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Research Article

A Comparative Study of Cytotoxic Effect in Three *Vitis vinifera* L. Varieties: King Ruby, Thompson and Crimson Against A-549 Lung Cancer Cell Line

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ABSTRACT

Grape (*Vitis vinifera* L.), family Vitaceae, is one of the most important plants used extensively in the cosmetics, pharmaceuticals, food sectors, and alternative medicine due to its high content of flavonoids. Leaves of three varieties namely King Ruby, Thompson, and Crimson, were assessed for their cytotoxic effect on the A-549 lung cancer cell line by the *in vitro* sulforhodamine B (SRB) assay. The results indicate that the tested varieties have weak cytotoxic activity based on cell viability. The King Ruby variety exerted the highest cytotoxic effect, followed by Crimson and Thompson with cell viability % values of 84.158±0.37, 87.613±0.25, and 91.994±0.72, respectively. The preliminary phytochemical screening of the leaves of the three varieties revealed the presence of carbohydrates, sterols/triterpenes, tannins, and flavonoids. Additionally, the determination of the contents of phenolics, flavonoids, and tannins showed that King Ruby was the richest in phenolics, flavonoids, and tannin contents. This motivate us to investigate the petroleum ether extract of King Ruby leaves by GC/MS to identify its phytochemical content.

Keywords: *Vitis vinifera* L., King Ruby, GC/MS, SRB assay, Cytotoxicity.

1. INTRODUCTION

The cultivation of grape (*Vitis vinifera* L.) is almost as old as civilization. Knowledge on grapes may be was discovered in the 4th century Egyptian hieroglyphics.[1] Egypt produces 1.7 million metric tons of grapes annually making them the 4th most important agricultural plant and the 14th most prolific nation in the world.[2] Numerous varieties were brought in and cultivated in Egypt such as Early Sweet, Superior, Thompson, Flame Seedless, Crimson, and Red Globe.[3]

The unique combination of polyphenolic chemicals found in grapes and products produced from them may have beneficial health effects according to recent studies. Grapes have many bioactive compounds including ergosterol, *β*-sitosterol, malic acid, tannic acid, dehydroascorbic acid, phenolic compounds, flavonoids, proanthocyanins, and stilbenoids.[4] These active constituents contribute to the health advantages of grape, which include hepatoprotective,[5] antiviral,[6] gastroprotective,[7] antibacterial,[8] and antidiabetic properties.[9] Also, condensed tannins and flavonoids may have a role in the anti-diabetic properties of *V. vinifera* L. leaves by inhibiting the activity of *α*-amylase.[10]

Moreover, published research highlighted that eating grapes may lower the risk of getting some malignancies, such as breast and colon cancer. The antioxidant, anti-inflammatory, and anti-proliferative characteristics of grape are principally responsible for their anticancer effects.[11] The present study focused on three varieties of *V. vinifera* L., namely King Ruby, Thompson and Crimson to evaluate their *in vitro* cytotoxic activity by sulforhodamine B (SRB) assay against lung cancer, which is thought to be the primary cause of cancer-related death globally and the most common malignant neoplasm in the majority of nations.[12] Existing drugs for lung cancer are ineffective and frequently have a

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variety of toxicities and side effects, making phytocompounds viable alternatives.[13]

Six Italian *V. vinifera* L. leaves varieties were evaluated using the A-549 cell line. Lung cancer was suppressed by all tested extracts in a concentration-dependent manner. Compared to the vinblastine used as a positive control, their findings are 0.7 times greater.[14]

2. METHODS

2.1. Plant Material

Leaves of the three varieties of *V. vinifera* L.: King Ruby, Thompson and Crimson **(Figure 1)** were collected from garden in Kotor-Tanta road, in August 2020. It was identified by Prof. Dr. Osama Kamal El-Abasy, Professor of Horticulture, Faculty of Agriculture, Tanta University and Prof. Dr. Nabil Ebrahim El-Sheery, Professor of Agriculture Botany, Faculty of Agriculture, Tanta University. Voucher samples were preserved at the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Tanta University. Voucher samples are No. PGA-8-1-2020 (King Ruby variety), No. PGA-8-2-2020 (Thompson variety) and No. PGA-8-3-2020 (Crimson variety).

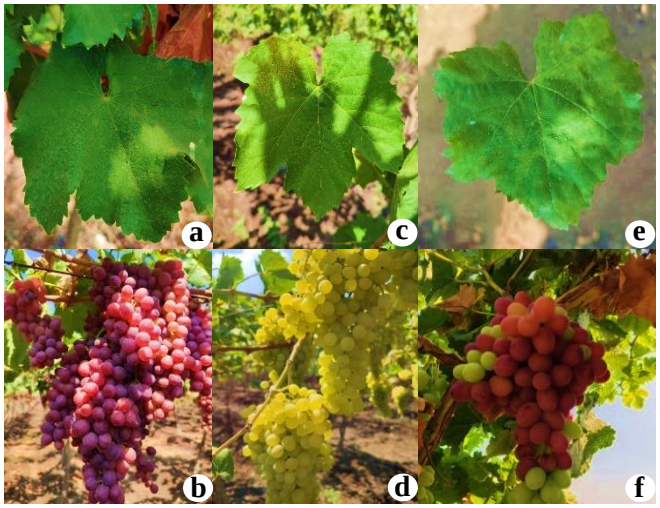

Figure 1. *V. vinifera* L. three selected varieties **(a)** King Ruby leaf**, (b)** King Ruby fruits, **(c)** Thompson leaf, **(d)** Thompson fruits, **(e)** Crimson leaf **(f)** Crimson fruits.

2.2. Chemicals and Reagents

Methanol (MeOH), petroleum ether (PE, 60-80 °C), ethyl acetate (EtOAc), diethyl ether, *n*-butanol (*n*-BuOH), acetone and methylene chloride (CH_2Cl_2) for extraction and fractionation. All solvents were of analytical grade and purchased from Iso-Chem Pharmaceutical Chemical Co., Egypt. The following: Gallic acid, Folin-Ciocalteu reagent, 1M Na_2CO_3 were purchased from Sigma Chemical Co. (St., Louis, USA) used for total phenolic contents. Rutin, 1.25% $AlCl_3$ 0.125 M sodium acetate were supplied from Merck (Germany) and used for total flavonoid contents. Tannic acid, 1% $FeCl_3$, 1% $K_4[Fe(CN)_6]$ obtained from Merck, India and used for total tannin contents. The following: alcoholic KOH (10%), anhydrous Na_2SO_4 (Iso-Chem Pharmaceutical Chemical Co., Egypt) and conc. HCl (Horus Co., Egypt) were used in the saponification of the PE. 1% Aqueous ferric chloride (Iso-Chem Pharmaceutical Chemical Co., Egypt), Alcoholic *α*-naphthol was used for Molisch's reagent, Dragendorff's reagent and Mayer's reagent were prepared in Pharmacognosy department laboratories, Faculty of Pharmacy, Tanta University. NaOH (Inter. Trade Co., Egypt) and di-nitro benzoic acid (ADWIC Co., Egypt) were used for preliminary phytochemical screening. Sulforhodamine B (SRB) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for cytotoxic assay. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, streptomycin and penicillin were purchased from Lonza Group Ltd. (Basel, Switzerland) and used for cell culture preservation.

2.3. Cell Culture

The lung cancer cell line A-549 was obtained from Nawah Scientific Inc. (Mokatam, Cairo, Egypt) and preserved in a medium composed of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/mL streptomycin, and 100 units/mL penicillin at 37 °C in a humidified 5% (v/v) CO_2 environment.

2.4. Plant Extraction

The dried powdered leaves of *V. vinifera* L. varieties (King Ruby, Thompson, and Crimson) were extracted, separately, by a cold maceration procedure with MeOH (2 × 5 L, 72 h for each variety), then filtered and concentrated under reduced pressure using rotary evaporator to produce crude extracts. The yields were King Ruby (200 g), Thompson (155 g), and Crimson (116 g). The extract were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* cytotoxic study.

2.5. *In Vitro* Cytotoxic Comparison of *V. vinifera* L. Varieties (SRB Assay)

Lung cancer cell line A-549 was humidified with 5% CO_2 at 37 °C. The SRB cell viability test was carried out. For a duration of 24 h, 96-well plates were cultured with a 100 µL cell solution (5×10^3), then 100 µL of media was mixed with five concentrations (0.03, 0.3, 3, 30, 300 µg/mL) of the tested extracts in dimethyl sulfoxide (DMSO) and then administered to the cells. After 72 h, 150 µL of 10% TCA was cultured in the medium, and the cells were then fixed by refrigerating for an hour. The cells were rinsed with purified water five times after the removal of the TCA solution. Following the addition of the cells, samples containing 70 µL of SRB solution (0.4% w/v) were incubated in dark and at room temperature for 10 min.

Plates were washed three times with 1% acetic acid and allowed to air dry for the full night. To dissolve the protein-bound SRB stain, 150 µL of trisaminomethane (TRIS) (10 mM) was applied. Using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany), the absorbance at 540 nm was determined for extracts and for DMSO as negative control.

2.6. Total Phenolic Contents

The total phenolic contents assessment was performed using the Folin Ciocalteu method.[15] In a 96-well microplate, 10 µL of the standard (gallic acid) and the tested samples (King Ruby, Thompson and Crimson) were incorporated separately with 100 µL Folin-Ciocalteu reagent (1:10 dilution). After that, 80 µL of 1M Na_2CO_3 was mixed, and the solution was let to sit at room temperature (25 °C) in the dark for 20 min. The hue of the blue complex was determined at 630 nm after incubation. The FluoStar Omega microplate reader was used to record the results (n=3), and the data are shown as means ± SD.

2.7. Total Flavonoid Contents

In a 96-well microplate, the total Flavonoid contents were assessed by modifying The aluminium chloride technique.[16] The microplates were filled with 15 µL of each sample (King Ruby, Thompson and Crimson) and standard (rutin) separately, followed by 175 µL of MeOH and 30 µL of 1.25% $AlCl_3$. Eventually, 5 min of incubation were spent after adding 30 µL of 0.125 M sodium acetate. At 420 nm, after incubation, the resulting yellow tint absorbance was measured. The FluoStar Omega microplate reader was used to record the results (n=3), and the data are shown as means ± SD.

2.8. Total Tannin Contents

With slight adjustments, the Ojha technique[17] for determining the total tannin contents, was employed. The process was carried out in a 96-well microplate. Tannic acid was used as a standard, 20 µL of each sample (King Ruby, Thompson and Crimson) was added separately. After that, 20 µL of 1% $FeCl_3$ and 20 µL of 1% $K_4[Fe(CN)_6]$ were added. Lastly, 200 µL of distilled water was added followed by incubation for 5 min. After incubation, the resultant yellow colour was measured at 720 nm using the FluoStar Omega microplate reader (n=3). The results are displayed as means ± SD.

2.9. Preparation of Unsaponifiable and Saponifiable Matter of the PE fraction

PE, CH_2Cl_2, EtOAc and n-BuOH were utilized in the fractionation of 100 g of the King Ruby variety's methanolic leaves extract. The PE fraction was further investigated for saponifiable (SAP) and unsaponifiable matter (USM).

Twelve grams of the PE (60–80 °C) fraction residue were saponified by heating in 180 mL of alcoholic KOH (10%) on a boiling water bath with an air condenser for 6 h. After the alcohol was distilled out of the saponified combination, the remaining liquid was combined with 120 mL of water and extracted four times with diethyl ether until exhaustion. In order to extract the USM of King Ruby leaves (8.5 g), the mix of ethereal extracts were washed with D.W., dried over anhydrous Na_2SO_4, and then evaporated. The USM fraction was investigated for further isolation of active constituents by column chromatography.

After USM extraction, conc. HCl was employed to acidify the alkaline aqueous solutions, and ether was then utilized to extract the released fatty acids. To obtain the fatty acid fraction, the ether extracts were washed with D.W., dehydrated over anhydrous Na_2SO_4, and then distilled.

Fatty acid fraction of the leaves (0.5 g) was dissolved in 25 mL absolute MeOH and 3 mL conc. H_2SO_4 and refluxed for 2 h. After the distillation of MeOH, the residue was extracted with diethyl ether till exhaustion. Following a dehydration process over anhydrous Na_2SO_4, The mixed ethereal extracts was distilled off to remove diethyl ether.

2.10. GC/MS Analysis

GC/MS analysis was performed to identify the methyl esters prepared. A GC/MS Finnigan mat. SSQ 7000, Trace GC 200 (thermo) GC mod. USA was used. One µL of methyl ester residue was mixed in analytical grade ether (1 mg/mL) for GC/MS analysis using Elite-5MS with a column diameter of 30 m × 0.25 mm ID, electron ionization (EI, 70 ev. energy) as ionization mode and helium as a carrier gas.

2.11. Statistical Analysis

All Statistical analysis was conducted by GraphPad Prism 8 (USA). Results were obtained as means ± SD and at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. *In Vitro* Cytotoxic Comparison of *V. vinifera* L. Varieties (SRB Assay)

Among the most frequently employed techniques for assessing *in vitro* cytotoxicity is the SRB test, since 1990, which is based on the binding ability of SRB with cell protein components that have been fixed on tissue culture plates with trichloroacetic acid (TCA). With two sulfonic groups, SRB is a vivid pink amino xanthene pigment that binds with basic amino acid residues in modestly acidic surroundings but splits in basic ones. The amount of dye recovered from stained cells is proportional to cell mass since SRB binding is stoichiometric.[18]

V. vinifera L. three varieties (King Ruby, Thompson and Crimson) leaves extracts were tested using the SRB routine in vitro cytotoxic assay against A-549 lung cancer cell line. The study about cytotoxic activity of *V. vinifera* L. on lung cancer is still limited. According to the IC_{50} value, the anticancer activity of IC_{50}: 100–1000 µg/mL is classified weakly active.[19] The IC_{50} results of the three varieties were > 300 µg/mL revealed that they have weak cytotoxic activity. As shown in **(Table 1, Figure 2)**, weak cytotoxic activity was demonstrated by all varieties according to % cell viability, the King Ruby leaves MeOH extract had the best activity at 300 µg/ml, on the A-549 lung cancer cell line when compared to the other two varieties. The data was presented as mean ± SD and $p < 0.05$. The % cell viability of DMSO is 100%.

Table 1. Cell viability result of SRB routine multidose cytotoxic assay of the *V. vinifera* L. three varieties extract on A-549 lung cancer cell line.

Sample Conc. (µg/mL)	% Cell viability		
	King Ruby	Thompson	Crimson
0.03	98.301 ± 0.04	99.27 ± 0.58	96.452 ± 1.91
0.3	92.021 ± 0.71	95.602 ± 0.43	96.192 ± 0.22
3	90.272 ± 0.75	95.228 ± 0.51	93.045 ± 0.42
30	86.357 ± 1.51	94.005 ± 0.97	92.844 ± 0.44
300	84.158 ± 0.37	91.994 ± 0.72	87.613 ± 0.25

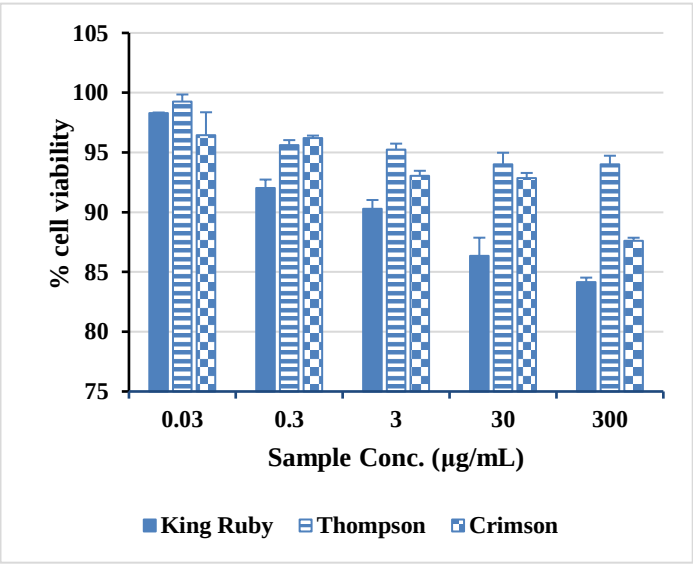

Figure 2. Cell viability results of SRB routine multidose in vitro cytotoxic assay of the V. vinifera L. leaves methanol extracts of three varieties (King Ruby, Thompson and Crimson) on A-549 lung cancer cell lines.

3.2. Preliminary Phytochemical Screening

According to **Table 2**, the three varieties contain carbohydrates, sterols/triterpenes, tannins, and flavonoids, while the cardenolides, saponins, anthraquinone and alkaloids were not detected.

Table 2. Result of preliminary screening of *V. vinifera* L. three varieties (K=King Ruby, T=Thompson and C=Crimson)

Result	Test	K	T	C
Carbohydrate	Molisch	+	+	+
Cardenolides	Keller-Killiani	–	–	–
Anthraquinone glycoside	Borntrager	–	–	–
Sterols and/or Triterpenes	Salkowski	+	+	+
Alkaloids	Mayer	–	–	–
Tannins	$FeCl_3$	+	+	+
Flavonoids	NaOH	+	+	+
Saponins	Froth	–	–	–

3.3 Total Phenolic Contents

The Folin-Ciocalteu reagent was used to test the phenolic contents results in accordance with the procedure.[15] The total polyphenol content data were represented using the gallic acid standard curve. **Table 3 and Figure 3** demonstrate that the extract from King Ruby leaves had the highest phenolic content at 165.37 ± 14.76 µg GAE/mg, followed by the extract from Thompson leaves at 147.97 ± 5.31 µg GAE/mg, and the extract from Crimson leaves at 88.07 ± 0.81 µg GAE/mg.

Table 3. Total phenolic compounds in *V. vinifera* L. leaves varieties (n=3).

V. vinifera L. varieties	Average reading at 630 nm	Total phenolic contents (µg Gallic acid per 1 mg extract)
King Ruby	1.5890	165.37 ± 14.76
Thompson	1.3664	147.97 ± 5.31
Crimson	0.8508	88.07 ± 0.81

3.4. Total Flavonoid Contents

Colorimetric analysis was used to determine the flavonoid content of the three *V. vinifera* L. varieties. The expression for the resulted flavonoid contents was rutin equivalent. The flavonoid contents in the extracts of Crimson and Thompson were 23.78 ± 1.06 and 38.18 ± 2.4 µg RE/mg extract, respectively as shown in **(Table 4, Figure 3)**. But King Ruby leaves extract showed flavonoid contents of 45.6 ± 1.45 µg RE/mg extract, which was comparatively higher than other varieties.

Table 4. Total flavonoid compounds in *V. vinifera* L. leaves varieties (n=3).

V. vinifera L. varieties	Average reading at 420 nm	Total flavonoid contents (µg Rutin per 1 mg extract)
King Ruby	0.4650	45.6 ± 1.45
Thompson	0.3908	38.18 ± 2.4
Crimson	0.2468	23.78 ± 1.06

Table 5. Total tannin compounds in *V. vinifera* L. leaves varieties (n=3).

V. vinifera L. varieties	Average reading at 720 nm	Total tannin contents (µg Tannic acid per 1 mg extract)
King Ruby	1.8421	62.57 ± 1.87
Thompson	1.7147	58.16 ± 2.12
Crimson	1.0210	34.16 ± 0.484

3.5. Total Tannin Contents

The standard curve of tannic acid equivalent was used to express the tannin concentration in the extracts of King Ruby, Thompson, and Crimson leaves. According to **(Table 5, Figure 3)**, the total tannin components in King Ruby leaves extract are higher than those in Thompson leaves extract and Crimson leaves extract, which are 62.573 ± 1.871, 58.161 ± 2.12, and 34.159 ± 0.484 µg TAE/mg extract, respectively.

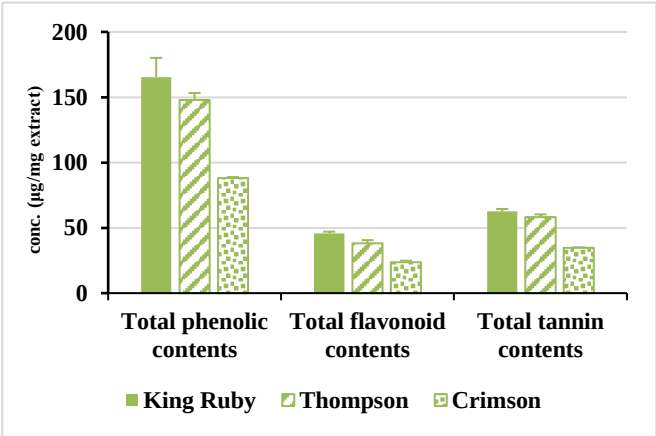

Figure 3. Comparison in phytochemical content between three *V. vinifera* L. leaves varieties: King Ruby, Thompson and Crimson (n=3).

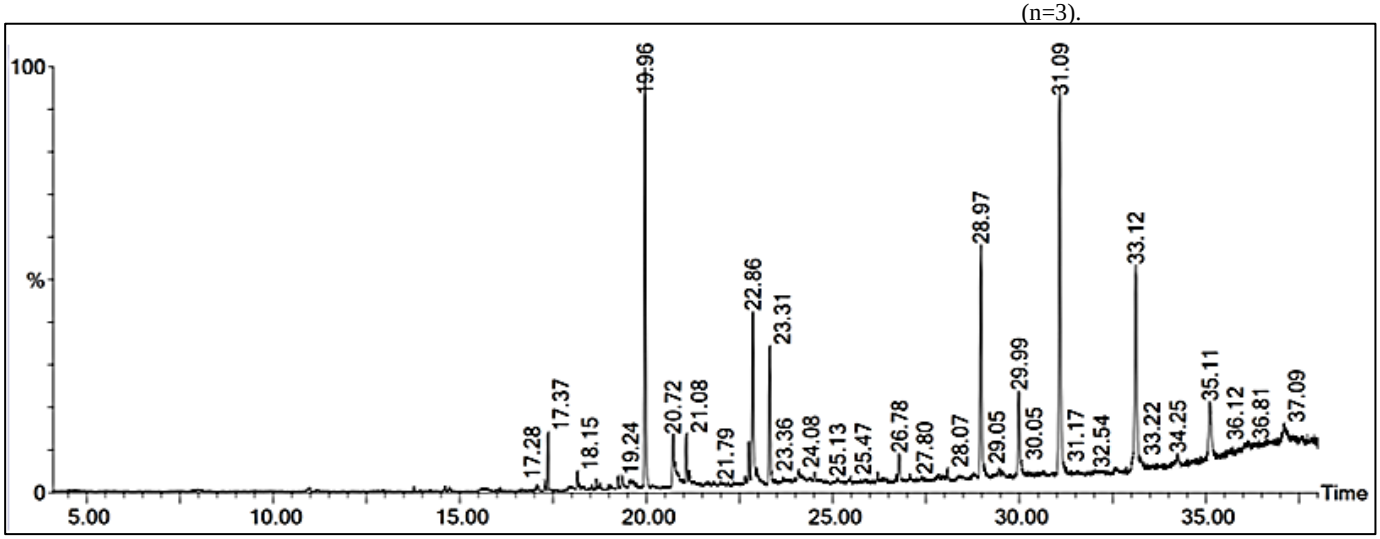

Figure 4. GC/MS chromatogram of the saponifiable matter of the PE fraction *V. vinifera* L. leaves extract King Ruby variety.

3.6. GC/MS Analysis of The Methylated Fatty Acids in *V. vinifera* L. King Ruby variety

According to the previous results, in which the King Ruby is the best variety, GC/MS is used for further study of the King Ruby variety. GC/MS was used to predict phytochemical compounds in the *V. vinifera* L. King Ruby variety (the most active variety), twenty-eight substances were identified. The retention times of the identified compounds and mass fragmentation patterns were directly compared to those of the available reference in GC/MS library, which had been tested under similar conditions. The results are shown in **Figure 4** and different compounds were detected and demonstrated in **Table 6** with their retention times, peak areas, molecular formula, molecular mass and biological activities.

Table 6. Compounds found in the saponifiable matter of the PE fraction of *V. vinifera* L. (King Ruby variety) using GC/MS.

Peak No.	R_t (min)	Peak area%	Name	Molecular formula	$[M]^+$ *m/z*	Reported biological activity
1	17.369	0.943	Cyclopentane undecanoic acid, methyl ester	$C_{17}H_{32}O_2$	268	Antioxidant, antibacterial activities.[20]
2	18.154	0.315	2-Ethyl-heptanoic acid	$C_9H_{18}O_2$	158	-
3	19.245	0.264	1-Octadecyne	$C_{18}H_{34}$	250	Antimicrobial, anti-inflammatory, antibacterial activities.[21, 22]
4	19.355	0.385	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	Antimicrobial, acaricide, antiseptic, pesticide activities.[23]
5	19.575	0.231	Dodecanoic acid, 2-hexen-1-yl ester	$C_{18}H_{34}O_2$	282	-
6	19.965	8.708	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	228	Antibacterial and anti-enteric activities.[24]
7	20.725	2.251	*n*-Decanoic acid	$C_{10}H_{20}O_2$	172	Antiviral and antibacterial activities.[25]
8	21.075	0.994	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	$C_{20}H_{40}O_2$	312	Antibacterial activity.[26]
9	21.160	0.290	Oxalic acid, allyl tridecyl ester	$C_{18}H_{32}O_4$	312	-
10	22.651	0.237	Oxalic acid, cyclobutyl octadecyl ester	$C_{24}H_{44}O_4$	396	-
11	22.751	0.844	1-Pentadecyne	$C_{15}H_{28}$	208	-
12	22.856	4.367	8-Nonynoic acid, methyl ester	$C_{10}H_{18}O_2$	170	Antifungal activity.[27]
13	22.966	0.537	Methyl 2-hydroxydodecanoate	$C_{13}H_{26}O_3$	230	-
14	23.306	3.709	Hexadecanoic acid, 15-methyl-, methyl ester	$C_{18}H_{36}O_2$	284	Antioxidant activity,[28] hypocholesterolaemic activities.[29]
15	24.147	0.239	Oxalic acid, cyclobutyl dodecyl ester	$C_{18}H_{32}O_4$	312	-
16	24.517	0.277	Oxalic acid, cyclobutyl tetradecyl ester	$C_{20}H_{36}O_4$	340	-
17	26.198	0.246	Octadecanoic acid, 2-oxo-, methyl ester	$C_{19}H_{36}O_3$	312	-
18	26.703	0.230	Hexadecanoic acid, 2-hydroxy-, methyl ester	$C_{17}H_{34}O_3$	286	-
19	26.778	0.938	2-Pentadecyl-1,3-dioxolane	$C_{18}H_{36}O_2$	284	-
20	28.969	6.663	Ethanedioic acid, bis(trimethylsilyl) ester	$C_8H_{18}O_4Si_2$	234	-
21	29.054	0.396	Oxalic acid, allyl pentadecyl ester	$C_{20}H_{36}O_4$	340	Anti-pest, antimicrobial and termiticidal activities.[30]
22	29.459	0.381	Oxalic acid, allyl decyl ester	$C_{15}H_{26}O_4$	270	-
23	29.989	3.161	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	Antimicrobial, antifungal activities.[31]
24	31.090	13.005	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	$C_{16}H_{22}O_4$	279	Anticancer,[32] antidiabetic, antioxidant, antimicrobial,[33] anti-scabies, antiviral, anti-inflammatory activities.[34]
25	33.120	8.281	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667	Antioxidant[35, 36], antifungal activities.[37]
26	34.246	0.627	Z-8-Pentadecen-1-ol acetate	$C_{17}H_{32}O_2$	286	-
27	36.042	0.442	2,4-Dimethoxycinnamic acid	$C_{11}H_{12}O_4$	208	-
28	37.092	1.331	3,4-Dimethoxycinnamic acid	$C_{11}H_{12}O_4$	208	Antioxidant, anticancer and neuroprotective activities.[38]

The phytochemicals in the King Ruby extract with the largest peak area percentage were compound **24** (13.005%), which has antimicrobial,[33] anticancer,[32] antidiabetic, antioxidant, anti-scabies, antiviral and anti-inflammatory activities,[34] compound **6** (8.708%), with antibacterial and anti-enteric activities,[24] while compound **25** (8.281%) has antioxidant,[35, 36] and antifungal activities.[37]

Many of the GC/MS-identified compounds, including compounds **(1), (14), (24), (25), and (28)** which are presented in **Table 6** may be responsible for the cytotoxic abilities of King Ruby leaves extract against lung cancer, due to their antioxidant effect as well as phenolic and flavonoid contents which present in grape leaves and known to be anticarcinogenic and antioxidants.[39, 40]

CONCLUSION

The biological and phytochemical investigations of leaves methanol extracts of three *V. vinifera* L. varieties (King Ruby, Thompson, and Crimson) results showed that King Ruby is the best variety as weak cytotoxic on A-549 lung cancer cell line compared to the other two varieties with cell viability % value of 84.158 ± 0.37 and highest flavonoid, phenolic and tannin contents.

Consequently, the saponifiable matter of the PE fraction of the King Ruby variety was investigated by the GC/MS analysis. This allowed us to identify 28 compounds with different biological activities.

The limitation of the study is the weak *in vitro* cytotoxic activity due to using of one cell line. So, we recommend *in vivo* study which is better indicator or using more lung cancer cell lines.

CONFLICT OF INTEREST

The authors have not declared any conflicts of interest.

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