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Genomic Instability Decreases in HIV Patient by Complementary Therapy with *Rosmarinus officinalis* Extracts

Blanca Patricia Lazalde-Ramos,[1] Ana Lourdes Zamora-Perez,[2] Aymée Ileana Ortega-Guerrero,[3] Saira Zulema Quintero-Fraire,[3] Omar Palacios-Lara,[4,5] Sol María Quirarte-Báez,[5] Carlos Galaviz-Hernández,[6] Martha Sosa-Macías,[6] Yveth Marlene Ortiz-García,[2] and Gabriela Morales-Velazquez[2]

[1]*Master of Science and Chemical Technology, Chemistry Department, Autonomous University of Zacatecas, Zacatecas, Mexico.*
[2]*Institute of Dental Research, University Center for Health Science, University of Guadalajara, Mexico.*
[3]*Pharmaceutical-Biological Chemistry Program, Chemistry Department, Autonomous University of Zacatecas, Zacatecas, Mexico.*
[4]*Outpatient Centers for the Prevention and Care of AIDS and Sexually Transmitted Infections and Care Services Integral Hospital, Secretary of Health, Zacatecas, Zacatecas, Mexico.*
[5]*Mexican Institute of Social Security, Zacatecas Delegation, Mexico.*
[6]*National Polytechnic Institute, CIIDIR-Durango, Academy of Genomics, Durango, Mexico.*

ABSTRACT Genomic instability is associated with increased oxidative stress in patients with human immunodeficiency virus (HIV). The aim of this study was to determine the effect of intake of methanolic and aqueous extracts *of Rosmarinus officinalis* on genomic instability in HIV patients. We studied 67 HIV patients under pharmacological treatment with ATRIPLA who were divided into three groups: group 1, patients under ATRIPLA antiretroviral therapy; group 2, patients with ATRIPLA and rosemary aqueous extract (4 g/L per day); and group 3, patients with ATRIPLA and rosemary methanolic extract (400 mg/day). The genomic instability was evaluated through the buccal micronucleus cytome assay. Oral epithelial cells were taken at the beginning and 1 and 4 months later. The groups that received the pharmacological therapy with ATRIPLA and the complementary therapy with *R. officinalis* extracts showed a decrease in the number of cells with micronuclei and nuclear abnormalities compared with the group that only received ATRIPLA. The complementary therapy with *R. officinalis* decreased the genomic instability in HIV patients.

KEYWORDS: • ***cytotoxicity*** • ***DNA instability*** • ***genotoxicity*** • ***HIV*** • ***nuclear abnormalities*** • **Rosmarinus officinalis**

INTRODUCTION

THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) represents a very complex public health problem. The antiretrovirals have effect on reducing morbidity and mortality, prolonging lives and improving the quality of life of many people living with HIV infection.[1,2]

However, it has been shown that there is an increase in reactive oxygen species (ROS) following the administration of antiretroviral therapy, even on a larger scale when compared with HIV individuals without treatment. These observations suggest that HIV infection alone or in combination with antiretrovirals induce oxidative stress, favoring their progression of pathogenesis by HIV.[3,4]

Current research focuses on ROS since they play a central role in the deterioration of systems and, consequently, they generate the progression of pathologies such as HIV.[5]

Increase of ROS is associated with the oxidation of nucleosides, which could cause DNA strand breaks,[6] producing teratogenic or carcinogenic consequences.[7] The use of plants with antioxidant property in HIV patients could restore the cellular balance.

Plants have efficient complex enzymatic antioxidant defense systems (such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) and nonenzymatic antioxidants (low-molecular-weight antioxidants, ascorbic acid, glutathione, proline, carotenoids, phenolic acids, flavonoids, and high-molecular-weight secondary metabolites such as tannins) to avoid the toxic effects of free radicals.[8,9]

Rosmarinus officinalis is a medicinal plant distributed throughout the world and is responsible for pharmacological

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Address correspondence to: Blanca Patricia Lazalde-Ramos, PhD, Maestria en Ciencias y Tecnología Química, Unidad Académica de Ciencias Químicas, Universidad Autónoma de Zacatecas, Campus UAZ Siglo XXI, Edif. L1, 3er piso, Carretera Zacatecas-Guadalajara Km 6, Ejido la Escondida, Zacatecas 98160, México, E-mail: blancalazalde@gmail.com

activities, such as anti-inflammatory, antioxidant, antimicrobial, antihyperglycemic, antiproliferative, antitumor, and protective, inhibitory, and attenuating activities.[10]

The best known phytocompounds of *R. officinalis* include caffeic acid, carnosic acid, chlorogenic acid, monomeric acid, oleanolic acid, rosmarinic acid, ursolic acid, alpha-pinene, camphor, carnosol, eucalyptol, rosmadial, rosmanol, rosmaquinones A and B, secohinokio, and eugenol and luteolin derivatives.[10]

The main constituents with antioxidant properties are carnosic acid and carnosol (responsible for 90% of the properties). Both are inhibitors of lipid peroxidation in liposomal and microsomal systems; they are good scavengers of peroxyl radicals, which reduce cytochrome c and scavenge hydroxyl radicals. Specifically, carnosic acid scavenges H_2O_2 but could also act as a substrate for the peroxidase system.[11]

Caffeic acid of rosemary activates the extracellular-signal-regulated kinase signaling pathway by a relatively low level of ROS, which block H_2O_2-induced DNA double-strand breaks and improve the viability of human liver cells.[12]

Rosmarinic acid increases the protein expression of Bax, and cleaved form of PARP-1 proapoptotic protein Bax and antiapoptotic protein Bcl-2 family bound to the mitochondrial membrane affect the intrinsic mitochondrial pathway of apoptosis by regulating efflux of cytochrome c from mitochondria to the cytoplasm, and subsequent activation of caspases such as caspase-3, -6, and -7 that are cysteine-aspartic proteases plays important roles in apoptosis. (PARP-1 is a poly-[ADP-ribosylating] enzyme necessary for DNA repair process.)[13,14]

The genomic instability can be evaluated through comet test (single-cell gel electrophoresis assay), FISH (fluorescence in situ hybridization), CGH (comparative genomic hybridization), TUNEL (terminal deoxynucleotidyl transferase [TdT] dUTP nick-end labeling) assay, micronucleus (MN) assay, and nuclear abnormalities (NAs), among others.[15] The buccal micronucleus cytome (BMCyT) assay is a minimally invasive cytological and interphase cytogenetic technique for measuring DNA damage and cell death biomarkers in the oral epithelium, the size, density, and distribution of chromatin experiment change, which lead to DNA damage.[16]

The present work aimed to determine the effect of ingestion of methanolic and aqueous extracts of *R. officinalis* on genomic instability in HIV patients through the BMCyT assay.

MATERIALS AND METHODS

Study design and participants

We studied 67 HIV patients of the Centro Ambulatorio para la Prevención y Atención en SIDA e Infecciones de Transmisión Sexual (CAPACIT), Zacatecas, Zacatecas, México.

The inclusion criteria included patients of legal age, indistinct sex, without complications due to the disease, and those who are only under pharmacological treatment with ATRIPLA (efavirenz 600 mg, emtricitabine 200 mg, and tenofovir disoproxil fumarate 300 mg).

The participants gave their informed written consent. The work was carried out in accordance with the Declaration of Helsinki, and it was approved by the Local Health Research Committee 3301 of the ''Hospital General Zona/MF 1 Zacatecas'' (Registration No. R-2019-3301-043) and also meets the registry before COMBIOETICA Committee (32 CEI 001 2017082) and COFEPRIS (17 CI 32 056 012)

The patients with HIV were divided into three groups: group 1, the follow-up group ($n = 22$), patients who only continued their pharmacological therapy with ATRIPLA. Group 2, patients who in addition to their pharmacological therapy with ATRIPLA had complementary therapy with rosemary aqueous extract at a dose of 4 g/L per day for 4 months ($n = 22$). Group 3, patients who in addition to their pharmacological therapy with ATRIPLA had complementary therapy with rosemary methanolic extract at a dose of 400 mg/day for 4 months ($n = 23$), and a control group consisting apparently healthy people was added ($n = 22$).

The dosage of the rosemary aqueous and methanolic extracts was based on previous studies reported by Lazalde-Ramos *et al.*[17,18]

Each patient underwent a clinical history, with emphasis on the development of HIV.

The samples of the oral mucosa were taken before starting the complementary therapy and every 30 days for 4 months

Plant material and preparation of the extract

The rosemary leaves used were acquired from Medicinal Plants of America, S.A. of C.V. Mexico, D.F. lot number 100210.

Rosemary aqueous extract: Infusion extraction method. A box containing 30 sachets of rosemary leaves (4 g each) were provided to each patient who belonged to the complementary therapy group with rosemary aqueous extract.

The rosemary leaves used were packaged in accordance with the Official Mexican Standard NOM-072-SSA1-2012 in the ethnopharmacology laboratory of the Autonomous University of Zacatecas. The preparation form was based on a previously published study by Lazalde-Ramos *et al.*,[17] in a liter of boiling water, add a bag of rosemary leaves and allow it to boil for 3 min and then pass through a sieve to remove the leaves. Patients were instructed to consume it as drinking water during the day.

Preparation of rosemary methanolic extract. The methanolic extract was extracted as previously described by Gutiérrez *et al.*[19] The dry leaves were pulverized into fine powder, macerated in methanol for 24 h, the proportion was 10 g of the rosemary leaf powder in 100 mL of methanol, and subjected to a reflux system for 2 h at 70°C. The chlorophyll was removed with activated carbon. Subsequently, it was distilled by means of a rotary evaporator system and precipitated with tridistilled water at −4°C, and the precipitate was dried and encapsulated according to NOM-072-SSA1-2012. Yield extraction was 14%.

BMCyT assay

From each participant, buccal mucosa samples were collected using a precoded slide. Subjects were asked to rinse their mouths with water, before taking the samples. Cells from the buccal mucosa were collected by scraping both cheeks with a slide with a ground edge. The obtained cells were spread directly on separate precoded slides in duplicate.[20]

The slides were air-dried and fixed with 80% methanol for 48 h and then stained with acridine orange (CAS No. 10127023; Sigma–Aldrich, St. Louis, MI).

Precoded slides were examined by one reader according to the criteria described by Thomas *et al.*[21] The cells were counted blindly as NAs including MN, binucleated cells (BN), cells with nuclear buds (NBs), karyolysis (KL), karyorrhexis (KR), abnormally condensed chromatin (CC), and pyknosis (PYK). The number of cells with NAs was evaluated among 2000 cells using an Olympus CX31 microscope equipped with epifluorescence and oil immersion objective (100×; Olympus, Tokyo, Japan). The results are presented as the number of cells with NAs per 2000 cells. NAs were evaluated by assessing the staining intensity, texture, and focal plane of the nucleus. Normal cells were identified as follows: intact and relative homogeneous cytoplasm, little or no contact with adjacent cells, and an intact homogeneous nucleus with a smooth and distinct nuclear perimeter.[21,22]

Statistical analysis

The results are presented as mean ± standard deviation (SD). The increase or decrease in percentage was calculated by dividing the final value by the initial one, and the value obtained was multiplied by 100 and the remainder was 100%.

Differences in MN and NA values were evaluated using the Wilcoxon signed-rank test for intragroup comparison, and the intergroup analysis was performed using the Mann–Whitney *U* test. All tests were performed using the Statistical Program for the Social Sciences (SPSS v.20) for Windows medical pack (SPSS, Chicago, IL). A *P*-value of <.05 was considered statistically significant.

RESULTS

The average age of the HIV patients was 40.5 ± 10.5 years, with a progression of the disease of 7.08 ± 5.27 years; all patients acquired the disease sexually.

The results obtained from the BMCyT assay in the study groups, at the different sampling times, are shown in Table 1.

The patients who received only the pharmacological therapy with ATRIPLA showed an increase at 120 days in the number of cells with MN (50.27%), BN (51.36%), and KR (12.71%), with statistically significant increase in the number of cells with BN.

The groups that received the pharmacological therapy with ATRIPLA and the complementary therapy with rosemary showed a decrease in the number of cells with MN and NAs, the decrease being greater in the patients who received the complementary therapy with rosemary methanolic extract (Table 1).

Figure 1 shows the results obtained from the difference between the baseline value and that obtained after 4 months of treatment in the study groups.

Table 1. Frequencies of Micronuclei and Nuclear Abnormalities in the Study Groups

		Micronuclei	*Cells with nuclear buds*	*Binucleated cells*	*Condensed chromatin cells*	*Karyolitic cells*	*Karyorrhectic cells*	*Pyknotic cells*
Control group	Basal	0.56 ± 0.58	2.43 ± 1.44	0.60 ± 0.65	2.91 ± 2.29	0.73 ± 1.35	1.17 ± 1.43	0.34 ± 0.57
HIV patients with	Basal	0.81 ± 1.0	48.50 ± 18.56	16.72 ± 12.59	146.18 ± 55.29	30.63 ± 28.28	52.90 ± 32.73	10.04 ± 13.37
ATRIPLA	120 days	1.22 ± 1.45	48.50 ± 22.21	25.31 ± 25.75	143.31 ± 60.00	29.72 ± 28.12	59.63 ± 34.10	8.40 ± 10.10
	P	NS	NS	.014[b]	NS	NS	NS	NS
	Increase or decrease ± (%)	+50.27	—	+51.36	−1.95	−2.96	+12.71	−16.28
HIV patients with	Basal	0.82 ± 0.98	16.65 ± 4.90	5.30 ± 3.64	77.00 ± 31.61	24.13 ± 20.22	21.78 ± 12.53	6.43 ± 4.46
ATRIPLA and	30 days	0.17 ± 0.38	12.78 ± 7.84	3.56 ± 3.14	56.34 ± 28.92	16.13 ± 8.76	11.56 ± 8.15	6.47 ± 3.85
rosemary	120 days	0.17 ± 0.38	9.43 ± 5.02	1.60 ± 2.29	35.30 ± 11.88	16.56 ± 11.83	10.39 ± 6.59	4.86 ± 4.93
methanolic extract	*P*	.008[a], .015[b]	.016[a], .0001[b]	.023[a], .0001[b], .006[c]	.002[a], .0001[b], .001[c]	NS	.0001[a,b]	NS
	Increase or decrease ± (%)	−79.05	−43.34	−69.68	−54.15	−31.35	−52.29	−24.32
HIV patients with	Basal	1.00 ± 1.77	27.18 ± 17.60	11.68 ± 9.33	100.13 ± 42.23	15.45 ± 22.42	36.27 ± 32.90	10.50 ± 16.32
ATRIPLA and	30 days	1.04 ± 1.25	21.90 ± 19.89	9.90 ± 7.32	77.81 ± 42.80	6.77 ± 9.99	26.00 ± 21.98	4.90 ± 11.40
rosemary tea	120 days	0.63 ± 1.36	18.72 ± 24.56	5.18 ± 5.08	48.40 ± 41.98	9.09 ± 13.17	19.54 ± 17.38	4.59 ± 8.33
	P	NS	.005[b]	.002[b], .007[c]	.024[a], .001[b,c]	.033[a]	.003[b]	.011[a], .025[b]
	Increase or decrease ± (%)	−36.4	−31.10	−55.64	−51.65	−41.18	−46.11	−56.28

Data are expressed as mean ± SD. Results are presented as the number of cells with MN and NAs per 2000 cells. Statistical significance was considered with $P < .05$. Differences in MN and NA values were evaluated using the Wilcoxon signed-rank test for intragroup comparison (basal value vs. 30 days[a], basal values vs. 120 days[b], and 30 days vs.120 days[c]). The increase or decrease was determined to the 120 days in relation to basal time.

MN, micronucleus; NAs, nuclear abnormalities; NS, not significant.

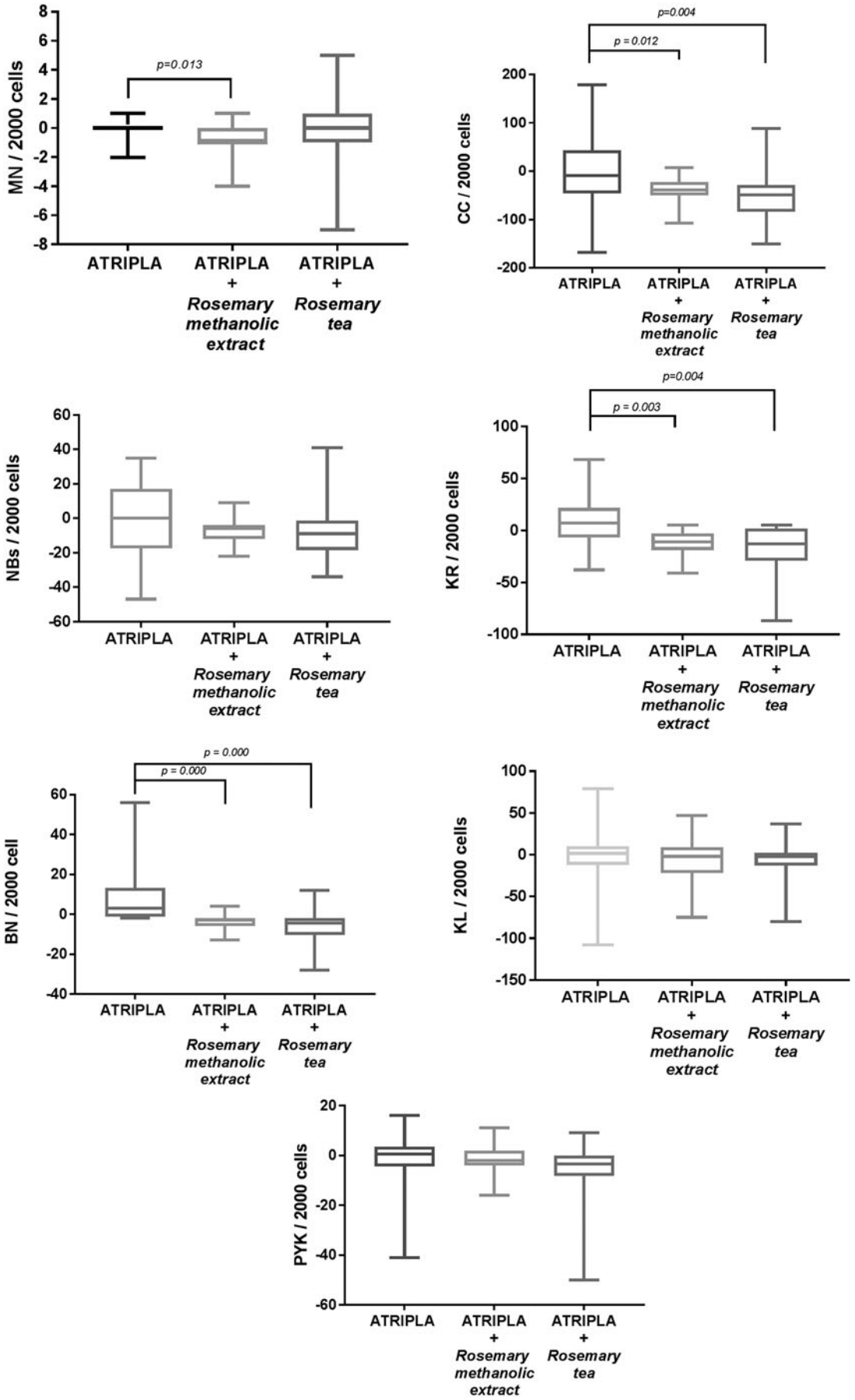

FIG. 1. Intergroup comparison between the differences obtained at 4 months and the baseline value of the number of cells with MN and NAs. The comparisons were performed using the Mann–Whitney *U* test; it was considered statistically significant when $P < 0.05$. BN, binucleated cells; CC, abnormally condensed chromatin; KL, karyolysis; KR, karyorrhexis; MN, micronucleus; NAs, nuclear abnormalities; NBs, nuclear buds; PYK, pyknosis.

The patients who received only the pharmacological therapy with ATRIPLA showed an increase in the number of cells with MN, BN, KR, and NBs; however, the patients who received the pharmacological therapy with ATRIPLA and the complementary therapy with rosemary showed a decrease in the number of these cells.

The number of cells with KL, CC, and PYK decreased in all the groups. The number of cells with CC decreased statistically in the patients who received the pharmacological therapy with ATRIPLA and the complementary therapy with rosemary. In relation to the number of cells with KL and PYK, only the group of patients who received the pharmacological therapy with ATRIPLA and the complementary therapy with rosemary aqueous extract showed a statistically significant decrease.

In the intergroup comparison, there was a statistically significant difference in the number of cells with MN, BN, CC, and KR between the patients who received the pharmacological therapy with ATRIPLA and those who received the pharmacological therapy with ATRIPLA and the complementary therapy with rosemary methanolic extract; this number being lower in the group of patients who received the complementary therapy with rosemary methanolic extract.

Similarly, in the group of patients who received the complementary therapy with rosemary aqueous extract, there was a statistically significant decrease in the number of cells with BN, CC, and KR when compared with the group of patients who received only the pharmacological therapy with ATRIPLA (Fig. 1)

DISCUSSION

HIV remains one of the most serious public health problems in the world, especially in developing countries.

The antiretroviral therapy in HIV individuals increases the time and quality of life and decreases the incidence of opportunistic diseases associated with the AIDS phase. Combined antiretroviral therapies increase the therapeutic efficacy and tolerability, decreasing the risk of developing resistance. However, the antiretroviral drugs have some disadvantages, since they have been associated with a redox imbalance, through the generation of ROS.[3] Complementary therapy with plants with antioxidant properties could help restore the redox imbalance.

In this work, we evaluated and compared the genomic instability in HIV patients under antiretroviral therapy with ATRIPLA and antiretroviral therapy (ATRIPLA) plus complementary therapy with aqueous or methanolic extract of *R. officinalis*.

The average age of the patients with HIV evaluated (40.5 years) was within the age range with the greatest distribution of the disease (80.5%) reported in Mexico from 1984 to 2018.[23]

The genomic instability was assessed by the BMCyT assay. The BMCyT assay has been repeatedly tested as a reliable biomarker for genotoxicity, cell instability, cell death, and cancer risk.[21,24–26]

The group of patients who received the pharmacological therapy with ATRIPLA showed an increase in the number of cells with MN, BN, and KR after 4 months of treatment.

This increase may be due to the three active substances (efavirenz, emtricitabine, and tenofovir) present in ATRIPLA (600 mg). Efavirenz is a nucleoside inhibitor of reverse transcriptase, which acts noncompetitively and reversibly at the catalytic center of reverse transcriptase, where once localized it causes the inhibition of gamma polymerase DNA, generating a decrease in the number of mitochondria and potentiating oxidative stress.[27–29]

The liver damage generated by efavirenz is associated with oxidative stress caused by mitochondrial damage.[30–34] Also, the consumption of efavirenz increased the PARP activity, decreased cell viability, and increased apoptosis and necrosis.[35]

Also, HIV has its own mechanism to potentiate the generation of ROS mediated by viral components such as the envelope protein Gp120, the Tat gene, Nef, Vpr, and the reverse transcriptase, each of them acting on different cellular levels but with purpose produce ROS.[35–39]

It has also been reported that the Vpr gene induces cell cycle abnormalities with accumulation in the G2/M phase and increased ploidy, inducing genomic instability.[40,41]

Our results agree with those previously published by Herd *et al.* They reported that HIV individuals receiving antiretroviral treatment as well as HIV individuals without treatment showed an increase in the number of cells with MN with respect to the follow-up group (consisting apparently healthy seronegative individuals), with the increase in MN being greater in the patients who took antiretroviral compared with those who did not.[28]

Lima *et al.* determined the number of cells with MN in oral mucosa in 30 HIV individuals under antiretroviral therapy and 30 patients without HIV. Patients who received antiretroviral therapy and low viral load values showed a higher frequency of multiple MN. The authors concluded that this may be due to the action of the Vpr gene.[42]

In contrast, the patients who received the pharmacological therapy with ATRIPLA and the complementary therapy with rosemary (methanolic or aqueous extract) showed a decrease in the number of cells with MN and NAs. This decrease may be due to the antioxidant effect of rosemary. Several studies show the antioxidant activity of *R. officinalis*.[43,44]

There are also reports that four diterpenes isolated from the leaves of *R. officinalis* (carnosic acid, rosmanol, carnosol, and epirosmanol) inhibit the production of superoxide anion in the xanthine oxidase system, showing a protective effect against oxidative stress.[43]

It has been shown that some components of rosemary (rosmarinic acid, carnosic acid, carnosol, and caffeic acid) can regulate the activity of peroxisome proliferator-activated receptors gamma (PPARγ).[45] PPARγ promotes the production of antioxidant enzymes and in turn modulates the expression of some genes responsible for inflammation and oxidation.[44] In addition, there is evidence to show that Tat gene response is attenuated by overexpression of PPARα or PPARγ.[46]

Furtado *et al.* reported the capacity of rosmarinic acid to prevent the breakage or loss of chromosomes induced chemically with doxorubicin.[47]

Our results agree with those previously published in patients with type 2 diabetes mellitus. Patients showed a significant decrease in the number of cells with MN, BN, CC, and Karyorrhectic cells after 30 days after the ingestion of rosemary aqueous extract from baseline.[17]

Likewise, the protective effect of methanolic extract of *R. officinalis* on DNA damage induced by cyclophosphamide was demonstrated, as well as the lack of genotoxicity and cytotoxicity of the extract in mouse peripheral blood by the micronucleus test.[18]

It was also shown that *R. officinalis* decreases damage to DNA, the anaphase-telophase bridges, and the arsenic-induced chromosomal aberrations.[48]

Based on our results, it can be concluded that the complementary therapy with *R. officinalis* in HIV patients can be an alternative in the genomic instability decrease generated by the increase in oxidative stress associated with both the disease and antiretroviral treatment.

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ETHICAL APPROVAL

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

The medical ethics review committee at the Universidad de Zacatecas, Zacatecas, Mexico, approved this study.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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