6α-Acetoxy-7α-Hydroxy-Vouacapan Isolated from *Pterodon pubescens* Benth. Fruit’s with Selective Activity against Prostate Cancer Cell Line: Artifact or Natural Product?

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**Abstract**

Challenges encountered in standardization of plant-derived drugs involves the determination of many parameters that implies quality control methods throughout the entire process from growing conditions to the final product. This study highlights the complexity of factors involved in the production of secondary metabolites in plants. Compound 6α-acetoxy-7α-hydroxy-vouacapan isolated from *Pterodon pubescens* Benth. (Fabaceae) fruit dichloromethane extract, with in vitro cytotoxic selectivity on human prostate cancer cell line was investigated to determine if the compound is an artifact produced overtime on plant storage or is characteristic of plant genotype.

The chemical composition of samples from São Carlos (São Paulo, Brazil) and Ponto Chique (Minas Gerais, Brazil) were monitored monthly during one year for geranylgeraniol, 6α-acetoxy-7α-hydroxy-vouacapan, 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester. In vitro cytotoxicity screening against human prostate cancer cell line, evaluating total growth inhibition parameter (TGI) displayed higher selectivity and potent anticancer activity with TGI 11.43 μg mL⁻¹ when higher 6α-acetoxy-7α-hydroxy-vouacapan over total vouacapan content ratio (3.14) was achieved. Nevertheless, 6α-acetoxy-7α-hydroxy-vouacapan maintained approximately the same content throughout the year among the samples in opposition to overall vouacapan content. Samples from São Carlos at time zero had 26% geranylgeraniol content whereas Minas Gerais samples contained the highest content of 1.3%.

Throughout the stability test geranylgeraniol concentration decreased with a straight relationship of overall increase of vouacapan content. The chemical variability data was evaluated using the statistical procedure based on Principal Component Analysis (PCA) to determine which of the extract’s components had a real impact on the in vitro antiproliferative activity.

Data presented herein did not suggest that 6α-acetoxy-7α-hydroxy-vouacapan is produced as artifact overtime in storage.

Further studies are needed to establish other markers involved with therapeutic activity to provide consistency of the development of products with quality, efficiency, and safety.

**Keywords:** *Pterodon pubescens* Benth.; Fabaceae; 6α-acetoxy-7α-hydroxy-vouacapan; Anticancer activity; Diterpene furans; Genotype

**Introduction**

Natural products have long demonstrated their important role as a rich source of new targets for drug discovery. The huge structural diversity of natural compounds together with unique mechanism of action has prompted promising applications in the fields of medicine, pharmacy and biology [1].

Among challenges encountered in product outcome is standardization of plant inputs for the pharmaceuticals industry: Factors such as soil, climate conditions, genotypes, harvesting, and post-harvest conditions have shown to have an important affect both on chemical and biological activity. Therefore, growth control of cultivars and their processing have a straight outcome on health benefits and economic issues. According to Ibáñez-Marcelo and Alarcon [2], Phenotypes arise from the combination of genotype together with the environment where the species is grown.

Previously our research group reported the antinociceptive properties of compounds isolated from *Pterodon pubescens* Benth. fruit (Fabaceae) that are commercially available in Brazil. The plant's crude alcoholic extract is used in folk medicine in anti-inflammatory, analgesic, and anti-rheumatic preparations. Phytochemical studies of *Pterodon* genus revealed the presence of alkaloids, isoflavones and diterpenes. Furan diterpenes were identified and isolated from *Pterodon* species. Authors [3-5] reported that furanoditerpenes possessing vouacapan skeleton are involved with the anti-inflammatory, antinoceptive and antiproliferative properties of *Pterodon pubescens* fruit crude extract. Among compounds reported involved with the antinoceptive and anticancer activity were geranylgeraniol, 6α-acetoxy-7α-hydroxyl-vouacapan, 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ester, and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester.
was used as carrier gas (0.7 bar, 1 mL min⁻¹). The MS were taken at 70 eV. Scanning speed was 0.84 scans s⁻¹ from 40 to 550. Sample volume was 1 μL. Split: 1:40. Column chromatography (CC): silica gel (0.063 x 40 mm, Merck®), UV detection and anisaldehyde solution. TLC (thin layer chromatography): precoated plates with 5% trichloroacetic acid and cell proliferation determined by spectrophotometric quantification (540 nm) of cellular protein content.

The chemical variability encountered in samples from different origins throughout the country prompted the study of the secondary metabolite 6α-acetoxy-7α-hydroxy-vouacapan variability among samples encountered in Minas Gerais and São Paulo states of Brazil, to determine if the compound was consequence of the production as an artifact throughout storage.

Material and Methods

Chemical analysis

IR spectra were recorded on a JASCO-FT/IR-410 spectrometer. ¹H, ¹³C NMR and 2D experiments were conducted on a Varian Inova-500 spectrometer (11 T). Chemical shifts were recorded in CDCl₃ solutions and quoted relative to TMS (δH 0.0, 1H NMR) and CHCl₃ (δC 77.0, 13C NMR). High-resolution electron impact ionization mass spectrometry (HREIMS) was recorded on a VG-AutoSpec high resolution mass spectrometer (70 eV) using direct probe. GC-MS was carried out using PC-03.

Phytodendron pubescens (Pp) fruits were previously collected in São Carlos, São Paulo in March 2012 and identified by Prof. Dr. Jorge Carlos, São Paulo in March 2012 and identified by comparison with an authentic voucher deposited at the University of Campinas (Unicamp) Herbarium, under number UEC 2012 and further deposited under number UEC 179140. The voucher specimen was deposited at the University of Campinas (Unicamp) and further deposited under number UEC 179140. The obtained fractions (FR) were: hexane (FR1) (0-450 mL); hexane/ethyl acetate (95:5) (FR2) (500-900 mL); hexane/ethyl acetate (60:40) (1400-1800 mL) (FR5-6); hexane/ethyl acetate (40:60) (1900-2300 mL) (FR7); rest flushed with methanol. The isolated compounds were obtained from the crude oil (18.2 g) that was purified on pre-column chromatography using silica gel (Merck 7734) (5 x 60 cm). The obtained fractions (FR) were: hexane (FR1) (0-450 mL); hexane/ethyl acetate (95:5) (FR2) (500-900 mL); hexane/ethyl acetate 1 (80:20) (FR3-4) (1000-1350 mL); hexane/ethyl acetate 2 (60:40) (1400-1800 mL) (FR5-6); hexane/ethyl acetate (40:60) (1900-2300 mL) (FR7); rest flushed with methanol. The resulting fractions were monitored by thin layer chromatography (TLC) in comparison to previously purified standards, exposed with anisaldehyde reagent (50 volume). 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester (Figure 1).

Further monthly sampling used fruits collected (1000 g), that were maintained at 40°С throughout the 330 days of analysis with exception of samples taken at time zero. A total of thirteen samples were analyzed from São Carlos and Minas Gerais origins respectively at times 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300 and 330 days. Every month the whole batch was homogenized and samples in triplicate were collected for analysis.

The extracts were monitored monthly for α-flavonoids, 6α-acetoxy-7α-hydroxy-vouacapan, 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ester, 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester and 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester content and total growth inhibition against prostate cancer cell lines (PC-03).

The extracts were monitored monthly for geranylgeraniol, 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester content and total growth inhibition against prostate cancer cell lines (PC-03).
using sulforhodamine B stain assay. Using the concentration-response curve for each cell line, TGI (concentration that produces total growth inhibition) was determined through non-linear regression analysis using software Origin 7.5 (OriginLab Corporation) [6].

### Principal Component Analysis (PCA)

The data correlations obtained from São Paulo (n=21) and Minas Gerais (n=22) samples were plotted in the light of PCA (Principal Component Analysis). The different parameters (variables) were analyzed by a multivariate approach considering TGI values compared to geranylgeraniol, isomers 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ether and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ether, 6α-acetoxy-7α-hydroxy-vouacapan, 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ether content, total vouacapan content (considering the sum of the vouacapans monitored) and geranylgeraniol over 6α-acetoxy-7α-hydroxy-vouacapan ratio content throughout twelve months maintained at 40°C. These variables in 43 columns were submitted to principal component analysis (PCA) with autoscaling pretreatment, resulting initially in a 43 x 7 data matrix. For analysis based on TGI potency a 21 x 7 (São Paulo) and 22 x 7 (Minas Gerais) data matrices were proposed. The Unscrambler 8.0 (CAMO, Norway) chemometrics package was employed for PCA calculations.

### Results and Discussion

The overall extraction yield was approximately the same ranging from 2.9-3.2% (m/m), without affecting overall 6α-acetoxy-7α-hydroxy-vouacapan content among different extraction process of the same sample. The extracts of Minas Gerais samples were monitored monthly for geranylgeraniol and vouacapans content and total growth inhibition against prostate cancer cell lines (PC-03) (Table 1).

Fraction 7 (10 g), containing vouacapan compounds was successively chromatographed by CC on silica-gel (70-230 mesh) (5 x 60 cm) and eluted with hexane/dichloromethane (7:3) (900-1800 mL) yielded 6α-acetoxy-7α-hydroxy-vouacapan (333 mg, 3% yield), Rf 0.75; hexane/dichloromethane (6:4) (1900-2600 mL) yielded 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ether and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ether (990 mg, 9.9% yield), Rf 0.56; hexane/dichloromethane (2:8) (2650-3100 mL) yielded geranylgeraniol (963 mg, 9.63% yield), Rf 0.29; (3350-4100 mL) yielded 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ether (1.2 g, 12% yield) Rf 0.17.

The different parameters analyzed by a multivariate approach considering TGI values compared to geranylgeraniol and vouacapans content, and geranylgeraniol over 6α-acetoxy-7α-hydroxy-vouacapan ratio content throughout twelve months maintained at 40°C. The original

#### Table 1: Minas Gerais sample Extracts monitored monthly for geranylgeraniol, 6α-acetoxy-7α-hydroxy-vouacapan, 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ether, 6α-7α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ether and total growth inhibition against prostate cancer cell lines (PC-03).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Extraction method</th>
<th>6α-acetoxy-7α-hydroxy-vouacapan</th>
<th>6α,7α-di-hydroxy-vouacapan-17α-oate methyl ether</th>
<th>6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ether and isomer</th>
<th>Total vouacapan compounds</th>
<th>TGI value (µg mL⁻¹)</th>
<th>Geranylgeraniol / vouacapan ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Soxhlet</td>
<td>1.40 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>7.82 ± 0.14</td>
<td>11.92 ± 0.15</td>
</tr>
<tr>
<td>30</td>
<td>Soxhlet</td>
<td>1.25 ± 0.20</td>
<td>0.23 ± 0.04</td>
<td>0.23 ± 0.04</td>
<td>0.05 ± 0.01</td>
<td>8.77 ± 1.47</td>
<td>12.64 ± 2.29</td>
</tr>
<tr>
<td>60</td>
<td>Soxhlet</td>
<td>1.04 ± 0.02</td>
<td>0.27 ± 0.07</td>
<td>0.27 ± 0.07</td>
<td>0.04 ± 0.01</td>
<td>6.69 ± 0.29</td>
<td>10.02 ± 0.78</td>
</tr>
<tr>
<td>90</td>
<td>Soxhlet</td>
<td>1.02 ± 0.08</td>
<td>0.25 ± 0.03</td>
<td>0.25 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>7.67 ± 0.66</td>
<td>9.92 ± 0.79</td>
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<tr>
<td>120</td>
<td>Soxhlet</td>
<td>1.28 ± 0.01</td>
<td>0.23 ± 0.04</td>
<td>0.23 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>6.07 ± 0.36</td>
<td>9.72 ± 0.74</td>
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<tr>
<td>150</td>
<td>Soxhlet</td>
<td>1.10 ± 0.12</td>
<td>0.27 ± 0.04</td>
<td>0.27 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>5.64 ± 0.17</td>
<td>8.81 ± 0.66</td>
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<tr>
<td>180</td>
<td>Soxhlet</td>
<td>1.14 ± 0.02</td>
<td>0.20 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>5.19 ± 0.10</td>
<td>8.19 ± 0.77</td>
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<tr>
<td>210</td>
<td>Soxhlet</td>
<td>0.82 ± 0.09</td>
<td>0.28 ± 0.03</td>
<td>0.28 ± 0.03</td>
<td>0.02 ± 0.01</td>
<td>4.85 ± 0.08</td>
<td>7.93 ± 0.43</td>
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<tr>
<td>240</td>
<td>Soxhlet</td>
<td>0.24 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>3.97 ± 0.15</td>
<td>4.37 ± 0.07</td>
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<tr>
<td>270</td>
<td>Soxhlet</td>
<td>0.35 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>3.17 ± 0.10</td>
<td>3.95 ± 0.07</td>
</tr>
<tr>
<td>300</td>
<td>Soxhlet</td>
<td>0.54 ± 0.06</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>2.88 ± 0.08</td>
<td>2.91 ± 0.06</td>
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<tr>
<td>330</td>
<td>Soxhlet</td>
<td>0.48 ± 0.04</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>2.68 ± 0.07</td>
<td>2.68 ± 0.07</td>
</tr>
</tbody>
</table>

Statistical evaluation of extraction methods (Soxhlet vs RST)

| ANOVA (F-test) | 0.0262 | 0.1155 | 0.0407 | 0.3976 | 0.1072 | - | - |
| Linear correlation (r) | 0.9598 | 0.8831 | 0.9887 | 0.7770 | 0.7640 | - | - |

* F<sub>crit</sub>=4.30 at 95% confidence level.
Considering all the samples (n=43) a complete separation based on the geographic location (São Paulo and Minas Gerais) was possible. The score plots separate groups by similarity and the loading plot indicate the variables that influence the separation. In accordance with scores plot (Figure 1S and Graphic a) a good separation could be observed of groups. The variables that contributed most significantly to the separation were the sum of isomers 6α-hydroxy-vouacapan and 6α-hydroxy-7α-oate methyl ester and the sum of isomers 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester and 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester content (Figure 1S and Graphic b).

Previous work reported by Cabral et al. [7] highlighted the chemical ESI(+)–MS fingerprinting profiles of samples from different geographical regions of Brazil, showing essentially the same constitutes with small variations in abundances. According to Cabral et al. [7] the results showed that PCA extracted three major principal components. The plot of PC1 versus PC2 versus PC3 accounted for ca. 99% of data variance (PC1=62.87%, PC2=32.62%, PC3=3.46%) and showed that all the oils from the different states in Brazil were clearly grouped. Samples from Sergipe and Mato Grosso states had a higher absolute 6α-acetoxy-7α-hydroxy-vouacapan content compared to samples from São Paulo, Minas Gerais and Bahia states. Previously our group [3] demonstrated that activity guided fractionation of Pterodon pubescens, purchased at commercial market, methylene chloride-soluble fraction afforded novel 6α-acetoxy-7α-hydroxy-vouacapan and 6α,7α-di-acetoxy-vouacapan, 7α-di-acetoxy-vouacapan and 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ester. The isolated compounds were evaluated for in vitro cytotoxic activities against human normal cells and tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCA-03 (ovarian), PC-03 (prostate), HT-29 (colon), 786-0 (renal), K562 (leukemia) and NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance). These compounds showed selectivity in a concentration-dependent way against human PC-03. The main component of that sample extract was 6α-acetoxy-7α-hydroxy-vouacapan. Further work by the same authors [3] with samples freshly collected at São Paulo state to continue with evaluation of the antinociceptive activity of the crude extract contribution demonstrated differently to contain mainly geranylgeraniol and 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ester with only traces of 6α-acetoxy-7α-hydroxy-vouacapan. Thereafter Servat et al. [8] demonstrated the antinociceptive properties of samples obtained at Minas Gerais state containing mainly isomers 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester. The variability observed among the samples prompted the investigation to determine if the differences observed were consequence of chemical modifications of the original secondary metabolites encountered in the fruits due to samples post-harvest conditions or if this was consequence of different genotypes among the species. In order to standardize an extract for the production of an herbal medicine those issues need to be well understood. Therefore this study monitored monthly during one year the geranylgeraniol, 6α-acetoxy-7α-hydroxy-vouacapan, 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester content of samples from São Carlos, São Paulo and Ponto Chique, Minas Gerais freshly picked and further observed monthly to verify if there was an increase of 6α-acetoxy-7α-hydroxy-vouacapan content overtime when maintained under 40°C temperature.

A statistical evaluation of the extraction procedures for Minas Gerais samples was performed by applying ANOVA and correlation analysis (Table 1), and we observed that there was no significant difference between the Soxhlet and RTS extraction methods (at 95% confidence level) with high linear correlation for both extraction methods.

In vitro cytotoxicity screening against human prostate cancer cell line displayed higher selectivity and potent anticancer activity with TGI 11.43 μg mL⁻¹ when higher 6α-acetoxy-7α-hydroxy-vouacapan over total vouacapan ratio (3.14) was achieved. Nevertheless, 6α-acetoxy-7α-hydroxy-vouacapan maintained approximately the same content throughout the year among the samples in opposition to overall vouacapan content (Figure 2).

Samples from São Carlos at time zero had 26% geranylgeraniol content whereas Minas Gerais samples only achieved the highest content of 1.3%. Throughout the year, the samples maintained at 40°C demonstrated geranylgeraniol concentration to decrease with a straight relationship with the overall increase of total vouacapan content. Among the chemometrics tools, the score plot of PCA analysis from Minas Gerais samples, which showed different group patterns of samples evaluated (Figure 3a), seem to demonstrate tendencies that

![Figure 2](image_url)
The development of an herbal medicine, one of the greatest challenges in the production of secondary metabolites in plants. To enable the in vitro antiproliferative activity differences observed.

The correlations of data obtained from Minas Gerais samples were plotted in the light of PCA. The different parameters were analyzed by a multivariate approach considering TGI values compared to geranylgeraniol, isomers 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester, 6α-acetoxy-7α-hydroxy-vouacapan and 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ester content throughout twelve months maintained at 40°C. As reported in the score plot (Figure 3a) the PCI includes most of the information (up to 51% of the total variance) due to 6α-acetoxy-7α-hydroxy-vouacapan ratio over total vouacapan content. The high loading values of geranylgeraniol, isomers 6α-hydroxy-7α-acetoxy-vouacapan-17α-oate methyl ester and 6α-acetoxy-7α-hydroxy-vouacapan-17α-oate methyl ester, 6α-acetoxy-7α-hydroxyl-vouacapan and 6α,7α-di-hydroxy-vouacapan-17α-oate methyl ester variables confirm, according to multivariate analysis, the major role of TGI potency where PC2 explains 27% of the total variance. The score plot reported in Figure 3a shows the inhibition of prostate cell line (PC-03) growth in the space of the two new variables PCI and PC2. Moving along PCI from left to right in the graph, we find different patterns of grouping with low anticancer potency (highest TGI values) close to the moderate anticancer potency samples separated, being observed the formation of different groups separation (Figure 2S).

Ultimately, the loadings plot reported in Figure 3b shows that variable with major influence in separation group with high anticancer potency (lowest TGI values) was 6α-acetoxy-7α-hydroxy-vouacapan, whereas the other variables were responsible for grouping samples with low and moderate anticancer potency. Samples from São Paulo (n=21) showed no obvious groups separation (Figure 2S).

This study highlights the complexity of factors involved in the production of secondary metabolites in plants. To enable the development of an herbal medicine, one of the greatest challenges is plant input standardization in order to meet efficacy, safety and reproducibility final product's requirements as recommended by Brazilian Sanitary Regulatory Agency (ANVISA).

Conclusion

The aim of this work was to interpret the analytical data for the understanding of *Pterodon pubescens* Benth. Fruit features necessary to enhance in vitro antiproliferative proprieties viewing plant input standardization. The combination of antiproliferative tests, comparative analyses, and chemometric evaluation provided information to characterize the species properties involved with important in vitro antiproliferative events of PC-03 cancer strains.

Data presented herein suggest that 6α-hydroxy-7α-acetoxy-vouacapan plays an important role over PC-03 cancer cell line growth inhibition. This furanditerpene over the time span studied was not affected by temperatures applied during extraction production process or storage temperature.

The significant 6α-hydroxy-7α-acetoxy-vouacapan concentrations detected among samples from different Brazilian regions could be attributed to different genotypes other than post-harvest and atmosphere conditions.

The application of PCA allowed the evaluation of similarities and differences between samples, being observed the formation of three main groups from Minas Gerais samples based on total growth inhibition (TGI) against prostate (PC-03) cell lines.

Further studies with microsatellite markers, one of the most informative and versatile DNA-based markers used in plant genetic research, are recommended to understand the variability among this species permitting to establish the most convenient phenotype for standardization of plant input.

The authors declare no competing financial interest.

Supplementary Information

Supplementary data relative additional PCA analysis.

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