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Research Article

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Identification and Quantitative LC-MS Analysis of the Main Flvonoids Present in Pomegranate Bark (*Punica granatum* L.)

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Abstract In this study, three different types of flavonoids ((3,3',4',5,7-pentahydroxy-2-phenylchromen-4-one (Quercetin), 5,7,3',4'-tetrahydroxy-flavone (Luteolin) and 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Kaempferol)) which in the pomegranate shell grown in South of Turkey, were measured by the help of liquid chromatography-mass spectroscopy (LC-MS) technique. In this measurement, ACE 5 C18 column systems, ammonium acetate: methylalchol (MeOH) mobile phase and diode-array detector were used. In 0.5 gr of dry pomegranate shell; 3.79 μg quercetin, 0.45 μg luteolin and 3.97 μg kaempferol were found.
After, the wool fabrics have been dyed using pomegranate shell in together mordanting and mordantless techniques. Fastness to light, washing and rubbing of the dyed fabrics were measured and discussed.

Keywords Punica granatum L.; Quercetin: Luteolin; Kaempferol: Natural dyes; dyeing, mordant, LC-MS

Introduction

Natural dyes/colourants have been used since ancient times. After the invention of synthetic dyes/colourants, usage of natural dyes/colourants has diminished rapidly. Natural dyes and colourants have been used for many years. For instance, they have been used coloring natural fibers like wool, cotton and silk, as well as fur and leather. In addition to this, they served to color cosmetic products and to produce inks, watercolors and artist's paints. Within the increase of an environmental awareness ecology and pollution control in the world, natural dyes become more attractive. Currently, Governments is compelling the dye industry to decrease poisonous waste and to cease the output of potential dangerous dyes and pigments [1].

Flavonoids, which find in natural plant foods, are polyphenolic compounds. They are exceedingly significant for the human health. They average intake of all flavonoids is estimated few grams per day [2]. They are found at C3 position of sugar bonds as o-glycosides. If the flavonoids has a diphenylpropane chain, they are known to be antimutagenic and anticarcinogenic [3-6]. They have both antimicrobial properties and restrain the oxidation of LDL [7-10]. They also have anti-inflammatory and anti-allergic effects [11]. *In vitro*, flavonoids have been shown to act as antioxidants [12] and inhibit growth of cancer cells from the colon [13-17], ovary [18] and gastrointestinal tract [19]. *In vivo*, it is found that soy isoflavones, like daidzin and genistein, decrease risk of breast cancer by the influence of biochemical processes [20-21]. Recent years, the pomegranate tree has been searched widely in point of pharmacological properties. Flower extracts reduced blood sugar levels [22], in rodents and humans juice was demonstrated to restrain LDL oxidation and the formation of atheromatous plaque [23]. When oil polyphenols were determinate to inhibit the eicosanoid enzyme cyclooxygenase and lipoxygenase, strong antioxidant properties of the fermented juice have been declared [24]. *In vitro*, there is an exertion in anti-proliferative effects on human breast cancer cells, by the half of fractions of the pomegranate; like crude seed oil, crude fermented and unfermented juice and peel extract [25]. Varieties of analytical methods have been improved so as to find their concentration in matrix scale, when the interest in flavonoid increased.

Two of the analytical methods are that GC/MS [26] and HPLC usage with MS [27], diode array [28] and electrochemical [29] detection.

Up to now, there are no enough measurements for the flavonoid quantitative determination to achieve the desired accuracy. In this paper, we used Liquid Chromatography-Mass Spectrophotometry (LC-MS) to present and accurate determination method for analyzing the main flavonoids of pomegranate bark (*Punica granatum* L.).

After, the wool fabrics have been dyed using pomegranate shell in together mordanting and mordantless techniques. Fastness to light, washing and rubbing of the dyed fabrics were measured and discussed.

2. Methods

2.1. Materials

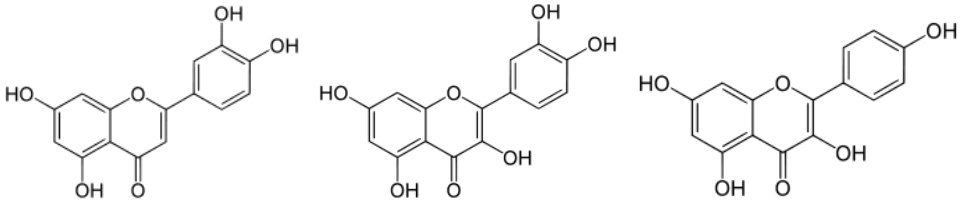
The aerial parts of pomegranate collected in Tekir (South of Turkey) were used. After drying, the plant was ground in a laboratory mill and then sifted. The three flavonoids used for the standard curves as well as the internal standard, routine, all being of analytical grade, were purchased from Aldrich. Buthylhydroxyanisol (BHA) was purchased by Sigma. Mordants ($KCr(SO_4)_2.12H_2O$, $Al_2(SO_4)_3.18H_2O$, $CoSO_4.7H_2O$, $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$) were purchased from Fluka.

2.2. Extraction of Aqueous pomegranate solution and acid hydrolysis

0.5 g of fresh pomegranate (*Punica granatum* L.) pericarps (peels) was extracted in a Soxhlet apparatus with 250 ml of water for 8 h. The extract was evaporated under vacuum to about 50 mL. This concentrated aqueous pomegranate pericarp extract was combined with three times ethylacetate (150 mL). The ethylacetate phase was separated from the aqueous phase and evaporated under nitrogen at 40 ^oC. The polyphenolic compounds were resuspended in 50 ml methanol [30-31]. Acid hydrolysis of aqueous pomegranate pericarp extract was performed as described previously, with only a few modifications [32-34]. For determination the polyphenolic compounds, 25 ml of 62.5% aqueous methanol containing BHA (as antioxidant, 2 g/ L) were added to 25 ml of the polyphenolic compounds. Subsequently, 10 mL of 5 M HCl were added to the solution. The reaction mixture was refluxed for 4 h, after which the solution was allowed to cool in the room temperature. The solution was diluted ten times with deionized water and filtered through a 0.45-µm filter Cartridges. This solution was extracted with three times ethylacetate (100 mL). The ethylacetate was evaporated under nitrogen. The solid phase was resuspended in 15 ml of methanol and were obtained as pure yellow solids through purification via flash column chromatography.

2.3 Chromatographic Analysis

LC-MS analysis was performed using an Agilent 1100 MSD gradient pump coupled to a HewlettPackard1100 diode-array detector. Flavonoid separation was carried out in a ACE 5 C18 (15 cm x 4.6 mm) column. Plant extracts were eluted at 0.6 mL/min. (20 µl injection volume) using as mobile phase binary solvent system consisting in methanol and 10 mM NH_4CH_3COO in 0.1 % formic acid. The gradient scheme is presented in Table1. Column flash chromatography was performed on glassware columns (20 mm, diameter) with sintered glass septa and Teflon taps. The columns were packed C18 60 A 35–70 µm (Isolera, Biotage), elution via acetone. Flavonoids were monitored by UV absorbance at 350 nm using e by a Perkin Elmer Lambda 45 spectrophotometer. Their UV/visible absorption spectra are presented in Fig.1. All chromatographic procedures were performed at 25 ^oC. The amount of quercetin, luteolin and kaempferol were estimated from standard curves obtained by analysis of various doses of authentic compounds. The chemical structures of three flavonoids are in Scheme.



Luteolin Quercetin Kaempferol

Scheme 1: Chemical structures of Luteolin, Quercetin and Kaempferol

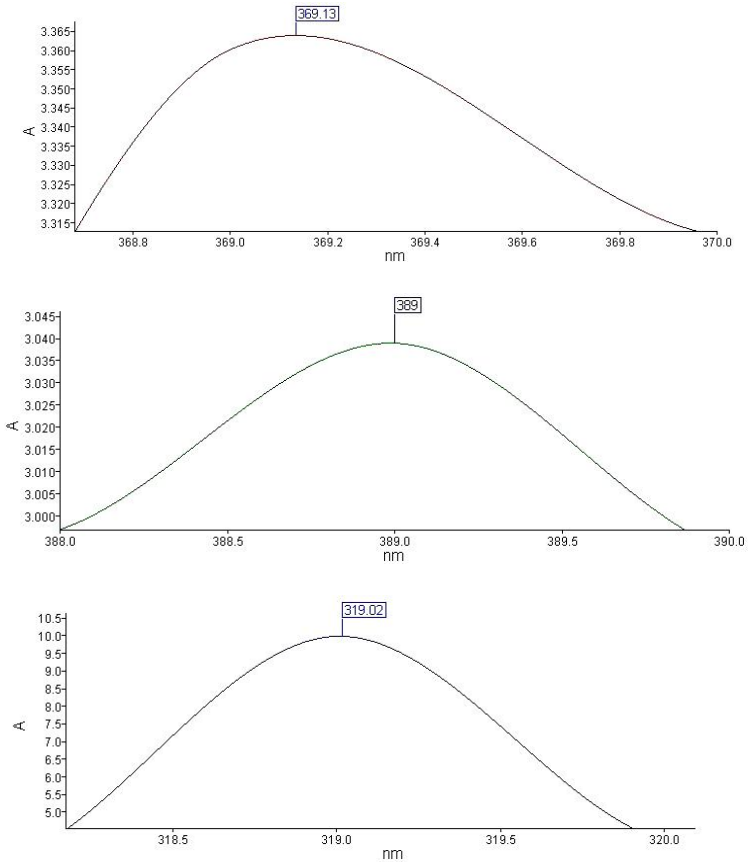


Figure 1: The UV-vis spectra of flavonoids of Qercetin, Kaempferol and Luteolin

Dyeing of wool fabrics

Dyeing without mordant

The wool fabrics (543 g/m^2) were dyed in a dye bath containing 3% of pomegranate bark. The dyeing was carried out at 97–98 °C for 60 min, after which 2% sodium chloride solution on the basis of material was added to the dye bath and the system was further kept at that temperature for 15 min. The dyed fabric was removed, rinsed with distilled water and air-dried.

Dyeing together with mordant

The together mordanting technique involved using 2% solutions each of mordants which were employed according to literature [35]. Mordanting was carried out for 30 min at 97–98 °C. The dyed fabric was removed, rinsed with distilled water and air-dried.

Measurement of fastness properties

Color fastness tests to light, washing and crocking or rubbing was carried out in a Xenotest Alph, Gyrowash and Crockmeter, respectively. The fastness ratings were given in grey scales [36].

Results and Discussions

Pomegranate shells are using as a natural dye in this region. Solutions of the pomegranate shells give different colors in different mordants. In this study, the amount of three different flavonoids used in natural dyeing actively, were quantitatively measured by the help of LC-MS. In addition to these, wool fibers were dyed with the pomegranate shells used in measurement then different types of mordants. Fastness tests were performed on to these wool fibers.

LC-MS Conditions

As were discussed in the experimental part, extracts of the pomegranate shells were analyzed by the use of LC-MS system with diode-array detector. ACE 5 C18 column were used in this analyze. Solution A; 10ml NH_4Ac

in 0.1 % formic acid and solution B; methanol: water gradient systems were used as a mobile phase for flavonoids.

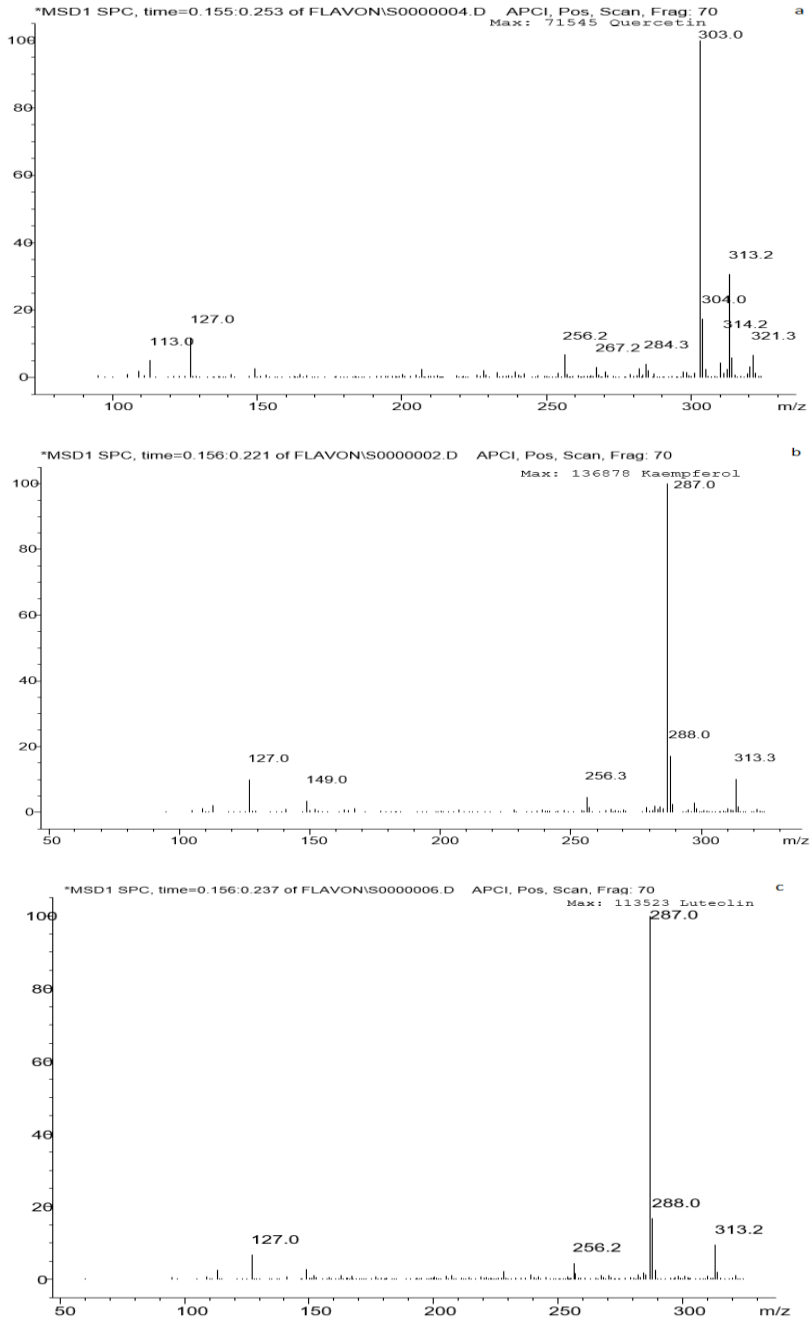


Figure 2: The mass spectra of flavonoids, A: Qercetin, B: Kaempferol, C: Luteolin

Gradient program was increased from 0 to 80 % B in ten minutes and from 10 to 100 % B in five minutes, furthermore turn into initial concentration in ten minutes. 0.6 ml/min was used as flow rate in analyze and measurements were made at 360 nm. The UV-Vis absorption spectra of three flavonoids are given in Figure 1. All of the wavelength of maximum absorbances (λ_{max}) well-adjusted as Mabry et all's book that title of "The Systematic Identification of Flavonoid" [37]. Figure 2, shows flavonoid's mass spectrums taken by the LC-MS system. As were shown by the mass spectrums, we see $[M+H]^+$ peaks at 303, 287, 287 for quercetin, luteolin and kaempferol, respectively. These results consist with the molecular weights of the flavonoids [37]. For the quantitative measurement of the flavonoids in extracts, we use standard reference materials to calibrate the device and we find peak areas. These curves are shown in the Figure 3. Regression analyze results and

validation parameters of the flavonoids calibration curves, are given in Table 2. The linear equation between the concentration of the standards injected and the peak area can be expressed as $y=mx+c$, where y is the concentration and x is the peak area of the standard, and m and c are constants. $y = 7.4\ x - 4.9.10^2$, $y = 1.6.10^1x - 6.8.10^2$ and $y=1.19.10^2\ x - 8.3.10^3$ for quercetin, kaempferol and luteolin, respectively. The retention times of the standard active materials in LC-MS spectrum were found as 10 min, 10.6 min, and 11.3 min for quercetin, kaempferol and luteolin, respectively. These results consist with the extract analyze and in this LC-MS system flavonoids are measured with the 0.8-1.3 % RSD. LC-MS chromatograms of the standard flavonoids are given in the Figure 4 and the pomegranate extraction is given in the Figure 5.

The linearity, limit of detection (LOD) and limit of quantitation (LOQ) for three flavonoids were investigated and the results are presented in Table 2. Detection limit is the lowest amount of the analytes in a sample that can be detected, but not necessarily quantitated. The lowest limit is usually evaluated as the signal to noise ratio that is equivalent to three times the standard deviation of the noise ($S/N = 3$). The LOD and LOQ were estimated in accordance with base line noise, which was evaluated by recording the detector response over a period of as much as the 10 times of the peak width. The instrument precision was measured by performing the intra-day and inter-day experiments by five replicate injections of flavonoids in three different working concentrations. The intra-day and inter-day RSDs of chromatographic determination were observed in the range of 1.30-3.91 % and 1.50-2.88 %, respectively (Table 3). The results showed good precision of the method. Quantitative results are expressed in terms of recovery percentage. The recoveries accomplished for flavonoids were in the range of 100.12-100.91 % (Table 4).

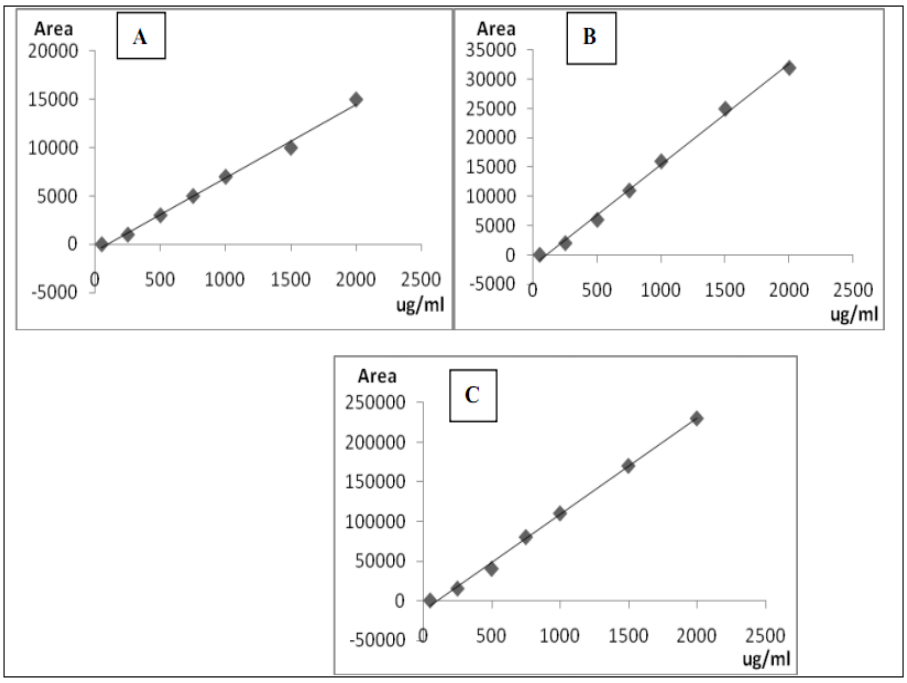


Figure 3: The calibration curves of flavonoids, A: Qercetin, B: Kaempferol, C: Luteolin

Effect of mordanting conditions

From initial experiments, it was observed that the together mordanting technique imparted better fastness properties to the fibres compared to post- and simultaneous mordanting techniques. Therefore, by adopting the together mordanting technique, the dyed fibres were mordanted.

Molecules of wool consist of amino acid units. Proteins are formed by amino acids which have free amino and carboxyl groups. Therefore, wool has an amphoteric formation [35]. In the dyeing of wool intermolecular hydrogen bonding occurs between the flavonoids and the amino group of wool. The metal ions coordinated to the $-NH_2$, –COOH, or –CO-NH- sites of the protein and donor groups of flavonoids. Addition of mordant leads to obtaining dye-lake on the fibre. These treatments of mordants have a great influence on the wool color. The

treatment of natural dyes with metal ions (e.g. Cr^{3+}, Cu^{2+}, Fe^{3+}) can improve light fastness and wash fastness properties [35]. These properties in most cases are accompanied by a bathochromic shift of the color of the dye. In this regard, the dyes often form ligand–metal complexes that are less soluble in water than the free ligand, which contributes to the observed improvement in wash fastness.

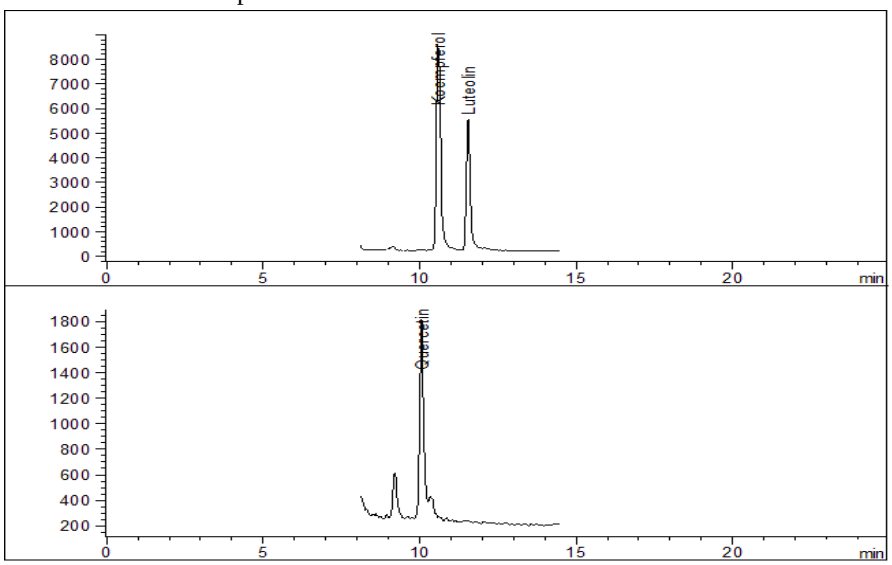


Figure 4: The LC-MS chromatograms of standard flavonoids

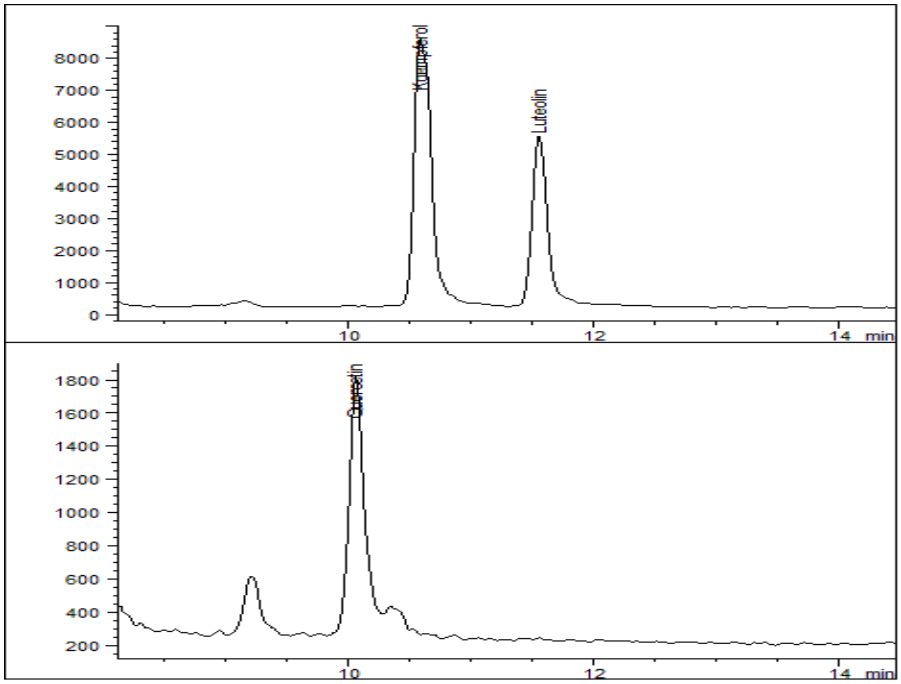


Figure 5: The LC-MS chromatograms of pomegranate extracts, A: Qercetin, B: Kaempferol, C: Luteolin

Table 1: LC-MS gradient scheme

Time	% MeOH
0.0	40
10	80
12	80
15	100
17	40

Table 2: Regression data of the calibration lines for quantitative determination of three flavonoids by LC-MS

	Qercetin	Kaempferol	Luteolin
Maximum absorbance (nm)	369	389	319
Linearity range (µg/ml)	75-2.10^3	75-2.10^3	75-2.10^3
Slope (µg)	7.43	1.61 x10^1	1.19 x10^2
Intercept (µg)	4.93x10^{-2}	6.75x10^{-2}	8.35x10^{-3}
Correlation coefficient	0.9972	0.9983	0.9979
LOD (µg /ml)	35	43	40
LOQ (µg /ml)	45	50	50

Table 3: Repeatability of intra-day and inter-day analysis

Compound	Concentration (µl/ml)	RSD (%)	
		Intra-day (n = 5)	Inter-day (n = 5)
Qercetin	100	1.31	1.88
	50	1.91	1.51
	25	2.18	2.63
Kaempferol	100	1.30	1.88
	50	2.61	1.51
	25	2.18	2.63
Luteolin	100	1.30	2.88
	50	3.91	1.50
	25	2.18	2.63

RSD = relative standard deviation; n = No of injections.

Table 4: Recovery data by standard addition

Compound	Concentration (µg/ml)	Added (µg/ml)	Obtained (µg/ml)	Recovery (%)
Qercetin	10.0	60	70.43	100.61
		80	90.66	100.73
		100	110.33	100.33
Kaempferol	10.0	60	70.58	100.83
		80	90.11	100.12
		100	110.67	100.60
Luteolin	10.0	60	70.54	100.77
		80	90.13	100.14
		100	111.00	100.91

Table 5: Color codes and fastness properties of dyed fabrics

Mordant	pH	Bath temp.	Dyeing period (min)	Colour code	Light	Croc dry	King wet	Wash
Mordantless	4.3	97	60	$Y_{80}M_{90}C_{80}$	3	3-4	3-4	3
$KCr(SO_4)_2.12H_2O$	5.7	97	60	$Y_{50}M_{60}C_{50}$	3	3	3	3
$Al_2(SO_4)_3.18H_2O$	6.0	98	60	$Y_{40}M_{40}C_{10}$	3	4	3	3
$CoSO_4.7H_2O$	3.0	98	60	$Y_{60}M_{70}C_{70}$	5	5	4-5	5
$CuSO_4.5H_2O$	4.0	97	60	$Y_{70}M_{99}C_{70}$	5	5	4-5	5
$ZnSO_4.7H_2O$	6.4	98	60	$Y_{80}M_{50}C_{00}$	4	5	4	5

In 5-grade scale; 1, the lowest; 5, the highest fastness.

The fastness tests and color codes [38] were applied to all mordanted and un-mordanted wool (Table 5). Dark colors were obtained at pH 4.3 and 6.4; pale colors were obtained at pH 3, 4, 5.7 and 6. Although the fabrics dyed without mordant showed poor fastness, the dyeing together with mordant had good light, washing and rubbing fastness. Moreover, the Cu^{2+} and Co^{2+} showed very good results for the light and rubbing fastness, but Al^{3+} and Cr^{3+} exhibited poor light fastness.The mordant activity of metal ions followed the sequence

Cu(II) → Co(II) → Zn(II) →Al(III) → Cr(III) in wool for *Punica granatum* L. The color intensity was found to be maximum when mordanted with Cu(II) and Co(II) as compared to Al(III) and Cr(III) for the fibres. Further, bright shades were obtained by using 2% of $CuSO_4$. $5H_2O$ and $CoSO_4.7H_2O$, which implied that the absorption of color by fibre was better when using Cu(II) and Co(II) as mordants. This might be due to the maximum absorption and strong interaction between metal ions and the wool.

Conclusions

The presented study, we realized quantitative determination of three different types of flavonoid (Quercetin, Luteolin and Kaempferol) belonging to pomegranate bark using LC-MS technique. In addition, the wool fabrics have been dyed using pomegranate shell in together mordanting and mordantless techniques. Fastness to light, washing and rubbing of the dyed fabrics were measured and discussed.

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