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Research Article

Proximate Analysis and Phytochemical Extraction from Grape Seeds and Applications of the Grape Seed Extract

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Abstract

The efficient method of extracting polyphenols from grape seeds have been a great interest to many of the investigators, due to its use in health care sectors to prevent various diseases. In this study, the extraction of oil was performed; thereafter the deoiled grape seed powder was used for the extraction of polyphenols using aqueous ethanol at different concentration. For this, various parameters like temperature, time and light sensitive environment was considered to be significant for the yield of polyphenols. The sample was subjected for proximate analysis like fat analysis, protein analysis, ash analysis, and Loss On Drying (LOD) using Soxhlet apparatus, Kjeldhal unit, muffle furnace and hot air oven respectively. Total polyphenols were measured using titration method to determine the percentage of polyphenols in the final product. The concentration of ethanol and temperature showed the significant impact on the yield and the percentage of polyphenols in the final product. Highest total polyphenol was obtained with 80% ethanol with 2 h of extraction at 40°C. In case of fat analysis, grape seed powder showed high amount of fat than the de-fatted sample and final product, where as in protein analysis de-fatted sample showed highest amount of protein than the other samples. Ash analysis and LOD analysis for grape seed powder and de-fatted grape seed powder showed 1.73, 1.91, 4.70 and 1.48% respectively.

Keywords: Grape seeds, polyphenols, proximate analysis, titration, ash analysis.

Introduction

Phytomedicine is an important historical aspect which has been used as a traditional medicine Grapes (*Vitis vinifera*) belongs to *Vitaceae* family and it is the second largest fruit grown in the world after orange (Pasqua and Simonetti, 2016; Simonetti *et al.,* 2020). The total production of grapes worldwide is about 60 million tons. The major producers of grapes include USA, China, Italy, and France. Grapes can be categorized based on the seeds available in the fruit, as grapes with edible seeds, seedless grapes, wine grapes, table grapes and raisin grapes (Aline *et al.,* 2016). Generally, a large number of grape seeds are obtained as a by-product from wine industries and also obtained from the waste of the processed fruits products like juice, jam or marmalade and boiled juice. The wastes of this industry, such as peels, seeds and pulps, represent about 50% to 60% of the raw processed fruit. Besides being a potentially valuable waste resource, they also aggravate serious disposal problems. Depending on the grape variety, the seed is between 2% to 6% of the weight of the berries and 1% to 4% of the weight of the grape.

The average yield of a seed is between 20-25% of the weight of the marc; marc dry seeds contain 40-65% and have an oil content of 12-22% (Mironeasa *et al.,* 2010). The grape seeds have attracted attention to develop a potential source of nutrients. Obviously, these kind of utilization leads to the production of various products of food as well as nutraceuticals to improve the standard of health because the grape seeds have abundance of phytochemicals that includes sugars, flavonoids, anthocyanins and proanthocyanins, organic acids, tannins, mineral salts and vitamins. Grapes skin, especially from the red and black species is rich in resveratrol which is a derivative of stilbene (Kanagarla *et al.,* 2013). Depending on the variety of the grape seed, the oil content varies with the degree of ripeness, soil, and climate etc., typically the grape seeds contain about 8-15% of oil which is rich in unsaturated fatty acids (Kallithraka *et al.,* 1995). Grape seeds are the sources of healthy fatty acids and dietary fibers. Some of the examples of unsaturated fatty acids are α-linolenic acid (ω-3) and γ-linolenic acid (ω-6), which constitute essential fatty acids as they are not produced by humans.

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The GSO is also rich in Vitamin E and other phytosterols that contributes to various biological activities on human consumption (Hussein and Abdrabba, 2015). GSO can be extracted through various methods like mechanical method by using oil extruder, cold pressing or through chemical method such as using organic solvents like petroleum ether, hexane using Soxhlet extraction method. Physicochemical properties of GSO play an important role for human consumption. Some of the properties are Iodine value, Saponification value, FFA%, peroxide value, thiobarbituric acid TBA value, ρ-Anisidine, Totox value, Total tocopherol content etc. helps in assuring the quality of the GSO for human consumption (Santos-Buelga *et al.,* 2015). The grape seeds contain various phytochemicals apart from lipids, protein and carbohydrates. Polyphenols are the phytochemical compound that constitutes about 5%-8% in the grape seeds; they are mainly flavonoids, including gallic acid, monomeric falvan-3-ols catechin, epicatechin, gallocatechin, epigallocatechin and epicatechin 3-O-gallate, procyanidin dimers, trimers and highly polymerized procyanidins (Fuleki and Da Silva, 1997). These compounds are polar in nature that contains multiple -OH groups. Recently, extraction of polyphenols from grape seeds has emerged as an opportune and vital business for the wine industry (Bonilla *et al.,* 1999). Conventionally, organic solvents such as methanol, acetone, acetonitrile, ethyl acetate, and others are used to extract polyphenols from grape seeds and the extracts are evaporated under vacuum to remove the solvent. Safety concerns associated with the use of organic solvents for industrial extractions include solvent residues in the product, exposure to workers, disposal of waste solvents, and pollution of the environment. Safer and more cost-efficient techniques to separate polyphenols from grape seeds are required. Some early studies showed ethanol and boiling water 4 to be effective for polyphenol extraction from food material. (John Shi *et al.,* 2003). The GSO and the polyphenols present in the GSE are the vital components that contribute to various biological activities in humans. GSO is predominant in linoleic acid, oleic acid, stearic acid which is otherwise termed as Poly Unsaturated Fatty Acids (PUFA). The GSO is also rich in Vitamin E and small percent of polyphenols (Aline *et al.,* 2016; Khan *et al.,* 2021). These molecules are responsible for various biological properties. PUFA acts as a cardioprotective agent, helps in reducing bad cholesterol, and they possess anti-inflammatory properties. On the other hand, Vitamin E acts as an antioxidant that helps in preventing the oxidative stress by acting against the free radicals. Polyphenols present in the grape seed oil acts as an antimicrobial agent that prevent various spoilage and pathogenic microorganisms. Considering the above points in view, this study investigated on the proximate analysis and phytochemical extraction from grape seeds and applications of the grape seed extract.

Materials and methods

Collection of grape seeds: Healthy, disease free grape seeds were collected from MVM Agro Tech industry, Tanisandra, Bangalore (Fig. 1).

Fig. 1. Black grapes at its natural habitat.

Extraction: The dried grape seeds were first subjected to oil extruder to remove the oil. Then the de-fatted grape seed powder was used for the extraction purpose (Fig. 2 & 3). About 25 g of de-fatted grape seed powder was extracted using the 60, 70, 80 and 93% ethanol in 1:4 volumes at 40°C using a mechanical stirrer. The extraction was done for 2 h in the light sensitive environment. The same process was repeated 3 times by collecting the spent from the previous extraction. Aftermath, the filtered extract is collected and dried using the Rotavac (Rotor evaporator). Then the dried powder obtained was used for further analysis (Fig. 4).

Fig. 2. Dried grape seeds.

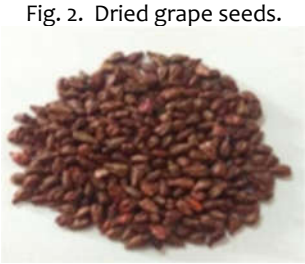

Fig. 3. De-fatted grape seed powder.

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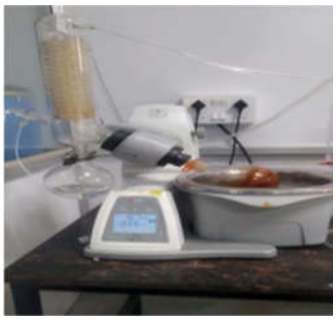
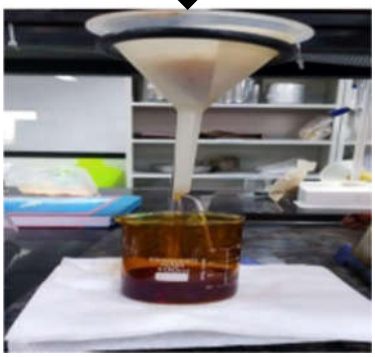
Fig. 4. Extraction of polyphenols.

De-fatted grape seed powder

Extraction using mechanical stirrer



Drying using Rotavac



Filtration of extract

Fat analysis: The grape seed powder/de-fatted grape seed powder/final product was used for the analysis of fat content in them. About 1 g of the sample was added to 100 mL of hexane in around bottom flask, then it was placed on the Soxhlet Extractor and the temperature was set for 40°C. The apparatus was kept for 2 h. After 2 h, the contents were filtered and the filtrate was taken in an evaporating dish and was kept on the heating mantle for complete evaporation of the filtrate. The weight of the empty evaporating dish was noted before and taken as W1; the dried filtrate in the dish is taken as W2. Then the fat% is calculated using the equation below.

$$\text{Fat\%} = \frac{\text{W2 - W1} \times 100}{\text{Wt. of the sample taken}}$$

Ash analysis: The grape seed powder/de-fatted grape seed powder was used for the ash analysis. About 2 g of the sample is weighed in the dried crucible, before weighing the weight of empty crucible is noted down as W1. Then the crucible is placed on the hot plate for 1 h and then placed in the muffle furnace at 630 C for 3 h till the sample is completely charred. The weight is noted down as W3. Then Ash% was calculated using the using the below equation.

$$\text{Ash \%} = \frac{\text{W3-W2} \times 100}{\text{W2-W1}}$$

Loss on drying (LOD): The grape seed powder/de-fatted grape seed powder was used for LOD analysis. First the weight of the empty petri plate is noted down as W1. Then 2 g of the sample is weighed accurately and taken as W2. Then, the petri plate was placed in the hot air oven for 2 h. Then the petri plate was taken out and allowed to cool down in the desiccator for 5 min and it is weighed using the weighing balance and noted as W3. The LOD% was calculated using the below equation.

$$\text{LOD\%} = \frac{\text{W3-W2} \times 100}{\text{W2-W1}}$$

Protein analysis: The grape seed powder/de-fatted grape seed powder and the final product were used for the analysis of crude protein. The sample of 200 mg is weighed and added to all digestion tubes. Then 3.5 g of catalyst mixture (potassium sulfate + copper sulfate) and 10 mL of concentrated H_2SO_4 is added to the digestion tubes and kept for digestion in Kjeldhal Digestor unit. Heat below the boiling point of the acids until frothing ceases, increase the heat until the acid boils vigorously and digest for 1 h. After complete digestion, the solution turns light green to blue colour and added 10-15 mL of de-mineralized water to cool the digestion tubes. Once digestion is completed, the digestion tubes are taken for distillation process.

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Then 40 mL of 40% NaOH and 20 mL of de-mineralized water is added to the digestion tubes and placed in the distillation unit. On the other hand, 20 mL of 2% boric acid is added in the conical flask and the tip tube is placed. Switch on the distillation unit, steam is provided until all the ammonia has passed to the conical flask containing 2% boric acid. This is called distillate. Then 2-3 drops of methyl red indicator are added to the distillate and titrated against 0.1N HCl till the pale pink colour appears which marks the end point. Similarly blank is carried out by omitting the sample. Then protein percentage is calculated using the equation:

Protein (%) = (A-B) X N X 1.401/W X 6.25

A - Volume of 0.1N HCl used for sample titration; B - Volume of 0.1N HCl used for blank titration; N - Normality of HCl; W - Weight of the sample 1.041- Factor containing the molecular mass of nitrogen 14.01 and the factor; 0.1 required for the conversion of the result to a percentage; 6.25 - protein–nitrogen conversion factor.

Determination of total polyphenols in final product: The sample of 0.1 g is weighed and dissolved in 750 mL of de-mineralized water and shaken well, then 20 mL of indigo carmine solution is added. Then this content is titrated against N/10 $KMnO_4$ solution until the blue colour solution changes to green and then golden yellow colour solution which marks the end point of the titration. Repeat the experiment with same quantity of reagent in the same manner but omitting the sample, this serves as blank. Then the difference between the two titrations represents the indigo carmine solution required to neutralize the tannin. Each mL of 0.1 N KMnO4 solution is equivalent to 0.004157g of tannin. Then the percentage of tannin is calculated using the formula.

$$\% \text{ Of Polyphenols} = \frac{(A-B) \times 0.004157 \times 100 \times 0.1}{\text{Wt. of the sample taken} \times N}$$

Where, A-Volume of KMnO4 solution used by the sample; B-Volume of KMnO4 solution used by blank; 0.004157-Tannin equivalent in 1 mL of 0.1N KMnO4; 100-percentage.

Solubility test for the final product: Take a pinch of final product in the test tubes arranged in the test tube stand and add 1ml of organic solvent to each of the test tube. Then note the solubility result as p positive for the solubility and negative for insolubility.

Results and discussion

Extraction of polyphenols: The polyphenols were extracted using ethanol as an organic solvent, the following Table 1 depicts the ethanol concentration, yield of the final product and the assay percentage obtained in the titration method. Table 1 depicts the percentage of polyphenols obtained using ethanol at different concentrations.

Table 1. Percentage of polyphenols with different ethanol concentration.

Trial No.	Ethanol concentration	Finished product (g)	Yield obtained (%)	Percentage of polyphenols (%)
1	60%	1.14	4.56%	54%
2	70%	2.16	8.64%	67.3%
3	80%	1.69	6.76%	78.21%
4	93%	-	-	55.95%
5	80%	1.6	6.4%	63.05%
6	80%	1.50	6%	82.07%
7	80%	1.71	6.84%	70.65%

Highest number of total polyphenols was obtained with 80% ethanol concentration which corresponds to 82.07%, percentage of polyphenols greatly vary with the ethanol concentration. Trial 4 corresponds to 93% ethanol concentration but did not yield the final product, but the percentage of polyphenols was determined using the combined filtrate obtained after the extraction process which corresponds to 55.95% of polyphenols. Polyphenols exhibited high sensitivity towards light, temperature as well as concentration of ethanol. Therefore, extraction of polyphenols is performed in light sensitive environment by using amber glass wares, temperature (40°C) and extraction time plays a vital role in the yield of the final product.

Fat analysis: The fat content in the grape seed meal de-fatted grape seed sample and final product was determined using Soxhlet extraction method using hexane as an organic solvent. As per the result obtained, the fat content was found to be higher in grape seed meal which corresponds to 7.5619% than in de-fatted grape seed sample and the final product. The final product had least amount of fat content compared to other samples. The findings show that temperature has an influence over the fat content by degrading the thermally unstable compounds present in the sample. Moreover, the fat analysis helps in determining the fat content in the sample that is used as one of the proximate analysis to assess the quantity of fat present in the sample.

Protein analysis: The crude protein analysis for the samples was analyzed using Kjeldhal method. The protein content was found to be higher in grape seed meal and de-fatted grape seed sample than the final product. The crude protein analysis provides the proximate content of protein present in the sample. Most cited values for protein in food composition databases is in fact derived from total nitrogen or total organic nitrogen values. The analysis of protein in the grape seed is an important criterion so as to provide the body with good quality protein through dietary supplement in the form of grape seed extracts.

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Ash and Loss on Drying (LOD) analysis: Ash analysis and LOD analysis is performed using muffle furnace and hot air oven respectively. Ash refers to the inorganic residue formed after complete burning or oxidation of organic matter in the food sample. Determining ash content in the food sample plays an important role in providing a measure of the total amount of minerals present in the food. The mineral content is important in analyzing the quality of food which mainly depends on the concentration and type of minerals present in the food or dietary supplement, whereas, the loss on drying is used to determine the moisture content present in the sample. This method is based on the thermogravimetric principle, where the sample is heated until no more weight is lost, i.e., it is completely dry. The moisture content plays a crucial role in maintaining the shelf life of the raw materials and products; therefore, it should be accurately determined in order to establish the quality for standard specifications. Table 2 depicts the ash content and loss on drying of the grape seed sample.

Table 2. Ash analysis and Loss on drying (LOD) of grape seed meal and de-fatted grape seed meal.

Sample	Ash content (%)	Loss on Drying (%)
Grape seed meal	1.737%	4.707%
De-fatted grape seed	1.919%	1.481%

Solubility test: Table 3 depicts the solubility of the final product with respective solvents. The solubility test was performed for the final product with various solvents such as ethanol, methanol, hexane, acetone, butanol and ethyl acetate. The final product was soluble in ethanol, methanol, butanol and acetone whereas, it was insoluble in hexane and ethyl acetate.

Table 3. Solubility test for final product using organic solvents.

S.No.	Organic solvents	Result
1	Ethanol	+
2	Methanol	+
3	Hexane	-
4	Acetone	+
5	Butanol	+
6	Ethyl acetate	-

Conclusion

The proximate analysis such as fat content, protein content, ash content and moisture content (LOD) was performed for grape seed samples, which plays a major role in the food manufacturing companies to ensure that the products meet appropriate laws and legal declaration as well as the safety aspects of the products released by the manufacturing industries to the consumer.

These factors play a key role in preparing the grape seed extracts and grape seed oil that act as a phytomedicine and used extensively in food industries, pharmaceutical industries as well as in cosmetic industries for their biological activities. The grape seed extract is used as a dietary supplement and nutraceuticals for their biological properties such as antioxidant, antimicrobial, anticancer, antidiabetic, antiaging and hepatoprotective effects. Hence, they are highly exploited by pharmaceutical industries, food industries for their biological functions in maintaining the human health.

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