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Arracacia xanthorrhiza Bancr compounds decrease β-actin, hypoxia-inducible factor-1 and nitric oxide production in HeLa cells.

Yenddy Carrero[1,2], Jenny Moya[2], Michael Acosta[2] and Jesús Mosquera-Sulbarán[3]

[1]Universidad Técnica de Ambato. Facultad Ciencias de la Salud. Ambato, Ecuador.
[2]Universidad Técnica de Ambato. Facultad de Ciencias de la Salud. Laboratorio de Biología Molecular y Celular. Ambato, Ecuador.
[3]Instituto de Investigaciones Clínicas "Dr. Américo Negrette". Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela.

Key words: actin; nitric oxide; HIF-1; *Arracacia xanthorrhiza Bancr*; Hela cells.

Abstract. The treatment of cancer patients with anti-cancer drugs is often accompanied by the presence of undesirable side effects. The use of natural plant derivatives alone, or in conjunction with existing anti-neoplastic drugs, has been suggested to obtain better results and decrease these side effects. Nitric oxide (NO•), the hypoxia-inducible factor-1 (HIF-1), and decreased concentration of actin play important roles in cancer progression. The beneficial effects of polyphenols in various organ disorders including cancer has been reported. The aim of this study was to determine the effect of *Arracacia xanthorrhiza Bancr* extracts, white (WAXB) and red (RAXB) variants (compounds rich in polyphenols) on the concentrations of β-actin, NO• and HIF-1 in Hela cells cultures, to uncover possible anti-neoplastic effects. Extracts from the plant leaves were added to Hela cell cultures at a concentration of 10^{-3} mg/mL, and after 24 hours of culture, the concentrations of β-actin, NO• and HIF-1 were determined by immunohistochemical, biochemical and western blot assays. Both extracts reduced the concentrations of β-actin, NO• and HIF-1 ($p<0.001$), similar to the methotrexate effect. These results suggest an antineoplastic effect of the studied plant extracts and highlight the possibility of their use in the treatment of neoplasms.

Corresponding author. Yenddy Carrero. Universidad Técnica de Ambato. Facultad Ciencias de la Salud. C/ Salvador entre México y Colombia, Ingahurco. Ambato 180150, Ecuador. Tel: + 59 3 33730268 ext. 5239.
E-mail: yenddycarrero@yahoo.es

Los componentes de la *Arracacia xanthorrhiza Bancr* disminuyen las producciones de la actina β, del factor-1 inducible por la hipoxia y del óxido nítrico en células Hela.

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Palabras clave: actina; óxido nítrico; HIF-1; *Arracacia xanthorrhiza Bancr;* células Hela.

Resumen. El tratamiento de pacientes con cáncer utilizando drogas-antineoplásicas presenta problemas relacionados con efectos colaterales indeseables. Se ha sugerido el uso de derivados de plantas naturales solas, o en combinación con drogas antineoplásicas existentes para obtener mejores resultados y disminuir los efectos colaterales. Así mismo, se ha reportado que el óxido nítrico (NO•), el factor-1 inducible por hipoxia (HIF-1) y la disminución de la expresión de la actina tienen un papel en la progresión del cáncer. También se ha reportado los efectos beneficiosos de lo polifenoles en varios desordenes orgánicos, incluyendo el cáncer. El objetivo de este estudio fue determinar los efectos de los extractos procedentes de la *Arracacia xanthorrhiza Bancr* blanca (AXBB) y la variedad roja (AXBR) (compuestos ricos en polifenoles) en las concentraciones de la actina-beta, el NO• y el HIF-1 en cultivo de células Hela, para destacar sus posibles efectos antineoplásicos. A los cultivos de células Hela se les agregaron los extractos de las hojas de AXBB o AXBR (10^{-3} mg/mL, concentración final) y después de 24 horas de cultivo se determinaron las concentraciones de la actina-beta, el NO• y el HIF-1 por métodos inmunohistoquímicos, bioquímicos y western blot. Ambos extractos disminuyeron las concentraciones de la actina-beta, el NO• y el HIF-1 ($p<0,001$) de una manera similar al efecto del metotrexato. Estos resultados sugieren un efecto antineoplásico de estos extractos y destacan la posibilidad de ser usados para el tratamiento de las neoplasias.

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INTRODUCTION

Cellular motility is the basis for cancer cell invasion and metastasis. Migration involves an intricate actin branched network. Regulation of cell migration is necessary for development, wound healing, and immune responses, whereas aberrant and uncontrolled cell motility is a feature in cancer cells [1]. Therefore, compounds capable of inducing degradation of actin, could be important in the production of anti-cancer drugs by decreasing the actin-dependent cell motility. In addition to the alterations of actin, nitric oxide (NO•), a vasodilator, has been shown to have a role in the regulation of cancer progression and metastasis [2]; effects that probably are mediated by angiogenesis, apoptosis and cellular motility [3,4]. In addition, NO• increases the hypoxia-inducible factor-1 (HIF-1), a transcription factor that enhances several hypoxia-inducible genes and induces cancer metastasis [5]. Previous studies have shown

that methotrexate is capable of inducing apoptosis [6], an important cytotoxic mechanism of anti-cancer drugs. It is well known that apoptosis is associated with alterations of the reorganization and concentration of actin [7, 8]. Therefore, drugs that target actin, NO•, and HIF-1may be important in the treatment of cancer. Thus, the aim of this study was determining the effect of two ecovariants of *Arracacia xanthorrhiza Bancr* extracts, white (WAXB) and red (RAXB) variants on the concentrations of β-actin, NO• and HIF-1 in Hela cells cultures (uterine cervix cancer cell line) and compared their effects with the effect of methotrexate, a well-known anti-cancer drug.

MATERIALS AND METHODS

Source of plants

Plants were obtained from the following localities: Red *Arracacia xanthorrhiza Bancr* (RAXB) from the province of Tungurahua, Quinchicoto (Municipality Tisaleo), Ecuador (Latitude: 1° 23' 54" S; Longitude: 78° 24' 41" W; Altitude: 3,260 meters above sea level). White *Arracacia xanthorrhiza Bancr* (WAXB) from El Triunfo (Municipality Baños), Ecuador (Latitude: 1°10' 18" S; Longitude: 78°32' 33" W; Altitude: 1,683 meters above sea level). Plant samples used for this research have been authorized by the Ecuadorian government under the framework of the United Nations Convention on Biodiversity.

Extract procedure

Fresh plant leaves were collected and dried in an oven at 40°C for 24 hours until a constant weight was obtained. The specimens were stored at room temperature in the dark prior to their extraction and subsequent testing. Stock solutions of RAXB and WAXB were prepared by dissolving 60 g of leaves in ethanol (20%) at 80°C for one hour. Ethanol was extracted by a rotary evaporator and active compounds remained in water. Extracts were grinded into a powder (VirTis Bench Top, SP Scientific, NY, USA), and then dissolved in PBS and filtered (0.22 μm). Filtered stock solutions (0.411 mg/mL) were stored at -80°C until use. Total phenolic content (TPC) and antioxidant activity (AA) assays in microplates were determined. An intra-laboratory validation was performed [9] of the Folin-Ciocalteu microplate method to measure TPC and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) microplate method to measure AA.

Cell culture

The human cervical cell line (HeLa) was obtained from the American Type Culture Collection (CCL-2). Cells were maintained in DMEM media, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100U/mL streptomycin. The cells were then incubated at 37°C with 5% CO2 saturation. Cells were used in the linear phase of growth with a passage number of 5 to 7.

Viability assay

The cytotoxic effect of WAXB and RAXB on HeLa cells was evaluated by MTT assay according to the manufacturer's instructions (Thermo Fisher, MA, USA). The cells were seeded at $1x10^4$ cells per well in a 96-well plate and incubated at 37°C for 24 h. Cells were treated with increasing doses of WAXB or RAXB by 2-fold dilution, starting with 10^{-1}mg/mL until 10^{-11}mg/mL. Cells were treated for 24h. The MTT assay results were obtained using a spectrophotometer plate reader (Victor X3; Perkin Elmer, USA) at 570 nm. Half maximal inhibitory concentration (IC50: 10^{-3} mg/mL) was obtained by no lineal regression analysis (Graphpad Prism 7.0 Software Inc., San Diego, CA, USA). All experiments were performed in triplicate to evaluate half-maximal inhibitory concentration for both plant extracts against the HeLa cell line.

Western Blot

Untreated and WAXB or RAXB- treated Hela cell cultures (10^{-3} mg/mL) and metho-

trexate-treated cultures (10 μM), were lysed with RIPA buffer for 30 min on ice. Then, cells were centrifuged at 1200 rpm for 15 min at 4°C, and the supernatant was collected. The protein concentration was measured by the Bradford protein assay. Similar amount of protein from each sample was separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 7% skimmed milk for 2 h at room temperature and incubated overnight at 4°C with a primary anti- β actin antibody (Santa Cruz Biotechnology Inc, Texas, USA). After washing three times with TBST(10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 0.05% Tween 20), the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000 dilution: Santa Cruz Biotechnology Inc, Texas, USA). Experiments were performed in triplicate and at least three independent experiments were done. Controls represent untreated HeLa cultures.

β-actin determination

HeLa cells were seeded on coverslips at $5x10^5$ cells per well. After 48 h of culture, cells were treated with WAXB, RAXB (10^{-3} mg/mL) or methotrexate (10 μM) for additional 24 h. Cells were fixed and permeabilized by a paraformaldehyde solution (4%, 15 min) and cold acetone (15 min), respectively. Then, cells were reacted with a monoclonal antibody against β-actin (β-Actin (C4): sc-47778 conjugated Alexa Fluor®647; Santa Cruz Biotechnology Inc, Texas, USA) for 30 min at 37°C. Thereafter, cells on coverslips were analyzed through a fluorescence microscopy (Leica-DMi8, Wetzlar, Germany). Experiments were performed in triplicate and at least three independent experiments were done. DAPI staining was used to identify nuclei. Results were expressed as fluorescence intensity units (502/530 nm) per x 630 field. Controls represent untreated HeLa cultures.

Nitric oxide determination

Nitric oxide was determined by detection of nitrite formed by the spontaneous oxidation of NO• using a Griess Reagent Kit (G-7921: Molecular Probe Inc. Eugene, USA) following the manufacture's indications. Results are expressed as μM per mg of protein.

Hypoxia-inducible factor-1 determination

HIF-1 was determined in WAXB, RAXB (10^{-3} mg/mL) treated Hela cell cultures using Image-iT™ Hypoxia Reagents (Invitrogen, Termo Fisher Scientific, Eugene, USA) following the manufacture's indications. Cells on coverslips were analyzed through a fluorescence microscopy (Leica-DMi8, Wetzlar, Germany). Experiments were performed in triplicate and at least three independent experiments were done. DAPI staining was used to identify nuclei. Results were expressed as fluorescence intensity units (502/530 nm) per x 630 field. Controls represent untreated HeLa cultures.

Statistical analysis

Experiments were in triplicate and performed at least three times. Results were expressed as the means ± standard deviation. Statistical analysis was performed using one way ANOVA and Bonferroni´s posttest using Graph Pad Prism software (version 7.0; Graph Pad Software Inc., San Diego, CA, USA). Statistical significance is expressed as $p<0.05$.

RESULTS

The antioxidant activity of WAXB and RAXB and their phenolic content are observed in Table 1. The cytotoxicity of both extract plants was determined by the MTT assay. The MTT assay indicated that both WAXB and RAXB have a half maximal inhibitory concentration (IC50) of 10^{-3} mg/mL (Fig. 1). Western blot analysis showed decreased thickness and intensity of the actin

Table 1
Phenolic content and antioxidant activity of *Arracacia xanthorrhiza Bancr* (white carrot and red carrot) extracts.

	WABX	RABX
Phenolic content (mg Eq AG/g powder)	78.6 ± 0.002	59.7 ± 0.001
Antioxidant activity		
(μg Eq Trolox/g powder)	0.595 ± 0.03	0.395 ± 0.02
(μmol Eq Trolox/100g powder)	237.7 ± 0.03	157.8 ± 0.02

WABX: White *Arracacia xanthorrhiza Bancr*; RAXB: Red *Arracacia xanthorrhiza Bancr.*

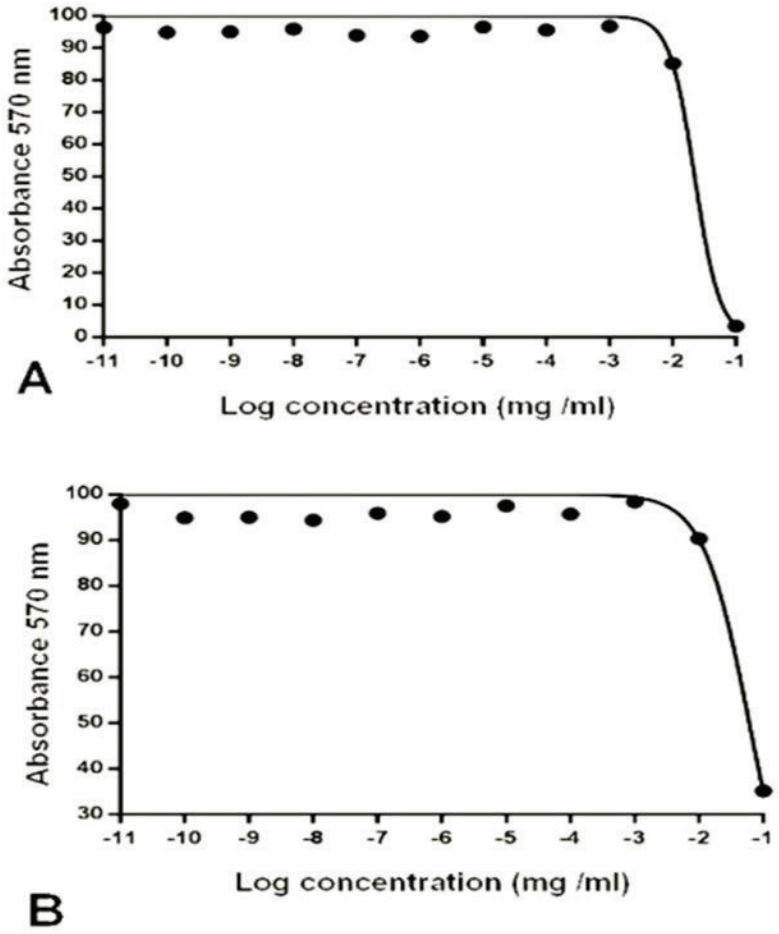

Fig. 1. Viability assay in Hela cell cultures. White (WABX) and red (RABX) *Arracacia xanthorrhiza Bancr* leaves cytotoxic effect on HeLa cells was evaluated by MTT assay. Cells were seeded at $1x10^4$ per well and treated with increased doses of WAXB (A) and RAXB (B) from 10^{-11}mg/mL to 10^{-1}mg/mL. Experiments were performed in triplicate to evaluate half-maximal inhibitory concentration (IC50: 10^{-3} mg/mL) for plant extracts.

bands in cultures treated with WAXB, RAXB or methotrexate compared with untreated cultures (Fig, 2). A similar anti-actin effect of plant extracts and methotrexate were observed. This finding was associated to decreased anti-β-actin antibody reactivity on Hela cells, as shown by immunofluorescence analysis (Fig. 3). WAXB, RAXB and methotrexate were capable of decreasing the nitrite (NO•) content in Hela cell cultures. Methotrexate had a higher NO• reducing effect when compared to plant extracts (Fig. 4). Hela cell cultures treated with WAXB, RAXB under culture conditions of 5% of CO_2, showed decreased expression of HIF-1in treated cultures compared to controls (Fig. 5).

DISCUSSION

Malignant cancer cells can invade surrounding tissues, progress to intravasation and, ultimately, to metastasize. Cellular membrane protrusion is related to local polymerization of actin filaments, and the molecules that link migratory signals to the actin cytoskeleton are upregulated in invasive and metastatic cancer cells [1]. Therefore, cellular actin cytoskeleton is very important for cancer cell migration during the formation of metastases [10]. Since modifications of the actin cytoskeleton are characteristic features of invasive tumor cells, it is likely that drugs inducing interactions of cytoskeletal proteins, can be useful to induce apoptosis

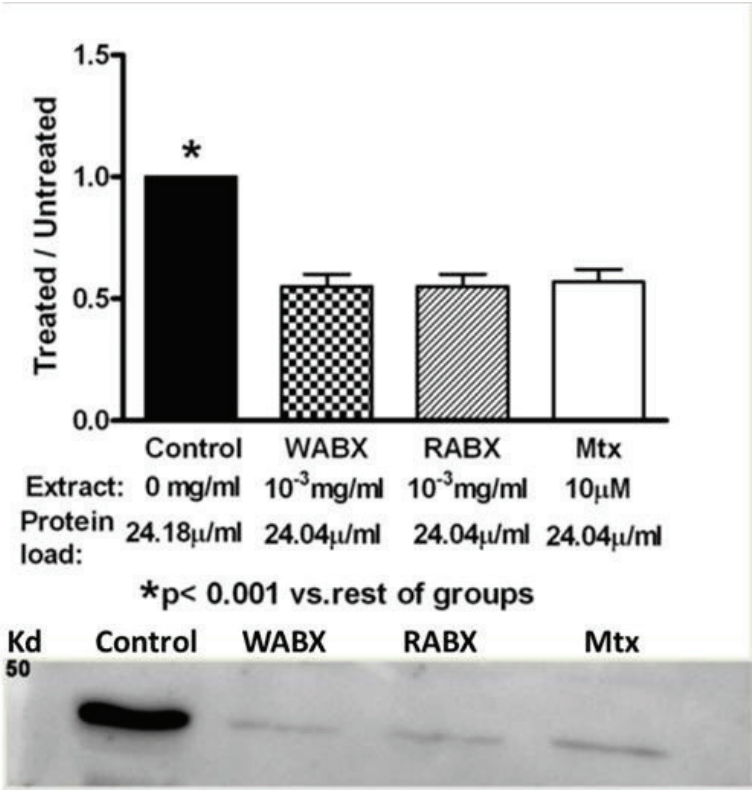

Fig. 2. β-actin expression in HeLa cell cultures treated with *Arracacia xanthorrhiza Bancr* extracts (10^{-3} mg/mL). In up panel: Decreased expression of β-actin was observed in plant extract treated cultures compared with untreated cultures. Similar effects of White *Arracacia xanthorrhiza Bancr* (WABX), Red *Arracacia xanthorrhiza Bancr* (RAXB) and methotrexate (Mtx: 10 μM) were observed. Low panel: Western blot analysis of β-actin in the same conditions observed in the upper panel. One way ANOVA with Bonferroni´s post-test.

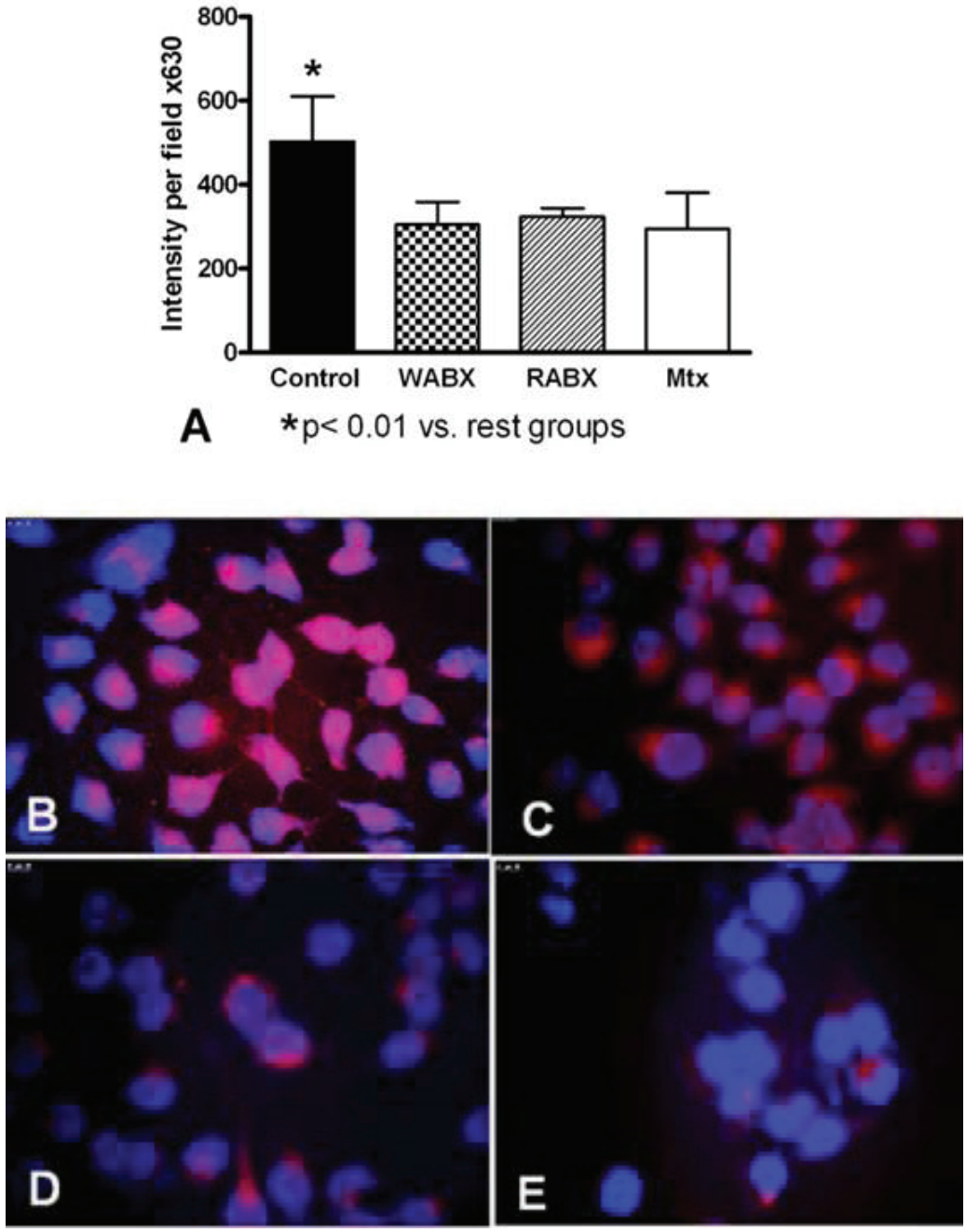

Fig. 3. β-actin expression in HeLa cell cultures treated with *Arracacia xanthorrhiza Bancr* extract. A) Decreased fluorescence intensity units were observed in White *Arracacia xanthorrhiza Bancr* (WABX), Red *Arracacia xanthorrhiza Bancr* (RAXB) (10^{-3} mg/mL) and methotrexate (Mtx: 10 μM) treated cultures compared with control without treatment. Low panel: β-actin positive cells in HeLa cell cultures. B) Control (untreated). C) WABX. D) RAXB. E) Methotrexate (10 μM). Monoclonal antibody against β-actin (β-Actin (C4): sc-47778 conjugated Alexa Fluor®647. One way ANOVA with Bonferroni´s post-test. Magnification X1000.

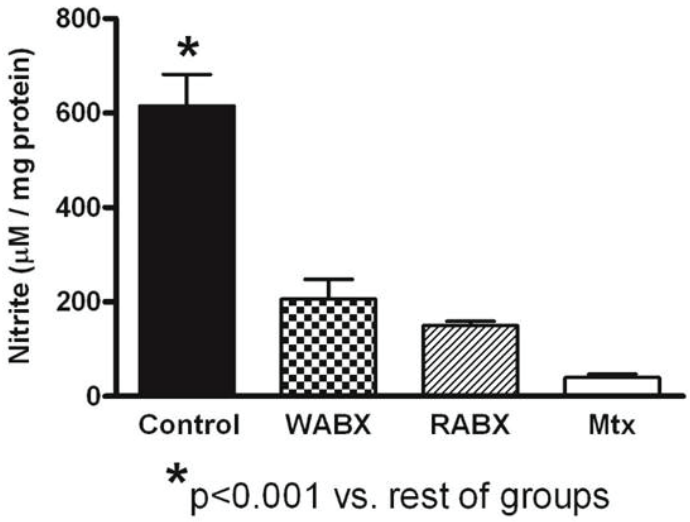

Fig. 4. Nitrite (NO•) production by HeLa cell cultures treated with *Arracacia xanthorrhiza Bancr* extracts. Decreased nitrite production was observed in White *Arracacia xanthorrhiza Bancr* (WABX), Red *Arracacia xanthorrhiza Bancr* (RAXB) (10^{-3} mg/mL) and methotrexate (Mtx: 10 μM) treated cultures compared with control (untreated). Methotrexate had higher reducer NO• effect compared to plant extracts. Griess Reagent Kit (G-7921: Molecular Probe Inc. Eugene, USA). One way ANOVA with Bonferroni´s post-test.

or inhibit cancer cell metastasis. In this regard, increased attention has been observed for drugs capable of modifying the state of actin (polymerization, microfilament organization) [11].

In this study, extracts from WAXB and RAXB could induce decreased expression of B-actin on Hela cells. The effects of WAXB and RAXB were like that observed using methotrexate. Methotrexate is an anti-folate used in the treatment of cancers and autoimmune diseases [12]. In previous a communication it has been reported that methotrexate is an actin affecting drug, acting mainly on its polymerized form [13]. In this study, all studied extracts and drug decreased the reactivity to an anti- β-actin antibody in Hela cells, suggesting an actin molecular alteration. We could not observe alterations in the actin cytoskeleton organization; however, some similar effects related to actin reorganization between methotrexate and extracts were observed. Methotrexate can induce alterations of the actin cytoskeleton in uterine cervix cancer cell line (CaSki) [6] as observed in Hela cells (uterine cervix cancer cell line) using extracts and methotrexate. Apoptosis is the major cytotoxic mechanism of anticancer therapies [8], and it is known that morphology alterations during apoptosis [7] correlate with the reorganization of the actin microfilaments and concentration of actin; in this study both, AXB extracts and methotrexate are capable of decreasing the actin concentration. These data suggest that the anticancer effects of WAXB and RAXB are like those observed in methotrexate. WAXB and RAXB actin degradation mechanisms remain unknown. Previous reports have shown that actin initiates apoptosis and that the final degradation of actin filaments enhances the apoptosis signaling [14]. A decreased actin band was observed in western blot analysis, suggesting degradation of actin molecule. Since plant extracts and methotrexate are capable of inducing apoptosis, activation of proteases during the apoptotic process may act on actin leading to its degradation. In

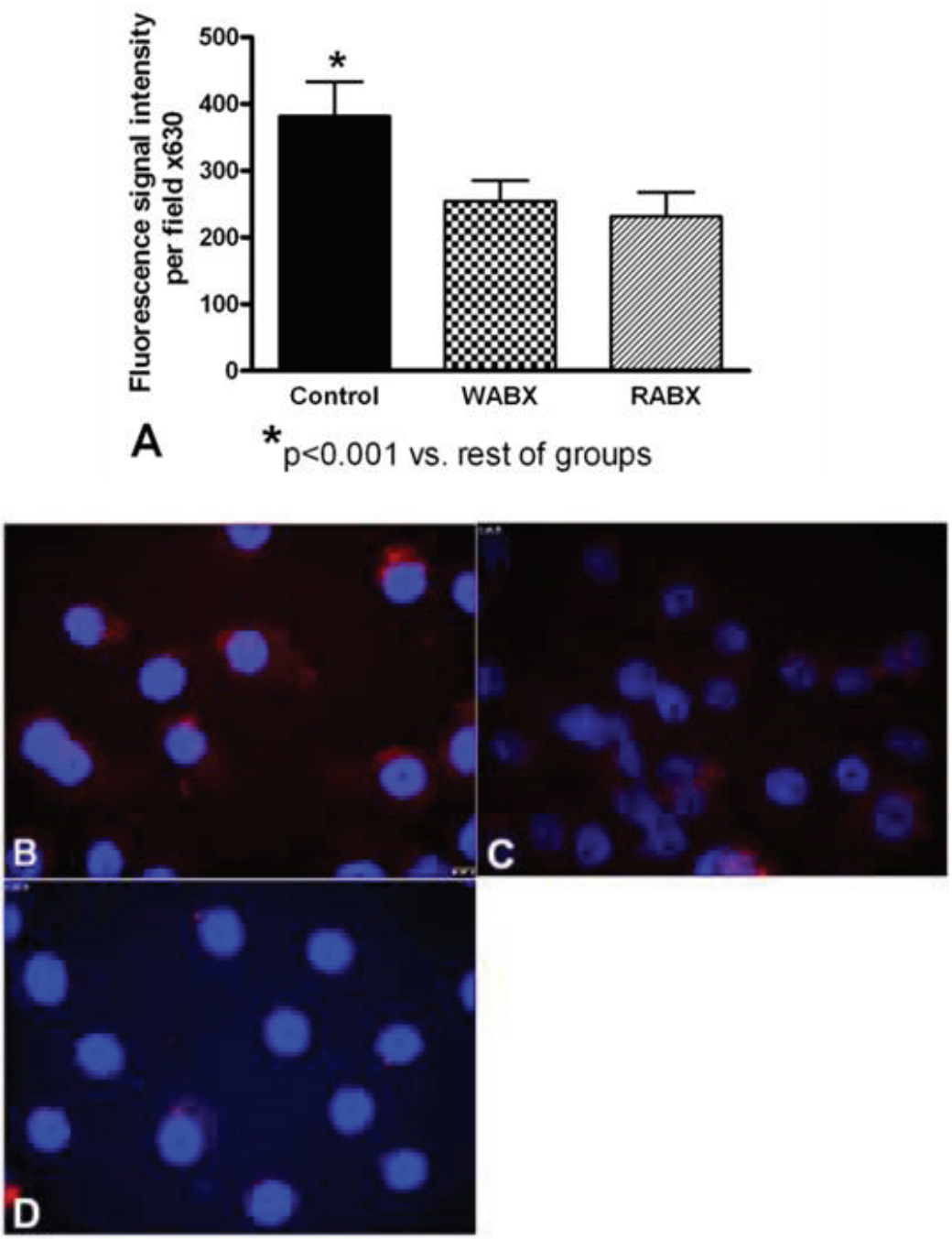

Fig. 5. Hypoxia-inducible factor-1expression (HIF-1)in HeLa cell cultures treated with *Arracacia xanthorrhiza Bancr* extracts. A) Decreased fluorescence intensity units were observed in White *Arracacia xanthorrhiza Bancr* (WABX) and Red *Arracacia xanthorrhiza Bancr* (RAXB) (10^{-3} mg/mL) treated cultures compared with control (untreated). Low panel: HIF-1positive cells in HeLa cell cultures. B) Control. C) WABX. D) RAXB. Image-iT™ Hypoxia Reagents (Invitrogen, Termo Fisher Scientific, Eugene, USA). One way ANOVA with Bonferroni´s post-test. Magnification X1000.

this regard, interleukin-1b-converting enzyme (ICE)-like family of proteases such as Nedd2 / Ich-1, CPP32 / Apopain / Yama, TX / ICE_{rel}II, TY / ICE_{rel}III, Mch 2, Mch 3 / ICE-LAP3, Mch 4, and FLICE are implicated in actin degradation during apoptosis [15].

Nitric oxide has been involved in the cancer biology probably by induction of angiogenesis, apoptosis, increased HIF-1 and alterations of cellular motility [3-5]. Some of these effects can induces cancer metastasis, spread and growth of tumor cells through angiogenesis and high production of HIF-1; however, other effects such as apoptosis and alterations of actin may impede or eliminate metastatic progression [2]. Angiogenesis is the growth of new blood vessels and is essential for tumor progression and metastasis [16]. It has been reported that increased activity of isoform enzyme iNOS (inducible nitric oxide synthase) facilitates tumor cell angiogenesis and metastasis [3], probably mediated by interleukin-33 [17].

HIF-1 is a transcription factor that regulates hundreds of genes, and it is activated by hypoxia (reduced oxygen availability) [18]. HIF-1 is stabilized by hypoxia-dependent or independent pathways and associated with pro-carcinogenic effects [19]. Stabilization of HIF-1 leads to changes in glycolysis, nutrient uptake, angiogenesis, cell migration and apoptosis promoting survival of tumor and metastasis [20, 21]. However, HIF can be activated by non-hypoxic pathways. In this regard, NO• can induce this transcription factor that enhances many hypoxia-inducible genes, via the PI3k/Akt pathway [5].

Previous studies have shown that plant extracts and polyphenolic compounds may have a beneficial role in several diseases including cancer [22-26]. Mechanisms related to the regulation of immune system, inactivation of arachidonic acids pathways, inactivation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), suppression of toll-like receptor and antioxidant activity [27] may be involved. Biochemical analysis of WAXB and RAXB shows that both are phenolic compounds and the effects previously reported for phenolic compounds probably are implicated in ours results. In this regard, WAXB and RAXB have antioxidant activity as shown by the Trolox assay. In addition, these plant extracts induced a decreased content of NO•, suggesting that these extract plants can act on nitrogen reactive species and may represent one of their anti-cancer mechanisms.

In conclusion, in this study Hela cells were capable of producing NO• and HIF-1, compounds that are related to cancer progression. WABX and RABX extracts induced decreased expressions of NO• and HIF-1 and actin-β in treated cultures. These effects associated with anti-cancer progression were similar to the effect of methotrexate, a well-known anti-cancer drug. As an important point, not included in this study, it is necessary to determine the effect of WAXB and RWABX on other types of cancer to determine if these plant extracts have a general effect on cancer. Further investigation should explore the clinical benefits of WAXB and RWABX (dietary or alcoholic extract) in human cancer.

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Declaration of conflict of interest

The manuscript has no conflict of interest.

Authors' ORCID numbers

- Yenddy Carrero (YC) 0000-0003-4050-4468
- Jenny Moya (JM) 0000-0002-9846-0122
- Michael Acosta (MA) 0000-0001-9437-828X
- Jesús Mosquera-Sulbaran (JM-S) 0000-0002-1496-5511

Authors' Contribution

- Conception and design of the work YC/JM/JM-S
- Experimental work YC/JM/MA
- Data Collection/obtaining results YC/JM/MA
- Data analysis and interpretation YC/JM/MA/JM-S
- Drafting the article. YC/JM-S
- Critical revision of the article YC/JM-S
- Final approval of the version to be published. YC/JM/MA/JM-S.

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