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ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACTS OF INDIGENOUS AND EUROPEAN GRAPE VARIETIES FROM PAKISTAN

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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 indigineous and European varieties of Pakistan. The results showed that grape leaves extract had clear antioxidants activities. The antioxidant activities of all the extracts were examined usung three complementary methods, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, Hydrogen peroxide (H_2O_2)scavenging assay and reducing power scavenging assay. Although all the varieties exhibited antioxidant activity at different extents but the Thompson showed maximum activity as compared to other varities in all methods.

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

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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-stressrelated 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 antioxidnatsattenion has to be paid to explore the natural and safer antioxidantsfor 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 (H_2O_2) 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; Naczk*et al*., 2003; Amaral*et al.,* 2005; Pari*et al*., 2007).

Our study is based on antioxidant activity of different varieties 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 antioxidantactivitieof seven different varieties 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 europeaon varieties 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 varieties were collected from different areas of Pakistan.

Sample Preparation and Extraction

For sample preparation,100 g of leaves of each variety 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 Whattman 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℃. To remove the extracting solution from the extracts rotary evaporation method was used. And then finally the remaining methnol was removed by freeze drying and the samples were kept at 4℃until use.

a. DPPH Scavanging Assay

For DPPH scavenging assay, 0.1 M DPPH solution was prepared in methanol. One ml sample of each variety was taken and mixed with 2 ml of DPPH solutionseperately. 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 sample (517nm) /X control (517nm)] ×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 solutionof 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 = (Control A –Sample A) / Control A × 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 50º 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 $FeCl_3$ (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.,* 2002). 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). Antioxidnats 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 dietrysupplemets 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 didnot 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 studies total phenolics in some edible leaves in range 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 (*Prunusavium*). It is worth emphasizing that the content of total phenolics in the extracts of Vitisvinifera leaves can be compared with those of the seeds of Vitisriparia and Vitisamurensis (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 varieties of grapes were evaluated by DPPH assay, Hydrogen peroxide scavenging assay and assay of reducing power. Said *et al*., 2015, prepared extratcts 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 ordr with increase concentration of the plant extract.DPPH radical (DPPH) scavenging

effects of leaf extract of7grape 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 µg/mL. The representative grape cultivars Red Globe, Automn 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 50µg/mL, the variety Thompson obtained the highest percent scavenging activity (17.75 ± 1.45%) among all the extracts ($p < 0.05$), while Kings ruby yielded the lowest (16.45 ± 0.43%). At the concentration of 100 µg/mL, all the varieties in Table.1 exhibited a 21.97±0.58%, 21.36±3.93%, 26.81±1.90%, 30.70±0.69%, 34.0±2.26%, 23.64±0.93% and 23.24±0.58% respectively. With regard to EC50, as shown in Table 1, amongst all varieties examined, the the variety Autumn Royal with the lowest range of EC50 (16.72 ± 0.44% to 60.88±1.11%, indicated the highest DPPH scavenging activity, followed by 'Crimson Seedless' (17.20 ± 0.48µg/mL to 60.97±1.43%), whilst 'thompson' with the highest EC50 value (73.25 ± 1.80µg/mL) exhibited the lowest scavenging activity. Overall, the DPPH scavenging activity was found to be in the order of: Autumn Royal>Crimson Seedless>Kings Ruby>Perlette>Red Globe>Sunderkhani>Thompson.Although there is limited information available in the literatures on the DPPH scavenging activity of all the above mentioned grape varieties, although EC50 values of gallic acid and Trolox are in a good agreement with somevarities of grapes 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 varieities were evaluated using the hydrogen peroxide (H_2O_2) scavenging activity. The highest percentages of H_2O_2 scavenging activity were obtained for two varieties (Thompson and Sunderkhani) with 10.0 at concentration of 50 µg/mL.the same concentration of extract of the variety 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 µg/mL) the hydrogen peroxide scavenging activity of all the varieties 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 varieties.

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 directly proportional to each other if the value of absorbance increases, the RP value also increase and vice bersa. The reducing ability of different concentration (50, 100, 200 and 400 µg/mL) ofextratcs of above mentioned 7 grape varieties are presented in Figure 1D. All the extracts were capable of reducing Fe3+ and did so in a linear dose-dependent manner. Grape leaf extract (50 µg/mL) from the variety Thompson exhibited the strongest RP (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 µg/mL. Sunder khani also showed prominent reductive capabilitiy at the same concentration, with the RP values of 26.24 ± 1.8. According to the values at highest concentration(400 µg/mL), the rank order of EC50 values for RP 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 varieties.As Red Globe and Crimson Seedless is a red, Autumn Royal is purple/pink, Thompson, Sunderkhani and Perlette are white varieties of grapes.

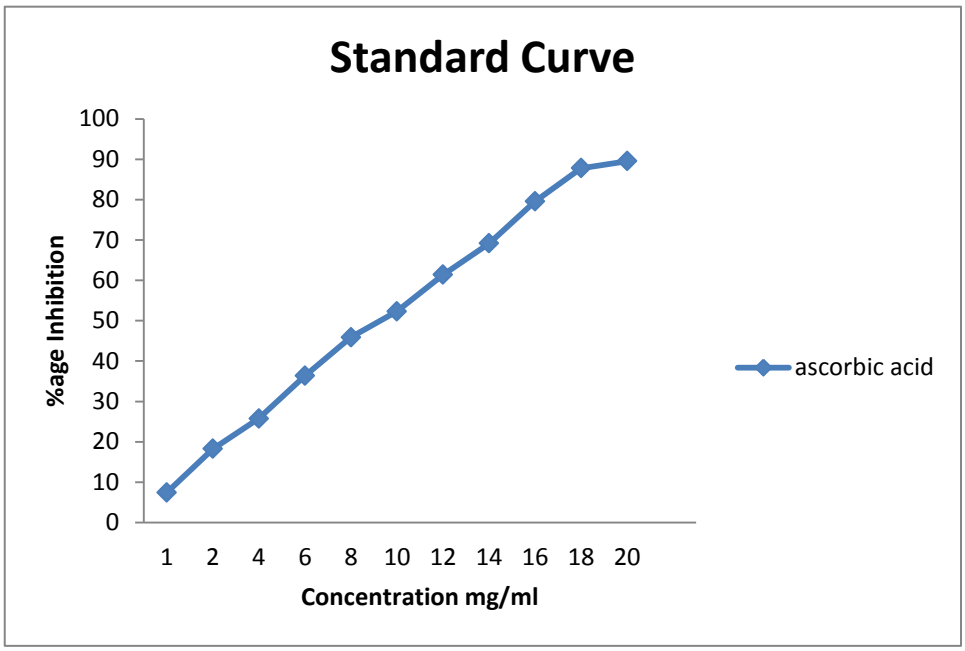
CONCLUSIONS:

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 varieties 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: EC50 values of grapes leaves extract in DPPH radicals, Hydrogen per oxide(H_2O_2) and Reducing Power(RP) Assays.

Varieties	Extract concentrations	DPPH EC50 (µg/mL)[b]	H_2O_2 EC50 (µg/mL)[b]	RP EC50 (µg/mL)[c]
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±0.00b	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±0.93i
	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±0.02i	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 ($p < 0.05$).
[b] EC50: effective concentration at which 50% radicals are scavenged.
[c] EC50: effective concentration at which the absorbance is 0.5.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Concentration mg/ml
ascorbic acid

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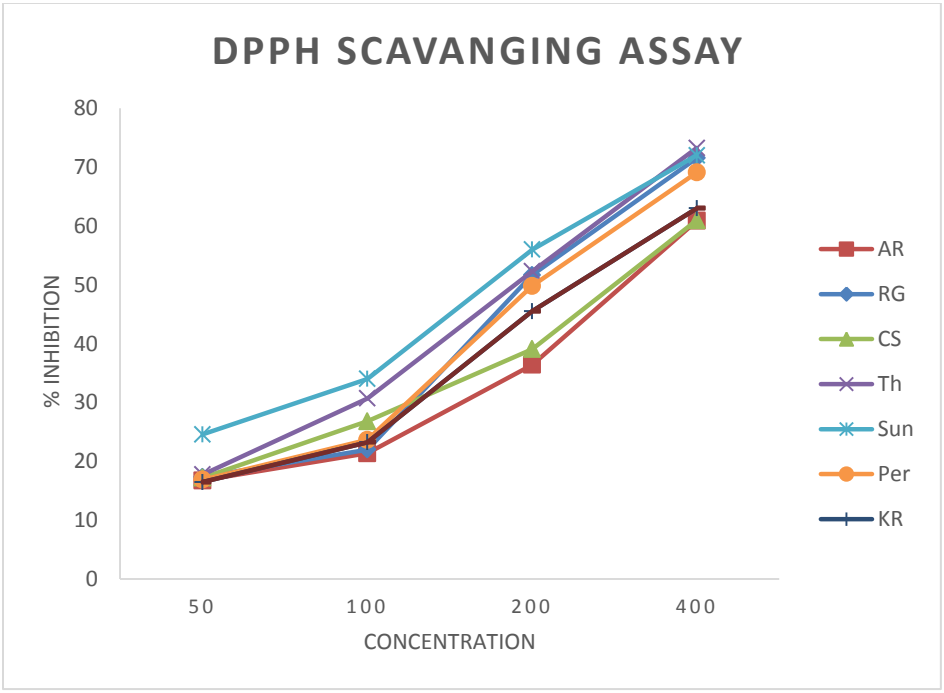
DPPH SCAVANGING ASSAY
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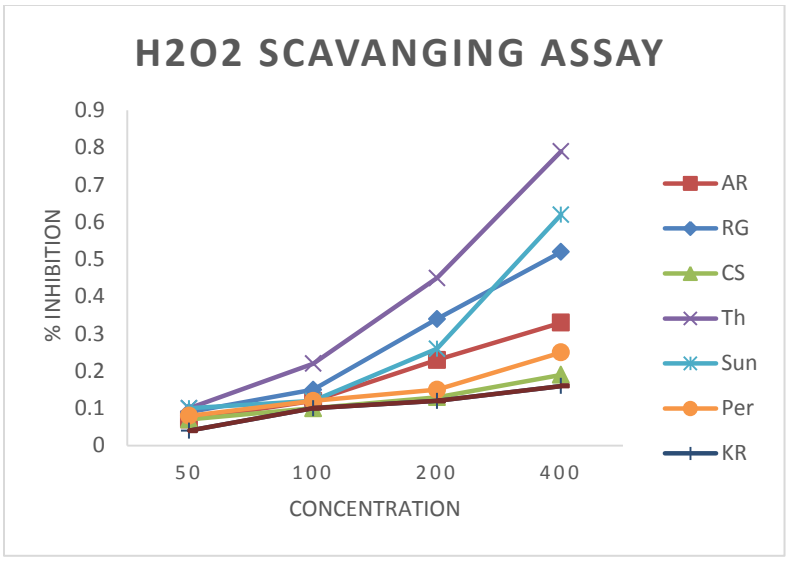

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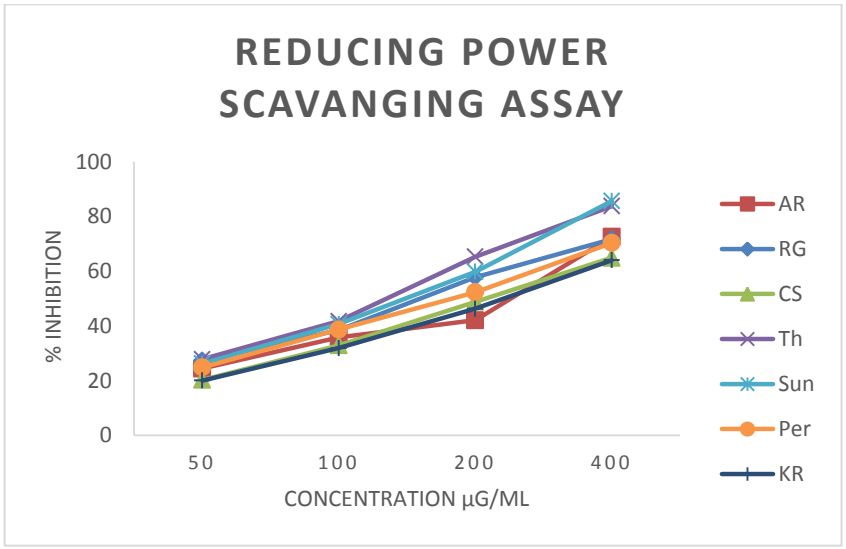

Figure 1:A: Standard calibration curve of ascorbic acid for determination of antioxidants B: Antioxidant acticity of Seven *Vitisvinifera* varieties using DPPH scavenging assay.Concentration in µg/mL.Data expressed as average of three determinations ± SD C; Antioxidant acticity of Seven *Vitisvinifera* varieties using Hydrogen peroxide scavenging assay.Concentration in µg/mL.Data expressed as average of three determinations ± SD D; Antioxidant acticity of Seven *Vitisvinifera* varieties using Reducing Power scavenging assay. Concentration in µg/mL.Data expressed as average of three determinations ± SD

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

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