CHARACTERIZATION OF GRAPE SEED AQUEOUS EXTRACT AND POSSIBLE APPLICATIONS IN BIOLOGICAL SYSTEMS

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Grapes (*Vitis vinifera* L.) belong to world's largest fruit crops. Since about 80% of the total amount is used in wine-making, the rest of 20% (about 10 million tons) of grape pomace arises within a few weeks from the harvest campaign. The seeds constitute a considerable ratio of the pomace, amounting to 38-52% on a dry matter basis. The results of certain studies have indicated that the polyphenols present in grape seeds in significant concentrations could be classified into two groups: flavonoids and non-flavonoids. The present study employed high-performance liquid chromatography (HPLC) to analyze the phenolic compounds in the seed extract. Spectrophotometric methods were employed for the determination of total phenolic content, total tannins, flavonoids, flavanols and antocyanins. The aqueous polyphenolic extract obtained from grape seeds was analyzed in germination tests, to evaluate the response of three different plants (oat, rape and maize) in terms of growth and development. Biometric measurements and quantitative determination of green biomass showed that grape seed extract amendments stimulated root elongation for oat and maize, as well as green biomass accumulation.

Keywords: polyphenols, separation, biological activity, modulators

INTRODUCTION

In recent years, naturally occurring plant phenolics in grape by-products have raised a lot of attention, due to their health-promoting effects and to the antioxidant role they play in biological and food systems.[1,2]

Apart from being a rich source of high-value fatty oil, grape seeds have also been appreciated due to their high content of phenolic compounds, such as gallic acid, catechin and epicatechin, and of a wide variety of procyanidins. The latter are also referred to as condensed tannins. Grape seed extracts and procyanidins have been a matter of intense investigations with respect to their potentially beneficial effects on human health.[3,4]

Phenolic compounds are secondary plant metabolites, especially important in the sensory and nutritional quality of fruits, vegetables and other plants.[5] Phenolics may act as phytoalexins, antifeedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light, among others.[6] Such bioactive properties explain the important role they play in plant growth and reproduction, providing an efficient protection against pathogens and predators.[7] As one of the most widely occurring groups of phytochemicals, these compounds are of considerable physiological and morphological importance in plants. As a large group of bioactive chemicals, they have diverse biological functions in plant growth and development cycle. The stimulatory or inhibitory effect on plant development depends on the concentration of polyphenolic compounds and on plant species.

Based on numerous evidence of the strong biological activity of phenolics in grape seeds, this study has been focused on determining their content in hot water extract and their possible applications as plant growth modulators.

EXPERIMENTAL

Extraction procedure

Aqueous extraction, carried out on 20 g of dried material, using 125 mL distilled water at 70 °C, for 45 min, was repeated three times, the extracts being cumulated to a volume of 500 mL, with distilled water. The aqueous extract obtained was concentrated to 30 mL, and fractionation by liquid-liquid extraction using ethyl acetate as solvent was carried out prior to HPLC analysis.

Spectrophotometric characterization

The total content of polyphenols, tannins, flavonoids, flavonols and antocyanins was determined by different colorimetric methods.

The total phenolic content of plant extracts was determined by FCR. About 1 mL of plant extracts was mixed with 500 µL of FCR, 2 mL of 10% sodium carbonate and 5 mL of water. The mixture was shaken thoroughly and allowed to stay for 90 min. Then, absorbance at 765 nm was determined against a blank containing all reagents, without the samples or the gallic acid, under the same conditions. The total phenolic content is expressed as the number of equivalents of gallic acid (GAE).

The total content of tannins was determined using a method based on the precipitation of tannins with casein. The content of tannins was determined by the FC method and expressed as the difference between the initial content of polyphenols and the content after precipitation with casein.[8]

The contents of flavonoids and flavonols were determined by the aluminium chloride method, using rutin as a reference compound, according to Makris,[9] respectively El-Sayed Saleh Abdel-Hameed.[8] The antocyanins content was determined by the pH differential method described by Ribereau-Gayon.[10]

HPLC determination

Chromatographic analyses were carried out on a Dionex UltiMate 3000, liquid chromatography apparatus, coupled to a diode array detector. Eluent (A) was 1% aqueous acetic acid, and eluent (B) – 1% acetic acid in MeOH, the flow rate being kept constant throughout the analysis at 0.5 mL/min. Injections were accomplished with a 10 µL fixed loop. The column was a Dionex Acclaim 120, C18 RP (4.6x150 mm, particle size 5 µm) and temperature was maintained at 30 °C. The elution programme used was from 10% B to 40% B, within 30 min. Chromatograms were monitored at 280 nm, and identification was based on retention times and on-line spectral data, comparatively with external standards.

Germination tests

The influence of grape seed extracts (188 mg/TPC/L extract) on plant growth and development was tested on rape, oat and maize, in germination experiments. Each Petri dish contained 10 rape seeds, 5 oat seeds and 5 maize seeds placed on a filter paper and 10 mL tested solution. The tested solutions were represented by tap water (control) and grape seed extract (GS). Petri dishes (5/each sample) were kept at dark into a thermostated chamber (25 °C) for 7 days. After 1 week, the Petri dishes were taken out and kept in daylight for 3 days, to allow chlorophyll assimilation. This experiment was done in triplicate.

Biometric measurements of plantlet elongation and quantitative determination of fresh weight (FW) biomass permit quantification of the growth index percentage *versus* the control sample.

Growth index % = 100 x (tested parameter value – control parameter value)/control parameter value.

The weighed fresh samples were extracted into 80% acetone. The absorbance values of the acetone extract were read at specific wavelengths: 470, 646, 663, on a Cintra UV-260 spectrophotometer, while the amount of assimilatory pigments (chlorophyll a, b and carotene) was calculated according to the formulas of Lichtentaler and Wellburn.[11]

RESULTS AND DISCUSSION

Estimation of total amount of phenolic compounds, tannins, flavonoids, flavonols and antocyanins

The concentrations of different classes of phenolic compounds were determined spectrophotometrically, using different colorimetric methods based on complex formation, oxidation and pH decreasing.

The Folin-Ciocalteu method for the determination of the total phenolic content depends on the reduction of FCR, by phenols, to a mixture of blue oxides, which have a maximal absorption in the 750 nm region.

The yield of extracted polyphenols in the case of grape seed extract was of 506.25 mg GAE/100 g, while the yield of total tannins was of 198.38 mg GAE/100 g.

The content of flavonoids and flavonols was determined with a method based on the formation of a complex flavonoid-aluminum having the absorption maximum at 510 nm for flavonoids, and at 440 nm, respectively, for flavonols. The total content of flavonoids was of 27.73 mgRE/100 g and that of flavonols was of about 7.11 mgRE/100 g. The concentration of total antocyanins was of 18.52 mg/100 g.

HPLC analysis

A reversed-phase high-performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds present in the grape seed extracts. To this end, a standard mixture solution of phenolic compounds was analysed (Fig. 1).

The phenolic profile of the aqueous grape seed extract is presented in Figure 2. The major compounds identified were gallic acid (6.12 mg/100 g) and catechine (44.36 mg/100 g). The presence of another major compound, possibly epicatechine, should be also observed at t_R = 10 min. Based on numerous proofs, the most important compounds found in grape seed extracts were gallic acid, catechine and epicatechine.[2] Considering different literature sources,[3,4,12,13] the methods of HPLC analysis, the eluents used and the retention time, one may presume that the third compound is epicatechine. Quantitative analysis of this compound will be performed in further studies.

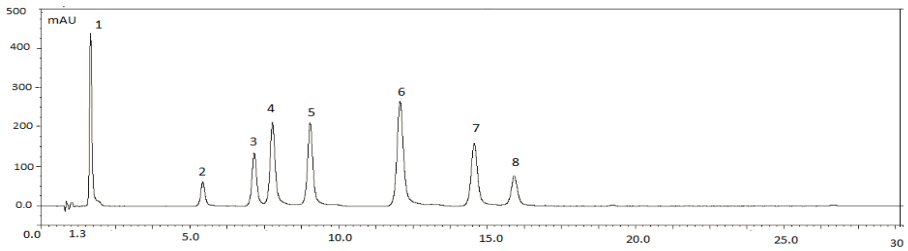

Figure 1: Typical chromatogram at 280 nm obtained for polyphenol standards. Identified compounds of peaks 1-8: gallic acid, catechine, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapic acid, respectively

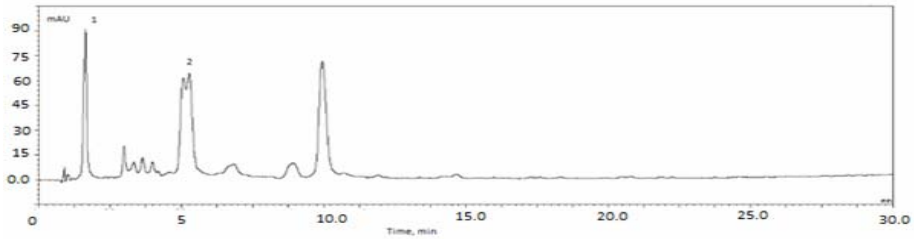

Figure 2: HPLC profile of grape seed aqueus extract. Identified compounds: 1 – gallic acid; 2 – catechine

Germination tests

Grape seed aqueous extract has stimulating effects on maize plantlet elongation, contributing to an increase of 41.8% in the length of hypocotyls, compared to the control (Fig. 3). Stimulatory effects of the grape seed aqueous extract were also registered for oat plantlets, but only in the case of rootlets and cotyledons. The presence of natural polyphenolic extracts in the growth medium had inhibitory effects on oat hypocotyl elongation and rape plantlet development.

If maize plantlet elongation was stimulated by the grape seed aqueous extract, contrary effects were registered as to green biomass accumulation, for the hypocotyl and cotyledon fresh mass. The grape seeds polyphenolic extract showed stimulatory effects on green biomass accumulation for rape and oat cotyledons, as well as on oat hypocotyls (Fig. 4). The stimulatory or inhibitory effects on plantlet growth and development depend on the plant species and on the interaction established between plant natural bioactive compounds and the nature of the polyphenolic compounds, which characterizes the grape seed aqueous extract.[14]

Different effects were also recorded for pigment accumulation in plantlet fresh cotyledons (Table 1).

It could be observed that the presence of the grape seed extract in the growth medium of the plants stimulated chlorophyll **a** (320.56 µg/g) and carotene (51.50 µg/g) biosynthesis in the rape plantlet cotyledon, an increasing trend of 12.79% being registered in chlorophyll **a** assimilation and of 5%, respectively, in carotene concentration, compared to the control.

Chlorophyll **a** and total chlorophyll

concentration of the maize plantlet was also positively influenced by the addition of the grape seed extract, presenting an increasing percentage of 29% for chlorophyll **a**, and of 7%, respectively, for the total chlorophyll photosynthesizing pigment, compared to the control.

Table 1
Influence of grape seed aqueous extract on concentration of assimilatory pigments for different plant species (µg/g)

Plant species	Tested solution	Chl a	Chl b	Chl a+b	Chl a/b	Carotene
Rape	Control (water)	284.20	294.30	578.51	0.96	48.66
	GS extract	320.56	164.38	484.94	1.95	51.10
Oat	Control (water)	661.10	339.51	1000.61	1.94	169.80
	GS extract	534.16	331.09	865.25	1.61	128.45
Maize	Control (water)	189.99	176.43	366.42	1.07	53.32
	GS extract	244.67	148.01	392.69	1.65	53.08

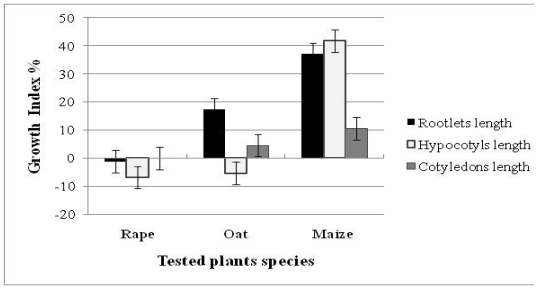

Figure 3: Influence of grape seed aqueous extract on plantlet elongation growth index, *versus* the control

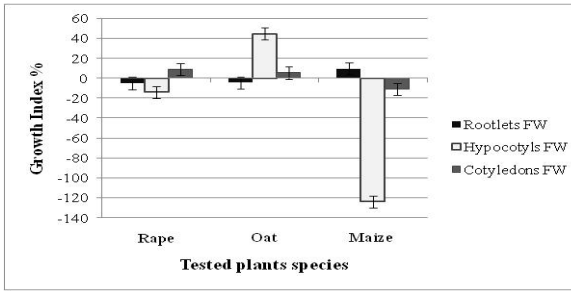

Figure 4: Influence of grape seed aqueous extract on plantlet fresh weight growth index reported to the control

Chlorophyll **a** and total chlorophyll concentration of the maize plantlet was also positively influenced by the addition of the grape seed extract, presenting an increasing percentage of 29% for chlorophyll **a**, and of 7%, respectively, for the total chlorophyll photosynthesizing pigment, compared to the control.

CONCLUSIONS

Grape seeds represent a valuable natural source of phenolic compounds. The aqueous extract seems to contain higher amounts of gallic acid and catechine. Although many researchers assert that distilled water is not a suitable extraction agent, it appears that, in the case of water-soluble phenolic compounds, when carrying out successive extractions at a relatively higher temperature in the presence of antioxidants, distilled water can be an efficient extraction agent and also a preferred solvent, being nontoxic, environmentally safe and inexpensive.

The grape seed aqueous extract had stimulatory effects on maize and oat plantlet elongation, but had an inhibitory effect on rape plantlets.

Green biomass accumulation was stimulated in the presence of grape seed extract in the case of rape and oat species. On the other hand, an inhibitory effect was observed for maize, contrary to the previous case, when plantlet elongation was considerably stimulated.

Spectrophotometric determinations evidenced that chlorophyll **a** assimilation was stimulated by a polyphenolic treatment for maize and rape plant, while the carotene pigments presented higher values only for rape plant.

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