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Research Article

**ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACTS OF
INDIGENOUS AND EUROPEAN GRAPE CULTIVARS FROM
PAKISTAN**

*Naila Ali, *Humera Afrasiab, *Farkhanda Jabeen

*Department of Botany, University of the Punjab, Lahore, Pakistan

Corr Author Email: wise.librans15@gmail.com**Abstract:**

Grapes are rich in phenolic compounds, both flavonoids and non-flavonoids. The aim of the present study is to evaluate antioxidant of methanol extract of Grape leaves of different indigeneous and European cultivars of Pakistan. The results showed that grape leaves extract had clear antioxidants activities. The antioxidant activities of all the extracts were examined using three complementary methods, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, Hydrogen peroxide (H₂O₂) scavenging assay and reducing power scavenging assay. Although all the cultivars exhibited antioxidant activity at different extents but the Thompson cv. showed maximum activity as compared to others in all methods.

Key words: Grapes, methanolic extract, antioxidant activity, DPPH, Hydrogen peroxide, Total reducing power.

Corresponding author:

*Naila Ali,

*Department of Botany,
University of the Punjab,
Lahore, PakistanEmail: wise.librans15@gmail.com

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INTRODUCTION:

Antioxidants can retard the oxidation process by scavenging free radicals. Recent epidemiological studies have proved the associations between the consumption of food rich in antioxidants and the prevention of oxidative-stress related diseases (Sies, 1997). However, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are also available but restricted by legislative rules. These synthetic antioxidants have recently been reported to be dangerous for human health because they are toxic and carcinogenic (Gupta and Sharma, 2006; Hao *et al.*, 2007). Therefore, the search for natural and effective compounds with antioxidant activity has been intensified since last few years. To protect human beings from harmful effects of synthetic antioxidants, attention has to be paid to explore the natural and safer antioxidants for human consumption. Antioxidants, phenolic compounds and secondary metabolites present in plants are proved to be safe and healthy for human consumption (Gupta and Sharma, 2006; Pandey and Rizvi, 2009). Antioxidants are known for their very important role against free radicals or reactive oxygen species (ROS) in body defense system. These ROS are produced as byproducts by the activity of normal cells during aerobic metabolism (Benmeziane, 2017).

Hydrogen peroxide (H₂O₂) is known as non-radical reactive oxygen species (ROS) in which has the ability to enter cell membranes, inactivate enzymes by oxidation of thiol groups, and initiate lipid peroxidation (Zhang *et al.*, 2011). Antioxidants cause oxidation of food material which prevent many physiological diseases because of this property antioxidants are popular among all the nutrients.

The literature data reported that plant leaves are a source of phenolic compounds exhibiting antioxidant activity (Nishino & Yoshida, 2002; Naczka *et al.*, 2003; Amaral *et al.*, 2005; Pari *et al.*, 2007).

Our study is based on antioxidant activity of different cultivars of grapes. In Pakistan, grapes rank at 10th position among fruits (Ali *et al.*, 2017). The aim of this study is to compare the potential antioxidant activity of seven different cultivars of grapevine leaf extracts from Pakistan in order to evaluate the grapes as a potential source of natural antioxidant for food preservative and pharmaceutical applications.

MATERIALS AND METHODS:**Sample Collection**

Seven different indigenous and european cultivars of grapes were selected for the experiment named as; Red Globe(RG), Th(Thompson), AR(Autumn Royal),

CS(Crimson Seedless), KR(Kings Ruby), Per(Perlette), Sun(Sunderkhani). These cultivars were collected from different areas of Pakistan.

Sample Preparation and Extraction

For sample preparation, 100 g of leaves of each cultivar of grapes were taken and washed thoroughly with distilled water. According to the procedure of Khamsah *et al.*, (2006), the drying process was done. The leaves were spread on paper sheet for 3-4 days in shade. Then these dried samples were crushed into fine powder and dipped into extracting solvent which was methanol for 24 hours. After this, the samples were filtered by using Whatman filter paper I. Soxhlet method was performed for extraction (Siddhuraju *et al.*, 2002). The samples were then dissolved in the extracting solvent (methanol) in a round bottom flask. The flask were then assembled in the Soxhlet apparatus and was left running for 12 hours at 60°C. To remove the extracting solution from the extracts rotary evaporation method was used. And then finally the remaining methanol was removed by freeze drying and the samples were kept at 4°C until use.

a. DPPH Scavenging Assay

For DPPH scavenging assay, 0.1 M DPPH solution was prepared in methanol. One ml sample of each cultivar was taken and mixed with 2 ml of DPPH solution separately. Immediately after mixing the extract with DPPH solution, change in color was observed, which indicated the scavenging activity of plant. After 30 minutes the samples were placed in the spectrophotometer and absorbance was recorded at 517 nm. (Prakash *et al.*, 2001, Molyneux, 2004 and Marksen *et al.*, 2007). DPPH solution alone was used as a negative control. The whole procedure was performed in triplicate and following formula was used for the calculation of inhibition percentage.

Effect of scavenging (%age Inhibition) = $[1 - X \text{ sample (517nm)} / X \text{ control (517nm)}] \times 100$

X= Absorbance

b. Scavenging assay for Hydrogen peroxide

For Scavenging assay for Hydrogen peroxide the protocol of Woisky and Salatino (1988) was followed. Standard Phosphate buffer pH 7.4 was prepared and then 2 mM solution of Hydrogen peroxide was made in this buffer. One ml of each sample was mixed with 0.6 ml of Hydrogen peroxide solution. After 10 minutes absorbance was recorded at 230 nm. For the control only phosphate buffer with sample was used without Hydrogen peroxide. The percentage inhibition of different extracts at different concentrations was calculated by the following formula and compared with ascorbic acid (standard).

%age scavenging = $(\text{Control A} - \text{Sample A}) / \text{Control A} \times 100$

A= Absorbance

c. Assay of reducing power

Assay of reducing power was found as described by Oyaizu, 1986. In test tubes, different grape extract solutions (50- 400 µg/l) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%, w/v), then was incubated at 50° C for 20 minutes. After incubation, 2.5 ml of tri-chloroacetic acid (100g/l) was added to the mixture, and then it was centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml FeCl₃ (g/l) and absorbance was measured at 700nm in UV-Visible spectrophotometer. Ascorbic acid was used as standard and phosphate buffer as blank solution. (Tagashira and Ohtake, 1998; Chang *et al.*, 1998). Higher absorbance indicated greater capacity which was calculated as follows:

$$RP = [A_m/A_b - 1] \times 100$$

A_m= absorbance of reaction mixer

A_b= absorbance of blank mixture

RESULTS AND DISCUSSION:

Antioxidants delay or prevent some types of cell damage (Yadav *et al.*, 2016). Antioxidants can be man-made or natural substances. They are found in many foods, both in fruits and vegetables. Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxides. Ancient people used traditional herbal medicines, dietary foods as a source of antioxidant that protected them from the harmness caused by free radicals. In dietary supplements antioxidants are widely used and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Antioxidants are also been used in food industry as preservative in foods and cosmetics as it prevents the degradation to rubber and gasoline.

Grape is a fruit used since ancient times by man for its therapeutic values due to its richness in antioxidant molecules that can replace synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ). These antioxidants are dangerous for human health.

The content of antioxidants and total phenolics in the extracts of grapevine leaves depended on the solvent used for extraction (Amarowicz *et al.*, 2008). The use of acetone in the extract resulted in the higher values (257 mg/g) than methanol (232 mg/g). Because of the

polarity of solvents its atoms have differing electronegativities. And the atoms of nonpolar liquids did not respond to the external electric forces and resulting in some atomic polarization. Therefore, the most common efficient solvents used in the extractions are aqueous mixtures of acetone, methanol and ethanol (Nanditha and Prabhasankar, 2009). Pandey *et al.*, (2014) also reported the methods of extraction using acidified acetone, methanol and ethanol. The acidified solvents rupture cell membranes and release anthocyanins; however, this harsh chemical treatment may break down the innate anthocyanin structure. It is therefore important that solvents should be acidified with organic acids (formic or acetic acid) rather than mineral acids (Rababah *et al.*, 2011). The same tendency was observed for results shown by dry and fresh leaves. However UV spectra have no significant effect on both extracts (Amarowicz & Weidner, 2001; Mabry *et al.*, 1970).

The presence of flavanols in grapevine leaves was reported before by Amarowicz *et al.* (2007). Durmaz *et al.* (2007) also studied total phenolics in some edible leaves ranging from 0.25 to 14.22 mg/g f.m. according to his results the highest amounts were found in the leaves of mulberry (*Morus alba*), quince (*Cydonia oblonga*), and cherry (*Prunus avium*). It is worth emphasizing that the content of total phenolics in the extracts of *Vitis vinifera* leaves can be compared with those of the seeds of *Vitis riparia* and *Vitis amurensis* (Wróbel *et al.*, 2005; Weidner *et al.*, 2007). Amarowicz *et al.*, 2008 found high level of total antioxidant activity in the extracts of grapevine leaves.

In the present study, antioxidant activity of seven different indigenous and European cultivars of grapes were evaluated by DPPH assay, Hydrogen peroxide scavenging assay and assay of reducing power. Said *et al.*, 2015, prepared extracts from the dried leaves of *M. Oleifera*. The dried leaves were powdered, sieved (No. 20) and extracted (100 g) successively with 600 mL of water in a Soxhlet extractor for 18-20 h. Then extract was concentrated to dryness under reduced pressure and controlled temperature (40-50°C) as performed in our work.

A. DPPH scavenging assay

The values of inhibition expressed in fig.1B showed increasing order with increase concentration of the plant extract. DPPH radical (DPPH) scavenging effects of leaf extract of all grape cvs. at the concentrations of 50, 100, 2000, and 400 µg/mL were investigated with the standard ascorbic acid

concentrations of 50, 100, 250, 500, 1000 $\mu\text{g/mL}$. The representative grape cultivars Red Globe, Autumn Royal, Crimson Seedless, Thompson, Sunderkhani, Perlette and Kings Ruby were selected to plot one graph of DPPH scavenging activity for comparison (Figure 1B). At a concentration of 50 $\mu\text{g/mL}$, the Thompson cv. obtained the highest percent scavenging activity ($17.75 \pm 1.45\%$) among all the extracts ($p < 0.05$), while Kings ruby yielded the lowest ($16.45 \pm 0.43\%$). At the concentration of 100 $\mu\text{g/mL}$, all the cultivars in Table.1 exhibited a $21.97 \pm 0.58\%$, $21.36 \pm 3.93\%$, $26.81 \pm 1.90\%$, $30.70 \pm 0.69\%$, $34.0 \pm 2.26\%$, $23.64 \pm 0.93\%$ and $23.24 \pm 0.58\%$ respectively. With regard to % inhibition, as shown in Table 1, amongst all cultivars examined, the the Autumn Royal with the lowest range of values ($16.72 \pm 0.44\%$ to $60.88 \pm 1.11\%$), indicated the lowest DPPH scavenging activity, whilst 'thompson' with the highest value ($73.25 \pm 1.80\mu\text{g/mL}$) exhibited the maximum scavenging activity. Overall, the DPPH scavenging activity was found to be in the order of: Thompson>Sunderkhani>RedGlobe>Perlette>Kings Ruby>Crimson Seedless >Autumn Royal. Although there is limited information available in the literature on the DPPH scavenging activity of all the above mentioned grape cultivars, although values of gallic acid and Trolox are in a good agreement with some grapes cvs. in the same experimental conditions reported by Zhang *et al.*, 2011.

B. Hydrogen peroxide scavenging assay

Antioxidant activity of different concentrations of methanolic grape extract of seven grape cvs. were evaluated using the hydrogen peroxide (H_2O_2) scavenging activity. The highest percentages of H_2O_2 scavenging activity were obtained for two cultivars (Thompson and Sunderkhani) with 10.0 at concentration of 50 $\mu\text{g/mL}$. The same concentration of extract of the cultivar Autumn Royal exhibited a percentage of 10.0 which is similar to that obtained for Crimson Seedless and higher than that obtained for Kings Ruby (4.0). At higher concentration of extract (400 $\mu\text{g/mL}$) the hydrogen peroxide scavenging activity of all the cultivars was found to be in order of Thompson>Sunderkhani> Red Globe > Autumn Royal >Perlette> Crimson Seedless > Kings Ruby. These results suggest that grape extracts may serve as a potential source of natural antioxidant for food preservative and pharmaceutical application despite of the cultivars.

C. Reducing Power (RP) Assay

Reductive capabilities exhibited by plant extracts during experiment can serve as a significant indicator of their potential antioxidant activities (Meir *et al*, 1995). The potassium ferricyanide reduction method or reducing power assay is a widely used method for evaluating the RP of plant polyphenols. In this assay, the presence of antioxidants in test samples resulted in the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form by donating an electron. The Fe^{2+} was then monitored by measuring the formation of Pearl's Prussian blue (Oyaizu, 1986). Values of absorbance at 700nm and the values of RP are contrariwise to each other if the value of absorbance increases, the RP value decreases and vice versa. The reducing ability of different concentration (50, 100, 200 and 400 $\mu\text{g/mL}$) of extrats of above mentioned grape cultivars are presented in Figure 1D. All the extracts were capable of reducing Fe^{3+} and did so in a linear dose-dependent manner. Grape leaf extract (50 $\mu\text{g/mL}$) of Thompson cv. exhibited the strongest RP value (27.33 ± 0.44) followed by Red Globe (27.33 ± 0.44) while 'Kings Ruby' yielded the weakest (20.08 ± 0.3) at a concentration of 50 $\mu\text{g/mL}$. Sunderkhani also showed prominent reductive capability at the same concentration, with the RP values of 26.24 ± 1.8 . According to the values at highest concentration (400 $\mu\text{g/mL}$), the rank order of RP values was: Sunderkhani> Thompson > Autumn Royal > Red Globe >Perlette> Crimson Seedless > Kings Ruby. These results suggest that grape extracts may serve as a potential source of natural antioxidant for food preservative and pharmaceutical application even purple, red or white cultivars. As Red Globe and Crimson Seedless is a red, Autumn Royal is purple/pink, Thompson, Sunderkhani and Perlette are white cultivars of grapes.

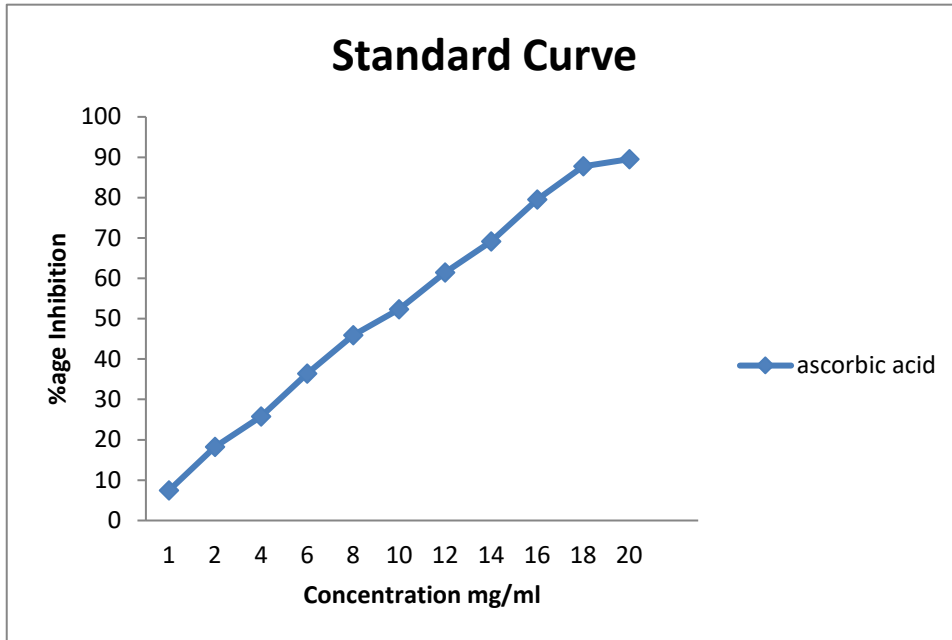
CONCLUSIONS:

The results of this study clearly indicate that methanolic extracts from grape leaves contain a considerable amount antioxidant, although the order of antioxidant potency of each cultivar evaluated by different methods does not follow the same pattern. In general, strong and positive presence of antioxidants were observed in all cultivars studied. With special attention to scavenging effects against different ROS, extracts efficiently respond to ascorbic acid. Hence, grape leaf extracts should be treated as potential free radical scavengers.

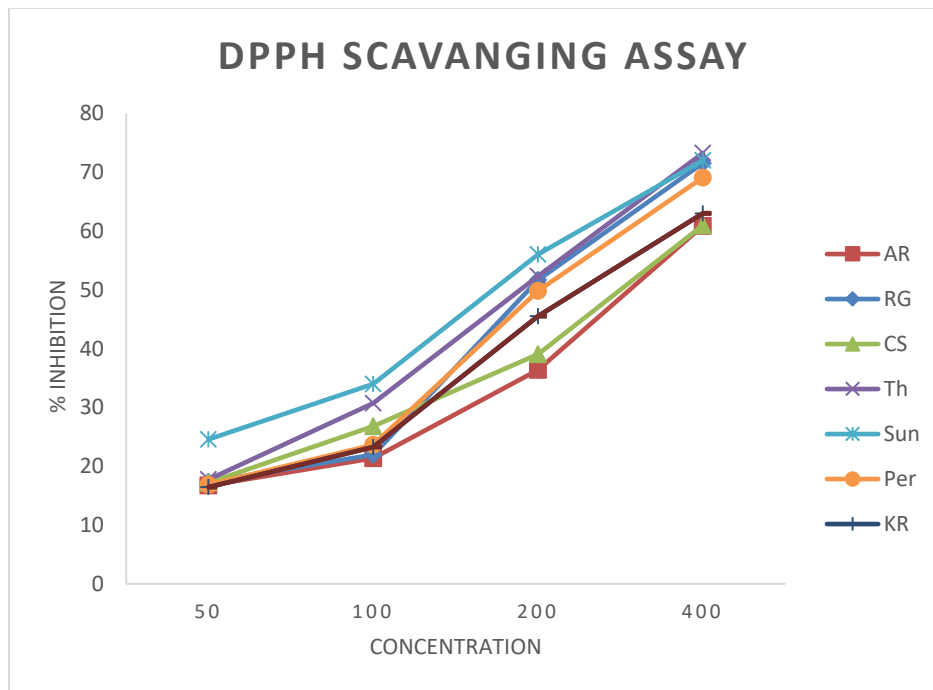
Table 1: %age inhibition values of grapes leaves extract in DPPH radicals, Hydrogen per oxide(H₂O₂) and Reducing Power(RP) Assays.

Cultivars	Extract concentrations	DPPH (%)	H ₂ O ₂ (%)	RP (%)
Red Globe	50(µg/mL)	17.34±0.48 ^g	9±0.00 ^{bcd}	27.33±0.44 ^b
	100 (µg/mL)	21.97±0.58 ^c	15±0.01 ^h	38.46±0.4 ^b
	200(µg/mL)	51.67±0.01 ^f	34±0.03 ^j	57.83±2.55 ^{hi}
	400(µg/mL)	71.55±1.91 ^g	52±0.01 ^g	71.68±3.65 ^g
Autumn Royal	50(µg/mL)	16.72±0.44 ^g	7±0.00 ^a	24.47±0.10 ^f
	100 (µg/mL)	21.36±3.93 ^c	12±0.00 ^{cd}	35.84±1.03 ^e
	200(µg/mL)	36.40±1.02 ^e	23±0.01 ^a	42.08±1.45 ^{fg}
	400(µg/mL)	60.88±1.11 ^h	33±0.02 ^b	72.56±2.65 ^g
Crimson Seedless	50(µg/mL)	17.20±0.48 ^g	7±0.00 ^b	20.28±0.20 ⁱ
	100 (µg/mL)	26.81±1.90 ^b	10±0.01 ^{cd}	32.95±0.56 ^b
	200(µg/mL)	39.06±1.22 ⁱ	13±0.01 ^{cde}	48.80±0.10 ^f
	400(µg/mL)	60.97±1.43 ⁱ	18±0.00 ^{fg}	64.91±2.02 ⁱ
Thompson	50(µg/mL)	17.75±1.45 ^{cd}	10±0.01 ^{def}	27.86±0.40 ^a
	100 (µg/mL)	30.70±0.69 ^d	12±0.00 ^{cd}	41.73±0.80 ^f
	200(µg/mL)	52.30±1.33 ^j	45±0.02 ^g	65.32±4.07 ^{ef}
	400(µg/mL)	73.25±1.80 ^k	79±0.50 ^e	83.77±2.15 ⁱ
Sunderkhani	50(µg/mL)	24.57±0.75 ^g	10±0.01 ^{def}	26.24±1.8 ^a
	100 (µg/mL)	34±2.26 ^c	12±0.01 ^{efg}	40.71±0.55 ^e
	200(µg/mL)	56±1.53 ^k	25±0.01 ^{cd}	59.83±0.93 ⁱ
	400(µg/mL)	72±3.65 ^{fg}	62±0.29 ^b	85.68±4.13 ^j
Perlette	50(µg/mL)	16.86±0.50 ^{cd}	8±0.00 ^{bc}	24.84±0.30 ^{bc}
	100 (µg/mL)	23.64±0.93 ^c	12±0.01 ^{cd}	38.70±1.03 ^e
	200(µg/mL)	49.80±0.90 ⁱ	13±0.01 ^{cde}	52.33±1.39 ^j
	400(µg/mL)	69.09±2.01 ^{efg}	25±0.02 ⁱ	70.32±3.65 ^f
Kings Ruby	50(µg/mL)	16.45±0.43 ^b	4±0.00 ^a	20.08±0.3 ^{bc}
	100 (µg/mL)	23.24±0.58 ^c	10±0.01 ^{cde}	31.95±3.93 ^a
	200(µg/mL)	45.51±1.43 ^{gh}	12±0.00 ^{bc}	46.33±1.11 ^h
	400(µg/mL)	63.0±3.25 ^{de}	16±0.00 ^{fg}	64.03±3.25 ^{de}

^a Values (mean ± SD, n = 3) with the same lowercases are not significantly different within each column according to Duncan's new multiple range test (p < 0.05).



A.



B.

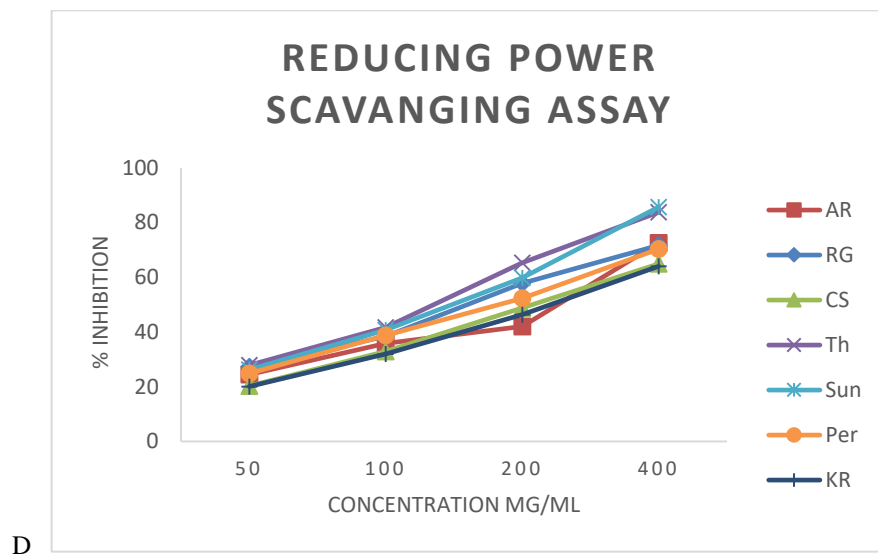
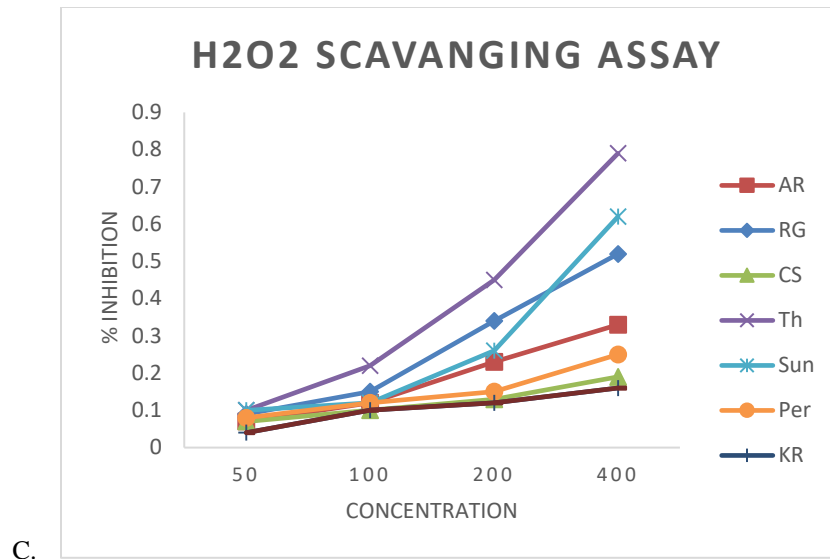


Figure 1: A: Standard calibration curve of ascorbic acid for determination of antioxidants B: Antioxidant activity of Seven *Vitis vinifera* cultivars using DPPH scavenging assay. Concentration in $\mu\text{g/mL}$. Data expressed as average of three determinations \pm SD C; Antioxidant activity of Seven *Vitis vinifera* cultivars using Hydrogen peroxide scavenging assay. Concentration in $\mu\text{g/mL}$. Data expressed as average of three determinations \pm SD D; Antioxidant activity of Seven *Vitis vinifera* cultivars using Reducing Power scavenging assay. Concentration in $\mu\text{g/mL}$. Data expressed as average of three determinations \pm SD

Legends: Red Globe(RG), Th(Thompson), AR(Autumn Royal), CS(Crimson Seedless), KR(Kings Ruby), Per(Perlette), Sun(Sunderkhani)

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