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PRELIMINARY ASSAYS FOR LEMONGRASS ESSENTIAL OIL ECOTOXICOLOGICAL TEST IN *D. SIMILIS* AND *C. SILVESTRII* SUBMITTED TO GAMMA RADIATION

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ABSTRACT

Pharmaceutical products are of great interest in ecotoxicological studies due to being found some of these products in the superficial waters and sediments, water and sewage treatment effluents. It was verified an increase of insect repellent chemical products in the aquatic environment because of the increase of diseases transmitted by mosquitoes like dengue. As these compounds show toxicity, the use of essential oils natural products with repellent properties is increasing and the literature about the impact in the aquatic environment is scarce. The hydric frame would suffer natural radiation and radiations from energy generation nuclear plants impacts *fall out* of tests and nuclear accidents. There is no universal definition of environmental protection and there are few studies on radiation effects in the aquatic environment. In this study was determined the lemon grass essential oil toxicity level as well as the lethal dose of ionizing radiation, LD_{50}, in aquatic organisms. Cytotoxicity test was performed by *in vitro* neutral red uptake method in NCTC clone L929 cell line. In the LD_{50} test aquatic organisms were submitted to gamma radiation. The essential oil of lemongrass *Cymbopogon flexuosus* showed cytotoxicity index IC_{50} about 50μg.mL^{-1}. The LD_{50} for *Daphnia similis* was 242 Gy and *Ceriodaphnia silvestrii* about 525 Gy. Studies will be continued with acute and chronic ecotoxicological tests of lemongrass essential oil in natural organisms and in organisms submitted to gamma radiation, utilizing the results obtained in this work.

1. INTRODUCTION

In the last 20 years occurred a resurgence of diseases caused by mosquito bite because population increase and urbanization, deforestation, precarious habitation situation , garbage and sewage systems, resistance to drugs and insecticides by vector and genetic changes in pathogens. These factors have contributed to the increase of mosquito populations and ease of contact between these animals and humans [1].
The intensive use of synthetic repellents has caused the increased release and contaminate in aquatic ecosystems because they are persistent organic compounds. These compounds are called “emerging contaminants” that are mainly pharmaceuticals products.

Recently, the monitoring of pharmaceuticals and personal care products in the environment has been gaining great interest in ecotoxicological studies because many of these substances

are frequently found in environment matrices such as surface water and sediments, effluent of sewage water treatment plants in the world [2].

Plant-based repellents have been used for generation as a personal protection measure against mosquito bites. Knowledge on traditional repellent plants obtained through ethnobotanical studies is valuable resource for the development of new natural products. Actually, commercial repellent products containing plant-based ingredients have gained increasing popularity among consumers, as these are commonly perceived as “safe” in comparison to long-established synthetic repellents [3].

As synthetic repellents are persistence organic compounds and show toxicity to humans, the use of essential oils natural products with repellent properties is increasing and can lead to greater release of these substances in the aquatic environment. The impact of these substances in the aquatic environment is scarce in the literature.

The environmental radioactivity or radioecology is a recent area which studies the effects of ionizing radiation on the environment [4].

Humans and animals are submitted to constant exposure to environmental radioactivity. Radioactive sources can be found in the background radiation, cosmic rays and radioactive substances present in the Earth’ crust, building materials, fall out of tests nuclear explosions, release of radioactive materials by nucleus energetic plants during the production of nuclear energy as well as in air and in food [6].

The absence of data about dose limits and reference dose of radiation is one of the reasons that concern environmental organizations in protecting against radiation. Many authors have developed studies with irradiated organisms to determine doses that cause interference in their life cycle. They are aquatic and terrestrial animals which must be considered keystone species in the environment in question.

This paper is part of a study to determine the toxicity of essentials oils with repellent properties in aquatic organisms used in ecotoxicological tests and compare with synthetic repellent.

Initially, the *in vitro* cytotoxicity test will be performed to determine the concentration of lemongrass essential oil that causes 50% death of cell population in order to be used this concentration as base to start ecotoxicological tests with aquatic invertebrates.

Gamma radiation DL_{50} assay will be used to determine the radiation dose level to be used in the lemongrass essential oil ecotoxicological test with irradiated and non irradiated organisms.

2. METHODOLOGY

The essential oil used in the study was obtained by Ferquima® produced from lemongrass *Cymbopogon flexuosus*. It was used two different lotes: L180 and L183.
The genus *Cymbopogon* includes about 30 species and many of these species are commercially important in the pharmaceutical, agricultural, personal care products and

cosmetics industries. Most are native to the tropical Old World and currently can be found in tropical and subtropical regions of the world [7].

2.1 *In vitro* cytotoxicity test

The assessment of cell death can be based in the integrity of cell membrane, ascertained by the uptake of foreign molecules into the cell, for example, neutral red. In this work the evaluation of cytotoxicity was performed by using neutral red uptake assay. Positive and negative controls are necessary to confirm the adequate performance of the test procedure [8,9].

The cytotoxicity assay was carried out with the exposure of cell culture to the lemongrass essential oil solution which stayed in contact for 24h. The cytotoxic effect was evaluated using the capacity of living cells uptake neutral red dye. The used mouse connective NCTC clone 929 cell line was acquired from American Type Culture Collection (ATCC) bank.
Two lots of lemongrass essential oil of *Cymbopogon flexuosus* were used in this paper and was diluted in dimethyl sulfoxide (DMSO) at concentration of $1x10^5$µg/mL. This solution was diluted in Minimun Eagle Medium (MEM) containing 10% fetal calf serum and 1% non-essential amino acids (work-MEM) at 1:100 (10^3µg/mL) to be used in the assay and the greater concentration was 500 µg/mL.

The cells were maintained in work-MEM in a humidified incubator with 5% CO_2 at 37ºC. The cells were detached by 0.2% trypsin and 0.2mL of the cells suspension, about $2.5x10^5$ cells/mL, were seeded in flat bottomed 96 microplate wells. The microplate was incubated for 24h at 37ºC in a CO_2 humidified incubator. After this period, the medium was discarded and replaced with 0.2mL of serially diluted essential oil solution (50, 25, 12.5 and 6.25%).
Control of cell culture wells were replaced by fresh work-MEM. In the same microplate extracts of positive control (natural rubber latex) and negative control (HDPE) were used. Samples and controls were tested in triplicates. The microplate was incubated again for 24h under the same conditions.

After the incubation period, the medium was replaced by neutral red solution (50µg/mL) and the microplate was maintained at 37ºC for 3h. Then the dye medium was discarded and the microplate was washed twice with phosphate buffered solution pH 7.4. The cells were washed with a solution of 1% $CaCl_2$ in 0.5% formaldehyde. The rupture of cells and neutral red release was obtained by addition of 0.2mL/well of extractant solution containing 50% ethanol in 1% acetic acid. The absorbances were read on an ELISA reader spectrophotometer Sunrise of Tecan with 540 nm filter. The viability percentage was calculated with the average of obtained optical density compared with control cells, considered 100%.

2.2 Gamma radiation lethal dose (LD_{50})

Gamma radiation LD_{50} is the irradiation dose which provokes the death of 50% of organisms population in the assay.

In this experiment was used freshwater microinvertebrates *Daphnia similis* and *Ceriodaphnia silvestrii* (Cladocera, Crustacea) which are commonly used for ecotoxicological testing of

both existing and new chemical substances. These aquatic organisms are known as water flea and are excellent representatives of Cladocera, a key group of organisms in freshwater systems. The disturbance of this population may have effects throughout the aquatic food chain. They are commonly used in toxicity tests worldwide.

Following the ABNT [10,11] guideline, organisms were maintained in continuous parthenogenetic reproduction conditions in the Laboratory of Ecotoxicology of the IPEN, São Paulo, Brazil.

D. similis and C. silvestrii were cultured in glass beakers in an incubator kept at 20ºC (± 2ºC) and 25ºC (± 2ºC) respectivelly, with 12 h-light to 12h-dark cycle. The culture medium was freshwater (pH 7.0-7.6; conductivity 120-150µS/cm; and hardness 40 to 48 mg $CaCO_3$ L^{-1}). Organisms were fed with an algal suspension *Pseudokirchneriella subcapitata* at $5x10^5$ cells mL^{-1} and a mixture of fermented fish food. The medium was renewed three times a week as feeding mixture.

The exposure to gamma radiation test was performed according to the methodologies of two studies from Sarapultseva & Bychkovskaya [12] and Gilbin *et al*. [13] with some modifications.

Three assays were performed using organisms exposed to different gamma radiation doses. In each experimental unit, four replicates with five organisms (<24h old) of each cladoceran species in polystyrene Falcon tube containing 10mL freshwater were irradiated by gamma rays from Co-60 source in Gammacell 200 at dose rate 1.70 kGy/h, with 100, 200, 400, 800 and 1600 Gy doses.

After irradiation the organisms *D. similis* were maintained in an incubator at 20ºC (± 2ºC) and *C. silvestrii* at 25ºC (± 2º C) both in the irradiated culture medium and the mortality was verified after 48h. Control animals were in the same conditions as experimental, but were not irradiated.

3. RESULTS

In the cytotoxicity test, with the average of optical density of each dilution of samples, negative and positive controls was calculated the viability percentage in relation to cell control (100%), presented in Table 1.

Table 1. Cell viability results of lemongrass essential oil in the cytotoxicity test.

Concentration of solution (%)	Cell viability ± cv (%)					
	Negative control	Positive control	Lemongrass essential oil			
			Lot 180 (1)	Lot 180 (2)	Lot 183 (1)	Lot 183 (2)
100	105±14	01±0	08±03	08±03	08±0	08±01
50	101±07	52±15	08±05	08±05	08±06	08±02
25	93±07	100±09	07±13	08±05	09±08	08±03
12.5	93±09	112±10	35±16	37±07	38±07	40±17
6.25	86±13	103±14	72±05	74±03	70±05	76±13

Plotting the percentage viability in relation to extract concentration was obtained the viabilities curves in the graphic, presented in Fig.1.

In this graphic, the cytotoxicity index, IC_{50}, can be obtained in the intersection of viability curve and IC_{50} line. IC_{50} is the extract concentration which injures or kills 50% of cell population in the assay.

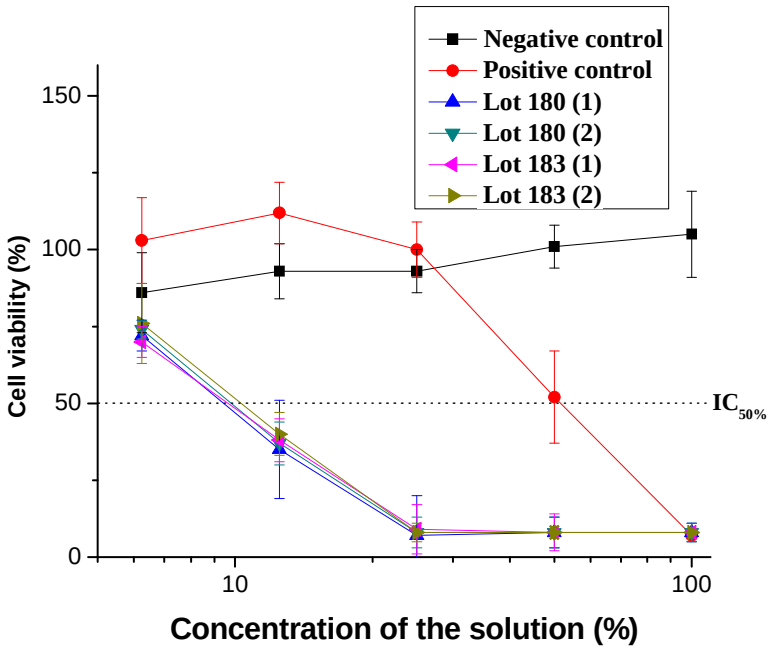

Figure 1. Cell viability curves of lemongrass essential oil in the cytotoxicity assay.

The tested samples of lemongrass essential oil showed toxic effect at around 10% solution concentration. The 100% extract concentration was 500 $\mu g.mL^{-1}$ and the IC_{50} of the samples were: Lot 180 (1) = 47$\mu g.mL^{-1}$; Lot 180 (2) = 49$\mu g.mL^{-1}$; Lot 183 (1) = 48$\mu g.mL^{-1}$; Lot 183$\mu g.mL^{-1}$ (2) = 52$\mu g.mL^{-1}$. The average of IC_{50} for lot 180 was 48μg/mL and for lot 183 was 50$\mu g.mL^{-1}$.

The results of organism survival in the gamma radiation lethal dose assay, LD_{50} were presented in Table 2.

Table 2. *C. silvestrii* and *D. similis* survival results in the gamma radiation LD_{50} assay.

Dose (Gy)	Survival ± cv (%)	
	C. silvestrii	***D. similis***
0 (Control)	100±06	100±03
100	100±03	102±0
200	100±03	61±20
400	72±09	08±08
800	02±03	0±0
1600	0±0	0±0

Plotting the survival percentage in relation to radiation dose it was obtained the dose-response curve, showed in Figure 2.

The radiation lethal dose (LD_{50}) is obtained in the intersection of dose-response curve and 50% survival line. LD_{50} is the gamma radiation dose which kills 50% of the organism population in the assay.

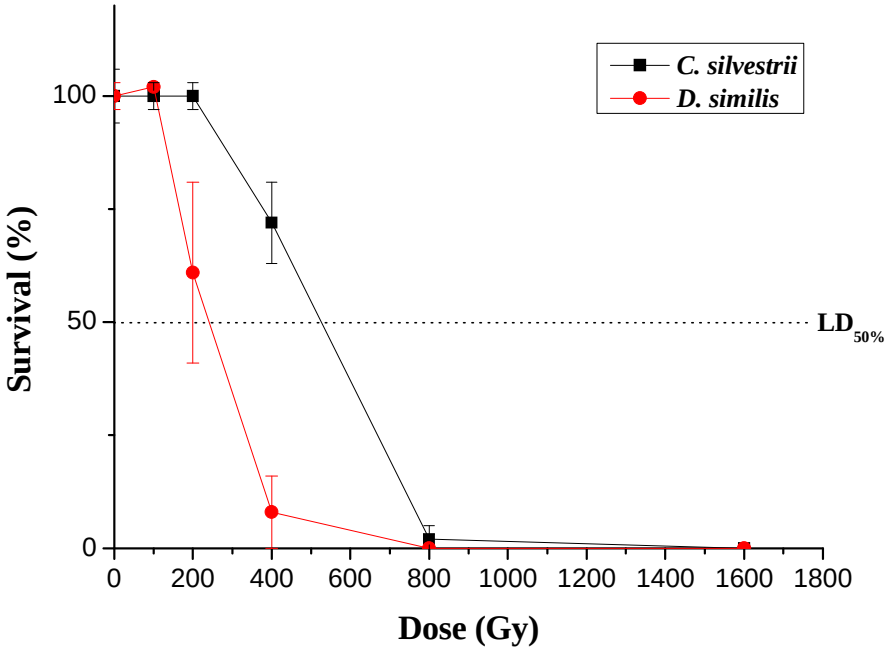

Figure 2. Dose-response curves of gamma radiation LD_{50} on *C. silvestrii* and *D. similis*.

The LD_{50} for *Ceriodaphnia silvestrii* was 525 Gy while for *Daphnia similis* was 242 Gy. In the sensitization assay utilizing standard reference substance, *C. silvestrii* showed higher sensibility than *D. similis*. But in the LD_{50} assay was contrary, *C. silvestrii* showed higher resistance to gamma radiation than *D. similis*.

The LD_{50} values of both species were much higher than the dose considered lethal of 100 Gy by Choppin *et al.* for the class Crustacea [14]. This difference would be due to the different methodology applied by authors as well as species of Cladocera used, radiation time and radiation dose rate.

Sarapultseva & Bychkovskaya determined the lethal doses for *Daphnia magna*: $LD_{80/30}$ = 100 Gy, $LD_{100/16}$ = 250 Gy e $LD_{100/4}$ = 600 Gy. The death of animal had been checked every day for 30 days. The authors observed the inheritance of the effect in first brood and elimination of damage in subsequent generations. Also detected which the effect does not increase with increasing dose in this dose range 0.1 to 20 Gy. These results support the idea of the universal nature of this unusual form of reaction [12].

Gilbin *et al.* kept *Daphnia magna* at low dose rates of external gamma radiation (from 0.4 to 31mGy.h^{-1}) over a 23 day period (i.e. 5 broods). In the paper were observed the at 31mGy.h^{-1}, decreased resistance of starvation in relation to dose and possible mechanisms of gamma radiotoxicity for daphnid reproduction [13].

Marshall detected the fecundity decrease at 300 $mGy.h^{-1}$ and death rate begins to rise rapidly above 50 roentgens h^{-1} for *Daphnia pulex*. The organisms were irradiated for an average of about 19 hours each day with radiation levels ranging from 20 to 75 $roentgens.h^{-1}$ [15].

Independent of applied methodologies the ionizing radiation affect the reproduction and resistance of neonates produced during the test.

3. CONCLUSIONS

The present study provide results to continue the study of "lemongrass essential oil ecotoxicological test in *D. similis* and *C. silvestrii* submitted to gamma radiation".

The cytotoxicity assay determined the lemongrass essential oil concentration to be used as a base to start ecotoxicological tests with aquatic invertebrates.

Gamma radiation DL_{50} determined the radiation dose level to be used in the lemongrass essential oil ecotoxicological test with irradiated and non irradiated organisms.

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