In vitro health beneficial activities of Pumpkin seeds from *Cucurbita moschata* cultivated in Lemnos

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Abstract

Pumpkin seeds are commonly consumed in Greece. Although *Cucurbita moschata* is locally grown in Lemnos and is traditionally used in pumpkin pies, the seeds are currently discarded after consumption of the fruit flesh. The aim of the present study was to investigate the nutritional functionality of pumpkin seeds from *Cucurbita moschata* grown in Lemnos.

Cucurbita moschatas' seeds, raw or roasted, were appropriately extracted and the results are presented for raw versus (vs) roasted seed extracts. The phenolic content was expressed as μg gallic acid/g of seeds according to Folin-Ciocalteau assay (370.3 $\pm$ 19.1 vs 551.0 $\pm$ 22.0). Antioxidant capacity was expressed as equivalent amount for 50% scavenging in mg of seeds for DPPH (50.03 $\pm$ 5.91 vs 25.82 $\pm$ 6.77) and ABTS (17.85 $\pm$ 0.77 vs 12.77 $\pm$ 0.76) assays, and as μmol of trolox/g of seeds for FRAP (1.19 $\pm$ 0.05 vs 2.50 $\pm$ 0.23) and CUPRAC (2.13 $\pm$ 0.11 vs 3.25 $\pm$ 0.06) assays. Anti-inflammatory/anti-thrombotic and anti-diabetic activities were expressed as mg of seeds for 50% inhibition of platelet activating factor (0.62 vs 0.15) and as μg of seeds for 25% inhibition of alpha-glycosidase (40.0 vs 61.0) activities respectively. Moreover, anti-atherogenic activity was expressed as the % increase in lag time of human plasma oxidation (62.7 versus 163.2)

Raw and roasted pumpkin seed extracts exert anti-oxidant, anti-thrombotic/anti-inflammatory, anti-atherogenic and antidiabetic activities. *Cucurbita moschata* seeds may represent a novel opportunity for development of functional foods, with a local interest in Lemnos that would contribute also to the regional public health improvement.

Keywords: Pumpkin seeds; *Cucurbita moschata*; Functional foods; *In vitro* nutritive value

Abbreviations and/or nomenclature

ABTS: 2, 2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
CUPRAC: Cupric Ion Reducing Antioxidant Capacity
DPPH: 2, 2-Diphenyl-1-picryhydrazyl
FRAP: Ferric Reducing Antioxidant Power
LDL: Low Density Lipoprotein
PAF: Platelet Activating Factor
PRP: Platelet Rich Plasma
SA_{50}:   Equivalent amount for 50% scavenging
IA_{50}:   Equivalent amount for 50% inhibition
IA_{25}:   Equivalent amount for 25% inhibition

1   Introduction

The four principal quality factors in foods are appearance, flavor, texture and nutrition. Traditionally, the nutrition quality factor comprises major nutrients such as carbohydrates, fats and proteins, as well as minor components such as minerals, vitamins, fibers (Bourne, 2002) and the well studied phenolics or not extensively studied various others (Jiang & Du, 2011; Koike, Li, Liu, Hata, & Nikaido, 2005; Li et al., 2009) or still unidentified minor food compounds.

In the last decades many studies have revealed that minor food components such as various lipids (Demopoulos, Karantonis, & Antonopoulou, 2003; Fragopoulou, Demopoulos, & Antonopoulou, 2009; Nasopoulou, Karantonis, Detopoulou, Demopoulos, & Zabetakis, 2014) and phenolics (Del Rio et al., 2013) exert beneficial effects in human health by preventing chronic disease development in humans such as cardiovascular diseases and diabetes type II.

In recent years it has been made clear that chronic diseases share common basic pathophysiological mechanisms such as inflammation, thrombosis and oxidative stress. Understanding of the processes of the above mechanisms would be helpful for the development of potential therapeutic approaches (Balagopal, 2006; Demopoulos et al., 2003; Kampoli, Tousoulis, Antoniades, Siasos, & Stefanadis, 2009; Libby, 2007). Interestingly, a link between those pathophysiological mechanisms and nutrition has been established (Nomikos, Fragopoulou, & Antonopoulou, 2007; Xanthopoulou et al., 2010).

Many plants, their extracts and isolated compounds have demonstrated a spectrum of biological activities and have been used as food supplements for various disorders (Kadam et al., 2012). One such plant, pumpkin is one of the vegetables that meet the requirements of healthy nutrition; it is a gourd-like squash of the genus *Cucurbita* and the family *Cucurbitaceae*. Pumpkins (*Cucurbita spp.*) are of local importance in traditional agricultural systems. They are cultivated throughout the world for their food, seed oil and medicinal value. *Cucurbita pepo* (squash), *Cucurbita maxima* (winter squash) and *Cucurbita moschata* (musk pumpin) are the species mostly grown in Central Europe (Kadam & Patil, 2014). The nutritional value of nuts is well recognized (Sabate, Ros, & Salas-Salvado, 2006). Among various nuts, pumpkin seeds are commonly consumed throughout the world including Greece (Merrick, 1990). In Greece pumpkin seeds are consumed either raw or roasted as component of the Mediterranean Diet. Several studies have dealt with the beneficial effects of the consumption of pumpkin flesh (Caili, Huan, & Quanhong, 2006), but limited studies exist on the pumpkin seeds.

Although *Cucurbita moschata* is locally grown in the Greek island of Lemnos and is traditionally used in pumpkin pies the seeds are currently discarded after consumption of pumpkin flesh. The purpose of this study was to evaluate the *in vitro* anti-oxidant, anti-thrombotic/anti-inflammatory, anti-atherogenic and anti-diabetic activity of *Cucurbita moschata* seed polar extracts in order to examine the possibility of using the seeds as a novel ingredient for the development of functional foods of local importance,

2 Materials and Methods

2.1 Reagents and chemicals

Folin-Ciocalteau reagent, di-sodium hydrogen phosphate dehydrate, absolute ethanol, acetone, and acetic acid were purchased from Merck (Darmstadt, Germany). Sodium carbonate anhydrous was purchased from SDS (Peypin, France). Gallic acid, 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH), Bovine serum albumin fatty acid free, β-acetyl-γ-O-hexadecyl-L-α-phosphatidylcholine (PAF), methanol and chloroform were purchased from Sigma-Aldrich Co. (St. Louis,MO). Trolox, neocuproine and Iron (III) chloride hexahydrate were purchased from from Acros Organics (New Jersey, USA). 2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ) was purchased from Fluka (Milwaukee, WI, USA). Ammonium acetate, sodium chloride, sodium dihydrogen phosphate dehydrate, sodium acetate, copper chloride dihydrate, were purchased from Penta (CZ Ltd., Chrudim, Czech Republic). 2, 2'-Azino-bis-(3-ethylbezothiazoline-6-sulphonic acid (ABTS) was purchased from Applichem (Darmstadt, Germany). Potassium persulfate was purchased from Chem-Lab (Zedelgem, Belgium). Copper sulphate pentahydrate was purchased from Alfa Aesar (Ward Hill, MA). The enzyme of alpha-glucozidase was purchased from MP Biomedicals (Illkirch, France).

2.2 Preparation of pumpkin seeds

Pumpkin (*Cucurbita moschata*) fruits cultivated in the island of Lemnos in Greece were obtained from a local market at Myrina. The seeds were hand selected from the interior of pumpkins and were manually dehusked in order to avoid damaging the kernels. 50 g of husked seeds were rinsed with water, treated with 1g of sodium chloride and roasted at 170 °C in a domestic oven. Roasted seeds were then dehusked. Raw and roasted dehusked kernels were kept at -40 °C for further analysis.

2.3 Extraction of the pumpkin seeds

An amount of 25.0 g of pumpkin seeds raw or roasted were homogenized for 5 min in 250.0 mL methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid using an homogenizer (Ultra Turrax, IKA Werke, Germany). The suspension was left at room temperature under magnetic agitation for 15 min and was then centrifuged for 10 min at 2000 × g in a Hermle Z 383 centrifuge (Hermle Labortechnik, Wehingen). The supernatant was collected in an Erlenmeyer flask, weighted and subjected to further separation into its hydrophilic and lipophilic components through a biphasic solvent system formation according to the method of Bligh and Dyer (1959) with slight modifications. Appropriate amounts of chloroform and water were added to the above collected supernatant to achieve a solvent ratio of chloroform/methanol/water equal to 1/1/0.9 (v/v/v). The mixture was stirred at ambient temperature for 15 min and then centrifuged for 10 min at 2000 × g for the separation of the two solvent phases of the biphasic system. The chloroform and water phase were collected separately in Erlenmeyer flasks, dried using a Lab Tech EV 311 Rotary evaporator (Lab Tech, Italy), and redissolved in a known volume of methanol/water 70/30 (v/v) plus acetic acid to 2% (v/v). The extracts were stored under nitrogen atmosphere at -40 °C until further analysis.

2.4 Determination of total phenolic content

Total phenolic content in the extracts of both raw and roasted pumpkin seeds was determined according to the method of Singleton and Rossi (1965) with minor modifications. Total phenolics assay was conducted by mixing 0.01 to 0.08 mL of extracts with 1.8 mL of distilled water and 0.1 mL Folin-Ciocalteu reagent. After that, samples were vigorously stirred and incubated for 2 min in the dark. Then a quantity of 0.3 mL of aqueous Na_2CO_3 20% (w/v) was added and the samples were vigorously stirred and incubated at 40°C in a water bath for 30 min. Absorbance was measured spectrophotometrically at 765 nm,

using a Spectrophotometer Lambda 25 (Perkin-Elmer, Norwalk, USA). A standard curve was prepared with gallic acid. Final results were given as μg gallic acid /g seeds. Every sample was tested in triplicate.

2.5 Evaluation of the antioxidant capacity

The antioxidant capacity of the extracts of both raw and roasted pumpkin seeds was assessed by the DPPH, ABTS, FRAP and CUPRAC assays.

DPPH Assay

The capacity of the pumpkin seed extracts to scavenge the free radical of DPPH was assessed by a modified method of Abe, Murata, and Hirota (1998). An aliquot of the extracts (0.05 to 0.15 mL) or appropriate standard solution of trolox was diluted with methanol up to 1.8 mL. Then, 0.2 mL of 0.6 mM DPPH reagent in methanol was added, followed by vigorous stirring. After standing for 15 min in the dark, the absorbance was measured spectrophotometrically at 515 nm against a reference sample containing methanol instead of a sample aliquot.

The results were expressed as a SA_{50} value that represents the amount of pumpkin seed in mg whose extract was able to scavenge the radical of DPPH by 50%. That amount was also expressed as nmol of trolox using the standard trolox solutions assayed at conditions similar to the sample extracts. Every sample was tested in triplicate.

ABTS assay

Determination of ABTS radical scavenging activity of extracts was performed by the method of Re et al. (1999) with minor modifications. ABTS radical cation ($ABTS^{+\bullet}$) was generated by the oxidation of ABTS with potassium persulfate ($K_2S_2O_8$). The $ABTS^{+\bullet}$ was produced by reacting 7 mmol/L stock solution of ABTS with 2.45 mmol/L potassium persulphate (final concentration). The $ABTS^{+\bullet}$ solution was diluted (with distilled water) to an absorbance of 0.7 $\pm$ 0.05 at 734 nm. Aliquots of raw and roasted pumpkin extracts (0.01 to 0.12 mL) or appropriate amounts of trolox standard solution were

dried under a stream of nitrogen followed by addition of 2.5 mL $ABTS^{+\bullet}$ and were vigorously stirred. Samples were incubated for 15 min in the dark at ambient temperature and the absorbance was measured spectrophotometrically at 734 nm. The ability of the extracts to scavenge the ABTS free cationic radical was evaluated relative to the reference sample that did not contain any extract. The results were expressed as a SA_{50} value that represents the amount of pumpkin seed in mg whose extract was able to scavenge $ABTS^{+\bullet}$ by 50%. That amount was also expressed as nmol of trolox using standard trolox solutions assayed at conditions similar to the sample extracts. Every sample was tested in triplicate.

FRAP Assay

The reducing potential of the sample was determined using the FRAP assay as described by Benzie and Strain (1996). The method is based on the reduction of the Fe^{3+}- tripyridyltriazine complex to its ferrous colored form at low pH in the presence of antioxidants. The FRAP reagent was freshly prepared daily and contained 0.2 mL of a 10 mM TPTZ (2,4,6- tripyridy-s-triazine) solution in 40 mM HCl plus 0.2 mL of 20 mM $FeCl_3\bullet6H_2O$ plus 0.2 mL of 3.0 M acetate buffer, pH 3.6. Aliquots of raw and roasted pumpkin extracts (0.02 to 0.12 mL) were transferred in glass test tubes, dried under a stream of nitrogen and dissolved in distilled water to a final volume of 1800 μL, followed by an addition of 600 μL of FRAP solution and vigorous stirring. The samples were incubated for 10 min in a 37 ˚C water bath and the absorbance was measured at 593 nm. A standard curve was prepared using different concentrations of trolox. The antioxidant activity of the pumpkin seeds was expressed as μmol trolox/g seeds. Every sample was tested in triplicate.

CUPRAC Assay

The reducing potential of the sample was determined using the CUPRAC assay as described by Apak, Guclu, Ozyurek, and Karademir (2004). Different volumes of the extracts were transferred in test tubes and were briefly dried under a stream of nitrogen. An ad-

dition of 600 μL CuCl$_2$•2H$_2$O 10 mM solution, 600 μL neocuproine 7.5 mM and 600 μL buffer CH$_3$COONH$_4$ 1M pH=7.0 was followed and a final volume of 2400 μL was reached with dH$_2$O. The samples were well stirred and incubated at room temperature for 30 min. After the incubation the absorbance of the samples was measured at 450 nm.

A standard curve was prepared using different concentrations of trolox. The antioxidant activity of the pumpkin seeds was expressed as μmol trolox/g seeds. Every sample was tested in triplicate.

2.6 *In vitro* anti-thrombotic/anti-inflammatory activity

The *in vitro* anti-thrombotic/anti-inflammatory activity of raw and roasted pumpkin seed extracts was evaluated on a Chrono-Log 500-Ca aggregometer (Chrono-Log Co., Havertown, PA, USA) connected to a computer (Aggro/Link software; Chrono-Log, Hawerton, PA), according to their ability to inhibit the thrombotic and inflammatory lipid mediator of platelet activating factor (PAF) towards human platelet rich plasma (PRP) as previously described (Karantonis et al., 2006). Aliquots of the extracts (0.1 to 5.0 μL) and PAF ethanolic solution were evaporated under a stream of nitrogen and reconstituted in bovine serum albumin (BSA) (2.5 mg/mL saline). After that, aliquots of 250 μL of PRP along with stir bars were placed in siliconized glass cuvettes and were incubated for 15 min in the incubation wells of the aggregometer at 37 ˚C. In the next step, the platelet response induced by PAF (10E-8 M, final concentration) in human platelet rich plasma before (considered as 0% inhibition) and after the addition of various amounts of the examined samples was recorded by the aggregometer upon stirring at 1200 rpm. The amount of extracts required for 50% inhibition (Equivalent amount for 50% inhibition; IA$_{50}$) of PAF activity was calculated using the linear plot of the percentage inhibition, ranging from 20 to 80 %, versus different amounts of the samples. The results were expressed as mg of raw or roasted seeds. Each sample was evaluated in

duplicate.

2.7 *In vitro* anti-atherogenic activity

In vitro antiatherogenic activity was evaluated by the human plasma oxidation inhibition assay according to Schnitzer et al. (1998) with minor modifications using a Spectrophotometer Lambda 25 (Perkin-Elmer, Norwalk, USA) equipped with an 8 position thermostated sample changer. Aliquots of raw and roasted pumpkin seed extracts (0.020 to 0.030 μL) were placed in UV-transparent disposable cuvettes (Brand, Wertheim, Germany) and dried under a stream of nitrogen. Then, 2200 μL of phosphate buffer solution, pH 7.4, 146 mM in NaCl and 50 μL of human plasma were added, followed by gentle shaking and incubation for 1 hour at ambient temperature. In the next step, the samples were placed into the thermostated chamber of the photometer and incubated for 10 min at 37 ˚C and the oxidation reaction was started by the addition of 250 μL of 1 mM CuSO$_4$•5H$_2$O. The absorbance was continuously recorded for 3 h at 245 nm and at a stable temperature of 37 ˚C. Lag times from the resultant sigmoid curves were calculated; time needed for the antioxidants to be oxidized, corresponding to the period of time that plasma resists oxidation. The percentage of the inhibition of *in vitro* plasma oxidation induced by the extracts was evaluated by comparing the lag time of each tested sample containing extract to a reference sample that did not contain extract. Every sample was tested in duplicate.

2.8 *In vitro* anti-diabetic activity

The anti-diabetic activity was determined by the alpha-glucosidase inhibition assay according to Vinholes et al. (2011). Aliquots of raw and roasted pumpkin seed extracts (0.020 to 3.5 uL) were transferred in polypropylene test tubes and dried under a stream of nitrogen. After that, 6.0 μL of a-glucosidase prepared in double distilled water (0.02 U per mL; final concentration) and sodium phosphate buffer solution (PBS) 0.1M pH equal to 6.8 were added to a final volume of 400 μL. The samples were incubated
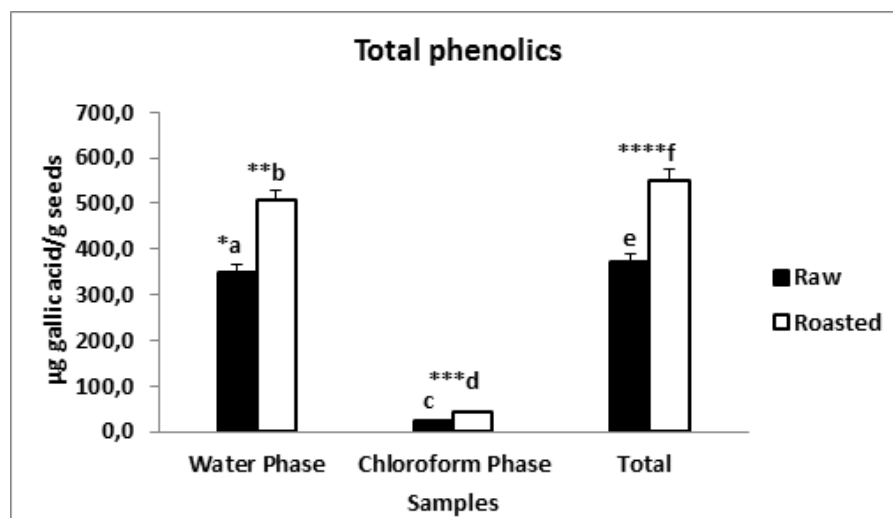
Figure 1: Total phenolic content for raw and roasted pumpkin seeds extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. The results are expressed as mean ± SD for triplicate determinations in water phase, chloroform phase and total extract. *: denotes significantly higher phenolic content compared to c and d. **: denotes significantly higher phenolic content compared to a, c and d. ***: denotes significantly higher phenolic content compared to c. ****: denotes significantly higher phenolic content compared to e. Differences with a value of p<0.05 were considered statistically significant

for 10 min at 37.5 ˚C. After the incubation step, 50 μL of p-nitrophenyl glucopyranoside (pNPG) 3 mM, used as enzyme substrate, was added to begin the enzyme reaction with a final concentration of alpha-glucosidase equal to 0.02 U/mL. The mixture was incubated for 10 min at 37.5 ˚C. The enzyme reaction was stopped by the addition of 800 μL Na$_2$CO$_3$ 1 M. The absorbance of the samples was measured at 405 nm.

For each different extract sample, a blank containing sample extract without alpha-glucosidase, and a reference containing alpha-glucosidase (without sample extract) were prepared. The results were expressed as the percentage inhibition of alpha-glucosidase activity and the IA25 values were determined as mg of seeds which inhibited alpha-glucosidase activity by 25%. Each sample was tested in triplicate.

2.9 Statistical analysis

Data are presented as mean ± standard deviation for triplicate determination or as mean for duplicate determinations. Statistical analysis was performed with SPSS version 21.0 (SPSS Inc, Chicago, IL). An independent t-test was performed to assess statistical significance. Differences with a value of p<0.05 were considered statistically significant.

3 Results and Discussion

3.1 Total phenolics

Phenolic compounds are associated with antioxidant activity and play an important role in stabilising lipid peroxidation (Yen, Duh, & Tsai, 1993). Content of total phenolics in extracts of *Curcubita moschata* seeds was expressed as μg of gallic acid per g of seeds. Total phenolics in the water and chloroform phases of raw

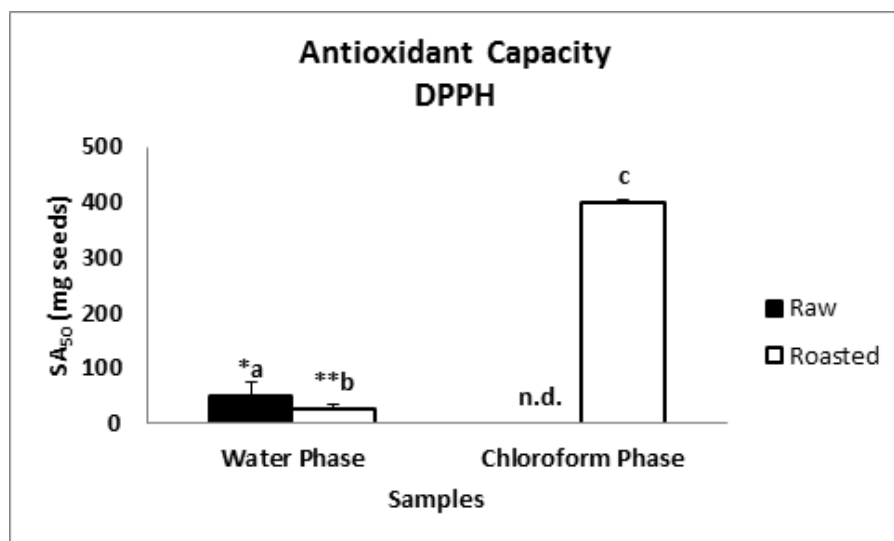Figure 2: Antioxidant capacity as assessed by the scavenging of the DPPH (2,2-Diphenyl-1-picryhydrazyl) radical. The results are expressed as mean ± SD for triplicate determinations in water phase and chloroform phase for raw and roasted pumpkin seeds after extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. SA_{50}: denotes the equivalent amount for 50% scavenging. n.d.: stands for not detected.*: denotes significantly higher antioxidant capacity compared to c. **: denotes significantly higher antioxidant capacity compared to a and c. Differences with a value of $p < 0.05$ were considered statistically significant

seed extract were 347.6 ± 17.4 and 22.7 ± 1.7 respectively, giving a total phenolic content for raw seeds equal to 370.3 ± 19.1 (Figure 1). On the other hand, total phenolics in the water and chloroform phases of roasted seeds were 506.8 ± 21.9 and 44.2 ± 1.0 respectively, giving a total phenolic content for roasted seeds equal to 551.0 ± 22.8. (Figure 1). The results indicate that pumpkin seeds contain both lipophilic and hydrophilic phenolics. The hydrophilic phenolics in both raw and roasted seeds were higher compared to the lipohilic ones (p<0.05). Hydrophilic and lipophilic phenolics were significantly higher in roasted seed extracts compared to raw seed extracts (p<0.05), resulting also in a significantly higher amount of total phenolics in roasted compared to raw seeds (p<0.05) (Figure 1). Increased phenolics after heating have been previously been reported in fruit seeds (Soong & Barlow, 2004). The authors suggested that the formation of phenolic compounds during

the heating process might be due to the availability of precursors of phenolic molecules, by non-enzymatic interconversion between phenolic molecules subjected to the effects of temperature.

3.2 Antioxidant Capacity

The plethora of different antioxidants in various foods, especially fruits and vegetables, make it difficult to measure each antioxidant component separately. Therefore, several methods have been developed to evaluate the total antioxidant capacity in various food samples including DPPH, ABTS, FRAP and CUPRAC assays.

Antioxidant Capacity

Results concerning the capacity of *Curcubita moschata* seed extracts to scavenge the free radical of DPPH were expressed as equivalent
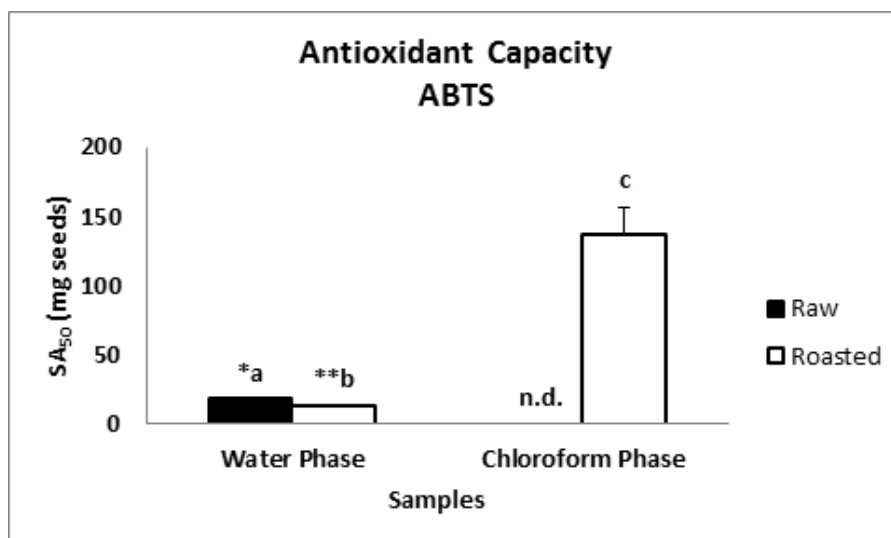
Figure 3: Antioxidant capacity as assessed by the scavenging of the ABTS (2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) radical cation. The results are expressed as mean $\pm$ SD for triplicate determinations in water phase and chloroform phase for raw and roasted pumpkin seeds after extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. SA_{50}: denotes the equivalent amount for 50% scavenging. n.d.: stands for not detected. *: denotes significantly higher antioxidant capacity compared to c. **: denotes significantly higher antioxidant capacity compared to a and c. Differences with a value of $p < 0.05$ were considered statistically significant

amount in mg of pumpkin seeds for 50% scavenging (SA_{50}), meaning that a lower value of SA_{50} corresponds to a higher scavenging activity for the extract. In raw seed extract, the SA_{50} for the water phase was equal to 50.03 ± 5.91 (mg of raw seeds), while no scavenging activity was detected in the corresponding chloroform phase (Figure 2). On the other hand, in roasted seed extract, the SA_{50} value for the water and chloroform phases were equal to 25.82 ± 6.77 and 400.80 ± 3.25 (mg of roasted seeds) respectively (Figure 2). The above values of SA_{50} were equivalent to 26 nmol of trolox based on a trolox standard curve for the assay. The results show that roasted seed extracts exerted significantly higher antioxidant capacity compared to the raw seed extracts ($p < 0.05$) in terms of DPPH radical scavenging. This result is in accordance with the higher total phenolic content determined in roasted compared to raw pumpkin seed extracts.

ABTS Assay

The same pattern was observed for the scavenging radical cation of ABTS. More specifically, in raw seed extract, the SA_{50} for the water phase was equal to 17.85 ± 0.77 (mg of raw seeds), while no scavenging activity was detected in the corresponding chloroform phase (Figure 3). On the other hand, in roasted seed extract, the SA_{50} value for the water and chloroform phases were equal to 12.77 ± 0.76 and 137.38 ± 19.38 (mg of roasted seeds) respectively (Figure 3). The above values of SA_{50} were equivalent to 33 nmol of trolox based on a standard curve for the assay. The results show that roasted seed extracts exerted significantly higher antioxidant capacity compared to the raw seed extracts ($p < 0.05$) in terms of ABTS cation radical scavenging. The results show also a higher capacity for scavenging the ABTS radical cation compared to the DPPH radical. This result is also in accordance with
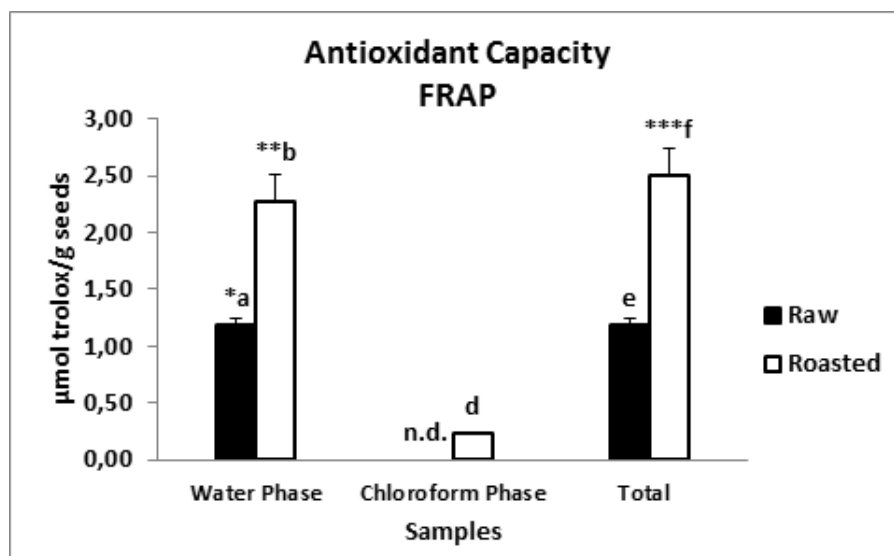
Figure 4: Antioxidant capacity as assessed by reduction of ferric to ferrous ions for raw and roasted pumpkin seeds extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. The results are expressed as mean ± SD for triplicate determinations in water phase, chloroform phase and total extract. n.d.: stands for not detected. *: denotes significantly higher antioxidant capacity compared to d **: denotes significantly higher antioxidant capacity compared to a and d. ***: denotes significantly higher antioxidant capacity compared to e. Differences with a value of $p < 0.05$ were considered statistically significant

the higher total phenolic content determined in roasted compared to raw pumpkin seed extracts.

FRAP Assay

The antioxidant activities of pumpkin seed extracts in terms of their capacity to reduce ferric to ferrous form of TPTZ were expressed as μmol trolox per g of seeds and are presented in Figure 4. In raw seeds, the estimated value from the FRAP assay was 1.19 ± 0.05 μmol trolox/g of seeds. This value comes from the water phase of the raw seed extract, since no antioxidant capacity was detected in the chloroform phase of this extract. On the other hand, antioxidant capacities in the water and chloroform phases of roasted seed extracts were 2.27 ± 0.24 and 0.23 ± 0.01 μmol trolox/g of seeds respectively. This gives a total antioxidant capacity for the roasted seeds equal to 2.50 ± 0.23 μmol trolox/g of seeds. The results show that antioxidant capacities in terms

of the FRAP assay were significantly higher in both phases of roasted seed extracts compared to those of raw seed extracts ($p < 0.05$), resulting also in a significantly higher antioxidant capacity in roasted compared to raw seeds in total ($p < 0.05$).

CUPRAC Assay

The antioxidant activities of pumpkin seed extracts in terms of their cupric ion reducing capacity were expressed as μmol trolox per g of seeds and are presented in Figure 5. Antioxidant capacities in the water and chloroform phases of raw seed extracts were 1.78 ± 0.11 and 0.35 ± 0.03 μmol trolox/g of seeds, respectively. This results to a total antioxidant capacity for raw seeds equal to 2.13 ± 0.11 μmol trolox/g seeds. On the other hand, antioxidant capacities in the water and chloroform phases of roasted seeds were 2.85 ± 0.05 and 0.40 ± 0.01 μmol trolox/g of seeds,
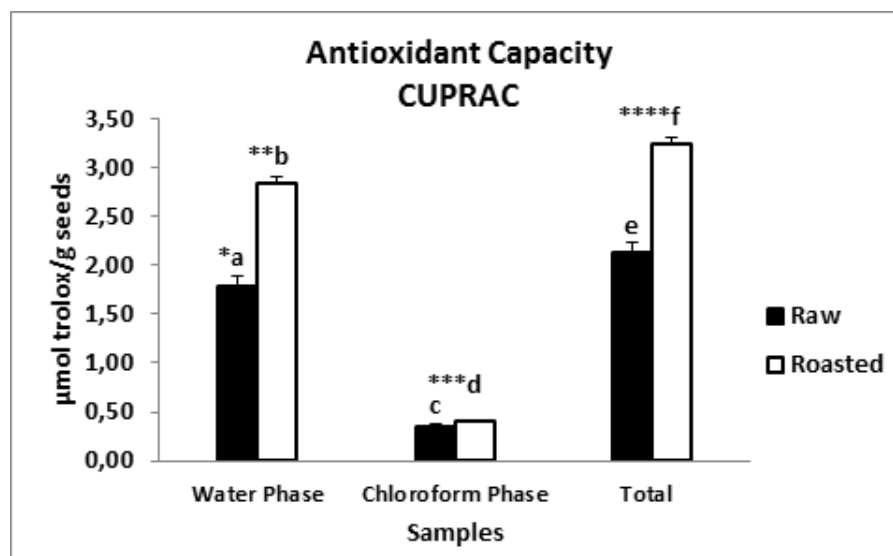
Figure 5: Antioxidant capacity as assessed by reduction of cupric to cuprous ions for raw and roasted pumpkin seeds extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. The results are expressed as mean ± SD for triplicate determinations in water phase, chloroform phase and total extract. *: denotes significantly higher antioxidant capacity compared to c and d. **: denotes significantly higher antioxidant capacity compared to a, c and d. ***: denotes significantly higher antioxidant capacity compared to c. ****: denotes significantly higher antioxidant capacity compared to e. Differences with a value of $p<0.05$ were considered statistically significant

respectively. This results in a total antioxidant capacity for roasted seeds equal to 3.25 ± 0.06 μmol trolox/g seeds. The results show that antioxidant capacity in terms of the CUPRAC assay were significantly higher in both phases of roasted seed extracts compared to those of raw seed extracts ($p<0.05$), resulting also in a significantly higher antioxidant capacity in roasted compared to those of raw seeds in total ($p<0.05$). Oxidative stress plays an important role in ageing and the pathogenesis of chronic diseases such as atherosclerosis and diabetes (Arzamastseva et al., 2007; Giacco & Brownlee, 2010). Many epidemiological studies suggest that consumption of a fruit- and vegetable-rich diet inversely correlates with the risk of chronic diseases. These chemoprotective effects are, at least in part, related to the antioxidant activities of phenolic compounds (Holt et al., 2009; Naelsen, Basu, Wolk, & Vessby, 2006).

3.3 In vitro anti-thrombotic/anti-inflammatory activity

The in vitro anti-thrombotic/anti-inflammatory activity of pumpkin seed extracts were expressed as the equivalent amount, in mg of seeds, required for 50% inhibition of PAF activity (IA_{50}) toward human platelet rich plasma (PRP). This means that a lower value of IA_{50} corresponds to a higher inhibitory activity for the extract. The IA_{50} value for the water phase of the raw seed extract was equal to 0.62 mg and was significantly higher compared to the value of 0.15 mg that corresponded to the respective phase of the roasted seed extract ($p<0.05$) (Figure 6). No inhibitory activity was detected in the chloroform phases of both raw and roasted seed extracts (Figure 6). The results show the existence of hydrophilic minor compounds in both
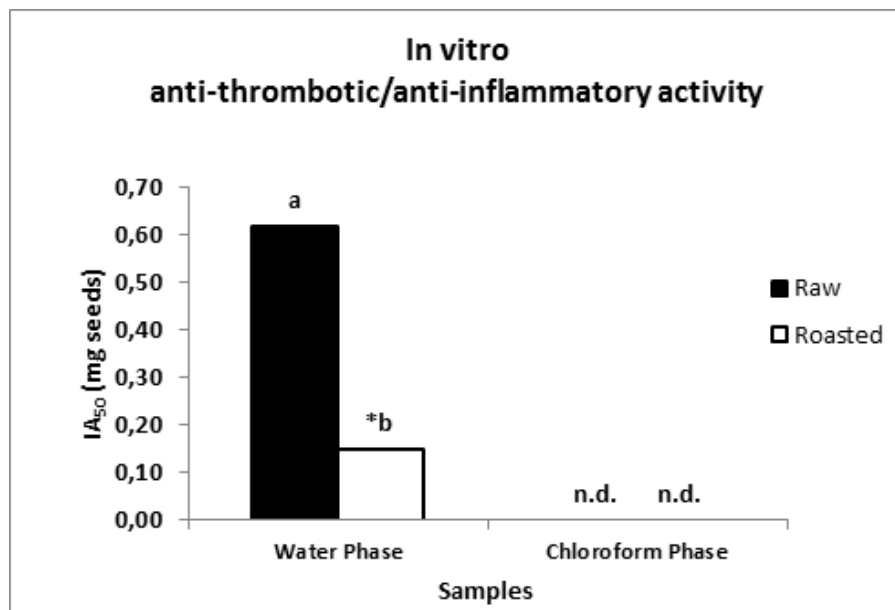
Figure 6: *in vitro* anti-thrombotic/anti-inflammatory capacity as assessed by inhibition of platelet activating factor (PAF) activity for raw and roasted pumpkin seeds extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. The results are expressed as mean value for duplicate determinations in water and chloroform phase. IA_{50}: stands for equivalent amount for 50% inhibition. n.d.: stands for not detected. *: denotes significantly higher anti-thrombotic/anti-inflammatory capacity a. Differences with a value of $p < 0.05$ were considered statistically significant

raw and roasted pumpkin seeds that exert anti-thrombotic/anti-inflammatory activity in terms of PAF inhibition toward human platelets. A higher activity was detected in the water phase of roasted seeds compared to the respective phase of raw seeds ($p < 0.05$). These results mirror what was found for the total phenolic content and antioxidant capacity of the seed extracts.

A fruit- and vegetable-rich diet has also been inversely correlated with reduced inflammation (Holt et al., 2009). Platelet activating factor (PAF) identified as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Demopoulos, Pinckard, & Hanahan, 1979) has been recognized as one of the most potent lipid inflammatory and thrombotic mediator that activates various cells through its specific receptor, such as platelets (Demopoulos et al., 2003). Activated platelets are important contributors to thrombosis and inflammation, and represent an important linkage between inflammation, thrombosis, and atherogenesis (Demopoulos et al., 2003; Fuentes et al., 2013; Gawaz, Langer, & May, 2005; Rondina, Weyrich, & Zimmerman, 2013). *In vitro* inhibition of PAF induced platelet activation from food components has been used as a research tool to investigate the nutritional value of those foods and their possible preventive effect against chronic disease development when consumed as part of a balanced diet (Karantonis, Antonopoulou, & Demopoulos, 2002, 2006; Karantonis et al., 2008; Nomikos, Karantonis, Skarvelis, Demopoulos, & Zabetakis, 2006; Nomikos et al., 2007).
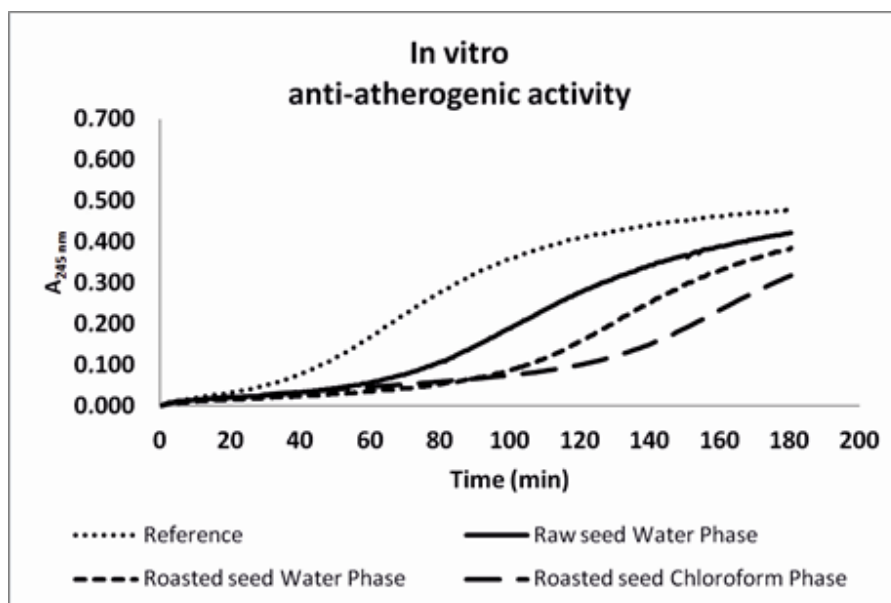
Figure 7: Representative graph for *in vitro* anti-atherogenic activity as assessed by inhibition of human plasma oxidation for raw and roasted pumpkin seeds extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. Amounts equivalent to other extract phased tested for Raw seed Chloroform phase did not exerted any inhibition in human plasma oxidation

3.4 *In vitro* anti-athirogenic activity

The *in vitro* anti-atherogenic activity of Curcubita moschata seed extracts was evaluated as inhibition of human plasma oxidation and expressed as % increase in lag time of human plasma oxidation in the presence of the tested sample compared to the reference sample with a lag time oxidation equal to 41.5 min. An amount of water phase extract that corresponded to 22.5 mg of raw seeds caused an inhibition of human plasma oxidation, resulting in a lag time of 67.5 min that corresponded to a 63% increase compared to the reference sample. Non inhibition was detected for the chloroform phase of this extract. On the other hand, the water phase of the roasted seed extract, in an amount that corresponded to 15.2 mg of roasted seeds, resulted in a lag time equal to 109.1 min that corresponded to a 163% increase compared to the reference sample. The chloroform phase of the roasted seed extract, in an amount that corresponded to 15.6 mg of roasted seeds, was also active, causing a lag time of 142 min that corresponded to a 242% increase compared to the reference sample (Figure 7). The results show a higher *in vitro* anti-atherogenic activity for the extracts of the roasted seeds ($p < 0.05$). These results mirror what was found for the total phenolic content and antioxidant capacity of the seed extracts.

The most common causes of death in westernized societies remain heart disease and stroke. Prevention of atherosclerosis is a major objective of modern medicine. Studies show that oxidation of plasma lipoproteins plays an important role in atherogenesis (Karten, Beisiegel, Gercken, & Kontush, 1997), since oxidatively modified molecules on oxidized LDLs initiate the events of atherosclerosis development at the molecular level (Demopoulos et al., 2003). Therefore, the delay of plasma oxidation is a contributing protective factor against ahterogenesis.
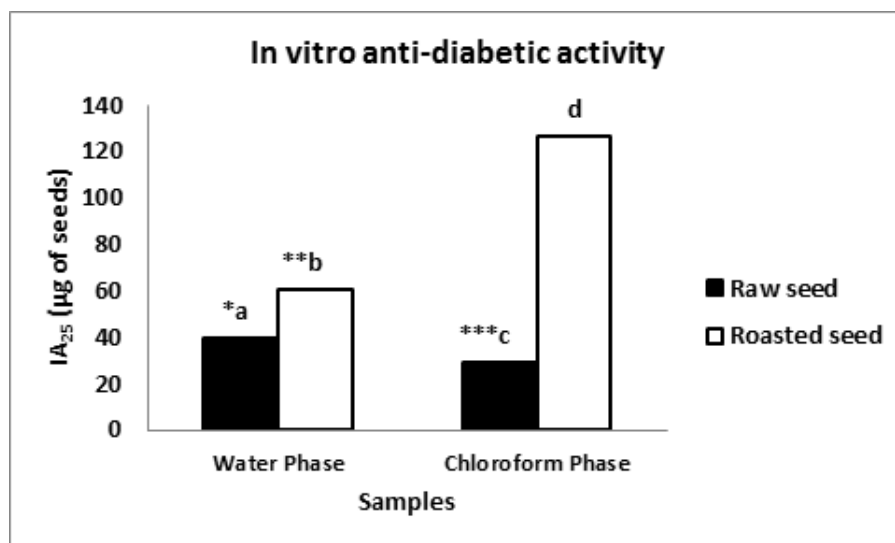
Figure 8: *in vitro* anti-diabetic activity as assessed by inhibition of alpha-glucosidase for raw and roasted pumpkin seeds extracted by methanol/water 70/30 (v/v) plus 2% (v/v) acetic acid and separated as hydrophilic components in water phase and lipophilic ones in chloroform phase. The results are expressed as mean value for duplicate determinations in water and chloroform phase. IA_{25}: stands for equivalent amount for 25% inhibition. *: denotes significantly higher anti-diabetic activity compared to b and d and a lower one compared to c. **: denotes significantly higher anti-diabetic activity compared to d and a significantly lower one compared to c. ***: denotes significantly higher anti-diabetic activity compared to d.Differences with a value of $p < 0.05$ were considered statistically significant

3.5 *In vitro* anti-diabetic activity

The results of the ability of the extracts to inhibit alpha-glucosidase activity were expressed as the equivalent amount, in μg of seeds, for 25% inhibition of the enzyme (IA_{25}). This means that a lower value of IA_{25} corresponds to a higher inhibitory activity for the extract. The IA_{25} value of 40.0 μg for the water phase of the raw seed extract was significantly lower compared to the value of 61.0 μg for the respective phase of the roasted seed extract ($p < 0.05$) (Figure 8). Similarly, IA_{25} of the raw seed extract chloroform phase, with a value equal to 29 μg of seeds, was significantly lower compared to 127 μg of seeds that corresponded to the IA_{25} value of the respective phase of the roasted seed extract ($p < 0.05$) (Figure 8). The results show the existence of both hydrophilic and lipophilic minor compounds in both raw and roasted pumpkin seeds that exert anti-diabetic activity in terms of alpha-glucosidase inhibition, with a significantly higher activity of raw seed extracts ($p < 0.05$). These results are in contrast to the rest of the results that show higher activities of roasted seed extracts compared to the raw ones.

One of the therapeutic approaches for treating diabetes is by controlling the postprandial hyperglycaemia by retarding the absorption of glucose. Enzyme alpha-glucosidase (EC 3.2.1.20), hydrolyses terminal non-reducing 1-4 linked alpha-glucose residues to release a single alpha- glucose molecule creating in this way absorbable monosaccharides. Inhibition of alpha-glucosidase in the digestive tract delays carbohydrate digestion and prolongs overall carbohydrate digestion time. This causes a reduction in the rate of glucose absorption and consequently blunts the postprandial blood glucose and insulin levels. Thus alpha-glucosidase inhibitors may be an attractive therapeutic modality in type II diabetic patients (Bischoff, 1994; Jiang & Du, 2011;

Krentz & Bailey, 2005; Lin et al., 2011; Oboh & Ademosun, 2011).

4 Conclusions

Raw seed extracts from *Cucurbita moschata* exert antioxidant, anti-inflammatory/anti-thrombotic, anti-atherogenic and anti-diabetic activities. Anti-diabetic activity was detected higher in raw seed extracts. In contrast, the rest of the studied activities were increased by roasting the seeds. Experimental conditions for the roasting of the pumpkin seeds that would optimize total phenolic content and the rest of the studied activities is an area for further research.

The results show that in Lemnos *Cucurbita moschata* seeds there exists hydrophilic and lipophilic compounds with *in vitro* functionalities related to heath-beneficial effects. Pumpkin seeds appear to be a suitable candidate ingredient that, as an additive, may boost anti-oxidant anti-throbotic/anti-inflammatory capacities in certain novel functional food production of regional interest. Such functional foods would contribute in a sustainable way to public health improvement by reducing oxidative stress, thrombosis/ inflammation and the related chronic disease development.

Acknowledgements

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