Vol.48, Special : pp. 57-61, October 2005
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

In Vitro Study of *Vellozia pusilla* Pohl (Velloziaceae), a Brazilian Plant Species: Antitumoral Activity and Labeling of Blood Elements

Ana Letícia Almeida Dantas[1,2*], Lígia Maria Marino Valente[2], Lilia Aparecida Salgado de Morais[3], Glaucio Feliciano[4] and Mário Bernardo-Filho[4]

[1]Instituto de Radioproteção e Dosimetria - IRD -CNEN; Serviço de Monitoração Interna; Av. Salvador Allende, s/n; Recreio dos Bandeirantes; 22780-160; adantas@ird.gov.br; Rio de Janeiro - RJ - Brasil. [2] Universidade Federal do Rio de Janeiro; Instituto de Química; Departamento Química Orgânica; Centro de Tecnologia; Bloco A; 21910-240; Rio de Janeiro - RJ - Brasil. [3]Instituto de Tecnologia em Fármacos; Fundação Oswaldo Cruz; Av. Brasil, 4365; Manguinhos; 21045-900; Rio de Janeiro - RJ - Brasil. [4]Universidade do Estado do Rio de Janeiro; Instituto de Biologia Roberto Alcântara Gomes; Departamento de Biofísica e Biometria; Av. 28 de setembro, 87; 20551-030; Rio de Janeiro - RJ - Brasil

ABSTRACT

Vellozia pusilla Pohl is a species of the botanic family Velloziaceae that occurs in the subtropical regions of South America and, although it lives under conditions of high solar irradiation and low water availability, shows great longevity. The methanol extract of roots, stem and leaf sheaths of this species showed an antitumoral activity through the inhibition of the enzyme Topoisomerase I when analyzed by an in vitro bioassay employing DNA repair or recombination deficient mutants of the yeast Saccharomyces cerevisiae. In the evaluation of the effect of Vellozia pusilla methanol extract on the labeling of RBC, blood of mice was treated with ^{99m}Tc tracer solutions. The percentage of radioactivity (%ATI) bound to plasma (P) and blood cells (BC) was determined. The %ATI in the insoluble fraction of plasma (IF) was also evaluate, and the results showed that there was a decrease in %ATI in this fraction that represents the plasmatic proteins.

Key words: Drug interaction, antitumoral activity, technetium-99m, blood elements, Velloziaceae

INTRODUCTION

The Velloziaceae constitutes a family of tropical plants occurring in South America and Africa. The Brazilian species occurring in the subtropical regions, especially in the state of Minas Gerias, Bahia, Goiás, Mato Grosso, Rio de Janeiro and coastal Santa Catarina. These plants grow in a characteristic ecosystem fully exposed on mountain sides in rocky or sandy soils. Although plants in the Velloziaceae family live under conditions of high solar irradiation and low water availability, they show surprising longevity (Ayensu, 1973). Previous studies on the specie *Vellozia pusilla* Pohl have afforded several terpenoids, including isopimarane nor-diterpenoid and diterpenoids, cleistantane diterpenoids and lupenone (Pinto et al.,1988; Dantas et al., 2003). There are reports concerning the use of some species of Velloziaceae in Brazilian and African folk medicine (Ayensu, 1973). Some Brazilian species of the genus *Vellozia* have been shown to

* Author for correspondence

be active against *Schistossoma mansoni, Trypanossoma cruzi* and Erlich carcinoma (Sampaio et al. 1984). A bioactive extract of *Vellozia candida* yielded a moderately DNA-damaging 6,7-*seco*-rosane diterpenoid through a bioassay-guided fractionation (Valente et al. 1997).

Based on the knowledge that many tumor cells have defects in their ability to repair damage to DNA as compared with normal cells, agents with selective toxicity towards repair-deficient cells might be potential anticancer agents. In this paper, studies of the MeOH extract of *Vellozia pusilla* of this botanical family was carried out using mechanism-based yeast bioassay that utilizes DNA repair- or recombination-deficient mutants of *Saccharomyces cerevisiae* yeast (Johnson et al., 1986) with the aim of discovering DNA-damaging and/or topoisomerase I and II inhibitor agents. This mechanism-based screening depends on differential responses of DNA repair-deficient and repair-proficient yeast strains to the sample under investigation (Gunatilaka et al. 1994). The methanol extract of *Vellozia pusilla* Pohl, collected in 1978, was tested and showed a significant activity. Based on these results, another collection was carried out in 1993, and the new methanol extract presented about the same bioactivity. This type of activity is similar to that of compounds like Camptothecin and Topotecan, both used in chemotherapy for solid tumor and hematologic malignancies (Cohen et al., 1999). The DNA Topoisomerase enzymes play a fundamental process in the cells, including replication and recombination and are an important therapeutical target in cancer chemotherapy.

Technetium 99m (^{99m}Tc) has been the radionuclide most utilized in clinical nuclear medicine procedures for diagnosis. It has also been used in basic research to study the influence of drugs on the labeling of red blood cells (RBC) and plasma proteins and in the biodistribution of this radionuclide in vivo. The labeling technique involves pre-tinning the RBC with stannous ions, followed by exposure to technetium 99m pertechnetate, which is reduced within the cell and remains trapped intracellulary by binding to the beta chain of hemoglobin. The use of medicinal plants or natural products has increased all over the world, and there are some studies about the effect of the medicinal plants on the labeling of RBC (Braga et al., 2000). In the present work, the effect of *Vellozia pusilla* extract on the labeling of RBC, plasma and cellular proteins with ^{99m}Tc was evaluated. Mouse blood was treated with ^{99m}Tc tracer solutions. The percentage of radioactivity (%ATI) bound to plasma (P) and blood cells (BC) was determined. The %ATI in the insoluble fraction of plasma was also evaluated.

MATERIALS AND METHODS

Plant material

The plants were collected at Serra do Cipó and Diamantina, Minas Gerais, Brazil. *Voucher* specimens were deposited at the Departamento de Botânica of the Universidade de São Paulo, SP, Brazil.

Extraction

The shade-dried and powdered whole plant materials were sequentially extracted with hexane, AcOEt and MeOH at room temperature. After the evaporation of the solvents under low pressure, some of the dried extracts were submitted to the *in vitro* bioassays.

Antitumoral in vitro bioassay

Four yeast strains have been used: RAD+, *rad6, rad52* and *rad52.top1*. The assay was performed by introducing the test sample (crude extract) dissolved in a 1:1 v/v mixture of MeOH/DMSO into a 100 μL well in agar plates separately impregnated with normal "wild-type" RAD+ yeast cells (strain derived from a mutation that makes the cell wall of the yeast more permeable to drugs but capable of DNA repair) and with mutant yeast cells and incubating the plates for 48 hrs at 30 ^{o}C. If the test sample contained a DNA-damaging agent or a topoisomerase inhibitor, then it would inhibit the growth of one or more mutant strains, producing a zone of inhibition. First, a single dose test of the crude extracts was carried out at the concentration of 2000 μg/mL. Those extracts that showed an inhibition zone ≥10 mm for the mutant strains and <10 mm for RAD+ were submitted to a dose response curve where four or five different doses were used for each sample, with the dose range dependent on the magnitude of the inhibition zone previously observed. The results are expressed as IC_{12} values that represent the concentration of the test sample (in μg/mL) required to produce an inhibition zone 12 mm in diameter. An active extract should have an IC_{12} for

the wild-type RAD+ strain equal to or greater than three times the IC_{12} for one of the mutant strains. For topoisomerase I inhibitors, the IC_{12} value for *rad52.top1* should be at least threefold larger than that for *rad52*, while for topoisomerase II inhibitors the reverse should be true. The activity of yeast in each plate was tested by the use of a known anticancer drug (positive control): for RAD+, *rad52* and *rad52.top1*, camptothecin (Johnson et al. 1986) and for *rad6*, streptonigrin (Gutanilaka et al. 1994).

Labeling of Blood Elements

The experimental procedure was accomplished without sacrificing the animals. Samples (0.5 mL) of mouse blood were incubated with the solutions of the methanol extract of *Vellozia pusilla* in NaCl 0.9 % for 60 minutes. The concentrations of the extract were 100; 50.0; 25.0; 12.5 and 6.25% of the stock solution containing 5.0 mg/mL. After this period, 1.2 ppm stannous chloride ($SnCl_2$) and 100 μL of ^{99m}Tc tracer solution (3.7 MBq) were added (this solution was recently milked from a $^{99}Mo/^{99m}Tc$ generator IPEN/CNEN). After incubating for 10 minutes, these samples were centrifuged and plasma (P) and blood cells (BC) were separated. Aliquots of 20 μL of P and BC were precipitated with 1.0 mL of 5% trichloroacetic acid (TCA), and the soluble (SF) and insoluble fractions (IF) were isolated. The ^{99m}Tc radioactivity was counted in a NaI (Tl) well counter (Automatic Gamma Counter, 1272 Clinigamma, LKB, Wallac, Finland). The percentage of radioactivity (%ATI) bound to P and BC was determined.

RESULTS AND DISCUSSION

The hexane extract of *V. pusilla* from the 1978 collection showed the same moderate anti-tumor activity that was confirmed for the similar extract from the 1993 collection. The results obtained from the comparison of the activity of the methanol extract of a *Vellozia pusilla* and Camptotecin compound, when analyzed by *in vitro* bioassay employing DNA repair or recombination deficient mutants of *Saccharomyces cerevisiae* (rad+, rad 52y and rad 321) are presented in Table 1. The MeOH extract of *V. pusilla* presented an IC_{12} value for *rad52.top1* three to four times larger than that for *rad52* and, although exhibiting a high degree of inhibition for the RAD+ strain, might be considered a potential topoisomerase I inhibitor.

The analysis of the influence of drugs on the labeling of red blood cells and plasma proteins (Table 2) showed that there is a decrease in %ATI in IF of plasma, which represents the plasmatic proteins, for all the concentrations of extract solutions.

Table 1 - The comparison of the activity of the methanol extract of *Vellozia pusilla* and Camptotecin when analyzed by *in vitro* bioassay employing DNA-repair or recombination-deficient mutants of the yeast *Saccharomyces cerevisiae* (rad+, rad 52y and rad 321)

	Yeast mutants (results in μg/mL)*			
Samples	rad +	rad 52y	rad 321	Relation
MeOH extract[a]	47	62	136	1:1:3
MeOH extract[b]	30	30	125	1:1:4
Camptotecin	36	0.92	5.8	39:1:6

*This results represents de necessary concentration of sample to inhibit the grow of the yeast
[1] extract obtained in 1978
[2] extract obtained in 1993

Table 2 - Distribution of the radioactivity on labeling of blood cells (Cell), in the fixation of ^{99m}Tc by the insoluble fraction of plasma (IFP) and in the fixation of ^{99m}Tc by the insoluble cell fraction (IFC), treated with different concentration of metanol extract of *Vellozia pusilla* Pohl.

Fraction	Control	100%	50%	25%	12,5%	6,25%
Cell	94 ± 2	89 ± 4	94 ± 1	96 ± 2	96 ± 4	85 ± 13
IFP	71 ± 4	51 ± 11	60 ± 9	48 ± 10	63 ± 2	49 ± 12
IFC	90 ± 2	89 ± 4	94 ± 2	92 ± 1	91 ± 3	89 ± 5

The lower concentration was probably sufficiently to alter the value from about 70 to 55 %. These results indicated an interaction between the substances in the methanol extract and the plasmatic proteins blocking the bonds with ^{99m}Tc. There was no interaction of the red blood cells with the substances in the extract, as shown by the values of (BC).

Results observed in the two experiments suggest a significant competition between ^{99m}Tc and the compounds present in the extract. The activity of the extract in the inhibition of the enzyme Topoisomerase I in the *in vitro* bioassay and the results observed in the distribution of the radioactivity in labeling of blood cells suggested a specific interaction with proteins. Future work will be performed with compounds like camptothecin with the aim of correlating these results

ACKNOWLEDGMENTS

The authors are grateful to Dr. Bernardo M. Dantas for revising the manuscript.

RESUMO

Vellozia pusilla Pohl é uma espécie de planta da família Velloziaceae que ocorre em regiões subtropicais da América do Sul e, apesar de viver sob condições de alta incidência solar e baixa disponibilidade de água, apresenta grande longevidade. O extrato metanólico das raízes, caule e folhas desta espécie apresentou atividade antitumoral através da inibição da enzima Topoisomerase I quando utilizado o bioensaio *in vitro* que emprega cepas mutantes *Saccharomyces cerevisiae* que apresentam deficiência na reparação ou recombinação do DNA. Na avaliação do efeito do extrato metanólico de *Vellozia pusilla* na marcação dos elementos sangüíneos com tecnécio 99m, sangue de rato foi tratado com solução de ^{99m}Tc como traçador sendo determinado o percentual de radioatividade (%ATI) no plasma (P) e nas células vermelhas (BC). As frações solúvel e insolúvel do plasma também foram avaliadas. Os resultados mostraram que houve um decréscimo de %ATI na fração insolúvel do plasma que é representada pelas proteínas plasmáticas.

REFERENCES

Ayensu, E. S. (1973), Biological and morphological aspects of the Velloziaceae. *Biotropica*, **5**, 135-149.

Braga, A. C. S.; Oliveira, M. B. N.; Feliciano, G. D.; Reiniger, I. W.; Oliveira, J. F.; Silva, C. R. and Bernardo-Filho, M. (2000), The effect of drugs on the labeling of blood elements with technetium^{-99m}. *Current Pharmaceutical Design*, **6** : (11), 1179-1191.

Cohen, D. P.; Adams, D. J.; Flowers, J. L.; Wall, M. E.; Wani, M. C.; Manikumar, G.; Colvin, O. M. and Silber, R. (1999), Pre-clinical Evaluation of SN-38 and Novel Camptothecin Analogs Against Human Chronic B-cell Lymphocytic Leukemia Lymphocytes. *Leukemia Research*, 23 : (11), 1061-1070.

Dantas, A. L. A. (1997), *Estudo do extrato metanólico bioativo e obtenção de padrões a partir do extrato hexânico não ativo de vellozia pusilla Pohl (Velloziaceae)*, Dissertação (Mestrado), Instituto de Química, UFRJ, Rio de Janeiro. 145 pp.

Dantas, A. L. A.; Valente, L. M. M. and Pinto, A. C. (2003), A new isopimarane diterpenoid fom *Vellozia pusilla* Pohl (Velloziaceae). *J. Braz. Chem. Soc*., **14**, 126-128.

Gunatilaka, A. A. L.; Kingston, D. G. I. and Johnson, R. K. (1994), Mechanism-based isolation and structures of some anticancer active natural products. *Pure & Appl. Chem.*, **66**, 2219-2222.

Johnson, R. K.; Bartus, H. F.; Hofmann, G. A.; Bartus, J. O.; Mong, S. M.; Faucettte, L. F.; McCabe, F.L.; Chan, J. A. and Mirabelli, C. K. (1986), Discovery of new DNA-reactive drugs. In: Hanka, L. J.; Kondo, T. and White, R. J. (Eds.). *In vitro* and *in vivo* models for detection of new antitumor drugs. In: International Congress of Chemotherapy, 14., Kyoto. *Proceedings…* Kyoto. pp. 15-26.

Pinto, A. C.; Valente, L. M. M. and Silva, R. S. (1988), "Norditerpenoids from *Vellozia pusilla*", *Phytochemistry*, **27** : (12), 3913-3915.

Sampaio, M. R. F. P.; Nakamura, I. T.; Oliveira Jr, B. S. and Oliveira, M. M. (1984), Modificação do método de DiPaolo & Moore para testes de extratos e substâncias anticancerígenas – Parte I. *Arq. Inst. Biol*., **51**, 35-39.

Valente, L. M. M.; Gunatilaka, A. A. L.; Kingston, D. G. I.; Patitucci, M. L. and Pinto, A. C. (1997), A bioactive *seco*-rosane diterpenoid from *Vellozia candida*. *J. Nat. Prod*., **60**, 478-481.

Received: July 05, 2005;
Revised: July 14, 2005;
Accepted: August 01, 2005.