Biological effects of grape stem extracts on human cancer cell lines

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Abstract. Grapes are rich in polyphenolic compounds, which are known for their beneficial effects on human health. Grape stems are byproducts of the winemaking procedure. They exert antioxidant effects; however, they can also become pollutants when discarded without control into the environment due to their high organic content. The main aim of the present study was to examine the potential prooxidant/anticancer effects of grape stem extracts, promoting the rationale of the scientific exploitation of such compounds. Therefore, three grape stem extracts derived from the native Greek vine varieties, Mavrodaphne, Muscat and Rhoditis were examined for their ability to alter the redox status [i.e., endogenous levels of reduced glutathione and reactive oxygen species (ROS)] of HeLa and HepG2 human cancer cell lines. The results revealed that the extracts from Muscat and Rhoditis exerted prooxidant effects on the tested cell lines, whereas the extract of Mavrodaphne did not exert any such effects. The fact that the extracts functioned as prooxidants appears to be detrimental for the cancer cells, since they are more susceptible to high concentrations of ROS, contrary to healthy cells that possess more robust antioxidant defense mechanisms. On the whole, as demonstrated in the present study, it appears that the Muscat and Rhoditis extracts may prove to be promising agents against cancer cells in the specific *in vitro* model. However, further studies are required to confirm these findings, in *in vivo* settings.

Introduction

Grapes are fruit that botanically belong to berries and to the plant genus *Vitis*. They consist of juice, pulp, skin, seeds and stem. Grapes exert established nutritional and medicinal properties, as they are an excellent source of water, carbohydrates, proteins and fat (1). The main properties grapes are attributed to their antioxidant, anticarcinogenic, immune-modulatory, antidiabetic, anti-atherogenic, neuroprotective, anti-obesity, anti-aging and anti-infection activities. Moreover, grapes represent an excellent source of water (82%), carbohydrates (12-18%), proteins (0.5-0.6%) and fats (0.3-0.4%). Lipids are mainly found in the seeds of grapes and include fatty acids, tocopherols, tocotrienols and phytosterols. Different grape varieties have different concentrations and profiles of fatty acids. Apart from the aforementioned properties, grapes are an essential source of molecules with potent antioxidant properties, mainly polyphenolic compounds (2). Polyphenols are abundant in grapes; they contribute to the defense of the plant against bacteria and environmental factors (3) and are synthesized through the phenylpropanoid pathway in response to biotic and abiotic stimuli (4). It has been reported that polyphenols are beneficial for human health due to their antioxidant activity, which in some cases is greater than that of vitamins (5-7).

Grapes and their products are widely consumed as dietary components worldwide and in particular, as fresh fruit, dried fruit, wine and juice (8). Wine is considered the major product of grapes, since it is a fundamental constituent for social, religious and cultural events (9). The polyphenolic compounds which are transferred from grapes to wine are dependent on the winemaking process (10). Polyphenolic compounds are major constituents of wine that largely determine the quality of the wine due to their direct effects on color, flavor, bitterness, astringency and aroma (11). Following the vinification process, large amounts of solid organic waste and byproducts are generated, including grape pomace (62%), wine lees (14%), grape stems (12%) and dewatered sludge (12%) (12). Additionally, a large number of polyphenols remain in the vinification waste, which form >13% of the processed grape weight (13). Thus, it is of utmost importance that the scientific community identifies strategies with which to exploit the byproducts from the winemaking procedure (i.e., stems), in order to diminish environmental pollution. The most common method of exploiting grape stems is the production of compost and energy; however, the polyphenols present in these are wasted through that process (14). It is noteworthy that the reintegration of wine byproducts into the food chain is of high

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economic, nutritional and environmental interest (15). Indeed, they can improve the nutritional value of baked, pastry and pasta products (16), whilst they can potentially be incorporated into dietary supplements (17).

It appears that byproducts of the winemaking procedure, such as grape stems and pomace are a pollutant when discarded to the environment due to their high organic load. Nevertheless, due to their bioactive compounds (i.e., polyphenols), they possess notable biological properties, mainly acting protectively against oxidative stress and related pathologies (18,19). Of note, it has been observed that grape (by)products improve the redox status of productive (20,21) and experimental animals (22), whereas they function as potent antioxidants *in vitro* (19,22-24). The content of polyphenols in grape stems is highly dependent on the variety and growing conditions (25). However, there is also evidence to indicate that polyphenols may function as prooxidants, a fact that is of utmost interest in terms of administration to cancer cells (26,27).

The present study is part of a greater project; the first article of this project examined the *in vitro* antioxidant and antimutagenic properties of the same grape stem extracts (19). According to the methodological practice recently proposed, the examination of the biological properties of plant extracts in the cellular environment constitutes the second line of screening for such compounds (28). To that end, the main objective of the present study was to determine the effects of the aforementioned extracts on the redox status [i.e., the levels of the endogenous reduced form of glutathione (GSH) and reactive oxygen species (ROS)] of two human cancer cell lines. The results of the present study are anticipated to provide new insight into the biological activity of the tested extracts, that may be further utilized by introducing them into animal and subsequently, the human diet (i.e., as components of bio-functional foods).

Materials and methods

Collection of plant material, preparation of plant extracts and determination of their chemical composition. The plant material was collected from the Northern Peloponnese (Patras) in Greece. Specifically, the grape stems used in the present study were collected manually from three grape varieties, namely Mavrodaphne, Muscat and Rhoditis. Subsequently, the stem samples were chemically processed according to the procedure that has been previously described (19). In brief, the samples underwent repeated extractions in methanol and following liquid chromatography analysis, the respective extracts were generated and used for the analyses described herein. The stem extract derived from the Mavrodaphne variety is rich in gallic acid and caffeic acid, hydroxybenzoic and hydroxycinnamic acids, respectively, whereas it contains higher amounts of the flavonol, quercetin, and quercitrin, a flavonol glycoside, compared to the other two extracts. The extract generated from the Muscat variety contains higher concentrations of gallocatechin, a flavanol, polydatin, which is a stilbene, and hesperidin, a flavanon glycoside, in comparison to the extracts derived from the varieties Mavrodaphne and Rhoditis. Finally, the chemical compounds detected in the extract of the Rhoditis variety in higher amounts compared to the Mavrodaphne and Muscat varieties are two members of proanthocyanidins (porcyanidin B1 and B2), the flavanols, catechin and epicatechin, two hydroxybenzoic acids (i.e., 2,5 dihydroxybenzoic acid, ellagic acid), the flavonol, rutin, quercitrin-3-b-glucoside, which is a flavonol glycoside, and trans-resveratrol, a well-known stilbene. The exact chemical composition of the extracts was determined by high-performance liquid chromatography (HPLC) and HPLC-electrospray ionization/mass spectrometry analysis and this has been published in a previous study (19).

Cell culture conditions. The cervical cancer cell line, HeLa, and the liver cancer cell line, HepG2, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. The cell lines used in this experiment, in accordance with the international guidelines for good cell culture practice, were examined for mycoplasma using PCR and were found to be mycoplasma-free (29,30). The working concentrations of the tested extracts were not found to be cytotoxic in any cell line (Table I). Furthermore, a morphological analysis, both at high and low culture densities using a microscope, was conducted to authenticate the state of the cells, through their phenotypic characteristics (data not shown). Finally, the passage number for each cell line did not exceed 30 population doublings.

Cell viability assay. Cell viability was assessed using the XTT assay kit (Roche Diagnostics GmbH). Briefly, $1x10^4$ HeLa cells/well were cultured in a 96-well plate in DMEM. Following a 24-h incubation (37˚C), a wide range of the grape stem extract concentrations (as shown in the figures) diluted in a serum-free DMEM were used to treat the cells for 24 h. Subsequently, 50 μl XTT reagent (50:1) were added to each well. Following 4 h of incubation (37˚C), the absorbance was monitored at 450 and at 630 nm as a reference wavelength using a BioTek ELx800 microplate reader (BioTek Instruments Inc.). As a negative control, samples containing serum-free DMEM only were used. In addition, the absorbance of the extracts alone in serum-free DMEM and XTT test solution was measured at 450 and 630 nm. The absorbance values of the extracts alone were subtracted from those derived from the absorbance of the cells treated with the test compounds. Data were calculated as the percentage of viability using the following equation: Viability(%)=[($OD_{control}$-OD_{sample})/$OD_{control}$] x100, where $OD_{control}$ and OD_{sample} indicate the OD of the negative control and the test compounds, respectively. The experimental protocols were conducted in triplicate (three repetitions), at three independent experiments.

Measurement of endogenous GSH levels in HeLa and HepG2 cell lines using flow cytometry. The intracellular GSH levels were assessed using the fluorescent dye, mercury orange (Merck KGaA). The cells were incubated (37˚C) for 24 h in FBS-free media including the test extracts. The cells were then used for the measurement of GSH levels using flow cytometry. According to the experimental procedure, a 400 μM stock solution of the dye was prepared in dimethyl sulfoxide (DMSO). The cells were trypsinized and centrifuged (1,200 x g, 5 min, 4˚C). Subsequently, the cell pellet

Table I. Cytotoxic concentrations of the test extracts that induced 25% cell death.

Cell line	Wine extracts	Cytotoxic concentration (μg/ml)
HepG2	Muscat	25
	Mavrodaphne	25
	Rhoditis	50
HeLa	Muscat	25
	Mavrodaphne	25
	Rhoditis	50

was resuspended in phosphate-buffered saline (PBS) at the concentration of $2x10^5$ cells/ml and incubated in the presence of mercury orange (40 μM) in 37˚C for 30 min. Following centrifugation (1,200 x g, 5 min, 4˚C), the supernatant was removed, and the pellet was resuspended in 350 μl PBS. The cells were then subjected to flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences) with excitation and emission wavelengths at 488 and 580 nm. The analysis was performed on 10,000 cells per sample, and the fluorescence intensities were measured on a logarithmic scale. Data were analyzed using BD Cell Quest software (BD Biosciences, version 6). Each experiment was repeated at least three times.

Measurement of endogenous ROS levels in HeLa and HepG2 cell lines using flow cytometry. The intracellular ROS levels were assessed using 2,7-dichlorofluorescein diacetate (DCF-DA), which is deacetylated within cells by esterases and is further converted into fluorescent DCF by the oxidative action of ROS. The cells were incubated (37˚C) for 24 h in FBS-free media including the test extracts. The cells were then used for the measurement of ROS using flow cytometry. A 20 mM stock solution of DCF-DA (Merck KGaA) was prepared in DMSO and a 10 μM solution of DCF (Merck KGaA) was added in each well and the cells were incubated at 37˚C for 45 min. The cells were then trypsinized and centrifuged (1,200 x g, 5 min, 4˚C). The supernatant was removed, and the pellet was resuspended in 350 μl PBS. The cells were then subjected to flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences) with excitation and emission wavelengths at 488 and 530 nm. The analysis was performed on 10,000 cells per sample, and the fluorescence intensities were measured on a logarithmic scale. Data were analyzed using BD Cell Quest software (BD Biosciences, version 6). Each experiment was repeated at least three independent times.

Statistical analysis. Data were analyzed using one-way ANOVA followed by Tukey's test for multiple pair wise comparisons and are presented as the mean ± standard error of the mean (SEM). A value of $P<0.05$ was considered to indicate a statistically significant difference. For the analyses, the statistical package for social sciences (version 21.0; IBM Corp.) was used.

Results

Effects of the extracts on cell viability. The extracts induced 25% cell death in the concentrations shown in Table I. Concentrations below these levels were selected for further analysis to determine their effects on the GSH and ROS levels in the human cancer cell lines.

Effects of incubation of the cells with the stem extract from the Mavrodaphne grape variety on endogenous GSH and ROS levels in HeLa and HepG2 cells. The GSH and ROS levels in HeLa (Figs. 1A, S1 and S2) and HepG2 (Figs. 1B, S3 and S4) cells were not significantly altered following incubation with the grape stem extract derived from the Mavrodaphne variety.

Effects of incubation of the cells with the stem extract from the Muscat grape variety on endogenous GSH and ROS levels in HeLa and HepG2 cells. The GSH levels in HeLa cells were increased compared to those in the untreated cells (control) following incubation with the extract concentrations equal to 1.56 and 6.25 μg/ml (Figs. 2A and S5). The ROS levels in HeLa cells were also increased compared to those in the control cells following incubation with the extract concentrations equal to 3.125 and 12.5 μg/ml (Figs. 2A and S6). As regards the HepG2 cells, the GSH and ROS levels were not significantly altered (Figs. 2B, S7 and S8).

Effects of incubation of the cells with the stem extract from the Rhoditis grape variety on endogenous GSH and ROS levels in HeLa and HepG2 cells. The GSH levels of the HeLa cells were increased compared to those of the control cells following incubation with extract concentrations equal to 25 μg/ml (Figs. 3A and S9). The ROS levels in HeLa cells were also increased compared to the controls following incubation with extract concentrations equal to 3.125, 6.25 and 25 μg/ml (Figs. 3A and S10). As regards the HepG2 cells, the GSH and ROS levels were increased compared to those in the control cells following incubation with extract concentrations equal to 25 μg/ml for both biomarkers (Figs. 3B, S11 and S12).

Discussion

The present study examined the effects of three grape stem extracts derived from Mavrodaphne, Muscat and Rhoditis, which are native Greek vine varieties, on the redox status of cancer cells. In particular, the endogenous levels of GSH and ROS were measured in two human cancer cell lines (HeLa and HepG2) following incubation with the extracts. Overall, the extract derived from the grapes of the variety Rhoditis exerted prominent prooxidant effects on both cell lines, as indicated by the increased levels of ROS. The extract from Muscat only exerted prooxidant effects on HeLa cells, whereas the examined parameters remained unaltered following incubation of both cell lines with the extract from Mavrodaphne. Thus, it is evident that the stem extracts derived from Rhoditis and Muscat appear to be promising compounds against cancer progression in the specific cellular environment.

Grape stems are byproducts of the winemaking procedure and studies have demonstrated that, due to their high polyphenolic content, they could potentially be considered as anticancer agents (31,32), whereas their antioxidant and antimutagenic properties have also been observed (33). Concomitantly, research evidence has demonstrated the prooxidant role of grape stems in the cellular level by increasing ROS levels in epithelial

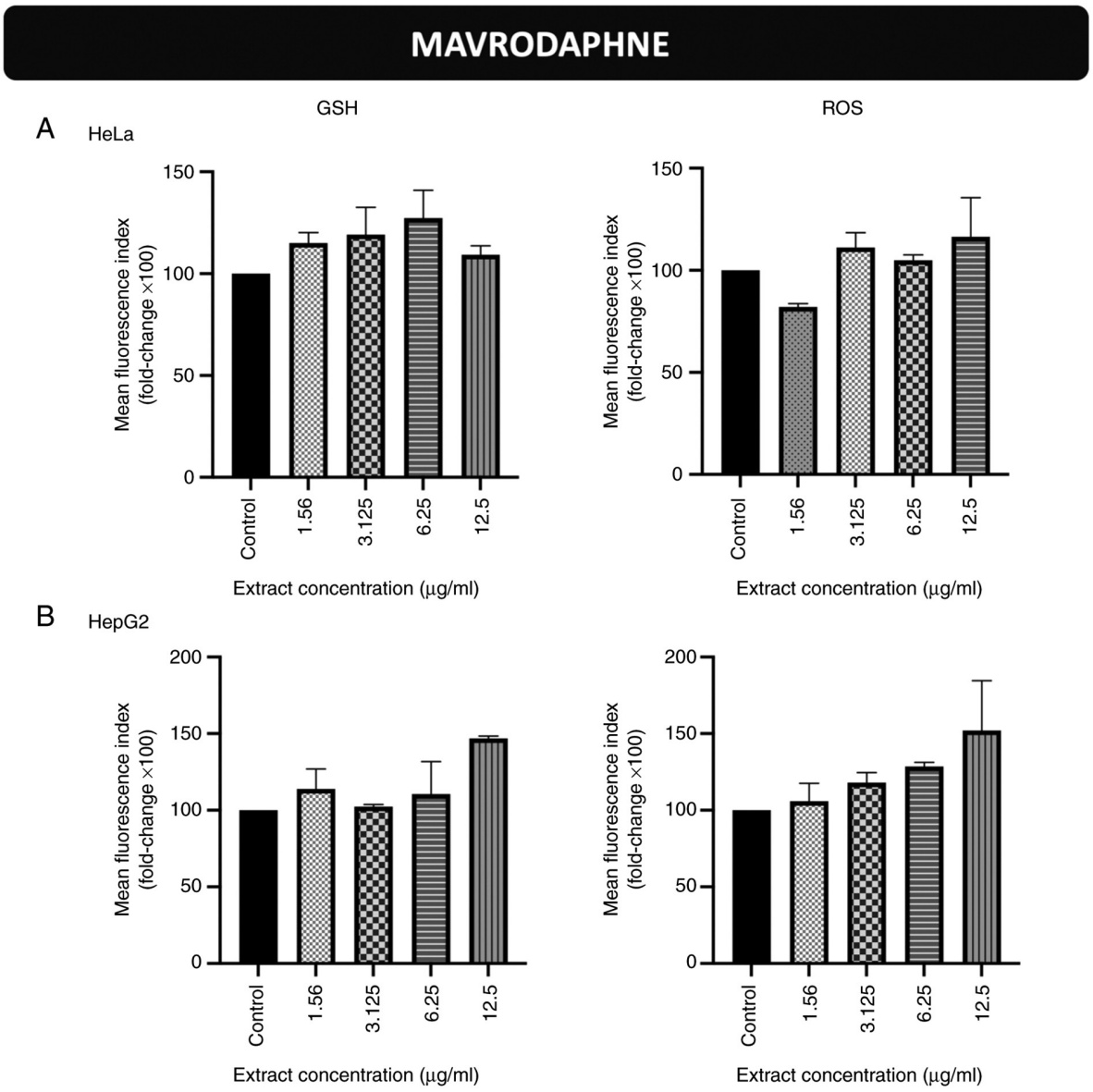

Figure 1. Effects of incubation of (A) HeLa and (B) HepG2 cell lines with the grape stem extract derived from the Mavrodaphne variety on endogenous GSH and ROS levels. GSH, glutathione; ROS, reactive oxygen species.

cells (34). Although the literature regarding stems is limited, there is sufficient evidence to indicate the prooxidant role of polyphenols in cancer cell lines. Numerous *in vitro* and *in vivo* studies have provided evidence towards the direction of adopting polyphenolic schemes for cancer prevention (35). For instance, it has been demonstrated that gallic acid exerts pro-oxidant effects on prostate cancer cells (36,37). Moreover, other studies have reported that quercetin can function either as an antioxidant or as a prooxidant depending on the cancer cell line (38-40). The anticancer properties of polyphenols have been attributed to their potent antioxidant activity (41,42). These results are in line with the findings presented herein. Indeed, the present study demonstrated that the extracts derived from the grape varieties Rhoditis and Muscat increased the levels of both ROS and GSH. Thus, it is suggested that these extracts function as prooxidant agents in the tested cancer cell lines, whereas GSH appears to be activated by the cells in order to overcome the potential oxidative modifications that are induced by the increase in ROS levels.

It has been found that increased levels of ROS activate the nuclear erythroid 2-related factor 2-antioxidant response element signaling pathway that regulates a plethora of genes whose products play crucial roles in the reinforcement of the antioxidant arsenal of cells, ensuring their survival (43). This may also be associated with the apoptosis of cancer cells and thus, with the prevention against cancer. In the apoptotic process, the role of GSH is controversial and is dependent on cell types and pro-apoptotic stimuli. Low intracellular GSH levels have been found to prevent apoptosis by compromising caspase activation in mouse hepatocytes (44). In addition, the depletion of intracellular GSH appears to prevent CD95-triggered apoptosis upstream of caspase-8 activation in T- and B-cells (45). Furthermore, cells undergoing apoptosis also appear to export GSH into the extracellular space (46,47). The production of ROS plays crucial role in the proapoptotic effects of polyphenols against cancer cell lines (48). ROS mediate the release of cytochrome *c* from the mitochondria,

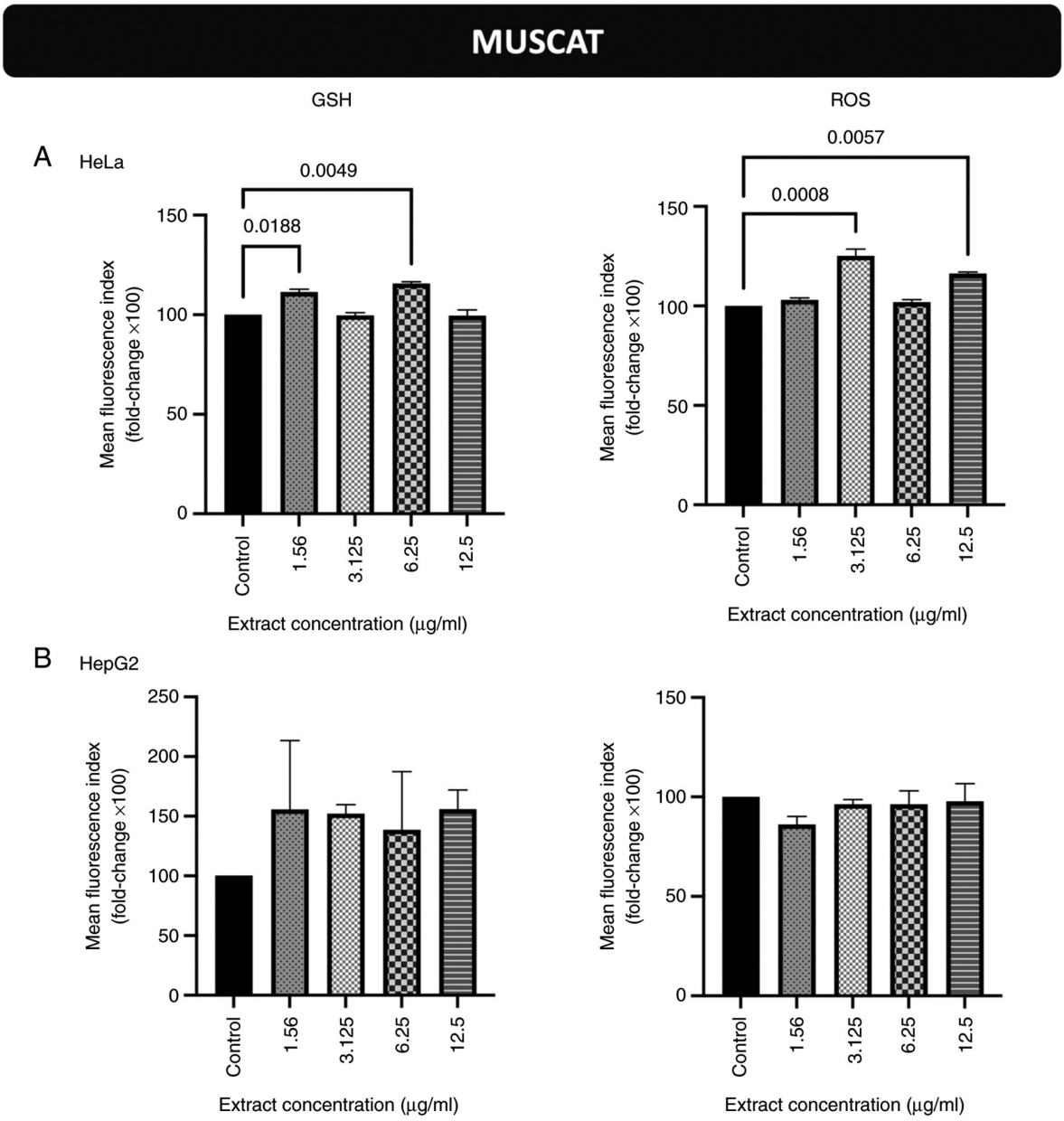

Figure 2. Effects of incubation of (A) HeLa and (B) HepG2 cell lines with the grape stem extract derived from the Muscat variety on endogenous GSH and ROS levels. The P-values obtained are indicated above the lines in the graphs. GSH, glutathione; ROS, reactive oxygen species.

which in turn leads to caspase activation and apoptosis (49). Therefore, the increased levels of GSH accompanied or induced by the elevated ROS levels observed in the present study may contribute to the apoptosis of cancer cells. Of note, the capacity of the test extracts to modulate the cell redox status does not correspond to their content in polyphenolic compounds. The stem extract from the variety Mavrodaphne has a higher total polyphenolic content in comparison to the two other extracts, as has been previously reported (19). However, it is the only extract that did not affect the redox status of the cells. This finding may be associated with the different capacities of the individual phenolics to penetrate the cell membrane, based on their polarity and size (50,51).

The present study demonstrated that grape stem extracts rich in polyphenolic compounds with potent antioxidant properties, do not appear to exert potent effects on the redox status of cancer cells. As aforementioned, polyphenols can function as prooxidants due to their antioxidant content (52,53). This is an interesting biological aspect of polyphenols, and it could be capitalized by researchers in the fight against cancer. Previous studies have demonstrated that antioxidants are detrimental against specific cancer types in animal models (54,55). The proposed mechanism is related to the fact that antioxidants scavenge free radicals, which are usually harmful to cancer cells. Indeed, free radicals can kill cancer cells as they have very weak antioxidant mechanisms compared to healthy cells (56). Therefore, free radicals appear to be 'allies' with anticancer therapies. The use of cancer cell lines is valuable to provide a repeatable source of biological activity estimation for experimental purposes. Therefore, the establishment of a novel cell line or normal primary cells would be more suitable, although a very complex process

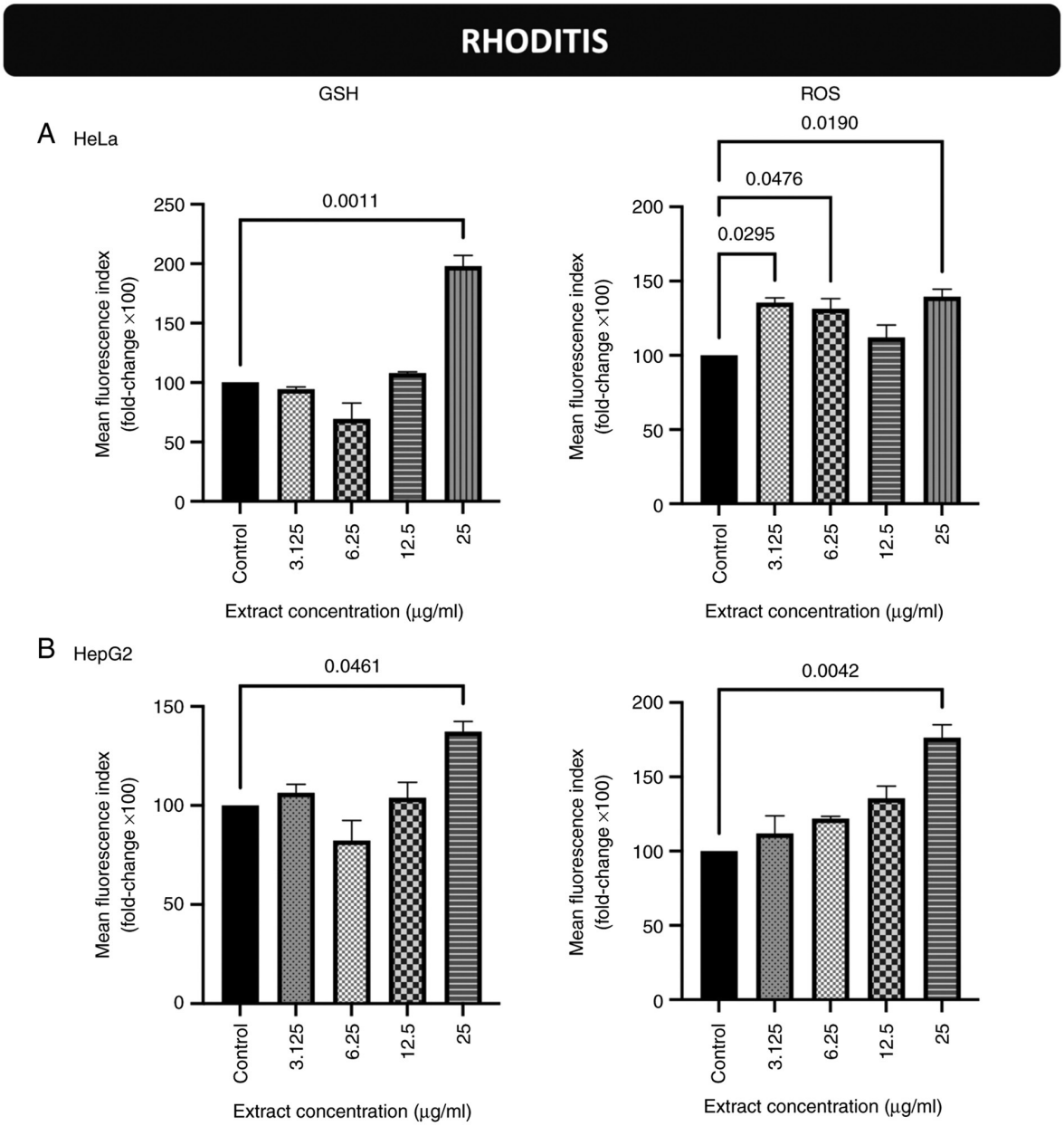

Figure 3. Effects of incubation of (A) HeLa and (B) HepG2 cell lines with the grape stem extract derived from the Rhoditis variety on endogenous GSH and ROS levels. GSH and ROS levels. The P-values obtained are indicated above the lines in the graphs. GSH, glutathione; ROS, reactive oxygen species.

that is still not well understood, a limitation of the present study. In conclusion, the role of the tested extracts appears to be promising; however, this is a research topic that warrants further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EV was involved in data curation, methodology and in the writing of the original draft. ASV was involved in the conceptualization of the study, as well as in data curation, in the writing of the original draft, and in the writing, review and editing of the manuscript. FT was involved in data curation, and in the writing, review and editing of the manuscript. ZS and KP were involved in the conceptualization of the study. SH was involved in the conceptualization and methodology of the study. DK was involved in the

conceptualization of the study, as well as in funding acquisition, project administration, study supervision, and in the writing, review and editing of the manuscript. All authors contributed to the interpretation of the data. All authors have read and agreed to the published version of the manuscript. EV and DK confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

SH and DK are Editors of the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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