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PHYSICOCHEMICAL PROPERTIES AND ANTIMICROBIAL EFFECTS OF ROSELLE COROLLA, ONION PEELS AND PEANUT SKINS ANTHOCYANINS

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ABSTRACT: Anthocyanins would make ideal natural food colourants with additional nutritional benefits however stability is a hindering factor. The stability, physicochemical properties and the biological activities of anthocyanins extracted from onion peels, peanut skins or roselle corolla were achieved. Crude anthocyanins were extracted using two different solvent systems (distilled water and acidified ethanol with HCl 1.5 N, 85:15, V/V). Roselle corolla pigment extracted by acidified ethanol was the highest in phenolic and anthocyanin contents compared with water extract content. The aqueous extract of roselle corolla, onion peels and peanut skin showed activities against Gram⁺ (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram⁻ (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria. Aqueous extract of roselle corolla and peanut skin inhibited mycelial growth of *Fusarium oxysporum*. About 78% of aqueous extract pigments of onion peels were retained when heated at 75°C. Stability under different light sources showed general decline in pigment retention of the samples over the time period with all extracts. However, roselle corolla extracted by acidified ethanol showed more stability under different light treatments compared with distilled water extract. The extracted pigments were stable against oxidizing agents, whereas it reduced gradually when treated with cane sugar or salt.

Key words: Anthocyanins, onion peels, peanut skins, roselle corolla, antibacterial, antifungal, physicochemical properties.

INTRODUCTION

Colour is one of the most important characteristic attributes affecting the consumer's acceptance of food since it gives the first impression of food quality. Natural colours are extracted from renewable sources such as plant materials, insects, algae, etc, while the synthetic colours are manufactured chemically. Nowadays, there is a drastic attention to polyphenols due to their positive effects on health by preventing cardiovascular, inflammatory and neurological diseases (Silva *et al.*, 2007). Many convenience foods such as confectionery products, gelatin desserts, snacks, cake, pudding, ice cream and beverages would be colorless, and would thus appear undesirable without the inclusion of colourants (Abou-Arab *et al.*, 2011).

Anthocyanins as a subsidiary of polyphenols have been under investigations in recent years, and sources of anthocyanin which are widely used in the food industry as natural colourants and as an alternative to synthetic colourants are considerable. In addition to their coloring efficiency, increasing evidence suggests that anthocyanins are not only nontoxicant mutagenic, but also have a wide range of therapeutic properties (Lozovskaya *et al.*, 2012). Anthocyanins are among the most important water soluble plant pigment found in higher plants. They contribute to the vibrant red, blue, purple, violet and orange colours of various plants. They can be found in numerous plant species such as red grapes, berries, purple fleshed sweet potato, radish and red cabbage (Kerio *et al.*, 2012).

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Anthocyanin pigments make a good source for a natural food colourants however, they are known to be very unstable. Factors that most commonly affect the stability are pH, temperature, light and storage. Additional benefits of using anthocyanins as food colourants is the biological roles that it plays such as antioxidant activity (Amr and Al-Tamimi, 2007). Ensuring the chemical stability of anthocyanins has become a focal point in recent studies, as there is an abundance of potential industrial applications. A major benefit would be to substitute synthetic colourants and dye and replace them with stable anthocyanins (Castaneda-Ovando *et al.*, 2009).

Hibiscus sabdariffa is an annual herbaceous shrub from the Malvaceae family (Mahadevan *et al.*, 2009), and is cultivated in both tropical and sub-tropical regions around the world. The plant is described as being red stemmed with serrated leaves and red corolla. Although *H. sabdariffa* is termed an under-utilized crop, it is used commonly in households for its traditional medicinal properties and produced into various edible products such as jam, jelly and tea (Sipahli *et al.*, 2017). *H. sabdariffa* is known for its various pharmaceutical, nutritional and traditional medicinal properties and has said to be a rich source of anthocyanins (Patel, 2014).

Onion (*Allium cepa* L.) is one of the oldest and most frequently cultivated food plants highly valued for its pharmacological properties, such as antioxidant, antimicrobial and antitumor ones, reduction of cancer risk and protection against cardiovascular diseases (Ly *et al.*, 2005). Though it is not specifically considered as a medicinal herb, the onion has shown health-promoting effects based on its secondary metabolites, such as flavonoids to which the strong antioxidant properties of onion have been attributed (Lachman *et al.*, 2003; Nuutila *et al.*, 2003).

By-products of the peanut industry which include peanut plant leaves, roots, hulls and skins have also been identified as rich sources of phytochemicals, suggesting that the bioactivity found in fruits and vegetables could possibly be present, although currently these plant parts have little economic value (Francisco and Resurreccion, 2008). Of these materials, peanut skins are most commonly used as low cost

fillers in animal feed but are known to have an astringent taste and anti-nutrient properties (Hill, 2002). The antioxidant activity of peanut skins has been reported (Ballard *et al.*, 2009; Nepote *et al.*, 2005), but there are no reports in the scientific literature regarding the relationship between antioxidants, their activity, and anti-inflammatory properties of peanut skins.

Anthocyanins would make ideal natural food colourants with additional nutritional benefits however stability is a hindering factor (Sipahli *et al.*, 2017). In view of the stability, the aim of the present study was to investigate the physicochemical properties, antibacterial and antifungal effects of the major anthocyanins extracted from roselle corolla, onion outer peels or peanut skins.

MATERIALS AND METHODS

Plant Materials

Roselle (*Hibiscus sabdariffa* L.), peanut (*Arachis hypogaea* L.) and onion (*Allium cepa* L.) were used as source of the natural anthocyanin. The dried corolla of Roselle were purchased from a local market at Zagazig, Egypt. Peanut skins were removed from peanut seeds. Dry outer peels of red onions were used for analysis. They are also obtained from local market at Zagazig, Egypt.

Extraction of pigments

Ethanol was acidified with 1.5N HCl (85:15, V/V) and distilled water which were used as a solvents for extraction of pigments from roselle corolla, dry outer peels of onions or peanut skins. Extracted pigments were obtained according to the procedure described by (Pouget *et al.*, 1990). Ten grams of each plant material powder were immersed in 200 ml of both tested solvent and kept at 4°C overnight. The mixture was filtered through a filter paper (Whatman No. 1), then the filtrates were collected and lyophilized. The yield of lyophilized extracts based on dry weight basis was calculated from the following equation:

$$\text{Yield (g/100 g dry plant material)} = [(W1 \times 100)]/W2$$

Where W1 was the weight of the extract after the evaporation of solvent and W2 was the weight of the residue.

Anthocyanin determination

Total anthocyanins content of roselle, onion peels or peanut skins were estimated according to the protocol described by **(Du and Francis, 1973)**, where a known volume of the filtered extract was diluted to 100 ml using the extracting solvent. The colour intensity was measured at 520 and 535 nm for water and acidified ethanol, respectively using Spectrophotometer (JENWAY –England 6405 UV/VIS). The total anthocyanins content referred to cyanidin-3-glucoside was calculated using the following equation:

$$\text{Total anthocyanin (mg/100g)} = \frac{(A \times D_f)}{(W_s \times 5.99)} \times 100$$

A: Absorbance, D_f: dilution factor, W_s: Sample weight

Total soluble solids

The total soluble solids (TSS) of samples were estimated according to **(Horwitz and Latimer, 2000)**.

Total phenolic content (TPC) determination

Total phenolic content was determined using the Folin-Ciocalteu assay **(Kähkönen et al., 1999)**. Samples (300 µl) were transferred into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% W/V). The tubes were allowed to stand for 30 min and the absorbance was measured at 765 nm. Total phenolic was expressed as gallic acid equivalent in mg per 100g of dry material. The calibration equation for gallic acid was $Y = 0.0009X + 0.214$ ($R^2 = 0.9679$), where Y is absorbance and X is concentration of gallic acid in µg/ml and R^2 is the correlation coefficient.

Total flavonoids content (TFC) determination

Total flavonoids content was measured according to the method of **(Ordonez et al., 2006)** with some modification. A 2 ml aliquot of 2 g/100 ml AlCl₃ ethanol solution was added to 500 µl of the extract (1000 µg/ml). After 60 min, the absorbance at 420 nm was recorded. Total flavonoids content expressed as quercetin equivalent (QE) was calculated based on the calibration curve using the following equation:

$$Y = 0.0012X + 0.008 \quad (R^2 = 0.944)$$

Where X is the concentration (µg QE), Y is the absorbance, and R^2 is the correlation coefficient.

Antimicrobial activity evaluation

Microbial and fungal strains were obtained from Plant Department, Faculty of Science, Zagazig University, Egypt. Extracts of acidified ethanol and distilled water at different concentrations were evaluated individually as antibacterial agents against two Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) by conventional well-diffusion assay **(Nanda and Saravanan, 2009)**. The pure cultures of bacterial strains were sub-cultured on nutrient broth at 37°C on a rotary shaker at 200 rpm. The exponential phase cultures of these strains were adjusted to a concentration of 1×10^9 CFU ml⁻¹. Each strain was spread uniformly onto the individual plates using sterile cotton swabs. Wells of 6-mm diameter were made on Müller Hinton Agar (MHA) plates using a gel puncturing tool. Aliquots (30 µl) of the extract solution (100, 200, 500, 1000 and 2000 µg/ml) were transferred into each well. After incubation at 37 °C for 24 hr., the diameter of the inhibition zone was measured using a transparent ruler. The effect of the same extracts on the mycelial growth of *Fusarium oxysporum* was evaluated also at different concentrations (100, 200, 500, 1000 and 2000 µg/ml) using the poisoned food technique **(Yahyazadeh et al., 2009)**. A 6 mm mycelial agar plug from a 7-day-old culture of *Fusarium oxysporum* was placed at the center of each Potato dextrose agar (PDA) plate and calculated volumes of the tested substances were added, to achieve the previously mentioned concentrations. Approximately, 0.05% (V/V) Tween-80 was then added to the media. Petri dishes were sealed with parafilm and incubated for 7 days at 25°C. The diameter (mm) of colony zone was measured with a caliper .

The extent of growth reduction (%) was calculated as follow:

$$\text{Growth reduction (\%)} = \frac{(C_{LG} - T_{LG})}{(C_{LG})} \times 100$$

C_{LG}: Linear growth of control, T_{LG}: Linear growth of treatment

Pigment stability

Stability of anthocyanins extracted either with distilled water or acidified ethanol from onion peels, peanut skins and roselle corolla were investigated according to (Tan et al., 2011; Sipahli et al., 2017).

Pigments stability under heat

The heat stability of 0.005 g/100 ml pigment solution was measured after treatment in a thermostatically controlled bath at 25, 50 and 75°C for different periods (0.5, 1 and 2 hr.). The samples were held at each temperature for specific time and then cooled immediately in an ice bath. Subsequently the absorption of the solutions was recorded at λ_{max} . Percentage retention of anthocyanins was calculated as follow:

Pigment retention (%) = (absorbance after heating / absorbance before heating) x 100

Pigments stability under light

The 0.005 g/100 ml pigment solution were held under natural light, dark place or under the ultraviolet-light far from 30 cm for specific time (1-4 days) and the absorbance was determined at λ_{max} .

Pigments stability under some chemicals stress

The effect of $KMnO_4$ or H_2O_2 on the stability of the pigment was measured. Ten ml of 5 mg/100 ml pigment solution and 50 ml of different concentrations of $KMnO_4$ (20-100 mg/ml), or H_2O_2 (10, 20 and 30%) were mixed, and then the absorbance of the homogenate was determined at λ_{max} .

The effects of sugar or salt effects on the stability of the pigment were measured. The solutions of cane sugar or salt (NaCl) were prepared by 0.5 g/100 ml, and then mixed with 10 ml of 5 mg/100 ml pigment solution. The absorbance λ_{max} of the solutions was measured every 20 min.

Statistical Analysis

All data were subjected to ANOVA using the MSTAT-C statistical package according to (Gad, 2001). Different letters in the tabulated data or above the bars in the figured data indicate significant differences by Fisher's Protected LSD test at ($P < 0.05$).

RESULTS AND DISCUSSION

Yield of Extract

Two solvents were compared in order to use the most effective one for extracting the pigments of roselle corolla, onion peels or peanut skin. The yield of anthocyanin pigments recovered from roselle, onion peels or peanut skin with two solvents (distilled water and acidified ethanol) are shown in Table 1. In general acidified ethanol was more effective than distilled water in case of onion peels or peanut skin. The highest yield of anthocyanin was observed in roselle corolla extracted by distilled water (27.43 mg/100g). These results agree with those reported by (Mattuk, 1998; Sipahli et al., 2017).

Total phenolic and flavonoid contents in extracted residue

Total phenolic contents (TPC) of all extracts were determined by Folin-Ciocalteu's method, and found to be varied (Fig. 1). The highest amount of total phenolic content was observed in roselle corolla pigment extracted by acidified ethanol (88.88 mg/ml) compared with distilled water extract. The present results were in keeping with the results obtained by Abou-Arab et al. (2011), who found that the total phenolic content of *H. sabdariffa* extracted by HCl acidified ethanol was the highest compared with other solvents. Whereas, (Sindi et al., 2014) reported that the lower phenolic content had been observed from *H. sabdariffa* anthocyanins extracted by methanol. Dry outer peels of onions showed the lowest amount of phenol content (4.44 mg/ml) among all acidified ethanol extracts. Total anthocyanin pigment extracted by distilled water showed a lower phenolic content in all tested plant materials compared with acidified ethanol extract. The same results were observed with flavonoid content, which the acidified ethanol extract showed the highest amount of flavonoid compared with distilled water extract.

Total anthocyanin and total soluble solid contents

Anthocyanin pigments and total soluble solid content recovered with the two different solvents are shown in Fig. 2. The highest amount of

Table 1. Yield of extract (mg/100g), of roselle corolla, onion peel and peanut skin using acidified ethanol and distilled water

Solvent	Roselle	Onion peel	Peanut skin
Acidified ethanol	20.4	20	11.7
Distilled water	27.43	3.4	5.7

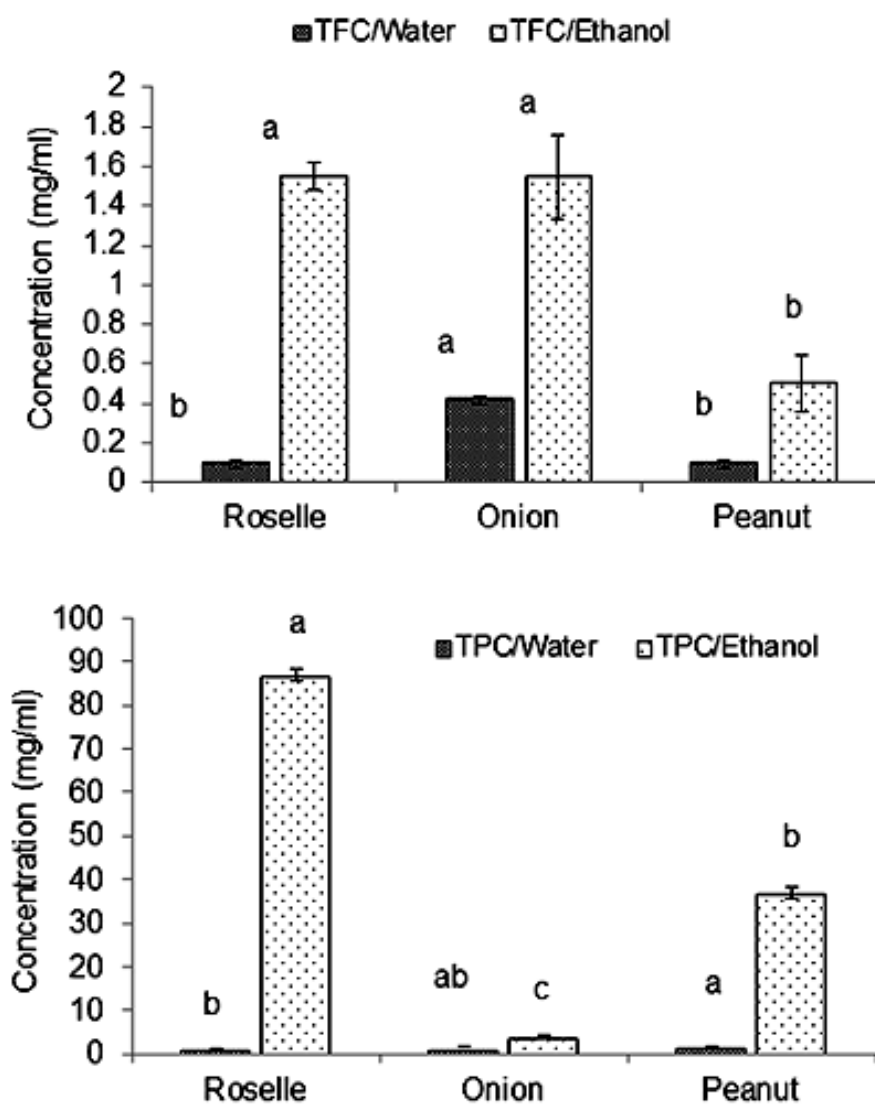


Fig. 1. Total phenolic (TPC) and flavonoid (TFC) contents in total anthocyanin pigment extracted from roselle or onion peel or peanut skin by distilled water or acidified ethanol

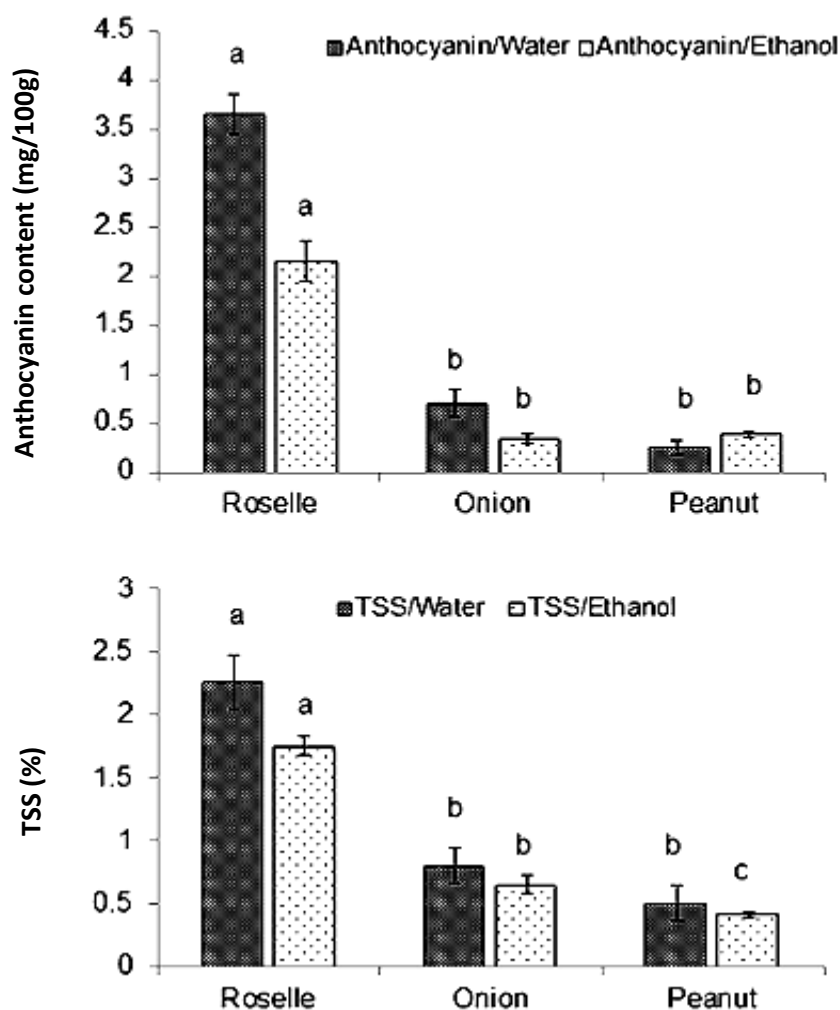


Fig. 2. Total anthocyanin and total soluble solid content extracted from roselle, onion peel and peanut skin by distilled water or acidified ethanol

anthocyanin were observed in roselle with acidified ethanol and distilled water (2.078 and 3.877 mg/100g, respectively) followed by aqueous extract of onion peel (0.635 mg/100g). Peanut skin showed the lowest amount of anthocyanin (0.208 mg/100g) with distilled water. The same trend was observed with the total soluble solids.

Antimicrobial Activity

The antibacterial activity of roselle, onion peel or peanut skin extracted by acidified ethanol was examined at different concentrations (100-2000 $\mu\text{g/ml}$) and the results are listed in Table 2. The minimum inhibition concentration (MIC) of roselle acidified ethanol extract against the four

studied bacteria was 200 $\mu\text{g/disc}$ whereas was 500 $\mu\text{g/disc}$ for onion peel acidified ethanol extract. MIC of peanut skin acidified ethanol extract against gram positive and gram negative bacteria were 200 $\mu\text{g/disc}$ and 500 $\mu\text{g/disc}$, respectively.

The antifungal activities (Fig. 3) of roselle corolla, onion peels or peanut skin aqueous extract against *Fusarium oxysporum* were examined at different concentrations (200, 500, 1000 and 2000 $\mu\text{g/ml}$).

The aqueous extract of roselle and peanut skin inhibited mycelial growth at a wide concentration range (500-2000 $\mu\text{g/ml}$) followed by onion peel (1000-2000 $\mu\text{g/ml}$).

Table 2. The Inhibition zones diameter (mm) induced in Gram⁺ and Gram⁻ bacteria using agar well diffusion assay under the influence of different concentrations (100-2000 µg/ml) of acidified ethanol extract from roselle, onion peels and peanut skin

Microorganism	Concentration (µg/ml)														
	Roselle corolla					Onion peel					Peanut skin				
	100	200	500	1000	2000	100	200	500	1000	2000	100	200	500	1000	2000
Gram +															
<i>Staphylococcus aureus</i>	-	15	20	30	45	-	-	12	14	22	-	8	12	18	26
<i>Bacillus subtilis</i>	-	10	13	16	29	-	-	9	15	25	-	8	16	21	29
Gram -															
<i>Pseudomonas aeruginosa</i>	-	8	14	18	27	-	-	10	17	20	-	-	9	11	23
<i>Escherichia coli</i>	-	8	10	12	22	-	-	11	22	30	-	-	10	16	24

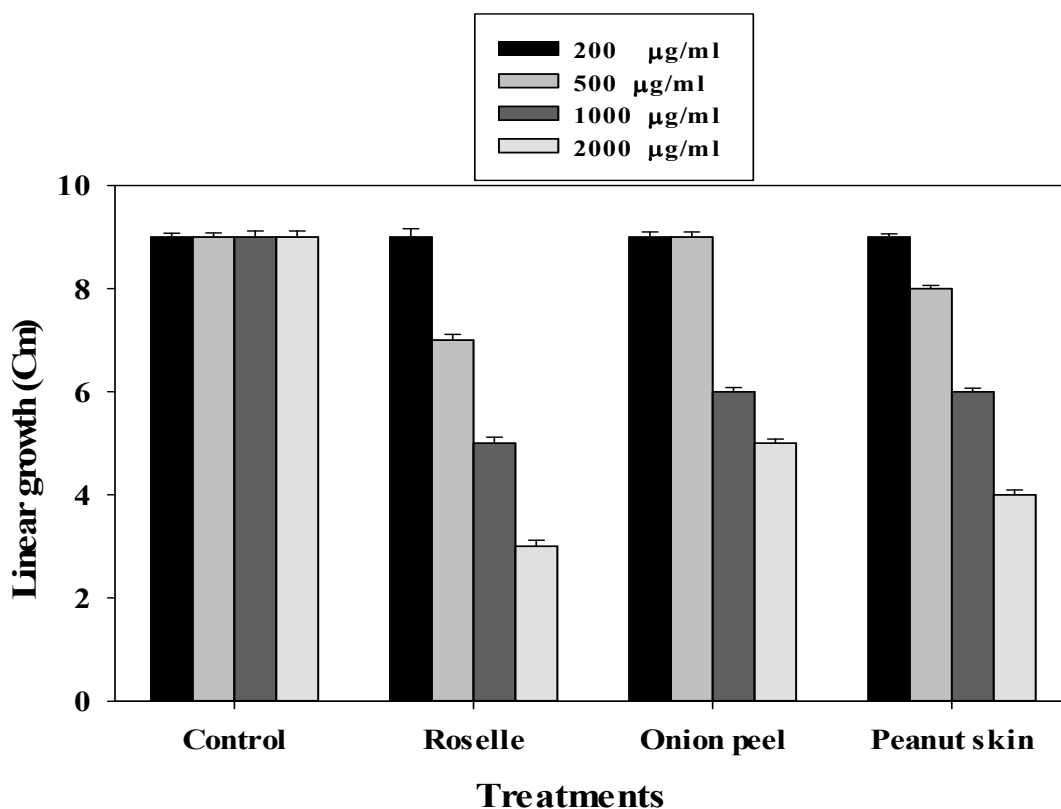


Fig. 3. Linear growth (Cm) of *Fusarium oxysporum* after 7 days at 25°C in the presence of distilled water extract of roselle, onion peels and peanut skin at different concentrations (200, 500, 1000 and 2000 µg/l).

Stability Studies

Stability of pigments under heat stress

The aqueous and acidified ethanol extracts from roselle, onion peel or peanut skin were heated at 25, 75 and 100°C for 0.5, 1 and 2 hr., and the pigment retention (PR) were spectrophotometrically measured (Fig. 4). There was no significant ($p < 0.05$) difference in pigment retention of roselle distilled water extract while, acidified ethanol extract showed to retain the most at 75 and 100°C of heat treatments (Fig. 4). Comparatively, there was significant ($p < 0.05$) difference in retention between onion peel distilled water extract and acidified ethanol extracts. Pigment retention of onion peel distilled water extract showed slightly degradation over time at 75 and 100°C compared with 25°C (Fig. 4), while acidified ethanol extract showed a significant decline especially at 75 and 100°C of heat treatments after 2 hr., (Fig. 4), it could be indicated imply that most of the pigments were degraded at 100°C (Amr and Al-Tamimi, 2007). The same results were observed for the pigment isolated from peanut skin with distilled water and acidified ethanol extract (Fig. 4). The rate of anthocyanin degradation when heated increases because of reacting molecules that come closer when the extract is concentrated (Kirca *et al.*, 2007). Colour changes were also observed upon heating over the time. In all of the samples, the colour decreased in intensity. Heat stability could possibly be improved by increasing the anthocyanin concentration, removal of oxygen and the inactivation of enzymes (Hellström *et al.*, 2013).

Stability of pigments under light stress

Stability of anthocyanins under light is a very important side because it aids in storage conditions (Sipahli *et al.*, 2017). Under different light treatments, the results showed general decline in pigment retention over the time period with all extracts (Fig. 5). However, roselle acidified ethanol extract showed more stability

under different light treatments compared with its distilled water extract (Fig. 5). Also peanut skin acidified ethanol extract observed increasing in stability of the pigment compared with its distilled water extract (Fig. 5). In contrast, onion skin distilled water extract showed significantly higher in stability of the pigment compared with acidified ethanol extract under different light conditions over the period time (Fig. 5). Amr and Al-Tamimi (2007) found that their samples retained 84% of the pigment which were treated to dark conditions for 10 days.

Effect of $KMnO_4$ or H_2O_2 stress on pigment stability

Effect of $KMnO_4$ at different concentrations

(20-100 mg/ml) on pigment stability extracted from roselle, onion peel and peanut skin with distilled water or acidified ethanol was observed and the results are shown in Fig. 6. Pigment retention gradually increased with the increasing $KMnO_4$ concentrations (20-100 mg/ml) for all tested samples. The same results were observed for all tested samples when treated with H_2O_2 (Fig. 7) at different concentrations (10, 20 and 30%). It can be concluded that all extracted pigments are stable to $KMnO_4$ and H_2O_2 .

Effect of sugar or salt (NaCl) stress on pigment stability

Effect of cane sugar (0.5%) and salt (0.5%) on the stability of the pigment stored at different periods (20-80 min) were investigated and the results are listed in Figures 8 and 9. Pigment retention was gradually reduced with increasing the storage time (20-80 min) for all tested samples when treated with cane sugar (0.5%). The same results were observed for all tested samples when treated with salt (0.5%).

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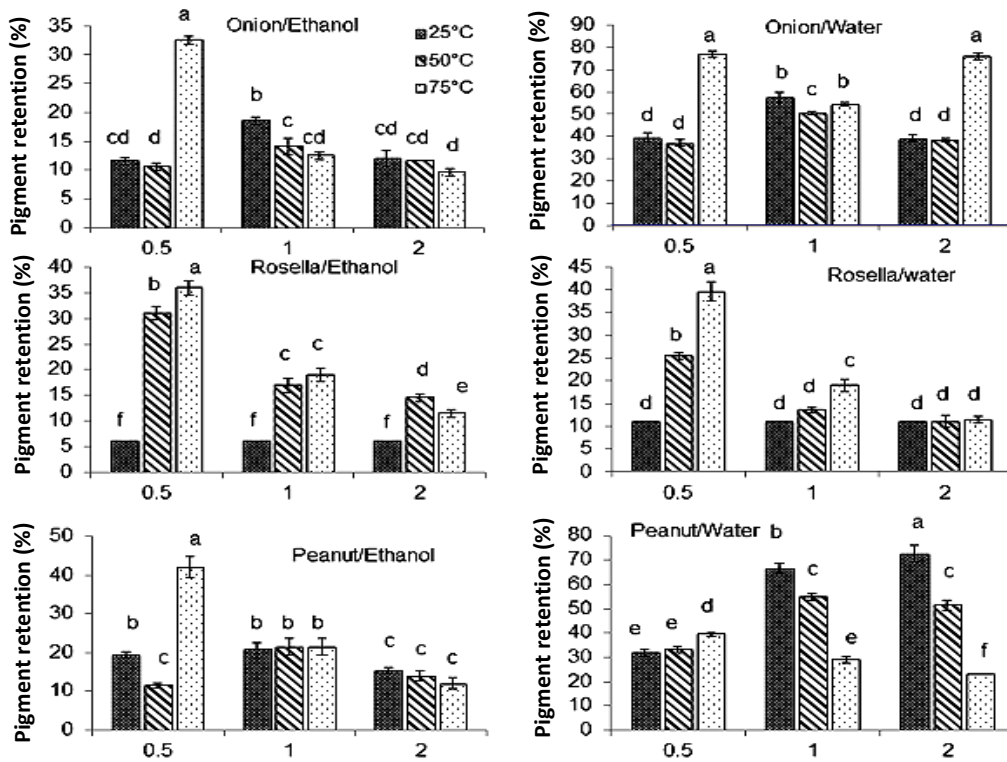


Fig. 4. Effect of heat on stability of roselle, peanut and onion anthocyanins extracted by distilled water or acidified ethanol at different temperatures: 25, 50 and 75°C, and at different time (0.5, 1 and 2 hr.).

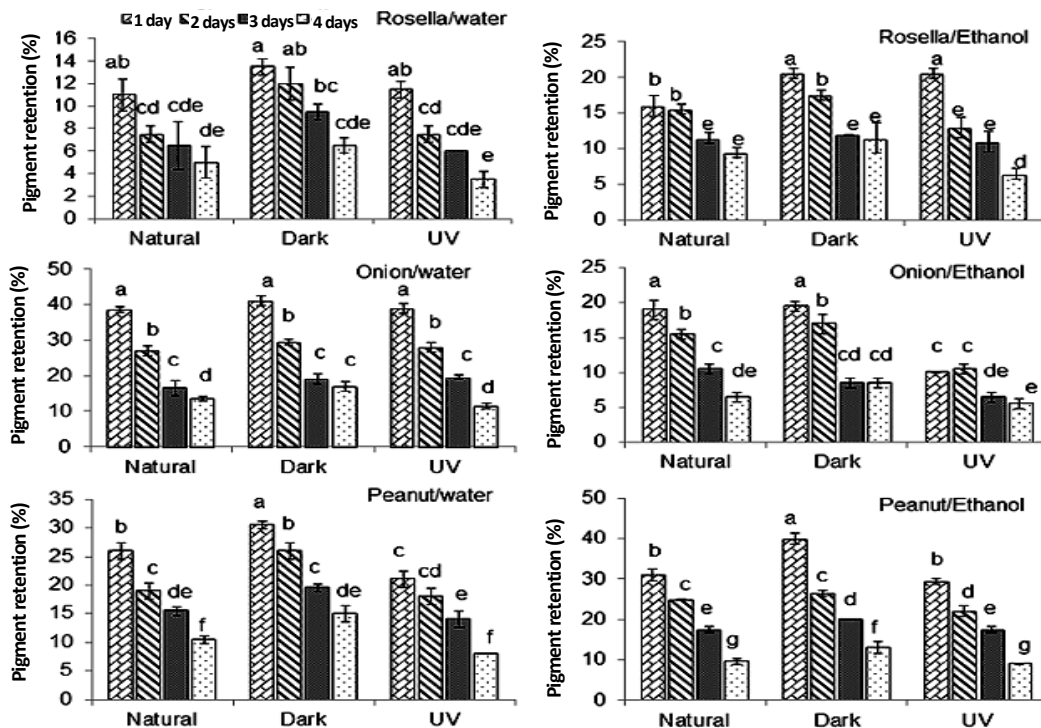


Fig. 5. Effect of light (natural light, dark place and ultraviolet-light) stress on the stability of the pigment at different periods (1-4 days)

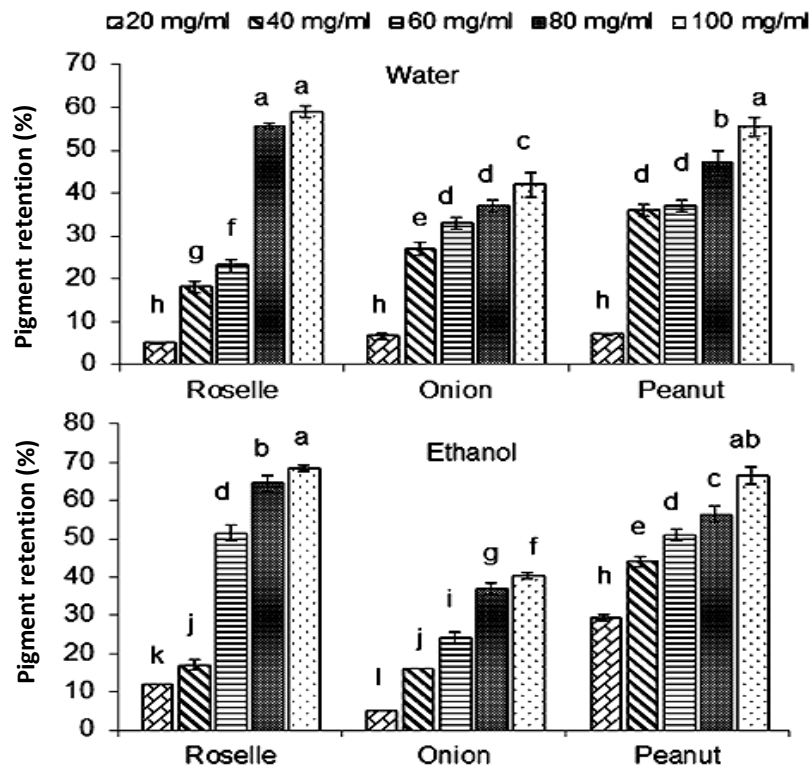


Fig. 6. Effect of $KMnO_4$ stress at different concentrations (20-100 mg/ml) on pigment stability

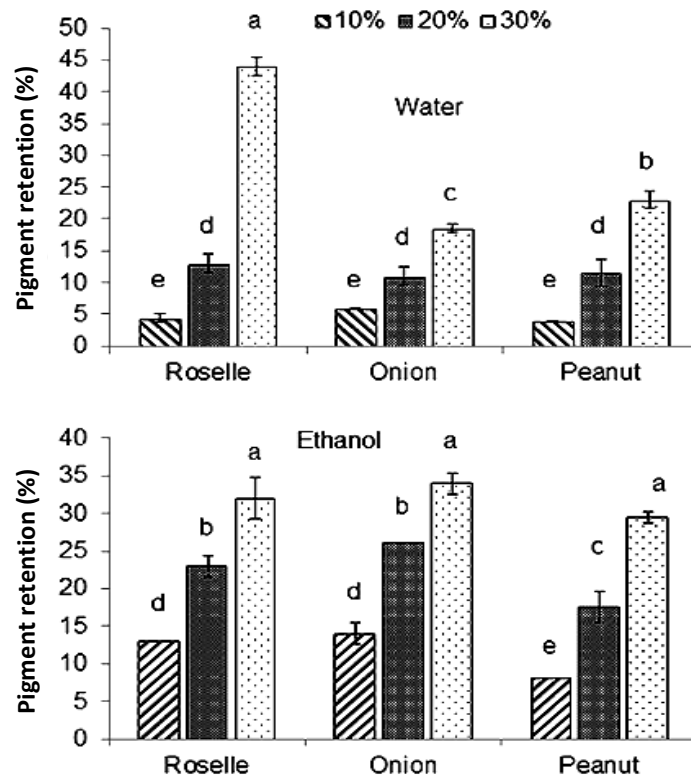


Fig. 7. Effect of H_2O_2 stress at different concentrations (10, 20 and 30%) on pigment stability

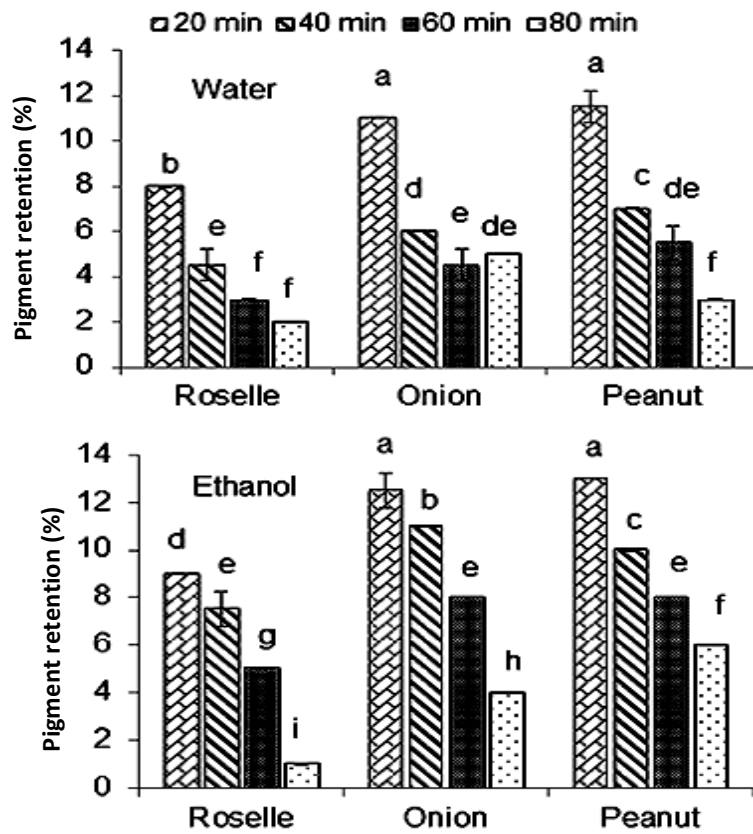


Fig. 8. Effect of cane sugar (0.5%) stress on pigment stability at different periods (20-80 min)

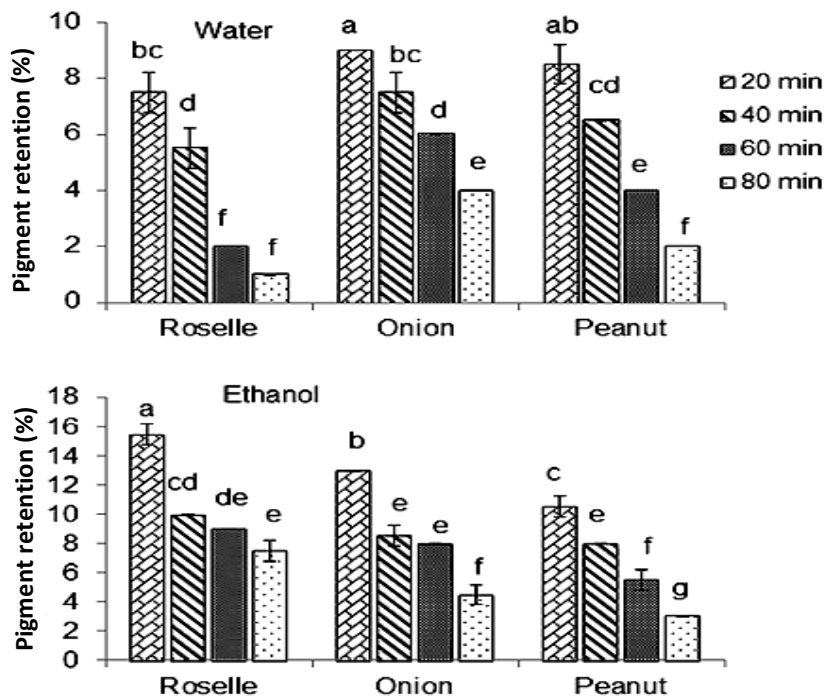


Fig. 9. Effect of salt (0.5%) stress on pigment stability at different periods (20-80 min)

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الخواص الفيزيوكيميائية والتضاد البكتيري والفطري للأنثوسيانينات المستخلصة من الكركدية وقشور البصل والقشرة الخارجية للفاول السوداني

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يخضع الأنثوسيانين كأحد مركبات البوليفينول للبحث في السنوات الأخيرة، حيث تستخدم مصادر الأنثوسيانين بشكل كبير في الصناعات الدوائية والغذائية والتجميلية، وعلاوة على ذلك، فإن الأنثوسيانين يمكن أن يضاف كمكونات غذائية طبيعية مثالية مع فوائد غذائية إضافية، ومع ذلك فإن ثبات الصبغة هو صفة هامة، الهدف من هذه الدراسة هو التركيز على الثبات وأختبار الأنشطة الحيوية للأنثوسيانين المستخلص من قشور البصل والقشرة الخارجية لبذور الفول السوداني أو بتلات الكركدية حيث تم استخلاص الأنثوسيانين الخام باستخدام اثنين من المذيبات المختلفة (الماء والإيثانول)، كانت صبغة الكركديه المستخرج مع الإيثانول المحمض هو أعلى في محتوى الفينولات ومحتوي الأنثوسيانين مقارنة مع الأصباغ الأخرى، وأظهر المستخلص المائي من الكركديه، وقشور البصل، والقشرة الخارجية للفول السوداني نشاطاً ضد البكتريا الموجبة لجرام (*Staphylococcus aureus* - *Bacillus subtilis*) والبكتيريا السالبة لجرام (*Escherichia coli* - *Pseudomonas aeruginosa*)، وقد أظهر المستخلص المائي للكركدية وقشرة الفول السوداني تثبيطاً لنمو الميسيليا لفطر *Fusarium oxysporum*، تم استرجاع 78% من المستخلص المائي لقشور البصل عند تسخينها عند درجة حرارة 75 درجة مئوية، وقد أظهرت نتائج تعرض مستخلصات الأنثوسيانين المختلفة للضوء وجود انخفاضاً عاماً في ثبات الصبغات خلال الفترة الزمنية مع جميع المستخلصات، ومع ذلك أظهر مستخلص الإيثانول المحمض من الكركدية مزيداً من الثبات تحت معاملات الضوء المختلفة مقارنة مع المستخلص المائي.

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