

Chromatographic Analysis of Natural Pigments Produced from *Datisca cannabina* L. and *Quercus infectoria* Oliv. Plants and their Antimicrobial Activity

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Summary: In this study, natural pigments from the hemp (*Datisca cannabina* L.) and dyer's oak (*Quercus infectoria* Oliv.) dye plants were prepared by using KAl(SO₄)₂·12H₂O (alum) mordant. A reversed-phase high performance liquid chromatography (RP-HPLC) with diode array detection (DAD) method was used in the identification of dyes in the natural pigments. The dye extractions from the natural pigments were carried out with 37% HCl/MeOH/H₂O (2:1:1 v/v/v) mixture. Also, antimicrobial activity of crude extracts of plants and pigments were investigated.

Keywords: natural pigment; hemp; dyer's oak; antimicrobial activity; RP-HPLC.

Introduction

The hemp (*D. cannabina* L.) is known as "gence" in Turkey. It is a robust, glabrous perennial plant that grows up to 1-2 m high in the Black Sea Region, West and South Anatolia [1-3]. The plant is very rich with flavonols; datiscetin, which is present in the form of a rutinoside and datiscin (amounting to 10% of the weight of the fresh leaves). Kaempferol, quercetin and galangin also exist [3]. Traditionally the plant showed bitter, diuretic, expectorant and purgative effects [4]. Experiments about Datisdirin which is isolated from *D. cannabina* showed activity against the ureases enzyme [5]. The dyer's oak or gallnut (*Q. infectoria* Oliv.) is a semi-deciduous small tree or a shrub that grows to 12 m height, distributed in Asia Minor and the Mediterranean countries as well as Southeastern Europe. The gallnuts contain as much as 50-70% of gallotannins [3]. *Q. infectoria* is used traditionally as astringent and styptic agents [4]. Scientific reports showed its astringent [6], antiparkinsonian, antitremorine [7], antidiabetic [8] and antioxidant [9] activity.

High performance liquid chromatography (HPLC) coupled to diode-array detection (DAD) is ideally suited for identification of natural dyes including organic pigments present in these materials [10-13]. Dyes obtained from plants can often inhibit

the growth of microorganisms; it is believed that these dyes can cause less allergic reaction than the chemically synthesized dyes [14,15]. The antimicrobial activity of the dyes can be due to the existence of phenol, tannin, gallic acid, ellagic acid, datiscetin etc. which are synthesized as secondary metabolites used in the growth, development and defence of plants. Therefore, substances which are able to inhibit or destroy pathogens without toxic impact on the host cells are obvious candidates for use in the development of new antimicrobial agents [16].

The aim of the present study is to use RP-HPLC-DAD to identify dyes found in the natural pigments prepared from hemp (*D. cannabina*) and dyer's oak (*Q. infectoria*) dye plants and to evaluate antimicrobial properties of these dyes against some bacterial and fungal strains.

Results and Discussion

HPLC Analysis

In the present study, the complexes formed by adding aluminium (III) solutions to hemp (*D. cannabina*) and dyer's oak (*Q. infectoria*) extracts which were obtained as natural pigments. The

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identification of dyes present in the natural pigments was analyzed qualitatively by a reversed phase high performance liquid chromatography (RP-HPLC) with diode array detection (DAD). The standard dyes used in the present study, gallic acid, ellagic acid and datiscetin, were also chromatographically and spectrophotometrically (UV-Vis) characterized. Absorbance maxima (nm) and retention times (min) related to datiscetin-3-*O*-[rhamnosyl(1-6)glucoside] dye were evaluated according to literature procedures [17] because the corresponding standard dyes were not exactly available. Absorbance maxima, which correspond to the one hemp and two dyer's oak component, appear to be similar and in good agreement with the spectral characteristics of gallic acid, ellagic acid and datiscetin, which are the main coloring components of the hemp and dyer's oak that can be found in the literatures [2,3]. Table-1 provides the results of HPLC-DAD analysis of the sample extracts, including retention times and corresponding absorbance maxima. The detection wavelength was selected according to the chemical nature of peaks present. In general, animal dyes were best analysed at 275 nm, whereas 255 nm was the optimal detection wavelength for vegetal mordant dyes and 288 nm for indigoids [18]. As shown in Fig. 1 (c), the main component (peak A) in the chromatogram of the non-hydrolysed hemp extract, was determined as datiscetin-3-*O*-[rhamnosyl(1-6)glucoside] [17]. In the chromatogram related to the same extract, peak B was identified as datiscetin. Additionally, peak B was also identified as datiscetin component in the chromatogram of the acid hydrolysed hemp extract related to Fig. 1 (d). As shown in Fig. 1 (a), gallic acid (peak C) was identified as a minor component while ellagic acid (peak F) was a major component. On the other hand, peak D was an ellagic acid derivative while peak E discloses the existence of a gallic acid derivative. Gallic acid (peak C) was determined as a main component in the chromatogram of the acid hydrolysed dyer's oak extract as shown in Fig. 1 (b), and ellagic acid (peak F) was also identified. Nevertheless, in the chromatogram related to the same extract, peaks G and H were identified as gallic acid and ellagic acid derivatives. In addition, it is worth mentioning that most gallic acid and datiscetin dyes present in the hydrolyzed chromatograms of the natural pigments (Fig. 2) were obtained by using (5:50:12.5 v/v/v) alum solution, hemp and dyer's oak extracts, respectively, while most of ellagic acid was observed by using (10:50:12.5 v/v/v) alum solution, hemp and dyer's oak extracts, respectively.

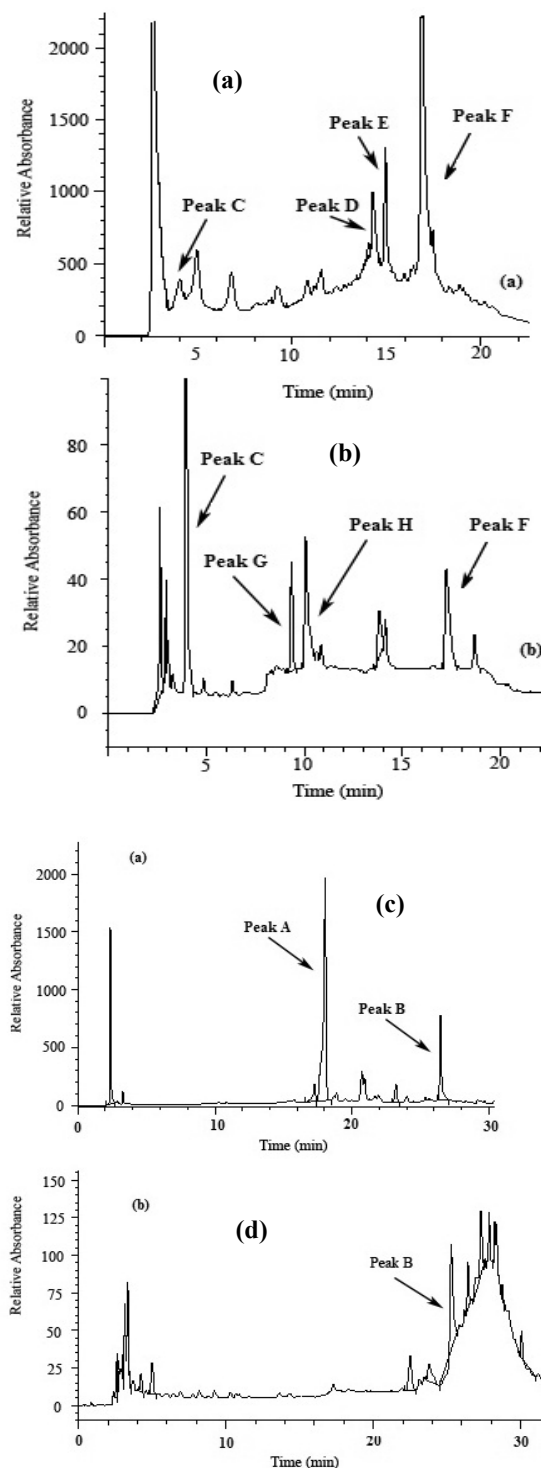


Fig. 1: Chromatograms of non-hydrolyzed (S.C), (at 268 nm)(a) and acid hydrolyzed (S.D), (b) dyer's oak and non-hydrolyzed (S.A), (c) and acid hydrolyzed (S.B) (d) hemp extracts.

Table-1: Chromatographic and spectral characteristics of the investigated sample extracts.

Sample number	Sample extract	Coloring components detected	Characteristics of the detected coloring components		
			R _f (min)	Peak	Absorbance maxima (nm)
S.A	Non-hydrolyzed hemp extract	Datisctin-3-O-[rhamnosyl (1-6)glucoside]	18.0	fig. 1 (c)	257, 305, 329
		Datisctin	26.4	fig. 1 (c)	257, 305, 347
S.B	Acid hydrolyzed hemp extract	Datisctin	25.2	fig. 1 (d)	255, 305, 347
		Gallic acid	4.0	fig. 1 (a)	269
S.C	Non-hydrolyzed dyer's oak extract	Ellagic acid derivative	14.2	fig. 1 (a)	251, 297, 359
		Gallic acid derivative	14.9	fig. 1 (a)	235, 275
		Ellagic acid	16.9	fig. 1 (a)	251, 301, 367
		Gallic acid	3.9	fig. 1 (b)	229, 243, 271
S.D	Acid hydrolyzed dyer's oak extract	Gallic acid derivative	9.3	fig. 1 (b)	229, 235, 243, 271
		Ellagic acid derivative	10.0	fig. 1 (b)	255, 373
		Ellagic acid	17.2	fig. 1 (b)	251, 307, 369
		Gallic acid	4.0	fig. 2	227, 271
S.1	Aluminium- dyer's oak-hemp pigment (prepared with 5 ml alum solution)	Gallic acid derivative	9.5	fig. 2	223, 271
		Ellagic acid	17.1	fig. 2	253, 305, 367
		Datisctin	25.1	fig. 2	257, 305, 347
		Gallic acid	3.9	fig. 2	213, 229, 271
S.2	Aluminium- dyer's oak-hemp pigment (prepared with 10 ml alum solution)	Gallic acid derivative	9.3	fig. 2	213, 229, 271
		Ellagic acid	17.1	fig. 2	251, 305, 367
		Datisctin	25.1	fig. 2	257, 305, 347
		Gallic acid	3.9	fig. 2	213, 229, 271
S.3	Aluminium- dyer's oak-hemp pigment (prepared with 15 ml alum solution)	Gallic acid derivative	9.3	fig. 2	213, 229, 271
		Ellagic acid	17.1	fig. 2	251, 305, 365
		Datisctin	25.1	fig. 2	253, 305, 347
		Gallic acid	3.9	fig. 2	213, 229, 271
S.4	Aluminium- dyer's oak-hemp pigment (prepared with 20 ml alum solution)	Gallic acid derivative	9.3	fig. 2	213, 229, 271
		Ellagic acid	17.1	fig. 2	251, 307, 361
		Datisctin	25.1	fig. 2	255, 307, 347
		Gallic acid	3.9	fig. 2	215, 229, 271
S.5	Aluminium- dyer's oak-hemp pigment (prepared with 25 ml alum solution)	Gallic acid derivative	9.3	fig. 2	213, 231, 273
		Ellagic acid	17.1	fig. 2	251, 325, 361
		Datisctin	25.2	fig. 2	255, 303, 347

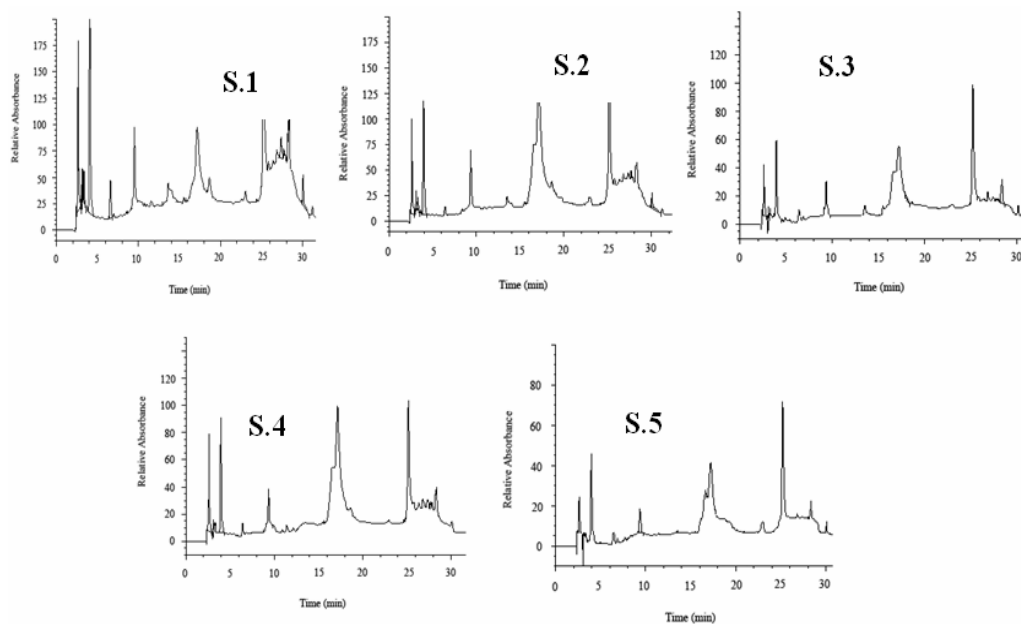


Fig. 2: Chromatograms of aluminum-dyer's oak-hemp natural pigments.

Antimicrobial Activity

The MIC results summarized in Table-2 showed that all the used dyes were able to prevent the growth of all tested microbial species. *D. cannabina* and *Q. infectoria* showed selective activities. Results

also revealed that their inhibitory effects were noted on 6 of the 7 (85.7%) studied organisms for the *D. cannabina* and *Q. infectoria*. These MIC results varied between 2.4 and 625 µg/g/ml. The lowest MIC value for *D. cannabina* (78 µg/ml) was obtained against *A. niger* and for *Q. infectoria* (156 µg/ml)

against *E. coli*, *S. typhimurium* and *C. albicans*. The corresponding value for dyes (2.4 µg/ml) was recorded with natural pigment (S.5) on *C. Albicans*, natural pigment (S.6) on *S. aureus* and natural pigment (S.8) on *S. typhimurium*. These results clearly indicated that natural pigment (S.7) was the most active sample, with MIC values lower than 50 µg/ml recorded on 100 % of the tested pathogens. MIC values lower than 10 µg/ml were also obtained with other dyes such as natural pigment (S.5) on *S. typhimurium* and *C. albicans*, natural pigment (S.6) on *S. aureus*, natural pigment (S.7) on *E. hirae* and natural pigment (S.8) on *E. hirae*, *P. aeruginosa* and *S. typhimurium*. The obtained activity could be considered as promising when that the tested bacteria was resistant to the first line antibiotics and when looking at the reference drugs MICs (6–10µg/ml).

Experimental

Dye Plant and Chemicals

Hemp (*D. cannabina*) and dyer's oak (*Q. infectoria*) were obtained from Turkish Cultural Foundation, Natural Dye Research and Development Laboratory (Istanbul, Turkey). Potassium carbonate; K₂CO₃, hydrochloric acid; HCl, methyl alcohol; CH₃OH and alum (potassium aluminum sulphate); KAl(SO₄)₂.12H₂O were purchased from Merck (Darmstadt, Germany).

Apparatus

Agilent 1200 series system, Elektro-mag M 420P Hot Air Sterilizer Laboratory Oven, WiseStir MSH-20A Daihan Scientific Co. Stirrer, Precisa XB 220A Gravimetrics AG. (Dietikan, Switzerland), Elga PureLab Option-Q and Microplate Photometer (USA) were used in the present study.

Extraction of Dyes from Hemp and Dyer's Oak

Hemp and dyer's oak extracts were prepared as previously described [10-13]. Forty grammas of

the highly granulated dyer's oak (*Q. infectoria*) plant were transferred into a 5000 ml beaker followed by the transfer of 80 g of the aerial parts of hemp (*D. cannabina*) plant into an another 5000 ml beaker. Then, 5000 ml of ultra pure water was added to each beaker and the mixtures were heated with stirring to 100 °C by using a magnetic stirrer and then at 75-80°C for 1 h. Finally, the mixtures were filtered to obtain the hemp and the dyer's oak extracts.

Procedure for the Preparation of Natural Pigments

A 15% KAl(SO₄)₂.12H₂O (alum) solution, hemp and dyer's oak extracts were heated separately to 90°C (for solutions) and 60°C (for extracts), respectively. Then, 5, 10, 15, 20 and 25 ml alum solutions at 90°C were separately added each to 50 ml of hemp extract mixed with 12.5 ml of the dyer's oak extract at 60°C; K₂CO₃ (0.1 M) solution was added to adjust the pH of the mixtures 6.5 and 7. The mixtures were cooled to room temperature to allow the precipitation of the aluminium-hemp-dyer's oak natural pigment. After settling down, the mixtures were filtered and the precipitates were washed with ultra pure water and dried on a filter paper at 100°C for 0.5 h. The dried aluminium-hemp-dyer's oak natural pigment precipitates were then powdered. The same procedure was repeated with the addition of 15 ml of alum solution to each of the 50 ml of hemp extract mixed with 12.5, 25, 37.5, 50 and 62.5 ml of dyer's oak extract and was then repeated by adding 15 ml of alum solution to each of 25, 50, 75, 100 and 125 ml of hemp extract mixed with 12.5 ml of dyer's oak extract. extract [(S.5): pigment prepared with (25:50:12.5 v/v/v) alum solution, hemp and dyer's oak extracts, (S.6): pigment prepared with (15:50:50 v/v/v) alum solution, hemp and dyer's oak extracts, (S.7): pigment prepared with (15:50:62.5 v/v/v) alum solution, hemp and dyer's oak extracts and (S.8): pigment prepared with (15:100:12.5 v/v/v) alum solution, hemp and dyer's oak extracts]

Table-2: The MIC values of *D. cannabina*, *Q. infectoria*, and dyes against the microorganisms tested in microdilution assay.

Tested samples	Tested microorganisms ^a						
	Gram-positive bacteria			Gram-negative bacteria			Fungi
	Sa	Eh	Ec	Pa	St	Ca	An
<i>D. cannabina</i>	312	156	312	625	>625	625	78
<i>Q. infectoria</i>	>625	625	156	625	156	156	312
S.5	78	39	78	39	9.75	2.4	19.5
S.6	2.4	19.5	625	78	19.5	19.5	39
S.7	39	4.8	19.7	9.75	39	19.5	9.75
S.8	156	2.4	39	4.8	2.4	39	78
Reference antibiotics							
Gentamycin	10	8	6	7	9	nt	nt
Nystatin	nt	nt	nt	nt	nt	8.5	9

nt: not tested.

^aMicroorganisms: Sa: *Staphylococcus aureus*, Eh: *Enterococcus hirae*, Ec: *Escherichia coli*, Pa: *Pseudomonas aeruginosa*, St: *Salmonella typhimurium*, Ca: *Candida albicans*, An: *Aspergillus niger*

Dye Extraction Procedure for HPLC Analysis

The dye extraction from the dye plant and the natural pigments was performed by using the previously mentioned method [10-13, 18].

The samples were prepared as follows:

For the dye extraction from hemp (*D. cannabina*) and dyer's oak (*Q. infectoria*) plant, two procedures (1° and 2°) were performed.

In the first procedure the dye extraction from hemp (10.2 mg) and dyer's oak (9.9 mg) dye plants was achieved in 400 µl of the mixture of MeOH: H₂O (2:1; v/v) in a conical glass tube without heating.

For the second procedure, hemp (7.3 mg) and dyer's oak (6.5 mg) dye plants were hydrolyzed by using 37% HCl: MeOH: H₂O (2:1:1; v/v/v; 400 µl) in a porcelain croze on a water-bath at 100°C to extract organic dyes. After rapid cooling under running cold water, the solution was evaporated just to dryness in a water-bath at 55-65°C under a gentle stream of nitrogen. The dry residues were dissolved in 400 µL of the mixture of MeOH: H₂O (2:1; v/v). For the acid hydrolysis of aluminium-hemp-dyer's oak, natural pigments (each one 7.0 mg) were achieved according to the procedure presented in the second step. Then, 40 µl of the supernatants were injected into the HPLC apparatus.

HPLC Equipment

Chromatographic experiments were carried out using an Agilent 1200 series system (Agilent Technologies, Hewlett-Packard, Germany) including a G1329A ALS autosampler, a G1315A diode-array detector. Chromatograms were obtained by scanning the sample from 191 to 799 nm with a resolution of 2 nm and the chromatographic peaks were monitored at 255 and 268 nm. A G1322A vacuum degasser and a G1316A thermostatted column compartment were used. The data were evaluated using Agilent Chemstation. A Nova-Pak C₁₈ analytical column (3.9 mm x 150 mm, 4 µm, Part No WAT 086344, Waters) protected by a guard column filled with the same material was used. Analytical and guard columns were maintained at 30°C. The HPLC gradient elution was performed by using the previously described method [19]. Chromatographic separation of the hydrolysed samples were achieved according to a published procedure by using a gradient elution program that utilizes two solvents: solvent A: H₂O - 0.1% TFA (trifluoroacetic acid) and solvent B:

CH₃CN (acetonitrile) - 0.1% TFA [19]. The flow rate was 0.5 ml/min and the applied elution program was the same as that previously performed program [10-13].

Antimicrobial Assays

Microbial Strains

Microbial strains, namely *Staphylococcus aureus* (ATCC 6533), *Enterococcus hirae* (ATCC 10541), (Gram-positive bacteria), *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella typhimurium* (ATCC 13311) (Gram-negative bacteria), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404) (Fungi) (X22) were obtained from the American Type Culture Collection (ATCC), Medical Microbiology Laboratory, Gazi University, Ankara, Turkey.

Microdilution Assay

The minimal inhibition concentration (MIC) values of the extracts and dyes were determined as follows: The test samples were first dissolved to give a final concentration of 625 µg/ml. This was serially diluted twofold to obtain concentration ranges of 625-1.22 µg/ml. A 100 µl amount of each concentration was added in a well (96-well microplate) containing 100 µl of Mueller Hinton Broth (MHB) and 10 µl of inoculum; the suspensions of microorganism were adjusted to 0.5 McFarland standard turbidity (10⁸ CFU /ml for bacteria and 10⁶ CFU / ml for fungal concentration). Negative control has been used for each one of the strips. The plates were covered with a sterile plate sealer, then agitated to mix the contents of the wells using a plate shaker and were then incubated at 37°C for 24 h. Microbial growth was determined by absorbance at 600 nm using the Microplate Photometer (Multiskan FC, USA). MIC was defined as the lowest concentration of the sample that prevented this change and exhibited complete inhibition of microbial growth.

Conclusion

In this study, the reaction of the dye present in hemp and dyer's oak plants with aluminium(III) has been used to prepare natural pigments. The dyes present in the natural pigments were analysed qualitatively by a reversed-phase high performance liquid chromatography (HPLC) with diode array detection (DAD) in comparison with the standard dyes. Moreover, experiments indicated that *D. cannabina*, *Q. infectoria* and natural dyes have antimicrobial activity.

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