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Qualitative Screening of Phytocompounds and Spectrophotometric Investigations of two Pumpkin Species †

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Abstract: Pumpkin (*Cucurbita maxima*) is a fruit packed with vitamins and nutrients beneficial to human health with numerous therapeutic uses: antiparasitic, antioxidant, helps lower the bad cholesterol, adjuvant in weight loss, improves cancer prevention, etc. Pumpkin is rich in beta-carotene, contains important amounts of lutein and zeaxanthin, antioxidants that can considerably prevent cataracts and macular degeneration. Worldwide, five pumpkin species are grown for their edible fruit and seeds. This paper describes the qualitative screening of phytocompounds and the quantitative determination of main bioactive compounds found in two pumpkin species: Valenciano and Waltham Butternut. The qualitative screening of phytochemicals is based on the visual change in color of the aqueous extracts upon adding known reactants. This allows a preliminary evaluation regarding the presence of different bioactive compounds such as saponins, alkaloids, tannins, flavonoids, etc. In order to determine the specific amount of different phytocompounds (e.g.,: total content of polyphenols, total content of flavonoids, etc.) UV—Vis spectra are recorded, in triplicate, at well—established wavelengths, thus obtaining an average absorbance. For example, a method widely applied for the determination of total polyphenolic content is the Folin—Ciocalteu (FC) reaction, which is basically an antioxidant analyses that relies on electron transfer, therefore measuring the reductive ability of a specific antioxidant. Briefly, FC reaction involved mixing 1 mL diluted aqueous extract with 5 mL FC reagent and adding, after 8 min, 4 mL Na_2CO_3. After 60 min incubation at room temperature, we recorded the absorptions at 765 nm, which corresponds to gallic acid curve calibration standard. Also, the antioxidant activity using the DPPH method was recorded for both aqueous extracts.

Keywords: phytocompounds; qualitative screening; pumpkin; quantitative determination

1. Introduction

Cucurbita maxima (pumpkin, squash, gourd) is an important representative of the Cucurbitaceae family and is among the top cultivated crops all around the world, especially in temperate and subtropical climate [1]. Squash pumpkin is constantly gaining importance in the past years mainly due to the nutritional and health benefits of its seeds that are packed with nutraceutical compounds

such as polysaccharides, carotene, minerals and vitamins [2]. The health benefits of different squash pumpkin species were previously reported showing that they could lower blood glucose [3] and could even prevent some types of diabetes and diminish their complications [4]. Squash pumpkin contains an impressive amount of vitamin A, 245% of the reference daily intake (RDI), is low in calories as it contains over 94% water, contains important amounts of antioxidants (e.g.,: alpha-carotene, beta-carotene and beta-cryptoxanthin) that can neutralize the action of free radicals [5].

Plants contain multiple and different radical scavenging molecules that are therapeutically active including alkaloids, amines, betalains, vitamins, phenolic acids, terpenoids. Most phytochemicals are antioxidant agents which essentially reduce the damages caused in tissue during physiological processes. The pharmaceutic value of plants depends on their bioactive compounds that exhibit different physiological effects on human health. Therefore, the screening for bioactive compounds allows the detection of various components that can be used as starting point for modern drugs that could treat different diseases [6]. These "metabolic chemicals" are better known as "secondary metabolites" [7] and they include alkaloids, flavonoids, coumarins, tannins, terpenes, terpenoids, phenols, polysaccharides and glycosides [8].

There are many known bioactive compounds, each with their own health action [9,10]:

- antioxidant, meaning that it protects the human cells from the oxidative stress;
- hormonal: especially isoflavones that can imitate human estrogens;
- antibacterial;
- physical: bioactive compounds can physically attach to cell walls

This paper describes the qualitative screening of phytocompounds and the quantitative determination of main bioactive compounds found in two pumpkin species: Valenciano and Waltham Butternut. The fresh samples were air-dried at room temperature for 10 days under no direct sunlight followed by an aqueous extraction at a controlled temperature of 4^0 C, in the refrigerator. The qualitative screening for bioactive compounds involves the color changing reaction that results when mixing different extracts with known reagents while the quantitative screening for bioactive compounds is based of spectrophotometric determinations at specific and well-established wavelengths. The results are presented in comparison for the two squash pumpkin species and clearly show that both Valenciano and Waltham Butternut are important sources of bioactive compounds with numerous health benefits.

2. Materials and Methods

2.1. Materials

DPPH (2,2—diphenyl—1—picryl—hydrazyl—hydrate), hydrochloric acid (HCl), sulphuric acid (H_2SO_4), copper sulphate ($CuSO_4$), copper acetate ($Cu(CH_3COO)_2$), silver nitrate ($AgNO_3$), aluminum chloride ($AlCl_3$), lead acetate ($Pb(Ch3COO)_2$), catechin standard, gallic acid standard, Folin-Ciocalteu reagent, ferric chloride ($FeCl_3$), glacial acetic acid (CH_3COOH), ammonium molybdate, Benedict and Millon reagents were purchased from Sigma—Aldricht. Ethanol (C_2H_5OH), methanol (CH_3OH), chloroform ($CHCl_3$) and sodium hydroxide (NaOH) were purchased from Scharlau. The distilled water was freshly prepared in the laboratory.

2.2. Preparation of the Aqueous Extracts from Cucurbita Maxima

Both types of pumpkin (Valenciano and Waltham Butternut, Figure 1) used in the present research study were taken from a local homemaker in Gradistea, Giurgiu, Romania that grew them in an eco-friendly environment with no chemicals or additives.

Figure 1. Valenciano squash pumpkin (**left**) and Waltham Butternut squash pumpkin (**right**).

All the component parts (e.g.,: shell, core and seeds and equal combinations between these parts) were carefully separated, thoroughly washed twice with tap water, thrice with freshly prepared distilled water, dried at room temperature for 10 days under no direct sunlight, finely grinded and used to prepare the corresponding aqueous extracts. The protocol used to prepare the aqueous extracts is the same for both *Cucurbita maxima* squash pumpkin species and involves the following steps:

- 25 g dried squash parts (e.g.,: shell, core, seeds and equal mixtures of them) were weighted, transferred into a glass extractor and infused with 250 mL distilled water;
- the solutions were kept 24 h in a refrigerator (4 °C) to infuse;
- the aqueous extracts were filtered until a clear liquid is obtained;
- the aqueous extracts are stable at 4 °C for more than 4 months.

2.3. Qualitative Screening for Bioactive Compounds

The qualitative screening for bioactive compounds uses standard analytical methods that rely on a color change reaction as a positive response [11].

Test for tannins: to 1 mL aqueous extract, 2 mL of 5% FeCl3 was added and the formation of a dark blue or greenish black solution confirms the presence of tannins.

Test for saponins: 2 mL aqueous extract is mixed with 2 mL distilled water and vigorously shaken lengthwise, using a graduated cylinder, for 15 min. The formation of a 1 cm foam layer confirms the presence of saponins

Test for alkaloids:

(a) Mayer test: to 2 mL aqueous extract, 2 mL of concentrated HCl was added followed by few drops of Mayer reagent (potassium mercuric iodide). The formation of green solution or white precipitate indicates the presence of alkaloids.
(b) Wagner test: to 3 mL aqueous extract, 1 mL Wagner reagent (iodine inpotassium iodide) was added. A reddish-brown precipitate indicated the presence of alkaloids.
(c) Hager test: to 3 mL aqueous extract, 1 mL Hager reagent (saturated picric acid solution) was added and, if a yellow precipitate is formed, then alkaloids are present.

Test for flavonoids: 2 mL aqueous extract was mixed with 1 mL 2N NaOH. The formation of a yellow color results that disappears after adding dilute HCl.

Test for proteins and aminoacids:

(a) Millon test: 1 mL aqueous extract reacts with 5–6 drops of Millon reagent and a white precipitate appears that changes its color to red at heating.
(b) Biuret test: to 3 mL aqueous extract, 3 mL 4% NaOH solution and few drops of 1% $CuSO_4$ are added and a purple solution is formed.
(c) Ninhydrin test: to 3 mL aqueous extract, 3 drops of 5% $Pb(CH_3COO)_2$ are added and heated for 10 min. A purple of blue color is a positive response.
(d) Cysteine test: to 5 mL aqueous extract, few drops of 40% NaOH and 5% are added and boiled for 5 min. The solution turns purple or blue or a black precipitate of lead sulphate is formed.
(e) Xantoprotein test: to 3 mL aqueous extract, 1 mL conc. H_2SO_4 is added. First a white precipitate is formed that turn yellow upon boiling and orange after adding 1 mL NH_4OH.

2.4. Quantitative determinations of bioactive compounds

The quantitative determination of bioactive compounds was also carried out to determine the total content of tannins (TCF), total content of flavonoids (TCF) and total content of polyphenols (TCP) [12,13].

The antioxidant activity (AA, %) was also determined and, for that, a 2,2—diphenyl—1—picryl—hydrazyl—hydrate (DPPH) solution was prepared in C_2H_5OH and 0.5 mL aqueous extract were mixed with 1 mL 0.02 mg/mL DPPH solution. The following step was recording the absorbance at 517 nm. A blank was also prepared: 0.5 mL distilled water were mixed with 1 mL 0.02 mg/mL DPPH solution. The antioxidant activity was calculated using the formula:

$$AA\ \% = [(A_{Control}{}^{TM}\ A_{Sample})/A_{Control}] \times 100,$$

where: $A_{Control}$ is the absorbance of the blank DPPH solution and A_{Sample} is the absorbance of the aqueous extract mixed with 0.02 mg/mL DPPH solution.

3. Results and Discussions

3.1. Preparation of the AQUEOUS extracts from Pumpkin Squash Species

The protocol used to prepare the aqueous extracts is the same for both Cucurbita maxima squash pumpkin species and is roughly presented in Scheme 1. For both species of squash pumpkin, 5 different aqueous extracts were prepared from 3 different parts of squash pumpkin as follows:

- three simple aqueous extracts from only one part, e.g., core, shell and seeds;
- two combined aqueous extracts from core and shell in equal amounts, respectively core, shell and seeds in equal amounts.

The resulted aqueous extracts were kept 24 h in a refrigerator (4^0 C) to infuse, filtered until a clear liquid is obtained and kept in the refrigerator for more than 4 months.

3.2. Qualitative Screening for Bioactive Compounds

The qualitative screening of tannins clearly shows that they are absent in both squash pumpkin species and the same conclusion can be drawn from the qualitative screening of steroids and triterpenoids.

The qualitative screening of flavonoids gives a positive response only in the case of the aqueous extract prepared from the seeds of Valenciano pumpkin while in the case of Waltham Butternut pumpkin the presence of flavonoids could be visually observed for the seeds and the complex aqueous extract prepared from all the three constituents.

Proteins are the main components of protoplasm and are involved in all the natural processes that happen in all the living cells. In nature, all proteins are colloidal and are irreversibly coagulated at a temperature higher than its boiling point. Proteins are not soluble in neutral salts (e.g.,: NaCl, $MgSO_4$) and only solubilize once the salts are diluted. On the other hand, aminoacids are, in their majority, soluble in water.

The qualitative analysis of aminoacids refers to a color change, precipitation or even ring formation that appears as a result of a modification in the structural configuration upon reacting with a reagent. The results for the qualitative screening of carbohydrates of both squash pumpkin species are detailed in Table 1 for Valenciano pumpkin and Table 2 for Waltham Butternut pumpkin.

Table 1. Qualitative screening of proteins and aminoacids for Valenciano pumpkin.

Phytochemical Test	Valenciano Shell	Valenciano Core	Valenciano Seeds	Valenciano Core and Shell	Valenciano Core, Shell and Seeds
Millon	+	++	+++	+++	+++
Biuret	+	-	+++	++	++
Xantoprotein	-	+++	-	++	+++

Ninhydrin	-	-	-	-	-
Cysteine	-	-	+	-	+

Table 2. Qualitative screening of proteins and aminoacids for Waltham—Butternut pumpkin.

Phytochemical Test	Waltham—Butternut Shell	Waltham—Butternut Core	Waltham—Butternut Seeds	Waltham—Butternut Core and Shell	Waltham—Butternut Core, Shell and Seeds
Millon	+++	+++	-	++	+++
Biuret	+	-	-	-	+
Xantoprotein	+	+	-	+	+
Ninhydrin	-	-	-	+	+
Cysteine	-	-	-	-	-

The qualitative screening for proteins and aminoacids of Waltham Butternut pumpkin squash clearly reveals that cysteine, a non-essential sulphur-containing aminoacid, is absent all the aqueous extracts while in the case of Valenciano pumpkin squash cysteine can be found only in the seeds and the complex mixture thereof. Analyzing Table 2 and, more specific, the aqueous extract prepared from the seeds of Waltham Butternut pumpkin squash, it can be easily concluded that they neither contain protein nor aminoacids.

Alkaloids can be analyzed as a bioactive compound using 3 different qualitative tests and the results are presented in Table 3 for Valenciano pumpkin squash and Table 4 for Waltham Butternut pumpkin squash.

Table 3. Qualitative screening of alkaloids for Valenciano pumpkin.

Phytochemical Test	Valenciano Shell	Valenciano Core	Valenciano Seeds	Valenciano Core and Shell	Valenciano Core, Shell and Seeds
Mayer	++	+	+++	+++	+++
Wagner	+	+	+	+	+
Hager	+	++	+++	+++	+++

Table 4. Qualitative screening of alkaloids for Waltham—Butternut pumpkin.

Phytochemical Test	Waltham—Butternut Shell	Waltham—Butternut Core	Waltham—Butternut Seeds	Waltham—Butternut Core and Shell	Waltham—Butternut Core, Shell and Seeds
Mayer	+	+	+	+	+
Wagner	+	+	+	+	+
Hager	++	+++	++	++	+++

The results detailed in Tables 3 and 4 prove that, whatever the qualitative test used to screen for alkaloids, they are present in all ten aqueous extracts and the only difference is the intensity of the resulted solution.

Saponins represent another bioactive compound that was screened for in all the aqueous extracts and the results show that they are present in 8 out of 10 of the aqueous extracts, the exception being the seeds of both pumpkin squash species.

3.3. Quantitative Determination of Bioactive Compounds

The quantitative determination of bioactive compounds was carried out to determine the total content of tannins (TCT), flavonoids (TCF) and total content of polyphenols (TCP) (Table 5).

Table 5. Quantitative methods for the determination of bioactive compounds.

Assay	Reagents	Reaction Parameters	Recordings
TCT	0.5 mL extract+3 mL 4% vanillin-MeOH and 1.5 mL HCl	15 min. incubation	Absorbance at 500 nm
TCF	1 mL extract+4 mL distilled water and 0.3 mL 5% $NaNO_2$; after 5 min: 0.3 mL 10% $AlCl_3$; after other 5 min: 2 mL 1M NaOH and 2.4 mL distilled water	30 min. incubation	Absorbance at 510 nm (catechin curve calibration standard)
TCP	1 mL diluted extract and 5 mL Folin-Ciocalteu reagent; after 8 min: 4 mL Na_2CO_3	60 min. incubation	Absorbance at 765 nm (gallic acid curve calibration standard)

The amount of total tannins and the total content of flavonoids are described as mg catechin/L and the ten aqueous extracts were analyzed in triplicate. The total content of polyphenols uses gallic acid as standard calibration curve. The results are detailed in the charts below.

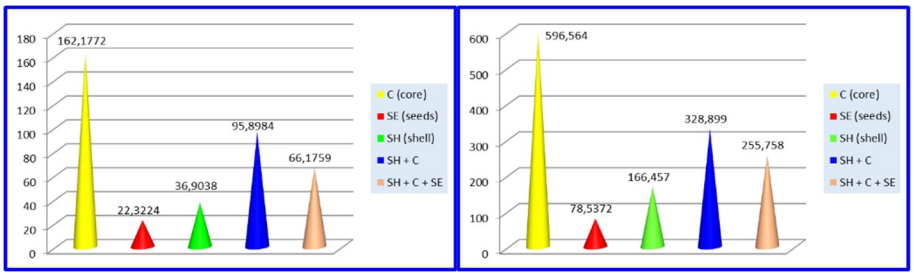

Figure 2. Total content of flavonoids (TCF) for Valenciano (**left**) and Waltham Butternut (**right**).

From the detailed chart that represents the total content of flavonoids (TCF) for both pumpkin squash species it can be concluded that, in the case of Valenciano pumpkin, TCF has the highest value for the aqueous extract prepared from core, followed by the mixture of core and shell, core and shell and seeds while seeds have the lowest concentration of TCF. In the case of Waltham Butternut pumpkin squash, the highest concentration was obtained for the core, followed by the mixture between core and shell.

The total content of tannins (TCT) was investigated as mg catechin/L and the results are presented in Figure 3.

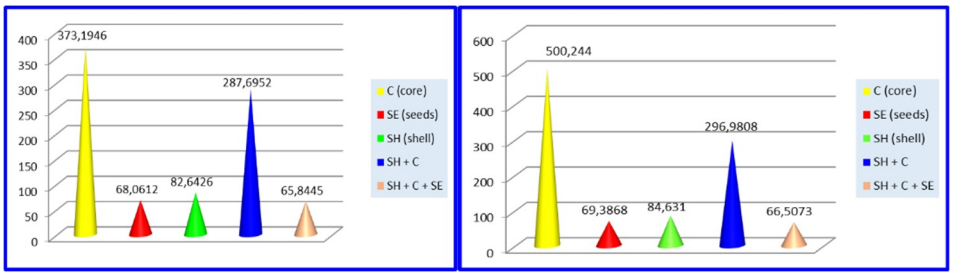

Figure 3. Total content of tannins (TCT) for Valenciano (**left**) and Waltham Butternut (**right**).

Analyzing the charts in Figure 5, it can be concluded that the highest concentration for TCT can be found in the core of both Valenciano and Waltham Butternut pumpkin squash (373,1946 mg/L for Valenciano and 500,244 mg/L for Waltham Butternut).

The total content of polyphenols (TCP) uses gallic acid as standard calibration curve and was investigated as mg gallic acid/L and are detailed in Figure 4.

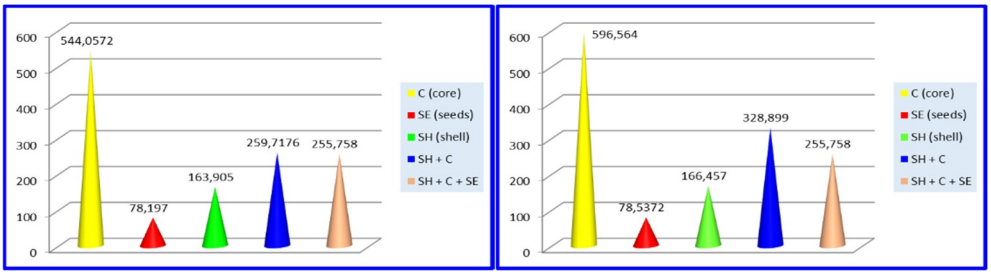

Figure 4. Total content of polyphenols (TCP) for Valenciano (**left**) and Waltham Butternut (**right**).

The antioxidant activity (AA, %) was measured using DPPH as standard:

$$AA\ \% = [(A_{Control} - A_{Sample})/A_{Control}] \times 100,$$

where: $A_{Control}$ is the absorbance of the blank DPPH solution and A_{Sample} is the absorbance of the aqueous extract mixed with 0.02 mg/mL DPPH solution. The results are presented in Table 6 as a comparison between the two pumpkin squash species.

Table 6. Antioxidant activity for pumpkin squash species.

AA (%)	Core	Shell	Seeds	Core and Shell	Core, Shell and Seeds
Valenciano	37.62	54.52	42.65	23.54	13.48
Waltham Butternut	37.22	54.12	41.85	21.73	12.87

From this table it can be concluded that the highest values are obtained for the shell aqueous extracts prepared from the shell of the two pumpkin squash species.

4. Conclusions

This research study describes two different species of squash pumpkin, Valenciano and Waltham Butternut, that are investigated by means of qualitative and quantitative phytochemical screening to determine the bioactive compounds. The qualitative screening for bioactive compounds involves the color change reaction that appears when mixing different extracts with known reagents and the quantitative screening for bioactive compounds is based of spectrophotometric determinations at well-established wavelengths.

The qualitative screening of tannins clearly shows that they are absent in both squash pumpkin species. The qualitative screening for proteins and aminoacids of Waltham Butternut pumpkin squash clearly reveals that cysteine is absent all the aqueous extracts. Alkaloids were also screened for and it was found that they are present in all ten aqueous extracts.

The quantitative determination of bioactive compounds was carried out to determine the total content of tannins (TCF), total content of flavonoids (TCF) and total content of polyphenols (TCP). In the case of Valenciano pumpkin, TCF has the highest value for the aqueous extract prepared from core (162,1772 mg/L), followed by the mixture of core and shell (95,8984 mg/L), core and shell and seeds (66,1759 mg/L) while seeds have the lowest concentration of TCF (22,3224 mg/L). In the case of Waltham Butternut pumpkin squash, the highest concentration was obtained for the core, followed by the mixture between core and shell.

The highest concentration for total content of tannins (TCT) can be found in the core of both Valenciano and Waltham Butternut pumpkin squash (373,1946 mg/L for Valenciano and 500,244 mg/L for Waltham Butternut). From the results obtained for the antioxidant activity it can be concluded that the highest values are obtained for the shell aqueous extracts prepared from the shell of the two pumpkin squash species (54,52716% for Valenciano pumpkin and 54,12475% for Waltham Butternut respectively).

The results presented clearly show that both Valenciano and Waltham Butternut are important sources of bioactive compounds with numerous health benefits.

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