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Original Article

Microscopic characterization and HPTLC of the leaves, stems and roots of *Fadogia agrestis* – an African folk medicinal plant

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ABSTRACT

Fadogia agrestis Schweinf. ex Hiern (*Vangueria agrestis* (Schweinf. ex Hiern) Lantz), Rubiaceae, is an African traditional medicinal plant also used as a dietary supplement in the US. The present paper is the first report of the pharmacognostic study of the leaf, stem and root of *F. agrestis* by microscopy, HPTLC and total phenolic/flavonoid content analyses. Noteworthy microscopic features that can help in identification and quality control are septate and lignified non-glandular trichomes on leaf and stem epidermises, paracytic stomata on leaf abaxial epidermis, numerous cells containing yellow substances of presumably phenolic compounds in leaf and stem, calcium oxalate druses and prismatic crystals in leaf and styloids in stem, primary phloem fibers in stem, brachysclereids in stem and root, spherical starch grains in root, and vessels with vestured pits and simple perforated end walls. In addition to microscopy, a total phenolic/flavonoid content determination and an HPTLC method were also developed for rapid chemical fingerprint analyses of *Fadogia* samples and dietary supplements.

Introduction

Fadogia agrestis Schweinf. ex Hiern, Rubiaceae, is a medicinal plant distributed from west tropical Africa to South Sudan. It is a dwarf shrub of up to 30 cm tall with woody stems and rootstocks. It has been reported to be used in the folk medicine of Africa as a diuretic and aphrodisiac as well as for the treatment for fever, kidney pain, diarrhea, stomach ache, blennorrhoea and toothache (Yakubu et al., 2005; Odugbemi, 2008).

Lantz and Bremer (2005) have transferred the species *Fadogia agrestis* to genus Vangueria based on phylogenetic analysis. According to these authors, the species *F. agrestis* was previously misplaced in *Fadogia* as it differs from the other species of *Fadogia* by its large ovate to triangular calyx lobes and consistently paired leaves (*Note*: the specimens used in the present study, however, had both ternate and paired leaves on the same twigs as seen in the type specimens). Therefore, the current accepted name for the species is *V. agrestis* (Schweinf. ex Hiern) Lantz (Lantz and Bremer, 2005; WCSP, 2017). However, this species is addressed in this paper by its popular synonym *F. agrestis*. The genus *Fadogia* is represented by about forty species, and the genus *Vangueria* consists of about 55 species.

F. agrestis is traded for its medicinal value. Several dietary supplement products purported to contain *F. agrestis* as a main ingredient are sold in the market. According to van Andel et al. (2012), thousands of kilograms of bark materials of *F. agrestis* are exported from Ghana to the USA, Ivory Coast, Nigeria and Burkina Faso markets.

There are no studies on the anatomy, authentication or quality control of *F. agrestis* available so far. In fact, no such study is available in the literature for any species in the genera *Fadogia* and *Vangueria*. The aim of the present study, therefore, was to provide an accurate analysis of the anatomy and micromorphology of *F. agrestis*. In addition to being the first microscopy report for the species, the paper also presents potential diagnostic characters that can help in taxonomy, future comparisons with other species and pharmacognosy and quality control of the botanical.

Several species of Rubiaceae have been reported to have toxic properties. *Fadogia homblei* De Wild. has long been known for its toxicity to domestic ruminants causing gousiekte, resulting in sudden death from cardiac failure of affected ruminants. The toxicity might be due to the presence of a polyamine, pavettamine (Van Elst et al., 2013). Two taxa of Fadogia, namely *F. homblei* De Wild. and *F. odorata* K.Krause (synonym of *F. stenophylla* subsp. *odorata*

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(K.Krause) Verdc.), and two Vangueria species, namely *V. thamnus* (Robyns) Lantz and *V. pygmaea* Schltr., have been included in the FDA Poisonous Plant Database. In the present study, the phenolic and flavonoid contents of twelve samples of *F. agrestis* were determined. A simple and fast HPTLC method was also developed for chemical fingerprint analyses of *F. agrestis* samples as well as seven dietary supplement products purported to contain *F. agrestis*. These methods are useful in establishing the quality and authenticity of herbal products claiming to contain *F. agrestis*.

Materials and methods

Plant materials and chemicals

Freshly collected samples of twigs with intact leaves (NCNPR # 20086, 20089) and stems (# 20087, 20092) as well as roots (# 20088, 20093) of *Fadogia agrestis* were obtained from Yaounde and Betare Oya in Cameroon. Representative samples and voucher specimens of these collections were deposited in the Botanical Repository of NCNPR in the University of Mississippi. The plant specimens were botanically identified by their morphological features (Oliver, 1877) and compared with herbarium specimens (E. Petit 5289, June 1964) collected from the region and deposited in the Cameroon National Herbarium (SRFK # 5289). Digital images of the original type specimens of *F. agrestis* (G. Schweinfurth 1312 and 1371, collected on 31st May 1869 and 4th April 1869, respectively) were accessed at the Royal Botanic Gardens, Kew website (http://apps.kew.org) and compared.

Folin–Ciocalteu reagent, gallic acid and quercetin standards, aluminum chloride and sodium carbonate were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Water was purified using a Milli-Q system (Millipore). Eight compounds, *i.e.* 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl pomolic acid (**1**), 2,4′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside (**2**), hyperoside (**3**), geniposidic acid (**4**), nicotiflorin (**5**), narcissin (**6**), randiasaponin IV (**7**) and rutin (**8**), which were used as reference standards in HPTLC analysis, were isolated from *F. agrestis* and identified in our lab.

Sample preparation for light microscopy

Fresh samples of leaves, stems and roots were fixed in FAA (Johansen, 1940) for two days and free-hand sections were prepared using razor blades. The sections were stained with toluidine blue (O'brien et al., 1964) or double stained with Astra blue and basic fuchsin (Kraus et al., 1998) and mounted on glass slides in a drop of glycerin. Some of the whole leaves were bleached in full strength household bleach (5% sodium hypochlorite) for 8–10 h or until cleared to translucent to study the epidermis, stomata, and crystals. Some of the FAA-fixed specimens were washed in distilled water and then dehydrated using a series of ethanol solutions (30, 50, 70, 95 and 100%) before passing through a graded series of xylene:ethanol solutions and then to 100% xylene. The specimens were then embedded in paraffin (Chamberlain, 1901; Ruzin, 1999) and specimen blocks were prepared using a Leica EG1150H embedding system (Leica Microsystems, Wetzlar, Germany). Sections 7–12 μm thick were prepared using a Leica RM2255 rotary microtome (Leica Microsystems, Wetzlar, Germany). Paraffin from the sections was removed using xylene. The sections were then rehydrated, stained with astra blue/basic fuchsin (Kraus et al., 1998), and mounted on glass slides in a drop of glycerin. The mounted sections were then analyzed and imaged using Nikon E600 and Nikon E600 POL microscope equipped with Nikon DS-Fiv camera systems and Nikon Elements imaging software (Nikon Inc., Tokyo, Japan).

Sample preparation for scanning electron microscopy

Fresh samples of leaves, stems and roots were fixed overnight in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4) and washed in distilled water. The samples were then dehydrated by passing through increasing concentrations of ethanol in water as per a standard procedure (Hayat, 2000). The specimens were then dried in a Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany) supplied with liquid CO_2. The fully dried samples were mounted on aluminum stubs using glued carbon tabs and sputter-coated with gold using a Hummer 6.2 sputter coater (Anatech USA, Union City, CA, USA). The specimens were analyzed and imaged using a JSM-5600 Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan).

Histochemical analyses

Freshly prepared sections of leaves, stems and roots were used for histochemical tests. The following standard solutions were employed: phloroglucinol/HCl to detect lignin (Sass, 1951); sudan III for lipophilic substances (Foster, 1949); ferric chloride (Johansen, 1940), potassium dichromate (Gabe, 1968) and potassium hydroxide (Costa, 1982) tests for phenolic contents; and 1% iodine solution was used to detect starch grains (Berlyn and Miksche, 1976).

Micrometrics

Measurements and numerical data for various microscopic features such as size of stomata, stomatal index, and sizes of cells in various tissues were prepared using standard methods. Ten to thirty cells/structures were measured from multiple sections/samples for each species and the measurements were averaged. Measurements were prepared and analyzed using Nikon Elements imaging software.

Sample preparation for total phenolic/flavonoid content and HPTLC

Dried and milled twelve samples of *Fadogia* plant (about 2 g) and seven dietary supplements purported to contain *F. agrestis* (1.5–2 g) were exhaustively sonicated in 80% methanol for 30 min and centrifuged at 983 × *g* for 10 min. The supernatants were pooled and the solvent was added to the residue for further extraction, following the same procedure four more times. The extract was filtered and dried in the speed vacuum to make dried powder. The dried weight ranged from 31 to 278 mg for plant samples and 87 to 444 mg for dietary supplements, respectively.

Estimation of the extracts for total phenolic content using the Folin–Ciocalteu method

The total phenolic contents of the different extracts were determined by a slightly modified Folin–Ciocalteu method (Folin and Ciocalteu, 1927). Briefly, 0.2 ml of sample extract (1 mg/ml) were made up to 0.8 ml with distilled water, then mixed vigorously with 0.2 ml of Folin–Ciocalteu reagent (1:1 diluted with water) for 5 min, followed by the addition of 1 ml of 8% (w/v) sodium carbonate and 1 ml of water. The mixture was allowed to stand for a further 30 min at room temperature in the dark, and absorbance was measured at 765 nm on a UV/Vis spectrophotometer (Evolution 220, Thermo Scientific, USA). The calibration curve was prepared with 0.1–150 μg/ml of gallic acid solution followed by the same procedure given above. The total phenolic content was calculated from the calibration curve (0.1–150 mg/l, $Y = 0.006x + 0.022$, $R^2 > 0.99$), and the results were expressed as mg of gallic acid equivalent (GAE) per g dry weight of plant extract. All determinations were carried

out in triplicate and results were expressed as mean ± standard deviation (S.D.).

Determination of total flavonoids using the $AlCl_3$ colorimetric method

The total flavonoid content of crude extract was determined by modified aluminum chloride colorimetric method (Woisky and Salatino, 1998). In brief, 1 ml of crude extract (1 mg/ml methanol) was mixed with 1 ml of 2% (w/v) $AlCl_3$ solution, and the mixture was incubated at room temperature for 60 min. The supernatant was taken and the absorbance was measured at 440 nm. The total flavonoid content was calculated from a calibration plot ($Y = 0.0453x + 0.0092$, $R^2 > 0.99$), which was prepared with quercetin solutions with a range of 0.1–40 μg/ml, and the result was expressed as mg quercetin equivalent (QE) per g dry weight of plant extract. All determinations were performed in triplicate and results were expressed as mean ± standard deviation (S.D.).

High performance thin layer chromatography (HPTLC) analysis

The extracts (5 mg each) of *Fadogia* plant materials and dietary supplement products were dissolved in 1 ml methanol/water (8/2), sonicated for 10 min and centrifuged at 983 × *g* for 5 min. The supernatants were used for HPTLC analysis, along with the reference standards prepared in methanol at 1 mg/ml. The samples were applied onto the HPTLC plates (Merck Silica gel 60 F_{254} 20 cm × 10 cm) using a Camag ATS 4 sampler with a Camag Microlitre syringe in band form with band length 8.5 mm, track distance 11.3 mm, distance from side edges 15 mm, and distance from the bottom edge 8 mm. A constant application rate of 150 nl/s was employed. The mobile phase consisted of chloroform/ethyl acetate/methanol/formic acid (3/6/2/0.8, v/v/v/v) was used for development. Linear ascending development was carried out in a twin trough glass chamber (20 cm × 10 cm; Analtech, USA) saturated with the mobile phase using a Camag Automated Development Chamber ADC2. The optimized chamber saturation time for the mobile phase was 20 min at room temperature (21 ± 1 °C) at a relative humidity of 35 ± 5%. The length of chromatogram run was 70 mm. Subsequent to the development, HPTLC plates were dried in the ADC2 chamber with the aid of dry heating air for 5 min. After drying, images were recorded with a CAMAG Visualizer under UV 366 nm first. The plates were then dipped into the derivatization reagent (anisaldehyde–sulfuric acid) using a Camag Immersion Device III (dipping time 3 s, dipping speed 5 cm/s) followed by heating at 100 °C for 3 min, and then images were recorded again under UV 366 nm and white light, respectively. The visionCATS software ver. 2.0 was used for device controlling and image processing.

Results

Leaf morpho-anatomy

The leaves of *F. agrestis* (Fig. 1) are covered on both sides with non-glandular trichomes (Fig. 1A–L), more densely on the abaxial side. In surface view of the leaf blade, the adaxial epidermis has straight anticlinal walls (Fig. 1C); whereas the abaxial epidermis has slightly wavy cell walls (Fig. 1D). Cuticle is thin and striated. The leaves are hypostomatic – having stomata only on the abaxial side. The stomata are of paracytic type, with an average length of 28.7 μm and width 6.2 μm. The average stomatal index is 19.3. The non-glandular trichomes are conical with pointed tip and slightly broadened base (Fig. 1E), straight or slightly curved. The trichomes are septate with 3–10 uniseriate cells and measure up to 500 μm long and 35 μm in diameter. The trichome walls are thick, lignified, and warty on the outer surface. Six to nine epidermal cells are arranged in a rosette around trichome base (Fig. 1E). Minute and solitary prismatic crystals of calcium oxalate (Fig. 1F), measuring about 5 × 1.5 μm, are found in the epidermal cells.

In cross-section (Fig. 1G–L), the upper (adaxial) and lower (abaxial) epidermises of leaves are unilayered and covered externally by a thin layer of cuticle. The cells of the upper epidermis are slightly larger than those of the lower epidermis. A noncontiguous layer of hypodermis is present on both sides (Fig. 1G–J). The stomata are confined to the lower surface and are located in the same level as the adjacent epidermal cells. The mesophyll is dorsiventral and formed of 1–2 layers of compactly arranged palisade cells and up to eight layers of spongy parenchyma cells, which are loosely arranged with large airspaces (Fig. 1G–J). Yellow substances (presumably phenolic compounds) are found in almost all the palisade cells and in two to three middle layers of spongy parenchyma cells. Minor collateral vascular bundles of secondary and tertiary veins traverse the mesophyll and are surrounded by a unilayered parenchyma sheath. Oil bodies, reacted positively with Sudan III, are observed in the mesophyll as well as the epidermal cells (Fig. 1H). Crystal idioblasts containing druses of calcium oxalate are occasionally found in the mesophyll, usually near vascular bundles (Fig. 1F, G and J).

In cross-section, the midrib (Fig. 1K and L) is biconvex in outline with a truncate base. The epidermis is unilayered and covered externally by thin cuticle. A major portion of the midrib is composed of ground tissue consisting mostly of parenchyma cells. Clusters of angular collenchyma cells occur along the upper and lower epidermises. The vascular system in the midrib consists of a 'U'-shaped single collateral vascular bundle with xylem toward the adaxial side and phloem toward the abaxial side (Fig. 1K–N). The xylem consists of vessels, tracheids and parenchyma cells; and the phloem consists of sieve cells and phloem parenchyma. A narrow cambiform tissue of about 3-layers occurs between xylem and phloem. An interrupted sheath of fibers, occurring in solitary or in groups of two to five or more, abuts the base of the phloem; fiber walls are thin or moderately thickened and slightly or moderately lignified. In cross section, the fibers are circular, oval or polygonal in shape, with wide lumina (Fig. 1N).

Idioblasts containing phenolic compounds are abundant in the phloem and are also common in the ground tissue and less frequent in the xylem. The phenolic contents react positively in the histochemical tests using ferric chloride, potassium dichromate and potassium hydroxide (Fig. 1K, M and N). Oil bodies are observed in the epidermal cells. Non-glandular trichomes are present. Calcium oxalate druses are occasionally found in the ground parenchyma.

The petiole is short, about 2–3 mm long, stout, elliptical in outline in cross section and has a short wing-like projection on either side (Fig. 1O). The epidermis shows features similar to that of the midrib. Non-glandular trichomes are present. The epidermal layer is followed by up to three layers of angular collenchyma. A major portion of the petiole is made up of parenchymatous ground tissue. A large, 'U'-shaped vascular bundle, similar to that of the midrib, is positioned at the center of the petiole. In addition, a pair of small circular bundles is present on either side of the central bundle. Similar to the midrib, idioblasts containing phenolic contents are found in the phloem, xylem and in the ground tissue. Druses as well as small styloid crystals are found in the ground parenchyma cells.

Stem anatomy

In cross section, the young stems are rectangular in shape and show incipient secondary growth (Fig. 2A and B); whereas the older stems are circular in outline and have well-developed secondary structures (Fig. 2C). Epidermis is unilayered and covered by a thick layer of striated cuticle. Stomata and non-glandular trichomes are present. Hypodermis is two-layered, made up of angular collenchyma. In older stems, the epidermis is pushed

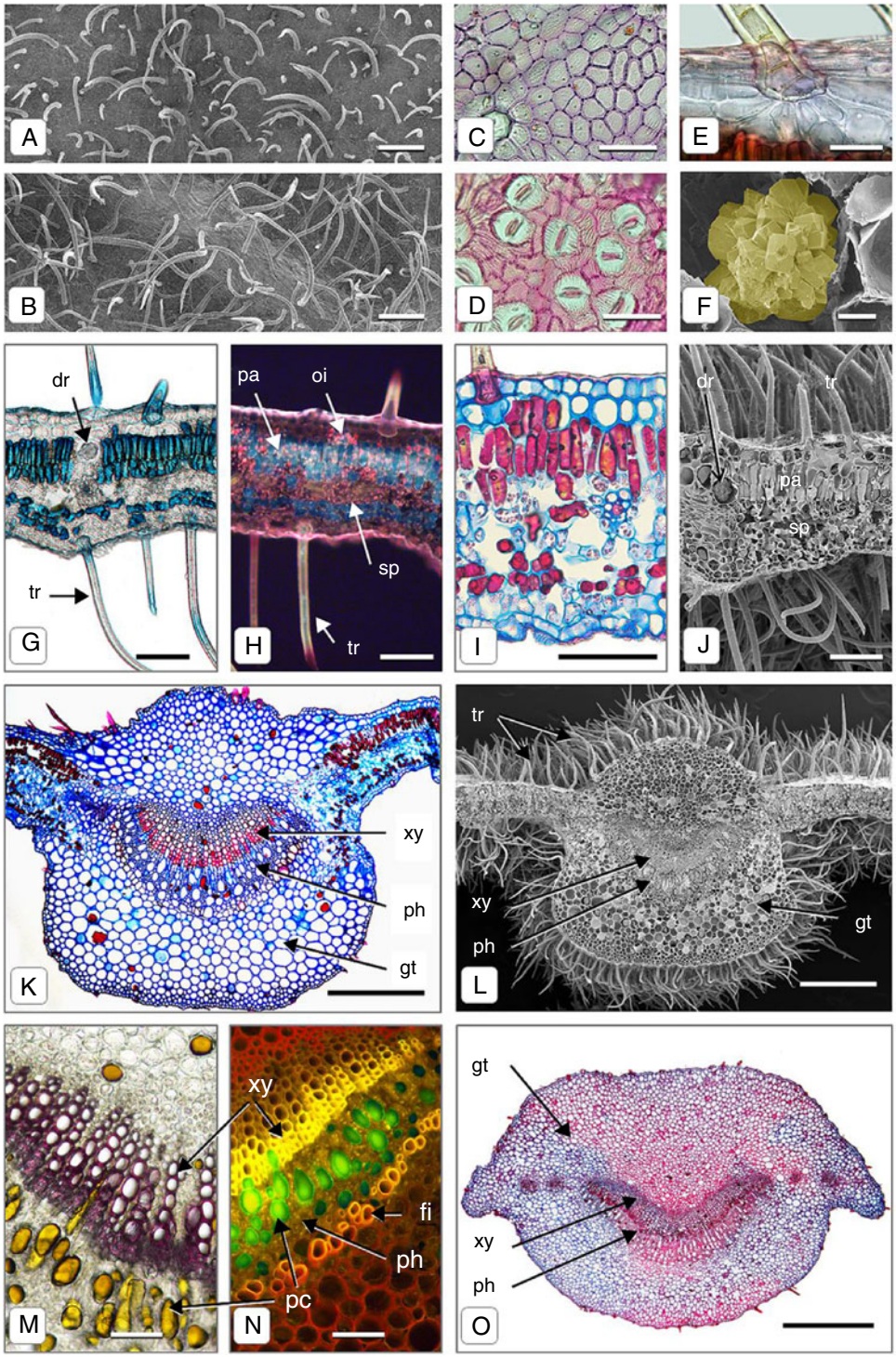

Fig. 1. Leaf anatomy of *Fadogia agrestis* [A, B, F, J, L: SEM images; C–E, G, I, K, M, O: normal light and H, N: fluorescent light microscopy; C, D, I, K, O: astra blue/basic fuchsin; F: colorized SEM; G: stained in toluidine blue; M: phloroglucinol/HCl]. A, C: adaxial and B, D: abaxial leaf surface; E: base of a septate trichome; F: druse crystal in mesophyll; G–J: leaf blade in cross section showing phenolic contents, oil bodies and druse crystals in the mesophyll; K–N: leaf midrib in cross section; M, N: portions of vascular bundle showing xylem, phloem with several idioblasts containing phenolic contents, and medullary fibers; o: petiole in cross section. (dr, druse crystal; gt, ground tissue; fi, fibers; oi, oil bodies; pa, palisade tissue; pc, phenolic contents; ph, phloem; sp, spongy tissue; tr, trichome; xy, xylem). Bars: A, B = 200 μm; C, D = 50 μm; E = 20 μm; F = 10 μm; G–J, M, N = 100 μm; K, L = 500 μm; O = 1 mm.

outwardly due to the formation of cork tissues that consist of radially arranged rectangular cells with brown contents. Phellogen is unilayered, made up of thin-walled cells with no brown inclusions. The parenchymatous cortex is about ten-layered, made up of somewhat tangentially elongated cells. Cortex region is followed by an interrupted ring of sclerenchyma consisting of primary phloem fibers and brachysclereids. In cross-section, the fibers are circular, oval or polygonal in shape, with thickened and lignified walls and narrow lumina. The sclereids are oval or circular in shape, and measure 10–50 μm in diameter; the walls are highly thickened, showing multiple layers appearing as concentric rings, lignified, and pitted with simple or ramiform pits (Fig. 2D–G).

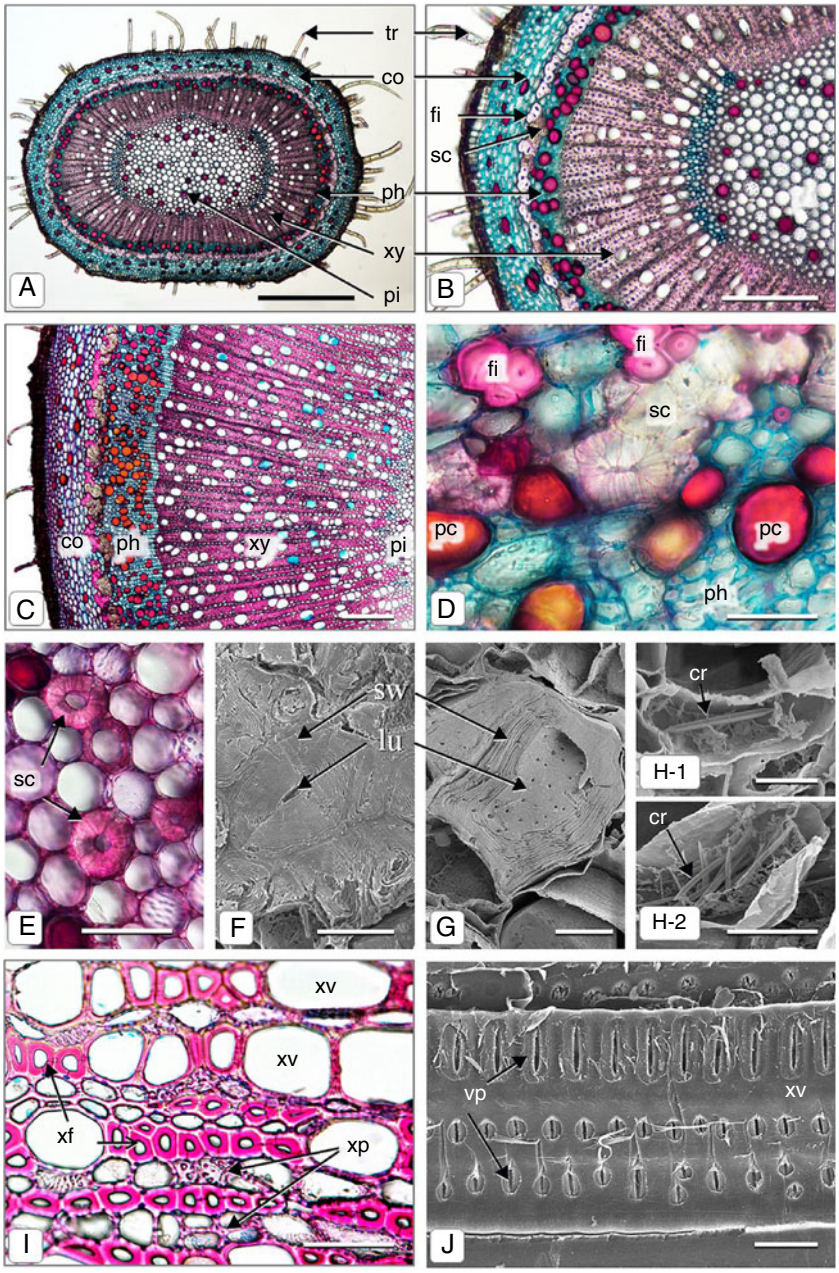

Fig. 2. Stem anatomy of *Fadogia agrestis* [F, G, H, J: SEM; all others: normal light microscopy, stained in astra blue/basic fuchsin]. A, B: cross section of young stem; C: cross section of mature stem; D: phloem region showing fibers, sclereids and idioblasts with phenolic contents; E: sclereids in the pith; F, G: sclereids in sectional view; H: calcium oxalate crystals in the phloem; I: xylem showing vessels, fibers and ray parenchyma; J: a vessel element showing vestured pits on its wall. (co, cortex; cr, crystal; fi, fiber; lu, lumen; pc, phenolic content; ph, phloem; pi, pith; sc, sclereid; tr, trichome; vp, vestured pit; wa, secondary wall; xf, xylem fiber; xp, ray parenchyma; xv, xylem vessel; xy, xylem). Bars: A = 500 μm; B = 200 μm; D, I = 50 μm; C, E = 100 μm; F, G = 20 μm; H-1, H-2, J = 10 μm.

The vascular system is composed of continuous rings of phloem, cambium, and xylem. Phloem is more or less as wide as the cortex and formed of sieve elements and thin-walled parenchyma cells. Phloem is characterized by the presence of large numbers of idioblasts containing phenolic contents (Fig. 2A–C). Cambium is narrow, and is made up of four to six layers of radially arranged narrowly rectangular cells. Xylem occupies a major part of the stem and is four to five times as wide as phloem. It is composed of vessels, fibers and xylem parenchyma (Fig. 2I). Vessel elements have thickened and lignified walls with vestured pits (Fig. 2J). Medullary rays are 1-cell wide and run radially from cambium through the xylem and merging into the pith. Isolated starch grains, measuring about 10 μm in diameter, are observed in the xylem and rarely in the pith. The central part of the stem is occupied by large more or less triangular-shaped pith. It is composed of circular or polygonal parenchyma cells arranged with intercellular spaces, with thickened, slightly lignified and pitted walls. Few 'donut' shaped sclereids are found, in solitary or two to three together, in the pith (Fig. 2E). Idioblasts containing phenolic compounds are present in various tissues throughout the stem but with largest amounts concentrated in the phloem. Styloid crystals (Fig. 2H-1, H-2) measuring 10–15 μm long and about 1.5 μm wide are present in the phloem and occasionally in the pith.

Root anatomy

The root is stout, cylindrical and dark brown. In cross-section (Fig. 3A–E), the root is circular in outline and has well-developed

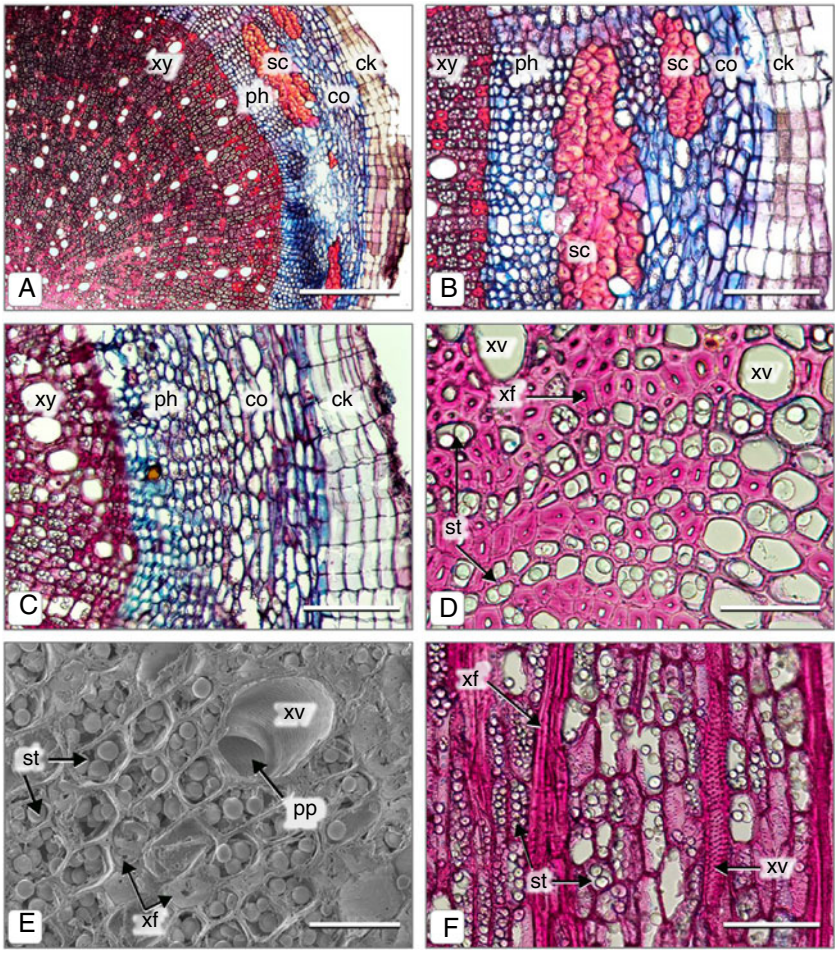

Fig. 3. Root anatomy of *Fadogia agrestis* [A–D, F: light microscopy, stained in astra blue/basic fuchsin; E: SEM]. Root in cross section (A–E) and longitudinal section (F). (ck, cork; co, cortex; fi, fiber; ph, phloem; pp, perforation plate; sc, sclereids; st, starch grains; xf, xylem fiber; xv, xylem vessel; xy, xylem). Bars: A = 500 μm; B, C = 200 μm; D, E = 50 μm; F = 100 μm.

cork, cortex, phloem and a central core of secondary xylem. The cork (Fig. 3A–C) or phellem is made up of up to seven layers of radially arranged rectangular cells with suberized walls and brown inclusions. Phellogen appears as a clear band and consists of a layer of thin-walled cells. The cortex consists of several layers of polygonal parenchyma characterized by the presence of large amounts of starch grains. Groups of brachysclereids forming large patches are distributed in the cortex. They are also rarely found as solitary or few in small groups. In cross section, the sclereids are oval, rounded or polygonal in shape and measure 18–50 μm in diameter. A narrow ring of phloem, with few layers of small parenchyma cells, occurs between cortex and xylem. Xylem (Fig. 3A–F) occurs as a central core in the root and occupies a major portion of it; and it consists of vessel elements, fibers and parenchyma—all have lignified and pitted walls. Starch grains (Fig. 3D–F) are abundant in the cortex as well as ray parenchyma. They are usually spherical, solitary or rarely in pairs, measuring 5–10 μm in diameter, and have a dot-like, radiating or cleft-shaped concentric hilum. Phenolic compounds, which occur abundantly in the leaves and stems, are uncommon in the roots.

Content of phenolic compounds and flavonoids

The Folin–Ciocalteu assay is a method which has been used for quantification of total phenolic contents of food products and dietary supplements. It is based on the electron transfer reaction from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in alkaline medium, which allows spectroscopic determination at 765 nm (Ainsworth and Gillespie, 2007). The total phenolic content (TPC) of the methanolic leaf extracts (NCNPR # 13511, 20086, 20089), calculated from the calibration curve ($R^2 > 0.99$) using gallic acid as a standard, was 58.1 ± 1.1, 40.6 ± 1.4, 149.1 ± 8.6 mg gallic acid equivalents (GAE)/g dry weight of plant extract, respectively. Leaf with stem (# 20102, 20103, 20104) extracts had 42.2 ± 0.4, 91.3 ± 0.5, 122.9 ± 8.2 mg GAE/g DW, stem (# 13512, 20087, 20092) extracts showed 101.7 ± 3.2, 82.3 ± 5.9, 76.2 ± 1.9 mg GAE/g DW and root (# 1472, 17094, 18299) extracts revealed 100.6 ± 1.3, 104.5 ± 0.5, 90.9 ± 1.1 mg GAE/g DW, respectively.

The total flavonoid content (TFC) estimation is based on a colorimetric method using aluminum chloride, which forms stable complexes with the C-4 keto groups and the hydroxyl groups at C-3 or C-5 of flavonoids in acid solution showing maximum absorbance at 415–440 nm (Chang et al., 2002). Leaf samples (# 13511, 20086, 20089) showed their total flavonoid content ($R^2 > 0.99$) as 19.1 ± 1.5, 17.8 ± 0.7, 31.4 ± 2.5 mg quercetin equivalents (QE)/g dry weight of plant extract, respectively. Stem with leaf (# 20102, 20103, 20104) have 10.5 ± 0.8, 26.8 ± 1.9, 24.3 ± 2.9 mg QE/g DW, stem (# 13512, 20087, 20092) revealed 3.0 ± 0.5, 6.9 ± 0.2, 8.4 ± 0.4 mg QE/g DW and root (# 1472, 17094, 18299) had 4.8 ± 0.4, 3.8 ± 0.4, 6.1 ± 0.3 mg QE/g DW, respectively. These results indicated that the leaf and the stem with leaf have significantly positive correlation ($R^2 > 0.99$ and 0.73, respectively) between TPC and TFC. In the case of the stem and the root,

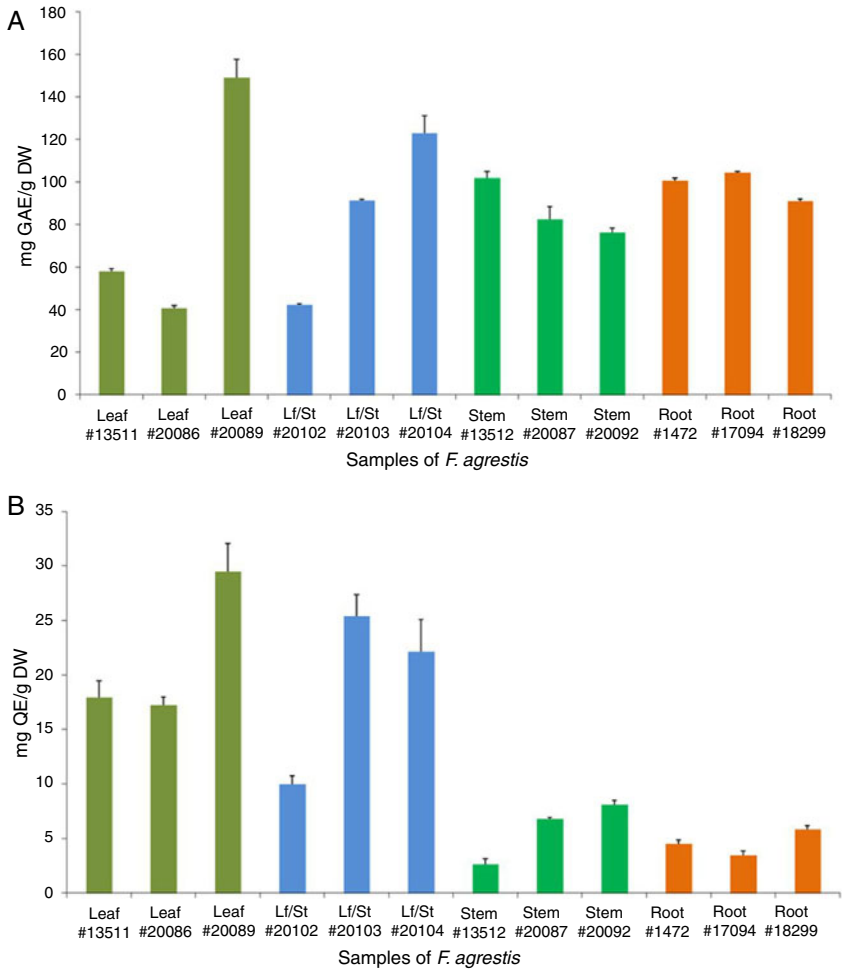

Fig. 4. Total phenolic content (A) and total flavonoid content (B) of *Fadogia agrestis*: Leaf (# 13511, 20086, 20089), leaf/stem (# 20102, 20103, 20104), stem (# 13512, 20087, 20089), and root (# 1472, 17094, 18299). Unit: mg GAE/g DW = mg gallic acid equivalent per g dry weight extract (total phenolic content), mg QE/g DW = mg quercetin equivalent per g dry weight extract (total flavonoids content).

non-significant correlation was found between TPC and TFC, showing relatively high amount of phenolic compounds compared to their low level of flavonoid content (Fig. 4).

HPTLC analysis of Fadogia agrestis samples and dietary supplement products

The composition of mobile phase for HPTLC analysis was optimized by testing different solvent systems to achieve the best separation for the constituents in the samples. The system containing chloroform/ethyl acetate/methanol/formic acid (3:6:2:0.8) produced the best band resolution for the reference standards and for both polar and nonpolar constituents in the samples, thus selected as the mobile phase. The developed HPTLC method was used to analyze the chemical constituents in three leaves (# 13511, 20086, 20089), three leaf/stem mixtures (# 20102, 20103, 20104), three stems (# 13512, 20087, 20092), and three roots (# 1472, 17094, 18299) of *F. agrestis* plant samples, as well as seven dietary supplement products (# 16838, 19300, 19303, 19305, 19307, 19308, 19309). Fig. 5A–C shows the HPTLC images as the results of analyses, of which Fig. 5A was obtained by observing under UV 366 nm wavelength before the derivatization. Fig. 5B and C was obtained by observing under UV 366 nm wavelength and white light, respectively, after the derivatization using *p*-anisaldehyde–sulfuric acid reagent. A phytochemical study being conducted in our lab has led to the isolation and identification of flavonoids and saponins from *F. agrestis*. Two saponins (tracks **1** and **7** in Fig. 5), five flavonoids/phenolics (**2**, **3**, **5**, **6** and **8**), and one iridoid (**4**) were selected as the reference standards for comparison purpose in the HPTLC analysis, and their HPTLC images are illustrated in Fig. 5 with the track numbers 1–8. All the references used in developing the HPTLC chromatograms (Fig. 5) have been isolated from the roots and the aerial parts of *F. agrestis* as part of the phytochemistry study (unpublished) conducted in our labs. These compounds were identified and their structures were elucidated based on their spectroscopic data, largely 1D and 2D NMR.

Discussion

The significance of anatomical studies in plant taxonomy and classification is well known. Anatomical studies also have applications in pharmacognosy and quality control of botanicals. For these reasons, numerous reports on the anatomy of plants are being published worldwide on regular basis. Nevertheless, detailed anatomical studies are not yet available for many taxa. No such details are available in the literature for the species in the genera *Fadogia* and *Vangueria*. The present paper is the first report of detailed anatomy of *F. agrestis* (synonym of *V. agrestis*).

Anatomy of *F. agrestis* reveals several noteworthy characteristics. The leaves are dorsiventral and hypostomatic with unilayered epidermis and paracytic or Rubiaceous type of stomata. Non-glandular septate trichomes with lignified walls are abundant on

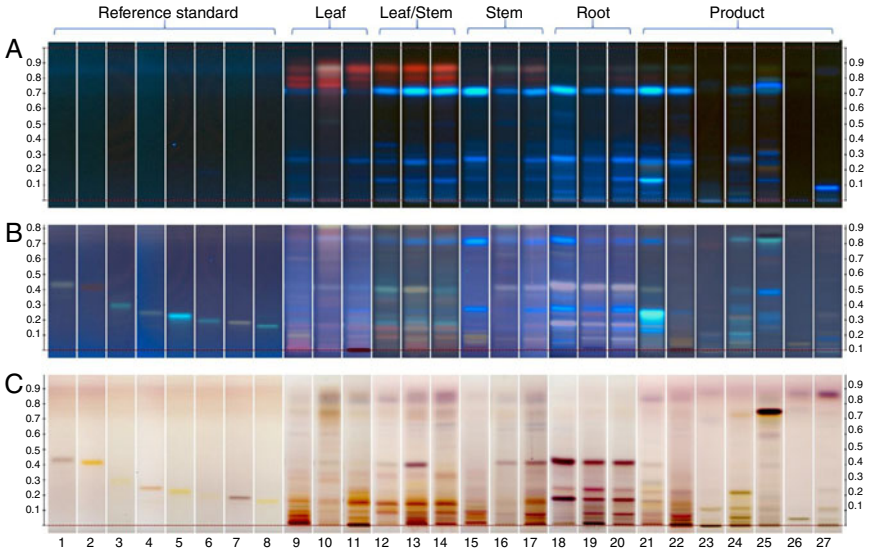

Fig. 5. HPTLC under UV 366 nm prior to derivatization (A), UV 366 nm after derivatization (B), and under white light after derivatization. Tracks 1–8: reference standards (1. 3-*O*-α-L-Rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl pomolic acid; 2. 2,4′,6-Trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside; 3. Hyperoside; 4. Geniposidic acid; 5. Nicotiflorin; 6. Narcissin; 7. Randiasaponin IV; 8. Rutin); tracks 9–11: leaf samples (9. #13511; 10. #20086; 11. #20089); tracks 12–14: leaf/stem samples (12. #20102; 13. #20103; 14. #20104); tracks 15–17: stem samples (15. #13512; 16. #20087; 17. #20092); tracks 18–20: root samples (18. #1472; 19. #17094; 20. #18299); tracks 21–27: dietary supplement products claiming to contain *Fadogia agrestis* (21. #16838; 22. #19300; 23. #19303; 24. #19305; 25. #19307; 26. #19308; 27. #19309).

the surfaces of leaves, petioles and stems. The midrib stele consists of a large, U-shaped vascular bundle. The phloem has a shallow sheath of fibers. In the petiole, an additional pair of smaller vascular bundles occurs on either side of the central bundle. Cells containing yellow substances, presumably phenolic contents, are abundant in the phloem, mesophyll and the ground tissue.

Calcium oxalate crystals are observed in the form of druses, prisms and styloids. Druses are common in the leaf mesophyll and in the ground tissue of midrib and petiole. Solitary minute prismatic crystals are observed in the leaf epidermal cells. Styloid crystals, occurring in isolation or a few in groups, are present in the stem phloem and pith. Raphide crystals are not observed. Verdcourt (1958) and Bremekamp (1966) divided the Rubiaceae into subfamilies on the basis of the presence or absence of raphides. According to them, raphides are absent in Vanguerieae, which include *F. agrestis*. Brachysclereids that provide mechanical strength are found in the stems and roots. In stems, they occur as an interrupted ring in the phloem as well as the pith. They have thick and lignified walls with simple or ramiform pits and narrow lumina. Sclereids in the pith are circular in cross section and have wider lumina.

The subfamily Vanguerieae, Rubiaceae, forms a group of closely related genera (Verdcourt, 1958) with more or less similar anatomy of their secondary wood (Koek-Noorman and Hogeweg, 1974). Vessels are usually solitary with simple perforations and transverse to slightly oblique end walls (Koek-Noorman and Hogeweg, 1974). Vessel elements show spiral thickenings on the inner walls and vestured pits on the outer walls; pits are alternately arranged and often radially elongated. Tracheids have numerous vestured pits on their radial and tangential walls. Secondary rays are predominantly uniseriate but sometimes biseriate, extending from vascular cambium through xylem and connecting the pith; often forming a reticulate pattern of uniseriate bands in the xylem.

The root can be distinguished from the stem by the absence of trichomes, primary phloem fibers and pith. Brachysclereids occur in large groups in the cortex of root; whereas in the stem, both primary phloem fibers and brachysclereids occur together in the phloem, forming a narrow and interrupted sclerenchymatous ring. Starch grains are abundant in root and are less common in leaf and stem.

The monophenolic and polyphenolic compounds in plants, including flavonoids, have a wide range of effects, essentially antioxidant, due to their phenolic hydroxyl groups which contribute to free radical scavenging activity. Total phenolic and flavonoid contents of different parts of *F. agrestis* were investigated because several idioblasts containing phenolic compounds were observed in microscopic studies (Figs. 1 and 2). The stem and root showed similar range of total phenolic content (76.2–104.5 mg GAE/g DW) regardless of their batch number but leaf and leaf with stem had wide variations among the samples. These variations could be due to different harvesting times or origins of the plant. Otherwise, total flavonoid content of each sample resulted in different patterning according to the parts analyzed. Leaf and leaf/stem showed higher flavonoid content while stem and root had relatively low flavonoid content. It is known that different phenolic compounds have different antioxidant responses due to their chemical structures (Saeed et al., 2012). *F. agrestis* stem extract is mostly used in dietary supplements on the online market for the purpose of testosterone support. It may be possible that the ingredients of stem which are responsible for the effect could be other compounds, not having reaction with aluminum chloride reagent used in this study.

HPTLC, as a method of chemical fingerprinting, can be used as a tool for rapid assessment of authenticity of plants and botanical products (Kruger et al., 2013). By comparing the HPTLC chromatograms (chemical fingerprints), the analysis enables one to detect the presence of aberrant chemical components from adulterants, as well as the changes of chemical composition in different plant parts. The significant differences of chemical profiles for the roots, leaves and stems of Fadogia plant samples were observed when comparing their HPTLC chromatograms as shown in Fig. 5, indicating that chemical compositions varied significantly across different plant parts. By comparing results with the reference standards, it is clear that saponins are the major components in the root, while the stem and leaf contain mainly phenolics/flavonoids, which are in agreement with the aforementioned observations. Nevertheless, a few common constituents can be observed for all the plant parts, such as the ones giving fluorescent bands at R_f 0.73 and 0.28 (see Fig. 5A and B), and further studies are needed to identify their structures.

For each of the plant parts [root, leaf, stem and aerial (leaf/stem)], at least three samples that were collected from different locations or seasons were investigated using HPTLC. Slight variations of chemical composition of samples sourced from different locations can be observed from the HPTLC chromatograms.

Many dietary supplements labeling *F. agrestis* as an ingredient are sold on the market. In the present study, seven commercial dietary supplement products were analyzed together with those authenticated *F. agrestis* plant parts by using HPTLC. Three products (#16838, 19300 and 19303) claim that *F. agrestis* extract is the only ingredient in the products according to their labels. By comparing their HPTLC fingerprints with those of the authenticated *F. agrestis* plant samples (Fig. 5), the two products (#16838 and 19300) demonstrate similarities in fingerprints to the authenticated samples, but product #19303 displays a significant difference, suggesting that product #19303 could possibly be adulterated due to the disagreement with the label claim. Four products (#19305, 19307, 19308 and 19309) purport to contain *F. agrestis* in their formulas based on their label information. The results of HPTLC analysis indicated that the two products #19305 and 19307 might possess *F. agrestis* in their formulas; but the other two products #19308 and 19309 are questionable in that their HPTLC fingerprints showed significant differences from those of the authenticated plant samples.

In conclusion, the present investigation has revealed noteworthy features in the anatomy of leaf, stem and root of *F. agrestis* that can be helpful in species identification, taxonomy and quality control. Septate non-glandular trichomes with lignified outer walls are abundant on the leaf, petiole and stem epidermises. Paracytic stomata are present on the abaxial leaf epidermis. Phenolic contents (as indicated by histochemical tests), occurring as yellow cell inclusions, are common in the mesophyll, midrib, petiole and stem and are particularly abundant in the phloem. Oil bodies are found in the leaf epidermis and mesophyll. Primary phloem fibers are present in the stem. Brachysclereids are found in association with the phloem in stems, but are located in cortex in the root. They are also observed in the pith in stem. Vessel elements have vestured pits on the radial walls, and their end walls have simple perforations. Calcium oxalate druses are common in the leaf and styloid crystals are present in the stem. Roots contain abundant starch grains, which are spherical, usually simple, with a concentric hilum. The HPTLC fingerprinting method developed in this study is simple, cost effective and fast. It is suitable for rapid decisive authentication and comparison of differences among samples of identical source and can be used for the routine quality control analysis. The results of phenolic and flavonoid contents analyses for the different plant parts support the existence of significant variation in the chemical composition of different plant parts. It is necessary to further study and elucidate the active components of *F. agrestis* stems, which are the main ingredients in the dietary supplements containing this species. In the present study, the stem samples showed high total phenolic content but relatively low total flavonoid content.

Authors' contributions

VR created the project. JMB and VR prepared specimens, conducted microscopy studies and wrote the article. JZ, JB and BA conducted HPTLC and UV Spectroscopy analyses. AGO and ZA isolated and identified the major compounds. IAK contributed to critical reading of the manuscript. All authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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