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The Effectiveness of the Functional Components of Grape (*Vitis vinifera*) Pomace as Antioxidant, Antimicrobial, and Antiviral Agents

Alaa A. Gaafar[1*], Mohsen S. Asker[2], Ali M.A.[3] and Zeinab A. Salama[1]

[1] *Plant Biochemistry Department, ID: 60014618;* [2]*Microbial Biotechnology Department, ID: 60014618;* [3]*Center of Scientific Excellence for Influenza Viruses, National Research Centre, ID: 60014618, Dokki, Giza, P.O. 12622 Egypt*

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Abstract

This research describes the effectiveness of the bioactive compounds of two varieties of grape pomace (Romy and Banaty) extracts as antioxidant, antimicrobial, and antiviral agents. The antioxidant activity was assessed using $DPPH^{\bullet}$, $ABTS^{\bullet+}$ assays reducing power and iron-chelating methods. The results show that the total flavonoid (TF), total phenolic (TP), and total tannin (TT) of the red grape pomace (Romy) were high (17.38 mg/g DW, 16.39 mg/g DW and 5.79 mg/g DW) respectively, while for the white grape pomace (Banaty), these were (11.09 mg/g DW, 6.61 mg/g DW and 2.45 mg/g DW respectively. When using HPLC for the phenolic profile detection, the red grape 80 % acetone extract exhibited a high content of phenolic compounds, above all benzoic and pyrogallol compounds (122.54 and 44.11 mg/100g, respectively). While the chlorogenic compounds were mostly found in the red pomace aqueous extract (34.20 mg/100g). The highest antioxidant and antimicrobial activities were spotted in the red pomace extract. The red grape pomace has an antiviral activity slightly higher than that of the white grape pomace and the aqueous extract at 400µg was much better than the ethanol and acetone extracts. This study highlights the possibility of using grape pomace to develop new potential sources in the pharmaceutical industry as an antioxidant, antimicrobial, and antiviral materials.

Keywords: Antimicrobial, Antioxidant, Antiviral activities, Grape pomace, Phenolics.

1. Introduction

Grapes are among the most considerable and widely consumed fruits around the world. The prominence of grapes and their products is on the rise because of the many health benefits of this fruit to humans. Different grape extracts have been considered to be industrial derivatives of whole grapes, which contain high concentrations of phenols, flavonoids, and linoleic acid (Rathi and Rajput, 2014). As a byproduct, the grape pomace results during juice and wine industries. It consists of several parts: 1) the skin and pulp (10-12 %), 2) the seeds (2-6 %), and 3) part of the stalks (2.5-7.5 %) (Yu and Ahmenda, 2013). Grape pomaces are distinguished by their high contents of polyphenols and health phytochemical compounds due to the insufficient extraction through the process of grape processing. These phenols are known to be effective secondary plant-metabolite compounds that have beneficial properties for human health due to their antioxidant, antimicrobial, antiviral, anticancer and anti-inflammatory activities. Also, the agro-industrial residue of the grape pomace is rich in effective compounds that can be used in the pharmaceutical, cosmetic and food additives (Fontana *et al*., 2013). Strong correlations were confirmed between the presence of bioactive phenolic compounds and previous biological activities (Peixoto *et al*., 2018). In this context, the aim of the present study is to determine the active phenolic ingredients of the grape pomace and their effectiveness as antioxidant, antimicrobial and antiviral agents.

2. Material and Methods

2.1. Chemicals

$ABTS^{\bullet+}$ (2, 2'-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid)), Folin–Ciocalteau reagents, Gallic acid, Quercetin, $DPPH^{\bullet}$ (2, 2-diphenyl-1-picrylhydrazyl), Ferrozine: (3-(2- pyridyl)- 5, 6-bis- (4-phenylsulfonic acid) -1,2,4-triazine, BHT: Butyl Hydroxytoluene and, potassium ferricyanide, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of Samples

Two varieties of grapes (*Vitis vinifera*) (Romy and Banaty) were obtained from the local market in Giza-Cairo. The grapes were pressed, and the pressed residues were considered the grape pomace. Furthermore, each variety of pomace was dried at 40 °C in an air-circulating

* Corresponding author. e-mail: dr.gaafar2308@hotmail.com.

oven for constant weight. The dried pomace was ground in a knife mill, and vacuum-packed and stored at – 4 °C until analysis.

2.3. Preparation of Red and White Grapes Pomace Extracts

The dried powder (100 g) of the grape pomace was dispensed separately in 1 L of three different solvents as distilled water, 80 % ethanol and 80 % acetone, overnight at room temperature using a shaker. Each mixture was filtered through Whatman No. 1 filter paper, and the extraction step was repeated three times. The ethanol and acetone filtrates were concentrated at 40 °C in a rotary evaporator under vacuum, and the water was completely dried by lyophilization. The dried crude extracts were stored in a refrigerator until analysis.

2.4. Chemical Studies

2.4.1. Total Phenolic

The total phenolic (TP) was determined by Folin Ciocalteu reagent assay using Gallic acid as standard according to Singleton and Rossi (1965). A suitable aliquot (1 mL) of both local grape varieties was added into a 25 mL volumetric flask, containing 9 mL of distilled water. One milliliter of Folin Ciocalteu`s phenol reagent was added to the mixture and shaken. After five minutes. 10 mL of the 7 % Na_2CO_3 solution was added to the mixture. The solution was diluted to 25 mL with distilled water and mixed. After incubation for ninety minutes at room temperature, the absorbance was determined at 750 nm with a spectrophotometer (Unicum UV 300) against the prepared reagent as blank. The total phenolic content in the samples was expressed as mg Gallic acid equivalents (GAE)/g dry weight. All samples were analyzed in triplicates.

2.4.2. Total Flavonoid

The total flavonoid (TF) was determined by the aluminum chloride method using quercetin as a standard (Zhishen *et al*., 1999). One mL of both of the local grape pomace extracts was added into a 10 mL volumetric flask, containing 4 mL of distilled water. To the flask, 0.3 mL of $NaNO_2$ (5 %) was added and after five minutes, 0.3 mL of $AlCl_3$ (10 %) was added. After the sixth minute, 2 mL of 1M NaOH was added, and the total volume was made up to 10 mL with distilled water. The solutions were mixed well and the absorbance was measured against a prepared reagent blank at 510 nm using a spectrophotometer (Unicum UV 300). The total flavonoids in the samples were expressed as mg quercetin equivalents (QE)/ g dry weight. The samples were analyzed in triplicates.

2.4.3. Total Tannins

The total tannin (TT) was measured using the Folin-Ciocalteu reagent according to Polshettiwar *et al*. (2007). One mL of both of the local grape pomace extracts was added to 7.5 mL of distilled water (dH_2O) and then 0.5 mL of Folin reagent and 1 ml of sodium carbonate solution (35 %) were added. The volume was made up for 10 mL with distilled water, and absorbance was measured against prepared reagent blank at 775 nm using a spectrophotometer (Unicom UV 300). The total tannins in the samples were expressed as mg tannic acid equivalent (TE)/g dry weight. All of the samples were analyzed in triplicates.

2.4.4. Identification and Quantitation of Phenolic Compounds by HPLC

The phenolic compounds in the grape pomace (aqueous, 80 % ethanol and 80 % acetone) extracts were identified using HPLC according to Ben-Hammouda *et al.* (1995). All chemicals and solvents used were of an HPLC spectral grade, and were obtained from Sigma (St. Louis, USA (and Merck –Shcuchrdt Munich, Germany).

The HPLC system is Agilent 1100 series coupled with DAD detector (G1315B) and (G1322A) DEGASSER. Sample injections of 5 µL were made from an Agilent 1100 series auto-sampler. The chromatographic separations were performed on ZORBAX-EclipseXDB-C18 column (4.6×250 mm, particle size 5 µm). A constant flow rate of 1 mL/min was used with mobile phases: (A) 0.5 % acetic acid in distilled water at pH 2.65; and solvent (B) 0.5 % acetic acid in 99.5 % acetonitrile. The elution gradient was linear starting with A and ending with B over fifty minutes, using a DAD detector set at a wavelength of 280 nm. The phenolic compounds of the grape pomace extracts were identified by comparing their retention times with those of the standard mixture chromatogram. The concentration of an individual compound was calculated on the basis of peak area measurements, and the results were expressed as mg phenolic/100 g dry weight.

2.5. Antioxidant Activity

2.5.1. DPPH· Free Radical Scavenging Activity

The determination of $DPPH^{\cdot}$ (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity was measured spectrophotometrically according to Chu *et al.* (2000). 0.1 mM of $DPPH^{\bullet}$ in methyl alcohol was prepared and 0.5 mL of this solution was added to 1 ml of three grape pomace extracts at different concentrations (25, 50, 75, 100 µg/mL). Methanol was used as a blank. The mixture was shaken vigorously, and allowed to stand at room temperature (for thirty minutes.). Butyl Hydroxytoluene (BHT, Sigma) was used as positive control; and negative control contained the entire reaction reagent except for the extracts. Then the absorbance was measured at 515 nm against a blank.

The capacity to scavenge the $DPPH^{\cdot}$ radical was calculated using the following equation:

$$DPPH^{\cdot} \text{ scavenging activity } \% = [(A_c - A_s) / A_c] \times 100$$

Where (A_c) is the absorbance of the negative control reaction, and (A_s) is the absorbance in the presence of the plant extracts. The results were expressed as IC_{50} (the concentration (µg/mL) of the grape pomace extracts that scavenge 50 % of $DPPH^{\cdot}$ radical).

2.5.2. ABTS·+ Scavenging Activity

The $ABTS^{\bullet+}$ assay was generated by the oxidation of $ABTS^{\bullet+}$ with potassium persulphate. Arnao *et al*., (2001). $ABTS^{\bullet+}$ was dissolved in deionized water to a 7.4 mM concentration, and potassium persulphate was added to a concentration of 2.6 mM. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for twelve to sixteen hours at room temperature in the dark. The solution was then diluted by mixing the 1mL $ABTS^{\bullet+}$ solution with 60 mL of methanol to obtain an absorbance of 1.1 ± 0.02 at 734 nm using a spectrophotometer. A fresh $ABTS^{\bullet+}$ solution was prepared for each assay. The grape pomace

extracts (150 µL) at different concentrations (25, 50, 75, 100 µg/mL) were allowed to react with 2850 µL of the $ABTS^{\bullet+}$ solution for two hours in the dark. Then the absorbance was taken at 734 nm using the spectrophotometer. Results were expressed in comparison with the standard Trolox. The activity to scavenge the $ABTS^{\bullet+}$ radicals was calculated using the following equation:

$ABTS^{\bullet+}$ scavenging activity % = $[(A_0 - A_1) / A_0] \times 100$

Where A_0 is the $ABTS^{\bullet+}$ absorbance of the control reaction, and A_1 is the $ABTS^{\bullet+}$ absorbance in the presence of the sample. The results were expressed as IC_{50} (the concentration µg/ml of the grape pomace extracts that scavenge 50 % of $ABTS^{\bullet+}$ radical)

2.5.3. Reducing Power

The reducing power was assayed spectrophotometrically (Kuda *et al*., 2005). One ml of the grape pomace extracts at different concentrations (25, 50, 75, 100 µg/mL) were mixed with 2.5 ml of phosphate buffer (50 mM, pH 7.0) and 2.5 mL of potassium ferricyanide (1 %). The mixture was then incubated at 50 °C for twenty minutes. After the addition of 2.5 mL of trichloroacetic acid (10 %) to the mixture, centrifugation at 3000 rpm for ten minutes was performed. Finally, 1.25 mL from the supernatant was mixed with 1.25 mL of distilled water and 0.25 mL of a $FeCl_3$ solution (0.1%, w/v). The absorbance was measured at 700 nm. BHT was used as a standard. The results were expressed as EC_{50} (the concentration µg/mL of the grape pomace extracts that provided the reading of 0.5 absorbance's at 700 nm).

2.5.4. Ferrous Chelating Activity

The ferrous ion chelating activities were assessed colorimetrically (Hsu *et al*., 2003). One ml of the grape pomace extracts or EDTA solution (as a positive control at different concentrations (25, 50, 75, 100 µg/mL) was spiked with 0.1 mL of 2 mM $FeCl_2$- $4H_2O$. 0.2 mL of a 5 mM ferrozine solution and 3.7 mL of methanol were mixed in a test tube and reacted for ten minutes at room temperature. The absorbance was then measured at 562 nm. The mixture without the extract was used as the control. A lower absorbance indicates a higher ferrous ion chelating capacity.

The percentage of the ferrous ion chelating ability was calculated using the following equation:

Chelating activity (Inhibition %) = $[(A_c - A_s) / A_c] \times 100$

Where (A_c) is the absorbance of the control reaction, and (A_s) is the absorbance in the presence of the plant extracts. The results were expressed as IC_{50} (the concentration (µg/mL) of the grape pomace extracts that chelate 50 % of Fe^{2+} ions).

2.6. Antimicrobial Activity

2.6.1. Microbial Strains

Bacillus subtilis NRRL B-94, Escherichia coli NRRL B-3703, Pseudomonas aeruginosa NRRL, Staphylococcus aureus NRRL, Aspergillus niger NRRL313, Aspergillus flavus NRC, Saccharomyces cerevisiae NRC and Candida albicans NRRL477. The bacterial strains were cultured on a nutrient medium, while the fungi and yeast strains were cultured on a malt medium and a yeast medium, respectively.

2.6.2. Antimicrobial Activity of Grape Pomace Extracts

The disk diffusion method was used to evaluate the antimicrobial activity of each of the grape pomace extracts. The grape pomace extract residues (200, 400 and 600 µg/mL) were re-dissolved in 1 ml of a corresponding solvent (water, ethanol, and acetone) sterilized through Millipore filter (0.22 mm). Ten mL of an agar medium (nutrient, malt, and yeast) was poured into sterile Petri dishes followed with 15 mL of a seeded medium previously inoculated with the bacterial suspension to attain a 10^5 CFU/mL of medium. These microorganisms were cultured and incubated at 37°C for twenty-four hours. The inoculums' suspension was spread uniformly over the agar plates using a spreader, for a uniform distribution of bacteria. Subsequently using a sterile borer, a well of 0.6 cm diameter was made in the inoculated media then 100 µL of each extract was added, and the control Ciprofloxacin (10 µg) was filled into the well. The solvent was used as negative control. The plates were kept in the fridge at 4°C for two hours to permit the grape pomace extract diffusion. They were incubated at 35°C for twenty-four hours. The presence of inhibition zones was measured and considered an indication of antibacterial activity (Scott, 1989).

2.6.3. Determination of Minimum Inhibitory Concentrations (MIC) of the Effective Extract

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after twenty-four hours of incubation on the agar plates (NCCLS, 1990). The most effective grape pomace extracts which exhibited a strong antibacterial activity at 10 mg/mL were manipulated to determine their MIC using the disk diffusion method. Different concentrations of the effective grape pomace extracts (100-600 µg/mL) were prepared separately, sterilized through Millipore filter and their requisite amount was loaded over sterilized filter paper discs (8 mm in diameter). Agar was poured unto the sterile Petri dishes seeded with the microbial suspensions of the pathogenic strains. The loaded filter paper discs with different concentrations of the effective grape pomace extracts were placed on the top of the agar plates. The plates were kept in the fridge at 4°C for two hours, and were then incubated at 35°C for twenty-four to forty-eight hours

2.6.4. Mode of Action

The effects of different concentrations of the ethanolic extract on some biochemical activities were studied. Immediately after incubating the flasks with *B. subtilis* (for twenty-four hours), the cells were harvested during the middle logarithmic growth phase, and an aqueous extract was applied in concentrations of 1/4 and 1/2 MIC. Each test was repeated three times. Subsequently, the flasks were shaken using a rotary shaker of 150 rpm at 30°C. The samples were withdrawn at the onset of the experiment and after incubation periods of 24, 48, 72, 96, 120 and 144 minutes. The bacterial cells were subjected to the following determinations: total acid-soluble phosphorus compounds (Hogeboom and Schneider, 1950; Chen *et al*., 1956), total lipids (Bligh and Dyer, 1959; Knight *et al*., 1972), total soluble protein (Daughaday *et al*., 1952; Bradford, 1976), and total nucleic acids (Burton, 1957).

2.7. Antiviral Bioassay:

2.7.1. MTT Cytotoxicity Assay (TC50)

The extracts were diluted with Dulbecco's Modified Eagle's Medium (DMEM). The stock solutions of the test extracts were prepared in 10 % DMSO in ddH_2O. The cytotoxic activity of the extracts was tested in Madin-Darby Canine kidney (MDCK) cells by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method with minor modification (Mossman, 1983). Briefly, the cells were seeded in 96 well plates (100 µL/well at a density of 3×10^5 cells/mL) and were incubated for twenty-four hours at 37 °C in 5 % CO_2. After twenty-four hours, the cells were treated with various concentrations of the tested extracts in triplicates. After further twenty-four hours, the supernatant was discarded, and the cell monolayers were washed with a sterile phosphate buffer saline (PBS) three times. The MTT solution (20 µL of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for four hours followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µL of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCl in 50 mL isopropanol). The absorbance of formazan solutions was measured at λmax 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation: $\%\ \text{Cytotoxicity} = [(A_0 - A_t) / A_0] \times 100$
Where A_0 is the absorbance of the cell without treatment, and A_t is the absorbance of the cell with treatment

The plot of % cytotoxicity versus the sample concentration was used to calculate the concentration which exhibited 50 % cytotoxicity (TC_{50})

2.7.2. Plaque Reduction Assay

The assay was carried out according to the method of Hayden *et al*., (1980) in a six-well plate where MDCK cells (10^5 cells/mL) were cultivated for twenty-four hours at 37°C. A/Chicken/ Egypt/M7217B/1/ (H5N1) virus was diluted to give a 104 PFU/ well, and was mixed with the safe concentration of the tested extracts and incubated for one hour at 37 °C before being added to the cells. The growth medium was removed from the cell culture plates and the cells were inoculated with (100 µL/well) virus. After one hour contact time for the virus adsorption, 3 mL of DMEM supplemented with 2 % agarose, and the tested compounds were added onto the cell monolayer. The plates were left to solidify and were incubated at 37 °C till the formation of viral plaques (3 to 4 days). Formalin (10 %) was added for two hours, then the plates were stained with 0.1 % crystal violet in distilled water. Control wells were included where the untreated virus was incubated with MDCK cells, and finally, the plaques were counted, and the percentage of reduction in the plaques' formation in comparison to the control wells was recorded as following
Inhibition (%) = viral count (untreated) -viral count (treated) / viral count (untreated) x100.

2.8. Statistical Analysis

Data were statistically analyzed using Costat statistical package (Anonymous, 1989).

3. Results and Discussion

3.1. Phenolics, Flavonoids, and Tannins Compounds

Table 1 shows that the red grape acetone (80 %) pomace extract had the highest level of TP, TF, and TT respectively. The lowest values of the same parameters were spotted with the ethanol (80 %) extract. The total phenolic content exhibited much higher TP (17.38 ± 0.19 mg/g) in the red grape acetone (80 %) pomace extract (0.81, 1.79, and 2.91 mg/g) than in the sesame cake, sugar beet pulp, and potato peels correspondingly (Mohdaly *et al*., 2010), and (5.60 and 4.90 mg/g) in blueberries and blackberries (Yu *et al*., 2005). It was extremely lower than that (62.00 mg /g) in tea leaves (Lee *et al*., 2005), (32.10-52.70 mg/g) and (15.00-20.30 mg/g) in grape seeds and grape skins (Yilmaz, 2002). There was a considerable distinction between the flavonoids content of grape pomace extracts for each variety. The very best level has been revealed in the acetone extract (16.39± 0.15 mg/g) followed by the aqueous and ethanolic extracts respectively. The white grape pomace aqueous extract manifested the very best amount (9.69± 0.12 mg/g) of flavonoids followed by the acetone and ethanol extracts. The Tannins content exhibited a similar tendency as TP and was the very best for the red and white grape pomace acetone extracts followed by the aqueous extracts, while the ethanolic extracts showed the lowest values for each variety (Table 1).

Table. 1. Bioactive compounds of grape pomace extracts

Extracts	TP (mg GAE/ g)		TF (mg QE/g)		TT (mg TE/g)	
	Red grape (Romy)	White grape (Banaty)	Red grape (Romy)	White grape (Banaty)	Red grape (Romy)	White grape (Banaty)
Aqueous	11.69 ^{b}± 0.12	7.02 ^{b}± 0.09	10.63 ^{b} ± 0.14	9.69 ^{c}± 0.12	4.38 ^{b} ± 0.04	3.22 ^{c} ± 0.04
Ethanol 80%	10.79 ^{a}± 0.23	5.81 ^{a}± 0.09	10.15 ^{a}± 0.14	5.89 ^{a} ± 0.20	3.32^{a}± 0.04	1.60 ^{a}± 0.03
Acetone 80%	17.38 ^{c}± 0.19	11.09 ^{c}± 0.19	16.39 ^{c}± 0.15	6.61 ^{b} ± 0.11	5.79 ^{c}± 0.03	2.45 ^{b} ± 0.02
LSD at 0.05	0.51	0.29	0.16	0.36	0.06	0.08

All values are the mean of three replicates ± S.D. Values with different letters are significantly different at $p \leq 0.05$.

A substantial diversity between the solvents at ($p \leq 0.05$) was discovered for the TP, TF, and TT contents. The separation and extraction of phenolic compounds from plant samples are influenced by the composition and sort of phenolic compounds as well as the polarity of the solvents used (Zhao *et al*., 2006). Condensed tannins (CT), which are also called proanthocyanidins, give too harsh tastes for grapes and wines (Alipour and Rouzbehan 2010; Fontoin *et al*., 2008). Prior *et al*. (2001) reported that about 32 to 54 % of the antioxidant capacity in blueberries, grapes, and cranberries can be accounted for by condensed tannins.

3.2. High- Performance Liquid Chromatograph (HPLC)

The HPLC could be the favored technique of separation and quantification of phenolic compounds (Lee *et al*., 2003). Considerable factors have an effect on the HPLC assessment of phenolics, which includes sample purification, column kinds, mobile phase, varieties and detectors (Katsube *et al*., 2004). Purified phenolics are utilized to an HPLC device making use of a reversed-phase C18 column (RP-C18), photograph diode array detector (PDA) (Lapornik *et al*., 2005). The phenolic compounds analyzed by HPLC are given in Table 2.

The results manifest that all extracts contained different components and contents of phenolic compounds.

Table 2. Identification of individual phenolics in different extracts of grape pomace

Phenolic compounds (mg/100g DW)	Red grape (Romy)			White grape (Banaty)		
	Aqueous	Ethanol 80%	Acetone 80%	Aqueous	Ethanol 80%	Acetone 80%
Ellagic	13.73	11.89	23.02	14.94	9.94	14.99
Tannic acid	2.04	7.16	17.52	2.59	3.42	6.34
Chlorogenic	34.32	19.82	-	5.90	16.54	-
Pyrogallol	44.04	38.68	44.11	36.77	7.62	9.23
Vanillic	-	-	4.87	-	-	4.22
quercetin	0.96	1.02	1.49	-	-	-
Benzoic	129.26	119.55	122.54	128.72	107.24	135.62
Rutin	-	-	-	0.08	0.07	0.08
Acacetin	0.20	0.18	0.27	0.20	0.19	0.10

It is clear from Table 2 that the red and white grape pomace contains a nearly similar phenolic profile. There is a component that is found in both varieties, but they differ in concentrations. The red pomace aqueous extract shows the highest content of phenolic compounds above all benzoic, pyrogallol, chlorogenic, and ellagic compounds followed by the acetone and ethanol extracts respectively. While the ethanol extract exhibited the lowest values of phenolic compounds. Benzoic, ellagic and pyrogallol compounds were found in the aqueous and acetone extract with high values in white grape pomace, respectively. Yilmaz and Toledo (2004) found that methanol was more effective than the aqueous extract in terms of the extraction of phenolics from Muscadine seeds. Rodtjer *et al*. (2006) maintain that the yields of phenolics depend on the solvent polarity and the variety and conditions of extraction.

3.3. Activity of Antioxidant

3.3.1. DPPH• Radical Scavenging Activity

The antiradical capability is resolved as the number of extracts requisite to depress the radical concentration by 50% (Ruberto *et al*., 2007; Wang *et al*., 2010). With respect to the DPPH• assay, the antioxidant capacity (IC_{50}) of the red grape pomace had the highest DPPH• radical scavenging activity compared to the white grape pomace. The red grape aqueous extract was found to be statistically superior to the white aqueous pomace extract when all results are compared (Table 3). These outcomes were higher than that formerly reported and conveyed by Anastasiadi *et al*. (2010) in the skin of the grape varieties of *Mandilaria*, *Voidomatis*, *Assyrtiko*, and *Aidani* with IC_{50} values 55.7, 177.5, 117.0 and 274.2 µg/mL, respectively. It was found that the results of the current study are higher than those obtained by Katalinic *et al*. (2010) in the grape skin extracts of seven *Vitis vinifera* red and white varieties with the DPPH• radical-scavenging ability (IC_{50}) of 156, 209, 239, 153, 58.0, 64.2 and 158 mg/L in *Vranac*, *Trnjak*, *Rudezusa*, *Merlot*, *Babic*, *Latin*, and *Plavina*, respectively.

Table 3. *In-vitro* antioxidant activity as (IC_{50}) for red and white grape pomaces

Sample	Extracts	DPPH• IC_{50} (µg / mL)	$ABTS^{•+}$ IC_{50} (µg / mL)	Reducing Power EC_{50} (µg /mL)	Fe^{2+}-chelating IC_{50} (µg / mL)
Red grape pomace	Aqueous	$2.67^{a} \pm 0.85$	$29.22^{c} \pm 0.57$	$59.45^{b} \pm 0.47$	$143.73^{b} \pm 5.17$
	Ethanol 80%	$33.29^{d} \pm 1.10$	$56.22^{d} \pm 0.45$	$160.97^{d} \pm 1.95$	$262.67^{d} \pm 0.35$
	Acetone 80%	$21.93^{c} \pm 0.61$	$16.32^{b} \pm 0.49$	$71.89^{c} \pm 1.06$	$199.77^{c} \pm 4.06$
	BHT	$7.61^{b} \pm 0.33$	-	$11.01^{a} \pm 0.32$	-
	Trolox	-	$4.28^{a} \pm 0.20$	-	-
	EDTA	-	-	-	$1.88^{a} \pm 0.46$
L.S.D at 0.05		1.74	0.88	1.15	0.76
White grape pomace	Aqueous	$24.31^{b} \pm 1.05$	$53.38^{c} \pm 0.79$	$126.69^{c} \pm 0.32$	$250.34^{d} \pm 5.03$
	Ethanol 80%	$54.02^{d} \pm 0.35$	$78.47^{d} \pm 0.79$	$141.50^{d} \pm 3.03$	$248.35^{c} \pm 1.92$
	Acetone 80%	$39.43^{c} \pm 0.40$	$37.31^{b} \pm 0.15$	$94.58^{b} \pm 0.58$	$203.14^{b} \pm 2.53$
	BHT	$7.61^{a} \pm 0.33$	-	$11.01^{a} \pm 0.32$	-
	Trolox	-	$4.28^{a} \pm 0.20$	-	-
	EDTA	-	-	-	$1.88^{a} \pm 0.46$
L.S.D at 0.05		1.13	1.31	1.25	0.47

All values demonstrated as mean ± S.D. Mean with different letters are significantly different at $p \leq 0.05$.

Hogan *et al*. (2009) inspected the efficiency of antioxidants for three fresh wine grapes from Virginia - Cabernet Franc clone1, Norton, and Cabernet Franc clone 313, and specified their DPPH$^{\bullet}$ scavenging activities as (8.8, 7.9, and 5.4μmol TE/g, respectively). The antioxidant activity of plants can be attributed to the flavonoid and polyphenolic compounds located in them (Yilmaz and Toledo 2004). Even so, the antioxidant capability also depends on numerous variables which include genetics, environmental circumstances, industrial methods employed, date of harvest and post-harvest and storage problems (Wang and Helliwell 2001). These outcomes are in accordance with Anastasiadi *et al*. (2010) and Ruberto *et al*. (2007) for red grape peels. Also, the outcomes of white grape pomace have been comparable to those reported by Katalinic *et al*. (2010).

3.3.2. ABTS$^{\bullet+}$ Radical Scavenging Activity

The outcomes show that the grape pomace has potent scavenging capacity for the ABTS$^{\bullet+}$ radical, and really should be explored as a potent antioxidant. Advanced reports have confirmed the radical scavenging activity of the red grape pomace from the seeds and outer skin extracts (Rockenbach *et al*., 2011). The IC_{50} values of ABTS$^{\bullet+}$ radical activity are varied significantly. The high activity was observed with the acetone (80 %) extract of both grape varieties parallel to Trolox as standard (Table 3). While the ethanol (80 %) extracts of the red and white pomaces showed lower antioxidant activity.

In the current research, the red and white grape pomace extracts showed a high anti-oxidant activity to scavenging ABTS$^{\bullet+}$ radical, compared to earlier studies conducted by (González-Centeno *et al*. 2013), who studied red and white grape pomaces (193–485 and 71–134 μmol TE/g).

3.3.3. Reducing Power

The reducing power of phytochemical compounds depends on their effectiveness in transmitting electrons. In the reducing power assay, the presence of antioxidant activity in the plant extracts improves the process of reduction of ferricyanide complex to the ferrous form by electron-donating. Therefore, the reducing ability of phytochemicals is fundamental evidence of its probable antioxidant activity. The occurrence of reductants in the samples would cause the reduction of (Fe^{3+}) to (Fe^{2+}) through the donation of an electron and the creation of the Perl Prussian blue complex. This kind of complex was estimated by measuring absorbance at 700 nm (Oyaizu, 1986). It was observed that the aqueous extract of the red grape and acetone extract of the white grape pomace had a higher reducing power (EC_{50} = 59.45 ± 0.47 and 94.58 ± 0.58 μg / mL), respectively (Table 3). This is probably due to the low viscosity of the solvent which possesses a low intensity and high diffusivity that allows them to be facilely diffused into the pores of the plant materials to create their way out of the bioactive matters (Sultana *et al*., 2007). Based on the results of this study, the extracts with the maximum antioxidant activity had the highest concentration of phenols (Katalinic *et al*. 2010).

3.3.4. Fe+2-chelating Activity

The chelation is a significant parameter because ferrous is desired to transport respiration oxygen and many enzyme activities. However, ferrous is an extremely invigorate metal and can stimulate oxidative changes in protein, lipid, and other ingredients in cells. Iron is essential to every single known living being, but increased iron can be unsafe, in light of the fact that free ferrous particles respond with peroxides to create free radicals, which can harm some neurological atoms, for example, genetics, proteins, lipids, and other cell segments (Wang *et al*., 2010).

The metals chelating capability is expressed as the percentage of suppression of the Fe^{2+} complex formation with various extracts Halliwell, and Gutteridge (1984). In the existent research, all of the extracts demonstrated a significant ability to chelate metal ions (Fe^{2+}). The capacity of grape pomace to chelate Fe^{2+} is given in Table 3. This study found that both varieties could chelate Fe^{2+} efficiently and reduce free ferrous realization. The IC_{50} values of both grape pomace extracts ranged from 143.73±5.17 to 262.67±0.35. The red grape pomace aqueous extract had the highest Fe^{+2} chelating capacity (IC_{50} =143.73±5.17 μg/mL), while the red grape pomace ethanol extract had the lowest capacity (262.67±0.35 μg/mL) (Table 3).

EDTA was used as a reference to this assay and its IC_{50} value for Fe^{2+}-chelation was 1.88 μg/mL. The results are in the range of those reported by Pal *et al*. (2015) and Maniyan *et al*. (2015) for kiwi byproducts. The results can be explained by the grape pomace's high content of phytochemical compounds acting as antioxidants such as anthocyanin, phenolics, flavonoids, and chlorophyll as previously reported by Cassano *et al*. (2006). Ethanol exhibited lower chelation activities than the aqueous and acetone extracts. The highest antioxidant activity of the aqueous and acetone extracts can be also attributed to the major components present in grape pomace, above all benzoic acid, and ellagic acid in the red Romy and white Banaty grape pomace extracts. This can be clarified by distinct factors, including the existence of diverse effective components in the plant which have the ability to mutate the antioxidant capacity, the synergistic effects of unlike components, the experimental circumstances, and the mechanisms of the methods utilized for antioxidant reactions (Cho *et al*., 2003).

3.4. Antimicrobial Activity of Grape pomace Extracts

Using natural products as antimicrobials for conserving food is on the rise. It has received an increasing awareness on the part of consumers regarding the appreciation of natural foods as well as increasing attention regarding microbial impedance by ordinary preservatives (Omidbeygi *et al*., 2007). Spices' antimicrobial properties have been recognized for thousands of years in the preservation of food. Biologically active molecules in food are important to stop dangerous outcomes to free radicals; they also prevent the degradation of foods resulting from the oxidation of lipid and microbial corruption. Spices are the most notable parts in the diets of humans to transfer the taste, color, and flavors of foods. Foodborne diseases due to food exhaustion contaminated by pathogenic toxins and/or bacteria are among the most serious public health concerns. Monitoring of pathogenic microorganisms can reduce outbreaks of foodborne diseases and ensure supplying consumers with nutritious, healthy, and safe foods. Plant extracts possess antimicrobial efficacy against distinct, pathogenic microorganisms (Luther *et al*., 2007; Allahghdri *et al*., 2010). Different microbial species were

utilized to recognize the antimicrobial capacity of extracts for both varieties of grape pomace. The study of antimicrobial activity of the red and white grape pomace extracts using the agar diffusion method revealed that the ethanolic extracts from the red and white grape can restrain the growth of all microorganisms tested as displayed in Table 4. The ethanol extract of the red and white grape pomace was the most effective extract inhibiting the microbial growth of all pathogenic microorganisms tested at 600 µg/mL, while the acetone and aqueous extracts of the red and white grape pomace were mediated by effective extract inhibition of the growth of microbials when testing all pathogenic microorganisms in all concentrations. The zone result of inhibition obtained in ethanolic extracts shows a higher inhibition at a concentration of 600 µg/mL. This is consistent with the previous reports of (Bradbury and Holloway, 1988; Furneri *et al*., 2002) which maintain that those action modes of phenolic compounds are concentration-dependent. The results of the present study are in part consistent with earlier studies of grape pomace extract which found that the extracts were efficient as anti-Gram+ve bacteria (Darra *et al*., 2012; Oliveira *et al*., 2013). The potent durability of Gram-ve bacteria to the grape pomace extracts in these results might be attributed to the double cell membrane and vigorous hydrophobicity of the exterior membranes of Gram-ve bacteria; this is in agreement with Smith-Palmer *et al*. (1998).

Table 4. Antimicrobial activity of Red and White grape pomace

Samples	Extracts	Conc. (µg/ml)	Inhibition Zone Diameter (mm)							
			Bacteria				Fungus		Yeast	
			B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*	*A. flavus*	*S. cerevisiae*	*C. albicans*
Red grape pomace	Aqueous	200	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
		400	11.55	10.86	09.87	10.95	09.76	10.20	11.47	10.30
		600	17.70	16.54	16.16	15.73	13.83	15.50	15.60	15.26
	Ethanol 80%	200	09.11	09.00	10.00	09.00	00.00	00.00	10.00	09.00
		400	17.20	15.30	15.00	14.00	12.67	11.42	14.00	15.43
		600	28.16	27.65	25.46	25.46	20.33	17.56	25.00	21.80
	Acetone 80%	200	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
		400	10.66	10.53	09.67	10.61	09.80	10.33	09.27	10.60
		600	15.32	14.17	15.23	15.86	13.65	12.76	14.22	14.44
White grape pomace	Aqueous	200	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
		400	12.63	13.70	11.00	12.00	10.62	11.30	10.56	11.43
		600	16.66	17.57	15.46	16.57	16.75	17.81	16.87	15.71
	Ethanol 80%	200	09.00	11.00	09.00	00.00	00.00	00.00	09.00	00.00
		400	15.50	17.55	15.00	13.80	11.70	11.00	12.87	11.76
		600	24.54	26.60	20.46	19.46	19.00	16.75	23.77	20.85
	Acetone 80%	200	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00
		400	11.66	11.73	11.00	09.00	09.64	10.30	11.62	10.87
		600	14.82	15.50	14.46	13.46	14.85	13.85	16.81	15.50

Inhibition zone diameter was measured as the clear area was centered on agar containing the sample; well with non-inhibition zone was record 0.00. The measurements are taken after 24 h incubation by yeast, 24 to 48 h with bacteria and 48 to 72 h with fungus.

3.4.1. Minimum Inhibitory Concentrations (MIC)

The effects of MIC of the white and red grape pomace extracts on behaving Gram-ve bacteria, Gram +ve bacteria, yeasts, and fungi were tested and the results are shown in Table 5.

The ethanolic and aqueous extracts of each red and white grape pomace display a vigorous antimicrobial vigor against *B. subtilis* NRRL B-94, *E. coli* NRRL B-3703, *P. aeruginosa* NRRL, *S. aureus* NRRL, *A. niger* NRRL313, *A. flavus* NRC, *S. cerevisiae* NRC, and *C. albicans* NRRL477. They possess spacious vision competence against Gram-ve bacteria, Gram +ve bacteria, and molds with MIC extending between 210 to 325 ppm. The distinction in the efficiency of the red and white grape extracts against different strains depends on permeability variances between the cells of these microbes and chemical contents of each extract; this is in harmony with Dorman and Deans, (2000).

Table 5. The MIC of red and white grape pomace

Samples	Extract	MIC (ppm)							
		Bacteria				Fungus		Yeast	
		B.subtilis	*S. aureus*	*E. coli*	*P. aueginosa*	*A. niger*	*A. fluves*	*S. cervisiae*	*C. albicans*
Red grape pomace	Aqueous	260	285	290	260	280	270	236	250
	Ethanol 80%	255	230	300	285	290	290	270	260
White grape pomace	Aqueous	265	312	260	285	310	325	320	300
	Ethanol 80%	210	215	220	227	256	260	227	224

3.4.2. Mode of Action

The effects of specific concentrations of the ethanolic extract of the white grape pomace were evaluated through the bio-synthesis of total lipids, DNA & RNA, acids soluble phosphorus, and protein in *B. subtilis* cells and the data are shown in Figures 1 and 2. The ethanolic white grape extract from the grape pomace had a significant impact on protein synthesis and total lipid in the cells of *B. subtilis* NRRL B-94 (Figure. 1 A and B). The effect is increased by increasing the concentration (1/4 and 1/2 MIC) and incubation duration. The ethanolic white grape pomace extract had a slight effect on the synthesis of DNA, RNA, and acid-soluble phosphorus (Figure 2 A- C). These results point out that the ethanolic white grape pomace extracts significantly affect protein biosynthesis by the control of a few steps in the compound interpretation processes. The ultimately remarkable antibiotics with the same procedure are tetracycline. Some chemotherapy agents are affected by the assembly of DNA or/and RNA or can join DNA or/and RNA so that their messages cannot be read. However, most drugs are non-selective, affecting animal and bacterial cells; therefore, they have no therapeutic enforcement (Sahu, 1997; Shuichi et al., 2000).

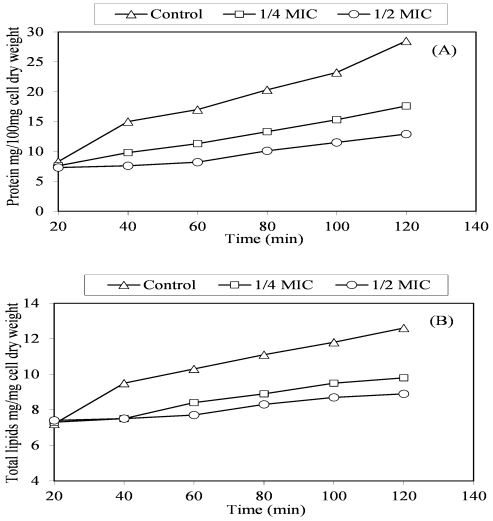

Figure 1. Effect of different concentrations of the ethanolic white grape extract on: (A) Biosynthesis of proteins. (B) Biosynthesis of total lipids in the cells of (*B. subtilis)* NRRL B 94.

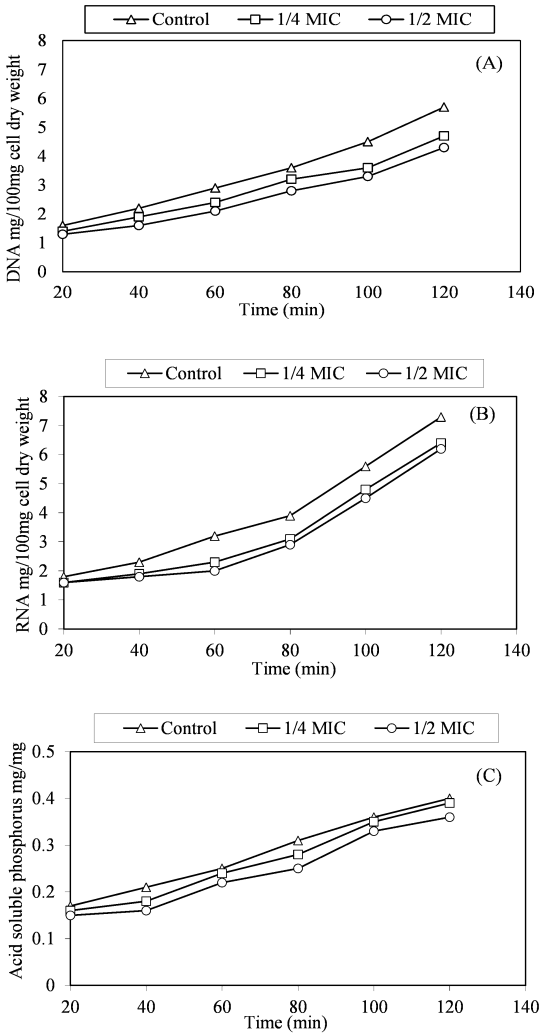

Figure 2. Effect of unlike concentrations of the white grape ethanolic extract on: (A) DNA synthesis in the cells of (*B. subtilis)* NRRL B-94. (B) RNA synthesis (C) Acid-soluble phosphorus synthesis

3.5. MTT Cytotoxicity

The cytotoxicity assay was carried on the studied extracts to choose the safe doses of the plant extract on cell culture Madin-Darby Canine kidney (MDCK) cells to be used for the antiviral activity assay. The results are presented in Table 6.

Table 6. MTT cytotoxicity

Samples	Extracts	Conc.µg/µL	Cytotoxicity TC_{50} µg/µL	Initial viral counts	Viral counts (PFU/mL)	Inhibition (%)
Red grape Pomace	Aqueous	200	443	7×10^6	4×10^6	43
		400			2.5×10^6	٦٥
	Ethanol	200	1596	10×10^6	5×10^6	٤0
		400			4×10^6	٦٠
	Acetone	200	796	10×10^6	6×10^6	٥0
		400			3×10^6	٦٤
White grape pomace	Aqueous	200	502	10×10^6	6×10^6	21
		400			4.5×10^6	71
	Ethanol	200	5539	7×10^6	5.5×10^6	٤0
		400			2×10^6	٥5
	Acetone	200	3901	10×10^6	4×10^6	٦0
		400			3.5×10^6	٦5
Standard	Oseltamivir	5	-	10×10^6	0	100

To explore the antiviral activity of the target extracts, the plaque infectivity reduction assay was used in two concentrations. The results reveal that the red grape extract has a slightly higher antiviral activity compared to the white one and the aqueous extract at 400 µg and is much better than the ethanol and acetone extracts, consistent with (Gaafar et al., 2015). The extracts are examined against AIV H5N1 (A / chicken / Egypt /M7217B / (H5N1) as shown in Figure 3.

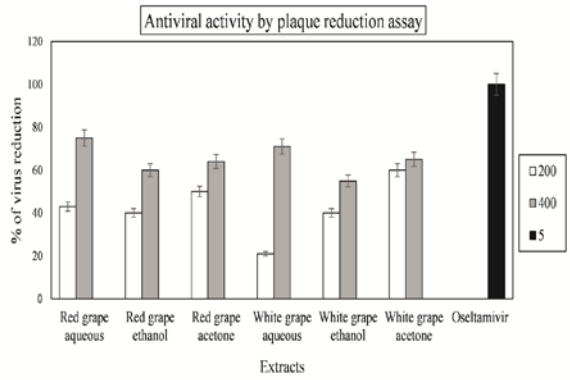

Figure 3. Antiviral activity (%) of pomace extracts expressed by the percentage of plaque reduction assay.

4. Conclusion

The considerable diversity of two grape pomace varieties (Romy and Banaty) with respect to the contents of TP, TF, TT, and antioxidant assays by $DPPH^{\bullet}$, $ABTS^{\bullet+}$, reducing power, and iron-chelating tests have been investigated in this study. The red grape pomace acetone extract displayed the highest quantity of phenolics and antioxidant activities. It was confirmed that all pomace extracts offered antimicrobial and antiviral activities. This study also shows that the grape pomace polyphenols exhibited activities against Gram+ve and Gram-ve bacteria which means that these underutilized grape pomaces could be a perfect provenance of antimicrobials for further utilization in the food manufacturing industries which oversight or deny food-borne pathogens. Red grape pomaces have been proven to have ~~a~~ great potential as sources of antioxidant, antimicrobial and antiviral agents. This investigation confirms the prospects of using grape pomaces to promote new potential sources of antioxidant, antimicrobial and antiviral materials in the pharmaceutical industry.

Conflict of Interests

The authors declare that there is no conflict of interest.

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