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Analysis of natural dyestuffs in *Achillea grandifolia* Friv. using HPLC-DAD and Q-TOF LC/MS

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The aim of this study was to analysis of natural dyestuffs from *Achillea grandifolia* Friv. leaves, flowers and stems in wool dyeing. The optimum dye extraction temperature and time were determined by spectrophotometrically. Natural dyeing was carried out with the optimized dye extract on unmordanted wool fabrics. The dyestuffs were analysed using electrospray ionization-quadrupole-time of flight liquid chromatography mass spectrometry (ESI-Q-TOF LC/MS) and high-performance liquid chromatography equipped with photodiode array detector (HPLC-DAD) methods. Thus, the purpose of this study was to identify and quantify of colouring constituents from the plant. As a result of the analysis, natural dyestuffs in *Achillea grandifolia* were determined as luteolin, rutin, quercetin, luteolin-7-*O* glucoside, quercetagetin 3,6-dimethyl ether, chlorogenic acid, caffeic acid, dicaffeoylquinic acid and their isomers. Finally, the colorimetric properties of the dyed fabrics were evaluated in CIELAB (Commission Internationale de l'Eclairage, CIE) system. L* (brightness), a* (red/green) and b* (yellow/blue) values of dyestuffs were measured.

Keywords: Natural dyestuffs, HPLC-DAD, *Achillea grandifolia* Friv.

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Natural dyes were used extensively throughout history in colouring clothing[1]. The ancient people were used them for colouring fabrics and other materials. However, synthetic dyes began to displace of natural dyes in the second half of the nineteenth century. Because, synthetic dyes give known results and they are commercial quantities in standardized potency presence of impurities. However, some researchers have recently demonstrated that synthetic dyes cause health problems, such as cancer, hypersensitivity or neurotoxicity[2,3]. So, there is growing interest in the use of natural dyes like in ancient times because of their less toxic, less allergenic and environmentally friendly products with reference to synthetic dyes[1,2]. Also, there is growing interest to natural dyes as an alternative source for food, textile, pharmaceutical and cosmetic industry[4]. Thus, the demand for natural dyes also equally increased for these reasons.

A survey of the literature reveals that some methods have been reported for determination of dyestuffs by UPLC, HPLC-UV-Vis-ESI-MS, spectral analysis of isolated dyestuffs by NMR and MS. Also the isolated compounds were used as standard phenolic compounds for HPLC-DAD-MS systems and the extracted dye from plant was also characterized by FTIR and UV spectrophotometer[5-13]. There are some studies are to investigate the optimum dyeing conditions (dyeing bath *p*H, time, temperature and mordant variety) for wool and silk fabrics with plants' extract[14-18]. But any analysis of dyestuff has been reported for *Achillea grandifolia* as a source of natural dye. *Achillea* L. species are used for natural dyeing owing to the flavonoids. *Achillea* (Asteraceae) species have 46 species in Turkey and 115 species in the world[19]. *A. grandifolia* is a flowering plant in the family Asteraceae, into distributed in Balkan, Peninsula and Turkey.

The aim of this study was to analysis for the first time of dyestuffs from leaves, flowers and stems of *A. grandifolia*. In this study, natural dyestuffs were extracted from *A. grandifolia* and the extracted dyes were examined by HPLC-DAD and Q-TOF LC/MS systems, thus identifying and quantifying their colouring constituents. Also, colour values and colour coordinates in terms of L*, a*, b* were examined with a Konica Minolta CM 2003d colorimeter.

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Methodology

Chemicals

Rutin, quercetin, caffeic acid, luteolin, chlorogenic acid and luteolin-7-*O* glucoside were obtained from Sigma Chemical Co. Quercetagetin 3,6-dimethyl eter were kindly donated by Prof Dr Ufuk Kolak. All the other reagents were of analytical grade. High purity water was obtained by Milli-Q treatment system (Millipore, Bedford, MA, USA).

Plant collection

A. grandifolia was collected from Kocaeli, Kartepe (altitude of 1520-1625 m) province of Turkey. The plant was authenticated by Prof Ertan Tuzlacı. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy (MARE 14583), Marmara University, Istanbul, Turkey.

Dyeing procedure

2.5 mg of *A. grandifolia* flowers, leaves and stems were added into test tubes with 10 mL of distilled water for preparation of dye baths. They were heated at 100 ^{o}C for from 5 to 60 min and then liquid extracts were decanted from solids by filtration through filter paper, after that they were measured spectrophotometrically. Absorbance results showed that 30 min of extraction time was sufficient for extract of dyestuffs from each parts of the plant. Then, to determine optimum extraction temperature for dye baths, 25, 50, 75 and 100 ^{o}C were tested during 30 min extraction, individually. After that, the liquid extracts were decanted from solids by filtration through filter paper and measured spectrophotometrically. The results showed that 100 ^{o}C was required for extraction of dyestuffs from each parts of the plant. For dyeing experiments, wool fibres (about 1.0 gm) were added to dye baths in 100 ^{o}C for 30 min. Then, the fibres were dyed without mordant (direct dyeing) in this dye baths containing 2 gm each one of flowers, leaves and stems from dried plants. After that dyed fibres were washed and dried at room temperature in the shade.

Dyestuffs extraction procedure from dyed wools

0.1960 gm leaves, 0.2291 gm flowers and 0.2291 gm stems in dyed wools were weighed accurately and transferred into separate test tubes. 2 mL dimethylformamide (DMF) were added to each test tube and shake for 1 min with vortex. They were extracted in water bath for 15 min and 30 min. The extracts were cooled and filtered by 1.0/0.45 μm membrane filter and injected 20 μL to HPLC. The extraction time of dyestuffs from the dyed wools was determined by measuring the peak areas of each individual dyestuff.

HPLC–DAD analyses

A Schimadzu HPLC system (Japan) equipped with DAD was carried out for chromatographic analyses. The separation was performed using a Nova-Pak C18 analytical column (3.9 x 150 mm, 5 μm, Part No WAT 086344, Waters), studied at a flow rate of 0.5 mL/min during a gradient. The HPLC gradient elution was performed water with trifluoroacetic acid (TFA) (0.1%) (phase A) and acetonitrile with trifluoroacetic acid (0.1%) (phase B) as mobile phase[20] and the solvent gradient changed in the following pattern: 0 min, 5% B; 1 min, 5% B; 20 min, 30% B; 25 min, 60% B; 28 min, 60% B; 33 min, 95% B, 35 min, 95% B, 40 min, 5% B. All the extracts and standards were prepared by dissolving in methanol/water (2:1; v/v) solution, then the obtained solutions were filtered through 0.45 μm syringe filter and the mobile phases were degassed before injection into the HPLC. The injection volume was 20 μL and the DAD acquisition range was 190-800 nm. 350 nm was chosen for the identification of dyestuffs and different wavelengths were utilized for their quantification as described in section "quantitative analysis of *A. grandifolia* dyestuffs".

Q-TOF LC/MS analyses

The Agilent 6530 quadrupole time of flight LC-MS system with ESI ion source, United States instrument, in the negative ion mode, (model G6530B, Agilent Technologies, USA), $[M - H]^-$ ions, was operated. The same gradient elution method was used for Q-TOF-MS analyses as used in HPLC. The mass range scanned was m/z 100 to 1000. According to this, the optimum values of ESI-Q-TOF-LC/MS parameters in negative mode were: capillary voltage, 3500 V; drying gas temperature, 350 °C; drying gas flow, 8 L/min; nebulizing gas pressure, 2 bar; and end-plate offset, −500 V. The accurate mass data for the molecular ions were processed using the Mass Hunter Workstation Software Version (B.O600 Build 6.0.633.10).

Results

Dyeing procedure

The absorption spectra of the extracts were recorded at 190-800 nm against distilled water. Measurements were made at 319 nm which the

absorption is maximum. As a result of photometric results, optimum time and temperature for extraction of dyestuffs from the dye baths were obtained at boiling temperature for 30 min for all parts of the plant are shown in Figs. 1&2.

Dyestuffs extraction procedure from dyed wools

Appropriate extraction time was determined by reverse phase HPLC quantitation of six main natural dyes in dyed wools with different parts of *A. grandifolia.* Our results showed that 15 min is the best time for maximum yield of investigated dyestuffs from flower and stem and 30 min from leaf portion of the plant. The results are shown in Table 1.

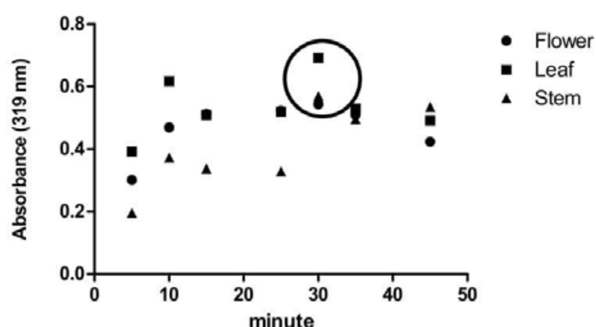

Fig. 1—Effect of time for extraction of natural dyestuffs from the dye bath.

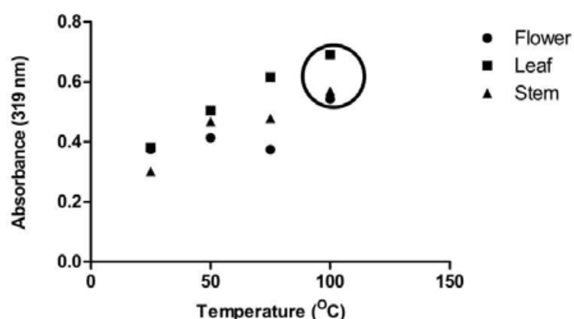

Fig. 2—Effect of temperature for extraction of natural dyestuffs from the dye bath.

Quantitative analysis of *A. grandifolia* dyestuffs

Each stock standard was chromatographed individually by injection 20 µL of 500 µg/mL. These stock solutions were further diluted to five known concentrations for all standards. Each standard solution was injected into HPLC-DAD column in triplicate. The calibration curve of each standard was obtained by plotting peak area versus the concentration of working standard solutions. HPLC-DAD spectra of each peak were recorded in the range of 200 to 800 nm where 354 nm for luteolin-7-*O* glucoside; 325 nm for chlorogenic acid; 353 nm for rutin; 323 nm for caffeic acid; 370 nm for quercetin; 347 nm for luteolin were used for quantitation of standards and the extracts. The limit of detection was calculated by $LOD=3.3\sigma/S$, where σ is the standard deviation of the intercept of the calibration curve and S is the slope of the calibration curve. The limit of quantitation was calculated as LOQ= 3xLOD. The linear range, regression equation and determination coefficient of each standard, LOD and LOQ values are shown in Table 2. Chromatographic identification of dyestuffs was based on comparing retention times with authentic standards and on-line ultraviolet absorption spectrums (Figs. 3-5).

The amount of dyestuffs in *A. grandifolia* flowers, leaves and stems were calculated according to the standards' calibration curves are shown in Table 2. As it is seen in Table 3 quantitation of dyestuffs from *A. grandifolia* are signed as mean ± standard deviation (Table 3).

Q-TOF LC/MS-MS analysis of the dyestuffs

After identification and quantification of dyestuffs, they were confirmed by LC/MS/MS with their retention time (Rt) and mass spectral characteristics (observed molecular formula and fragment ions) of all standards and the dyestuffs. Mass spectral characteristics of the dyestuffs are summarised in Table 4. The content of dicaffeoylquinic acid with its

Table 1—Effect of time on main dyestuffs yields from dyed wools with *A. grandifolia*

Dyestuffs	Flowers (Ā)		Leaves (Ā)		Stems (Ā)	
	15 min	30 min	15 min	30 min	15 min	30 min
Rutin	335991	310909	65040	74814	-	-
Luteolin-7-*O* glu	4346666	4023432	224481	241638	87112	86529
Luteolin	7345191	6389455	-	-	-	-
Quercetin	-	-	1250520	1276682	-	-
Caffeic acid	-	-	160039	172602	-	-
Chlorogenic acid	-	-	249571	697937	-	-

Ā: Mean value of peak area of three parallel measurements by HPLC-DAD method.

isomers and quercetagetin 3,6-dimethyl ether were not quantified due to the lack of standards. So, their structure was identified by MS/MS (Table 4).

The fragmentation of the $[M-H]^-$ molecular ion at *m/z* 353.0878 gave product ions at *m/z* 191 and 179 with respect to quinic through the loss of a caffeoyl and through the loss of a quinic respectively, was identified as chlorogenic acid[21]. Luteolin-7-*O* glucoside showed $[M-H]^-$ value at *m/z* 447.0933 and the product ion at *m/z* 285 representing luteolin aglycone through the absence of a glucoside moiety (447-162)[22]. Luteolin gave $[M-H]^-$ value at *m/z* 285.0405 and the product ions at *m/z* 175 [M-H-110], 151 [M-H-134] and 133 [M-H-152]. Caffeic acid showed $[M-H]^-$ value at *m/z* 179.0329. It was found that the fragment ions at *m/z* 161and 135 by losses of a H_2O molecule and a CO_2 molecule, respectively. Rutin showed $[M-H]^-$ value at *m/z* 609.2080 gave product ions at *m/z* 301 by losses rutinose. The fragmentation of the $[M-H]^-$ molecular ion at *m/z* 300.9021 gave product ions 151, 121 which was attributed to the aglycone quercetin. Quercetagetin 3,6-dimethyl ether showed $[M-H]^-$ value at *m/z* 345.0616 and gave product ions at *m/z* 330 [M-H-CH_3], 315 [M-H-CH_3-CH_3] and 287 [M-H-CH_3-CH_3-CO]. The fragmentation of the $[M-H]^-$ molecular ion at *m/z* 515.1277 and 515.1113 with fragment ions 353 (chlorogenic acid), 191, 179 was identified as dicaffeoylquinic acid[23]. Trifluoroacetic acid (TFA) was used as the mobile phase adducted to dicaffeoylquinic acid. Because the $[M-H]^-$ value was observed to be *m/z* 629.0977 (515+114).

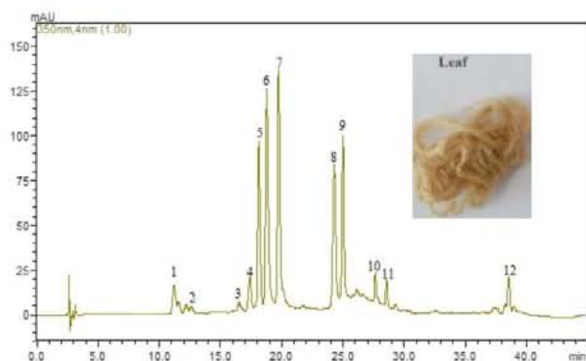

Fig. 3—HPLC-DAD chromatogram of dyestuffs extract of dyed wool with *A. grandifolia* leaves. Chlorogenic acid (1), caffeic acid (2), rutin (3), luteolin-7-*O* glucoside (4), dicaffeoylquinic acid (5), Isomer of dicaffeoylquinic acid (6), Isomer of dicaffeoy lquinic acid (7), quercetin (8), quercetagetin 3,6-dimethyl ether (9), not identified (10-12)

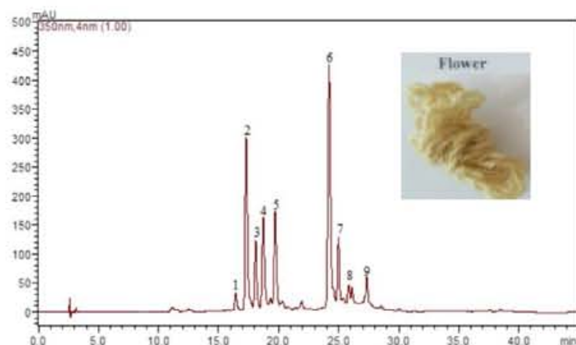

Fig. 4—HPLC-DAD chromatogram of dyestuffs extract of dyed wool with *A. grandifolia* flowers. Rutin (1), luteolin-7-*O* glucoside (2), dicaffeoylquinic acid (3), Isomer of dicaffeoylquinic acid (4), Isomer of dicaffeoylquinic acid (5), luteolin (6), quercetagetin 3,6-dimethyl ether (7), not identified (8,9).

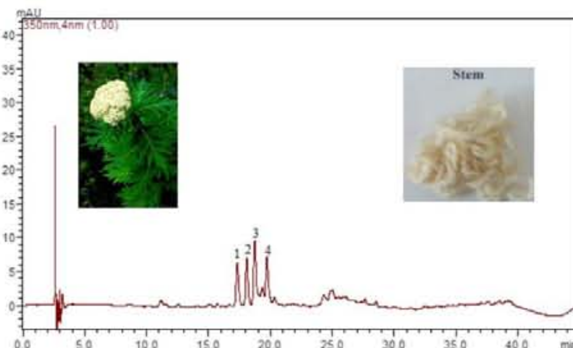

Fig. 5—HPLC-DAD chromatogram of dyestuffs extract of dyed wool with *A. grandifolia* stems. Luteolin-7-*O* glucoside (1), dicaffeoylquinic acid (2), Isomer of dicaffeoylquinic acid (3), Isomer of dicaffeoylquinic acid (4).

Table 2—Quantitative determination of six standards using HPLC-DAD.

Dyestuffs	Regression equation	R^{2a}	Linear range (µg/mL)	LOD[b] (µg/mL)	LOQ[b] (µg/mL)
Caffeic acid	y=277241x-188308	0.9994	5.0-40	1.10	3.36
Chlorogenic acid	y= 406496x+279519	0.9998	5.0-30	0.46	1.38
Rutin	y = 64641x - 5024.6	0.9966	5.0-30	2.15	6.53
Luteolin7-*O* glu	y = 17067x + 26827	0.9983	10-75	3.45	10.46
Luteolin	y= 203849x+ 2013.9	0.9986	5.0-50	2.22	6.75
Quercetin	y= 163014x+205756	0.9959	5.0-50	3.83	11.62

[a]R^2: Determination coefficient
[b]LOD/LOQ (µg/mL): Limit of detection/limit of quantitation

Table 3—Quantitation of dyestuffs in *A. grandifolia*[a]

Dyestuffs	Flower Ā	Conc. (µg/mL)	Leaf Ā	Conc. (µg/mL)	Stem Ā	Conc. (µg/mL)
Caffeic acid	-	-	172602	1.30±0.01	-	-
Chlorogenic acid	-	-	697937	2.40±0.32	-	-
Rutin	335991	5.28±0.58	74814	1.24± 0.07	-	-
Luteolin-7-*O* glu	4096666	241.61±0.79	251638	16.32±0.83	87112	6.68±0.24
Luteolin	7045191	34.56±1.04	-	-	-	-
Quercetin	-	-	1276682	9.09±0.49	-	-

[a] :Values are the mean ± standard deviation of three parallel measurements

Table 4—List of dyestuffs identified in *A. grandifolia* by LC/ESI-MS/MS

Compounds	Rt(min)[a]	λmax (nm)	$[M-H]^-$ (*m/z*)	Molecular formula	Fragmentions	Ref./Std.
Chlorogenic acid	10.99	218, 235, 325	353.0913	$C_{16}H_{18}O_9$	191, 179	Std.
Caffeic acid	11.66	217, 234, 323	179.0350	$C_9H_8O_4$	135	Std.
Rutin	16.46	255, 353	609.1875	$C_{27}H_{30}O_{16}$	301	Std.
Luteolin-7-*O* glucoside	17.02	255, 354	447.0876	$C_{21}H_{20}O_{11}$	285	Std.
Luteolin	23.60	252, 347	285.0368	$C_{15}H_{10}O_6$	133, 151, 175, 199, 217	Std.
Quercetin	24.02	254, 370	300.9021	$C_{15}H_{10}O_7$	151, 121	Std.
Dicaffeoylquinic acid I	18.19	216, 234, 327	515.1113	$C_{25}H_{24}O_{12}$	353,191, 179	Ref.[23]
Dicaffeoylquinic acid II	18.71	216, 234, 327	515.1277	$C_{25}H_{24}O_{12}$	353,191, 179	Ref. [23]
Dicaffeoylquinic acid III	19.10	216, 234, 327	629.0977	$C_{25}H_{24}O_{12}$	515, 353, 179	Ref. [23]
Quercetagetin 3,6-dimethyl ether	24.67	258, 350	345.0565	$C_{17}H_{14}O_8$	330, 315, 287	Std.

[a] Rt (min): Retention time.
Ref: Literature reference; Std: Standards

Table 5—Mean standard deviation of CIE L*a*b* values measured in *A. grandifolia* flower, leaf and stem

	CIE L*a*b* values[a]		
A. grandifolia	L*	a*	b*
Flower	72.59±0.79	1.83±0.20	37.47±0.59
Leaf	66.58±0.30	2.28±0.28	38.28±0.65
Stem	83.53±0.14	0.64±0.04	31.42±0.64

[a] Values are the mean ± standard deviation of three parallel measurements

CIELAB measurements

The colours on dyed materials were evaluated based on (L*, a*, b*) CIE LAB colour system which are given as CIELAB values in Table 5.

Discussion

Natural dyestuffs are eco-friendly, healthy, renewable and biodegradable. The rich biodiversity of Turkey has provided us raw materials for natural dyeing. According to the available literature, there is only one report on the natural dyeing potential of *A. millefolium*[24]. This study is based on the investigation of dyeing properties from *A. millefolium* by using some metal mordant. However, in our current study, we investigated the potential of natural direct dye source of *A. grandifolia* on wool fabric without using any mordant. For this purpose, the optimization of dye baths temperature and time was carried out with *A. grandifolia* flowers, leaves and stems individually. Since, we used distilled water for the dye bath preparation, it is simple, economical and readily available method. Additionally, the analysis of dyestuffs were performed and ten dyestuffs in the DMF extracts of dyed wool with *A. grandifolia* were identified for the first time by HPLC-DAD and confirmed with ESI-Q-TOF LC/MS methods. Hence, this study is an opportunity to reveal aspects of use *A. grandifolia* as a natural direct dye source and this plant could be used as an alternative dye source for food, textile, pharmaceutical and cosmetic industry.

Conclusion

This study was carried out in order to analyse of natural dyestuffs in *A. grandifolia*. The dyestuffs in the dyed wools with *A. grandifolia* were analysed by ESI-Q-TOF LC/MS and HPLC-DAD. The HPLC studies showed that an optimal and sufficient extraction time of dyestuffs from dyed wools with flower and stem was 15 min and with leaf was 30 min. The dyestuffs in flower were identified as luteolin, quercetagetin 3,6-dimethyl ether, rutin and luteolin-7-*O* glucoside as flavonoids, chlorogenic acid, caffeic acid and dicaffeoylquinic acids as phenolic acids; in leaf were identified as quercetagetin 3,6-dimethyl ether, quercetin, rutin, caffeic acid,

chlorogenic acid, dicaffeoylquinic acids and finally in stem were identified as chlorogenic acid, luteolin-7-*O* glucoside and dicaffeoylquinic acids. Hence, ten dyestuffs were positively identified by the LC-MS/MS and six main dyestuffs were quantified by the HPLC-DAD. Also, the process of extraction dyestuffs from *A. grandifolia* is environmentally friendly and it is concluded that *A. grandifolia* can be used as a natural direct dye source for wool dyeing. Furthermore, this study provides preliminary information aimed at a more detailed investigation for using of *A. grandifolia* in the other industries such as pharmaceutical and cosmetic industry as a natural dye source.

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