



Research Article

HPTLC Fingerprinting and Cytotoxicity of Secondary Metabolites of *Equisetum Diffusum* D. Don Extracts

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Abstract

The aerial parts of *Equisetum diffusum* D. Don (Equisetaceae ED), a Vietnamese folk medicine used for hypertensive, anti-inflammatory, diuretic and hemostatic properties, were collected in Northern Vietnam. The secondary metabolites of the sequential n-hexane, ethyl acetate and methanol extracts were profiled by HPTLC with chromatographic conditions and derivatization reagents characteristic for flavonoids, polyphenols and terpenoids/steroids.

All these metabolite classes were present in the methanol extract whereas the ethyl acetate extract comprised some polyphenols and flavonoids; no characteristic compound class could be identified in the n-hexane extract. HPLC allowed to determine the concentration of isoquercitroside, the major flavonoid of the methanol extract (1.60 ± 0.04 mg/g dry weight; n=3).

In a preliminary assessment of *Equisetum diffusum* aerial parts safety, the cytotoxicity of these 3 ED extracts was investigated on the FHs 74 Int human intestinal epithelial cell line, a non-cancerous, non-transformed normal cell line. The ethyl acetate extract was shown to be the most toxic with an IC₅₀ of 8.7 ± 2.3 µg/mL, followed by the n-hexane and methanol extracts, with an IC₅₀ of 46.1 ± 3.6 µg/mL and 124.7 ± 23.0 µg/mL, respectively.

Keywords: *Equisetum diffusum* D.Don; *Equisetum arvense* L.; Fingerprint; HPTLC; HPLC; Secondary metabolites; Isoquercitroside

1. Introduction

Medicinal herbs have a long history of development in the world. Their traditional use is documented in many handbooks of phytotherapy and is more and more popular in the field of alternative and complementary medicine [1]. The medicinal herbs market grows every year and will reach a CAGR (Compound annual growth rate) of 5.34 % from 2021 to 2028 to reach 426.43 billion US dollars by 2028 [2]. Ethnopharmacological inquiries provide us with important information about traditional medicinal plants and countless papers study their various effects, properties and characteristics. Nevertheless, our knowledge of the field is still largely imperfect and many questions remain on the safety of traditional remedies [3].

The demand for medicinal herbs and complementary medicines is increasing worldwide and, consequently, the number of low-quality herbs on the market is steadily increasing. Therefore, methods for authentication of herbal material and standardization

of their active ingredients are very important and necessary. Until recently, medicinal herbs were often identified according to the morphology of used parts, such as the roots, leaves, flowers, or stems [4, 5]. However, medicinal herbs on the market are often chopped or even powdered for sale, making such identification difficult. Nowadays, DNA barcoding technology can be applied to define plants in general and medicinal plants in particular [5-7].

This method gives accurate results, sometimes at the level of species or even variety, but has not been widely applied because of the high cost and the requirement for specific techniques and instruments. Also, DNA analyses give no clues on the quality of the identified material, notably its content in active compounds. Fingerprinting methods using High-Performance Thin-Layer Chromatography (HPTLC) represent an elegant alternative to both identify species and assess the quality of medicinal plants and extracts. This method begins with the extraction of the plant and continues with the chromatography of the extract. The results are presented in the form of a "fingerprint", i.e. a specific sequence of peaks or zones due to the migration of known or unknown components of the extract in the selected chromatographic conditions.

The fingerprint of botanically authenticated raw material serves as a primary reference to all unknown material that needs to be characterized. Identification can also be performed on the basis of chemical reference compounds whenever the sample is expected to contain those. One of the advantages of HPTLC fingerprints is that it mostly relies on a visual impression, yielding a rapid and easy interpretation. A

broad spectrum of constituents can be detected and described without the need to know the chemical nature of each of the zones visualized on the chromatogram [8].

Equisetum diffusum D.Don (*ED*) [synonyms, *Equisetum arvense* subsp. *diffusum* (D.Don) Fraser-Jenk., *Equisetum diffusum* var. *caespitosum* Milde, *Equisetum diffusum* var. *nudum* Milde, *Equisetum diffusum* var. *paucidentatum* C.N.Page, *Equisetum diffusum* var. *polystachyum* Milde, *Equisetum diffusum* var. *ramosum* Milde, *Equisetum mekongense* C.N.Page, *Equisetum wallichianum* C.N.Page] [9] is known in Vietnamese as “Mộc tặc trãi”; this Equisetaceae is distributed in Vietnam, Bangladesh, China, Himalaya, India, Laos, Myanmar, Nepal, Pakistan, Thailand and Tibet. In Vietnam, it is used in traditional medicine to treat low blood pressure and inflammation, and as a diuretic and hemostatic [10]. So far, very few studies have been published on *Equisetum diffusum* but another species, *Equisetum arvense* L. (*EA*), commonly known as "horsetail", has long been used in European and Chinese traditional medicines to treat different disorders, including bone pathologies [11], based on its alleged content in silica and supposedly important organo-silicon compounds [12].

Hydroalcoholic extracts of *EA* stems demonstrated antioxidant [13, 14], antinociceptive and anti-inflammatory properties [15, 16]. Other activities such as sedative, anticonvulsant [17], antiproliferative

[18], antibacterial [18, 19], anticancer [20], and antidiabetic [21] have also been demonstrated for aerial parts of *EA*.

It has been shown that TLC fingerprinting allows distinguishing *EA* from other *Equisetum* species, including *ED* [7]. Therefore, in this study, we aim to further develop fingerprinting techniques, using both HPTLC and HPLC. The sequential n-hexane, ethyl acetate, and methanol extracts of aerial parts of *ED* will be subjected to HPTLC with different mobile phases and detection reagents to separate and visualize the bioactive compounds flavonoids, polyphenols, and terpenoids; in addition, the major flavonoid, isoquercitroside, will be quantified by High Performance Liquid Chromatography (HPLC).

In a preliminary assessment of *Equisetum diffusum* aerial parts safety, the cytotoxicity of the 3 *ED* extracts will be investigated on the FHs 74 Int human intestinal epithelial cell line, a non-cancerous, non-transformed normal cell line.

2. Material and Methods

2.1 Plant material

Aerial parts of *ED* were collected in October 2018 from Sa Pa (22°21'05.8"N 103°48'56.5"E), Lao Cai province, Northern Vietnam. A voucher specimen was deposited in the Museum of Biology, Faculty of Biology, VNU-University of Science, Hanoi, Vietnam, where it was identified by Dr Kim-Thanh Thi NGUYEN.



Figure 1: *Equisetum diffusum* D. Don. Sapa, Lao Cai province, Northern Vietnam.

2.2 Chemicals

n-Hexane, ethyl acetate (EtOAc), methanol (MeOH), acetonitrile, and formic acid were purchased from VWR (Radnor, PA, USA); the Hybri-Care[®] from ATCC (Rockville, MD, USA); FBS (fetal bovine serum), EGF (human recombinant growth factor) and PBS from Gibco (Thermo Scientific, Waltham, MA, USA); and accutase from Corning (Mediatech, Manassas, VA, USA). HPTLC analyses were performed on silica gel 60 F₂₅₄ HPTLC plates (Merck, Germany). Sodium bicarbonate, p-anisaldehyde, 2-aminoethyl diphenylborinate, polyethylene glycol 400 (macrogol), bismuth nitrate, potassium iodide, myricetin, rutin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and o-dianisidine bis(diazotized) zinc double salt (fast blue salt B) were purchased from Sigma (St. Louis, MO, USA); isoquercitroside, quercetin, caffeine, noscapine, vanillin, and ferulic acid from the European Directorate for the Quality of Medicines

(EDQM, Strasbourg, France); sinapic acid, oleanoic acid, maslinic acid, betulinic acid, geniposide from Extrasynthese (Genay, France); Folin-Ciocalteu reagent from Merck (Merck, Germany).

The p-anisaldehyde-sulfuric acid reagent was prepared by mixing in the following order, 0.5 mL of p-anisaldehyde, 10 mL of glacial acetic acid, 85 mL of methanol and 5 mL sulfuric acid. The Dragendorff's reagent was prepared by mixing 5 mL Solution A (0.17 g of bismuth nitrate in 10 mL of a 20 % acetic acid aqueous solution), 5 mL Solution B (4 g potassium iodide in 10 mL of water), 20 mL acetic acid and 70 mL water. The FHs 74 Int cells (ATCC: CCL-241) were obtained from ATCC (Rockville, MD, USA) and cultured in Hybri-Care[®] medium, supplemented with 10 % FBS, 1.5 g/L sodium bicarbonate, and 0.0025 % EGF, at 37°C under 5 % CO₂.

2.3 Plant extraction

The aerial parts of *ED* were collected, cleaned, and washed with water and then with distilled water, dried in a 40°C oven and ground to powder (size smaller than 0.5 mm). The extraction protocol follows a previously described procedure [22] with some modifications. Dried powder of *ED* (50 g) was grinded with sand and n-hexane, sonicated for 15 min and extracted successively (for each solvent: 500 mL, 3 times, 20 h, agitation 360 rpm at room temperature (RT) with n-hexane, ethyl acetate, and methanol.

The extracts were filtered and the solvents were evaporated under reduced pressure in a Rotavapor to obtain the respective crude extracts (n-hexane, ethyl acetate and methanol extract). These extracts were stored at -20°C until use. After sequential extraction, the yields of the n-hexane, ethyl acetate, and methanol extracts were 1.96, 1.78 and 3.35 expressed in % of dry weight, respectively.

2.4 Qualitative and quantitative phytochemical analysis

Secondary metabolites of plants have been classified into major chemical groups such as alkaloids, flavonoids, terpenoids, polyphenols, or steroids. In the first step, a qualitative analysis was performed to identify the presence of these groups in the extracts of *ED*, following standard methods [23]. Polyphenols were researched from their coloration with FeCl_3 ; flavonoids from their coloration change according to pH; terpenoids and steroids from their reaction with CHCl_3 and concentrated sulfuric acid; alkaloids from their precipitation by the Dragendroff's reagent.

The total polyphenol content was estimated by mixing

1 mL of a 1 mg/mL solution of extract in methanol with 2.5 mL of Folin-Ciocalteu reagent and 2.5 mL of 2 % Na_2CO_3 , heating at 45°C for 45 min and measuring the absorbance at 765 nm; a blank was prepared using 1 mL of MeOH instead of the extract. The total polyphenol content was expressed as Gallic Acid Equivalent (GAE, mg/g dry weight) using the following equation based on the calibration curve: $y = 0.0039x - 0.0197$ ($R^2 = 0.9911$) where y was the absorbance and x the gallic acid concentration ($\mu\text{g/mL}$). The total flavonoid content was estimated by mixing 1 mL of a 1 mg/mL solution of extract in methanol with 1 mL of 2 % AlCl_3 , incubating 60 min at room temperature and measuring the absorbance at 415 nm; a blank was prepared using 1 mL of MeOH instead of the extract. The total flavonoid content of the extract was expressed as Quercetin Equivalent (QE, mg/g dry weight) using the following equation based on the calibration curve: $y = 0.012x + 0.4965$ ($R^2 = 0.9541$) where y was the absorbance and x the quercetin concentration ($\mu\text{g/mL}$).

2.5 MTT assay

The MTT assay was performed following a previously described protocol [24] with some modifications. Firstly, cells were seeded into 96-well plates at a density of 4000 cells/well. After 24 h of incubation, the extracts were added. After 48 h, 200 μL of a 0.5 mg/mL MTT solution were added into each well and incubated for 4 h at 37°C, 5 % CO_2 .

The MTT solution was then carefully eliminated, 100 μL of DMSO were added to dissolve the formed formazan and, the plate was measured at 570 nm and 690 nm. The cell viability (%) was calculated using the following equation:

$$\text{cell viability (\%)} = \frac{(A_{570 \text{ nm}} - A_{690 \text{ nm}})_{\text{sample}}}{\text{average}[(A_{570 \text{ nm}} - A_{690 \text{ nm}})_{\text{blank}}]} \times 100$$

The experiment was performed in triplicate and expressed as mean +/- standard deviation; IC₅₀, the concentration of drug or extract required for 50 % cell viability reduction, was determined by fitting a curve to experimental points, using the GraphPad 5.0 software. The fitted curves “% of cell viability” versus “log (catalyst concentration)”.

2.6 High-performance thin-layer chromatography (HPTLC) profiling

HPTLC was performed according to the procedure of the European Pharmacopeia 10 [25], using Camag

Automatic TLC Sampler (ATS 4), Automatic Developing Chamber 2 (ADC 2), Derivatizer and TLC Visualizer 2. The Camag systems were driven by the software visionCATS version 2.5.

The HPTLC was performed on silica gel 60 F₂₅₄ HPTLC plates (Merck, Germany); 10 µL of samples were applied in 8-mm wide bands, the plates were activated on MgCl₂ (~ 33 % RH) and the tank saturated for 20 min; the solvent systems and derivatizing solutions [23] are described in Table 1. All images of the plates were recorded under white light, UV₂₅₄ and UV₃₆₅ illumination.

Compound classes	Mobile phase	Spray reagent	Heat	Visible light	UV (365 nm)
Alkaloids	Mb1 = chloroform-methanol (30 : 13, v/v)	Dragendorff's reagent	RT for 10 min	Yellow	Dark blue, dark violet
Flavonoids	Mb2 = formic acid - water - methyl ethyl ketone - ethyl acetate (1 : 1 : 3 : 5, v/v/v/v)	NP ^(a) and PEG ^(b)	100°C for 3 min	Yellow, red	Yellow, green
Polyphenols	Mb3 = chloroform - ethyl acetate - methanol - water (15 : 50 : 22 : 10, v/v/v/v)	Fast blue salt B ^(c)	RT for max 10 min	Purple, orange, brown	Dark violet
Terpenoids	Mb4 = toluene - ethyl acetate - methanol - formic acid - acetic acid (100 : 15 : 10 : 2 : 1, v/v/v/v/v) Mb5 = chloroform - ethyl acetate - methanol - water (15 : 50 : 22 : 10, v/v/v/v)	10 % sulfuric acid (v/v) in methanol	100°C for 3 min	Violet, purple	Violet, purple
Antioxidant	Mb2 and Mb4	DPPH ^(d) reagent	No	White band on a purple background	No

^(a) NP reagent: 1 g of 2-aminoethyl diphenylborinate in 100 mL of methanol

^(b) PEG reagent: 5 g of polyethylene glycol 400 (macrogol) in 100 mL of ethanol (96 % v/v).

^(c) Fast blue salt B reagent: 0.5 g of fast blue salt B in 100 mL of water

^(d) DPPH reagent: 0.5 g of diphenyl-1-picrylhydrazyl (DPPH) in 100 mL of methanol (100 % v/v)

Table 1: Secondary metabolites classes, mobile phases, spray reagents used for HPTLC profiling of *Equisetum diffusum*.

2.7 High-performance liquid chromatography (HPLC)

HPLC analyses were carried out on an HPLC Agilent (model 1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA) system equipped with a diode array detector, using a C18 column Infinity Lab Poroshell 100 × 4.6 mm I.D., 4 μm (Agilent Technologies, Santa Clara, CA, USA). The injection volume was 5 μL and the detection wavelengths were set at 254.4 nm and 326.4 nm. The separation of compounds was achieved at a flow rate of 1.5 mL/min using 1.0 % v/v formic acid in water (solvent A) and acetonitrile (solvent B) as mobile phases with an elution gradient as follows: 0–2 min, 5 % B; 2–6 min, 5–15 % B; 6–20 min, 15–36 % B; 20–27 min, 35–95 % B; 27–29 min,

95–100 % B; 29–35 min, 100 % B. The isoquercitroside was determined from the calibration curve: $y = 7757.4x + 45.091$ ($R^2 = 0.9999$), where y was the area of isoquercitroside peak and x the isoquercitroside concentration (mg/mL).

3. Results

3.1 Qualitative analysis

Table 2 shows the results of the phytochemical screening detecting the presence of secondary metabolites classes. Most of tested metabolite classes were present in the methanol extract whereas the ethyl acetate extract comprised mainly polyphenols and flavonoids; no characteristic compound class could be identified in the n-hexane extract.

Bioactive compounds classes	n-Hexane extract	Ethyl-acetate extract	Methanol extract
Alkaloids	-	-	-
Flavonoids	-	+/-	+++
Polyphenols/tannins	-	+/-	+++
Terpenoids	-	-	+
Steroids	-	-	+

Table 2: Qualitative analysis of the extracts of *ED*.

3.2 Quantitative analysis

Table 3 present the total phenolics and flavonoids content of *ED* methanolic extract. Humidity of plant's powder is 4.04 %. There were no measurable

polyphenols and flavonoids in the n-hexane and ethyl acetate extracts, despite a faint colorimetric detection in the ethyl acetate extract (Table 2).

Extraction yield (%)	Total phenolics content (GAE mg/g DW plant)	Total phenolics content (GAE mg/g DW extract)	Flavonoids content (QE mg/g DW plant)	Flavonoids content (QE mg/g DW extract)
3.49	1.09 ± 0.08	32.7 ± 2.3	1.58 ± 0.12	47.2 ± 3.6

Table 3: Total phenolic and flavonoid content of *ED* methanolic extract.

3.3 HPTLC profiling of antioxidant compounds

The yellowish bands on a blue DPPH background (Figures 2 and 3) indicate the presence of antioxidants

in the methanol and n-hexane extracts of *ED* aerial parts.

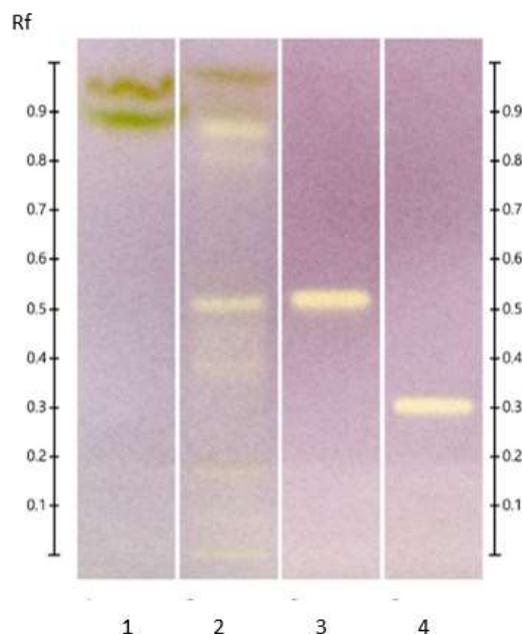


Figure 2: HPTLC antioxidant profile of *ED* aerial parts extracts; silica gel 60 F_{254} plate; mobile phase: formic acid - water - methyl ethyl ketone - ethyl acetate (1:1:3:5 v/v/v/v); the plate was sprayed with DPPH and visualized under visible light. Tracks: EtOAc extract (1); MeOH extract (2); isoquercitroside (3); and rutin (4).

3.4 HPTLC profiling

3.4.1 Flavonoids: Flavonoids are an important class of natural products widely distributed in the plant kingdom that correspond to a class of low-molecular-weight phenolic compounds. They have favorable biochemical and antioxidant effects associated with a protective effect against several diseases such as cancer, Alzheimer's disease, atherosclerosis, etc. They constitute one of the most characteristic classes of compounds in higher plants [26].

3.4.2 Terpenoids: Terpenoids are the largest and most diverse group of secondary metabolites from natural sources. Many terpenoids are biologically active and are used worldwide for the treatment of diseases such as cancer, malaria, etc.

An interesting example of this group are taxol and its derivatives [27].

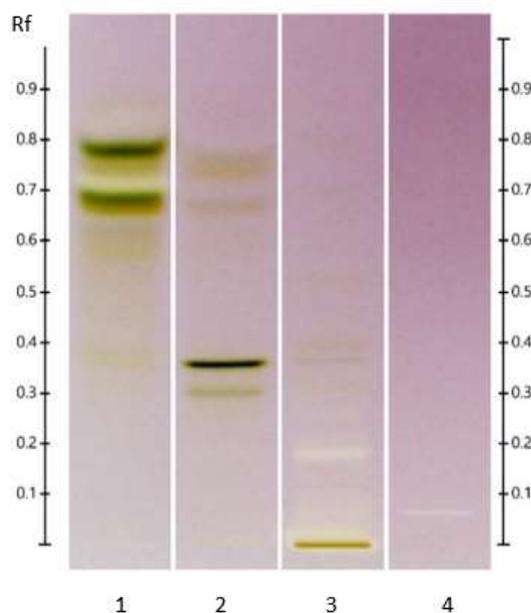


Figure 3: HPTLC antioxidant profile of *ED* aerial parts extracts; silica gel 60 F_{254} plate; mobile phase: toluene - ethyl acetate - methanol - formic acid - acetic acid (100:15:10:2:1 v/v/v/v/v); the plate was sprayed with DPPH and visualized under visible light. Tracks: n-hexane extract (1); EtOAc extract (2); MeOH extract (3); and rutin (4).

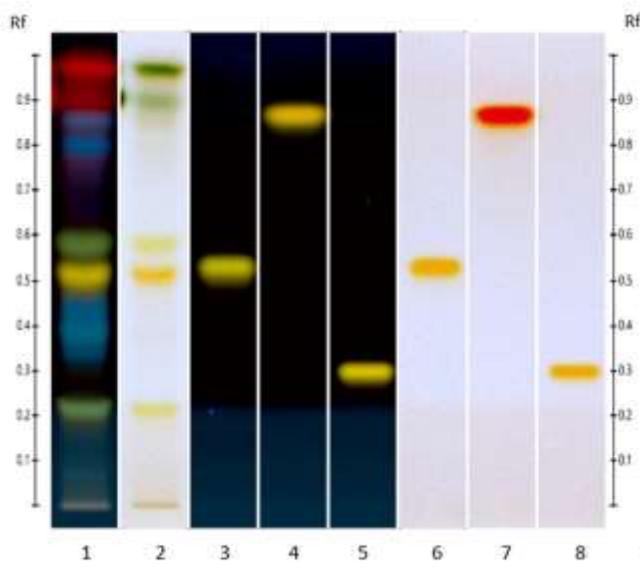


Figure 4: HPTLC profiles of *ED* aerial parts extracts; silica gel 60 F_{254} plate; mobile phase: formic acid - water - methyl ethyl ketone - ethyl acetate (1:1:3:5 v/v/v/v); the plate was heated at 100°C for 3 min, sprayed with NP and PEG reagents and visualized under UV365 (tracks 1, 3, 4, 5) and visible (tracks 2, 6, 7, 8) light. Tracks: methanolic extract (1 and 2); isoquercitroside (3 and 6); myricetin (4 and 7) and rutin (5 and 8).

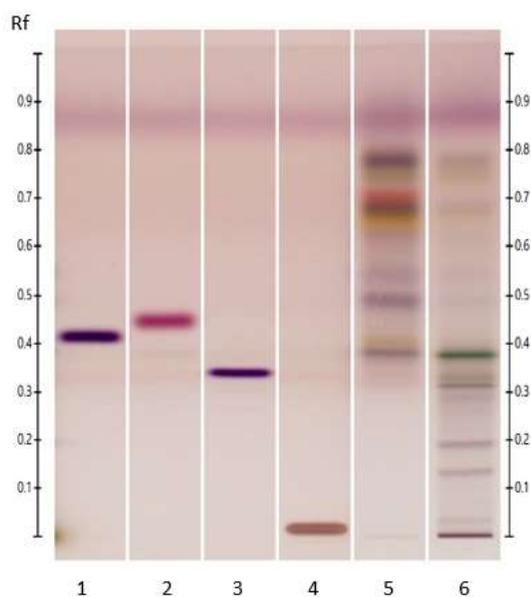


Figure 5: HPTLC profile of *ED* aerial parts extracts; silica gel 60 F_{254} plate; mobile phase: toluene – ethyl acetate – methanol – formic acid – glacial acetic acid (10:1.5:1:0.2:0.1 v/v/v/v/v), the plate was heated at 100°C for 3 min, sprayed with 10 % acid sulfuric in methanol and visualized under visible light. Tracks: oleanolic acid (1); betulinic acid (2); maslinic acid (3); geniposide (4); n-hexane extract (5); ethyl acetate extract (6).

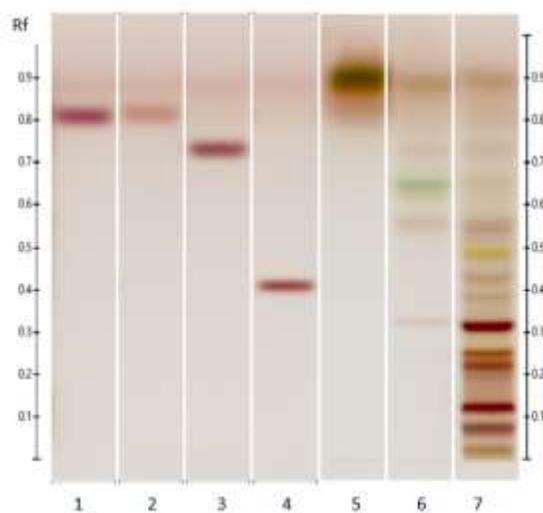


Figure 6: HPTLC profile of *ED* aerial parts extracts; silica gel 60 F_{254} plate; mobile phase: chloroform – ethyl acetate – methanol – water (15: 50: 22:10 v/v/v/v), the plate was heated at 100°C for 3 min, sprayed with 10 % acid sulfuric in methanol and visualized under visible light. Tracks: oleanolic acid (1); betulinic acid (2); maslinic acid (3); geniposide (4); n-hexane extract (5); ethyl acetate extract (6); methanolic extract (7).

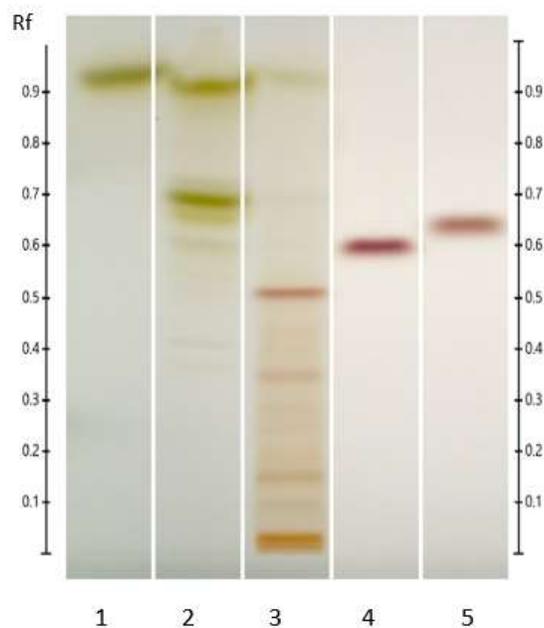


Figure 7: HPTLC profile of *ED* aerial parts extracts; silica gel 60 F₂₅₄ plate; mobile phase: chloroform – ethyl acetate – methanol – water (15: 50: 22:10 v/v/v/v); the plate was sprayed with fast blue salt B reagent and visualized under visible light. Tracks: n-hexane extract (1); ethyl acetate extract (2); methanolic extract (3); sinapic acid (4); ferulic acid (5).

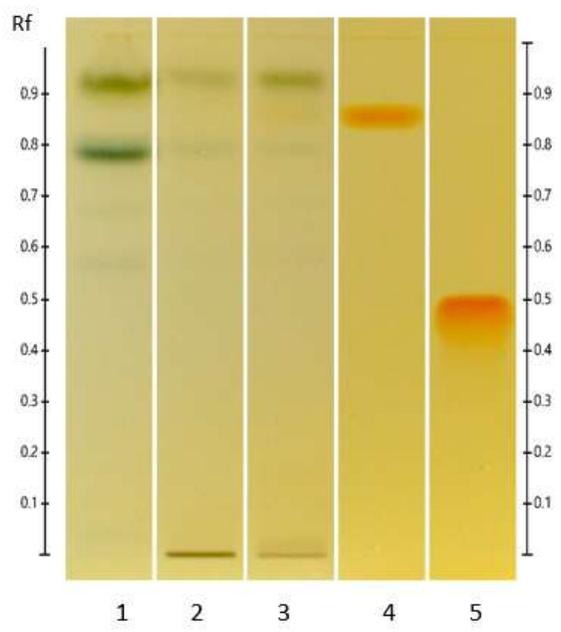


Figure 8: HPTLC profile of *ED* aerial parts extracts; silica gel 60 F₂₅₄ plate; mobile phase: chloroform – methanol (30: 13 v/v); the plate was sprayed with Dragendorff's reagent and visualized under visible light. Tracks: n-hexane extract (1); ethyl acetate extract (2); methanolic extract (3); quinine sulfate (4); theophylline (5).

3.4.3 Phenolic compounds: Phenolic compounds are a heterogeneous group of phytochemicals comprising phenol rings bearing one or more hydroxyl groups and can be divided into several classes. The main groups of phenolic compounds include flavonoids, phenolic acids, tannins, stilbenes, and lignans. Therefore, they are the most abundant secondary metabolites in plants [28].

3.4.4 Alkaloids: Consistently with the results obtained in the phytochemical screening, no yellow or orange bands were detected in the different *ED* extracts upon spraying the plate with Dragendorff's

reagent (Figure 8) confirming the absence of alkaloids in all three extracts.

3.5 High-performance liquid chromatography (HPLC)

To profile flavonoids and evaluate the concentration of isoquercitroside in *ED* aerial parts, the methanolic extract was analyzed by HPLC (Figures 9-10). From an isoquercitroside standard curve, its concentration was calculated in the methanol extract (1.60 ± 0.04 mg/g dry weight plant, i.e. 47.9 ± 1.2 mg Isoquercitroside/g dry extract weight).

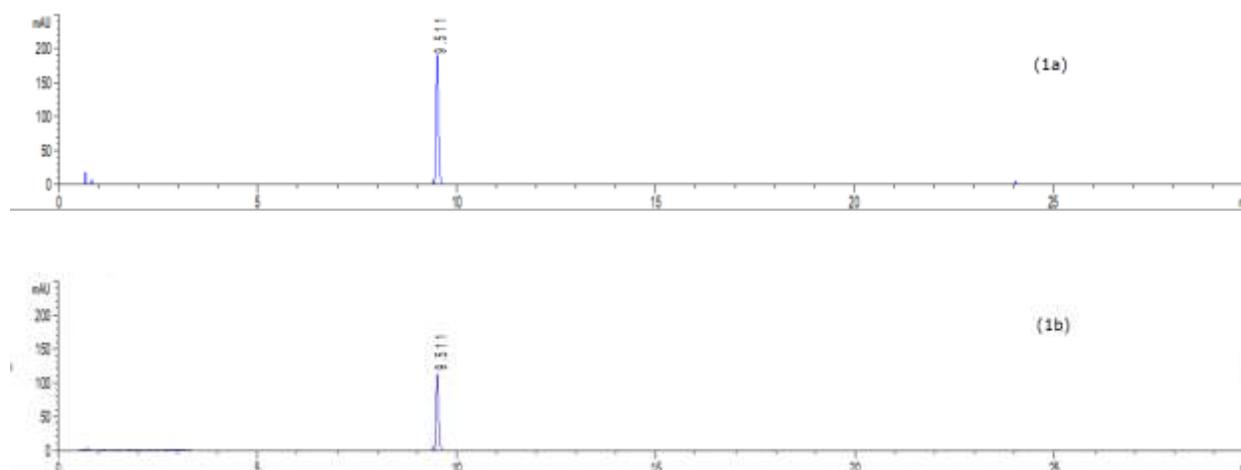


Figure 9: HPLC chromatogram of isoquercitroside at 254.4 nm (1a) and 326.4 nm (1b).

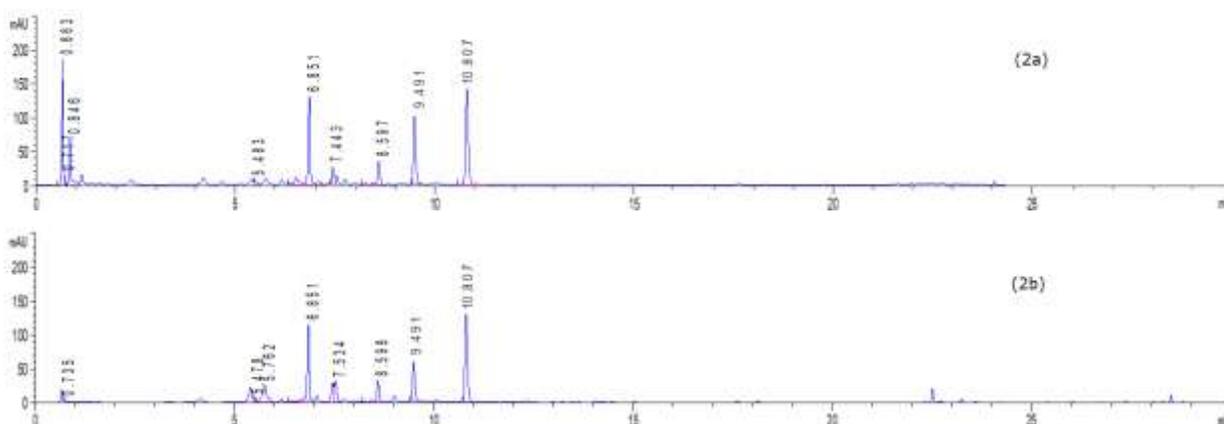


Figure 10: HPLC chromatogram of ED methanolic extract at 254.4 nm (2a) and 326.4 nm (2b).

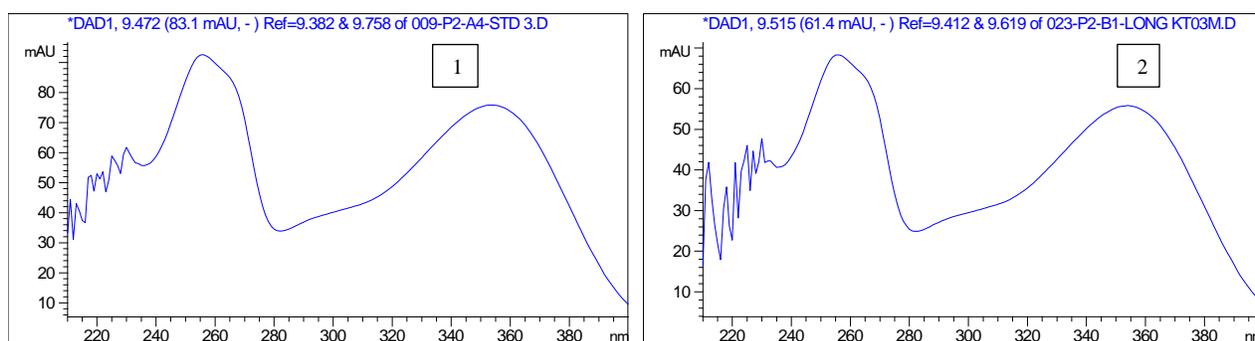


Figure 11: The UV spectra of the isoquercitroside's peak in reference solution (1) and in ED methanolic extract (2).

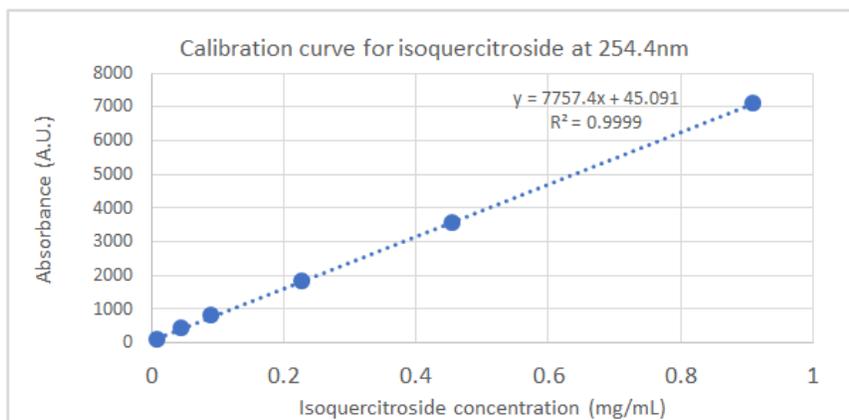
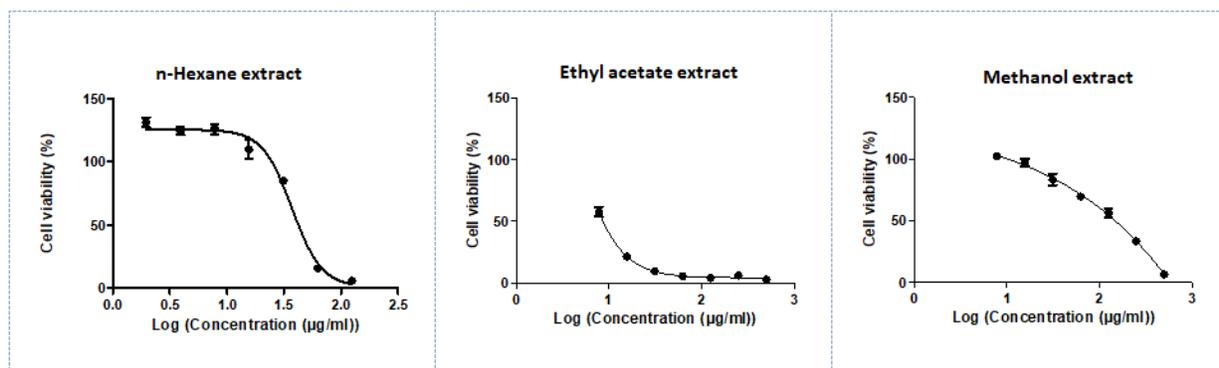


Figure 12: Calibration curve for isoquercitroside at 254.4 nm.

3.6 Cytotoxicity

The effect of *ED* extracts on FHs 74 Int cells viability was evaluated with the MTT assay over 48 h contact. The EtOAc extract appears to be the most toxic with

an IC_{50} of $8.66 \pm 2.31 \mu\text{g/mL}$, followed by the n-hexane and methanol extracts, with IC_{50} of $46.13 \pm 3.63 \mu\text{g/mL}$ and $124.73 \pm 22.97 \mu\text{g/mL}$, respectively.



4. Discussion

Some 33,400 plants are used for traditional medicine around the world [29]. The first and major step when using a herb resides in the authentication of the material. In a recent research, Lagoudakis et al. present a method to identify common horsetail by using DNA barcoding, focusing on the distinction between *Equisetum arvense* and other *Equisetum* species. According to these authors, the genus *Equisetum* is resolved in 2 major clades, each comprising seven species and corresponding (i) to the two subgenera *Equisetum* and *Hippochaete*; and (ii) in a sister species, *Equisetum bogotense* Kunth. Except for *E. diffusum* D. Don and *E. sylvaticum*, all species were recovered as monophyletic, including *E. arvense* and *E. palustre* [7].

TLC chemical profiles (according to Identification C of the European Pharmacopoeia monograph for *E. arvense*) positively identify *E. arvense* (all marker bands were detected) and discriminate it from all

tested species; the *ED* chromatogram shows additional bands in both high and low R_f zones [7]. In our study, the HPTLC fingerprint of *ED* methanol extract (system Mb2; Figure 4) is consistent with this previously described profile.

Our phytochemical screening of *ED* revealed the presence of a series of secondary metabolites. Our analyses indicate that the methanol extract (3.35 %) is the extract containing the most phytochemical compounds, with a noticeable content in total phenolic compounds ($1.09 \pm 0.08 \text{ mg GAE/g dry weight}$), flavonoids ($1.58 \pm 0.12 \text{ mg QE/g dry weight}$) and isoquercitroside ($1.60 \pm 0.04 \text{ mg/g dry weight}$).

A previous screening by Subba et al. indicates the presence, in *ED* aerial parts methanol extract, of alkaloids, flavonoids, tannins, phenols, steroids, phlorotannins, saponins, glucosides, and carbohydrates and the absence of terpenoids, anthraquinones, and proteins [30]; Still in the study of

Subba et al, the methanol extract of *ED* has investigated the total tannins (37.80 ± 0.05 mg GAE/g dry extract wt), phenols (17.40 ± 0.06 mg GAE/g dry extract wt), flavonoids (~ 17 mg rutin equivalent/g dry extract wt) and flavonols contents (~ 47 mg rutin equivalents/g) [30].

The stems of *E. arvense* are also described to contain minerals, potassium and calcium, various flavonoids, phenolic, triterpenoids, phytosterols, and low amounts of essential oil [19]. In a study by Grundemann et al. [31], the amount of isoquercitroside was 43.1 ± 0.7 $\mu\text{g/mL}$ methanol extract. Radulovic et al. (2006) identified 25 volatile compounds in *EA* essential oil, accounting for 54.23 % of peak areas [19]. A further study identified 52 constituents in the *ED* essential oil; the major compounds were phytol (35.63 %), hexacosane (8.04 %), cadin-4-ene-7-ol (cis) (5.32 %), n-decane (3.31 %), heptacosane (2.92 %), phytone (2.95 %), 4-heptanone-2-methyl (2.24 %) and n-nonane (2.24 %), accounting for 55.27 % of peaks areas [32].

The extracts of *EA* aerial parts have been shown to present anticancer, antioxidant, anti-inflammatory and antibacterial activity [13-16], [18]. Carsten et al. have shown that *EA* extract inhibits T cells in a dose-dependent manner without the induction of apoptosis or necrosis [31]. In a study by Dukic et al., the *EA* antioxidant activity and phenolic compounds were determined in 3 extracts (n-BuOH, EtOAc, and water); the highest and lowest antioxidant activities were expressed by the EtOAc and the water extract, respectively. Whereas the major flavonoid of both the *EA* EtOAc and n-BuOH extracts was also isoquercitroside, a high proportion of phenolic acids

and a low percentage of flavonoids were detected in the water extract [14]. This major flavonoid of *EA* and *ED*, isoquercitroside, presents properties of cardio-angio- and neuro-protection, is anti-inflammatory and was shown to prevent diabetic complications [31, 33].

Our cytotoxicity study of *ED* extracts on small intestine cells (FHs 74 Int cell line) indicates that the ethyl acetate extract presents the highest toxicity, followed by the n-hexane extract and finally the methanol extract. From IC_{50} of extracts, the n-hexane and EtOAc extracts were classified as moderately cytotoxic ($2 < \text{IC}_{50} < 90$ $\mu\text{g/mL}$) whereas the MeOH extract is not cytotoxic ($\text{IC}_{50} > 90$ $\mu\text{g/mL}$) [34, 35].

5. Conclusions

Medicinal herbs have been used for a very long time but are still often identified according to the morphology of whole parts of a plant. HPTLC fingerprinting has the advantages of a low-cost method that gives a quasi-immediate image of the characteristic phytochemicals in a plant and, therefore, is more and more applied. From the present study, chromatographic profiles (HPTLC and HPLC) of *ED* using different chromatographic conditions allow clear profiles for identification of the plant, also conforming to a previous method shown to be species-specific. Except for alkaloids, several groups of secondary metabolites were found, especially in the methanol extract of *ED*, isoquercitroside being the major flavonoid (1.60 ± 0.04 mg/g). In a preliminary assessment of *Equisetum diffusum* aerial parts safety, the cytotoxicity of these 3 *ED* extracts was investigated on the FHs 74 Int human intestinal epithelial cell line, a non-cancerous, non-transformed normal cell line. The ethyl acetate extract was shown

to be the most toxic with an IC_{50} of $8.7 \pm 2.3 \mu\text{g/mL}$, followed by the n-hexane and methanol extracts, with an IC_{50} of $46.1 \pm 3.6 \mu\text{g/mL}$ and $124.7 \pm 23.0 \mu\text{g/mL}$, respectively.

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