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PALYNOLOGY OF TAXA BELONGING TO *ANTHEMIS* L., AND *COTA* J. GAY GENERA THAT GROW AT ÇANAKKALE PROVINCE

Sunay ALTAN[a*], Hanife AKYALÇIN[b]

[a]*Çanakkale Onsekiz Mart University, Faculty of ScienceandArts, Department of Biology, Çanakkale,Turkey.*

[b]*Çanakkale Onsekiz Mart University, Institute of Natural &AppliedSciences, Çanakkale, Turkey.*

***Corresponding co-authors**: sunayaltan@hotmail.com

ABSTRACT

Pollen morphology of Anthemis L. and Cota J. GAY taxa that were collected from various localities at Çanakkale province in 2015 are examined in this article. Wodehouse (1935) and Asetoliz (1960) methods were used in this study. Pollen morphologies of 5 taxa (2 species and 1 subspecies of Anthemis L. genus and 1 species and 1 variety of Cota J. GAY genus) were identified according to LM and SEM. Pollen grains are monad, tricolporate, isopolar and they have radial symmetry under light microscope. Pollen grains are in the shape of suboblate and oblatae-spheroidae. Amb are in the shape of semiangular-circular and interhexagonal-subtriangular. Ornamentation of Anthemis L. and Cota J. GAY are echinate-reticulate under light micrograph. Also, in SEM micrograph, ornamentation of Anthemis L. and Cota J. GAY genera are echinate-microperforate. Morphological properties of pollen grains belonging to Anthemis L. and Cota J. GAY have some similarities. Sexine2 is more apparent in pollen grains of Cota genus. Base length of spine is longer than spine length in both genera. In addition, exines of Anthemis L. and Cota J. GAY prominently have laminar structure. Palynological properties of Anthemis auriculata Boiss., Anthemis tomentosa L. subsp. tomentosa taxa are examined for the first time in this study. Also, according to type records in Flora of Turkey, Anthemis cotula L. is collected from Çanakkale for the first time.

***Keywords:**, Asteraceae, Anthemis L., Cota J. GAY, Pollen morphology, Çanakkale, Turkey.*

1. INTRODUCTION

Asteraceae family has wide distribution around of the world except for Anthartica. Among the flowering plants, it is in the first place in terms of diversity with more than 1.100 genera and more than 20.000 species. There are perennial and annual species exist in this family.The most characteristic of this family is the flower structure of the flower called the

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capitulum (Yıldız & Aktoklu, 2010). Plants mostly locate in mountain vegetation, open meadowlands and glades. They are found fewer in the moisture tropical forests (Kadereit & Jeffrey, 2007).

Pollen morphologies of *Anthemis cotula* L., *Anthemis auriculata* Boiss., *Anthemis tomentosa* L. subsp. *tomentosa, Cota tinctoria* (L.) J. GAY ex Guss. var. *tinctoria* and *Cota altissima* (L.) J. GAY belonging to Asteraceae family that grow at Çanakkale province were examined comprehensively in this study. *Anthemis* L. which is number 42 genus of Anthemideae tribe is in Asteraceae family. *Anthemis* L. has 80 taxa under 3 sections (Anthemis, Maruta, Cota) that are registered in 5th volume of Flora of Turkey (Davis, 1975). *Anthemis karacae*, *Anthemis kotschyana* var. *gypsicola* and *Anthemis kotschyana* var. *kotschyana* endemic species were added to *Anthemis* genus at 11th volume (additional volume 2) (Güner et al., 2000). *Cota* section of *Anthemis* genus was enhanced the level of genus and was called as *Cota* genus at List of Turkish Plants. For this reason, *Anthemis tinctoria* L. var. *tinctoria* was specified as *Cota tinctoria* (L.) J. GAY ex Guss. var. *tinctoria* and *Anthemis altissima* was specified as *Cota altissima* (L.) J. GAY. This genus has 17 species and 22 taxa (Güner et al., 2012).

Punt & Hoen (2009) described their studied species belonging to *Anthemis* genus as ‘‘Anthemis arvensis’’ type. Measurements belonging to *Anthemis arvensis*, *Anthemis austriaca, Anthemis tinctoria* species and LM-SEM micrographs belonging to *Anthemis arvensis* were published. Vezey et al. (1994) described pollen types according to ornamentation and internal exine elements of 45 pollen grains in Anthemideae (Asteraceae) tribe and identified Anthemoid pollen type. Oberprieler (1998) has also examined pollen characteristics in his study about systematics of North Africa *Anthemis.* It was investigated pollen morphology of 235 taxa belonging to Asteraceae family by Stix (1960). Pollen grains belonging to these taxa have been classified into 45 pollen types according to their exine characteristics. Erdtman et al. (1961) has evaluated Asteraceae family into 9 pollen types and examined *Anthemis* genus in Anthemis (Achillea) type. Pollen grains were described as echinate for *Anthemis* genus. Skvarla & Turner (1966) has showed *Anthemis* genus in Anthemideae according to pollen wall morphology at tribal level. Same researchers explained pollen morphology and exine structure of *Anthemis nobilis* L. species in their study with light and electron microscope in 1971. Wodehouse (1935) has formed morphological keys of pollen of some taxa belonging to Anthemideae tribes and specified characteristics of pollen grains. İnceoğlu & Karamustafa (1997) have presented LM measurements of pollen morphology of various genus belonging to Compositae family in their studies.

Özbek et al. (2016) investigated pollen morphological properties of 22 taxa of *Cota* (Asteraceae) using LM and SEM which is naturally distributed in Turkey. Koyuncu et al. (2013) were studied *Cota fulvida* (Grierson) Houlb. which is rare endemic for Turkey and collected from Türkmen Mountain in Eskişehir province.

The purpose of this study is to examine detailed pollen morphologies of *Anthemis* and *Cota* taxa belonging to Asteraceae family that grow at Çanakkale province, to create data for systematic studies including these taxa and for preparing Pollen Atlas of Turkish Plants.

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2. MATERIALS AND METHODS

Flowering plant samples of *Anthemis* and *Cota* species were collected in 2015 in Çanakkale province by field trips from different localities (Table 1).

Plant samples were dried conveniently and made into herbarium specimens. Pollen grains taken from these samples were used for preparing pollen slides. Plant samples were identified by Dr. Bayram YILDIZ and Assoc. Prof. Dr. Gül KUŞAKSIZ (Uludağ University Faculty of Arts and Science, Department of Biology). Plant herbarium samples are kept at ÇOMU, Faculty of Arts and Science, Department of Biology, Palynology Laboratory and Ege University, Faculty of Pharmacy, IZEF Herbarium.

The pollen slides for LM and SEM were made by using Wodehouse (W) (1935) and Asetholize (E) (Erdtman, 1960) methods .The measurements of pollen grains were made with LEICA 2500 DM Light Microscope. Also microphotographs of pollen grains were taken by Cence 2.0 MP microscope camera. Immersion oil, ocular 10X and lens 100X were used at light microscope measurements (exclude spines). By the light microscope, measurements were made with average on 50 sample for each taxa excluding spines. The polar axis (P), equatorial axis (E), P/E ratio, Meso (mesocolpium), t (Length of the one side of the triangular polar area), Amb (the length of diameter in the polar view of the pollen), Clg (Colpus length), Clt (Colpus width), Plt (endoaperture pore diameter) Nexine, Sexine1, Sexine3, Sbwe (spine base length equatorial view), Sbwp (spine base length polar view), Sle (spine length equatorial view), Slp (spine length polar view), An (aperture number) of the pollen grains belonging to *Anthemis* and *Cota* were measured by LM.

For electron microscopy studies, the pollen was placed on a stap with double-sided adhesive tape according to the Acetolysis method (Erdtman, 1960). The stubs were covered with gold. JEOL SM 7100F SEM located at Çanakkale Onsekiz Mart University Science and Technology Application and Research Center (ÇOBİLTUM) were used in SEM studies. Surface ornamentation, polar and equatorial views, and spin characteristics of pollen grains were studied detailed and their micrographs were taken. SLE (spine length at equatorial), SBDE (spine base diameter at equatorial), FPSE (flat part of spine at equatorial), PWSBE (pore width of spine base at equatorial), DBSE (distance between spins at equatorial), SLPA (spine length at polar area), SBDPA (spine base diameter at polar area), FPSPA (flat part of spine at polar area), PWSBPA (pore width of spine base at polar area), DBSPA (distance between spins at polar area), SN (spine number per 100 μm^2) characteristics of pollen grains at SEM micrographs were measured by Image J 1.36b.

The mean, standard deviation and min-max values of measurements belonging to LM views were performed by IBM SPSS Statistics 22. The pollen grains terminology follows mainly Punt & Hoen (2009), Punt et al. (2007), Faegri & Iverson (1992), Moore & Webb (1983), Erdtman (1943, 1960, 1969), Skvarla & Turner (1966, 1971). The pollen slides were deposited in the Palynology Laboratory of Çanakkale Onsekiz Mart University, in Turkey.

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Table 1. Plant samples collected from different localities of Çanakkale province.

Taxa	Localities	The date collected	Collected by	Identified by
A. cotula	Çanakkale Science High School, Çınarlı Village-Çanakkale, 35447402 D. 4435386 K., 98 m.	07.06.2015	Hanife AKYALÇIN Sunay ALTAN	Bayram YILDIZ
A. auriculata	Dümrek Village -Çanakkale, 35445310 D. 4426339 K., 81m.	11.06.2015	Hanife AKYALÇIN Sunay ALTAN	Bayram YILDIZ
A. tomentosa subsp. *tomentosa*	Dümrek Village -Çanakkale, 35444674 D. 4426026 K., 92 m.	11.06.2015	Hanife AKYALÇIN Sunay ALTAN	Bayram YILDIZ
C. tinctoria var. *tinctoria*	Çomü Terzioğlu Campus-Çanakkale, 35450261 D. 4440328K., 76 m.	05.06.2015	Hanife AKYALÇIN Sunay ALTAN	Gül TARIMCILAR
C. altissima	Dümrek Village -Çanakkale, 35443765 D. 4426584 K., 129 m.	11.06.2015	Hanife AKYALÇIN Sunay ALTAN	Bayram YILDIZ

3.RESULTS

The main palynological features of the species examined in this study are summarized in Tables 2–5 and shown in Figures1-5.

3.1. The Palynological Characteristics of *Anthemis* L.

The pollen grains are radial symmetry, monad and isopolar in 3 taxa of belonging to *Anthemis* genus. All taxa are in the shape of oblate spheroid at slides prepared according to Wodehouse and Erdtman methods. They have tricolporate aperture. The shape of amb is semiangular-circular in pollen grains but their equatorial view is circular. Ornamentation is echinate-reticulate according to studies made with LM. The mean ratio of P/E is between 0.89-0.90 (E) and 0.90-0.98 (W). Values of equatorial axis belonging to pollen grains are between 19-26 (W) and 21-25 (E). Polar axis values of pollen grains are between 18-23 (W) and 19-22 (E). The pollen grains are mostly small (Table 2). Operculum is located on pore in examined pollen slides (Fig. 2; 2, 10) and these pores are seemed as circular elliptic. Pore diameter can be changed between 5.08-5.33µm with Acetolysis methods (E) and 5.15-5.54 µm with Wodehouse methods (W). Nexine thickness is between 0.99-1.06 µm (E), 0.96-0.97 µm (W). Sexine has a laminar structure. Sexine1 has 2.08-2.12 µm (E), 1.90-2.66 µm (W) thickness and sexine3 has 0.45-0.56 µm (E), 0.50 µm (W) thickness. Sexine2 thickness cannot be measured because of its extra thin structure (Table 2). Observations made by LM are given at Table 2.

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According to palynological studies made with SEM, ornamentation is echinate and tectum surface (interspine area) is microperforate structure. Perforations range from dense to light in region between spines. Perforations are not homogenius (Fig. 3). Exine structure (Fig. 1, 2, 3) can be distinguished specifically as nexine, sexine1, sexine3 and sexine4 (spines included). The ends of the spines are acute or convoluted and half of spines or more have smooth surface. The appearance of spine ends are perpendicular or curved to different directions. The perforations at spine base are usually bigger and more irregular (Fig. 3). Measurements made by SEM micrographs are given at Table 3. Mean spine number at polar region of taxa belonging to *Anthemis* genus varies from 4.60 to 7.00 per 100 μm^2 (Fig. 3; 1, 5, 6, 9).

3.2. The Palynological Characteristics of *Cota* J. GAY

The pollen grains are radial symmetry, monad and isopolar in 2 taxa of *Cota*. All taxa are in the shape of oblate spheroid at slides prepared according to Wodehouse and Erdtman methods. They have tricolporate aperture. The shape of amb is semiangular-circular in pollen grains but their equatorial view is circular. Ornamentation is echinate-reticulate according to studies made with LM. The mean ratio of P/E is between 0.92-0.99 (E) and 0.95-0.98 (W). Values of equatorial axis of pollen grains are between 20-27 (W) and 22-26 (E). Polar axis values of pollen grains are between 20-26 (W) and 20-26 (E). The pollen grains are mostly small. Operculum is located on pore at examined slides and these pores are seemed as circular elliptic. Pore diameter can be changed between 4.87-5.37µm with Erdtman methods (E) and 4.90-5.28 µm with Wodehouse methods (W). Nexine thickness is between 1.00-1.08 µm (E), 1.00-1.01 µm (W). Sexine has a laminar structure. Sexine1 has 2.00-2.60 µm (E), 1.82-2.64 µm (W) thickness and sexine3 has 0.50-0.58 µm (E), 0.53-0.60 µm (W) thickness. Sexine2 thickness cannot be measured because of its extra thin structure (Table 4).

According to palynological studies made with SEM, ornamentation is echinate structure and tectum surface (interspine area) is microperforate structure. Perforations range from dense to light in region between spines. Perforations are not homogenius (Fig. 4). Exine structure can be distinguished specifically as nexine, sexine1, sexine2, sexine3 and sexine4 (spines included) in Figure. 4. The ends of the spines are acute or convoluted and half of spines or more have smooth surface (Fig.4; 9, 10). The appeareance of spine ends or curved to different directions. The perforations at spine base are usually bigger and more irregular. Measurements made by SEM micrographs are given at Table 5. Mean spine number at polar region of taxa belonging to *Cota* genus varies from 3.17 to 7.00 per 100 μm^2 (Fig. 4; 2, 8).

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Table 2. Pollen morphological data of *Anthemis* taxa in LM analyses

				POLAR AXIS			EQUATORIAL AXIS			MEAN OF MEASUREMENTS													
TAXA	Methods	P/E	Pollen Shape	Mean	Std. Deviation	Min-Max	Mean	Std. Deviation	Min-Max	Meso	t	amb	Clt	Clg	Plt	Nexine	Sexine1	Sexine3	Sbwe	Sbwp	Sle	Slp	An
A. cotula	E	0.89	oblatae spheroidae	20.72	0.72	20-22	23.24	1.04	22-25	13.04	11.88	22.10	6.16	13.26	5.18	1.05	2.08	0.56	3.86	3.38	3.27	3.09	3
	W	0.98	oblatae spheroidae	20.57	0.97	19-23	20.98	1.05	19-24	13.35	9.40	21.14	6.25	13.30	5.15	0.97	1.90	0.50	3.67	3.54	3.33	3.33	3
A. auriculata Boiss.	E	0.90	oblatae spheroidae	19.91	0.68	19-21	22.04	0.86	21-24	12.74	10.02	20.12	6.08	12.52	5.08	0.99	2.08	0.45	3.9	3.52	3.38	3.20	3
	W	0.92	oblatae spheroidae	19.96	0.44	18-21	21.74	0.98	20-25	13.07	9.53	21.37	6.55	13.50	5.54	0.97	1.94	0.50	3.43	3.34	3.31	3.18	3
A. tomentosa subsp. *tomentosa*	E	0.89	oblatae spheroidae	20.52	0.70	20-22	22.94	0.84	22-24	12.87	10.36	21.42	6.29	13.66	5.33	1.06	2.12	0.56	3.88	3.66	3.22	3.16	3
	W	0.90	oblatae spheroidae	20.41	0.80	19-22	22.71	0.90	22-26	13.86	9.51	21.51	6.73	13.8	5.25	0.96	2.66	0.50	3.10	3.25	3.51	3.20	3

Meso, mesocolpium; t, length of the one side of the triangular polar area; Amb, the length of diameter in the polar view of the pollen; Clt, colpus width; Clg, colpus length; Plt, endoaperture pore diameter; Sbwe, spine base length equatorial view; Sbwp, spine base length polar view; Sle, spine length equatorial view; Slp, spine length polar view; An, aperture number, all measurements in µm.

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Table 3. Ornamentation and spine measurements of *Anthemis* species in SEM analyses.

TAXA	SLE	SBDE	FPSE	PWSBE	DBSE	SLPA	SBDPA	FPSPA	PWSBPA	DBSPA	SN	Ornamentation
	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Mean	
A. cotula	2.36-4.30	2.19-3.71	1.38-1.71	0.18-0.51	0.74-1.81	2.08-4.43	2.22-3.78	1.49-1.69	0.18-0.51	0.50-1.73	7	Echinate-microperforate
A. auriculata	2.03-4.38	2.74-3.87	1.38-2.31	0.15-0.59	0.59-1.84	2.10-3.70	2.79-3.66	1.23-2.01	0.17-0.39	0.82-1.81	5.7	Echinate-microperforate
A. tomentosa subsp. *tomentosa*	2.13-3.97	2.08-3.93	1.28-2.17	0.14-0.45	0.77-1.66	2.29-3.84	2.89-3.80	1.13-1.97	0.14-0.42	0.86-1.39	4.6	Echinate-microperforate

SLE, spine length at equatorial; SBDE, spine base diameter at equatorial; FPSE, flat part of spine at equatorial; PWSBE, pore width of spine base at equatorial; DBSE, distance between spins at equatorial; SLPA, spine length at polar area; SBDPA, spine base diameter at polar area; FPSPA, flat part of spine at polar area; PWSBPA, pore width of spine base at polar area; DBSPA, distance between spins at polar area; SN, spine number per 100 µm² ; all measurements in µm.

Table 4. Pollen morphological data of *Cota* taxa in LM analyses.

				POLAR AXIS			EQUATORIAL AXIS			MEAN OF MEASUREMENTS												
TAXA	Methods	P/E	Pollen Shape	Mean	Std. Deviation	Min-Max	Mean	Std. Deviation	Min-Max	Meso	t	amb	Clt	Clg	Plt	Nexine	Sexine1	Sexine3	Sbwe	Sbwp	Sle	Slp	An
C. tinctoria var. *tinctoria*	E	0.92	oblatae spheroidae	21.64	1.02	20-24	23.47	0.95	22-25	13.36	11.12	21.82	6.15	13.45	4.87	1	2	0.50	3.76	3.73	3.24	3.21	3
	W	0.98	oblatae spheroidae	21.67	0.94	20-23	22.02	1.10	20-24	14.32	9.96	21.38	6.46	15.11	4.90	1	1.82	0.53	3.60	3.42	3.20	3	3
C. altissima	E	0.99	oblatae spheroidae	24.35	0.91	23-26	24.61	0.80	23-26	14.57	12.62	23.74	6.24	14.78	5.37	1.08	2.6	0.58	4.27	4.22	4.01	3.90	3
	W	0.95	oblatae spheroidae	24.04	0.97	22-26	25.25	0.68	24-27	15.10	11.56	22.68	6.59	15.1	5.28	1.01	2.64	0.6	4.08	3.94	3.98	3.90	3

Meso, mesocolpium; t, length of the one side of the triangular polar area; Amb, the length of diameter in the polar view of the pollen; Clt, colpus width; Clg, colpus length; Plt, Pore diameter; Sbwe, spine base length equatorial view; Sbwp, spine base length polar view; Sle, spine length equatorial view; Slp, spine length polar view; An, aperture number, all measurements in µm.

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Table 5.Ornamentation and spine measurements of *Cota* Species in SEM analyses.

TAXA	SLE	SBDE	FPSE	PWSBE	DBSE	SLPA	SBDPA	FPSPA	PWSBPA	DBSPA	SN	Ornamentation
	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Min-Max	Mean	
C. tinctoria var. *tinctoria*	3.24-3.55	2.89-3.44	0.18-1.85	0.15-0.34	0.85-0.92	2.24-3.49	2.61-3.39	1.10-1.99	0.14-0.36	0.64-1.02	6	Echinate-microperforate
C. altissima	3.17-4.83	3.54-4.95	2.04-2.99	0.20-0.47	0.87-1.92	—	—	—	—	—	3.2	Echinate-microperforate

SLE, spine length at equatorial; SBDE, spine base diameter at equatorial; FPSE, flat part of spine at equatorial; PWSBE, pore width of spine base at equatorial; DBSE, distance between spins at equatorial; SLPA, spine length at polar area; SBDPA, spine base diameter at polar area; FPSPA, flat part of spine at polar area; PWSBPA, pore width of spine base at polar area; DBSPA, distance between spins at polar area; SN, spine number per 100 μm^2; –,unmeasured, all measurements in µm.

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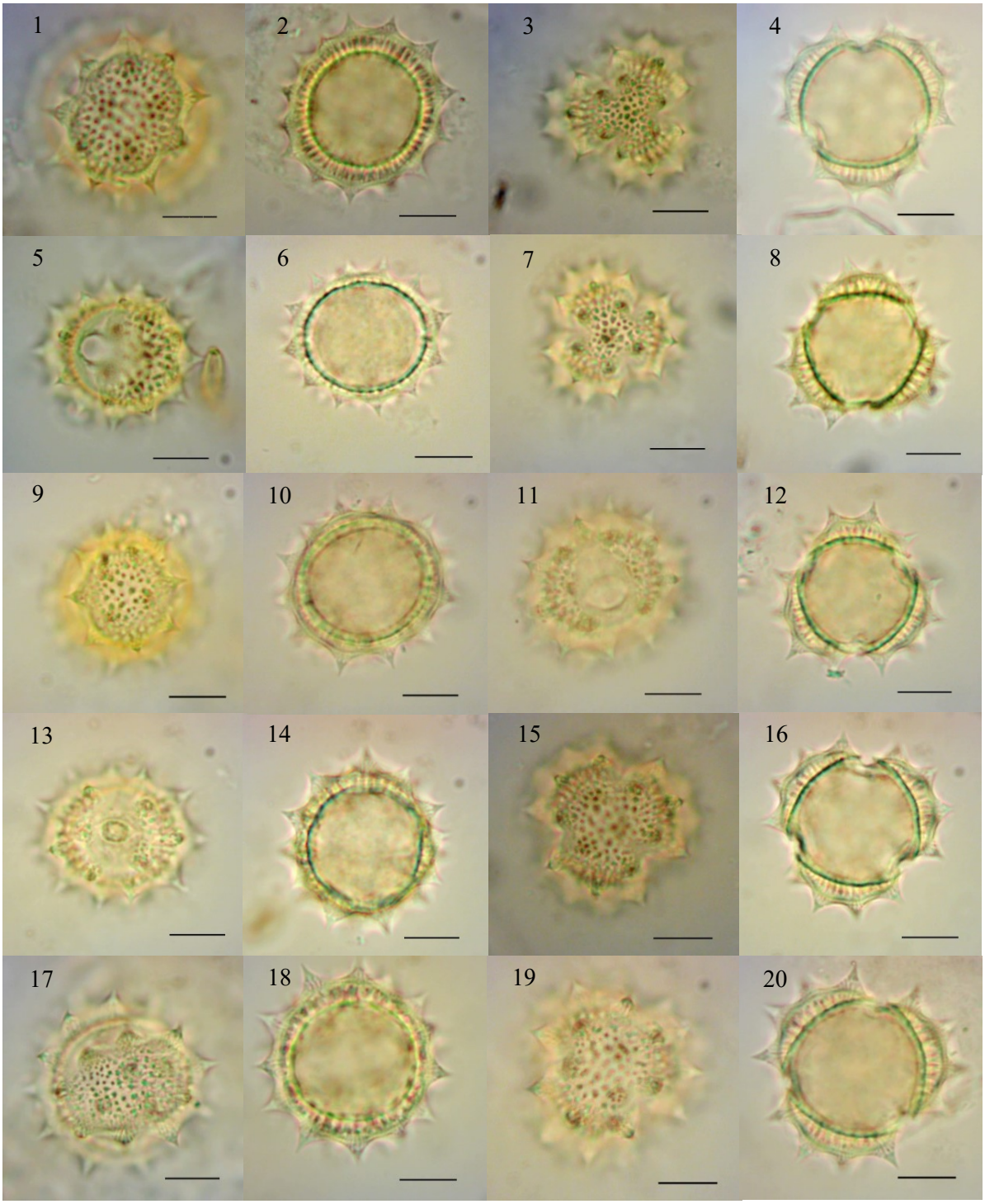

Figure 1. Pollen grains by light microscopy (E) of *Anthemis* and *Cota.* 1-4 *A. cotula*.;5-8 *A. auriculate*; 9-12 *A. tomentosa* subsp.; 13-16 *C. tinctoria* var. *tinctoria*; 17-20 *C. altissima* Scala bar 10 µm.

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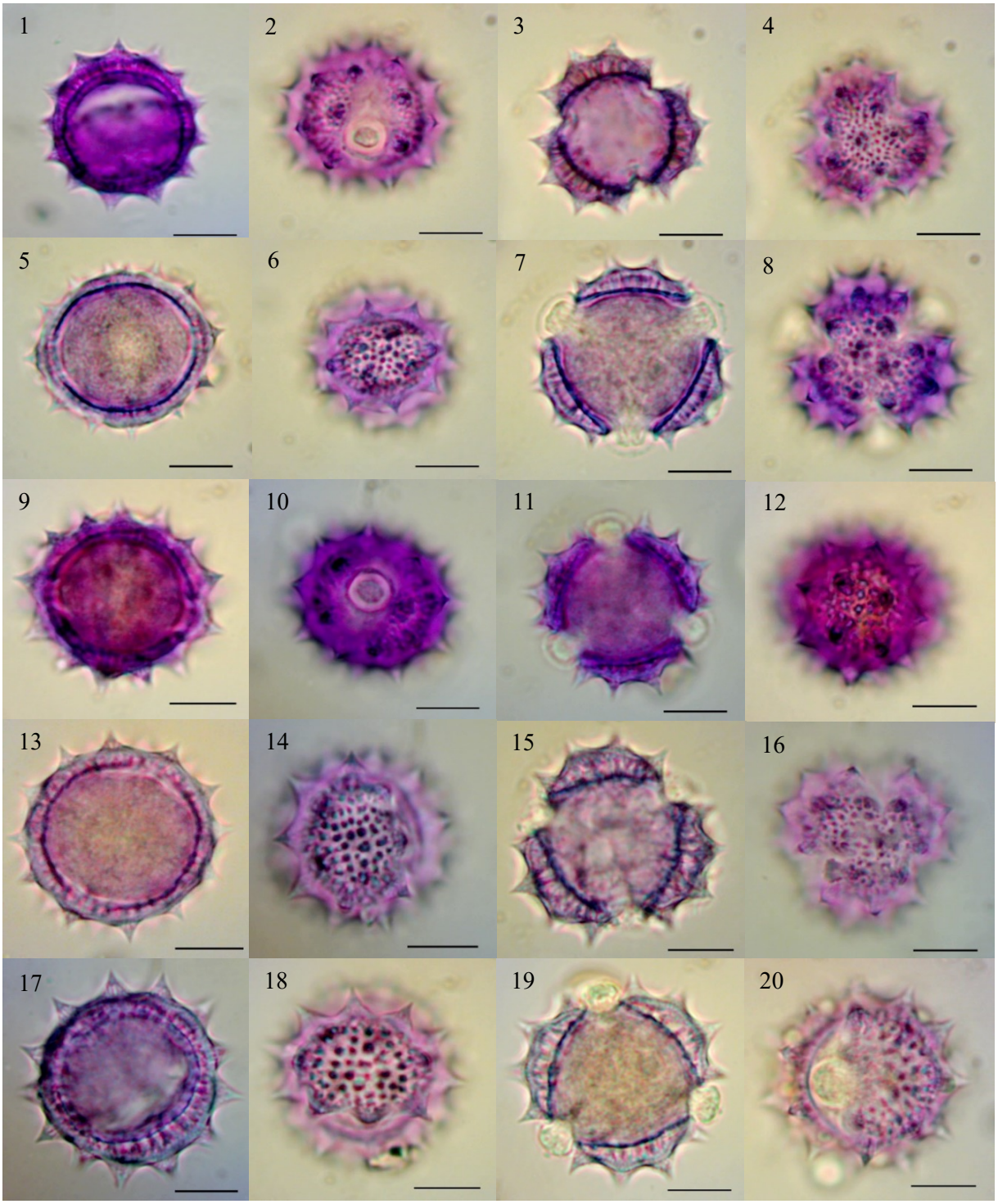

Figure 2. Pollen grains by light microscopy (W) of *Anthemis* and *Cota*. 1-4 *A. cotula*; 5-8 *A. auriculate* 9-12 *A. tomentosa subsp. tomentosa*; 13-16 *C. tinctoria* var. *tinctoria*; 17-20 *C. altissima* Scala bar 10 µm.

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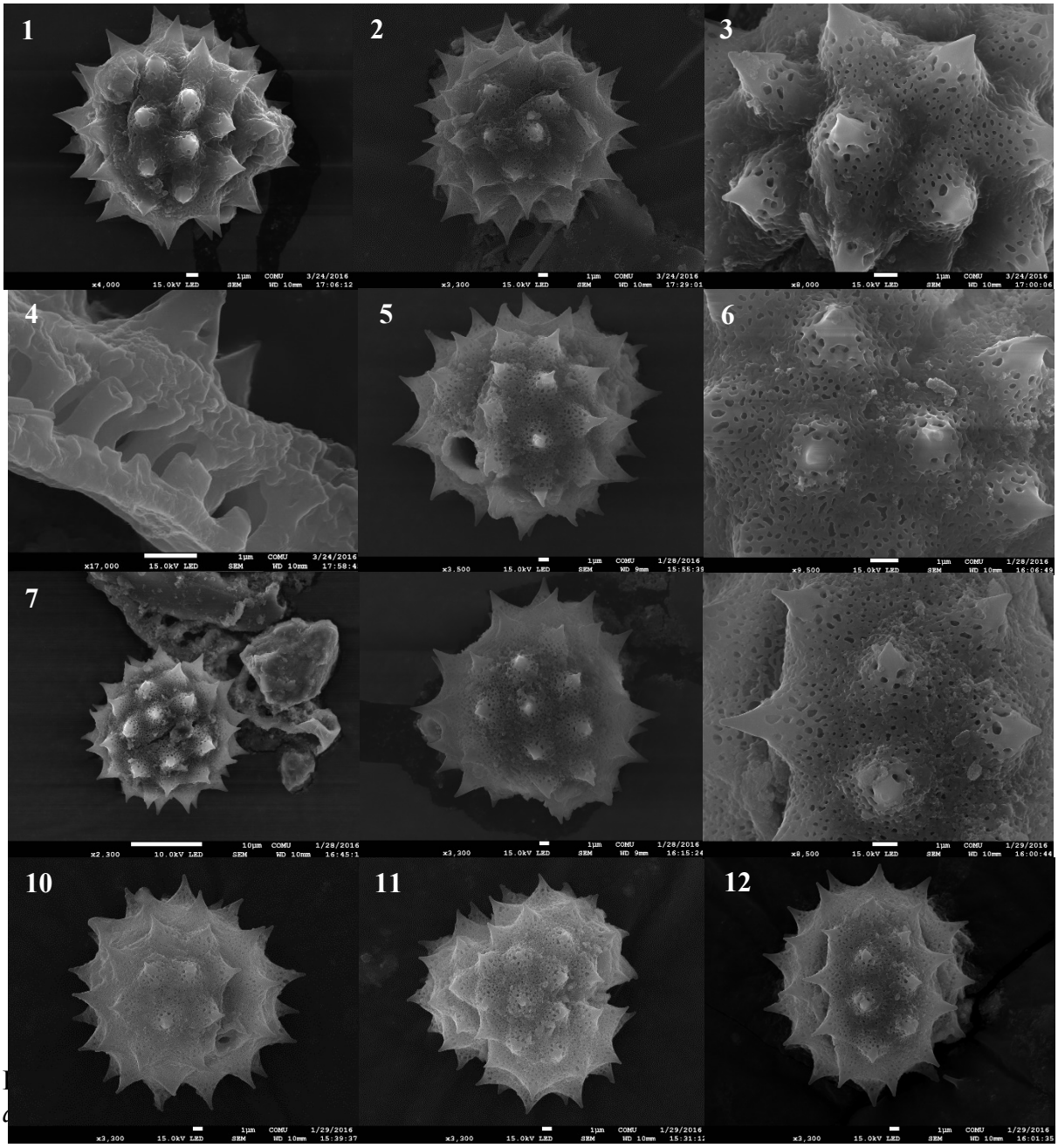

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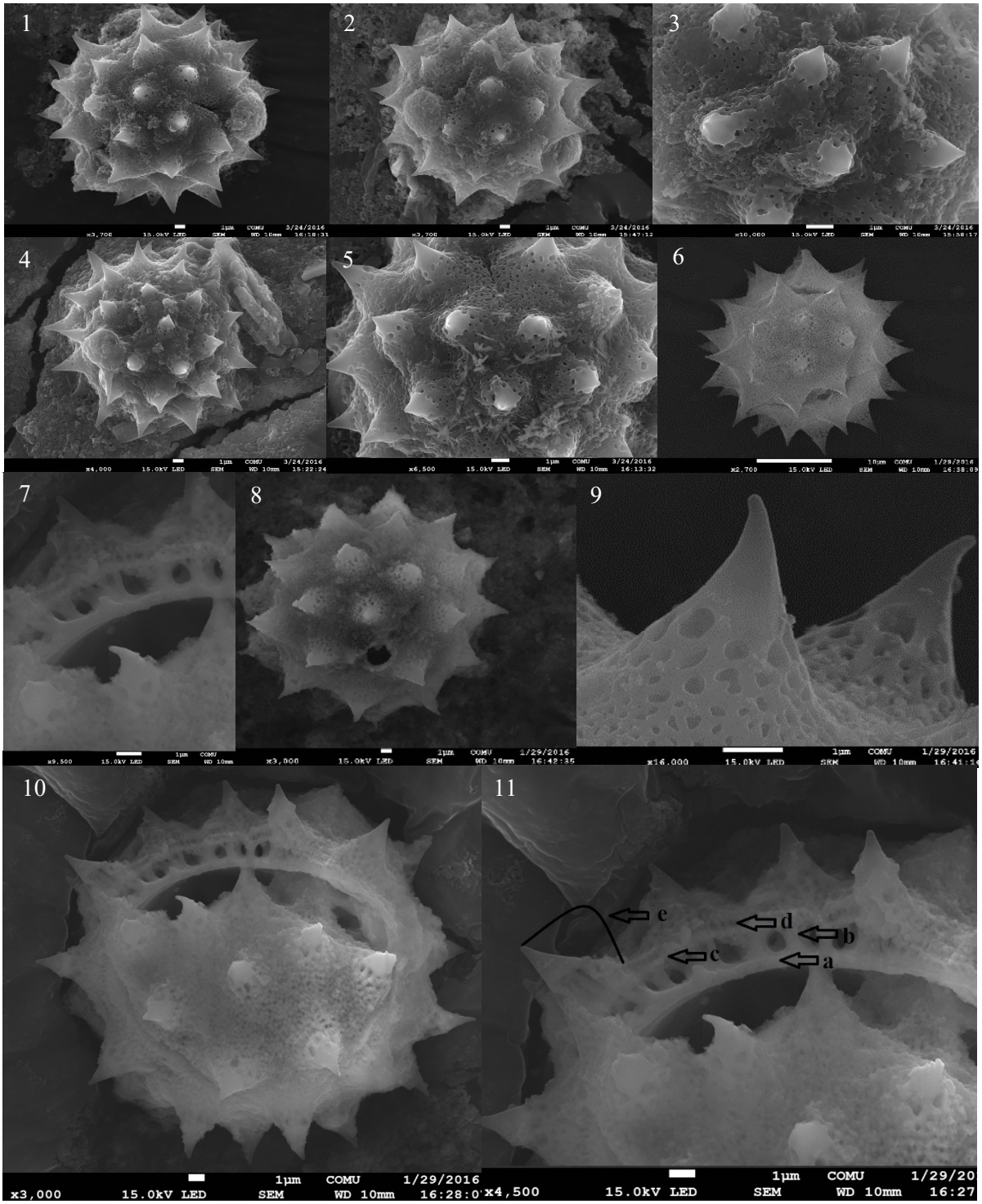

Figure 4. Pollen morphology of *Cota* by scanning electron microscopy. 1-5 *C. tinctoria* var. *tinctoria*; 6-11 *Cota altissima*, (a-foot layer and endexine, b-sexine1, c-sexine2, d-sexine3, e-sexine4).

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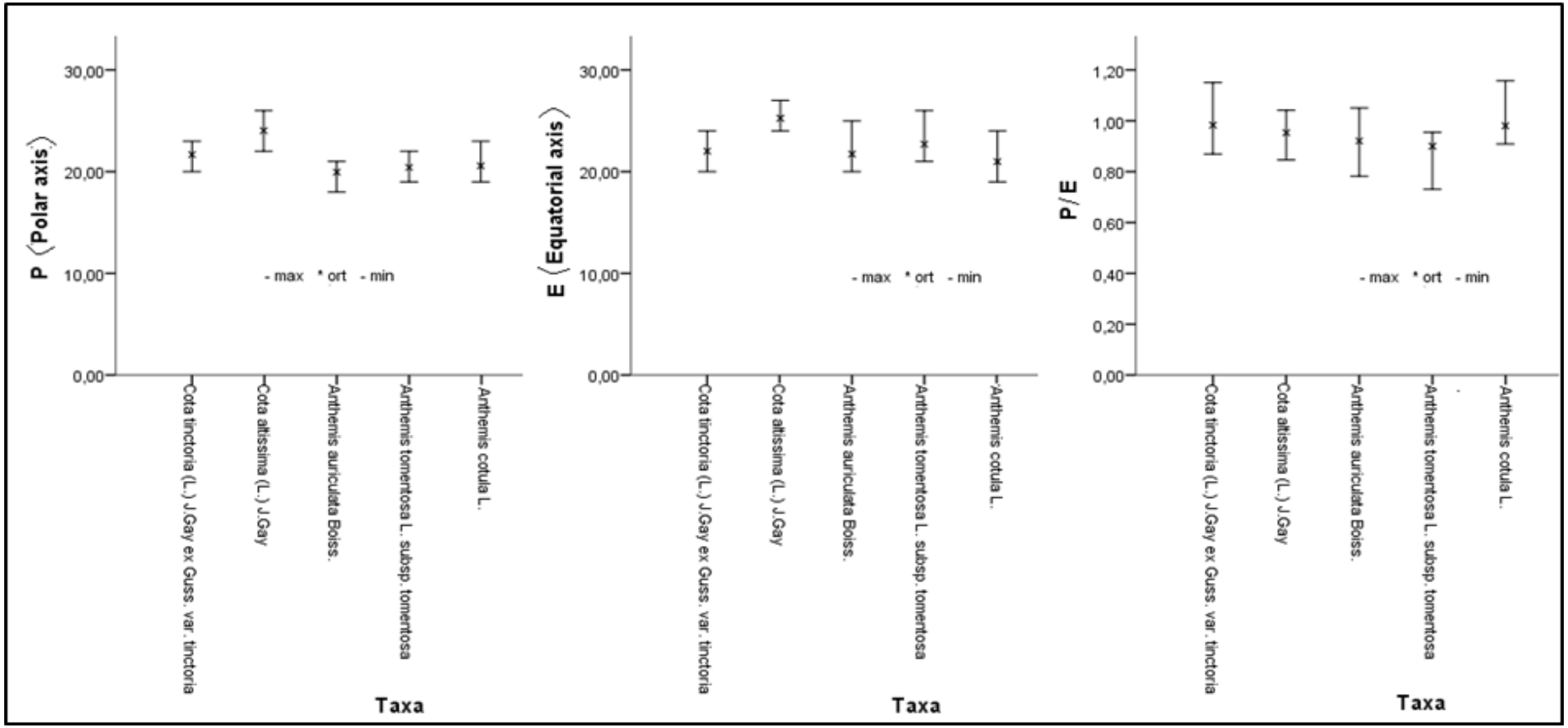

Figure 5. Polar axis, Equatorial axis and P/E value distribution of taxa examined at this study at IBM SPSS Statistics 22 by means of Wodehouse method.

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4. DISCUSSION

Pollen grains belonging to *Anthemis* are tricolporate, isopolar and they have radial symmetry. Pollen shape is oblatae-spheroidal, amb is semiangular-circular in pollen grains but their equatorial view is circular. Ornamentation is echinate-reticulate under LM.

Small variations were observed in the measurements that examined pollen grains of *Anthemis cotula* in t value, spine base width in equatorial region and spine length in polar region according to both methods (E,W) (Table 2). This species were studied and defined in Anthemoid pollen type by Vezey et al. (1994). Punt & Hoen (2009) were described *A. cotula* in ‘‘Anthemis arvensis’’ type. They specified palynological characteristics and measurements belonging to this species. According to Punt & Hoen (2009), although P mean value was 24.5 µm, E mean value was 25 µm and P/E mean value was 0.98, P mean value was observed 20.72 µm (E)-20.57 µm (W), E mean value was observed 23.24 µm (E)–20.98 µm (W), and P/E mean value was observed 0.89 (E)–0.98 (W) in our study. According to the mean value of P obtained in our measurements, the polar axis lengths are shorter than those of Punt & Hoen. Stix (1960) described *Anthemis* pollen grains as “Anthemis” type. Oberprieler (1998) presented P and E values of *A. cotula* pollen as 16.17-16.58 µm. The P and E values of *A. cotula* pollen grains collected from Çanakkale were measured as 20.72-23.24 µm. These values are higher than Oberprieler’s values (1998) in our study. Results of Punt & Hoen (2009) support our findings about *A. cotula.* According to type register in Flora of Turkey, *A. cotula* is collected from Çanakkale for the first time for this study (Davis, 1975). The pollen grains of *Anthemis airuculata* are parallel with palynological properties of the genus. No study about the pollen grains of this species could not be encountered Dauti et al. (2014) were studied *Anthemis tomentosa* in their palynological studies on *Anthemis.* The pollen morphology of *Anthemis tomentosa* subsp. *tomentosa* is similar to general characteristics of *Anthemis* based on pollen morphology. *Anthemis tomentosa* subsp. t*omentosa* was investigated at taxon level first time with this study. Also pollen morphological characteristics of *A. tomentosa* subsp. *tomentosa* are similar to those of two other *Anthemis* species studied. Wodehouse (1935), Skvarla & Turner (1971), İnceoğlu & Karamustafa (1977) and Jafari & Ghanbarian (2007) studied the palynological features of *Anthemis nobilis*, *Anthemis cretica* and *Anthemis pseudocotula* respectively. The palynological results of these researchers on *Anthemis* support our results, too.

According to light microscope measurement (E,W) of *Anthemis*, small variations were observed in pollen shape, properties of exine and aperture, spine measurement, mesocolpium and polar region (Table 2, 3; Fig 5).

In SEM micrographs of *Anthemis*, ornamentation is echinate and tectum surface (interspine area) is microperforate. Spine based perforations are usually larger and more irregular. The ends of the spines are acute or convoluted and half of spines or more have smooth surface. Appearances of spine are perpendicular or curved to different directions (Fig.3; 5, 10, 12). The min-max values belong to equatorial and polar view of pollen images by SEM were measured between spine length 2.03-4.38µm, spine base diameter 2.08-3.87 µm, flat part of spine 1.13-2.31µm, pore width of spine base 0.15-0.59 µm, distance between spines 0.50-1.84 µm, spine number per 100 µm^2 de 4.6-7 (Image J 1.36b). Measurements

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belonging to SEM micrographs about researches conducted on *Anthemis* could not encountered.

Pollen grains of *Anthemis* was described as "Anthemis type" by Erdtman et al. (1961) and Stix (1960), Anthemideae by Skvarla (1966, 1977) and ''Anthemis arvensis'' type by Punt & Hoen (2009) respectively. According to our thoughts these authors have used generally Erdtman terminology while they entitle the pollen wall structure (Exine) of *Anthemis*. Pollen morphological characteristics of *Anthemis* have generally similarities when considering the terminology based on exine structure in this study. Nexine, Sexine1, Sexine3 and Sexine4 can be especially distinguished at fracture pollen wall structure of *Anthemis* pollen grains in SEM micrographs (Fig 1, 3). Sexine2 could not be measured under LM because of its thin structure as Punt & Hoen (2009) say (Fig 1,2, Table 2). Also, it is difficult to distinguish it in SEM micrographs (Fig 3; 4).

As seen in the micrographs, results match with ''Anthemis arvensis'' type defining of Punt & Hoen (2009). Layers described as tectum (intratectal) and bacula according to Erdtman (1969) were defined as ''double tectum'' (Anthemoid pattern) and large basal columella by Vezey et al. (1994). As stated by Oberprieler (1998), Vezey et al. (1994) preferred a different method to entitle the layers of pollen wall structure.

The palynological studies related to *Cota* are few number. All taxa in *Cota* section located in *Anthemis* L. genus are raised *Cota* J. GAY genus level by Güner et al. (2012). For this reason, pollen characteristics of *Cota tinctoria* var. *tinctoria* and *Cota altissima* were compared with species called *Anthemis tinctoria* and *Anthemis altissima* in the previous studies. While E and P values were measured as 27.46 µm and 25.54 µm in *Cota tinctoria* var. *tinctoria*, these values were 29.77µm and 29.06µm in pollen grains of *Cota altissima* by Özbek (2016). On the other hand, E and P values of *C. tinctoria* var. *tinctoria* is 22.02 µm and 21.67 µm, E and P value of *C. altissima* is 25.25 µm and 24.04 µm in our study. There are significant differences in the P/E, Plt, Clt, t, and Amb measurements between the results we obtained and the results of the study of Özbek (2016), as in E, P values given above (Table 4). Dauti et al. (2014) examined pollen characteristics of *Anthemis tinctoria* and *Anthemis altissima.* In our stuy, morphological characteristics of *Cota tinctoria* var. *tinctoria* and *Cota altissima* in are similar to the results in Dauti et al. (2014). Pollen grains of *Anthemis tinctoria* was studied by the researchers İnceoğlu & Karamustafa (1977) and Punt & Hoen (2009) and similar results have been found like in our study. Erdtman et al. (1961) and Stix (1960) evaluated *Anthemis* genus as Anthemis (Achillea) type. The results of *Cota* is in harmony with the results of Erdtman and Stix no variations were observed in the measurements of pollen shape, exine, aperture, spine, mesocolpium, and polar region properties of *Cota* according to LM in this research (Fig. 1, 2, 4, Table 4, 5).

Pollen grains belong to *Cota* ornamentation is echinate and tectum surface (interspine area) is microperforate in analysis of SEM micrographs. Half of spines are perforated. Spine based perforations are usually larger and more irregular. The ends of the spines are acute or convoluted and half of spines or more have smooth surface. Appearances of spine are perpendicular or curved to different directions (Fig. 4; 2, 9, 10). The min-max values belong to equatorial and polar view of pollen images by SEM were measured between spine length

2.24-4.38 µm, spine base diameter 2.61-4.95 µm, flat part of spine 0.18-2.99 µm, pore width of spine base 0.14-0.47 µm, distance between spins 0.85-1.92 µm, spine number per 100 μm^2 de 3.2-6 (Image J 1.36b). Measurements belonging to SEM micrographs about researches conducted on *Cota* could not encountered. Pollen grains of *Cota* (the taxa of previous *Cota* section in *Anthemis* genera) was described as "Anthemis type" by Erdtman et al. (1961) and Stix (1960), "Anthemideae" by Skvarla (1966) and ''Anthemis arvensis'' type by Punt & Hoen (2009). Pollen morphological characteristics of *Cota* have generally similarities when considering the terminology based on exine structure in this study. The pollen grains of Cota can be evaluated in the "Anthemis arvensis" type in our study.

Nexine, Sexine1, Sexine2 (internal tectum), Sexine3 and Sexine4 can be especially distinguished at fracture pollen wall structure of *Cota* pollen grains in SEM micrographs (Fig.4; 7, 10, 11). Sexine2 could not be measured under LM because of its thin structure as Punt & Hoen (2009) say (Fig 1, 2, Table 3), but it can be clearly distinguished in SEM micrographs (Fig 3; 4). As seen in the SEM micrographs, the pollen grains of *Cota* can be evaluated in the *"Anthemis arvensis*" type in our study.

As a result, ornamentation of Anthemis and *Cota* examined in this study has echinate microperforate structure in SEM micrographs. Pollen morphological characteristics belonging to *Anthemis* and *Cota* have similarities in LM micrographs. As Hesse et al. say (2009), the pollen grains are mostly small. Spine base length is wider than spine length in both genera. According to SEM micrographs, while Sexine2 can not be seen clearly in the structure of pollen grains in *Anthemis*, it can be distinguished apparently in the structure of *Cota* (Fig. 3; 4, Fig. 4; 7, 10, 11). The stratified structure properties of the exine described by Punt & Hoen (2009) were also seen in *Anthemis* and *Cota* species studied in this research.

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Note: "The Pollen Morphology of Three Taxa Belonging to *Anthemi*s L. (Asteraceae) Genus Under Light Microscope" was presented as a poster at the TÜB Graduate Student Congress and abstract published in the congress book.

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VOLTAMMETRIC DETERMINATION OF CLOZAPINE FROMITS DRUG FORM

Reyhan EKER[a], Selehattin YILMAZ[a,]*, Sultan YAĞMUR[a] ,

Ozlem TONGUC YAYINTAS[b],

[a]*CanakkaleOnsekiz Mart University, Faculty of Science and Arts, Department of Chemistry 17020, Canakkale, Turkey*

[b]*CanakkaleOnsekiz Mart University, CanakkaleApplied Science of College, Fisheries Technology, 17020, Canakkale, Turkey*

***Corresponding author:** seletyilmaz@hotmail.com

ABSTRACT

A voltammetry method was developed for the direct quantitative determination of clozapine in tablet dosage forms based on its oxidation behavior. The electrochemical determination of clozapine was easily carried out on glassy carbon electrode (GCE) using a variety of voltammetry techniques. The electrochemical measurements were carried out on GCE in various buffer solutions in the pH range from 3.00 to 12.00 by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques. The dependence of pH on the anodic peak current and peak potential was investigated. Acetate buffer (pH 5.50) was selected for analytical purposes. The diffusion-controlled nature of the peak was established. A linear calibration curve for DPV analysis was constructed in the clozapine concentration range from $3x10^{-6}$mol L^{-1} to $1x10^{-5}$mol L^{-1}. Limit of detection (LOD) and limit of quantification (LOQ) were obtained as $4.082x10^{-7}$mol $L^{-1}$and $1.361x10^{-6}$mol L^{-1}respectively. The applied voltammetric method was validated.

***Keywords**: clozapine, determination, voltammetry, GCE, drug forms.*

1. INTRODUCTION

Schizophrenia is a serious, chronic, and debilitating disease characterized by positive symptoms (hallucinations), negative symptoms (withdrawn behaviors, emotional expressions) and cognitive disorders. Clozapine (Figure 1) is a dibenzodiazepine derivative used in treatment-resistant schizophrenia patients (Centorrino*et al*., 2002;Baldessarini*et al*., 2001). Clozapine treatment suppresses the abnormal movements of tardive dyskinesia as well as TAPs, and may treat clozapine movement disorders differently. Clozapine alone or in

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combination with other psychotropic; are used in psychoactive disorders such as seizure disorders, severe bipolar disorder, early periods of schizophrenia, borderline personality disorder and Parkinson's disease (Lieberman et al., 2005; Fitton et al., 1990).

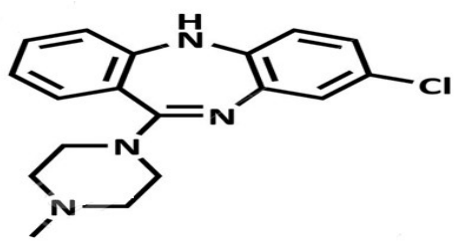

Figure 1. Chemical structure of clozapine

The purpose of this study is to investigate the electrochemical properties of clozapine using a glassy carbon electrode and to quantitate rapidly and precisely the amount of drug dosage forms by voltammetry technique.

2. MATERIAL ANDMETHOD

2.1. Apparatus

A Model Metrohm 757 VA Trace Analyzer (Herisau, Switzerland) was used to the voltammetric measurements, with a three-electrode system consisting of glassy carbon working electrode (GCE; φ = 3 mm, Metrohm), a platinum wire auxiliary electrode and Ag/AgCl (KCl 3 M, Metrohm) reference electrode. The glassy carbon working electrode was polished with alumina (prepared from φ = 0.01µm aluminum oxide) on alumina polish pad before each experiment and then, rinsed with ultra-pure deionized water and ethanol. The firstly, the deoxygenation process of the supporting electrolyte solutions were carried out with argon gas for 5 min before all experiments. Then, the argon gas was also passed from the solutions for 60 s after the addition of each sample solution in the experiments. In each new experiment, a new bare electrode surface was used. All pH measurements were made with Model Metrohm 744 pH meter (Herisau, Switzerland. All measurements were carried out at ambient temperature of the laboratory (15-20°C) For the analytical application, the following parameters were employed: pulse amplitude 50 mV; pulse time 0.04 s, voltage step 0.009 V, voltage step time 0.04, potential step 10 mV (DPV); the scan rate in the range 10-1000 mVs^{-1} (CV).

2.2. Reagents and materials

Clozapine and leponex were kindly supplied by (Novartis, Istanbul, Turkey). A stock solution of $1.0x10^{-2}$ M of clozapine was prepared by dissolving an accurate mass of the drug in an appropriate volume of ethanol kept in the refrigerator. The working solutions for the voltammetric investigations were prepared by dilution of the stock solution. All solutions were protected from light and were used within 24 h to avoid decomposition. 0.067 mol L^{-1} phosphate buffer; pH:4.50-8.00 (sodium hydrogen phosphate (Na_2HPO_4, Riedel, Seelze, Germany, and sodiumdihydrogen phosphate NaH_2PO_4, Riedel, Seelze, Germany). 0.2 mol L^{-1} acetate buffer; pH:3.50-5.50 (acetic acid: Riedel, Seelze, Germany, 100 m/m % and sodium

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hydroxide: Riedel, Seelze, Germany) and 0.04 mol L^{-1} Britton Robinson buffer; pH:2.02-12.00 (acetic acid: Riedel, Seelze, Germany, 100 m/m %; boric acid; Merck, Darmstadt, Germany, and phosphoric acid, Carlo Erba, Rodeno, France, 85 m/m %) were used to the supporting electrolyte solutions. Ultra-pure-deionized (0.055 µS/cm) water obtained from TKA Smart 2 model was used to prepare supporting electrolytes. Other chemicals, all of the analytical-reagent grade (Merck) were used.

2.3. Calibration graph for quantitative determination

The stock solution of clozapine was diluted with ethanol to obtain different clozapine concentrations. Using the optimum conditions described in the experimental section, a linear calibration curve was constructed in the clozapine concentration range $3x10^{-6}$-$1x10^{-5}$mol $L^{-1.}$ The repeatability, accuracy, and precision were checked.

2.4. Working voltammetric procedure of spiked tablet dosage forms

Ten tablets were weighed and ground to a fine powder. An adequate amount of this powder, corresponding to a stock solution of concentration $1x10^{-2}$ M was weighed and transferred 10 mL calibrated flask and the volume was adjusted with ethanol. The content of the flask was centrifuged for 20 min at 4000 rpm to affect complete dissolution and then diluted to volume with the same solvent. Appropriate solutions were prepared by taking suitable aliquots of the clear supernatant liquor and diluting with selected supporting electrolyte solutions. Each solution was transferred to the voltammetric cell. The nominal content of the corresponding regression equations was compared with previously platted calibration plats.

3. RESULTS AND DISCUSSION

3.1. Electrochemical oxidation behavior of clozapine

The electrochemical oxidation process and the determination using this electrode were firstly carried out by CV and DPV techniques. CV measurements performed with clozapine $1x10^{-4}$ M at scan rates between 10 – 1000 mVs^{-1} on GCE in 0.2 mol L^{-1} acetate buffer (pH 5.50) are given in Figure 2.

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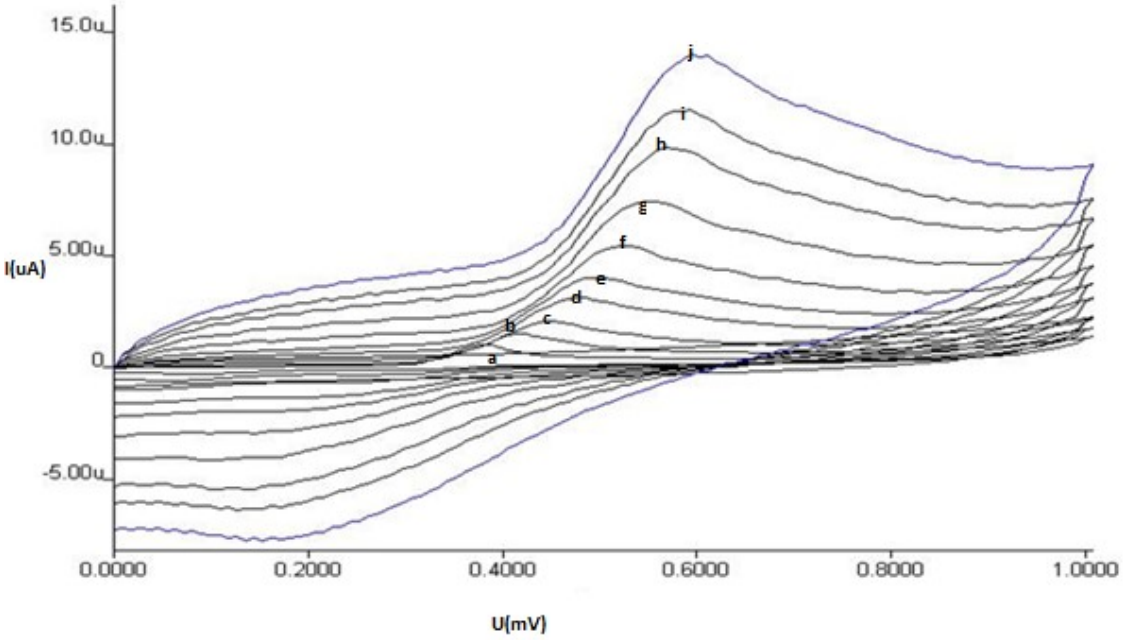

Figure 2. The cyclic voltammograms of $1x10^{-4}$ M clozapine in 0.2 M acetate buffer (pH5.50) on GCE . Scan rate,mV s^{-1} a) 10, b) 25, c) 50 , d) 100, e) 150, f) 250, g) 400, h) 600, i) 750, j) 1000

The linear relationship existing between peak current and the square root of the scan rate between 10-1000 mV s-1 (Ip(µA) = 0.2426v1/2 -0.2578, correlation coefficient 0.9993) were observed. The correlation coefficient is closed to 1 indicated that the oxidation process is predominantly diffusion-controlled. In addition, a plot of the logarithm of peak current versus the logarithm of scan rate gave a straight line (correlation coefficient 0.9993) with a slope of 0.2426, which is the expected value for an ideal reaction of solution species (Çıtak*et al.,* 2007; Skrzypek*et al*., 2005;Yılmaz*et al*., 2013).

The cyclic voltammogram of clozapine exhibited only one anodic peak, with no peak on the reverse scan, indicating the totally irreversible nature of the electrode reaction. In addition, for an irreversible oxidation process, the peak potential Ep shifts to less negative values with the increasing of scan rate. Therefore, the oxidation process of clozapine was proved to be irreversible(Çıtak*et al*., 2007; Skrzypek*et al*., 2005; Yılmaz*et al*., 2013).

3.2. Effect of pH on peak current and peak potential of clozapine

The voltammetric response was strongly pH dependent. The DPV peak current of the oxidation shifted with increasing pH Fig. (3a). The effect of pH on the peak potential was shown in Figure (3b). The maximum current was observed at the 0.2 mol L^{-1} acetate buffer (pH 5.50). Therefore, this pH value and supporting electrolyte were chosen to carry out the electroanalytical determination of clozapine.

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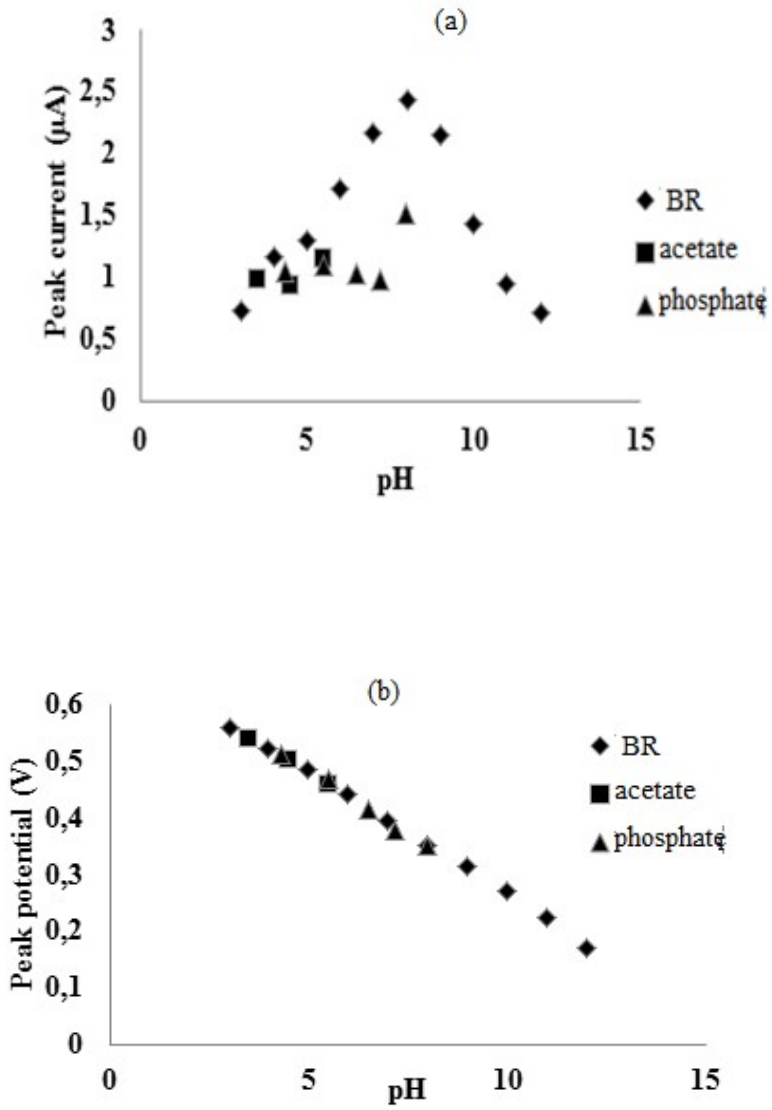

Figure 3. The changing of pH on the peak current (a) and peak potential (b) of $7x10^{-6}$ M clozapine in various supporting electrolyte by DPV voltammograms.

3.3. Determination of clozapine

DPV technique was used to develop a voltammetric methodology for determination of the drug in pharmaceutical. Under the optimized experimental conditions,linear relationship between the oxidation peak currentof clozapineat GCE and concentration can be established in the range of $3x10^{-6}$-$1x10^{-5}$mol L^{-1} (Figure 4).

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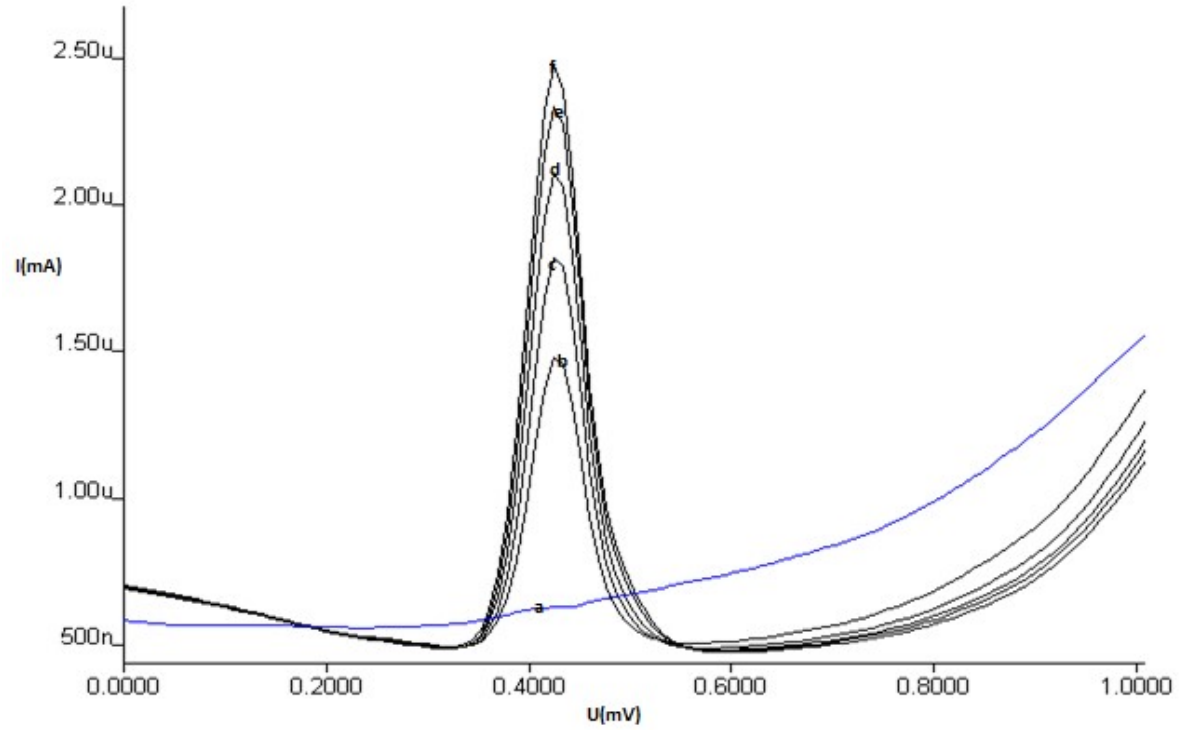

Figure 4. The calibration voltammograms at different concentrations of clozapine in 0.2 mol L^{-1} acetate buffer (pH5.50)on GCE by DPV. a) supportingelectrolyte, b) $3x10^{-6}$ c) $5x10^{-6}$d) $7x10^{-6}$e)$9x10^{-5}$f)$1x10^{-5}$(mol L^{-1}).

Validation of the procedure for the quantitative determination of the clozapine was examined via evaluation of the limit of detection (LOD), limit of quantification (LOQ), precision (repeatability and reproducibility)in Table 1, accuracy (bias) and recovery values in Table 2. LOD and LOQ were calculated on theoxidation peak current using the following equations: LOD = 3 s / m, LOQ = 10 s / m (s is the standard deviation of the peak currents (five runs, m is the slope of the calibration curve) (Çıtak*et al.,* 2007; Skrzypek*et al*., 2005;Yılmaz*et al*., 2013; Yagmur*et al*., 2017).

The LOD and LOQ were calculated as $4.082x10^{-7}$ and $1.361x10^{-6}$mol L^{-1} respectively. A good repeatability andreproducibility of the peak current and potential were calculated from five independent measurements for 5 $x10^{-6}$mol L^{-1}clozapine (Skrzypek*et al*., 2005; Çıtak*et al.,* 2007;Yılmaz*et al*., 2013; Yagmur*et al*., 2017).

Repeatability of peak current and peak potential (R.S.D %) were found as 1.77 and 0.423 respectively. Reproducibility of peak current and peak potential (R.S.D %) were found as 1.79 and 0.423 respectively (Table 1).

The equation of the linear regression plots was Ip(µA)= $1.40x10^{4}$ C (mol L^{-1}) +0.558 correlation coefficient, r=0.993; n=5 repeat measurements. Standard deviations for intercept and slope of the calibration curve are given in Table 1.

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Table 1. Regression data of the calibration lines for the quantitative determination of clozapine. The calibration plots were obtanied in 0.2mol L^{-1} acetate buffer (pH5.50)on GCEby DPV technique.

Parameter	Results
Measured potential (V)	0.418
Linear concentration range (mol L^{-1})	$3x10^{-6}$-$1x10^{-5}$
Slope (µA mol L^{-1})	$1.40x10^{4}$
SD of slope	698.0
Intercept (nA)	0.558
SD of intercept	0.071
Correlation coefficient, r	0.993
Number of measurements, n	5
LOD (mol L^{-1})	$4.08x10^{-7}$
LOQ (mol L^{-1})	$1.36x10^{-6}$
Repeatability of peak current (R.S.D %)	1.77 for $5x10^{-6}$mol L^{-1}
Repeatability of peak potential (R.S.D %)	0.423 for $5x10^{-6}$mol L^{-1}
Reproducibility of peak current (R.S.D %)	1.79 for $5x10^{-6}$mol L^{-1}
Reproducibility of peak potential (R.S.D %)	0.423 for $5x10^{-6}$mol L^{-1}

3.4. Determination of clozapine in leponex®tablets byvoltammery techniques

The amount of clozapine in lopenex commercial tablets was calculated by calibration plots. The results obtained are given in Table 2. To determine whether excipients in the tablets interfered with the analysis, the accuracy of the proposed methods were evaluated by recovery tests after the addition of a certain amount of pure drug to pre-analyzed formulations of clozapine (Table 2). The results showed the validity of the proposed techniques for the quantitative determination of clozapine in tablets.

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Table 2. Application of the DPV technique for the assay of clozapine in leponex tablets and mean recoveries on GCE.

Parameter	Results
Labeled clozapine (mg)	25.00
Amount Found (mg)	25.50
Relative Standard deviation, R.S.D.%	0.98
Bias %	2.00
clozapine (mg)	5.00
Found(mg)	4.92
Number of measurement, n	5.00
recovery (%)	98.30
Relative standard deviation of recovery, R.S.D. %	0.20
Bias %	0.02

The detection limits reported fornon-electrochemical method and electrochemical methodsare given in Table 3.

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Table 3. Comparison of linear range and detection limits for clozapine to different known methods

Linear range	Limit of detection *(LOD)*	Limit of quantification *(LOQ)*	Method	Reference
0.5-45 µM	–	–	voltammetry	Marshhadizadeh andAfhsar (2013)
1.0×10^{-9}-1×10^{7} mol L^{-1}	2.08×10^{-10} mol L^{-1}	6.95×10^{-10} mol L^{-1}	voltammetry	ArvandandShiraz (2011)
1-3.5 mmol L^{-1}	–	–	voltammetry	Manjunatha*et al.* (2011)
25-50 µg mL^{-1}	–	–	voltammetry	Farhadi*et al.* (2007)
1×10^{-6}- 1×10^{-5}mol L^{-1}	1.7×10^{-7} mol L^{-1}	5.6×10^{-7} mol L^{-1}	voltammetry	Blankert*et al.* (2007)
–	4.5×10^{-1}_1.5×10^{-10}mol L^{-1}	–	voltammetry	Hammam*et al.*(2004)
$3x10^{-6}$-$1x10^{-5}$mol L^{-1}	$4.082x10^{-7}$ mol L^{-1}	$1.361x10^{-6}$ mol L^{-1}	voltammetry	**Thisstudy**
0.1-2 µM	30 nM	-	Voltammetry	Fat'hiandAlmasifar. (2017)
3-70 nM	1.53 nM	-	Voltammetry	Tammari*et al.*(2017)
–	23.6 µg L^{-1}	–	chromatography	Dural *et al.* (2015)
25-2000 $ngmL^{-1}$	–	–	chromatography	Wongsinsup*et al.* (2010)
0.1-0.5 µg	–	–	chromatography	Patil andGhosh (2009)
4-200 µg	1.12-1.76-2.22-0.95-13.26 µg mL^{-1}	–	Spectrometry	Darwish*et al.* (2005)

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4. CONCLUSIONS

A simple, sensitive, selective DPV technique for the quantitative determination of clozapine based on the electrochemical oxidation at GCE was established. From the CV and DPV measurements, it is understood that electrode reaction process is irreversible and pH dependent. Clozapine was successfully determined in 0.2 mol L^{-1} acetate buffer in tablets dosage by DPV technique.

The principal advantage of the DPV technique over the other techniques is that it may be applied directly to the analysis of pharmaceutical dosage form without the need for extensive sample preparation since there was no interference from the excipients and endogenous substances. Another advantage is that the developed DPV technique is rapid, requiring about 5 min to run any sample and involves no sample preparing other than dissolving, diluting, precipitating, centrifuging and transferring an aliquot to the supporting electrolyte.

Conflict of Interest Statement

The authors declare no conflict of financial, academic, commercial, political, or personal interests.

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IN VITRO SCREENING OF ANTIBACTERIAL ACTIVITY OF HONEY SAMPLES COLLECTED FROM KOSOVO

Tülay BİCAN SÜERDEM*, Hanife AKYALÇIN

Çanakkale Onsekiz Mart University, Faculty of Arts and Sciences, Department of Biology, 17020, Çanakkale, TURKEY

***Corresponding-author:** tbican@comu.edu.tr

ABSTRACT

Since the ancient times, it is well known that honey has a therapeutic effects on human health. However, the effectiveness of antibiotics is diminished as resistant pathogens develop and spread. So in this case, we need alternative antimicrobial agents and it is important to use medicinal important materials such as plants, plant based products including honey to struggle this situation. There are too many studies conducted on antimicrobial activity and researchers have been reported both bacteriostatic and bactericidal effects of honey. The aim of this research is screening the antimicrobial effects of six different honey samples from Kosovo against some gram-positive *(Enterobacter faecalis ATCC 29212, Staphylococcus aureus, S. aureus ATCC 29213, S. aureus ATCC 6538P, S. aureus ATCC 25923, Bacillus cereus)*and gram-negative *(Escherichia coli ATCC 25922, Salmonella tyhimurium ATCC 51812) bacteria by using the agar well diffusion method on Mueller Hinton Agar. According to our results all the honey samples have effectively high antibacterial activity against especially Staphylococcus strains and Salmonella typhimurium ATCC 51812 when comparing the reference antibiotics used in the study.*

Keywords: *Antibacterial activity, antimicrobial activity, honey, agar well diffusion method, Kosovo*

1. INTRODUCTION

Honey is a very important and special product which is produced from flower nectars (floral honey), combined with an enzyme secreted by honey-bees. Bees gather these sugary substances, enrich them with their own substances and store them in the honeycombs. Honey is a valuable nutritive food used for its antibacterial activity and widely in the food industry, which provides energy to the organism due its high percentage of carbohydrates, which are easily assimilated (Akçiçek and Yücel, 2015; Cenet et.al., 2017). The use of honey has become popular again today due to its strong antibacterial activity against to resistant bacteria in vitro and the usage of as an antibacterial agent in chronic wound infections that unresponsive to antibiotic treatment.

Also, due to phenolic compounds and other valuable compounds in honey, it has many medicinal properties such as antioxidant, anti-inflammatory, antimutagenic, antitumor

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and antimicrobial activity (Suarez et al., 2013). In addition, recent studies reported that honey can exert anti-proliferative effects against cancer cells (Suarez et al., 2014). The antibacterial activity of honey have been related to well known antibacterial factors in honey like the high sugar concentration, osmoloarity, hydrogen peroxide, the low pH-asidity and more recently methylglyoxal and the antimicrobial peptide bee defensin-1 were identified as important antibacterial compounds in honey (Kwakman et al., 2010; Kwakman et al., 2011; Kwakman and Zaat, 2012). However, another kind of honey, called non-peroxide honey (manuka honey), displays significant antibacterial effects even when the hydrogen peroxide activity is blocked. Its mechanism may be related to the low pH level of honey and its high sugar content (Deb Mandal and Mandal, 2011). The floral source of honey plays an important role on its biological properties (Molan, 2002). The rich multiflora of honey increases not only its nutritional quality as well as antimicrobial potential on various clinically important microorganisms (Cenet et al., 2017). According to Eteraf-Oskouei and Najafi (2013) researchers have been reported both bacteriostatic and bactericidal effects of honey and they are especially effective on pathogenic strains like *Klebsiella pneumonia*, *S. aureus*, *Salmonella typhimurium* etc. Honey has been used as a topical antibacterial agent for the treatment of surface infections such as ulcers and bed sores and those resulting from burns, injuries and surgical wounds.

Honey is composed of about 181 components and is basically a solution supersaturated in sugars, of which fructose (38%) and glucose (31%) are the most important (Nagai et al., 2006). In addition, there is a great variety of minor components, including phenolic acids, flavonoids, the enzymes glucose oxidase and catalase, ascorbic acid, carotenoids, organic acids, amino acids, proteins and alfa-tocopherol (Carina et al., 2014). However, the composition of honey varies depending on many factors such as the floral source, climate, environmental conditions and the processing it undergoes as pasteurization or storage (Gheldof et al., 2002).

The aim of the research is to determine the in-vitro antimicrobial effects of six different honey samples from Kosovo against some gram-positive and gram-negative bacteria by using agar well diffusion method and then to analyse the zones of inhibition around the wells as qualitative and finally to compare the results of inhibition zones with standard antibiotics as used in the research.

2. MATERIALS AND METHODS

2.1. Preparation honey samples for antibacterial activity

6 different honey samples from Kosovo were obtained from the bee-keepers on January-February 2016, transferred to Palynology Laboratory in Çanakkale Onsekiz Mart University, Biology Department, in glass jars and stored at room temperature in the dark. They were described as mountain honey, floral honey, meadow flowers honey and pinus honey by the bee-keepers. The localities are given in **Table 1.** A map of Kosovo indicating the location of the honey samples is shown in **Figure 1.**

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Table 1. Localities of honey samples and honey types from Kosovo

Sample Number	Country	District	Honey types determined by bee keepers
B1	Kosovo	Srecka-Prizren	Mountain
B2	Kosovo	Giakove	Floral
B3	Kosovo	Prizren	Meadow flowers
B4	Kosovo	Rahovec	Pinus
B5	Kosovo	Mitrovice	Floral
B6	Kosovo	Prishtine	Floral

2.2. Test microorganisms

Some gram-positive *(Enterobacter faecalis ATCC 29212, Staphylococcus aureus, S. aureus ATCC 29213, S. aureus ATCC 6538P, S. aureus ATCC 25923, Bacillus cereus)*and gram-negative *(Escherichia coli ATCC 25922, Salmonella tyhimurium ATCC 51812)* bacteria were used to evaluate the antibacterial activity of honey samples. Bacterial strains obtained from first researcher's personal culture collection from Basic and Industrial Microbiology Research Laboratory in Çanakkale Onsekiz Mart University, Biology Department.

2.3. Screening of antibacterial activity

Agar well diffusion method was used to screen antibacterial activity of honey samples (Moussa et al., 2012; Balouri et al., 2016; Cenet et al., 2017). They were dissolved in distilled water (1:1) and then kept in +4^{o}C until the experiment. All the bacterial strains were incubated for over-night at 37^{o}C after their inoculation into Tyriptic Soy Broth (TSB-Merck). Bacterial inoculum was set up to 0.5 Mac Farland (10^{6} bacterial cells/mL) before transferred to petri dishes containing Mueller Hinton Agar (MHA-Merck) and100 µL inoculum was spread on MHA.Wells on MHA were made after the bacterial inoculation on MHA and honey samples were filled into wells approx. 50-60 µL. Inhibition zones formed around the wells on agar plates were measured by inhibition zone ruler (Bioanalyse) in mm to determine the activity and they were analysed as qualitatively. While Mueller Hinton Agar was used as medium, penicillin (P10), streptomycin (S10) and ampisillin (AM10) were used as standard reference antibiotics for positive control. Studies were performed in triplicate.

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Figure 1. Research area (Kosovo) and stations of honey samples that were obtained (https://www.ezilon.com/maps/europe/kosovo-physical-maps.html)

3. RESULTS AND DISCUSSION

In this study totally six different types of honey samples collected from different localities from Kosovo were evaluated for their antibacterial activity against some gram-positive *(Enterobacter faecalis ATCC 29212, Staphylococcus aureus, S. aureus ATCC 29213, S. aureus ATCC 6538P, S. aureus ATCC 25923, Bacillus cereus)* and gram-negative *Escherichia coli ATCC 25922, Salmonella tyhimurium ATCC 51812)* bacteria. As a result, antibacterial activity was determined in different values against different microorganisms in all the honey samples and the inhibition zones were varied from 10 mm to 40 mm in diameters. But none of the honey samples were effective against two bacteria *Enterobacter faecalis* and *E. coli* ATCC 25922. However, the honey samples used in our study showed

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more antibacterial activity than the antibiotics used in some species, even higher than the strains of *S. aureus* and S. *tyhimurium.* So, it can be said *Enterobacter faecalis* and *E. coli* ATCC 25922 are more resistant to honey samples than other microorganisms used in the study.

Among the honey samples floral honey from Giakove-Kosovo (sample no:B2) forms the maximum inhibition zone 40 mm in diameters against *Salmonella tyhimurium ATCC 51812.* The results of antimicrobial activity were given in **Table 2** according to the inhibition zone diameters formed around the wells. In generally, two honey samples (B4 and B5) have antibacterial activity against six bacteria and they are more effective than the others. The least effective honey sample is B1.

Table 2. Antibacterial activity of honey samples from Kosovo

Microorganisms	Inhibition zones (mm)*								
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**P(10)**	**S(10)**	**AM(10)**
E. faecalis	-	-	-	-	-	-	-	-	-
***S.aureus* ATCC 29213**	-	10	12	18	14	12	12	16	12
***S.aureus* ATCC 6538 p**	-	-	12	14	14	10	42	22	40
S.aureus	-	-	10	18	10	-	42	26	40
***S.aureus* ATCC 25923**	36	34	38	34	34	32	12	14	14
***E.coli* ATCC 25922**	-	-	-	-	-	-	8	20	12
B.cereus	-	-	-	12	10	-	-	26	8
***Salmonella typhimurium* ATCC 51812**	36	40	36	36	36	36	16	16	20

(-): no inhibition P (10): Penicillin G (10 U), S (10): Streptomycin (10 µg), AM (10): Ampicillin (10 µg) *: inhibition zones includes 6 mm disk diameter, data are average of three measurements

The high activity of honey samples and reference antibiotics tested in-vitro are shown in **Figure 2** as inhibition zones formed around the wells.

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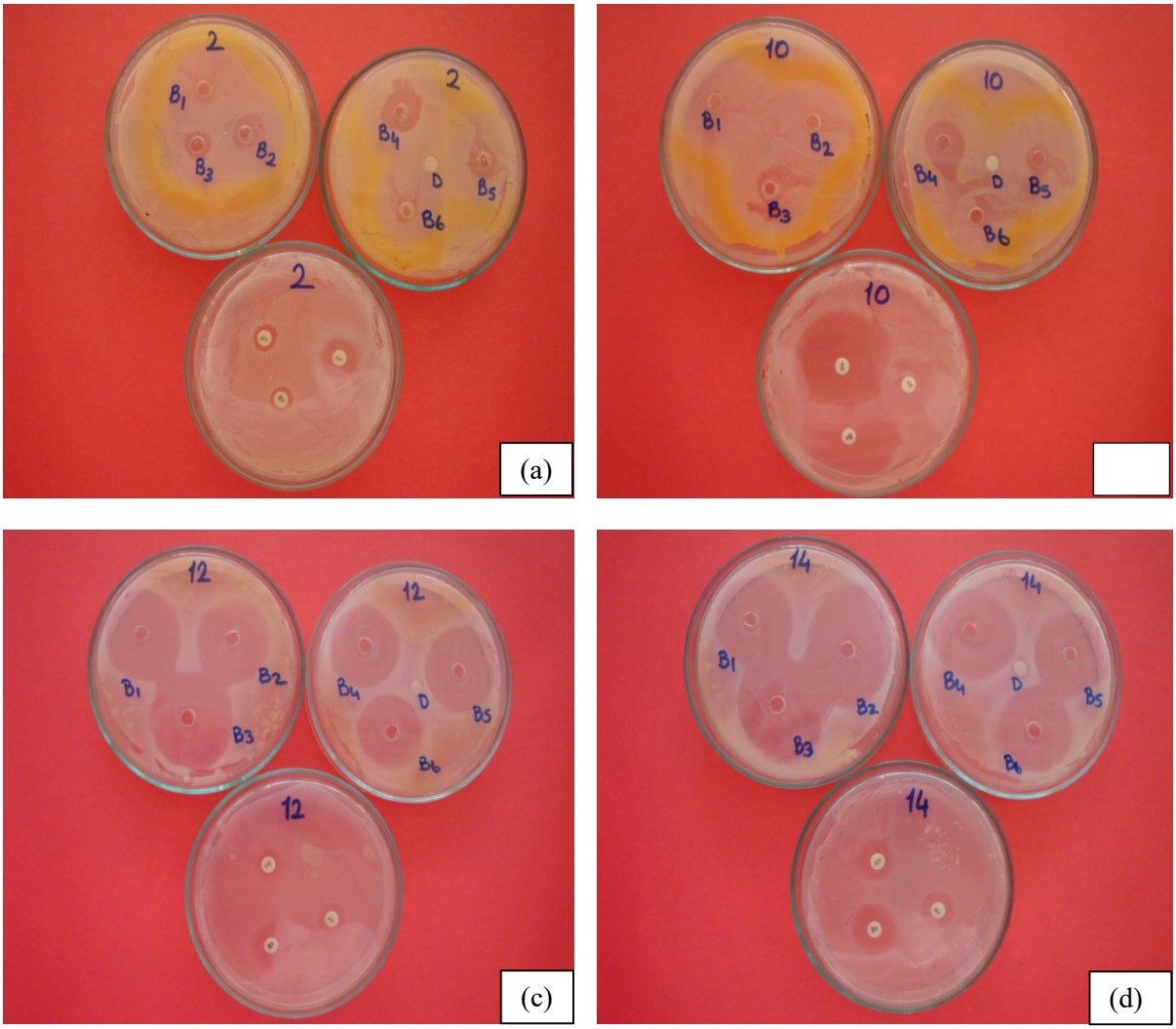

Figure 2: In-vitro tested honey samples (a) *Staphylococcus aureus* ATCC29213 (b) *S. aureus* (c) *S.aureus* ATCC25923 (d) *Salmonella typhimurium* ATCC51812 bakterilerine karşı inhibisyon etkileri. (B1): Srecka (B2) Giakove (B3): Prizren (B4): Rahovec (B5): Mitrovice (B6): Prishtine P (10): Penicillin G (10 U), S (10): Streptomycin (10 µg), AM (10): Ampicillin (10 µg) *: inhibition zones includes 6 mm disk diameter.

Mahendran and Kumarasamy (2015) in their study, a total of twelve honey samples from different origins were evaluated for their antibacterial activity against the Gram positive species such as *Staphylococcus aureus, Streptococcus pyogenes*, and the Gram negative species such as *Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis*. Among the twelve honey samples studied S1(summer honey) and W1(winter honey) honey samples show maximum antibacterial activity especially against *Staphylococcus aureus*.

According to Rani et al., (2017) both Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA) isolates were found sensitive to honey. But MRSA were resistant to all antimicrobials tested except linezolid where as MSSA were sensitive to all except penicillin.

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Cenet et al. (2017), investigated honey specimens from Southeastern Anatolia and found that they were effective on five bacterial species like *S. aureus* 29213, *S. aureus* BAA-977, *E. faecalis* 29212, *E. coli* 25922, *E. hormaechei* 700323.

The activity against *S. aureus*, *E. coli* and *Salmonella sp*. has been expressed despite honey originating from different floral sources and countries. The inhibition of growth in those bacteria is principally due to the peroxide effect, which is very common in honey worldwide, and as it is a derivative compound from bees, it is expected that it is present in all honeys (Molan, 1992; Carina et al., 2014; Deb Mandal and Mandal, 2011). As seen in our study, nearly all types of honey from Kosovo have high activity against *S. aureus* strains and *Salmonella typhimurium* and the findings of our study together with three of our previous studies show similarities (Bican Süerdem et al., 2013; Bican Süerdem et al. 2014; Bican Süerdem et al., 2016). It can be one of the reasons for the high antibacterial activity which exceptionally rich in plant and tree species considering Kosovo's relatively small area (http://pdf.usait.gov/pdf_docs/Pnact 349.pdf).

Finally, the activity of all honey samples against *S. aureus* ATCC 25923 and *Salmonella tyhimurium* ATCC 51812 are nearly three times higher than that of standard antibiotics discussed as reference in our study (Table 2.). It is therefore very likely that honey can be used as a potential alternative antibiotic to treat bacterial infections caused by these species especially of the skin and soft tissue.

4. CONCLUSION

Because of many negative situations such as; increasing bacterial resistance to antibiotics day by day, side effects after antibiotic usage, high cost of production and long process, alternative treatment methods like apiteraphy (treatment with bee products like honey, pollen, propolis etc.) should be re-activated again. It is advisable to use honey as an alternative natural product, generally in children and older people and should be used in medicine and pharmaceutical industry. In all the researches about antibacterial activity of honeys, the common point is that the potency of the antibacterial activity can vary very markedly. The number of variable factors involved makes it impossible to predict with any certainty that a particular honey will have a high antibacterial activity. Because of this, honeys preferred for therapeutic use should be tested for their antibacterial activity against the pathogens to be get sure. In addition to many benefits of honey, there are some critical points while consuming honey. Firstly, it is necessary to guarantee its quality, secondly to consider the lesion type and the patient state (risk of allergy, diabetes, etc.).

Such preliminary researches should be carried out further on the subject of identifying the antimicrobial properties of the substances in honey. The determination of botanical origins of different types of honeys makes these studies even more meaningful.

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