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Isolation of Colour Components from *Rubia tinctorum* L.: Chromatographic Determination, Spectrophotometric Investigation, Dyeing Properties and Antimicrobial Activity

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In this paper, a sensitive quantification high performance liquid chromatographic method for analysis of alizarin in madder root (*Rubia tinctorum* L.) obtained from South of Anatolia,Turkey is reported. The alizarin is separated on Zorbax SB C_{18} column with a water-acetonitrile gradient as eluent and measured with UV detection at 250 nm. With this method the aglycone alizarin can be analyzed. Regression equation that obtained from the calibration curve, revealed a linear relationship ($r^2 = 0.9981$) between the mass of alizarin injected and the peak area. After, the colour components responsible for dyeing were determinated and its chemical constituents were established based on chemical and spectroscopic investigations. Afterwards, the wool fabrics have been dyed with combined mordanting and mordantless techniques. Fastness to light, washing and rubbing of the dyed fabrics were measured and discussed. Additionally, extracts (ethanolic and aqueous) of *R. tinctorum* L. root and dyed materials were investigated for their antimicrobial activities against eight pathogens (*Aeromanas hydrophila, Bacillus megaterium, Corynebacterium xenosis, Pseudomonas aeruginosa, Micrococcus luteus, Enterococcus faecalis, Stapylococcus aureus* and *Escherichia coli*). The extracts and dyed materials were not effective against the growth of *Escherichia coli*. The fabric dyed, however, showed less antimicrobial activity, as uptake of this dye in textile material is below minimum inhibitory concentration (MIC).

Key Words: *Rubia tinctorum* L., HPLC, Natural dye, Mordant, Biological activity.

INTRODUCTION

The natural dyes are generally environmental friendly and have many advantages over synthetic dyes with respect to the production and application[1]. In recent years, there has been an interest manifested towards the application

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of these dyes due to their bio-degradability and higher compatibility with the environment[2,3]. In the last decade, investigations about possible use of natural dyes in textile dyeing processes have been performed by various research groups. The dyeing of cotton and jute with tea as a natural dye using alum, copper sulfate, or ferrous sulfate mordants has been studied by Deo and Desai[4]. Bhattacharya *et al.*[5] investigated the properties of selected natural dyes on jute. Nishida and Kobayashi[6] reported properties of natural dyes on silk, cotton and cashimilon using alum or ferrous sulfate mordants. Brückner *et al.*[7] investigated the colour depth and fastness properties of selected natural dyes on wool and on synthetic fibers, *e.g.*, polyester, polyamide and polyacrylonitrile. Lokhande and Dorugade[8] presented results with selected natural dyes on polyamide using various mordants, *e.g.*, alum, ferrous sulfate, stannous chloride and tannic acid. The dyeing procedures are mainly two-bath dyeings including a separated mordanting step, so such processes are rather difficult to be handled in a modern dyehouse. In the study of Brückner[7], the final conclusion focuses on the unsatisfying fastness properties of the natural dyes, which must be understood as an indicator for a distinct need for research to overcome these problems. In the application of the dyes, different techniques of mordanting and post-treatment were used to improve colour fastness properties[3-6,9]. As a result, a broad set of variations in the dyeing recipes is given in the literatures and an optimization of the dyeing conditions with regard to the type of natural dye is quite common.

Turkey has a rich flora due to its geographical position and climate. It is well known that plant based dyes were widely used by the Turks in both Central Asia and Anatolia throughout the history. The plant based dyeing is very popular all over Anatolia and people grow dye plants for this purpose. Among these plants are walnut, sumac, valonia oak, pomagranade, indigo grass, euphorbia, onion, corn poppy, dyer's rocket, madder and daisy[10].

Rubia tinctorum L. (*Rubiaceae*) is widely distributed in Southern and Southeastern Europe, Mediterranean region and in Central Asia[10]. Its reddish roots (madder roots) contain various secondary metabolites, mainly hydroxyanthraquinones and these compounds are used for the dyeing of textiles and in the treatment of kidney and bladder stones. There are many reports on the genotoxicity of this plant with or without the addition of a metabolic-activating system[11-15]. *R. tinctorum* L. produces various kinds of anthraquinones colourant, such as purpurin, xanthopurpurin and alizarin, the latter being the most abundant of them[16]. Anthraquinones are metabolites of commercial and pharmacological interest and their biosynthesis in elicited cultures would represent an interesting approach due to lower cost than synthetic production, renewed interest in natural dyes and environmental concerns.

The madder extract is hydrolyzed and the identify of alizarin is characterized in different regions and different researcher groups. Kino-oka *et al.*[17] investigated the production and release of anthraquinone pigments by hairy roots of madder (*R. tinctorum* L.) under improved culture conditions. Krizsan *et al.*[18] developed a sensitive and reproducible RP-HPLC method of anthraquinone derivatives in *R. tinctorum* L. and its cell cultures. Angelini *et al.*[19] offered for HPLC determination of alizarin using RP C_{18} LiChroCART (250 × 4 mm, 5 mm, Merck) column and methanol 10 % aqueous acetic acid (60:40) as solvent. They have dyed cotton, wool and silk yarns using *R. tinctorum* L. extract belong to different areas. But, the experimental conditions for HPLC, require a lot of reaction steps. In addition, the author did not used any mordant techniques in dyeing procedures. Goverdina *et al.*[20] applied Waters 600E HPLC-systems for madder root extracts with water and acetonitrile gradient systems. Goverdina *et al.*[21] carried out for the separation of some anthraquinones in *R. tinctorum* L. using liquid chromatography technique. Bechtold *et al.*[22] presented some dyeing procedures with 50 different types of natural dyes. However, authors did not advice any mechanisms between natural dye and cellulosic material. Balakina *et al.*[23] investigated of the red dye obtained from an ancient Pazyryk textile. Manojlovic *et al.*[24] reported antifungal activity of *R. tinctorum* L. extract and standard alizarin against some bacterias.

Alizarin (1,2-dihydroxy-9,10-anthraquinone) is a natural dye that acts as an acid-base indicator and its chemical structure has been given in Fig. 1. It is one of the oldest dyestuffs known to human beings[3]. Alizarin has, for centuries, been obtained from a variety of plant sources such as *Rubia alkane* L. (tropical species, Africa, Asia, East Indies, South America), *Rubia cordifolia* L. (Indian Madder) (Far East, India, China, Korea) and *Rubia yunnanensis* L. (Franch. ex Diels) (Europe)[25].

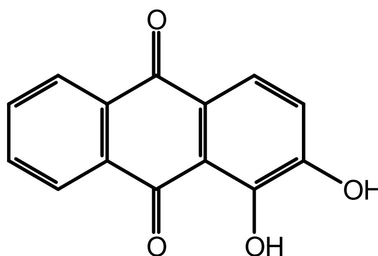

Fig. 1. Chemical structure of alizarin

The objectives of present study were to: (1) a sensitive quantification method for analyzing the alizarin of madder root (*Rubia tinctorum* L.) from Turkey, based on HPLC method according to literature[20] but, using different

colon systems. This colon systems, it is possible to inject the whole plant extract and so determine alizarin content alone in the extract more shorter retention time. This method has been developed and validated as prescribed in good analytical practice[26], (2) extract colour component from this plant, (3) evaluate the chemical constituent of the colour component, (4) examine the dyeing characteristics on wool, (5) give the mechanisms between wool and dye component and (6) antimicrobial activity of colour component and dyeing materials.

EXPERIMENTAL

Standard alizarin was obtained from Sigma. All organic solvents were of HPLC grade from Merck or Sigma. Mordants ($KCr(SO_4)_2 \cdot 12H_2O$, $Al_2(SO_4)_3 \cdot 18H_2O$, $CoSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$) were purchased from Fluka.

Elemental analyses (C, H, N) were performed by the TÜBITAK Instrumental Analysis Laboratory (Ankara, Turkey) using a Carlo Erber 1106 elemental analyzer. Infrared spectra were obtained using KBr discs (4000-400 cm^{-1}) on a Shimadzu 8300 FT-IR spectrophotometer. ^1H NMR spectra were taken on a Bruker Avance DPX-400 NMR instrument. TMS was used as internal standard and deuterated dimethyl sulfoxide as solvent. Mass spectra were recorded on an Agilent 1100 MSD LC/MS spectrometer. The HPLC measurements were carried out on a Agilent (HP) 1100, a crystal 250 diode-array UV-Vis detector and a Rheodyne Model 7125 injector with a 5 μL sample loop. Zorbax SB C_{18} analytical column (Merck, 5 μm, 4.6 mm × 250 mm, specific surface area of 500 m^2/g, a specific pore volume of 0.95 mL/g and a pore diameter of 8 nm) was used. Chromatography was carried out using two solvents: Double-distilled water (A); acetonitrile (B) in a linear gradient programme (Table-1). The electronic spectra were recorded on a Shimadzu 160 A spectrophotometer. Mettler Toledo MP 220 pH meter was used for the pH measurements. Analytical balance was used for determining the total dyestuff. All dyeing were done in an dyeing apparatus. The light fastness was determined using artificial illumination with Xenon

TABLE-1
GRADIENT TABLE FOR HPLC ANALYSIS

Time (min)	Solvent A (%)*	Solvent B (%)**
Initial	70	30
6	70	30
20	30	70
35	30	70
40	70	30
45	70	30

*Solvent A = Double-distilled water, ** Solvent B = Acetonitrile.

are light according to DIN EN ISO 105 B02 (Xenotest 150F, TÜBITAK, Bursa, Turkey) and was related to the standard grey scale. Rubbing and washing fastness were determined using standard methods of the ISO 105-X12 (Gyrowash) and ISO 105 CO6 A2S (Crockmeter) (Textile Factories of Matesa and Kipas, Kahramanmaras, Turkey), respectively.

Sample preparation for HPLC: Sample preparation for HPLC has been carried out according to literature[20]. Dried and powdered two year-old root material of *Rubia tinctorum* L. (2.5 g) that picked up Belen, Hatay, Turkey in September 2003 was refluxed with 100 mL water-ethanol mixture (75:25, v/v). After 6 h 200 µL of the extract was filtered over a Büchner filter. 100 µL of the filtered extract was diluted with 900 µL water-methanol (1:1) and analyzed by HPLC.

Method validation

Calibration curve: The stock solution (1 mg/mL) of standard alizarin was freshly prepared in a mixture of water-ethanol (75:25, v/v) and desired concentrations were obtained by serial dilution for standard curve preparation. The calibration graphs (Fig. 2) were plotted after linear regression of the peak area *versus* concentration and both detection limits (LODs) and quantitation limits (LOQs) were measured following the standard methods[26,27].

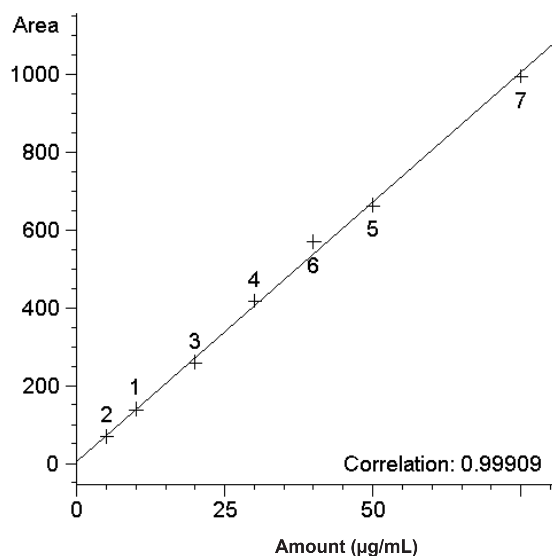

Fig. 2. Calibration curve of alizarin

Repeatability: The precision of the chromatographic determination for the proposed method, expressed as a relative standard deviation (RSD), was calculated by five replicate injections of compound (intra-day and

inter-day). The standard solutions used for repeatability experiments were the same as used in the calibration curve experiment.

Recovery: For percent recovery, three sets of water-ethanol mixture extract of *Rubia tinctorum* L. were prepared from one of the location (1 mg/mL). Three different concentrations of standard alizarin (25, 50 and 100 µg/mL) were prepared. The sample, to which standard was added, was pretreated and analyzed using the developed HPLC method. 20 µL of concentration was injected into the HPLC for measuring the percentage recovery.

Extraction technique adopted for isolation of colour component: The colour component isolated from roots of *Rubia tinctorum* L. showed positive Borntregar reaction, a characteristic for an anthraquinone[28]. Dried and powdered two year-old root material of *Rubia tinctorum* L. (500 g) was refluxed with 1000 mL water-ethanol mixture (750:250, v/v) for 6 h in a conical flask (2000 mL). The extract was collected, fresh distilled water was added and the same procedure was repeated until the colour in the extract was negligible. The total extract was then concentrated under reduced pressure over a water bath to get a solid mass. The solid mass was subjected to extraction with 70:30 (v/v) ethanol:water mixture and filtered. The filtrate was evaporated under reduced pressure to get a concentrated mass of the colour component. The concentrated colour mass (55 g) obtained from stems of *Rubia tinctorum* L. roots was subsequently extracted successively with chloroform and were chromatographed over an open column of silica gel (60-120 mesh)[29]. The colour component was obtained as red-purple crystals, m.p. 277-278 °C. UV λ_{max} (MeOH) nm: 244, 259, 271, 330, 433; IR (KBr, ν_{max}, cm^{-1}): 3339 (-OH), 1661, 1625 (-C=O), 1578 (aromatic -C=C-); ^{1}H NMR δ (DMSO-d_6): 7.18 (1H, d, *J* = 8 Hz, H-3), 7.70 (1H, d, *J* = 8.5 Hz, H-4), 7.79-7.80 (2H, m, H-6 and H-7), 8.20-8.33 (2H, m, H-5 and H-8); MS: m/z 240 M^+, 100 %. Anal for $C_{14}H_8O_4$: Found (%) C 69.95, H 3.33, Calcd. (%) C 70.00 and H 3.36. These results showed the compound to be Alizarin[30].

Determination of absorbance and colour strength measurements: Dye solutions of 1-5 % for the extracted dye was prepared and definite amount was taken in the dye bath by maintaining M:L ratio at 1:10. The absorbance of the dye solutions was recorded before and after dyeing on an UV/Vis spectrophotometer. An average of 5 measurements at the concentration was recorded. The amount of dye absorbed was calculated by using the relation[31]:

$$\%\ \text{Dye absorbance} = \frac{\text{Absorbance before dyeing} - \text{Absorbance after dyeing}}{\text{Absorbance before dyeing}} \times 100$$

The colour strength (K/S) values of the dye solutions as well as the dyed samples were evaluated by light reflectance technique and the values were assessed using the Kubelka-Munk equation[32]

$$K/S = (1 - R)^2/2R$$

where R = the observed reflectance; K = the absorption coefficient and S = the light scattering coefficient.

Statistical analyses: The absorbance (%) and K/S values of dye were analyzed by analysis of variance and treatment was replicated three times in a completely randomized design. Treatment means were separated by a F test[33]. The standard error of the difference (SEd±) was calculated by $SEd(\pm) = \sqrt{(2 \cdot \text{error mean square/number of replication})}$. The critical difference (CD) was calculated to test the differences among the treatments as $CD\ (5\ \%) = SEd(\pm) \cdot t$, where t = 5 % tabulated value of 't' at error degree of freedom.

Dyeing of wool fabrics

Dyeing without mordant: The wool fabrics (543 g/m^2) were dyed in a dye bath containing 3 % of *Rubia tinctorum* L. The dyeing was carried out at 97-98 °C for 1 h, 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[34]. Mordanting was carried out for 0.5 h at 97-98 °C. The dyed fabric was removed, rinsed with distilled water and air-dried.

Measurement of fastness properties: Colourfastness tests to light, washing and crocking or rubbing was carried out in a Xenotest Alpha, Gyrowash and Crockmeter, respectively. The fastness ratings were given in grey scales[35].

Test organisms: The growth inhibitory activity of the chemical matter was tested against eight bacteria (*Aeromanas hydrophila* A199, *Bacillus megaterium* DSM 32, *Corynebacterium xenosis*, *Pseudomonas aeruginosa*, *Micrococcus luteus* LA 2971, *Enterococcus faecalis* ATTC 15753, *Stapylococcus aureus* and *Escherichia coli*). These microorganisms were provided from Microbiology Laboratory Culture Collection, Department of Biology, Kahramanmaras Sütçü Imam University, Turkey.

Antimicrobial screening test: Antimicrobial screening tests have carried out according to previous studies[36-38]. Nutrient agar medium (g/L: peptone 5.0; beef extract 1.5; yeast extract 1.5; NaCl 5.0; agar 20; pH 7.5) was prepared and autoclaved at 121 °C for 20 min. Sterilized petriplates were prepared with an equal thickness of nutrient agar. Test organisms were grown overnight at 37 °C, 120 rpm in 10 mL nutrient broth. This broth was used for seeding the agar plates. 10 μg of each extract was impregnated onto a small disc of filter paper (diameter 5.0 mm) and placed on top of the seeded medium. After overnight incubation at 37 °C, the zones of inhibition

were measured. In the second set of experiments, concentration of dye impregnated (5-40 μg) onto a disc of filter paper was varied to study its effect on the growth of microbes and MIC of dye. The antimicrobial efficacy of a compound will vary when it is present in solution and when it is held intimately by a textile substrate. In the next set of experiments the antimicrobial activity of dyed wool specimens was tested. The 1-4 $inch^2$ dyed fabrics were introduced in the 100 mL nutrient broth inoculated with the desired microbe and incubated at 37 °C overnight (16 h). The reduction of bacterial growth by dye was expressed as follows:

$$R = \frac{B - A}{A} \times 100$$

where R = % reduction in bacterial population; B = absorbance (558 nm) of the media inoculated with microbe and undyed fabric; A = absorbance (558 nm) of the media inoculated with microbe and dyed fabric.

RESULTS AND DISCUSSION

HPLC quantitative determination for a large number of hydroxyanthraquinone derivatives from *Rubia tinctorum* L. is well known[17-21]; however, present studies have two advantages than the other studies. Firstly, other columns (C_{18} silica column[18], I.D. column containing ODS Hypersil 5[39], RP C_{18} Nova-pak™[40], C_{18}-RP column[20] and Alltima end-capped C_{18} column[21]) are more expensive than Zorbax SB C_{18} column. Secondly, while the retention time (t_r) is between 20-25 min in other studies, is found to be 6.5 min in present study. This result does due to the difference of the using colon and show that study will be finished in shorter time as practically. In addition, quantitative determination have not been carry out in *Rubia tinctorum* L. of Turkey. Therefore, the present study was aimed at developing a simple and reliable HPLC method for the chromatographic determination of alizarin in *Rubia tinctorum* L. from Southern Turkey. It was well seperated with a Zorbax C_{18} column and eluted within 6.5 min. A liquid chromatogram and UV-Vis spectra of water-ethanol mixture extract and standard alizarin are shown in Figs. 3 and 4, respectively. Linear calibration graphs, based on the peak area with good correlation (Table-2) were obtained for alizarin. The highest sensitivity was obtained by monitoring at 250 nm, the wavelength where an alizarin shows its maximum absorbance.

TABLE-2
PARAMETERS OF THE LINEARITY, DETECTION LIMIT AND QUANTITATION LIMIT FOR ALIZARIN

Compd.	Linearity range (μg/mL)	Linear equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
Alizarin	10-150	$y = 1334x + 4.63$	0.9981	0.71	0.97

r^2 = Correlation coefficient.

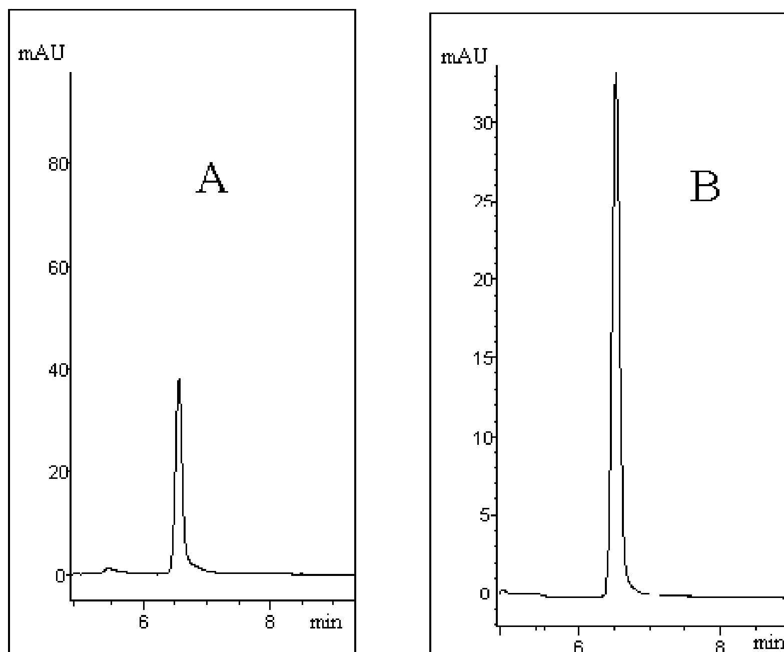

Fig. 3. HPLC chromatogram of *Rubia tinctorum* L. extract (A) and standard alizarin (B)

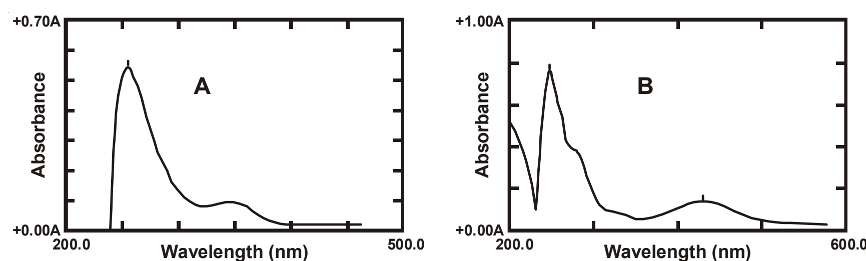

Fig. 4. UV-Vis spectra of *Rubia tinctorum* L. extract (A) and standard alizarin (B)

The linearity, limit of detection (LOD) and limit of quantitation (LOQ) for alizarin was investigated and the results are presented 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. A good linearity was found 0.9980 for alizarin. Detection limit is the lowest amount of the analyte 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 LOD for alizarin was 0.71 μg/mL. The LOQ, which is defined as the lowest concentration that can be determined with acceptable accuracy and precision, can be established at a signal to noise ratio of 10. The LOQ for alizarin was experimentally verified by eight injections and was found 0.97 μg/mL. The instrument precision was measured by performing the intra-day and inter-day experiments by five replicate injections of alizarin in three different working concentrations. The intra-day and inter-day RSDs of chromatographic determination were observed in the range of 2.01-3.16 and 2.51-4.10 %, respectively (Table-3). The results showed good precision of the method. Quantitative results are expressed in terms of recovery percentage. The recoveries accomplished for alizarin was in the range of 100.11-100.66 % (Table-4).

TABLE-3
REPEATABILITY OF INTRA-DAY AND INTER-DAY ANALYSIS

Compound	Concentration (μL/mL)	RSD (%)	
		Intra-day (n = 5)	Inter-day (n = 5)
Alizarin	100	2.30	2.98
	50	2.01	2.51
	25	3.16	4.10

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 (%)
Alizarin	10.0	60	70.34	100.49
		80	90.59	100.66
		100	110.12	100.11

Alizarin in the water-ethanol mixture extract of the *Rubia tinctorum* L. was quantitated using developed HPLC method. The calculated percentage of alizarin in *Rubia tinctorum* L. that picked up Belen was 0.93 %.

Effect of concentration of dye on absorption and colour strength: The absorption of dye (%) increased with increasing in concentration and reached a maximum at 1 % concentration for *Rubia tinctorum* L. Similarly, K/S values also increased with an increase in the dye concentration (Table-5). Though the K/S values continued to increase, maximum absorption (45.350 % for *Rubia tinctorum* L.) was observed at 1 %. Therefore, this concentration of the dye in the dye bath might be taken as optimal.

TABLE-5
ABSORPTION (%) AND K/S VALUES OF DIFFERENT CONCENTRATIONS OF THE NATURAL DYE

Plant	Wavelength	Dye concentration (%)	Absorbance		Absorption (%)	K/S
			Before dyeing	After dyeing		
Rubia tinctorum L.	403	1	0.1870	0.159	14.970	12.010
		2	0.2030	0.167	17.730	14.230
		3	0.2850	0.219	23.160	18.580
		4	0.3130	0.223	28.750	23.070
		5	0.4390	0.299	31.890	25.580
SEd(±)			0.1010	0.056	0.071	0.057
CD (5 %)			0.2330	0.129	0.164	0.132

Effect of mordanting conditions: From initial experiments, it was observed that the combined mordant and mordantless technique imparted better fastness properties to the fibres compared to post- and simultaneous mordanting techniques. Therefore, by adopting the tohether 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[28]. In the dyeing of wool intermolecular hydrogen bonding occurs between the alizarin and the amino group of wool (Fig. 5). The metal ions coordinated to the $-NH_2$, -COOH or -CO-NH- sites of the protein and donor groups of alizarin (Fig. 6). Addition of mordant leads to obtaining dye-lake on the fibre. This treatment of mordants have a great influence on the wool colour. The treatment of natural dyes with metal ions (*e.g.* Cr^{3+}, Cu^{2+}, Fe^{3+}) can improve light fastness and wash fastness properties[41,42]. These properties in most cases are accompanied by a bathocromic shift of the colour of the dye. In this regard, the dyes often form ligand-metal complexes that are less soluble in water than the free ligands, which contributes to the observed improvement in wash fastness.

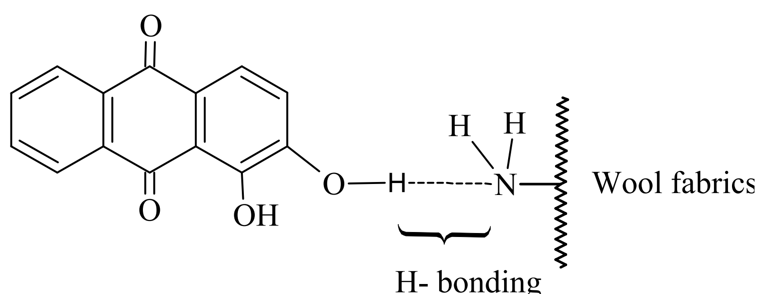

Fig. 5. Proposed H-bonding with alizarin

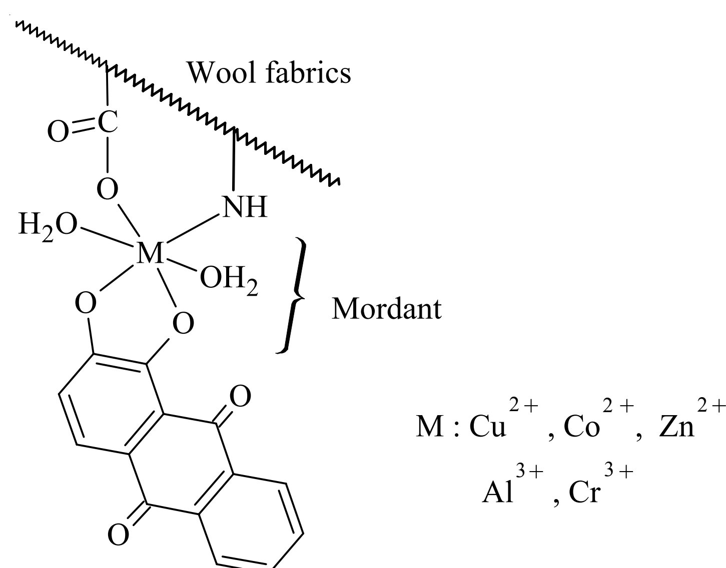

Fig. 6. Intractions of the metal ions with alizarin

The fastness tests and colour codes[43] were applied to all mordanted and un-mordanted wool (Table-6). Dark colours were obtained at pH 5-7; pale colours were obtained at pH 4 and 3. 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 good results for the light and rubbing fastness, but Al^{3+} and Cr^{3+} exhibited poor light fastness.

TABLE-6
COLOUR CODES AND FASTNESS PROPERTIES OF DYED FABRICS

Mordant	pH	Bath temp. (ºC)	Dyeing period (min)	Colour code	Light	Croc dye	King wet	Wash
Mordantless	4.3	97	60	$Y_{60}M_{90}C_{60}$	2/3	3	3	3
$KCr(SO_4)_2\cdot 12H_2O$	5.7	97	60	$Y_{70}M_{70}C_{50}$	3	4	4	4
$Al_2(SO_4)_3\cdot 18H_2O$	6.0	98	60	$Y_{60}M_{50}C_{00}$	3	4	3	4
$CoSO_4\cdot 7H_2O$	3.0	98	60	$Y_{80}M_{80}C_{70}$	4	3/4	4	5
$CuSO_4\cdot 5H_2O$	4.0	97	60	$Y_{50}M_{99}C_{70}$	5	5	4/5	5
$ZnSO_4\cdot 7H_2O$	6.4	98	60	$Y_{40}M_{50}C_{00}$	5	5	5	5

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

The mordant activity of metal ions followed the sequences Cu(II) → Co(II) → Zn(II) → Al(III) → Cr(III) in wool for *Rubia tinctorum* L. The colour 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 $CuSO_4 \cdot 5H_2O$ and $CoSO_4 \cdot 7H_2O$, which implied that the absorption of colour 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.

Antimicrobial activity of natural dye in solution[44]: Aqua and ethanolic solutions of colour component were screened for its antimicrobial activity against selected microbes. They were effective against all microbes tested except *Escherichia coli*. The effect of concentration of extracts on antimicrobial activity were studied further and results are summarized in Table-7. The zone of inhibition (diameter) was recorded in each case. It was observed that increase in dye concentration leads to increased inhibition reflected by enhancement in diameter. It may be concluded that the extracts are highly effective antimicrobial agents as the MIC for most of these lies in region of 5-40 μg in two solutions. The extracts of *R. tinctorium* L. in ethanol and aqua have much higher resistance against the *Aeromonas hydrophila* (21-16 mm inhibition zones). It is also clear from the results that ethanol extract has an antibacterial effect against, *Bacillus megaterium, Corynebacterium xerosis, Micrococcus luteus, Pseudomonas aeruginosa, Stapylococcus aureus* and *Enterococcus faecalis* showing an inhibition zones of 16-22 mm. The aqua extract inhibited the growth of *Bacillus megaterium, Corynebacterium xerosis, Micrococcus luteus, Pseudomonas aeruginosa, Stapylococcus aureus* and *Enterococcus faecalis* tested with zones of inhibition between 12-20 mm. In comparasion with the inhibition zones of reference antibiotics, the ethanol and aqua extracts of *R. tinctorium* L. are usually somewhat higher. The differences observed can arise from the differences observed in the chemical composition of the solvents.

Antimicrobial activity of natural dyes on substrate: Since aqua and ethanolic solutions of colour component showed good antimicrobial activity against selected microbes. The wool samples dyed with combined mordant and mordantless technique. As seen in the antimicrobial results of the aqua extract and dyed materials, a reduction of 10-15 % in bacterial growth is seen on a wool sample dyed with $KCr(SO_4)_2 \cdot 12H_2O$ and a reduction of 15-25 % on wool samples dyed with $CuSO_4 \cdot 5H_2O$ against all bacteria. An interesting observation is that $CuSO_4 \cdot 5H_2O$, which exhibit high bactericidal activity in solution found to be bacteriostatic when dyed onto wool fibre. This may be because the concentration of dye (5.3 % owf) on fabric samples is not sufficient enough for bactericidal activity. Even a 9.2 % owf $KCr(SO_4)_2$ mordant is not sufficiently effective. Although both medium dyes are anthraquinone (alizarin) based, their antimicrobial activity differs greatly. This is an interesting finding and requires more in-depth investigation into the

TABLE-7
ANTIMICROBIAL ACTIVITIES OF EXTRACTS OR WOOL SAMPLES

Extracts or wool samples	Conc. (µg)[a] or wool amount ($inch^2$)[b]	Zone of inhibition (diameter in cm)							
		AA	BB	CC	DD	EE	FF	GG	HH
Ethanol	5	[c]5	4	-	2	6	3	3	-
	10	9	8	3	4	9	6	7	-
	20	16	14	7	11	15	11	12	-
	40	30	28	12	18	30	25	20	-
Aqua	5	6	5	3	3	4	7	4	-
	10	10	11	5	9	10	12	9	-
	20	21	19	12	18	20	19	19	-
	40	40	36	20	38	40	40	40	-
Mordantless	1	2	3	1	1	3	5	2	
	2	4	8	2	6	5	7	6	
	3	10	15	9	15	12	18	15	
	4	20	29	15	30	25	30	30	
$KCr(SO_4)_2.12H_2O$	1	5	3	2	1	5	2	3	-
	2	9	7	3	4	8	5	6	-
	3	19	12	6	10	13	10	11	-
	4	25	25	11	16	25	23	17	-
$Al_2(SO_4)_3.18H_2O$	1	3	2	1	1	4	2	3	-
	2	7	6	2	2	7	4	4	-
	3	16	10	5	8	11	9	10	-
	4	20	23	9	14	21	21	16	-
$CoSO_4.7H_2O$	1	3	4	2	2	3	5	3	-
	2	6	7	3	6	6	10	6	-
	3	12	14	10	14	15	14	14	-
	4	25	30	15	28	30	28	29	-
$CuSO_4.5H_2O$	1	4	4	2	2	3	6	3	-
	2	7	7	4	7	8	11	7	-
	3	12	15	11	16	19	17	15	-
	4	26	30	17	30	32	33	30	-
$ZnSO_4.7H_2O$	1	3	4	2	2	3	5	3	-
	2	7	7	3	6	6	10	6	-
	3	12	14	10	16	15	14	17	-
	4	26	29	16	28	30	30	29	-
Standard[d] A-10	10	17	20	15	10	16	14	12	10
Control (ethanol)		[e]-	-	-	-	-	-	-	-
Control (aqua)		-	-	-	-	-	-	-	-

AA = *A. hydrophila*; BB = *B. megaterium*; CC = *C. xerosis*; DD = *P. aeruginosa*; EE = *M. luteus*; FF = *E. faecalis*; GG = *S. aureus*; HH = *E. coli*.
[a]Extracts; [b]Dyed wool; [c]Inhibition zone, mm ; [d]A-10; Ampicillin 10 mcg;
[e](-); No inhibition zone

effect of dye-mordant structure on antimicrobial property. It is obvious that antimicrobial properties are closely related to the dye-mordant structure.

Conclusion

Rubia tinctorum L. was found to have good agronomic source as a dye material in the South of Turkey. According to HPLC analysis, one gram root contains 9.34 mg alizarin content. All dyed samples showed good beter fastness properties. Extracts (aqua and ethanol) and dyed material have good antimicrobial activity against common pathogenic bacterias. These textiles dyed with these natural dyes can be very useful in developing clothing for infants, elderly and infirm people to protect them against common infections. They will be equally useful in bed linen, carpets and other home textiles, which are major propagators of common infections.

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