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In vitro and *in vivo* toxicity studies of *Agrobacterium radiobacter* k84 biopolymer (ARB)

Estudos *in vitro* e *in vivo* de toxicidade de biopolímero de *Agrobacterium radiobacter* k84 (ARB)

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Abstract

Sugar cane molasses is a cheaper carbon source alternative than glucose traditionally used in fermentation processes. In the present study a biopolymer soluble from *Agrobacterium radiobacter* k84 (ARB) was obtained by fermentation using sugar cane molasses as a carbon source in a process with yield of 10.0 $g.L^{-1}$. The ARB is composed by minerals (40%), carbohydrate (35%) and protein (15%). *In vitro* test of the cytotoxic effect of ARB at concentrations 2.5 mg/mL, 5.0 mg/mL and 10.0 mg/mL in LLC MK_2 (Rhesus Monkey Kidney) cells revealed a 50% cytotoxic concentration (CC_{50}) of 9.32 mg/mL. In a 30-day *in vivo* oral toxicity study, Swiss mice were administered ARB by gavage at 5 mg/mL, 15 mg/mL, 50 mg/mL and 150 mg/mL (approximately 25 mg/kg/day, 75 mg/kg/day, 250 mg/kg/day and 750 mg/kg/day). The results did not present any hematological or histopathological signs of adverse effects, leading us to define the no observed adverse effect level (NOAEL) as 150 mg/mL (approximately 750 mg/kg/day).

Key words: Cane molasses, fermentation, cytotoxicity

Resumo

O melaço de cana-de-açúcar é uma fonte de carbono alternativa de menor custo que a glicose tradicionalmente utilizada em processos fermentativos. No presente estudo, um biopolímero solúvel de *Agrobacterium radiobacter* k84 (ARB) foi obtido por fermentação utilizando melaço de cana de açúcar como fonte de carbono em um processo com rendimento de 10,0 $g.L^{-1}$. O ARB é composto de minerais (40%), carboidratos (35%) e proteínas (15%). O teste do efeito citotóxico do ARB *in vitro* nas concentrações de 2,5 mg/mL, 5,0 mg/mL e 10,0 mg/mL em células LLC MK_2 (Rim de Macaco Rhesus) revelou uma concentração citotóxica 50% (CC_{50}) de 9,32 mg/mL. Em estudo *in vivo* de toxicidade oral durante 30 dias, camundongos Swiss receberam por gavagem soluções de ARB nas concentrações de 5 mg/mL, 15 mg/mL, 50 mg/mL e 150 mg/mL (aproximadamente 25 mg/kg/dia, 75 mg/kg/dia, 250 mg/kg/dia e 750 mg/kg/dia). Os resultados não apresentaram sinais hematológicos ou histopatológicos de efeitos adversos, levando a definir a dose sem efeito adverso observado (NOAEL) como 150 mg/mL (aproximadamente 750 mg/kg/dia).

Palavras-chave: Melaço de cana, fermentação, citotoxicidade

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Agrobacterium radiobacter, first identified as *Alcaligenes faecalis* var. myxogenes, is a non-pathogenic, Gram-negative, aerobic bacterium, typically found in soil, with optimum growth occurring in the temperature range between 25 - 30ºC and the pH range of 6 – 9; this bacterium is known as a producer of exopolysaccharides curdlan and succinoglucan (DUSSAP; DE VITA; PONS, 1991).

Curdlan is a safety insoluble β-1,3 linked glucose polymer which is commercialized as a heat-gelling and water-binding agent, thickener, stabilizer, emulsifier and fat substitute. Succinoglucan is a soluble heteropolysaccharide, industrially produced with applications as emulsion-stabilizing, suspending and thickening agent (MCINTOSH; STONE; STANISICH, 2005). Other potential application is in the agronomic area, since the agrocin produced by *A. radiobacter* is toxic to *Agrobacterium tumefaciens*, the bacterium that causes crown gall disease, common in peach trees (PENALVER et al., 1994).

In the last years, several studies to obtaining biopolymers from *A. radiobacter* fermentation by varying the carbon source and process conditions has been described. It is clear that *A. radiobacter* fermentation has potential in food ingredient development or compounds with other applications, especially as we look to renewable resources as alternatives to products of the chemical industry (PORTILHO et al., 2006).

In biotechnological products obtained by fermentation, between 38% and 73% of the total production cost comes from the raw materials, especially from the organic carbon source. Sugar cane molasses is a cheaper carbon source alternative than glucose traditionally used to make commercial products from *Agrobacterium* cultivation. Its advantages lie in its high concentration of sucrose and a great variety of mineral salts, necessary for industrial metabolite production (OLIVEIRA et al., 2007).

In this work, we evaluated the cytotoxicity of a novel *A. radiobacter* k84 biopolymer (ARB) produced using sugar cane molasses as a carbon source. The cytotoxicity of ARB was determined in LLC MK_2 (Rhesus Monkey Kidney) cells, and the 30-day oral toxicity of ARB was determined in mice.

To be used as substrate for biopolymer production, sugar cane molasses was kindly provided by COROL, Rolândia, Paraná, Brazil. The *A. radiobacter* k84 strain was supplied by Departamento de Ciência e Tecnologia de Alimentos, Universidade Estadual de Londrina, Parana, Brazil.

ARB production - *A. radiobacter* k84 was maintained in a slant of mannitol yeast agar. The culture broth, composed of sugar cane molasses 4ºBRIX, 1.2% yeast extract and traces of mineral salts was used to prepare a 48 h pre-inoculum and a 24 h inoculum of the bacterium at 28ºC and 150 rpm (CUNHA, 2002). A new medium was inoculated (10% v/v) and incubated at 28°C, 150 rpm, for 120 h to produce ARB. The biomass was separated by centrifugation at 12000 x g, for 30 min at 4ºC, and ARB was precipitated 24 h from the supernatant using cold absolute ethanol (3:1 v/v). The ARB were dehydrated at 45ºC overnight, weighed and milled to 60 mesh. ARB centesimal composition and dietary fiber were determined by official AOAC methods (AOAC, 1995).

Cytotoxicity - *In vitro* toxicity was evaluated by the MTT test (Sigma®) using LLC MK2 cells (Rhesus Monkey Kidney Epithelial Cells) in a 96-well plate, according to the manufacturer's instructions. ARB solutions were prepared in sterile Dulbecco's modified eagle's medium to achieve concentrations of 2.5 mg/mL, 5.0 mg/mL and 10.0 mg/mL. The cells reacted with ARB for 72 h and cells grown without test substances were used as controls.

Oral toxicity - ARB solutions were prepared with sterile distilled water at room temperature under agitation and kept under refrigeration until use. Twenty-five male Swiss mice, eight weeks old and free from pathogens were acclimated to a 12/12

h light/dark cycle for one week. The mice were housed in a polystyrene cage with free access to feed and sterile tap water. Mice were divided into five groups of five animals per cage and identified with picric acid solution. ARB solutions (200 µL) of 5 mg/mL, 15 mg/mL, 50 mg/mL and 150 mg/mL, (approximately 25, 75, 250 and 750 mg/kg/day respectively) were administered daily by gavage for 30 days. The control group received 200µL of sterile distilled water. The animals were weighed before treatment, weekly during treatment, and again at the end of the study. Behavior and mortality were observed daily and feed consumption was recorded weekly.

Hematology - At the end of the experiment, animals were anesthetized in an ether chamber, and blood was collected by cardiac puncture. Analysis of red blood cell count (RBC), hematocrit (Hct), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and differential cells count were conduced.

Histology - Necropsies were performed on all study animals. Liver and kidney were analyzed macroscopically. For microscopic examination tissues were fixed in aqueous Bouin, processed, embedded in paraplast, sectioned to a thickness of 7 µm, and stained with hematoxylin and eosin. Histological analysis of organs was done by three specialists considering a damage scale from 0-4 (GERMOLEC et al., 2004).

Statistical analysis - Data were presented as mean +/- SD of three genuine experiments. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis of the dose-response curve. In the 30-day oral toxicity, treated and control groups were compared by ANOVA with significance $p \leq 0.05$, using Statistica 7.1 (STATISTICA, 2006).

Agrobacterium radiobacter k84 growing in molasses 4°BRIX medium yielded approximately 10 $g.L^{-1}$ of a brown powder with 40% minerals, 35% carbohydrate, 15% protein and 10% moisture. Approximately 3 $g.L^{-1}$ of biomass were recovered from the cultivation broth after ARB production conduced three times without variation. The carbohydrate identification of ARB revealed exclusive composition by glucose and dietary fiber analysis detected only soluble fiber.

After 72 hours of exposure to ARB cell proliferation decreased in a dose-dependent manner. Treatment with 2.5 mg/mL ARB in the cell culture medium decreased cellular proliferation by 5% as compared to untreated controls. At the concentration of 5.0 mg/mL, 23.48% of the cells died, and when 10.0 mg/mL was applied, the loss of cellular viability was 55.91%. From this analysis, a CC_{50} of 9.32 mg/mL was calculated; this concentration reduced the cell viability by 50%.

Every animal survived until the end of the experiment, and none of the animals presented altered behavior. Body weight gains (Table 1) and feed consumption in all ARB concentrations tested were comparable to control group values.

Table 1. Mean weekly body weight (grams) of mice administered ARB by gavage for 30 days.

Day	Dose (mg/mL)				
	0	**5**	**15**	**50**	**150**
Initial (Day 0)	35.51 ± 3.1	38.84 ± 1.6	39.12 ± 4.9	39.36 ± 1.5	34.22 ± 4.1
7	36.40 ± 2.4	37.30 ± 2.8	38.66 ± 2.8	40.20 ± 1.7	32.82 ± 5.1
14	38.94 ± 2.7	41.62 ± 1.2	42.26 ± 3.6	44.20 ± 3.3	36.08 ± 3.5
21	40.30 ± 2.4	42.90 ± 1.5	44.12 ± 4.7	45.90 ± 4.1	39.42 ± 3.5
30	40.88 ± 2.8	44.32 ± 1.6	44.08 ± 5.1	45.04 ± 4.2	41.44 ± 3.9

No treatment-related macroscopic findings were identified in treated animals at necropsy, and light microscopic examinations did not show significant differences between organs of ARB-treated and control group animals.

Hematological parameters (Table 2 and Table 3) of treated animals exposed to all doses of ARB were considered normal, as these values were comparable to those of the control group and were similar to values considered normal for Swiss mice.

Table 2. Means of red blood cell parameters of mice administered ARB by gavage for 30 days.

Parameter	Dose (mg/mL)				
	0	5	15	50	150
Red blood cells (millions/ mm^3)	9.1 ± 0.4	7.6 ± 0.4	6.3 ± 0.9	6.8 ± 1.5	8.5 ± 0.4
Hemoglobin (g/dL)	13.4 ± 2.2	13.1 ± 1.4	13.3 ± 1.0	15.0 ± 2.0	18.0 ± 3.5
Hematocrit (%)	49.5 ± 5.5	51.0 ± 7.0	49.3 ± 3.7	47.3 ± 4.1	46.5 ± 2.4
Mean corpuscular volume (μ^3)	50.8 ± 2.4	70.2 ± 11.5	74.6 ± 7.5	73.9 ± 11.5	52.9 ± 2.6
Mean corpuscular hemoglobin (pg)	17.2 ± 1.3	17.8 ± 1.2	21.2 ± 5.0	20.8 ± 3.8	21.6 ± 8.0
Mean corpuscular hemoglobin concentration (%)	28.0 ± 6.1	27.0 ± 4.6	27.0 ± 3.2	31.0 ± 3.1	39.0 ± 9.7

Table 3. Means of white blood cell parameters of mice administered ARB by gavage for 30 days.

Parameter	Dose (mg/mL)				
	0	5	15	50	150
Neutrophfils (%)	11.0 ± 2.8	13.0 ± 6.3	11.2 ± 5.1	13.6 ± 8.9	14.8 ± 9.9
Lymphocytes (%)	83.6 ± 2.9	81.0 ± 7.4	83.0 ± 5.0	80.6 ± 9.2	80.0 ± 8.9
Monocytes (%)	4.6 ± 1.9	4.8 ± 1.5	4.2 ± 1.8	4.4 ± 1.7	4.2 ± 1.6
Eosinophils (%)	0.8 ± 1.1	1.3 ± 0.9	1.6 ± 0.9	1.4 ± 1.1	1.3 ± 1.3

From the results of the 30-day toxicity ARB study in mice, the oral no observed adverse effect level (NOAEL) was determined to be the highest concentration administered (150 mg/mL, approximately 750 mg/kg/day).

The novel crude biopolymer named ARB (*Agrobacterium radiobacter* k84 biopolymer) is ethanol precipitated from the extracellular substances generated through the fermentation of *A. radiobacter* k84, using sugar cane molasses as a carbon source. The ARB obtaining process generates a product of economical interest with the property to form viscous solutions when mixed with water at room temperature with a significant increase in viscosity due the gelation under heating. The ARB composition showed a polymer different from curdlan and succinoglucan.

The composition of the ARB can be interesting to health because of its mineral, soluble fiber and protein contents. In humans, minerals are important regulators of enzymatic activity, they facilitate the transport of essential compounds through

cell membranes, and they maintain both the acid-base equilibrium and osmotic pressure (SINGH, 2004). Soluble fibers are known to benefit human heath by maintaining the normal function of the gastrointestinal tract, reducing serum cholesterol and inducing satiety at meal time (MANNING; GIBSON, 2004). Protein is also important compound for the human body. Considering that ARB has interesting rheological properties and potential health benefits, the toxicological data is essential as a question of food safety.

In the present study the dose-response curve obtained from MTT test was similar to the 50% inhibitory concentrations observed for vegetal extracts like garlic, cinnamon and ginger (CHARLES et al., 2002). These findings were reported as the initiation of a database tool for methods developed to assess comparative toxicity of foodstuffs, without the indication that these foods are inherently unsafe.

The lack of negative effects of ARB on behavior, body weight, hematology, and organ histology suggests that it is safe for consumption in mice. From the NOAEL of 150 mg/mL (approximately 750 mg/kg/day) defined in the ARB 30-day oral toxicity study the Acceptable Daily Intake (ADI = NOAEL/100) was calculated as 7.5 mg/kg/day (NESTMANN; LYNCH, 2007). The daily amount of ARB allowed to an adult weighting 70 kg would be 525 mg/day. The absence of toxic effects in mice represents a favorable perspective for the use of ARB, considering that animal models are usually good predictors for human toxicities of around 70–80% (EISENBRAND et al., 2002).

Regarding the correlation between cytotoxicity and oral toxicity results obtained in this work, it is important to consider that *in vitro* studies are helpful in describing the cellular effects of the substances, but that they cannot determine the magnitude of animal or human risks (BRENT, 2004). The *in vitro* to *in vivo* extrapolation in tests with mixtures such as ARB, is more complicated due to varying bioavailability and the possibility of interactions between constituents (AUFDERHEIDE, 2008). However, ARB safety could be expected, seeing as *A. radiobacter* strain k84 has been registered since 1979 with no reports of adverse effects to the environment and to the living species (EPA, 2005). Additionally, there are no adverse effects reported about the polymers curdlan and succinoglucan produced by this bacterium.

The results showed that the sugar cane molasses, a cheaper carbon source, can be used in fermentation processes to obtaining an *Agrobacterium radiobacter* k84 (ARB) biopolymer with technological properties and without *in vitro* and *in vivo* toxicity.

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