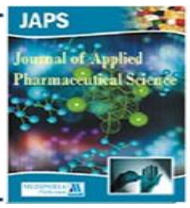
Journal of Applied Pharmaceutical Science
Available online at www.japsonline.com



ISSN: 2231-3354
Received on: 12-07-2012
Revised on: 12-08-2012
Accepted on: 17-08-2011
DOI: 10.7324/JAPS.2012.2828

Micro-morphology of *Achillea clypeolata*: contribution to the pharmacognostical profile

Anely Nedelcheva

Anely Nedelcheva
Department of Botany
Sofia University "St. Kliment Ohridski", Faculty of Biology
blvd. Dragan Tzankov 8, Sofia–1164
Bulgaria

For Correspondence
Anely Nedelcheva
Phone: (+359 2) 8167 /304/
Email: *aneli_nedelcheva@yahoo.com*

ABSTRACT

Achillea clypeolata Sibth. & Sm. is Balkan endemic species, commonly known as yellow yarrow and traditionally used in Bulgarian folk medicine. The purpose of this study was to establish pharmacognostical profile of *A. clypeolata*, including macroscopic and microscopic morphological characteristics. The study also aims to propose a method for micromorphological investigation and pharmacognostical identification. As a result were established morphological characters necessary for identification of Achilleae clypeolatae flos and Achilleae clypeolatae herba – morphology of stem leaves (lanceolate in shape, pinnately lobed-margin, acute-apex with lamina), ligule petals (2.7-3 mm), involucre bracts (4.5x2 mm) and achenes (1.5-1.7 mm); micromorphology of lamina (110-130 µm), epidermal cells, stomata, glandular, non-glandular trichomes and 3-colporate, echinate pollen grains (20.5-20.8 µm). This study presents new micromorphological data. The used staining method dyed various tissues; makes visible the outlines of structures, tissues and some inclusions; allows by one procedure to visualize important diagnostic features; unlike many other methods, allowing pollen to be used as a characteristic of herbal substances. The results are useful for quality control methods for medicinal plant materials and contributes to the implementation of good practices for plant identification for the herbal industry, food, nutrient supplements and other plant based products.

Keywords: Yellow yarrow, Herba, Flos, Pharmacognosy, Balkan endemic, Bulgaria.

INTRODUCTION

Genus *Achillea* L. is one of the most polymorphic genera and difficult for interpretation in the family *Asteraceae* and is object of recent taxonomical comments on the basis of multidisciplinary studies (Ehrendorfer and Guo, 2005). Biosystematic studies of *Achillea* have a tradition of long standing in Bulgarian botany (Kuzmanov, 1984, Nedelcheva, 1998, Saukel *et al.*, 2003). According to the latest data, the genus comprises about 20 (3+17) species of perennial herbs distributed in Bulgaria, belonging to two sections: sect. *Anthemoideae* (DC.) Heimerl s.l. and sect. *Achillea* s.l. [= sect. *Millefolium* (Adanson) Koch s.l., incl. Sect. *Filipendulinae* (DC.) Afan.]. In Bulgaria are known 6 species with yellow flowers, (*A. clypeolata* Sibth. & Sm., *A. coarctata* Poir., *A. pseudopectinata* Janka s.lat., *A. chrysocoma* Friv., *A. thracica* Velen. and *A. ochroleuca* Ehrh.) (Saukel *et al.*, 2003, Nedelcheva and Tzonev, 2006).

Achillea clypeolata is a Balkan endemic species and ranges from North and Central Greece to South Albania, Macedonia, Serbia, Bulgaria, South-east Romania, West and European Turkey. Together with *Achillea aegyptiaca* L. (incl. *Achillea taygetea* Boiss.) they form the dominantly diploid and Balkan centered group of *A. clypeolata*, characterized by morphometric and phytochemical features. *A. clypeolata* is most morphological close to *Achillea coarctata* (Richardson, 1976, Saukel *et al.*, 2003).

Among species with yellow flowers, mostly *A. clypeolata* commonly known as "yellow yarrow" and traditionally used in Bulgarian folk medicine to treat hemorrhoids, wounds, bleeding, gastro-intestinal atony, bed wetting, kidney inflammation, amenorrhoea and inflamed gums, liver deseases. Its medicinal effect is like white yarrow, which is more widespread and better known in folk medicine. Bulgarian folk names: "zhult ravnets", "strumski ravnets", "div pelin", "zhulta eniovitsa", "skalen ravnets", "ramchets", "glavoch" and "gushterka". The large number and variety of folk names showed that the plant is well known and wide used. They are based mainly on its yellow color, shape of the inflorescence, bitter taste like mugwort and rocky habitats (Ahtarov *et al*., 1939; Nedelcheva and Dogan, 2009).

Some of the current research focuses on the pharmacological properties of the yellow yarrow (Konyalioglu and Karamenderes, 2005; Karaalp *et al*., 2009). Significantly more are the data about white yarrow as herbal drug (herbal substance) (Rauchensteiner *et al.*, 2004; Nirmala and Karthiyayini, 2010).

Of central importance in the practice is correct identification of the species concerned, whether in the fresh, dried or powdered state, because one of the criticisms of herbal medicine is lack of standardization and quality control profiles. The macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such materials (identity tests) and should be carried out before any tests to be undertaken (WHO, 1998). Therefore, more nowadays studies are conducted on micromorhology of herbal drugs for their correct identification (Jayeola, 2009; HMPC, 2010; HMPC, 2010a). For *A. clypeolata* there is no pharmacopoeial monograph and in control of starting herbal material (including mother plant identification) should be followed detailed specification of the quality controls (EDQM, 2011; Nikam *et al.*, 2012). The wide use of the yellow yarrow as a herb in Bulgaria, its place in folk medicine and its use today in the herbal remedies, along with limited data as a plant substance justify the purpose of this study: to establish pharmacognostical profile of *A. clypeolata*, including macroscopic and microscopic morphological characteristics. The study also aims to propose a method for micromorphological investigation and pharmacognostical identification of the herbal substances which is informative, fast and with easy laboratory performance as well as applicable in the system of herbal gathering and herb production, preparation and implementation of marketing, quality control of herbal preparations, food, nutrient supplements and other plant based products.

MATERIALS AND METHODS

Preparation of the plant material and samples

Aboveground parts of the plant are used herbal substance: Achilleae clypeolatae flos and Achilleae clypeolatae herba (Nikolov, 2006). The present study is examination of the dry and grounded plant material collected from natural areas and commercial herb samples (product contains dried herbs) (Table 1). Medium representative sampling were separated and processed. Warm water (up to 30ºC) is poured over a certain amount of dried herb, covered, and allowed to steep for 20-40 minutes. For temporary or permanent microscope observation small amount of wet herbal fragments were placed on microscope slides and mixed with 1-2 drops from differential staining mixture and fixed with cover slips. The cover-slips of slides intended for storage were sealed with paraffin wax to prevent excessive evaporation of the mounting medium. The mounts were kept in laboratory oven at 50ºC for 24 hr. Mounts stored for over a 6 months (refrigerator 2-6ºC) showed no loss of staining.

Table 1: List of the specimens used in morphological investigations.

	Natural areas herbal samples	Commercial herbal samples
Achilleae clypeolatae flos	AN/1/645; AN/1/304; AN/1/077; AN/1/532; AN/1/062	Bilki Eood/ 2010 Karmen/2005 Suni Yambol EOOD/2010
Achilleae clypeolatae herba	AN/2/005; AN/2/112; AN/2/452; AN/2/33; AN/2/076	Bilki Eood/ 2010; Karmen Solara /2009

The stain was prepared according to Alexander (1969) from various constituents: malachite green, glycerol, phenol, chloral hydrate, acid fuchsin, orange G, glacial acetic acid, glycerol, 95% alcohol, distilled water. The amount of glacial acetic acid added to mixture is (4 ml to 100 ml stain mixture), because thick-walled and spiny pollen grains as well as 5 mg acid fuchsin additionally with aim to improve contrast (Nedelcheva, 1998). In the staining process malachite green is used for staining cell walls and pollen walls.

Acid fuchsin in the present mixture is included for staining the protoplasm and also colored non aborted pollen grains in red to red-purple. These characteristics gave reason to experiment with this stain solution and on plant tissues, not only to differential staining of aborted and non aborted pollen grains which was the target of Alexander (1969).

For morphological investigations related size measures, twenty plant individuals from each population or samples were studied. Dry herbal substances were analyzed and photographed using the light microscope Olympus BX with digital camera Olympus SP-510UZ.

Voucher specimens was deposited in the personal author collection in Department of Botany and in the Herbarium of University of Sofia (SO).

Study area

Bulgaria (42°41′0″N, 23°19′0″E) is a country in the Balkans in south-eastern Europe. It borders five other countries: Romana to the north (mostly along the River Danube), Serbia and the Republic of Macedonia to the west, and Greece and Turkey to the south. The Black Sea defines the extent of the country to the east. Phytogeographically, Bulgaria straddles the Illyrian and Euxinian provinces of the Circumboreal region.

Bulgarian flora comprises 159 families, 906 genera and 4030 species, 12.8% are endemics (Petrova and Vladimirov, 2010). There are about 770 species of medicinal plants constituting 20% of the Bulgarian flora. Of these, 200 are currently in use and over 250 herbal drugs are derived from them and presently used. The plant is included in the list of medicinal plants in Bulgaria (MOEW, 2000). Achilleae clypeolatae flos is wild collected herbal substance (drug), wich export quantities officially declared by licenses (2001-2005) is range 100-250 kg. Achilleae millefolii flos is more than 36 000 kg (Evstatieva *et al.*, 2007). Both species are not cultivated in Bulgaria.

RESULTS AND DISCUSSION

The structure of the herbal substances was established after weight examination of the plant fragments with different origin. In both of them (flos and herba) is counted prevalence of the involucrum and florets fragments, followed by leaves fragments and with well distinguished glandular and non glandular trichomes as well as pollen grains (Fig. 1).

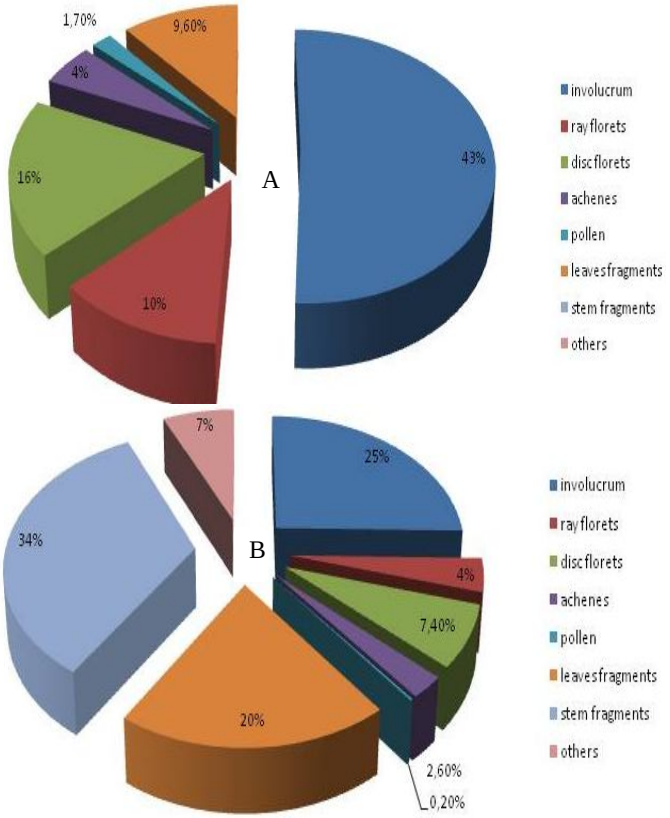


Fig. 1: Structure of the composition of the herbal substances Achilleae clypeolatae flos (**A**) and Achilleae clypeolatae herba (**B**).

Based on this preliminary analysis was performed micromorphological investigation on most involved characteristics with diagnostic value and last but not least susceptible to dying by the proposed method. Inflorescences are yellow, dense, flat-topped and 3-5(7) cm wide corymbs. They are composed of many 50-150(250) flower heads (capitulum) obovate in outline and variable in range 3-4(6) mm long and 2-3(4) mm wide. **Capitulim** is composed of both ray (2-5) and disk (10-20) florets; in the base with conic or convex receptacle, surrounded by imbricate bracts in several series (Fig. 2).

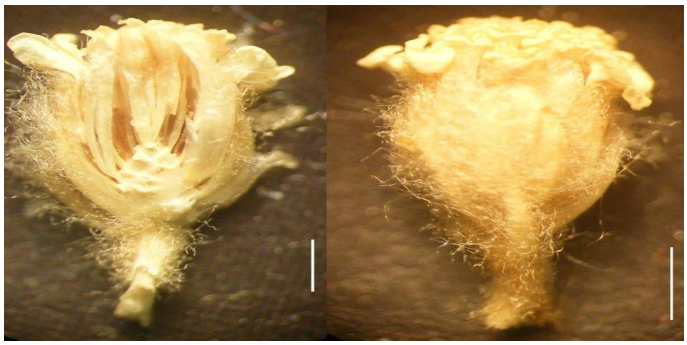


Fig. 2: The morphology and structure of capitulum. Bar = 1mm.

Involucrum is obovate-oblong, imbricate, 2-3(4) mm long and 2-3(4) mm wide, squamous, with narrow transparent membranous margin or without such, yellowish to brownish, outer bracts are shorter and narrower than outline cover bracts (Fig. 3). In the involucrum bracts are visible elongated epidermall cells, on the abaxial surface with ± covering and glandule trichomes similar to those on leaves. Margins of the involucrum bracts are irregular as outline (Fig. 4).

Fig. 3: The morphology of involucrum bracts. Bar = 1mm.

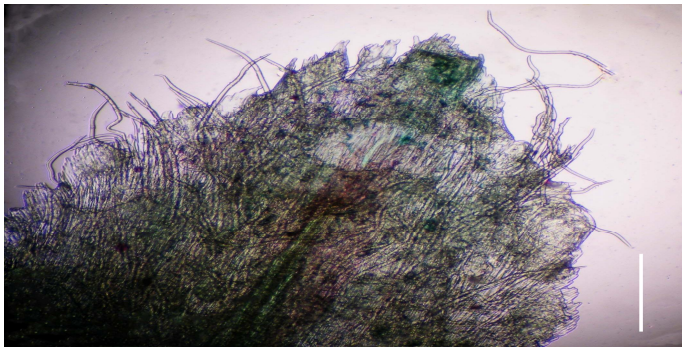


Fig. 4: Fragment of lamina with non-glandular trichomes. Bar = 0.25mm.

Ray florets are 2-4(5) per head, pistillate, fertile; corollas are yellow, lamina is elliptical 0.5-1.0(1.5) x 1.5-2(3) mm. Ligule is deeply tripartite in outline, long to ¼ of the length of involucrum. Ray florets are morphologically variable (Fig. 5). On the outside of the corolla tube are visible glandular trichomes similar to those on leaves. Adaxial epidermal cells of the ligulae are isodiametric, papillose and striated and with elongated abaxial epidermal cells; **Stigma** is 2-lobed, with papillosed surface. Forked stigma lobes are 175-225 µm long (Fig. 6). The yellow color of ray florets is a good diagnostic feature, but in some habitats, in some hybrids can be pale yellow as well as in improperly stored herbal substance (Nedelcheva , 1998).

Fig. 5: The morphology of ray florets. Bar = 1mm.

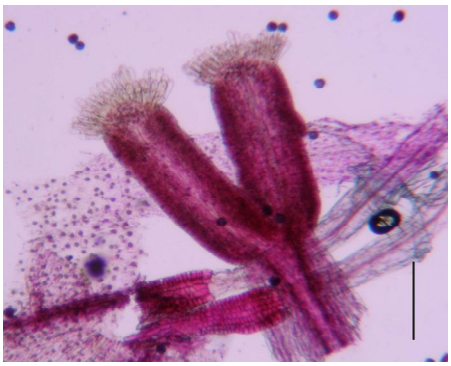


Fig. 6: Stigma (two-lobed) with papillosed surface. Bar = 0.1mm.

Achenes in the herbal substances are not maturated. They are light brownish in color, not well developed white margin and their average length/width ratio is 2. Undeveloped achena is 0.9-1.1 mm long and 0.5 mm wide, obovoid-oblong in shape, translucent, with a ring of sclereids at apex and base, without glandular trichomes (Fig. 7). Taxonomic value of the achene micromorphology and anathomy is well known for *Achillea* species (Akcin and Akcin, 2010).

Glandular trichomes are biseriate and have at full growth a subcuticular large spherical chamber a space coming out when the two layers of the cuticle become detached from the pecto-cellulosic wall (Fahn, 2000) (Fig. 8). Some of the glandular trichomes appear in blue (Fig. 9), blue-green or brownish, whereas any other or missing colour. Proazulenes can be easely detected by simply heating some florets in a mixture of chloralhydrate (60%) and phosphoric acid (85%). During this process proazulenes are transformed to coloured chamazulenes (Saukel, 1993, Rauchensteiner *et al.*, 2004). In this study, the herbal samples were tested for proazulene positive glands and gave the negative result. The missing colour indicates a different composition within the sesquiterpene lactones. These data confirm previous phytochemical reports for sesquiterpene content and there is no info for proasulenes (Ivancheva and Nedelcheva, 1998, Nedelcheva and Ivancheva, 1998, Todorova and Tzankova, 1999, Saukel *et al.*, 2003, Nikolova and Ivancheva, 2006).

Fig. 7: Micromorphology of undeveloped achena. Bar = 1mm.

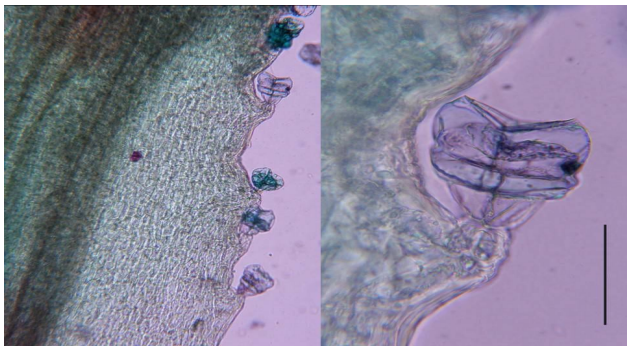


Fig. 8: Glandular trichome (s) in ray florets outline without color. Bar = 50µm

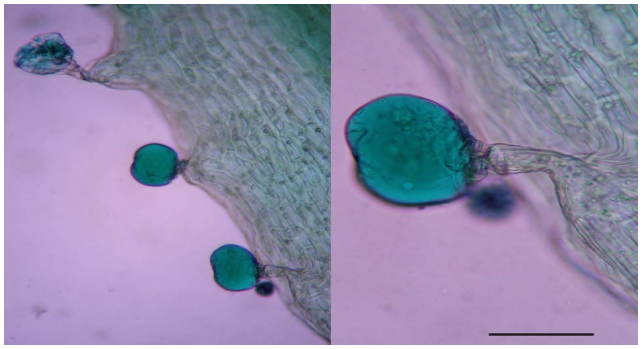


Fig. 9: Glandular trichome (s) in ray florets outline with blue-green color. Bar = 50µm

From **disc florets** are well distinguished elongated epidermal cells and mesophyll with small oxalate druses (Fig.10). In outline have papillose triangular lobes of corolla. Biseriate glandular trichomes are frequent on the outside of the floral tube and rarely on the lobes. Connate **anthers** contain pollen into the interior of the anther tube. Anther sac is filled with fertile pollen grains. In Fig. 11 are visible pollenkitt fragments (oily grains in yellow) in which the pollen grains are suspended during the period from anther opening (Pacini and Hesse, 2005).

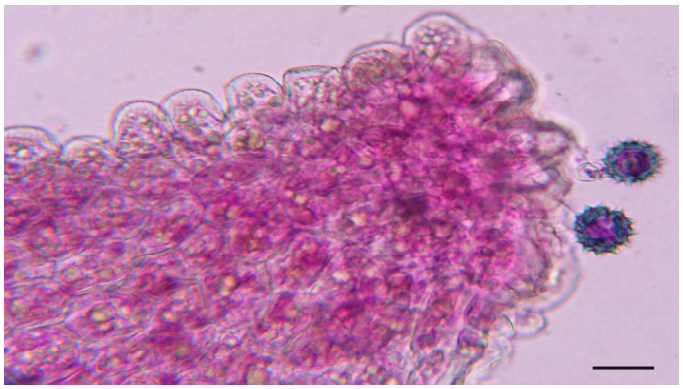


Fig. 10: Micromorphology of disc florets. Bar = 50µm.

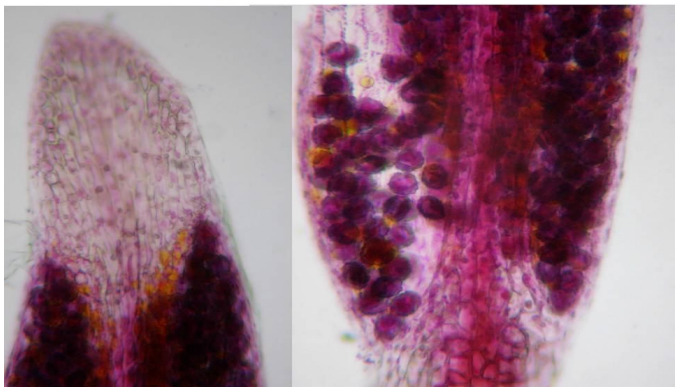


Fig. 11: Stained pollen grains in nondehiscent anthers with an oily, sticky materal (pollenkitt). Bar = 50µm

After applied staining fertille and sterille **pollen grains** are well distinguished. The aborted pollen is stained blue (blue-green) and the non aborted pollen grain is red to red-purple, because of protoplasm (Fig. 12). In both cases the pollen wall is well visible. Pollen grains are tricolporate sheroidal with spiny exine, and 18-22 µm in diameter (not include spines). According to Nedelcheva (1998), this pollen size meets the measured size of pollen prepared by acetolysis method of Erdtman (1960). Presented results showed the significance of the pollen morphology in herbal substance identification.

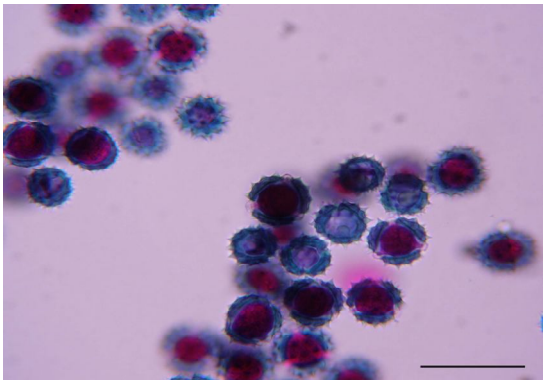


Fig. 12: Aborted and nonaborted pollen grains. Bar = 50µm.

In the studied samples participation of leaves is limited which is according with general morphology of *A. clypeolata*. The basal leaves give the main biomass of leaves, but they are not in the composition of the both herbal substances: flos and herba. The stem leaves are up to 4-7 on the flowering stem, oblong to lanceolate in shape, pinnately lobed to pectinate, 3-5(10) cm in length and 1-1.5(2) cm in width, grey-green in color. When hang drying the aerial parts the stem leaves are often scattered and found in very small quantities in the samples. In contrast is Achilleae millefolii herba, where the leaves are significant part in the final drug, and therefore the leaves are subject to more detailed studies (Nirmala and Karthiyayini, 2010). Non-glandular trichomes are also frequently occurring in the epidermal cells of the leaves and stem.

The trichomes are uniseriate, of varying length, commonly with swollen basal cells or seated on pedestals of several epidermal cells (Fig. 13). It is 650 µm in length. Swollen basal cell is 30 µm in diameter and tip of the trichome is 15 µm in diameter. Epidermal cells are lobed and amoeboid in outline. The stoma is consisting of an elliptical pore in the epidermis and surrounded by two specialized, kidney-shaped epidermal cells (Fig. 14). These data confirm previous reports for *Achillea* trichomes morphology (Nedelcheva, 1998, Nirmala and Karthiyayini, 2010).

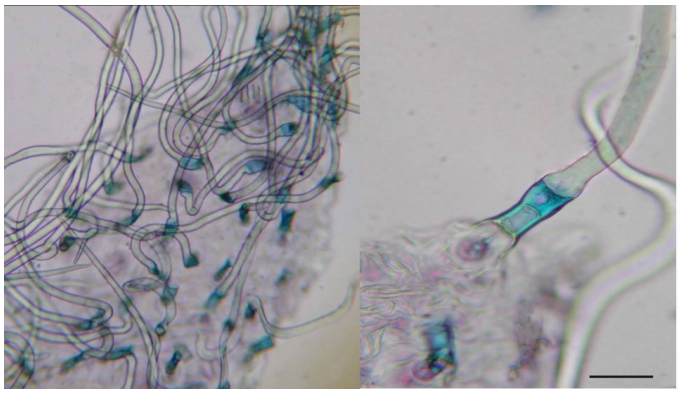


Fig. 13: Non-glandular trichomes on the surface view. Bar = 50µm

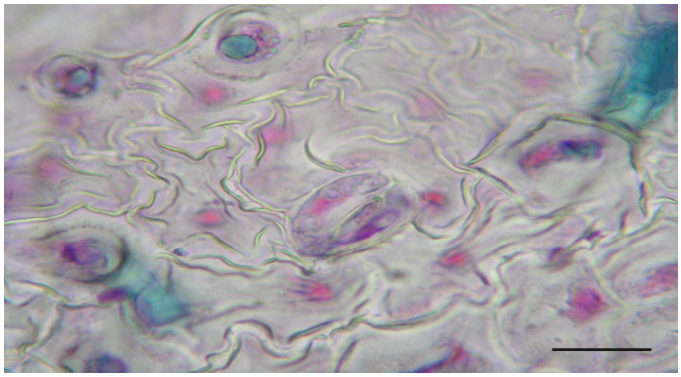


Fig. 14: Fragment of abaxial epidermis with stomata. Bar = 50µm

CONCLUSION

Determination of the diagnostic characters (macro- and micro morphological) is the referential information for the correct identification of the herbal substances. This study presents new micromorphological data about Balkan endemic *A. clypeolata* and is contribution to pharmacognostical profile of yellow yarrow based herbal substances. The used staining method does not require

complicated preparation of herbal samples for testing. By contrast it is dyed various tissues; makes visible the outlines of structures, tissues and some inclusions; allows by one procedure to visualize important diagnostic features; unlike many other methods, allowing pollen to be used as a characteristic of herbal substances. The present paper describes a new utilization of a staining technique originated by Alexander (1969) and modified for this study. It makes visible the outlines of structures, tissues and some inclusions; allows by one procedure to visualize important diagnostic features; unlike many other methods, allowing pollen to be used as a characteristic of herbal substances. Applied staining method is firstly used as micromorphological and pharmacognostical approach.

The present study is a contribution to the pharmacognostical description of the *A. clypeolata*, quality control methods for medicinal plant materials and contributes to the implementation of good practices for plant identification for the herbal industry, quality control of herbal preparations, food, nutrient supplements and other plant based products.

REFERENCES

Ahtarov B., Davidov B. and Javashev A. Materials for Bulgarian Botanical Glossary. Bulgarian Academy of Sciences, Pridvorna Pechatnitza, Sofia (1939)

Akcin T.A. and Akcin A. Morphological and anatomical characteristics and taxonomical significance of achene micromorphology of *Achillea phrygia* and *A. gypsicola* (Asteraceae), endemic to Turkey. Nord J Bot. 2010; 28(1): 65-73

Alexander M.P. Differential staining of aborted and nonaborted pollen. Stain Technol. 1969; 44(3): 117-122

EDQM, European Pharmacopoeia - 7th Edition. European Directorate for the Quality of Medicines & HealthCare, Strasbourg, 2011, from the database of European Directorate for the Quality of Medicines & HealthCare (EDQM) (http://www.edqm.eu/en/European-Pharmacopoeia-1401.html)

Ehrendorfer F. and Guo Y.-P. Changes in the circumscription of the genus *Achillea* (*Compositae-Anthemideae*) and its subdivision. Willdenowia. 2005; 35: 49-54

Erdtman G. The acetolysis method, revised description. Svensk Bot Tidskr. 1960; 54: 561-564

Evstatieva L., Hardalova R. and Stoyanova K. Medicinal plants in Bulgaria: diversity, legislation, conservation and trade. Phytol Balc. 2007; 13(3): 415-427

Fahn A. Structure and function of secretory cells, In: Hallahon D.L., Gray J.C. (Eds.) Advances in botanical research. Incorporating advances in plant pathology. Plant trichomes. Academic Press, London (2000)

HMPC, List of references supporting the assessment of *Achillea millefolium* L., herba, European Medicines Agency, Committee on Herbal Medicinal Products (HMPC), EMA/HMPC/290282/2009, 2010, from the EMEA database (http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_List_of_references_supporting_the_assessment_report/2011/09/WC500115472.pdf)

HMPC, List of references supporting the assessment of *Achillea millefolium* L., flos. European Medicines Agency, Committee on Herbal Medicinal Products (HMPC), EMA/HMPC/ 149469/2010, 2010a, from the EMEA database (http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_List_of_references_supporting_the_assessment_report/2011/09/WC500115471.pdf)

Ivancheva S. and Nedelcheva A. (1998) Exudate flavonoid aglycones of *Achillea* species from sect. *Millefolium* and sect. *Filipendulinae* - Comparative study. In N. Randelovic (Ed.) Zbornik Radova V Symposium o Flori Jugoistočne Srbije (pp. 183-189). Niš: Pergament

Jayeola A.A. Micromorphological study of plant fragments in some powdered medicinal plants. J Med Plants Res. 2009; 3(5): 438-442

Karaalp C., Yurtman A.N. and Karabay Yavasoglu N.U. Evaluation of antimicrobial properties of *Achillea* L. flower head extracts. Pharm Biol. 2009; 47(1): 86-91

Konyalioglu S. and Karamenderes C. The protective effects of *Achillea* L. species native in Turkey against H_2O_2-induced oxidative damage in human erythrocytes and leucocytes. J Ethnopharmacol. 2005; 102(2): 221-227

Kuzmanov B., Taxonomy and Evolution of the Bulgarian Compositae. Doctor thesis, Bulgarian Academy of Sciences, Sofia, Bulgaria (1984) (in Bulgarian)

MOEW, The Ministry of Environment & Water **(**MOEW), Bulgaria. Law of the medicinal plants. DW/29-2000, 2000, from the database of the Bulgarian Biodiversity Foundation (http://www.biodiversity.bg/files/File/zak_bg_lech-rast.pdf).

Nedelcheva A. and Ivancheva S. Quantitative study of flavonoids content in species from genus *Achillea* L. sect. *Filipendulinae* (DC.) Afan. (Asteraceae). Ann Sofia Univ Fac Biol. 1998; 88(4): 101-106

Nedelcheva A. and Tzonev R. *Achillea ochroleuca* (Asteraceae): a new species for the Bulgarian flora. Phytol Balc. 2006; 12(3): 371-376

Nedelcheva A. Biosystematic investigations of genus *Achillea* L., sect. *Filipendulinae* (DC) Afan. (Asteraceae) in Bulgaria PhD thesis. Sofia University, Sofia, Bulgaria (1998) (in Bulgarian)

Nedelcheva A.M., Dogan Y. (2009) Folk Botanical Nomenclature and Classification in Bulgarian Traditional Knowledge. In J.-P Morel. and A.M. Mercuri (Eds.) Plants and Culture: seeds of the cultural heritage of Europe. Bari: Edipuglia

Nikam P.H., Kareparamban J., Jandav A. and Kadam V. Future Trends in Standardization of Herbal Drugs. JAPS. 2012; 02(06): 38-44

Nikolov S. Specializirana enciclopedija na lechebnite rastenija v Bulgaria. Trud, Sofia (2006)

Nikolova M. and Ivancheva S. Distribution of surface flavonoids in Bulgarian plants. Nat Prod Commun. 2006; 1(11): 1029-1035

Nirmala S. and Karthiyayini T. Pharmacognostical Studies on leaves of *Achillea millefolium* L. J Pharm Res. 2010; 3(5): 1114-1118

Pacini E and, Hesse M., Pollenkitt - its composition, forms and functions. Flora. 2005; 200(5): 399-415

Petrova A. and Vladimirov V. Balkan endemics in the Bulgarian flora. Phytol Balc. 2010; 16(2): 293-311

Rauchensteiner F., Nejati Sh. and Saukel J. The *Achillea millefolium* group (Asteraceae) in Middle Europe and the Balkans: a diverse source for the crude drug Herba Millefolii. J Trad Med. 2004; 21: 113-119

Richardson I.B. (1976). *Achillea* L. In T.G. Tutin et al. (Eds.) Flora Europaea. Cambridge: Univ Press Cambridge.

Saukel J., Anchev M., Guo Y.-P., Vitkova A., Nedelcheva A., Goranova V., Konakchiev A., Lambrou M., Nejati S., Rauchensteiner F. and Ehrendorfer F. Comments on the Biosystematics of *Achillea* (*Asteraceae/Anthemidae*) in Bulgaria. Phytol Balc. 2003; 9(3): 361-400

Saukel J., Schnellnachweis von Proazulenen und anderen sesquiterpenen in mikroskopischen Präparaten. Sci Pharm. 1993; 61: 59-63

Todorova M. and Tzankova E. New sesquiterpenoids from *Achillea clypeolata*. Phytochemistry. 1999; 52: 1515-1518

WHO, Quality Control Methods for Medicinal Plant materials, World Health Organization, Geneva, 1998, from the WHO database (http://whqlibdoc.who.int/publications/1998/9241545100.pdf)