CYTO AND GENOTOXIC EVALUATION OF THE AQUEOUS EXTRACT FROM *Psacalium* **peltatum (MATARIQUE) IN HUMAN LYMPHOCYTE CULTURES**

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Summary

It has been demonstrated experimentally that the aqueous extract from "matarique", *P. peltatum,* diminishes the glucose concentration in rat blood and also that it is cytotoxic to prostate cancer cells *in vitro*. In order to determine the cytotoxicity and genotoxicity of this extract on normal cells, human lymphocyte cultures were exposed to different concentrations of that extract for 48 h. Results showed that the mitotic index (MI) increased in a significant manner at the extract concentration of 10 µg/ ml but it did not act as a mitogenic agent. Its possible effect might be on Phtytohemaglutinin, the mitogenic added to stimulate lymphocyte proliferation. The extract was cytotoxic at 100 µg/ ml since the MI decreased significantly. The kinetic proliferation remained unaltered as well as the chromosome diploid number and structure. Although the proportion of cells with DNA damage and the DNA migration length significantly increased from concentration 10 µg/ ml, the highest value registered fall into the minimum damage range. Thus, we concluded that the aqueous extract from *P. peltatum* is partially cytotoxic and genotoxic.

Key words: *P. peltatum.* Aqueous extract, cytotoxicity, genotoxicity.

Psacalium peltatum is a plant traditionally used because of its hypoglycemic activity and is consumed in the form of decoction (1, 2). The aqueous fraction of this species also has bacteriostatic effects on *Bacilus subtilis*, *Escherichia coli* SOS, E. coli (ATCC 8739), *Proteus mirabilis* (NCTC 2896), *Salmonella typhy* (ATCC 6539) and *Staphilococus aureus* (ATCC6538). More recently it has been also found that the same fraction is cytotoxic to human prostate cancer cells *in vitro* (3).

There are different experimental models to test the toxicity of a given agent *in vitro*. Human lymphocyte cultures from peripheral blood have been preferred because of the relative availability, they are easy to handle and give results in a short time (4, 5). The most frequently cellular and DNA damage markers used with this model are: the mitotic index (MI) which gives information about cell survival to the exposition to a particular agent and if they keep proliferating normally (6); through the replication index (RI), it is possible to determine if the exposed cells replicate in the same period of time as the unexposed, that is to say, cell proliferation kinetics (CPK). Changes in the diploid chromosome number indicate disruption of the mechanism of chromosome distribution during cell division (mitosis) while chromosome structural changes (AC) reflect chromatin damage (7). By means of the "comet assay" (single cell electrophoresis) it is possible to determine if the agent induces single DNA chain breaks (8, 9, 10). In this paper we evaluated the cytotoxic and genotoxic potential of the aqueous extract of *P. pelatatum*, *in vitro*.

Materials and methods

Whole blood samples from 10 adult-healthy donors (5 female and 5 male) were taken. Aliquots of 0.3 ml of whole blood were placed into plastic sterile tubes (Nunc) containing 2.5 ml of Mc Coy 5a culture medium (Microlab) added with 4 % Phytohemaglutinin M (PHA; Microlab) and 0.4 % Antibac (Microlab). Cultures were incubated at 37° C for 72 h. After the firs 24 h of incubation, from each donor's sample 4 groups of cultures were formed: a control and 3 experimental ones which were exposed to the following final concentrations of aqueous extract of *P. peltatum*: 1 μg/ ml, 10 μg/ ml y 100 μg/ ml, respectively. Then all culture tubes were re-incubated at 37° C for 48 h more. The extract was previously sterilized by filtration.

Three cultures were taken from each group of each donor to determine the MI. Before cell harvesting, cultures were added with 0.06 ml of Colcemid (Microlab) to arrest cells in metaphase. Cells were separated from the culture medium by centrifugation, then they were exposed to a hypotonic treatment (0.4 % KCl ; J. T. Baker) for 20 min and fixed with Carnoy's solution (acetic acid:methanol, 1:3; J. T. Baker). Slides were prepared by the air dried technique and stained with 10 % Giemsa (Merck). The MI was calculated from a total of 6000 cells (dividing + non dividing cells) for each group per donor.

Variations in chromosome number and structure were studied using the same slides. The numbers of chromosomes present in each of 120 mitoses/group/donor; modal numbers were calculated afterwards. 60 mitoses of excellent quality were analysed to determine the frequency of single and double gaps and breaks.

The three cultures from each group/donor destined to the CPK analysis were added with 200 μg/ ml 5-BrdU (Sigma) 48 h before harvesting. Cells were also treated with Colcemid and collected as previously described. Slides were stained following the procedure of fluorescence + Giemsa: forst, they were immersed in Hoechst 33258 (Sigma) for 40 min, mounted with Sörensen's buffer (pH 6.8) and exposed to dark light for 2 h and then, stained with 10% Giemsa (Merck) in Sörensen's buffer (pH 6.8). The frequency of cells in the first, second and third round of replication were recorded in an average number of 113 cells/group/donor in order to calculate the RI.

The single cell electrophoresis method was applied to the last 3 cultures from each group. Cells were washed twice with PBS, mixed with 0.5 % agarose (LMP; Sigma) and placed onto fully frosted slides which surface had been previously covered with agarose; when the gel solidified a third layer of agarose was added. Slides were immersed into a lysis solution for at least 1 h at 4 °C; then they were transferred to the electrophoretic chamber containing the electrophoresis buffer for 20 min and finally subject to an electric current of 25 V, 300 mA for 20 min more. After being washed with neutralization buffer, the slides were stained with ethidium bromide (Sigma) and kept in darkness at 4 ° C overnight. Microscope analysis was performed with a fluorescence microscope (Olympus) connected to an image analyser and a computer with the VIDS software. From a total of 300 cells/group/donor, the proportion of cells with DNA damage as well as the DNA average migration length was calculated.

Results and Discussion

The average MI determined for the control and experimental groups is shown in Table 1. There is a significant increase (Tukey, $p<0.05$) of around 20 % in cultures exposed to the final concentration of 10 μg/ml which led us to consider the possibility of the extract having a mitogenic effect. Although the difference between the control and the group exposed to 100 μg/ml is no statistically significant (Tukey, $p>0.05$), we did not discard that part of the cellular population was sensitive to this concentration or that they lost their capability to respond to the PHA stimulus.

In order to test the mitogenic activity of the aqueous extract from *P. peltatum*, we cultured blood samples from other 6 healthy donors and formed 2 groups: the control stimulated with PHA (Microlab), the experimental one stimulated with 10 μg/ml extract and determined the MI for both. Data showed that the extract is not mitogenic since the MI in cultures without PHA was zero. Possibly the extract enhanced the PHA stimulating potential in the first experiment and, because of this, the MI was higher.

Table 1. Average Mitotic Index (MI) registered in human lymphocyte cultures exposed to different concentrations of the aqueous extract from *P. peltatum* (EE, Standard Error).

	Control	1 μg/ml	10 μg/ml	100 μg/ml
MI + EE	3.48 + 0.32	3.06 + 0.46	4.23 + 0.69	2.63 + 0.23

Human diploid chromosome number, 2n = 46, was present in all groups thus indicating that the aqueous fraction from matarique did not perturb chromosome distribution during mitosis; neither the frequency of chromosomal aberrations was altered. Thus, we concluded that this extract is neither aneuploidogenic nor clastogenic.

Table 2 shows the CPK results. It can be observed that in all groups, most of the cells were in their second replication cycle and the IR calculated is similar among groups. The differences are not significant (χ^2, $p>0.05$). This observation suggests that cells that survive to the presence of the extract do not reduce nor extend their life cycle span although the amount of proliferating cells can increase or decrease as seen with the MI. These results support the idea that not all cells survive to the presence of the extract at 100 μg/ml since it does not delay replication.

Table 2. CPK and Replication Index (RI) registered in human lymphocyte cultures exposed to different concentrations of the aqueous extract from *P. peltatum* (M1, M2 and M3, frequency of cells in 1st., 2nd. and 3rd . cycle of replication, respectively).

Group	M1	M2	M3	IR
Control	31	66	3	1.69
1 μg/ml	52	73	3	2.06
10 μg/ml	47	61	2	1.75
100 μg/ml	39	76	3	2.0

With regard to the comet assay, data in Table 3 show that the fraction of cells with single chain breaks increases in a significant way from the group of cultures exposed to 10 μg/ml (χ^2, $p< 0.05$) and the average migration length is also significantly longer from the same concentration (Tukey, $p<0.05$). Nevertheless, when analysing the distribution of migration length in all groups it is noted that the maximum value registered falls into the range of lower damage and the possibility exists for the cell to repair it. On the basis of these results, the aqueous fraction of *P. peltatum,* is not genotoxic.

Table 3. Proportion of lymphocytes with damaged DNA after exposition to to different concentrations of the aqueous extract from *P. peltatum.* The electrophoretic migration length of the DNA molecule is also presented.

	Control	1 μg/ml	10 μg/ml	100 μg/ml
Cells with damaged DNA (%)	44.76	53.20	58.64	59.33
Average migration length (μm ± EE)	13.42 ± 1.44	14.03 ± 1.95	17.21 ± 2.05	12.44 ± 0.81
Migration length between 1-20 μm (% cells)	88	84.3	72.44	91.64

Conclusions

The aqueous extract from matarique, *P. peltatum*, is partially cytotoxic and genotoxic from concentration of 10 μg/ml since it alters MI and increases the fraction of cells with DNA damaged but the extract does not affect CPK, nor is it aneuploidogenic or clastogenic with any of the concentrations tested.

References

1. Hernández-Galicia E, Aguilar-Contreras A, Aguilar-Santamaría L, Roman-Ramos R, Chavez-Miranda AA, García-Vega L M, Flores-Saenz JL. Studies on Hypoglycemic Activity of Mexican Medicinal Plants. Proc West Pharmacol Soc 2002; 45:118- 124.
2. Contreras-Weber C, Pérez-Gutiérrez S Alarcón-Aguilar FJ, Román-Ramos R. Anti-Hyperglicemic Effect of *Psacalium peltatum*. Proc West Pharmacol Soc 2002; 45:134- 136.
3. Almanza–Pérez J. Efecto de tres extractos orgánicos y dos acuosos de *Psacalium peltatum* sobre la proliferación *in vitro* de células hematopoyéticas y células humanas transformadas. Tesis de Licenciatura en Biología Experimental. Universidad Autónoma Metropolitana-Iztapalapa. México. 2004.
4. Ballantyne B, Marrs T, Turner P, eds. General and Applied Toxicology, USA: M Stocton Press, 1994.
5. Gonsebatt M, Herrera L, Ostrosky-Wegman P Lymphocyte proliferation as a biomarker in environmental monitoring. In: Butterworth, F., ed. Biomonitors and biomarkers as indicators of environmental change, USA: Plenum Press, 1995.
6. Ostrosky-Wegman P, El índice mitótico y la cinética de proliferación linfocitaria en el monitoreo biológico. Gac Med Mex 1994; 130:432-437.
7. Miller OJ, Therman E, Human Chromosomes, USA: Springer, 2001.
8. Singh N, McCoy M, Tice R, Schneider E, A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 178: 184-191.

9. Tice R Applications of the single cell gel assay to environmental biomonitoring for genotoxic pollutants. In: Butterworth, F, ed. Biomonitors and biomarkers as indicators of environmental change, USA: Plenum Press, 1995.
10. Tice R, Agurell E, Anderson D, et al. Single cell/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 2000: 35:206-221.