Anatomical study of Japanese Knotweed (*Reynoutria japonica* Houtt., Polygonaceae): key insights for identifying an invasive species with medicinal traits

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The macroscopic and microscopic characteristics of the above- and underground organs, along with features of powdered plant material of the invasive species *Reynoutria japonica* Hout., Polygonaceae, as a potential raw material for the pharmaceutical industry, were examined. No specific characteristics of the stem, root, and rhizome were identified that could independently allow for species determination. However, identifying features include the leaf midrib with its protrusions, vascular bundle arrangement and trichomes. The leaf powder is characterized by presence of epidermal cells with wavy walls, anisocytic stomata, numerous crystal druses, and conical unicellular trichomes. The elements present in the rhizome and root powder are crystal druses, cork fragments, vessels, macrosclereids, and grouped starch grains. The use of *R. japonica* as medicinal raw material could help to reduce the negative impact of this species on habitats and biodiversity in Serbia.

Keywords: *Fallopia japonica*, invasive plant, microscopy, identification, rhizome, midrib

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1. INTRODUCTION

Japanese knotweed, *Reynoutria japonica* Houtt., Polygonaceae, (synonyms: *Fallopia japonica* (Houtt.) Ronse Decr., *Polygonum cuspidatum* Siebold & Zucc.), is a perennial, semi-woody plant (hemicryptophyte) native to East Asia. It differs from other allochthonous species of this genus that occur in Serbia (*R. sahalinensis* (F. Schmidt) Nakai and *R. x bohemica* Chrtek & Chrtková) by the presence of red spots on the stem, a truncate leaf base and pointed leaf tips. In Serbia almost exclusively female individuals (with sterile stamens) are present in the distribution area - sexual reproduction does not occur (Stojanović et al., 2021).

R. japonica was introduced to Europe as an ornamental plant in the middle of the 19th century. At the beginning of the 20th century, the number of naturalized populations increased sharply, which was a consequence of its frequent planting as an exotic, fast-growing ornamental plant. In the second half of the 20th century, it became established in the Balkans, and towards the end of the century its presence was confirmed in ruderal habitats of Belgrade. Today, the species is widespread in Serbia. It is registered in 270 georeferenced locations. (Jovanović et al., 2022).

The International Union for Conservation of Nature (IUCN) has classified *R. japonica* as one of the 100 most invasive species in the world due to its ability to rapidly colonize large areas in non-native environments and severely disrupt natural ecosystems. By outcompeting native species for resources, invasive species generally pose a significant threat to indigenous biodiversity. They also cause economic damage, particularly in agriculture and forestry, by reducing yields and increasing management costs. Controlling and preventing their spread is essential for maintaining ecological balance and protecting habitats (Lowe et al., 2000). Once established, it is almost impossible to remove *R. japonica* from a habitat. Due to vegetative propagation, the species has a remarkable regenerative ability. Its root system is extraordinarily well developed, with rhizomes up to 20 m long and penetrating up to 3 m deep into the soil. Even very small rhizome fragments, no larger than 1 cm, can generate new individuals, and if buried, they can even sprout from a depth of 2 m. Seedlings can even penetrate asphalt (Alberternst and Böhmer, 2011; Barney et al., 2006; Stojanović et al., 2021).

However, *R. japonica* is used in traditional Chinese and Japanese medicine and is also a common food plant in Japan (Khalil et al., 2020; Stojanović et al., 2021). The rhizomes and roots of *R. japonica* have been reported to exhibit a spectrum of beneficial properties, such as antitumor, anti-inflammatory and antibacterial activities (Liu et al., 2022).

In China, the use of *R. japonica* dates back to ancient times. It was traditionally prepared as a decoction, powder or infusion. In a monograph on pediatric treatment from 1119, its use for treating fever and night sweats in children is described. Over the centuries, *R. japonica* was included in 77 prescriptions, either alone or as a part of herbal mixtures (Dong et al., 2016).

Since 1977, Japanese knotweed has been included in the Pharmacopoeia of the People's Republic of China (under the name *Polygoni cuspidati Rhizoma et Radix*), and its root is used as an active ingredient in more than 100 formulations (Peng et al., 2013). It is also official according to the Taiwanese pharmacopoeia. The traditional use of this plant is widespread and includes the treatment of diseases of the liver, the cardiovascular system and the endocrine system (Liu et al., 2022).

In the cosmetic industry, this plant is used as a source of yellow pigment, which serve not only as a colorant but also exhibits antioxidant properties helping to protect the skin from oxidative stress and premature aging (Liu et al., 2022).

One hundred and ten biologically active compounds have been isolated and identified from the rhizome and roots of *R. japonica*, the most important of which are anthraquinones, flavonoids and stilbenes (Liu et al., 2022). Anthraquinone emodin has been shown to exhibit antiviral, antibacterial, antiallergic, immunosuppressive and hepatoprotective effects (Dong et al., 2016). The flavonoids in the root of Japanese knotweed include flavonols such as quercetin, quercitrin, isoquercitrin and hyperoside, while the leaves contain reynoutrin (quercetin-3-D-xyloside). The methanol extract of the flowers is rich in rutin, kaempferol and quercetin. These compounds contribute significantly to the antibacterial and antiviral effects, particularly rutin and quercetin, which also protect the cardiovascular system. In addition, the procyanidins isolated from the root bark enhance the overall antioxidant effect. The gallic and protocatechuic acids contained in the plant demonstrate the ability to reduce blood sugar levels and protect against cardiovascular diseases such as atherosclerosis and coronary artery disease (Liu et al., 2022).

The underground organs of *R. japonica* contain relatively high concentrations of stilbenes, including resveratrol and polydatin, both of which have important biological activity. Resveratrol is a natural plant polyphenol found in significant concentrations in grapes, berries and peanuts (Kovarova et al., 2010). Both resveratrol and its glycoside derivative polydatin exhibit beneficial effects on cancer prevention, inhibition of platelet aggregation and antioxidant protection (Liu et al., 2022).

In addition, extracts from this species have been shown to protect crops from fungal infections. The species also has the ability to accumulate significant amounts of zinc, copper and cadmium, making it a potential candidate for the decontamination of heavy metals in soil (Stojanović et al., 2021).

Considering that currently, the rhizome and root of *R. japonica* are a major source of natural resveratrol used in dietary supplements worldwide (Chen et al., 2013) this species with its widespread distribution and high utilization potential represents a remarkable opportunity as a medicinal raw material, and its harvesting could simultaneously mitigate its impact on native ecosystems. It may be more effective to control the spread of this invasive plant species by intensifying its use rather than relying on eradication measures, which can involve significant costs.

The trichome morphology in the section *Reynoutria* in Korea was studied by Moon et al. (2011), who identified three main types of trichomes: conical unicellular trichomes, uniseriate filiform trichomes, and peltate glandular trichomes. Recently, a comparative anatomical and chemical study of Korean native *Reynoutria* species was conducted by Khalil et al. (2020), however, there are certain inconsistencies concerning the anatomy of underground organs in this paper. On the other hand, Vinogradova et al. (2021) conducted a study of the taxonomic characteristics of the vegetative organs of invasive *Reynoutria* species in Central and Northern Europe, focusing on the micromorphological features of the leaf epidermis; however, they did not address internal anatomy, particularly that of underground organs.

In this paper, to characterize the organs of *R. japonica* both macroscopically and microscopically as potential medicinal raw material, we provide detailed information on the anatomy of the aboveground and underground vegetative organs (stem, leaf, rhizome and root) of this species as well as the noteworthy characteristics of the powdered plant material (pulvis).

2. MATERIALS AND METHODS

2.1. Plant material

The underground and above-ground parts of several specimens of *R. japonica* were collected on 09.11.2023. from a ruderal habitat in Serbia, near Kumodraž (on the outskirts of Belgrade) (44°45'02"N 20°29'59"E). The collected material was identified by Assistant Professor Dr. Miloš Zbiljić from the Department of Botany, Faculty of Pharmacy, University of Belgrade. The voucher specimen has been deposited in the Herbarium of the Department of Botany, Faculty of Pharmacy, University of Belgrade (HFF), under the number 4293. The collected plant material was separated into plant organs. The rhizome and root were cleaned of soil and impurities, washed and cut into slices about 1 cm long. One part of the fresh material was left to dry in a dark, well-ventilated place at room temperature, while the other, smaller part, was fixed in 50% ethanol.

2.2. Macroscopic and microscopic analysis

The anatomical features of the plant organs of *R. japonica* were studied in the laboratory of the Department of Botany, Faculty of Pharmacy, University of Belgrade. Temporary and permanent slides of the rhizome, root, stem and leaves were prepared from plant material previously fixed in 50% ethanol. For each organ, six consecutive cross-sections, approximately 15 μm thick, were obtained using the sliding microtome (Reichert, Vienna). For temporary slides, sections were stained with a general reagent according to Tucakov (Kundaković et al., 2017). This complex reagent differentially stains various plant structures according to their chemical nature. Aniline sulfate from reagent stains lignified structures yellow, while Sudan III stains suberized structures reddish-brown. It was also used to determine the characteristic features of powdered plant material. The dried plant organs were ground into a powder and then sampled using the tip of a needle and immersed in the reagent. For permanent slides, sections were decolorized with sodium hypochlorite (NaClO), rinsed, and then stained with safranin (Merck, Darmstadt, Germany) (1% w/v in 50% ethanol) for lignified and alcian blue (Acros Organics, New Jersey, USA) (1% w/v in water) for unlignified structures. Excess stain was removed by passing the sections through a series of ethanol solutions of increasing concentrations (50%, 70%, 96%, and absolute ethanol) (Sani-hem, Novi Sad, Serbia). Finally, the

sections were mounted in D.P.X. mountant (HiMedia Laboratories Pvt. Ltd. Mumbai, India) and analyzed.
Representative samples of the plant material were examined using an Olympus SZ61 stereomicroscope, and the anatomical sections, along with the features of the powdered material, were studied with a trinocular microscope Olympus BX31 and photographed with an Olympus SC30 color camera for light microscopy (Olympus Corporation, Tokyo, Japan).

3. RESULTS

3.1. Stem anatomy

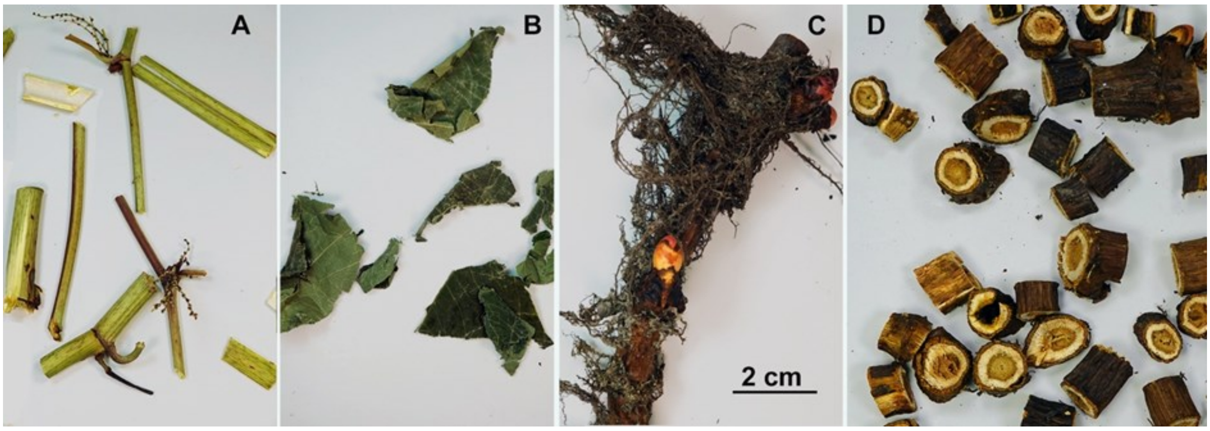

Fig. 1. Desicated vegetative organs of *R. Japonica;* A - annual stem; B – leaf; C - rhizome with roots; D - pieces of rhizome.

Macroscopically, the annual stem of *R. japonica* is glabrous, green with red spots, cylindrical, jointed and hollow (Figure 1A).
Microscopically in a cross-section, sparse unicellular trichomes can be observed at the stem surface. The epidermal cells have a slightly convex outer wall covered with a cuticle. Few layers of collenchyma are located under the epidermis with the lowest transitioning into chlorenchyma. Clusters of calcium oxalate – crystal *druses* (about 50 µm in diameter) are scattered in the cells of the primary cortex parenchyma as well. The pericycle consists of well-developed continuous sclerenchyma, with closed collateral vascular bundles attached to its inner side. The bundles are arranged in a circular pattern, divided by sclerenchymatic parenchyma, leaving practically no medullary rays of parenchyma. Inner parenchyma of the central cylinder is consisting of large round cells with a significant number of crystal druses. In the very center, there is a cavity/canal formed by the rupture of large parenchymatous cells (Figure 2A, 2B).

In the powder obtained from the stem, fragments of sclerenchyma fiber bundles (Figure 2C), clusters of calcium oxalate in the form of crystal druses (Figure 2D), as well as elements of the xylem vessels with annular and pitted thickenings of the cell walls, can be identified (Figure 2E).

3.2. Leaf anatomy

Macroscopically, the leaf is heart-shaped, with entire margins, a truncate leaf base and pinnate-reticulate semicraspedodromous venation (Figure 3A). Leaf lamina is dark green on the upper surface, and grayish-green underside (Figure 1B). Leaf veins are yellowish, prominent on both the axial and adaxial sides. The leaf is glabrous, but non-glandular trichomes can be observed on the main and secondary veins, more frequently on the upper surface under a stereomicroscope (Figure 3B). Longitudinally along the midrib at the abaxial side two protrusion lines are observable. Additionally, small brown spots representing glands are sporadically visible on the lower surface of the leaf (Figure 3C).
Microscopically the cross-section reveals a typical dorsiventral structure of the leaf. The cells of the upper epidermis are twice as large as those of the lower epidermis. The palisade tissue is single-layered and is approximately equally represented as the spongy parenchyma. Between these layers, large cells containing crystal druses are present, spaced apart from one another (Figure 4A). The midrib rises in a triangular shape on the upper side, while it is semicircular on the lower side. Two distinct central protrusions and two faint lateral ones are visible on the lower side. On the upper triangular protrusion, there are conical unicellular trichomes (around 50 µm long) and rare multicellular, uniseriate filiform trichomes (around

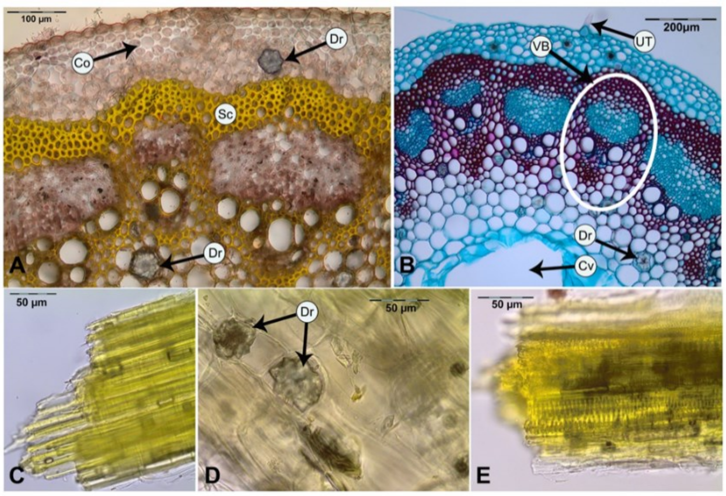

Fig. 2. Anatomical features of the stem of *R. Japonica;* A – cross section of the stem stained with general reagent by Tucakov; B - cross section of the stem stained with safranin and alcian blue; C –sclerenchyma fibers in powdered stem; D - details of the powdered stem with crystal druses. E – fragment of xylem vessels with pitted and annular thickenings. *Abbreviations*: Co – collenchyma; Dr – druse; Sc – sclerenchyma/pericycle; VB - vascular bundle; Ut – uniceluler trichome; CV – cavity/canal in the center of the stem.

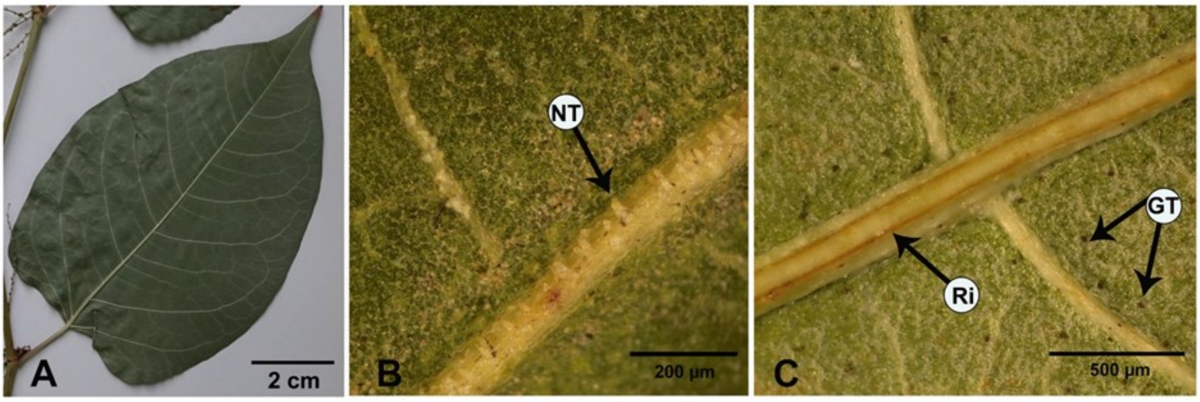

Fig. 3. Leaf of *R. Japonica;* A – shape and venation of the leaf; B – detail of the upper surface with trichomatous prominent midrib; C – midrib at the lower side with scattered glands on the surface. *Abbreviations*: Nt – nonglandular trichomes; Ri – midrib protrusion at lower side; GT – glandular trichomes.

150 µm). At the midrib, the epidermis on both the upper and lower sides is single-layered, composed of rectangular cells with a convex outer wall covered by a thin layer of cuticle. Beneath the epidermis, there are two to three layers of collenchymatous cells, and the majority of the cross-section is occupied by parenchyma with numerous scattered idioblasts containing calcium oxalate druses (app. 50 µm in diameter). Four collateral vascular bundles are arranged in a circular pattern, with the xylem oriented towards the center of the section. The largest bundle, crescent-shaped, is located on the lower side, with a smaller one opposite it on the upper side, and two small bundles on the sides. Two layers of lignified fibers are located next to the phloem (Figure 4B). Glandular trichomes of the peltate type with consisting of four-celled head and about 50 µm in diameter, are sporadically observed in the lower epidermis (Figure 4A, 4C).

In the powder obtained from the leaf, the cells of the lower epidermis with wavy anticlinal walls and anisocytic stomata (Figure 4D), druses and tracheids (Figure 4E), and striated conical unicellular trichomes (Figure 4F) can be identified.

3.3. Rhizome and root anatomy

Macroscopically, the rhizome is hard, woody, and predominantly cylindrical. The surface is slightly longitudinally grooved, dark brown in color, covered with numerous adventitious roots (Figure 1C). When sliced into short pieces, at a transversely cut surface, the bark is relatively thin, the wood is broad, white-yellowish, with radial rays. The pith is yellowish-brown, entire or cracked, rarely hollow (Figure 1D).

Microscopically, in cross-section, the outer part of the secondary cortex of the rhizome consists of the periderm with cork, four to five layers thick, which peels off at the periphery upon contact with the soil. Secondary cortex parenchyma contains some intercellular spaces resembling aerenchyma to some extent (Figure 5A). A large number of starch grains (and elaioplasts) are observed in the parenchyma of the secondary cortex at a fresh section stained with a general reagent (Figure 5B), while round bundles of sclerenchyma fibers are visible at different levels adjacent to the secondary phloem (Figure 5A, C). Crystal druses are scattered throughout the cortical parenchyma, but in the medullary rays of the phloem, they are often arranged in radial rows (Figure 5A). In the xylem, tracheids dominate the cross-section, with scattered individual vessels

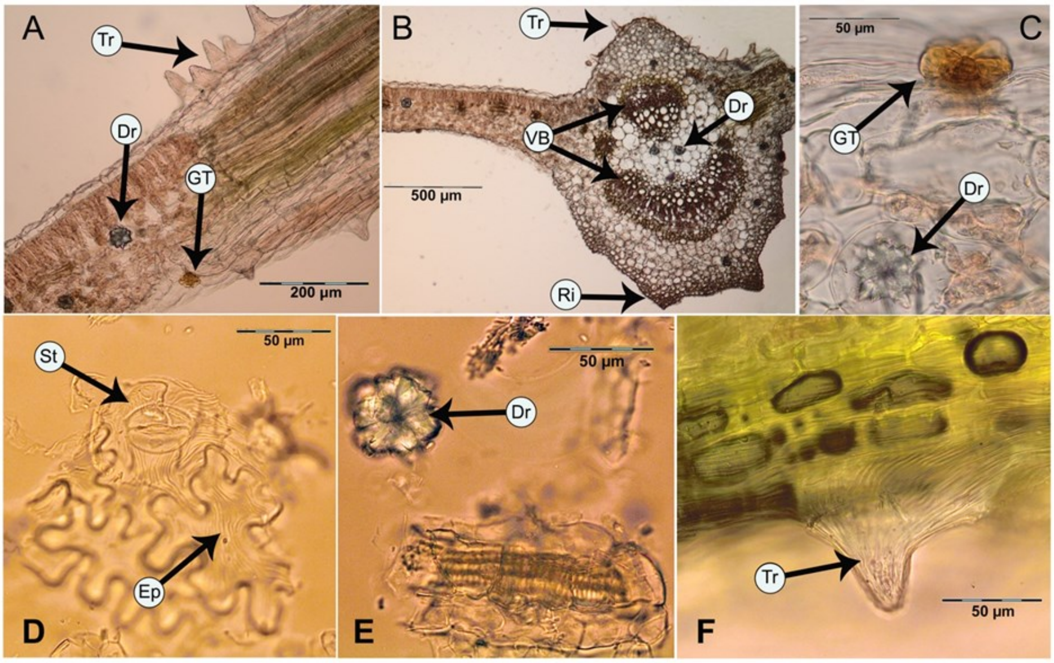

Fig. 4. Anatomical features of the leaf of *R. japonica;* A - cross section of the leaf lamina stained with general reagent by Tucakov; B - cross section of the midrib; C – lower epidermis with glandular trichome; D, E, F – details of the powdered leaf. Abbreviations: Tr – trichome; Dr – crystal druse; GT –glandular trichome/gland; VB – vascular bundles; Ri – midrib protrusion; St – anisocytic stoma; Ep – epidermal cell.

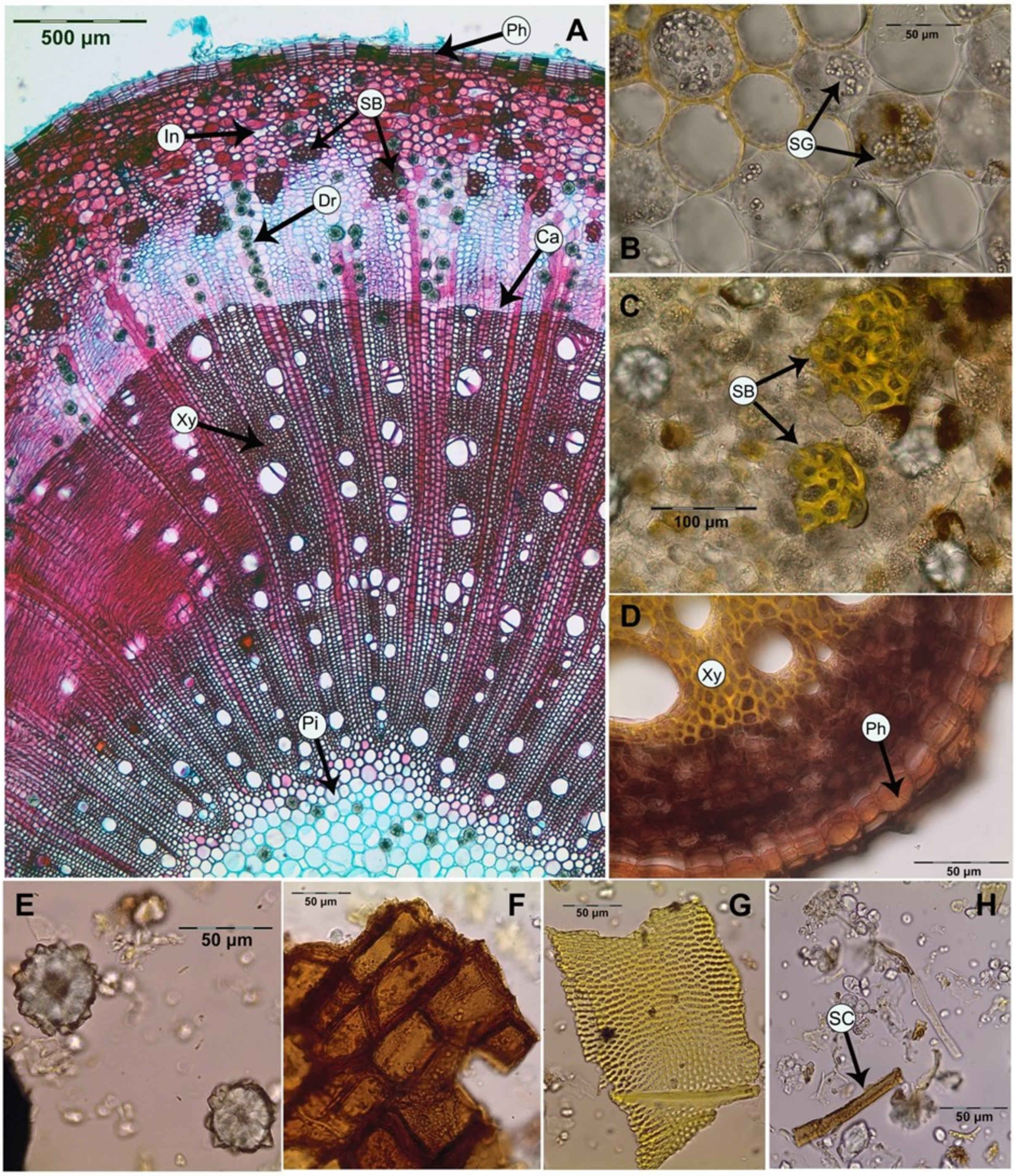

Fig. 5. Anatomical features of underground organs of *R. japonica;* A - cross section of the rhizome; B – secondary cortex parenchyma with starch grains; C – scelerenchyma fiber bundles; D - cross section of the adventitous root; E – crystal druses; F – fragments of cork tissue; G - pitted vessels; H - cylindrical stone cells. Abbreviations: Ph – cork (phelem); In – intercellular space; SG – starch grains; SB – sclerenchyma bundles; Dr – crystal druses; Ca – cambium; Xy – xylem; Pi – pith; St – starch grains in parenchyma; SC - stone cell (macrosclereid).

or, occasionally, groups of two to three. The diameter of vessels is 60–100 µm. Growth rings are hardly visible. The pith parenchyma is intact and rich in idioblasts containing large crystal druses (Figure 5A).

Unlike the rhizome, in cross-section, the adventitious root with secondary growth lacks the pith in the center, with the xylem derived from a radial vascular bundle in the core. The cortical parenchyma is rich in starch grains, with numerous scattered idioblasts containing crystal druses, and the surface is covered by periderm (Figure 5D).

In the powder obtained from the rhizome and roots, clusters of calcium oxalate in form of druses are frequent (Figure 5E), along with large fragments of cork tissue (phelem) with subpolygonal to subsquare cells (Figure 5F), as well as segments of pitted vessels from the xylem (Figure 5G). Cylindrical stone cells, approximately 50 µm in length with simple pits, are present, along with numerous individual round or elliptical starch grains often grouped into compound granules composed of 2–4 units (Figure 5H).

4. DISCUSION

The anatomical features of the above- and underground vegetative organs of *Reynoutria japonica* reflect on the one hand the life form and adaptation to moist (ruderal) habitats, and on the other hand they represent a number of characteristics that are typical for the genus *Reynoutria* and for this particular species. As a hemicryptophyte, *R. japonica* has an annual, aboveground primary stem that is hollow in the center, while its overwintering organ, the perennial rhizome, has features of the secondary stem, as well as adaptations to habitats with moist, soft soils.

The parenchyma of the secondary cortex of the rhizome contains intercellular spaces and numerous starch grains. "Enveloped" starch grains, often grouped into compound granules of 2 to 4 units, are a characteristic feature of the starch of species from the Polygonaceae family (Czaja, 1978). Vessels with a diameter of more than 100 µm are found in the xylem, which is common in the genera *Reynoutria* and *Fallopia* within the family Polygonaceae. The relatively small number of vessels in the xylem is also a characteristic of the genus *Reynoutria* (Schweingruber et al., 2011). In addition, the scattered bundles of lignified fibers in the secondary phloem represent a synapomorphy for the genus *Reynoutria* (Khalil et al., 2020). It is interesting that Khalil et al. (2020) mistakenly described the structure of the rhizome as root anatomy, even though pith parenchyma with numerous crystal druses is present in the center of the cross-section.

However, as features of the rhizome can vary depending on age and environmental conditions of the habitat, they cannot be reliably used as diagnostic features for species identification. One of the most important anatomical diagnostic features of *R. japonica* is the shape of the leaf midrib in cross-section (Khalil et al., 2020). In particular, the prominent triangular protrusion on the upper side of the midrib, together with three to four protrusions on the lower side (two of which are even visible to the naked eye), in combination with the number and position of vascular bundles, serves as a reliable identification feature for the leaves of *R. japonica*. As suggested by Moon et al. (2011) the conical unicellular trichomes and uniseriate filiform trichomes on the midrib as well as peltate glandular trichomes with a four-celled head on the abaxial leaf surface are additional anatomical characteristics that can aid in the identification of *R. japonica*.

Since the rhizome and roots of this species are widely used in traditional medicine in East Asia and serve as a source for the extraction of active ingredients, the combined presence of various structures observed in the powdered drug may be important for quality control. For the identification of rhizome and root powder, it is necessary to microscopically identify the specific pitted vessels with a diameter of about 100 µm, crystal druses, sclereids, granules with 2–4 starch grains and segments of cork tissue.

5. CONCLUSION

Reynoutria japonica Houtt. (Polygonaceae), the highly invasive species in Serbia was collected in a typical ruderal habitat in Kumodraž, a southern suburb of Belgrade. Given the importance of the species as a source of resveratrol and other biologically active compounds, the plant organs were dried and examined macroscopically and microscopically using stereo and light microscopy to determine characteristics important for the identification of the plant as a potential medicinal raw material.

Macroscopically, the green, hollow annual stem lacks specific identifying features. However, the leaf, even if fragmented, can be identified by the yellow midrib, which is raised on the upper side with conical, non-glandular trichomes. On the underside, the midrib has two raised distinctive lines, while the flat area of the leaf contains numerous small, dark, glandular trichomes, while non-glandular trichomes are absent. The most reliable microscopic features for leaf and species identification are the shape of the midrib in cross-section and the arrangement of the vascular bundles.

Characteristic fragments in the stem powder are sclerenchyma fibers, xylem elements with annular and pitted thickenings of the cell walls as well as groups of calcium oxalate crystals in the form of large druses. In the leaf powder, the epidermal cells of the abaxial surface with waved anticlinal walls and anisocytic stomata, numerous crystal druses and conical unicellular trichomes with striated cuticular thickenings are common features.

When cleaned from adventitious roots and sliced, rhizome with its thin bark and broad white-yellowish wood macroscopically resembles many similar drugs. Microscopically, noticeable features include pith rays filled with rows of crystal druses, the wood consisting mainly of tracheids and numerous bundles of sclerenchyma fibers. Both the rhizome and the roots contain starch and numerous calcium oxalate druses. Characteristic fragments of the rhizome and root powder include crystal druses, cork fragments, pitted xylem vessels and cylindrical sclereids as well as simple starch grains, often arranged in pairs, triples or quadruples. Harvesting and utilizing the rhizomes and roots of *R. japonica* as medicinal raw material would also help to mitigate the negative impact of this species on biodiversity and ecosystems in Serbia.

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CONFLICT OF INTEREST

The authors declare that they have no financial and commercial conflicts of interest.

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