International Journal of Food Science and Nutrition
ISSN: 2455-4898; Impact Factor: RJIF 5.14
Received: 22-05-2020; Accepted: 09-06-2020; Published: 23-06-2020
www.foodsciencejournal.com
Volume 5; Issue 3; 2020; Page No. 150-154

In-vitro antioxidant activities of free, esterified and bound phenolics fractions from the peel and seed of two pumpkins (*Cucurbita moschata*) varieties

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Abstract
This work sought to measure the contents of free, esterified, and bound phenolics (phenol and flavonoid) in peel and seed of two pumpkin varieties (sakaka and nagdi) and evaluate the antioxidant properties. The total phenol and flavonoid contents were evaluated using the Folin–Ciocalteu method, and the aluminum chloride colorimetric assay, respectively. The antioxidant activities of the two varieties were assessed using TEAC and reducing power assays. The free phenol content of nagdi peel was higher than that of sakaka peel and seed of two pumpkins, while esterified and bound phenol contents in nagdi seed were the highest among all samples examined. In the case of flavonoid content, the sakaka peel had the highest contents of free, esterified, and bound flavonoid. Combining the three fractions, the high contents of free, esterified and bound phenolics were found in nagdi peel. In the case of antioxidants properties, Free fraction from nagdi peel showed the highest antioxidant activity properties in both assays. Whereas, esterified fraction and bound fraction from nagdi seed and sakaka peel showed the highest TEAC and reducing power values, respectively. Generally, the Phenolics extracts of pumpkin by-products showed antioxidant properties. Therefore, these extracts could potentially in nutraceuticals preparation and functional food formulations.

Keywords: pumpkin peel and seed, phenol and flavonoid contents and antioxidant properties

1. Introduction
Reactive oxygen species (ROS) are unstable and react with other substances including lipids, proteins, and DNA. The unchecked activities of ROS are associated with health disorders such as cancer, diabetes mellitus, gastric ulcers, hypertension, neurodegenerative, arthritis, reperfusion, and inflammatory diseases [1]. There are found that vegetables are an excellent source for antioxidants which are well known to neutralize ROS and free radicals [2]. Pumpkin (*Cucurbita moschata)* is one of the important types of vegetables in agricultural systems, t is widely cultivated in several countries due to its high nutritional value and its health benefits [3]. Pumpkin can be used for nutritional food as well as medicine in many countries. It is used in processed foods such as syrups, jams, jellies, and purees, This vegetable very popular with its abundant nutrition, low-calorie count, and soft texture [4]. In particular, there are found that the three parts of the pumpkin are a good source for phenol, flavonoid, a-tocopherol, carotenoids, vitamin C, vitamin A, carbohydrates, and amino acids [5]. What is more, numerous experiment results revealed that pumpkin and its by-products have health benefits such as anti-diabetes [6], anti-cancer [7], and antioxidant properties [3].
The health benefits of plant and agriculture by-products have been correlated with the existence of bioactive components, mainly polyphenols. The benefits of phenolics are due to their antioxidant activity against numerous radicals such as superoxide radicals, singlet oxygen, and 2,20-azino-bis (3 ethylbenzothia zoline-6-sulphonic acid) diammonium salt radical cation [8]. Therefore, phenolics such as phenol and flavonoid have attracted attention to the studies of plant and agriculture by-products [9]. Interestingly, the phenolics are presented in three forms include free, esterified and bound phenolics forms, the free and esterified phenolics are extractable by a solvents solution such as acetone, methanol, ethanol and water which are conjugated to carbohydrates and low-molecular-mass components, whereas bound phenolics are connected to structural components of the cell wall through covalent bond and they are usually extracted by residue alkalization after free and esterified phenolics extraction [10, 11, 12]. Overall, the evidences have displayed a positive correlation between the content of phenolics and the antioxidant potential of the edible plants [9]. However, there is little evidence about the existence of phenolics in pumpkin *Cucurbita moschata* (peel and seed). Furthermore, to the best of our knowledge, no studies were carried out on the free, esterified, and bound phenolics of *Cucurbita moschata* planted in Saudi Arabia. *Cucurbita moschata* is planted in different environments and areas which display different properties and composition of nutrients [4]. thus, the current study examined free, esterified, and bound phenolics in peel and seed of two pumpkins (sakaka and nagdi) varieties grown in Saudi Arabia and then the antioxidant properties of these phenolics were examined in vitro to understand their medicinal properties.

2. Materials and Methods
2.1 Materials
Two varieties (sakaka and nagdi) of the summer pumpkin, (*Cucurbita moschata*) are procured from the Department of Plant Production, College of Food and Agriculture Sciences,

King Saud University, Saudi Arabia. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carbxylic acid), 2, 2 0-Azobis (2-methylpropionamidine) hydrochloride (AAPH); 3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Folin Ciocalteau's phenol reagent**,** gallic acid, quercetin, organic solvents and reagents, and ferric chloride were procured from Sigma Aldrich USA.

2.2. Sample preparation
Pumpkin samples (peel and seed) were separated, cut, and dried. For further examination, the dried samples were ground and passed through a 0.5 mm sieve, vacuum-packed, and stored at -20 C °.

2.3 Extraction and fractionation of the free, esterified and bound phenolics
Dried Pumpkin (peel and seed) was mixed with 150 ml of a mixture of methanol-acetone-water (7:7:6 v/v/v). After ultrasonic for 20 min at 30 C°, the slurries were centrifuged at 4000 g for 5 min and the supernatants were collected. The residue was re-extracted and centrifuged under the same conditions. The combined supernatants were evaporated under vacuum at 40 C° to remove the organic solvents. After adjusting the pH of the aqueous phase to 2, the interfering lipids in the aqueous phase were removed through the extraction with hexane. The free phenolics (FPS) in the aqueous phase were extracted with diethyl ether-ethyl acetate (1:1 v/ v) 4 times, dried under vacuum using a rotary evaporator, and the extract was dissolved in 5 ml of 80 % methanol. The esterified phenolics (EPS) remaining in the aqueous phase were hydrolyzed with 4M sodium hydroxide and the liberated phenolics were extracted, dried, and dissolved in 5 ml methanol as in the case of FPS. The residues were initially dispersed in 50 ml of 4 M sodium hydroxide and stirred for 4 h under nitrogen. The solution was then acidified to pH 2, centrifuged and the bound phenolics (BPS) were extracted, dried, and dissolved in 5 ml methanol as in the case of FPS and EPS [2].

2.4 Estimation total of free, esterified and bound phenol (FP, EP, and BP)
In brief, the fractions (0.5 ml) were mixed with 0.5ml Folin Ciocalteu's phenol reagent (FCR)**.** After mixing, 1ml of sodium carbonate and 10 ml of distilled water were added. Followed by incubation of the mixtures at room temperature for 45 min. After centrifugation for 5min at 4000g, the Absorbance of the supernatants was subsequently detected at 725 nm (MOD. 4050, Biochrom, and Cambridge, UK). The standard curve was prepared using a Gallic acid solution. Total phenol content; C (expressed as mg GAE/100g dry weight) was calculated using an Eq. (1);

$C = C_1 \times (V/m) \times 100$ (1)

where C1 is the Gallic acid concentration established from the calibration curve in mg/ml; V is the volume aqueous phase extract in ml; m is the weight of sample in grams [13]

2.5 Determination total of free, esterified and bound flavonoid (FF, EF, and BF)
Total flavonoid contents of FF, EF, and BF were measured by the aluminum chloride colorimetric assay (1). 1 ml of extract or standard solution was mixed with 4 ml distilled water, and 0.3 ml of 5% sodium nitrite. The 0.3 ml 10% aluminum chloride and 2 ml of 1 M sodium hydroxide were added after 5 and 6 min, respectively. After dilution, the absorbance was measured at 510 nm (MOD. 4050, Biochrom, and Cambridge, UK). Total flavonoid content (expressed as mg Quercetin/g 100 dry weight) was calculated as mentioned above.

2.6. In vitro antioxidant assays
2.6.1. Total antioxidant capacity by Trolox equivalent antioxidant capacity (TEAC)
The TEAC test is based on scavenging of 2, 2 0-azinobis-(3-ethylbenzothiazoline -6-sulphonate) radical cation (ABTS*). Briefly, a solution of ABTS* was generated by mixing 2.0 mM ABTS stock solution with 2.5 mM (AAPH) at 1:1 (v/v). The ABTS* solution was heated for 12 min at 60 C°, filled in the dark bottle, and stored at room temperature. The absorbance of the radical solution at 734 nm was adjusted to 0.700 using ethanol. To measure TEAC values, the fractions (40 µl) or standard (Trolox) were mixed with ABTS* solution (1.96 ml). After 6 min incubation at room temperature, the absorbance was subsequently recorded at 734 nm (MOD. 4050, Biochrom, and Cambridge, UK). TEAC values were presented as µmol of Trolox equivalents (TE) per 100 grams of a dried sample [14].

2.6.2. Measurement of reducing property
The reducing property of the fractions was measured by evaluating the ability of the fractions to reduce the ferric chloride ($FeCl_3$) solution**.** Briefly, 1 ml fraction was mixed with 2.5 ml of phosphate buffer (0.2 mol /l pH 6.6) and 2.5 ml of potassium ferricyanide (1%). After incubation for 20 min at 50 C°, 2.5 ml of 10% trichloroacetic acid was added. Subsequently, 2.5 ml of solution was moved to a new tube, 2.5 ml of distilled water, and 0.5 ml of 0.1% $FeCl_3$ were added. After mixing, the absorbance was detected in UV-VIS Spectrophotometer at 700 nm (MOD. 4050, Biochrom, and Cambridge, UK). The standard curve was set using known concentrations of Trolox [1].

2.7. Statistical analysis
All experiment data in this study were analyzed by using one-way analysis (ANOVA) and Duncan test for comparison between means using the SAS statistical analysis software program (SAS software version 9.2 (SAS Institute Inc., 2008). Significance was determined at $p \leq 0.05$ level. All data were reported as the mean ± standard deviation (S.D.).

3. Results and discussion
3.1 Total phenol and flavonoid
The contents of the FP, EP, and BP in tested samples are presented in Fig. 1. The extracts of pumpkin by-products showed FP, EP, and BP values ranging from 67.90 to 104.70, 21.50 to 32.80, and 11.80 to 24.50 mg GAE /100g dried sample, respectively. In the free fractions, the nagdi peel fraction displayed the highest ($P<0.05$) FP, followed by sakaka peel and nagdi seed, while FP content in sakaka seed fraction was significantly lesser than that of nagdi seed and peel of two pumpkins (Fig.1A). In the esterified fractions, the high ($P<0.05$) EP content was found in the seed of nagdi pumpkin, while sakaka peel fraction contained the least ($P<0.05$) amount of EP among all samples (Fig.1B). In the bound fractions, the nagdi seed fraction contained the highest ($P<0.05$) amount of BP among all samples examined, while low ($P<0.05$) BP amount was found in sakaka peel fraction. No significant difference in the content

of BP between sakaka seed and nagdi peel (Fig.1C). Combining the three fractions, the high (P<0.05) FP, EP and BP content was found in nagdi peel followed by nagdi peel> nagdi seed > sakaka seed > sakaka peel (Fig.1D). What is more, the sum phenol content (FP, EP, and BP) in by-products of nagdi pumpkin was higher than that in by-products of sakaka pumpkin.

In the case of flavonoid content, the extracts of pumpkin by-products showed FF, EF, and BF values ranging from 18.10 to 25.40, 4.00 to 18.70, and 22.40 to 3 0.00 mg Quercetin /100g dried sample, respectively. The free flavonoid content in the free fraction from peel sakaka was higher (P<0.05) than that of nagdi peel and seed of two pumpkins (Fig.2A). In esterified fractions and bound fractions, the sakaka peel fraction had the highest (P<0.05) contents of EF and BF (Fig.2B, C), while FF, EF and BF quantities in nagdi seed fraction were lower (P<0.05) than those in sakaka seed and peel of two pumpkins (Fig.2A, B, C). Combining the three fractions, the higher FF, EF and BF contents were in sakaka peel followed by nagdi peel, sakaka seed > nagdi seed (Fig.2D). Combining the phenol and flavonoid contents, the high (P<0.05) FPS content was found in a fraction of nagdi peel (Fig.3A). In the esterified and bound fractions, the sakaka peel showed the highest (P<0.05) EPS and BPS contents (Fig.3B, C). The sum of FPS, EPS, and BPS in nagdi peel was higher than that in sakaka peel and the seed of two pumpkins (Fig.3D). What is more, the sum of FPS, EPS, and BPS in by-products of nagdi pumpkin was higher than those in by-products of sakaka pumpkin. Interestingly, it is observed that the phenolics quantities in pumpkin and their by-products vary among different varieties [15, 16, 17] as it was also found in our study, this difference may be mainly attributed to harvest location and genotypes which affect the phenolics accumulation by synthesizing different types and quantities of phenolics [2]. However, affect the growing conditions (season, fertilizer, soil type, amount of sunlight received and climatic conditions), vegetable maturity when harvested, storage, and analysis methods on the content phenolic compounds cannot be ruled out. Moreover, the total phenolics content observed in this study was higher than phenolics observed in the existing literature [18, 15, 19]. This noticeably highlights the importance of the esterified and bound phenolics in the determination of total phenolics. This illustrates notably the importance of the esterified and bound phenolics in determining total phenolics.

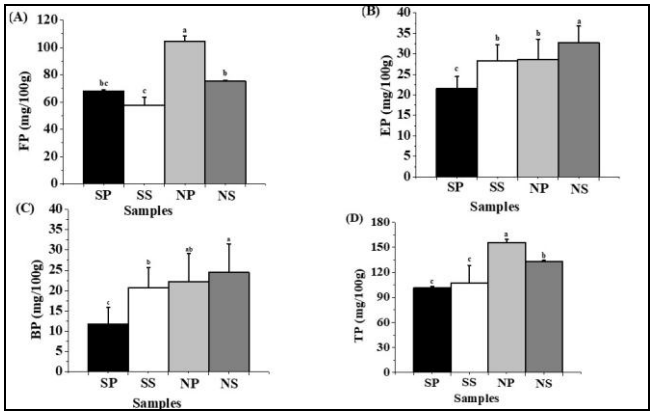


Fig 1: Total phenol (mg GAE /100g dried sample) of pumpkin extracts, A, B, C, D for free phenol (FP), esterified phenol (EP), bound phenol (BP), and the sum phenol content (FP, EP, and BP), respectively. SP = sakaka peel, SS = sakaka seed, NP = nagdi peel, NS = nagdi seed, and GAE = gallic acid equivalents

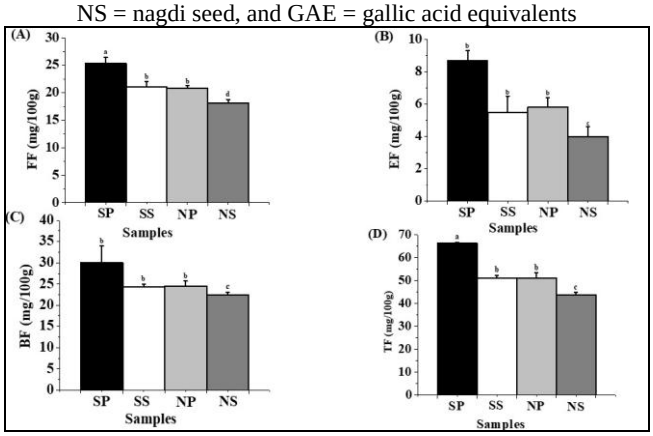


Fig 2: Total flavonoids (mg Quercetin /100g dried sample) of pumpkin extracts. A, B, C, D for free flavonoid (FF), esterified flavonoid (EF), bound flavonoid (BF), and the sum flavonoid content (FF, EF, and BF), respectively. SP = sakaka peel, SS = sakaka seed, NP = nagdi peel, and NS = nagdi seed.

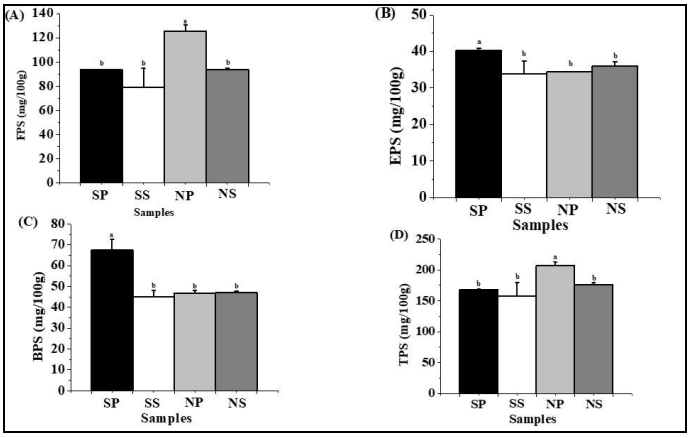


Fig 3: Total phenolics (mg /100g dried sample) of pumpkin extracts. A, B, C, D for free phenolics (FPS), esterified phenolics (EPS), bound phenolics (BPS), and the sum of free, esterified, and bound phenolics (TPS), respectively. SP = sakaka peel, SS = sakaka seed, NP = nagdi peel, and NS = nagdi seed.

3.2 Antioxidant activities

Phenolics display numerous functional properties, acting as hydrogen donors, reducing agents, reactive oxygen and/or nitrogen varieties (ROS/RNS) quenchers, transition metal chelators, up regulators and/or protectors of endogenous defense systems and enzymes inhibitors involved in

oxidative stress [20]. Due to the different antioxidant mechanisms, the best conclusions could be drawn if the study employs at least two assays. Taking this into account, the antioxidant activities of the fractions were measured using two assays (F.4 A, B, C), including TEAC and reducing power.

In general, Fractions that had the highest phenolics were most influential as free radical inhibitors (Fig.4. In the free fractions. The fraction from nagdi peel displayed the highest ($p \leq 0.05$) antioxidant activity in both TEAC and reducing power tests; while fractions from the seed of the two pumpkins showed the lowest activity in both tests (Fig.4A). In the esterified fractions, the first fraction with the strongest antioxidant activity in TEAC and reducing power tests were nagdi seed fraction and sakaka peel fraction, respectively, whereas nagdi peel fraction showed the lowest activity in both tests (Fig.4B). In the bound fractions, the fractions from nagdi seed and sakaka peel displayed the highest antioxidant activity in TEAC and reducing power tests, respectively, whereas fractions from sakaka seed had the least antioxidant activity in both assays (Fig. 4C). However, the trend in the TEAC values was agreed with reducing power values of the most tested samples. Overall, some fractions such as the esterified fraction of sakaka seed that had lower total phenolics than the esterified fraction of nagdi peel showed higher antioxidants capacities than an esterified fraction of nagdi peel. This may be due to the differences in the chemical constituents which contribute to the scavenging activity [21]. The efficiency of phenolics as antioxidants, extracted from foods of plant, frequently varies; however, this does not always influence by their amounts, but may well be dictated by the chemical structures of their components [2, 22]. In addition, the antioxidant activity of the pumpkin by-products in this study was higher than that reported by Saavedra, *et al*., [16] & Aziah, A. H. *et al*., [23]. This difference possibly attributed to the content of antioxidants components in the pumpkin by-products which influence by several factors as mentioned above in the discussion of phenolics data.

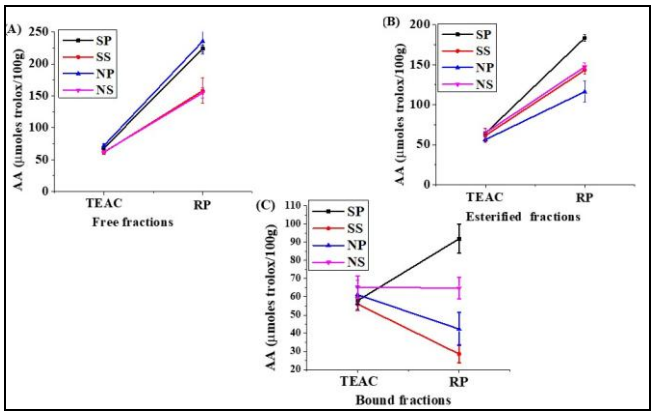
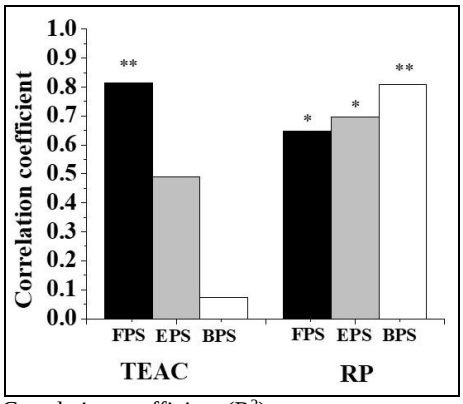


Fig 4: Trolox equivalent antioxidant capacities and reducing power of pumpkin extracts. A, B, C, D for free fractions, esterified fractions and bound fractions respectively. AA = antioxidant activity, TEAC = trolox equivalent antioxidant capacity, RP = reducing power, SP = sakaka peel, SS = sakaka seed, NP = nagdi peel and NS = nagdi seed

3.3 Correlations between the total phenolics and their antioxidant activity

In order to shed light on the contribution of phenolics to antioxidant capacity detected by various methods, the correlations between the total FPS, EPS, and BPS and their antioxidant properties were tested using Pearson correlation test (Fig. 5). In brief, the total FPS, EPS, and BPS contents positively correlated with antioxidants properties, except the BPS content had no linear relationship with the TEAC test (Fig.5). What is more, the FPS and BPS content strongly correlated with TEA C ($R2 = 0.814$, $p < 0.01$) and reducing power ($R2 = 0.810$, $p < 0.01$) tests respectively. The positive correlation indicates that the higher phenolics content resulted in higher antioxidant activity. Furthermore, the results of the present study indicated that TEAC and reducing power assays may preferably be a better way to assess the antioxidant compounds in pumpkin by-products.



Correlation coefficient (R^2)
* Significantly different $p \leq 0.05$.
** Significantly different $p \leq 0.01$.

Fig 5: Correlation between the total free, esterified and bound phenolics with antioxidant activities, free phenolics (FPS), esterified phenolics (EPS), bound phenolics (BPS), TEAC = trolox equivalent antioxidant capacity and RP = reducing power

4. Conclusion

In this study, the results revealed that the extracts of pumpkin peel and seed contain several antioxidant compounds that can efficiently scavenge numerous free radicals /reactive oxygen varieties. Therefore, pumpkin peel and seed could be considered as a good source for natural antioxidants and may be beneficial for inhibiting diseases of oxidative stress and preparing ingredients of functional food. The present study is the first to examine all three phenolics forms (free, esterified, and bound) in pumpkin by-products and their antioxidative capacities and noticeably highlights the importance of esterified and bound phenolics in the reporting and analysis of total phenolic compounds.

5. Acknowledgment

This research was supported by King Saud University, Deanship of Scientific Research, college of food, and Agricultural Sciences Research Center.

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