

# Plant-derived extracts P2Et and Anamu-SC affect NO and ROS levels in leukemic cells

Juan José Arévalo-Ferrin<sup>1</sup>, Jimmy Alejandro García-Ortiz<sup>1</sup>, Cindy Mayerli Arévalo-Olaya<sup>1</sup>, Sandra Milena Quijano-Gómez<sup>1</sup>, Susana Fiorentino-Gómez<sup>1</sup>, Viviana Marcela Rodríguez-Pardo\*<sup>1</sup>

## Edited by

Angela Johana Espejo Mojica  
editorus@javeriana.edu.co

1. Grupo de Inmunobiología y Biología Celular, Facultad de Ciencias, Pontificia Universidad Javeriana. Bogotá D.C., Colombia.

\*vivianar@javeriana.edu.co

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## Abstract

Leukemic cells often show high nitric oxide (NO) and reactive oxygen species (ROS) levels. These can lead to resistance to apoptosis and therapy and increased proliferation. Plant-derived extracts decrease chemoresistance in cancer cells. In this study, we evaluated the effects of the plant-derived extracts P2Et (*Caesalpinia spinosa*) and Anamu-SC (*Petiveria alliacea*) and their combination with chemotherapeutic agents on NO and ROS levels in leukemic cell lines K562 and Reh. NO and ROS were determined using the DAF-FM DA and H2DCFDA probes. The mean fluorescence intensity for each variable was measured by flow cytometry. The extracts showed an antioxidant effect on both cell lines leading to a significant decrease in ROS levels without decreasing cell viability. Anamu-SC also increased NO levels in K562 cells when combined with idarubicin. Both extracts reduced the number of leukemic cells after 12 hours of treatment. Further studies are necessary to evaluate their effect on primary human leukemia cells. These findings suggest the potential of P2Et and Anamu-SC as adjuncts in leukemia treatment.

**Keywords:** *Caesalpinia spinosa*, flavonoids, leukemia, nitric oxide, *Petiveria alliacea*, reactive oxygen species.

## 1. Introduction

Acute leukemias (AL) are tumors of hematopoietic origin, generated by the malignant transformation and proliferation of hematopoietic stem cells or hematological progenitors. Because of their malignant status, tumor cell clones harbor genetic alterations providing advantages of growth, improvement, and expansion, and facilitating their suppression and replacement of normal hematopoiesis [1, 2].

In cancer, nitric oxide (NO) and reactive oxygen species (ROS) have been shown to have a dual effect that depends on their concentration, cellular origin, tumor stage, and microenvironment [3]. Low endogenous concentrations of NO in tumor cells promote proliferation, angiogenesis, and metastasis, whereas high levels induce cell cycle arrest, glycolytic efflux inhibition, and cell death [4]. Similarly, ROS effects are dose-dependent, such that low levels induce cell cycle progression, epithelial-mesenchymal transition (EMT), and metastasis; and high ROS levels promote senescence and apoptosis [5]. In hematological neoplasms, such as leukemias, modulating NO and ROS levels could represent a therapeutic option [6, 7].

An increase of NO levels in acute myeloid leukemia (AML) cells decreases the expression of c-myc and c-myb mRNAs, reducing cell proliferation and viability [8]. In addition, a high level of NO serves as an adjuvant to chemotherapeutic agents that induce leukemic cell apoptosis [9]. Acute lymphoid leukemia (ALL) cells have a high nitric oxide synthase-2 (NOS2) mRNA expression; therefore, the use of NO inhibitors promotes the apoptosis of these cells [8]. Regarding ROS levels, acute leukemia cells revealed overstimulated glycolysis and oxidative phosphorylation to meet their proliferation needs. Consequently, these hematological neoplasm types have high levels of ROS and efficient antioxidant systems hindering the toxic effects of such endogenous ROS levels. However, it has been shown that conventional drugs, such as arsenic trioxide ( $As_2O_3$ ) to treat acute promyelocyte leukemia, cytarabine (Ara-C) for acute myeloid leukemia, or bortezomib for multiple myeloma, increase ROS levels to a toxic status that induces cell death [10].

The modulation of NO and ROS in leukemic cells constitutes a therapeutic option and/or a coadjuvant for conventional chemotherapy. Due to their multi-molecular nature, plant-derived extracts have been shown to have several effects on tumor cell metabolism, such as modulating NO and ROS levels [11, 12]. In our group, we have been working with plant-derived extracts with antitumoral properties. The P2Et extract, derived from the tara tree *Caesalpinia spinosa*, is rich in polyphenols, flavonoids, and triterpenes [13], exerting a high antioxidant capacity and cytotoxic activity on leukemia cell lines that over-express resistance proteins, thus acting synergistically with drugs such as doxorubicin [13, 14, 15]. The extract Anamu-SC, derived from *Petiveria alliacea*, is composed of several secondary metabolites such as benzaldehyde, dibenzyl disulfide (DDS), and dibenzyl trisulfide (DTS) [16]. Anamu-SC can modulate the glycolytic flux [17], thereby decreasing the expression of the  $\beta$ -F1-ATPase [18] and reducing the viability of tumor cell lines [16] and leukemic blasts isolated from patients with AML and ALL [14].

In this study, we evaluated the effect of the plant-derived extracts P2Et and Anamu-SC, alone and in combination with chemotherapeutic agents, on the levels of NO and ROS in the leukemic cell lines K562 and Reh. The aim of this investigation was to evaluate the bioactivity of these extracts as NO and ROS modulators or as a coadjuvant to conventional chemotherapeutic agents used in acute leukemia.

## 2. Materials and methods

### 2.1. Culture conditions of leukemic cells

The K562 (Chronic Myeloid Leukemia in myeloid-erythroid blast crisis) and Reh (Pre-B Acute Lymphoid Leukemia) cell lines were obtained from American Type Culture Collection (ATCC, USA) and were cultivated in RPMI 1640 (GIBCO<sup>®</sup>, USA) medium supplemented with L-glutamine (2 mM, Eurobio<sup>®</sup>, France), HEPES (10 mM, Eurobio<sup>®</sup>, France), sodium pyruvate (1 mM, Eurobio<sup>®</sup>, France), Fetal Bovine Serum (FBS, 10 % Eurobio<sup>®</sup>, France), and Penicillin/Streptomycin (1 % Corning<sup>®</sup>, USA) at 37 °C and 5 % CO<sub>2</sub>.

### 2.2. Obtaining the plant-derived extracts P2Et and Anamu-SC

Plant extracts were obtained from *Caesalpinia spinosa* (P2Et) and *Petiveria alliacea* (Anamu-SC). For the P2Et fraction, *Caesalpinia spinosa* pods and fruits were collected in Villa de Leyva, Boyacá (Colombia), within the polygon delimited by the following coordinates: between 5°37'95" and 5°39'17" North and between 73°32'19" and 73°34'63" West. The specimens were identified by Carlos Alberto Parra of the Colombian National Herbarium (voucher specimen number COL

588448). The P2Et extract was produced following good manufacturing practices and was chemically characterized [13]. For the Anamu-SC extract, *Petiveria alliacea* leaves were collected in Quipile, Cundinamarca (Colombia). This plant material was identified by Antonio Luis Mejia at the Colombian National Herbarium (voucher specimen number COL 333406). The Anamu-SC extract was obtained through supercritical fluids at 60 °C, 400 bar, flow rate 30 kg h<sup>-1</sup>, and 15 % ethyl acetate as a co-solvent [14]. For each test, extracts were reconstituted with 96 % ethanol.

### 2.3. Determination of NO and ROS levels in K562 and Reh cells

To evaluate intracellular NO and ROS levels,  $1.5 \times 10^5$  cells mL<sup>-1</sup> and  $2.0 \times 10^5$  cells mL<sup>-1</sup> to  $2.5 \times 10^5$  cells mL<sup>-1</sup> respectively, were treated for 12 h with IC50 of P2Et, Anamu-SC, and chemotherapy. K562 cells were treated with P2Et (178 µg mL<sup>-1</sup>), Anamu-SC (294 µg mL<sup>-1</sup>), idarubicin (IDA) (0.13 µM), and Ara-C (2 mM). Reh cells were treated with P2Et (254 µg mL<sup>-1</sup>), Anamu-SC (54 µg mL<sup>-1</sup>), doxorubicin (Doxo) (60 nM), vincristine (VCR) (10 nM), and methotrexate (MTX) (13 µM). As a negative control, extract diluents (ethanol, DMSO, H<sub>2</sub>O) were used.

NO and ROS levels were determined with DAF-FM DA (2.5 µM, Sigma-Aldrich, USA) and H2DCFDA (1 µM, Sigma-Aldrich, USA), respectively. Data were acquired in a FACS Aria II-flow cytometer (Becton Dickinson®) and analyzed with the FlowJo V 10.0 software (Tree Star, Inc®). The cytotoxic effect of the extracts and each of the chemotherapeutics on tumor cells was assessed by the methyl thiazole tetrazolium (MTT) assay (Sigma-Aldrich, Saint Louis, MO, USA) as previously reported [19].

### 2.4. Statistical analysis

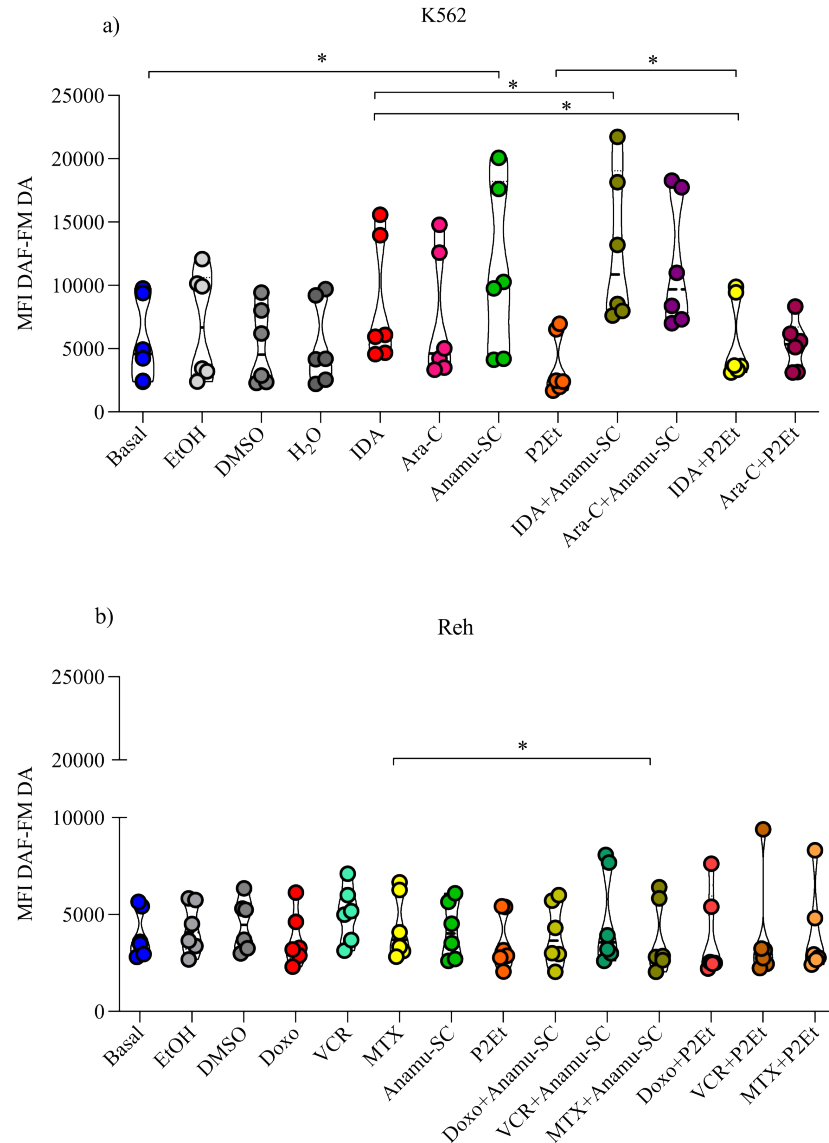
Data were analyzed in the SPSS® program (IBM® version 28.0.1.1). Graphs were made using GraphPad Prism 8 software (GraphPad Software, Inc®). Wilcoxon tests was performed for all statistical analyses. Statistical significance was established at  $p < 0.05$ .

## 3. Results

**Anamu-SC and its combination with idarubicin increased NO levels in K562 cells.** Anamu-SC significantly increased NO levels in K562 cells with respect to baseline. In addition, the combination of this extract with idarubicin significantly increased NO levels, compared to cells treated with idarubicin alone. Regarding the P2Et treatment, this extract in combination with idarubicin decreased NO levels compared to the effect of the drug alone. The treatment of K562 cells with P2Et alone did not modify NO levels (Figure 1a,  $p < 0.05$ ). As for Reh cells, a significant difference was observed in the NO levels induced by the combination of Anamu-SC and methotrexate compared to the effect of the drug alone (Figure 1b,  $p < 0.05$ ).

**P2Et and Anamu-SC had an antioxidant effect on K562 and Reh cells.** After a 12-h treatment with Anamu-SC and P2Et, a decrease in intracellular ROS levels was observed compared to baseline levels and Ara-C and idarubicin in K562 cells (Figure 2a,  $p < 0.05$ ). In addition, the combination of P2Et and Anamu-SC with the chemotherapeutic agents decreased the pro-oxidant effect of the drugs in K562 cells (Figure 2a). With respect to Reh cells, P2Et and Anamu-SC also decreased ROS levels with respect to basal levels and chemotherapy only (Figure 2b,  $p < 0.05$ ).

**Anamu-SC and P2Et decreased the number of K562 and Reh cells after 12 hours of treatment.** The treatment of K562 and Reh cells with P2Et and Anamu-SC, as well as their combination with chemotherapeutic agents, decreased cell numbers (Figure 3). Interestingly, in K562 cells, a marked

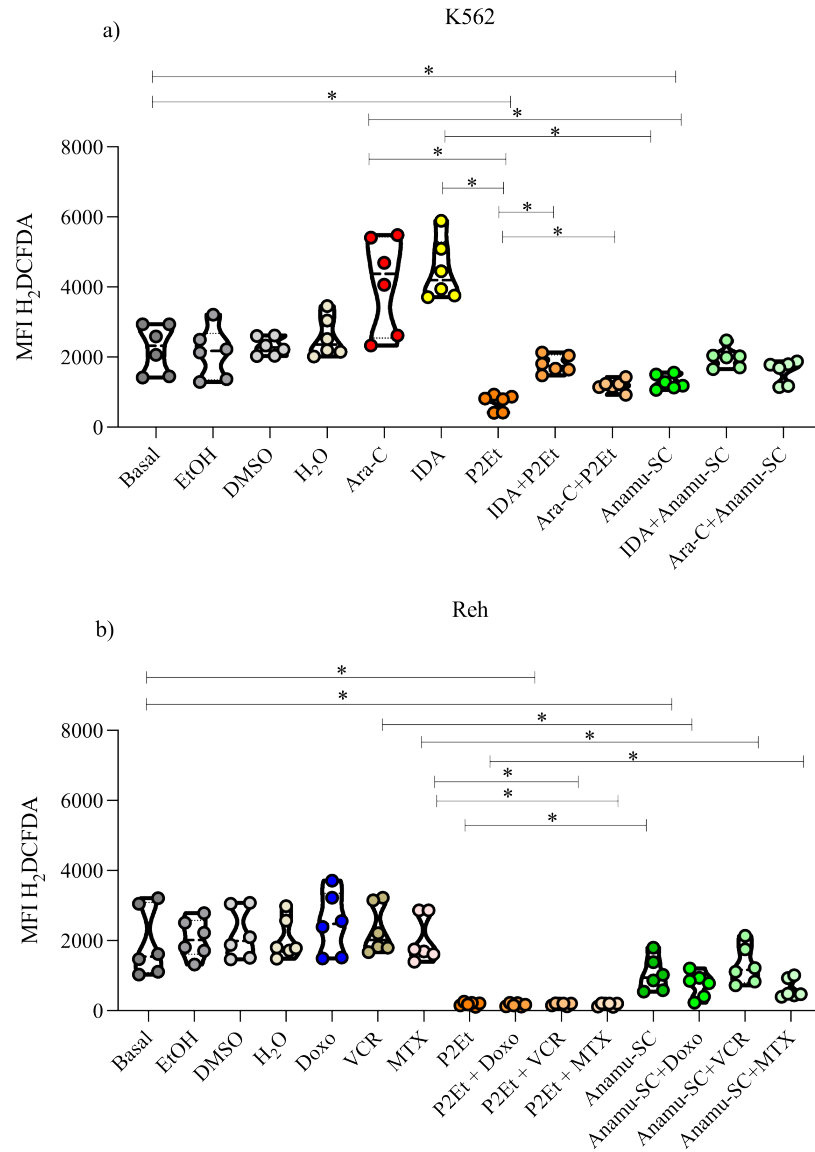


**Figure 1.** NO levels in K562 and Reh cells treated with P2Et, Anamu-SC, and chemotherapeutics. (a) K562. (b) Reh. Mean fluorescence intensity (MFI) is presented using GraphPad Prism 8 software (GraphPad Software, Inc.®). (\* $p < 0.05$ ).

decrease in cell counts was observed when the cells were treated with extracts in combination with the chemotherapeutic agents, compared to treatment with the extracts alone (Figure 3a,  $p < 0.05$ ). No decrease in cell viability was observed in any cell line after 12 hours of treatment (data not shown).

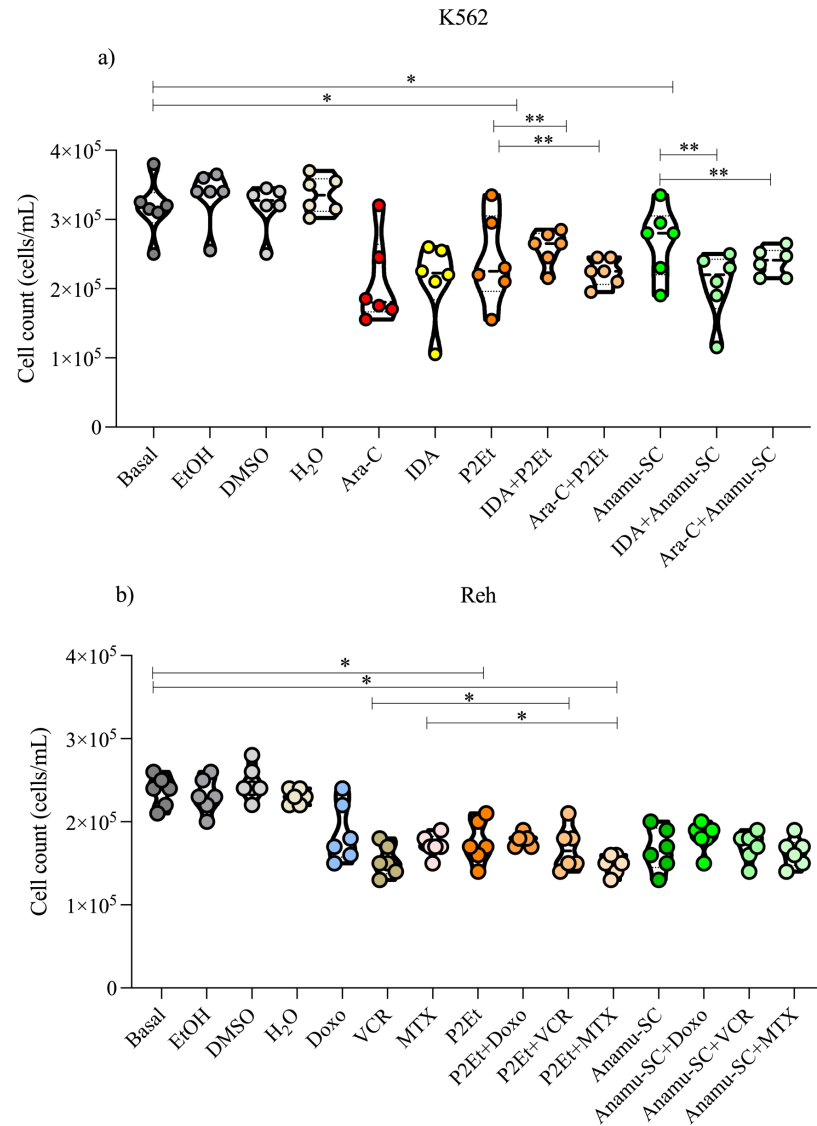
#### 4. Discussion

NO and ROS are related to tumorigenesis and tumor progression in acute leukemias, making them attractive therapeutic targets [20, 21]. Scarcely any reports have shown NO modulation by plant-derived extracts or isolated compounds in hematopoietic tumor cells with mixed results.



**Figure 2.** ROS levels in K562 and Reh cells treated with P2Et, Anamu-SC, and chemotherapeutics. (a) K562. (b) Reh. Mean fluorescence intensity (MFI) is presented using GraphPad Prism 8 software (GraphPad Software, Inc.®). (\* $p < 0.05$ ).

We show that the Anamu-SC extract obtained from *Petiveria alliacea* increases NO levels in the K562 myeloid leukemia line. Anamu-SC has been previously chemically characterized and has a high content of flavonoids and sulfur compounds [19]. On the one hand, isolated flavonoid-type compounds such as curcumin and quercetin, which have a potent antioxidant effect, can increase NO after 4 hours in lymphoid cell lines. Such an increase is likely protective since NO can prevent depolarization of the mitochondrial membrane due to its reversible binding capacity to cytochrome C oxidase [22]. However, Pan et al. isolated 20 compounds from *Melia azedarach L.*, of which some limonoids presented cytotoxic activity and inhibitory capacity of NO production in the leukemic cell line of myeloid origin HL60 [23]. Likewise, the cytotoxic effect induced by resveratrol, a phenolic compound found in more than 70 plant species, on tumor lymphoid cells was attributed to the reduction of NO and the expression of BCL-2 [24].



**Figure 3.** Cell count of K562 and Reh cells treated with P2Et, Anamu-SC, and chemotherapeutics. (a) K562. (b) Reh. Total counts are presented in cells/mL using GraphPad Prism 8 software (GraphPad Software, Inc.®). (\* $p < 0.05$ ).

The flavonoid quercetin-3-methyl ether (Q-3-ME), extracted from *Larrea divaricata*, induces an increase in NO and iNOS and alters the mitochondrial membrane potential with consequent activation of the intrinsic pathway of apoptosis in lymphoma cells [25]. Also, fasinin, a flavonoid widely distributed in plants, fruits, and vegetables, induces an increase in NO in K562, U937, and THP-1 myeloid leukemia cells, alters the mTORC signaling pathway, and activates the intrinsic and extrinsic pathways of apoptosis [9]. In addition, the sulfur compounds in the Anamu-SC extract can modify the cytoskeleton of tumor cells [26]. Such a mechanism is associated with the modulation of transcription factors that regulate the expression of iNOS, increasing NO levels [9, 27].

Most studies show the modulation of NO on hematopoietic tumor cells using isolated compounds and not complex extracts; therefore, this study proposes Anamu-SC as one of the few, if not the first, extracts that induces a change in intracellular NO levels in the acute leukemia model. The use of extracts offers a therapeutic advantage since the mixture of multiple bioactive compounds can influence different biological activities simultaneously [28].

Further study of NO modulation in these cells is required to assess whether there is a phenomenon that depends on the type of hematologic lineage (myeloid vs. lymphoid), cell differentiation stage, or whether it is a response depending mainly on the type of genetic alterations present in the leukemic cells, as part of the clonal origin or tumor evolution.

The role of ROS in tumor cells has been extensively studied in leukemia cells of myeloid origin. These cells express more effective antioxidant systems than cells of lymphoid lineage, displaying improved protection against the harmful effects of ROS [29]. Also, known BCR/ABL oncogene alterations in K562 cells can favor Nrf2 factor translocation to the nucleus, which increases the expression of the p67phox, p47phox, and p40phox complexes that interact to form the NOX enzymatic complex and thus favor ROS generation [30].

We observed that P2Et and Anamu-SC have an antioxidant effect on K562 and Reh cells, decreasing their ROS levels compared to baseline or chemotherapy treatments. Interestingly, the combination of the plant-derived extracts P2Et and Anamu-SC with chemotherapeutic agents tended to counteract the pro-oxidant effect of the drugs, although without significant differences. The P2Et extract is rich in gallic acid and ethyl gallate [12] and is related to a high antioxidant capacity, depolarization of the mitochondrial membrane, and caspase3 activation [13], and the Anamu-SC extract is rich in sulfur compounds and polyphenols, which can exert their antioxidant effect directly as ROS scavengers and cellular oxidative stress detoxifiers. In addition, the sulfhydryl compounds in Anamu-SC can become polysulfides and other reactive sulfur species in the presence of ROS, which are highly sensitive to the action of antioxidant enzymes such as superoxide dismutase (SOD) [14]. However, in other tumor models, such as melanoma, Anamu-SC has a pro-oxidant effect, implying that the induced change in ROS levels may be cell-dependent (unpublished data).

Several natural products exert antileukemic effects through ROS-dependent actions. *Allium senescens* L. extracts can increase ROS, through MAPK phosphorylation, in T-ALL cells and lead to an antiproliferative outcome [28]. Likewise, Wang et al. (2016) demonstrated that the alkaloid cathachunine obtained from *Catharanthus roseus* induces ROS-dependent apoptosis and increases the expression of BCL-2 in the HL60 and K562 lines [31]. El Houry et al. (2020) showed that *M. pseudolavatera* leaf extracts have a pro-apoptotic effect generated by the induction of ROS, an increase in the Bax/Bcl-2 ratio, and the release of cytochrome c from mitochondria in myeloid cell lines such as U937 [32].

The antioxidant effect of natural compounds has been better described in models other than leukemia. Hydroalcoholic compounds, extracted from *Trigonella foenum-graecum*, *Cassia acutifolia*, and *Rhazya stricta* and rich in flavonoids and phenols, demonstrated an antioxidant effect on hepatocarcinoma cells [33].

Interestingly, some compounds with arguably broad antioxidant potential in the leukemia model, such as curcumin, are likely pro-oxidants [34, 35]. Thus, their differential effect on ROS intracellular levels can be attributed to concentration or treatment time variation. Sun et al. used the flavonoid isoliquiritigenin at low concentrations to demonstrate its antioxidant activity; however, when used at higher doses, its cytotoxic capacity was evidenced through the induction of ROS in hepatocellular carcinoma lines [36].

Although an increase in NO and ROS levels favors the death of leukemia cells, it is worth considering that NO and ROS homeostasis can also facilitate the maintenance of these cells. In this study, after 12 hours of treatment with P2Et or Anamu-SC plant extracts, on their own and combined with chemotherapeutics, cell counts decreased compared to the control. It is possible that during the 12 hours of treatment, the increase in NO levels in K562 cells and the decrease in ROS levels in the two cell lines had both cytostatic and non-cytotoxic effects. It is necessary to evaluate the outcomes of these treatments for more than 12 hours and to explore other consequences, such as early cell cycle arrest or metabolic alterations that could affect the viability of leukemia cells over extended periods. In Melanoma, *Petiveria alliacea* extracts can induce an arrest in the cell cycle in the G2/M phase [19].

Finally, phytochemicals such as quercetin, sulforaphane, genistein, and epigallocatechin-3-gallate have been shown to modulate the expression of enzymes involved in NO metabolism in cervical cancer cells [37]. Similarly, in a breast cancer model, olive leaf extracts were shown to affect the expression of antioxidant enzymes such as superoxide dismutase and catalase [38]. Following this work, the modulating effect of P2Et and Anamu-SC on the expression and activity of the enzymes involved in NO and ROS metabolism should be investigated applying different analytical techniques.

## 5. Conclusion

We demonstrated the modulating role of the plant-derived extracts P2Et and Anamu-SC on NO and ROS levels in K562 and Reh cells. It is necessary to study the effect of these extracts on the modulation of NO and ROS in primary human leukemic cells according to their lineage (myeloid vs. lymphoid), genetic alterations, metabolic dysfunction, and tumor stage to elucidate their role as possible therapeutic agents or as adjuvants in leukemia therapy.

## 6. Acknowledgements

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## 7. Conflict of interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## 8. Data and material availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## 9. Ethical Approval

The study was reviewed and approved by the Ethics Committee of the Faculty of Sciences of the Pontificia Universidad Javeriana (Bogotá, Colombia).

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### Los extractos derivados de plantas P2Et y Anamu-SC afectan los niveles de NO y ROS en células leucémicas

**Resumen:** Las células leucémicas suelen presentar altos niveles de óxido nítrico (NO) y especies reactivas de oxígeno (ROS). Éstos pueden conducir a resistencia a la apoptosis y a la terapia, así como a un aumento en la proliferación. Los extractos derivados de plantas disminuyen la quimiorresistencia en las células cancerosas. En este estudio, evaluamos los efectos de los extractos derivados de plantas P2Et (*Caesalpinia spinosa*) y Anamu-SC (*Petiveria alliacea*), así como su combinación con agentes quimioterapéuticos, sobre los niveles de NO y ROS en las líneas celulares leucémicas K562 y Reh. Los niveles de NO y el ROS se determinaron utilizando las sondas DAF-FM DA y H2DCFDA. La intensidad media de fluorescencia para cada variable se midió mediante citometría de flujo. Los extractos mostraron un efecto antioxidante en ambas líneas celulares, lo que llevó a una disminución significativa en los niveles de ROS sin disminuir la viabilidad celular. El extracto de Anamu-SC también aumentó los niveles de NO en las células K562 cuando se combinó con idarubicina. Ambos extractos redujeron el número de células leucémicas después de 12 horas de tratamiento. Se necesitan más estudios para evaluar su efecto en células primarias de leucemia humana. Estos hallazgos sugieren el potencial de P2Et y Anamu-SC como adyuvantes en el tratamiento de la leucemia.

**Palabras Clave:** *Caesalpinia spinosa*; Flavonoides; Leucemia; Óxido nítrico; *Petiveria alliacea*; Especies reactivas de oxígeno.

### Extratos derivados de plantas P2Et e Anamu-SC afetam os níveis de NO e ROS em células leucêmicas.

**Resumo:** As células leucêmicas frequentemente apresentam níveis elevados de óxido nítrico (NO) e espécies reativas de oxigênio (ROS). Esses elementos podem levar à resistência à apoptose e à terapia, além de promover a proliferação celular. Extratos derivados de plantas reduzem a quimiorresistência em células cancerígenas. Neste estudo, avaliamos os efeitos dos extratos derivados de plantas P2Et (*Caesalpinia spinosa*) e Anamu-SC (*Petiveria alliacea*), bem como sua combinação com agentes quimioterápicos, nos níveis de NO e ROS em linhagens celulares leucêmicas K562 e Reh. Os níveis de NO e ROS foram determinados utilizando as sondas DAF-FM DA e H2DCFDA. A intensidade média de fluorescência para cada variável foi medida por citometria de fluxo. Os extratos mostraram um efeito antioxidante em ambas as linhagens celulares, levando a uma diminuição significativa nos níveis de ROS sem diminuir a viabilidade celular. O extrato de Anamu-SC também aumentou os níveis de NO nas células K562 quando combinado com idarubicina. Ambos os extratos reduziram o número de células leucêmicas após 12 horas de tratamento. Estudos adicionais são necessários para avaliar seu efeito em células primárias de leucemia humana. Essas descobertas sugerem o potencial de P2Et e Anamu-SC como coadjuvantes no tratamento da leucemia.

**Palavras-chave:** *Caesalpinia spinosa*; flavonoides; leucemia; óxido nítrico; *Petiveria alliacea*; espécies reativas de oxigênio.

**Juan Jose Arevalo Ferrin BSc** Bacteriologist from Pontificia Universidad Javeriana, Bogotá, Colombia.

ORCID: 0000-0002-8868-2186

**Jimmy Alejandro Garcia Ortiz BSc** Bacteriologist from Pontificia Universidad Javeriana, Bogotá, Colombia. Bacteriologist, Blood Center Karl Landsteiner In Memoriam, Bogotá D.C, Colombia. Bacteriologist. ESE Hospital San José de Guaduas, Guaduas, Cundinamarca, Colombia.

ORCID: 0009-0003-5882-7481

**Cindy Mayerly Arévalo Olaya BSc, Spc, (c)Ph.D** Bacteriologist B.Sc. Pontificia Universidad Javeriana, Bogotá, D.C., Colombia. Spc. Hematología en el laboratorio clínico y manejo del banco de sangre. Pontificia Universidad Javeriana, Bogotá, D.C., Colombia. Spc. Docencia Universitaria. Universidad El Bosque, Bogotá, D.C., Colombia. (c)Ph.D en Ciencias Biológicas. Pontificia Universidad Javeriana, Bogotá, D.C., Colombia.

ORCID: 0000-0003-4229-0918

**Sandra Milena Quijano Gomez BSc, MSc, PhD** Pontificia Universidad Javeriana, Bogotá, Colombia. MSc Microbiology. Pontificia Universidad Javeriana, Bogotá, Colombia. MSc Molecular Oncology. European School of Oncology. Centro Nacional de Investigaciones Oncológicas (CNIO) Madrid-España. PhD Centro de Investigación del Cáncer. Programa de Doctorado Biología y Clínica del Cáncer. Salamanca-España. Professor Department of Microbiology. Pontificia Universidad Javeriana, Bogotá, Colombia. Scientific advisor. Cytometry Laboratory. Fundación Santa Fe de Bogotá and Hospital Universitario San Ignacio, Bogotá, Colombia.

ORCID: 0000-0003-4452-9291

**Susana Fiorentino BSc, MSc, PhD** from the University of Paris VI. Professor at the Faculty of Sciences of the Pontificia Universidad Javeriana. Two postdoctoral stays of 4 years in total, at the Necker Hospital and the Saint Louis Hospital in Paris. Her areas of interest are Immunology, Oncology and particularly the R&D of polymolecular drugs against cancer that regulate tumor metabolism and activate the immune response.

ORCID: 0000-0002-4664-0682

**Viviana Marcela Rodríguez Pardo, BSc, MSc, PhD** is an professor of hematology at the Pontificia Universidad Javeriana in Bogotá, Colombia. Her research is dedicated to studying the complex biology of human stem cells, including mesenchymal cells, skeletal cells and iPSCs, as well as exploring their therapeutic applications and their impact in haematological malignancies. In addition to her scientific contributions, he has played a key role in developing national and international standards related to these cell types.

ORCID: 0000-0003-1881-9367