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Bioactive compounds in European ferns: inhibition of pro-inflammatory enzymes and cytotoxic effects on cancer cells

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Abstract

Background Ferns, among the oldest vascular plants, have been widely used in traditional medicine worldwide. While pharmacological studies predominantly focus on tropical and subtropical species, European ferns remain underexplored despite their potential to yield novel bioactive compounds. Given the limitations and side effects of existing anti-inflammatory and anticancer agents, European ferns represent a promising source of safer, naturally derived therapeutic options.

Methods This study investigated the anti-inflammatory and anticancer properties of methanol extracts from 16 European fern species. The extracts were tested for their ability to inhibit pro-inflammatory enzymes of the arachidonic acid cascade, specifically cyclooxygenases (COX-1, COX-2) and 5-lipoxygenase (5-LOX). Cytotoxicity assays on SW480 human colon cancer cells, HeLa cervical cancer cells, CCD 841 CoN colorectal epithelial cells, and HepaRG liver cancer/non-cancer cells were performed to evaluate anticancer activity and toxicity. Flavonoid content analysis was conducted for selected species using LC/MS.

Results Most fern extracts at low concentration of 10 µg/mL effectively inhibited COX-1, with *Dryopteris cambrensis* and *Athyrium distentifolium* achieving inhibition comparable to Ibuprofen (92% and 91%, respectively). Moderate inhibition of 5-LOX (over 50%) was observed across many species, while only a few, including *Blechnum spicant*, *Dryopteris expansa*, and *D. aemula*, moderately inhibited COX-2. Cytotoxicity screening identified five species, notably *D. aemula* and *D. borreri*, with significant activity against SW480 cells (IC50 = 79 and 115 µg/mL). *A. distentifolium* exhibited notable activity against HeLa cells. Despite cytotoxicity of active species toward non-cancerous CCD 841 CoN cells, HepaRG cell assays suggested a more favorable toxicity profile for most active species.

Conclusions This study reveals the significant anti-inflammatory and anticancer potential of European ferns, particularly their selective cytotoxicity in HepaRG cancer cells. These findings highlight European ferns as promising sources of therapeutic agents and encourage further exploration of their bioactive compounds for drug development.

Keywords Anti-inflammatory, Anticancer, Cytotoxicity, Cyclooxygenase, Flavonoids, Ferns

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Introduction

Ferns, the oldest vascular plants on Earth, are used in traditional medicine in many parts of the world. These plants of Division *Pteridophyta* include 12 573 accepted species according to World Flora Online Plant List [1] with more than 400 species reported for ethnomedicinal uses, out of which only 34 species were used in Europe [2]. Although several studies had demonstrated the pharmacological efficacy of fern species, most of these studies included predominantly species from tropical and subtropical climatic zones [3]. It is not surprising regarding the fact that almost half of the known fern species grow in wet tropical regions of Southeast Asia and ethnomedicinal use of ferns is reported mostly in China, India, and Malaysia [2]. Thus, the European species present a potential source of unknown compounds with so far unrecognized bioactivity.

Within *Pteridophytes*, the families *Aspleniaceae* and *Polypodiaceae* are the two of the highest diversity in Europe [4]. To these families belong fern species of genera *Asplenium*, *Athyrium*, *Polypodium*, *Dryopteris* or *Polystichum*. From *Asplenium*, *A. trichomanes* and *A. scolopendrium* are the most represented in Europe, while from *Athyrium*, it is *A. filix-femina*. From *Polypodium* and *Polystichum*, especially *Polypodium vulgare* and *Polystichum aculeatum* are the widespread species, whereas *Dryopteris* genera is more diverse with *D. filix-mas*, *D. carthusiana*, *D. dilatata* and other being rather common in Europe.

Our previous study revealed high antioxidant capacity of the majority of European fern species (37 species tested) [5, 6] and thus encouraged a search for bioactive compounds among these mostly unexplored ferns. The two families of *Dryopteridaceae* and *Athyriaceae* appeared to be the most interesting from a pharmacological point of view. Thus, 16 fern species were selected to be tested in this study. Some of these fern species have been explored for its chemical content or pharmacological properties; however, the knowledge about them is scarce in comparison with non-European species. For example, an extract Primiplex™ prepared from *Athyrium filix-femina* was reported to inhibit diverse human solid tumour malignancies in vitro [7]. However, there is no evidence of its clinical use and its chemical characterization is missing. There is only one study that reports the presence of caffeic acid derivatives in fronds of *(A) filix-femina* [8]. In 1971, a very sweet compound called osladin was isolated from the rhizome of *P.vulgare* [9], which is used commercially as a sweetener. The rhizome also contains a range of ecdysteroids and triterpenoids [10, 11]. *P. vulgare*, was proposed for use in cosmetics due to its beneficial activity against harmful microorganisms [12]. A study of Naz SB et al. [13] approved the traditional use of *P. vulgare* as a smooth muscles relaxant. From another *Polypodium* sp., *P. leucotomos*, the polyphenol enriched extract – Fernblock– found a commercial use in cosmetics and as a dietary supplement with photoprotective effects. The extract was studied and confirmed also for its chemopreventive activity against skin cancer [3].

The *Dryopteris* species were extensively investigated for the content of phloroglucinol compounds [14–16]. Phloroglucinols are well known for their nematocidal activity [17–19], but also antibacterial [20], antiviral [15], and anticancer [21] activities were observed for them. Moreover, triterpenes form *D. crassirhizoma* were reported to exert inhibitory activity against HIV-1 protease [22]. Furthermore, Cao et al. [23] showed antioxidant, anticancer and acetylcholinesterase inhibition activities of flavonoids extracted from *D. erythrosora*. The methanol extracts of *D. affinis* and another European fern species, *Polystichum aculeatum*, showed antibacterial potential against *Staphylococcus aureus* [24].

From other fern species, especially *Blechnum spicant* is found in Europe. Its chemical characterization was carried out back in 1974 by Jizba and Herout [25]; however, its pharmacological effect remain unexplored. On the other hand, several non-European *Blechnum* species were studied. For example, the extract of *Blechnum occidentale* was reported to exhibit anti-inflammatory and antinociceptive activity [26]. In addition, rosmarinic acid isolated from *(B) brasiliense* showed its potential as an agent against neurodegenerative diseases [27]. Moreover, *B. orientale* extracts were found effective against colon cancer [28].

Inflammation

is a response of organisms to injury caused by the influx of inflammation mediators to the site of injury, which is manifested with symptoms such as heat, redness, swelling, pain and loss of function. Among enzymes involved in inflammation belong cyclooxygenase (COX) and lipoxygenase (LOX) enzymes of the arachidonic acid cascade [29], which is the target of commonly used pain-killers and anti-inflammatory drugs called non-steroidal anti-inflammatory drugs (NSAIDs). However, NSAIDs are known to have side effects causing gastric damage and other serious health problems that may lead to the progression of cancer [30]. Ferns might be promising source of safer anti-inflammatory agents because about 45% of fern species were reported to be used in ethnomedicine to treat gastrointestinal disorders [2].

From the enzymes involved in the arachidonic acid metabolism, COX-2 is considered as a key pro-inflammatory enzyme, which is over-expressed in most sites of inflammation and several tumours, and is responsible for the characteristic inflammatory symptoms (redness, pain, oedema, fever and loss of function) [31]. There is a continuous effort to develop safer anti-inflammatory

agents, that has led to the development of dual COX-2/5-LOX inhibitors [32] which are also considered as a promising target for cancer chemoprevention [33]. Selective COX-1 inhibitors, on the other hand, are involved in platelet aggregation and are proposed to have beneficial effects in the prevention of neuroinflammatory [34], cardiovascular [35], and cancer diseases [31]. Finally, 5-LOX is crucial for the biosynthesis of leukotrienes, which are implicated in asthma, ulcerative colitis, rheumatoid arthritis, and cardiovascular pathologies [36–38]. The pharmacological intervention targeting 5-LOX has been proposed as a treatment strategy for allergic and inflammatory diseases, atherosclerosis, and cancer [36, 39–41].

Cancer

is one the leading causes of death worldwide, with incidence and mortality increasing at an alarming rate [42]. Although several efforts have been made to identify potent and safe pharmacological agents to treat carcinomas, most of the chemotherapies still cause harsh side effects. Therefore, the attention turned from the synthesis of new chemotherapeutic agents to the development of more effective strategies [43]. Nevertheless, plants are recognized as endless pool of biologically active compounds that present a potential source of chemopreventive or chemotherapeutic agents [44]. When combined with conventional cancer therapy, certain natural compounds can reduce resistance to cancer therapy and provide chemoprotective effects [45]. In case of ferns, it has been demonstrated that their extracts possess the capacity to act as effective free radical scavengers [6]. Consequently, fern-derived natural compounds may play a pivotal role in the prevention of cancer invasion, migration, and proliferation [46].

Moreover, the cancer treatment with antitumor drugs combined with COX or LOX inhibitors was taken up as a promising strategy [31, 47–52]. The COX-1 overexpression was observed in various malignancies, including ovarian carcinoma, renal cell carcinoma and cervical carcinomas [48, 53, 54]. The overexpression of both, COX-1 and COX-2 was observed in urinary bladder carcinoma [55], or in mouse lung tumors [56]. The overexpression of COX-2 was observed in breast, colon, prostate, hypopharyngeal or lung cancer [57–60], where it correlates with a poor prognosis. The expression patterns of COX-1 and COX-2 vary across different tumor types, indicating that their roles may be context-dependent in cancer pathophysiology [31]. The elevated expression of 5-LOX has also been observed in various tumors, including colon cancer [61], pancreatic cancer [52], adenocarcinoma [62], and cervical cancer [51, 63]. In several cancers, such as esophageal squamous cell carcinoma or colon adenoma, 5-LOX overexpression was associated with tumor malignant transformation, lymph node metastasis and poor prognosis [64, 65]. COX or LOX inhibition offers a promising strategy for cancer treatment, especially in combination with other therapies. Such inhibition has been shown to induce apoptosis and increase the sensitivity of cancer cells to radiotherapy [51, 52, 61, 66, 67].

The objective of this study was to investigate the anti-inflammatory and anticancer properties of methanol extracts from 16 European fern species. Specifically, the study sought to evaluate their ability to inhibit pro-inflammatory enzymes (COX-1, COX-2, and 5-LOX) and to assess their cytotoxic potential against various cancerous and non-cancerous cell lines. In addition, the study sought to analyze the flavonoid content of selected fern species to explore their potential as sources of novel bioactive compounds for therapeutic applications.

Materials and methods

Plant material

Collection

For the purpose of this study, we selected 16 European fern species from 4 families (Table 1) based on our previous research [5]. The leaves of studied ferns were collected in a private fern collection garden of RNDr. Libor Ekrt, Ph.D. (coordinates: 49∘11‘19.385“N, 15∘27‘11.701“E; W), or in Botanical Garden of the Faculty of Science, Charles University, Prague (coordinates: 50∘4′ 14.988“N, 14∘25′ 14.621“E), or from horticultural plants cultivated in pots at the Institute of Experimental Botany in Prague. Botanical identification was provided by RNDr. Libor Ekrt, Ph.D. (from the University of South Bohemia, Czech Republic). All collected materials were freeze-dried and homogenized before extraction in MeOH.

Extraction

Plant material soaked in MeOH was sonicated for 30 min, incubated at room temperature in dark for 24 h and then centrifuged at 4000 rpm for 15 min. Supernatants were filtrated with PVDF 0.45 µm (ProFill, Fisher, Czech Republic) and evaporated in vacuum. The extracts were re-dissolved in DMSO to the concentration of 100 $\mu g.mL^{-1}$ and stored at -80 °C until analyses [6].

Inhibition of cyclooxygenases and 5 Lipoxygenase

Cyclooxygenase enzymes (ovine COX-1, Lot: SLBV6690, or human recombinant COX-2, Lot: SLBV9863; Sigma-Aldrich, Czech Republic) were added to a reaction mixture of cofactors (5 µM porcine haematin, 18 mM L-epinephrine 50 µM Na_2EDTA) in 100 mM of Tris buffer (pH 8.0), and 10 µL of tested substance. The tested substance included individual fern extracts at a final concentration of 10 µg/mL in each well. (*S*)-(+)-Ibuprofen was used (Sigma-Aldrich, Prague, Czech Republic) as a reference compound at 50 µM final concentration in a

Table 1 List of analysed fern species and source of plant material collection

Family	Species	Source of plant material	Distribution in Europe and CZ
Aspleniaceae Newman	*Athyrium distentifolium* Tausch ex Opiz	Fern collection garden in Telč	native in Europe incl. CZ
	Athyrium filix-femina (L.) Roth	Institute of Experimental Botany	native in Europe incl. CZ
Blechnaceae Newman	*Blechnum spicant* (L.) Roth	Institute of Experimental Botany	native in Europe incl. CZ
Davaliaceae M.R.Schomb. ex A.B.Frank	*Davallia canariensis* (L.) Sm.	Fern collection garden in Telč	native only in Iberian Peninsula
Polypodiaceae J.Presl & C.Presl	*Dryopteris aemula* (Aiton) Kuntze	Fern collection garden in Telč	native in Europe, not in CZ
	Dryopteris borreri (Newman) Fraser-Jenk.	Fern collection garden in Telč	native in Europe incl. CZ, subspecies D. affinis subsp. borreri
	Dryopteris cambrensis (Fraser-Jenk.) Beitel & W.R.Buck	Fern collection garden in Telč	native in Europe incl. CZ
	Dryopteris caucasica (A.Braun) Fraser-Jenk. & Corley	Fern collection garden in Telč	native only in Turkey
	Dryopteris dilatata (Hoffm.) A.Gray	Institute of Experimental Botany	native in Europe incl. CZ
	Dryopteris expansa (C.Presl) Fraser-Jenk. & Jermy	Fern collection garden in Telč	native in Europe incl. CZ
	Dryopteris oreades Fomin	Fern collection garden in Telč	native in Europe, not in CZ
	Dryopteris remota (A.Braun ex Doll) Druce	Fern collection garden in Telč	native in Europe, extinct in CZ
	Polystichum acrostichoides (Michx.) Schott	Institute of Experimental Botany	introduced to EU (Belgium)
	Polystichum aculeatum (L.) Roth	Botanical garden of the Charles University in Prague	native in Europe incl. CZ
	Polystichum setiferum (Forssk.) T.Moore ex Woynar	Fern collection garden in Telč	native in Europe, introduced to CZ
	Polypodium vulgare L.	Botanical garden of the Charles University in Prague	native in Europe incl. CZ

Accepted botanical names according to World Checklist of Vascular Plants [68]; Fern collection garden in Telč (private fern collection garden of RNDr. Libor Ekrt, Ph.D. in Telč, Czech Republic); Institute of Experimental Botany (Prague, Czech Republic); Botanical Garden of the Faculty of Science, Charles University, Prague (Czech Republic); *CZ* Czech Republic

well. The Ibuprofen was dissolved in DMSO, in a manner consistent with the procedure employed for the tested samples. The reaction was initiated with 10 µM arachidonic acid as a substrate and it was stopped after 20 min incubation at 37 °C with 10% (v/v) formic acid [69, 70]. The reaction mixture was diluted 1:15 in assay buffer and the concentration of prostaglandin E_2 (PGE_2) produced by the reaction was determined by a PGE_2 ELISA kit (Lot: 03181618B; Enzo Life Sciences, USA) according to the manufacturer's instructions.

The 5-lipoxygenase enzyme (human recombinant 5-LOX, Lot: 0542041; Cayman Chemical, MI. USA) was added to a reaction mixture in phosphate buffer saline (pH 7.4) with 1 mM EDTA, 40 mM ATP (Sigma-Aldrich, Prague, Czech Republic), and 10 µL of tested substance – fern extract at concentration of 10 $\mu g \cdot mL^{-1}$. Zileuton (Farmak a.s., Olomouc, Czech Republic) was used as a reference compound at 45 µM final concentration in a well. The Zileuton was dissolved in DMSO, in a manner consistent with the procedure employed for the tested samples. Then 80 mM $CaCl_2$ was added after 10 min of incubation at 4 °C and the reaction was initiated by 800 µM arachidonic acid. The reaction was stopped after 10 min of incubation at 37 °C by adding 10% (v/v) of formic acid [71]. All samples were diluted 1:15 in assay buffer, and the main product of the reaction, leukotriene B_4 (LTB_4), was quantified using commercial LTB_4 ENZO kit (Lot: 04041613; Enzo Life Sciences, Farmingdale NY, USA) according to the manufacturer's instructions.

The absorbance of final products, PGE_2 or LTB_4, was measured by a microplate reader Tecan Infinite 200 PRO (Tecan Group, Männedrof, Switzerland) at 405 nm and the inhibitory activity was calculated as percentual inhibition compared to blank (DMSO).

Cell line cultures

Cytotoxicity of fern extracts was measured in colon adenocarcinoma SW480 cells, colorectal epithelial cells CCD 841 CoN (both ATCC, LGC Standards, Warszawa, Poland), HeLa cervical cancer cells, and HepaRG nondifferentiated (cancer) and differentiated (normal) hepatic cells (Biopredic, Rennes, France). The HeLa cell line was maintained within cell culture flasks in Modified Eagle's media (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) along with 100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL of fungizone (obtained from Separations Scientific (Pty) Ltd., Johannesburg, South Africa). The SW480 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium - High Glucose) medium supplemented with 10% FBS and 1% penicillin/streptomycin. The CCD 841 CoN cells were maintained in DMEM medium supplemented with 10% FBS, 1% (v/v) sodium pyruvate, 1% non-essential amino acids and 1% penicillin/streptomycin. The HepaRG cells

were maintained in Williams E Medium with GlutaMAX™ supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin and 50 µM hydrocortisone hemisuccinate. The cells were seeded according to the suppliers' protocols in 96-well plates for the viability assays and kept in a humidified atmosphere containing 5% CO_2 at 37 °C. For differentiation, the HepaRG cells were cultured for two weeks in the respective medium supplemented with 1.7% DMSO, replaced every 2–3 days The differentiation of HepaRG cells was monitored microscopically and, at the same time the expression of CYP3A4 was determined [72, 73].

All cell cultures except HeLa cells were kept and anticancer assays were performed at the Faculty of Pharmacy in Hradec Kralove, Charles University, Czech Republic (CU). The HeLa cell line was kept and anticancer assays were performed at the Department of Plant and Soil Sciences, University of Pretoria, South Africa (UP). Cells were sub-cultured at 80% surface confluency with the use of 0.25% trypsin-EDTA.

In vitro cell viability

The evaluation of cytotoxicity in cell lines (SW480, CCD 841 CoN, and HepaRGs) at the CU was made by WST-1 colorimetric assay (Sigma-Aldrich, Prague, Czech Republic). This assay is based on the conversion of tetrazolium salt to formazan by mitochondrial dehydrogenase enzyme in alive cells. Cells were seeded as recommended by the supplier in 96-well plates and allowed to adhere for 24 h and exposed to fern extracts for 24, 48 and 72 h. Initially, the fern extracts pre-dissolved in DMSO were tested in two concentrations (100 and 10 µg/mL) in respective medium. For IC_{50} evaluation, four concentrations were analyzed (10, 50, 100, 200 µg/mL). In all samples the concentration of DMSO was 0.1% as in the control. At the end of the incubated period, medium was removed using an Aspiration Station and replaced with 100 µL of 40× diluted WST-1 in respective medium. The absorbance was measured at 450 nm with 650 nm of reference wavelength by Tecan M200 microplate reader (Tecan Group, Switzerland) after 2 h.

The cytotoxicity in HeLa cells at UP was evaluated using the PrestoBlue™ Cell Viability Reagent (PB) (ThermoFisher Scientific, Johannesburg, South Africa). This reagent is quickly reduced by metabolically active cells, providing a quantitative measure of viability and cytotoxicity. The in vitro cell viability of HeLa cells was measured as follows. Hundred µl of the cell suspension was seeded within 96-well plates at a concentration of 1.0×10^6 cells/mL and allowed to adhere for 24 h. A stock solution of each fern extract was prepared at a concentration of 20 mg/mL. It was then serially diluted, with 100 µl of each dilution added to the 96-well plate to reach a final concentration ranging from 3.125 to 400 µg/mL. The controls included were 2% DMSO vehicle control, cells grown in complete media only, as well as Actinomycin D (ActD) at final concentrations ranging from 3.9×10^4 to 0.05 µg/·mL. Sample and control exposed cells were then incubated for further 72 h, after which 20 µl of PB was added to each well and incubated for 2 h. The absorbance was measured at 570 nm (reference wavelength of 600 nm).

The samples were tested in triplicates with cell viability being calculated as follows:

% Viability = ((Abs sample – Abs PB)/(Abs control – Abs PB)) × 100.

Abs sample was either for the plant extracts or ActD and Abs control was either DMSO (for the plant samples), or media only (for ActD) to account for the solvent. Abs PB was deducted from all values to minus the color of the dye itself.

Statistical analysis

For the anti-inflammatory activity assessment, all samples were measured in triplicate, with at least three independent measurements performed. Results were expressed as means ± SD (standard deviation). Data were analysed by STATISTICA software (StatSoft, TIBCO Software Inc., Palo Alto, CA, USA), subjected to one-way analysis of variance (ANOVA). Tukey's test with significant difference at $p \leq 0.05$ level was used to separate the means.

The cytotoxicity was expressed as the 50% inhibitory concentrations (IC_{50}). Statistical significance (evaluated using a one-way ANOVA) was acceptable to a level of $p < 0.05$. The concentration inducing a 50% decrease of cell viability as compared to control (IC_{50}) was calculated using GraphPad Prism 10.4 nonlinear regression (GraphPad Software Inc., USA).

Chemical characterization

Extracts were dissolved in 950 µL of 100% MeOH and 50 µL of internal standard solution (10 µg/mL fluconazole in 100% MeOH) was added. Samples were stored at -18 °C for one day and then centrifuged (4,755 × g, 10 min, 4 °C). Supernatants were then collected, and 100 times dissolved with MeOH prior the LC/MS analysis. A UHPLC/MS-HRAM system consisting of an Ultimate 3000 ultra-high-performance chromatograph (Thermo Fisher Scientific, Sannyvale, CA, USA) and an Impact II high-accuracy mass Q-TOF spectrometer (HRAM) (Bruker Daltonik, Bremen, Germany) were used for analysis. Separation was carried out on Acclaim RSLC 120 C18 column (2.2 µm, 2.1 × 100 mm, Thermo-Fischer Scientific, Sannyvale, CA, USA) and mobile phases used were: (A) formic acid in water (0.2% v/v) and (B) MeOH (100%) with gradient elution. The gradient started at 2%

B (0–2 min), then increased to 100% B in 15 min and held at 100% for 5 min, returned to starting conditions (2%) in 21 min and equilibrated for 5 min. The flow rate of the mobile phase was constant at 250 µL/min and column temperature was kept at 35 °C. MS analysis was performed by electrospray ionization (ESI) in positive mode with sampling frequency of 1 Hz and mass resolution of 60,000. Injection volume of the sample was 5 µL. oTof Control 4.0, HyStar 3.2 and DataAnalysis 4.3 software (all Bruker Daltonik, Bremen, Germany) were used for data acquisition and preprocessing. Polyphenols were quantified using commercially available standards (all Sigma-Aldrich, Prague, Czech Republic). The relative amount of the tentatively identified compounds was expressed as the ratio of the peak area of the analyte and the internal standard. The identification of the measured metabolites was carried out by their exact mass, isotopic profile and MS/MS spectra and, if possible, also by comparison with the retention times of commercial standards.

Results

Anti-inflammatory activity

Fern extracts at concentration of 10 µg/mL were tested to inhibit the activity of COX-1, COX-2 and 5-LOX pro-inflammatory enzymes. The inhibitory activity expressed in percents for individual fern species is shown in Table 2.

Fern extracts inhibited COX-1 more effectively than COX-2. In several cases, extracts inhibited COX-1 comparably to the reference compound Ibuprofen. Extracts of eight fern species caused more than 80% inhibition of COX-1. The most effective were extracts of *D. cambrensis* (92%), *(A) distentifolium* (91%), *D. remota* (90%), and *(B) spicant* (89%). On the other hand, only low to moderate inhibition of COX-2 was observed, with *B. spicant*, *D. expansa*, and *D. aemula* giving the best results (44–48% inhibition).

In case of 5-LOX, eleven out of all tested fern species caused at least 50% inhibition of 5-LOX. The best results were observed for four *Dryopteris* species: *D. cambrensis* (69%), *D. caucasica* (68%), *D. borreri*, and *D. oreades* (both 66%).

Anticancer activity

The anticancer activity of fern extracts was tested on cervical cancer cell line (HeLa), colorectal cancer cell line (SW480) and hepatic cancer cell line (HepaRG nondifferentiated). The toxicity to noncancerous cells was tested using colorectal epithelial cell line (CCD 841 CoN) and human hepatic cells (HepaRG differentiated). The viability of tested cell lines upon the addition of fern extracts was determined. Firstly, fern extracts were tested at concentrations of 100 and 10 µg/mL (data not shown). Extracts of several fern species were inactive in these cytotoxicity assays, such as *D. canariensis*, *P. vulgare*, some *Dryopteris* spp. (*dilatata*, *expansa*, *oreades*), and all *Polystichum* sp. Next, the 50% inhibitory concentrations (IC_{50} values) were evaluated only for those fern species that proved activity in preliminary screening (Table 3).

From the tested fern species, *Athyrium*, *Blechnum* and *Dryopteris* species exhibited potential to exert anticancer activity in selected cancer cell lines. The IC_{50} values in cancer cell lines ranged from 69.2 µg/mL to 314 µg/mL. The highest IC_{50} values were observed for HeLa cancer cell line. In this case, the most effective was *A. distentifolium* with $IC_{50} = 209$ µg/mL[1]. In case of SW480 colorectal cancer cells, the lowest IC_{50} value was determined for *D. aemula* (79 µg/mL), but also *D. borreri*, *D. remota*, *(A) distentifolium* and *(B) spicant* exerted IC_{50} values below 200 µg/mL. However, except for *A. distentifolium*, all these fern extracts exhibited higher toxicity to noncancerous CCD 841 CoN colorectal epithelial cells. On the other hand, in HepaRG cancerous vs. noncancerous cells, all active fern extracts exhibited desired toxicity ratio. The viability of hepatic cancer cells was inhibited by extracts of *A. distentifolium*, *A. filix-femina*, *D. aemula*, *D. borreri* and *D. cambrensis*. Considering all cancer cell lines, the lowest IC_{50} values were determined for *D. aemula* and *A. distentifolium*, with *A. distentifolium* being more effective in cancer cell lines than toxic for noncancerous cell lines.

Chemical characterization of biologically active fern extracts

Based on the results of anti-inflammatory activity and cytotoxicity, we selected 5 fern species as promising holders of bioactive compounds: *(A) distentifolium*, *(B) spicant*, *D. aemula*, *D. borreri*, and *D. cambrensis*. The methanolic extracts of these selected ferns were analysed using UHPLC/MS-HRAM system to quantify known flavonoids and phenolic acids (Table 4; Supplementary Fig. 1) and additionally, a tentative identification of other compounds was performed for *Dryopteris* species (Supplementary Table 1).

In general, all analyzed fern extracts were rich in chlorogenic acid, but the exact amounts were very distinctive. Extremely high content of chlorogenic acid was observed in *A. distentifolium* (12498 µg/g). On the other hand, *D. aemula* contained the lowest amount of chlorogenic acid (311 µg/g). Other phenolic compounds present in *(A) distentifolium* extract in higher amounts than in other fern species were vitexin, taxifolin and 3-hydroxybenzoic acid. *(B) spicant* was rich in rutin, chlorogenic acid and *p*-coumaric acid. *D. aemula* had the lowest content of quantified phenolic compounds from the selected fern species, but it contained the highest amount of luteolin. *D. borreri* was rich in chlorogenic acid, protocatechuic acid, rutin and kaempferol. It contained the highest amount of protocatechuic acid and kaempferol from the selected ferns.

Table 2 Inhibition of cyclooxygenases and 5-lipoxygenase by fern methanol extracts at a concentration of 10 µg/mL

Species	COX-1 inhibition (%)				COX-2 inhibition (%)				5-LOX inhibition (%)			
Athyrium distentifolium	91.18	±	1.07	a	29.34	±	8.49	cde	57.45	±	12.31	bcdef
Athyrium filix-femina	60.95	±	4.66	cd	NA				57.59	±	4.54	bcdef
Blechnum spicant	88.89	±	5.07	a	48.19	±	11.96	b	55.83	±	9.45	bcdef
Davallia canariensis	86.63	±	6.27	ab	19.39	±	3.57	de	33.08	±	11.08	h
Dryopteris aemula	87.07	±	2.32	ab	43.63	±	4.00	bc	49.30	±	7.55	efgh
Dryopteris borreri	82.53	±	3.89	ab	17.25	±	4.21	e	66.32	±	6.60	bcd
Dryopteris cambrensis	92.46	±	1.23	a	24.85	±	6.17	de	68.50	±	7.64	bc
Dryopteris caucasica	52.57	±	11.96	d	NA				68.32	±	4.38	bc
Dryopteris dilatata	81.61	±	2.42	ab	26.82	±	3.86	cde	35.30	±	6.82	gh
Dryopteris expansa	29.68	±	13.92	e	47.54	±	17.51	b	53.14	±	10.23	cdef
Dryopteris oreades	32.28	±	12.35	e	NA				65.57	±	5.14	bcde
Dryopteris remota	90.07	±	0.94	a	35.75	±	11.52	bcd	13.83	±	15.10	i
Polystichum acrostichoides	71.80	±	6.60	bc	NA				51.68	±	7.56	defg
Polystichum aculeatum	63.42	±	8.39	cd	NA				42.85	±	9.47	fgh
Polystichum setiferum	NA				NA				53.74	±	7.16	cdef
Polypodium vulgare	NA				20.19	±	12.75	de	57.63	±	15.83	bcdef
Ibuprofen	93.80	±	1.78	a	91.60	±	2.99	a	NT			
Zileuton	NT				NT				91.24	±	3.87	a

NA not active, extract that reached less than 10% inhibition at 10 µg/mL concentration; *NT* not tested. Small letters represent statistical differences evaluated by ANOVA Tukey HSD test at $p \geq 0.05$

Table 3 Antiproliferative activity (IC_{50} values in µg/mL) determined for fern extracts against cancer and normal cell lines after 72 h of incubation

Cell line	HeLa	SW480	CCD 841 CoN	HepaRG nondiff.	HepaRG diff.
Fern Species	$IC_{50} \pm SD$ (µg·mL^{-1})				
Athyrium distentifolium	208.83 ± 38.84	130.40 ± 6.43	198.36 ± 18.36	69.20 ± 15.6	88.57 ± 9.40
Athyrium filix-femina	303.17 ± 88.93	> 200	> 200	123.10 ± 18.40	> 200
Blechnum spicant	> 400	193.00 ± 8.21	83.41 ± 8.20	> 200	> 200
Dryopteris aemula	NT	78.7 ± 5.38	64.30 ± 6.38	103.20 ± 5.45	> 200
Dryopteris borreri	314.43 ± 29.44	115.35 ± 14.47	109.9 ± 17.56	181.00 ± 64.10	> 200
Dryopteris cambrensis	306.03 ± 47.23	> 200	183.36 ± 33.39	179.6 ± 12.20	> 200
Dryopteris caucasica	220.30 ± 56.70	ND	ND	ND	ND
Dryopteris remota	> 400	188.30 ± 35.89	124.49 ± 20.08	> 200	> 200

ND Not determined due to inactivity

Table 4 The content of polyphenolic compounds in the selected fern species determined by UHPLC/MS

Compound	*Athyrium distentifolium*	*Blechnum spicant*	*Dryopteris aemula*	*Dryopteris borreri*	*Dryopteris cambrensis*
	µg·g^{-1} dry extract				
3-hydroxybenzoic a.	71.27	tr.	15.60	tr.	4.38
caffeic a.	tr.	tr.	33.27	15.11	
chlorogenic a.	12498.31	2580.27	311.13	2821.03	4882.09
ferulic a.		0.50			
kaempferol	10.74	1.51	44.18	117.13	99.59
luteolin			95.35		5.95
luteolin glucoside				34.58	tr.
p-coumaric a.		319.17	22.15	10.59	
protokatechuic a.	80.82	58.52	31.42	444.39	36.76
quercetin	2.09	18.67	2.84	5.61	4.73
rutin	tr.	3660.54		152.18	1634.60
taxifolin	20.87		2.62		tr.
vitexin	75.15			tr.	tr.

* tr. = trace amount, under limit of quantification; a. = acid

D. cambrensis also contained rather high amount of kaempferol when compared to other selected ferns, and it was rich in rutin and chlorogenic acid.

Despite *D. aemula* having the lowest content of identified polyphenols, its UHPLC analysis revealed a number of peaks that remained undetermined and were subjected to tentative identification. The most significant peak was observed for ion with $(M+H)^+$ of 447.2 that was unique for *D. aemula*. A tentative identification of minor compounds in the extracts was carried out also for other *Dryopteris* species (Supplementary Table 1). In general, they showed very similar UHPLC profiles suggesting similar chemical composition. Most of the minor compounds were identified as phloroglucinols, such as desaspidin, flavaspidic acid, abbreviatin, albaspidin and aspidin derivatives.

Discussion

Previously, we studied the antioxidant capacity and the content of nutraceutical compounds in European fern species [5]. Based on the obtained results, we selected 16 fern species, which extracts were tested for the anti-inflammatory and anti-cancer activity. The chemical characterization was carried out for those extracts that gave the most significant results.

Identification of compound present in biologically active fern extracts

The chemical characterization confirmed the presence of various phenolic compounds, such as phenolic acids (caffeic, chlorogenic, *p*-coumaric, 3-hydroxybenzoic, ferulic and protocatechuic) and flavonoids (kaempferol, luteolin, quercetin, rutin, taxifolin and vitexin). These compounds are excellent antioxidants as well as anti-inflammatory agents. Their antioxidant effect is related to the number of hydroxyls in the molecule and their position within the structure. In case of ferns, their remarkable antioxidant capacity that exceeds those of fruits, vegetables and other medicinal plants is predominantly attributed to the presence of hydroxycinnamic acids (coumaric, chlorogenic and ferulic; [74, 75]). On the other hand, the anti-inflammatory activity of fern extracts is attributed to the presence of flavonoids with 3,4-catechol moiety (luteolin, quercetin, rutin, taxifolin; [74, 76]). Phenolic compounds, which were identified in our study as the components of fern extracts (Fig. 1), were often studied in various models of inflammation either in vitro or in vivo. It has been shown that they inhibit the expression of various pro-inflammatory markers, such as interleukins, TNFα, COX-2, *i*NOS, or NF-κB. Many of them has also proven to have anti-cancer properties [77–82].

Tentative identification of minor compounds in *Dryopteris* species

Tentative identification of other compounds presents in *Dryopteris* extracts confirmed the presence of phloroglucinols (Supplementary Fig. 2). This is in accordance

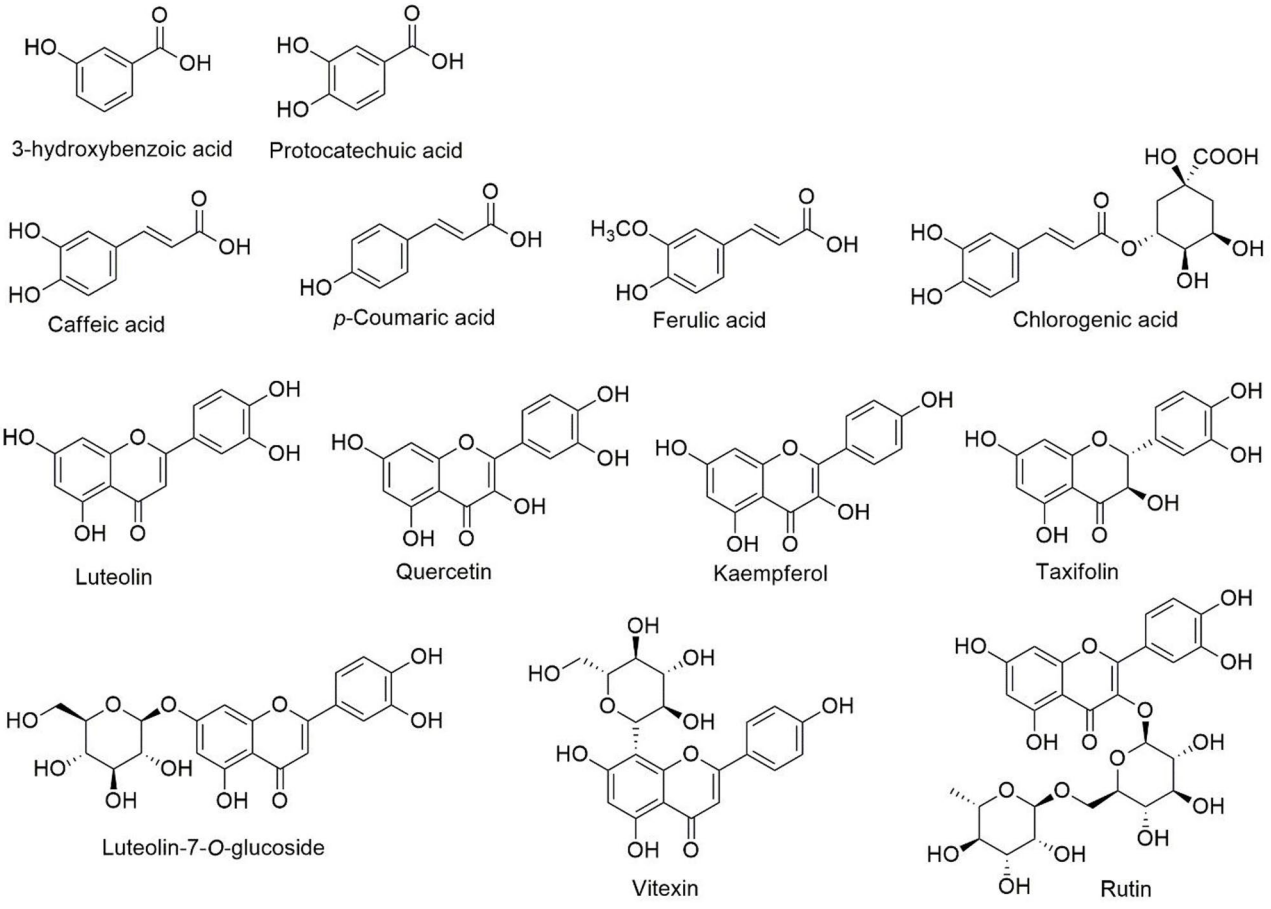

Fig. 1 The chemical structures of polyphenolic compounds in the selected fern species determined by UHPLC/MS. Structures were created by ChemDraw software

with the knowledge that phloroglucinols are compounds that seem to appear exclusively in suborder *Polypodiineae* (Eupolypods I) and predominantly in the family *Dryopteridaceae* [16, 83]. Phloroglucinol compounds were detected in probably all the species of the genus *Dryopteris* and in a few species of the genus *Polystichum* [16]. Phloroglucinols tentatively identified in our extracts are desaspidinol, aspidinol, albaspidins, aspidins, desaspidins, flavaspidic acids, filixic acids and others. All phloroglucinols are well known as anthelmintics.

Inhibition of pro-inflammatory enzymes

The IC_{50} values or percentual inhibition of COX and LOX enzymes determined in various studies for polyphenol compounds are compared in Supplementary Table 2. Direct comparison of COX and 15-LOX inhibitory activity of several common flavonoids by Lee and Kim [84] showed that most of the studied flavonoids were able to inhibit COX-2 at IC_{50} values below 100 μM with the lowest IC_{50} value (59.9 μM) obtained for luteolin, followed by kaempferol and quercetin, and that weak COX-1 inhibition was observed for kaempferol and quercetin. Quercetin and luteolin also inhibited 15-LOX at 100 μM [84]. There were no reports on the ability of phenolic acids to inhibit COX-1 and there are rather big differences in obtained IC_{50} values among all the studies (Supplementary Table 2). Despite these discrepancies it seems that phenolic acids are rather weak COX-2 inhibitors, whereas flavonoids are moderate inhibitors. Kaempferol seems to be moderate COX-1 inhibitor and quercetin strong 5-LOX inhibitor [85].

In addition, the study on the effect of polyphenols on angiogenesis, luteolin and quercetin showed remarkable anti-angiogenic effects, both effectively suppressing the COX-1 expression [86]. Luteolin is one of the most studied flavonoids. It acts as a COX-2 inhibitor when by inhibiting IL-1β-induced JNK, ERK, IκB, and p-NF-κB, downregulates COX-2 expression. Luteolin preferentially inhibits MAPK signaling pathway compared to NF-κB pathway [81]. Quercetin regulates the expression of pro-inflammatory markers via disturbing PI3K/Akt/m-TOR signaling pathway, thus being effective against hepatotoxicity, cardiovascular diseases, neuroinflammation, diabetes or cancer [87]. Rutin (quercetin-3-*O*-rutinoside)

was observed to down-regulate pro-inflammatory markers including COX-2 in rats with cyclophosphamide-induced hepatotoxicity [79]. Actually, quite a number of studies exerted the effectivity of rutin against nephrotoxicity, hepatotoxicity and cardiovascular diseases [88]. Similarly, kaempferol exerts protective effects against a variety of toxins, thus being implicated in the protection against inflammatory bowel diseases, mastitis, lung and liver injuries, cardiovascular events or neurotoxicity in vitro as well as in vivo [89]. Kaempferol inhibits oxidative stress and the expression of inflammatory markers (interleukins, TNF-α, COX-2, NF-κB, etc.), while it modulates apoptosis and MAPK-signaling pathway [89]. Vitexin, 8-*C*-glucoside of apigenin, exerts multiple pharmacological activities, including anti-inflammatory and anticancer [78]. It is able to inhibit most of the pro-inflammatory markers, including COX-2 expression. Taxifolin, a dihydroquercetin, is a strong antioxidant that also exhibited anti-inflammatory activity in various models. Regarding the arachidonic acid pathway, it was found to inhibit COX-2 expression in cerebral ischemia-reperfusion injury or in bone marrow-derived mast cells [82]. Chlorogenic acid, an isomer of caffeoylquinic acid, as well as protocatechuic acid, both have also been shown to exert anti-inflammatory activity in various studies [90]. For example, the ability of chlorogenic acid to inhibit the production of pro-inflammatory markers including down-regulation of COX-2 expression in LPS-stimulated cells has been demonstrated [91]. Protocatechuic acid inhibited COX-2 expression in vitro in models of osteoclast differentiation and photoaging as well as in vivo in models of inflammatory bowel disease, paw edema, arthritis, nephrotoxicity or spinal cord injury [92, 93]. The experimental findings on anti-inflammatory and analgesic (pain-relieving) activity of protocatechuic acid were comparable to standard drugs [93]. The strong antioxidant and anti-inflammatory properties of these acids are associated with the presence of vicinal hydroxyl groups in their molecules.

From these results it seems that predominantly quercetin, luteolin and kaempferol would be responsible for the activity of fern extracts on COXs and 5-LOX, but a contribution of phenolic acids cannot be ruled out.

Regarding the COX and 5-LOX inhibitory activity of the fern extracts tested herein, our results show their potential as the sources of anti-inflammatory agents. The extracts of most of the fern species strongly inhibited COX-1, and moderately 5-LOX. On the contrary, only three fern species exerted low to moderate potency to inhibit COX-2. COX-1 was established as an anti-inflammatory target relatively recently, when its role in the primary response to inflammatory stimuli was uncovered. Moreover, COX-1 was found to play a key role in neuroinflammation, atherosclerosis, and cancer, as well as to modulate response to chemotherapy [94]. 5-LOX, on the contrary, modulates the immune response and plays a role in atherosclerosis, inflammatory bowel diseases, rheumatoid arthritis, and asthma [95]. From the results, it seems that those species that may be regarded as good sources of anti-inflammatory compounds are *(A) distentifolium*, *(B) spicant*, *D. aemula*, *D. borreri*, *D. cambrensis*, and *D. expansa*. Especially, *B. spicant* exerted one of the best percentual inhibition of all three enzymes. *B. spicant* extract contained high amounts of chlorogenic acid, rutin and *p*-coumaric acid, which altogether may be responsible for the activity. Similarly, *D. cambrensis* exhibited the highest inhibition of both COX-1 and 5-LOX enzymes and its extract was also rich in chlorogenic acid and rutin, and also in kaempferol. Other extracts potently inhibited at least one of the enzymes tested.

Anticancer activity

The ability of phenolic compounds identified in our fern extracts to inhibit viability of cancer cells is summarized in Supplementary Table 3. The phenolic acids seem to be rather weak anticancer agents. Only the publications by Nawaz et al. [96], Yao et al. [97], and El Molla [98] suggest moderate activity of chlorogenic acid against HeLa and HepG2 cancer cell lines with IC_{50} values around 25 μM and of *p*-coumaric acid against a panel of cancer cell lines with IC_{50} values between 27 and 95 μM. Flavonoids seem to be more potent anticancer agents than phenolic acids as most of them exhibit moderate IC_{50} values against various cancer cell lines (Supplementary Table 3). In case of SW480 cancer cell line, which was included in our study, the best IC_{50} values were recorded for luteolin and quercetin (Supplementary Table 3). In addition, their inhibitory effects on MAPK-signaling pathway implicates them in the regulation of apoptosis. The regulation of the expression of cytokines and transcription factors further enhances their potency.

Regarding the HepaRG nondifferentiated cancer cell line used in this study, it may be compared with HepG2 cancer cell line on which several flavonoids were tested. From the Supplementary Table 3, it is obvious that most of the flavonoids identified in the fern extract exert good to moderate activity on HepG2 cells. The lowest IC_{50} values were again reported for quercetin and luteolin, but also taxifolin, vitexin and kaempferol were good inhibitors of HepG2 viability.

The evaluation of anticancer activity of fern extracts revealed that there are fern species with pronounced antiproliferative activity, while some are completely inactive. The inactive extracts were from *Davallia*, *Polypodium* and *Polystichum* spp., but also several from *Dryopteris* species. This is in accordance with some studies that reported inactivity of fern species, such as

Osmunda, *Pteridium*, *Polypodium* or *Asplenium*, in anticancer studies [99–101].

On the other hand, the extracts of *Athyrium*, *Blechnum* and several *Dryopteris* species exerted moderate activity against tested cancer cell lines. Herein, the extract of *A. distentifolium* emerged as the most potent with lowest IC_{50} values for the inhibition of viability of cancer cells rather than that of noncancerous cells. It was the only fern species that inhibited the viability of colon cancer cell line (SW480) with lower IC_{50} value than the IC_{50} value for the inhibition of the viability of non-cancer colorectal epithelial cells (CCD 841 CoN). In addition, it most potently inhibited the viability of hepatic cancer cell line (HepaRG nondiff). Chlorogenic acid, vitexin and taxifolin all may contribute to the pronounced anticancer activity of *A. distentifolium* extract, despite the fact that chlorogenic acid is present in surpassing amount (12.5 mg/g) and its moderate anticancer activity was observed only in one study [96]. In our previous study, *Athyrium distentifolium* exhibited a distinctive antioxidant capacity that was comparable to that of the positive control Trolox [5]. However, there are not any other reports on medicinal use or biological activity of *A. distentifolium*, neither for any of its synonyms listed in the World Checklist of Vascular Plants.

Moreover, all active *Dryopteris* sp. inhibited the viability of hepatic cancer cell line with higher potency than its noncancerous cell line, but they were rather toxic to normal colon tissue. Particularly, *D. aemula* showed high safety towards normal hepatic cells since the highest concentration applied (200 µg/mL) did not notably decrease the cell viability, and at the same time, it exerted pronounced activity against cancerous cell lines. Luteolin may stand behind its pharmacological properties; however, potential contribution of minor compounds may be expected as *D. aemula* extract contained wide range of them and its overall content of polyphenols was rather low. These minor compounds were tentatively identified as phloroglucinols. The peaks with the highest response were of $(M + H)^+$ ion of 447 and unique for *D. aemula*. They may correspond to several phloroglucinol compounds, such as Albaspidin PB, Aspidin PB, Desaspidin BB, Ortho-desaspidin BB, Flavaspidic acid AA, Flavaspidic acid BB, Phloraspidinol BB, or Margaspidin BB. The anticancer properties of a panel of phloroglucinols were tested on Epstein-Barr virus antigen activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. Aspidin and desaspidin were the most active compounds in this assay, and aspidin was more potent in vivo against skin carcinogenesis [102]. In more recent studies, aspidins were reported to inhibit the proliferation and viability of osteosarcoma, and various other cancer cell lines (HepG2, HeLa, HCT-8, HO-8910, MCF7, A549, CEM and PC3) in a dose-dependent manner with IC_{50} values between 10 and 36 µM. They affected cell cycle regulators (p53, p21, cyclins and CDKs), inhibited Bcl-2 and PI3K expression as well as Akt phosphorylation, and induced Bax expression, which led to the activation of caspase-9 and caspase-3 cascades [21, 103, 104]. In addition, aspidinols were found to inhibit the viability of A549, Hela and MCF7 cancer cell lines with IC_{50} values between 10 and 60 µM [105, 106]. Phloroglucinols were also found to inhibit fatty acid synthase, a potential target in the treatment of cancer, with IC_{50} values between 23 and 72 µM [107].

D. cambrensis and *D. borreri* exerted similar pharmacological activities in our assays. They both contained the highest amount of kaempferol among tested species and had similar profile of minor compounds tentatively identified as desaspidinol, aspidinol, albaspidins, desaspidins and filixic acids. *D. cambrensis* was also rich in chlorogenic acid and rutin. High amounts of chlorogenic acid and rutin were also detected in *B. spicant* extract which; however, exerted only weak activity against cancer cell lines. Thus, it is possible that these two compounds do not contribute or contribute very little to the anticancer activity of the extracts. Traditionally, *B. spicant* is used to treat skin and stomach disorders [74, 108]. However, no pharmacological activity was studied for *Blechnum spicant* extracts. On the other hand, extracts of another *Blechnum* spp., *B. orientale* exerted moderate anticancer activity on colon cancer cell lines H29 and HCT-116 [109].

In case of HeLa cervical cancer cell line, the IC_{50} values determined for fern extracts were rather low and much higher than their IC_{50} values determined on noncancerous cell lines. To HeLa cell, most potent was the extract of *A. distentifolium*, together with that of *D. caucasica* for which no toxicity effect was observed for any other tested cell line. On the other hand, the safety of fern extracts to sheep hepatocytes has previously been confirmed using the lactate dehydrogenase assay [5].

So far, there are not any fern extracts evaluated against SW480 colorectal adenocarcinoma to compare our results to. Neither there are many evaluated against other colon cancer cell lines. From those, the antiproliferative activity of *Athyrium hohenackerianum* ethanol extract was tested on HCT-116 colon cancer cell line, MCF7 breast cancer cell line and A549 lung cancer cell line [110]. It inhibited best the viability of HCT-116 cells with IC_{50} = 123.90 µg/mL, then MCF-7 cells with IC_{50} = 149.92 µg/mL, and A549 cells with IC_{50} = 179.74 µg/mL. The positive control, cisplatin, inhibited viability of cancer cells with IC_{50} values between 26 and 44 µg/mL. The fractions of *Blechnum orientale* methanol extract were found active against HT29 and HCT-116 colon cancer cell lines with IC_{50} values between 27.5 and 93.9 µg/mL, while against others (MCF7, K562, Chang) were

inactive (IC_{50} ≤ 100 µg/mL). The positive control, curcumin, exhibited IC_{50} values of 5.5 µg/mL [109]. From other edible and medicinal plants, a few plant leaf alcoholic extracts were evaluated against SW480. From these, only two exerted good activity. *Dodonaea viscosa* methanolic extract reached IC_{50} value of 37 µg/mL [111], when the positive control, 5-fluorouracil, exhibited IC_{50} value of 22.7 µg/mL. Similarly, the leaf extract of *Sageretia thea* exerted IC_{50} value of 48.5 µg/mL [112]. The effect of the extract was more pronounced than the effect of the positive control, 5-fluorouracil. In case of liver cancer, an extract of *Dryopteris ramosa* was evaluated against HepG2 cancer cell line. It inhibited its viability with IC_{50} = 85.7 µg/mL, while it was found inactive on PC3 prostate cancer cell line [113]. In addition, an ethanol extract of *Athyrium multidentatum* inhibited the viability of HepG2 cells with IC_{50} = 114 µg/mL, while its IC_{50} value for normal human liver cells Hl7702 was estimated to be 304 µg/mL [114]. The methanol extracts of two species of water fern *Azolla*, *A. caroliniana* and *A. filiculoides*, were tested for their antiproliferative activity on the HepG2 cell line. They exerted IC_{50} values of 217 and 421 µg/mL, respectively, while the positive control, doxorubicin, had IC_{50} = 0.303 µg/mL [115]. From other medicinal plants, *Azadirachta indica*, for example, inhibited the viability of HepG2 cells with IC_{50} = 81.2 µg/mL [116], while an extract of *Anchusa officinalis* L. exhibited IC_{50} value of 72.5 µg/mL [117].

There are also few studies on the activity of fern extracts against HeLa cervical cancer. For example, the methanol extract of *Pteridium aquilinum* fronds exhibited IC_{50} value of 470 µg/mL on HeLa cells [118]. The anticancer activity of the extracts of three *Asplenium* species, *A. ceterach*, *A. scolopendrium* and *A. trichomanes*, were also tested on HeLa cells. They inhibited HeLa cells viability with IC_{50} values between 40 and 205 µg/mL with *A. ceterach* being the most active. The extracts were inactive on A549 cancer cell line and noncancerous cell lines [119]. Moreover, the fractions of ethylacetate and ethanol extracts of *Stenochlaena palustris* all exerted IC_{50} ≤ 50 µg/mL on HeLa cells [120]. The positive control, doxorubicin hydrochloride, had IC_{50} = 2.21 µg/mL. The activity of *Dryopteris juxtapostia* extracts depended on the part of plant and extraction solvents used. While root extracts exerted superior activity to shoot extract against HeLa, dichloromethane extracts were superior to methanol extracts. The IC_{50} values ranged from 17.1 to 143.6 µg/mL for HeLa cancer cell line [121]. The positive control, doxorubicin, exerted IC_{50} = 0.9 and 1.9 µg/mL, respectively.

From this comparison, it is obvious that the anticancer activity of individual fern extracts (IC_{50} = 69.2–314 µg/mL) does not swerve from those obtained in other studies or those of other medicinal plants, suggesting their potential in further studies concerning their fractionalizations and compound isolations. The results revealed that especially the *Dryopteris* species together with *A. distentifolium* may be compelling for further exploration. The tests of their anticancer activity may be extended to other cancer cell lines overexpressing COX and/or 5-LOX, for example to HT29 (colorectal cancer, COX-1, COX-2 and 5-LOX), LoVo (colorectal cancer, 5-LOX), OVCAR-3 and SKOV-3 (ovarian cancer, COX-1), MDA-MB-23 (breast cancer, COX-2), DBU-145 and PC3 (Prostate cancer, COX-2), PANC-1, AsPC-1, and MiaPaCa2 (pancreatic cancer, 5-LOX) [54, 58, 61, 122–126]. In addition, an activity-guided fractionation of the extracts may reveal new substances responsible for COX/LOX inhibitory activity.

Conclusion

This study provides novel insights into the anti-inflammatory and anticancer potential of European fern species. The methanol extracts of selected fern species were profoundly active on COX-1 with moderate activity towards 5-LOX. In particular, *A. distentifolium* and *Dryopteris cambrensis* exerted the highest activities against the two enzymes. *(A) distentifolium* extract is extremely rich in taxifolin and 3-hydroxybenzoic acid. *D. cambrensis*, on the other hand, contains high amounts of chlorogenic acid, rutin and kaempferol. In regards to COX-2, the fern extracts were rather weak inhibitors, with *(B) spicant* being the most active.

The extract of *A. distentifolium* also exerted the best inhibitory activity against cancer cell lines, inhibiting the viability of all three tested cancer cell lines (HeLa, SW480, HepaRG non-diff.) with the highest potency while sparing normal noncancerous cells. In addition, the extracts of *Dryopteris* species exhibited selective cytotoxicity against hepatic cancer cells. The presence of luteolin and kaempferol in these extracts underscores their potential as chemopreventive agents.

This study accentuates the untapped potential of European ferns in pharmacology, encouraging further exploration and utilization of these ancient plants in modern medicine. Future research should focus on further fractionalization and detailed chemical characterization of the active extracts, in order to identify the compounds responsible for COX-1 and 5-LOX inhibitory activity as well as the anticancer activity. Such studies could provide new treatments for inflammation, neuroinflammation, and cardiovascular diseases, as well as chemopreventive agents for cancer therapy.

Abbreviations

5-LOX	5 lipoxygenase
a.	acid
ActD	Actinomycin D
COX-1	Cyclooxygenase 1

COX-2	Cyclooxygenase 2
CU	Charles University
CZ	Czech Republic
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization
FBS	Fetal bovine serum
IC50	Half-maximal inhibitory concentration
LTB_4	Leukotriene B_4
MEM	Modified Eagle´s Medium
NA	Not active
ND	Not determined
NSAIDs	Non-steroidal anti-inflammatory drugs
NT	Not tested
PB	PrestoBlue
PGE_2	Prostaglandin E_2
tr.	Trace amount, under limit of quantification
UP	University of Pretoria

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12906-026-05282-w.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Supporting information

Additional supporting information may be found in the online version of the article at the publisher´s website.

Authors' contributions

LL: Conceptualization; Methodology; Formal Analysis; Resources; Funding Acquisition; Supervision; Writing – original draft preparation. MD: Writing – original draft preparation; Visualization. PM: Methodology; Investigation. AP: Investigation; Writing – original draft preparation. PM: Methodology; Investigation. TE: Investigation. NL: Supervision; Funding Acquisition; Writing – review and editing. BS: Project Administration; Supervision; Resources; Writing – review and editing.

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Data availability

The data that support the findings of this study are available from the corresponding author (langhansova@ueb.cas.cz) upon reasonable request.

Declarations

Competing interests

The authors declare no competing interests.

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