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Diagnostic features of raw materials of related *Equisetum* species of Ukrainian flora

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ABSTRACT: A comparative morphological and anatomical study of six *Equisetum* species (*E. arvense* L., E. *fluviatile* L., *E. palustre* L., *E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh.) was carried out using light and scanning electron microscopy to investigate the species-specific diagnostic features of their raw organs for distinguishing between the taxa. The main diagnostic features that allow identifying the belonging of whole (uncrushed) raw materials of *Equisetum* are the presence/absence of spore-bearing organs and spores, the number of branches ribs, the ratio of the length of the branch internode to leaf whorl of stem and the ratio of the central cavity diameter of the stem to the stem diameter. The ultrastructure of the surface of stems and branches of model species contains a number of diagnostic features, which together allow determining the species affiliation of raw materials correctly. The most significant diagnostic characteristics of *Equisetum* species are cuticle structure, location, and shape of mamillae and papillae, presence and localization of stomata, features of silica warts on stomata subsidiary cells, siliceous outgrowths on the ribs top (ridge) of stem and branches. The linear dimensions of epidermal cells, stomata and silica structures, the size of the ribs and the central cavity of the stem have secondary importance for the identification of individual species.

KEYWORDS: *Equisetum*, raw materials, diagnostic features, papillae, mamillae, silica warts.

1. INTRODUCTION

The genus *Equisetum* L. is represented in the flora of Ukraine by 9 species belonging to two subgenera *Equisetum* and *Hippochaete* (Milde) Baker. [1] and all of them contain biologically active compounds with significant resource potential in Ukraine. According to the data of the State Pharmacopoeia of Ukraine [2], only the herbal horsetail (*E. arvense* L.) is approved for use in official medicine. Raw materials of other horsetail-like species are used in traditional medicine [3]. Morphologically and anatomically similar to *Equisetum arvense* are 5 species: *E. fluviatile* L., *E. palustre* L., *E. pratense* Ehrh., *E. sylvaticum* L. and *E. telmateia* Ehrh. The raw materials of these species may be mistakenly included in the raw materials of horsetail.

The species of the horsetail family can be easily distinguished on whole plants, but it is more difficult to determine the species affiliation of raw materials. The macromorphological characteristics of *Equisetum* species and hybrids are described in floristic summaries and monographs [4]. Recently, increasing attention has been paid to the study of the morphological and anatomical characteristics of *Equisetum* species for the identification of medicinal raw materials [5,6].

This paper presents a comparative macromorphological, anatomical and micromorphological study of diagnostic characters of six common Ukrainian *Equisetum* species.

2. RESULTS AND DISCUSSION

After analyzing the results of previous studies and conducting a series of our own morphological and anatomical studies on model species of Equisetum, a number of macromorphological and micromorphological characteristics were identified that can be used to identify the raw materials of each species. These characteristics are presented comparatively below.

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The presence of morphologically distinct dimorphic shoots is characteristic of the species *E. arvense*, *E. telmateia*, *E. pratense*, and *E. sylvaticum*. In *E. arvense* and *E. telmateia*, the chlorophyll-free, spore-bearing shoot develops in early spring and dies after sporulation, after which a vegetative, photosynthetic shoot develops that is used as a medicinal raw material. In *E. pratense* and *E. sylvaticum*, a photosynthetic, spore-bearing shoot develops in early spring and branches during sporulation, leaving a dry strobil at the tip or dies. *E. fluviatile* and *E. palustre* are characterized by monomorphic shoots; vegetative and spore-bearing shoots differ only in the presence of a strobil at the tip of the shoot and sometimes the upper branches.

The main diagnostic features that allow identifying the species of whole (uncrushed) raw materials of *Equisetum* species are the presence/absence of spore-bearing organs and spores, the number of branch ribs, and the ratio of the length of the first internode of the branch and the corresponding leaf whorl (leaf cluster) of the stem. As the officially accepted medicinal raw material is summer green shoots of *E. arvense*, the presence of strobile fragments and spores in the raw material indicates that it may be another species. The number of branch ribs in *E. arvense* is mainly 4 (rarely 3, 5), in *E. pratense* – 3 (rarely 4, 5), in *E. palustre* 4-7, and in *E. fluviatile* – 4-11; the lateral branches of the first-order of *E. telmateia* and *E. sylvaticum* have 4-5 ribs each (Figures 1a-1f), in addition, *E. sylvaticum* has 3-faceted branches of the second-order (ramulus). In *E. arvense* the first internode of the branch of the lowest node is expressed longer than the corresponding leaf whorl of the branch (Figure 1g). The first internode of the *E. palustre* branch is shorter than the corresponding leaf whorl of the stem in all nodes (Figure 1i). They are approximately the same length in other species (Figures 1h, 1j, 1k, 1l).

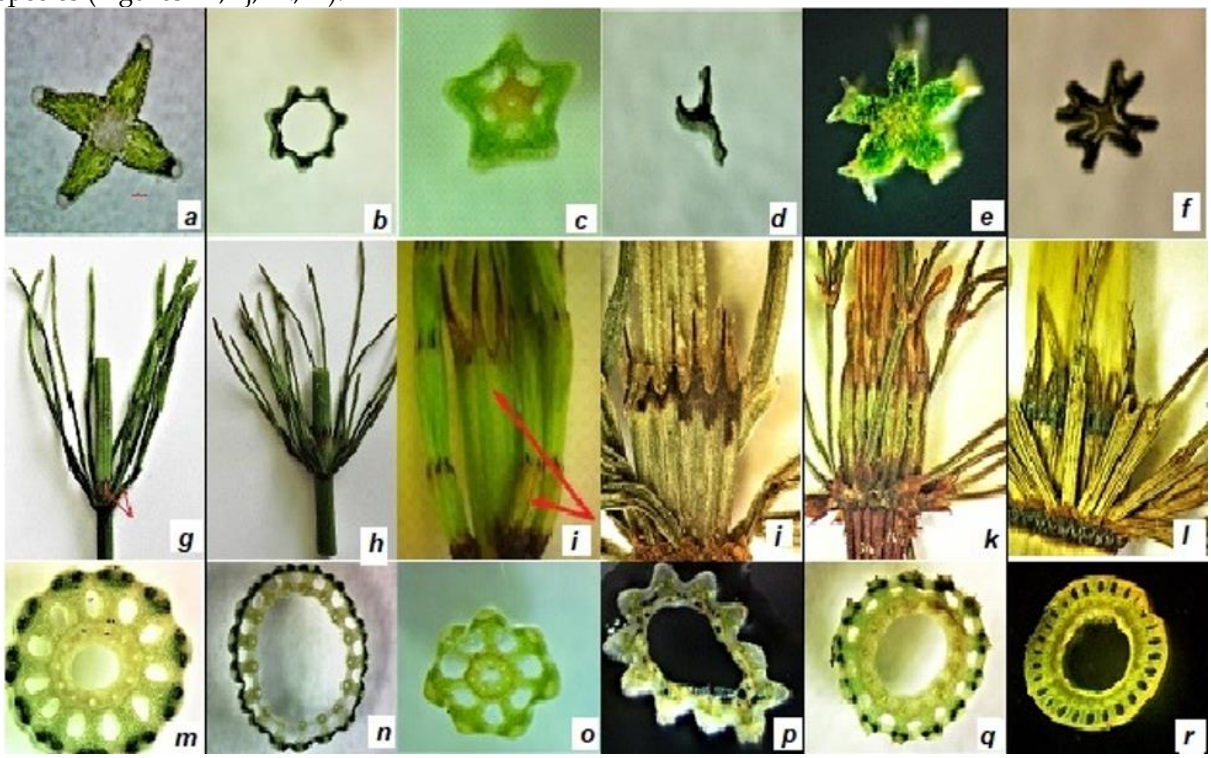

Figure 1. Diagnostic features of raw material of *Equisetum* species: a-f – branch; g-l – node; m-s – a cross-section of the stem; a, g, m – *E. arvense*; b, h, n – *E. fluviatile*; c, i, o – *E. palustre*; d, j, p – *E. pratense*; e, k, q – *E. sylvaticum*; f, l, r – *E. telmateia*.

Important diagnostic features of the species affiliation of raw materials of studied horsetail species are the size of the stem central cavity and surface structure (including epidermal cell size, cuticle structure, location and morphology of stomata, features of structure and location of silica warts).

In *E. arvense*, *E. palustre*, *E. pratense* and *E. sylvaticum*, the diameter of the main stem in the middle part of the plant varies mainly within 2-3 mm, rarely 4-5 mm (except *E. pratense*), while the diameter of the stem in *E. fluviatile* and *E. telmateia is* 4-8 mm and 5-12 mm, respectively. This characteristic is not significant among the diagnostic features of the raw material because the range of variations in stem diameter as well as the branch diameter may coincide or overlap in different species.

The stems of all the studied species in the cross-section are characterized by a uniformly ribbed or wavy contour with a central cavity of different sizes (Figures 1m-1r). The height of the stem ribs is a species-specific characteristic and the stem ribs are higher in *E. pratense* (Figure 1p). The number of ribs of the main

stem is not a stable feature, this indicator is more important for branches, while the ratio of the diameter of the central stem cavity to the diameter of the stem is an important diagnostic feature [7]. In our study this ratio is 0.26-0.36 for *E. arvense*, similar to *E. palustre* (0.26-0.3); in *E. pratense* – 0.43-0.54, *E. sylvaticum* – 0.44-0.62, *E. telmateia* – 0.37-0.69, and *E. fluviatile* – 0.68-0.71. *E. pratense* (1: 1.2-2.5) and *E. palustre* (1: 1.8-2.6) are more similar in the ratio of the branch diameter to the stem diameter. This feature distinguishes the raw materials of *E. arvense* (1: 1.5-3.4) from *E. telmateia* (1: 5.1-13.2) and *E. fluviatile* (1: 3.3-5.4). This feature overlaps on the lower or upper limit for other species, so it is less diagnostic for identifying the raw materials.

The structure of branches epidermis of *Equisetum* species, including the presence and location of stomata, is an important diagnostic species-specific feature and can be used to identify the species affiliation of horsetail raw materials. The epidermis cells of stem internodes of all studied species have rectangular elongated form, the anticline cells walls are straight or slightly wavy, unevenly thickened; moreover, the epidermal cells are longer on the facets than in furrows. The longest epidermal cells were found in *E. palustre* and *E. sylvaticum*, and the thickest cell membranes – in *E. palustre* and *E. fluviatile* (Table 1).

Table 1. The size of the epidermal cells of the stem of *Equisetum* species.

	Length, µм	**Width, µм**	**Anticline wall thickness, µм**
E. arvense	79-218	18-32	3-6
E. fluviatile	50-224	16-33	3-11
E. palustre	99- 302	25-41	5-13
E. pratense	88-185	12-28	2-7
E. sylvaticum	99-269	16-32	2-9
E. telmateia	96-231	17-28	4-9

The epidermis surface of stems and branches of studied species is markedly cutinized, but the structure of the cuticle has some specific features. Epicuticular wax is represented mainly by multidirectional vertically arranged plates, which densely cover the surface of ribs of stems and branches in *E. arvense* (Figures 2a-2b), and in furrows, the cuticle can be smooth (Figure 2c). The high density of small wax plates was found mainly on the branch's ribs of *E. fluviatile*, *E. palustre*, *E. pratense* (Figures 2d, 2e, 2f). The stems surface of these species is mostly covered with a smooth (Figures 2g, 2h, 2i) or slightly layered cuticle. The cuticle of *E. sylvaticum* stem differs from the previous species by the presence of flat wax plates with raised edges (Figure 2j), while its branch's surface is abundantly covered with vertically placed small (0.4–0.6 µm) wax plates, as in the previous species. The cuticle of *E. telmateia* stems is mostly smooth with numerous large granules of wax near the mamilla (Figure 2k), and the cuticle of the branches is similar to the previous species (Figure 2l).

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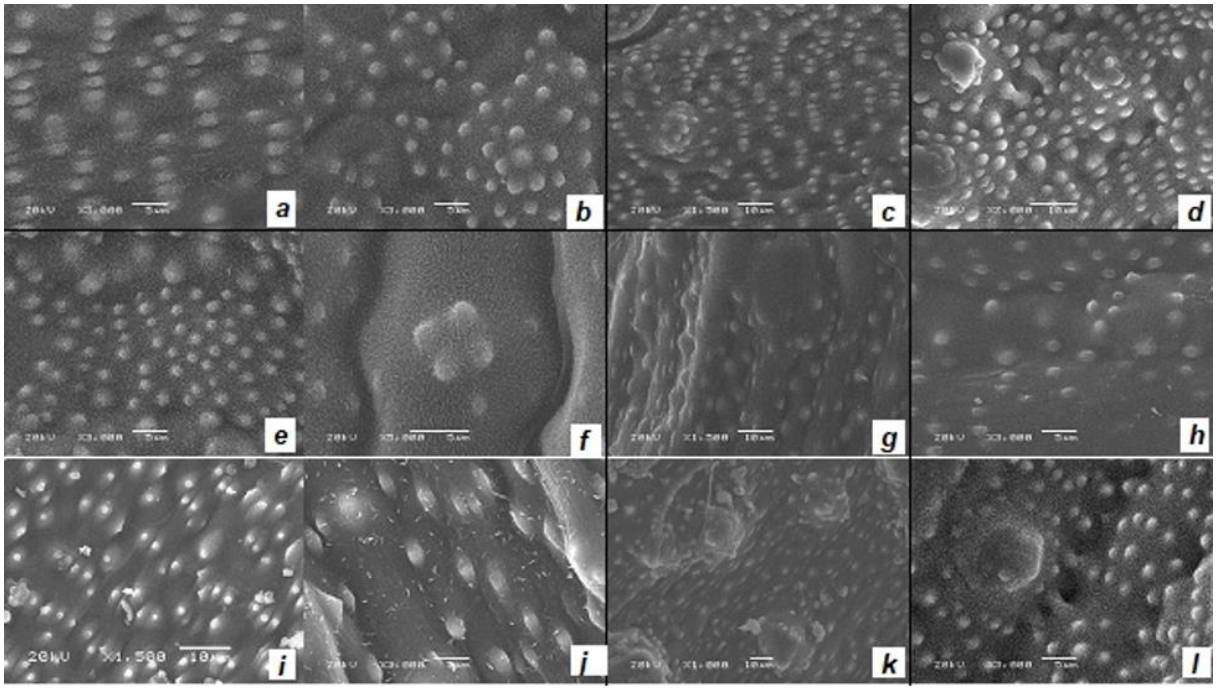

Figure 2. The cuticle structure of *Equisetum* species: a – stem surface of *E. arvense,* b-c – *E. arvense* branch, d – *E. fluviatile* branch, *e* – *E. palustre* branch, f – *E. pratense* branch, g – *E. fluviatile* stem, h –*E. palustre* stem, i – *E. pratense* stem, j – *E. sylvaticum* stem, k– *E. telmateia* stem, l – *E. telmateia* branch

The most prominent features of the ultrastructure of the surface of stems and branches of studied *Equisetum* species are stomata, small silica papillae and aggregated papillae (mamillae). The names of silica structures on the surface of the horsetail epidermis are characterized by considerable variety. They are often named tubercles, silica nobs, nodules, pustules, spicules, papilla, mamilla, pilulae, warts or are differentiated into microtubercles, mesotubercles and megatubercles [8]. Describing the surface structure of model species, we use the term "silica papillae" or "papillae" for small siliceous outgrowths and "mamillae" – for large papillae aggregations. Papillae usually have a dome-shaped apex 1.7–5 μm wide; abundantly cover the facets, the top of ribs and furrows. They can be located individually or grouped in several horizontal or diagonal lines or arranged in axial rows (Figure 3). Papillae are usually more distinct on the subsidiary cells of the stomata, they are smaller and are located densely than on other epidermal cells. The structure and location of papillae are similar in the studied species and do not play a significant diagnostic role.

Mamillae are cylindrical or conical structures 12-17 μm in diameter, more or less abundantly covered with small silica papillae, sometimes with a smoothed apex. Their structure and location on surfaces of stems and branches are species-specific. The mamillae are scattered on the facets and in the furrows of *E. arvense* stems (Figure 3a), they form dense axial rows on the branches (Figure 3b). They occasionally merge 3-8 into transverse colliculus on the stem and more often on the surface of *E. fluviatile* branches (Figures 3c, 3d). The same flat-topped colliculus was found on *E. palustre* branches (Figure 3e). The mamillae form dense axial rows in the furrows and are scattered between the stomata on *E. pratense* branches (Figure 3f). The mamillae and papillae are merged into longitudinal smooth strands on *E. sylvaticum* stem (Figure 3g). These strands are much narrower than on *E. palustre* and *E. pratense* branches. The mamillae form dense axial rows on *E. sylvaticum* branches, same as on *E. pratense* branches (Figure 3h). The stem surface of *E. telmateia* is abundantly dotted with silica papillae arranged in the axial rows. There are also numerous mamillae with a flat top, which form convex silica structures of round or indeterminate shape (Figure 3i). Mamillae on *E. telmateia* branches are located in the correct order in the furrows along the lines of the stomata and occasionally between the stomata (Figure 3j).

Silica outgrowths on the ribs top (ridge) of horsetails stem and branches also have a diagnostic role. They have a nodular-serrated shape on the ribs of *E. arvense* branches (Figure 3k), or serrated shape in *E. telmateia* (Figure 3j). These outgrowths are formed by transverse colliculus with a flat surface in *E. fluviatile*, *E. palustre*, and *E. pratense*, (Figures. 3l, 3m, 3n). The top of the ribs of *E. sylvaticum* branches is smooth (Figure 3o), and there are finger-shaped outgrowths, arranged in 2-3 rows along the top of stem ribs (Figure 3p).

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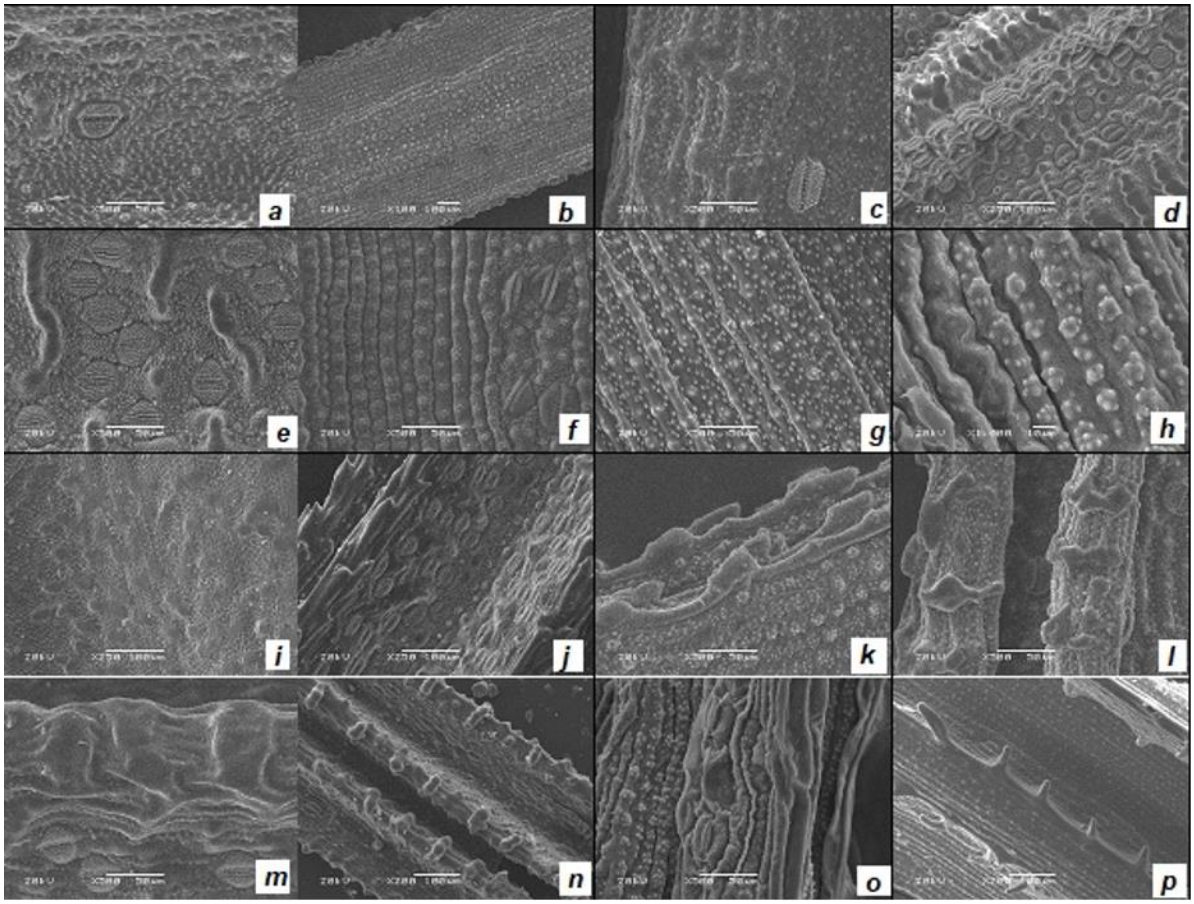

Figure 3. The silica structures on the stems and branches of species of *Equisetum* genus: papillae and mamillae on the surface of stem (a, c, g, i) and branches (b, d, e. f, h, j): a, b – *Equisetum arvense*, c, d – *E. fluviatile*, e – *E. palustre*, f – *E. pratense*, g, h – *E. sylvaticum*, i, j – *E. telmateia*; silicon outgrowths on the ribs ridge of branches (k-o) and stem (p): k – *E. arvense*, l – *E. fluviatile*, m – *E. palustre*, n – *E. pratense*, o, p – *E. sylvaticum*

The stomatal apparatus of *Equisetum* species is taxonomically significant in distinguishing the two subgenera [9]. The stomata are paracitic with two subsidiary cells in all studied species. These subsidiary cells differ significantly from other epidermal cells in their structure, because their formation involves not only guard stomatal cells but also subsidiary epidermal cells, which are superimposed on top of the guard cells. Subsidiary cells are characterized by dense silicified thickenings of the outer membrane and radial ridge-like thickenings on the inner tangential cell walls, which close the ostiole from above (Figures 4a-4c). It is believed that these are cellulose membrane outgrowths covered with silica [10]. In our study, the longest ridge-like silica membrane thickenings of subsidiary cells were found in the stomata of *E. palustre* stem (Figure 4c). A distinctive feature of the stomata subsidiary cells of *E. pratense* stem is the uneven silica thickening of the membrane, which increases in the direction of the ostiole (Figure 4d) and as in *E. arvense* (Figures 4a, 4f), *E. fluviatile* (Figure 4b, 4g) and *E. sylvaticum* (Figure 4e) the row of densely spaced papillae along the perimeter of the guard cells is absent.

The two stomata guard cells also form an internal protrusion and thickening on their walls, but they are not usually visible under the subsidiary cells. Therefore, the stomata of horsetails are double-closed, which is an important adaptation for maintaining internal moisture. The stomata on the branches are similar to those on the axial stem, although generally, they are slightly smaller. Silica outgrowths of *E. sylvaticum* branches form a dense row around the perimeter of the subsidiary cells with randomly placed small papillae in the middle, and they form a wavy roller, clearly raised above the surface of the subsidiary cells on the ostiole side (Figure 4j). The stomata on *E. telmateia* stem are almost absent, but they abundantly cover the facets and furrows of branches in 8-12 rows (Figure 4k), which has an important diagnostic role. Stomata of the branches have several rows of elongated tightly closed papillae on subsidiary cells walls along the ostiole (Figure 4l).

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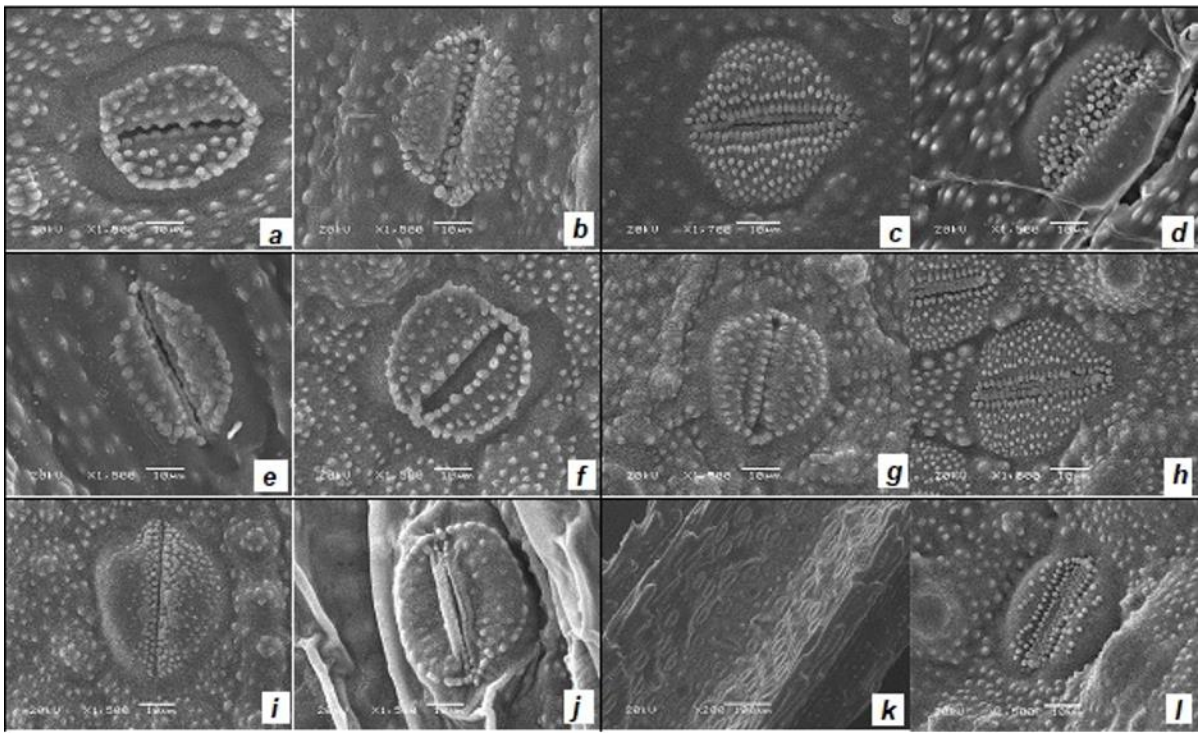

Figure 4. The stomata structure of stem (a-e) and branches (f-l) of *Equisetum* species: a, f – *E. arvense*; b, g – *E. fluviatile*; c, h – *E. palustre*; d, i – *E. pratense*; e, j – *E. sylvaticum*, k, l – *E. telmateia.*

The sizes of mature stomata of the studied horsetail species are characterized by insignificant variability and may overlap in a certain range (Table 2). They are smaller on branches than on stems.

Table 2. Linear dimensions of stomata on the stem of *Equisetum* species.

	E. arvense	*E. fluviatile*	*E. palustre*	*E. pratense*	*E. sylvaticum*
Lengt, μм	46-82	42-73	51-79	45-75	68-99
Width, μм	40-71	32-47	36-55	30-50	41-69

The stomata of horsetails are unidirectional, they are placed in longitudinal rows between the ridges (in the furrows and at the bottom of ribs facets). The stomata are present on the axial stem and branches in most of the studied species, except for *E. telmateia*. The number of stomata rows and their localization on the stem is an important species-specific feature of *Equisetum* species. On the stems and lateral branches of studied *E. arvense* specimens' stomata are located in the furrows and the lower part of the rib's facets in form of 3-4 indistinct lines (Figures 5a, 5f); there are 8-12 scattered rows of stomata on the stem and branches of *E. fluviatile* (Figures 5b, 5g); and 5-6 (10) rows – in *E. palustre* (Figure 5c). Moreover, lines of stomata on the branches intersect with transverse rollers of different lengths of mamillae, abundantly covered with small silica papillae (Figure 5h). The stomata are located scattered on the facets and occasionally in the furrows of *E. pratense* stem (Figure 5d), on ribs of branches facets they are densely arranged in lines in 1-2 rows mostly (Figure 5i). *E. sylvaticum* stomata are placed on the stem in one or two rows on the rib's facets, and on branches – in one clear row (Figures 5e, 5j).

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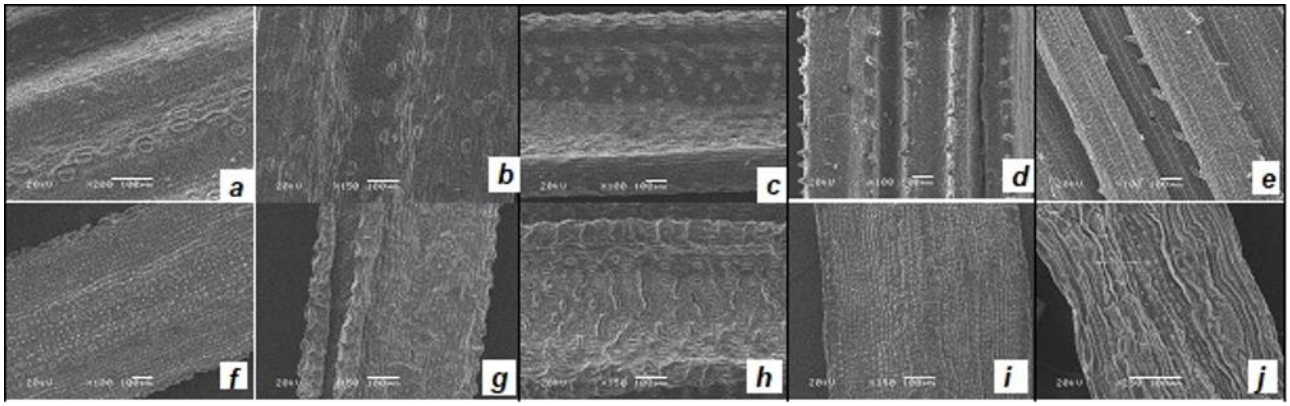

Figure 5. Stomata placement on the surface of the stem (a-e) and branches (f-j) of *Equisetum* species: a, f – *E. arvense*; b, g – *E. fluviatile*; c, h – *E. palustre*; d, i –*E. pratense*; e, j – *E. sylvaticum*.

We have developed the diagnostic key to differentiate the raw materials of the studied species (Table 3).

Table 3. The diagnostic key for differentiation of the raw materials of the studied species.

1	The branches do not branch…….....**2**
	The branches are branched twice or thrice…….**6**
2	Ascending branches directed upwards……..……..**3**
	Horizontal branches or arched downward……**5**
3	The first internode of the branch of the lowest node is shorter than or equal to the appropriate leaf sheath of stem……..……..…..**4**
	The first internode of the branch of the lowest node is longer than the appropriate leaf sheath of stem ……...***E. arvense***
4	The number of stem ribs is 14-20 (30), the ratio of the central cavity diameter of the stem to the stem diameter is 0.68-0.71: 1, the stomata are placed on the facets and in the furrows in 8-12 rows, on the outer periclinal walls of the subsidiary cells of the stomata the papillae are scattered, along the stomatal aperture are organized in 1 row ……..……..…….....***E. fluviatile***
	The number of stem ribs is 5-8 (10), the ratio of the central cavity diameter of the stem to the stem diameter is 0.26-0.3: 1, the stomata are placed on the facets in 5-6 rows, on the outer periclinal walls of the subsidiary cells of the stomata the papillae are densely arranged, along the stomatal aperture are club-shaped and organized in 2-3 clear rows …….. ***E. palustre***
5	Stem diameter is 2-3 mm, the ratio of the branch diameter to the stem diameter is 1:1.2–2.5, the number of stomata rows on the stem is 1 (rarely 2), on the outer periclinal walls of the subsidiary cells of the stomata the papillae are scattered only near the stomatal aperture, on the crest of the ribs of the stem there are transverse rounded smooth silicon outgrowths……....***E. pratense***
	Stem diameter is 5-12 mm, the ratio of the branch diameter to the stem diameter is 1:5.1–13.2, the stomata on the stem and the silicon outgrowths at the crest of the ribs of the stem are abscent ……...***E. telmateia***
6	The ratio of the central cavity diameter of the stem to the stem diameter is 0.26-0.36: 1, the number of stomata rows on the stem is 3-4, on the outer periclinal walls of the cells of the stomata the papillae form a series around the perimeter and are scattered in the middle, on the crest of the ribs of the stem there are transverse rounded silicon outgrowths occasionally covered with papillae……..***E. arvense***
	The ratio of the central cavity diameter of the stem to the stem diameter is 0.44-0.62: 1, the number of stomata rows on the stem is 1(rarely 2) on the outer periclinal walls of the subsidiary cells of the stomata the papillae form a series around the perimeter and occasionally placed in the middle, on the crest of the ribs of the stem there are 2-3 rows of silicon finger-like outgrowths ……..……...***E. sylvaticum***

3. CONCLUSION

The most important diagnostic features that can be used to determine the affiliation of whole (uncrushed) *Equisetum* raw materials are the presence or absence of spore-bearing organs and spores, the number of branchial ribs, the ratio of the lenght of the branch internode to leaf whorl of stem, and the ratio of the central cavity diameter of the stem to the stem diameter. A comprehensive analysis of the

ultrastructural features of the epidermis of stems and branches of *Equisetum* species, including the features of the cuticle, the position and shape of the mammillae and papillae, the presence and localization of stomata guard cells, the characteristics of siliceous warts on stomata, siliceous outgrowths on the tip of the rib (ridge) of stems and branches, makes it possible to unambiguously determine the species affiliation of crushed and whole raw materials of horsetails on the basis of a number of characteristics.

4. MATERIALS AND METHODS

The research was based on own materials of *Equisetum* collected by authors in their natural habitats during field research in 2019–2021 in different regions of Ukraine and also the herbarium specimens deposited at the National Herbarium of Ukraine (KW) were used (Table 4).

Table 4. Specimens of the *Equisetum* species analyzed in the study.

Species	Locality	Collecting date	Collectors, Herbarium code
Equisetum arvense	Kyiv region, Obukhiv district, village Kozyn, floodplain meadow of Kozynka river	2.07.2019	Tymchenko I.A., Dvirna T.S., KW №155539
	Kyiv region, Makariv district, near the village of Nikolaevka, the abandoned fields	15.06.2020	Minarchenko V.M., KW №155537
Equisetum fluviatile	Kyiv region, Obukhiv district, village Kozyn, the bank of Kozynka river	10.06.2019	Tymchenko I.A., KW №155544
	Vinnytsia region, Khmilnyk district, near the village of Shyroka Hreblya, the bank of the Pivdennyi Buh river	2.08.2014	Kolomiychuk V. P., Orlov O.O., KW №113885
Equisetum palustre	Vinnytsia region, Lityn district, near the village of Bahrynivtsi, the floodplain of the Zhar river	30.06.2003	Chorna G.A., KW №039146
	Ivano-Frankivsk region, Yaremcha district, near the village of Yablunytsya, Yablunytskyj Pass, 940 m a.l.s.	6.07.2019	Minarchenko V.M., KW №155545
Equisetum pratense	Rivne region, Dubno district, near the village of Busha	15.06.1985	Shumilova A.V., KW №095648
	Ternopil region, town of Kremenets, Divochi skeli	8.07.2003	Burkalova D., Herasymchuk D., Krasylenko Yu., KW №040942
Equisetum sylvaticum	Zakarpattya region, Perechyn district, near the village of Lumshory, edge of forest	3.09.2008	Shevera M.V., KW №090804
	Kyiv region, Makariv district, oak forest near the village of Nikolaevka	15.06.2021	Minarchenko V.M., Makhynia L.M., KW №155542
Equisetum telmateia	Cherkasy region, the town of Uman, arboretum «Sofiyivsky Park»	1.07.2019	Kuzemko A.A., KW №155543
	Kyiv region, Obukhiv district, the village of Stayky, near the stream along the road	15.07.2020	Konaikova V.O., KW №155541

The specimens of model species at the mature stage of development were collected and soaked in an alcohol solution. The microscopical study of raw organs of *Equisetum* species was focused on morphological and anatomical features of the axial stem and branches of each species. For morphological and anatomical analysis of raw materials, at least 10 specimens of each species were studied, from which fragments of summer green shoots in the middle part of their length were selected; they were boiled in water or 5% sodium hydrochloride solution for 2–5 min or immersed in the macerating solution for 7–14 days and prepared for light microscopy. In some cases, the microslides were prepared from fresh plants. Semipermanent slides for the microscopic studies were carried out according to the usual techniques [11, 12].

Microscopic studies of the specimens were performed on multiple occasions for each organ. The photomicrographs were prepared using the Olympus CX23 light microscope, Philip Harris stereomicroscope, and Levenhuk M1000 PLUS camera software. The ultrastructure study of raw organs surfaces was performed using a scanning electron microscope (SEM) (JSM-6060LA, Japan) according to the standard method: dehydrated objects were fixed on brass tables, sprayed with a thin layer of a mixture of

gold and platinum in a vacuum chamber. The linear dimensions of micro-objects (epidermal cells, stomata, papillae) were determined using Levenhuk Lite and Axio Vision 4.8 software, the sample size was 50 measurements for each parameter. A digital microscope Sigeta Superior 10-220x LCD 1080P HDMI/USB/TV was used for macromorphological research.

The description of diagnostic features of studied *Equisetum* specimens is based on the usual terminologies [8,13,14,15]. At the same time, simple terms that do not require explanation are included in the definition of specific characteristics of some structures.

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