

Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts

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Abstract

Grape seeds were powdered and the fatty material was extracted in a Soxhlet extractor with petroleum ether (60–80 °C) for 6 h. The defatted powder was extracted with acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) for 8 h each separately. The extracts were concentrated under vacuum to obtain crude extracts, which were analyzed by high performance liquid chromatography with UV detection at 280 nm. Monomeric procyanidin was found to be the major compound being at 48 and 40% in acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) extracts, respectively. These extracts were tested for antibacterial activity by pour plate method against *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. It was found that, Gram-positive bacteria were completely inhibited at 850–1000 ppm, while Gram-negative bacteria were inhibited at 1250–1500 ppm concentration. Radical-scavenging activity of grape seed extracts of acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) were compared with BHA at 25 and 50 ppm concentrations by HPLC method using 1, 1-diphenyl-2-picrylhydrazyl (DPPH). The antioxidant capacities of grape seed extracts were determined by the formation of phosphomolybdenum complex method. It was found that acetone:water:acetic acid (90:9.5:0.5) extract was better radical scavenger than methanol:water:acetic acid (90:9.5:0.5) extract.

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1. Introduction

Microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Concern over pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks of food borne disease (Tauxe, 1997). Currently there is a growing interest to use natural antibacterial compounds, like plant extracts of herbs and spices for the preservation of foods, as these possess a characteristic flavour and sometimes show antioxidant activity as well as antimicrobial activity (Smid & Gorris, 1999).

Consumption of foods containing significant amounts of polyunsaturated fatty acids has increased the importance and use of the antioxidants to prevent oxidation. The addition of antioxidants is a method of increasing the shelf life, especially of lipids and lipid containing foods. Synthetic antioxidants, such as butylated hydroxyanisole

(BHA) and butylated hydroxytoluene (BHT), have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic (Madavi & Salunkhe, 1995). Therefore, the importance of search for natural antioxidants, especially of plant origin, has greatly increased in recent years (Jayaprakasha & Jaganmohan Rao, 2000).

Grapes (*Vitis vinifera*) are considered as the world's largest fruit crops, with an approximate annual production of 58 million metric tonnes (FAO, 1997). Grape seeds are rich sources of monomeric phenolic compounds, such as (+)-catechins, (–)-epicatechin and (–)-epicatechin-3-O-gallate and dimeric, trimeric and tetrameric procyanidins and these compounds act as anti-mutagenic and antiviral agents (Saito, Hosoyama, Ariga, Kataoka, & Yamaji, 1998). Phenolics in grapes and red wines have been reported to inhibit oxidation of human low-density lipoproteins (LDL) in vitro (Frankel, Waterhouse, & Teissedre, 1995; Teissedre, Frankel, Waterhouse, Peleg, and German, 1996). Recognition of such health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary supplement (Laparra, Michaud, & Masquelier, 1979; Soleas,

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Diamandis, & Goldberg, 1997). Phenolic compounds extracted from 12 different varieties of grapes showed antioxidant activity towards LDL oxidation in vitro (Mayer, Ock-Sook Yi, Person, Waterhouse, & Frankel, 1997). Recently, Jayaprakasha, Singh, and Sakariah (2001) reported the antioxidant activity of grape seed extracts using β -carotene-linoleate and linoleic acid peroxidation methods. The procyanidin composition of grape seeds has been determined (Lee & Jaworski, 1987). Escribano-Baiton, Gutierrez-Fernandez, Rivas-Gonzalo, and Santos-Buelga (1992) have reported 17 chemical constituents in *V. vinifera* (Tintal del pais) grape seeds. Gabetta et al. (2000) reported the presence of monomers, dimers, trimers, tetramers, pentamers, hexamers, heptamers and their gallates in grape seeds. In the present work, we report the investigation on antibacterial and antioxidant activities of grape seeds, a by-product of juice and wine manufacture, to exploit its potential as a natural preservative.

2. Materials and methods

2.1. Materials

All solvents/chemicals used were of analytical/HPLC grade and obtained from E-Merck, Mumbai, India. Catechin and DPPH was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Standard procyanidins were generously provided by Dr. Sheryl A. Lazarus, Mars, Incorporated, NJ, USA.

2.2. Extraction

V. vinifera variety Bangalore blue grapes are widely grown in the States of Karnataka and Tamil Nadu, India (The Wealth of India, 1976). Grape seeds were collected from local juice processing industries. Dried grape seeds were powdered and extracted in a Soxhlet extractor with petroleum ether (60–80 °C for 6 h) to extract the fatty material. The defatted grape seed powder (100 g) was extracted in a Soxhlet apparatus for 8 h separately with 150 ml of acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5). The extracts were filtered and concentrated under vacuum (Buchi, Switzerland) to get crude extracts. The acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) extracts yielded 5.6% (w/w) and 6.1% (w/w) respectively and were stored in a desiccator.

2.3. Determination of total phenolics

The concentration of phenolics in the extracts was determined by the method of Jayaprakasha and Jaganmohan Rao (2000) and results were expressed as (+)

catechin equivalents. 5mg of each dried grape seed extract was dissolved in a 10 ml mixture of acetone and water (6:4 v/v). Samples (0.2 ml) were mixed with 1.0 ml of 10-fold diluted Folin-Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 765 nm using Genesys-5 UV-visible spectrophotometer (Milton Roy, NY, USA). The estimation of phenolic compounds in the extracts was carried out in triplicate.

2.4. HPLC analyses

Monomeric and oligomeric procyanidins was determined by the method of Adamson et al. (1999). The chromatographic system consisted of a Shimadzu LC-6A model (Shimadzu, Tokyo, Japan), fitted with a Phenomenex (Torrance, CA) 5 μ m silica column (250 \times 4.6 mm I.D.) at 37 °C. The injection system used was a 20 μ l sample loop. Detection was done by an UV-visible spectrophotometer SPD-6AV set at a sensitivity of 0.04 AUFS and a wavelength of 280 nm. Elution was carried out at a flow rate of 1.0 ml/min. The binary mobile phase consisted of (A) dichloromethane:MeOH:water:acetic acid (82:14:2:2 v/v) and (B) MeOH:water:acetic acid (96:2:2 v/v). The elution was starting with 0–17.6% B in A, 0–30 min; 17.6–30.7% B in A, 30–45 min; 30.7–87.8% B in A, 45–50 min. The column was equilibrated between injections for 10 min with initial mobile phase. The compounds were quantified using a Shimadzu C-R4A Chromatopak data processor at chart speed of 2.5 mm/min.

2.5. Determination of procyanidins in grape seed extracts

A known volume of (10 μ l) of the grape seed extract (1mg/ml) in water:EtOH (6:4) was injected on to the HPLC column. The concentration of individual procyanidins were obtained directly from the peak area and by application of the dilution factor. The concentration of procyanidins in the grape seed extract was expressed as g/100 g of grape seed extract.

2.6. Bacterial cultures

Bacterial cultures namely *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the Department of Food Microbiology, CFTRI, Mysore. The above cultures were grown in nutrient agar media (HiMedia, Mumbai, India) at 37 °C. Each bacterial strain was transferred from stored slants at 4–5 °C to 10 ml nutrient broth and cultivated at 37 °C for 24 h. Pre-culture was prepared by transferring 1 ml of this culture to 9 ml nutrient broth and cultivated for

48 h. The bacterial cells were harvested by centrifugation (1200 g, 5 min), followed by washing with saline and finally it was suspended in 9.9 ml of sterilized saline.

2.7. Growth inhibition assay

Effect of grape seed extracts on the growth of different bacteria was studied by the method of Jayaprakasha, Negi, Sagarika sikder, Jaganmohan Rao, and Sakariah (2000). Appropriate quantities of grape seed extracts in propylene glycol were transferred into different flasks containing 20 ml of melted nutrient agar to obtain final concentrations of 250, 500, 750, 850, 900, 1000, 1250 and 1500 ppm. A control sample was prepared by transferring an equivalent amount of propylene glycol to 20 ml of melted nutrient agar. 100 μ l (about 10^4 cfu/ml) of each bacterium was inoculated into flasks under aseptic conditions. The medium was then poured into sterilized petri-plates in quadruplet and incubated at 37 °C for 20–24 h. The colonies developed after incubation were counted and expressed as colony forming units per ml of culture (cfu/ml). The inhibitory effect was calculated using the following formula, % Inhibition = $(1 - T/C) \times 100$, where T = cfu/ml of test sample and C = cfu/ml of control. The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium tested.

2.8. Measurement of the radical-scavenging activity

Radical scavenging activity of grape seed extracts was assayed according to DPPH-HPLC method of Choi, Song, Vkrda, and Sawamura (2000). The 200 μ l of grape seed extracts (equivalent to 25 and 50 ppm concentration) were incubated in 0.5 mM DPPH–MeOH solution (1 ml) and 100 mM Tris–HCl buffer (800 μ l) pH 7.4 for 20 min at room temperature in the dark. The reaction mixture was then subjected to a reverse phase HPLC analysis. The chromatographic system consisted of a Hewlett-Packard HPLC model HP 1100 Series (Hewlett-Packard, CA, USA), fitted with a Waters μ -BondapakTM (Waters Corporation, Milford, MA, USA) C₁₈ column (300 \times 4.6 mm I.D). The injection system used was a 20 μ l sample loop. Detection was done by a HP 1100 Series Variable Wavelength Detector at 517 nm. The compounds were quantified using HP ChemStations software. MeOH:water (7:3) was used as the mobile phase at a flow rate of 1 ml/min under isocratic condition. The Tris–HCl buffer (1 ml) incubated in the DPPH solution (1 ml) was analyzed as a control and BHA as a standard. Radical scavenging activity was calculated from the following equation, % Radical scavenging activity = $(1 - \text{area of sample or BHA} / \text{area of control}) \times 100$.

2.9. Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of grape seed extracts were evaluated by the method of Prieto, Pineda, and Aguilar (1999). An aliquot of 0.1 ml of sample solution (equivalent to 25 and 50 ppm) was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank 0.1 ml of methanol was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in Genesys-5-UV-visible spectrophotometer (Milton Roy, New York, USA). For samples of unknown composition, water soluble antioxidant capacity was expressed as equivalents of ascorbic acid (μ mol/g of extract).

3. Results and discussion

The percentage of phenolics (catechin equivalents) in acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) extracts were found to be $46 \pm 1.6\%$ (w/w) and $38 \pm 1.4\%$ (w/w) respectively. Both extracts were injected into HPLC and peaks were compared with standard procyanidins. The identification of each procyanidin was achieved by comparing with the retention times of the standards. The percentage of chemical composition of acetone:water:acetic acid (90:9.5:0.5) extract and methanol:water:acetic acid (90:9.5:0.5) extract of grape seed are presented in Table 1. Nine compounds were identified, which constituted 95% of the extract. Monomeric procyanidins was found to be major compound present in both extracts. Extraction with methanol:water:acetic acid (90:9.5:0.5) gave a high yield of the extract with low phenolic compounds, whereas acetone:water:acetic acid (90:9.5:0.5) gave a low yield with

Table 1
Chemical composition (%) of procyanidins in grape seed extracts by HPLC^a

Oligomers	Acetone:water:acetic acid (90:9.5:0.5) extract	Methanol:water:acetic acid (90:9.5:0.5) extract
Monomers	48.0 \pm 2.98	40.4 \pm 3.67
Dimers	16.5 \pm 3.11	26.3 \pm 4.63
Trimers	10.8 \pm 1.91	15.1 \pm 3.12
Tetramers	8.1 \pm 0.92	5.5 \pm 1.21
Pentamers	5.3 \pm 0.83	3.4 \pm 0.92
Hexamers	3.2 \pm 0.052	2.8 \pm 0.67
Heptamers	2.7 \pm 0.079	1.4 \pm 0.34
Octamers	3.0 \pm 0.083	0.99 \pm 0.08
Nanomers	1.5 \pm 0.067	–

^a Values expressed are mean \pm S.D. of three experiments.

high phenolic compounds. Dumon (1990) has reported that an acetone–water mixture gives a better extraction of procyanidins from grape seeds in comparison with other extractants. The present study has confirmed that the use of acetone:water:acetic acid (90:9.5:0.5) selectively extracts more phenolics than methanol:water:acetic acid (90:9.5:0.5) in 6–8 h.

The MIC levels of grape seed extracts determined by the number of colonies developed after incubation was taken as index of growth inhibition. The results have shown that the grape seed extracts exhibited antibacterial effect against all bacteria tested as shown in

Fig. 1. Both extracts were found to be the most effective antibacterial fraction against Gram-positive bacteria when compared to Gram-negative bacteria. The MIC with methanol:water:acetic acid (90:9.5:0.5) extract was found to be 900 ppm for *B. cereus*, *B. subtilis* and *B. coagulans* and 1000 ppm for *S. aureus*. Similarly, for *E. coli* and *P. aeruginosa* the MIC was found to be 1250 and 1500 ppm respectively. On the other hand, with Acetone:water:acetic acid (90:9.5:0.5) extract the MIC for *B. cereus*, *B. subtilis* and *B. coagulans* were found to be 850 ppm and for other organisms the MIC was same as methanol:water:acetic acid (90:9.5:0.5) extract.

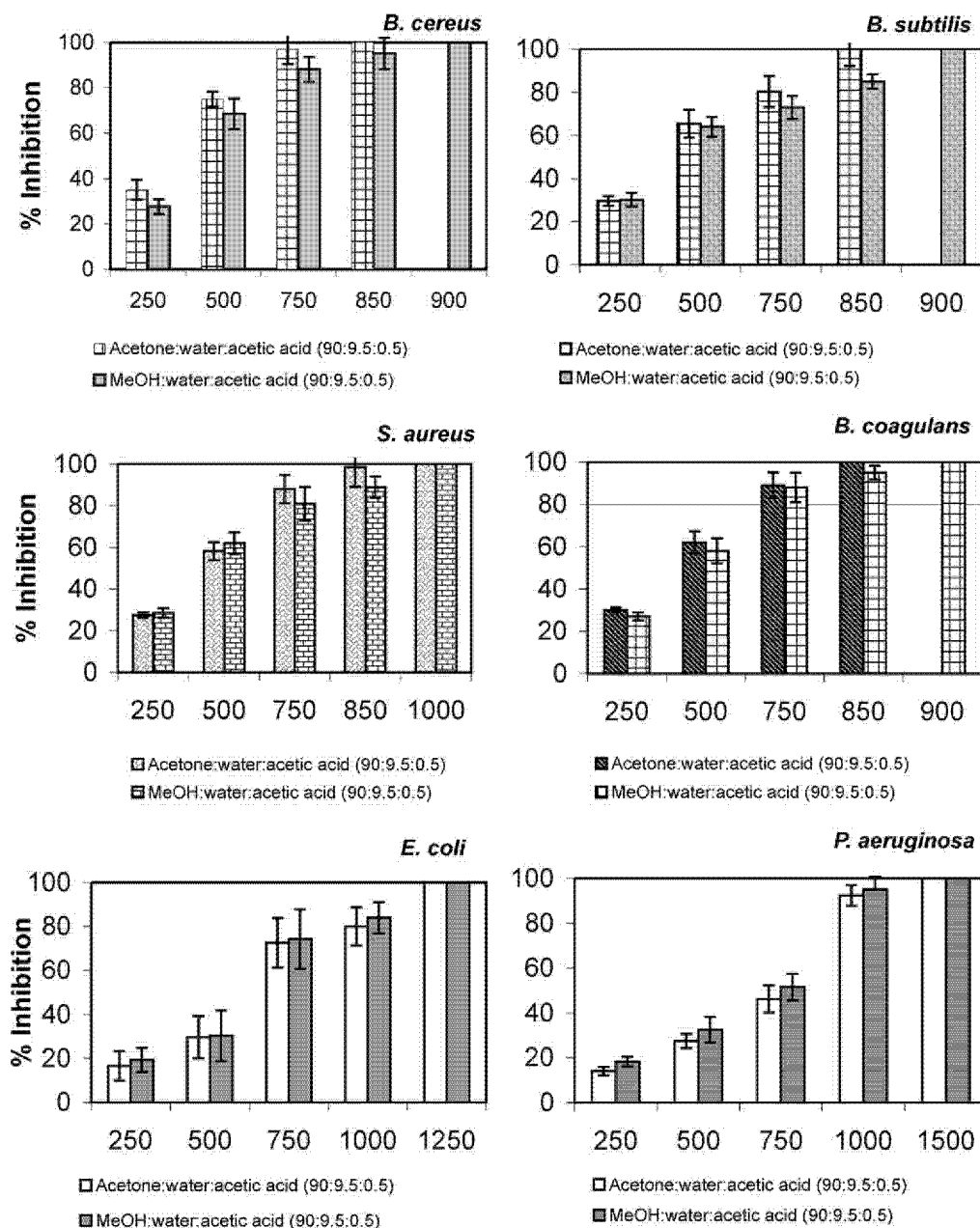


Fig. 1. Effect of grape seed extracts on growth of different bacteria at different concentrations (ppm).

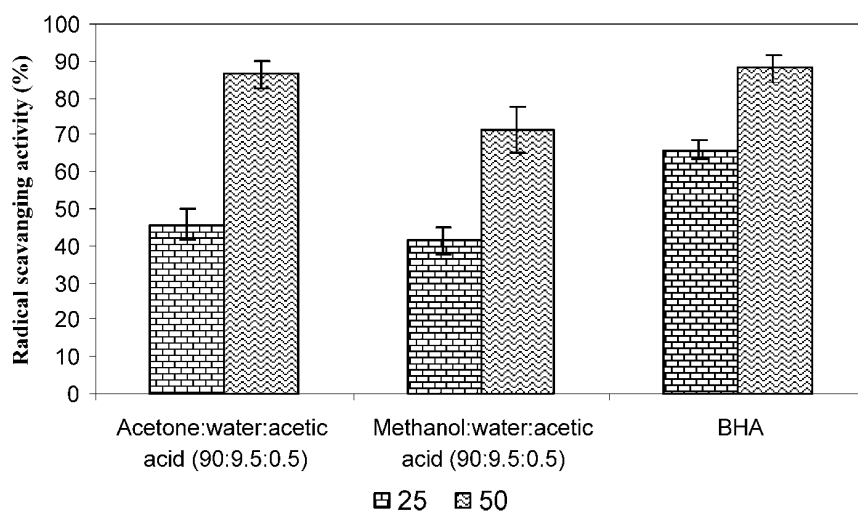


Fig. 2. Radical scavenging activity of grape seed extracts at different concentrations (ppm) by DPPH method.

Table 2

Antioxidant capacity of grape seed extracts as equivalent to ascorbic acid ($\mu\text{mol/g}$ grape seeds)^a

Extracts	25 ppm	50 ppm
Acetone:water:acetic acid (90:9.5:0.5) extract	215.6 \pm 18.2	237.1 \pm 12.3
Methanol:water:acetic acid (90:9.5:0.5) extract	208.4 \pm 13.4	233.2 \pm 21.2

^a Values expressed are mean \pm S.D. of three experiments.

Shoko et al. (1999) have reported the antimicrobial activity of methanol extract from grape seeds. The active compound for the inhibition of *E. coli* and *Salmonella enteritidis* was identified as gallic acid. Structural activity of correlation assays revealed that three hydroxyl groups of the compounds were effective for antibacterial activity and all the substituents of the benzene rings were effective against *S. aureus*.

The free radical scavenging activity of grape seed extracts was evaluated by the decrease in the peak area of the DPPH radical at 517 nm. The amount of DPPH radical decreased in the presence of grape seed extracts. Fig. 2 shows the radical scavenging activity of the two different extracts along with BHA. The radical scavenging activity of acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) extracts showed from 45.6 and 41.3%, respectively at 25 ppm concentration. A sharp increase in radical scavenging activity with an increase in the concentration of extract was observed at 50 ppm concentration. The activity of the extracts is attributed to their hydrogen donating ability (Shimada, Fujikawa, & Nakamura, 1992). It is well known that free radicals cause auto-oxidation of unsaturated lipids in food (Kaur & Perkins, 1991). On the other hand, antioxidants are believed to intercept

the free radical chain of oxidation and to give hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid (Sherwin, 1998). The data obtained reveal that the extracts are free radical inhibitors and primary antioxidants that react with free radicals.

The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex with a maximal absorption at 695 nm. The different grape seed extracts exhibited various degrees of antioxidant capacity (Table 2). The antioxidant capacity of acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) extracts showed 237 \pm 12.3 and 233.2 \pm 21.2 $\mu\text{mol/g}$ of grape seeds (as equivalent to ascorbic acid) respectively at 50 ppm concentration.

The reducing properties are generally associated with the presence of reductones (Pin-Der Duh, 1998). Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The data presented here indicated that the marked antioxidant activity of pomegranate extracts seems to be due to presence of polyphenols which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction.

Acknowledgements

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