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Cytotoxic activity of gallic acid and myricetin against ovarian cancer cells by production of reactive oxygen species †

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Abstract: Some studies demonstrate that gallic acid (GA) and myricetin (MYR) isolated from *Rhus trilobata* provide the therapeutic activity of the plant against cancer. However, few reports demonstrate that both compounds also could have therapeutic potential in ovarian cancer. Therefore, evaluate the cytotoxic activity of GA and MYR against ovarian cancer cells and determine the possible action mechanism present, is important. For this purpose, SKOV- 3 cells (ovarian adenocarcinoma; HTB-77™, ATCC®) were cultivated according to supplier's instructions (37°C and 5% CO_2), to determine the biological activity of GA and MYR by confocal/transmission electron microscopy, PI-flow cytometry, H_2DCF-DA, MTT, and Annexin-V assays. Possible molecular targets of the compounds were determined by the Similarity Ensemble Approach-model. Results showed that GA and MYR treatments decreased viability of SKOV-3 cells at 50 and 166 µg/mL respectively ($p \leq 0.05$, ANOVA vs vehicle group); As well as morphological changed (cytoplasmic reduction, nuclear chromatin condensation, cytoplasmic vesicles increment, polymerized actin, and stabilized tubulin), cell cycle arrest (GA: 8.3% G_2/M and MYR: 78% G_1), and apoptosis induction (GA: 18.9% and MYR: 8.1%), because to ROS generation (34 to 42%) for 24 h ($p \leq 0.05$, ANOVA vs vehicle group). *In silico* studies demonstrated that GA and MYR interact with carbonic anhydrase and PI3K, respectively. As conclusions, GA and MYR show cytotoxic activity against SKOV-3 cells through ROS production, which modify the cytoskeleton and induces apoptosis, mainly. Therefore, GA and MYR could be considered as base compounds for the development of new treatments in the chemotherapy of ovarian cancer.

Keywords: Biomedicine; Cancer therapeutics; Cytotoxic activity; Gallic acid; Myricetin; Reactive oxygen species; SKOV-3

1. Introduction

Ovarian cancer is the sixth most frequent tumor in women and represents the fourth cause of death in Mexico due to gynecological tumors [1]. The principal treatment for this disease is surgical resection, followed by a complementary treatment with chemotherapy in which cytotoxic or cytostatic drugs are

administered with antitumor activity [1,2]. However, chemotherapy for ovarian cancer has shown limited success and generation of resistance in neoplastic tissue, whereby search for alternative treatments or new therapeutic agents for this disease is necessary. Plants used for alternative medicine represent an option in the search for active compounds for cancer treatment. Some examples of plant-compounds with antineoplastic action and used in the treatment of ovarian cancer are paclitaxel (*Taxus brevifolia*) [3], vincristine (*Catharanthus roseus*) [4], and curcumin (*Curcuma longa*) [5], among others [1,2].

Polyphenols are another type of compound found in plants, which have attracted attention in recent decades for their beneficial effects on health because polyphenols can fight diseases such as cancer through induction of oxidative stress by the generation of reactive oxygen species (ROS) [6]. In the same sense, several studies have demonstrated that the ROS can take action as second messengers and regulate different biologic processes in the cells, as the cytoskeleton restructured, the division, and the death, mainly [7]. Some examples of polyphenols with anti-cancer properties are gallic acid (GA), quercetin, fisetin, kaempferol, myricetin (MYR), among others [8]. However, several findings have demonstrated that particularly GA and MYR, could have interesting applications in ovarian cancer treatment [9, 10].

Therefore, this study has aimed to evaluate the cytotoxic activity of GA and MYR against ovarian cancer cells and to determine the possible action mechanism present.

2. Materials and Methods

2.1. Compounds studied

Compounds evaluated were GA (G7384) and MYR (M6760) from Sigma-Aldrich© (≥ 96 % purity, HPLC-grade). Controls used were paclitaxel (T7402, Sigma®) because is a drug utilized as treatment of ovarian cancer, 0.3 % hydrogen peroxide (H_2O_2; H1009, Sigma®) as oxidative stress inducer, and 0.5 % DMSO/1X PBS as a vehicle (D2650, Sigma®) for dilution of compounds. Reagents and equipment utilized complementary in the study are mentions in the text.

2.2. Cell culture protocol

The cell line used was SKOV-3 (ovarian adenocarcinoma, HTB-77™) acquired from ATCC® (Virginia, U.S.). The cell culture made was according to the supplier's instructions (37 °C and 5 % CO_2). The cell collections were with 1X trypsin-EDTA (Sigma®), and the determination of density was with 0.4% Trypan blue (Sigma®).

2.3. Determination of cell viability by Formazan salts

Cultures of 20,000 cells/well were cultivated with 200 µL of supplemented medium (Sigma®), under 96-well plates (Corning®), and incubated for 24 h. Treatments on adherent cells were with 10 to 200 µg/mL of samples or controls for 24 h. At the end of treatments, 5 mg/mL MTT (Sigma®) was added for 4 h, and optical density was measured at λ: 590 nm in VariosKan® Flash (Thermo-Scientific®). Half-maximal inhibitory concentration (IC_{50}) was calculated by regression analysis (percentage survival vs log concentration). Verification of results was by phase-contrast microscopy (LSM 700, Zeiss®) [8].

2.4. Measurement of ROS and apoptosis

Cultures of 20,000 cells/well were cultivated with 200 µL of supplemented medium (Sigma®), under 96-well black plates (Corning®), and incubated for 24 h. Adherent cells were treated with IC_{50} of samples or controls for 24 h. Then, the culture medium was removed, and ROS/apoptosis was determined on independent assays as follows: i) ROS: 25 µM H_2-DCF-DA (Sigma®), for 15 min, at 37 °C, and λ: $488_{ex}/529_{em}$ nm; ii) Apoptosis: 200 µL 1X binding buffer, 2 µL AnV-FITC (BioVision™), for 15 min, at 37 °C, λ: $485_{ex}/538_{em}$ nm. The optical density measured was in VariosKan® Flash (Thermo-Scientific®). Verification of results was by confocal microscopy (LSM 700, Zeiss®).

2.5. Evaluation of cell cycle by flow cytometry and cell morphology by immunofluorescence/TEM

Cultures of 50,000 cells/well were cultivated with 2 mL of supplemented medium (Sigma®), under 6-well plates (Corning®), and incubated for 24 h. Adherent cells were treated with IC_{50} of samples or controls for 24 h. Then, the culture medium was removed, and cell cycle, cell morphology, or cell ultrastructure were determined on independent assays by flow cytometry, immunofluorescence, and transmission electron microscopy (TEM) respectively, according to the method disclosed by Varela-Rodríguez *et al.* (2019) [8].

2.6. In-silico analysis.

Identification of target pharmacophores was made with the Similarity Ensemble Approach-model (SEA) (http://sea.bkslab.org/) [11] to find proteins with binding sites for the active compounds through an inverse protein-ligand approach. The parameters used were as follows: *pKi*, *P-Value,* or *Max TC*, for selecting the possible target protein.

2.7. Statistical analysis.

The presentation of results was as mean ± S.D. of 3 independent assays (n = 3; triplicates). The statistical analysis was performed for parametric data with normal distribution using one-way ANOVA and made comparisons among means with the negative control (vehicle group without treatment) through Tukey and Dunnett tests (Minitab®, version 18). Differences founded were taken as significant when $p \leq 0.05$.

3. Results and Discussion

Some studies demonstrate that polyphenols can induce oxidative stress in cancer cells and alter various biological processes [6]. Whereby in SKOV-3 cells, were determined cell viability, apoptosis, and ROS after treatment with AG and MYR for 24 h. GA and MYR presented an IC_{50} of 50 and 166 µg/mL respectively, in comparison with the negative control (vehicle group) ($p \leq 0.05$, Dunnett) (Figure 1A). However, these results were not like the exhibited for paclitaxel, as a positive control (reference group of cancer drug) with IC_{50} of 5 µg/mL. In the same conditions was detected, cell death by apoptosis for the presence of phosphatidylserine in the external cell membrane (Figure 1D). GA and MYR induced 18.9 and 8.1 % of apoptosis comparison to the vehicle group ($p \leq 0.05$, Dunnett) (Figure 1B). The apoptosis induction was like the observed with paclitaxel but less intensity for both compounds (Figure 1D). Additionally, GA and MYR increased intracellular-ROS levels among 42 and 34 % comparison with 3.5 % observed in the vehicle group ($p \leq 0.05$, Dunnett) or 76.5 % with the H_2O_2 group (Figure 1B and D).

Several studies have demonstrated that ROS can act as second messengers and modify the cytoskeleton restructured and the cell division [7]. Therefore, analyses of cytoskeleton protein as actin and tubulin were made, as well as different phases of the cell cycle during treatments with GA and MYR in SKOV-3 for 24 h. Some morphological changes observed in treatments with both compounds were rounding and individualization cellular (PCM at 40X; Figure 1D), as well as cytoplasmatic reduction, chromatin condensation in the nucleus, and numerous cytoplasmic vesicles (TEM at 1,000X; Figure 1D). These features were similar to the paclitaxel group; However, the characteristics found differed with the vehicle group that present mitotic division and chromosomal segregation (TEM at 1,000X; Figure 1D). Additionally, these changes suggest the presence of apoptotic events and corroborate the results mentioned above. Subsequently, microfilaments and microtubules network were analyzed in the cell cytoskeleton through immunofluorescence to observe changes in the structuration of actin and tubulin of SKOV-3 cells (Figure 2). Both compounds in actin caused polymerization, and a decrease of membrane prolongations or filaments, while in tubulin caused stabilization and an increase of microtubules (Figure 2). These changes were found in the paclitaxel group but absented in the vehicle group (Figure 2); Whereby, these findings can have implications

important in the compromise of cell functions such as transport, traffic, support, and division [7], however further studies to verify this claim are needed.

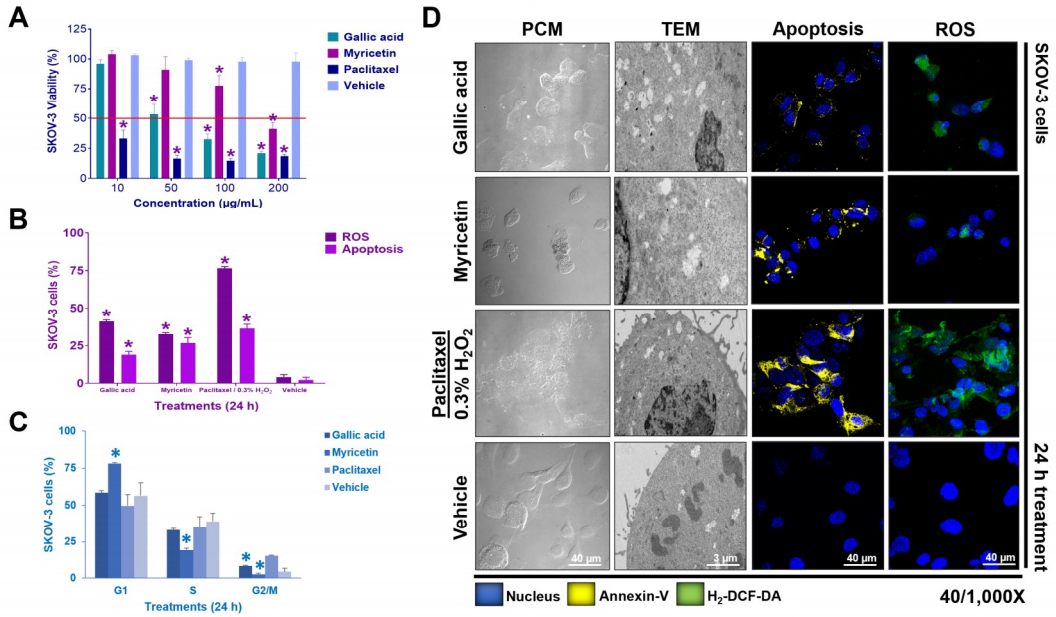

Figure 1. Biological activity of GA and MYR in SKOV-3 cells. The IC_{50} of both compounds was acquired of 24 h-viability curves in SKOV-3 cells by MTT assay (**A**). Induction of oxidative stress and apoptosis were measured in SKOV-3 cells by H_2-DCF-DA and annexin-V assays respectively, after 24 h of treatment with GA (50 µg/mL) and Myr (166 µg/mL) (**B**). DNA content was measured by PI and flow cytometry in the same conditions mentioned above to determine phases of the cell cycle (**C**). Morphological and ultrastructural changes were observed in SKOV-3 cells by PCM and TEM, respectively (**D**). Results represent mean ± S.D. of 3 independent replicates (n = 3; triplicates); * Difference significative respect to vehicle group (cells treated with 0.5 % DMSO/1X PBS) ($p \leq 0.05$, ANOVA). Paclitaxel and H_2O_2 were positive controls used according to the assay. IC_{50}, half-maximal inhibitory concentration; GA, gallic acid; MYR, myricetin; ROS, reactive oxygen species; PI, propidium iodide; PCM, phase-contrast microscopy; TEM, transmission electron microscopy.

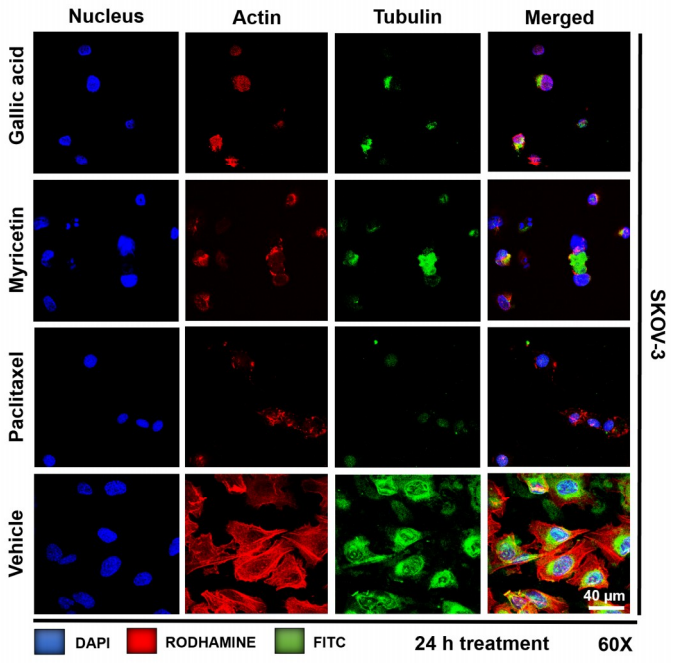

Figure 2. Cytoskeletal alterations in SKOV-3 cells during treatments with GA and MYR. Morphological changes observed in actin microfilaments or tubulin microtubules in SKOV-3 cells after 24 h of treatment with GA (50 μg/mL) and Myr (166 μg/mL) were by immunofluorescence with Rhodamine-Phalloidin and α-tubulin primary polyclonal antibody. Preparations were mounted with VectaShield®/DAPI and observed in confocal microscopy at 60X magnification. Results are representative of 3 independent replicates (n = 3; triplicates). Paclitaxel and 0.5 % DMSO/1X PBS were positive and negative controls used in studies.

On the other hand, changes in phases of the cell cycle were observed by flow cytometry (Figure 1C). GA increased the G_2/M phase (8.3 %) while MYR increased the G_0/G_1 phase (78 %), in comparison with the vehicle group, or the paclitaxel group that increased the G_2/M phase (15.3 %) ($p \leq 0.05$, Tukey) (Figure 1C). Possibly, these findings can associate with the foul of cell division detected by TEM, morphological changed seen by PCM, and the increase of intracellular ROS observed in SKOV-3 cells (Figure 1B and D). Finally, *In-silico* analyses were performed with the chemical structures of GA and MYR in SEA to find the protein targets of these compounds. Results indicate that GA could inhibit carbonic anhydrase-IX through ROS induction [10], while MYR appears to be a protein kinases inhibitor, such as PI3K [9] (Table 1). The carbonic anhydrase-IX is an enzyme responsible for regulating the internal pH of cells through catalysis [12]; While PI3K is an enzyme capable of phosphorylating inositol (phosphatidylinositol), activate AKT, and regulate diverse cell functions such as growth, proliferation, motility, survival, and intracellular trafficking [13] (Table 1). However, none of these molecular mechanisms has been studied in-depth; Whereby, the proposed interaction of GA-carbonic anhydrase-IX or MYR-PI3K requires more in-depth studies that can confirm and demonstrate them.

Table 1. Therapeutic targets of GA and MYR.

Compound name	Target key	Target protein	Organism	Description	pKi (L.E.)	*P*-Value	Max Tc * (affinity)
GA (ZINC1504)	CAH9_HUMAN+5 (SP: Q16790)	Carbonic anhydrase IX (CA9)	Eukaryote (Human)	Enzyme (E-lyase)	5.13 (0.60 kcal/mol/atom)	9.984 x10^{-5}	1 (6,990 nM)
MYR (ZINC3874317)	PIK3CG_HUMAN+5 (SP: P48736)	PI3K	Eukaryote (Human)	Enzyme (E-other)	5.33 (0.32 kcal/mol/atom)	0.5057	1

* Affinity binding of compound vs protein ≤ 10 μM. GA, gallic acid; MYR, myricetin; SP, Swiss-Prot protein sequence database (UniProt); PI3K, Phosphatidylinositol 4,5-bisphosphate 3-kinase.

4. Conclusions

GA and MYR demonstrated are capable of act against SKOV-3 ovarian adenocarcinoma cells through ROS production, which modifies the actin/tubulin cytoskeleton, induces cell cycle arrest, and activation of cell death by apoptosis, mainly. In silico studies with the SEA model allowed us to propose that carbonic anhydrase-IX and PI3K enzymes could be the targets for GA and MYR, respectively. However, docking and experimental studies are necessary to confirm this proposal. Therefore, GA and MYR could be considered as base compounds for the development of new treatments in the chemotherapy of ovarian cancer.

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