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Evaluation of antioxidant and anti-initiating activities of crude polyphenolic extracts from seedless and seeded Indian grapes

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ABSTRACT

The extracts of crude polyphenols (seeds, pulp + skin, whole) from four different cultivars of Indian grapes were used in this study. The total polyphenolic contents of grape polyphenolic extracts (GPEs) were determined and their *in vitro* antioxidant and anti-initiating activities evaluated. The total polyphenolic contents, expressed in terms of gallic acid/catechin/procyanidin B3 equivalents, were found to vary significantly. Antioxidant activity of GPEs, particularly the seedless variety, was evident from significant dose-dependent inhibition of lipid peroxidation and DPPH activity. GPEs and catechin inhibited the microsome-catalysed activity of cytochrome P450 isozymes (1A1, 1A2, 2B1) in a dose-dependent manner, by the decreased formation of resorufin. The inhibitory activity of GPEs on nitrite-mediated *N*-nitrosation of dimethylamine and *N*-methylaniline appears to correlate significantly with the total polyphenolic contents. Furthermore, six individual polyphenols present in GPEs were quantitated by HPLC, wherein procyanidin B3 was a major constituent.

1. Introduction

Grapes are rich in polyphenols, of which about 8% or less are present in pulp, 46–69% in the seeds, and 12–50% in the skin (Amering & Joslyn, 1967, chap. 6). Anthocyanins, catechin, epicatechin and resveratrol are some of the characteristic polyphenols present in grapes, especially grape seeds (Bartolome et al., 1996; Zhao, Wang, Chen, & Agarwal, 1999). Some of these polyphenols are known to exert chemoprotective effects in different experimental systems (Bagchi et al., 2000; Lambert, Hong, Yang, Liao, & Yang, 2005; Zhao et al., 1999). Extract of grape seed polyphenols has been reported to exhibit antioxidant activity, scavenging both free radicals and reactive oxygen species, both *in vivo* and *in vitro* (Ahn et al., 2002; Bagchi et al., 2000). In addition, the antioxidant effects of red and white wines have been demonstrated in many experimental systems (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999). However, the effects of grape polyphenols on the activities of metabolizing enzymes involved during carcinogenesis are limited (Seo, Jung, Park, Song, & Choung, 2001), as some of the characteristic polyphenols have been related to these properties (Moon, Wang, & Morris, 2006). Resveratrol, a natural product derived from wine grapes, is known to be a selective inhibitor of human cytochrome P450 (CYP) isozymes in liver microsomes (Chang, Lee, & Ko, 2000).

Most of the experimental chemopreventive studies on grapes pertain to grape seeds and red wine or its components, while relatively little information is available on the bioactivity of crude polyphenolic fractions in grape pulp and skin, as well as in widely-consumed seedless grapes. The present study focuses on the influence of polyphenols extracted from green and black varieties of Indian grapes (whole, pulp with skin and seeds) on *in vitro* antioxidant properties which are measured by the lipid peroxidation and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assays (Sanchez-Moreno et al., 1999), and activities of CYP enzymes involved in the activation of procarcinogens. The inhibitory activity of different grape polyphenolic extracts on nitrite-mediated *N*-nitrosation of the secondary amines dimethylamine (DMA), *N*-methylaniline (NMA) and morpholine (Tanaka, Hayatsu, Negishi, & Hayatsu, 1998) was also studied. Furthermore, the individual polyphenols in the different grape polyphenolic extracts were quantitated by high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Chemicals and reagents

Catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, procyanidin B3, resveratrol, 2,2′-diphenyl-1-picrylhydrazyl

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(DPPH), thiobarbituric acid, adenosine diphosphate (ADP), nicotinamide adenine dinucleotide phosphate reduced (NADPH), resorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, tetraethoxypropane or malondialdehyde, gallic acid, vitamin C, vitamin E and *N*-methylaniline were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Dimethylamine (DMA) and morpholine were purchased from Thomas Baker Chemicals (Mumbai, India) and Aroclor 1254 from Monsanto Chemicals, (St. Louis, MO). Folin–Ciocalteu reagent was obtained from SD Fine Chemicals, Mumbai, India. All other chemicals and solvents were of analytical or HPLC grades.

2.2. Collection and processing of grape samples

Four different cultivars of Indian grapes (*Vitis vinifera)*, seeded Bangalore Blue (BB) and Pandhari Sahebi (PS) (green), seedless Sharad (SS) (black) and Thompson (TS) (green) were obtained in batches from the National Research Centre for Grapes (Indian Council of Agriculture Research), Pune, India during the peak season (February–March). Immediately after collection, the grape samples were washed thoroughly to remove pesticide residues (confirmed by HPLC and GC analysis). Seeded grape varieties were separated into three parts as whole grapes, pulp + skin and seeds. These samples, as well as whole grapes of seedless varieties, were individually crushed and completely lyophilised. The lyophilised powders were used for the extraction of crude polyphenols.

2.3. Extraction of crude polyphenols

Extraction of crude grape polyphenols was carried out using a standard solvent extraction procedure (Zhao et al., 1999). Briefly, 100 g each of freeze-dried grape samples, which corresponds to approximately 400 g of fresh grapes, were macerated three times with 800 ml of 100 mM acetate buffer, pH 4.8 in water/acetone (30:70 v/v) for 12 h at room temperature, each time. The three macerates were combined and concentrated *in vacuo* at <35 °C. The concentrated solution was then extracted four times with 200 ml of ethyl acetate each time. The four ethyl acetate extracts were combined and evaporated to dryness, and the powdered crude grape polyphenolic extracts (GPEs) thus obtained were stored in the dark at −20 °C until tested. This extraction procedure was carried out for all the samples to maintain uniformity in obtaining crude polyphenolic extracts. The yield of crude polyphenolic extract was calculated based on considering freeze-dried grape samples as 100%.

2.4. Determination of total polyphenolic content

The total polyphenolic content of each grape polyphenolic extract expressed in terms of gallic acid was measured by the modified Folin–Ciocalteu procedure (Singleton & Rossi, 1965). Briefly, 10 ml of distilled water, 1 ml of GPE in either distilled water or 50% aqueous methanol and 1 ml of Folin–Ciocalteu reagent were added to a 25-ml volumetric flask. After 5 min incubation in the dark, 10 ml of a 7% sodium carbonate solution was added and the volume was made up to 25 ml with distilled water. The solutions were mixed and allowed to stand in the dark at room temperature for 30 min. The absorbance was measured at 760 nm, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard.

The total polyphenolic content was also determined in terms of catechin and procyanidin B3 by a vanillin-hydrochloric acid colorimetric method (Sun, Ricardo-da-Silva, & Spranger, 1998). In brief, 2 ml of 10% w/v vanillin in methanol and 1 ml of 35% hydrochloric acid in water were mixed to generate a reagent. Different GPEs in 50% aqueous methanol (0.4 ml) were added to 1.2 ml of the above reagent mixture. The absorbance of the solutions was recorded at 500 nm after 15 min incubation, which were then matched with standard curves of catechin and procyanidin B3. The results are the mean of duplicate analyses for each sample and expressed as mg gallic acid/catechin/procyanidin B3 equivalents per g of crude polyphenolic extract.

2.5. Determination of antioxidant activity

2.5.1. Preparation of liver microsomes from mice and rats

After approval from the Institutional Animal Ethics Committee as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) guidelines, animals were obtained from the animal colony of ACTREC, India. Six to eight weeks old male Sprague–Dawley rats as well as Swiss albino mice were housed in polypropylene cages (five of the same gender/cage), kept in a controlled environment at 24 ± 2 °C with 50 ± 10% relative humidity under 12 h light/dark cycles and provided with food pellets and drinking water *ad libitum*.

Male Sprague–Dawley rats were injected intraperitoneally with a single dose of Aroclor 1254 (500 mg/kg body weight) dissolved in 100 μl of corn oil. These animals as well as normal Swiss albino mice were fasted for 12–15 h prior to sacrifice by employing CO_2 asphyxiation. Livers from treated rats as well as normal mice were perfused *in situ*, excised, crushed and subjected to differential centrifugation for preparation of microsomes. The protein content of the microsomes was determined and aliquots were stored at −80 °C until use.

2.5.2. In-vitro assay for lipid peroxidation

The generation of malondialdehyde (MDA) was employed as a marker for lipid peroxidation which was estimated by the modified method of Wright, Colby, and Miles (1981). Briefly, varying concentrations of GPEs were incubated with normal mouse liver microsomes (1 mg protein) suspended in 0.15 M KCl, in the presence of 2 mM ferric chloride and 5 mM adenosine di-phosphate, at 37 °C for 1 h. The reaction was stopped by addition of 10% trichloroacetic acid and 0.5% thiobarbituric acid (TBA) followed by incubation at 90 °C for 20 min. After cooling, *n*-butanol was added to each assay mix to extract the MDA–TBA complex. The *n*-butanol layer (pink chromogen) was separated by centrifugation and the absorbance was measured at 532 nm. The amount of malondialdehyde generated was calculated by using tetraethoxypropane as external standard. Inhibition of lipid peroxidation (expressed as percent inhibition) by the test compounds was measured by a decrease in the amount of MDA generation. The inhibitory concentration at 50% (IC_{50}) was obtained from the curve plotted against extract concentration. The results are mean of duplicate analyses for each sample.

2.5.3. DPPH assay

DPPH-scavenging activity of different GPEs was measured according to the method described by Brand-Williams, Cuvelier, and Berset (1995). Scavenging of DPPH represents the free radical reducing activity of compounds based on one-electron reduction. Briefly, 500 μl of each sample of GPE dissolved in either methanol or distilled water or aqueous methanol was added to 500 μl of DPPH (0.1 mg/ml methanol). After mixing vigorously for 10 s, the solution was transferred into a cuvette. At exactly 3 min readings were recorded at 517 nm. Vitamins C and E, catechin, gallic acid and procyanidin B3 were used as reference standards. Percentage of DPPH scavenged by GPEs at different concentrations was calculated as follows:

$$\% \text{ Inhibition} = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the control reaction containing all reagents except the sample and A_{sample} is the absorbance of the sample. Extract concentration providing 50% inhibition (IC_{50}) of DPPH was calculated from the graph of inhibition percentage against extract concentration. The results are mean ± SE of three determinations from two independent assays.

2.6. Determination of anti-initiating activity

2.6.1. Determination of activities of CYP isozymes

The activities of the isozymes of CYP, *viz* 1A1, 1A2 and 2B1, *in vitro*, were determined by monitoring the formation of resorufin, employing isoform-specific probe drugs, i.e., ethoxyresorufin (ER), methoxyresorufin (MR), and pentoxyresorufin (PR), respectively as described previously (Burke et al., 1985). Standard curve for resorufin was prepared prior to evaluating the activities of these isozymes of CYP. Briefly, the assay mix contained 0.1 M sodium phosphate buffer (pH 7.4), 6.25 mM $MgSO_4$, 60 μM EDTA, 5 μM ethoxyresorufin, methoxyresorufin or pentoxyresorufin, 1 mg microsomal protein (Aroclor-induced rat liver microsomes) and 100–250 μM NADPH in 1 ml total volume. Varying concentrations of GPEs as well as catechin dissolved in 0.1 M sodium phosphate buffer, pH 7.4, were incorporated in 10-μl volumes to the assay mix. The reaction time was 5 min with a pre-incubation period of 5 min without NADPH. The reaction was stopped by the addition of 2 ml chilled methanol. The precipitated protein was centrifuged down and the fluorescence of the supernatant (resorufin formed from O-dealkylation of ethoxyresorufin, methoxyresorufin and pentoxyresorufin) was measured in a spectrofluorimeter at an excitation wavelength of 550 nm and emission wavelength of 585 nm. The results expressed as pmol resorufin formed/min/mg protein are mean ± SE of at least four observations. IC_{50} values were determined from the graph plotted as concentration (μg/ml) against percent inhibition.

2.6.2. Nitrosamine formation of secondary amines in vitro

Inhibition of nitrosamine formation of secondary amines, i.e., DMA, NMA and morpholine by crude GPEs was studied, according to the method reported by Tanaka et al., 1998 with slight modification. DMA (500 mM), NMA (10 mM) and morpholine (20 mM) were incubated with sodium nitrite (10–80 mM) in the presence or absence of an inhibitory agent (GPEs, vitamin C, catechin). The medium used was 50 mM sodium citrate, and the reaction mixture (15 ml) was maintained at 37 °C. The nitrosation was started by addition of 60% perchloric acid to adjust the pH value to 3.5. For every series of inhibition experiments, a control was run in which no inhibitors were added. When the incubation was completed, the pH was adjusted to13.00 with 5 ml of 1 N NaOH to stop the reaction. The incubation periods and concentrations of sodium nitrite are specific for each amine, i.e., 5 h/80 mM for DMA, 30 min/10 mM for NMA, 1 h/20 mM for morpholine. After addition of NaCl (5 g), the mixture was extracted two times with 20 ml of dichloromethane each time. The dichloromethane solution was then dried with sodium sulfate and filtered. Sodium sulfate was washed with more dichloromethane and the total combined solution was made to 50 ml volume with dichloromethane. The quantification of nitrosamine formation was performed by directly measuring the absorbance of the solution at a wavelength characteristic of the nitrosamine group (DMA – 350 nm, NMA – 380 nm, morpholine – 360 nm). The results, expressed as percent inhibition, are the mean of three determinations of each sample.

2.7. Analysis of the main polyphenolic constituents of GPEs by HPLC

The grape polyphenolic extracts were analysed for the levels of individual polyphenols using an HPLC (Waters, Milford, MA) equipped with tertiary pump system (Waters Model 510), automated gradient controller (Waters Model 680), injection port (Waters Model U6K), LC detector (Waters Lambda Max Model 481), and data recorder (Waters Model 745). The separation was completed on a μ Bondapak column (300 mm × 3.9 mm × 10 μm; Waters). The mobile phase consisted of solvent **A** (5% v/v acetonitrile in 100 mM sodium acetate buffer, pH 4.8) and solvent **B** (70% v/v acetonitrile in 100 mM sodium acetate buffer, pH 4.8). Elution was performed at room temperature with the linear gradient as follows: 0–5 min, 100% solvent **A**; 5–10 min, 100% solvent **A** to 75% solvent **A**; 10–35 min, 75% solvent **A** to 50% solvent **A**; 35–40 min, 50% solvent **A** to 30% solvent **A**; 45–50 min, 30% solvent **A** to 100% solvent **B**; 51 min, stop of run. The flow rate was 1.0 ml/min. The column eluate was monitored at 270 nm. The individual polyphenols were quantitated by comparing HPLC retention times with known amounts of authentic standards.

2.8. Statistical analysis

One-way ANOVA followed by Tukey's HSD test or paired Student *t*-test was used to assess the statistical significance of changes in all indices with the level of significant difference set at $p < 0.05$. The correlation between two variants was analysed by the Pearson test. Statistical analysis software (SPSS version 15.0; SPSS Inc., Chicago, IL) was used for all analyses.

3. Results and discussion

3.1. Preparation of crude GPEs and determination of the total polyphenolic content

The objective of our study was to compare the chemopreventive effects of seeded grapes with those of seedless varieties. Hence, a

Table 1
Yield of crude polyphenolic extracts and total polyphenolic contents in different varieties of Indian grapes.

Grape variety	% Yield of crude polyphenolic extracts	Total polyphenolic contents (mg/g of crude polyphenolic extract)		
		Gallic acid	Catechin	Procyanidin B3
Bangalore Blue				
Whole grapes	51 ± 6.32^{a}	2.36 ± 0.01^{a}	3.42 ± 0.58^{a}	4.60 ± 0.77^{a}
Pulp + skin	41 ± 0.64^{b}	1.17 ± 0.02^{ab}	1.36 ± 0.80^{bc}	1.44 ± 0.40^{bc}
Seeds	6 ± 0.01^{abc}	64.7 ± 0.01^{abc}	527 ± 100^{abc}	694 ± 132^{abc}
Pandhari Sahebi				
Whole grapes	39 ± 0.50^{cd}	1.50 ± 0.06^{acd}	0.47 ± 0.01^{cd}	0.63 ± 0.01^{cd}
Pulp + skin	33 ± 2.00^{ace}	1.53 ± 0.03^{abce}	2.00 ± 0.14^{ce}	2.69 ± 0.15^{ce}
Seeds	3 ± 0.01^{abdef}	221 ± 0.05^{abcdef}	701 ± 190^{abdef}	938 ± 250^{abdef}
Thompson seedless	14 ± 0.25^{abdef}	0.70 ± 0.09^{abcdefg}	1.49 ± 0.03^{cf}	2.00 ± 0.02^{cf}
Sharad seedless	32 ± 0.90^{acf}	4.60 ± 0.03^{abcdefg}	28.3 ± 2.03^{cf}	39.6 ± 1.86^{cf}

Values are mean ± SE.
Different superscripts column-wise are significant among varieties ($p \leqslant 0.01$).

uniform method of standard solvent extraction procedure was employed for the extraction of polyphenols from all the varieties of grapes and their components. The results presented in Table 1 revealed significant variation (3–51%) in the yield of crude polyphenolic extracts from whole grapes and their components (pulp + skin and seeds). The total polyphenolic contents determined, using the vanillin-hydrochloric acid method for catechin/procyanidin B3 contents and Folin–Ciocalteu assay for gallic acid determination, were found to vary significantly in all the grape polyphenolic extracts. The polyphenolic contents expressed in mg/g of crude polyphenolic extract for gallic acid ranged from 0.70 to 221, for catechin from 0.47 to 701 and in the case of procyanidin B3 ranged from 0.63 to 938. The concentrations in seeds of Pandhari Sahebi and Bangalore Blue were more pronounced when compared with the values measured in whole grapes of seedless and seeded varieties, and also pulp + skin of Pandhari Sahebi and Bangalore Blue. Thus, there exists quite a large variation in the yield and polyphenolic contents among different

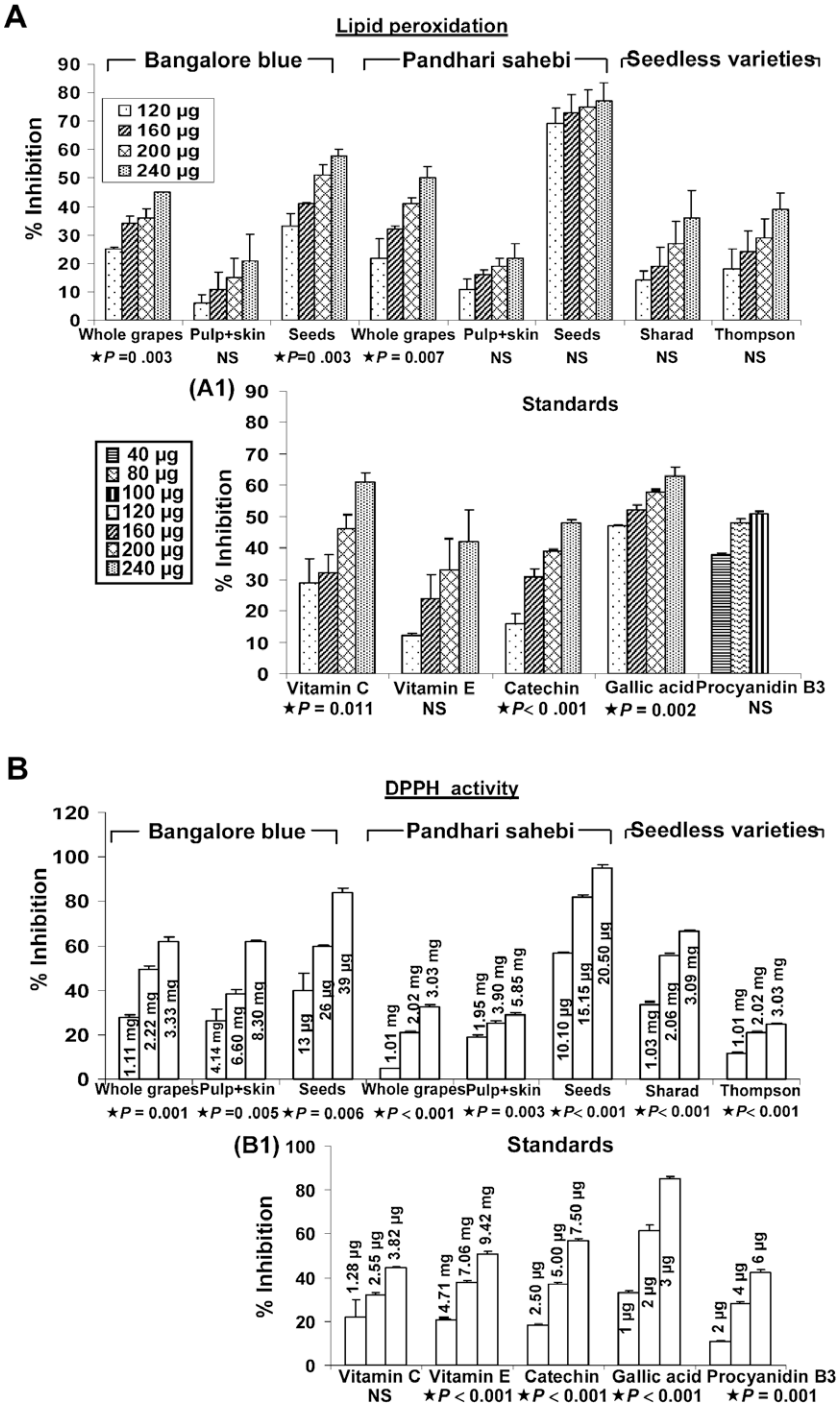

Fig. 1. Antioxidant activities of different grape polyphenolic extracts determined by lipid peroxidation assay. (A) Grape polyphenolic extracts, (A1) Standards for lipid peroxidation; and DPPH assay (B) Grape polyphenolic extracts, (B1) Standards for DPPH assay. The concentrations of GPEs and standards used for lipid peroxidation assay were in terms of µg/ml and for DPPH assay, µg/ml and/or mg/ml. Bars represent SE of mean of duplicate analyses of each sample. ★ – Test for trend in means using ANOVA; NS – not significant.

GPEs. The hierarchy in terms of percent yield of crude polyphenolic extracts of grape varieties was in the order BB whole grapes > BB pulp + skin > PS whole grapes ⩾ PS pulp + skin ⩾ SS > TS > BB seeds > PS seeds. In case of total polyphenolic contents the ranking was PS seeds > BB seeds > SS > BB whole grapes > PS pulp + skin ⩾ BB pulp + skin ⩾ TS > PS whole grapes.

3.2. Determination of antioxidant activities of GPEs

The hydroxyl radical is an extremely reactive free radical formed in biological systems, capable of damaging almost every molecule found in living cells. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity. In biological systems lipid peroxidation generates a number of degradation products, such as malondialdehyde, which have been measured as markers for oxidative stress (Gutteridge, 1995).The DPPH radical has been widely used to test the free radical-scavenging ability of various natural products (Brand-Williams et al., 1995). In the present study, antioxidant activities of crude polyphenols extracted from Indian grapes and their components were evaluated by lipid peroxidation and DPPH assays.

3.2.1. Inhibition of lipid peroxidation

The ability of grape polyphenolic extracts to inhibit microsomal lipid peroxidation was evaluated by measuring a decrease in the amount of malondialdehyde generation. As seen from Fig. 1A, both seedless and seeded GPEs displayed a rather linear dose-dependent inhibition of lipid peroxidation *in vitro*. The significant inhibitory effects were observed with extracts from seeds and whole grapes of Bangalore Blue and whole grapes of Pandhari Sahebi which were comparable with vitamin C, catechin and gallic acid, used as reference standards [Fig. 1(A1)]. The IC_{50} values of GPEs (Table 2) in descending order of their ability to inhibit lipid peroxidation were PS seeds > BB seeds > PS whole grapes ⩾ BB whole grapes ⩾ SS ⩾ TS. These values were similar to known antioxidants used as standards. The polyphenolic extracts from seedless grape varieties were more effective than pulp + skin from seeded cultivars in inhibiting microsomal lipid peroxidation (Fig. 1A, Table 2). The two possible mechanisms postulated for the inhibition of lipid peroxidation by grape polyphenolic extracts are scavenging of superoxide radicals, which are responsible for the reduction of ferric to ferrous, catalysed by the Fenton reaction, and the iron chelating activity. The observations indicate that GPEs reduced peroxyl radical formation, which was in accordance with the inhibition of NADPH and ADP-Fe^{2+} dependent microsomal lipid peroxidation by flavonoids (Middleton, Kandaswami, & Theoharides, 2000).

3.2.2. DPPH scavenging activity

Determination of free radical (DPPH) scavenging activity of different GPEs is illustrated in Fig. 1B. It is clear from the figure that all the GPEs caused significant loss in DPPH activity in a concentration-dependent manner, similar to known antioxidants like vitamins C and E, catechin, gallic acid and procyanidin B3 [Fig. 1(B1)]. Furthermore, the DPPH scavenging activity of certain GPEs was ranked based upon their IC_{50} values (Table 2). The ranking for seeded grapes and their components was PS seeds > BB seeds > BB whole grapes > PS whole grapes > PS pulp + skin > BB pulp + skin. Seed polyphenolic extracts from both varieties showed the strongest activity, comparable to vitamin C, catechin and procyanidin B3, than the corresponding pulp + skin and whole grapes. Among seedless grape cultivars, extracts from Sharad seedless were more potent as DPPH scavengers than Thompson seedless and pulp + skin of the seeded varieties. Interestingly, all GPEs were more efficient in anti-radical activity than vitamin E, which is a known strong endogenous antioxidant. It has been reported by others that vitamin E has a low free radical scavenging activity by the DPPH method (Brand-Williams et al., 1995). The reason for its poor activity in DPPH assay is that not all antioxidants show positive activity in aqueous-based assay systems since molecules like vitamin E requires tissue or cell membrane systems, based on physicochemical properties of certain lipophilicity, partitioning etc.

The polyphenolic extracts from seedless grape varieties were more effective than pulp + skin from the seeded cultivars, in inhibiting microsomal lipid peroxidation and DPPH activity. This observation appears to indicate that human consumption of seedless grape varieties may have a protective effect from oxidative stress and seedless cultivars now make up the overwhelming majority of table grape plantings. Further comparison of the results of antioxidant assays revealed that the efficiency of grape polyphenols to

Table 2
In vitro effects of Indian grape polyphenolic extracts in terms of inhibitory concentration (IC_{50} = μg/ml) on various parameters.

Sample	Lipid peroxidation	DPPH radical	Xenobiotic enzymes		
			CYP 1A1	CYP 1A2	CYP 2B1
Bangalore Blue					
Whole grapes	265 ± 5^{a}	2225 ± 125^{a}	970 ± 139^{a}	920 ± 122^{a}	310 ± 21^{a}
Pulp + skin	538 ± 88^{ab}	7300 ± 500^{ab}	–	–	–
Seeds	203 ± 13^{bc}	23 ± 1.3^{abc}	380 ± 54^{ab}	400 ± 53^{ab}	170 ± 12^{b}
Pandhari Sahebi					
Whole grapes	245 ± 10^{bd}	4750 ± 250^{abcd}	2010 ± 20^{abc}	770 ± 38^{bc}	150 ± 10^{c}
Pulp + skin	505 ± 45^{acde}	5000 ± 200^{abce}	–	–	–
Seeds	138 ± 8^{be}	9.8 ± 0.8^{abdef}	300 ± 5^{acd}	325 ± 4^{ac}	160 ± 4^{d}
Seedless varieties					
Sharad seedless	320 ± 35^{bf}	2100 ± 200^{bdegh}	390 ± 56^{abcde}	660 ± 88	100 ± 8^{aef}
Thompson seedless	290 ± 20^{bg}	5225 ± 525^{acfg}	830 ± 101^{acef}	560 ± 40	280 ± 15^{e}
Standards					
Vitamin C	208 ± 8^{be}	4.3 ± 0.1^{abdeg}	–	–	–
Vitamin E	260 ± 25^{be}	9500 ± 790^{abcdefgh}	–	–	–
Catechin	255 ± 5^{be}	6.9 ± 0.2^{abdeg}	870 ± 35^{bcdf}	440 ± 21^{a}	470 ± 26^{bcdef}
Gallic acid	163 ± 8^{be}	1.65 ± 0.05^{abdeg}	–	–	–
Procyanidin B3	97 ± 3^{befg}	5.1 ± 0.3^{abdeg}	–	–	–

Values are mean ± SE.
Means bearing different superscript(s) column-wise are significantly different ($p \leqslant 0.01$).

scavenge DPPH and to inhibit lipid peroxidation was not always the same, which could be ascribed to the types of reactions occurring in each assay and the difference in the total polyphenolic contents (Schwarz et al., 2001).

3.3. Determination of anti-initiating activity of GPEs

3.3.1. Inhibition of CYP 1A1, 1A2 and 2B1 activities

Cytochrome P450 (CYP) isozymes are xenobiotic metabolising enzymes involved in the metabolism of polycyclic aromatic hydrocarbons, nitrosamines, and other endogenous and exogenous compounds (Conney, 2003). Fruits, vegetables, herbs and other foodstuffs (as well as inedible plants) contain numerous chemical constituents known to modulate the metabolic activation of chemical carcinogens, thus providing the opportunity for selective inhibition of the carcinogenicity and toxicity of known chemicals by specific inhibition of CYP enzymes (Conney, 2003). Thus, modulation of these enzymes, which can influence the metabolism of xenobiotics, is important in terms of human health, producing effects of pharmacological and toxicological importance.

The effects of GPEs on the activities of different isozymes of CYP, *viz*, 1A1, 1A2 and 2B1 were assessed employing isoform-specific probe drugs as substrates. The observations depicted in Fig. 2 illustrate the potent inhibitory effect of GPEs on the activities of specific CYP isozymes in Aroclor 1254-treated rat liver microsomes. Varying concentrations of GPEs as well as catechin significantly brought about a dose dependent inhibition in the dealkylase activities of CYP 1A1, 1A2 and 2B1 isozymes, as judged by the formation of resorufin from ER, MR and PR, respectively (Fig. 2A–C).

To compare the efficacy of various GPEs within themselves as well as with that of catechin and also to check preferential inhibition of any specific isozymes of CYP, IC_{50} values are presented in Table 2. It is clear from the data that relatively low IC_{50} values were observed for all the GPEs and catechin in significantly inhibiting CYP 2B1 than CYP 1A1 and/or CYP 1A2 activity. Among seeded varieties of GPEs, inhibition for CYP 2B1 activity was in the order: PS whole grapes > PS seeds > BB seeds > BB whole grapes, whereas inhibition for CYP 1A1/CYP 1A2 activities both followed the order, i.e., PS seeds > BB seeds > BB whole grapes > PS whole grapes. Thompson seedless appeared to be less effective than Sharad seedless for inhibiting activity of all three isozymes of CYP. These results suggest that grape polyphenolic extracts reduce the formation of reactive intermediates of carcinogens and are in accordance with those from some *in vitro* experiments wherein other phenolic constituents especially from tea and resveratrol analogues had inhibitory effects on a microsomal CYP enzyme system (Chang et al., 2000; Ciolino & Yeh, 1999; Krishnan & Maru, 2005). Some flavonoids, which are concentration–dependent, alter CYP activity through binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acting as either AhR agonists or antagonists (Ciolino & Yeh, 1999). Inhibition of CYP 450 enzymes, including CYP 1A1, 1A2, 2E1 and 3A4, by competitive or mechanism-based systems also occurs (Moon et al., 2006).

3.3.2. Inhibition of N-nitrosamine formation

N-nitroso compounds which are widespread environmental contaminants, have received considerable attention owing to their mutagenicity and carcinogenicity. Carcinogenic *N*-nitroso compounds are formed from the reaction of naturally-occurring amines and nitrites during processing, storage and preparation of foods or are produced by bacterial reduction of nitrate and in the mammalian stomach. Human exposure to endogenously formed *N*-nitroso compounds has been related to an increased risk of gastric, oesophageal, nasopharyngeal, and bladder cancer (Mirvish, 1995). Many dietary constituents, such as vitamins C and E, polyphenols and complex mixtures, such as fruit juices, have been shown to inhibit the nitrosation reaction. Factors that influence the rates of nitrosation reactions include pH, temperature, catalysts, and inhibitors. Predictions of the extent of nitrosation are complicated by these factors. *In vitro* studies with green tea showed a strong inhibitory effect on the formation of *N*-nitroso compounds, after incubation of nitrite with salt-preserved fish extracts and green tea (Tanaka et al., 1998; Wu, Wang, Li, & Han, 1993).

Inhibition of nitrite-mediated *N*-nitrosation of DMA, NMA and morpholine by grape polyphenolic extracts was studied by quantifying the nitrosamines formed on reaction of these amines with

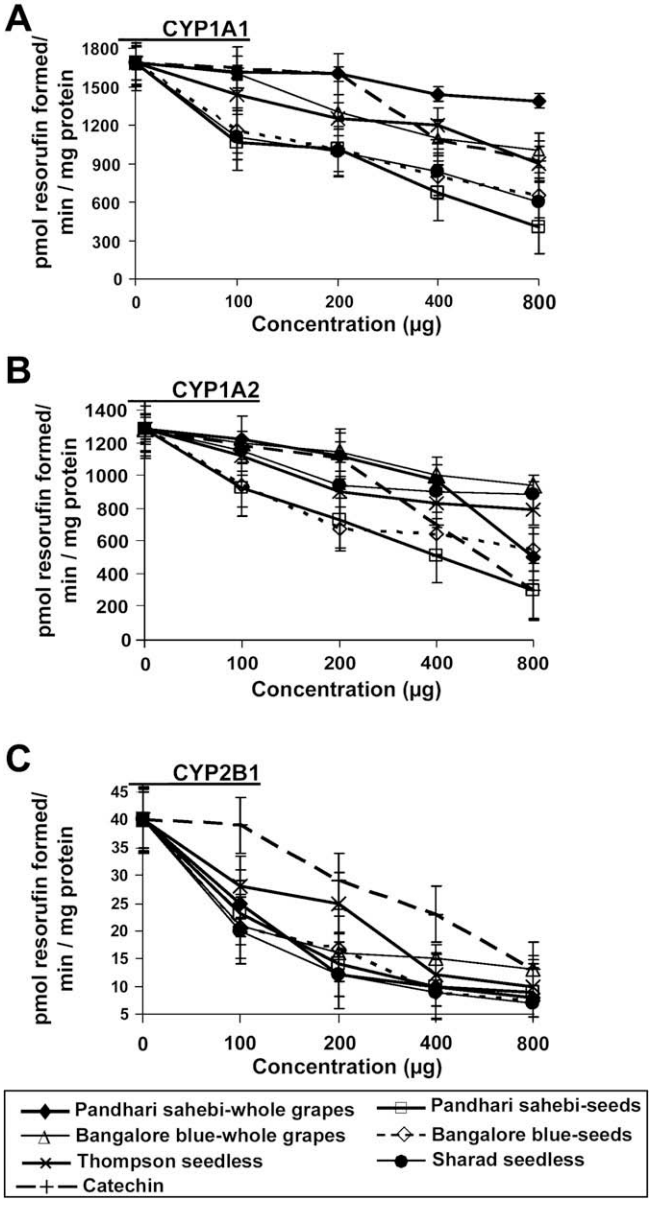

Fig. 2. Effect of grape polyphenolic extracts at different concentrations (µg/ml), on the activities of CYP 1A1, CYP 1A2 and CYP 2B1 *in vitro* in microsomes from liver of Aroclor 1254-treated rats. Data expressed as pmol resorufin formed/min/mg protein are mean ± SE of four observations. *P* values were calculated using ANOVA with test for trend in means. (A) CYP 1A1 – Pandhari Sahebi whole grapes, Pandhari Sahebi seeds, Bangalore Blue whole grapes, Pandhari Sahebi seeds, Thompson seedless, catechin – $p < 0.0001$; Sharad seedless – $p < 0.001$. (B) CYP 1A2 – Pandhari Sahebi whole grapes, Pandhari Sahebi seeds, Bangalore Blue whole grapes, Bangalore Blue seeds, catechin – $p < 0.0001$; Thompson seedless – $p = 0.001$; Sharad seedless – $p = 0.004$. (C) CYP 2B1 – Pandhari Sahebi whole grapes, Pandhari Sahebi seeds, Bangalore Blue whole grapes, Bangalore Blue seeds – $p < 0.0001$; Thompson seedless – $p = 0.001$; Sharad seedless – $p = 0.002$.

nitrite. Vitamin C and catechin (reference standards) were also tested for their inhibitory activities. All the GPEs with the exception of seeds showed inhibition of *N*-nitrosation of DMA with 500 mg/15 ml and 400 mg/15 ml concentrations, for seeded (whole grapes, pulp + skin) and seedless varieties, respectively (Table 3). In the case of *N*-nitrosation of NMA, grape polyphenolic extracts (300 mg/15 ml concentration) of both seeded and seedless varieties showed similar inhibition to that observed with DMA, while seed polyphenolic extracts of Bangalore Blue and Pandhari Sahebi showed much more inhibition with 20 mg/15 ml concentration. However, GPEs had no effect on decomposition of the *N*-nitroso derivatives of morpholine, in contrast to the reported observation of an *in vitro* inhibitory effect of high concentrations of green tea extracts on formation of *N*-nitrosomorpholine (Wu et al., 1993). The extent of inhibitory activity of grape polyphenolic extracts on *N*-nitrosation of DMA, NMA and morpholine may be due either to the structural differences and the position of amino groups in these compounds or probably as a result of catalytic effects of polyphenols on nitrosation or of another, yet unknown mechanism.

3.4. Correlation of antioxidant and anti-initiating activities with the total polyphenolic content

Total phenolic contents of several plant-derived extracts have been usually found to correlate highly with DPPH and O^{2-} scavenging activities, as well as with inhibition of carcinogenic N-nitroso compounds formation. Phenolics from plant extracts were therefore considered as the main contributors responsible for the above activities (Katsube et al., 2004; Tanaka et al., 1998). However, it has been reported that the phenolic content is not the only factor influencing the antioxidant capacity of grape juices and wines; there are other bioactive compounds such as minerals and vitamins inducing synergistic effects (Sanchez-Moreno et al., 1999). In accordance with these observations, from the present study, there exists a significant correlation between the total polyphenolic contents of a particular GPE in terms of catechin and procyanidin B3 and its DPPH scavenging ability ($r = -0.77$, $p < 0.05$). Caillet, Salmieri, and Lacroix (2006) investigated the free radical-scavenging properties of commercial grape phenol extracts and observed a good correlation between total phenolic contents and free radical-scavenging activities of the different grape extracts. Seed extract possessed the most important free radical-scavenging properties, similar to those observed in the present study, whereas the whole grape extract showed the lowest free radical-scavenging capacity.

No significant correlation was observed between total polyphenolic content of different GPEs and IC_{50} values for inhibition of lipid peroxidation ($r = -0.643$, $p = 0.09$). The total polyphenolic content of certain GPEs significantly correlated with the IC_{50} value for inhibition of only CYP 1A2 activity ($r = -0.849$, $p < 0.01$) and not in the case of CYP 1A1 or CYP 2B1 activities. These results suggest that some specific phenolic components rather than total polyphenols

Table 3
Inhibitory effects of Indian grape polyphenolic extracts in terms of *in vitro N*-nitrosation of secondary amines.

Sample	DMA (500 mM)		NMA (10 mM)	
	Concentration (mg/15 ml)[a]	% Inhibition	Concentration (mg/15 ml)[a]	% Inhibition
Bangalore Blue				
Whole grapes	500	9.52 ± 0.69	300	7.11 ± 0.84
Pulp + skin	500	17.0 ± 1.36	300	2.00 ± 0.00
Seeds	20–1000	No effect	20	15.4 ± 1.10
Pandhari Sahebi				
Whole grapes	500	16.1 ± 1.95	300	11.2 ± 0.71
Pulp + skin	500	6.80 ± 0.86	300	7.82 ± 1.57
Seeds	20–1000	No effect	20	19.7 ± 0.87
Seedless varieties				
Sharad seedless	400	11.6 ± 0.57	300	10.7 ± 1.13
Thompson seedless	400	13.4 ± 1.59	300	4.58 ± 0.83
Standards				
Vitamin C	50	33.5 ± 2.08	50	45.7 ± 0.66
Catechin	90	38.2 ± 3.56	–	–

Values are mean ± SE.
Concentration of $NaNO_2$ used was 80 mM for DMA and 10 mM for NMA.
[a] The concentrations of each GPE was for the total volume (15 ml) of reaction mixture.

Table 4
Concentrations of individual polyphenols present in grape polyphenolic extracts by HPLC.

Grape variety	Catechin	EC	ECG	EGCG	Procyanidin B3	Resveratrol	Total
Bangalore Blue							
Whole grapes	1.74 ± 0.12^{a}	1.98 ± 0.08^{a}	1.82 ± 0.10^{a}	ND	13.9 ± 0.15^{a}	0.34 ± 0.01^{a}	19.7 ± 0.39^{a}
Pulp + skin	2.32 ± 0.04^{ab}	1.30 ± 0.01^{b}	0.73 ± 0.03^{ab}	0.81 ± 0.03^{a}	12.9 ± 0.24^{b}	0.27 ± 0.01^{b}	18.6 ± 0.16^{b}
Seeds	8.06 ± 0.06^{abc}	28.1 ± 0.20^{abc}	0.91 ± 0.02^{ac}	29.4 ± 0.30^{b}	23.4 ± 0.47^{abc}	ND	89.9 ± 1.05^{abc}
Pandhari Sahebi							
Whole grapes	0.45 ± 0.02^{abcd}	1.06 ± 0.02^{cd}	0.85 ± 0.04^{ad}	ND	1.18 ± 0.04^{abcd}	0.17 ± 0.01^{ac}	3.71 ± 0.01^{abcd}
Pulp + skin	3.35 ± 0.15^{abcd}	ND	2.09 ± 0.05^{abcde}	0.53 ± 0.02^{bc}	12.9 ± 0.20^{cde}	0.11 ± 0.01^{abd}	18.93 ± 0.31^{acd}
Seeds	23.0 ± 0.05^{abcd}	44.3 ± 0.54^{abcde}	30.2 ± 0.11^{abcde}	17.5 ± 0.42^{abcd}	91.4 ± 1.12^{abcde}	ND	211 ± 4.62^{abcde}
Thompson seedless	1.20 ± 0.08^{abcdef}	0.61 ± 0.03^{ac}	0.89 ± 0.02^{ae}	0.37 ± 0.003^{acd}	3.60 ± 0.24^{abce}	0.03 ± 0.01^{abc}	8.05 ± 0.06^{abce}
Sharad seedless	1.82 ± 0.03^{bcdef}	1.58 ± 0.02^{ce}	0.83 ± 0.01^{ae}	0.93 ± 0.02^{cd}	2.35 ± 0.15^{abce}	0.54 ± 0.05^{abcd}	6.77 ± 0.33^{abce}

Values are mean ± SE expressed as mg/g of crude polyphenolic extract.
ND = not detected; EC – Epicatechin; ECG – Epicatechin gallate; EGCG – Epigallocatechin gallate.
Means bearing different superscript(s) column-wise are significantly different ($p < 0.01$).

were the critical determinants for the scavenging capacity of certain indices among grape polyphenolic extracts. Moreover, the inhibitory activities of different GPEs on *N*-nitrosation of DMA and NMA showed very high and significant correlation with catechin and procyanidin B3 contents ($r = -0.857$, $p = 0.006$ for both amines), whereas lower correlation was observed with gallic acid ($r = -0.732$ and $p = 0.039$ for DMA; $r = 0.811$and $p = 0.015$ for NMA).

3.5. Quantitation of individual polyphenolic constituents of GPEs

The grape polyphenolic extracts showed strong antioxidant and anti-initiating activities which appear to correlate significantly with the total polyphenolic contents as mentioned above. Zhao et al., 1999 had separated individual polyphenolic compounds from grape seed polyphenolic fraction using reverse phase analytical HPLC and compared retention times with those of authentic standards. By using the method mentioned above, we have separated and analysed for the levels of six main individual polyphenols, such as catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, resveratrol and procyanidin B3, in grape polyphenolic extracts. The extracts from grape seeds showed higher concentrations of the above individual polyphenolic constituents expressed as mg/g of crude polyphenolic extracts (Table 4). Moreover, the level of procyanidin B3 was significantly higher than that of the other constituents in almost all grape polyphenolic extracts. The structural identification of individual polyphenols in grape seed fraction has been carried out by Zhao et al., 1999 using HPLC profiles, physicochemical properties and spectral analysis. Hence, further studies are needed to elucidate the different mechanisms of individual polyphenolic constituents and the existence of possible synergism, if any, among these compounds.

4. Conclusions

Together with the inhibition of anti-radical (lipid peroxidation and DPPH), CYP isozyme activities and *N*-nitrosamine formation, the present study clearly demonstrates the selective suppressive effects of grape polyphenolic extracts on various indices. Polyphenolic extracts from Sharad seedless, seeds from Bangalore Blue and Pandhari Sahebi showed the strongest suppressing activity. These findings indicate that grape polyphenolic extracts, particularly from the widely-consumed seedless varieties appear to exhibit chemoprotective effects which could be attributed to their antioxidant and anti-initiating activities. These may have potential role(s) in disease protection including cancer.

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