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RESEARCH ARTICLE

Antioxidant and Anti-cancer effects of crude extracts from (*Vitis vinifera* L.) leaves on melanoma cells (SK-Mel and A375)

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ABSTRACT

This study aimed to estimated total phenol, the antioxidant and antiproliferative activities of crude extract derived from grape leaves (*Vitis vinifera.* L.). Total phenol of grape leaves (*Vitis vinifera.* L.) was extracted using aqueous and methanol solvent and dosed with Folin-Ciocalteu using spectrophotometry. The antioxidant activity was estimated by spectrophotometry in the existence of the DPPH radical, when the antiproliferative activity was evaluated by using MTT test on two melanoma cells (A375 and SK-MEL) as compared to the effect of Cisplatinum. Results showed that extracts of grape leaves (*Vitis vinifera.* L.) were rich in total phenols. Indeed, the extracts exhibited an antioxidant activity, when 1.98 mg/mL of methanol and aqueous crude extracts could inhibit 90.20 % and 77.78 % of DPPH* radical respectively. The proliferation of Melanoma cells A375 and SK-MEL is decreased with increasing concentration of water and methanolic extracts (1.136, 2.27, and 4.54 mg/mL) included in the culture throughout 72 h. Results offered that extract from grape leaves (*Vitis vinifera.* L.) characterize with biological effect compounds such as phenols, with promise antioxidant activity, revealing an antiproliferative effect impact on A375 and SK-MEL malignant tumor cells compete with the synthetic molecule Cisplatinium.

Keywords: *Vitis vinifera*; phenols; antioxidant; anti-melanoma; A375 and SK-MEL

INTRODUCTION

Oxidative stress is defined as an unbalance between the production of free radicals and reactive metabolites, called oxidants or reactive oxygen species (ROS), and their exclusion by protective mechanisms, named antioxidants (Reuter et al., 2010).

Antioxidants are defined as "any endogenous or exogenous substance which in low concentration relative to the oxidizable substrate prevents or slows the oxidation of that substrate" (Pastre and Priymenko, 2007). Antioxidants now appear as the keys to longevity and our allies to fight against modern diseases such cancer, diabetes and ageing. These are protective elements that act as free radical scavengers (Bartosz, 2003). There is now a renewed attention in phytochemicals as sources of bio-antioxidants. The purpose is to exploit them in foods and pharmaceutical preparations to supplant synthetic antioxidants, which cause potential health risks due to their carcinogenic or mutagenic effects (Le Cren, 2012). Such as olive oil has been proposed by many experts as an important source of fat in the Mediterranean diet, it can form a fundamental influence on health benefits of Non-alcoholic fatty liver disease patients (Abenavoli et al., 2019).

Recently, the increased consumption of fruits and vegetables it's raw and processed form, or their various types of antioxidants containing as nutraceuticals, pharmaceuticals, and phytoceuticals may decrease the risk of the progress of chronic human diseases (Jideani et al., 2020). Contrary following a recent study suggest a controlled consumption of citrus juice could limit the risk of skin cancer non-melanoma in postmenopausal women (Sakaki et al., 2021).

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Skin cancer refers to the three most common types of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin (collectively called non-melanoma skin cancer) and melanoma. (Yokoyama et al., 2011).Melanoma is the most invasive skin cancer, difficult to treat and potentially capable of metastasizing (Jaszewska et al., 2009). Since the mid-1960s, the incidence of melanoma has increased from 3 to 8 % each year in most people of European descent, with the largest increases in older men (Thompson et al., 2005).

Melanoma accounts for only 4 % of all skin cancers, but 80% of skin cancer deaths. When UV exposure is the primary cause of melanoma, only 14% of patients with metastatic melanoma survive for 5 years (Arlo et al., 2006). Direct mutagenicity on DNA, stimulates cellular components of the skin to produce growth factors, reduces the immune defenses of the skin, promotes melanin of reactive oxygen species (ROS), induces and prevents DNA damage, promotes malignant changes in the skin (Thompson et al., 2005).

Like the all cancers, genetic predispositions are identified in patients with a family history Avilés JA, (High and Lázaro 2006, Pho, 2006 and Robinson, 2007). Usually the first treatment used is surgical removal. Chemotherapy, radiation therapy and immunotherapy may also be used. New treatments like ipilimumab (Yervoy) are particularly effective against melanoma in advanced stage (Hodi et al., 2010).

Cisplatin complex is clinically used as adjunct tumor therapy to induce cancer cell death and tumor lesions through the induction of apoptosis (Floreaet Büsselberg., 2011). Cisplatin has been clinically proven to fight several types of cancer, including sarcomas, soft tissue, bone, muscle, and vascular cancers. Despite the fact that such malignancies have recently gotten a better prognosis and treatment options (Dasari et Tchounwou, 2014).

Although plant extracts showed high anti-cancer and anti-proliferative activity (Chang et al., 2002; Nam et al.; 2003 and Lu et al., 2012). The isolation of the alkaloid from *Catharanthus roseus* (Apocynaceae) opened a new era in the use of plant materials as anticancer agents (Shoeb, 2006 and Manju et al., 2012). These compounds interfere with many aspects of tumor formation and progression in both *in vitro* and *in vivo* experiments. They induce cell cycle arrest in G1 or G2/M phase and inhibit the growth of various tumor cell lines by apoptosis (Lu et al., 2012).

Diarrhea and bleeding can be treated with grape leaves (Zargari, 1993). Grapes were also used to cure constipation, gastritis, enteritis, gout, and hemorrhagic diarrhea (Afzalzadeh et al., 2013). Different parts of grape vine have been employed since antiquity because phenols are naturally present in many of these biological features.

DelCastilloAlonso et al., 2020 were able to identify 47 phenolic compounds in the skins and wines of Tempranillo grapevines exposed or non-exposed to close-to-ambient solar UV levels using appropriate filters, including flavonols, anthocyanins, flavanols, stilbenes, and hydroxycinnamic and hydroxybenzoic acids. In addition, 25 anthocyanins, 17 flavonols, 7 hydroxycinnamic acid derivatives, 2 stilbenes, and many flavan-3-ols have been found and measured in berry sections from two unique *Vitis vinifera* L. red grape genotypes (Moribel and Tinto Fragoso) (Pérez-Navarro et al., 2019).

The aims of this work are to estimate the total phenols extracted by aqueous and methanol from grape leaves (*Vitis vinifera* L.), to evaluate of their antioxidant potential by using the DPPH* test and antiproliferative activities by using MTT test on two melanoma cells (A375 and SK-MEL) as compared the effect of Cisplatinum.

MATERIAL AND METHODS

Plant material

In August, mature leaves from the apical section of *Vitis vinifera* L. were harvested in Medea (warm temperate climate, average temperature 14.4 C°), Algeria. The leaves were cleaned under running water and dried at room temperature (25°C). Finally, they were ground into a fine powder and stored in a sterile bag under vacuum in the dark at 5 °C (Ferhi et al., 2019).

Extraction

Aqueous crude extract

The extraction is done as indicated (Sanogo et*al*., 2006) with a few changes. It was performed at room temperature by macerating 40 g of the leaf-powder into one liter ofsterile distilled water and stirring during 24 h. Then, the supernatant was filtered and frozen at – 30 °C.

Methanol crude extract

The methanol extract was performed as indicated (Mokale Kognou et al., 2011) with little changes. Ten gr of dried leaves were macerated in 50 mL of methanol/water 80 ° (v/v) during 24 h. Then, the solution was filtered with 0, 45 µm syringe Millipore filter and conserved at 4°C until needed.

Total phenolic (TP) content

The modified Folin-Ciocalteu technique was used to determine the total phenolic content (Ferhi et al., 2019).

1 mL of each extract was vortexed and centrifuged with 9 mL of ethanol (80%) (1:10 w/v). The Folin-Ciocalteu reagent (1mL) was then added to 200 µL of each extract. A spectrophotometer was used to measure the absorbance at 760 nm (8453 Agilent Technologies, Santa Clara, CA, USA).

On the basis of a Gallic acid calibration curve (50 to 500 mg/L with R2 = 0.996), the results were reported as milligrams of Gallic acid equivalent/g of dry weight.

Antioxidant activity

The scavenging activity was evaluated using the DPPH* test (Choi et al., 2002). The extracts' absorbance at 518 nm reduced as the concentration of extracts increased. The ability of the extracts to scavenge free radicals improved that the concentration of the extracts rose (Azad et al., 2015).

The test solution was prepared by adding 30 µL of each extract at different concentrations (10, 20, 25, 50, 100, 200 mg/mL) to 3 mL of 0.3 mM DPPH methanol solution. However, to prepare the control solution, 3 mL of methanol was added to 30 µL of the sample solution at all concentrations. Then, 3 ml of DPPH solution was added to 30 µL for negative control. The blank for this solution is methanol. The solution was kept in the dark for 30 minutes at room temperature. The absorbance values were measured at 518 nm and converted to a percentage of antioxidant activity according to the following formula:

Antioxidant activity (%) = [(absorbance solution - absorbance control) ÷ absorbance control] ×100

Cell culture

SK-MEL and A375 melanoma cell line preserved in the culture medium (RPMI) derives from The Roswell Park Memorial Institute 10 % fetal bovine serum (FBS) RPMI medium growing in T25 culture flask. Cultures were incubated at 37 °C in 5 % CO_2 incubator. While cells reach confluence need to be detached by trypsinization and to be "splitted", transferred to a new flask at a lower density and keep them growing.

MTT viability assay

In order to evaluate the antiproliferative activity of methanol and aqueous crude extracts of *Vitis vinifera* L. leaves on melanoma cells; a cell viability test is useful. The MTT assay is a colorimetric assay based on the ability of functional mitochondria of cells to reduce by succinate dehydrogenase enzyme (Mosmann, 1983). The MTT formazan crystals produced in detectable visible spectrometry after dissolution in DMSO (Diméthylsufoxide). Then, the amount of living cells surviving in the assessed cell culture is then used to determine the effects of the treatments studied on the overall growth of a given cell population.

Proliferation assay

The cells following (A375, SK-Mel) as used as $3*10^3$/well in 200 µl of RPMI medium on a 96 well plate. Before, it needed to count cells by using (Talitm Image Based Cytometer Invitrogen Life Technology) and let them grow over night at 37 °C in a 5 % CO_2 incubator. After 24 hours substitute growth RPMI medium with new RPMI medium containing test compounds (methanol and aqueous crude extract) at final concentration of 4,54 mg/mL, 2,27 mg/mL and 1.136 mg/mL knowing that the mixture as follows 10 µL of extract and 190 µL of RPMI medium. The negative control is prepared only by adding RPMI medium but positive control is prepared by Cis-Platinium 10 µM dilute in RPMI medium. It performed a test by replacing the extracts with methanol in the RPMI medium in order to see how methanol affected the two cell lines that were employed. Every test is performed three times. After one day, it substituted again RPMI medium with or without compounds and repeat the same treatment (treatment 2). Finally, after 48 hours since the first treatment, observed cells at the microscope and stopping viability with assay (MTT method) by adding 20 µL of MTT solution, 5 mg/mL in Phosphate-buffered saline (PBS) to each well, Incubate at 37 °C in the 5 % CO_2 incubator for 3-4 hours and remove the RPMI medium by aspiration then added 100 µL/well DMSO, Shaking for 10 min with a shaker (ThermomexerConfortEppendorf) at room temperature. Read absorbance in a Microtiter plate (Sunrise_basicTecan) reader at 570 nm. The relative percentage of growth cells (viability of cells) is calculated by contribution to the negative control.

Relative percentage viability = Absorbance of sample/ Absorbance of negative control× 100

Statistical analysis

Data were expressed as mean ± standard deviation. ANOVA test with Duncan's multiple range testing approach was used for statistical analysis (DMRT). This study was conducted using the SPSS 25.0 Windows program. P values <0.05 were considered significant.

RESULTS

Total phenolic content and DPPH Radical scavenging activity

Table 1 showed values of IC50 and Total phenols (TP).

In comparison, the aqueous crude extract to the methanol crude extract had much more TP (approximately 1.5 times) (P <0.05). The observed TP content discrepancy could be caused by the polarity of the methanol.

In the presence of the DPPH radical, the antioxidant activity of the leaf extracts was measured using spectrophotometry.

The methanol and aqueous crude extract of grape leaves (*Vitis vinifera*. L.) exihibited an antioxidant activity and able to trap the DPPH radical. The antioxidant capacity is presented by the IC50 coefficient, it is the quantity of antioxidant required to reduce 50 % of the concentration of the free radical DPPH. The IC50 is calculated from the regression line formed from percentages of inhibitions. For the calculation of these values, Microsoft Excel software was used.

The percentage of scavenging is related to the crude extract concentration ($R^2 > 0.90$).

The maximum trapping DPPH radical (90.20 %) is given by the methanol extract 1.98 mg/mL. The same concentration gave (77.78 %) with the aqueous crude extract. The values of IC50 are inversely related to antioxidant capacity, the lower IC50 means higher antioxidant capacity of compounds. The least effective extracts are those of higher IC50 values. Indeed, with IC50s of 0.76 mg/mL and 1.1 mg/mL, respectively, methanol extract had stronger free radical scavenging activity than aqueous extract ($P < 0.05$) (Table 1).

Effect of grape leaves (*Vitis vinifera.* L.) methanol and aqueous crude extracts on Cell Proliferation

The antiproliferative activity of grape leaves (*Vitis vinifera*. L.) using MTT test on melanoma cells (A375 and SK-MEL) are documented in Fig. (1, 2), Photos (1, 2).

The MTT assay assesses mitochondrial activity and was chosen as a biochemical test to evaluate cell viability. In this test, a Tétrazolium salts is used as a substrate. Dehydrogenase enzymes in active mitochondria cleave the Tétrazolium ring, contucted to the production of formazan.

Thus, the yellow substrate is transformed in a blue-colored product. The survival cells after treament with methanolic and water extract appereated with blue and death cells appeared no-colored or like water.

(W1, W2, W3: cells treated with different doses of aqueous extract from highest to lowest dose, M1, M2, M3: cells treated with different doses of methanol extract from highest to lowest dose, Cis: cells treatead with Cisplatinium,OH: cells treated only with methanol, Blank: only midium).

Table 1: IC50 and TP of methanol and aqueous crude extract of grape leaves (*Vitis vinifera* L.)

Extracts	TP (mg GAE/g DW)±SD	IC50 (mg/mL)±SD	R^2
Methanol	23.0 ± 21.3[a]	0,76 ± 0.08[a]	0.991
Aqueous	16.35 ± 2.06[b]	1,10 ± 0.12[b]	0.984

TP: Total Phenols, GAE: Gallic acid equivalent; DW: Dry weight; SD: standard deviation; sample concentration at which 50% of the free radical activity was inhibited, R2: the correlation coefficient, The values with dissimilar letters are significantly different at P 0.05

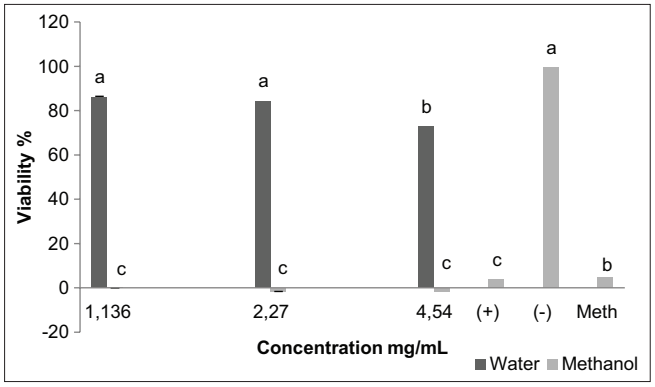

Fig 1. Effect of Methanolic and Aqueous crude extract on SK-Mel cell proliferation (untreated group: concentration = (-), group treated by Cisplatinium: (+), Methanol: (Meth)). Data are expressed as mean ±SD, n = 3. According to Duncan's Multiple Range Test, bars with dissimilar letters within a group are substantially different at P <0.05. (DMRT).

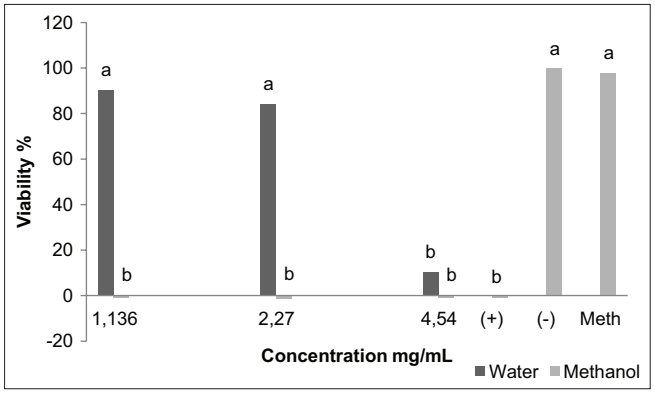

Fig 2. Effect of Methanolic and Aqueous crude extract on A375 cell proliferation (untreated group: concentration = (-), group treated by Cisplatinium: (+), Methanol: (Meth)). According to Duncan's Multiple Range Test, bars with dissimilar letters within a group are substantially different at P <0.05. (DMRT).

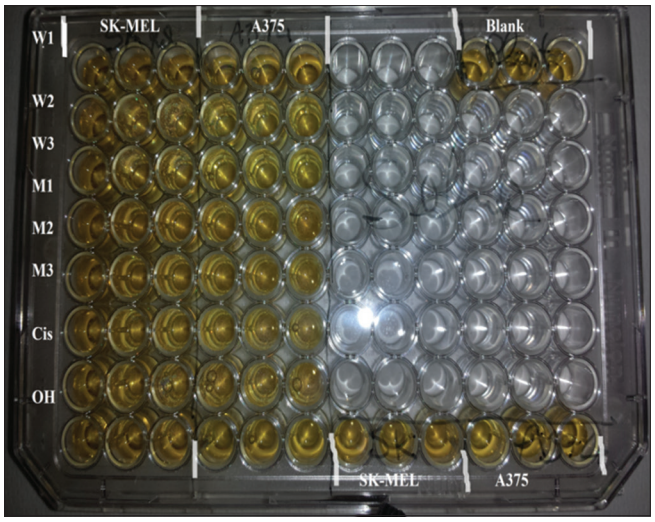

Photo 1. Plate with MTT before incubation

Effect of methanolic and aqueous extracts of grape leaves (*Vitis vinifera*. L.) on SK-Mel Proliferation

SK-Mel cell viability was significantly reduced after incubation with methanol extract (P = 0.001) (cell proliferation is expressed as the average percentage of viable and untreated cells). (Photo 2). While, the aqueous extract was not toxic for SK-Mel cells, the cell proliferation reduced but insignificantly (P > 0.05); exceptionally the maximum dose (4.54 mg/mL) could inhibit significantly SK-Mel cell proliferation. The maximum growth inhibition was obtained using methanol extract (~100 %) followed by Cisplatinum (> 97 %), representing the positive control, and finally the aqueous extract 4.54 mg/mL (> 72 %). The methanol was toxic for SK-Mel cells and could inhibit significantly (P < 0.05) growth of but less than methanol extract. We can conclude that SK-Mel cells were sensitive to methanol (Photo 2).

Effect of methanolic and aqueous crude extracts of grape leaves (*Vitis vinifera.* L.) on A375 cell Proliferation

Both crude extracts significantly (P<0.05) decreased A375 cell multiplication, similar to what was seen in SK-Mel cells (Figure 2). Specifically, that methanol extract was more efficacious on cells than water extract exceptionally the highest dose of water extract (4.45 mg/mL),when its effect was powerful on A375 cell prolefiration. Maximum growth inhibition rate was obtained by methanol extract (100 %), followed by Cisplatinium and the highest dose of water extracts (Figure 2). The A375 cells showed more resistant to methanol than SK-Mel cells also it was not toxic on A375 cells. In this case, it can be deduced that the methanol extract of grape leaves (*Vitis vinifera* L.) had a similar or better effect on the growth of A375 cells than that of cisplatin.

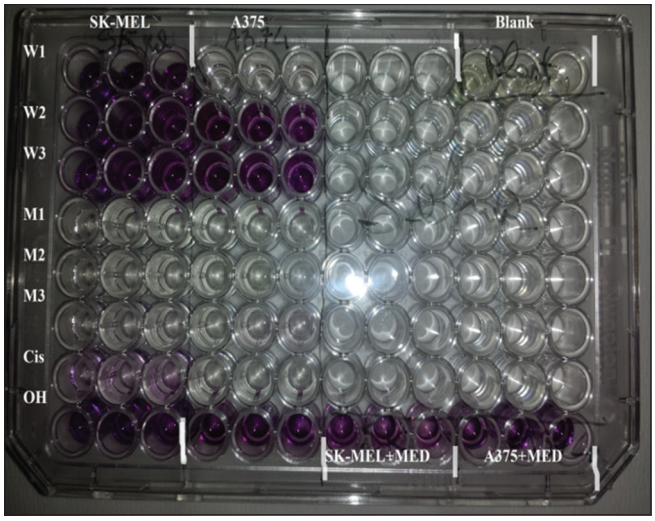

Photo 2. Plate with MTT after incubation

DISCUSSION

Antioxidants now appear as the keys to longevity and our allies to fight against modern diseases. These are protective elements that act as free radical scavengers (Bartosz, 2003). The interesting activity of grape leaves (*Vitis vinifera.* L.) crude extract is explained by the presence of phenolics compounds, these results are accorded to those obtained previousely by Nassr-Allah et al, 2009, Popovici et al, 2009, Ba et al, 2010, Zheng et al., 2010 and Katalinic et al., (2009 and 2013). These discoveries have given interest in grape leaves (*Vitis vinifera* L.) as a possible source of chemicals as nutritional and biological value.When, Monagas et al., 2006[a] and Ferhi et al., 2019[1] found that grape leaves (*Vitis vinifera.* L.) contained high level of phenols; anthocyanins, flavonols and trans-caftaric acid.

The anticancer activities of essential oils, aqueous and organic solvent extracts of many aromatic plants were evaluated *in vivo* and *in vitro*, *Luffaaegyptiaca*(sponge gourd), *Cassia italica*(Senegal senna), *Ocimumbasillcum*(basil), *Colocasiaantiguorum*(taro), *Beta vulgaris* (beet), fruit of *Capsicum frutescens*(chili pepper) andfruit, leaves, root of *Morindacitrifolia*(Brown, 2012). Also, the extracts from *Urticamembranacea* (Urticaceae), *Artemesiamonosperma*(Asterac eae), and *Origanumdayi post* (Labiatae) demonstrated toxicity in a variety of human tumor cell lines and primary cultures derived from patient biopsies. (Solowey et al., 2014).

Similarly, the methanol extracts of *Dendrosicyossocotrana, Withaninaaduensis, Withaniariebeckii, Dracenacinnabari* and *Buxushildebrandtii*showed a high toxicity on all tumor cell lines with IC_{50} values ranging between 0.29 and 5.54 mg/mL (Mothana et al., 2007). In all cell lines, *Scutellaria baicalensis* exhibited a significant dose-dependent growth reduction, with IC50 values of 1.1, 0.9, 0.52, 0.82, 1.1, 1.5, 1.0, and 1.2 mg/mL on HepG2, MCF-7, PC-3, LNCaP, KM-12, HCT- 15, KB, and SCC-25 cells, respectively (Ye et al., 2002). Other plants like *Inulagraveolens, Salvia dominica*, *Conyzacanadiensis*and *Achilleasantolina*showed also showed strong antiproliferative activity (Abu-Dahaband et Afifi, 2007).

In addition, at IC50 of 30 Mm (9.5 µg/mL), the phenolic compounds extracted from *Rabdosia japonica* proved cytotoxicity against the murine B16-F10 melanoma cell line (Nitoda et al., 2008). The aqueous extracts of aerial part of *Tetraenagaetulaand* root bark of *Berberishispanica* (at 80 and 300 mg/kg, respectively) had a strong antiproliferative activity (El Youbi et al., 2012).

habitually, thymol is used as an inhibitor of the enzymatic system of melanin production, otherwise, it proved moderate cytotoxicity with an IC50 value of 400 µM but

no antimelanogenic action on B16-F10 melanoma cells (60.09 μg/mL) (Satooka and Kubo, 2012).

Inhibition of 72.58 % of melanoma metastatic colony formation in the lungs was obtained by treating animals with Withania extract at 20 mg/dose/animal, while intraperitoneally injection of 500 g/dose/animal of Withanolide D inhibited 69.84 percent of B16F-10 development (Leyon and Kuttan, 2004).

Another study found that Triterpene Extract from Mistletoe, with an IC50 of 2.675 mg/mL, suppressed the growth of B16.F10 cells in a dose-dependent manner (Struh et al., 2012).

The 200 μg/mL of 1,1-dimethylallyl caffeate (DMAC), 3-methyl-3-butenyl caffeate, pinocembrin, benzyl ferulate, benzyl isoferulate and tectochrysin provided excellent antiproliferative activity against all human gastrointestinal cancer cell lines (> 80%) (DLD-1 colon adenocarcinoma, HCT-116 colon carcinoma, KYSE-30 oesophageal squamous cancer, and NCI-N87 gastric carcinoma); while Pinobanksin-3-O-acetate, 5-phenyl-Penta-2,4-dienoic acid, and pinostrobinchalcone showed moderate to high activity against all cell lines except KYSE30, however, p-coumaric acid was inactive Catchpole et al., (2015).

Finally, Ferhi et al., (2019) provide that the extract of grape leaves (*Vitis vinifera.* L.) grown in (Algeria) showed power antiproliferative effect on MCF-7 breast cancer cells and HepG2 hepatocarcinoma cells.

CONCLUSION

For the first time, it discovered in this research that the methanolic and aqueous crude extracts of grape leaves (*Vitis vinifera)* grown in Medea area in Algeria promoted an interesting source of not dangerous bioactive molecules like phenols who provided antioxidant and anti-melanoma activities, these can even conquer the effect of Cisplatin. So, it suggested that these extracts might use as available source of bio-antioxidants and as matrix for preparing medicaments countering cancer cells proliferation.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

There are no conflicts of interest declared by the authors.

Acknowledgement/Disclaimers

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AUTHORS' CONTRIBUTIONS

S.F. is the leading author, who developed the idea, practical works, wrote and revised the article. T.C. helped to discuss the results, S.G. helped to find journal and submitted the work, R.M.C and G.D. provided technical guidance during simulations and experiments, the necessary technical tools for the realization of this work S.Z. is the supervisor of this work. G.D. supervised the work and is the coordinators of the guesting Institute

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