Pak. J. Bot., 58(2) February, 2026.

ARTICLE DOI: http://dx.doi.org/10.30848/PJB2026-2(19)

ECOLOGICAL-ANATOMICAL STUDY ON THE PHYTOCONTAMINATION PROFILE OF THE MEDICINALLY IMPORTANT SPECIES *TRIBULUS TERRESTRIS* L. (ZYGOPHYLLACEAE R.BR.) DISTRIBUTED IN THE POST-CONFLICT TERRITORY OF AZERBAIJAN

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Abstract

The aim of the study is to investigate, from an ecological-anatomical perspective, the anatomical adaptation features formed in the pharmaceutically significant species *Tribulus terrestris* L. under phytocontamination conditions, thereby scientifically determining its stress-resistance mechanisms, ecological plasticity potential, and bioindicator capacities. The study demonstrated that this plant exhibits high tolerance to the phytocontamination process and maintains its ability to develop normally in areas containing various pollutants. Collected plant specimens were fixed, processed with appropriate reagents in the laboratory, and prepared for sectioning. Transverse and longitudinal sections obtained from the specimens were transferred to permanent slides following histological staining procedures. Anatomical analyses were performed using modern LED microscopes. As a result of the phytocontamination process, pigmentized anomalous black-colored intercellular and dark yellow intracellular accumulations (likely phytotoxins) were identified in all vegetative organs. Additionally, the accumulation of pigmentized callose (presumably phytotoxic callose) in the phloem elements of the vascular systems was recorded. Numerous large pigmentized druse crystals (likely druse-toxins) were observed in the leaf mesophyll, parenchyma cells of the petiole, and in the pith and cortex of the stem. Thickening of cell walls in the epidermal tissue surrounding vegetative organs and modification of the cuticle layer were interpreted as stress-response reactions to contaminants. Collenchymatous sclerenchyma areas located at the boundary with the phloem of conductive bundles in the leaf and petiole can be explained as the transformation of modified collenchyma into sclerenchyma. For the first time in the flora of Azerbaijan, the study identified the presence of a Kranz anatomical structure in the leaflets as a specific anatomical characteristic of *T. terrestris*. The scientific and practical significance of the ecological-anatomical investigation, which determined the tolerance structure and its identification under phytotoxic conditions, may serve as a biomarker for adaptive changes that could occur in other plant species under similar conditions.

Key words: Anomalous intercellular and intracellular inclusions, Collenchyma differentiation, Columellar structure, Kranz anatomy, Pericyclic vascular system, Phytocontamination

Introduction

T. terrestris species belongs to the Zygophyllaceae family showing natural distribution within the territory of the Republic of Azerbaijan (Qurbanov, 2024). It is an annual herbaceous plant, morphologically characterized by numerous branching and sprawling stems. The long and slender roots enable the plant to survive in arid areas by utilizing moisture from deeper soil layers. In general this plant is capable of expanding its phytogeographical range (Tripathi *et al*., 2019). The trichomes are formed across the entire surface of the plant and fruits have hard spines. The trichomes and hard spines serve as defense mechanisms for the plant. The compound leaves of the plant are pinnately bipartite and arranged oppositely on the stem. The small yellow flowers are five-petaled and each fruit formed results from the fusion of five carpels. The development of carpels can vary depending on the degree of development of the seeds and effectiveness of double fertilization process; after maturation, the carpels dry and separate from each other.

The plant contains saponins, mainly furostanol saponins (Protodioscin), which have testosterone-boosting and adaptogenic effects. The steroidal saponins present in this plant are the main compounds regulating hormonal balance (Adaikan & Gauthaman, 2001; Affaf *et al*., 2019). The plant contains alkaloids which stimulate the nervous system and help reduce stress. The plant contains kaempferol, quercetin, and rutin flavonoids, which are strong antioxidants. There are also beta-sitosterol phytosterols that help regulate cholesterol levels and prevent cardiovascular diseases. The plant also contains minerals like; zinc, iron, calcium, phosphorus, and others, which play an important role in cellular metabolism, vitamin C content supports the immune system wheeras the tannins exert antibacterial and anti-inflammatory effects; the linoleic and oleic acids are beneficial for skin regeneration and protection of the cell membrane (Patil & Lade, 2016). However, the effects of these constituents vary depending on the growth conditions and ecological factors. *T. terrestris* is a widely used medicinal plant, especially in Azerbaijan, traditionally used in folk medicine for kidney and cardiovascular diseases, immune system restoration, anti-inflammatory purposes, stimulation of sexual function, and other phytotherapeutic features (Zhu *et al*., 2017; Ibadullayeva, 2024).

Received: 13-05-2025 Revised: 04-07-2025 Accepted: 12-07-2025 Online: 02-12-2025

This ecological-anatomical study was undertaken to enlighten the latest research directions such as phytocontamination and bioremediation, the adaptation capabilities of these plants in the uptake and elimination of toxins from the natural environment (Khan *et al*., 2023; Sahoo, 2024). As determined in other studies conducted on various plants, contamination with heavy metals affects plants at different levels (Bilal *et al*., 2024; Danish *et al*., 2024; Ejaz *et al*., 2024; Karim *et al*., 2024). As in the ecological anatomical studies carried out by the author on other related medicinal plants (Sardarova, 2025a, 2025b, 2025c, In press), the investigation of the ecological adaptation characteristics of the *T. terrestris* species holds high significance. Especially from the perspective of its medicinal importance, the structural and compositional variations resulting from growth in contaminated areas in this species can play a key role in determining its raw material usage potential and safety.

Material and Methods

The voucher specimens of *T. Terrestris* were deposited in the herbarium of Department of Biology, Valida Tutayuq, Azerbaijan State Agricultural University (voucher number: ASAU-TT-2024-AGHDAM4).

Laboratory studies: Plant specimens of *T. terrestris* were collected in June 2024 from the contaminated area of the Aghdam Industrial Park in Karabakh (Fig. 1). The specimens were fixed using an appropriate fixation method (Chamberlain, 2020; Criswell *et al*., 2025) with high-concentration FAA and Kraf III fixatives and subsequently processed under laboratory conditions. Paraffin (BW Blended Waxes, Inc. US) was used as an auxiliary medium both for the infiltration of the material and during the sectioning process. The thickness of the sections was calibrated and measured using the micrometric adjustment screw on a modern hand microtome (RADICAL, RMT-5, India). The thickness of the sections ranged from 6-9 µm.

Histochemical techniques were performed using differential staining with methylene blue (KimyaLab, Turkey), delafield's hematoxylin, toluidine blue, calcofluor, fast green, safranin O, Sudan III, and iodine dyes (Innovating Science, USA). The staining process applied for the selective coloring of tissue components was conducted step-by-step using the decolorization method (Peterson *et al*., 2008; Engin *et al*., 2024). The sequential use of different histological stains allowed for more precise identification of the ecological-anatomical structural components of *T. terrestris*. During the section processing, histochemical reagents such as aniline sulfate, strontium chloride, sodium hydroxide, and phloroglucinol (KimyaLab, Turkey) were also used. The permanent slides of the stained sections were prepared using Canada balsam (INOVATING SCIENCE, US). The obtained preparations were placed in a specialized incubator device (Carolina, USA) maintained at a constant temperature of 20-25°C to ensure complete drying of the Canada balsam. Permanent preparations made from transverse and longitudinal sections of the plant were subjected to microscopic analysis.

Microscopic analysis: Latest digital and more universal microscopes available at the Department of Biology of the Azerbaijan State Agricultural University were used. Specifically, "Carl Zeiss, Axio Imager A2" (ZEISS, Germany) microscope was used for accurate analysis of structural elements and micrometric measurements. Video observations were recorded and digital micrographs of the specimens from the vegetative organs of *T. terrestris* were prepared. During the study, an LCD Digital Microscope NLCD-307B (Wincom Company Ltd., China) was used to monitor the quality and histological staining efficiency of the transverse and longitudinal sections of plant specimens prior to their transfer to permanent slides. Final analyses, micrograph acquisition, and statistical measurements of the prepared slides were performed using a Carl Zeiss Axio Imager A2 microscope. In order to ensure the precise identification of the anatomical structure of the organs, all magnification settings of the objective (4×, 10×, 40×, 60×, 100×) were used. Additionally, observations were made using a 100× objective with the application of immersion oil (RMY, US). The stereoscopic microscopes (Stereo Zeiss Stemi508 (ZEISS, Germany), Stereo YK-SM067B2 (Wincom Company Ltd., China)) were used for macroscopic analysis of the samples.

During the analyses, ocular and stage micrometers (MUHVA, China) were used to measure tissue structures. In the initial stage, calibration of the ocular micrometer was performed using the stage micrometer (Moyo *et al*., 2015), after which the dimensions of anatomical elements in the microscopic preparations were calculated based on the micrometer scale readings. To enhance measurement accuracy, an automated micron measurement system integrated with the microscope was employed. Prior to anatomical sectioning, the standardization of organ dimensions under investigation was precisely conducted using a digital micrometer (Jiavarry, China) in accordance with micrometric principles.

Statistical analysis: Eight individuals from the same population were collected from the contaminated site. From each vegetative organ, 15 sections were prepared, and the most representative ones were selected to create permanent slides for further investigation. The micrometric data obtained during the study were analyzed using the statistical software Jamovi (version 2.6.26, University of Sydney, Australia). Mean values and standard deviations (SD) were calculated to assess the variability and central tendencies of the measured parameters.

Results

The results of the microscopic analysis conducted on the transverse and longitudinal sections obtained from the different vegetative organs of *T. terrestris* are as follows:

Peduncle: The peduncle possesses a nodal stele, with the vascular bundles forming its conductive system surrounding the pith (Fig. 2A, B). Collateral-type vascular bundles are separated from one another by parenchyma cells that form the medullary rays (Fig. 2C). The bundles are poorly developed and contain xylem tissue consisting of a few primary protoxylem elements. Between this tissue and the phloem, the fascicular cambium is observed, composed of somewhat narrow, elongated meristematic cells. The phloem tissue of the bundles is relatively well-developed. Microscopic observations revealed the accumulation of pigmented callose (likely toxic callose) in the sieve tubes of the phloem. In the part of the phloem adjacent to the cortex, angular collenchyma tissue has formed (Fig. 2E).

The vascular bundles are structured such that the xylem is oriented toward the pith, while the phloem is oriented toward the cortex, surrounded by parenchyma tissue. The cortex is located external to the vascular system. The isodiametric parenchyma cells of the cortex are, on average, 1.82 times smaller than the pith parenchyma cells. Parenchyma cells in the cortex adjacent to the epidermis exhibit lower morphometric values (Fig. 2D, F). Both the cortex and pith parenchyma cells, as well as the structural elements of the vascular system, show accumulation of ergastic and constitutional substances, along with non-specific structural inclusions. In the peduncle of *T. terrestris*, the pigmentation of thin, prozomatic trichomes on the epidermis - serving a protective function - is interpreted as an indicator of phytotoxic stress in the contaminated environment (Fig. 2G, H). Stomata are also present on the epidermis, with the guard cells' walls thickened, especially on the sides facing the inner epidermis. The outer walls of these cells, adjacent to the stomatal pore, bear typical papillae (He & Liang, 2018). Epidermal cells are slightly ellipsoidal and small in size. Their outer periclinal walls are thickened and covered with a cuticular layer, whereas thickening of the inner and anticlinal walls is much less pronounced. Accumulation of ergastic and constitutional substances, as well as anomalous intracellular inclusions (likely phytotoxins), within the epidermal cells was observed during anatomical analysis.

Leaflet: In *T. terrestris*, the leaflet exhibits a bifacial structure and is amphistomatic (Semerdjieva, 2011). As observed in the cross-section, the leaflet is relatively thick, with a protrusion formed in the central vein region oriented toward the abaxial side (Fig. 3A). Within this protruding area, beneath the central vascular bundle, two to three rows of parenchyma cells lacking chloroplasts are arranged (Fig. 3B). These cells are large and isodiametric, serving as the main tissue responsible for water storage in the leaflet. A key structural feature of the leaflet is the presence of Kranz anatomy, which represents one of the principal adaptations allowing the plant to survive under adverse conditions (Lauterbach *et al*., 2019). Around both the central vascular bundle and the lateral bundles, large bundle sheath cells are positioned in accordance with the Kranz anatomical arrangement. Slight thickening of the cell walls in these cells can be observed, likely resulting from phytocontamination processes. As shown in the photomicrographs, chloroplasts within these cells are localized near the vascular bundle in a clustered manner (Fig. 3E, F).

The vascular bundles consist of adaxial xylem and abaxial phloem. Microscopic analysis of the central and lateral bundles revealed the spiral arrangement of xylem vessels forming the xylem tissue in longitudinal structure. Pigmented callose accumulation (likely phytotoxic callose) was also detected in the phloem tissue. In the central bundle, both xylem and phloem elements were actively developed. Collenchymatous sclerenchyma regions composed of cells with intensively thickened walls were located adaxially to the xylem and abaxially to the phloem. The dorsal side of the leaflet contains compact palisade tissue, whereas the ventral side exhibits both palisade and spongy parenchyma. In the transverse section of *T. terrestris* leaflets, the dominance of palisade parenchyma in the mesophyll is characterized as an anatomical indicator of the plant's xerophytic traits (Fig. 3C). Dark-colored anomalous intercellular inclusions (likely phytotoxins) were observed, particularly in the spongy parenchyma. In addition, large pigmented druse crystals (likely druse-toxins) were recorded within this tissue and around the central vascular bundle (Fig. 3D). The enclosing cells of the vascular bundles contained a few pigmented raphide crystals (likely raphide-toxins).

Overall, microscopic observations indicate that anatomical-diagnostic signs of contamination are present throughout the mesophyll tissue system of the leaflet. Tectorial trichomes were observed on both the adaxial and abaxial epidermises (Fig. 3H). Lignification was observed in the walls of these trichomes, serving as an anatomical indicator of the plant's protective potential in a contaminated environment (Samy *et al*., 2012). Quantitative analysis revealed that the height of adaxial epidermal cells was on average 14.85% smaller than that of abaxial epidermal cells. In both epidermal layers, the outer periclinal walls were intensely thickened and covered with a cuticular layer. Guard cells of the stomata were smaller than the surrounding epidermal cells and had thickened walls. Sub-stomatal cavity formed from the stomata toward the inner side of the leaflet mesophyll facilitate efficient aeration (Fig. 3G).

Petiole: In the examined *T. terrestris* specimens, the petiole shows a slightly circular structure in transverse section, with two lateral exogenous protrusions recorded on its upper surface (Fig. 4A). In the central part of the petiole, four collateral-type vascular bundles are present, with the xylem tissues oriented toward the center (Gabr & Ragab, 2023). The xylem vessels of medium and small diameter are relatively densely arranged. Additionally, two peripheral vascular bundles are located within the petiole; these are much smaller in size and positioned parallel to the surface in the exogenous protruding regions. Around the petiolar vascular bundles-particularly at the boundary with the phloem tissue-small-sized cells with intensely thickened walls have formed. Based on microscopic analyses, these structures in *T. terrestris* were interpreted as representing the transformation of collenchyma into sclerenchyma. In some of these cells, the initial stage of lignification was also observed (Fig. 4B).

Between the peripheral bundles situated near the exogenous protrusions of the petiole and the epidermis, two layers of columellar-structured cells were recorded, which are characterized as an indicator of the plant's xerophytic adaptation (Fig. 4E). Inside these cells, intensive accumulation of yellow pigmented, drop-shaped anomalous intracellular inclusions (possibly phytotoxins) was observed. As a primary sign of the phytocontamination process, numerous large pigmented druses (likely druse-toxins) were identified within the parenchyma tissue of the petiole (Fig. 4C, D, G). Compactly structured, small epidermal cells encircle the petiole externally. Uniform and active thickening of epidermal cell walls is considered an anatomical indicator of the phytocontamination process. Numerous thin, prozomatic trichomes were formed intensively on the epidermis, within which anomalous intracellular inclusions (presumably phytotoxins) were observed under microscopic examination (Fig. 4H). The formation of stomata in the epidermal tissue of the petiole was also recorded (Fig. 4F). Due to the influence of the contaminated environment, non-specific accumulations were observed in the stomatal and substomatal regions during microscopic investigations. Based on morphometric data, significant thickening of the walls of the small guard cells was recorded. Microscopic studies also revealed the presence of modified callose aggregations in the sieve elements of the phloem within the vascular bundles of the petiole, identified as contamination indicators under ecological stress.

Fig. 1. **A** - General view of *Tribulus terrestris*, **B** - Location of the site Aghdam Industrial Park.

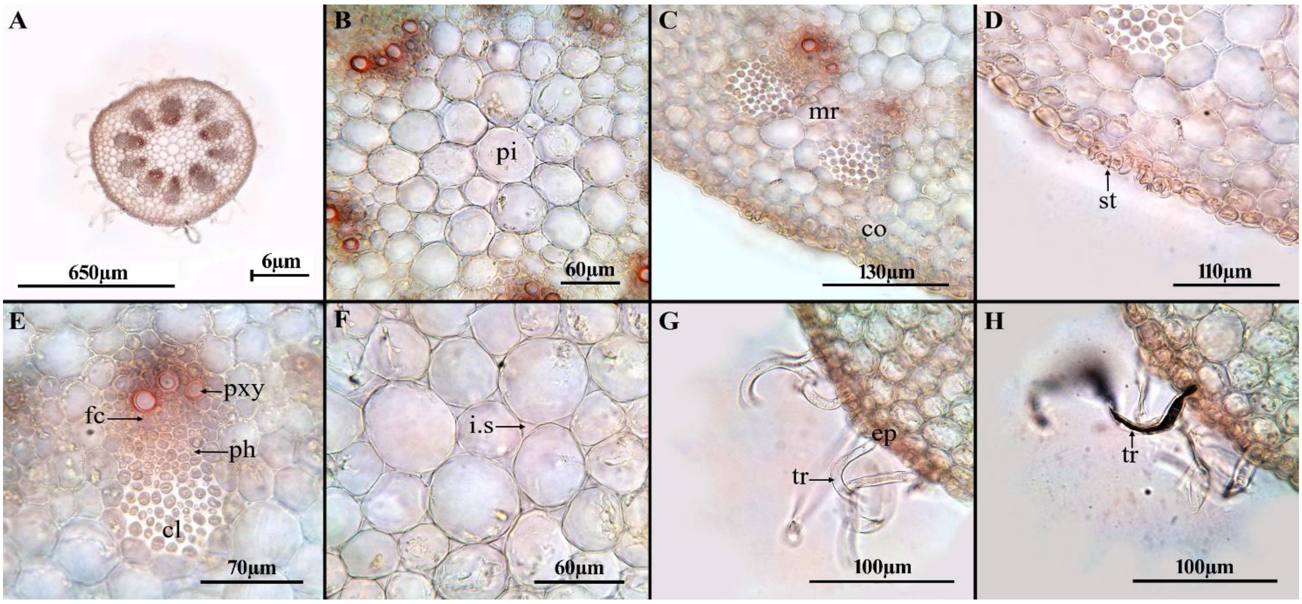

Fig. 2. *Tribulus terrestris.* Transverse section of the peduncle. **A** - General structure, **B** - Pith, **C** - Cortex and bundles, **D** - Cortex, **E** - Collateral bundle, **F** - Pith, **G**, **H** - Prozomatic trichomes on the epidermis. **ep**-epidermis; **cl**-collenchyma; **st**-stomata; **pi**-pith; **mr**-medullary ray; **co**-cortex; **i.s**-intercellular space; **pxy**-protoxylem; **ph**-phloem; **fc**-fascicular cambium; **tr**-trichome; **er**-ergastic substrances; **cns**-constitutional substances; **a.ina.i**-anomalous intracellular inclusions (likely phytotoxins).

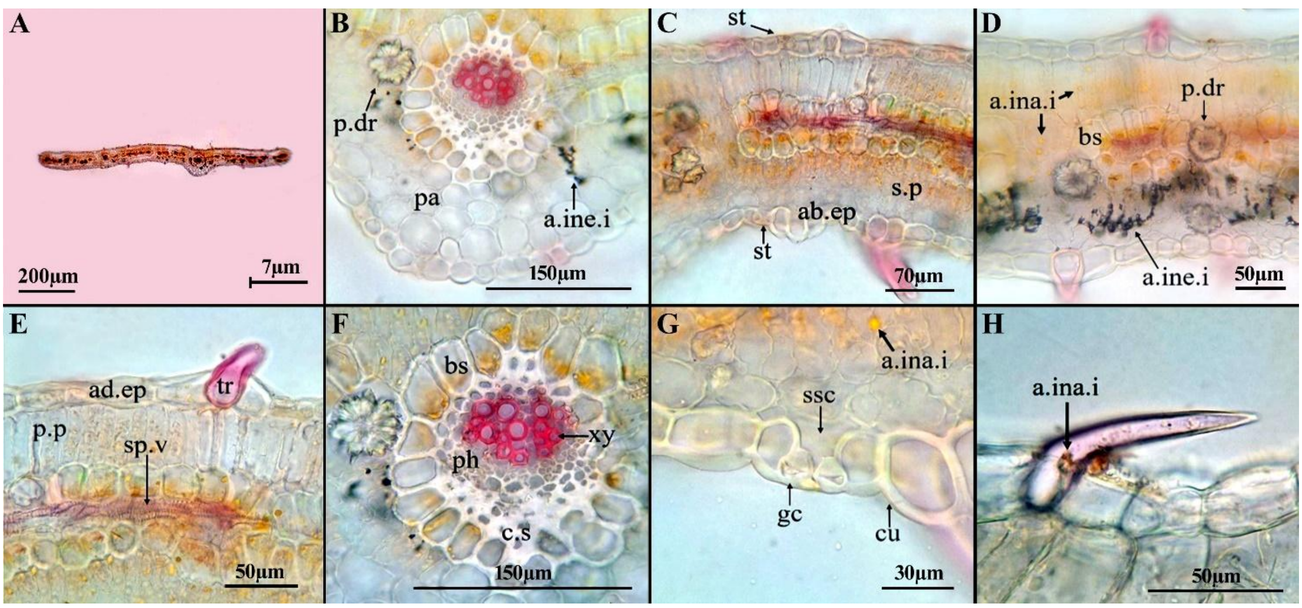

Fig. 3. *Tribulus terrestris.* Transverse section of the leaf**. A -** General structure, **B -** Midrib region, **C, D, E -** Parts of mesophyll, **F -** Central bundle, **G -** Abaxial stoma, **H -** Tectorial trichome on the adaxial epidermis. **ad.ep**-adaxial epidermis; **ab.ep**-abaxial epidermis; **c.s**-collenchymatous sclerenchyma; **s.p**-spongy parenchyma; **p.p**-palisade parenchyma; **bs**-bundle sheath; **cu**-cuticle; **sp.v**-spiral vessel; **ph**-phloem; **xy**-xylem; **pa**-parenchyma; **st**-stoma; **ssc**-sub-stomatal cavity; **gc**-guard cell; **tr**-tectorial trichome; **p.dr**-pigmented druse (likely druse toxin); **a.ine.i**-anomalous intercellular inclusions; **a.ina.i**-anomalous intracellular inclusions (likely phytotoxins).

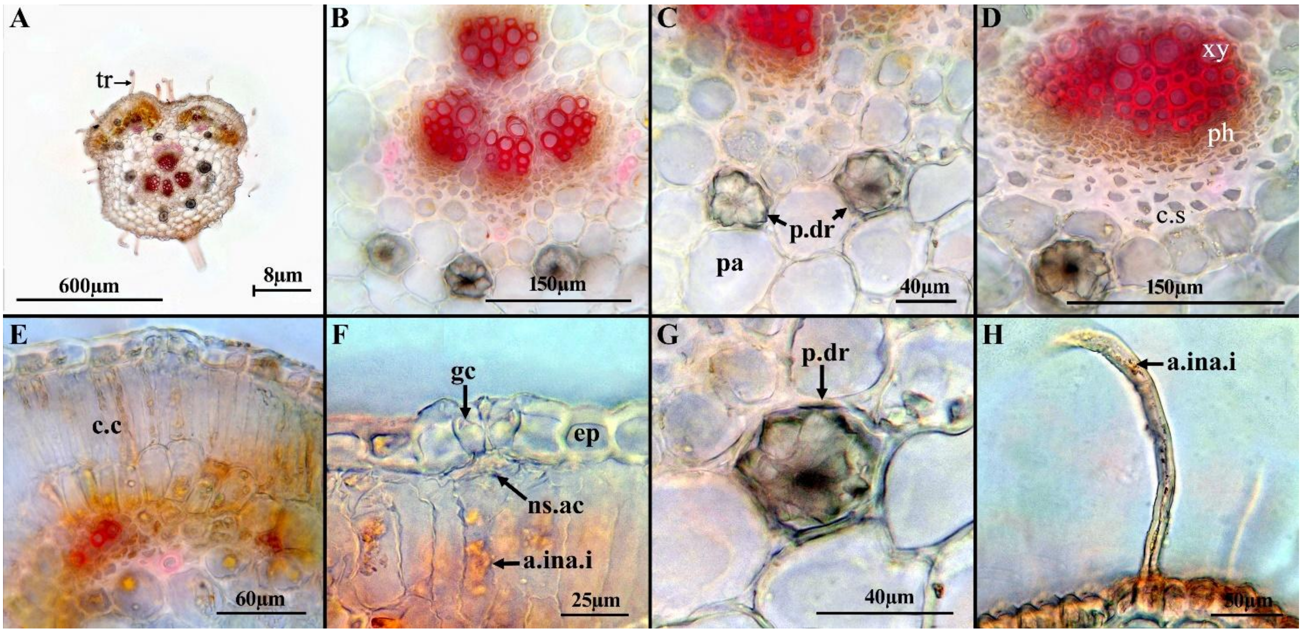

Fig. 4. *Tribulus terrestris.* Transverse section of the petiole. **A** - General structure, **B** - Vascular bundles, **C** - Pigmented druses in parenchyma, **D** - Collateral bundle, **E** - The region with columellar cells, **F** - Stoma structure, **G** - Pigmented druse in parenchyma, **H** - Prozomatic trichome on the epidermis. **ep**-epidermis; **c.s**-collenchymatous sclerenchyma; **xy**-xylem; **ph**-phloem; **tr**-trichome; **gc**-guard cell; **c.c**-columellar cells; **pa**-parenchyma; **a.ina.i**-anomalous intracellular inclusions (likely phytotoxins); **p.dr**-pigmented druses (likely druse-toxins); **ns.ac**-non-specific accumulations.

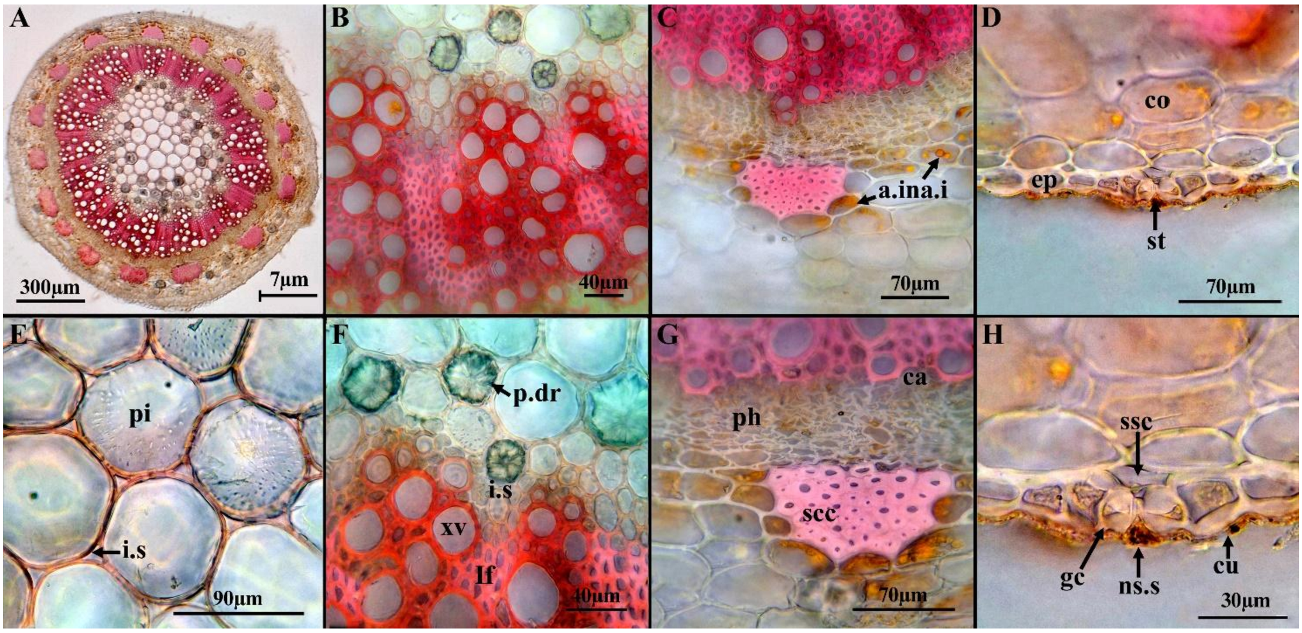

Fig. 5. *Tribulus terrestris.* Transverse section of the stem. **A** - General structure, **B**, **C** - Vascular elements, **D** - Cortex, **E** - Pith, **F** - Perimedullary region, **G** - A part of the vascular system and cortex, **H** - Structure of the stomatal apparatus. **cu**-cuticle; **ep**-epidermis; **scc**-sclerenchyma; **pi**-pith; **co**-cortex; **xv**-xylem vessels; **lf**-libriform fibres; **st**-stoma; **ssc**-sub-stomatal cavity; **gc**-guard cell; **i.s**-intercellular spaces; **ph**-phloem; **ca**-cambium; **p.dr**-pigmented druses (likely druse-toxins); **a.ina.i**-anomalous intracellular inclusions (likely phytotoxins); **ns.s**-non-specific structure.

Stem (Transverse Section). In the transverse structure of the eustele-type stem, a ring-shaped xylem tissue surrounding the pith can be observed (Fig. 5A). At the boundary between the xylem tissue and the pith, there are small-diameter and weakly developed protoxylem elements. The other xylem vessels are larger and possess well-thickened walls. The spaces between the xylem vessels are filled with libriform fibers, which provide mechanical support (Fig. 5B). The cambium, composed of fusiform initials, surrounds the xylem on the cortical side. Due to the exarch activity of this meristematic tissue, a continuous phloem ring is formed toward the cortex. Around the phloem, within the cortex, there are mechanical tissue clusters arranged at regular intervals (Elkamali *et al*., 2016).

The presence of yellow-orange anomalous intracellular inclusions (presumably phytotoxins) in the parenchyma cells surrounding these clusters composed of lignified-walled sclerenchymatous fibers, as well as in other parenchymatic cells, was identified through microscopic observations. Additionally, pigmented callose deposits (likely phytotoxic callose) were detected in the phloem tissue (Fig. 5C, G).The cortical cells are of medium size and somewhat elongated, with slightly thickened walls in the subepidermal zone (Fig. 5D). Both the cortex and the pith contain pigmented druses (possibly druse-toxins). The pith parenchyma cells are on average 1.27 times larger and isodiametric in shape, with intercellular spaces observed between them (Fig. 5E, F).

The epidermal cells of the stem are small, with intensely thickened walls. A deformed and thick cuticle layer is visible on the epidermal surface, regarded as an anatomical indicator of environmental contamination effects. Both trichomes and stomata are present on the epidermis. In the stems of *T. terrestris* exposed to ecological pollution, intense wall thickening and accumulation of contaminants were observed around the stomatal aperture (Fig. 5H). In the contaminated environment, the plug-like structure or deformation of the stomatal aperture affected the morphometric parameters of the epidermal cells.

Stem (Longitudinal Section): In the longitudinal section of the stem of *T. terrestris*, the specific structure of the pith parenchyma is clearly distinguishable. The parenchyma cells, which appear isodiametric in the transverse section, are elongated and horizontally oriented in the longitudinal direction, forming consecutive layers in the ventral region (Fig. 6A, B). In the longitudinal section of the stem, the internal organization of the xylem tissue within the vascular system is more distinct. The primary protoxylem elements located adjacent to the pith display a spiral structure, whereas the remaining xylem vessels exhibit a pitted pattern. Among these vessels, long and narrow fiber-like cells are observed, representing libriform fibers with thickened, lignified walls. Within the xylem tissue, regions composed of small, brick-shaped cells with slightly lignified walls - identified as medullary rays - are also evident (Fig. 6C, D). From the xylem toward the cortex, fusiform initials of the cambium, elongated cells of the phloem, and long sclerenchymatous fibers with lignified walls are clearly visible (Fig. 6E, F).

It is noteworthy that the cortical parenchyma cells differ in appearance between the transverse and longitudinal sections: in the longitudinal view, they appear more elongated horizontally, with straighter walls and slightly angular or polygonal corners (Fig. 6G). Yellow-orange anomalous intracellular inclusions (likely phytotoxins) are present in the cortex, while pigmented druses (likely druse-toxins) are found in both the cortex and pith. Trichomes are developed on the epidermis, serving protective and covering functions. The tectorial trichomes are large in diameter, with their bases located in the subepidermal region of the epidermis (Fig. 6H). Numerous small prozomatic trichomes are also present on the epidermal surface.

Root: In the transverse section of the root of *T. terrestris*, a broad xylem tissue occupies the central region. The arrangement of protoxylem elements at the center indicates that the initial stage of root development is diarch. The large metaxylem vessels, representing secondary xylem elements, are formed throughout the xylem tissue and constitute its main portion. The areas between these relatively compact elements are composed of lignified and sclerified libriform fibers (Fig. 7A, B). Microscopic analysis revealed the accumulation of anomalous intracellular inclusions (presumably phytotoxins) within the fusiform initials of the cambial layer at the surface of the xylem tissue, as well as the presence of pigmented callose (possibly phytotoxic callose) in the phloem located adjacent to the cambium (Fig. 7C).

Table 1. Micrometric characteristics of different tissue types in the analyzed organs of species *Tribulus terrestris.*

Organs			(Mean ± SD)
1. Thickness of the epidermis layer (μm)			
Organs			(Mean ± SD)
Peduncle			15,38 ± 2,83
Leaflet	adaxial epidermis		22,54 ± 2,16
	abaxial epidermis		26,47 ± 2,35
Petiole			22,14 ± 1,31
Stem			22,64 ± 1,63
2. Thickness of the outer wall and cuticle of the epidermis (μm)			
Organs			(Mean ± SD)
Peduncle			3,96 ± 0,79 / 1,13 ± 0,11
Leaflet	adaxial epidermis		4,39 ± 0,61 / 0,98 ± 0,17
	abaxial epidermis		4,41 ± 0,52 / 0,85 ± 0,13
Petiole			5,21 ± 0,84 / 1,09 ± 0,15
Stem			7,32 ± 0,58 / 1,32 ± 0,23
3. Diameter of the parenchyma cell (μm)			
Organs			(Mean ± SD)
Petiole			63,41 ± 5,36
Peduncle	cortex parenchyma		28,18 ± 1,84
	pith parenchyma		51,21 ± 3,57
Stem (transverse section)	cortex parenchyma		67,46 ± 7,59
	pith parenchyma		85,93 ± 5,04
Stem (longitudinal section; parenchyma cells along the vertical axis of the stem)	cortex parenchyma	height	25,52 ± 5,19
		width	55,98 ± 4,52
	pith parenchyma	height	18,72 ± 6,33
		width	83,67 ± 8,19
4. Height of palisade cells (μm)			
Organs			(Mean ± SD)
Leaflet			58,82 ± 4,56
Petiole			58,63 ± 4,85
5. Size of vascular bundles (μm)			
Organs			(Mean ± SD)
Peduncle			121,65 ± 7,54
Leaflet (central bundle)			146,53 ± 8,57
Petiole			122,73 ± 8,25
6. Diameter of the xylem vessel (μm)			
Organs			(Mean ± SD)
Peduncle			17,28 ± 1,36
Leaflet			17,46 ± 1,47
Petiole			18,71 ± 2,17
Stem			39,84 ± 5,13
Root			58,91 ± 7,89
7. Size of druse crystal (μm)			
Organs			(Mean ± SD)
Leaflet			46,73 ± 3,48
Petiole			47,78 ± 3,21
Stem			35,05 ± 4,62

Note: SD - standard deviation

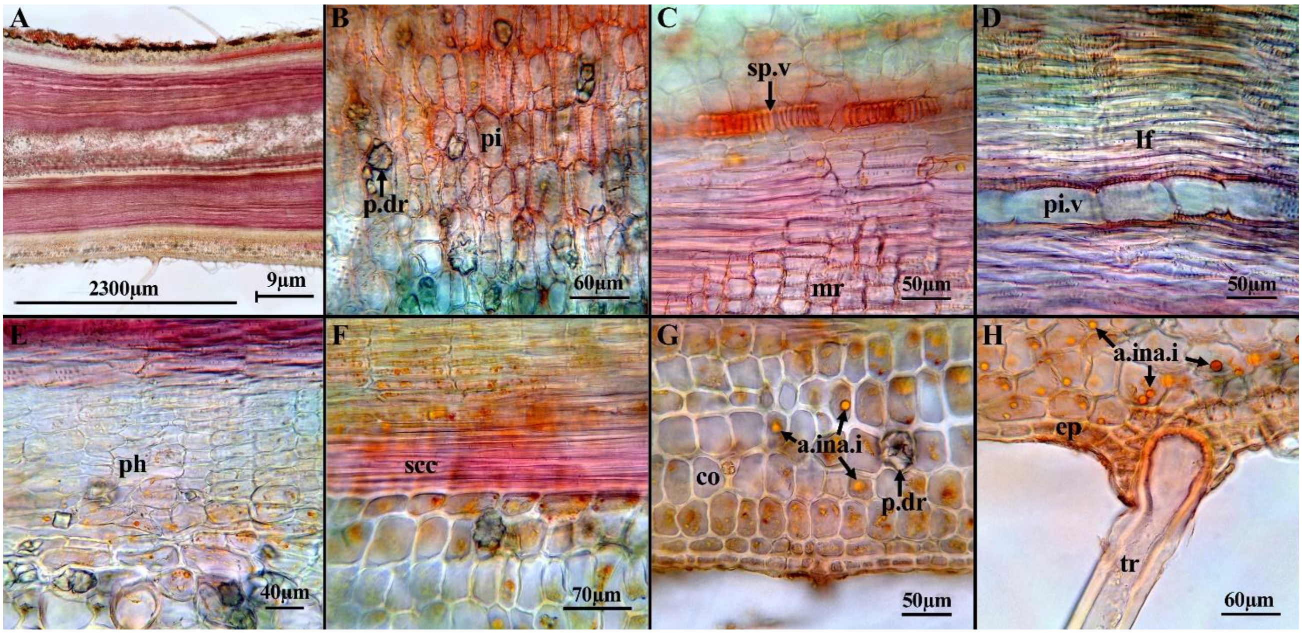

Fig. 6. *Tribulus terrestris.* Longitudinal section of the stem. **A** - General structure, **B** - Pith, **C**, **D** - Xylem elements, **E** - Phloem, **F** - Sclerenchyma fibers, **G** - Cortex, **H** - Tecrotial trichome on the epidermis. **ep**-epidermis; **scc**-sclerenchyma; **pi**-pith; **co**-cortex; **sp.v**-spiral vessel; **pi.v**-pitted vessel; **lf**-libriform fibres; **ph**-phloem; **mr**-medullary ray; **tr**-trichome; **a.ina.i**-anomalous intracellular inclusions (likely phytotoxins); **p.dr**-pigmented druses (likely druse-toxins).

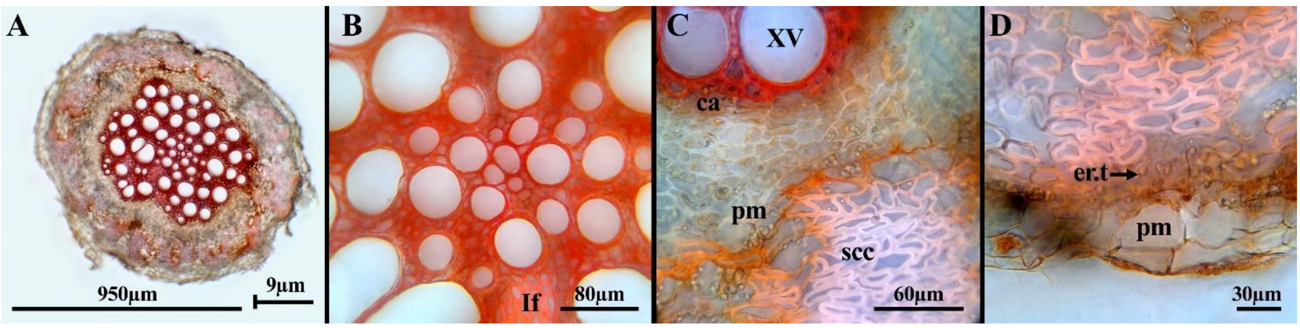

Fig. 7. *Tribulus terrestris.* Transverse section of the root. **A** - General structure, **B** - Xylem tissue, **C** - Vascular and mechanical tissues, **D** - Cortex. **pr**-periderm; **scc**-sclerenchyma; **lf**-libriform fibres; **ph**-phloem; **ca**-cambium; **pxy**-protoxylem; **mxy**-metaxylem; **p.er**-pigmented ergastic substances (presumably ergastic toxins); **a.i**-anomalous inclusions (presumably phytotoxins).

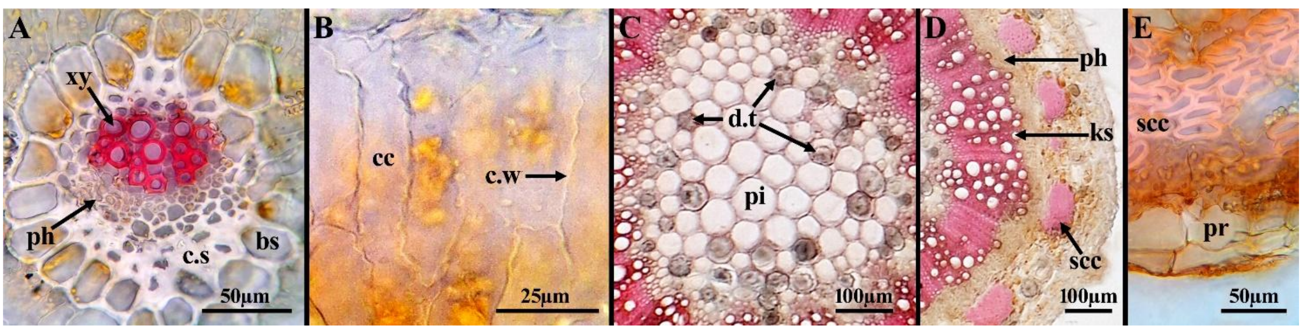

Fig. 8. Anatomical structural characteristics of *Tribulus terrestris* under ecological stress conditions. **A** - central vascular bundle of the leaf, **B** - columellar cells of the petiole, **C** - pith of the stem, **D** - part of the stem, **E** - part of the root. **scc**-sclerenchyma; **bs**-bundle sheath; **c.s**-collenchymatous sclerenchyma; **c.c**-columellar cells; **c.w**-cell wall; **ph**-phloem; **xy**-xylem; **pi**-pith; **p.dr**-pigmented druse (likely druse toxin); **pr**-periderm.

Due to the high degree of sclerification, a large portion of the cortex consists of sclerenchyma tissue (Nikolova & Vassilev, 2011). The mechanical cells forming the sclerenchyma of the root deviate slightly from the typical sclerenchymatous structure. This tissue, characterized by thick cellulose-lignified cell walls, is densely packed with poorly developed intercellular spaces, displaying a compressed structural organization (Fig. 7D). This represents a structural indicator of phytocontamination effects under ecological stress conditions.

Between the sclerenchymatous regions, a few cortical parenchyma cells contain pigmented ergastic substances (likely ergastic toxins). The root is surrounded by a periderm consisting of large phellem cells, within which anomalous inclusions (presumably phytotoxins) also accumulate.

The results of quantitative micrometric measurements performed on various tissues and structures of the vegetative organs of *T. terrestris* during the study are presented in Table 1.

Discussion

The anatomical analysis of the leaflet of *T. terrestris* showed that it possesses complex structural characteristics. In the peripheral zones of the leaf, a compact, single-layered palisade parenchyma adjacent to the dorsal epidermis is developed. Within the palisade cells, larger as well as narrow and elongated forms are present. These structural features are favorable for the effective distribution of sunlight and enhancement of photosynthetic activity. On the ventral side, in the subepidermal zone, a more complex and differentiated structure is formed. Here, spongy and palisade parenchyma cells are jointly involved. The spongy parenchyma cells are directly adjacent to the ventral epidermis and gradually decrease in size towards the peripheral areas, transforming into smaller and more densely packed cells. The palisade parenchyma cells, on the other hand, are formed in a position leaning against the bundle sheath cells surrounding the vascular system on the ventral side. This structural feature, namely the concentration and adherence of the palisade parenchyma cells around the vascular system, corresponds to the Kranz-type anatomical structural characteristic. Such a Kranz-like structure plays an important role in the localization of photosynthetic activity and the effective regulation of water and gas exchanges. The special differentiation observed on both the dorsal and ventral sides of the leaflet indicates a high adaptation of the *T. terrestris* species to a xerophytic lifestyle. The formation of such complex anatomical adaptations is for both an effective conservation of water and optimization of photosynthesis under arid conditions which increases its ecological adaptability. Based on these features, it is scientifically possible to confirm that the leaflet of this species is a modified dorsoventral leaflet with a Kranz-like structure. The leaflets exhibit typical kranz anatomy, which is a morpho-anatomical feature of the C4 photosynthesis pathway and an important structural characteristic that enhances the efficiency of carbon assimilation under high light and temperature conditions (Cutler *et al*., 2007). Our findings indicate that significant changes have occurred in the mechanical tissues adjacent to the vascular system, both in the leaflet mesophyll and in the petiole, in the phytocontaminated plants. Specifically, signs of lignification were observed in the structure of the collenchyma cells, which resulted in their differentiation into sclerenchyma (Fig. 8A). As such, a transition-type mechanical tissue is formed, with lignification being more intense in the perivascular areas of the vascular structures. The thickening of collenchyma cell walls, activation of lignin biosynthesis, and acceleration of sclerenchymatization of mechanical tissue are regarded as anatomical structural features of tolerance under contaminated conditions. Such changes are regarded as an adaptive mechanism aimed at maintaining the stable functioning of the vascular bundles and occur within the framework of stress-induced differentiation in plant tissues. This collenchyma-sclerenchyma transformation is the main structural adaptation that ensures increased resilience in the vascular system and adjacent areas against the mechanical and physiological stress induced by phytocontamination.

From an ecological-anatomical perspective, the subepidermal structure of the petiole of these plants is characterized by specific structures. Immediately beneath the epidermal layer in the petiole, there are columellar cells, which play a crucial role in providing mechanical stability and adaptation of plants to xerophytic environments. The close arrangement and thick cell walls of the columellar cells enhance their protective and supportive functions (Fig. 8B). This structure holds adaptive significance for this species, which is widespread in arid and semi-arid ecosystems. The degree of development of the subepidermal columellar cells can be regarded as an ecological indicator of mechanical resilience and the regulation of photosynthetic activity under water deficit conditions.

In the stems, a pericyclic vascular system is formed, exhibiting a strong and stable structure. This type of vascular system maintains hydraulic conductivity stability, ensures the rigidity of the stem under eco-pressing conditions, and contributes to compensation in the water loss. The presence of numerous druse-type calcium oxalate crystals in the stem's pith and cortex (Fig. 8C) is related to the plant's ion regulation and allelopathic defense mechanisms. The intensity of druse formation can be explained as an important ecophysiological mechanism aimed at both the neutralization of ecotoxic elements and the immobilization of metabolic waste products. The observation of pigmented raphides (likely raphide toxin) in the bundle sheath (Kranz) cells of the leaf mesophyll can be interpreted as a regulatory stimulator of this process. The well-developed sclerenchyma elements are observed in groups in both the root and stem cortex (Fig. 8D, E), which not only ensures the mechanical stability of the plant but also plays a key adaptive role in its adaptation to arid and semi-arid ecosystems. The localized arrangement of sclerenchyma cells, especially in the cortex tissue, enhance its protective barrier function.

Microscopic analysis has revealed that the medicinally important *T. terrestris* is exposed to phytocontamination, resulting in notable anatomical changes in its vegetative organs (leaflets, petiole, stem, flower peduncle, and root). Variations, deformations, and in some cases differentiations were observed in all tissue complexes of the plant's vegetative organs (including epidermal cells, stomatal structures, periderm, and xylem and phloem elements). Under contaminated conditions, increased developmental activity in sclerenchyma cells, as well as an increase in the number and size of ergastic substances - particularly druses - was recorded. These changes can be interpreted as an adaptive response of the plant to ecological stress factors, particularly the presence of heavy metals, salts, and other contaminants in the soil (Okcu *et* *al*., 2012; Glavač *et al*., 2017; Hlihor *et al*., 2022; Nouha *et al*., 2024; Ullah *et al*., 2024). The intense formation of sclerenchyma and crystals can be associated with the activation of the plant's defense mechanism. It is known that one of the functions of druses is the neutralization or storage of toxins, and under phytocontamination conditions, the increased intensity of druse formation in the plant can be associated with this process. The micrographs confirm these anatomical changes and clearly show that a pathological condition has developed at the tissue level in the plants exposed to the effects of ecological toxicants. These results demonstrate the plant's phytoremediation potential and its anatomical adaptability to toxic environments. The microphotographs (Fig. 9-14) are a visual expression of these changes and prove that the anomalous inclusions formed in the plant under polluted conditions permeate into all tissue layers. In the epidermal layer, the cell shape changes, and the stomata are deformed (Fig. 9, 10, 12D). This is an early indicator of disruption in gas exchange processes.

In the longitudinal section of the stem (Fig. 13), the structure of the cortical parenchyma cells is unevenly developed, and xylem and phloem elements are distributed disproportionately, indicating difficulties in the transport of water and nutrients. In the stem, the detoxification functionality of druses can be characterized by the pigmentation of the druses (Tutuncu Konyar *et al.,* 2014; Asiminicesei *et al.,* 2024). The accumulation of anomalous intercellular and intracellular inclusions (presumably phytotoxins) in the epidermal and subperidermal zones results in the disruption of cell structures, cytoplasmic hardening, and changes in the vacuoles.

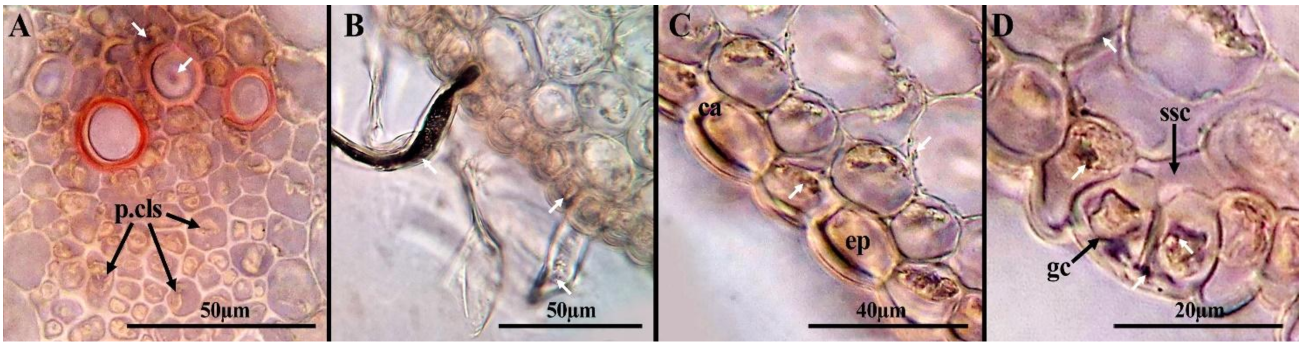

Fig. 9. *Tribulus terrestris.* Anomalous inclusions (likely toxins) in the peduncle tissues (marked with white arrows). **A** - in the vascular bundle, **B** - in the trichomes, **C** - in the epidermic and parenchyma, **D** - in the stomatal apparatus. **p.cls**-pigmented callose (likely phytotoxic callose); **ep**-epidermis; **ssc**-sub-stomatal cavity; **gc**-guard cell.

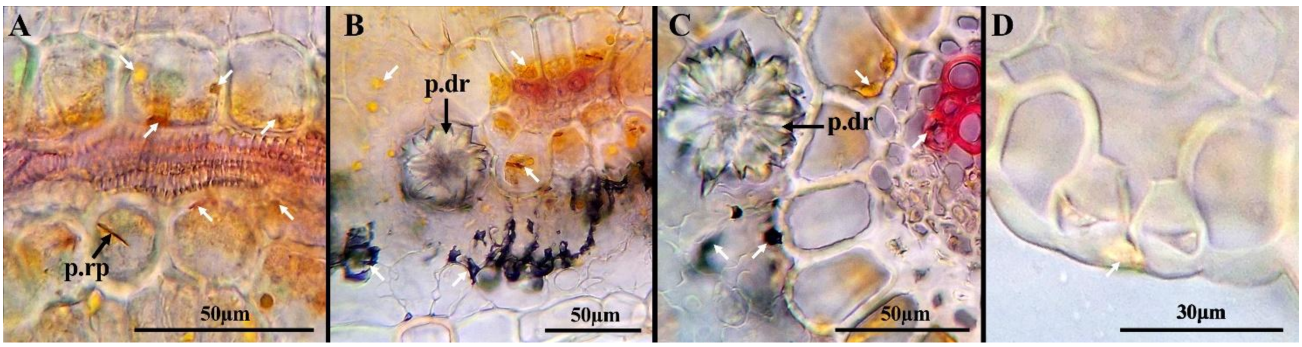

Fig. 10. *Tribulus terrestris.* Anomalous inclusions (presumably toxins) in the leaf mesophyll (marked with white arrows). **A, B -** in the palisad parenchyma and bundle sheath cells, **C -** in the central vascular bundle and nearby tissues, **D -** in the stomatal apparatus. **p.rp**-pigmented raphide (presumably raphide toxin); **p.dr-**pigmented druse (presumably druse toxin).

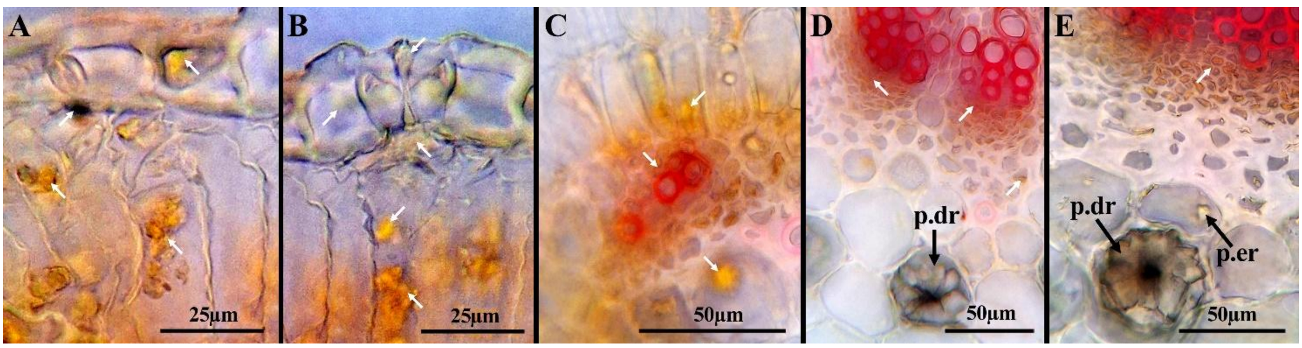

Fig. 11. *Tribulus terrestris.* Anomalous inclusions (possibly toxins) in the petiole (marked with white arrows). **A**, **B** - in the epidermis and columellar cells, **C** - in the in the peripheral vascular bundle, **D**, **E** - in the central vascular bundles and nearby parenchyma tissue. **p.dr-**pigmented druse (likely druse toxin); **p.er-**pigmented ergastic substances (presumably ergastic toxins).

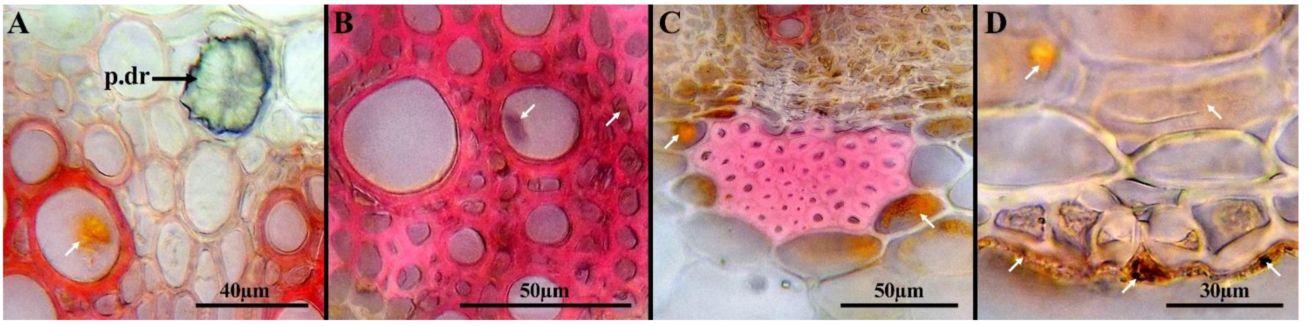

Fig. 12. *Tribulus terrestris.* Anomalous inclusions (presumably toxins) within the internal structure of the stem in transverse section (marked with white arrows). **A** - in the perimedullary region, **B -** in the xylem tissue, **C** - in the parenchyma cells nearby sclerechyma tissue, **D** - in the stomatal apparatus and cortical parenchyma. **p.dr**-pigmented druse (presumably druse toxin).

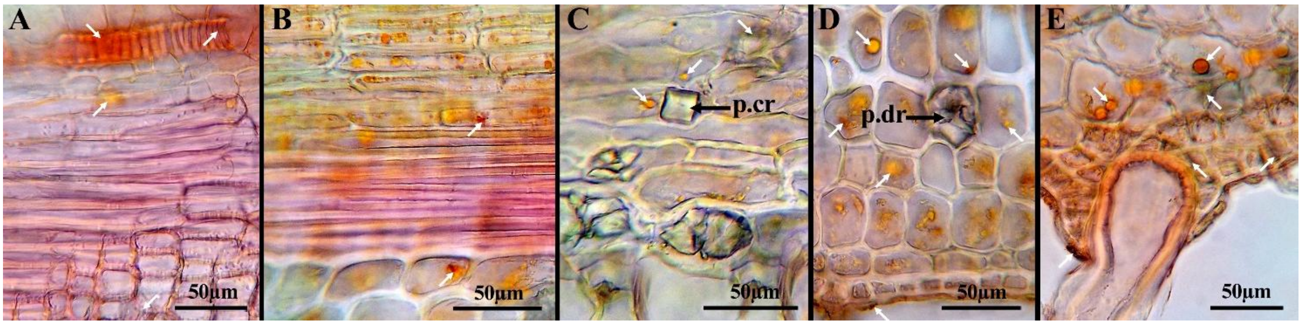

Fig. 13. *Tribulus terrestris.* Anomalous inclusions (likely toxins) observed in the stem in longitudinal section (marked with white arrows). **A** - in the xylem tissue, **B** - in the phloem, **C**, **D** - in the cortical parenchyma, **E** - in the subepidermal region and epidermis. **p.cr** -pigmented crystal (presumably crystal toxin); **p.dr**-pigmented druse (presumably druse toxin).

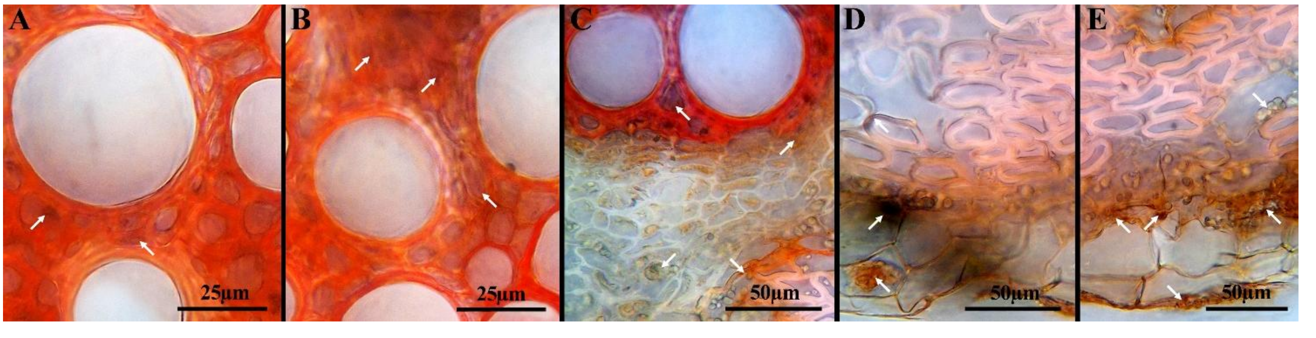

Fig. 14. *Tribulus terrestris.* Anomalous inclusions (presumably toxins) in the root (marked with white arrows). **A, B** - in the xylem tissue, **C** - in the phloem and surrounding tissue, **D, E** - in the cortex and periderm.

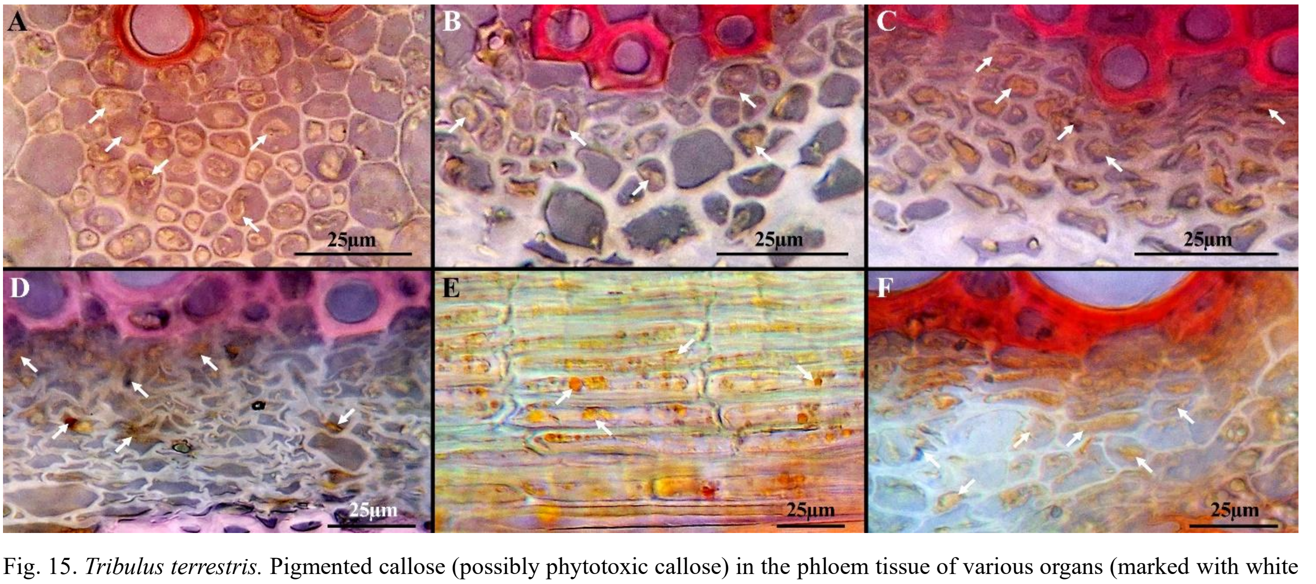

Fig. 15. *Tribulus terrestris.* Pigmented callose (possibly phytotoxic callose) in the phloem tissue of various organs (marked with white arrows)**. A -** peduncle**, B -** leaf, **C -** petiole, **D -** stem (transverse section), **E** - stem (longitudinal section), **F** - root.

In general, callose accumulates in the plasmodesmata and sieve-like tubes of the phloem under stress conditions, due to phytocontamination. In areas exposed to industrial pollution, such as the Aghdam Industrial Park, the accumulation of callose can intensify as a response to heavy metals. In particular, the accumulation of pigmented callose (presumably of phytotoxic origin) in the phloem of *T. terrestris* is regarded as a toxic stress response resulting from phytocontamination, associated with a reduction in translocation and activation of defense mechanisms (Fig. 15). Microscopic observations revealed the formation of darkly pigmented spots within the callose structures, which can be explained by the adsorption of industrial heavy metal ions onto the callose matrix, leading to the formation of partially necrotic callose complexes (Nedukha, 2015; Wang *et al*., 2021).

The results indicate that during the use of *T. terrestris* specimens exposed to phytocontamination as medicinal raw material, their phytochemical composition and toxicological profile must be carefully investigated (Hussein *et al.,* 2009; Tawfeeq *et al*., 2024). The pharmacists and pharmacognosy experts should conduct additional research to determine the safety and therapeutic efficacy of such samples. Our results prove that when *T. terrestris* is exposed to phytocontamination, adaptive mechanisms at the anatomical level are activated, particularly in the mechanical tissue, crystal formation, vascular tissues, and stomata, such changes formed under ecotoxic conditions affect not only the structural adaptation of these plants but also its phytotherapeutic potential directly and indirectly. The accumulation of anomalous inclusions in the epidermis and vascular tissues, as well as the observed increase in ergastic substances (particularly calcium oxalate crystals), can affect the synthesis and metabolic balance of bioactive compounds (saponins, flavonoids, and alkaloids) (Asgari Lajayer *et al*., 2017; Yadav *et al.*, 2021; Sardarova, 2022). Such changes could alter the quality and safety indicators of the plant material used in phytotherapy. As a result of toxin accumulation, the likelihood of toxic substances present in the extracts obtained from the plant increase, limiting its therapeutic use and affecting its pharmacological efficacy. Therefore, it is necessary to separately assess the pharmacological-anatomical and phytochemical profiles of samples growing under ecological stress.

Elkamali *et al*., (2016) has identified similar characteristics during his comparative anatomical analysis of the leaflets and stems of *Tribulus longipetalous*, *T. pentandrus*, and *T. terrestris* plant species collected from the Khartoum region of Sudan. In the stem, the presence of a uniseriate epidermis, collateral vascular bundles, the positioning of pericyclic fibers on these bundles, and the structure of the bundles consisting of primary and secondary xylem and phloem were noted by the authors. Our findings show that, among the 3 species compared, *T. terrestris* has the lowest trichome density on stem and leaf surface. Furthermore, unlike the other two species, sclerenchymatic fibers were not observed in the pith of the stem in this species. It is noted that the leaflet has a dorsiventral structure, the presence of cranial anatomical features, and the formation of druse crystals.

Gabr & Ragab (2023) have studied the leaf and stem anatomies of 12 species belonging to 3 genera of the Zygophyllaceae family. Distinctive features were identified in the representatives of the *Tribulus* genus from the plants collected from the eastern region of Saudi Arabia, including the presence of a bundle structure in the stem, the cortex being composed of parenchyma tissue, and the number of vascular bundles located centrally in the petiole. Additionally, the presence of a trichome covering on the epidermis of the leaf, petiole, and stem can be observed from the presented photomicrographs. The distinctive features have been identified for the genus as determined by us in the *T. terrestris*.

Nikolova & Vassilev (2011) have analyzed the influence of the anatomical features of the root, stem, and leaves of this plant on its ecological adaptation, and xeromorphic adaptation characteristics of the leaf, the isobilateral and amphistomatic structure of the mesophyll, the presence of Kranz anatomy, the small size and low density of stomata, and the presence of numerous unicellular trichomes on the lower epidermis have been identified. Authors emphasize that the vascular tissues in the root and stem are well developed and a strong sclerification occurs. In our study, it was observed that the periphery of the leaflet consists of complete palisade cells on the dorsal side, and on the ventral side, in the subepidermal region, a single row of spongy cells is located, followed internally by palisade cells that are positioned adjacent to the bundle sheath cells surrounding the vascular bundle. Therefore our studies demonstrate that the leaflet of *T. terrestris* exhibits an even more differentiated dorsiventral structure.

Semerdjieva (2011) conducted an anatomical analysis of leaf samples of *T. terrestris* collected from three different populations in the Trans-asian floristic region. In general, the epidermal cells in the examined samples were small and isodiametric in shape, with an amphistomatic leaf structure, the formation of long trichomes in the epidermis, a thick cuticle, strong development of palisade parenchyma located on both the dorsal and ventral sides. The findings reported by other authors are relatively consistent with our findings. In terms of environmental tolerance, different structural characteristics (differentiation and modification processes) were observed, both due to the different climatic conditions of different countries and the fact that our study was conducted under phytocontamination conditions.

Conclusions

For the first time in *Tribulus terrestris*, a modified dorsoventral and Kranz-type leaf anatomy has been identified. In the leaf mesophyll, isodiametric parenchyma surrounding the central vein, a fully developed palisade layer on the dorsal side, and a complex arrangement of both palisade and spongy parenchyma on the ventral side indicate the formation of a Kranz structure. This anatomical organization represents a key anatomical-physiological adaptation mechanism, reducing water loss under xerophytic conditions and maintaining photosynthetic efficiency.

It was also newly observed that subepidermal cells with a columellar structure develop beneath the petiole epidermis. In both leaves and petioles, collenchyma cells located near the vascular system undergo lignification, resulting in their transformation into sclerenchyma. The formation of this transitional mechanical tissue is interpreted as a local structural adaptation under ecological stress conditions.

Received: 13-05-2025 Revised: 04-07-2025 Accepted: 12-07-2025 Online: 02-12-2025

During this first ecological-anatomical study of the Azerbaijani flora, grouped sclerenchyma structures were identified in the cortical zone of the stem and root organs of *T. terrestris*, while pigmented druses (presumably druse-toxins) were observed in the pith and cortex. In leaflets, the presence of Kranz anatomy, consistent with C4-type photosynthesis, and pigmented raphides (presumably raphide-toxins) in the bundle sheath cells serve as anatomical indicators of the species' high tolerance to ecological pressure.

In specimens collected from contaminated areas of Karabakh, the formation of pigmented callose in the phloem tissue (likely indicative of phytotoxic stress) was observed for the first time. Based on microscopic analyses, the accumulation of pigmented callose under ecotoxic stress is considered an anatomical marker of necrotic structural changes.

Changes observed in the epidermis, vascular system, sclerenchyma, and crystal structures under phytocontamination conditions demonstrate the plant's active defense response to toxic stress in contaminated environments. Additionally, the local accumulation of ergastic and constitutional substances in the organs reflects the species' metabolic and phytotherapeutic potential. However, the accumulation of anomalous inclusions (presumably toxins) in tissues used for medicinal purposes represents a biosanitary risk and necessitates toxicological evaluation. The results of this study provide important preliminary scientific and practical data for pharmaceutical toxicology research.

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