Tarla Bitkileri Merkez Araştırma Enstitüsü Dergisi, 2017, 26 (1):1-10
Araştırma Makalesi (Research Article)

Effect of Different Sowing Times on Leaf Characteristics and Indican Content of Some *Isatis* Species

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Geliş Tarihi (Received): 14.11.2016 Kabul Tarihi (Accepted): 13.04.2017

Abstract

In this study, effects of different sowing dates on the some agronomic properties of 4 *Isatis* species (*Isatis tinctoria* L., *Isatis candolleana* BOISS. (endemic), *Isatis tinctoria* L. subsp. *corymbosa* (BOISS.) DAVIS and *Isatis buschiana* SCHISCHKIN were investigated in ecological conditions of Kahramanmaras in 2008 and 2009 growing season. Optimum sowing time for *I. tinctoria* leaf yield was spring and for the other species were autumn. The highest leaf yield was obtained in 2008 for all species and the values were 15185 kg ha^{-1} in *I. tinctoria*, 15109 kg ha^{-1}, 19299 and 10909 kg ha^{-1} in *I. tinctoria* subsp. *corymbosa*, *I. buschiana* and *I. candolleana*, respectively. The highest indican contents were found 23.5, 21.4, 18.2 and 15.2 mg g^{-1} in *I. buschiana*, *I. tinctoria* subsp. *corymbosa, I. tinctoria and I. candolleana* respectively, by using ultrasonic extraction and DAD detector analysis of HPLC.

Keywords: HPLC-DAD, *Isatis*, indican, leaf yield, sowing date

Bazı *Isatis* Türlerinin Yaprak Karakterleri ve İndikan İçeriği Üzerine Farklı Ekim Zamanlarının Etkisi

Öz

Bu çalışmada Kahramanmaraş ekolojik koşullarında 2008 ve 2009 yetiştirme sezonlarında yetiştirilen 4 *Isatis* türünün (*Isatis tinctoria* L., *Isatis candolleana* BOISS. (endemik), *Isatis tinctoria* L. subsp. *corymbosa* (BOISS.) DAVIS ve *Isatis buschiana* SCHISCHKIN) bazı agronomik özellikleri üzerine farklı ekim zamanlarının etkileri incelenmiştir. *I. tinctoria* bitkisinden yaprak verimi almak için optimum ekim zamanı ilkbaharken diğer türler için sonbahar olmuştur. *I. tinctoria*'dan 15185 kg ha^{-1} *I. tinctoria* subsp. *corymbosa*'dan 15109 kg ha^{-1}, I. *buschiana*'dan 19299 ve *I. candolleana*'dan 10909 kg ha^{-1} olmak üzere, tüm türler için en yüksek yaprak verimleri 2008 yılında elde edilmiştir. HPLC'de yapılan analizler sonucunda, *I. buschiana, I. tinctoria* subsp. *corymbosa, I. tinctoria* ve *I. candolleana*'da en yüksek indikan içerikleri sırasıyla 23.5, 21.4, 18.2 ve 15.2 mg g^{-1} olmak üzere ultrasonikasyonla yapılan ve DAD dedektörle yapılan okumalardan elde edilmiştir.

Anahtar Kelimeler: Ekim zamanı, HPLC-DAD, *Isatis*, indikan, yaprak verimi

Introduction

Indigo was the most universally important dyestuff (Gilbert and Cook 2001) and natural indigo as a textile dye have been used since Bronze Age (-7000) (Pawlak et al. 2006). Natural indigo can be derived from many plants belonging to different species, genera and families but the most commonly used species was *Isatis tinctoria* in temperate climates (Gilbert and Cook 2001). In addition to being a dye plant, *I. tinctoria* is also known as a medicinal plant. Leaves, roots and seeds of *I. tinctoria* contain indole-derived compounds (principally glucosinolate) with anti-inflammatory and anti-tumoral properties (Frechard et al. 2001; Hamburger 2002; Oberthür et al. 2005). The roots are used for pharyngitis, laryngitis, erysipelas, and carbuncle, and to prevent hepatitis A, epidemic meningitis, cancer and inflammation and also antibiotic, antiseptic and anti-viral activities were also reported (Han et al. 2011). Although *Isatis* is a natural source of indigo, breeding programme for *I. tinctoria* attracted limited scientific interest (Angelini et al. 2007). The production of low-cost and high-quality plant material in great amounts is a significant factor for all of the commercial

DOI: 10.21566/tarbitderg.323555

plants. Hence, it is essential to develop effective cultivation methods for important dye plants (Kızıl 2006). Agriculture for woad was achieved using wild-type *Isatis* seeds. Thus, high variability for agronomic and genetic traits was frequently observed (Spataro and Negri 2008). The renewed interest in natural dyes showed that the potentials in cultivations of woad in marginal lands and their usage in medicine and cosmetic industry could make it interesting crop in the near future (Rocha et al. 2011).

In this study, wild species of *I. buschiana, I. candolleana, I. tinctoria* subsp. *corymbosa* and a culture form *I. tinctoria* were cultivated with the aim of (i) to evaluate differences among the sowing dates in leaf yield and characteristics of *Isatis* species grown in a field experiment; (ii) to determine the amount of indican (iii) to define potential high yielding plants to investigate in further breeding programs.

Material and Method

Plant Material

Wild-types of *I. buschiana, I. candolleana* (endemic)*, I. tinctoria* subsp. *corymbosa* and a culture form of *I. tinctoria* were investigated. *I. tinctoria* seeds were provided from Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. Wild *I. candolleana* (endemic) plants and seeds were collected from Ahir Mountain in Kahramanmaraş (city center) at June (altitude 960 m). Wild *I. buschiana* and *I. tinctoria* subsp. *corymbosa* plants and seeds were collected from native stands in Göksun-Kahramanmaras in summer vegetation (altitude of 1300-1400 m and 1200-1250 m, respectively). Plants were identified according to Flora of Turkey (Davis 1982). The city center and Göksun County in Kahramanmaras province where the wild plants and seeds were collected have a Mediteranean climate and a Mediterranean-Terrestrial climate, respectively (Table 1).

Field Trials

Field studies were carried out during two sequential years (from September 2007 to July 2009) at the trial areas of Kahramanmaras, Turkey. This cultivation location has also typical Mediterranean–type climate conditions (37º 35' N latitude; 36º 56' E longitude). The soil was characterized by 30-60 cm deep and has loamy texture, pH 7.54, 0.081% salt, 26.73% $CaCO_3$, 1.93% organic matter, 45 and 682 kg ha^{-1} P_2O_5 and K_2O, respectively (Comlekcioglu, 2011).

In each experiment, treatments were constructed in a randomized complete block design with three replications with a plot size as 6.3 m^2 (2.1 x 3 m) with 8 rows. Plant density was about 14 plants/m^2, with inter-row and intra-row spacing of 0.3 x 0.3 m. Sowing dates for the first trial year were made in October 26th, November 30th (2007), February 27th and March 25th (2008), and for second trial year, were made in October 24th, November 19th (2008), March 8th and March 31th (2009). Sowing was done in hole, established by hoe at depth of 2-3 cm manually at the rate of 4-5 siliqua drops in each hole. Thinning was done after emergence, leaving one plant in each hole at 4-5 leaves stage.

Plants were keeped under same fertilizer regimes. Mineral fertilizer was performed at pre-planting at rates of 5/5/0 kg ha^{-1} of N/P/K 50 kg ha^{-1} of N (urea) were supplied as higher fertilizer. When plants had attained the rosette stage, fresh leaves were harvested manually with scissors in different time according to species. Production measurements (yield of plot) were applied on total plot, excluding outer rows (Sales et al. 2006). Leaf width-length and fresh leaf weight were evaluated from 15 plants per species and analysed statistically, using ANOVA. Data obtained were evaluated with one way variance analysis and independent two sample t-test. When appropriate, differences among mean of treatments were analyzed using Turkey.

Table 1. Plant material that used in this study
Çizelge 1. Bu çalışmada kullanılan bitki materyalleri

Species	Life cycle	Distribution	Altitude	Location
I. tinctoria L.	Culture	Culture	-	-
I. tinctoria L. subsp. *corymbosa* (BOISS.) DAVIS	Biennial or perennual	Not Endemic	1300-1400	Püren Passage-Göksun/K.Maraş
I. candolleana BOISS.	Biennial or perennual	Endemic	960	Ahirdagi/ K.Maraş
I. buschiana SCHISCHKIN	Perennual	Not Endemic	1200-1250	Çardak Village-Göksun/K.Maraş

Post-Harvest Treatments

After cutting, fresh leaf specimens from each experimental plot were sampled randomly. Leaf samples were cut with scissors into small pieces of 2-3 cm length. The cutted leaf samples freezed with liquid N_2 and immediately freeze dried in a lyophilizator (CHRIST Freeze Dreyer, Alpha 1-2 LD). The freeze dried leaf samples stored in a deep freezer at -80 ºC (Mohn et al. 2009).

Indican Extraction and Sample Preparation for HPLC

Extraction of indican and preparation of samples were carried out according to Oberthür et al. (2004) with several modifications as described before (Comlekcioglu et al., 2013). Accelerated solvent extraction (ASE) and ultrasonication were carried out by using Dionex ASE 350 and Bandelin Sonopuls, respectively.

HPLC-DAD Conditions

The HPLC system (Shimadzu, Kyoto, Japan) used includes a Shimadzu binary gradient pump (Shimadzu LC-10AT) and DAD detector. The sample injections were carried out by using automatic sampling system (SIL 20AC). The separations and determination of indican were achieved on an Inertsil ODS-C18 column (250 mm x 4.6 mm, particle size, 5 µm). $CH_3CN/H_2O/HCOOH$ (32%/68%/0.1%) was eluted isocratically (flowrate 0.7 ml min^{-1}). Temperature of column was maintained stabile at 40 ºC. Detection of DAD was 270 nm.

Standard Indican by HPLC-DAD

Synthetic indican (97% purity) was used as standard and purchased from Sigma. Indican showed a retention time (RT) of 4.5 min in HPLC-DAD. Quantification of indican was performed with a calibration curve. Quantification of indican was performed with a calibration curve obtained from measurements of a series indican standard (0, 0.05, 0.1, 0.25, 0.5 and 1 mg ml^{-1}) and it was given in Figure 1.

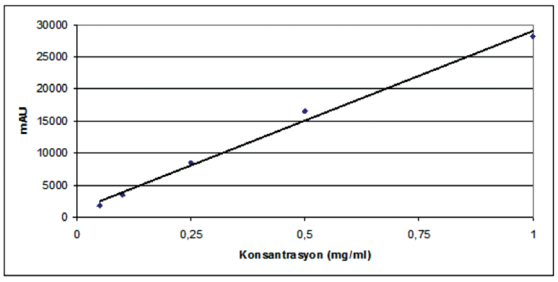

Figure 1. Calibration curve of indican peaks
Şekil 1. İndikan piklerinin kalibrasyon eğrisi

Weather Conditions

Total rainfall and temperature per month in the first and second year compared to long time data (1975-2007) are presented in Table 2. Rainfall quantity and distribution was varied substantially in the two years of trial (620.3 and 859.2 mm from October to September in first and second trial years, respectively) and in terms of typical long-term period (721.0 mm from October to September). The second trial year was characterized by rainy spring, especially rainfall of February and March months higher than in previous trial year and the long-term trend.

Mean temperatures were similar to long-term trend both in first and second trial years. Air temperatures increased from March to August, with a decreasing trend observed thereafter. As the temperature was under 10 ºC in winter, it increased from March to September. November sowing was negatively affected by the frost event in which temperatures fell below zero degrees in the winter months. As a result, the first year of the trial was more arid than second year of trial. Plant emergence and development were negatively affected by frost events that occurred from November to February.

Results and Discussion

Leaf Characteristics

Analysis of variance of the four *Isatis* spp. showed significant differences in leaf number, leaf length, leaf width, fresh leaf weight and leaf yield (Table 3 and 4). The findings from statistical analysis of data are given with reference to the main source of variation (sowing dates). The effect of sowing times on leaf number per plant was significant in the first year for all *Isatis* spp. except *I. tinctoria* ($P<0.01$). In the second year, the effect of sowing times on leaf number per plant was significant for *I. tinctoria* and *I. buschiana* ($P<0.01$).

These results could be explained by differences in the weather conditions especially total rainfall amount and distribution between years. Number of leaves per plant varied between 75 and 147.6 in *I. tinctoria.* In previous studies, it was reported that leaf number of *I. tinctoria* changed between 14.36, 14.15 and 99.5 in Tansı (1998), Kızıl (2000), Tansı and Karaman (2005), respectively. Leaf number in this study was higher than those of Tansı (1998) and Kızıl (2000)'s results. For *I. tinctoria*

Table 2. Climate data of Kahramanmaraş for trial months in 2007, 2008 and 2009, in respect to long term period (1975-2007)

Çizelge 2. Kahramanmaraş İli, 2007, 2008 ve 2009 yılları deneme ayları ve uzun yıllar (1975-2007) ortalamalarına ilişkin bazı iklim verileri

Months	Sowing years	Mean temperature (ºC)	Mean relative humidity (%)	Total rainfall (mm)
October	2007	20.6	54.2	19.1
	2008	19.3	54.6	13.8
	Long Terms	19.0	55	51.2
November	2007	11.9	65.9	101.7
	2008	13.2	64.1	105.9
	Long Terms	11.4	64.0	90.2
December	2007	6.3	66.8	125.6
	2008	6.1	65.5	96.2
	Long Terms	6.6	71.0	128.1
January	2008	3.3	55.0	78.6
	2009	4.5	69.0	107.5
	Long Terms	4.9	70.0	122.6
February	2008	5.5	61.4	121.5
	2009	7.2	78.8	221.2
	Long Terms	6.3	65.0	110.1
March	2008	14.4	59.6	69.5
	2009	9.4	67.2	158.0
	Long Terms	10.4	60.0	95.0
April	2008	18.1	55.5	54.7
	2009	15.1	59.4	82.5
	Long Terms	15.3	58.0	76.3
May	2008	20.2	56.5	23.7
	2009	20.5	51.9	43.4
	Long Terms	20.4	54.0	39.9
June	2008	27.3	49.8	-
	2009	26.8	48.2	3.7
	Long Terms	25.1	50.0	6.2
July	2008	29.9	58.3	-
	2009	28.5	56.9	6.9
	Long Terms	28.3	52.0	0.9
August	2008	30.1	59.7	2.3
	2009	28.8	52.9	0.6
	Long Terms	28.3	54.0	0.5
September	2008	25.1	61.4	23.6
	2009	23.6	51.3	19.5
	Long Terms	25.1	51.0	6.6
Annual	2007	17.9	59.1	690.6
	2008	17.7	58.5	589.8
	2009	17.2	61.3	1059.3
	Long Terms	16.8	58	727.6

mean leaf length in first trial year (19.1 cm) was higher than second trial year (16.1 cm) while mean leaf widths were similar in both trial years (3.1cm). Leaf width and length were reported as 1.15 and 14.37 cm (Tansı, 1998), 4.18 and 14.34 cm (Kızıl, 2000), 2.69 and 13.76 cm (Tansı and Karaman, 2005), 5.23 and 15.7 cm (Akar, 2006), respectively. Mean value of two trial years for leaf length (17.6 cm) was found to be higher than the other studies (Akar, 2006; Kızıl, 2000; Tansi and Karaman, 2005).

In first trial year, differences in yields of plot were found to be important in all sowing times and species ($P<0.01$). Plant output rate affected the plot yield, which were 89% and 30% in October and November sowings, respectively. In first trial year leaf number, leaf length, width and fresh leaf weight values were maximum in February sowing in *I. buschiana*. However, yield of plot value was higher (1929.9 g/m^2) in October sowing. Plant output rate was 83%, 52%, 23% and 7% in October, November,

Table 3. Results of analysis of variance and means of some morphological characters obtained from *Isatis* spp. in the first trial year (2008)

Çizelge 3. Birinci deneme yılında (2008) Isatis türlerinden elde edilen bazı morfolojik karakterlerin ortalamaları ve varyans analizi sonuçları

Sowing Times	Number of days from sowing to harvest	Leaf number per plant	Leaf Length (cm)	Leaf Width (cm)	Fresh leaf weight (g/plant)	Yield of plot (kg ha^{-1})	Plant output rate (%)
			I. tinctoria				
October	-	-	-	-	-	-	
November	-	-	-	-	-	-	
February	68	146.7 ± 18.0 a	18.8 ± 0.6 a	2.9 ± 0.1 b	29.8 ± 3.3 b	1800.1 ± 15.4 b	69
March	60	147.6 ± 20.6 a	19.4 ± 0.4 a	3.4 ± 0.1 a	136.1 ± 25.5 a	15185 ± 41.1 a	68
Mean		147.2 ± 9.0	19.1 ± 0.4	3.1 ± 0.1 **	82.8 ± 26.4 *	8493 ± 404.5 **	
			I. tinctoria subsp. *corymbosa*				
October	190	169.8 ± 11.4 a	17.2 ± 0.6 a	4.2 ± 0.2 a	102.4 ± 4.0 a	15109 ± 39.2 a	89
November	160	119.1 ± 9.5 b	16.3 ± 0.5 a	4.4 ± 0.2 a	93.9 ± 10.0 a	5834 ± 58.1 b	30
February	-	-	-	-	-	-	
March	-	-	-	-	-	-	
Mean		144.6 ± 5.1 **	16.7 ± 0.4	4.3 ± 0.1	98.2 ± 5.1	10472 ± 281.8 **	
			I. candolleana				
October	190	104.8 ± 7.0 a	13.6 ± 0.4 a	6.2 ± 0.3 b	246.0 ± 24.1 a	10909 ± 67.5 a	17
November	140	87.5 ± 7.3 ab	14.7 ± 0.3 a	7.1 ± 0.2 a	175.2 ± 5.1 ab	4409 ± 52.2 b	10
February	75	60.7 ± 17.5 b	14.4 ± 1.0 a	6.2 ± 0.4 b	120.5 ± 5.8 b	1173 ± 17.5 c	4
March	60	59.4 ± 13.2 b	14.8 ± 0.5 a	6.6 ± 0.3 ab	196.1 ± 52.6 ab	1774 ± 22.8 c	3
Mean		77.1 ± 6.2 **	14.4 ± 0.2	6.5 ± 0.1*	189.8 ± 18.8*	4566 ± 158.3**	
			I. buschiana				
October	190	28.8 ± 2.9 b	25.4 ± 0.8 b	6.8 ± 0.2 a	136.9 ± 39.3 a	19299 ± 142.3 a	83
November	160	32.5 ± 1.9 ab	21.8 ± 0.5 c	5.5 ± 0.2 b	127.1 ± 13.2 a	11099 ± 53.9 b	52
February	75	38.6 ± 5.0 a	27.4 ± 0.7 a	7.3± 0.2 a	142.5 ± 21.5 a	8839 ± 57.2 c	23
March	60	16.1 ± 1.9 c	19.7 ± 0.8 d	6.1 ± 0.3 b	62.3 ± 13.6 b	1199 ± 17.9 d	7
Mean		29.0 ± 1.6**	23.6 ± 0.4**	6.4 ± 0.1**	117.1 ± 14.2*	10109 ± 264.9**	

*: P<0.05; **: P<0.01

February and March sowings, respectively. The highest leaf length (mean 23.6 and 24.4 cm in 2008 and 2009, respectively) was obtained in *I. buschiana* (Table 2 and 3). Yildirimli (1988) reported that leaf length and width of *I. buschiana* varied between 8-20 cm and 1.2-3 cm, respectively. In this study, leaf length and width of *I. buschiana* varied between 19.7-28 and 5.5-8 cm, respectively.

The maximum and minimum values of leaf number per plant, leaf length and width, fresh leaf weight and yield of plot of *I. candolleana* were obtained in October sowings and February sowings, respectively, in 2008 trial. The output rate was decreased in spring sowings (4-3%) and the plants could not survive. In this study, leaf length and width of *I. candolleana* varied between 13.6-18.4 cm and 5.9-7.1 cm, respectively. Yildirimli (1988) reported that the leaf length and width of *I. candolleana* were 4-15 cm and 4-15 cm, respectively. On the other hand, Akar (2006) determined that the leaf length and width of *I. candolleana* were 11-27 cm and 3.8-12 cm, respectively, in Kahramanmaraş conditions.

Sowing dates affected the leaf yeald in all of the *Isatis* spp. significantly. Leaf width and fresh leaf weight were statistically significant for all *Isatis* spp. except *I. tinctoria* subsp. *corymbosa* (first trial year) and *I. tinctoria* (second trial year). In second trial year the differences in leaf length between sowing dates were statistically

Table 4. Results of analysis of variance and means of some morphological characters obtained from *Isatis* spp. in the second trial year

Çizelge 4. İkinci deneme yılında Isatis türlerinden elde edilen bazı morfolojik karakterler ve varyans analizi sonuçları

Sowing Times	Number of days from sowing to harvest	Leaf number per plant	Leaf Length (cm)	Leaf Width (cm)	Fresh leaf weight (g/plant)	Yield of plot (kg ha^{-1})	Plant output rate (%)
			I. tinctoria				
October	-	-	-	-	-	-	-
November	-	-	-	-	-	-	-
February	55	75.2 ± 6.8 b	18.0 ± 0.3 a	3.1 ± 0.1 a	100.2± 6.0 a	6676 ± 11.9 a	50
March	35	110.7 ± 6.9 a	14.3 ± 0.3 b	3.1 ± 0.1 a	86.2 ± 8.7 a	5417 ± 29.4 b	35
Mean		95.0 ± 5.3 **	16.1 ± 0.2 **	3.1 ± 0.04	93.2 ± 5.7	6047 ± 40.6 **	
			I. tinctoria subsp. *corymbosa*				
October	-	-	-	-	-	-	-
November	170	177.5 ± 13.8	19.3 ± 0.4	4.7 ± 0.1	246.1 ± 18.4	9294 ± 31.2	30
February	-	-	-	-	-	-	-
March	-	-	-	-	-	-	-
Mean		177.5 ± 13.8	19.3 ± 0.4	4.7 ± 0.1	246.1 ± 18.4	9294 ± 31.2	
			I. candolleana				
October	-	-	-	-	-	-	-
November	175	39.7 ± 3.1	18.4 ± 0.3	5.9 ± 0.1	234.6 ± 41.0	10457 ± 37.2	35
February	-	-	-	-	-	-	-
March	-	-	-	-	-	-	-
Mean		39.7 ± 3.1	18.4 ± 0.3	5.9 ± 0.1	234.6 ± 41.0	10457 ± 37.2	
			I. buschiana				
October	210	19.2 ± 1.2	20.7 ± 0.5 c	6.2 ± 0.2 c	67.2 ± 2.1 b	3541 ± 34.3 c	20
November	175	34.1 ± 2.7	28.0 ± 0.5 a	7.0 ± 0.2 b	227.5 ± 12.7 a	11899 ± 42.7 a	40
February	75	28.1 ± 2.9	24.5 ± 0.5 b	8.0 ± 0.2 a	213.4 ± 21.8 a	4960 ± 40.2 b	10
March	-	-	-	-	-	-	-
Mean		27.1 ± 1.5 **	24.4 ± 0.3**	7.1 ± 0.1**	169.4 ± 26.6**	6800 ± 175.4**	

*: P<0.05; **: P<0.01

significant for *I. tinctoria* and *I. buschiana* (second trial year). *I. tinctoria* plants grown from seeds sown in both October and November started flowering at the end of March. Therefore leaf yield could not be obtained for these sowing dates in both trial years. In the first trial year, leaf yields decreased in February sowing, because of stalk formation in most *I. tinctoria* plants. Seeds of *I. tinctoria* subsp. *corymbosa* sown in spring did not germinate; leaf yield could not be obtained in the second trial year for October sowing. Leaf yield was not also obtained for *I. candolleana* in all of sowing dates at second trial year except November sowing.

Average yield of plot for *I. tinctoria* was recorded 8493 kg ha^{-1} in first trial year and 6047 kg ha^{-1} in second trial year. Tansı and Karaman (2005) and Kızıl (2000) determined 6000 and 7000 kg ha^{-1} in Çukurova and Diyarbakır conditions, respectively. Sales et al (2006) investigated agronomic factors, such as sowing date, plant density, affecting production of indigo, from *I. tinctoria* crops in Spain. Sales et al. (2006) found that leaf yield of *I. tinctoria* sown in February and March was 66300 and 68200 kg ha^{-1} in 2002, respectively, and 32800 and 33100 kg ha^{-1} in 2003, respectively. There is a difference between years. Angelini et al. (2007) reported the fresh leaf yield as 15000 kg ha^{-1}. Leaf yields obtained in this study ranged between 1801-15185 kg ha^{-1} and the average is 7270 kg ha^{-1}. These values are lower than the studies conducted in Europe conditions, but similar to Çukurova and Diyarbakir conditions. Decrease in growth and development, delay

in flowering, shortening in vegetative and generative development phase, increase in sensitivity to frost damage, and decrease in yield were reported as a result of the delay in sowing time (Christensen et al., 1985; Öztürk, 2000; Saran and Giri, 1987). Similar results were obtained in this study. Yield was affected from environmental conditions such as temperature and rainfall (Beğbağa and Kaya, 2008).

Indican Contents

All leaf samples harvested in July 2010 and stored at -80ºC until extraction experiments. ASE (and ultrasonication methods were used for extraction trials. Aqueous extracts of woad leaf materials and samples of indican standard were analysed using HPLC-DAD. The traces obtained from this type of analysis showed

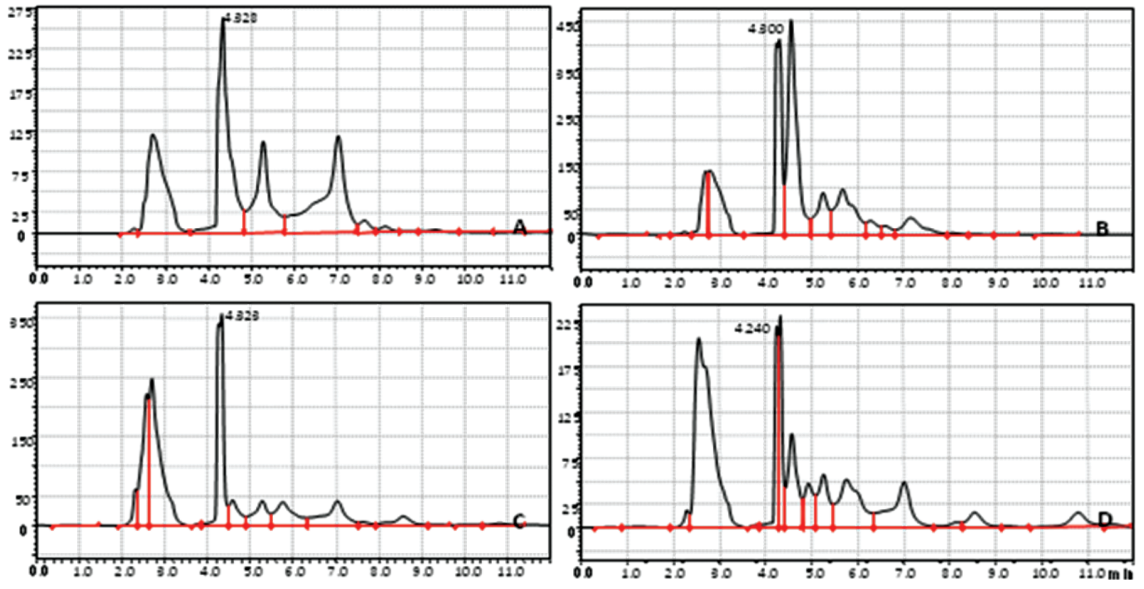

Figure 2. HPLC-DAD results acquired from ASE Extraction. The indican peak was identified at tR 4.3. (A) *I. tinctoria*, (B) *I. buschiana*, (C) *I. tinctoria* subsp.*corymbosa*, (D) *I. candolleana.*

Şekil 2. ASE Ekstraksiyonundan elde edilen HPLC-DAD kromatogramları. 4.3'uncu dakikada çıkan pik indikan olarak belirlenmiştir (A) I. tinctoria, (B) I. buschiana, (C) I. tinctoria subsp.corymbosa, (D) I. candolleana

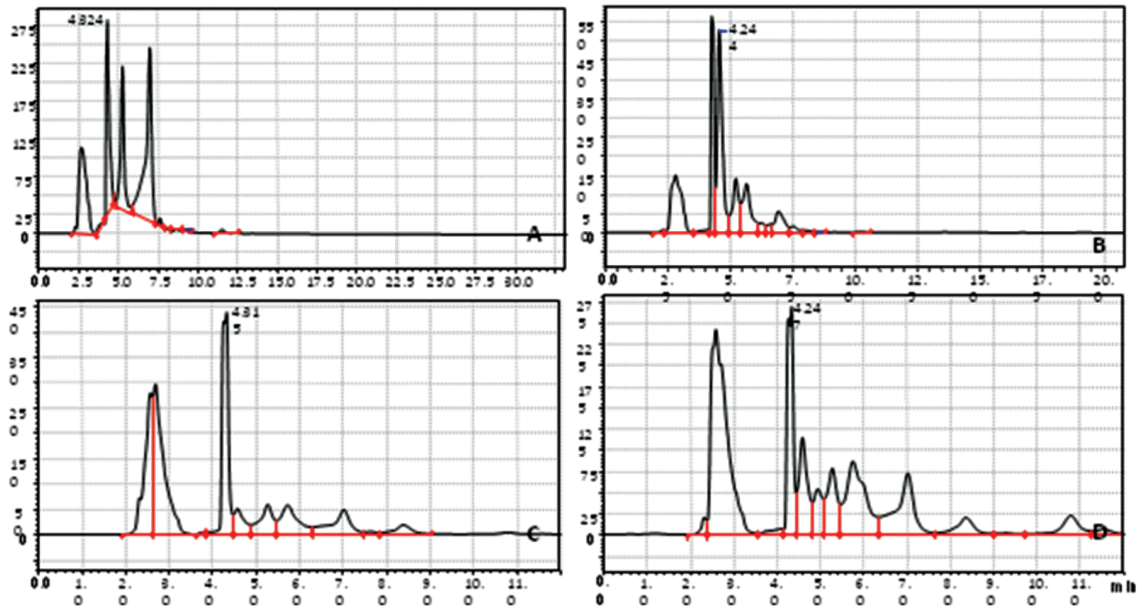

Figure 3. HPLC-DAD results acquired from ultrasonication. The indican peak was identified at tR 4.3. (A) *I. tinctoria*, (B) *I. buschiana*, (C) *I. tinctoria* subsp. *corymbosa*, (D) *I. candolleana.*

Şekil 3. Ultrasonik ekstraksiyondan elde edilen HPLC-DAD kromatogramları. 4.3'uncu dakikada çıkan pik indikan olarak belirlenmiştir (A) I. tinctoria, (B) I. buschiana, (C) I. tinctoria subsp.corymbosa, (D) I. candolleana.

that indican was present in all plant extracts, identified by direct comparison of their retention times with the standard compounds. HPLC-DAD data indicated that all *Isatis* spp. contain indican. The results of HPLC analysis are given in Figure 2 and 3.

The second peak is determined as indican, with a retention time (tR) of 4.3 min by using ASE and Ultrasonic extraction methods (Figure 2 and 3, respectively). Extracts obtained from ASE and ultrasonication gave very similar peaks in the chromatograms. Same solvent used in extraction systems may be the reason of the identical chromatograms. Indican has extracted using various solvents. Ethyl acetate, acidified acetone, methanol and water were reported as extraction solvents (Zhou et al. 2007) Extraction methods used in this study reduced the time in extraction and enhanced the efficiency of extraction. The concentrations of indican are shown in Table 5.

Ultrasonication resulted the highest indican concentrations in *I. buschiana, I. tinctoria* subsp. *corymbosa* and *I. candolleana*, while ASE was found to be more efficient for *I. tinctoria* (Table 4). The indican amounts differed in terms of species and extraction techniques. This variation may be related to species, cultivation methods and harvest time (Zou and Koh, 2007).

Kızıl (2000), Akar (2006) and Campeol et al. (2006), investigated *I. tinctoria* for its indican content and they reported that indican concentrations were determined as 0,034, 11.34 and 1.2-6.0 mg g^{-1}, respectively. The young and old leaves of *I. tinctoria* contained 5.94 mg g^{-1} and 3.90 mg g^{-1} of indican (Kokubun et al., 1998). Indican concentrations of *I. tinctoria* was reported as 0.45, 0,50 and 0,38 mg g^{-1} in 2001, 2002 and 2003, respectively (Angelini et al., 2007). The indican concentrations extractedboth from ASE and Ultrasonication in this study were higher than these results (Kokubun et al., 1998; Kızıl, 2000; Akar, 2006). Environmental conditions influenced the production of indigo precursors in *I. tinctoria* (Campeol et al., 2006).

Conclusions

This work represents the indican values from *I. buschiana, I. tinctoria, I. tinctoria* subsp. *corymbosa* and *I. candolleana.* It is the first assay that determines indican content and potential of indigo production in wild *Isatis* species. Both ultrasonication and ASE extraction are generated selective and sensitive resultsin HPLC. However, ultrasonication enhanced the efficiency of extraction. Hence, it could be suggested that ultrasonication was more accurate extraction method for *Isatis* spp. In this study, it was observed that *Isatis* spp. grew slowly in the trial field and they found to be weak competitors against weeds. Therefore they should be planted in a well-prepared field, and weeds must be removed continuously. It is important to use herbicide for cultivation in large areas. The variation of leaf yield between sowing dates could be affected by the difference in plant output rate. Plant output rates decreased with the delay in sowing time, therefore the yield reduced in parallel with the decrease in plant number (%). Because of vernalisation requirement of the plants, plant output rates in spring sowings were especially lower than autumn sowings in native *Isatis* spp. The poor germination could be a result of seed dormancy. In this study, high rainfall and low temperature influenced the plant output and development negatively.

In conclusion, output times differed according to species and sowing time. The highest leaf yield was obtained in February and March sowings in *I. tinctoria*. In contrast, autumn sowings gave the highest yield in wild type *Isatis* species. The maximum leaf length was obtained in *I. buschiana* and the minimum leaf length was determined in *I. candolleana.* The highest leaf width was determined in *I. buschiana* and *I. candolleana.*

Acknowledgement

This paper was prepared by using a part of Nazan ÇÖMLEKCİOĞLU's PhD thesis results.

Table 5. Amounts of indican (mg g^{-1}) extracted by ASE and ultrasonication

Çizelge 5. ASE ve ultrasonikasyon ile ekstrakte edilen indikan miktarları (mg g^{-1})

	ASE	Ultrasonication
I. tinctoria	22.0 ± 0.25	18.2 ± 0.21
I. tinctoria subsp. *corymbosa*	18.7 ± 0.33	21.4 ± 0.27
I. candolleana	10.1 ± 0.19	15.2 ± 0.18
I. buschiana	20.4 ± 0.22	23.5 ± 0.24

References

Akar D., 2006. Investigations of dyeing properties and dyestuff contents of some *Isatis* (woad) species growing as natural from East Mediterranean region. Master Thesis. K.S.U. Institute of Science. Kahramanmaras

Angelini L.G., Tozzi S. and Nassi N., 2007. Differences in leaf yield and indigo precursors production in woad (*Isatis tinctoria* L.) and Chinese woad (*Isatis indigotica* Fort) genotypes. Field Crops Research, 101: 285-295

Beğbağa M. and Öztürk Ö., 2008. The effects of different sowing dates on the yield, yield components and quality of some winter rapeseed varieties under agean region conditions. Selcuk University Journal of the Faculty of Agriculture, 22 (44): 84-98

Campeol E., Angelini L., Tozzi S. and Bertolacci M., 2006. Seasonal variation of indigo precursors in *Isatis tinctoria* L. and *Polygonum tinctorium* Ait. as affected by water deficit. Environmental and Experimental Botany, 58: 223-233

Christensen J.V., Legge W.G., Depauw R.M., Hennig A.M.F., Mckenzie J.S., Siemens B. and Thomos J.B., 1985. Effect of seeding date, nitrogen and phosphate fertilizer on growth, yield and quality of rapeseed in Northwest Alberta. Canadian Journal of Plant Science, 65: 275-284

Comlekcioglu N., 2011. Kahramanmaraş'ta yayılış gösteren bazı *Isatis* spp. (çivitotu) türlerinde farklı ekim zamanlarının verim unsurlarına etkisi ile boyama özellikleri ve boyarmadde miktarının saptanması. PhD Thesis. K.S.U. Institute of Science. Kahramanmaras

Comlekcioglu N., Efe L. and Karaman S. 2013. Comparison of different extraction methods for the determination of indican precursor from four *Isatis* spp. by HPLC-UV. EJBS, 7(1): 21-26

Davis P.H., 1982. Flora of Turkey and The East Aegean Islands, Edinburg at the University Press, I: 287-366

Frechard A., Fabre N., Pean C., Montaut S., Favvel M., Rollin P. and Fauraste L., 2001. Novel indole-type glucosinolates from woad (*Isatis tinctoria* L). Tetrahedron Letters, 42(51): 9015-9017

Gilbert G.K. and Cooke D.T., 2001. Dyes from plants: Past usage, present understanding and potential. Plant Growth Regulation, 34: 57-69

Hamburger M., 2002. *Isatis tinctoria* – from the rediscovery of an ancient medicinal plant towards a novel anti-inflammatory phytopharmaceutical. Phytochemistry Reviews, 1: 333-344

Han J., Jiang X. and Zhang L., 2011. Optimisation of extraction conditions for polysaccharides from the roots of *Isatis tinctoria* L. by response surface methodology and their *in vitro* free radicals scavenging activities and effects on IL-4 and IFN-γ mRNA expression in chicken lymphocytes. Carbohydrate Polymers, 86 (3): 1320-1326

Kızıl S., 2000. Investigations on determination of suitable sowing density and dyeing properties of some woad species (*Isatis tinctoria* L., *Isatis constricta* Davis). PhD Thesis. Ankara University. Institute of Science. Ankara

Kızıl S. and Kayabaşı N., 2005. A study on the determination of dyeing properties of weld (*Reseda lutea* L.). Akdeniz University Journal of the Faculty of Agriculture, 18(2): 195-200

Kızıl S., 2006. Morphological and agronomical characteristics of some wild and cultivated *Isatis* species. Journal of Central European Agriculture, 7(3): 479-484

Kokubun T., Edmonds J. and John P., 1998. Indoxyl derivatives in woad in relation to medieval indigo production. Phytochemistry, 49 (1): 79-87

Mohn, T., Plitzko, I. and Hamburger, M. 2009. A comprehensive metabolite profiling of *Isatis tinctoria* leaf extracts. Phytochemistry, 70(7): 924-934

Oberthür C., Graf H. and Hamburger M., 2004. The content of indigo precursors in *Isatis tinctoria* leaves – a comparative study of selected study of selected accessions and post-harvest treatments. Phytochemistry, 65: 3261-3268

Oberthür C., Jäggi R. and Hamburger M., 2005. HPLC based activity profiling for 5-lipoxygenase inhibitory activity in *Isatis tinctoria* leaf extracts. Fitoterapia, 76 (3): 324-332

Öztürk Ö., 2000. Effects of different sowing dates and row spacings on the yield, yield components and quality of some winter rapeseed varieties. PhD Thesis. Selçuk University, Department of Field Crops. Konya

Pawlak K., Puchalska M., Miszczak A., Rosloniec E. and Jarosz M., 2006. Blue natural organic dyestuffs-from textile dyeing to mural painting. Separation and characterization of coloring matters present in elderberry, lowood and indigo. Journal of Mass Spectrometry, 41: 613-622

Rocha L., Carvalho C., Martins S., Braga F. and Carnide V., 2011. Morpho-agronomic characterization and variation of indigo precursors in woad (*Isatis tinctoria* L.) accessions. Plant Genetic Resources, 9(2): 206-209

Sales E., Kanhonou R., Baixauli C., Giner A., Cooke D., Gilbert K., Arrilaga I., Segura J. and Ros R., 2006. Sowing date, transplanting, plant density and nitrogen fertilization affect indigo production from *Isatis* species in a Mediterranean region of Spain. Industrial Crops and Products, 23: 29-39

Saran G. and Giri G., 1987. Influence of dates of sowing on *Brassica* species under semi-arid rainfed conditions of North-West India. Journal of Agricultural Science, 109(3): 561-566

Spataro G. and Negri V., 2008. Assessment of the reproductive system of *Isatis tinctoria* L. Euphytica, 159: 229-231

Tansı S., 1998. The research on plant performance of woad (*Isatis tinctoria* L.) under the Cukurova conditions. Journal of Field Crops Central Research Institute, 7: 38-43

Tansı S. and Karaman S., 2005. Çukurova bölgesinde doğal olarak bulunan çivitotu (*Isatis*) türlerinin kültüre alınma olanakları ile boyarmaddelerin incelenmesi. Çukurova University, Agriculture Faculty, Department of Field Crops, Project Report ZF2000-31, pp. 10

Yildirimli S., 1988. The revision of the genus *Isatis* L. (Cruciferae) in Western half and North of Turkey. Doğa TU J Botany, 12(3): 332-400

Zou P. and Koh H.L., 2007. Determination of indican, isatin, indirubin and indigotin in *Isatis indigotica* by liquid chromatography /electrospray ionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 21: 1239-1246

Zhou L.H., Zheng T.L., Wang X., Ye J.L., Tian Y. and Hong H.S., 2007. Effect of five Chinese traditional medicines on the biological activity of a red-tide causing alga-*Alexandrium tamarense*. Harmful Algae, 6: 354-360