SP-9

Phytochemical Analysis of Methanol Extract of Grape Seed - Vitis Vinifera L.

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Abstract: Grape (*Vitis vinifera L.*) is an important fruit crop grown and consumed worldwide. Grape seed the major solid waste from wine industry are rich in phenolic compounds especially proanthocyanidins that possess varied potential health benefits. The present study was aimed to analyse the photochemical constituents of methanolic extract of grape seeds. Methods: Methanol extraction was performed by cold percolation method. The phytochemicals were qualitatively and quantitatively analysed by appropriate methods viz., Spectrophotometric methods, TLC and HPLC. Results: The qualitative analysis of the phytochemicals revealed the presence of alkaloids, flavonoids, polyphenols and proanthocyanidin. Nevertheless, steroids were below detectable level in qualitative analysis. Proanthocyanidin content was high (367.75 mg/g) in the grape seed extract. This suggests that methanol extract of grape seed is a rich source of proanthocyanidin, a potential therapeutic agent. However, further clinical and animal studies need to be carried out to ascertain the same.

Key words: *Vitis vinifera,* grape seed, proanthocyanidin, phytochemical analysis.

INTRODUCTION

Grapes *(Vitis sps.)* is the widely cultivated fruit crop in the tropical and subtropical areas around the world, mainly for wine production. In India, grapes are widely grown in Delhi, Meerut in Uttar Pradesh, Hissar and Jind in Haryana, Ludhiana and Ferozpur in Punjab, Kolar and Bangalore in Karnataka, Chitoor in Andhra Pradesh, Madurai, Theni, Dindigul and Coimbatore in Tamil Nadu.[1] Grape seeds are the industrial solid waste during grape juice and wine production. Grape seeds are rich in bioactive compounds with promising applications in promoting health and pharmacological benefits.[2] Increasing evidence supports, grape seeds to be rich in proteins, fiber, lipids (omega-6 fatty acid), vitamins, complex carbohydrates and phenolics (70% of total extractable compounds).[2-5] Phenols and phenolic compounds are the amplest secondary metabolites, widely distributed in plant kingdom.[6] The most abundant phenolic compounds in grape seeds include phenolic acids (gallic acid), flavonoids- flavan-3-ol monomers (catechin, Epicatechin and epicatechin-3-O-gallate), and non-flavonoids (stilbenes and procyanidins).[4] Majority of the phenolic compounds (except gallic acid) present in grape seeds, are less soluble in water Also they possess low stability in biological fluids and hence when orally administered there is decreased bioavailability at the target sites.[7-8] From industrial perspective, grape seeds are considered as one of the predominant natural renewable resources of flavanols and polyphenols, because of their low cost and ampleness.[9] Proanthocyanidins are plant derived polyphenols abundantly available in grapes. Approximately 30% of the proanthocyanidin content is present in the seeds, nevertheless the cell walls need to be broken for the complete extraction of the proanthocyanidins.[10-11] Proanthocyanidins are also termed as condensed tannins or catechin tannins, are the biologically active constituent in grape seed extract.[12] It consists of polymers or oligomers of flavan-3-ol units that belong to the wide group of polyphenolic compounds and are byproduct of flavonoid biosynthetic pathway.[13] Based on the intraflavanic linkages, proanthocyanidins are either B-type proanthocyanidins, wherein the monomeric units are typically linked by C-C bonds (mainly C-4→C-8, less frequently C-4→C-6)) or A-type proanthocyanidins characterized by an additional C-O-C bond between C2 → O7 with a wide structural diversity.[14] Grape seed proanthocyanidins belong to type B**.** Proanthocyanidins being condensed tannins are not readily hydrolysed and are decomposable in acidic - alcoholic environment producing insoluble phlobaphenes and anthocyanidins [15-17]. Bioavailability of a compound refers to the proportion of a compound that reaches the systemic circulation following ingestion, digestion and absorption [18]. While, bioaccessibility refers to the sequential reactions including, digestive system transformations, tissue diffusion and biological activity, intestinal and hepatic metabolism, and assimilation into intestinal epithelium cells.[19] Thus, bioavailability is largely dependent on the bio-accessibility.[20] On the other hand, the degree of polymerization largely determines the bioavailability of proanthocyanidins. Proanthocyanidins are broadly classified into oligomeric (2–4 monomers) and polymeric proanthocyanidins (> 5 monomers) based on the number of monomeric flavan-3-ol units contained within it [13].GSPE are reported to possess an array of pharmacological potential including, antioxidant, anticarcinogenic, antibacterial, antiviral, antiproliferative, anti-allergic, anti-inflammatory, vasodilatory, cardioprotective neuroprotective, hypoglycemic hypolipidemic and immunostimulatory properties.[13] Owing to these biological properties, grape seed extracts have been standardized and commercially manufactured in many countries around the globe. Grape seed extracted proanthocyanidins are widely used as a food additive / nutritional supplement in Japan, China, Australia, United States, Korea, as well as in many other

countries.[21-22] Proanthocyanidins of grape seeds are potent antioxidants with higher potency than Vitamin C (20 times) and vitamin E (50 times).[6] Grape seed PCAs exhibit free radical scavenging capacity by effectively modulating the production of intracellular and epithelial nitric oxide, cell apoptotic pathways, lipoxygenase and cyclooxygenase pathways.[23] Proanthocyanidin has the potential to prevent body from sun damage, to promote collagen formation aids in improvement of vision, strengthens blood vessels and connective tissues to improve microcirculation**,** improve immune functions protect cells against drug, chemical and environmental pollutants toxicity.[13, 22, 24] French paradox, the lower incidence of cardiovascular diseases among French people though they consume a high fat diet has been associated with the increased consumption of red wine by those people. Various studies have suggested that the higher concentration of proanthocyanidin in red wine could be the plausible explanation for the French Paradox. Hence, recent studies have largely turned their focus onto complex polyphenols of grape seeds.[25] Selection of the appropriate solvent and the exact solvent:sample ratio is crucial for the higher recovery of phenolic compounds from natural plant products. Though, various organic solvents vis a vis alcohols (methanol, ethanol and alcohol:water mixture), acetone and ethyl acetate have been employed for the extraction processes, methanol is reported to be the ideal solvent for the maximum extraction of phytochemical constituents.[6] Hence, the present study was designed to evaluate the phytochemical constituents of methanolic extract of grape seed and to estimate the concentration of proanthocyanidin in the methanol extract of grape seed.

MATERIALS AND METHODS:

Preparation of Herbal Extracts:

Grape seed powder (*Vitis vinifera*) were procured from InLife Pharma Pvt Ltd., India. Grape seed extract was prepared (2:1 w/v) by cold percolation method using methanol.[26] The herbal extract was evaporated to dryness and the stock solution (100mg/mL) of the extract was prepared using 10% DMSO. The stock solutions were sterilized using Sartorius Minisart syringe filters (0.45 µm).

Phytochemical Analysis of Grape Seed Extract

Thin layer chromatography was employed to qualitatively analyze the presence of alkaloids, flavonoids and polyphenolic compounds, while, High-performance liquid chromatography (HPLC) was performed to estimate the concentration of alkaloids, flavonoids and polyphenolic compounds- proanthocyanidins in the grape seed extract. Spectrophotometric methods were employed for the determination the presence of steroids.

Analysis of Alkaloids:

An isocratic elution system containing acetonitrile and mono basic potassium phosphate was used as a mobile phase. Briefly, 9.93 gm of monobasic potassium phosphate was liquefied in 730 mL of distilled water to which 270 mL of acetonitrile was added mixed thoroughly.[27] The mixture was filtered using a 0.45 µm filter and was degassed. Standard solution was prepared with 0.2 mg of each USP Reference Standard per ml of Methanol: water (1:1 V/V). Equal volumes (10 µL) of the standard solution and the test solution were inoculated into the chromatograph separately. The liquid chromatograph, 4.6-mm × 150-mm column that was packed with L1 was adjusted at a flow rate of 1.8 mL/min. and the detection was performed at the wavelength of 235 nm. The chromatograms were documented and the major peaks areas were measured.

Analysis of Flavonoids:

Quercetin, kaempferol and isorhamnetin was purchased and used as standard in HPLC. Ten grams of the sample was refluxed with the mixture (78 mL) of alcohol, water and Hydrochloric acid (50:20:8) in a hot water bath for 135 minutes followed by the addition of methanol (20 mL) and sonication for 30 minutes.[28] The extract was filtered and the volume was made up to 100mL with Methanol. HPLC analysis was performed with a column 4.6 mm × 25 cm packing contains L1 and a detector with 270 nm at room temperature. Elution was done using the mixture of Methanol, water and Phosphoric acid (100:100:1) and the flow rate was maintained about 1.5 ml/min. The standard solutions and the test solution (20 µL) were separately injected into the chromatograph, the chromatograms were recorded, and the major peaks areas were measured and the amount of total flavonoid in sample was calculated.

Analysis of Total Phenols:

The stock solution was prepared by liquefying 30 mg of dried extract in 25 mL of methanol and water (30:70 V/V)[29] and the mixture was filtered through a sterile membrane (0.45µm). The reference standard (Chlorogenic acid CRS) solution was prepared in methanol (5mg/50mL). The working standard solution was prepared by diluting 5 mL of the reference standard in methanol and water (5:20 V/V). The test sample (30 mg) was diluted in the solvent mixture to obtain 25 mL. HPLC was performed using Shimadzu (Shimadzu, Kyoto, Japan) LC20AT system with UV detection by binary gradient method. Octadecylsilyl silica gel was used as the stationary phase in a column (4.6 mm × 25 cm) maintained at 40°C. Phosphoric acid, water (0.5:99.5 V/V) and phosphoric acid, acetonitrile (0.5:99.5 V/V) were used as mobile phase A and B respectively. The standard solutions and the test solution (25 µL) were separately injected into the system. The flow rate was set to 1.2 mL/min for a total run time of 35 min and the peaks were detected at a wavelength of 330nm. The major peaks areas were measured and recorded. The total phenols present in the sample was calculated using the formula, $A_1 \times m_2 \times p \times 0.125 / A_2 \times m_1$, where

A_1 is the peak area of the test solution, A_2 is the peak area of the reference solution, m_1 is the mass of the test extract (in mg), m_2 is mass of chlorogenic acid CRS (in mg), p is the percentage content of chlorogenic acid in chlorogenic acid CRS. Gradient elution program was proceeded with the following process.

No.	Time (min.)	Mobile Phase A % (V/ V)	Mobile Phase B % (V / V)
1.	0 – 1	92	8
2.	1– 20	92 - 75	8 – 25
3.	20 - 33	75	25
4.	33 - 35	75 - 0	25 – 100

Analysis of Steroids

The stock reference standard (Betamethasone) solution was prepared in aldehyde - free ethanol and finally diluted to obtain a working solution of the steroid (10µg / mL). The test sample solution was prepared in aldehyde free ethanol to obtain a concentration of 1 gm, aldehyde-free ethanol was included as the blank.[30] Briefly 20 mL of the blank, standard (10µg / mL) and test (1 gm) were taken in three separate screw capped test tubes. Two mL of blue tetrazolium solution and 2 mL of tetramethyl ammonium hydroxide solution (10% solution in aldehyde - free ethanol) was added to all the 3 tubes. The tubes were mixed and incubated in the dark at room temperature (25°C - 35°C) for 90 minutes. After incubation, 1 mL of glacial acetic acid was added to all the tubes and mixed thoroughly. The absorbances of the test solution and the standard solution was measured spectrophotometrically with DLAB SP-UV1000 SPECTROPHOTOMETER (CHINA) at a wavelength of 525 nm with reagent blank as reference. The total steroids in 20 mL of the test solution were calculated using the formula, At / As X Cs, Where At was the test solution absorbance; As, was the standard solution absorbance; Cs denotes the concentration of the standard (x mg/20 mL).

ANALYSIS OF PROANTHOCYANIDIN

The stock solution of the extract was prepared by liquefying 1 g of dried extract in 25 mL of the solvent mixture, HCl:CH_3OH (2:98V/V) [31] and diluting it with dilute phosphoric acid (5 ml in 20 mL), filtered and used as working solution. The reference standard solution was prepared by dissolving 10 mg of cyanidin chloride CRS in 25 mL of the solvent mixture, HCl:CH_3OH (2:98V/V) and further diluted with dilute phosphoric acid (2 ml in 100 mL) for the working solution.HPLC analysis was performed by using Shimadzu LC with 370 nm detector. The column C18 (4.6 mm × 25 cm) packed with 5µm Octadecylsilylsilica gel (stationary phase) and maintained at 30° C. The volume (10µL) of the working solution of the extract, reference standard- cyanidin chloride CRS) was injected and flow rate was set at 1 mL/min. The gradient elution was accomplished using solvent A (anhydrous formic acid and water (8.5:91.5 V/V)) and solvent B (anhydrous formic acid, acetonitrile, methanol and water (8.5:22.5:22.5:41.5 V/V)). The chromatograms were recorded, and the major peaks areas were measured. The total proanthocyanidins in the sample was calculated by the formula, $A_1 \times m_2 \times 100 \times p / m_1 \times A_2 \times 1250$, where A_1 is the peak area of test solution, A_2 is the peak area of reference solution, m_1 is the mass of the extract (in grams), m_2 is mass of chlorogenic acid CRS (in grams), p is the percentage of cyanidin chloride CRS. Gradient elution program was accomplished with the following process.

No.	Time (min.)	Mobile Phase A % (V/ V)	Mobile Phase B % (V / V)
1.	0 – 35	93 - 75	7 – 25
2.	35 – 45	75 - 35	25 – 65
3.	45 – 46	35 - 0	65 – 100
4.	46 – 50	0	100

RESULTS:

The phytochemicals such as alkaloids, flavonoids, phenols and polyphenols qualitatively analysed by thin layer chromatography revealed the presence of a high content of polyphenols (++++++) followed by flavonoids (+++++), phenols (++++) and alkaloids (++++). UV spectrophotometric analysis of revealed that steroids were below the detectable level. Quantitative assessment of the phytochemicals, alkaloids and flavonoids by high performance liquid chromatography, indicated that alkaloids were higher in concentration (12.66 mg) than the flavonoids (4.56 mg) (Fig 1, 2).

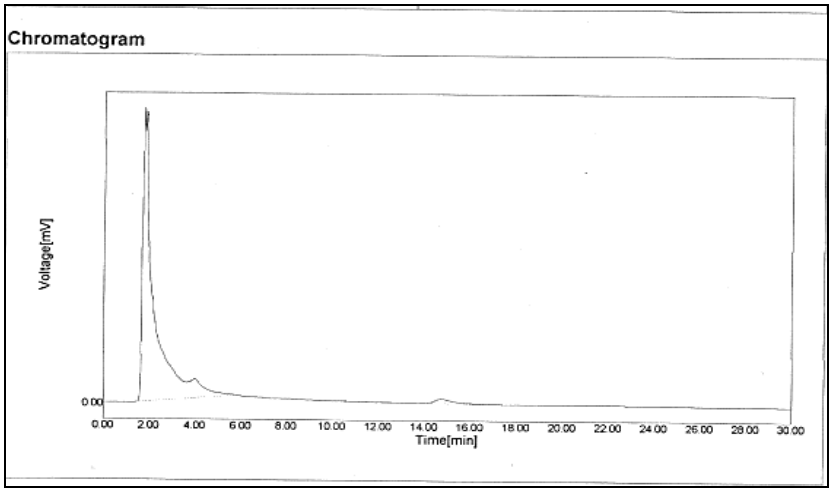

Fig 1: ANALYSIS OF ALKALOIDS - CHROMATOGRAM

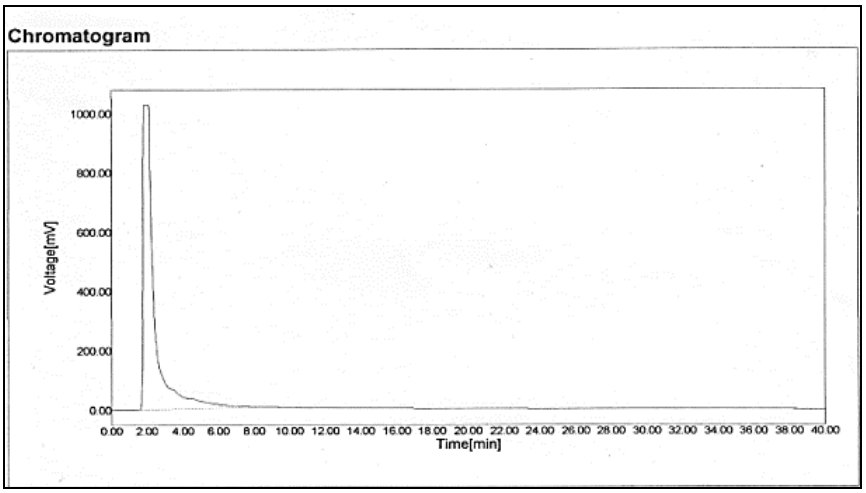

Fig 2: ANALYSIS OF FLAVONOIDS - CHROMATOGRAM

Total Phenols:

The phenols were qualitatively analysed by thin layer chromatography and quantitatively analysed by high performance liquid chromatography. A very high amount of poly phenols was indicated in TLC. The polyphenol content in the methanol extract of grape seed was found to be 22. 55 mg (Fig 3).

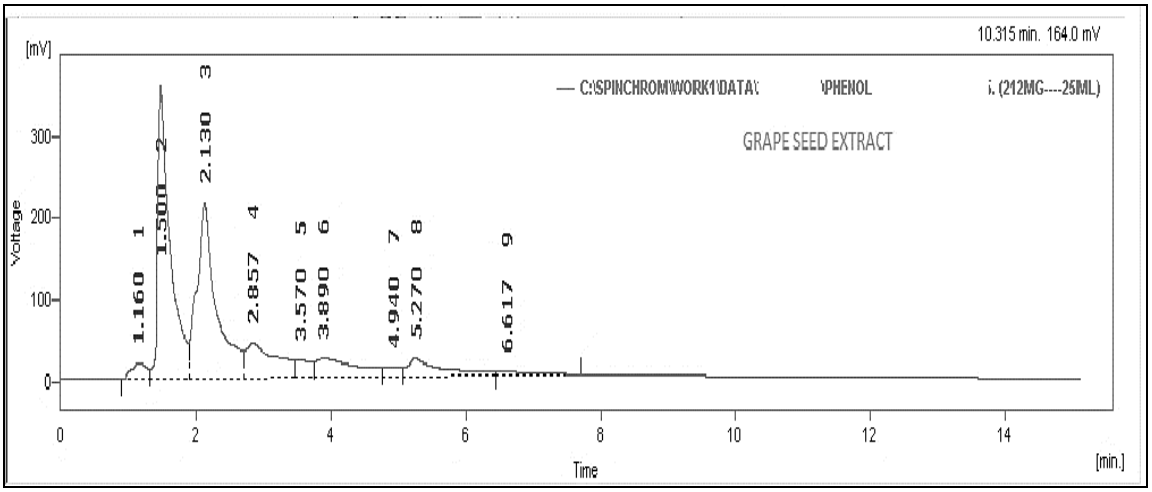

Fig 3: ANALYSIS OF TOTAL PHENOLS - CHROMATOGRAM

PROANTHOCYANIDIN:

The proanthocyanidin was analysed by high performance liquid chromatography. The retention time of the standard peak was 14.7 min/mV which corresponds with the grape seed extract sample peak 15.08 min/mV and the content of proanthocyanidin in grape seed extract was reported as 367.75 mg/g (Fig 4).

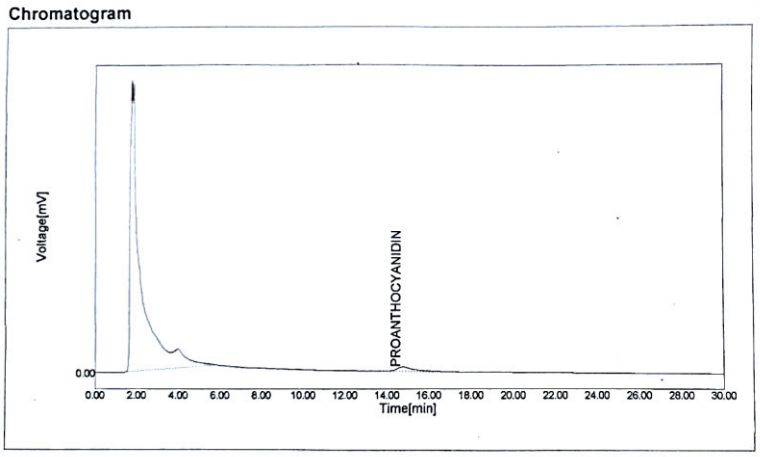

Fig 4: ANALYSIS OF PROANTHOCYANIDIN - CHROMATOGRAM

DISCUSSION

Grape is one of the widely cultivated fruit crops around the world and rich in bioactive components with health promoting and disease managing properties. Grape seeds have 70% of total extractable phenolic compounds, lipids and vitamins. The most commonly found phenolic compounds are phenolic acids, flavonoids and non-flavonoids (tannins and stilbenes). The grape seed has increased amount of total phenolics compounds followed by skin (28-35%) and pulp (≤ 10%).[13] In our study, a high content of polyphenols (22.55 mg) was noted while steroids were less in the methanol extract of grape seed. The phytochemical constituents of the methanol extract of grape seed included, polyphenols, alkaloids and flavonoids. Owing to their sour taste, proanthocyanidins are used in fruit juices and beverages to increase their shelf life. Proanthocyanidins are used as food additives to increase salivary viscosity and microbial stability, to improve heat stability and oxidative stability and foamability.[13] Various studies have documented that grape seed proanthocyandin exhibits a wide range of chemoprotective, biological and pharmacological properties and helps the cells to defend against environmental toxic pollutants, drugs and chemicals which leads to certain forms of cancer.[32] In this study, proanthocyanidin content was found to be higher (367.75 mg/g). Food and Drug Administration (FDA) has approved grape seed extract as generally recognised as safe (GRAS) and commercially being marketed as health supplement on Everything Added to Food in the United States (EAFUS) database.[24] Oral administration of grape seed proanthocyandins (200-300 mg / day) is reported to prevent epigastric pain. Notably it reduces the intensity and recurrence of pain and thereby reduces the usage of analgesics.[13]

CONCLUSION:

Phytochemical analysis of methanol extract of grape seed - *vitis vinifera l.* revealed the presence of a high content of polyphenols followed by flavonoids, phenols and alkaloids. HPLC analysis indicated that alkaloids were higher in concentration (12.66 mg) than the flavonoids (4.56 mg) while, steroids were below the detectable level. Of note, proanthocyanidin content was high (367.75 mg/g) in the grape seed extract. This suggests that methanol extract of grape seed is a rich source of proanthocyanidin, a potential therapeutic agent. However, further clinical and animal studies need to be carried out to ascertain the same.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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