HPLC-DAD analysis of *Thymus serpyllum* based natural pigments and investigation of their antimicrobial properties

Emrah Çakmakçi
Department of Organic Chemistry, Marmara University, Istanbul, Turkey

Ozan Deveoglu
Department of Analytical Chemistry, Marmara University, Istanbul, Turkey

Ahmed Muhammed
Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey

Ali Fouad
Department of Medical Microbiology, Gazi University, Ankara, Turkey

Emine Torgan
Natural Dye Research and Development Laboratory, Turkish Cultural Foundation, Istanbul, Turkey, and

Recep Karadag
Laboratory for Natural Dyes, Marmara University, Istanbul, Turkey

Abstract

Purpose – In this study, it was aimed to investigate the antibacterial properties of natural pigments prepared from *Thymus serpyllum*.

Design/methodology/approach – Al (III), Fe (II), Sn (II) and Cu (II) complexed natural pigments were obtained by using a precipitation method and the main constituents in the pigments were identified with HPLC-DAD. Also FTIR analysis was performed for further structural characterization. Moreover, the thermal stability and thermal degradation properties of the pigments were analyzed by thermogravimetric analyses (TGA). The antimicrobial activity of the thyme plant-extracted pigments was evaluated by measuring the minimal inhibitory concentration.

Findings – Apigenin and luteolin flavones were detected as the main components of the natural dyes. Thermal degradation behaviour of the pigments was determined by means of TGA. All pigments showed high char yields and it was attributed to the high complexation between the metal and the ligand species. The antimicrobial activity of the thyme plant-extracted pigments was measured and it was found that all pigments had high antimicrobial activity. Aluminum-thymus pigments showed the highest antimicrobial efficiency among other pigments used in this study.

Originality/value – The obtained pigments have high antimicrobial activities, and therefore, they can be used for the production of antimicrobial textiles. Furthermore, Thymus-based natural pigments might have potential applications in coating, paint, plastic industries, etc.

Keywords Pigments, Antimicrobial, HPLC-DAD, HPLC-DAD, Minimal inhibition concentration, *Thymus serpyllum*

Paper type Research paper

Introduction

In recent years, organic pigments which are also known as lakes, have attracted significant attention in the field of chemistry, as well as the restoration and conservation of works of art such as tapestries, carpets, miniatures and paintings (Favaro *et al.*, 2007; Amat *et al.*, 2010). Lakes are synthetic metal-organic complexes obtained by adding metal salts to plant or insect extracted dyestuffs solutions (Amat *et al.*, 2010).

Thymus serpyllum, which belongs to Lamiaceae family, has been widely used in traditional and folk medicine. This plant is also known as wild thyme and it comprises about 400 species (Aziz and Ur-Rehman, 2008; De Martino *et al.*, 2009). The family of thymus plant are of significant importance for their pharmacological properties, such as spasmolytic, antiseptic, antitussive and expectorant (Evans, 1998). Also thyme oils have high antimicrobial and antioxidant properties (Kuresh and Stanley, 1999).

In Turkey, the genus Thymus is represented by 39 species and the ratio of endemism in the genus is 53 per cent (Karaman *et al.*, 2001; Tumen *et al.*, 1998).

In literature, studies on thyme plants mainly focused on the analysis of the main components of the plant extracts and on the investigation of their antimicrobial properties. GC-MS and HPLC techniques are the most widely used tools for the determination of the chemical composition of the plant constituents and extracts. For instance, the composition of

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several Thymus species were identified by HPLC analysis and 5,6-dihydroxy-7,3′,4′-trimethoxyflavone was detected in most of the tested samples (Marin *et al.*, 2003). Moreover, polyphenolic compounds in Thymus species were determined by HPLC-Mass spectrometry (Boros *et al.*, 2010).

Experimental

Materials

Thymus serpyllum was obtained from Laboratory for Natural Dyes, Faculty of Fine Arts, Marmara University. The plant was identified by Prof. Dr Recep Karadag. Standard organic dyes: luteolin (5,7,3′,4′ tetrahydroxyflavone) and apigenin (5,7,4′-trihydroxy-flavone) were obtained from Carl Roth (Karlsruhe, Germany). HCl, CH_3OH, $SnCl_2.2H_2O$, $FeSO_4.7H_2O$, $KAl(SO_4)_2.12H_2O$ and K_2CO_3 were obtained from Merck (Darmstadt, Germany). $CuSO_4.5H_2O$ was obtained from Rafineks Kimya Sanayi ve Tic. A.Ş.

Microbial strains

Microbial strains namely *Staphylococcus aureus* (American type culture collection (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) were obtained from the ATCC, Medical Microbiology Laboratory, Gazi University, Ankara, Turkey.

Method for the preparation of *Thymus serpyllum* extract

Fifteen gram of dried and grounded *Thymus serpyllum* were transferred into a 1,000 mL beaker. Seven hundred and fifty millilitre distilled water was then added. The mixture was heated to 100°C for one hour. Finally, the mixture was filtered to obtain the thyme extract (nonhydrolysed).

Method for the preparation of natural pigments

Fifteen per cent $KAl(SO_4)_2.12H_2O$ (alum) aqueous solution and 50 mL thyme extract were heated separately to 90 and 60°C, respectively. Twenty millilitre of alum solution at 90°C was added to thyme extract at 60°C. K_2CO_3 solution (0.3M) was added to neutralise the mixture. The mixture was cooled to room temperature to precipitate the natural pigment. After settling down, the mixture was filtered and the precipitate was washed with distilled water and dried on a filter paper at 100°C for 30 minutes. The dried aluminium natural pigment precipitate was powdered. The same process was repeated by adding 40 mL of alum solution to 50 mL of thyme extract. These experiments were repeated to precipitate the natural pigments by using 3 per cent $FeSO_4.7H_2O$, 3 per cent $SnCl_2.2H_2O$ and 3 per cent $CuSO_4.5H_2O$ solutions.

Method for the determination of antibacterial activity

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial substance that inhibits microorganism growth (the lower the value of MIC, the higher the antimicrobial activity). MIC values of the extract and dyes were determined as follows: the test samples were first dissolved in distilled water to give a final concentration of 625 μg/mL. These solutions were serially diluted twofold to obtain concentration ranges of 1.22-625 μg/mL. Hundred millilitre 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 (10^8 CFU/mL for bacteria and 10^6 CFU/mL for fungal concentration). Negative control have 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 incubated at 37°C for 24 hours for bacteria and for 72 hours for fungi. Microbial growth was determined by measuring the absorbance at 600 nm using Microplate Photometer (Multiskan, FC, USA).

Characterisation

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. The spectra 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 nm. A G1322A vacuum degasser and a G1316A thermostatted column compartment were used. The data were analysed using Agilent Chemstation. A Nova-Pak C18 analytical column (3.9 × 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 using the previously described method (Halpine, 1996; Karapanagiotis *et al.*, 2005). Chromatographic separations of the hydrolysed samples were performed using a gradient elution program that utilizes two solvents: solvent A: H_2O-0.1 per cent TFA (trifluoroacetic acid) and solvent B: CH_3CN-0.1 per cent TFA. The flow rate was 0.5 mL/min and the elution programme was as described earlier (Halpine, 1996; Karapanagiotis *et al.*, 2005).

Thymus serpyllum extract was prepared as described previously (Deveoglu *et al.*, 2009, 2010a, b, 2012).

For chromatographic experiment, the precipitated pigments were treated as follows: natural pigment samples (each one approximately 5.5 mg) were first hydrolysed by mixing them with a solution of H_2O: MeOH: 37 HCl (1:1:2; v/v/v; 400 μL) in conical glass tubes which were than kept for precisely eight minutes in a water-bath at 100°C. After rapid cooling under running cold water, the solution was evaporated to dryness in a water-bath at 50-65°C under a gentle stream of nitrogen. The dry pigment residues were dissolved in 200 μL of MeOH: H_2O (2:1; v/v) mixture.

FT-IR spectrum was recorded on Perkin Elmer Spectrum100 ATR-FTIR spectrophotometer. Grounded and dried samples were put on ATR crystal and samples were then pressed with sufficient force.

Thermogravimetric analyses (TGA) of the dyestuff were performed using a Perkin-Elmer Thermogravimetric analyser Pyris 1 TGA model. Approximately, 10 mg of dried pigment was placed into TGA crucible. Samples were heated from 30 to 900°C with a heating rate of 10°C/min under nitrogen atmosphere.

Results and discussion

HPLC analysis

In the present study, novel natural pigments were obtained from the complexes formed by adding aluminium(III), iron(II), tin(II) and copper(II) solutions to thymus extract. The identification of organic colorants present in the natural pigments was performed qualitatively by reversed phase high performance liquid chromatography (RP-HPLC). Results from the HPLC analysis of iron – thyme pigment showed that luteolin was the main colorant in these pigments. Apigenin was determined in tin – thyme natural pigment. Both luteolin and apigenin were identified in copper-thyme and aluminium-thyme natural pigments. The HPLC chromatograms of acid hydrolysed pigments are shown in Figure 1. Furthermore, as an example, the spectra of standard apigenin and luteolin samples are given along with the absorbance peaks detected in the HPLC chromatogram of copper-thyme natural pigment (Figure 2). Figure 3 shows the chemical structures of both apigenin and luteolin (A) and also shows a one possible metal-flavone complex (B). Coordination of flavones with metals may differ with respect to the metal cation type and pH of the medium. For instance, it was shown that apigenin forms a three to one, ligand to metal (aluminium or iron) complex (Erdogan *et al.*, 2011) while it was found that 3-hydroxyflavone could form 1:1 metal-ligand complex with Al^{3+} (Santos *et al.*, 2002).

ATR-FTIR analysis

The ATR-FTIR spectra of the natural pigments obtained from thyme plant by using Fe, Al, Sn and Cu cations are shown in Figure 4. As determined by HPLC analysis the organic moeities of the pigments are flavonoids. In all spectra the peaks around 3,350 cm^{-1} are attributed to the hydroxyl stretching of absorbed water and to the phenolic –OH groups of the flavones. The bands in the 1,650-1,050 cm^{-1} range are characteristic flavone skeleton (Mishra *et al.*, 2007).

In the ATR-FTIR spectrum of alum precipitated pigment, the absorption band seen in the region between 750 and 400 cm^{-1} can be attributed to the Al-O vibrations. FTIR spectrum of the Cu precipitated pigment shows peaks between 480 and 540 cm^{-1} which can be assigned to Cu-O bonds (Iordanescu, 2011).

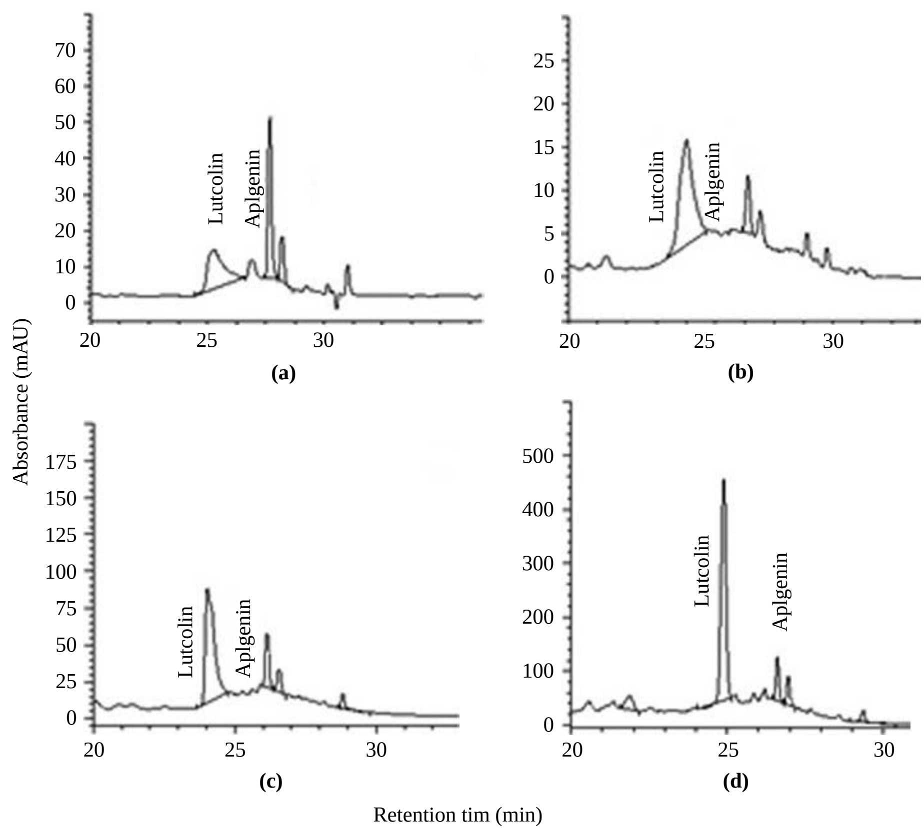
Fe precipitated pigment shows a peak at 598 cm^{-1} which can be related to Fe-O bond. The absorption band at 1,420 cm^{-1} is attributed to the CO_3^{2-} group. Thus, it can be concluded that carbonate co-precipitates with iron (Miliani *et al.*, 2008). Same peak can also be seen in the spectrum of the Sn precipitated pigment.

Also in the FTIR spectra of pigments, the peaks at $\sim$1,020 and 1,060 cm^{-1} correspond to C-O stretchings. In all spectra, the absence of the peaks at around 2,915 and 2,850 cm^{-1} which are due to asymmetric C-H stretching in alkyl hydrocarbons indicates that none of the pigments contain glycosides or impurities with alkyl groups. Moreover, no peaks were detected due to the SO_4^{2-} for all four samples.

TGA analysis

TGA thermograms of pigments are shown in Figure 5. In all four thermograms, high inorganic content was observed. This situation was attributed to the high complexation between the metal and the ligand species and also it was attributed to the aromatic structure of the organic domains. The weight

Figure 1 The HPLC-DAD chromatogram of the acid hydrolysed



Notes: (a) Tin-thymus natural pigment; (b) iron-thymus natural pigment; (c) copperthymus natural pigment and (d) aluminium-thymus natural pigment

Figure 2 (a) Photodiode array spectra of (– – –) luteolin standard compound and (– – –) peak at 24.1 minutes from chromatogram in Figure 1(c); (b) photodiode array spectra of (– – –) apigenin standard compound and (– – –) peak at 26.1 minutes from chromatogram in Figure 1(c)

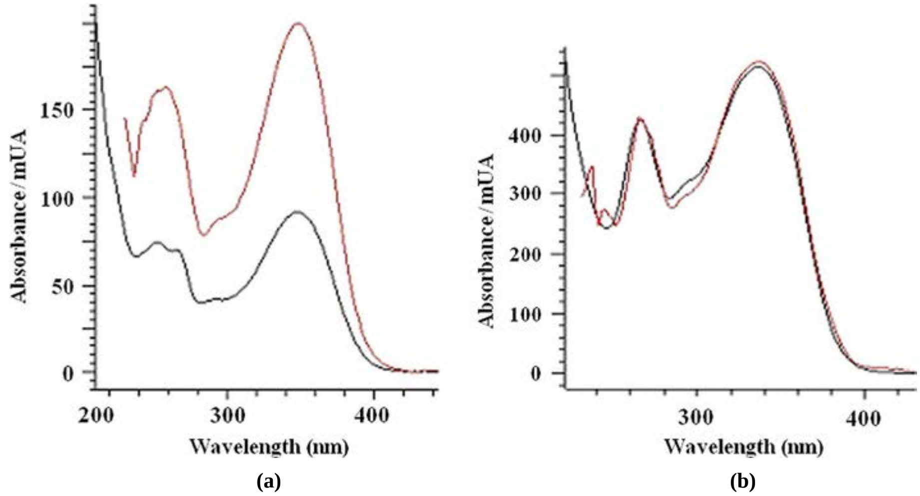
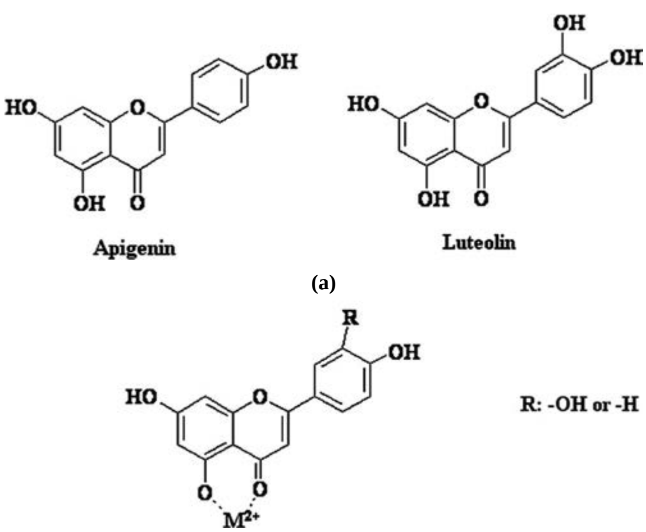


Figure 3 Chemical structures of apigenin and luteolin (a) and a representative image of metal-luteolin or metal-apigenin complex (the image shows only one possible metal-flavone complex, coordination of flavones may differ with respect to the metal cation type)



loss between 30 and 200°C is due to the absorbed water. After 200°C a slow degradation is observed. The weight loss during the period between 200 and 500°C is attributed to the degradation of organic moiety. A third weight loss was also observed for Sn and Fe. This weight loss can be related to the release of carbon dioxide due to the presence of precipitated CO_3^{2-} (Serifaki *et al.*, 2009).

However, char yields were found as 67, 51, 41 and 40 for Sn, Al, Cu and Fe, respectively. These char yields also indicate high metal chelating capacity of the flavones.

It was previously determined that luteolin and apigenin which belonged to the class of flavonoids were present at high amounts in the extracts of different Thymus species (Marin *et al.*, 2003, 2005). Flavonoids are polyphenolic substances which are present in most plants and over 8,000 types of flavonoids are known up to date (De Groot and Raven, 1998; Tapas *et al.*, 2008).

These compounds were found to have antioxidant, antibacterial, antimicrobial and antiviral activities (Tapas *et al.*, 2008). Specifically, both apigenin and luteolin have been reported to have anti-inflammatory activity (Farmica and Regelson, 1995). Moreover, it has been shown that apigenin is a potential candidate for cancer treatment (Xu *et al.*, 2011).

Antimicrobial activity

The MIC results are summarised in Table I. Results showed it can be said that all the dyes were able to prevent the growth of all tested microbial species. Thymus showed selective activities. Their inhibitory effects were noted on six of the seven (85.7 per cent) studied organisms for the thymus. These MIC results varied between 2.4 and 625 μg/mL. The lowest MIC value for thymus (78 μg/mL) was obtained against *S. aureus*. The corresponding value for organic dyes (2.4 μg/mL) was recorded with tin-thymus (prepared with 40 mL) on *P. aeruginosa,* copper-thymus (prepared with 20 and 40 mL) on *S. typhimurium* and aluminium-thymus (prepared with 40 mL) on *E. hirae*. These results clearly indicated that the aluminium-thymus pigment was the most active sample, with MIC values lower than 50 μg/mL recorded on 100 per cent of the tested pathogens. MIC values lower than 10 μg/mL were also obtained with other dyes such as iron-thymus (prepared with 40 mL) on *P. aeruginosa*, tin-thymus (prepared with 20 and 40 mL) on *P. aeruginosa* and *A. niger*, copper-thymus (prepared with 20 mL) on *S. typhimurium*, copper-thymus (prepared with 40 mL) on *S. typhimurium* and *A. niger*, aluminium-thymus (prepared with 20 mL) on *E. hirae*, and the aluminium-thymus on *S. aureus* and *E. hirae*. When it is considered that the tested bacteria. When it is considered that the tested bacteria were resistant to the first line antibiotics it can be seen that *Thymus Serpyllum* based natural pigments could be considered as promising compounds for their high antimicrobial activity. Furthermore, when the antimicrobial activity of the reference drugs (MIC values of 6-10 μg/mL) was compared with the activity of the natural pigments, the effectiveness and the selectivity of these novel pigments could be seen.

Figure 4 ATR-FTIR spectra of the thyme plant pigments

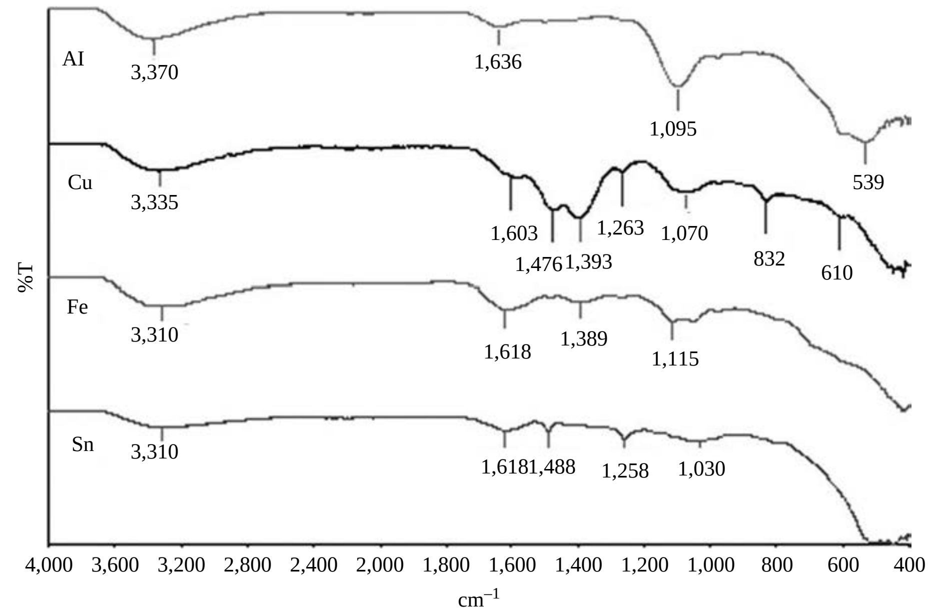


Figure 5 TGA spectra of pigments

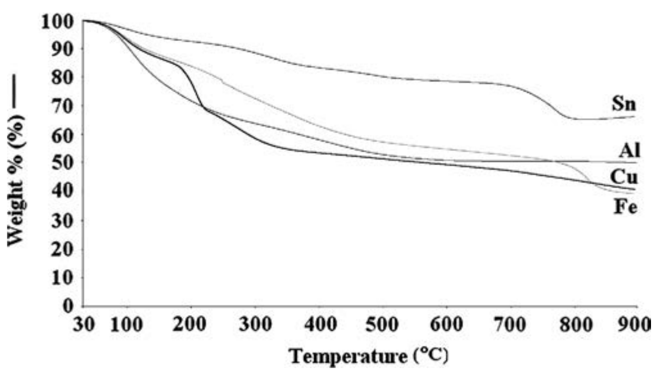


Table I The MIC values of Thymus and organic dyes against the microorganisms tested in microdilution assay

	Tested microorganisms[a]						
	Gram-positive bacteria		Gram-negative bacteria			Fungi	
Tested samples	**Sa**	**Eh**	**Ec**	**Pa**	**St**	**Ca**	**An**
Thymus	78	312	625	>625	312	312	156
20 mL Fe-thymus	312	39	156	39	78	156	312
40 mL Fe-thymus	78	156	78	*9.75*	19.7	156	78
20 mL Sn-thymus	78	156	625	*9.75*	>625	19.5	*9.75*
40 mL Sn-thymus	39	19.5	78	*2.4*	156	19.5	*4.8*
20 mL Cu-thymus	625	156	156	625	*2.4*	156	39
40 mL Cu-thymus	156	78	39	78	*2.4*	39	*4.8*
20 mL Al-thymus	19.5	*9.75*	19.5	312	9.75	39	78
40 mL Al-thymus	*4.8*	*2.4*	9.75	19.5	39	39	39
Reference antibiotics							
Gentamycin	6	9	7.5	7	10	nt	nt
Nystatin	nt	nt	nt	nt	nt	9	9.5

Notes: [a]Microorganisms: Sa – *Staphylococcus aureus*, Eh – *Enterococcus hirae*, Ec – *Escherichia coli*, Pa – *Pseudomonas aeruginosa*, St – *Salmonella typhimurium*, Ca – *Candida albicans*, An – *Aspergillus niger*, nt – not tested

Conclusions

In this study, novel natural pigments were prepared from Thymus plant. The pigments were then structurally characterised and their thermal and antimicrobial properties were investigated. According to thermogravimetric analysis results all pigments showed high char yields which was attributed to the high complexation between the metal and the ligand species. Luteolin and apigenin were determined as the main components of the natural pigments. The antimicrobial activity of the crude extract and dyes against some microbial strains were evaluated based on the minimal inhibition concentration (MIC) values. The MIC results showed that, all the used dyes were able to prevent the growth of all tested microbial species. Furthermore, when the antimicrobial activity of the reference drugs was compared with the activity of the natural pigments, natural pigments were found to be highly effective. Aluminium-thymus pigments showed the highest antimicrobial efficiency among other pigments used in this study.

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About the authors

Emrah Çakmakçi is a Research Assistant in the Department of Organic Chemistry at Marmara University. His research focuses on polymer science, photocurable coatings, thiol-ene polymerization systems, biopolymers, electrospinning, polyimides and natural pigments. Emrah Çakmakçi is the corresponding author and can be contacted at: emrah.cakmakci@marmara.edu.tr

Ozan Deveoglu obtained his BSc degree from the Department of Chemistry, Faculty of Science and Letters, Balıkesir University in 2002 and his Master's degree in analytical chemistry from Marmara University in 2008. In the same year, he was accepted to study for a PhD in analytical chemistry. Since 2009, he is a Research Assistant in the Department of Analytical Chemistry at Marmara University. His research interests include the characterisation of the natural dyed textiles, the analysis of lake pigments and natural dyes, and the natural dyeing of the textiles.

Ahmed Muhammed is currently a post-doctoral Fellow at Physiology Department, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey. He obtained his PhD (2012) from Ankara University. His current research interests include the use of traditional medicinal plants as an alternative for chemical drugs in the treatment of various diseases and ailments (especially, diabetes mellitus, cancer, peptic ulcer, burns, and microbial infections) through *in vitro* and *in vivo* experiments.

Ali Fouad is currently a graduate student in the Department of Medical Microbiology, Gazi University, Ankara, Turkey. He obtained his MSc (2010) in the same department. His current research interests include microbial diversity and mechanisms of antibiotic resistance.

Emine Torgan obtained her BSc degree in chemistry education in 2006 and her MSc degree in analytical chemistry in 2008, both from Marmara University. Her work with the Turkish Cultural Foundation, Research and Development Laboratory for Natural Dyes, concerns dye analysis in historical textiles and natural pigments.

Recep Karadag has a PhD in analytical chemistry (Marmara University, 1994). He became an Associate Professor in textile technology at Marmara University, Faculty for Fine Arts, Laboratory for Natural Dyes in 2004 and Full Professor in 2009. He works in the Marmara University Natural Dyes Research and Development Project (DOBAG), has studied dye analysis in historical textiles and has over 60 publications.