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Contributions to the flora and vegetation of Binalood mountain range, NE Iran: Floristic and chorological studies in Fereizi region

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Abstract

The mountainous area of Fereizi is located in northwestern Binalood range, Khorassan, north east of Iran. Special climatic and geographical characteristics of the area make it a suitable habitat for various plant species. As a part of a research poject on the flora and vegetation of Binalood mountain range, the floristic composition of this area, life forms and chorology of vascular plants have been determined with emphasis on endemism. During several collection excursions in 2005 to 2007, a total number of 484 vascular plant taxa were identified in the area belonging to 229 genera and 59 plant families. The largest plant families in the area are Asteraceae (63 species), Poaceae (51 species), Fabaceae (50 species) and Brassicaceae (43 species). *Astragalus* (25 species) and *Allium* (13 species) are the richest genera. The dominant life forms are hemicryptophytes and therophytes. The floristic composition of the area is strongly influenced by Irano-Turanian elements (56.8%). The analysis of endemism shows that the area inhabited by 49 subendemic taxa and 21 Iranian endemic species from which 13 species are endemic to northeast of Iran and three rare and threatened species. *Ferula flabelliloba*, *Cousinia termei* and *Sisymbrium integerrimum* are local endemics to Binalood mountain range. *Geranium charlesii* is recorded as a new species for the flora of Iran.

Key words: floristic composition, life form, chorology, phytogeography, endemism, new records, Fereizi, Binalood, Khorassan

Introduction

Iran with *ca.* 7300 plant species (Akhani, 2006), after Turkey, is the second richest country of plant diversity in SW Asia. The rich flora of the country is the consequence of locating among three main phytochoria in the Old World including Irano-Turanian, Euro-Siberian and Sahara-Sindian, and influencing by Mediterranean and Somalia-Masaei species (Zohary, 1973; Léonard, 1988, 1993; White and Léonard, 1991).

In 2004, the former Khorassan province located in northeast and east of Iran with a surface area of 313,335 km^2, was divided into three smaller provinces viz. North Khorassan, Razavi Khorassan and South Khorassan. Tabas County was recently influenced by this division and joined to Yazd province. The floristic composition of Khorassan is influenced partly by the Hyrcanian elements in northwest, Irano-Turanian desert plants from west and southwest, some Sahara-Sindian elements from south and southeast, eastern Irano-Turanian elements along the borders with Afghanistan, Aralo-Caspian species from north, and many local endemic and regional endemic taxa specially originated in Khorassan- Kopet Dagh floristic province. According to the Flora Iranica (Rechinger, 1963-2008) and subsequent botanical inventories (Ghahremaninejad *et al.* 2005; Joharchi and Akhani, 2006; Joharchi *et al.* 2007), the total number of vascular plants in the former Khorassan province is *ca.* 2300 species, comprising about 31.5 percent of the Iranian flora.

Flora of Binalood mountain ranges has not been comprehensively studied hitherto. Several occational plant collections done by foreign and Iranian botanists have been recorded in Flora Iranica (Rechinger, 1963-2008) and Flora of Iran (Assadi *et al*. 1988-2005). In a floristic study, Ghahreman *et al.* (2006) listed 487 plant species from SW slopes of Binalood.

Floristic survey of an area is a prerequisite for any vegetation and ecological surveys and conservation management. This paper provides the floristic composition of vascular plants and phytogeography of Fereizi, an area scarcely studied hitherto, based on 2005-2007 botanical collections as a part of a research program on vegetation and conservation of the flora of Binalood mountain range.

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Material and Methods

Physical geography of the study area

Geography: Fereizi village is located in Golmakan section of Chenaran County in northern part of Razavi Khorassan province. The studied area is a mountainous region situated in Fereizi river catchment area between 36° 25' and 36° 33' northern latitudes and 58° 51' and 59° 04' eastern longitudes. The area covering *ca.* 20,000 hectares belongs to the north-facing slopes of northwestern parts of Binalood mountains with a minimum altitude of 1430 m in northeast of the area between Fereizi and Abghad villages and a maximum altitude up to 2500 m in south of Fereizi river on northern slopes of Jaji mount (Figure 1). The main peak of Binalood (3211 m) is located near the southern border of the studied area. Fereizi, Kandelan, Oshak, Kalat and Dermeh are the main deep valleys where in there are many apple, cherry, plum, peach and walnut orchards.

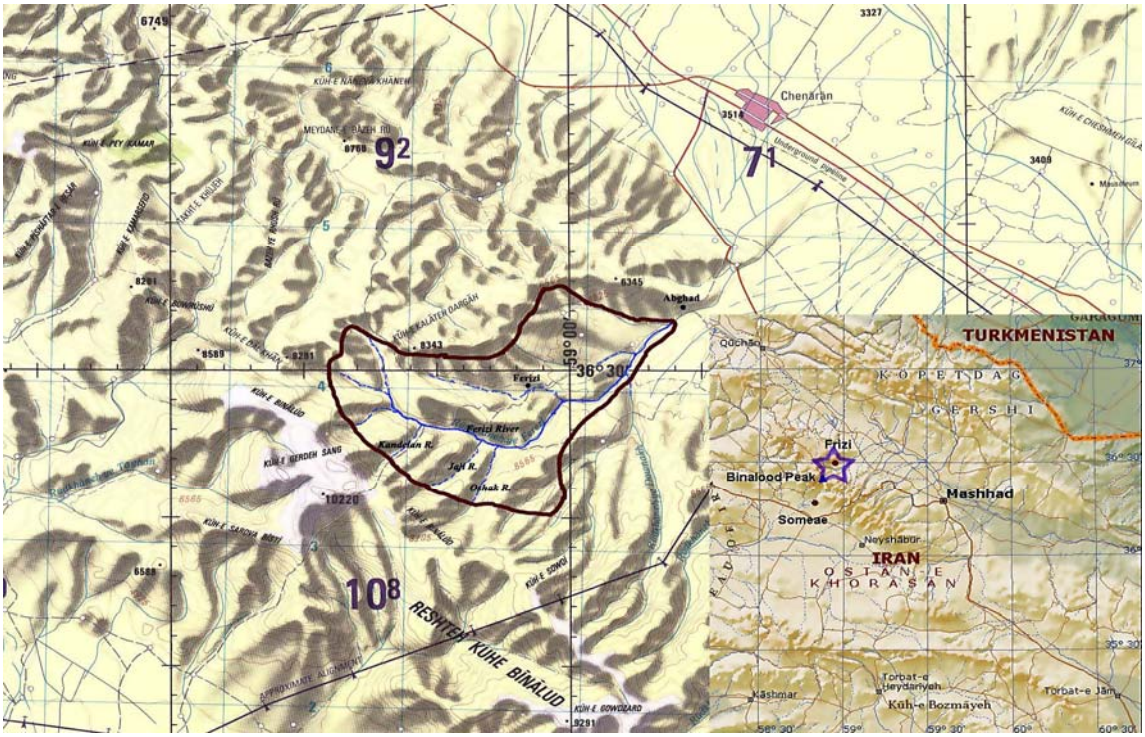

Figure 1. Map of Fereizi river catchment area showing its position in Khorassan.

Geology: Binalood zone, an eastern segment of Alborz Mountains, is restricted to Paleo-Tethys remnants and metamorphic rocks and Permian turbid sediments from the north, and Neo-Tethys ophiolitic rocks in the south. Binalood mountain range is characterized by thin-skinned tectonics due to NE-SW thrust faulting and composed of thick sequence of slate and phylitic rocks of Triassic-Jurassic age, and over thrust slices of Paleozoic sedimentary rocks, mainly Cambrian-Silurian and Devonian rocks (Darvishzade, 1991; Alavi, 1992). Based on geological maps, the main part of Fereizi river catchment area is composed of Jurassic age shale and dark grey Mashhad phylite, and partly in northeast of Fereizi village toward Abghad with Jurassic sandstone, shale and conglomerate and in upper layer with marl and limestone. Recent alluvium of Quaternary forms the river beds and water channels (Geological Survey of Iran, 1986).

Climate: According to available data from the nearest climatic station in Golmakan during 1987-2005 (Anonymous, 2006), the mean maximum temperature of the warmest month (July) and the mean minimum temperature of the coldest month (January) are 32.8°C and -3.1°C, respectively. Annual precipitation ranges between 129 mm (in 2000) and 313 mm (in 2003) with the mean annual precipitation of 212.6 mm. Wet season starts late November and lasts until April, and the maximum monthly rain is in March (Figure 2). According to Emberger's method, the station is located in a cold semi-arid area.

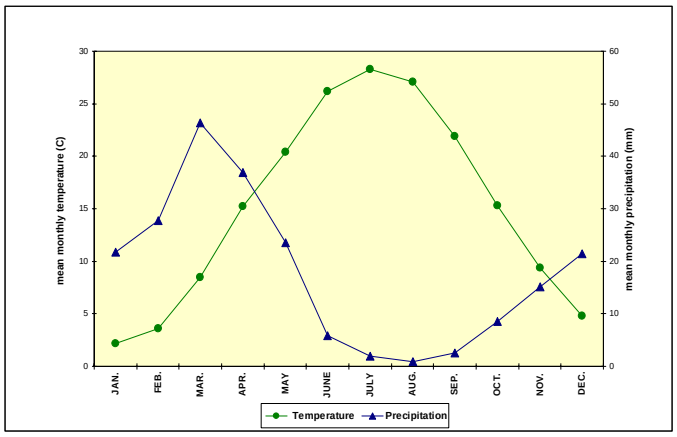

Figure 2. The climatic diagram based on data from Golmakan meteorological station during 1987-2005.

Methods: During several botanical excursions in growing seasons from 2005 to 2007, about 1200 vascular plant specimens were collected using normal random collecting method in different habitat types and elevations of the area. The exact coordinates and elevation were registered using GPS for collecting locations. The plants were dried and labeled in laboratory for providing herbarium specimens and then were identified using available Floras and literature (Rechinger, 1963-2008; Assadi *et al*., 1988-2008; Davis, 1965-1988, Komarov, 1934-1958; Maassoumi, 1986-2006). All voucher specimens are avaiable in Herbarium of Ferdowsi University of Mashhad (FUMH) under sheet numbers 35840 to 37038. The life forms were classified based on Raunkiaer (1934) and the chorology of each taxon was determined by using distribution data in the Floras and other published literature (Akhani, 1998; Browicz, 1983-1996; Léonard, 1988).

Results

Flora: A total number of 484 species/infraspecific vascular plant taxa has been identified in the study area which belong to 229 genera and 59 families. In appendix of the paper, a checklist of all vascular plants identified in Fereizi region is listed with information about their life forms and chorological types. The Dicotyledons with 49 families, 177 genera and 396 species/infraspecific taxa are the most diverse plant group followed by Monocotyledons with 7 families (considering Liliaceae in its wider concept *sensu* Flora Iranica (Rechinger, 1963-2008)), 49 genera and 84 species/infraspecific taxa, Gymnosperms with 2 families, 2 genera and 3 species and Pteridophytes with only one family, genus and species.

The important rich families are Asteraceae (63 species), Poaceae (51 species), Fabaceae (50 species) and Brassicaceae (43 species), Lamiaceae (29 species), Apiaceae (28 species), Boraginaceae (23 species), Liliaceae *s.l.* (22 species) and Caryophyllaceae (21 species). These 9 families compose about 70 percent of the flora in the area. The genera *Astragalus* (25 species) and *Allium* (13 species) are the richest followed by *Silene* and *Polygonum* (each with 8 species), *Artemisia* and *Bromus* (each with 6 species), and *Geranium* and *Vicia* (each with 5 species).

Geranium charlesii, a tuberous geophyte species hitherto known from Afghanistan, Pakistan (Chitral) and southern Pamir-Alai mountains, is recorded as a new species for the flora of Iran. *Marrubium procerum*, a critically endangered plant, is reported after 150 years from *locus classicus*.

Life forms: The dominant life forms in the flora of study area are hemicryptophytes (176 species) including rosettes and small stemed plants, tall herbs and Umbelliferae-like herbs and graminoids, and therophytes or annual plants (176 species), each composes 36.4% of the flora. 13.8% of the flora consists of geophytes mainly including bulbous, tuberous and rhizomatous species. Chamaephytes comprise 9.7% of the flora (Figure 3).

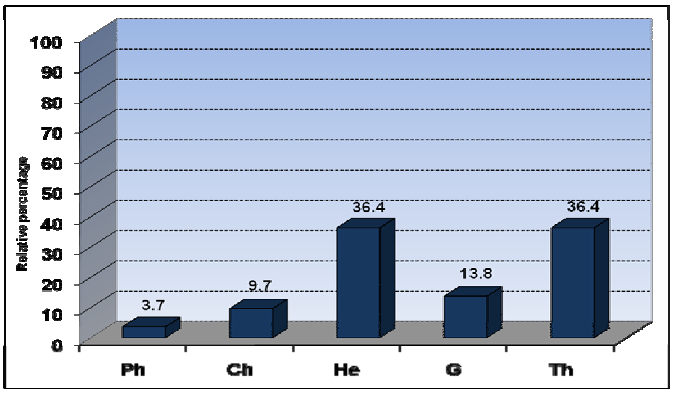

Figure 3. Life form spectrum of plants in Fereizi region (Ph: Phanerophytes, Ch: Chamaephytes, He: Hemicryptophytes, G: Geophytes, Th: Therophytes).

Chorology: A considerable number of species (56.8%) belongs to Irano-Turanian (IT) phytochorion. The flora of the area is influenced by Pluriregional and common Irano-Turanian, Euro-Siberian and Mediterranean elements. Mediterranean (M) and Euro-Siberian (ES) regions have more influence on these two- or three-regional chorological types than Sahara-Sindian (SS) (Figure 4).

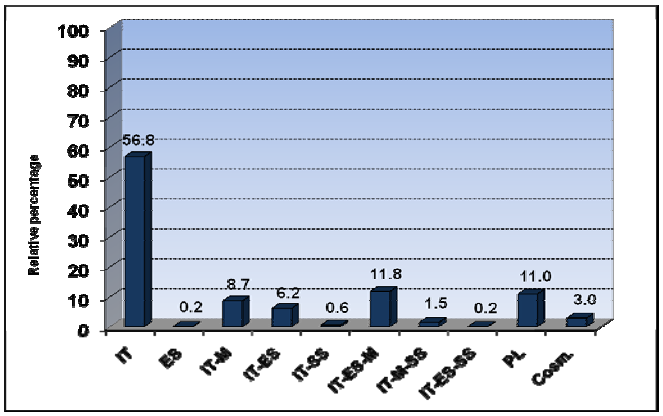

Figure 4. Different chorological types and their relative percentage in flora of Fereizi region (IT: Irano-Turanian, ES: Euro-Siberian, SS: Sahara-Sindian, PL: Pluriregional, Cosm.: Cosmopolitan).

Endemism: Among 484 recorded vascular plant taxa, 21 species (4.3%) are endemic to Iran and 49 taxa (10.1%) are subendemics or regional endemics:

a) The Iranian endemic species belong to 10 families and 16 genera and genus *Astragalus* has the most number of endemic species in the area. Among the Iranian endemic species, *Ferula flabelliloba* (Apiaceae), *Cousinia termei* (Asteraceae) and *Sisymbrium integerrimum* (Brassicaceae) are narrow local endemics to Binalood mountains. Several taxa have a restricted distribution mostly in NE Iran, including *Tanacetum khorassanicum, Aethionema trinervium var. apterocarpum, Graellsia integrifolia, Dianthus binaludensis, Diaphanoptera khorasanica, Astragalus assadii, A. nephtonensis, A. meschedensis, Allium kuhsorkhense* (a very recently described *Allium* species by Fritsch *et al.* 2006) and *Acantholimon pterostegium*. So, overall 13 taxa (more than 60%) of the Iranian endemics of the area are only found in NE Iran. The other endemics occur in a wider geographical extent in Iran: *Zeravschania aucheri, Scorzonera stenocephala, Scabiosa flavida, Astragalus pseudoindurascens, A. sumbari, A. campylotrichus, Marrubium procerum* and *Nepeta glomerulosa*.

b) Endemic to Iran and Turkmenistan (mainly in Khorassan- Kopet Dagh floristic province): *Chaerophyllum khorassanicum, Cephalorrhynchus kossinskyi, Cousinia freynii, Crepis turcomanica, Jurinea monocephala* subsp. *sintenisii, Onosma longilobum, Cleome khorassanica, Astragalus fuhsii, A. raddei, Nepeta ucrainica* subsp. *kopetdaghensis, Salvia chloroleuca, Allium monophyllum, Tulipa micheliana, Bromus*

kopetdaghensis, Delphinium turkmenum, Amygdalus spinosissima subsp. *turcomanica, Cruciata taurica* subsp. *persica, and Hymenocrater calycinus.*

c) Endemic to Iran and Afghanistan: *Eryngium bungei, Pseudotrachydium vesiculoso-alatum, Artemisia khorassanica, Senecio paulsenii* subsp. *khorassanicus, Trichodesma bamianicum, Cicer chorassanicum* and *Eremostachys labiosiformis.*

d) Endemic to Iran, Afghanistan and Turkmenistan: *Artemisia ciniformis, Artemisia kopetdaghensis, Artemisia turcomanica, Chamaegeron bungei, Erysimum ischnostylum, Stroganowia affghana, Dianthus crinitus* subsp. *turcomanicus, Lagochilus cabulicus, Nepeta saccharata, Phlomis cancellata, Salvia nemorosa, Eremurus luteus, Ranunculus leptorrhynchus, Cerasus pseudoprostrata, Asperula glomerata* subsp. *turcomanica, Iris kopetdaghensis* and *Onobrychis chorassanica.*

e) Endemic to Iran and the Middle Asia: *Clausia turkestanica, Acantholimon raddeanum, Rumex tianschanicus, Hyoscyamus turcomanicus, Allium oschaninii, Allium barsczewskii* and *Allium tenuicaule*. The later two *Allium* species have been recently recorded as new species for the flora of Iran (Memariani *et al.* 2007). All of these species group, in their southwestern distribution extent, occur in Khorassan, NE Iran.

Discussion

Among 484 identified plants of the study area, 107 taxa which have not been reported in previous published works are newly recorded for the flora of Binalood mountains (see the appendix). Ghahreman *et al.* (2006) recorded 487 taxa from the same altitude range but from southwestern slopes of Binalood in an area between Baghshangach village and Kharv town with very larger surface area of 50,000 hectares and with more characteristic xerophytic plant species. 49.5 percent of plant species identified in the present study from northern slopes of Binalood were not recorded by Ghahreman *et al.* (2006). These results indicate the difference of floristic composition between north and south slopes of Binalood.

Dominant life forms of plant species clearly reflect the climate of the study area. According to cold semi-arid climate of the area, hemicryptophytes can survive in cold season by their buds below and near soil surface or in dried rosette leaves at soil surface, and therophytes complete their life-cycle during favorable season and survive in the form of seeds in the arid condition of summer and the cold winter. Geophytes survive with resting buds on their subterranean stems in dry and cold seasons. Chamaephytes are adapted to dry, high radiation and windswept conditions in high altitudes of the area as thorny and cushion-like growth forms.

Excluding cultivated orchard tree and shrub species, phanerophytes make up a small portion of life forms (3.7%) in natural flora of the area, mainly as *Lonicera-Cotoneaster-Cerasus* shrubland belt in northern slopes of Jaji Mount, scattered *Berberis* and *Rosa* shrubs in valleys and very scattered *Juniperus* trees in western part of the area. So the life form spectrum of the flora thoroughly demonstrates the dominant montane and subalpine steppe vegetation type of the area.

Considering the considerable number of Irano-Turanian species (56.8%), according to Léonard (1988, 1993) the study area is situated in "Irano-Turanian regional centre of endemism". The influence of Sahara-Sindian regional zone in chorotypes gradually decreases when traveling from the south to north of Iran and from low elevations to montane and alpine zones (Léonard, 1988, 1993; White and Léonard, 1991). Joharchi & Memariani (2006) recorded almost similar phytogeographical spectra in flora of Tandooreh National Park in mountainous areas of central Kopet-Dagh.

Pluriregional (PL, 11%) and cosmopolitan (Cosm., 3%) species are mainly invasive and ruderals or weeds which reflect the anthropogenic origin of some habitats in the area specially agricultural and horticultural locations in several valleys of the Fereizi. The proportion of these two chorotypes is usually low in well protected natural areas such as Tandooreh National Park (Joharchi & Memariani, 2006). Hydrophytes or truly aquatic plant species do not occur in the study area, although some subaquatic or hygrophilous species of the area have been included into the other groups like graminoid hemicryptophytes and rhizomatous geophytes.

According to Jalili & Jamzad (1999), the average number of endemics per million hectares of the whole country is 10.46 species. Presence of 21 Iranian endemic species demonstrates a high plant endemism in such a small studied area as well as 31 plant taxa (6.4% of the flora) endemic to Khorassan- Kopet Dagh floristic province in mountainous areas of NE Iran and S Turkmenistan.

Binalood mountain range, with its rich flora and high number of endemic and subendemic plants, has a considerable significance in plant diversity of Khorassan. Local endemics with narrow distributions and Khorassan-Kopet Dagh floristic elements show an important role in its flora and vegetation. Moreover, several plant species occurring mainly in Alborz mountain range or in the Middle Asian mountains, especially Pamir and

Tien Shan, have isolated disjunct populations in Binalood. Regarding the unique plant diversity and endemism, the urgent conservation of the area is suggested because of human settlements, extensive agricultural activities, increasing overgrazing of vegetation, gathering medicinal species, and absence of any protected area especially in north-facing slopes.

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Appendix. Checklist of vascular plant taxa in Fereizi region, Binalood mountain range, NE Iran.

Species / Infraspecific taxa	Chorotype[1]	Life form[1]	Herbarium No. (FUMH)[2]	Records from Binalood Mnts.[3]
Amaranthaceae				
Amaranthus retroflexus L.	PL	Th	37012	4
Amaryllidaceae				
Ixiolirion tataricum (Pall.) Herb.	IT-M	G.b	35977	1, 4
Anacardiaceae				
Rhus coriaria L.	PL	Ph	s.n.	4, 5
Apiaceae				
Astrodaucus orientalis (L.) Drude	IT-ES	G.t	36666, 36844	2, 5
Bunium cylindricum (Boiss. & Hohen.) Drude	IT	G.t	36050	1, 4, 5
B. persicum (Boiss.) B.Fedtsch.	IT	G.t	36160, 36664	1, 4
B. wolffii Kljuykov	IT	G.t	36567	*
Bupleurum exaltatum M.Bieb.	IT	Ch	36835	1, 4, 5
B. rotundifolium L.	IT-M	Th	36827	1
Caucalis platycarpos L.	IT-M	Th	36170, 36293	2, 4
Chaerophyllum khorassanicum Czerniak. ex Schischk.	IT	He	36566	1, 5
Ch. macrospermum (Spreng.) Fisch. & C.A.Mey.	IT	He	36935	5
Conium maculatum L.	IT-ES	He	36397, 36618	4, 5
Eryngium bungei Boiss.	IT	He	36759, 36823	1, 4, 5
Ferula flabelliloba Rech.f. & Aell.	IT	He	36024, 36072, 36652	1, 4, 5
F. ovina (Boiss.) Boiss.	IT	He	36071, 36665	1, 4, 5
F. szowitsiana DC.	IT	He	36432	1, 5
Galagania platypoda (Aitch. & Hemsl.) M.G.Vassiljeva & Pimenov	IT	G.t	36555	*
Heracleum persicum Desf. ex Fischer	IT	He	36378, 36677	1, 5
Johreniopsis seseloides (C.A.Mey.) Pimenov	IT-ES	He	36721	2, 5
Pimpinella affinis Ledeb.	IT-ES	G.t	36668, 36750	1, 4, 5
Prangos bungei Boiss.	IT	G.t	35915	5
Pr. latiloba Korov.	IT	He	35937, 35985, 36148	1, 4, 5
Scandix aucheri Boiss.	IT-ES	Th	35991	1, 4
S. pectin-veneris L.	IT-ES-M	Th	35982B, 36015	*
S. stellata Banks & Sol.	IT-M	Th	35990, 36049, 36146	1, 4
Torilis leptophylla Rchb.f.	IT-ES	Th	36171, 36419, 36579	4, 5
Pseudotrachydium vesiculoso-alatum (Rech.f.) Pimenov & Kljuykov	IT	Ch	36542	5
Turgenia latifolia (L.) Hoffm.	IT-M	Th	36328, 36459, 36606	1, 4, 5
Zeravschania aucheri (Boiss.) Pimenov	IT-ES	He	36646,	5
Zosima absinthifolia (Vent.) Link.	IT	He	35913, 36033, 36319	1, 4
Araceae				
Arum kotschyi Boiss. & Hohen. ex Boiss.	IT-ES	G.t	36138	4
Asclepiadaceae				
Cynanchum acutum L.	IT-M-SS	He	36876	4
Vincetoxicum pumilum Decne.	IT	He	36546	1, 4, 5
Asteraceae				
Achillea biebersteinii Afan.	IT-ES	He	36209, 36341, 36789	1, 4
A. wilhelmsii K.Koch	IT	He	36554	1
Acroptilon repens (L.) DC. subsp. *australe* (Iljin) Rech.f.	IT	He	36768	1, 4
Arctium lappa L.	IT-ES	He	36942	*
Artemisia biennis Willd.	PL	He	36972	1
A. ciniformis Krasch. & Popov ex Poljakov	IT	Ch	36988, 37006, 37021	*
A. khorassanica Podl.	IT	Ch	36986, 37033	1

A. kopetdaghensis Krasch., Popov & Lincz. ex Poljakov	IT	Ch	36989, 36991, 37005	1
A. scoparia Waldst. & Kit.	IT-ES-M	Ch	36993	1, 4
A. turcomanica Gand.	IT	Ch	36987	*
Carduus pycnocephalus L.	IT-M	Th	35971	4
Centaurea behen L.	IT	He	36833	1
Centaurea depressa M.Bieb.	IT	Th	36418	4
Centaurea virgata Lam.	IT	He	36549	1, 4
Cephalorrhynchus kossinskyi (Krasch.) Kirp.	IT	G.t	36139	1
C. polycladus (Boiss.) Kirp.	IT	He	36617	1, 4
Chamaegeron bungei (Boiss.) Botsch.	IT	He	37036	*
Chardinia orientalis Kuntze	IT	Th	35999, 36064, 36306	4
Chondrilla juncea L.	IT-ES-M	He	36880, 36976	4
Cichorium intybus L.	PL	He	36774, 36881	4
Cirsium arvense (L.) Scop. var. *incanum* (S.G.Gmel.) Ledeb.	PL	He	36607, 36769	1, 4
C. strigosum M.Bieb.	IT	He	36975	1
Cnicus benedictus L.	IT-M	Th	35844, 35970	4
Codonocephalum peacockianum Aitch. & Hemsl.	IT	He	36594	1
Cousinia freynii Bornm.	IT	He	36762	1
C. microcarpa Boiss.	IT	He	36686, 36776	1, 4
C. termei Rech.f.	IT	He	36974	1
C. umbrosa Bunge	IT	He	36616	1, 4
Crepis pulchra L. subsp. *turkestanica* Babcock	IT	Th	35995, 36094, 36767	1, 4
Cr. sancta (L.) Babcock subsp. *iranica* Rech.f.	IT	Th	35889, 35969	4
Cr. turcomanica Krasch.	IT	G.r	36662	*
Cymbolaena griffithii (A.Grey) Wagenitz	IT	Th	36448	1
Filago arvensis L.	IT-ES-M	Th	36444	1
F. pyramidata L.	IT-ES-M	Th	35981, 36446	1, 4
Garhadiolus angulosus Jaub. & Spach	IT	Th	35896	4
Gundelia tournefortii L.	IT	He	36452	4
Heteropappus altaicus (Willd.) Novopokr. var. *canescens* (Nees) Serg.	IT	He	36999	1, 4
Jurinea monocephala Aitch. & Hemsl. subsp. *sintenisii* (Bornm.) Wagenitz	IT	He	36404	1
Koelpinia linearis Pall.	IT-SS	Th	35996, 36436, 36450	1, 4
Lactuca serriola L.	IT-ES-M	He	37025	4
Lapsana communis L.	IT-ES	He	36971	2
Leontodon asperrimus (Willd.) Boiss. ex Ball.	IT	He	36547	1, 4
Pulicaria gnaphalodes (Vent.) Boiss.	IT	Ch	37003	4
Scariola orientalis (Boiss.) Sojak subsp. *orientalis*	IT	Ch	36926, 36981	1, 4
Scorzonera laciniata L.	IT-ES-M	He	36592	*
Sc. stenocephala Boiss.	IT	G.t	35968, 36059, 36181	**
Senecio paulsenii O.Hoffm. subsp. *khorasanicus* (Rech.f. & Aell.) B.Nord.	IT	He	36363, 36187	*
Serratula latifolia Boiss.	IT	He	36484, 36584	1
Sonchus asper (L.) Hill	IT-M	He	36602	*
So. oleraceus L.	PL	Th	36892, 36963, 37032	4
Steptorhamphus persicus O.Fedtsch. & B.Fedtsch.	IT	G.t	36076	1, 4
Tanacetum khorassanicum (Krasch.) Parsa	IT	He	36633	1
T. partheninm (L.) Sch.Bip.	PL	He	36304, 36627, 36679	1, 4
Taraxacum montanum (C.A.Mey.) DC.	IT	He	36920, 36980	2
T. nevskii Juz.	IT	He	36389	*
T. pseudo-calocephalum Soest	IT	He	36108	*
T. wallichi DC.	IT	He	35894	*
Tragopogon gaudanicus Boriss.	IT	He	36361	*
Tr. graminifolius DC.	IT	He	35972	4
Tr. longirostris Bisch.	IT-M-SS	He	36104, 36175, 36308	4

Tripleurospermum disciforme (C.A.Mey.) Sch.Bip.	IT	He	36208, 36406	*
Varthemia persica DC.	IT	Ch	36922, 36977	1
Xeranthemum longipapposum Fisch. & C.A.Mey.	IT	Th	36603, 36683	4
Berberidaceae				
Berberis integerrima Bunge	IT	Ph	35966	1, 4
Boraginaceae				
Anchusa arvensis (L.) M.Bieb. subsp. *orientalis* (L.) Nordh.	IT-M	Th	36394, 36423, 36784	*
A. italica Retz.	IT-M	He	36006	4, 5
Asperugo procumbens L.	PL	Th	35867	4, 5
Buglossoides arvensis (L.) Johnston	IT-ES	Th	36017, 36107	1
Caccinia macranthera (Banks & Soland.) Brand var. *crassifolia* (Vent.) Brand	IT	He	35887	4
Echium italicum L.	IT-M	He	36593, 37023	1, 2, 4, 5
Heliotropium europaeum L.	IT-ES-M	Th	36697	4, 5
Heterocaryum laevigatum A. DC.	IT	Th	36358	*
H. subsessile Vatke	IT	Th	35850, 36053	*
Lappula barbata Gürke	IT	He	36421, 36657	4, 5
L. microcarpa Gürke	IT	Th	35918, 36152	1, 5
L. sinaica (A.DC.) Asch. ex Schweinf.	IT	Th	35849, 36291, 36656	5
Lithospermum officinale L.	IT-ES-M	He	36375, 36783, 36895	1, 5
Nonnea caspica (Willd.) G.Don	IT	Th	35914, 36185	4
Onosma dichroanthum Boiss.	IT	He	36194	1, 5
Onosma longilobum Bunge.	IT	He	36178, 36724	1, 4, 5
Rochelia bungei Trautv.	IT	Th	35861, 36031	*
R. cardiosepala Bunge.	IT	Th	35984, 36057, 36420	1, 4, 5
R. disperma (L.f.) K.Koch	IT	Th	35866	*
R. persica Bunge. ex Boiss.	IT	Th	36068, 36386, 36624	1
Solenanthus circinnatus Ledeb.	IT	He	36137	1
Trichodesma bamianicum Rech.f. & Riedl	IT	He	36647, 36696	4, 5
Trichodesma incanum (Bunge.) A.DC.	IT	He	36722, 36930	1, 5
Brassicaceae				
Aethionema carneum (Banks & Soland.) B.Fedtsch.	IT	Th	35873, 36056	1
Ae. trinervium (DC.) Boiss. var. *apterocarpum* (Rech.f. & Aell.) Hedge	IT	Ch	35951	*
Alliaria petiolata (M.Bieb.) Cavara & Grande	IT-ES-M	He	36009, 36112	1
Alyssum meniocoides Boiss.	IT	Th	36069	4
A. stapfii Vierh.	IT	Th	35854, 35945, 36086	1, 4
Barbarea plantaginea DC.	IT	He	35878, 36113, 36712	1, 4
Brassica napus (L.) Koch	PL	He	35874, 36894	2
Br. rapa L. subsp. *campestris* (L.) A.R.Clapham	PL	He	36121, 37011, 37037	*
Buchingera axillaris Boiss.	IT	Th	35931, 36120	1
Camelina rumelica Velen.	IT-ES-M	Th	35974, 36000, 36327	*
Capsella bursa-pastoris (L.) Medicus	Cosm.	Th	35934, 36143	1, 4
Cardaria draba (L.) Desv.	Cosm.	He	35973, 36343	1, 4
Chorispora tenella (Pall.) DC.	IT	Th	35884, 36106, 36622	4
Clausia turkestanica Lipsky	IT	He	35933, 36371	*
Clypeola jonthlaspi L.	IT-M	Th	35977	1, 4
C. microcarpa Moris.	IT-M	Th	35855, 36614	*
Conringia perfoliata (C.A.Mey.) Busch	IT	Th	35998, 36101	4
Crambe kotschyana Boiss.	IT	He	36025	1, 4
Crucihimalaya wallichii (Hook.f. & Thoms.) Al-Shehbaz, O'Kane & R.A.Price (Syn.: *Arabidopsis wallichii*)	PL	He	36631	1, 4
Descurainia sophia (L.) Webb & Berth.	IT-ES-M	Th	36345	4
Diptychocarpus strictus (Fisch.) Trautv.	IT	Th	35856	1
Drabopsis verna K.Koch	IT	Th	35857, 36619	4
Erophila verna (L.) Besser	IT-ES-M	Th	36620	1

Erysimum ischnostylum Freyn. & Sint.	IT	He	36020, 36189	1
Euclidium syriacum (L.) R.Br.	IT	Th	36578	1
Eu. tenuissimum (Pall.) B.Fedtsch.	IT	Th	35954, 36193, 36422	4
Fibigia suffruticosa (Vent.) Sweet	IT	He	36407	1
Goldbachia laevigata (M.Bieb.) DC.	IT	Th	36580	4
Graellsia integrifolia (Rech.f.) Rech.f.	IT	He	35935, 36131	1
Lepidium latifolium L.	IT-ES-M	He	36779	4
Nasturtium officinale R.Br.	PL	He	36562	4
Neotorularia dentata (Kitam) Hedge & J.Léonard	IT	Th	35872	*
Neslia apiculata Fisch., C.A.Mey. & Avé-Lall.	IT-ES-M	Th	36008, 36192	4
Olimarabidopsis pumila (Stephan) Al-Shehbaz, O'Kane & R.A.Price (Syn.: *Arabidopsis pumila*)	PL	Th	35880, 35975, 36147	1
Pachypterygium brevipes Bunge.	IT	Th	36200, 36651	1, 4
Sisymbrium integerrimum Rech.f. & Aell.	IT	He	36190	1
S. loeselii L.	IT-ES	He	36114, 36928	4
S. septulatum DC.	IT	Th	35853, 35876	4
Strigosella africana (L.) Botsch. var. *africana* (Syn.: *Malcolmia africana* var. *africana*)	IT-M-SS	Th	35882	4
Stroganowia affghana (Boiss.) Pavlov	IT	He	36719, 36979	1
Tauscheria lasiocarpa Fisch. ex DC.	IT	Th	36188, 36356	1
Thlaspi arvense L.	PL	Th	36144, 36376	2
Th. perfoliatum L.	IT-ES-M	Th	35932, 36655	2, 4
Capparidaceae				
Buhsea trinervia Fresen	IT	G.r	36550	5
Capparis spinosa L.	PL	He	36434	4, 5
Cleome khorassanica Bunge. & Bien. ex Boiss.	IT	Th	36824	4, 5
Caprifoliaceae				
Lonicera nummulariifolia Jaub. & Spach	IT	Ph	36318, 36924	5
Caryophyllaceae				
Acanthophyllum glandulosum Bunge ex Boiss.	IT	Ch	36890	1, 4
A. mucronatum C.A.Mey.	IT	Ch	36755	1
Arenaria leptoclados (Rchb.) Guss.	IT-ES-M	Th	35976, 36684	1, 4
Cerastium inflatum Link ex Desf.	IT	Th	35886, 36126	1, 4
Dianthus binaludensis Rech.f.	IT	Ch	36481, 36552	1, 4
D. crinitus Sm. subsp. *turcomanicus* (Schischk.) Rech.f.	IT	Ch	36553	1
D. orientalis Adams	IT	Ch	36653	1, 4
Diaphanoptera khorasanica Rech.f.	IT	Ch	36430	*
Lepyrodiclis stellarioides Schrenk ex Fisch. & C.A.Mey.	IT	Th	35960, 36401	1, 4
Mesostemma kotschyanum (Schischk.) Vved. subsp. *kotschyanum*	IT	He	36307	1, 4
Minuartia meyeri (Boiss.) Bornm.	IT	Th	36095, 36337	*
Saponaria orientalis L.	IT	Th	36297	1
Silene brahuica Boiss.	IT	Ch	36551	**
S. chaetodonta Boiss.	IT	Th	36454	4
S. coniflora Nees ex Otth	IT-M	Th	36346	*
S. conoidea L.	IT-M	Th	36331	1, 4
S. latifolia Poir. subsp. *persica* (Boiss. & Buhse) Melzh.	IT-ES-M	He	36005, 36136, 36352	1, 4
S. noctiflora L.	IT-ES-M	He	36882	3
S. swertiifolia Boiss.	IT	He	36310, 36557	1, 4
S. viscosa (L.) Pers.	IT-ES	He	s.n.	*
Stellaria media (L.) Vill.	Cosm.	Th	36116	1, 4
Chenopodiaceae				
Chenopodium botrys L.	PL	Th	36925	1, 4
Ch. foliosum Asch.	PL	Th	36703	*
Krascheninnikovia ceratoides (L.) Gueldenst.	IT-M	Ch	36556, 36997	*
Noaea mucronata (Forssk.) Asch. & Schweinf.	IT-M-SS	Ch	36899	1, 4
Salsola kali L.	PL	Th	37004	1, 4

Convolvulaceae				
Convolvulus pseudocantabricus Schrenk	IT	He	36558	*
C. arvensis L.	Cosm.	G.r	36722	4
C. lineatus L.	IT-ES-M	He	36372, 36591	4, 5
Crassulaceae				
Pseudosedum multicaule (Boiss. & Buhse) Boriss.	IT	He	s.n.	1, 5
Cupressaceae				
Juniperus excelsa M. Bieb.	IT	Ph	s.n.	1
Cyperaceae				
Bolboschoenus affinis (Roth) Drob.	PL	G.r	36471	1
Carex stenophylla Wahlenb.	PL	He	35859	*
Scirpoides holoschoenus (L.) Sojak	PL	G.r	36475	4
Dipsacaceae				
Cephalaria kotschyi Boiss. & Hohen.	IT	He	36720	*
Dipsacus strigosus Willd.	ES	He	36936	1
Scabiosa flavida Boiss. & Hausskn.	IT	Th	36958	2
S. olivieri Coult.	IT	Th	35848, 36451	1, 4, 5
S. rotata M.Bieb.	IT	Th	35957, 36158, 36445	4, 5
Elaeagnaceae				
Elaeagnus angustifolia L.	IT-M	Ph	36435	4
Ephedraceae				
Ephedra intermedia Schrenk & C.A.Mey.	IT	Ch	36753	*
E. major Host	IT-ES-M	Ph	36947	1
Equisetaceae				
Equisetum palustre L.	PL	G.r	36470, 36787	*
Euphorbiaceae				
Euphorbia buhsei Boiss.	IT	He	36440	*
E. bungei Boiss.	IT	He	35942, 36204, 36340	1, 4
E. szovitsii Fisch. & Mey.	IT	Th	36058, 36295, 36681	1
Fabaceae				
Astragalus (Alopecuroidei) schahrudensis Bunge	IT	He	36339	1
A. (Annulares) campylorrhynchus Fisch. & C.A.Mey.	IT	Th	35899, 36124, 36437	4
A. (Anthylloidei) fuhsii Freyn. & Sint.	IT	Ch	36344, 36578	*
A. (Anthylloidei) raddei Basil.	IT	He	36545	1
A. (Astragalus) basineri Trautv.	IT	He	36002	1, 4
A. (Astragalus) sieversianus Pall.	IT	He	35928, 36218	1, 4
A. (Caprini) assadii Maassoumi & Podlech	IT	He	36323, 36673	5
A. (Caprini) nephtonensis Freyn	IT	He	35851	4
A. (Caprini) pseudoindurascens Sirj. & Rech.f.	IT	He	36476	1
A. (Cremoceras) ochreatus Bunge	IT	He	35907, 36400, 36669	4
A. (Dipelta) dipelta Bunge	IT	Th	36196, 36765	1, 4
A. (Dissitiflori) sumbari Popov	IT	He	36544	6
A. (Erioceras) anacamptus Bunge	IT	He	35908, 36573	**
A. (Heterodontus) campylotrichus Bunge	IT	Th	36047	*
A. (Hymenostegis) chrysostachys Boiss.	IT	Ch	36588	1
A. (Incani) ackerbergensis Freyn	IT	He	36021, 36206, 36333	6
A. (Malacothrix) suluklensis Freyn & Sint.	IT	He	36159, 36438	6
A. (Onobrychioidei) brevidens Bunge	IT	He	36582	6
A. (Oxyglottis) oxyglottis Steven	IT-M	Th	36052	1
A. (Oxyglottis) schmalhausenii Bunge	IT	Th	36355	1
A. (Oxyglottis) vicarius Lipsky	IT	Th	36001, 36167	5
A. (Platonychium) meschedensis Bunge	IT	Ch	36477	6
A. (Platyglottis) camptoceras Bunge	IT	Th	35898, 36166, 36205	*
A. (Sesamei) perspolitanus Boiss.	IT	Th	35906	1
A. (Theiochrus) siliquosus Boiss. subsp. *siliquosus*	IT	He	36385	1

Cicer chorassanicum Popov	IT	Th	36164, 36460	4
C. tragacanthoides Jaub. & Spach var. *tragacanthoides*	IT	Th	36695	1, 4, 5
Colutea buhsei (Boiss.) Shapar.	IT-ES	Ph	36316, 36671	1, 4
Glycyrrhiza glabra L. var. *glandulifera* (Waldst. & Kit.) Boiss.	IT-ES-M	G.r	36877	4
Lathyrus aphaca L. var. *aphaca*	IT-ES-M	Th	36568	1, 4
L. inconspicuus L.	IT-M	Th	36098, 36179	4
Lens cyanea (Boiss. & Hohen.) Alef.	IT	Th	35905, 36091	5
L. orientalis (Boiss.) Hand. -Mzt.	IT	He	36153, 36680	1
Lotus corniculatus L. subsp. *corniculatus*	PL	He	36382, 36756	4
Medicago lupulina L.	PL	He	36311	4
Melilotus indicus (L.) All.	PL	Th	37030	*
M. officinalis (L.) Pall.	IT-ES-M	He	36388, 36771	1, 4
Meristotropis xanthioides Vassilcz.	IT	G.r	36480	1
Onobrychis chorassanica Bunge	IT	He	36548	1
O. cornuta (L.) Desv.	IT	Ch	s.n.	*
Pisum sativum L.	PL	Th	36299	*
Trifolium pratense L.	IT-ES-M	He	36127	1, 4
T. repens L.	IT-ES-M	He	36048	1
Trigonella monantha C.A.Mey. subsp. *noeana* (Boiss.) Huber. -Morath	IT	Th	35900	*
Vicia ervilia (L.) Willd.	IT-M	Th	36169	*
V. hyrcanica Fisch. & C.A.Mey.	IT	Th	36413	1
V. peregrina L.	IT-ES	Th	35901, 36336	1, 4
V. sativa L. var. *angustifolia* L.	IT-ES-M	Th	36157	1
V. sativa L. var. *sativa*	IT-ES-M	Th	36165	*
V. subvillosa (Ledeb.) Trautv.	IT	Th	35927, 36685	1, 4
Fumariaceae				
Fumaria asepala Boiss.	IT-M	Th	35891	*
F. vaillantii Loisel.	IT-ES-M	Th	36090	1, 4
Geraniaceae				
Biebersteinia multifida DC.	IT	G.t	35940	1, 4
Erodium ciconium (Jusl.) L'Her. ex Aiton	IT-M	Th	35917, 35962	*
E. cicutarium (L.) L'Her. ex Aiton	IT-ES-M	He	35910	1, 4
Geraniam charlesii (Aitch. & Hemsl.) Vved. ex Nevski	IT	G.t	35909, 35986	***
G. divaricatum Ehrh.	IT-ES-M	Th	35963	*
G. kotschyi Boiss.	IT	G.t	35925	1, 4
G. pusillum L.	IT-ES-M	Th	36466, 37038	**
G. rotundifolium L.	IT-ES-M	Th	36485	1, 4
Hypericaceae				
Hypericum perforatum L.	PL	He	36780	4, 5
H. scabrum L.	IT	He	36314	1, 4, 5
Iridaceae				
Gladiolus atroviolaceus Boiss.	IT-M	G.c	36060	5
Iris fosteriana Aitch. & Baker	IT	G.r	35840	1, 5
I. kopetdagensis (Vved.) B.Mathew & Wendelbo	IT	G.r	35862	1, 4, 5
Juglandaceae				
Juglans regia L.	PL	Ph	36927	1, 4
Juncaceae				
Juncus bufonius L.	Cosm.	Th	36623, 36709	*
J. rigidus Desf.	PL	G.r	36788	*
Lamiaceae				
Acinos graveolens (M.Bieb.) Link.	IT-M	Th	36066, 36140, 36302	1
Drepanocaryum sewerzowii (Regel) Pojark.	IT	Th	35979, 36041	1
Eremostachys labiosa Bunge	IT	He	35890, 36054	1
E. labiosiformis (Popov.) Knorr.	IT	He	35987	1, 4

E. macrophylla Montbr. & Auch.	IT	He	35841	*
Hymenocrater calycinus (Boiss.) Benth.	IT	Ch	36051, 36300, 36639	1, 4
Lagochilus cabulicus Benth.	IT	Ch	36543	1
Lallemantia baldschuanica Gontsch.	IT	Th	35983	*
L. iberica (Stev.) Fisch. & C.A.Mey.	IT-M	Th	36305, 36399, 36690	2
L. royleana Benth.	IT	Th	36141	1
Lamium amplexicaule L.	Cosm.	Th	35922, 36142	1, 4
Marrubium anisodon K.Koch	IT	He	36800	1, 4
M. procerum Bunge	IT	He	36932	1
Mentha longifolia (L.) Hudson	PL	He	36764, 37028	4
Nepeta bracteata Benth.	IT	Th	36301, 36609	1, 4
N. glomerulosa Boiss.	IT	He	36796	1, 4
N. saccharata Bunge	IT	Th	35846, 36172, 36467	*
N. ucrainica L. subsp. *kopetdaghensis* (Pojark.) Rech.f.	IT	He	36583	*
Perovskia abrotanoides Karel.	IT	Ch	36763, 36829	1, 4
Phlomis cancellata Bunge	IT	He	36431, 36717	1, 4
Salvia ceratophylla L.	IT	He	36207	*
S. chloroleuca Rech. f. & Aell.	IT	He	36186, 36309, 36670	1, 4
S. nemorosa L.	IT-ES	He	36414, 36605	1
Scutellaria litwinowii Bornm. & Sint. ex Bornm.	IT	Ch	36154, 36676	1, 4
Stachys lavandulifolia Vahl.	IT	Ch	36014	1, 4
St. setifera C.A.Mey.	IT	He	36654, 36834	4
Teucrium polium L.	IT-M	Ch	s.n.	1, 4
Ziziphora clinopodioides Lam.	IT	Ch	36838	1
Z. tenuior L.	IT	Th	35847, 36029	1
Liliaceae *s.l.*				
Allium altissimum Regel	IT	G.b	36379	1
A. ampeloprasum L.	IT-ES-M	G.b	36757	*
A. atroviolaceum Boiss.	IT-ES	G.b	36773	1, 4
A. barsczewskii Lipsky	IT	G.b	26072A	7
A. fibrosum Regel	IT	G.b	36034	4
A. iranicum (Wendebo) Wendelbo	IT	G.b	36642	*
A. kuhsorkhense R.M.Fritsch & Joharchi	IT	G.b	35978, 36335	8
A. monophyllum Vved.	IT	G.b	36026	*
A. oschaninii O.Fedtsch.	IT	G.b	36663, 36840	1
A. rubellum M.Bieb.	IT	G.b	36070	4
A. tenuicaule Regel	IT	G.b	26072B	7
A. umbilicatum Boiss.	IT	G.b	36585	1
A. xiphopetalum Aitch. & Baker	IT	G.b	36087, 36405	1, 4
Colchicum kotschyi Boiss.	IT	G.b	37009, 37027	*
Eremurus luteus Baker	IT	G.r	36028	1, 4
E. spectabilis M.Bieb.	IT	G.r	36370	*
Fritillaria gibbosa Boiss.	IT	G.b	36199, 36360	1, 4
Gagea stipitata Merckl. ex Bunge	IT	G.b	35852, 35936	4
Muscari neglectum Guss.	IT-ES-M	G.b	35920, 36027	1, 4
Polygonatum sewerzowii Regel	IT	G.r	36641	*
Tulipa biebersteinia Schult. f.	IT-ES	G.b	35858	*
T. micheliana Hoog	IT	G.b	35916, 36381	1, 4
Malvaceae				
Alcea angulata (Freyn & Sint.) Freyn & Sint. ex Iljin	IT	He	36716	*
Althaea cannabina L.	IT-M	He	36843	1
Malva sylvestris L.	PL	He	36395	*
Onagraceae				
Epilobium hirsutum L.	PL	G.r	36777	1, 4, 5
E. minutiflorum Hausskn.	IT	G.r	36961	1, 5

E. tetragonum L.	IT-ES-M	G.r	36628	*
Orchidaceae				
Epipactis persica (Soó) Nannf.	IT-ES	G.r	36883	2
Listera ovata (L.) R.Br.	PL	G.r	36135	*
Orobanchaceae				
Orobanche mutelii F.W.Schultz	IT-M	G.p	36324	*
Papaveraceae				
Glaucium elegans Fisch. & C.A.Mey.	IT	Th	35870	4
Hypecoum pendulum L.	IT-M	Th	35868, 36063	4
Papaver dubium L.	PL	Th	35989, 36330	1, 4
Roemeria hybrida (L.) DC. subsp. *dodecandra* (Forssk.) Maire	IT-M-SS	Th	35912, 36329	1, 4
R. refracta DC.	IT	Th	36040, 36338	1, 4
Plantaginaceae				
Plantago lanceolata L.	IT-ES-M	He	36184, 36934	4
P. major L.	Cosm.	He	36960, 37034	4
Plumbaginaceae				
Acantholimon erinaceum (Jaub. & Spach) Lincz.	IT	Ch	36982	4, 5
Ac.raddeanum Czernjak.	IT	Ch	36896	4, 5
Ac. pterostegium Bunge	IT	Ch	36161, 36348	1, 4, 5
Poaceae				
Aegilops tauschii Coss.	IT	Th	36457	4
Ae. triuncialis L.	IT-M	Th	37417	4
Aeluropus littoralis (Gouan) Parl.	IT-M-SS	He	36879	*
Alopecurus arundinaceus Poir. var. *arundinaceus*	PL	He	36469, 36706	1, 4
Arrhenatherum kotschyi Boiss.	IT	He	36080, 36162, 36392	1, 4
Avena sativa L.	Cosm.	Th	36785	*
Boissiera squarrosa (Banks & Sol.) Nevski	IT	Th	36151	1
Bothriochloa ischaemum (L.) Keng	PL	He	36898	1, 4
Bromus danthoniae Trin.	IT	Th	35994, 36598	1, 4
Br. japonicus Thunb. var. *japonicus*	PL	Th	36426	4
Br. kopetdaghensis Drobov	IT	He	36368, 36571	1
Br. pseudodanthoniae Drobov	IT	Th	36130, 36409	*
Br. scoparius L.	IT-ES-M	Th	36428	1, 4
Br. sterilis L.	IT-ES-M	Th	36042, 36315, 36700	4
Br. tectorum L.	PL	Th	36082, 36458	4
Calamagrostis epigejos (L.) Roth	PL	He	36761	2
C. pseudophragmites (Hall.f.) Koeler	IT-ES-M	He	36713, 36793	4
Catabrosa aquatica (L.) P.Beauv.	PL	He	36704	*
Dactylis glomerata L.	PL	He	36210	1, 4
Echinochloa crus-galli (L.) P.Beauv. var. *crus-galli*	Cosm.	Th	37015	4
Elymus hispidus (Opiz.) Melderis subsp. *hispidus*	IT-ES-M	He	36599	*
El. repens (L.) Gould subsp. *elongatiformis* (Drobov) Melderis	PL	He	36940	4
El. transhyrcanus (Nevski) Tzvelev	IT	He	36586	*
Eremopoa persica (Trin.) Roshev.	IT-M	Th	36374, 36638	*
Eremopyrum bonaepartis (Spreng.) Nevski var. *bonaepartis*	IT-M	Th	36043	4
E. distans (K.Koch) Nevski	IT	Th	35845, 36044	*
Festuca arundinacea Schreb.	IT-ES	He	36464	*
F. pratensis Huds.	IT-ES	He	36212, 36778	1
Glyceria plicata (Fries) Fries	IT-ES-M	He	36702	*
Henrardia persica (Boiss.) C.E.Hubb.	IT	Th	36453	*
Heteranthelium piliferum Hochst. ex Jaub. & Spach	IT	Th	36213, 36569	4
Hordeum murinum L. subsp. *leporinum* (Link) Arcang.	IT-M	Th	36037	**
H. murinum L. subsp. *glaucum* (Steud.) Tzvelev	IT-M	Th	36801	4
Lolium rigidum Gaudin	IT-ES-M	Th	37026	1

Melica persica Kunth subsp. *canescens* (Regel) P.H.Davis	IT	He	36163, 36390	*
M. persica Kunth subsp. *jacquemontii* (Decne. ex Jaquem.) P.H.Davis	IT	He	36802	*
Pennisetum orientale Rich.	IT-SS	He	36878	1, 4
Poa angustifolia L.	IT-ES	He	36211, 36391	4
P. bulbosa L.	IT-ES-M	He	35923, 36111	1, 4
P. pratensis L.	PL	He	36110	1, 4
P. trivialis L.	PL	He	36132, 36403, 36707	1
Polypogon monspliensis (L.) Desf.	PL	Th	36781	*
Rostraria cristata (L.) Tsvelev (Syn.*: Lophochloa phleoides* (Vill.) Rchb.)	PL	Th	36455	4
Setaria glauca (L.) P.Beauv.	PL	Th	36968, 37010	1, 4
S. viridis (L.) P.Beauv.	PL	Th	36943	4
Stipa arabica Trin. & Rupr.	IT	He	36347, 36570	1
St. hohenackeriana Trin. & Rupr.	IT	He	36797	*
Taeniatherum caput-medusae (L.) Nevski	IT-M	Th	36045, 36215, 36798	1
Triticum aestivum L.	Cosm.	Th	36795	*
Vulpia myuros (L.) C.C.Gmel.	IT-M	Th	36442	4
V. persica (Boiss. & Buhse) V.I.Krecz. & Bobr.	IT	Th	36202	*
Podophyllaceae				
Bongardia chrysogonum (L.) Spach	IT	G.t	35947	1, 4
Leontice leontopetalum L. subsp. *ewersmannii* (Bunge) Coode	IT	G.b	35949, 36125	*
Polygonaceae				
Atraphaxis spinosa L.	IT	Ch	35964, 36987	1, 4
Polygonum arenastrum Boreau	Cosm.	Th	36705, 37002	*
P. argyrocoleon Steud. ex Kunze	IT	Th	36939	*
P. aviculare L.	Cosm.	Th	36799, 37035	*
P. convolvulus L.	PL	Th	36884, 36970	2, 4
P. mite Schrank	IT-ES-M	Th	37016	2
P. paronychioides C.A.Mey. ex Hohen.	IT	He	36478	*
P. patulum M.Bieb.	IT-M	Th	36790	4
P. polycnemoides Jaub. & Spach	IT	Th	36298, 36692, 36938	1, 4
Rheum ribes L.	IT	G.r	36078, 36667	1, 4
Rumex tianschanicus Los.-Losinsk.	IT	G.r	36380	1
R. tuberosus L.	IT-M	G.t	36079, 36668	1, 4
Primulaceae				
Anagalis arvensis L. subsp. *arvensis* var. *caerulea* (L.) Gouan	PL	Th	36629	4
Androsace maxima L.	IT-ES-M	Th	35956	4
Ranunculaceae				
Adonis aestivalis L.	IT-ES-M	Th	35843, 36410	*
A. scorbiculata Boiss. subsp. *scorbiculata*	IT	Th	35952, 36065	1
Anemone petiolulosa Juz.	IT	G.t	35950	4
Ceratocephala falcata (L.) Pers.	IT-ES-M	Th	35842, 36097	4
C. testiculata (Crantz) Roth	IT-ES	Th	35941, 36145	4
Clematis orientalis L.	IT	Ch	36842, 37007	4
Consolida orientalis (J.Gay) Schrödinger	IT-M	Th	36424	1, 4
Delphinium turkmenum Lipsky	IT	Th	36449, 36636	1, 4
Nigella integrifolia Regel	IT	Th	36575	*
Ranunculus arvensis L.	IT-ES-M	Th	36004, 36174, 36411	*
R. leptorrhynchus Aitch. & Hemsl.	IT	G.r	35943, 35980	1
R. oxyspermus Willd.	IT-ES	G.r	36003, 36173	4
Thalictrum isopyroides C.A.Mey.	IT	G.r	35926	4
Resedaceae				
Reseda lutea L.	IT-ES-M	He	35885, 36439	1, 4, 5
Rhamnaceae				

Rhamnus pallasii Fisch. & C.A.Mey.	IT-ES	Ph	s.n.	*
Rosaceae				
Agrimonia eupatoria L.	IT-ES-M	He	36766, 36830	1, 4
Amygdalus spinosissima Bunge. subsp. *turcomanica* (Lincz.) Browicz	IT	Ph	36085, 36441	4
Cerasus microcarpa (C.A.Mey.) Boiss. subsp. *microcarpa*	IT	Ph	36077, 36367, 36775	4
C. pseudoprostrata Pojark.	IT	Ch	36387, 36660, 36931	4
Cotoneaster nummularius Fisch. & C.A.Mey.	IT	Ph	36364, 36839	5
Crataegus pseudoheterophylla Pojark. subsp. *turkestanica* (Pojark.) K.I.Chri.	IT-ES	Ph	36923	4
Geum urbanum L.	IT-ES	He	36119, 36682	2, 4
Rosa beggeriana Schrenk	IT	Ph	36416, 37024	1, 4
R. canina L.	IT-ES-M	Ph	36415, 36675, 36836	1, 4
R. persica Michx. ex Juss.	IT	Ch	35967, 36201	1, 4
Rubus caesius L.	IT-ES-M	Ch	36672	4
Sanguisorba minor Scop.	IT-ES-M	He	36134	4
Rubiaceae				
Asperula glomerata (M.Bieb.) Griseb. subsp. *turcomanica* (Pobed.) Ehrend.	IT	He	36155, 36342	4
Asperula setosa Jaub. & Spach	IT	Th	36067, 36577	1
Callipeltis cucullaria (L.) DC.	IT-M	Th	36099, 36197	1, 4
Crucianella gilanica Trin.	IT	He	36590	1
Cruciata taurica (Pall. ex Willd.) Ehrend. subsp. *persica* (DC.) Ehrend.	IT	Ch	36384	1
Galium aparine L.	PL	Th	36122	1, 4
G. humifusum M. Bieb.	IT-M	He	36600, 36725	*
G. spurium L.	IT-ES-M	Th	36635	1
G. tricornutum Dandy	IT-ES-SS	Th	36123, 36156	1, 4
Rubia florida Boiss.	IT	Ch	35965, 36828	1
Rutaceae				
Haplophyllum acutifolium (DC.) G.Don	IT	He	36317, 36758	5
Salicaceae				
Salix pycnostachya N.J.Andersson	IT	Ph	35953, 36219	1, 4
Santalaceae				
Thesium kotschyanum Boiss.	IT	G.r	36604	1
Scrophulariaceae				
Euphrasia pectinata Ten.	IT-M	G.p	35877, 36176	*
Leptorhabdos parviflora (Benth.) Benth.	IT	Th	36596	4
Linaria simplex (Willd.) DC.	IT-M	Th	35893, 35959	*
Scrophularia striata Boiss.	IT	Ch	36644, 36941	1, 4
S. umbrosa Dumort.	IT-ES	Ch	36643, 36933	1, 4
S. variegata M.Bieb.	IT	Ch	36296	1, 4
Verbascum cheiranthifolium Boiss.	IT	He	36595, 36825	4
V. macrocarpum Boiss.	IT	He	36473, 37014	1
Veronica anagallis-aquatica L.	IT	G.r	36313, 36564, 36944	1, 4
V. hederifolia L.	IT-ES-M	Th	35929, 36115	1
V. rubrifolia Boiss.	IT	Th	35863	4
Solanaceae				
Hyoscyamus pusillus L.	IT-SS	He	35892	1, 4
H. turcomanicus Pojark.	IT	He	36023, 36294	*
Solanum nigrum L.	Cosm.	Th	37029	4, 5
Tamaricaceae				
Myricaria germanica (L.) Desv.	IT-ES	Ph	36626	4
Tamarix ramosissima Ledeb.	PL	Ph	36658	4, 5
Thymelaeaceae				
Diarthron vesiculosum C.A.Mey.	IT	Th	s.n.	1, 5
Urticaceae				

Parietaria judaica L.	IT-ES-M	Ch	s.n.	1, 4, 5
Urtica dioica L. subsp. *dioica*	Cosm.	He	36320	4, 5
Valerianaceae				
Valeriana ficariifolia Boiss.	IT	He	36198, 36351	1, 5
Valerianella tuberculata Boiss.	IT	Th	36012, 36092	5
Verbenaceae				
Verbena officinalis L.	PL	He	36841	4, 5
Violaceae				
Viola occulta Lehm.	IT	Th	35946, 36019	*
Zygophyllaceae				
Peganum harmala L.	IT-M-SS	He	s.n.	4
Zygophyllum atriplicoides Fisch. & C.A.Mey.	IT	Ch	s.n.	*

1. Abbreviations as text and Figures 3 and 4, Geophytes more subdivided to G.b: bulbous, G.c: with corms, G.p: root parasit, G.r: rhizomatous, G.t: tuberous.

2. Main collectors: F. Memariani, H. Zangooei and Kh. Emadzade

3. The numbers and symbols refer to: 1: Rechinger (1963-2008), 2: Ghahremaninejad *et al.* (2005), 3: Joharchi *et al.* (2007), 4: Ghahreman et al. (2006), 5: Assadi *et al.* (1988-2008), 6: Maassoumi (1986-2006), 7: Memariani *et al.* (2007), Fritsch *et al.* (2006), * new records for Binalood mountain range, ** new records for Khorassan, *** new record for Iran.

Scanning electron microscopy of scales in cyprinid fish, *Alburnoides bipunctatus* (Blotch, 1782)

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Abstract

The normal and lateral line cycloid scales of a cyprinid fish; *Alburnoides bipunctatus* (Blotch, 1782) have been subjected to SEM to study their detailed structure. It shows the general architectural pattern of a cycloid cyprinid scale. The focus of the scale is clear and sharp located in the anterior field. Circuli are observed in all fields. Primary radii which originate from very near the focus divide the posterior field of scale into compartments. Originating far away from the focus, the secondary radii are seen. But the tertiary radii are few. The anterior radii are absent. Lepidonts (teeth-like structures) are absent or are very week on the circuli. Tubercles (granules) at the posterior filed of scale are not well developed. Many resorbtion regions are seen on the scale. Presence of a long, straight, S-shape or J-shape central canal originating from the upper margin of anterior region which in most cases extending down to the posterior margin is characteristic feature of lateral line scale. The architectural specification of scales such as focus shape and position, circuli, chromatophore, lepidonts and lateral line canal might be used as important taxonomic tools.

Keywords: scale, scanning electron microscopy, lepidont, *Alburnoides bipunctatus*

Introduction

Scales, the dermal derivatives of fish body are important structures used as a versatile research material (Kaur and Dua, 2004). Scale morphology has proved to be a useful tool in fish classification, determining the diet of piscivorous predators (Lekuona et al., 1998; Campos et al., 2002) or in the paleontological analysis (Meunier and Poplin, 1995; Jawad and Al-Jufail, 2007). It could provide complete knowledge of life history, age at recruitment, growth rates, age at first maturity and average life span of fishes (Tzeng et al., 1994). Detailed structures of the fish scale can be helpful in identification of fishes up to major group (Lagler, 1947; Van Oosten, 1957; Norman, 1957) or species level (Chu, 1935; Das, 1959; Lanzing and Higginbotham, 1974). It could be also used in study of fish phylogeny (Kobayashi, 1951; Kobayashi, 1952), sexual dimorphism (Esmaeili, 2001), past environment experienced by fish, discriminating between hatchery reared and wild populations, migration and pathology of fish scale (Kaur and Dua, 2004; Campos et al., 2002; Lekuon et al., 1998; Esmaeili, 2001). So the role of hard parts, especially scale cannot be over-looked for effective fishery management's practices (Johal and Sawhney, 1999; Johal and Bansal, 2000). The importance of scale morphology used in classification was strengthened with the introduction and development of SEM (Scanning Electron Microscopy) (De lamator and courtenay, 1973, 1974; Jawad, 2005 a,b; Jawad and Al-Jufail, 2007). During the perusal of literature it has been found that the age and growth studies on cyprinid native fishes of Iran are rarely opted and some taxa are completely ignored. So an attempt has been made here to study the ultrastructure of the scale of a widely distributed cyprinid freshwater fish, *A. bipunctuatus* by using scanning electron microscopy. In Iran it is called under different names such as tailoress fish, possibly from the lateral line pattern like stitches, lapak, parak, sima, kuli, shebeh zury (Coad, 2008). Due to its small size, this fish has no fisheries value but it has aesthetics value because of pretty colors and pattern on the body.

Material and Methods

To study the ultrastructure of the scale of *A. bipunctatus*, the fishes were collected by the authors from Sarab-e- Beyza spring stream (29° 57′ 41.8″ N, 52° 21′ 11.1″ E), Kor River basin, southwest Iran (Figure 1) using dipnet and electrofishing device in 2007. The scales of fishes were gently removed with fine forceps from the left side of body below the dorsal fin preferably the

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third or forth row and also from lateral line from the middle of the lateral line (Figure 2). Immediately, the scales were washed thoroughly with water by gently rubbing them between the tips of the fingers and cleared with 3% potassium hydroxide solution too. The cleaned scales were dehydrated in 30, 50, 70 and 90% ethanol for 30 minutes respectively and dried on filter paper to avoid curling (Lippitsch, 1990). Then the scales were kept between the two microslides for 2-3 days. The scales were not put in absolute alcohol as 100% ethanol curls the scale margins (Esmaeili, 2001). The cleaned and dried scaled were mounted on metallic stubbs by double adhesive tape with dorsal surface upward and ventral surface sticking to the tape and coated with a 100˚A thick layer of gold in a vacuum in a gold coating unit (SC7640 SPUTTER COATER, Model: FISONS) (Esmaeili, 2001). The scales were viewed under vacuum in a Leica Cambridge scanning electron microscope at an accelerating voltage of 20kv at low probe current. Various images of the scales were taken and were saved in the computer attached to the scanning electron microscope. When gold-coated scales were not being viewed, the stubbs were stored in a desiccator to avoid moisture.

Figure 1. Map of Iran and Fars province showing fish collection site.

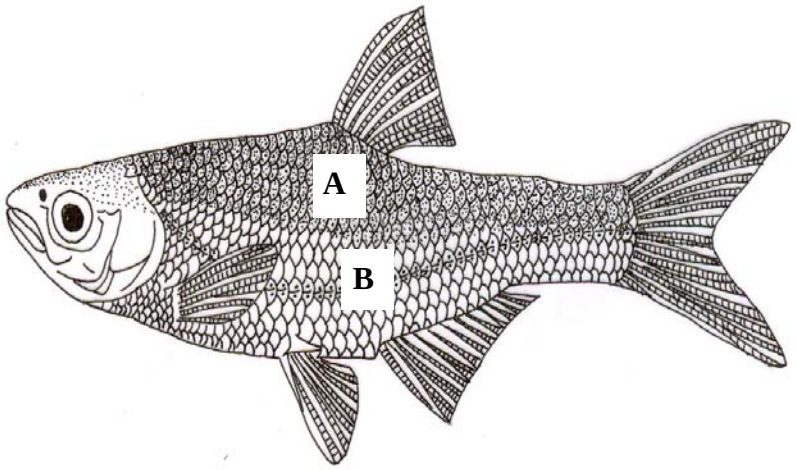


Figure 2. Schematic drawing of *Alburnoides bipunctatus* (total length, 52.8 mm) showing location of key scales using for scanning electron microscopy. A, scale below dorsal fin; B, lateral line scale.

Results

The general structure of normal and lateral line scales of hypothetical fish and that of *A. bipunctatus* is given in Figure 3. Like most of the cyprinid scales, there is no cteni at the posterior part of the scale of *Alburnoides bipunctatus* (Blotch, 1782) hence it is a cycloid scale. The scales maintain the same morphological proportions located on the different parts of the body. The scales of the lateral line and below the dorsal fin are largest respectively. The scales on the other parts of fish are smaller in size. As the scales below dorsal fin depict all the features, these scales have been designated as key scales. The dorsal part of scale is rough, convex and has distinct structures, which consists of ridges, grooves and granules (tubercles) and the ventral part of scale is shiny and smooth. Each scale has a focus. Focus is the first part of the scale to be formed during ontogenesis and has different locations in different species (Kaur and Dua, 2004; Esmaeili, 2001) (Figure 3). In this species, the focus is distinct and lies in the anterior part of the scale divides the scale into anterior, rostral or cephalic (A), posterior or caudal (P) and lateral fields (L). The shape of focus is oval or round (Figures 3a, b, 4b). Few mocus pores are found in the focus region. From the focus lines of growth (the ridges) start appearing which are named as circuli (Figures 3, 4). The space between circuli are called inter circular space. Circuli (the lines of growth) are the elevated ridges present on the surface of scale and show differences with regard to thickness, arrangement and relative spacing (Kaur and Dua, 2004). The intercirculus space is maximum in the posterior part and minimum in the anterior part. It is due to anterior location of the focus on the scale. Intercirculus space is intermediate in the lateral parts (Figure 3a, b). Each circuli is wedge shaped, having broad base and pointed upper part (Figure 4). The anterior part is overlapped by the posterior part of the preceding scale (Johal and Bansal, 2000; Esmaeili, 2001). Hence the anterior part is soft and uncoated whereas the posterior part of the scale is covered with a thick layer of epidermis. The posterior part occupies more than 70% of the total surface area of the scale. Presence of lepidonts, the scale denticles or tooth-like process is another characteristic feature of some circuli of this fish (Figure 4c, d, e). There is very minute lepidonts on the circuli of the posterior part of the scale under high magnification (Figure 4d). The majority of circuli is without lepidonts and smooth in considered species (Figure 4i, j). Distinct breaks in the circuli points towards the formation of annuli are observed in scale of *A. bipunctatus*. The annuli indicate the fish age. Annuli clearly marked in rostral and lateral fields by alternate wide light and dark, narrow spaces that correspond to summer and winter growth in many fishes (Jawad and Al-Jufail, 2007).

In the posterior and lateral parts, the circuli are partitioned by deep and narrow grooves that run radially towards the focus. They are called radii which are categorized into three types depending upon their point of origin on the scale including: Primary radii, originating from the focus, reaching the margin of the scale; secondary radii, originating midway between focus and margin and tertiary radii originating between midway and margin (Figure 3a, b). The radii cut the circuli and annuli at right angle. Primary radii are present on posterior and lateral fields of the scale. They are absent in the anterior part of the scale. The relative number of primary and secondary radii is more as compared to the tertiary radii. Some of scales are without radii. These are called simple scales, the term which has been used by Lippitsch (1990). The scales with well developed radii are here called "sectioned". The posterior part of the scale confers color to the fish body due to the presence of chromatophore which lies on the tubercles. Tubercles are not clear in this species. Scales of *Alburnoides bipunctatus* (Blotch 1782) shows severe signs of resorbtion (Figure 4, f and h). Numerous cavities were present on the calcified layer surface. Schematic drawing of lateral line scale is showed in (Figure 3b).

Lateral line scale of this fish also is divided into anterior (rostral) and posterior (caudal) parts. Scanning electron microscopy of lateral line scale of *A.bipunctatus* showed presence of a long, straight, S-shape or J-shape central canal originating from the upper margin of anterior region or focus in most cases extending down to the posterior margin. The canal opening is open and round in two sides in all cases. The posterior opening of the lateral line canal is wider than the anterior opening (Figure 5h, i). Anterior, posterior and lateral parts of lateral line have several mucus pores (Figure 5h). In the posterior part, the circuli are spaced (Figure 5f). There is no clear granulation on the posterior portion of lateral line scale. Another character of lateral line canal is the presence of a few accessory pores in its wall in considered species (Figure 5a). We observed no obvious difference between male and female in scale structures.

Discussion

This paper describes the scale morphology of a cyprinid fish, *Alburnoides bipunctatus* (Blotch, 1782) from Iran. It shows the general architectural pattern of a cycloid cyprinid scale having focus, circuli and radii. The focus of the scale is clear and sharp located in the anterior field and is the first part of the scale to be formed during ontogenesis. Circuli are observed in all fields. The arrangement of the circuli corresponds to the scale shape (Esmaeili, 2001).The circuli formation is due to the excess calcium salts secreted by the skin and their subsequent deposition on the scale and distance between circuli indicates fast and slow growth period. Lepidonts (teeth-like structures) are absent or are very week on the circuli. Lepidonts are important structures known to support species distinctness (Kaur and Dua, 2004; Jawad and Al-Jufail, 2007; Esmaeili, 2001). The taxa usually differ with regard to shape, texture, attachment and orientation of lepidonts on the crest of circuli (Kaur and Dua, 2004). Lepidnots of different size and shape have been reported in many fish species (Lippitsch, 1990; Delmater and Courtenay, 1974; Jawad and Jufaili, 2007). They might characterize genera and may even distinguish some taxa at the specific level (Delmater and Courtenay, 1974). Lepidonts are not homologous to breeding tubercles and contact organs (Delmater and Courtenay, 1974). Radii are present on the lateral and posterior parts of scales of *A. bipunctatus*. There is no significant relationship between number of radii and scale size, as the numbers of radii depend on location of the scale on the fish body. However, in some other teleotes such as *Mullus surmuletus* L., 1758 and *M. barbatus* L., 1758, the number of radii is correlated to fish size (Jawad and Jufaili, 2007). The presence of primary and secondary radii is a growth phenomenon and obviously only weakly influenced by genetic factors (Lippitsch, 1990). The radii formation is considered to be related to the accommodation power of the large surface area of the anterior and lateral parts of the scale in the lesser space as these two parts of the scale are overlapped by the posterior part of the preceding scale. The higher number of radii is correlated with the better nutritive conditions of the fish (Johal et al., 1984; Tandon and Johal, 1996). Radii represent the line of scale flexibility.

Tubercles are not clear in this species. No earlier attempt has been made to study the importance of tubercles in species specificity. The shape of tubercles in other species varies from round to oval, semi-oval and oblong structure. Tubercule are formed by the aggregation of epithelial layer of the skin which covers the posterior part of the scale. They impart specific color to fish as they contain chromotophores in the outer surface. Presence of chromatophore on the posterior part of scale is a characteristic feature of the cycloid and ctenoid scales of carps and perches respectively (Tandon and Johal, 1996; Johal et al., 1984; Johal et al., 1996; Johal and Agarwal, 1997).

Severe signs of resorbtion were found on the scale of this cyprinid fish. This resorbtion in teleost scales occurs under various physiological and experimental conditions and has been suggested to be initiated during periods of increased calcium demand such as during sexual maturation when estradiols induce vitellogenesis (Crichton, 1935; Jarvi and Menzies, 1936; Van Someren, 1937; Takagi, 1990; Persson et al., 1995, 1998) and lack of food (Esmaeili, 2001).

Presence of a long, straight, S-shape or J-shape central canal originating from the upper margin of anterior region which in most cases extending down to the posterior margin is characteristic feature of lateral line scale of *A. bipunctatus*. Lateral line scales prove their potential in fish classification and taxonomy. Number, position of canal, its alignment viz. straight or oblique, perforation in anterior, posterior or lateral are important features for fish classification (Kaur and Dua, 2004). According to Delmater & Courtenay (1973) scanning electron microscopy of lateral line scales of teleost fishes demonstrates a wide range of structural variation of lateral line canal from a simple direct or slightly oblique perforation to an extended canal with or without simple to highly complex cantilevered extensions covering the anterior opening.

The above observations regarding the architectural specification of scales such as focus shape and position, circuli, chromatophore, lepidonts and lateral line canal might be used as important taxonomic tools.

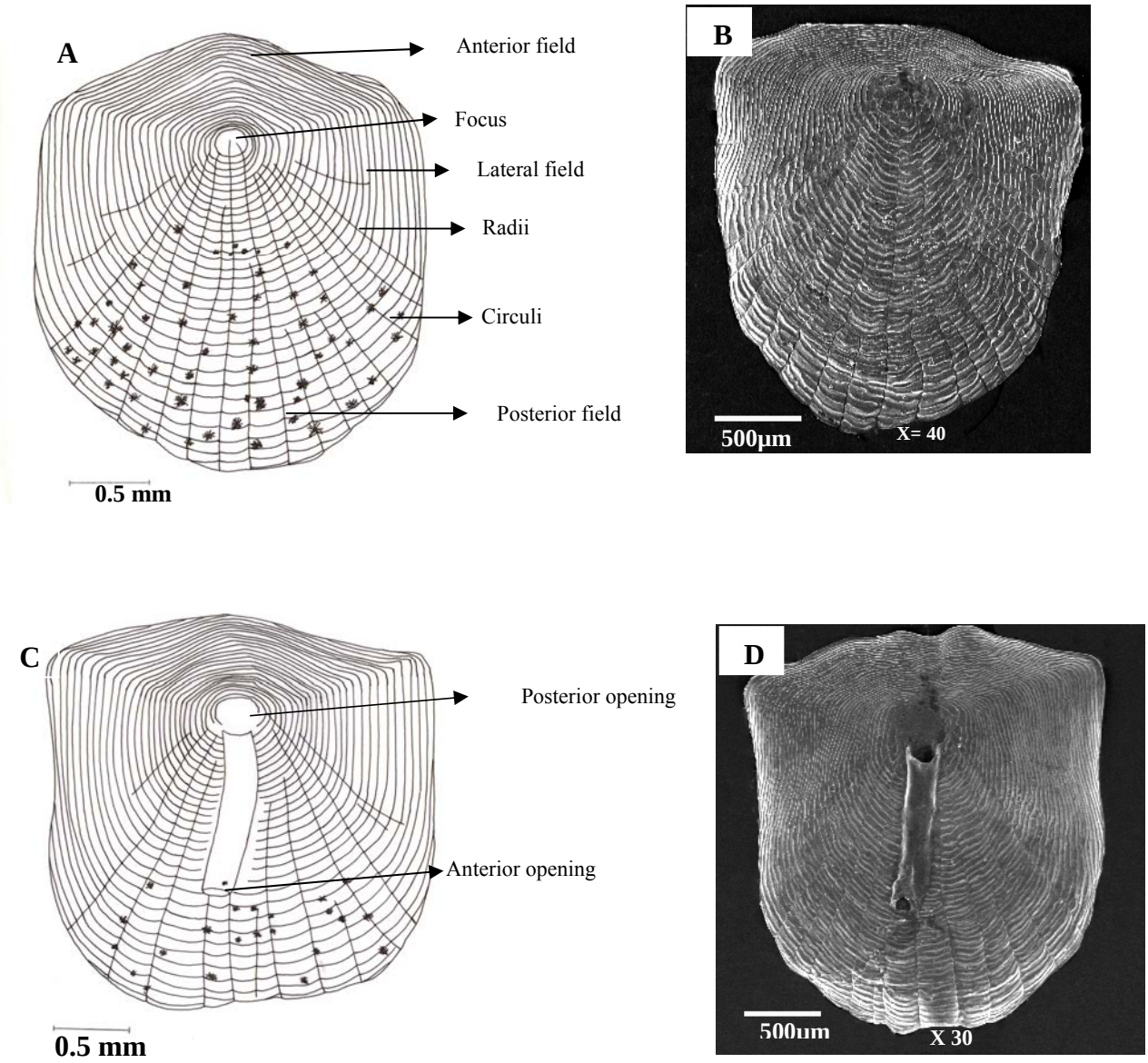


Figure 3. A, Schematic drawing of a sectioned cyprinid scale. B, SEM microphotograph of a normal *A.bipunctatus* scale. C, schematic drawing of a sectioned cyprinid lateral line scale. D, SEM micrograph of *A.bipunctatus* lateral line scale.

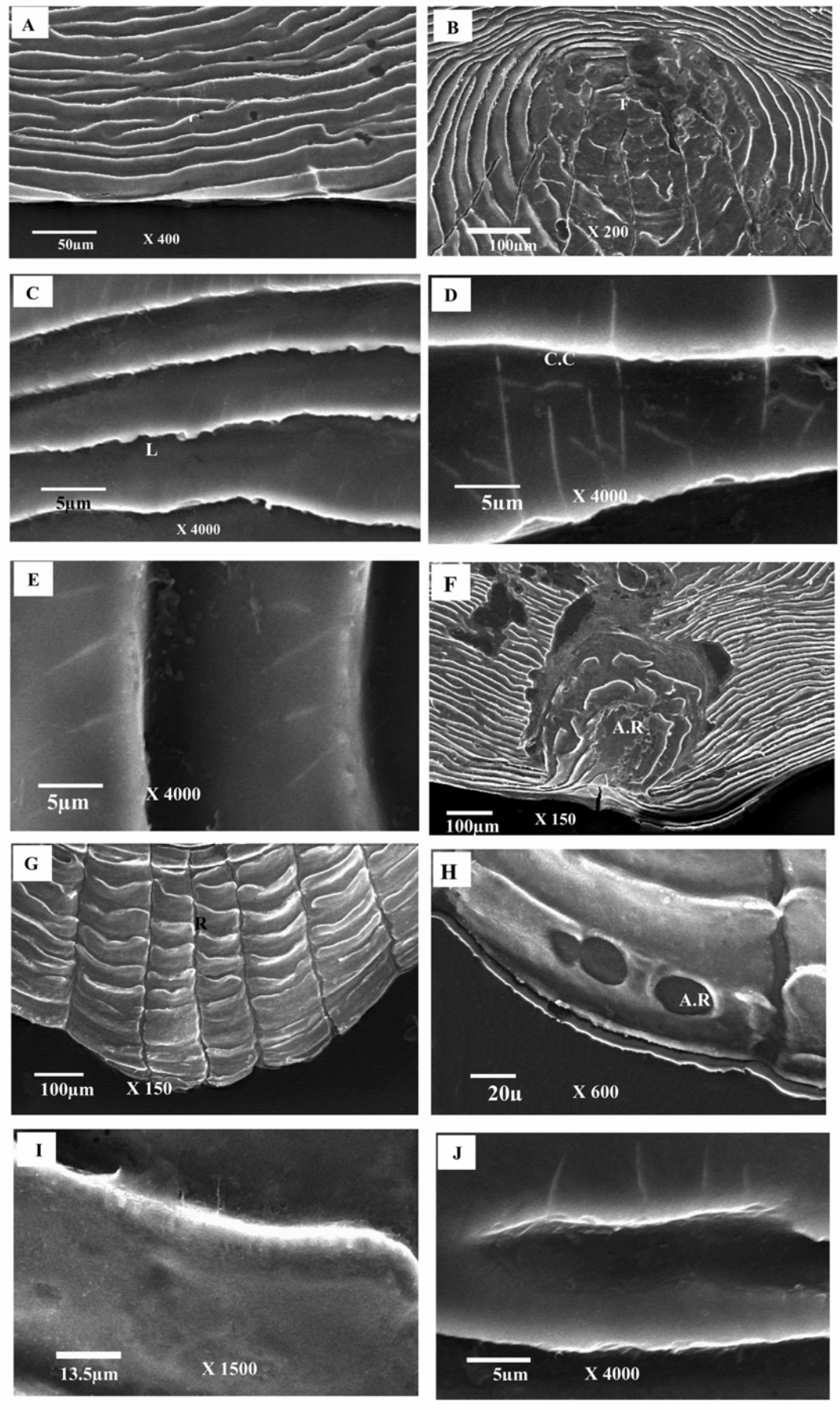
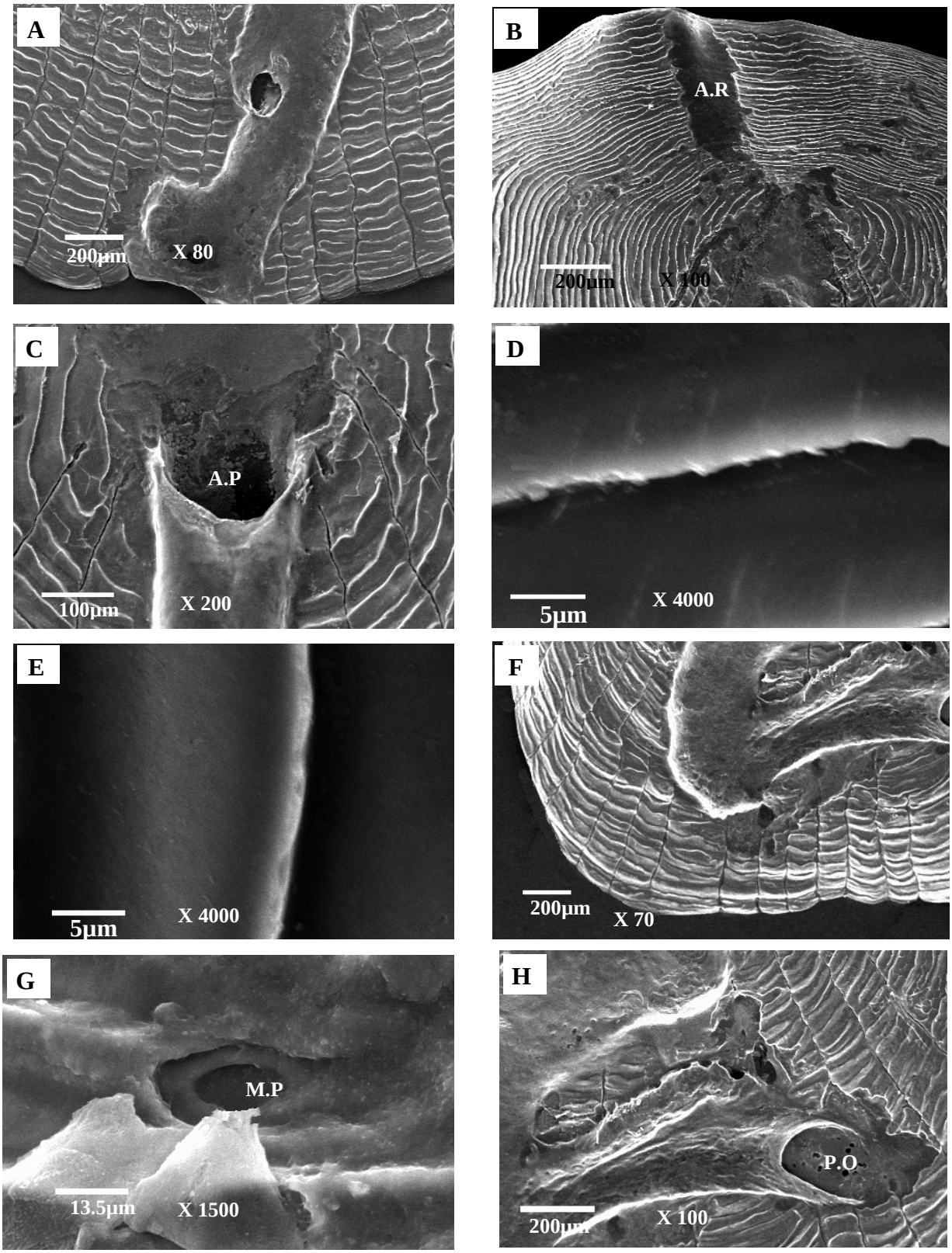


Figure 4. SEM microphotographs of *A.bipunctatus* (R), circuli (C), lepidont (L), focus (F), crest of circuli (C.C), Absorbtion Region(A.R).


A
200µm
X 80
B
A.R
200µm
X 100
C
A.P
100µm
X 200
D
5µm
X 4000
E
5µm
X 4000
F
200µm
X 70
G
M.P
13.5µm
X 1500
H
P.O
200µm
X 100

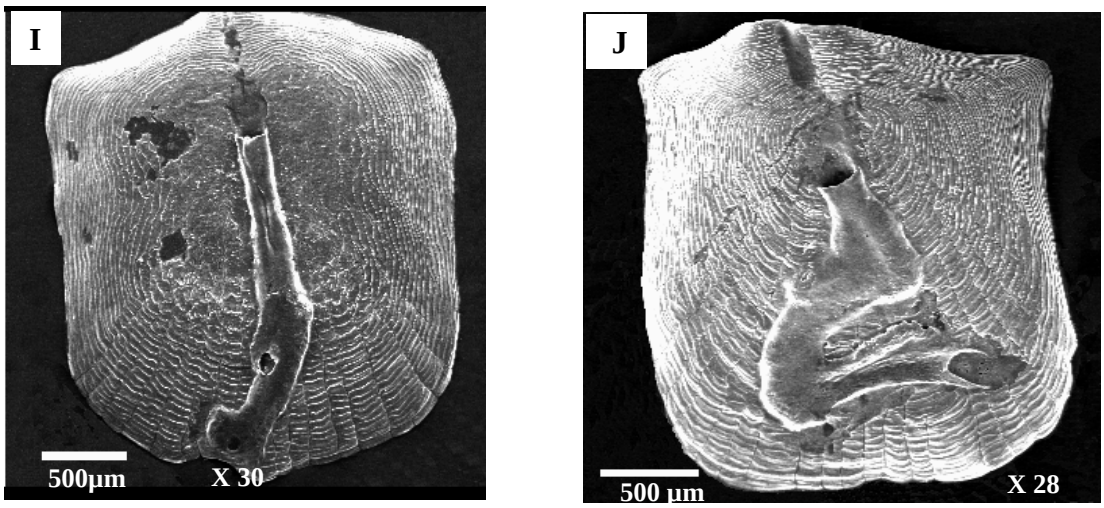


Figure 5. SEM microphotographs of lateral line scale of *A*.bipunctatus circuli (C), crest of circuli (C.C), circular groove (C. lateral circuli (l. C), primery radii (P. R), secondary radii (S. R), line grown (L. G). Anterior pore (A. P), Posterior pore (P. O), Tubercle (T) and Mocus pore (M. P).

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Neuroprotective effects of *Equisetum telmateia* in rat

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Abstract

Equisetum telmateia (Equisetaceae) seems to have anti inflammatory and antioxidant properties. In the present study, the neuroprotective effects of organic and inorganic silica were investigated on spinal cord alpha motoneuron of rats after injury of sciatic nerve. After highly compression of sciatic nerve in 42 Wistar rats, the injured rats were divided into sham (n= 6) and two experimental groups which each were divided into 3 subgroups (n= 6). The first subgroups received 3, 6 or 9 injections (15 mg/kg/injection, ip) of horse tail extract and the second subgroups received 3, 6 or 9 injections (6 mg/kg/injection, ip) of sodium meta silicate, respectively. The first injection was made after sciatic nerve injury and the others by 72 hours intervals. After a month, the rats were sacrificed and their spinal cord lumber segment sampled, processed for histological preparation and analyzed stereologicaly (the *dissector* technique) for estimation of numerical density of alpha motoneurons. The results showed significant decrease in the numerical density of alpha motoneurons in shams ($p < 0.05$) and no significant differences between experimental and control groups. This may suggest the neuroprotective effects of silica on the survival of alpha motoneurons.

Key words: neuroprotective, antioxidant, *Equisetum telmateia*, horsetail, rat

Introduction

The medicinal properties of aerial parts of Equisetaceae family in the treatment of acne, rheumatism, pain in broken bones, diuretic, expectorant, kidney stones and in strengthening hair, skin and nails (Uzun et al., 2004) have made these plants useful natural drug in traditional medicine. The hydroalcoholic extract of *E. arvense* stem has an antinociceptive property, which is not related to the opioid system, and also anti inflammatory effect in mice (Do Monte et al., 2004). Chronic administration of the hydroalcoholic extract from stems of *E. arvense* improves the cognitive deficits in aged rats, and this effect can be due, at least in part, to its antioxidant action (Dos Santos et al., 2005a). Between three species of Equisetaceae, *E. arvense*, *E. ramosissimum* and *E. telmateia*, it has been shown that the *E. telmateia* extract demonstrates the most relevant scavenger and antioxidant properties (Stajner et al., 2006).

Traumatic events of intense mechanical compression of the mammalian peripheral nerves lead to axotomized motoneurons regenerate their axons and if this happens shortly after nerve injury, the cell body usually returns to its former appearance (Seniuk, 1992). Failure to contact a new target cell leads to the neuronal atrophy and death (Crouch et al., 1994). Secondary injury, which is partly due to oxygen radicals released from neutrophils, further contributes to worsening of CNS function (McTigue and Tripathi, 2008; Bagdatoglu et al., 2002; Marin et al., 1998). In rat after peripheral axotomy, some motoneurons survive and undergo typical reactive changes typical for chromatolysis while the others undergo changes that lead to cell death (Behnam Rassouli et al., 2000).

In the hope of promoting the survival rate of neurons and axonal regeneration, this paper explains a possible supportive effect of extract from a horsetail plant. Since *E. telmateia* has most relevant scavenger and antioxidant properties this study was designed, by comparing the beneficial effects of SM by HT, to explore whether this property is due to HT high silica content.

Material and methods

Animal: Fortyeight male Albino Wistar rats weighing 250-300 g (supplied by Razi Institute,

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Mashhad, Iran) were used in the study. At the time of injury the animals were two to three months old. The animals were housed in plastic cages in the animal house and given rat chow (Javaneh Khorassan, Iran) and tap water *ad libitum* and maintained under conditions of controlled lighting (lights on: 6 a.m. to 6 p.m.) and ambient temperature 22±2°C. Animals were cared for and handled in accordance with the Iranian Society of Animal Care (member of International Animal Care Society).

Sciatic nerve injury process: At the time of surgery all rats were anesthetized for sterile surgery by intera peritoneal injection of 0.2 ml of a mixture (1:2) of 10% ketamin (Bayer, Germany) and 2% xylazine (Boxtel, Holland). After exposure of the left sciatic nerve through a gluteal muscle splitting incision, the sciatic nerve of 42 rats were crushed for 30 seconds period between prongs of #5 clamp forceps (Behnam Rassouli et al.,2000). On the remaining 6 rats, a sham operation was performed which exposed the sciatic nerve but did not disturb it. The muscle and skin were then closed with 14 mm stainless steel sutures.

Experimental design: The lesion rats were then divided into control (n=6) and two treating groups; horsetail stem extract (HT) and sodium metasilicate (SM) treated groups. The HT group were further divided into 3 subgroups (n=6); receiving 3, 6 or 9 injections (13.65 mg/kg, ip), respectively. Also the SM group further divided into 3 subgroups (n=6) and received 3, 6 or 9 injections (6 mg/kg, ip), respectively. The first injection was made after sciatic nerve injury and the others by 72 hours intervals. At the end of experimental period (one month) the animals were anesthetized and transcardially perfused with 10% formaldehyde. Immediately following perfusion, the L4 to L6 spinal segments with associated dorsal roots of sciatic nerve were dissected and post fixed for 2 h or overnight. The spinal blocks were processed for histological preparation and embedded in paraffin and then sectioned serially at 7 µm diameter. To sample the sections, a uniform random sampling scheme was employed so that about 10 sections from each block were sampled (Gundersen and Jensen, 1987). Sections were stained with toluidine blue with special buffer of acetic acid 1N (1 ml), sodium acetate 1N (1 ml) and distilled water (98 ml), pH 4.65. After permanent mounting, the numerical density (Nv) of motoneurons in the left and right sides of ventrolateral regions of spinal cord were estimated, using stereological counting technique; the physical *dissector* (Sterio, 1984; Gundersen, 1986; Cruz Orive, 1987).

Preparation of sodium metasilicate: In order to prepare the sodium metasilicate solution, 180 mg sodium metasilicate powder (Aldrich Chemical Co.) was dissolved in 100 ml of distilled water and the pH of the solution neutralized (pH=7.4) by adding 1N hydrochloric acid. Since the administrational dose of silica is varies up to 40 µg/g rat chow (Seaborn and Nielsen, 2002, 1994) we administrated a supplemental dose of 6 mg/kg/injection SM. Therefore, by using a sterile syringe and 22 gauge needle approximately 1 ml of the solution was injected intra peritoneally in every injection.

Plant material and preparation of aqueous extract: Horsetail was collected from Syah Roodbar forest in the north of Iran during the summer of 2005 and identified by Mr. Jouharchi, Herbarium Centre, Ferdowsi University of Mashhad, as *Equisetum telmateia* (voucher no. 31401). The leaves and stems of the plant were separated, dried and homogenized to a fine powder and then stored at room temperature in opaque screwtop jars until use. In order to prepare an aqueous extract of leave and stem, 5 g powdered materials were placed in 250 ml boiling (distilled) water for 2 hours and concentrated to half of the volume by boiling in a water bath. The suspensions were filtered (Whatman no. 1) and the filtrated volumes adjusted to 130 ml with distilled water and neutralized (pH=7.4) by adding 1N NaOH. To determine the amount of silica, as various compounds in crude extracts, a sample of each extract was analysed by Atomic Absorption Spectrometry (AAS) method in the Analytical Chemistry Lab., Dept. of Chemistry, Ferdowsi University of Mashhad, in acetylene flame and N2O and C2H2. The results showed that the amount of silica in the stem of horsetail is higher than leaves (Figure 1). Therefore it was decided to treat the injured rats by stem extract. After that the amount of dried material in the above horsetail stem extract was measured (4.55 mg/ml). Since the administrational dose of the horsetail stem extract varies between 10 to 400 mg/kg body weight (Dos Santos et al., 2005b; Do Monte et al., 2004) the dose selected for the treatment of animals was 4.55 mg/kg body weight/day (or 13.65 mg/kg body weight/injection).

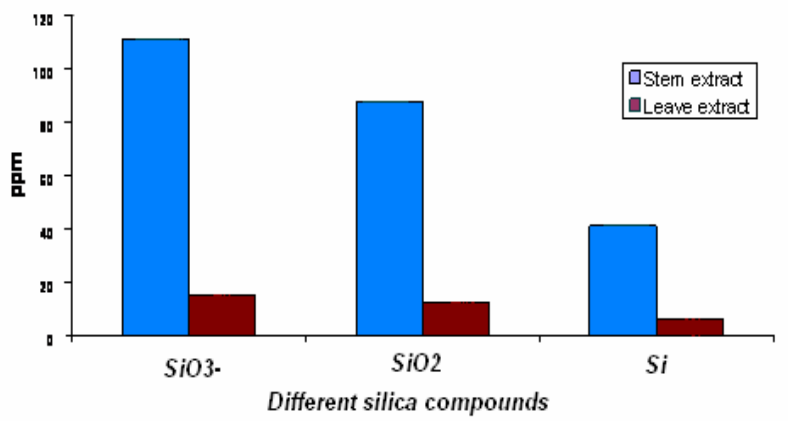

Figure 1. The amount (ppm) of different silican compound in the extract of Equisetum telmateia stem and leave, measured by the Atomatic Absorption Spectrometry technique.

Statistical analysis: One-way single factor ANOVA was used to compare more than two groups followed by student test (Microsoft Office Excel software) to detect differences between groups. For all test, $P<0.05$ was considered statistically significant. All results are expressed as mean ± S.E.M.

Results

The results of the Atomic Absorption Spectrometry assays are presented in Figure 1. These data showed that the total amount of different silica compounds (SiO3 --, SiO2 and Si) in the stem extract of *Equisetum telmateia* is higher than leaves (the total amount silica was 240.42 ppm vs. 32.71 ppm). The results of the estimation of numerical density of alpha motoneurons in the ventral horn of L4 to L6 segments of spinal cord are indicated in table 1. As seen in the table after sciatic nerve crush, the spinal ventral horn motoneuron counts show a decline in number (962.72 /mm3 in controls vs. 1466.13/mm3 in shams). Statistical analyses show that the reduction in the motoneurons of controls, when compared with shams and all experimental groups, is significant ($p<0.05$) (table 1). Although the numerical density of motoneurons in all experimental groups is lower than control group but comparison of numerical density among control and all experimental groups was only significant ($p<0.05$) in HT treated subgroups who received 3 and 6 injections of extract (table 1). Also inter group comparison of numerical density of motoneurons among the similar HT and SM treated subgroups as well as intra group comparison of numerical density between different HT treated subgroups and between different SM treated subgroups showed no significant differences.

Table 1. The numerical density (no/mm^3) of motoneurons in control, sham and experimental groups at 30th post operation day.

		Sodium meta silicate (6mg/kg/injection)			Horse tail extract (13.65mg/kg/injection)		
		3 injection	6 injection	9 injection	3 injection	6 injection	9 injection
		1338.82 (±79.33)	1421.88 (±93.12)	1265.58 (±75.36)	1177.29 (±108.37)	1226.96 (±96.18)	1394.73 (±110.17)
Control	962.72 (±32.75) ●●●	**	***	**	*	*	**
sham	1466.13 (±80.30)	ns	ns	ns	●	●	ns

Data are presented as mean (±SEM). Statistical analyses were performed using one-way ANOVA, followed by Dunnett test. ●P<0.05; ●●●P<0.001 compared with the control group, *P<0.05; **P<0.01; ***P<0.001 compare with the sham group. (ns) no significant differences.

Discussion

After injury the production of reactive oxygen species may increase leading to tissue damage via several different molecular pathways (McTigue and Tripathi, 2008). Radicals can cause damage to cardinal cellular components such as lipids, proteins, and nucleic acids (e.g., DNA), leading to subsequent cell death by modes of necrosis or apoptosis. The damage can become more widespread due to weakened cellular antioxidant defense systems. Flavonoids, a naturally occurring plant substance and currently available for the treatment of acute CNS injuries (Gilgun Sherki et al., 2002) is known as an antioxidant and free radical scavenger. Thus some of the protective effect of horse tail may be due to flovonoid. To our knowledge the present study is the first documentation in which the probable neuroprotective effect of inorganic silica (SM) were compared by horse tail extract.

The results obtained from the present study indicate that intra peritoneal injection of HT and SM at the time of injury and afterwards may prevent or delay the onset of neuronal loss in the spinal cord. Statistical analyses of data indicate that, in comparison with sham and all experimental groups, the numerical density of motoneurons in controls is significantly reduced while, except the HT treated subgroups who received 3 and 6 injections, there is no remarkable difference between experimental groups and shams. Similarly, there was no clear difference between the HT and SM treated groups.

It is obvious that damage to the nervous system does not stop immediately after the initial injury, but continues in the hours following trauma, a process called secondary injury (Gilgun Sherki et al., 2002). Reduction in the endogenous antioxidant defense system due to environmental and genetic factors may contribute to oxidative stress evolution. Antioxidants of varying chemical structures have been investigated as therapeutic agents in the treatment of acute CNS injury. The secondary injuries can be the result of a number of auto destructive phenomena such as neutrophilic infiltration (Genovese et al., 2005; Tonai et al., 2001). The resistance of CNS to regeneration might be related to the restriction in the numbers of macrophages recruited and activated by the injured CNS (Lazarov Spiegler et al., 1996) but the activated neutrophils may be implicated in the worsening of nerve injury and release of oxygen radicals which is toxic to the cell membrane component and free radical induced lipid peroxidation (Bagdatoglu *et al.,*2002; Marin *et al.,*1998; Konat and Wiggins, 1985). Between three different species of Equisetaceae; *E. arvense*, *E. ramosissimum* and *E. telmateia*, it has been shown that the free radical scavenging activity of the *E. telmateia* is higher than the other two and Electron Spin Resonance signal of DMPOOH radical adducts in the presence of *E. telmateia* phosphate buffer extract is reduced to 98.9% (Stajner et al., 2006). In the case of inorganic silica it has been reported that intra peritoneal injection of silica dust at the time of a compression injury to the spinal cord produces a delay of one to two days in the onset of secondary functional loss below the level of injury (Blight, 1994). The results obtained from the present research are in consistent with the above observations.

In conclusion, in the case of SM treated animals it seems that silica is the active agent but whether silica is the only constituent of HT which exerts HT neuroprotective effects is not certain. By applying a silica chelator it is possible to draw out the silica from the HT and then test if silica is the active agent. Also in vitro evaluation of silica anti-oxidant property and in vivo evaluation of motor function and measuring nerve conduction velocity could be performed to confirm the therapeutic benefits of silica in future studies.

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Investigation of the effect of Curcumin on Inflammatory Biomarkers in Arthritic Rats

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by joint swelling, synovial inflammation and joint destruction. Curcumin (diferuolymethane) is the most active component of *Curcuma longa* L. Several clinical trials have indicated curcumin to be a notable anti-inflammatory and antioxidant compound. Therefore the aim of this study is investigating the effects of curcumin on levels of inflammation and inflammatory biomarkers in arthritic rats.

Arthritis was induced by subcutaneous injection of Freund's complete adjuvant (0.5 mL) into the right hind paw of Wistar male rats. Animals were divided into four groups (n=8). GroupI acted as control, group II arthritic rats received vehicle, group III arthritic rats were treated with curcumin (30 mg/kg, orally) and another group arthritic rats were treated with indomethacin (3 mg/kg, orally) seven days after injection of Freund's Complete Adjuvant for 15 days. The changes caused by chronic inflammation were evaluated by measurement of the ankle circumference three times per week. At the end of the experimental period, blood samples were collected by cardiac puncture to determine erythrocyte sedimentation rate, C.reactive protein levels and White blood cells count.

An increase in erythrocyte sedimentation rate, C. reactive protein concentrations, White blood cells count and ankle circumference was observed in arthritic rats compared with control rats ($p<0.05$). Curcumin significantly decreased inflammation and inflammatory biomarkers in arthritic rats ($p<0.05$).These results suggest that curcumin can possess beneficial effects in alleviating arthritic symptoms in Adjuvant-Induced Arthritis model.

Keywords: Curcumin, Freund's Complete Adjuvant, Rheumatoid arthritis, inflammation

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint swelling, synovial inflammation and cartilage destruction which commonly leads to significant disability and a consequent reduction in quality of life (Gabriel, 2001). Epidemiology of the arthritis in female: male is 3:1 and the prevalence is 0.5-1.0% of the world population (Gabriel, 2001; Narendhirakannan et al., 2007). RA caused by number of pro-inflammatory molecules released by macrophages (Henderson et al., 1987). These are including the reactive oxygen species and eicosanoids such as prostaglandins, leukotrienes and cytokines (interleukin-1, 6 [1L-1β, IL-6], and tumor necrosis factor α [TNFα]). The regulation of these mediators secreted by macrophages and other immune cells therefore may control the chronic inflammatory conditions (Joe and Lokesh, 2000).. The nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) mediated cytokine pathways and the cyclooxygenase-2 (COX-2) prostaglandin cascade are the most well studied pathways (Chun and Surh 2004).

The acute phase response develops in the setting of a wide range of acute and chronic inflammatory conditions severe bacterial, viral, or fungal infections; rheumatic and other inflammatory diseases. These conditions elicit a response in which IL-1, IL-6 and other cytokines trigger the synthesis by the liver of a variety of plasma proteins, including C-reactive protein (CRP) and fibrinogen. Because fibrinogen and certain other acute phase proteins (not including CRP) bind to erythrocytes and increase their sedimentation rate, the erythrocyte sedimentation rate (ESR) is a measure of the acute or chronic phase response. Monitoring CRP and ESR levels can provide useful

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information on the activity of diseases such as rheumatoid arthritis (Imboden et al., 2004).

Recent evidence suggests that various herbal extracts including turmeric (*Curcuma longa* rhizomes) have potent anti-inflammatory activity in a variety of inflammatory diseases (Ammon and Wahl, 1991). Curcumin (difteruloylmethane) is the most active component of turmeric. It is believed that curcumin is a potent antioxidant and anti-inflammatory agent (Aggarwal et al., 2003), (Figure1). Some experimental studies indicate that curcumin has similar anti-inflammatory activity as some of the common nonsteroidal anti-inflammatory drugs (NSAIDs) like indomethacin, Vioxx, Celebrex, and ibuprofen, but it has no side effects, such as gastrointestinal distress and cardiovascular complications(Graumlich, 2001).

The molecular basis of the anti-inflammatory properties of curcumin is linked to its effects on several targets, including transcription factors, growth regulators, and cellular signaling molecules (Han et al., 2002). Curcumin is reported to directly influence the activity of various inflammatory regulators; it has been shown to reduce NF-kB activation, AP-1 binding to DNA, as well as to decrease the production of the enzyme COX-2, all of which play a pivotal role in the inflammatory cascade (Han et al., 2002). In addition, several studies have shown that curcumin can indirectly inhibit these inflammatory regulators through its ability to scavenge free radicals (Biswas et al., 2005).

Adjuvant-Induced Arthritis (AIA) is the most widely used model for studying the pathogenesis of RA and for screening the new drugs for treatment of rheumatoid disease, which shares some features with human RA, such as swelling, cartilage degradation and loss of joint function. It has been previously reported that administration of Freund's Complete Adjuvant (FCA) increased ankle circumference, CRP, ESR levels and White blood cells count (WBC) in arthritic rats (Simoes et al., 2005; Cai et al., 2006; Funk et al., 2006 a). Therefore, the present study, using this model, is designed to investigate the effects of curcumin on inflammation, plasma CRP, ESR levels and WBC count in comparison with indomethacin which has provided experimental evidence for its therapeutic efficacy in the treatment of RA.

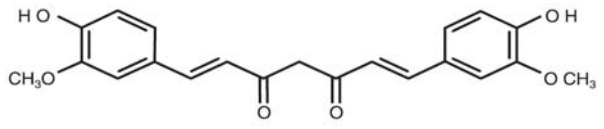

Figure 1. Chemical structure of curcumin (Wang et al., 2008)

Methods and Materials

Materials: Curcumin, Freund's Complete Adjuvant (FCA), Indomethacin were purchased from Sigma–Aldrich, Ether was purchased from Merck. Ketamin (Germany Rotex Medica), Xylasin (Holland Alfasan Woerden).

Animals: 32 adult male Wistar albino rats (180-220 g, from Laboratory Animals Unit, Faculty of Science, Urmia University) were used in the experiment. The rats were housed under standard conditions and received food and water *ad libitum*; the temperature was maintained at 22±2ºC and relative humidity (40-60%) with 12h light/ dark cycle (in the departmental animal house). Rats were acclimated to their surroundings over 1 week to eliminate the effect of stress prior to initiation of the experiments**.** In addition, this research was approved by the animal experiment Ethics Committee of this University.

Induction of arthritis: The method described by Newbould in 1963 was employed with some modifications (Newbould, 1963). To induce adjuvant arthritis, rats were anesthetized with Ketamin (70mg/kg intraperitoneally [I.P]) and Xylazin (5 mg/kg I.P) and adjuvant arthritis was induced by subcutaneous injection of 0.5 ml FCA (suspension of heat-killed *Mycobacterium tuberculosis* in mineral oil) into sub plantar tissue of the right hind paw of each rat in the test groups using a 1 ml glass syringe with a 21 GA needle, while, the control rats were injected with 0.5 ml of normal saline.

Experimental setup: Animals were divided into four groups of eight animals in each group as follows:

Group I: Control rats (untreated)

Group II: Adjuvant induced arthritic (AIA) rats (0.2 ml normal saline)

Group III: Arthritis treated with curcumin (30 mg/kg/day, orally) for 15 days.

Group IV: Arthritis treated with indomethacin (3 mg/kg/day, orally, as reference drug) for 15 days by gavage starting 7 days after adjuvant injection. At the end of the experimental period (on day 22nd), rats were fasted overnight and killed after general anesthesia by inhalation of ether. Blood samples were collected by cardiac puncture to determine ESR, CRP and WBC count.

Measurement of ankle circumference: Ankle circumference (mm) was measured for paws with a flexible strip three times per week.

Hematological examination: CRP factor was measured by means of an antibody to purified CRP by latex slide test in serum by use of a kit (Holland Medco-ERP Ltd). ESR was determined by a modified method based on ICSH (International

Council for Standardization in Hematology) selected methods (Bull et al., 1993). Briefly, 120µL of blood sample was taken directly and dropped into 30µl of 0.109 mol/L sodium citrate mixed well, and then transferred into a 1.0 mm×100 mm capillary tube. The tubes were held obliquely at an angle of 45ºc and the results were recorded at 15 min. Leukocytes count was determined with a picoscale hematological analyzer.

Statistical analysis: All the results were expressed as mean ± standard error (S.E.M). Data were analyzed using One-way ANOVA followed by Tukey test .$p<0.05$ was considered as statistically significant.

Result

Figure 2 depicts the anti-inflammatory effect of curcumin and indomethacin on the changes in ankle edema of control and experimental animals. Swelling and redness developed over a 24 h period in the foot injected with adjuvant. The finding of this study showed that on 7th day after FCA injection, the ankle circumference was significantly increased compared to the day one in the RA groups ($p<0.05$). The increasing of circumference was significant till the end of study (day 21) ($p<0.05$). Rats injected with saline did not show any joint swelling at any point in the study. Upon curcumin and indomethacin administration, the inflammation was started to decrease significantly when compared by AIA rats ($p<0.05$).

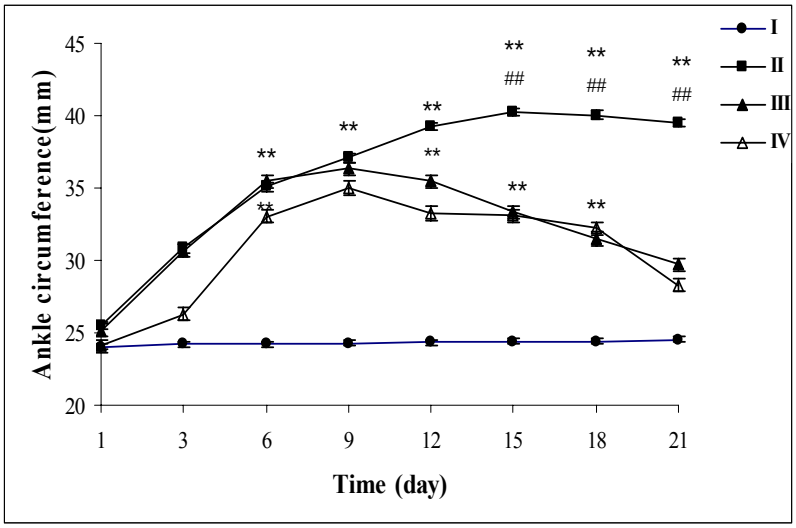

Figure 2. Ankle Circumference changes in arthritic rats. I: control group (-♦-), II: AIA rats (-■-), III: arthritic rats treated with 30 mg/kg curcumin (-▲-) and IV: arthritic rats treated with 3 mg/kg indomethacin (-Δ-). Values represent means ± standard error of the mean (S.E.M). ## $p<0.05$ compared with arthritic rat treated with drugs, ** $p<0.05$ compared with control rats (n=8).

Serum CRP and ESR levels increased by FCA administration (Figure 3A, 3B) in arthritic rats. In comparison with control values, CRP and ESR were raised in arthritic groups ($p<0.05$). These factors decreased significantly in arthritic rats after treated with curcumin and indomethacin ($p<0.05$).

Figure 3C shows that total leukocyte count was significantly increased in the arthritic rats as compared with the control rats ($p<0.05$) and in treated animals with curcumin and indomethacin was significantly decreased when compared with AIA group (II) ($p<0.05$).

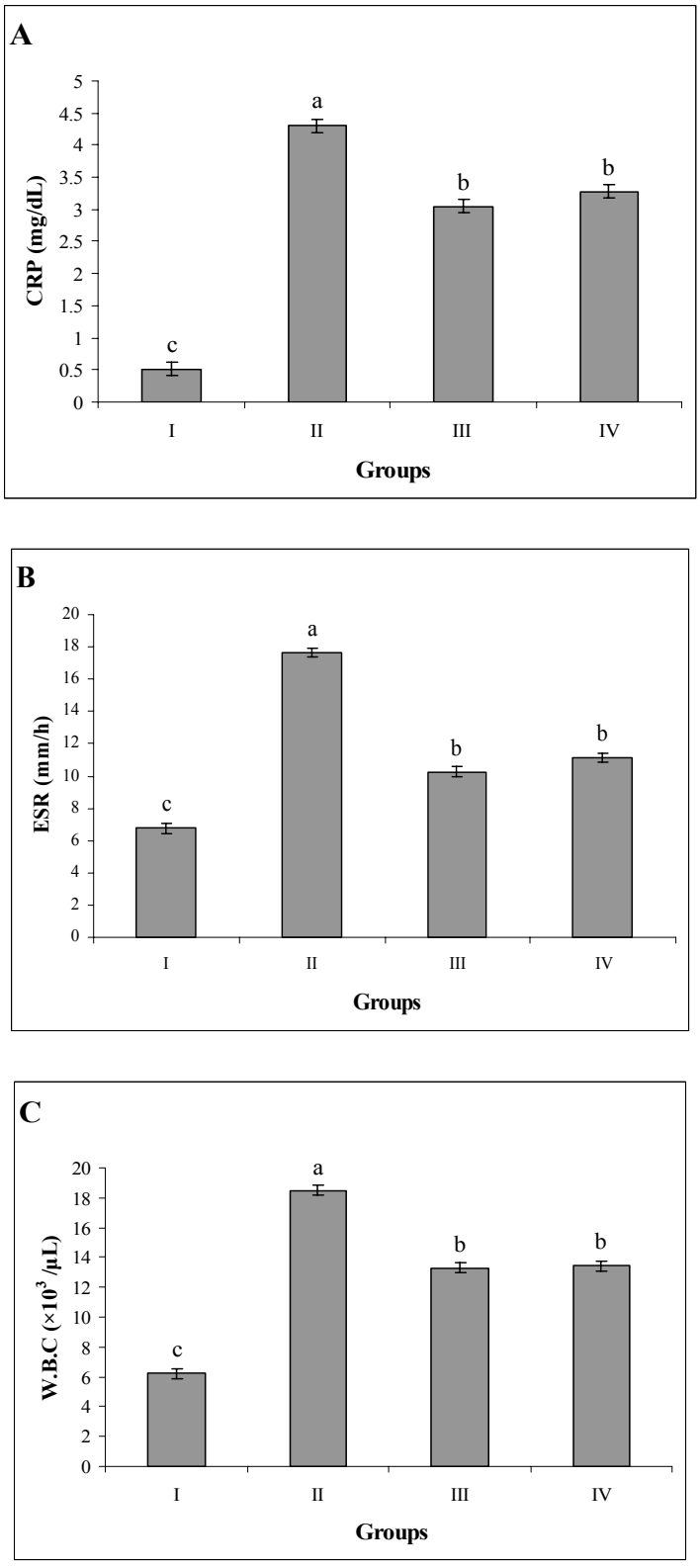

Figure 3. Effect of curcumin and indomethacin on serum CRP (A), ESR (B) and WBC(C) in arthritic rats. I: control group, II: AIA rats, III: arthritic rats treated with 30 mg/kg curcumin and IV: arthritic rats treated with 3 mg/kg indomethacin. Values represent means ± the S.E.M. Same words aren't significant in $p<0.05$ in Tukey test (n=8).

Discussion

Freund's complete adjuvant is generally used to induce arthritis in animal models. Adjuvant arthritis in rat shows several clinical and histological similarities to human RA. In the present study, following a single injection of FCA at the plantar surface, rats developed pronounced arthritis in the paws and ankles, showing 100% incidence. FCA injection resulted in a significant increase in ankle circumference with in 24h. The results of this study indicated that the curcumin exhibits anti-inflammatory properties in adjuvant-induced arthritic rats. There are several similar reports, for example, oral administration of curcumin has been shown to decrease elevated levels of the glycoprotein Gp A72, with concomitant lowering of paw inflammation in arthritic rats (Joe et al., 1997). Funk et al. determined the in vivo efficacy of curcumin in the prevention or treatment of arthritis using streptococcal cell well-induced arthritis, a model of RA (Funk et al., 2006 b). In this model, curcumin prevented joint inflammation when treatment was started before, but not after, the onset of joint inflammation. Results of this study supported the anti-inflammatory effect of curcumin. The molecular basis of the anti-inflammatory properties of curcumin has been attributed to its effects on several targets including transcription factors, enzymes, and cellular signaling molecules, including NF-kB, AP-1, and COX-2. Curcumin has been shown to directly inhibit activation of transcription factors NF-kB and AP-1 (Han et al., 2002;Kang et al., 2004). Curcumin has also been reported to suppress COX-2, the key enzyme in the formation of prostaglandins, a family of compounds derived from arachidonic acid through the COX pathway (Kang et al., 2004; Chun et al., 2003). Prostaglandins are potent mediators in the inflammatory response.

In this AIA model, both ESR and CRP were found to be markedly associated with the development of the disease, significantly elevated ESR and CRP levels were observed in arthritic rats in comparison with the control rats. Administration of curcumin similar to indomethacin 7 days after arthritis induction exhibited an inhibition on over produced plasma CRP and ESR levels, and statistically significant differences were indicated only in the arthritic group (II). IL-1β, IL-6 and TNFα are pro-inflammatory cytokines released from activated macrophages at the site of inflammation, and influencing hepatic metabolism by up regulating acute phase protein gene expression (Baumann and Gauldie, 1994). Elevated IL-1 and IL-6 levels have been reported in isolated spleen cells from rats having elevated CRP levels following the induction of CFA arthritis (Giffen et al., 2003). This kinetic change of the serum IL-1β and IL-6 levels was associated with the elevated ESR and CRP levels. Curcumin can decrease the expression and activity of these cytokines (Biswas et al., 2005), thus can decreased CRP and ESR levels in the blood. In all arthritic groups we observed a markedly higher leukocyte count as compared with the healthy rats. Franch *et al.* and Carlson *et al.* reported that leukocytosis, neutrophilia and an increase in the number of lymphocytes were observed after 21 and 49 days of adjuvant-induced arthritis in rats (Franchand et al., 1994; Calosn et al., 1985). These changes agree with our results in this study. In the present study, leukocytosis was significantly inhibited by curcumin ($p<0.05$). There was no significant difference between treated animals with curcumin and indomethacin concomitantly. However, Funk *et al.* reported that curcumin decreased WBC count in SCW-induced arthritis (Funk et al., 2006 a), which agrees with our results in this study.

In conclusion, these results suggest that curcumin can possess beneficial effects in alleviating arthritic symptoms in AIA model.

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Ozone inhalation can induce chromosomal abnormalities in bone marrow cells of Wistar rats

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Abstract

Due to the wide range of applications for ozone and its increasing use for medical and industrial purposes, studying its effects has become a very important line of research. The ozone has been suspected to be a carsinogen. Because of the increasing use of ozone, the human could be more and more exposed to this gas. In this study the effects of ozone inhalation on chromosomes and its clastogenic consequences have been investigated using *in vivo* micronucleus assay in bone marrow cells of treated rats.

Animals were treated for 6 hours a day at 3 ppm of ozone during 10 consecutive days. The micronucleus assay was performed immediately and 11 days after the last exposure. The frequency of micronucleated polychromatic erythrocyte of bone marrow (MNPCE) increased in both groups compared to the control. Such increase confirmed the clastogenic effects of ozone. The elevated frequency of MNPCE did not decrease after 11 days of the last ozone exposure.

Results indicate that ozone inhalation could induce persistent chromosomal damages even to bone marrow cells which were not in direct contact to it. Also, once more, the results confirmed the usefulness of the micronucleus assay in toxicological studies.

Key words: ozone inhalation, micronucleus assay**,** chromosomes

Introduction

Studying the substances and factors polluting the environment is getting more and more important in our industrialized life and environment. One of those widely used factors is ozone. Ozone is used for sterilization of operating rooms and surgical tools, as a direct or indirect antiseptic agent of drinking water and preservative of food. Also due to its very strong oxidative capability, it is considered as a very good agent in removal of natural organic substances of swimming pools (Matilainen, 2006; Murphy, 2006).

Because of its special chemical and physical properties, in aqueous solution, ozone is capable of producing free radicals which could cause wide range of damages to cells and tissues. Different studies have presented various results on the effects of ozone on living organism (Victorin, 1992).

Ozone is a very strong oxidant with the ability to interact with biomolecules. Its disintegration in aqueous solutions leads to formation of various free radicals of oxygen such as oxygen super oxide (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^0), and very active single oxygen of superoxide (O^0). These free radicals, in turn, can take part in secondary reactions which produce an oxidative stress (Victorin, 1992). Free oxygen radicals eventually destroy DNA by inducing cleavage in the deoxyribose-phosphate backbone and the chromosome breakages. Also ozone could directly react with DNA and destroy or modify its organic bases (Cataldo, 2006, Ito, 2005). The results of these changes in cells and tissues are oxidative destructions which are effective on aging, cell deformation, mutation, cancer, and eventually cell death and necrosis. In some particular concentrations ozone inhibits DNA replication. The tissue destruction caused by ozone is mostly due to its destructive effects on lipids of cell memebranes

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(Steinberg, 1990). It leads to decomposition of fatty acids of cell membrane and inflict serious damages to the airway cells (Leikauft, 1995). Analysis the ozone exposure on airway tissues of several test animal species and humans with regards to the ozone concentration suggest that time and frequency of exposure have a very important role on its induced-destructive effects (Ratto, 2006). The elevated level of ozone in living and working environments may cause serious damages to living cells and chromosomes of the individuals involved in such conditions, and may explain the increasing risk of subsequent lung cancer (Chen, 2006).

In nature ozone is found in concentrations of 0.01 to 0.05 ppm which reaches 10 to 20 ppm in the ozone layer high above the ground. In natural conditions it is produced as a result of solar radiation and thunder storms. The industrial methods of producing ozone are: using UV irradiation on pure oxygen or air and/or passing air or oxygen through electric arch chambers (Sundell, 1996).

Considering the destructive effects of ozone on cells and tissues, there has been extensive research on its mechanisms of inducing damages and effective doses. These studies have mostly performed on target tissues such as cells of pulmonary systems or treated cells in culture media (Leikauf, 1995; Ratto, 2006; Chorvatovicova; 2000). There has been no report concerning the effects of this gas on other tissues which are in no direct contact with it in *in-vivo* conditions.

Due to the extended uses of ozone and its known effects on tissues we decided to study the effects of ozone inhalation in long-term treatment on the chromosomes of rat bone marrow cells which are not in direct contact to inhaled ozone. In this study the micronucleus assay has been performed.

The *in vivo* micronucleus assay was introduced by Schmid in 1975. The *in vivo* micronucleus is able to reveal the structural and numerical chromosomal damages induced by physical or chemical stimuli. The *in vivo* micronucleus has significant advantages over analysis of metaphase chromosome. In terms of preparation and scoring the probable damages, this method is easier and faster than metaphase chromosomal analysis while keeping the accuracy intact (Heddle 1973). It is widely used in toxological study for analysis the effect of physical and chemical agents in our environment. In this method, any damages to chromosomes which may lead to chromosome breakage or loss, could be detected by scoring the small nucleous (micronucleus) in cytoplasm of the damaged cell (Heddle, 1991; Gocke, 1996; Hayashi, 1994; Mutsuki, 1993). The frequency of micronucleus reflects the rate of chromosomal damages. In *in vivo* systems this method is applicable to sample from different tissues such as skin, spleen, bone marrow, and blood (Abramsson-Zetterberg, 1999).

Materials and Methods

Male Wistar rats with the age of 7 to 8 weeks and weight of 250-300 grams were kept in animal house with the standard condition of 12h dark/12h light with the temperature of 20 ± 2 °C for a week to adapt before treatment. They were divided into three groups of control, treatment1 and treatment2. Total of 12 rats were divided into these three groups. All the experimental procedure were performed according to the Guide for the Care and Use of Laboratory Animals by National Academy Press Washington, D.C. in 1996.

Ozone treatment: To provide the required dose of ozone, the ozone producing chamber (Teb-e-Razi Mashhad) was used which could produce ozone by electrical discharge of the air. The dose of 3 ppm of ozone was used in this study. The calibration of 3 ppm ozone was performed by chemical titration of iodine released from KI solution (recommended by the manufacturer). Briefly, passing the ozone gas through KI solution resulted in releasing of iodine according to the following formula:

$$O_3 + 2KI + H_2O \rightarrow I_2 + 2KOH + O_2$$

The sodium thiosulfate is capable to scavenge the iodine from the above solution. Decrease of the free iodine would change the solution color from yellow to blue. The dose of ozone used to free the iodine from KI could be calculated by measuring the amount of sodium thiosulfate solution consumed to scavenge the iodine.

The treatment chamber with dimensions of 70×110×50 cm was used for ozone exposure. The samples were treated separately inside the chamber. The rats from each treatment groups 1 and 2 were exposed to 3ppm of ozone to inhale for 10 consecutive days and 6 hours daily from 12:00 to 18:00.

Sampling: Micronucleus assay were performed on control as well as treatment groups 1 and 2. The rats from treatment group 1 were sacrificed immediately after last ozone inhalation. The rats of the group 2 were sacrificed 11 days after the last ozone inhalation.

The micronucleus assay was performed according to Hayashi et al (1994), and Schmid (1975); briefly, rats were euthanized by chloroform inhalation. The femoral bone marrow cells were gently flushed out by a 5 ml syringe containing 3 ml fetal bovine serum (gibco) and smeared on clean slides. The smeared cells were left 24 h to air dry and fixed with absolute methanol for 5 min and stained according to May-Grünwald – Gimsa technique. Observations were made within 24 h. The coverslipped slides were blindly scored on coded slides at ×1000 magnification. At least 2000 polychromatic erythrocytes (PCEs) with or without micronuclei and normochromatic erythrocytes (NCE) were scored per slide. At least two slides per animal were scored. The ratio of micronucleated polychromatic erythrocytes (MNPCEs) to PCEs was calculated after simultaneously recording both PCE and MNPCE on each slide.

Statistical analysis: The statistical analysis was performed using software MINITAB. The differences between treated groups and control and also between treated groups themselves were analyzed by the one way analysis of variance (ANOVA).

Results

Treatment with ozone had a significant effect on increased frequency of micronucleus in bone marrow cells compared to the control group (Figures 1, 2). Micronucleus frequency in the control group was 1.12%. Long term treatment with ozone could significantly elevate the micronucleus frequency to 4.88% (Table 1). The rats treated with similar dose and time of ozone, the elevated frequency of micronucleus did not decreased even after 11 days of the last exposure. The frequency of micronucleus in treatment 1 and 2 did not show any significant differences.

In both treatment groups 1 and 2 the ratio of polychromatic erythrocytes to the total number of normochromatic and polychromatic cells was decreased significantly, representing the toxicity of the ozone inhalation in bone marrow cells (Table1). Comparing the values from the two treatment groups showed a significant increase for the treatment group 2, representing recovery from ozone treatment and returning to natural cellular conditions in bone marrow after 11 days of last exposure.

Table1: Frequency of MNPCE** in Rat Bone Marrow Cells

	MnPCE/100 PCE	PCE/NCE***+PCE
Control	1.12±0.29	54.45±0.092
Treatment 1	4.88*±0.88	46.34*±1.02
Treatment 2	5.62*±1.34	51.77*±0.88

*Significant difference with control (0.05)

** Micronucleated polychromatic erythrocyte

*** Normochromatic erythrocyte

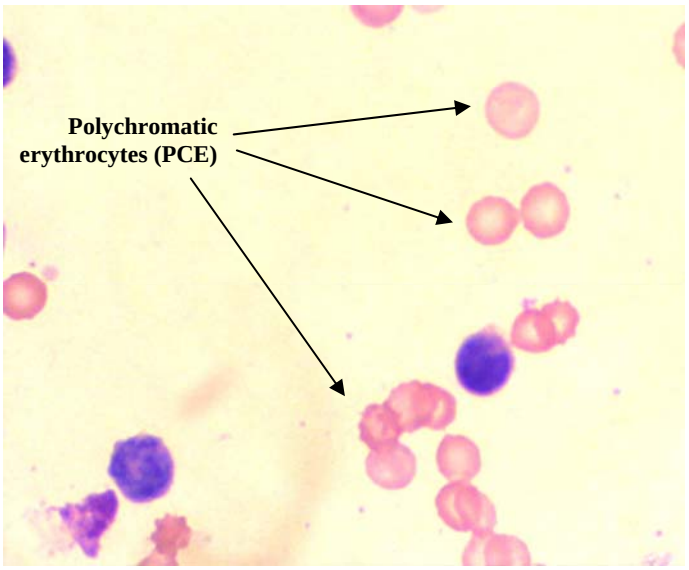

Figure 1: Bone marrow smear from control rat. PCE are stained light purple.

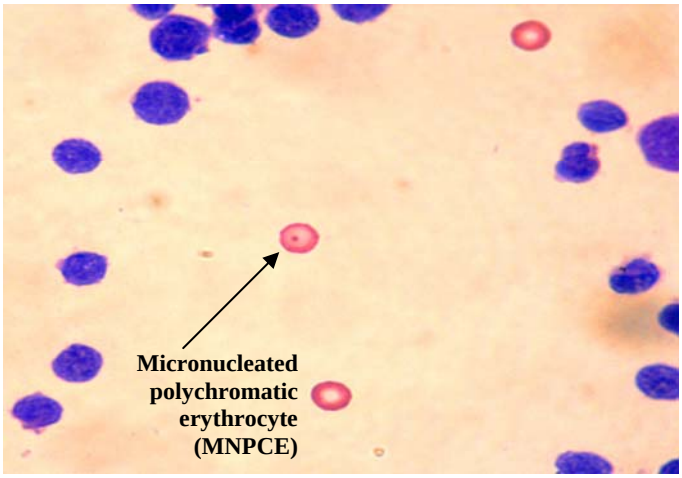

Figure 2: Bone marrow smear of treated rat. MNPCE is at the center.

Discussion

Due to the way of exposure of living organisms to ozone, a ubiquitous air pollutant, the study of clastogeneic effect of this gas in *in vivo* are mostly devoted to the cells and tissues directly exposed to it (Haney et al., 1999; Chorvatovicova, 2000). In this study the cytogenetic effects of long term ozone inhalation were analysied on bone marrow cells of rat. The cells studied here are not directly exposed to ozone.

The frequency of micronucleus in PCE of control rats was 1.12. In toxicological studies on rats the reported frequency for control group covers a wide range. Such studies report frequencies of 0.01 to 1 percent (Suzuki, 2006; Zhong, 2000). Therefore the observed base line micronucleus frequency in the present study is in the range reported by others.

In this study ozone inhalation clearly increased the frequency of micronucleus in polychromatic erythrocyte of rat bone marrow. In *in vivo* micronucleus assay increase in the frequency of MN represents the structural and numerical chromosomal damages in cells affected by the stimuli. Lost or broken parts of chromosome in cytoplasm of the cell form a small nuclei which is visible as micronucleus. Here the small size of MN suggests the structural damages to the chromosomes (Wakata and Sasaki, 1987). Due to its chemical and physical properties, ozone is capable of producing free radicals when it comes in contact with biologic systems (Victorin, 1992).

Free oxygen radicals may destroy DNA, cleave the deoxyribos-phosphate bonds of DNA, and break the chromosomes. Clastogenic properties of ozone through its ability to break DNA chain have already been established in different studies (Haney, 1999; Diaz-Liera, 2002; Bornholdt 2002). Increased frequency of micronucleus in rats of this experiment represents the ozone-caused structural damages to chromosomes by direct or indirect exposure to free radicals induced by long term ozone inhalation which could reach the bone marrow cells.

In-vitro studies of effects of ozone on leucocytes of peripheral blood have demonstrated temporary effects of ozone in inducing damages to the DNA. The clastogenic effect of ozone treatment was reversed when treatment stopped (Diaz-Liera, 2002). What is significant here and has not been reported before is the persistent harmful effect of long time ozone treatment. The stable frequency of induced micronucleus even after 11 days from last ozone exposure is a proof that the damages induced to chromosomes or bone marrow cells are some how permanent. It is expected that frequency of micronucleus will decrease when the induction of chromosome damages is stopped. It is reported in other studies that the effects of chromosomal damaging factors on micronucleus frequency are reversible and reduces as factors creating chromosome disorders are eliminated (Haddad et al., 2004; Malvandi et al., 2006). Reduction of micronucleus frequency after stopping the induced-damages to chromosome is due to the following reasons:

- Random integration of micronucleus to one of the main nuclei (Gustavino, 1994)
- Disintegration of micronucleus by cytoplasm nucleases (Granetto, 1996)
- Replacement and repairing of the tubulin reservoir and remaking of the dividing spindle needed for correct chromosome separation in the following cell division (Nichol, 1988)
- Activities of monitoring mechanism for preventing the division of damaged cells and induction of apoptosis in cells unable of repair (Sablina, 1998)

Due to the persistence elevated level of micronucleus frequency after 11 days of the last ozone exposure, it can be concluded that despite stopping the ozone exposure there are still factors inducing chromosome damages inside the bone marrow of the treated rats. Induction of chromosome damages, which is due to the attack of the free radicals to DNA, might be because of the deposit of free radicals in fat tissues of rats. The ability to induce clastogenic damages to bone marrow cells and the long lasting effects of ozone inhalation both refer to the existence of a mechanism that transfers ozone and/or ozone-produced free radicals to internal tissues of the body and continuously induces the damages to the chromosome structure.

Although more investigation regarding the analysis of the induced abnormalities to the tissues which are not in direct contact to inhaled ozone are required. In general the proposed model demonstrates that people who are exposed to ozone inhalation because of their jobs requirement may suffer chromosomal damages which may remain for long period of time. Thus care must be taken to not be exposed to ozone inhalation in particular cases such as planning for pregnancy.

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Antibacterial activity of a malodor neutralizer containing silver nanoparticles

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Abstract

In this study bactericidal activity of a malodor neutralizer containing silver nanoparticles manufactured in Iran has been tested. For this purpose different concentrations of the product encounter with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus* then the activity were evaluated in different contact times. The products containing at least 200 ppm of silver nanoparticles were effective on all of bacterial strains and higher exposure time increased its antibacterial activity**.** Bactericidal activity against spore of *Bacillus cereus* was less obvious in comparison with other bacteria. It seems that silver nanoparticle is a valuable antibacterial agent even in presence of aromatic fragments and could be applied as disinfectant in many situations.

Key words: silver nanoparticle, bactericidal, aromatic, disinfectant

Introduction

Since ancient times, it has been known that silver and its compounds are effective antimicrobial agents (Klasen, 2000). Because of the recent advances on metal nanotechnology, silver nanoparticles have received renewed attention as a possible antimicrobial agent (Lee et al., 2008; Melaiye et al., 2005; Sondi and Salopek-Sondi, 2004; Landsdown, 2002).The great interest arouse when recently silver nanoparticles claim as new antibacterial compound which rarely develop resistant bacteria (Landsdown, 2002; Baker et al., 2005; Lock et al., 2007). It has been shown that the LD50* of silver nanoparticles either by ingestion or injection is very high (1266 mg/kg & 284 mg/kg respectively) and inhalation of a high doses (1.32 x 10(6) particles/cm3, 61 microg/m3) of silver nanoparticles is safe, so it can be classified as a non-toxic substance (Ji et al., 2007; Fu et al., 2006). This and some other properties make silver nanoparticles very suitable for disinfecting usage.

The acceptability of each product for a defined purpose cannot be determined without valid tests. Therefore, the product should be subjected to relevant tests in order to evaluate their activity under conditions recommended for their intended use (Zhao and Stevens, 1998; ISIRI 10504, 2008). The aim of this study was to evaluate basic bactericidal activity of a fragrant product containing silver nanoparticles.

Nanotechnology deals with preparation of uniform nanosized silver particles with specific requirements in terms of size, shape, and physical nanomaterials which are very useful both in scientific and commercial applications (Sondi and Salopek-Sondi, 2004; Landsdown, 2002).

Material and Methods

For assessment of bactericidal activity of this product and determination of minimum inhibitory concentration, we prepared serial dilutions of products containing 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 ppm of silver nanoparticles (Nanocid, Iran). Size of silver nanoparticles that we used was around 4.5 nanometers.

Each dilution of product encountered with 1.5×10^8 CFU of bacteria prepared in Muller Hinton broth (Merck, Germany). Bacterial strains that we used in this study were *Staphylococcus aureus* (PTCC[*]1112) as an indicator of Gram positive bacteria and *Pseudomonas aeruginosa* (PTCC 1073) as a Gram negative and and resistant bacteria. *Bacillus cereus* (PTCC 1014) was also included in this study as a spore forming bacteria.

In this study contact time was 5, 15, 30 minutes and 24 hours and the temperature during the exposure was 25^oc. After each contact 10 µl of bacterial suspension and the disinfecting product have been

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[*] Lethal dose for 50 percent of subjects

[*] Persian Type Culture Collection

taken and inoculated on Muller Hinton agar medium and incubated at 37°c for 24 hours. Then each plate was inspected and the results were reported. We used distilled water without any disinfectant as negative control for each suspension. Also Carrier test is performed for evaluation of antibacterial activity of this substance which is to apply on surfaces. This test mimics a condition similar to the real application of disinfecting agent. In this test, 1.5×10^7 CFU of *E. coli* (PTCC: 1389) was applied to a sterile surface. Then three dilutions of disinfecting agent which contained 200, 100 and 50 ppm of silver nanoparticles were sprayed on this surface. Samples were taken by sterile swabs from this surface after 5 minutes and inoculated on to Muller Hinton agar medium (Merck, Germany). After 24 hours incubation in 37°c, the plates were inspected for bacterial growth.

Results

The bactericidal activity of this disinfecting agent on *S. aureus* was presented in table 1. The product with concentrations greater than 12.5 ppm of active ingredient demonstrated at least a 5 decimal log reduction in bacterial population when tested and there is a significant difference in bacterial number between before and after usage of this product with corresponding concentrations ($p<0.01$).

Table 1: Number of remaining bacteria after exposure with different concentration of silver nanoparticles which presents bactericidal activity on *S.aureus*

Concentration / time	200	100	50	25	12.5	6.25	3.12	1.56	0.78
5 min	0	0	0	0	0	$5.7*10^3$	$27*10^3$	$44*10^3$	$>10^5$
15 min	0	0	0	0	0	$3.3*10^3$	$19*10^3$	$30*10^3$	$>10^5$
30 min	0	0	0	0	0	0	$15*10^3$	$21*10^3$	$>10^5$
24 h	0	0	0	0	0	0	$0.3*10^3$	$4*10^3$	$>10^5$

Results of bactericidal activity of different concentration of this disinfecting agent on *P. aeruginosa* were presented in Table 2. The product with concentrations greater than 100 ppm of active ingredient and more than 5 minutes exposure demonstrated at least a 5 decimal log reduction in bacterial population when tested and there is a significant difference in bacterial number between before and after usage of this product with corresponding concentrations ($p<0.01$).

Table 2: Number of remaining bacteria after exposure with different concentration of silver nanoparticles which presents bactericidal activity on P. aeruginosa

Concentration / time	200	100	50	25	12.5	6.25	3.12	1.56	0.78
5 min	$0.3*10^3$	$14*10^3$	$21*10^3$	$>10^4$	$>10^4$	$>10^5$	$>10^5$	$>10^5$	$>10^5$
15 min	0	0	$19*10^3$	$24*10^3$	$>10^4$	$>10^5$	$>10^5$	$>10^5$	$>10^5$
30 min	0	0	$2.3*10^3$	$3*10^3$	$7*10^3$	$14.6*10^3$	$15.3*10^4$	$>10^4$	$>10^5$
24 h	0	0	0	0	0	0	0	$6.5*10^3$	$28.5*10^3$

Results of bactericidal activity of different concentration of this disinfecting agent on *B.cereus* were presented in Table 3. No tested concentration of the product demonstrated a 5 decimal log reduction in bacterial population although there is a significant difference in bacterial number between before and after usage of this product ($p<0.01$).

Table 3: Number of remaining bacteria after exposure with different concentration of silver nanoparticles which presents bactericidal activity on *B. cereus*

Concentration / time	200	100	50	25	12.5	6.25	3.12	1.56	0.78
5 min	$24*10^3$	$33*10^3$	$>10^4$	$>10^4$	$>10^5$	$> 10^5$	$> 10^5$	$> 10^5$	$> 10^5$
15 min	$33*10^3$	$40*10^3$	$43*10^3$	$>10^4$	$>10^4$	$> 10^5$	$> 10^5$	$> 10^5$	$> 10^5$
30 min	$41*10^3$	$43*10^3$	$54*10^3$	$>10^4$	$>10^4$	$> 10^5$	$>10^5$	$>10^5$	$> 10^5$
24 h	$25*10^3$	$30*10^3$	$>10^4$	$>10^4$	$> 10^4$	$> 10^5$	$> 10^5$	$> 10^5$	$> 10^5$

Carrier test show that 200 ppm of silver nanoparticles destroyed all bacteria so has good disinfecting property when were sprayed on surfaces

Discussion

This product which contains silver nanoparticles (200 ppm), perfume (0.02 -0.05%), FOT (1.5% - 2%) and water has good bactericidal activity on Gram positive bacteria like *S.aureus*. The longer contact time has additive effect on bactericidal activity of this product. This fact is also true for Gram negative bacteria like *P. aeruginosa*.

In this study, although higher concentrations of silver nanoparticles seem to decrease the number of *B. cereus*, but longer contact time doesn't seem to cause significant decrease in the number of this bacterium so it sounds that this product is not an effective disinfectant on spores. Our previous study shows that presence of FOT as a malodor neutralizer in composition of this product have slight negative effect on antibacterial activity of silver nanoparticles. Optimal concentration that is a minimum concentration which demonstrates a five log reduction in the test conditions were 12.5, 100 and 200 ppm of silver nanoparticles for *S.aureus, P. auerogenosa* and *B. cereus*, respectively. The silver nanoparticles are stable in environment, thus concentration of it will increase in environment after each usage.

It is believed that silver nanoparticles destroyed bacteria by two mechanisms: ion mechanism and catalectic mechanism (Jia et al., 2008; Kim et al., 2008; Kim et al., 2008). In ion mechanism, silver nanoparticles gradually radiate Ag+ ions. These ions during replacement reaction change HS- bands in microorganism's membrane and enzymes into Ag-S bands, in this way the nano-silver suppresses respiration, basal metabolism of electron transfer system, and transport of substrate in the microbial cell membrane. The result of this reaction, are denaturation and wasting of the microorganism (Landsdown, 2002; Panacek et al., 2006).

In Catalectic mechanism, silver nanoparticles were put on semi conductor bases, such as TiO_2 or SiO_2. In this case particle acts like an electro chemical pile which produces O_2^- radicals and OH ion that both are active bases that are amoung the strongest antibacterial agent. (Lok et al., 2007, Jia et al., 2007; Jeon et al., 2003)

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Cytotoxic activity of *Isatis campylocarpa*, an Iranian endemic plant, on human cancer cell lines

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Abstract

The antineoplastic activities of alcoholic and aqueous extracts of the roots, stems and leaves of *Isatis campylocarpa* an endemic species of the Brassicaceae family from Iran, investigated on the growth of Jurkat as an acute lymphocytic leukemia cell line, K562 as a chronic myelogenous leukemia cell line and Fen as a bladder cancer cell line using colorimetric assay. Results showed that 1 to 200 µg/ml concentrations of all the extracts inhibited the proliferation of the cells and may be it relate to the Indirubin compounds. The maximum effect on the Jurkat cells observed for the aqueous root extract. The effect of the extracts on the Jurkat cells was greater than on the K562 cells, which may be indicate more sensitivity to lymphocytic cells than myeloid ones.

Key words: cytotoxic, *Isatis campylocarpa*, cancer, leukemia, indirubin

Introduction

Antineoplastic drugs prevent cancerous cell division in chemotherapy. Inhibition efficiency depends upon drug type and concentration. Chemotherapy has several side effects but natural compounds use in medicine, with fewer side effects. Sixty percent of antitumor and anti-infection drugs existing in market or under clinical studies have natural origins. Most of these compounds can not be synthesized chemically and they should be extracted from plants or produced by cell cultures (King and Robins, 2006; Asghari, 2006).

Isatis is a plant genus of the Brassicaceae family mainly distributed in the Irano-Turanian region and only a few species of them distributed in the Europe-Siberia and the Mediterranean phytogeography regions (Sajedi et al., 2005). One species of the genus i.e. *Isatis tinctoria* has been used in China as traditionally medicine in chronic myelogenous leukemia treatment (Xiao et al., 2002).

Indirubin, a compound found in *I. tinctoria* roots, has undergone screening for anti-cancer activity. Indirubin thought to be inhibiting DNA replication in neoplastic cells without causing significant bone marrow suppression (Hoessel et al., 1999). The *Isatis* contains a number of indole compounds, which thought to have anti-cancer effects and may be help to explain the traditional uses of *Isatis* in the treatment of cancer. Indirubin competes with ATP for binding to catalytic sites of cyclin dependent kinases (CDKs) and block cell proliferation in the late-G_1 and G_2/M phases of the cell cycle, as well inhibits the assembly of microtubules. The crystal structures of CDK in complex with two Indirubin derivatives reveal the atomic interactions of these inhibitors with the kinases ATP-binding site (Hoessel et al., 1999; Meijer et al., 2006).

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is a standard colorimetric assay to determine cytotoxicity of potential medicinal agents (Hay, 1988). MTT is water-soluble and reduced in living cells because mitochondrial reductase enzyme (dehydrogenase) activate and convert MTT into the purple crystals of formazan. These crystals are insoluble in water and their amounts will be directly related to the quantity of living cells (Masmann, 1983; Freshney, 1992).

The aim of the present study is to investigate the cytotoxic activity of the alcoholic and aqueous extracts of the roots, stems and leaves of *Isatis campylocarpa*, an Iranian endemic species.

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Material and Methods

***Preparation of the extracts*:** Appropriate amounts of fresh materials of *I. campylocarpa* obtained from the north of Fars Province in Iran. A voucher specimen deposited in Shiraz University Herbarium. Different parts of the plant including the roots, stems and leaves completely separated and dried by air in the shade, powdered and subjected to extraction (both aqueous and alcoholic extracts). Different concentrations of aqueous extracts using acidic (pH=3) solution of RPMI 1640 medium (Sigma, St, Louis, USA) and alcoholic extracts using dimethyl sulphoxide (DMSO) were prepared from stock samples (20 mg/ml). Germinated seedlings as well used for aqueous extract. The solutions were centrifuged (3000 rpm for 10 min) to remove insoluble ingredients, and the supernatants passed through 0.22 µm filters for sterilization.

***Cell lines*:** Suspension culture of Jurkat (T cell leukemia) and K562 (myelogenous leukemia) cell lines and monolayer culture of Fen (bladder carcinoma) cell line used. The cell lines obtained from an Iranian cell bank. All the cell lines were kept in RPMI 1640 medium (Sigma, St, Louis, USA) supplemented with 10% fetal calf serum (Gibco, Germany) in culture flasks at 37˚c in 5% humidified CO_2 incubator. The cells fed until confluence (2×10^6) and were expanded by trypsinization (for adherent cells) and subcultured at lower numbers in new culture flasks. Cells viability determined by trypan blue dye test.

***MTT colorimetric assay*:** Briefly, 90 µl of each cell suspension containing 40000 Jurkat cells and 20000 K562 cells separately added into the 96 well culture plates. Then 10 µl of various prepared concentrations (2000, 1000, 500, 100, 10 µg/ml) from stock samples of the extracts were added to each row of wells and so the final concentrations of the extracts reached to 200, 100, 50, 10 and 1 µg/ml in each well. We used 10 µl of DMSO or acidic RPMI as negative control and 10 µl of doxorubicin (an anticancer agent) as positive control. In the case of monolayer cultures, 90 µl of cell suspension containing 15000 Fen cells placed into the wells, and incubated in CO_2 incubator for 24 h at 37˚C and subsequently plant extracts added. Plates of suspension and monolayer cultures placed in the CO_2 incubator at 37˚C for 48 h at humidified atmosphere. After the addition of 10 µl of MTT solution to each well, the plates transferred to the incubator and retained for 3-4 h. Supernatant removed only from the monolayer culture and then 100 µl of DMSO solutions added for dissolving of purple crystals of formazan, however in suspension cultures the supernatant did not remove. Then, 100 µl acidic isopropanol was added and the plates were placed in an incubator (5 min at 37˚c) to remove bubbles from wells. Absorbance of each well was measured using ELIZA reader at 570 nm and 630 nm wavelength.

***Analysis*:** Growth inhibition percentage (Inh%) for each concentration of the extracts was calculated as:

$$\text{Inh \%} = \left(1 - \frac{\text{Mean of light absorptions for test samples}}{\text{Mean of light absorptions for negative control}}\right) \times 100$$

The 50% inhibitory concentrations (IC50) estimated by probit analysis (Minitab statistical software 13.30; Minitab Inc.). The assays carried out with three replications. Raw data imported to Microsoft Excel for calculations and graphical representation. SPSS version 11.5 and ANOVA used for analysis of variance and comparison of means by Duncan's method at $P < 0.05$.

Results

According to National Cancer Institute (NCI) protocol, significant cytotoxic activity of different extracts interpreted by IC50 (Caideron, 2003). Cytotoxic activity will be considerable if IC50 is lower 25 µg/ml and will be weak if IC50 is in the range of 25-100 µg/ml. The extract has no significant toxicity if IC50 is more than 100 µg/ml. Therefore, with respect to this guideline, root aqueous extract of *I. campylocarpa* has a considerable toxicity (IC50=10.2 µg/ml) and leaf aqueous extract (IC50=50.1 µg/ml) has a weak cellular toxicity on Jurkat cells. In the case of Fen cells, root aqueous extract (IC50=72.44 µg/ml), root alcoholic extract (IC50=39.8 µg/ml), stem alcoholic extract (IC50=97.7 µg/ml), and leaf alcoholic extract (IC50=85.11 µg/ml) has weak cytotoxic activity. According to IC50 value, other extract samples did not showe significant toxicity. IC50 values of three examined cell lines which

affected by different extracts are showed in Table 1. Inhibitory effects of different aqueous and alcoholic extracts of root, stem and leaf on Jurkat, K562 and Fen cell lines showed in Figure 1.

Discussion

Some researches have demonstrated that plant extracts possess various biological activities including anti tumor and anti leukemia activity (Tsan et al, 2002; Valente et al., 2004; Moongkarndi, 2004; Kanadaswami et al., 2005; Vasilev, 2005; Amirghofran et al., 2006). Therefore, plant substances continue to serve as viable source of anticancer drugs for the world population and several plant-based anticancer drugs are in clinical use (Heinrich et al., 2006). In the present study alcoholic and aqueous extracts of the roots, stems and leaves of *Isatis campylocarpa,* an Iranian endemic species, examined for cytotoxic activity against different cancerous cell lines. We found that based on IC50, all of the extracts except stem aqueous extract and seedling aqueous extract more or less have the capacity to decrease the proliferation of cancerous cells. Among the plant extracts, the highest activity againt the Jurkat cells was root aqueous extract and againt the K562 cells was root alcoholic extract. The sensitivity of the Jurkat cells was more than K562 cells, which may be indicates more sensitivity of lymphocytic cells than myeloid ones and may be it relate to Indirubin compounds. Average inhibition of Jurkat, K562 and Fen cells treated with alcoholic and aqueous extracts of the different parts of *Isatis campylocarpa* was 130.6, 178.4 and 104.4 based on IC50 and so Fen cells are more sensitive to this plant extracts.

Isatis tinctoria has used in China as traditionally medicine in chronic myelogenous leukemia treatment (Xiao, 2002), but the results presented, indicate that the extracts of *Isatis campylocarpa* used in this study have low antineoplastic activities against the cells that originated from leukemia. Leukemia is one of the most common malignancies causing death worldwide, and, although chemotherapy is the standard method of treatment for leukemia patients but is very expensive and has not been fully effective, and therefore studies on another species of *Isatis* plant in Iran remains important to reduce the rate of mortality.

It has showed that the mechanism of action of antineoplastic agents can be due to two distinct processes of necrosis or apoptosis in the cells. Cell death by necrosis is a more passive form of cell death that is characterize by organelle and cell swelling, loss of membrane integrity, rupture of the plasma membrane and cell lysis (Dive et al., 1992). Necrosis is often associated with extensive tissue damage and an intense inflammatory response (Alison et al., 1995). Apoptosis, on the other hand, is an active process that involves the activation of various cell-signaling cascades which results in characteristic morphological and biochemical changes such as chromatin condensation, DNA fragmentation, membrane blebbing, and cell shrinkage (Rieux-Laucat et al., 2003). The cell is eventually broken down into smaller membrane-bound vesicles termed apoptotic bodies that engulfed by surrounding cells without initiating an inflammatory response (Rieux-Laucat et al., 2005).

Specific compounds in the extracts of *Isatis tinctoria* from China have different effects on cell lines, for example, roots containing high values of Indirubin and leaves have indicant and isatan B and C (Wu, 1982; Maugard, 2001). Different sensitivities of cell lines and steps in our experiments could also contribute to a variation of results.

However many modern drugs have their origin in the traditional medicine, plants are cheap and safe in comparison to synthetic compounds, especially endemic medicinal plants which are more available than foreign medicinal plants. Moreover, some additional compounds in plant extracts can prevent the side effects of the synthetic drug.

In conclusion, we observed the anti cancer activity of *I. campylocarpa* plant against different tumor cell lines. More in vitro and in vivo studies need to discover the chemical composition and anti cancer characteristics of the extracts and further more mechanistic work is essential to prove the compounds of the extracts as a one of the specific cancer drug.

Table 1. IC50 values of *I. campylocarpa* extracts on three examined cell lines

Extract \ Cell line	IC50 (µg/ml)		
	Jurkat	K562	Fen
Stem alcoholic extract	128.8	>200	97.7
Root alcoholic extract	125.8	123.02	39.8
Leaf alcoholic extract	199.5	125.89	85.11
Stem aqueous extract	>200	>200	107.15
Root aqueous extract	10.2	>200	72.44
Leaf aqueous extract	50.1	>200	154.88
Seedling aqueous extract	>200	>200	173.78
Positive control	<5	<5	<5

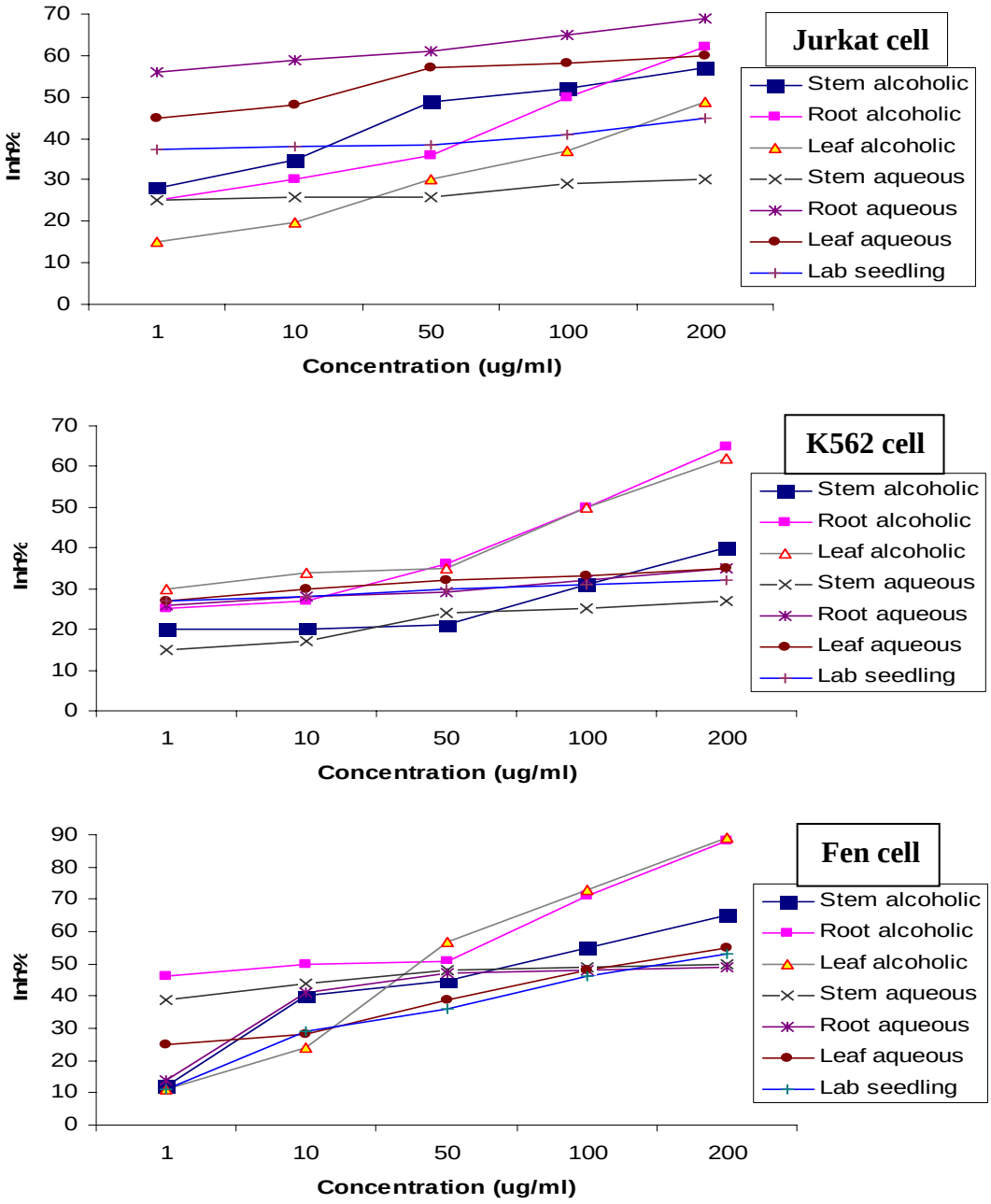

Figure 1. Effect of different concentrations of *I. campylocarpa* extracts on cells growth. Values represent the mean of three experiments.

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Effects of salicylic acid on photosynthetic pigment content in *Ocimum basilicum* L. under UV-C radiation stress

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Abstract

Basil plants (*Ocimum basilicum* L.) were sprayed with salicylic acid (1mM) and exposed to Ultraviolet-C (UV-C) radiation (40 Wm^{-2}) after emergence of six developed leaves. Plants were grown for 18 days and exposed to UV-C for 5 $mind^{-1}$ alternatively. Chlorophyll a, chlorophyll b and carotenoide were considered for analysis of photosynthetic pigments. The results showed that the photosynthetic pigments were decreased under UV-C radiation. Decreasing of photosynthetic pigments under UV-C radiation was significantly alleviated by salicylic acid treatment.

Key words: basil, UV radiation, salicylic acid, photosynthetic pigments

Introduction

The role of salicylic acid (SA) as a defense signal in plants has been well established in tobacco and *Arabidopsis* (Delaney et al., 1994). As an important endogenous signal molecule, SA has been proven to be a major component in signal transduction systems, which can induce particular enzymes catalyzing biosynthetic reactions and is essential for the development of systemic acquired resistance (Van Loon and Antoniw, 1982). In addition to its role in plant pathogenesis (Shulaev et al., 1997), SA is also believed to play role in plant responses to abiotic stresses such as ozone and ultraviolet (UV) light (Yalpani et al., 1994; Sharma et al., 1996; Rao and Davis, 1999), heat (Dat et al., 1998; Senaratna et al., 2000**;** Larkindale and Knight, 2002), chilling, drought stresses (Senaratna et al., 2000), salt and osmotic (Borsani et al., 2001). These studies suggest that while moderate doses of SA enhance the antioxidant status and induce stress resistance, higher concentrations activate a hypersensitive cell death pathway and increase stress sensitivity. Besides, parallel increases in SA and pathogenesis-related proteins have been reported in plants exposed to UV-C light and ozone, which suggests a common signal transduction pathway in plant responses to biotic and abiotic stresses (Yalpani et al., 1994).

Plants use sunlight for photosynthesis and, as a consequence, are exposed to the ultraviolet radiation that is present in sunlight. UV radiation is generally divided into three classes: UV-C, UV-B, and UV-A. The UV-C region of the UV spectrum includes wavelengths below 280 nm; these highly energetic wavelengths are effectively absorbed by ozone in the strato- sphere and, thus, are not present in sunlight at the earth surface. UV-C wavelengths will be removed from the light reaching the earth's surface so long as there is any ozone presents (Caldwell et al 1989).

The aim of this work is to determine the effect of SA treatment on basil plants under UV stress. The present study was aimed at evaluating a putative relationship between mechanisms of photo- and antioxidative protection and SA accumulation in UV stress.

Material and methods

***Plant material*:** Seeds of sweet basil (*Ocimum basilicum* L.) were grown in pots filled with sandy loam soil in the greenhouse at 22/20˚C (day/night), and 16/8 h light/dark photoperiod for 56 days. These pots were irrigated daily by Hoagland solution. The plants were divided into four groups: 1. control plants; 2. plants treated with SA; 3. plants treated with UV-C; 4. plants treated with SA and UV-C. Salicylic acid (1 mM) was sprayed on the leaves to the six leaves stage for 4 successive days. Basil plants were exposed to UV-C radiation after emergence of six developed leaves. UV-C was produced by a UV lamp (250 nm) that providing irradiation dose of approximately 40 Wm^{-2} from 50

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cm distance. Plants were grown for 18 days and exposed to UV-C radiation for 5 $mind^{-1}$, alternatively.

Assay of photosynthetic pigments: Photosynthetic pigments were extracted from leaves in 80 % aqueous acetone and content of chlorophyll a, b, carotenoide were estimated spectrophotomertrically in 662, 645 and 470 nm as described by Lichtenthaler (Lichtenthaler and Wellburn, 1985).

Results

Figure 1 shows the effect of UV-C and SA on photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoide) of leaves in basil. Results indicated that the chlorophyll a and chlorophyll b content were decreased under UV-C stress. Foliar spray of SA in concentration of 1mM alleviated the reduction in chlorophyll a and b content in both UV treated and control plants. Carotenoid content of plants were exposed to UV-C decreased but this decrease was not significant. SA treatment moderated carotenoide reduction.

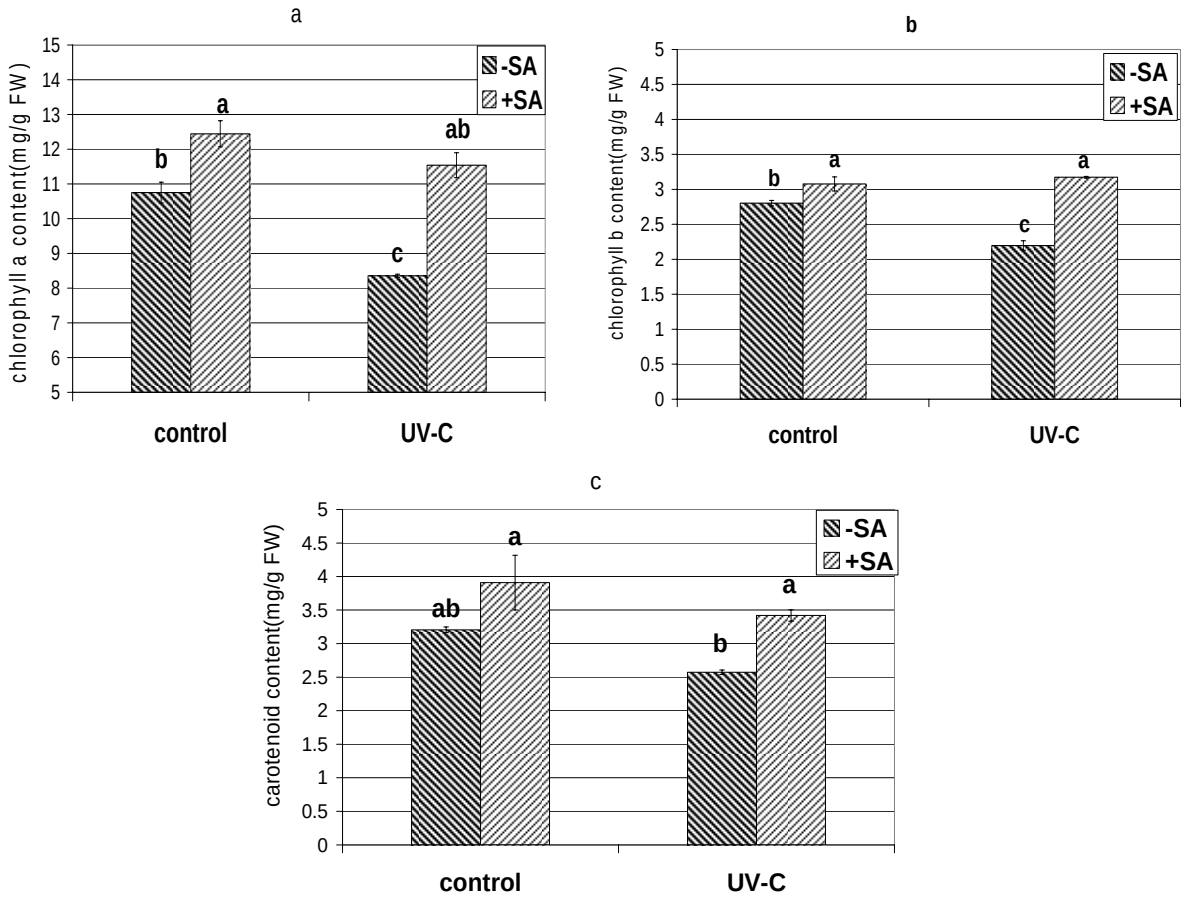

Figure 1: Effects of salicylic acid on the photosynthetic pigments (chlorophyll a (a), chlorophyll b (b) and carotenoide (c)) of basil plants under UV-C radiation. Different superscripts on bars (each duration) showed significant ($P < 0.05$) difference between the means according to Duncan test.

Discussion

Decrease in contents of photosynthetic pigments under UV-C stress is in agreement with findings of Mahdavian *et al.* (2008) in pepper (Mahdavian et al., 2008) and Takeuchi et al. (2002) in rice (Takeuchi et al., 2002). UV light causes multiple effects on the photosynthesis machinery, including loss of plastoquinone, Rubisco and chlorophylls, and degradation of phycobiliproteins in cyanobacteria. Electron transport through PSII is highly sensitive to UV light and the inhibition of electron transport through PSII under UV light is accompanied by lowering of variabled fluorescence chlorophyll *a* and lowering of the intensities of the thermoluminescence Q and B bands (Esa, 2008). UV damages chloroplasts that led to decrease of chlorophyll content (Teramura and Briggs, 1996)

Caldwell et al. (1995) concluded that in sensitive plants, UV-B and UV-C significantly decreased chlorophyll contents, primarily because UV destroyed the structure of chloroplast, inhibited synthesis of chlorophyll and increased the rate of chlorophyll degradation (Caldwell et al. 1995). Rahmatzade and Khara showed that UV-C radiation reduced chlorophyll a, b and carotenoides in wheat plants (Rahmatzadeh and Khara, 2007). Du and Jin (2000) showed that the carotenoids and chlorophyll decreased with treatment of UV-C irradiation (Du and Jin, 2000).

Foliar spray of SA in concentration of 1 mM increased photosynthetic pigments in both UV treated and control plants (Figure 1). It is consistent with the result of Mahdavian et al., 2008 (Takeuchi et al., 2002). Sinha et al. (2003) concluded SA-treated maize plants contained more chlorophyll and carotenoide than the control plants (Sinha et al., 1993). Zhao et al. (1995) reported that photosynthetic pigment contents increased in soybean plants treated with SA (Zhao et al., 1995). According to the results of Ervin et al. (2004) and Janda et al. (1999), foliar application of SA may alleviate the decline in photochemical efficiency and turf quality (Ervin et al., 2004; Janda et al. 1999).

There are not good experimental evidences for the roles played by SA in stability of chlorophylls in plant tissues. However, there is two ways for increasing the level of a certain compound in tissues: promotion of *de novo* synthesis and suppression of degenerative processes. It seems that SA inhibits the synthesis of ROS and also can inactivate them. Removal of ROS can protect chloroplast membranes and may stabilize chlorophylls subsequently. Increasing of photosynthetic pigments in UV- stressed plants in response to SA may be related to the induction of antioxidant responses that protect the plant from damage. Cheng *et al.* (1996) suggested a hypothesis for the *in vivo* antioxidant activity of salicylic acid (Cheng et al., 1996). Moreover SA might serve as a regulator of biogenesis of chloroplasts (Uzunova and Popova, 2000).

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Acknowledgments. This optional part should include a statement thanking those who assisted substantially with work relevant to the study. Grant support should be included in this section.

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Magurran A. E. (1988). Ecological Diversity and its Measurement. Cambridge University Press, 179 pp.

Tothmeresz B. (1995). Comparison of Different Methods for Diversity Ordering. Journal of Vegetation Science, 6: 283-290.

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