Int J Pharm Bio Sci 2013 July; 4(3): (P) 609 - 617

Research Article **Pharmacognosy**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

SCREENING AND IDENTIFICATION*IN VITRO* ANTIOXIDANT ACTIVITIES OF PHYTOCHEMICAL COMPOUNDS IN ETHANOLIC GRAPE (*VITIS VINIFERA*) SEED EXTRACT

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ABSTARCT

Grape (*Vitis vinifera*) seed extract found to be exhibits wide range of beneficial effects insights to screen the potential compounds which includes polyphenolic compounds both flavonoids and non-flavonoids that show antioxidant effects. In this present study phytochemical screening of alkaloids, flavonoids, glycosides, polyphenol and flavonoids, tannins, and sterols were presented in the ethanolic grape seed extract. The *In vitro*antioxidant activity was estimated by IC_{50} value and the values are65.44 µg/ml for DPPH radical scavenging assay and inhibition of hydroxyl radical, superoxide anions, hydrogen peroxide anions and lipid peroxide inhibition assay with IC_{50} values of 77.33, 92.43, 88.15 and 83.54µg/ml, respectively. The results obtained from this study clearly indicate that ethanolic grape (*Vitis vinifera*) seed extract possesses antioxidant activity.

KEYWORDS: Grape (*Vitis vinifera*) seed extract, phyto-chemical screening, polyphenolic compounds,*In vitro*antioxidant.

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INTRODUCTION

Grape seed extract exhibits wide beneficial characteristic features initiate to screening the phytochemicals which is used remedy for conditions related to the heart and blood vessels, such as atherosclerosis (hardening of the arteries), high blood pressure, high cholesterol, and poor circulation; complications related to diabetes, swelling after an injury or surgery; cancer prevention; and wound healing. The role of oxygen radicals has been implicated in several diseases, including cancer, and diabetes mellitus[1]. Antioxidants are vital reducing substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. There is an increasing interest in natural antioxidants that present in medicinal and dietary plants, which might help to prevent oxidative damage[2]. There is a growing interest in natural and safer antioxidants, especially of plant origin [3]. More attention has been paid to the study of the antioxidative and anti-lipid peroxidation activity of natural dietary antioxidant and their protective effects against drug-induced toxicities especially whenever free radical generation is involved [4]. Grapes are one of the most widely grown fruit crops throughout the world. Grapes are antimutagenic, antineoplastic and reduce human low-density lipoprotein (LDL) oxidation and allergic inflammation [5]. Grape peel and seeds are rich sources of functional components such as phenolics and anthocyanins which have antioxidant and radical scavenging activities [6, 7]. Phenolics may also act selectively at very low concentrations to inhibit LDL oxidation *in vitro*[8, 9]. These natural antioxidants have favorable effects on human health by decreasing the heart disease risks, and being anti-carcinogenic in nature [10]. The aim of this study was to assess the phenolic compounds content and the *in vitro* antioxidant activity of seed extract of purple black grape variety from local market, with a view to exploiting its potential as a source of natural antioxidants.

MATERIALS AND METHODS

Plant material

The grapes (*Vitis vinifera*) were procured from local fruit market (Titupati, India). Seeds were isolated from grapes and dried in shadow. The dried seeds were grind to powder.

Extraction

1 kg of grape seed powder was soaked in ethanol (4 L) using with soxhlater. The solution was filtered through Whatman No.1 filter paper. The filtrate was concentrated under reduced pressure at 50°C using rotary evaporator. The crude grape seed extract was subjected to *in vitro* antioxidant and preliminary phytochemical screening.

Photochemical screening

Qualitative analysis of phytochemicals

The obtained ethanol grape seed extract were subjected to preliminary phytochemical screening and the following tests were done to check the presence of phytoconstituents [11]. Test for Alkaloids (Mayer's test), Flavonoids (Shinoda's test), Glycosides (Keller Kilani test), Saponins, Tannins, Monoterpenoid (Trim-Hill reagent test), steroids, Lignins, Phenols (Ellagic test), Cardiac glycosides (Raymond's test).

In vitro antioxidant studies

Determination of DPPH (1-1-diphenyl 2-picrylhydrazyl) radical scavenging activity

The DPPH free radical scavenging activity of ethanolic grape seed extract was measured by the method of [12]**.** 0.1 mM of DPPH solution was prepared in ethanol and 1.0 ml of this solution was added to 1.0 ml different concentrations (10-200 µg/ml) of ethanolic grape seed extract. Thirty minutes later, the absorbance was measured at 517nm in a spectrophotometer (Shimadzu UV-1201; Shimadzu, Kyoto, Japan)against blank. Vitamin C was used as the reference standard. Radical scavenging activity was expressed as percentage of inhibition and was calculated using the following formula:

$$\% \text{ of inhibition} = (A_0 - A_t)/A_0 \times 100)$$

Here, A_o was the absorbance of the blank (without plant extract) and A_t was the absorbance of ethanolic grape seed extract. All the tests were performed in triplicate.

Determination of Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured using modified method of [13]. Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), Ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of different concentrations of (10-150 µg/ml) ethanolic grape seed extract dissolved in ethanol, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37^0C for 1 h. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of (10%) TCA and 1.0 ml of (0.5%) TBA to develop the pink colour measured at 532 nm in a spectrophotometer (Shimadzu UV-1201; Shimadzu, Kyoto, Japan) against blank. The hydroxyl radical scavenging activity of ethanolic grape seed extract was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the following equation:

$$\% \text{ of inhibition} = (A_0 - A_t)/A_0 \times 100$$

Where, A_o was the absorbance of blank (without plant extract) and A_t was the absorbance in the presence of ethanolic grape seed extract. All the tests were performed in triplicate. Vitamin E was used as a positive control.

Determination of Superoxide radical scavenging activity

The superoxide radical scavenging activity was measured using NBT (nitroblue tetrazolium reagent) method as described by [14]. Different concentrations of (10-150 µg/ml) ethanolic grape seed extract was dissolved in ethanol and taken into ~~the~~ test tube. To this, reaction mixture consisting of 1 ml of (50 mM) sodium carbonate, 0.2ml of (12 mM) NBT and 0.2 ml of 0.1 mM EDTA solutions were added to the test tube and immediate reading was taken at 560 nm in a spectrophotometer (Shimadzu UV-1201; Shimadzu, Kyoto, Japan). Vitamin C was used as the standard control. The absorbance was recorded and the percentage of inhibition was calculated according to the following equation:

$$\% \text{ of inhibition} = (A_0 - A_t)/A_0 \times 100$$

Where, A_o was the absorbance of the blank (without extract) and A_t was the absorbance ethanolic grape seed extract. All the tests were performed in triplicate.

Hydrogen peroxide scavenging activity

The ability of the ethanolic grape seeds extract to hydrogen peroxide scavenging activity was determined according to the method of[15]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Ethanolic grape seed different concentrations (10–200 µg/ml) in ethanol were added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer (Shimadzu UV-1201; Shimadzu, Kyoto, Japan) against a blank solution containing in phosphate buffer without hydrogen peroxide after ten minutes. The percentage of scavenging of hydrogen peroxide of ethanolic grape seed extract and Vitamin C was calculated using the following equation:

$$\% \text{ of inhibition} = (A_0 - A_t)/A_0 \times 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the sample of ethanolic grape seed and standards.

Anti-lipid peroxidation assay (ALP)

The anti-lipid peroxidation assay was measured using liver homogenate of mice by the standard method[16]followed by slight modification. 2.8 ml of 10% mice liver homogenate, 0.1 ml of 50 mM $FeSO_4$ and 0.1 ml of different concentration of ethanolic grape seed was mixed. The reaction mixture was incubated for 30 min. at 37˚C. 1 ml of reaction mixture was taken with 2 ml 10% TCA-0.67% TBA in acetic acid (50%) for stopped the reaction. Then the mixture was boiled for 1 hour at 100˚C and centrifuged at 10,000 rpm for 5 min, supernatant was taken for absorbance at 535 nm in a spectrophotometer (Shimadzu UV-1201; Shimadzu, Kyoto, Japan) was against a blank (expect liver homogenate and ethanolic grape seed extract). Identical experiments were performed to determine the control (without extract and $FeSO_4$) and induced (without extract). Vitamin E was used for standard. Anti-lipid peroxidation percentage was calculated using the following formula. IC_{50} values of all experiment were calculated using different concentration of ethanolic grape seed extract.

$$\% \text{ ALP} = \frac{\text{Absorbance of Fe2 + induced peroxidation} - \text{Absorbance of sample}}{\text{Absorbance of Fe2 + induced peroxidation} - \text{Absorbance of control}} \text{ X } 100$$

RESULTS

Phytochemical analysis of grape seed extract

Phytochemical analysis of grape seed extract revealed the presence of Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids, flavonoids, glycosides. Cardiac glycosides was absent in grape seed extract (Table 1).

Table 1
Phytochemical constituents of Grape seed extract

Phytochemicals	Grape seed
Alkaloids	+
Flavonoids	+
Glycosides	+
Saponins	+
Tannins	+
Monoterpenoids	+
Steroids	+
Lignins	+
Cardiacglycosides	_
Phenols	+

+ - Presence, -- - Absence

In vitro antioxidant activity

Effect of ethanolic grape seed extract on the DPPH radical scavenging activity

The free radical scavenging activity of ethanolic grape seed extract against DPPH radical is shown in (Table 2). A 200 µg/ml of ethanolic grape seed extract and Vitamin C exhibited 79.87% and 89.43% inhibition, respectively, and it was observed that the IC_{50} value of the ethanolic grape seed extract was 65.44µg/ml

compared with that of standard Vitamin C which was 58.01µg/ml. The different concentrations of ethanolic grape seed extract (10, 20, 30, 40, 50, 100, 150 and 200 µg/ml) showed antioxidant activities in a dose dependent manner (29.23%, 32.57%, 39.65%, 45.67%, 56.12%, 64.67%, 72.73% and 79.87% of inhibition, respectively) in the DPPH radical scavenging activity assay.

Table 2
DPPH radical scavenging activity of the ethanolic grape seed extract and standard (Vitamin C)

Concentration (µg/ml)	% of inhibition of ethanolic grape seed extract	% of inhibition of Ascorbic acid
10	29.23 ± 0.42	23.12 ± 0.25
20	32.57 ± 0.35	28.34 ± 0.22
30	39.65 ± 0.26	39.6 4± 0.32
40	45.67 ± 0.41	51.45 ± 0.37
50	56.12 ± 0.32	59.25 ± 0.39
100	64.67 ± 0.13	70.44 ± 0.27
150	72.73 ± 0.32	84.45 ± 0.44
200	79.87 ± 0.24	89.43 ± 0.22
IC_{50}	**65.44 ±---**	**58.01** ± ---

Values are mean ± S.D three replicates.

Effect of ethanolic grape seed extract on the Hydroxyl radical scavenging activity

The free radical scavenging activity of the ethanolic grape seed extract against hydroxyl radical scavenging activity is shown in (Table 3). The % of inhibition of ethanolic grape seed extract (10-200 µg/ml) on hydroxyl radical scavenging was found to be 15.35%, 23.57%, 35.71%, 40.23%, 54.62%, 67.87%, 74.35% and 82.46% respectively. A 200 µg/ml of ethanolic grape seed extract and Vitamin E exhibited 82.46% and 85.65% inhibition, respectively and IC_{50}value was found to be 77.33µg/ml and 50.88µg/ml respectively. All results showed that antioxidant activity in dose dependent manner.

Table 3
Hydroxyl radical scavenging activity of the ethanolic grape seed extract and standard (Vitamin E)

Concentration (µg/ml)	% of inhibition of ethanolic grape seed extract	% of inhibition of Vitamin E
10	15.35 ± 0.44	23.45 ± 0.34
20	23.57 ± 0.56	38.58 ± 0.65
30	35.71 ± 0.64	42.53 ± 0.64
40	40.23 ± 0.46	50.38 ± 0.32
50	54.62 ± 0.75	63.75 ± 0.26
100	67.87 ± 0.45	70.87 ± 0.75
150	74.35 ± 0.28	81.67 ± 0.43
200	82.46 ± 0.86	85.65 ± 0.23
IC_{50}	**77.33 ± 0.25**	**50.88 ± 0.19**

Values are mean ± S.D three replicates

Effect of ethanolic grape seed extract on the Superoxide scavenging activity

The superoxide scavenging activity of ethanolic grape seed extract was shown in (Table 4). Different concentrations of ethanolic grape seed extract (10-200 µg/ml) has strong superoxide scavenging activity exhibiting 19.45%, 25.32%, 36.44%, 42.74%, 51.89%, 54.14%, 62.52% and 73.42% inhibition, respectively. The IC_{50} Value of the ethanolic grape seed extract was 92.43

µg/ml compared with that of standard Vitamin C which was 50.17 µg/ml respectively. All results showed scavenging activity in dose dependent manner.

Table 4
Super oxide scavenging activity of the ethanolic grape seed extract and standard (Vitamin C)

Concentration (µg/ml)	% of inhibition of ethanolic grape seed extract	% of inhibition of Vitamin C
10	19.45 ± 0.25	28.44 ± 0.33
20	25.32 ± 0.26	37.76 ± 0.42
30	36.44 ± 0.36	48.23 ± 0.26
40	42.74 ±0.32	54.53 ± 0.19
50	51.89 ± 0.25	60.34 ± 0.23
100	54.14 ± 0.23	63.15 ± 0.32
150	62.52 ± 0.45	73.82 ± 0.29
200	73.42 ± 0.51	79.34 ± 0.37
IC_{50}	**92.43 ± 0.27**	**50.17 ± 0.22**

Values are mean ± S.D three replicates

Effect of ethanolic grape seed extract on Hydrogen Peroxide scavenging activity

The free radical scavenging activity of the ethanolic grape seed extractagainst Hydroxyl radical scavenging activity is shown in (Table 5). The various concentrations of ethanolic grape seed extract (10-200 µg/ml) showed 21.25%, 28.56%, 36.67%, 43.76%, 49.33%, 55.62%, 63.45 %, and 75.67% inhibition, respectively. Results was shown that the percentage of inhibition in a dose dependent manner. A 200 µg/ml of ethanolic grape seed extract and Vitamin C exhibiting 88.15% and 79.27% inhibition, respectively and the concentration of ethanolic grape seed extract needed of 50 % of inhibition was found to be 88.15 µg/ml and 62.51 µg/ml was needed for Vitamin C.

Table 5
Hydrogen peroxide scavenging activity of the ethanolic grape seed extract and standard (Vitamin C)

Concentration (µg/ml)	% of inhibition of ethanolic grape seed extract	% of inhibition of Vitamin C
10	21.25 ± 0.12	26.69 ± 0.15
20	28.56 ± 0.22	34.62 ± 0.12
30	36.67 ± 0.35	43.67 ± 0.23
40	43.76 ± 0.22	48.33 ± 0.32
50	49.33 ± 0.19	54.54 ± 0.42
100	55.62 ± 0.21	64.46 ± 0.24
150	63.45 ± 0.48	74.35 ± 0.43
200	75.67 ± 0.24	79.27 ± 0.33
IC_{50}	**88.15 ± 0.29**	**62.51 ± 0.24**

Values are mean ± S.D three replicates

Effect of ethanolic grape seed extract on the lipid peroxidation inhibition activity

The lipid peroxidation inhibition activity of ethanolic grape seed extract was shown in (Table 6). A 200 µg/ml of ethanolic grape seed extract and Vitamin E exhibited 74.83% and 82.56% inhibition, respectively, and it was observed that the IC_{50} Value of the ethanolic

grape seed extract was 83.54µg/ml compared with that of standard Vitamin E which was 63.71 µg/ml. The different concentrations of ethanolic grape seed extract (10, 20, 30, 40, 50, 100 and 150 µg/ml) showed antioxidant activities in a dose dependent manner (16.98%, 27.12%, 35.54%, 46.21%, 52.47%, 60.69%, 68.25% and 74.83% of inhibition, respectively) in the lipid peroxidation inhibition activity assay.

Table 6
Lipid peroxidation inhibition activity of the ethanolic grape seed extract and standard (Vitamin E)

Concentration (µg/ml)	% of inhibition of ethanolic grape seed extract	% of inhibition of Vitamin E
10	16.98 ± 0.23	24.13 ± 0.45
20	27.12 ± 0.45	31.34 ± 0.34
30	35.54 ± 0.55	43.23 ± 0.23
40	46.21 ± 0.23	50.42 ± 0.64
50	52.47 ± 0.23	58.56 ± 0.46
100	60.69 ± 0.35	63.28 ± 0.46
150	68.25 ± 0.46	70.37 ± 0.23
200	74.83 ± 0.34	82.56 ± 0.45
IC_{50}	**44.48 ± 0.37**	**35.18 ± 0.27**

Values are mean ± S.D three replicates

DISCUSSION

The phytochemical analysis conducted on ethanolic grape seed extract revealed the presence of tannins, flavonoids, steroids and saponins. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention and anticancer [17]. Thus, ethanolic grape seed extract containing this compound may serve as a potential source of bioactive compounds in the treatment of cancer. Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 [18] and this property may explain the mechanisms of antioxidative action of ethanolic grape seed extract. Flavonoids serve as health promoting compound as a results of its anion radicals [19]. The plant extract was also positive for steroids which are very important compounds especially due to their relationship with compounds such as sex hormone [20]. The presence of these phenolic compounds in this plant contributed to their antioxidative properties and thus the usefulness of these plants in herbal medicament. Phenols have been found to be useful in the preparation of some antimicrobial compounds such as dettol and cresol. This plant is used routinely among many tribes in Africa for the treatment of various diseases. The result of DPPH scavenging activity assay in this study indicates that the plant was potently active. This suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. The ability of this plant extract to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS+. The scavenging activity of ABTS+ radical by the plant extract was found to be appreciable; this implies that the plant extract may be useful for treating radical-related pathological damage especially at higher

concentration [21]. Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated [22]. The scavenging activity of this radical by the plant extract compared favorably with the standard reagents such as gallic acid suggesting that the plant is also a potent scavenger of superoxide radical. Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell [21]. Scavenging of H_2O_2 by the plant extracts may be attributed to their phenolics, which donate electron to H_2O_2, thus reducing it to water. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner.

CONCLUSION

This study affirms the *in vitro* antioxidant potential of crude extract of ethanolic grape seed extract with results comparable to those of the standard compounds such as Vitamin C and E. Further studies are needed to clarify the *in vivo* potential of this plant in the management of human diseases.

ACKNOWLEDGEMENT

We are highly thankful to DST purse programme for supporting me by providing financial assistance through fellowship in department of Zoology, Sri Venkateswara University,Tirupati.

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