAS
Pan-American Journal of Aquatic Sciences

Ecotoxicological analysis of cashew nut industry effluents, specifically two of its major phenolic components, cardol and cardanol

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Abstract. Cashew nut processing plant is a major industry in almost all northeastern States of Brazil. The technical cashew nut shell liquid (CNSL), which contains mainly cardanol, cardol, polymeric material, and traces of methyl-cardol, is the most abundant by-product of this process. The high level of CNSL in the effluent generated during production is a potential environmental toxin. This is the first study that has assessed the toxicity of this industrial effluent, specifically two of its major components, cardol and cardanol, using the brine shrimp (*Artemia* sp.) lethality assay. Effluents were collected at a cashew nut processing plant located in Fortaleza, Ceará, Brazil. Cardol and cardanol were isolated from the technical CNSL. The LC_{50} of cardol was 0.56 and 0.41 mg/L after 24 and 48 hr exposures, respectively, and of cardanol was 1.59 and 0.42 mg/L. The LC_{50} values for crude effluent were 1.38 and 0.60 % after 24 and 48 hr exposures, respectively, and were 2.16 and 0.88 % for treated effluent. Data from this study suggested that the cashew nut industry effluents are highly toxic to the environment. The current treatment strategy to minimize the toxicity of this industry's effluent is insufficient and must be improved.

Keywords: acute toxicity test, *Artemia* sp., phenols, CNSL, effluent treatment

Resumo. Caracterização ecotoxicológica dos efluentes da indústria de beneficiamento da castanha de caju, especificamente de dois dos seus componentes fenólicos: cardol e cardanol. O beneficiamento da castanha de caju é uma das mais desenvolvidas atividades em quase todos os estados do Nordeste brasileiro, em especial no Ceará. O líquido da casca da castanha (LCC), no qual estão presentes cardóis, cardanóis, material polimérico e traços de metil-cardol, é o mais abundante sub-produto e o alto teor de LCC torna potencialmente tóxico o efluente gerado durante o processo. Este é o primeiro estudo em que a toxicidade deste efluente é avaliada, assim como de dois dos seus componentes (cardol e cardanol), utilizando o teste de toxicidade aguda com *Artemia* sp. Os efluentes foram coletados em uma indústria localizada em Fortaleza, Ceará, Brasil. O cardol e o cardanol foram isolados do LCC técnico. A CL_{50} média do cardol foi 0,56 e 0,41 mg/L após 24 e 48 h de exposição, respectivamente, e a do cardanol foi 1,59 e 0,42 mg/L. Os valores de CL_{50} obtidos dos experimentos com o efluente bruto foram 1,38 e 0,60% após 24 e 48 h, respectivamente, e 2,16 e 0,88% para o efluente tratado. Os resultados do estudo sugerem que os efluentes desta indústria são altamente tóxicos. A estratégia de tratamento atualmente empregada para minimizar a sua toxicidade é insuficiente e deve ser revista.

Palavras-Chave: teste de toxicidade aguda, *Artemia* sp., fenóis, LCC, tratamento de efluentes

Introduction

The cashew (*Anacardium occidentale* L.) is a well-known member of the ANACARDIACEA family and is commonly found in northeast Brazil. The cashew nut has been commercially exploited since colonization. Brazil, India and Mozambique, are the leading cashew nut producers in the world (Paramashivappa *et al.* 2001, Kumar *et al.* 2002).

The process of the improvement of the cashew involves four basic stages: stockpiling, structuring of the chestnut for use, extraction of the almond, and refinement (Moura *et al.* 2005). The technical cashew nut shell liquid (CNSL), containing the phenolic compounds, cardanol (60-65%), cardol (15-20%), polymeric material (10%), and traces of methyl-cardol, is the most abundant by-product of cashew improvement (Paramashivappa *et al.* 2001, Kumar *et al.* 2002, Trevisan *et al.* 2006). CNSL is used for industrial technological applications, biological/pharmaceutical applications, friction dust production by the automobile industry, and in certain polymeric/surface coating applications (Stasiuk *et al.* 2008).

Analysis of potential mutagenic, carcinogenic and cocarcinogenic activities of CNLS demonstrated that it may be a weak promoter of carcinogenesis but presented no mutagenic or cocarcinogenic activity (George & Kuttan 1997). Epidemiological studies suggested that CNSL may contribute to oral submucous fibrosis (Varghese *et al.* 1986). In addition, its phenolic components exerted several biological activities, including antioxidant (Trevisan *et al.* 2006, Façanha *et al.* 2007), inhibition of acetylcholinesterase (Stasiuk *et al.* 2008) and membrane perturbation (Stasiuk & Kozubek 2008).

There is no direct evidence regarding the toxicity of CNSL or its major phenolic components. However, the effluent generated during the improvement of the cashew nut could be considered potentially harmful to the environment due to the high CNSL content. Previous studies have not fully characterized these effluents. Moura *et al.* (2005) described the physical-chemical properties of the effluents obtained at different stages of the process, and concluded that among the analyzed parameters, the tenor of oil and greases is about two times the value allowed by environmental legislation standards (CONAMA 2005). The pH, alkalinity, turbidity, and the COD/BOD ratio were considered acceptable according to environmental legislation. There are no data available regarding the toxicity of this effluent using an ecotoxicological approach.

The analysis of the hazards of effluents should include ecotoxicological bioassays (Environmental Canada 1999, U.S. EPA 2002, CONAMA 2005), so that the potential toxicity to the environment can be assessed. This study is the first to assess the toxicity of the cashew nut improvement industry effluent and two of its major components, cardol and cardanol.

To assess the toxicity of these components, a brine shrimp (*Artemia* sp.) lethality assay was employed. The intrinsic features of the *Artemia* genus make it a suitable organism for ecotoxicological assays, as it is a robust and cost-effective model system (Nunes *et al.* 2006). Therefore, it is an effective model system for large scale analysis of industrial effluents (Guerra 2001).

Material and Methods

Effluent collection

The effluents were collected at a cashew nut improvement industry located in Fortaleza, Ceará, Brazil. The factory has a small sewage treatment plant, which filters, minimally treats and decants the effluent before releasing it into the environment. The samples were collected every week from April through June of 2006 before (crude effluent) and after treatment (treated effluent). A total of 40 samples were collected, which included 20 crude and 20 treated effluents, and they were placed in 100 mL amber glass flasks. The samples where stored at 4°C until analysis.

Cardol and cardanol isolation

Cardol and cardanol were isolated from the technical cashew nut shell liquid according to the procedures described by Paramashivappa *et al.* (2001) and Kumar *et al.* (2002). Technical CNSL was dissolved in methanol, ammonium hydroxide (25%) was added, and the solution was stirred for 15 min. The cardanol was extracted by adding hexane (4 times), followed by a 5% HCl wash of the organic layer. The hexane fraction was then dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure to obtain pure cardanol. The methanolic ammonia solution was extracted with ethyl acetate/hexane (4:1), followed by a 5% HCl wash of the resulting organic layer, and a distilled water wash. The remaining fraction was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure to obtain pure cardol.

Brine shrimp lethality assay

Brine shrimp (*Artemia* sp. Leach) eggs were hatched in a beaker filled with seawater under constant aeration. After 48 hr, the nauplii were collected by pipetting and were counted macroscopically in the stem of the pipette using a lighted background. Ten nauplii were transferred to each well of a 24-multi-well plate containing the samples. The crude and treated effluent concentrations ranged from 0.06 to 32 %, from 0.01 to 30 mg/L for cardanol, and from 0.001 to 3 mg/L for cardol. The plates were incubated under illumination. Survivors were counted after 24 and 48 hr of incubation, and the percentage of deaths at each dose and control (seawater plus vehicle) was determined (Veiga & Vital 2002).

Sodium dodecyl sulfate (SDS, BIORAD) was used as reference toxicant as suggested by Veiga & Vital (2002). A stock solution of 1000 mg/L was prepared, and concentrations of 15, 21, 27, 34, 42, 51 and 61 mg/L were used in the assay.

Statistical analysis

In accordance with U.S. E.P.A. standards (U.S. EPA 2002), three experimental replicates were used for each dilution and for control tests. Data were analyzed as means ± standard deviation (SD). The LC_{50} (median lethal concentration) values and their 95% confidence intervals (CI 95%) were obtained using the Trimmed Spearman-Karber test (Hamilton *et al.* 1977). LC_{50} values were compared using paired (same sample with different exposure time of 24 or 48 hours) or unpaired (different) samples, with the Student's t-test with a 5% significance level.

Results and discussion

Negative controls showed nauplii survival greater than 90% in all assays. SDS was used as a positive control, and its effects were highly reproducible across experiments (Figure 1). The mean LC_{50} ± SD was 24.8 ± 2.2 mg/L (n = 6, coefficient of variation of 9.0%) and the data were within the acceptable LC_{50} range (Veiga & Vital 2002). Physical-chemical analysis of the control water and test samples demonstrated that DO, pH and salinity were within the desirable range for use in toxicity testing (data not shown).

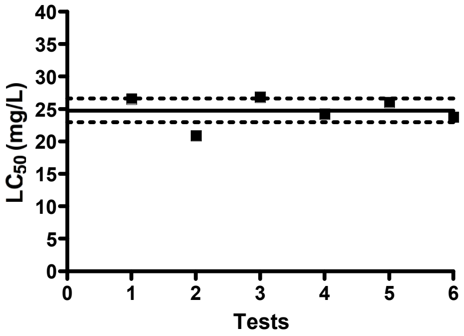

Figure 1. Mean LC_{50} values and the upper and lower intervals (mean ± 2SD) obtained from the acute toxicity test with *Artemia* sp. with sodium dodecyl sulfate after 24 hours.

The acute toxicity test using *Artemia* sp. nauplii is a major worldwide assay for measuring the toxicity of chemical substances (Barahona & Sánchez-Fortún 1996, Guerra 2001, Svensson *et al.* 2005, Nunes *et al.* 2006). Previous studies indicate that *Artemia* nauplii are highly sensitive to phenolic compounds, which are the major constituents of cashew nut industry effluents. In 1996, Barahona and Sánchez-Fortún (1996) assessed the sensitivity of three age classes of *Artemia salina* nauplii to eight phenolic compounds: pentachlorophenol (PCP), 2,6-dichloroindophenol (2,6-DCIP), 2,4-dinitrophenol (2,4-DNP), o-nitrophenol (o-NP), p-nitrophenol (p-NP), diamidophenol, diaminophenol and 2,6-dimethylphenol (2,6-DMP). The most sensitive to these phenolic compounds was the 48 hr age class, which had an LC_{50}s that ranged from 0.3 mg/L for PCP to 2.4 mg/L for p-NP.

Due to their increased sensitivity, 48 hr old *Artemia* sp. nauplii were used in this study to evaluate the toxicity of cardol and cardanol, which are two of the major components of cashew nut industry effluents. It should be noted that there are no studies assessing the toxicity of these compounds in the *Artemia* sp. model. Figure 2 shows the variation of LC_{50} values obtained from the acute toxicity test with cardanol and cardol in *Artemia* sp. in different experiments.

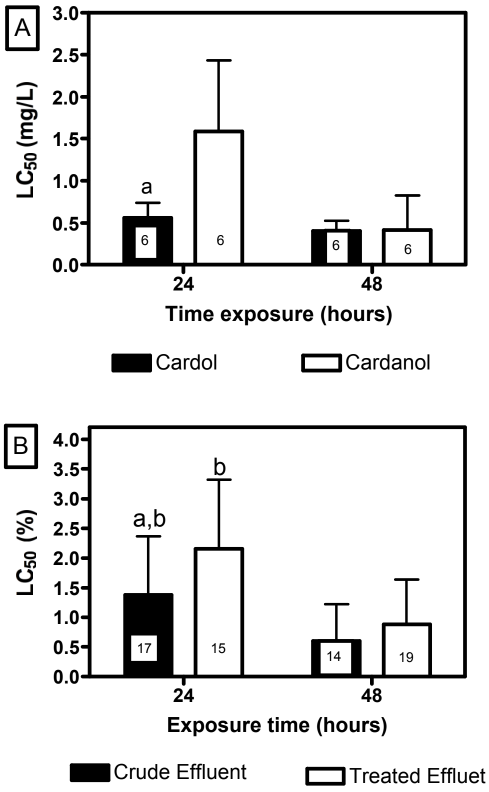

Figure 2. Effect of cardanol and cardol (A) or the cashew nut industry effluents (B) in the acute toxicity test with *Artemia* sp. after 24 and 48 hours incubation. Data are presented as mean ± SD from n experiments (n showed inside the bars). a, $p < 0.05$, t-test comparing cardol and cardanol or crude and treated effluents at the same exposure time. b, $p < 0.05$, paired t-test comparing different exposure time for the same compound or effluent.

These data indicated that these phenolic compounds were highly toxic under these assay conditions. Cardol had an LC$_{50}$ of 0.56 mg/L and 0.41 mg/L after 24 and 48 hr exposures, respectively. Cardanol had an LC$_{50}$ of 1.59 mg/L and 0.42 mg/L after 24 and 48 hr exposures, respectively. At 24 hr of exposure, cardol was significantly more toxic than cardanol ($p < 0.05$), but after 48 hr of exposure, there was no difference in toxicity ($p > 0.05$).

The hazardous effects of phenolic compounds have been extensively studied. According to Veeresh *et al.* (2005), a concentration above 1 mg/L can affect aquatic life, while Newman and Unger (2003) recognize deleterious effects at concentrations as low as 1 µg/mL. Au *et al.* (2003) demonstrated that phenolic concentration of 0.1 mg/L affect the spermatogenesis of the sea urchin *Anthocidaris crassispina* impairing the reproduction. According to them, phenolic compound disrupted the spermatozoid membrane structure. The acute toxicity of phenol was also demonstrated using *Baetis rhodani* larvae with a LC$_{50}$ around 2.1mg/L after 11 days of incubation (Khatami *et al.* 1998). As noticed in the present work, the lethal effects in *Artemia* nauplii occurred in the same concentration range, reinforcing the high sensitivity and robustness of this *Artemia* nauplii assay in measuring the toxicity of phenolic compounds, and support the idea of using this assay to assess and monitor the toxicity of the cashew nut industry effluent.

Both crude and treated cashew nut industry effluent was toxic to *Artemia* nauplii (Figure 2). The LC$_{50}$ values for the crude effluent were 1.38 ± 0.99 % and 0.60 ± 0.62% after 24 and 48-hr exposures, respectively, and were 2.16 ± 1.16 % and 0.88 ±0.76 %, respectively, for treated effluent. These LC$_{50}$ values indicated there is a statistically significant ($p < 0.05$) difference between the toxicity of crude and treated effluent after 24 hr of exposure, with the treated effluent exhibiting a slightly lower toxicity (Figure 2).

However, there was no significant difference after 48 hr ($p > 0.05$). These data indicate that the industrial treatment of the effluent is not efficient in removing the toxicity. As mentioned previously, this treatment includes a filtration, a small primary treatment and a decantation step. Table I summarizes the toxicities of several other effluents obtained with the *Artemia* nauplii model.

Table I. *Artemia's* sensitivity to several types of raw effluents in the literature.

Test Species	Exposure period and endpoint	Effluent	Toxicity value (LC$_{50}$)	Reference
Artemia salina	24 hr	Chemical plant.	2.73 – 35.5%	Guerra (2001)
Artemia sp.	24 hr	Olive oil mill	4.5 %	Aggelis et al. (2003)
Artemia salina	24 hr	Oilfield	1.2 %	Campos et al.(2002)
Artemia salina	24 hr	Landfill leachate	70 – 80%	Svensson et al. (2005)
Artemia salina	24 hr	Alcohol distillery	1.5%	Santana and Machado (2008)
Artemia salina	24 hr	Textile	55%	Souza et al. (2007)
Artemia sp.	24 hr	Cashew nut industry	1.38 - 2.16 %	Present work

These data indicate that *Artemia*'s sensitivity is rather variable, with LC_{50} values ranging from 1.2% for waste water from an oilfield to almost 80% for a landfill leachate. In fact, the toxicity is intimately associated with the chemical composition of the tested effluent. Studies with leachate water from landfills suggested that ammonium and ammonia are responsible for the acute toxicity observed in *Artemia salina* (Aggelis *et al.* 2003, Svensson *et al.* 2005). In comparison, according to Guerra (2001) and Aggelis *et al.* (2003), the toxicity of chemical plant and olive mill effluents, respectively, is due to the presence of phenolic compounds. The use of a bioreactor, inoculated with the fungi, *Pleurotus ostreatus,* for the treatment of olive mill wastewaster was efficient in removing phenolic compounds and toxicity, suggesting that a correlation exists between these two parameters (Aggelis *et al.* 2003). As previously mentioned, data on the chemical composition of the cashew nut industry effluent are scarce, but preliminary analysis showed high phenol content due to the CNLS contents of cardol, cardanol and anacardic acid. The high toxicity observed for the isolated phenols, cardol and cardanol, potentially contributed to the toxicity of the cashew nut industry effluent.

Conclusion

Overall, these data demonstrate that cashew nut industry effluents are highly toxic in the *Artemia* sp. model and are potentially harmful to the environment. Thus, the acute toxicity test using *Artemia* nauplii could be considered a sensitive, practical and feasible method to monitor the toxicity of effluent generated by the cashew nut improvement industry. Since the crude and treated toxicities exhibited similar toxicities after 48 hr, it can be concluded that the current industrial treatment is inefficient in removing toxic components. An efficient treatment strategy must be adopted by the industry to reduce the environmental impact that results from the production process.

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Received June 2009
Accepted August 2009