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# Morphological and Morphometric Analysis, Apoptosis and Oxidative Stress Study in Hep 2 Cells Treated by Resveratrol or Quercetine

## Análisis Morfológico y Morfométrico, Estudio de Apoptosis y Estrés Oxidativo en Células Hep 2 Tratadas con Resveratrol o Quercetina

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**SUMMARY:** Resveratrol (RES) and quercetine (QRC), is a promising agent relevant for both cancer chemoprevention and treatment via several signaling pathways, involved in their anticancer activity related to its chemotherapeutic potential, associated with the induction of ROS generation in cancer cells, leading to apoptosis. In our study, we have summarized the mechanisms of action of RES and QRC, and their pharmacological implications and potential therapeutic applications in cancer therapy. After treatment of Hep 2 cells with QRC or RES, the death pathways such as the cytochrome c release, ERK1/2 and IRS-1 pathways were upregulated, while cell survival pathway, including PI3K/AKT were downregulated. The RES and QRC caused oncosis, cells hypertrophy, hypercondensatin of chromatin, rupture of the plasma membrane and nuclear membrane, and formation of apoptotic bodies. Morphometric measurements of some cellular and nuclear parameters showed that RES and QRC induced an increase in cells and nuclear size, the nucleocytoplasmic ratio remained below 1 (N-Cyt R < 1), sign of low nuclear activity. The RES and QRC induced apoptosis of Hep2 cells by increasing of oxidative stress markers, MDA, and by modulating detoxifying enzymes, CAT and SOD. Our study results prove antiproliferative and proapoptotic properties of quercetin and resveratrol with regard to larynx cancer.

**KEY WORDS: Hep2 cells; Morphology; Apoptosis; Oxidative stress; Cell hypertrophy.**

## INTRODUCTION

Cancer is a highly malignant disease, it is urgent to explore effective drugs effect for cancer treatment or adjuvant therapy. Resveratrol and quercetin induced cell growth inhibition, DNA damage, and oxydative stress in Hep2 cells but not in normal cells (Boumaza *et al*., 2018). RES and QRC treatment causes apoptosis in cancer cells and it could be an incisive strategy against cancer. Based on multiple scientific studies, the links between nutrition and the risk of developing or avoiding many cancers, are well known. Cancer cells use nutrients, survive stress, and during this process lead to excessive reactive oxygen species (ROS) production. The increase in ROS has diverse biological consequences. On one hand, it promotes tumorigenesis and at the other hand causes irreparable damage to cancer cells leading to cell death (Tiwari & Mishra, 2023). Resveratrol (RES) and quercetin (QRC), two therapeutic molecules with a redox effect, are commonly used as anticancer molecules (Del Follo-Martinez *et al*., 2013). Quercetin, a plant flavonol, is contained in many plants, including onions, broccoli, raspberries, apples, citrus, Nelumbo nucifera, and leafy greens. QRC is an excellent free radical scavenging antioxidant. This antioxidant property is attributed to its catechol (3',4'-dihydroxy) group in the B-ring, its double bond between carbon 2 and 3 in the C-ring conjugated with the keto group at position 4, and the hydroxyl group substituents at position 3 of the C-ring and position 5 of the A-ring, which allows it to directly neutralize free radicals and bind metal ions (León-González *et al*., 2015). The powerful bioactivity and modulatory role of quercetin has

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prompted extensive research into the chemical, which has identified a number of pathways that potentially work together to prevent cancer, alongside, there is also extensive evidence of QRC use as a therapeutic agent in cancer stem cells (Biswas *et al*., 2022). Resveratrol, an active stilbene, is comprised of various pharmacological activities, including anticancer activity. RES exhibits antioxidant properties acting as a potent direct scavenger of free radicals or by increasing intracellular expression of enzymatic antioxidants. Other studies have shown that resveratrol can prevent LDL oxidation (Tino *et al*., 2016). Flavonoids are polyphenolic phytochemicals, which occur naturally in plants and possess both anti-oxidant and pro-oxidant properties. Flavonoids are gaining increasing popularity in the pharmaceutical industry as healthy and cost-effective compounds. Flavonoids show beneficial pharmacological activities in the treatment and prevention of various types of diseases. They are natural and less toxic agents for cancer chemotherapy and radiotherapy via regulation of multiple cell signaling pathways and pro-oxidant effects (Tiwari & Mishra, 2023).

In this study, we will discuss the effects of RES and QRC in apoptosis and oxidative stress on cancer Hep2 cells and various other mechanisms leading to cancer cell death. Finally, we will discuss the strategies to target the oxidative stress pathway for the treatment of cancer. The present study was undertaken to examine the effect of quercetin and resveratrol on cell viability, and to determine the molecular mechanism of quercetin- and resveratrol-induced cell death by investigating the cytochrome C released, AKt, ERK1/2, DNA and RNA. Some markers of oxidative stress, MDA, CAT, and SOD have evaluated. Morphologic and morphometric analysis were also analyzed in Hep2 cells.

## MATERIAL AND METHOD

**Hep-2 Cancer Cells (Human laryngeal epidermoid carcinoma):** Hep-2 cells are characterized by a marked viability due to their resistance to nutritional and environmental changes and are considered to be a substrate of choice for studies performed in vitro (Sun *et al*., 2012).

**Resveratrol (trans-3,4',5-trihydroxystilbene) and Quercetine (3, 3', 4', 5,7-pentahydroxyflavanone):** The Resveratrol (RES, 40 mg, box/60 Tablets; Biovea, France) and quercetin (QRC, Sigma) were used. The antioxidant (anti-radical) power of RES and QRC corresponds to the effective concentration that reduced the DPPH by 50 %. The EC50 of RES was evaluated at 375 µM and the EC50 of QRC was evaluated at 0.083 µM. The RES and QRC were added to the culture medium, and were preserved at 4 °C until its use (Boumaza *et al*., 2018).

**Hep2 Cell Culture:** Hep2 cells were cultured in DMEM (Gibco, USA), supplemented with 10 % foetal calf serum (SVF) (SIGMA, USA), streptomycin (5 µg/ml), penicillin (50 IU/ml) and glutamine at 200 mM (Gibco, USA) at 37°C under air: CO2 (95 %: 5 %) atmosphere until they reached confluence. After trypsinisation (0,1 % of trypsin; Gibco, USA), they were sown in plaques of 6 wells at 106 cells/well. At confluence, the cells were treated with resveratrol 375 µM or quercetin 0,083 µM for 24 h. A control cells plaque was submitted to the same conditions.

**Cell proliferation and viability assay:** After treatment with RES and QRC, cells were trypsinized and the rate of proliferation carried out on the cellular suspension by counting using the Mallasez cells. The rate of viability cells was estimated by trypan blue staining, and presented as the percentage of living cells.

**Morphological and morphometric study:** After treatment with RES and QRC, cells were washed with a phosphate-buffered saline (PBS, 1X) then fixed in aqueous bath and colored with either a May Grunwald-Giemsa (MGG; V/V, 1/1) or with acridine orange (100 µg/ml). The observation was made with an inverted microscope (Zeiss) for MGG coloring and a fluorescence microscope (Zeiss, blue filter) for the coloring with orange acridine. The measurement of the fluorescence (green) was performed on photos taken by the fluorescence microscope after staining by acridine orange by Mesurim software. The morphometric study was performed with TSView software after calibration at x1000 magnification. Cellular and nuclear measurements were made; major and small axes (MA, SA), cellular and nuclear area (CA, NA), nucleocytoplasmic ratio (N-Cyt R) estimation, counting of apoptotic bodies (AB) and cytoplasmic vacuoles (Cyt V). For each parameter analyzed, measurements were performed for 100 cells. The nucleocytoplasmic ratio is the ratio of the surface of the nucleus to that of the cytoplasm. This ratio assesses the nucleus activity (N-Cyt R < 1 : low nuclear avtivity, N-Cyt R > 1 : important nuclear activity). It is calculated according to the formula:

$$\text{N Cyt R} = \frac{\text{CA}}{\text{NA}}$$

**Evaluation of cytochrome c, Akt, ERK1/2, IRS-1, DNA and RNA concentration (ELISA):** The assessment was determined by immunoenzymatic assay. The ICC, of Hep2 cells, controls and treated with Res or Qrc, were collected and cytochrome c, Akt, ERK1/2, and IRS-1, DNA and RNA concentration were directly measured by ELISA (Invitrogen) for the quantitative detection according to the manufacturers' instructions using a microplate reader (BioTek Instruments). The assays were performed in duplicate.

**Proportioning of tiobarbituric Acid Reactive Substances (TBARs):** After the reaction with thiobarbituric acid (TBA) (Sigma) in the presence of 10 % trichloroacetic acid (TCA), the TBARs were measured in the ICC of Hep2 cells controls and treated with RES or QRC. The MDA contained in the supernatant of our samples caused the formation of a red complex estimated at 532 nm (Heath & Packer, 1968).

**Enzymatic activity of catalase (CAT) Assay:** The enzymatic activity of catalase (CAT) was determined using the method of Greenwald (1985). The principle of this method was based on the disappearance of hydrogen peroxide ($H_2O_2$) in the presence of the enzyme (catalase) source at 25 °C. The $H_2O_2$ (0.33 Mm; freshly prepared) was added to K-phosphate buffer (KH2PO4 50 mM; pH 7) and ICC of our samples. Absorbance was estimated at 240 nm in two time points, t0 and after two min. The enzymatic activity of catalase was evaluated in IU/min/µg of total proteins/$10^6$ cells.

**Superoxide Dismutase (SOD) Activity Assay:** The evaluation of the SOD activity was performed according to the procedure adopted by Giannopolitis & Ries (1977). in the ICC and ECC of our samples. Reagent A (50 mM of K-phosphate buffer (pH 7.8), methionine 13 mM, NBT 75 mM, EDTA 0.1 mM, riboflavin 4 mM) was added to ICC of our samples. The mixture of reagent A and samples was exposed to a light bulb (15 Watt) for 10 min. Absorbance was measured at 560 nm. The values were expressed in IU/106 cells. One unit of SOD activity was fined as the amount of enzyme required to cause 50 % inhibition of the NBT reduction.

**Statistical analysis:** PRISM software (GraphPad Prism 9.0, USA) was used for statistical analyses. Normality was verified with Shapiro Wilk test and homogeneity of variances with Bartlett's test. Ordinary one way ANOVA, or Welch ANOVA test, or Kruskal-Wallis test with Tamhane's T2 multiple comparison test, or Tukey's multiple comparisons test, or Holm-Sidak's multiple comparisons test, or Dunn's multiple comparisons test were used to compare three groups (Hep2 C *vs* Hep2 RES *vs* Hep2 QRC). When appropriate, repeated measures two way ANOVA with Tuckey's multiple comparisons test were used. The results were expressed as means ± SD. Differences with a $p$ value <0.05 were considered to be statistically significant.

## RESULTS

Resveratrol and Quercetine decreased viability and induced apoptosis of Hep 2 cells: The proliferation rate and Trypan blue staining showed a decrease in the number of living cells and an increase in dead cells after treatment with RES or QRC compared to pilot cells (p = 0.0003 ; p<0.0001) (Fig. 1). Our results revealed that the rate of proliferation was 0.55 ± 0.06 x $10^6$ cell and 0.35 ± 0.08 x $10^6$ cell in Hep 2 treated respectively with RES or QRC *vs* 2.48 ± 0.61 x $10^6$ cell in controls Hep 2. Moreover, after Trypan blue staining, we recorded an increase of cell death of 71.4 % and 55.8 % in Hep2 treated with respectively with RES or QRC compared to 98.6 % in corresponding Hep 2 controls. Furthermore, we observed a high percentage of detached cells (data not shown). We performed cytochrome c released, Akt, ERK1/2 and IRS-1 ELISA assay to determine how RES and QRC affect the proliferation and the viability and apoptosis of Hep 2 cancer cell. As shown in Figure 2, the cytochrome c released, p38 MAPK, ERK1/2 and IRS-1 showed a significant increase in Hep 2 treated with RES or QRC compared to control Hep 2 cells (Cyt C : p < 0.0001 ; MAPK : p < 0.0001 ; ERK1/2 : p < 0.0001 ; IRS1 : p < 0.0001). However, the AKt evaluation showed a significant decrease after RES or QRC treatment of Hep 2 versus control cells (p < 0.0001).

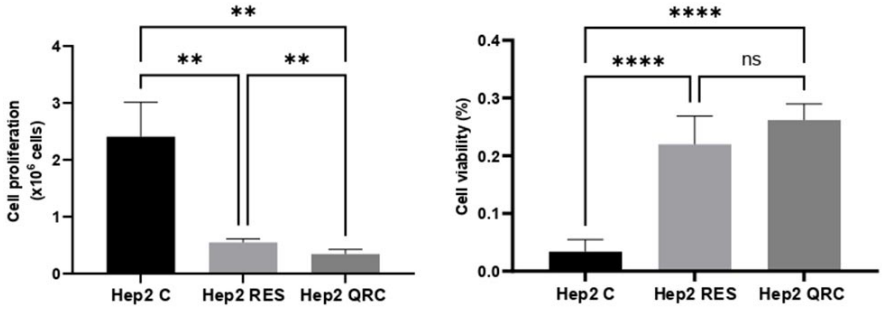


Fig. 1. Cell proliferation (x 106 cells) and cell viability (%) in Hep 2 cells treated by RES or QRC for 24 h. Cell proliferation : Welch ANOVA test (*** : p = 0,0003). Tamhane's T2 multiple comparaison test, individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES ** : p = 0,0067 ; Hep2 C *vs* Hep2 QRC ** p = 0,0043 ; Hep2 RES *vs* Hep2 QRC ** p = 0,0066). Cell viability : Ordinary one way ANOVA (**** p<0,0001). Tukey's multiple comparaisons test, with a single pooled variance (Hep2 C *vs* Hep2 RES **** : p <0,0001 ; Hep2 C *vs* Hep2 QRC **** p <0,0001 ; Hep2 RES *vs* Hep2 QRC ns p = 0,1763).

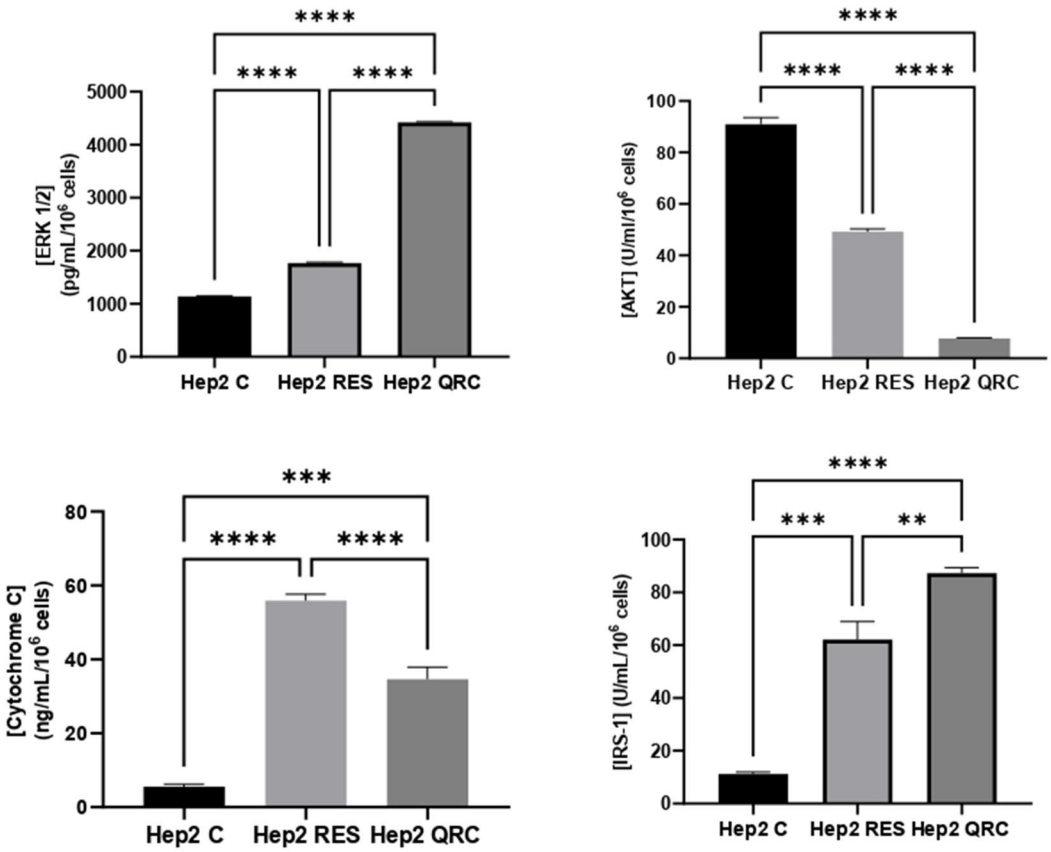


Fig. 2. ERK 1/2 (pg/mL/$10^6$ cells), Cytochrome C (ng/mL/$10^6$ cells), AKT (U/mL/$10^6$ cells) and IRS-1 (U/mL/$10^6$ cells) rates in Hep 2 cells treated with RES or QRC for 24h. ERK1/2 : Welch ANOVA test (**** : $p < 0,0001$). Holm-Sidak's multiple comparaisons test, with individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES **** : $p < 0,0001$ ; Hep2 C *vs* Hep2 QRC **** : $p < 0,0001$ ; Hep2 RES *vs* Hep2 QRC **** : $p < 0,0001$). Cyt C : Welch ANOVA test (**** : $p < 0,0001$). Tamhane's T2 multiple comparaison test, individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES **** : $p < 0,0001$ ; Hep2 C *vs* Hep2 QRC **** : $p < 0,0001$ ; Hep2 RES *vs* Hep2 QRC **** : $p < 0,0001$). AKT : Welch ANOVA test (**** : $p < 0,0001$). Holm-Sidak's multiple comparaisons test, with individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES **** : $p < 0,0001$ ; Hep2 C *vs* Hep2 QRC **** : $p < 0,0001$ ; Hep2 RES *vs* Hep2 QRC **** : $p < 0,0001$). IRS1 : Welch ANOVA test (**** : $p < 0,0001$). Tamhane's T2 multiple comparaison test, individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES *** : $p = 0,0002$ ; Hep2 C *vs* Hep2 QRC **** : $p < 0,0001$ ; Hep2 RES *vs* Hep2 QRC ** : $p < 0,0016$).

Resveratrol and Quercetine induced morphological and morphometric changes in Hep 2 cells : The fluorescence analysis of cells after 24 h of treatment of Hep 2 cells with RES or QRC showed an intense fluorescence in the perinuclear area, marking the hypercondensation of chromatin, a nuclei fragmentation and formation of apoptotic bodies compared to the corresponding controls (Figs. 3 A, B and C). The measurement of the fluorescence (green) was performed by Mesurim software on the fluorescence microscope after OA staining of our cells. Our results showed an increase in fluorescence intensity in Hep 2 cells treated with RES (16.1 %) or QRC (19.2 %) compared to Hep 2 controls (6.27 %) (Fig. 3D).

The morphological analysis of the apoptotic cells was performed after 24 h of treatment of Hep2 with RES or QRC. Compared to the controls Hep 2, we observed a vacuolization of the cytoplasm, sign of oncosis, cells hypertrophy and hypercondensation of chromatin, sign of apoptosis (Fig. 4). Moreover, the Hep 2 cells experimented showed a rupture of the plasma membrane and nuclear membrane as well as a nuclei fragmentation marking the formation of apoptotic bodies (Fig. 4). Morphological study was reinforced by morphometric measurements of some cellular and nuclear parameters. As illustrated in Table I, RES and QRC induced a significant increase in the cellular and nuclear major axis (MA), small axis (SA) and cellular

and nuclear area (CA, NA). Moreover, we reported an increased number of cytoplasmic vacuoles and apoptotic bodies per cell (Cyt-V/cell and AB/cell) after treatment with RES or QRC. However, the nucleocytoplasmic ratio (N-Cyt R) decreased in Hep 2 treated with RES or QRC during 24 h versus Hep 2 controls (Table I). We reported 0,45 ± 0,08 and 0,45 ± 0,11 in Hep 2 treated respectively with RES or QRC *vs* 0,68 ± 0,14 in control group. The N-Cyt R remained below 1 (N-Cyt R < 1) in different experimental groups, sign of low nuclear activity. Our results were confirmed by ELISA quantitative detection of DNA and RNA. As shown in Table I, the ELISA assay revealed that RES and QRC caused a decrease in the amount of DNA(ns ; p=0,6369) and RNA (p = 0,0010).

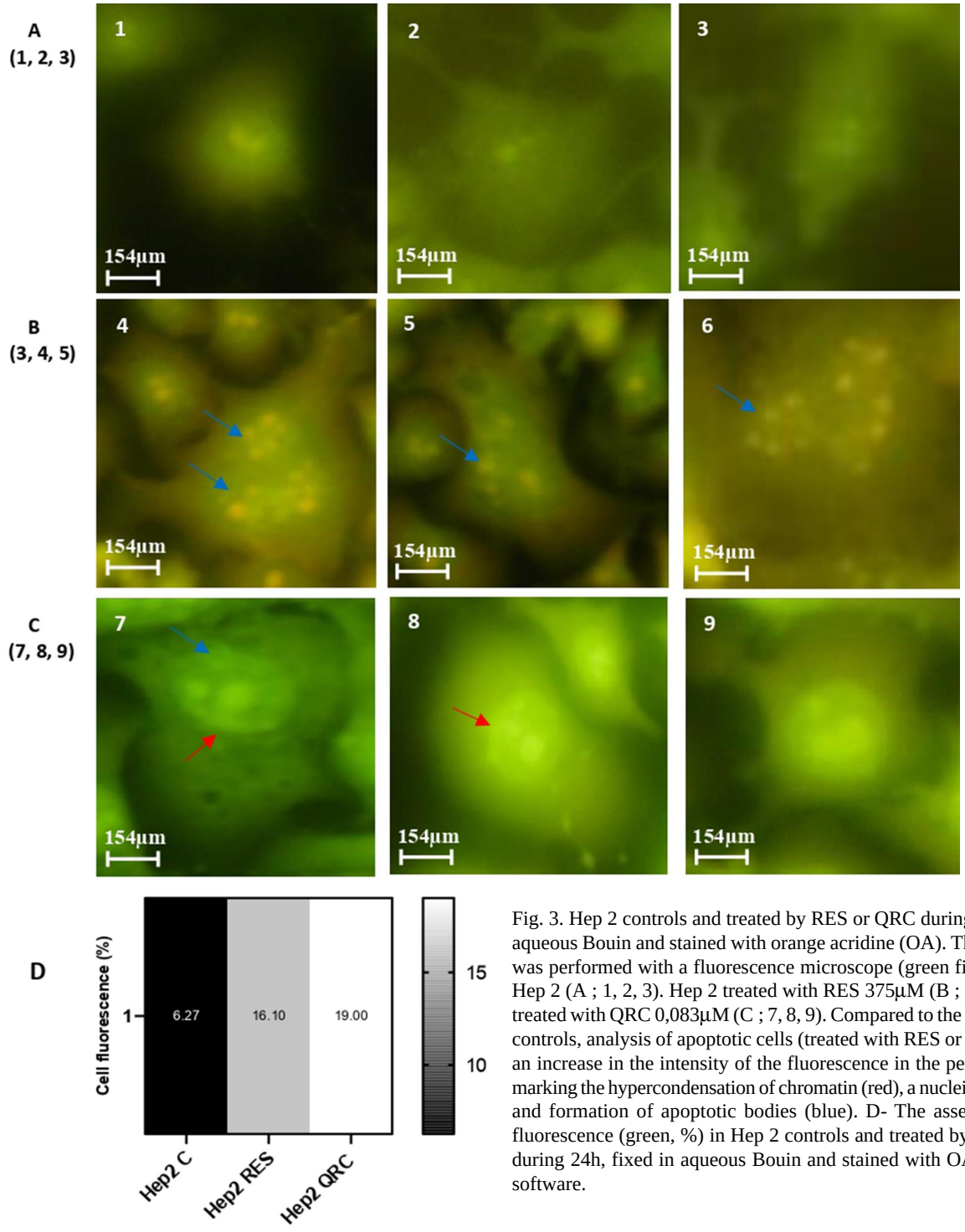


Fig. 3. Hep 2 controls and treated by RES or QRC during 24h, fixed in aqueous Bouin and stained with orange acridine (OA). The observation was performed with a fluorescence microscope (green filter). Controls Hep 2 (A ; 1, 2, 3). Hep 2 treated with RES 375µM (B ; 4, 5, 6). Hep 2 treated with QRC 0,083µM (C ; 7, 8, 9). Compared to the corresponding controls, analysis of apoptotic cells (treated with RES or QRC) showed an increase in the intensity of the fluorescence in the perinuclear area, marking the hypercondensation of chromatin (red), a nuclei fragmentation and formation of apoptotic bodies (blue). D- The assessment of the fluorescence (green, %) in Hep 2 controls and treated by RES or QRC during 24h, fixed in aqueous Bouin and stained with OA by Mesurim software.

Table I. Morphometric study of some cellular and nuclear parameters and DNA and RNA (µg/mL) in Hep 2 controls and treated with RES or QRC for 24 h.

| | | Hep2 C | Hep2 RES | Hep2 QRC |
|---|---|---|---|---|
| Cellular parameters | MA (µm) | 64,98 ± 7,43 | 116,20 ± 15,52**** | 113,21 ± 14,80$^{\$\$\$\#\#}$ |
| | SA (µm) | 47,39 ± 5,73 | 94,89 ± 11,73 **** | 94,39 ± 11,98 $^{\$\$\$}$ |
| | CA (µm_) | 5514,14 ± 219,22 | 10692,73± 728,22**** | 10675,53±726,73$^{\$\$\$}$ |
| | Cyt V (/cell) | 0,09 ± 0,29 | 1,31 ± 0,51**** | 1,18 ± 0,39$^{\$\$\$\#\#}$ |
| | AB (/cell) | 0,1 ± 0,30 | 0,96 ± 0,20**** | 1,09 ± 0,29$^{\$\$\$\#\#\#}$ |
| Nuclear parameters | MA (µm) | 28,11 ± 5,50 | 41,29 ± 8,31**** | 39,80 ± 7,01$^{\$\$\$}$ |
| | SA (µm) | 23,96 ± 4,24 | 35,85 ± 7,76**** | 35,15 ± 7,25$^{\$\$\$}$ |
| | NA (µm_) | 1736,49 ± 823,60 | 3242,86 ± 686,71**** | 3179,51 ± 642,06$^{\$\$\$}$ |
| N-Cyt R = NA/CA | | 0,68 ± 0,14 | 0,45 ± 0,08**** | 0,45 ± 0,12$^{\$\$\$}$ |
| DNA (µg/mL) | | 0,18 ± 0,03 | 0,17 ± 0,07 | 0,15 ± 0,01 |
| RNA (µg/mL) | | 1,14 ± 0,10 | 0,32 ± 0,13** | 0,25 ± 0,13$^{\$\$}$ |

The values were expressed as means ± SEM. Morphometric study was performed with TSView software after calibration at x1000 magnification. Cellular and nuclear measurements: major and small axes (MA, SA), cellular and nuclear area (CA, NA), nucleocytoplasmic ratio (N-Cyt R) estimation, counting of apoptotic bodies (AB) and cytoplasmic vacuoles (Cyt V). For each parameter analyzed, measurements were performed for 100 cells. Cellular and nuclear parameters : RM (repeated mesures) two way ANOVA with Geisser-Greenhouse correction, matched values are both stacked and spread across a row. Tuckey's multiple comparaisons test, with individual variances computed for each comparaison. N-Cyt R : Kruskal-Wallis test. Dunn's multiple comparaisons test. DNA and RNA: Ordinary One Way ANOVA test with Tuckey's multiple comparaisons test. * Hep 2 Controls *vs* Hep 2 treated by RES ; $ : Hep 2 controls *vs* Hep 2 treated by QRC ; # : Hep 2 treated by RES *vs* Hep 2 treated by QRC.

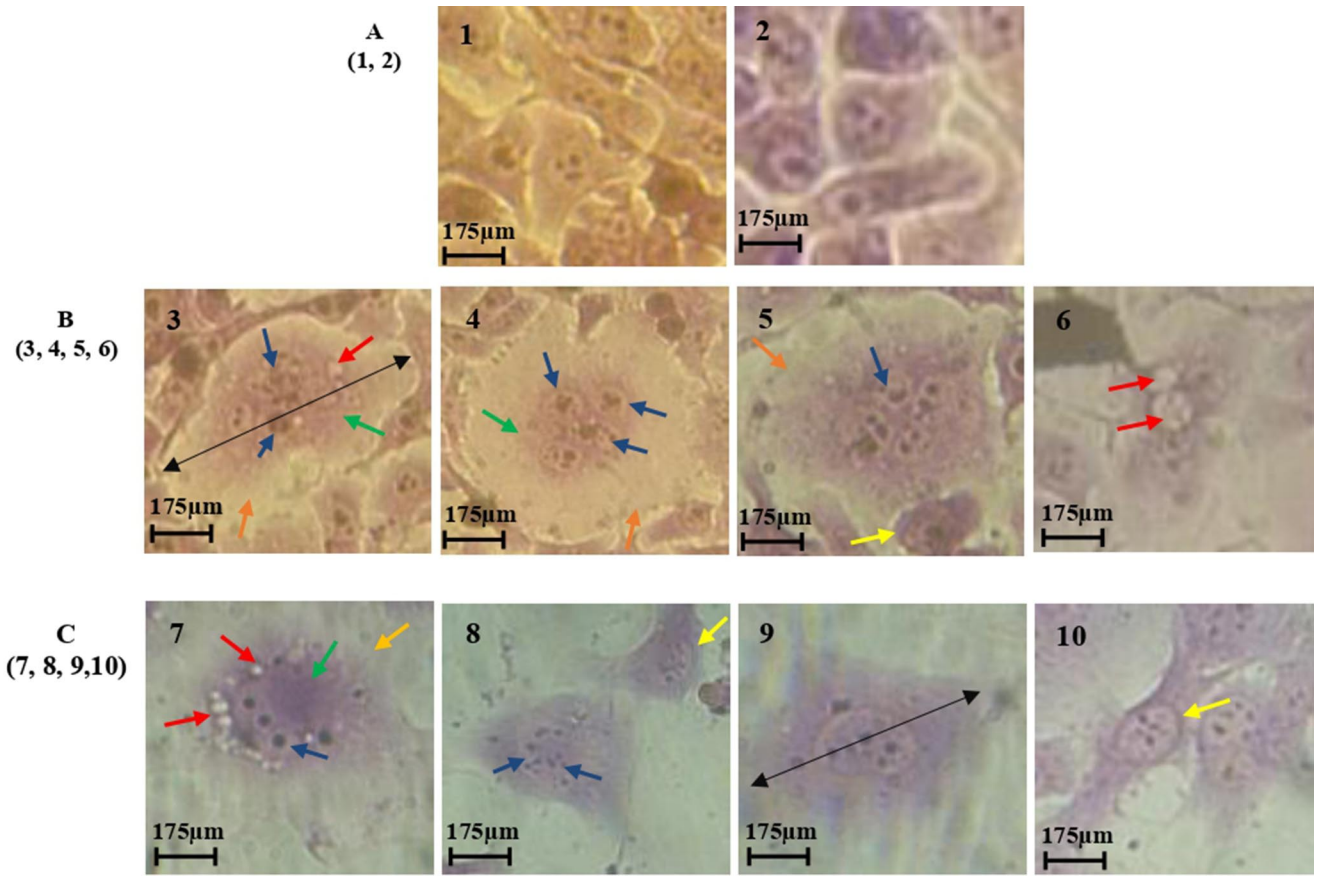


Fig. 4. May Grunwald Giemsa (MGG) staining of Hep 2 treated by RES or QRC during 24 h, fixed in aqueous Bouin. The observation was made with an inverted microscope (Zeiss). Controls Hep 2 (A ; 1, 2), Hep 2 treated with RES (B ; 3, 4, 5, 6), Hep 2 treated with QRC (C ; 7, 8, 9, 10). Morphological analysis Showed a vacuolization of the cytoplasm (red), sign of oncosis, cells hypertrophy (black) and hypercondensatin of chromatin (yellow), sign of apoptosis, degradation of the cytoplasmic membrane (orange) and nuclear membrane (green) as well as a nuclei fragmentation marking the formation of apoptotic bodies (blue).

**RES and QRC induces oxidative stress damage:** Our results showed that RES and QRC caused significantly increased expression oxidative stress markers including, MDA (in ICC : p<0,0001 ; in ECC : p<0,0001), CAT activity (p = 0,0001), SOD activity (p<0,0001), PC (p<0,0001) and AOPP (p<0,0001) compared to controls Hep 2 (Fig. 5).

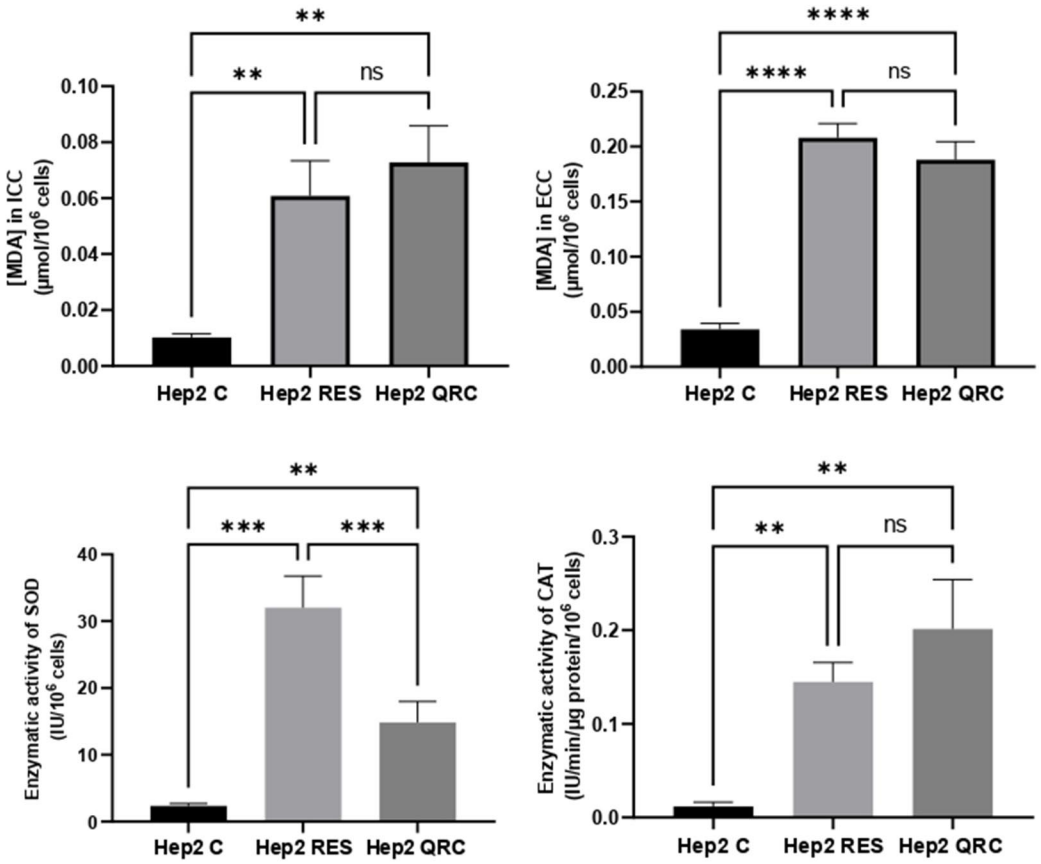


Fig. 5. MDA in ICC and ECC (µmole/$10^6$ cells), enzymatic activity of CAT (IU/min/µg protein/ 106 cells) and SOD (IU/$10^6$ cells) in Hep 2 cells treated by RES or QRC for 24h. MDA in ICC:Welch ANOVA test (**** :p < 0,0001). Tamhane's T2 multiple comparaison test, individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES ** : p = 0,0017; Hep2 C *vs* Hep2 QRC *** : p = 0,0009; Hep2 RES *vs* Hep2 QRC ns : p = 0,3502). MDA in ECC : Ordinary one way ANOVA (**** : p < 0,0001). Tukey's multiple comparaisons test, with a single pooled variance (Hep2 C *vs* Hep2 RES **** : p < 0,0001 ; Hep2 C *vs* Hep2 QRC **** : p < 0,0001; Hep2 RES *vs* Hep2 QRC ns : p = 0,0640). Enzymatic activity of CAT : Welch ANOVA test (*** : p = 0,0001). Holm-Sidak's multiple comparaisons test, with individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES ** : p = 0,0022 ; Hep2 C *vs* Hep2 QRC ** : p = 0,0025; Hep2 RES *vs* Hep2 QRC ns : p = 0,0755). Enzymatic activity of SOD : Welch ANOVA test (**** : p < 0,0001). Tamhane's T2 multiple comparaison test, individual variances computed for each comparaison (Hep2 C *vs* Hep2 RES *** : p = 0,0003 ; Hep2 C *vs* Hep2 QRC ** : p = 0,0017; Hep2 RES *vs* Hep2 QRC *** : p = 0,0007).

## DISCUSSION

Quercetin (QRC) and resveratrol (RES) have been indicated as a prominent target in investigations of alternative therapies against cancer. A better understanding of the mechanism of cancer cell responses is needed to develop new therapeutic strategies. The major challenge is to reduce the incidence of cancer, which is constantly increasing. The strategy of preventing cancer cells or inhibiting their growth by inducing apoptosis was widely studied (Tiwari & Mishra,

2023). In our study, QRC and RES were studied to treat larynx cancer and investigate the anticancer therapeutic effect. The morphological and morphometric changes, apoptosis and oxidative stress-stimulating properties of RES and QRC on Hep 2 cancer cells were studied.

Our results showed that the RES and QRC caused Hep 2 cells apoptosis by reducing cell viability and increasing cell death. This pathway is supported by activation of signaling pathways leading to cell death, including the release of cytochrome c. Cytochrom c is an apoptotic marker which subsequently leads to apoptosome formation, cleavage of targeting proteins and DNA fragmentation initiating and executing the process of apoptosis. Moreover, the ERK1/2 and IRS-1 pathways were activated. We suggested that activation of these pathways leading to apoptosis of Hep 2 cancer cell. In the present study, we showed that PI3K/Akt pathway were reduced after RES or QRC treatment of Hep 2 cancer cells. Numerous studies have shown that the anti-proliferative effect of polyphenols, including RES and QRC, is through cell cycle arrest and apoptosis in several cancer cell lines in different phases (Stervbo *et al*., 2006). Del Follo-Martinez *et al*. (2013) showed that RES and QRC in combination (1:1 ratio) inhibited growth in human leukemia cells. Our study showed that RES and QRC caused apoptosis of Hep 2 cell via PI3K/Akt, ERK1/2 and IRS-1 pathways. According to Berger *et al*., (2021), the cancer cells possess a highly stable inherited chance to persist (CTP) during therapy and this CTP is drug specific. This CTP has a unimodal distribution ranging from 0 to almost 100 %, so some cells resist and others succumb to death after RES and QRC treatment as shown in our study. Berger *et al*., (2021), showed that differential serine/threonine phosphorylation of the insulin receptor substrate 1 (IRS1) protein determines the CTP of lung, of head and neck cancer cells under epidermal growth factor receptor inhibition, both in vitro and *in vivo*.

Quercetin plays a crucial role in several signal transduction pathways. QRC, directly, activates the MAPK/ERK-mediated pathways leading to the apoptosis process of the A549 cell line, resulted in a DNA fragmentation, a decrease in total PI3K/Akt protein and phosphorylation of Akt (Nguyen *et al*., 2004). In addition, Yang *et al*. (2016) showed that the quercetin induced apoptosis of HeLa cells and HT-29 colon cancer cells by inhibition of PI3K/Akt pathway. The quercetin derivative called 7-O-granuestine quercetin (GQ) induced apoptosis of gastric cancer cells (SGC-7901 and MGC-803) via increases ROS production and cytochrome c releases (Zhu *et al*., 2017), and decreases of PI3K/AKT expression and inceases of ERK1/2 signaling pathways (Asgharian *et al*., 2022).

The morphological analysis of the apoptotic cells showed that RES and QRC caused oncosis marked by a vacuolization of the cytoplasm, apoptosis activation via cells hypertrophy, hypercondensation of chromatin, rupture of the plasma membrane and nuclear membrane, nuclei fragmentation and formation of apoptotic bodies. Theresults of a study performed by Ba¸dziul *et al*. (2014), reported that the microscopic observations of Hep-2 cells treated with QRC for 24 h, showed an increased number of apoptotic cells. Morphometric measurements of some cellular and nuclear parameters showed that RES and QRC induced a significant increase in the cellular and nuclear major axis (MA), small axis (SA), surface area (SA), number of cytoplasmic vacuoles and apoptotic bodies per cell (Cyt-V/cell and AB/cell). According to the study of Stervbo *et al*. (2006), the RES induced an increase in nuclear size and granularity in the G1 and S phases of a cell cycle arrest in different phases of HL-60 treated and HepG2-treated cells. Furthermore, in our study, the nucleocytoplasmic ratio (N-Cyt R) decreased after RES and QRC treatment of Hep 2 cell and remained below 1 (N-Cyt R < 1) in both Hep 2 controls and treated with RES or QRC, sign of low nuclear activity. These results were confirmed by ELISA quantitative detection of DNA and RNA. We noted a non-significant decrease in the amount of DNA and RNA in Hep 2 cells treated with RES or QRC *vs* control cells. These findings are consistent with previous studies of Stervbo *et al*. (2006), they reported that the resveratrol affects DNA-synthesis in HL-60 and HepG2 cells. RES inhibited DNA synthesis in a concentration-dependent manner and caused cell apoptosis. Combination of RES and QRC reduced cell growth, caused DNA damage, S-phase cell cycle arrest, and cell death in Cal-33 cells. The DNA damage caused by RES and QRC occurs through reactive oxygen species (ROS) generation (Singh *et al*., 2020).

As chemopreventive agents, resveratrol and quercetine supplementation alter the pro-oxidant/anti-oxidant balance in Hep 2 cells. RES and QRC caused significant CAT, SOD and MDA increase. According to Vásquez-Garzón *et al*. (2009), quercetin inhibits hepatocarcinogenesis through upregulation of enzymatic (SOD, glutathione peroxidase, and CAT) and nonenzymatic (GSH and total glutathione) antioxidant defense system. According to Chang *et al*. (2006), QRC increased ROS and lipid peroxidation in human hepatoma cells. Diverse experimental studys discovered that overexpression of endogenous antioxidants such as SOD is balanced in oxidative stress of various cancer cells. It is worth noting that the RES exposure of cancer cells increased activity of antioxidative enzymes, analogically to the results of Khan *et al*. (2013), on prostate, hepatic, and breast cancer cells. The enzymatic activity of CAT and SOD, although still

controversial, some promising results are being obtained. Previous studies have shown that reduced amounts of antioxidant enzymes, especially SOD, are found in a variety of cancer cells, but not all tumors. The results of Miku?a-Pietrasik *et al*. (2015) showing that the generation of ROS increased and CAT and SOD activity decreased in ovarian cancer cells exposed to RES. The cancer cells produced excessive intrinsic pro-oxidant such as H2O2 free radicals. The accumulation of H2O2 is a crucial step in the induction of cancer cell death and the cytotoxic activity of these therapies accumulation of H2O2. Quercetin is well known for its antioxidant and cell protective effects. However, quercetin also displays strong pro-oxidant effects and increases the cellular levels of ROS to cytotoxic levels in B16F10 melanoma cells and many other cancer cells (Rafiq *et al*., 2015).

In our study, we previously reported that RES and QRC caused redox status disorder. Our results showed that RES and QRC caused significantly increased MDA amounts. According to Zhang *et al*. (2020), ROS at high levels, are known to induce oxidative damage to biomolecules, including DNA, lipids and proteins in prostate cancer cells. ROS can react with the polyunsaturated fatty acids of lipid membranes and induce lipid peroxidation. QRC induced ROS in hepatocellular cancer and increased the MDA production which causes membrane damage (Zhang *et al*., 2020). Therefore, QRC may be used to selectively eliminate cancer cells and be therapeutically useful. During oxidative stress conditions, quercetin reacts with hydrogen peroxide (H2O2) to form o-semiquinone radical and quercetin–quinone products (QQ) (Awad *et al*., 2000), which are highly unstable and toxic for cell proliferation and for which QRC takes action as a pro-oxidant than antioxidant. QQ products are cytotoxic and induce Human breast carcinoma cell death through their interaction with DNA leading to DNA damage and thereby causes cellular damage and apoptosis (Awad *et al*., 2000). According to our results, we suggest that the pro-oxidant effect of quercetin overdominates its antioxidant effect, resulting in cell death. Therefore, quercetin may be used to selectively eliminating cancer cells and be therapeutically useful.

## CONCLUSION

Our study results prove antiproliferative and pro-apoptotic properties of quercetin and resveratrol with regard to larynx cancer. Upon treatment of Hep 2 cancer cells with QRC or RES, the proteins related to death pathways such as the cytochrome c release, ERK1/2 and IRS-1 pathways were upregulated, while proteins associated with cell survival, including PI3K/AKT were downregulated. The morphological analysis of the apoptotic cells showed that RES and QRC caused oncosis, apoptosis via cells hypertrophy, hypercondensation of chromatin, rupture of the plasma membrane and nuclear membrane, nuclei fragmentation and formation of apoptotic bodies. The nucleocytoplasmic ratio remained below 1 (N-Cyt R < 1), sign of low nuclear activity. The RES and QRC anticancer properties are linked to their ability to modulate anioxidant levels, CAT and SOD, and their capability to increase their toxicity as pro-oxidants by increasing oxidative stress markers, MDA.

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**RESUMEN:** Resveratrol (RES) y quercetina (QRC), es un agente prometedor y relevante tanto para la quimioprevención como para el tratamiento del cáncer a través de varias vías de señalización, involucrado en su actividad anticancerígena relacionada con su potencial quimioterapéutico, asociado con la inducción de la generación de especies reactivas del oxígeno (ROS) en células cancerosas, lo que lleva a apoptosis. En nuestro estudio, hemos resumido los mecanismos de acción de RES y QRC, y sus implicaciones farmacológicas y posibles aplicaciones terapéuticas en la terapia del cáncer. Después del tratamiento de las células Hep 2 con QRC o RES, las vías de muerte, tal como la liberación de citocromo c, las vías ERK1/2 e IRS-1, se regulaban positivamente, mientras que la vía de supervivencia celular, incluida PI3K/AKT, se regulaba negativamente. El RES y el QRC provocaron oncosis, hipertrofia celular, hipercondensación de la cromatina, rotura de la membrana plasmática y nuclear y formación de cuerpos apoptóticos. Las mediciones morfométricas de algunos parámetros celulares y nucleares mostraron que RES y QRC indujeron un aumento en las células y el tamaño nuclear, la proporción nucleocitoplasmática se mantuvo por debajo de 1 (N-Cyt R <1), signo de baja actividad nuclear. RES y QRC indujeron la apoptosis de las células Hep2 aumentando los marcadores de estrés oxidativo, MDA, y modulando las enzimas desintoxicantes, CAT y SOD. Los resultados de nuestro estudio demuestran las propiedades antiproliferativas y proapoptóticas de la quercetina y el resveratrol con respecto al cáncer de laringe.

**PALABRAS CLAVE: Células Hep2; Morfología; Apoptosis; Estrés oxidativo; Hipertrofia celular.**

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