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Phytochemicals, Antioxidant Activity and Identification of Phenolic Compounds by HPLC of Pomegranate (*Punica granatum* L.) Peel Extracts

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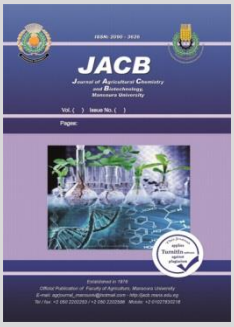

ABSTRACT

Pomegranate peel is food waste unfit to eat parts obtained during the production of pomegranate juice. The aim of this work is to study the effect of four different solvents (ethanol, isopropanol, hot and cold water) on phytochemicals screening, total phenolics content, total flavonoid content and antioxidants activity in pomegranate peels, as well as identification of phenolic compounds in extracts by HPLC. Phenolics, flavonoids, alkaloids, saponins, tannins, steroids and terpenoids were detected in all extracts. The highest total phenolics and total flavonoids were observed in ethanol extract of pomegranate peels (161.5 mg GAE/g and 70.65 mg Rutin/g), respectively. Furthermore, the ethanol extract of pomegranate peels showed the highest DPPH scavenging activity with the lowest IC_{50} value 14.6 µg/ml compared to other extracts. The high antioxidant activities may be due to the high contents of phenolics and flavonoids in pomegranate peel ethanol extract. HPLC analysis was used for the identification and quantitative determination of phenolic compounds in pomegranate peels extracts. The results revealed nine polyphenolic compounds including protocatechuic acid, p-coumaric acid, caffeic acid, ellagic acid, cinnamic acid, quinic acid, benzoic acid, syringic acid and iso-ferulic acid in pomegranate peels extracts. The obtained results confirmed that ethanol extract was the most abundant of phenolics compounds as compared to the other extracts. Finally, it can be concluded that pomegranate peel extracts can be used in various fields for being rich in natural antioxidants that have a medicinal and therapeutic impact.

Keywords: Pomegranate peel extracts, Phytochemicals, total phenolic, antioxidant activity, HPLC

INTRODUCTION

Pomegranate (*Punica granatum* L.) is an important source of vital compounds and at the past it has been used as a medicinal for prevention and cure many diseases in the last centuries (Li *et al*., 2006). The pomegranate is vastly grown in many tropical and subtropical regions and is an important source of antioxidants, anticancer and antimicrobial compounds that allows it to become a light point in the center of many studies. It's known to contain a significant amount of phenolics (Machado *et al*., 2002). So, there is increasing emphasis that phenolic components or their derivatives may cause other beneficial effects, independent of their antioxidant capacities, by directly influencing the activities of enzymes (McDougall *et al*. 2005). Pomegranate fruits revealed beneficial health effects due to the vital component in flowers, seeds, arils and peels. Pomegranate peel is an unfit to eat parts obtained during production of pomegranate juice. Pomegranate peel is a rich source of flavonoids, tannins and other phenolic compounds (Jaiswal *et al*., 2010 and Mehder, 2013). The peels represent approximately 40 % of the whole fruit and are rich in derivatives of ellagic acid such as the ellagitannins, punicalagin, and punicalin. In addition, some ellagic acid derivatives (ellagic acid hexoside, pentoside, etc.) are also present, although in lesser amounts (Seeram *et al*., 2005). Several previous studies mentioned that phytochemicals were identified in the different parts of the pomegranate fruit: juice, seeds, and peels (Singh *et al*., 2002 and Elfalleh *et al*., 2009). Farag *et al*. (2014) revealed that thirty polyphenolic compounds were separated from pomegranate peels by HPLC such as gallic acid, protocatechuic acid, caffeic acid, ferulic acid and coumarin. Also, Pomegranate peels contain many affective compounds such as ellagic tannins, iso-ferulic acid and ellagic acid are among important antioxidant which more effective against many diseases (Ali *et al*., 2014). Solvent extraction is a technique used to isolate plant antioxidants (Sultana *et al*., 2009). Phenolic contents and antioxidant activity of different plant materials depend on the nature of the solvent used due to its various chemical and polar properties (Mohammedi and Atik, 2011). Several studies have found that the phenolic contents in plants differ with solvent polarity (Ali *et al*., 2014). Recently, Pomegranate peels extracts have attracted interest because of their potential uses as natural food preservatives (Negi *et al*., 2003). Therefore, this study was carried out to investigate the effect of four different solvents (ethanol, isopropanol, hot and cold water) on phytochemicals screening, total phenolics content, total flavonoids content and antioxidants activity in pomegranate peels, as well as identification of phenolic compounds in extracts by HPLC.

MATERIALS AND METHODS

Plant materials

Pomegranate fruits were collected from the local market of Cairo, Egypt. Then pomegranate peels were removed carefully by a knife. Peels of pomegranate were washed thoroughly with distilled water. The pomegranate peel samples were dried at room temperature, dried peels were ground to a fine powder then the powder kept in a refrigerator until use.

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Chemical analysis

Chemical composition of Pomegranate peels powder was carried out by Near-Infrared analyzer (NIR) Spectroscopy apparatus, model DA1650, which manufactured by FOSS Corporation (Zhao *et al*., 2005). Total carbohydrates were calculated according to (AOAC, 2010) by difference.

Preparation of extracts

Ten gram of Pomegranate peel powder was extracted by soaking in 100 ml of ethanol, isopropanol, hot water (80ºC) for a half-hour, and cold water at room temperature for 12 hours with constant stirring, after that the extracts were filtered by Whatman no.1 filter paper. The filtered extracts were centrifuged 4000 rpm for 15 minutes and kept at 4ºC until assay.

Qualitative phytochemical screening of pomegranate peel

Peels of Pomegranate were screened for the presence or absence of various secondary metabolites, Phenolics were assessed by ferric chloride test (Cai *et al*., 2011). Flavonoids were investigated by Alkaline reagent (Tiwari *et al*., 2011). Alkaloids were evaluated by Wagner's test (Sasidharan *et al*., 2011). Saponins were analyzed by Froth's Test (Savithramma *et al*., 2011). Tannins were detected by Braemer's test (Sasidharan *et al*., 2011). Steroids and terpenoids were detections by Salkowski test according to the methods reported by Savithramma *et al*. (2011) and Sasidharan *et al*. (2011), respectively.

Total phenolics

Total phenolics were determined in the extracts by the Folin-Ciocalteu method as described by Singleton and Rossi (1965). Gallic acid was used as a standard, the data were expressed as; mg of gallic acid equivalents (GAE) per gram dry weight.

Total flavonoids

According to the aluminum chloride colorimetric method, the Flavonoids contents were determined as described by Matyuschenko and Stepanova (2003). The data were expressed as: mg rutin equivalents per g dry weight.

Antioxidant activity by DPPH free-radical scavenging assay:

Determination of the antioxidant activity of pomegranate peels extracts was performed by DPPH radical scavenging according to Yen and Duh (1994). Through the following equation the inhibition of DPPH free radical was calculated; The percentage Inhibition (PI %) = $(AC - AT)/(AC) \times 100$ Where *AC*= absorbance of the control reaction at=0 min, *AT*= the absorbance of the test extract + DPPH at t=16 min.

IC_{50} = the concentration required to inhibit DPPH radical by 50%, was estimated from graphic plots of the dose response curve.

Identification of phenolic compounds using HPLC

HPLC system consisting E-Chrom Tech Model LC 1620 A Liquid chromatography equipped with a UV detector at wavelength 280 nm. The analysis was achieved on Column C 18: Shodex C18-120-5 4 E (250*4.6 mm), Pump: P 1620A Pump, Software: PA Station 2015 ChemStation Version 2.0, Flow Rate: 1 ml/minute, Eluent: Methanol : water : tetrahydrofuran : acetic acid (23 : 75 : 1 : 1), respectively.

Statistical analysis

Statistical analysis was carried out using Microsoft excel program. All experiments were performed in triplicate. Results are presented as mean ± standard division.

RESULTS AND DISCUSSION

Chemical composition of pomegranate peels

The proximate chemical compositions of pomegranate peels are given in Table 1. Results showed that pomegranate peels contained fat 0.85%, ash 4.22, moisture 6.95%, protein 8.97% and fiber 19.41 whereas total carbohydrates in the sample were calculated to be 59.60%. The results observed that pomegranate peels are a good source of carbohydrates and fiber while the percentage of fat was very low. These results are consistent with those found by **Ranjitha *et al*. (2018)** who found that pomegranate peels contain fat 0.85%, ash 4.32%, moisture 7.27%, protein 3.74%, fiber 17.31% and carbohydrates 66.51%.

Table 1. Chemical composition of pomegranate peels

Parameters	Quantity (%)
Fat	0.85
Ash	4.22
Moisture	6.95
Protein	8.97
Fiber	19.41
Total carbohydrates	59.60

Phytochemical screening of pomegranate peel extracts

Phytochemical screening tests showed that constituents of pomegranate peels extracts were not differ with the different extracts used. Extracts of pomegranate peels of ethanol, isopropanol, hot water, and cold water showed similar results in all results of phytochemical screening carried out. Phenolics, flavonoids, alkaloids, saponins, tannins, steroids and terpenoids were found in all extracts (Table 2). These compounds, especially flavonoids, saponins, alkaloids and tannins have great activity against pathogens (Usman *et al*., 2009). Alkaloids possess antimicrobial properties while flavonoids possess anti-inflammatory and antitumoral activities (Omulokoli *et al*., 1997; Ferrandiz and Alcaraz, 1991; Gil *et al*., 1994). Saponins cause lowering cholesterol in the blood by preventing its absorption. Also, it has anti-tumor activity and helps the immune system in human against viruses and bacteria (Roa *et al*., 1995; Prohp and Onoagbe, 2012). Similar results were obtained by Farag *et al*. (2014).

Table 2. Qualitative phytochemical screening of pomegranate peel extracts

Phytochemical test	Ethanol	Isopropanol	Hot water	Cold water
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Alkaloids	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Steroids	+	+	+	+
Terpenoids	+	+	+	+

(+) present; (-) absent

Total phenolics and total flavonoids

The results in table 3 showed that ethanolic extract of pomegranate peels contained a significantly higher amount of total phenolics and total flavonoids (161.5 mg GAE/g and 70.65 mg Rutin/g) when compared to hot water

extract (143.4 mg GAE/g and 58.85 mg Rutin/g), cold water extract (126 mg GAE/g and 53 mg Rutin/g) and isopropanol extract (95.8 mg GAE/g and 49.22 mg Rutin/g), respectively. Phenolics are considered compounds derived from the secondary metabolism of plants and are widely exploited because of their biological effects including their effects as antioxidants. Flavonoids are also antioxidants that have biological and chemical activities, the most important of which is scavenging free radicals (García-Cruz *et al*., 2012 and Sankhalkar, 2014). Previous results showed that a high amount of phenolics and flavonoids in pomegranate peels extracts, especially the ethanol extract; therefore, pomegranate peels can be considered as an important source for antioxidants. These results agree with Pande and Akoh (2009); Elfalleh *et al*. (2012).

Antioxidant activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical assay was used to evaluate the antioxidant activity of pomegranate peels extracts. The free radical scavenging activity measured by DPPH was expressed as the IC_{50} (Table 3 and figure 1).

Table 3. Total phenolics, total flavonoids and antioxidant activity of pomegranate peel extracts

pomegranate peel extracts	Total phenolics (mg GAE/g)	Total Flavonoids (mg Rutin/g)	Antioxidant Activity DPPH method (IC50) (µg/ml)
Ethanol	161.5 ± 0.5	70.65 ± 4.05	14.6 ±1.2
Isopropanol	95.80 ± 1	49.22 ± 3.25	23.9±1.3
Hot water	143.4 ± 5.2	58.85 ± 0.4	23.5±2.7
Cold water	126.0 ± 1	53.00 ± 1.35	27.6±2.9

Each value is presented as mean ± SD (n = 3).

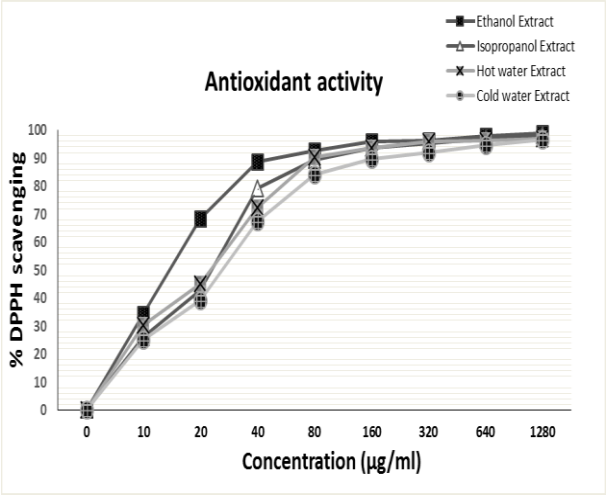

Fig. 1. Antioxidant activity in pomegranate peel extracts

The lower IC_{50} values of pomegranate peel extracts mean stronger scavenging of DPPH free radical. The ethanol extract of pomegranate peels showed the highest DPPH scavenging activity with the lowest IC_{50} value 14.6 µg/ml. Also, results showed that both isopropanol and hot water extracts exhibited good antioxidant activities with IC_{50} values 23.9 µg/ml and 23.5 µg/ml, respectively. Nevertheless, the cold water extract showed the lowest DPPH scavenging activity compared to other extracts with IC_{50} value 27.6 µg/ml. From these results, we can suggest a significant relationship between the activity of antioxidants in scavenging free radicals and the content of total phenolics and flavonoids present in extracts of pomegranate peel, for example the ethanol extract achieved the highest content of total phenolic and flavonoids with the highest antioxidant activity compared to other extracts (Noda *et al*., 2002 and Ghasemzadeh *et al*., 2010). The present results are in accord with Russo *et al*. (2018) and Ali *et al*. (2014) who attributed the high of antioxidant activities to the high contents of total phenolics and flavonoids of pomegranate peel extracts.

Identification of phenolic compounds by HPLC

HPLC (High- performance liquid chromatography) analysis was used for identification and quantitative analysis of some various phenolic compounds in pomegranate peels extracts. Table 4 and figure 2 revealed six polyphenolic compounds including protocatechuic acid, p-coumaric acid, caffeic acid, ellagic acid, cinnamic acid, and quinic acid in ethanol extract of pomegranate peels. Cinnamic acid was the most abundant (33.08 mg/ml) followed by quinic acid (20 mg/ml) and p-coumaric acid (18.43 mg/ml), while caffeic acid (9.55 mg/ml) and ellagic acid (8.14 mg/ml) were the lowest abundant in ethanol extract.

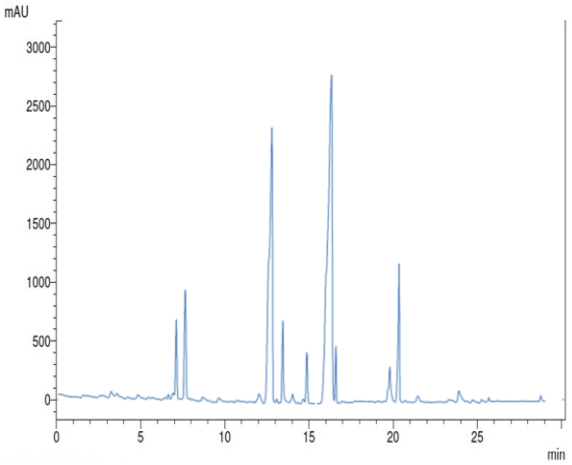

Fig .2. HPLC chromatogram of phenolic compounds identified in ethanol extract of pomegranate peels

Table 4. Concentrations of phenolic compounds identified in ethanol extract of pomegranate peels by HPLC.

Compound	$R_{t\ (min)}$	Concentration (mg/ml)
Protocatechuic acid	7.8	10.26
p-coumaric acid	12.2	18.43
Caffeic acid	13.5	9.55
Ellagic acid	15.0	8.14
Cinnamic acid	16.1	33.08
Quinic acid	20.1	20.0

$R_{t\ (min)}$; Retention time by minute

Moreover, syringic acid was the most abundant polyphenolics in isopropanol extract of pomegranate peels which constituted (15.12 mg/ml) while the rest was protocatechuic acid (9.55 mg/ml) and benzoic acid (7.43 mg/ml) as shown in table 5 and figure 3.

On the other hand, four phenolic compounds identified in hot water extract of pomegranate peels are shown in table 6 and figure 4. HPLC analysis revealed that ellagic acid was the main polyphenolics of hot water extract, it constituted (25.33 mg/ml) followed by Iso-ferulic acid (12.36 mg/ml) while syringic acid and protocatechuic acid

were found by lower quantities in hot water extract which contained (5.22 mg/ml) and (2.02 mg/ml), respectively.

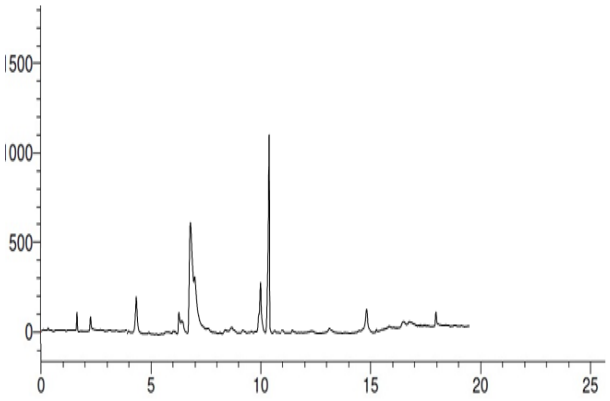

Fig.3. HPLC chromatogram of phenolic compounds identified in isopropanol extract of pomegranate peels

Table 5. Concentrations of phenolic compounds identified in isopropanol extract of pomegranate peels by HPLC.

Compound	$R_{t\,(min)}$	Concentration (mg/ml)
Protocatechuic acid	7.8	9.55
Benzoic acid	9.3	7.43
Syringic acid	10.11	15.12

$R_{t\,(min)}$; Retention time by minute

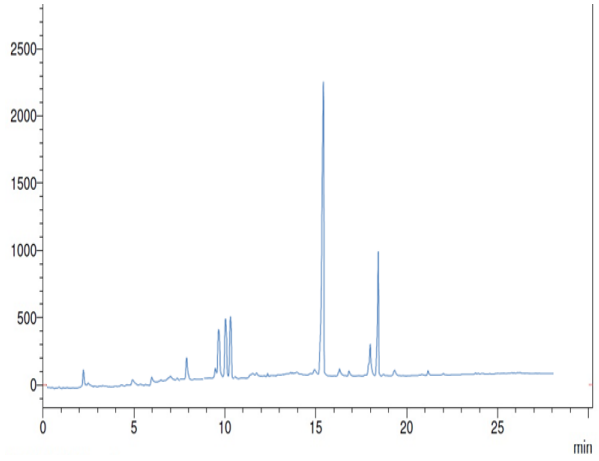

Fig.4. HPLC chromatogram of phenolic compounds identified in hot water extract of pomegranate peels .

Table 6. Concentrations of phenolic compounds identified in hot water extract of pomegranate peels by HPLC.

Compound	$R_{t\,(min)}$	Concentration (mg/ml)
Protocatechuic acid	7.8	2.02
Syringic acid	10.11	5.22
Ellagic acid	15.0	25.33
Iso-ferulic acid	18.61	12.36

$R_{t\,(min)}$; Retention time by minute

Data in table 7 and figure 5 revealed the presence of three phenolic compounds such as protocatechuic acid, syringic acid and ellagic acid in cold water extract of pomegranate peels. syringic acid was the most abundant (19.41 mg/ml) in comparison with ellagic acid (11.06 mg/ml) and protocatechuic acid (10.25 mg/ml).

In this concern, the previous studies upon extracts of different plants containing polyphenolic compounds (including ellagic acid, ferulic acid, quinic acid, protocatechuic acid, p-coumaric acid, caffeic acid, syringic acid, and cinnamic acid) act as antioxidants (Reddy *et al*., 2007) and act as antibacterial (Salih *et al*., 2017) and are used in cosmetics and pharmaceutical industry (Maldini *et al*., 2009). The obtained results from this work confirmed that ethanol extract of pomegranate peels was the most abundant of polyphenolics as compared to the other extracts. Protocatechuic acid appeared in all extracts followed by ellagic acid which appeared in three extracts and was absent in isopropanol extract. Also, syringic acid was found in three extracts and was absent in ethanol extract. Different phenolic compounds were extracted from pomegranate peels have been shown to possess an antioxidants property (Li *et al*., 2006). These results are in agreement with those obtained by Mansour *et al*. (2013) showed the presence of ellagic acid, quercetin, gallic acid, vanillic acid, caffeic acid and p-coumaric acid in pomegranate peel extract. Also, Ali *et al*. (2014) revealed the presence of cinnamic acid, benzoic acid, ferulic acid, coumaric acid, pyrogallol and chlorogenic acid in methanolic extract of pomegranate peel by HPLC.

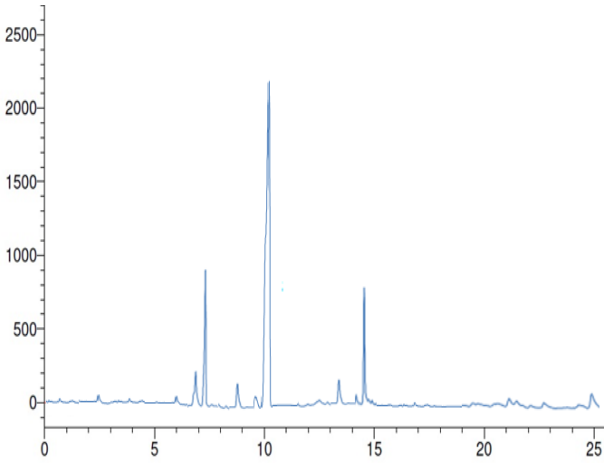

Fig.5. HPLC chromatogram of phenolic compounds identified in cold water extract of pomegranate peels.

Table 7. Concentrations of phenolic compounds identified in cold water extract of pomegranate peels by HPLC.

Compound	$R_{t\,(min)}$	Concentration (mg/ml)
Protocatechuic acid	7.6	10.25
Syringic acid	10.11	19.41
Ellagic acid	14.87	11.06

$R_{t\,(min)}$; Retention time by minute

CONCLUSION

In this work, pomegranate peel extracts contain many phytochemicals such as phenolics, flavonoids, alkaloids, saponins, tannins, steroids and terpenoids. The ethanol extract showed the highest total phenolics, total flavonoids and the highest antioxidant activity with the lowest value IC_{50} compared to the other extracts. Furthermore, these results revealed the presence of nine polyphenolic compounds: protocatechuic acid, p-coumaric acid, caffeic acid, ellagic acid, cinnamic acid, quinic acid, benzoic acid, syringic acid and iso-ferulic acid by HPLC in

pomegranate peels extracts. It can be concluded that pomegranate peel extracts can be used in various fields for being rich in natural antioxidants that have a medicinal and therapeutic impact.

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المواد الفيتوكيميائية ونشاط مضادات الأكسدة وتحديد المركبات الفينولية بواسطة جهاز HPLC لمستخلصات قشر الرمان

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قشور الرمان هي مخلفات غذائية غير صالحة للأكل تم الحصول عليها أثناء إنتاج عصير الرمان. الهدف من هذا العمل هو دراسة تأثير أربع مذيبات مختلفة (الإيثانول، الأيزوبروبانول، الماء الساخن والبارد) على فحص المواد الكيميائية النباتية، محتوى الفينولات الكلية، محتوى الفلافونويدات الكلية ونشاط مضادات الأكسدة في قشور الرمان، وكذلك تحديد المركبات الفينولية في المستخلصات بواسطة جهاز HPLC. تم اكتشاف الفينولات والفلافونويدات والقلويدات والصابونين والتانينات والاستيرويدات والتربينويدات في جميع المستخلصات. أعلى محتوي من الفينولات الكلية والفلافونيدات الكلية تم ملاحظتها في المستخلص الإيثانولي لقشور الرمان (١٦١,٥ ملجم GAE/جم و٧٠,٦٥ ملجم Rutin/ جم)، على التوالي. علاوة على ذلك، أظهر المستخلص الإيثانولي لقشور الرمان أعلى نشاط لمضادات الاكسدة باستخدام طريقة DPPH مع أقل قيمة (IC_{50}= 14.6 ميكروجرام / مل) مقارنة مع المستخلصات الأخرى. قد تكون زيادة النشاط لمضادات الأكسدة بسبب المحتوى العالي من الفينولات والفلافونويدات في المستخلص إلايثانولي لقشور الرمان. تم اجراء تحليل بواسطة جهاز HPLC للتعرف والتقدير الكمي للمركبات الفينولية في مستخلصات قشور الرمان. كشفت النتائج عن تسع مركبات فينولية مثل حمض البروتوكاتيشيويك – حمض البارا كيوماريك - حمض الكافييك - حمض الإيلاجيك - حمض السينابيك - حمض الكينيك - حمض البنزويك - حمض السرينجيك – حامض الايزو فيريوليك في مستخلصات قشور الرمان. أكدت النتائج المتحصل عليها أن مستخلص الإيثانول كان أكثر وفرة بالمركبات الفينولية وأكثر نشاطاً كمضادات اكسدة مقارنة بالمستخلصات الأخرى. أخيرًا، نستنتج أن مستخلصات قشر الرمان يمكن استخدامها في مجالات مختلفة لكونها غنية بمضادات الأكسدة الطبيعية التي لها تأثير طبي وعلاجي.