**Arracacia Xanthorrhiza Bancr (White Carrot) Extract Induces Increased Expression of Caspases 3/7, Bax, Apoptosis and Anti-Oxidative Effects in Hela Cells**

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**Abstract**

The use of natural products has been one of the most effective ways to fight malignancies. Induction of apoptosis has been shown as a relevant strategic in cancer therapy. Several studies have revealed the mechanism by which continued oxidative stress can lead to chronic inflammation, which in turn could mediate chronic diseases including cancer. The aim of this study was to determine the effect of *Arracacia Xanthorrhiza Bancr* (AXB) alcoholic extract on apoptosis and oxidative stress in HeLa cell cultures. Apoptosis was determined by TUNEL assay. Caspase 3/7, Bax, Bcl2, mitochondrial superoxide anion, reactive oxygen species and glutathione were analyzed by commercial kits following manufacturer’s instructions and by western blot. AXB treated cultures showed increased apoptosis, activation of caspase 3/7 and increased Bax expression accompanied by decreased expression of Bcl2 (p<0.001). In addition, decreased production of radical oxygen species, mitochondrial superoxide anion and increased production of glutathione were also found in AXB treated cultures (p<0.001). These properties of AXB highlight its potential effect as an anti-neoplasia compound. Further large-scale cohort studies are required to confirm the anticancer effect of AXB in oncologic patients.

**Keywords:** Apoptosis; Cancer cervical; Antioxidant; Diet.

**Manuscript Information:** Received: March 13, 2020; Accepted: April 10, 2020; Published: April 14, 2020

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Introduction

The induction of apoptosis and the ability to favor recognition and elimination of dying tumor cells by phagocytes in association with the release of pro-inflammatory molecules (such as cytokines and high-mobility group box-1) [1] highlight the potential value of the apoptosis inducers in cancer immunotherapy protocols. Despite the remarkable progress in cancer therapy in recent years, this disease remains as a serious public health concern. The use of natural products has been and continues to be one of the most effective ways to fight malignancies. Various compounds from African flora displayed prominent cytotoxic effects in vitro in many cancer cells lines [2,3]. In fact, over 25% of pharmaceutical drugs used in medicine today are derived from plants [4]. For instance, plant secondary metabolites such as terpenes, phenolics and alkaloids, are extensively exploited in cancer research [5,6]. *Arracacia Xanthorrhiza* Bancr (AXB) (*Umbelliferae*. Arracacha. Peruvian Carrot) is plant that has been cultivated and used as a food from early times in the cooler mountainous districts of northern South America, where the roots form a staple diet of the inhabitants [7,8,9]. There is no information regarding to the effect of this plant on the viability of cancer cells. HeLa cells, a cervical cancer cells, have been used to detect the apoptotic effect of some plants [10]; therefore, the aim of this study was to determine the apoptotic and anti-oxidant effects of AXB leaf extract on HeLa cells and the ability of this plant to activate different apoptosis inducers.

Aim of the study

In this study, we investigated the potential role of *Arracacia Xanthorrhiza* Bancr in the apoptosis and oxidative stress in HeLa cell cultures by analyzing the capacities of this plant to increase apoptosis and decrease oxidative stress in cancer cells.

Materials and methods

Source and storage of plants: Dried plants (leaf) of AXB were obtained from crops in the province of Tungurahua (Ecuador). Fresh plant materials 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 AXB 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. Extract was ground into 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 was determined. For instance, plant secondary metabolites such as terpenes, phenolics and alkaloids, are extensively exploited in cancer research [5,6]. *Arracacia Xanthorrhiza* Bancr (AXB) (*Umbelliferae*. Arracacha. Peruvian Carrot) is plant that has been cultivated and used as a food from early times in the cooler mountainous districts of northern South America, where the roots form a staple diet of the inhabitants [7,8,9]. There is no information regarding to the effect of this plant on the viability of cancer cells. HeLa cells, a cervical cancer cells, have been used to detect the apoptotic effect of some plants [10]; therefore, the aim of this study was to determine the apoptotic and anti-oxidant effects of AXB leaf extract on HeLa cells and the ability of this plant to activate different apoptosis inducers.

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 AXB on HeLa cells was evaluated by MTT assay according to manufacturer’s instructions (Thermo Fisher, MA, USA). The cells were seeded at 1x10⁶ cells per well in a 96-well plate and incubated at 37°C for 24 h. Cells were treated with increasing doses of AXB by 2-fold dilution starting with 10⁻³ mg/ml until 10⁻⁷ 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: 10⁻⁷ 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 AXB against the HeLa cell line.

Caspase 3/7 and oxidative stress determinations: The activation of caspases 3/7 was determined by a commercial kit following manufacture’s instructions (CellEvent Caspase 3/7 Green Detection Reagent, MA, USA). HeLa cells were seeded on coverslips at 5x10⁵ cells per well. After 48 h of culture, cells were treated with AXB (10⁻⁵ mg/ml) for additional 24 h. Cells were fixed and permeabilized by paraformaldehyde solution (4%,15 min ) and Triton X-100 (0.1 %, 15 min ) respectively. Then, caspase reagent (5 µM) was added to cells and incubated 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 3 independent experiments were done. As a positive control nickel chloride to induce caspase activation was used at an optimal dose (10⁻⁵ mg/ml). DAPI staining was used to identify nuclei. Results were expressed as fluorescence intensity units (502/530 nm) and number of caspase positive cells per x630 field. To determine oxidative stress, HeLa cultures treated as describe above, were reacted with Cell ROX Oxidative Stress Reagent to determine ROS production. MitosoxRed mitochondrial superoxide indicator (M36008) to determine mitochondrial superoxide anion or Thiol Tracker Violet: Glutathione Detection Reagent (T10095) to determine glutathione production. All reagents were obtained from Thermo Fisher Scientific (MA, USA). Thereafter, cells on coverslips were analyzed through a fluorescence microscopy (Leica-DMi8, Wetzlar, Germany). Experiments were performed in triplicate and at least 3 independent experiments were done. Results were expressed as fluorescence intensity units (502/530 nm) and number of positive cells per x630 field. Controls represent AXB untreated HeLa cultures.

Apoptosis determination: Late apoptosis was determined by a commercial kit (APO-BrdU TUNEL assay kit; Invitrogen, CA, USA) [12]. HeLa cells were seeded on coverslips at 1x10⁶ cells per well. After 24 h of culture, cells were treated with AXB (10⁻³ mg/ml) for additional 24 h. Cells were fixed with paraformaldehyde solution (1%, 15 min ). Thereafter, cells were incubated with ethanol (70 %) for 18 h at -20°C. Final detection of BrdU incorporation at DNA break sites in apoptotic cells was achieved through TUNEL assay kit following manufacturer’s instructions. As a positive control methotrexate (10 µM) an inducer of apoptosis was treated with AXB (10⁻³ mg/ml) for additional 24 h. Cells were treated with increasing doses of AXB by 2-fold dilution starting with 10⁻³ mg/ml until 10⁻⁷ 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: 10⁻⁷ 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 AXB against the HeLa cell line.
Western Blot: Untreated and AXB treated Hela cell cultures as described above (10⁴ mg/ml) 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-bax antibody (Santa Cruz Biotechnology Inc, Texas, USA). After washing with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for three times, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000 dilution). Anti-β-actin antibody was used as a loading control.

Statistical analysis: Experiments were in triplicate and performed at least three times. Results were expressed as the means value ± standard deviation. Statistical analysis was performed using one-way ANOVA and Bonferroni’s post-test or unpaired t-test. Analysis was performed 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 phenolic content and antioxidant activity of AXB are observed in Table 1. The cytotoxicity of AXB was determined by MTT assay. The MTT assay results indicated that AXB treatment markedly decreased the cell viability of HeLa cells (Figure 1). Under our experimental conditions, increased apoptotic cells were observed in AXB treated cultures compared with control or methotrexate (10 µM) treated cultures. Methotrexate was capable of inducing apoptosis, however AXB extract had higher apoptosis inducer effect than methotrexate (Figure 2). Related to apoptotic inducer effect, increased fluorescence intensity units and number of caspase 3/7 positive cells in AXB treated cultures were observed (Figure 3). The number of positive caspase 3/7 cells was similar in extract or nickel (caspase inducer) treated cultures. In addition, increased fluorescence intensity units and number of Bax positive cells (Figure 4) accompanied by decreased Bcl2 expression (Figure 5) were observed in AXB treated cultures.

Hela cultures showed oxidative stress since increased production of ROS, and mitochondrial superoxide anion were observed (Figures 6 and 7). In addition to apoptotic effect, AXB was capable to reduce oxidative stress in HeLa cultures. Accordingly, decreased productions of ROS (Figure 6) and mitochondrial superoxide anion (Figure 7) were observed, suggesting an antioxidant effect. Together with these effects, increased production of glutathione (Figure 8) in AXB treated cultures was found.

Discussion

Traditional medicine plays a critical role in treatment of many chronic conditions and diseases, including cancer [13]. Since, the severity of side effects and acquired resistant of recent chemotherapeutic agents remain as important problems, to investigate for other anticancer treatments is necessary [13,14].
effect is expected when anti-oxidant status is presented decreasing mutagenic effect of ROS [33,34]; however, in murine lung cancer model, antioxidant treatment increases tumor progression and reduces mouse survival leading to reduced ROS levels, p53 expression and DNA damage in cancer cells [35]. Taken together, these data could show a bimodal effect of oxidant/anti-oxidant status and may be related to cancer cell type.

As important points, not included in this study, it is necessary to determine the effect of AXB on normal cervical cells to define its specificity on neoplastic cells and on other types of cancer to determine if AXB has a general effect on cancer.

Conclusion

In conclusion, the present study highlights the AXB potential as a source of cytotoxic phytochemicals to combat malignant diseases. AXB was capable of inducing apoptosis to HeLa cells, probably mediated by activation of caspase 3/7, increased expression of bax and diminishing the mutagenic effect of oxidative stress by its anti-oxidant capacity. Further investigation should explore the clinical beneficial of AXB (dietary or alcoholic extract) in human cancer.

Figures

**Figure 1:** Viability assay in Hela cell cultures. *Arracacia xanthorrhiza Bancr* (AXB) cytotoxic effect on HeLa cells was evaluated by MTT assay. Cells were seeded at 1x10⁴ per well and treated with increased doses of AXB from 10⁻³ mg/ml to 10⁻¹ mg/ml. Experiments were performed in triplicate to evaluate half-maximal inhibitory concentration for AXB.

**Figure 2:** Apoptosis expression in HeLa cell cultures treated with *Arracacia xanthorrhiza Bancr* extract (10⁻³ mg/ml). In up panel: Increased number of apoptotic cells was observed in extract treated cultures compared with methotrexate (MTX: 10 µM) and control without treatment. Higher apoptosis inducer effect in the extract than in MTX was observed. Low panel: Tunel reactive cells in HeLa cell cultures. A) Control (no treated). B) MTX treated cultures. C) Extract treated cultures. Arrows: Tunel positive cells. APO-BrdU TUNEL assay. One-way ANOVA, Bonferroni’s post-test. Magnification X1000.
Figure 3: Caspase 3/7 expression in HeLa cell cultures treated with *Arracacia xanthorrhiza Banca* extract. In up panel: Increased fluorescence intensity units and number of caspase 3/7 positive cells were observed in extract (10^{-3} mg/ml) and nickel (10^{-7} mg/ml) treated cultures compared with control without treatment. Low panel: Caspase 3/7 positive cells in HeLa cell cultures. A and B) Control (no treated). C and D) Nickel treated cultures. E and F) Extract treated cultures. Arrows: Caspase 3/7 positive cells. Cell Event Caspase 3/7 Green Detection Reagent. One-way ANOVA, Bonferroni’s post-test. Magnification X1000.

Figure 4: Bax expression in HeLa cell cultures treated with *Arracacia xanthorrhiza Banca* extract (10^{-3} mg/ml). In up panel: Increased intensity units (A) and number of bax positive cells (B) in extract treated cultures were observed when compared with controls (untreated cultures). C) Untreated HeLa cell culture. Arrow shows bax positive cells. D) Extract treated cultures. Arrows show bax positive cells. Indirect Immunohistochemistry. E) Western blot quantification. Unpaired t test. Magnification X1000.
Figure 5: Bcl2 expression in HeLa cell cultures treated with _Arracacia xanthorrhiza_ Bancr extract (10^{-3} mg/ml). In up panel: Increased intensity units (A) and number of bax positive cells (B) in extract treated cultures were observed when compared with controls (untreated cultures). C) Untreated HeLa cell culture. Arrow shows Bcl2 positive cells. D) Extract treated cultures. Arrows show Bcl2 positive cells. Indirect Immunohistochemistry. Unpaired t test. Magnification X1000.

Figure 6: Reactive Oxygen Species (ROS) expression in HeLa cell cultures treated with _Arracacia xanthorrhiza_ Bancr extract (10^{-3} mg/ml). In up panel: Decreased intensity units (A) and number of ROS positive cells (B) were observed in extract treated cultures compared with controls (untreated cultures). C) Untreated HeLa cell culture. Arrows show ROS positive cells. D) Extract treated cultures. Arrow shows ROS positive cell. Cell ROX Oxidative Stress Reagent. Unpaired t test. Magnification X1000.

Figure 7: Mitochondrial Superoxide Anion (MSA) expression in HeLa cell cultures treated with _Arracacia xanthorrhiza_ Bancr extract (10^{-3} mg/ml). In up panel: Decreased intensity units (A) and number of ROS positive cells (B) were observed in extract treated cultures compared with controls (untreated cultures). C) Untreated HeLa cell culture. Arrows show MSA positive cells. D) Extract treated cultures. Arrow shows MSA positive cell. MitosoxRed mitochondrial superoxide indicator (M36008). Unpaired t test. Magnification X1000.

Figure 8: Glutathione expression in HeLa cell cultures treated with _Arracacia xanthorrhiza_ Bancr extract (10^{-3} mg/ml). In up panel: Increased intensity units (A) and number of glutathione positive cells (B) were observed in extract treated cultures compared with controls (untreated cultures). C) Untreated HeLa cell culture. Arrow shows glutathione positive cells. D) Extract treated cultures. Arrows show glutathione positive cells. Thiol Tracker Violet: Glutathione Detection Reagent (T10095). Unpaired t test. Magnification X1000.
Table 1: Phenolic content and antioxidant activity of *Arracacia xanthorrhiza* (White carrot) extract.

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<thead>
<tr>
<th>Phenolic content</th>
<th>Antioxidant activity</th>
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<tr>
<td>(mg Eq AG/g powder)</td>
<td>78.561 ± 0.002</td>
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<tr>
<td>(μg Eq Trolox/g powder)</td>
<td>0.595 ± 0.03</td>
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<tr>
<td>(μmol Eq Trolox/100g powder)</td>
<td>237.690 ± 0.03</td>
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**Author's contributions**

Y. Carrero and J. Mosquera conceived and designed the experiments. M. Davila, J. Moya, I. Nuñez, M. Acosta, C. Aranda and I. Manjarres were involved in acquisition of data (performed the experiments). Y. Carrero and J. Mosquera were involved in analysis and interpretation of data and were responsible for writing and reviewing of the manuscript.

**Acknowledgments**

This research was supported by a grant from Dirección de Investigación y Desarrollo de la Universidad Técnica de Ambato, Ecuador (grant number: 0452-CU-P-2016).

**References**


