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In vitro assessment of cytotoxic, antioxidant and antimicrobial activities of leaves from two grape varieties collected from arid and temperate regions in Palestine

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ABSTRACT

Grape leaves (*Vitis vinifera* L.) are widely consumed in Palestine, and other Mediterranean countries. Positive health effects of grape products are reported by various studies and pharmaceutical preparations from grape leaves are patented and commercialized as drugs. The aim of this *in vitro* study is to assess the therapeutic potential of leaf extracts; their cytotoxicity against lung cancerous cells, their antioxidant and antimicrobial activity against several human pathogenic bacterial strains, and according to geographical location, to examine the overall effect of annual rainfall on the aforementioned activities.

Leaves from Shami and Baituni grapes, collected from Dahria (an arid region) and Beit Omar (a temperate region) in Palestine were ground to powder using liquid nitrogen and a pestle and mortar. Cytotoxicity was measured against lung cancer cells and muscle cells. Antioxidants potential of leaf extracts and antimicrobial activity against five human pathogenic bacterial strains were assessed.

Results showed Shami leaves from Beit Omar inhibited the proliferation of lung cancer cells. Cytotoxicity assessment against lung cancer cells showed leaves from Baituni grapes are ineffective. Antioxidant capacity of the leaf extracts of both genotypes from both locations gave high levels of antioxidants, but no significant differences recorded between treatments. Leaf extracts of both grape genotypes were effective against *S. aureus* and slightly effective against *P. aeruginosa* bacteria. However, these extracts were ineffective against *L. monocytogenes*, *S. typimurium* and *E. coli*.

Results show the therapeutic potential of leaves of Shami and Baituni might be related to their phytochemical composition. Our findings suggest effective cytotoxic activity of Shami grape leaves against lung cancer cells and provide a preliminary view of the effect of annual rainfall on the grape leaves' anticancer and antimicrobial activities.

Keywords: Grape, cancer, antioxidants, antimicrobial, Palestine

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1. INTRODUCTION

Wild and cultivated edible plants are commonly consumed in the eastern region of the Mediterranean, including Palestine[1]. Wild edible herbs and plant parts from trees and vines (e.g. grape leaves) constitute a main part of traditional diets, and are famous for their health and medicinal qualities among local communities and indigenous people. In Palestine, table grapes (*Vitis vinifera* L.) are widely produced, and a large number of grape genotypes exist[2], some of which are believed to be very old. Referring to the Mediterranean diet, it is known that natural products from plants have chemopreventative potential[3]. In this respect, it is reported that nutraceuticals in grape products include vitamins, minerals, carbohydrates, edible fibers and other phytochemicals (e.g. polyphenols), which may contribute positively to human health. Of most significance are secondary metabolites, which belong to several phytochemical groups such as antibiotics, terpenes, sterols and phenolics[4]. Their diversity in structure, function and occurrence has been the focus of significant research. For example, the phytoalexin, resveratrol received great attention due to its proposed chemopreventative and therapeutic effects against many diseases[5–7]. It was also found that anthocyanins of Concord grape juice enhanced neurocognitive function in older adults with mild memory decline[8,9]. In this sense, the phytochemical composition of grapes has been intensely studied, and it varies among grape genotypes and grape parts. As an example, anthocyanins were reported to exist mainly in red grape skins as pigments, whereas flavanols are primarily found in the stems and seeds of grapes[10]. Such diversity may explain the large number of studies that addressed their health impact[11]. Accordingly, several modern and common diseases have been reported to be inhibited by such phenolic compounds, amongst these being cardiovascular diseases[12,13], certain types of cancer[14–16] and reversing neuronal and behavioral aging [17,18]. In addition, the anticancer activity of grapes has received attention, as cancer is considered a growing epidemic[19], predicted to surpass heart diseases to become the most deadly disease worldwide in future years. Therefore, the study of preventative methods, including the use of plant products and extracts, is an active field of research. In this respect, several studies reported the effect of grape extracts on various types of cancer. In one study, it was reported that grape skin extract induces apoptosis of prostate tumor cell lines[6]. In another study, grape pomace proved to have significant antiproliferative effect on human cancer adenocarcinoma cells[14,20]. Furthermore, it was reported that proanthocyanidins and catechins inhibit breast cancer metastasis[21,22], whereas resveratrol from grapes induced apoptotic and antiproliferative effects of prostate cancer cell lines[23]. As for lung cancer, which is the leading cause of cancer death in men and women worldwide[24], it is estimated that 80% of lung cancer patients suffer from non-small cell lung cancer (NSCLC including squamous cell carcinomas, adenocarcinomas, and large-cell carcinomas[25]. While lung cancer is significantly related to cigarette smoking[26], it was seen in some patients that chemotherapy and radiation were ineffective due to the acquired or intrinsic resistance to the drugs used[27]. Accordingly, lung cancer has been the topic of studies that aimed to develop therapeutic agents and therapies that can significantly affect tumor growth and apoptosis in lung cancer patients. In this respect, natural plant products present an opportunity for developing effective chemotherapeutic agents. A wealth of evidence suggests cytotoxic effects of grape products against several cancer cell lines. For example, the effect of red grape skin extracts on lung cancer cell lines has been studied, and shows a significant reduction in the number of metastatic nodules on the surface of lungs in Swiss mice[3]. In addition, grape seed proanthocyanidins were seen to induce apoptosis of NSCLS cells, A549 and H1299 cells *in vitro*.[28]

As for antioxidant potential, plant secondary metabolites, particularly in grapes, have been a major field of research due to their wide health-promoting effects. It was elucidated that phenolic compounds, including polyphenols, had free radical scavenging properties and antioxidant activities. It was reported that procyanidin from grape seeds were 20 times more effective as reducing agents than vitamin C and 50 times more than vitamin E[29]. Another study found that phenolics present in red wine could inhibit myoglobin, iron ascorbate and cytochrome C that catalyzes lipid peroxidation[30]. Further studies showed the potential of procyanidins from grape seeds in reducing the oxidation of polyunsaturated fatty acids in mouse liver microsomes[31]. In addition to anticancer and antioxidant activities grape products have been reported to have antimicrobial effects on pathogenic strains. It was reported that grape products inhibited microbial growth of *Escherichia coli*[32], *Salmonella typhimurium*[33] and *Listeria monocytogenes*[34].

2. MATERIALS AND METHODS

2.1. Plant material

Grape leaves used in this study were from Baituni and Shami varieties. For each variety, leaves based on morphological characteristics were harvested from two different geographic regions in Palestine, namely, Dahriah (arid region) and Beit Omar (temperate region). These regions were selected as they significantly differ in annual rainfall rates, and it is known that plants in Dahria region suffer from drought. The collection time was during the main flush of vegetative growth (June). Samples were taken in triplicate. Directly after harvest, leaves were shock-frozen in liquid nitrogen and stored at −80°C.

2.2. Anticancer assessment

2.2.1. Extract preparation

Two grams from each sample was added to 10 ml of 50% (ethanol/distilled water) solution and left for 2 minutes at 100°C. The crude extract was passed through 0.22 μm filter. Ten ml of the extract was air-dried, and the concentration calculated. The extracts were t diluted to the required concentrations of 500, 250, 125, 62.5, 32, 16, and 8.0 $\mu g.ml^{-1}$.[35]

2.2.2. Cell lines

Cell lines used were lung cancerous cells (A549) from lung epithelial tissue (Sigma-Aldrich Israel Ltd) and normal muscle cells L6 from skeletal muscle (ATCC number: CRL-1458). Cells were cultured in RPMI media1640 and DMEM media, respectively, supplemented with 10% fetal calf serum, 1% penicillin streptomycin, 1% amphotericin B, 1% nonessential amino acids, and 1% L-glutamin. Cell lines were maintained in a humidified atmosphere with 5% CO2 at 37°C.

2.2.3. Cytotoxicity assessment using MTT assay

Cells with 70-80% confluence were detached from the culture flask using 0.05% trypsin- EDTA, and plated into 96-well plates at a density of 2.0×10^4 $cells.well^{-1}$ (100 $\mu l.well^{-1}$). After 24 hours, cells were either treated for another 24 hours with the leaf extracts, a solution of 50% ethanol/distilled water, or not treated with the extract. Cell viability was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (Sigma Aldrich Israel Ltd) at a concentration of 0.5 $mg.ml^{-1}$ and incubated at 37°C for 4 hours. MTT solution was removed, and isopropanol (0.1 N HCl) was added for Formazan solubilization for 15 minutes in darkness. Sample absorption was determined using an ELISA plate reader (Model 680, microplate reader, Bio-Rad) at 570 nm. For each extract the method was repeated three times and averaged to produce graphs of OD% vs conc. of extract ($\mu g.ml^{-1}$). IC50 (50% inhibitory concentration) values were extrapolated from the resulting graphs.[36]

2.3. Total antioxidants capacity

Half a gram from each leaf powder was extracted with 5 ml of acetone/water/acetic acid (70: 29.5: 0.5, v/v/v) solution. Mixtures shaken at 300 rpm at room temperature for 3 hours and left overnight in darkness, supernatants were removed. A second extraction, using an additional 5 ml of solvent for each precipitated sample, extracts were combined and stored at 4°C until analysis within 2 days. The total antioxidant activity was determined by Follin-Ciocalteu assay, as reported by Xu and Chang[37], using gallic acid as standard. Samples of the extracts, 50 μl, was added to 3 ml of distilled water, 250 μl of Follin-Ciocalteu reagent and 750 μl of 7% of NaCO3 were vortexed and left for 8 minutes at room temperature. Distilled water, 950 μl, was added to the mixture and left to stand for 2 hours at room temperature. Absorbance was measured at 765 nm against distilled water. Antioxidant activity was measured as gallic acid equivalents (GAE $\mu g.g^{-1}$ sample) by referring to a calibration curve of gallic acid. Linearity range of the calibration curve was 50 to 1000 $\mu g.ml^{-1}$ ($r = 0.9967$). For each extract the method was repeated three times.

2.4. Antimicrobial tests

The bacterial strains used were *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus (*ATCC 6538), *Listeria monocytogenes* (ATCC 19115), *Salmonella typhimurium* (ATCC 14028), and *Escherichia coli* (0157) (ATTC 700728). Bacterial strains were stored at −80°C and revived on Mueller Hinton agar. Fresh inoculants were used for testing the antimicrobial activity of the grape leaf extracts through the agar well diffusion assay.[38] The bacterial strains were cultured on petridishes. In each hole 50 μl leaf

extract (concentration 1.0 mg.ml^{-1}) was applied, and plates were kept in an incubator (37(°C) overnight. The inhibition zone radius was recorded after 24 hours and compared against the negative control, 3:1 acetone/water (v:v) solution and the positive controls, ampicillin and gentamicin antibiotics, diluted to 10 μg.ml^{-1}. For each extract the method was repeated three times.

2.5. Statistical analysis

The CoStat statistical package (CoHort Software, Monterey, USA) was used for analysis of variance (ANOVA), and the comparison of the means was conducted using the Student-Newman-Keuls test at $P \leq 0.05$. Moreover, standard error (SE) values were calculated and included ($n = 3$).

3. RESULTS AND DISCUSSION

3.1. Anticancer activity of grape leaf extracts

The effects of grape leaf extracts on the lung cancerous cell line (A549 cells) were dose dependent for all leaf extracts. In order to deduce the extract effectiveness results were compared to normal muscle cells (L6 cells). Hence, it was possible to monitor the cytotoxicity effect of the grape leaf extracts' concentration on the proliferation of cell lines. The optical density (OD%) was plotted against the concentration of the leaf extract (Fig. 1). Moreover, the concentration of each leaf extract that reduced the percentage of cell proliferation into half (IC50) was determined graphically and shown in Table 1.

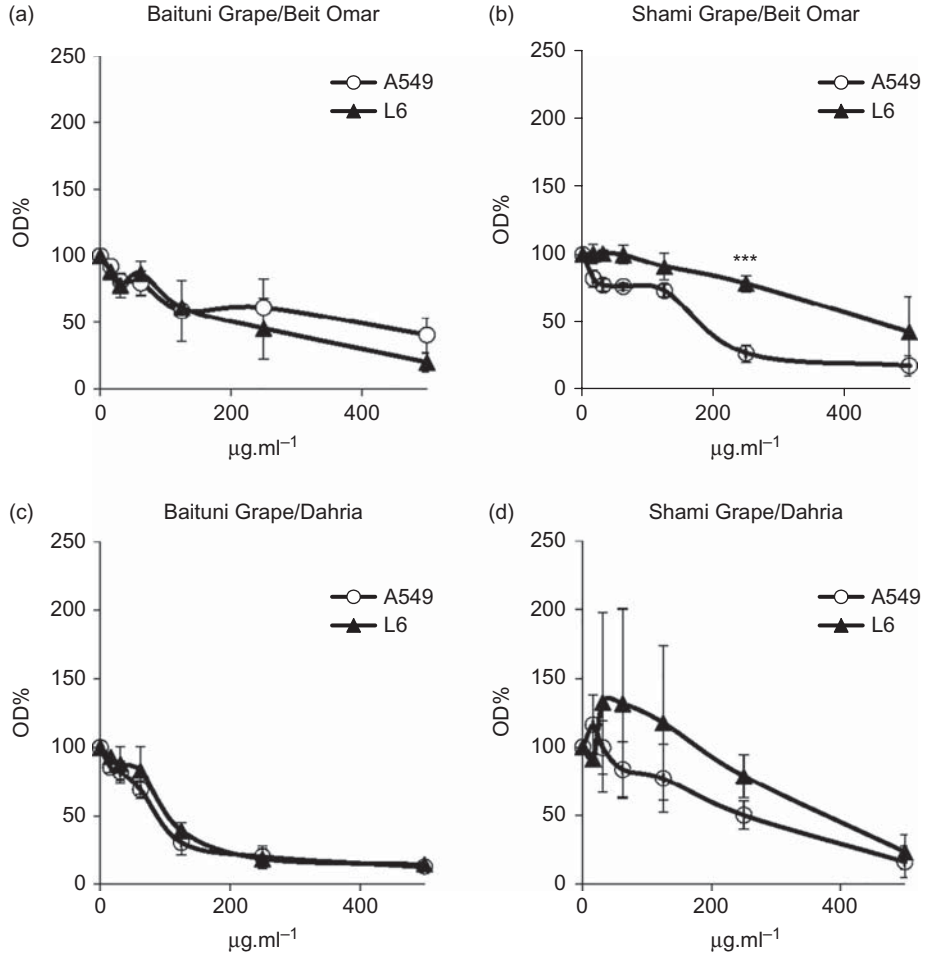

Figure 1. Effect of leaf extracts (μg.ml^{-1}) from grape varieties, collected from two locations in Palestine, on the proliferation percentage of A549 cells (lung cancerous cells) in comparison with L6 cells (muscle cells). OD = Optical density. ***$p < 0.001$.

As seen in Fig. 1a, an extract of Baituni leaves taken from Beit Omar gave no significant difference in proliferation between A549 cells and L6 cells. The extract exhibited cytotoxic activity on the human cell lines with IC50 of 90 μg.ml^{-1} and 130 μg.ml^{-1}, respectively (Table 1). Moreover, Baituni leaves taken

Table 1. The cytotoxicity (IC50; $\mu g.ml^{-1}$) of leaf extracts from grape varieties, collected from two locations in Palestine, on the proliferation of A549 cells (lung cancerous cells) in comparison to L6 cells (muscle cells).

		Cell lines	
Genotype	Region	A549	L6
Baituni	Beit Omar	90	130
Baituni	Dahria	85	100
Shami	Beit Omar	140	440
Shami	Dahria	165	320

from Dahria exhibited similar results, and no significant differences were detected between cancer and normal cells (Fig. 1c); the IC50 values for A549 cells and L6 cells were 85 $\mu g.ml^{-1}$ and 100 $\mu g.ml^{-1}$, respectively. These cytotoxic activites were similar to those of the Baituni leaves from Beit Omar. Alternatively, leaves of Shami grapes from Dahria (arid region), as seen in Fig. 1d, gave a high standard error. Therefore, preliminary results indicate no significant differences were detected between A549 cells and L6 cells however, further analysis is required to provide conclusive results of the effect of these grape leaves on the tested cell lines. Conversely, Shami grape leave extract, from Beit Omar (temperate region), was effective in reducing the proliferation of A549 cells when compared to L6 cells, and consequently exhibited a highly significant effect ($P < 0.001$) (Fig. 1b). This is supported by the high difference in IC50 values upon comparing the two cell lines; the IC50 values were 140 $\mu g.ml^{-1}$ for A549 cells and 440 $\mu g.ml^{-1}$ for L6 cells. This indicates the cytotoxic activity of the Shami Beit Omar leaves is selective towards A549 cells.

The efficacy of grape products against various cancer types, particularly lung cancer[35], is well known. Most studies addressed grape seed extract (GSE) and berries, whereas studies that addressed grape leaves are lacking. However it is possible to correlate this study with others based on the predominate phytochemicals found in grape leaves. For example, in a running study results clearly show that Shami and Beituni leaves contain flavonols, in particular myricetin, quercetin, kaempferol and isorhamnetin derivatives.[39] Other studies have clearly demonstrated the effect of such metabolites in various fields. Flavanoids were found to reduce cell proliferation in HT-29 cells against murine colonocytes based on the nonhydroxylated core structure of the metabolites that act as a selective inhibitor of proliferation.[40] It also showed that particular effect of both myricetin and quercetin in comparison to several phenolic compounds, on B16F10 cells (melanoma) in a 72 hour assay against non-transformed melanocytes. One of several explanations given for the activity of polyhydroxylated flavanoids is the unsaturated C2 and C3 bond[41]. It was suggested by Agullo et. al[42] that the presence of adjacent hydroxyl groups results in elevated antiproliferative power, this power seems to increase as the number of hydroxyl groups rose to three, as seen in myricetin. In conclusion, the presence of these metabolites explains the antiproliferative activity of the genotypes studied. Moreover, it has been found that water deficiencies affect phytochemical concentrations in grapes,[43] this explains the preliminary differences between the Shami leaves collected from different locations.[39]

3.2. Antioxidants activity

Grape leaves exhibited relatively high levels of natural antioxidants that are free radical scavengers. As depicted in Fig. 2, the antioxidant capacities in grape leaf extracts ranged from 818 to 1023 μg of gallic acid equivalents (GAE).g^{-1}; gallic acid is a powerful antioxidant against cancer.[44] However, there were no significant differences amongst grape genotypes or between locations (Fig. 2). It is worth noting that water deficit stress is known to reduce crop yield, but clearly enhances flavor and quality characteristics of berries.[45] Consequently, antioxidant variation amongst genotypes and locations is thought to be evident, though results from this study show no significant differences. This can be explained by the affect attributed to the phytochemical variation between genotypes and locations.[43] In this sense, differences between grape genotype appear to be qualitative rather than quantitative. In a study from Morocco, which has similar environmental conditions to Palestine, such a trend was evident.[39] Several studies confirm the antioxidant activity of phytochemicals. For instance, polyhydroxylated flavonoids that bear 4-6 OH groups act as strong antioxidants in an aqueous milieu, opposing those with more or fewer OH groups, that show low or no antioxidant activity.[46] Directly concerning the predominant composition of Shami and Baituni leaves, the antioxidant effect of flavonols is due to OH groups in the

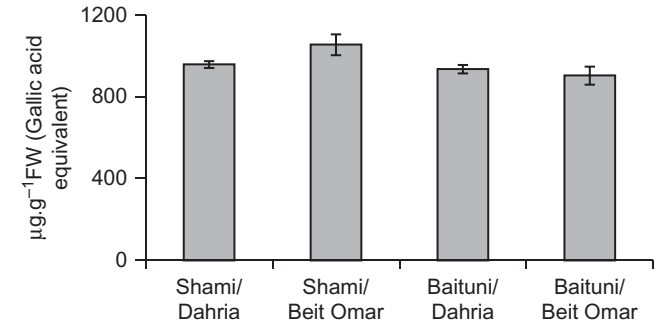

Figure 2. The antioxidant capacity of grape leaf extracts taken from different geographic locations relative to gallic acid equivalents (GAE; µg.g^{-1} FW).

ortho-position at ring B, besides the presence of an unsaturated bond between C2 and C3, together with the carbonyl function in ring C. This structure, as mentioned previously, also contributes to the antiproliferative affect of these metabolites.[47]

3.3. Antimicrobial activity

Five pathogenic strains were selected to test the antimicrobial effect of the grape leaf extracts. Our findings showed that leaf extracts were effective against *Staphylococcus aureus* and slightly effective against *Pseudomonas aeruginosa* bacteria. However they were ineffective against *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli (0157)* bacteria. As seen in Fig. 3, the extracts exhibited an effect on *Pseudomonas aeruginosa* growth relative to the negative control. There is a high significant difference between the extracts' effects ($P < 0.01$), with Baituni leaves from Dahria the most effective. In addition, an effect was observed in *Staphylococcus aureus* growth (Fig. 4), and significant differences ($P < 0.05$) between various leaf extracts were recorded, with the highest effect for Baituni Dahria leaf extract.

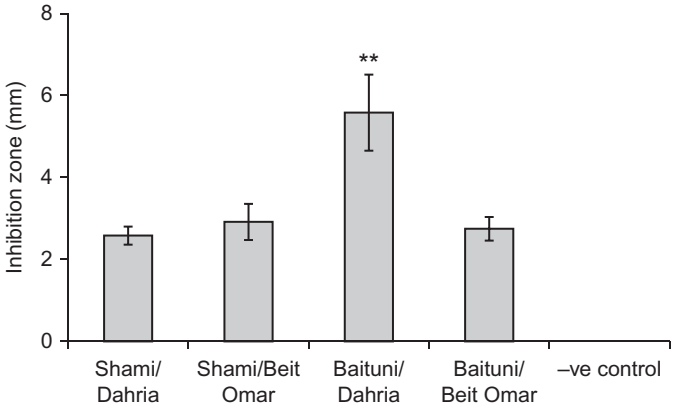

Figure 3. Effect of grape leaf extracts on the growth of *Pseudomonas aeruginosa*. **p < 0.01.

These results prove the antibacterial activity of grape leaf extract, specifically Baituni Dahria leaves against certain human pathogens. It is worth noting the grape variety with the highest anticancer activity gave opposing results regarding antimicrobial assessment. This is based on the possible differential phytochemical composition of the two genotypes.[39,48] This suggests the impact of water stress on grapes cultivated in Dahria region might have induced the biosynthesis of certain phytochemicals that are powerful against microbes but not against cancer cells. A number of studies have investigated the action of phytochemicals, specifically flavonoids, against bacterial strains. Papadopoulou et al.[49] stated the effect of total phenolic content on the antimicrobial activity of wine extracts, mostly effective against *Staphylococcus aureus* but less effective against *Escherichia coli*. Moreover. It explained that flavonoid activity is likely due to their ability to form complexes with extracellular and soluble proteins and with bacterial cell walls, eventually leading to their destruction.[50] The effect of phytochemicals acts in a dose dependent manner, for example kaempferol, quercetin, and

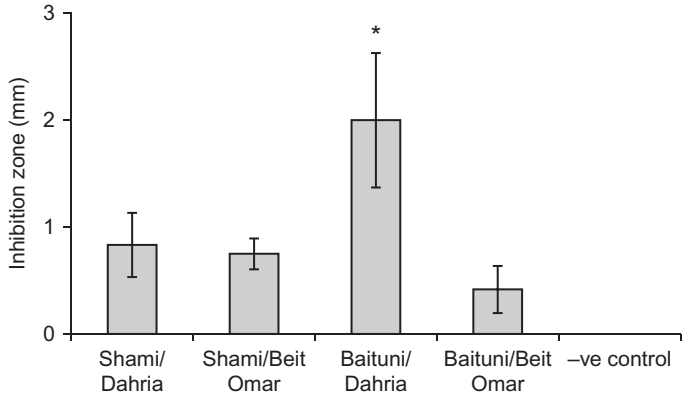

Figure 4. Effect of grape leaf extracts on the growth of *Staphylococcus aureus*. $*p < 0.05$.

rutin had no effect on bacterial growth in concentrations under 5 $mg.ml^{-1}$, exhibiting the importance of concentration for such flavonoids.[51] Puupponen-Pimiä et al.[52] reported gram-positive and gram-negative bacteria exhibit different sensitivities towards specific phenolics. *Pseudomonas aeruginosa and Staphylococcus aureus* were inhibited by catechin in a dose dependent manner. However unlike *Pseudomonas aeruginosa, Staphylococcus aureus* was more susceptible to quercetin at lower concentrations. Therefore, the phytochemical composition of Shami and Baituni leaves and the overall levels of certain antimicrobial chemicals define the antimicrobial activity of these leaves.

4. CONCLUSION

By studying the anticancer, antioxidants and antimicrobial potential of grape leaves, it is evident that the natural phytochemical composition of grape leaves is beneficial to human health. Results obtained in this study indicate that leaves of Shami grapes collected from Beit Omar region are significantly effective against A549 lung cancerous cells. Leaves of the Baituni grape collected from Dahria were effective against bacterial human pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Concerning grape leaf antioxidants, grape leaves exhibited high antioxidant capacities, ranging from 818 to 1023 μg of gallic acid equivalents (GAE).g^{-1}. This study opens the field for further research and analyses the active compounds in the assessed activities and their route of action regarding cytotoxicity and antimicrobial activity.

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Competing interests

The authors declare no competing interests.

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