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HPLC Analysis and DPPH Assay of Some Bioactive Compounds in Pomegranate Peel Extracts

Adel A. M. Saeed[1*], Omnia Hussein Abdu[2], Taha Abu-Bakr Fadhl Salem[3]
[1]Departement of Chemistry/Faculty of Science, University of Aden
[2]Departement of Pharmaceutical Chemistry/Faculty of Pharmacy, University of Aden
[3]Departement of Chemistry/ Faculty of Aden Education, University of Aden

Corresponding Author
E-mail Id:- adel_saeed73@yahoo.com

ABSTRACT

Pomegranate (Punica granatum L.) and their derivative parts contain various phytochemical compounds. The pomegranate peels had the highest antioxidant activity as compared to other parts of pomegranate fruit. HPLC-UV technique was used to identify and quantify the individual ascorbic acid and bioactive compounds (i.e. gallic acid, procatachouic acid, quercetin, and kaempferol) in solvents extract of peels of pomegranate cultivated in Yemen, while scavenging assay of DPPH of dried and undried peel extracts were used to measure antioxidant capacity expression as EC_{50} value. The resulted findings by HPLC analysis showed that the amount of ascorbic acid was higher than the phenolic acids (gallic acid and protocatechuic acid), and flavonoids (quercetin and kaempferol) in all extracts and the highest amount of ascorbic acid was found in aqueous extract followed by methanolic whereas the last one was in ethanol solvent. While the phenolic acids were higher than the flavonoids in all extracts, the amount of protocatechuic acid in aqueous extract was higher than the amount of other polyphenols, and the quercetin amount was the lowest one. To compare the anti-oxidative activity of dried and undried peel extract by acetone and water, the EC_{50} value of dried and undried peel acetonic extracts were 1.2±0.35 and 5±1.8 µg/ml, respectively, which were higher than aqueous extracts of dried and undried peel (8.1±0.66 and 7.9 ±0.08 µg/ml) respectively.

Keywords:-*Pomegranate peels, phytochemical and bioactive compounds, hplc, anti-oxidation activity*

INTRODUCTION

Punica granatum L fruit commonly known as pomegranate, is an important source of vitamins, phytochemical and bioactive compounds (e.g. phenolic acids, flavonoids, and tannins) that has been used as a traditional micronutrient medicine for along times [1-6]. Pomegranate fruit pericarp (peels) has a promising treatment for several diseases without induce any side effects [7] and the peels regarded as waste, constitute 40% of all pomegranates fruit has high amount of phenolic compounds and the peel extract has many useful effects such as using as antioxidant, antibacterial, antiviral…etc. [8-10]. As a result, the field of pomegranate researches has experienced remarkable growth [11].

Phenolic compounds (or polyphenols) can be classified according to their abundance as dietary sources into two groups: The least abundant (phenolic acids) and the most abundant (flavonoids). Flavonoids, with more than 4000 substances, are classified as anthocyanins, flavones, isoflavones, flavanones, flavonols, and flavanols. [12].

Ascorbic acid (L-threo-Hex-2-enono-1,4-lactone, ascorbate or vitamin C) is one of

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the important essential nutrient and water soluble vitamins found in various foods. It has health beneficial uses such as helping immune system, repairing the tissue (wound healing), preventing common cold and functioning as an antioxidant [13-16].

Phenolic acids, one of the most widely occurring groups of phytochemicals, are secondary metabolites that naturally occurring compounds found in plant kingdom with unique structural similarities, presence of carboxylic group as in, gallic acid (3,4,5-trihydroxybenzoic acid) and protocatechuic acid (PCA, 2,4-dihydroxy benzoic acid). These compounds play a vital role in growth and reproduction, providing protection against pathogens and predators, and sensory characteristics of could be a major determinant of antioxidant potentials of foods, and could therefore be a natural source of antioxidants [17,18].

Gallic acid has important pharmacological properties attributed to its antioxidant and anti-inflammatory potentials. Addition, gallic acid and its derivatives demonstrated a broad range of beneficial effects on several disorders [19].

Several researches have shown that protocatechuic acid is a major metabolite of complex polyphenols, especially anthocyanins. Anthocyanins have been shown to affect a variety of physiological activities which are of great benefit to health [20].

Flavonoids are the most widely distributed polyphenols compounds in foods and the main bioactive compounds found in fruits. Kaempferol (3,4′,5,7-tetrahydroxyflavone, kaempferol-3 or kaempferide), is a major flavonoid compound found in many plants that has been shown to be antioxidant, antimicrobial, neuroprotective, antihypertensive, cardioprotective, anti-inflammatory, antidiabetic, antitumor, and anticancer activities [21-23]. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is a plant flavonol that is one of the most abundant dietary flavonoid groups of polyphenols with several medicinal and clinical activities [24].

HPLC, a shortcut name for the term high-pressure liquid chromatography or high-performance liquid chromatography can be useful for identifying sample composition. HPLC with UV-Vis detector can be selective and the use of a DAD (diode array detector for UV measurements) can provide UV spectra of the analytes, which in certain cases can be diagnostic in the sense that once the UV spectrum of a compound is known, it can be useful for positive identification. However (with a few exceptions), this detector is not useful for the identification of unknown compounds. HPLC is a versatile, robust, and widely used technique for the isolation and quantitation of analytes in samples such as pharmaceuticals, environmental samples, pollutants, biological samples, food and agricultural products, and many other materials and/or processes. HPLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components such as polyphenols and bioactive compounds. [25-31].

Superfoods are nutrient rich foods and are particularly beneficial toward health according to their high components of polyphenols and antioxidants. Antioxidants are the compounds, which combat the free radicals by intervening at any one of the three major steps of the free radical mediated oxidative process namely, initiation, propagation and termination and oxidative stress is due to the imbalance between the generation of reactive oxygen species (ROS) alongside reactive nitrogen

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species (RNS) and the antioxidant defenses [32,33].
Free radicals and reactive oxygen species (ROS) are fundamentally the main cause of numerous disorders in humans and cause direct oxidative damage to easily oxidized biological molecules such as proteins, DNA, and lipids [33-35].

Scavenging of DPPH (α,α-diphenyl-β-picryl-hydrazyl) radical is the basis of the popular DPPH antioxidant assay. based on the ability of the antioxidants to reduce it to its radical form, DPPH·, a stable nitrogen radical producing a violet color with the decrease in color monitored by absorbance at 520 nm or by electron spin resonance. The results are reported as EC50, which measures a 50% decrease in the DPPH· concentration, which is proportional to the antioxidant concentration [34,36].

Nowadays. the worldwide considerable attention to the coronavirus (COVID-19) epidemic has been largely limited to monitoring/containment and clinical trials should be dive into recent findings about possible and affordable treatment options in using plants as a sources of vitamins, phytochemical and bioactive compounds such as using zinc ionophore activity of quercetin, kaempferol and other polyphenols [37-41].

This paper intends to identify and quantify some bioactive compounds (i.e. Vit. C and some polyphenols) in pomegranate peel extracts by HPLC analysis. Also to compare the non-enzymatic antioxidant effect of pomegranate peel extract by DPPH assay between aqueous and acetonic extracts of dried and undried pomegranate peels.

EXPERIMENTAL

Materials and Methods

Gallic acid (Alfa Aesar, England, 99%), protocauchouic acid (Spectrochem, India, >97%), quercetin (Sigma Aldrich, India, ≥95% (HPLC)) and kaempferol (Sigma Aldrich, India, ≥90% (HPLC)), L-ascorbic acid (Fine chem, India, 99% (Laboratory Reagent-LR)) and perchloric acid (Fine chem, India, 70% (Diamond Grade, AR)), α,α-diphenyl-β-picryl-hydrazyl (DPPH, Sigma, USA, 95%). All solvents and other reagents (Loba cheme and Labtech chemicals, India) were of analytical grade without any further purification. Double-distilled and deionized water (specific conductance (1–2) x 10^{-6} $S.cm^{-1}$) was used throughout.

Integrated HPLC systems LC-2010AHT from Shimadzu Corporation (Chromatographic and Spectrophotometric Division, Kyoto, Japan) consisted of a 4-liquid gradient system, high speed auto-sampler, column oven, and UV-vis detector. Chromatograms were recorded and integrated on a PC installed with Shimadzu chromatographic software.

Metaspect Pro Spectrophotometer model UV5500 from (Taizhou Juhao Import and Export Co., Ltd., China) with large LCD screen, auto zero and blank, and tungsten lamp and deuterium lamp to make broad scan in Uv-Vis field. Absorbance was recorded and integrated on a PC installed with UV-Professional software to provide complete control of the spectrophotometer from a computer through the USB port.

Collection and Processing of Plant

Fresh fruit peels of *Punica granatum* cultivated in Saada Governorate were collected from Aden's Markets. The peels were removed manually from fruits, washed in tap water then dried by keeping them in dark place at room temperature for three weeks then pulverized.

Preparation of Crude Extract by Soxhlet Extraction Method

P. granatum fruit peel powder (100 g) was filled in the thimble and subjected to

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continuous hot percolation for 6 h using 300ml of distilled water as a solvent. Then, extract concentrated under vacuum and dried at 50 ^{0} C in oven. The soxhlet extraction was repeated using 300ml of other solvents (*i.e.* methanol, ethanol, and acetone) [42].

Identification of Phenolic Compounds

As reported by [43], 25mg of the sample was solubilized in 5 ml of sterile double-distilled water. Three drops of 0.1% freshly prepared ferric chloride solution was added. The formation of a dark blue-black color solution indicated the presence of the phenolic compound.

Identification of Flavonoids

25mg of extract dissolved in lead acetate solution and to 100 ml, NaOH solution was added. The existence of flavonoids was indicated by the formation of a yellow-colored precipitate [43].

Identification and Quantification of Ascorbic Acid and Polyphenols by HPLC Analysis

Before subjecting samples to HPLC separation, the stainless steel column (cyano analytical, Symmetry (C_{18}, 250x4.6 mm, 5µm) from Waters, USA), as stationary phase was washed with 60% methanol. The detection was carried out using variable wavelength UV–Vis detector set at 280 nm. HPLC system default pressure was set at 2000 psi, while chromatographic fractionation was done under 1800– 1950 psi pressure. 14-20 HPLC fractions were multiply collected and analyzed. Elution was performed using a mobile phase consists of mobile phase (A) buffer (0.2% perchloric acid) and mobile phase (B) acetonitrile (ACN). Starting with a 20% B increase to levels of 80 % for 20 min then back to 20% in 40min. The solutions were chromatographed at a steady flow rate of 0.8 ml/min, extracts samples and mobile phases were filtered through millipore membrane 0.45 µm porosity before HPLC analysis. All pertinent analyses were made at ambient room temperature and the volume of solutions injected onto the column was 10µl.

Quantification of bioactive compounds was carried out using authentic ascorbic acid, gallic acid, protocatechuic acid, quercetin and kaempferol. Peak areas were recorded for all peaks. Respective peak areas were taken into account to quantitate the label amounts by using the following formula:

$$\frac{R_u}{R_s} \text{ x } \frac{W_s}{W_u} \; x\, 100 \qquad (1)$$

where R_u is peak area obtained for investigated solution, R_s are the peak areas obtained for the standard solution. W_s is the weight, in µg, of respective reference standards taken to prepare standard solution; W_u is the weight, in µg, of test sample.

Anti-oxidation Analysis by DPPH Assay

Anti-oxidation analysis of acetonic and aqueous extract of the dried and undried peel was carried out as follow. The free radical scavenging activity of all extracts was evaluated by α,α-diphenyl-β-picryl-hydrazyl (DPPH) according to the effective method reported in [32,44-46] .In recent study, the acetonic and aqueous extracts of dried pomegranate peel and that of undried pomegranate peel was compared. Briefly, a 0.1mM solution of DPPH in methanol was prepared, and a 1ml of this solution was added to 3 ml of the solution of all extracts in methanol at different concentrations (*i.e.* 2,4,6,8 and 10 µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 518nm a spectrophotometer. Less absorbance values of the reaction mixture indicated high free radical scavenging activity. The capability of scavenging the DPPH radical (percentage of inhibition, *I%*) was

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calculated by using the following formula:

$$I\ \% = \frac{A_0 - A_1}{A_0} x100 \qquad (2)$$

where A_0 is the absorbance of all reagents except the test compound (*i.e.* the control reaction; 0.1mM DPPH sol./3ml methanol) and A_1 is the absorbance of the extract samples (the test compounds). All the tests were performed in triplicates and the results were averaged.

Statistical Analysis

One-way analysis of variance (ANOVA) and Duncan triplicates range test ($P<0.05$ level significance) were appointed to data and subjected to correlation coefficient by using Graphped prism version 6 statistical analysis software. The results were expressed as mean ± SD.

RESULTS AND DISCUSSION

Identification of Total Polyphenols and Flavonoids

All medium extracts (Table 1) of pomegranate peels were given positive tests for total polyphenols and flavonoids.

Table 1. *Identification of total polyphenols and flavonoids*

Extract / Substance	Aqueous	Methanolic	Ethanolic	Acetonic
Polyphenols	+	+	+	+
Flavonoids	+	+	+	+

Identification and Quantification of Bioactive Compounds by HPLC Analysis

Figure 1 and Table 2 show the retention times and area of the authentic ascorbic acid and polyphenols that used to characterize the same bioactive compounds in pomegranate peel extracts under study.

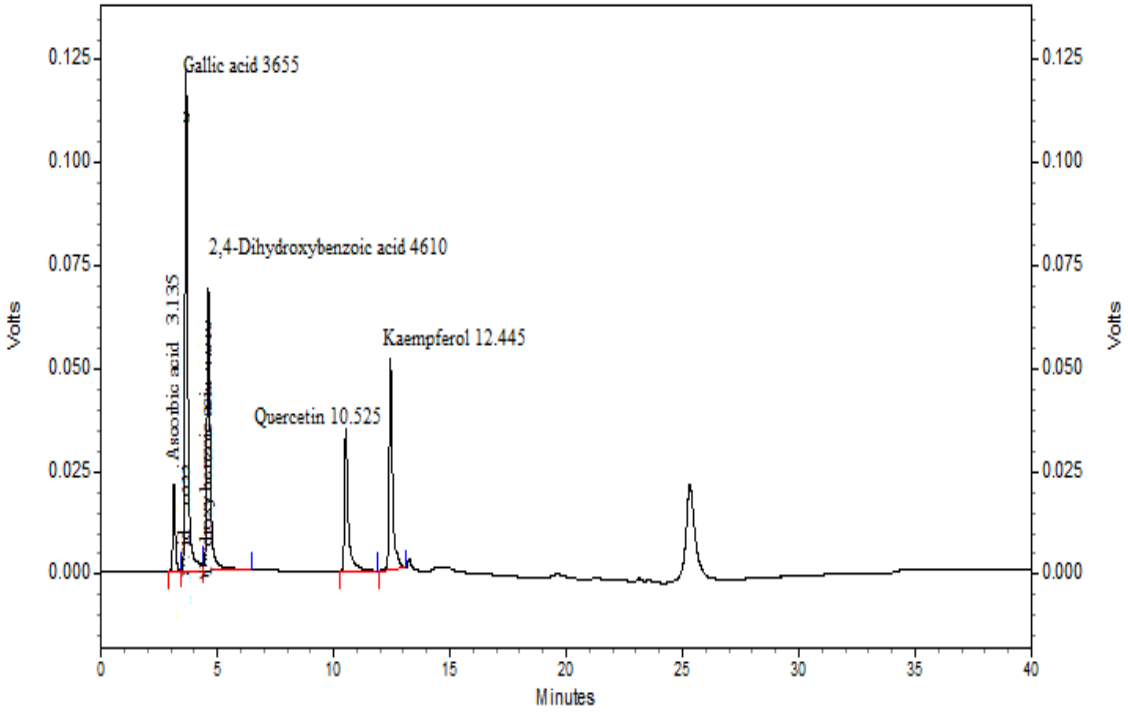

Fig.1:-*HPLC Chromatogram of standards on C18 column and at 280nm; Peaks appeared with its retention time sequentially of each compound*

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Table 2:-*Retention times and area of the peak the available authentic ascorbic acid and phenolic components*

No.	Standards	Retention Time	Area	Area Percent
1	Ascorbic acid	3.135	139615	5.32
2	Gallic acid	3.655	974000	37.09
3	Procatachouic acid (2,4-Dihydroxy benzoic acid)	4.610	660439	25.15
4	Quercetin	10.525	373205	14.21
5	Kaempferol	12.445	478751	18.23
	Total		2626010	100

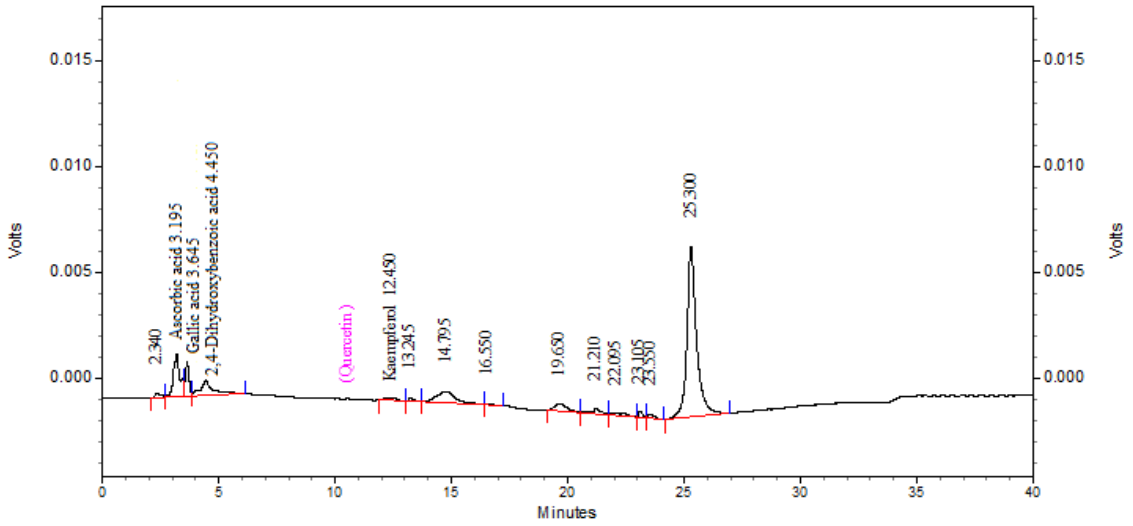

Fig.2:-*The chromatogram of aqueous extract of pomegranate peel (C18 column, 280nm)*

Table 3:-*Retention times and area of the peak of Aqueous extract of pomegranate peel*

No.	Substances	Retention Time	Area	Area Percent
1	Unknown	2.340	9391	0.87
2	Ascorbic acid	3.195	106372	9.86
3	Gallic acid	3.645	47359	4.39
4	Procatachouic acid (2,4-Dihydroxy benzoic acid)	4.450	78290	7.26
5	Kaempferol	12.450	13236	1.23
6	Unknown	13.245	8392	0.78
7	Unknown	14.795	94485	8.76
8	Unknown	16.550	4062	0.38
9	Unknown	19.650	32159	2.98
10	Unknown	21.210	18045	1.67
11	Unknown	22.095	14874	1.38
12	Unknown	23.105	10968	1.02
13	Unknown	23.550	11499	1.07
14	Unknown	25.300	629692	58.37
	Total		1078824	100

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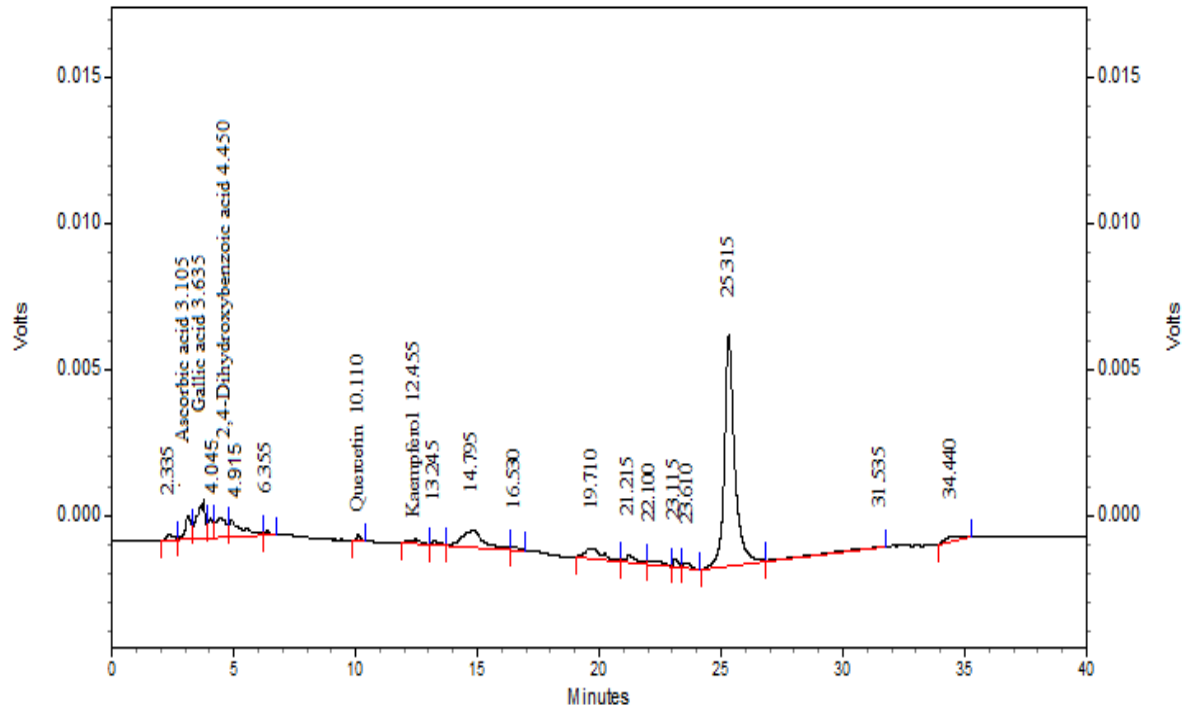

Fig.3:-*The chromatogram of methanolic extract of pomegranate peel (C18 column, 280nm)*

Table 4:-*Retention times and area of the peak of methanol extract of pomegranate peel*

No.	Substances	Retention Time	Area	Area Percent
1	Unknown	2.335	9817	0.82
2	Ascorbic acid	3.105	38091	3.19
3	Gallic acid	3.635	75650	6.33
4	Unknown	4.045	29924	2.50
5	Procatachouic acid (2,4-Dihydroxy benzoic acid)	4.450	52450	4.39
6	Unknown	4.915	54190	4.54
7	Unknown	6.355	3605	0.30
8	Quercetin	10.110	5599	0.47
9	Kaempferol	12.455	13448	1.13
10	Unknown	13.245	7897	0.66
11	Unknown	14.795	95361	7.98
12	Unknown	16.530	4876	0.41
13	Unknown	19.710	44171	3.70
14	Unknown	21.215	20459	1.71
15	Unknown	22.100	15432	1.29
16	Unknown	23.115	12143	1.02
17	Unknown	23.610	10857	0.91
18	Unknown	25.315	640893	53.64
19	Unknown	31.535	38686	3.24
20	Unknown	34.440	21199	1.77
	Total		1194748	100

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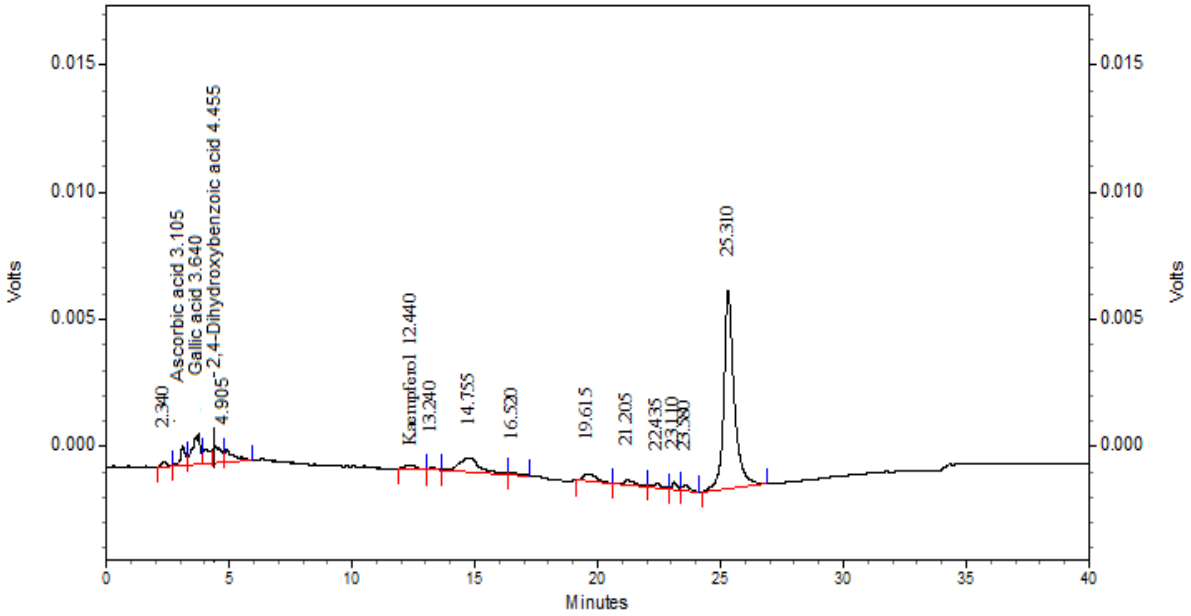

Fig.4:-*The chromatogram of ethanolic extract of pomegranate peel (C18 column, 280nm)*

Table 5:-*Retention times and area of the peak of ethanol extract of pomegranate peel*

No.	Substances	Retention Time	Area	Area Percent
1	Unknown	2.340	7027	0.69
2	Ascorbic acid	3.105	30120	2.96
3	Gallic acid	3.640	65903	6.47
4	Procatachouic acid (2,4-Dihydroxy benzoic acid)	4.455	40498	3.98
5	Unknown	4.905	33806	3.32
6	Kaempferol	12.440	13835	1.36
7	Unknown	13.240	6033	0.59
8	Unknown	14.755	92210	9.06
9	Unknown	16.520	5151	0.51
10	Unknown	19.615	33299	3.27
11	Unknown	21.205	22964	2.26
12	Unknown	22.435	13701	1.35
13	Unknown	23.110	11648	1.14
14	Unknown	23.580	12170	1.20
15	Unknown	25.310	629497	61.85
	Total		1017862	100

Table 6:-*Shows present the composition of polyphenolic compounds of extract samples of pomegranate peel*

Substances	Aqueous Extract	Methanolic Extract	Ethanolic Extract
	(%)		
Ascorbic acid	19.05	6.82	5.39
Gallic acid	1.22	1.94	1.69
Protocachouic acid	2.96	1.98	1.53
Quercetin	ND*	0.38	ND*
Kaempferol	0.69	0.70	0.72

ND*: Not Detectable.

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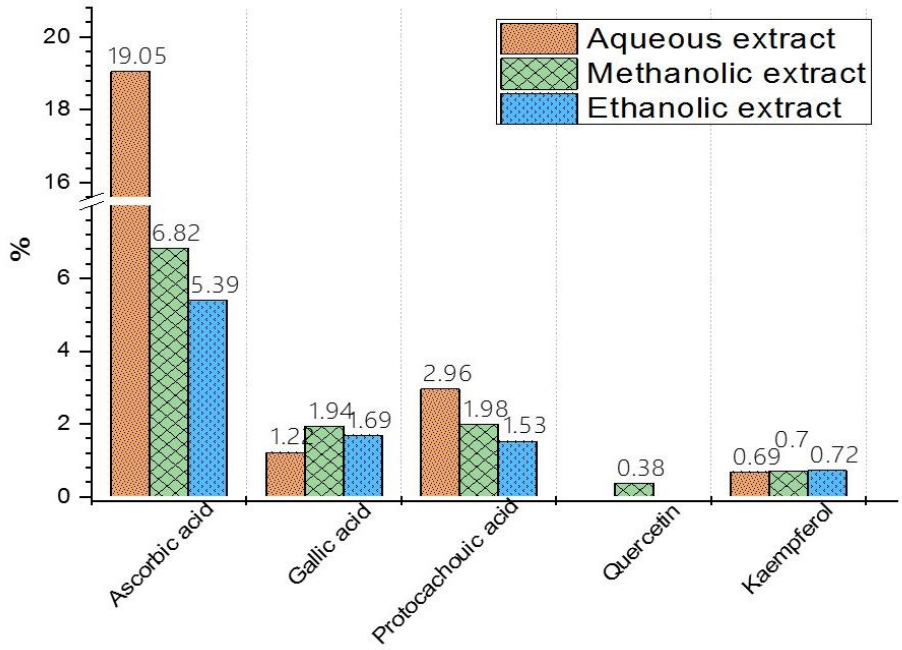

Fig.5:-*The composition of ascorbic acid & polyphenolic compounds of pomegranate peel extract.*

High-performance liquid chromatography (HPLC) was used for the identification and quantitative analysis of polyphenolic compounds of pomegranate peel. Figures 2-4 and Tables 3-5 represent the components of pomegranate peel in aqueous, methanolic, and ethanolic extracts were fractionated into 14,15 and 20 different peaks, respectively by HPLC. Unfortunately, the deficiency of certain technique (*i.e.*, it is not connected with mass spectrometer device) prevented further identification and quantification of the pomegranate peel components.

In current study, only five components were identified (ascorbic acid, gallic acid, protocatchoic acid, quercetin, and kaempferol) in the three extracts. Table 6 and Figure 5 show that the amount of ascorbic acid was higher than the phenolic acid (gallic acid, protocatechuic acid), and flavonoids (quercetin, kaempferol) in all extracts and the highest amount of ascorbic acid was found in aqueous extract followed by methanolic and finally ethanolic extract. While the phenolic acids were higher than the flavonoids in all extracts, the amount of protocatechuic acid in all extracts showed higher amount than gallic acid. The aqueous extract contains the highest value of protocatechuic acid followed by methanolic and ethanolic extract. Whereas the highest amount of gallic acid was found in methanolic extract followed by ethanolic and aqueous extract respectively. For the flavonoids the result represented their small amounts especially quercetin compound which was not detected in aqueous and in ethanolic extracts but hardly found in methanolic extract only. The kaempferol was detected in all extract but less than other investigated components.

Reference [47] stated that the amount of protocatechuic acid was higher than gallic acid and quercetin in pomegranate juice which resembled the result in our study. However, the amount of those components in our investigation on pomegranate peel

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extracts were higher than that in pomegranate juice

Antioxidation Activity by DPPH Assay

On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the decrease intensity of violet color and the degree of discoloration indicates the scavenging potentials of the antioxidant extract. Signifying the DPPH radical by R• and the donor molecule by DH, the primary reaction is

R• + DH = RH + D• (3)

The effective concentration of extract required to inhibit 50% of the initial DPPH free radical can be expressed as EC_{50} and those considered values denotes the effective concentration of a sample required to decrease the absorbance at 518 nm to half. All measurements were performed in triplicate. EC_{50} were calculated from regression equations of calibration plots next:

$Y=7.059x+41.489$, $R^2 = 0.8747$ (4)

$Y=5.460x+22.680$, $R^2 = 0.9805$ (5)

$Y =5.275x+7.051$, $R^2 = 0.9902$ (6)

and

$Y=4.765x+12.490$, $R^2 = 0.9935$ (7)

where, Y= EC_{50} in µg/ml; eq. (4) for acetonic dried peels (DAE); (5) for acetonic non-dried peels (NDAE); eq. (6) for aqueous dried peels (DWE) and; eq. (7) for aqueous non-dried peels (NDWE), respectively. The results of are shown in Table7.

Table 7:-*Antioxidation activity by DPPH assay*

Extract	Aqueous extract of undried peel (NDWE)	Aqueous extract of dried Peel (DWE)	Acetonic extract of undried peel (NDAE)	Acetonic extract of dried Peel (DAE)
DPPH at ($EC_{50\%}$) (µg/ml)	7.9 ± 0.08*	8.1 ± 0.66	5 .1 ± 1.8	1.2 ± 0.35

*values represented as mean ± SD (n = 3)

As mentioned in Table 7, values of dried and undried acetonic extracts were 1.2 ± 0.35 and 5 .1 ± 1.8 µg/ml, respectively, which were higher than aqueous extracts of dried and undried peel 8.1 ± 0.66 and 7.9 ± 0.08 µg/ml respectively. Besides, relative antioxidation activity of dried acetonic extract was higher than undried acetonic extract. And, anti-oxidation activity of undried aqueous extract was higher than dried aqueous extract.

The loss of antioxidants caused by hot air drying could be attributed to the possible polymerization of bioactive compounds at high temperatures [48] and enzymatic action [49]. For these reasons, the aqueous extract of dried peel contained less amount of ascorbic acid/polyphenols so less scavenging activity.

On the other hand, for acetonic extracts of dried peel the drying led to increase the scavenging activity of the extract. The acetone solvent is more effective for radical scavenging activity than water where the acetonic extract contained high bioactive compounds. Recent results are in agreement with the study of [50,51].

CONCLUSIONS

Yemeni pomegranate peel contains several bioactive compounds and the amount of investigated compounds were in the order: ascorbic acid > protocatechuic acid > gallic acid > kaempferol >> quercetin.

The acetonic and aqueous extracts for dried and undried pomegranate peel displayed good antioxidant activities where the highest scavenging potential was in acetonic extract of dried peel followed by acetonic extract of undried peel, then aqueous undried peel and the last one aqueous dried peel extracts.

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Although pomegranate peel showed small amounts of investigated compounds, further studies required to complete image for finding and using bioactive substances that have promise to heal several recent diseases such as covid 19.

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