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SHORT COMMUNICATION

Alpha-spinasterol in the roots of *Impatiens glandulifera* and its effects on the viability of human cells

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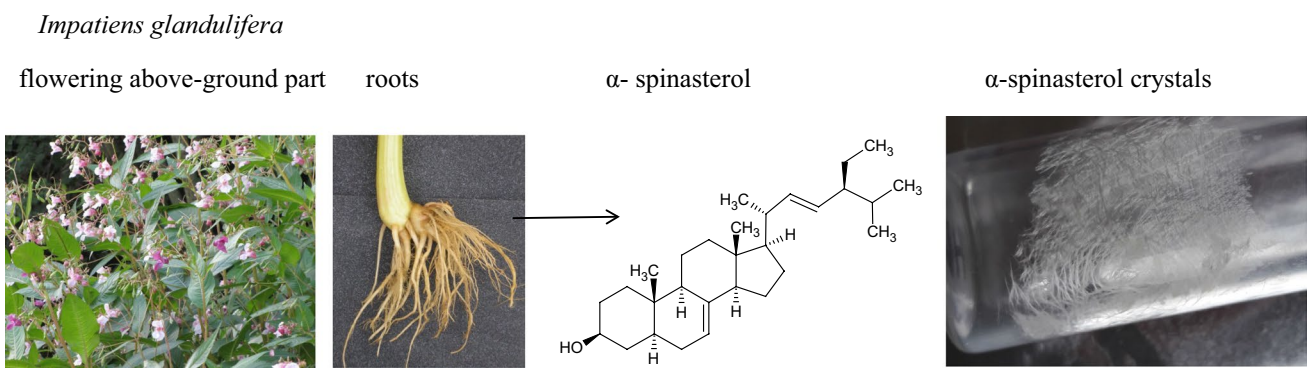
Abstract

The roots of *Impatiens glandulifera* belong to plants, or part of plants with a high content of α-spinasterol. Chromatographic analysis of roots hexane extract using GC–MS revealed that α-spinasterol is the dominant substance in the freeze-dried roots of *Impatiens glandulifera* and its content reaches ca. 1 wt%. When the hexane extracts are concentrated, α-spinasterol crystals fall out in the form of tiny needles, so the isolation of this substance from the roots of *Impatiens glandulifera* is very easy. Regarding the easy isolation and usage of phytosterols as anticancer and antimicrobial agents, cytotoxicity tests were performed using hepatic Hep G2 cells and blood HL-60 cells. Since hexane is an organic solvent, the cytotoxicity of the used hexane concentrations was measured, too.

Graphical abstract



Keywords *Impatiens glandulifera* · Roots · α-Spinasterol · Cytotoxicity

Introduction

Impatiens glandulifera is an invasive plant in Europe, whose metabolites, especially phenolic compounds, show various biological properties; for example, phytotoxic properties (Vrchotová et al. 2011), repellent properties (Pavela et al. 2009), or antioxidant and cytotoxic abilities of plant lipophilic components (Szewczyk et al. 2018). Of the lipophilic substances contained in *Impatiens glandulifera*, α-spinasterol alone among the sterols has interesting biological properties, but it does not occur in large quantities in plants. It got its name from spinach, where it was first identified by Collinson and Smedley-MacLean (1931),

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its structure was further refined in other works (Hart and Heyl 1932; Obata et al. 1955). Alpha-spinasterol is chemically (3β,5α,22E)-stigmasta-7,22-dien-3-ol (syn. hitodesterol; bessisterol). The occurrence of α-spinasterol in various plants and its biological activities are summarized in Table 1.

Recently, an overview of all important pharmacological properties of α-spinasterol known to date was published by Majeed et al. (2022). Let us mention at least some of them to illustrate further possible developments in research regarding the biological properties of α-spinasterol. Jeong et al. (2004) described that α-spinasterol has an inhibitory potency on glomerular mesangial cell proliferation about 1000 times higher than that of simvastatin, which is administered in the treatment of diabetic nephropathy. A recent study showed that α-spinasterol could inhibit intestinal absorption of glucose with an IC_{50} value of 8.6 µg/mL for inhibition activity of α-glucosidase, which was not significantly different from that of the standard α-glucosidase inhibitor, acarbose (Lawal et al. 2020). Alpha-spinasterol can be also used as a novel selective antagonist of transient receptor potential vanilloid 1 receptor (TRPV1) for the treatment of mental disorders such as depression and anxiety (Trevisan et al. 2012; Socała and Wlaź, 2016). In recent years, α-spinasterol has been among selected natural substances tested for antiviral activity against SARS-CoV-2 (Siddiqui et al. 2020; Zubair et al. 2021).

The content of α-spinasterol is not very high in plants. Hart and Heyl (1932) state that the content of α-spinasterol is 0.01% of dried materials of *Spinacia oleracea* leaves. Fernholz and Moore (1939) obtained 0.02 wt% of α-spinasterol from dehydrated alfalfa meal and state that alfalfa meal is probably the most accessible source for this sterol. Wang et al. (2019) report in their work the results of α-spinasterol content measurement in the seeds and nuts of 13 plants. They found the most bound and free α-spinasterol in pumpkin seeds (0.024% and 0.018% of dry weight) and watermelon seeds (0.033% and 0.017% of dry weight). Kalač and Moudrý (2000) state that α-spinasterol constitutes 50% of the sum of dry sterols in Amaranthus seeds.

The method of extraction of α-spinasterol depends mostly on the part of the plant that is extracted. In the stems and especially in the leaves, α-spinasterol is accompanied by a number of other substances that pass into the extract, e.g. chlorophyll, and it is necessary to purify the extract, especially by chromatographic methods. From this point of view, the roots are more suitable for the isolation of α-spinasterol. Roots of different plants were extracted using chloroform (Jeong et al. 2004), petroleum ether (Szewczyk et al. 2018), 95% ethanol, then fractionated between water and hexane following silica gel column chromatography (Wang et al. 2011), shaking roots in 100% ethanol for 3 days, 80% methanol, then aqueous residue after evaporation of methanol was extracted with dichloromethane, ethyl acetate, and butanol. Ethyl acetate fraction was further purified with column chromatography over Sephadex LH-20 (Krasteva et al. 2008). In the case of oils obtained from amaranth seeds (*Amaranthus cruentus*), either supercritical extraction with carbon dioxide under a working pressure of 306 atm at 50 °C or mixture of chloroform/methanol 2:1 (v/v) was used (Czaplicki et al. 2012).

The main goal of this work was to determine the amount of α-spinasterol in freeze-dried roots of *Impatiens glandulifera* and to isolate this substance from the extract in the simplest possible way. Once α-spinasterol was isolated, the cytotoxicity of this sterol and of its solvent (hexane) was examined to verify the potential further usage of α-spinasterol.

Material and methods

Plant material

The roots of *Impatiens glandulifera* (Royle) were collected on the outskirts of the city of České Budějovice, Czech Republic, at the place called "Luční jez", altitude 381 m a.s.l., 48.9658131 N, 14.4659969 E. 1 kg of fresh roots was collected. The roots were cleaned, frozen, and then lyophilized. After lyophilization, the material was stored at − 18 °C.

Chemicals

n-Hexane for gas chromatography MS Suprasolv, purity 98.0% (Merck, Praha, Czech Republic) (referred to as hexane in the text), tetracosane, purity 99.0%, and β-sitosterol, purity ≥ 70.0%, both Sigma-Aldrich, Praha, Czech Republic.

Preparation of the plant material

Prior to extraction, the freeze-dried roots were ground to a very fine powder for hexane extraction. Two samples (A and B) were prepared, each weighing 0.1 g. 3 mL of hexane was added to the plant material, the extraction took place in the dark at room temperature for 1 h with shaking. Then, the extract was removed and the extraction with hexane was repeated four times more. Samples A1–A5 and B1–B5 were thus obtained. Hexane was evaporated from the fractions with nitrogen and the evaporates were dissolved in exactly 3 mL of hexane.

Sample preparation

Samples A1, B1, A2, and B2 were used directly: 200 µL of the sample was taken from the extract and 8 µL of the

Table 1 Brief overview of plants containing α-spinasterol

Plants	Parts of plants	Activity and amount of α-spinasterol	Literature
Acacia auriculiformis	Leaves	Cholinesterase inhibitory effect	Lawal et al. (2020)
Albizia schimperiana	Stem bark	Antimicrobial activity	Fufa et al. (2018)
Amaranthus cruentus	Seeds	Sum of α-spinasterol and sitosterol = 50% of sum sterols	Czaplicki et al. (2012)
Amaranthus spinosus	Seeds		Billah et al. (2013)
Amaranthus spinosus	Whole plant	α-Spinasterol; spinasterol-3-*O*-β-glucopyranoside	Tuyen et al. (2019)
Anacardium occidentale	Nuts		Wang et al. (2019)
Angelica sinensis	Rhizomes		Xin et al. (2019)
Arachis hypogadea	Nuts		Wang et al. (2019)
Ardisia pyramidalis	Leaves	Suppressed blood wessel branching	Raga et al. (2017)
Argania spinosa	Oil	α-Spinasterol about 30% of total phytosterols	El Kharrassi et al. (2018)
Beta vulgaris	Beet pulp	α-Spinasterol obtained from its glucoside	Obata et al. (1955)
Cactaceae spp. (columnar species)		Inhibitory activity against COX-1, COX-2, *Helicobacter pylori*, HeLa	Salazar et al. (2020)
Carya sp.	Nuts		Wang et al. (2019)
Castanea sp.	Nuts		Wang et al. (2019)
Citrullus colocynthis	Leaves	α-Spinasterol + dihydrospinasterol; aphicidal activity	Ahmed et al. (2022)
Citrullus lanatus	Seeds		Wang et al. (2019)
Corulus sp.	Nuts		Wang et al. (2019)
Cucurbita sp.	Seeds		Wang et al. (2019)
Cucurbita maxima	Flowers	Anticarcinogenity activity	Villaseñor and Domingo (2000)
Cucurbita pepo	Seeds		Hrabovski et al. (2012)
Filicium decipiens	Stem bark	From 5 kg of dry material obtained 32 mg of α-spinasterol	Muthia et al. (2015)
Gypsophyla trichotoma	Roots		Krasteva et al. (2008)
Impatiens balsamina	Roots, leaves	Antibacterial activity	Wang et al. (2011)
Impatiens balsamina	Roots		Panichayupakaranant et al. (1995)
Impatiens glandulifera	Rootseed, leaf		Szewczyk et al. (2018)
Impatiens noli-tangere	Rootseed, leaf		Szewczyk et al. (2018)
Juglans sp.	Nuts		Wang et al. (2019)
Koreana stewartia	Leaves	Procollagen production, inhibition of matrix metalloproteinase-1 expression	Lee et al. (2011)
Macadamia sp.	Nuts		Wang et al. (2019)
Manilkara zapota	Bark		Chunhakant and Chaicharoenpong (2019)
Mautabea quianensis	Stems	Allelopatic activity against common weeds of the Amazon region	Ricardo Filho et al. (2012)
Medicago sativa	Seeds		King and Ball (1942)
Melandrium firmum	Whole plants	Prevent benign prostatic hyperplasia	Lee et al. (2014)
Physospermum verticillatum	Aerial parts	Antibacterial activity	Boulacel et al. (2017)
Phytolacca americana	Roots	Therapeutic potential for diabetic nephropathy	Jeong et al. (2004)
Pistachia vera	Nuts		Wang et al. (2019)
Polygala sp.		Antifungal activity	Johann et al. (2011)
Prunus amygdalus	Nuts		Wang et al. (2019)
Pueraria lobata	Roots	Antitumor activity	Jeon et al. (2005)
Pueraria mirifica	Roots	Antitumor activity	Jeon et al. (2005)
Sheareria nana	Aerial parts	α-Spinasterol-3-*O*-β-D-glucoside	Meng et al. (2018)
Spinacia oleracea	Leaves	α-Spinasterol = 0.010% of dried material	Hart and Heyl (1932)
Stegnosperma halimifolium	Stems, leaves	Antiproliferative activity in the cervical cancers, murine macrophage cancer cells	Meneses-Sagrero et al. (2017)

Table 1 (continued)

Plants	Parts of plants	Activity and amount of α-spinasterol	Literature
Pure compounds		Antidepressant, anxiolytic effect	Socała and Wlaź (2016)
Pure compounds		Antagonist TRPV1, cyclooxygenase inhibitor, antinociceptive, antidepressant	Fischer et al. (2020)
Pure compounds		α-Spinasterol resembles the membrane behaviour of cholesterol	Haralampiev et al. (2017)

internal standard solution—tetracosane (C_{24}) with a concentration of 50 µg/mL was added. Samples A3, B3, A4, B4, A5, and B5 were blown dry with nitrogen and then diluted to 0.5 mL with hexane. Then 200 µL was taken into the insert, blown dry with nitrogen, and diluted with 50 µL of hexane. Then 2 µL of the internal standard solution—tetracosane (C_{24}) with a concentration of 50 µg/mL was added.

GC–MS analysis

GC–MS analyses were performed on a Finnigan GCQ instrument, column Rxi–5 ms (Restek Co., Bellefonte, PA, USA), 30 m × 0.25 mm × 0.25 µm. Conditions for the analysis were as follows: injection volume was 1 µL, injection temperature 250 °C, splitless time 1.00 min., split flow 50 mL/min, and carrier gas flow (helium) 1 mL/min. The initial column temperature was set to 120 °C and increased at a rate of 20 °C/min up to a temperature of 260 °C, which was held for 2 min. It was then increased up to 310 °C at a rate of 3 °C/min. The temperature of 310 °C was maintained for 2 min. The samples were measured in full scan mode in the range of 50–750 amu from 3.00 to 28.5 min.

NMR data

α-Spinasterol. 1H-NMR (CDCl3): δ (ppm) 5.16 (dd, 15.0, 8.9, 1H, H-22), 5.15 (bm, 1H, H-7), 5.02 (dd, 15.0, 8.9, 1H, H-23), 3.57 (m, 1H, H-3), 1.02 (d, 6.6, 3H, H-21), 0.85 (d, 6.4, 3H, H-27), 0.81 (t, 7.2, 3H, H-29), 0.80 (d, 6.0, 3H, H-26), 0.79 (s, 3H, H-19), 0.55 (s, 3H, H-18).

The 1H NMR spectrum was referenced to the solvent signal (CDCl3, 7.32 ppm). 1H and 2D experiments (COSY, HSQC, and TOCSY) were measured on a Varian INOVA 500 MHz spectrometer equipped with a standard broadband probe at 25 °C. Only the dominant signals could be undoubtedly signed. The measured record matched the literature data of Ahmed et al. (2022).

Cytotoxic test

Human hepatic cells Hep G2 (kindly provided by the Department of Medicinal Biology, Faculty of Science, University of South Bohemia) and human peripheral blood cells (lymphocytes) HL-60 (ATCC CCL-240) were maintained in DMEM medium (Biosera) supplemented with 10% foetal bovine serum (Cytiva), 1% L-glutamine, and 1% antibiotics (penicillin 100 units/mL and streptomycin 100 µg/mL, all Biowest) at 37 °C with 5% CO_2 atmosphere. Cells were seeded at the concentration of 10,000 cells/well into 96-well plates. After 6 h of adhesion, the medium was changed with the medium enhanced with 10%, 7.5%, 5%, 2.5%, 1%, 0.75%, 0.5% 0.25%, and 0.1% hexane. Cells were incubated for 1, 2, and 4 days to determine the cytotoxic effect of hexane. At selected intervals, the viability of cells was measured using the alamarBlue assay. A new medium with alamarBlue reagent 10:1 v/v (Invitrogen) was added to cells and incubated for 2 h at 37 °C in 5% CO_2 atmosphere. Thereafter, fluorescence (excitation 550 nm, emission 590 nm) was measured using Infinite M200 (Tecan). The results of three replicates were compared to the untreated control without hexane supplementation. The experiment was performed in three independent repetitions.

The cytotoxic effect α-spinasterol was verified the same way. Cells were seeded, adhered, and medium with 48.5, 4.85, 2.4, 0.48, and 0.24 µg/mL of α-spinasterol in 1%, 0.1%, 0.05%, 0.01%, and 0.005% hexane, respectively, was added. Cell viability was measured similarly as described previously. Results were compared to controls consisting of the same hexane supplementation as examined concentrations of α-spinasterol.

Statistical analyses were performed using GraphPad Prism version 10.2.0. Inhibitory concentrations IC_{50} were calculated using nonlinear regression with the dose–response–inhibition equation. The significance of the decrease in cell viability was calculated using two-way ANOVA with Dunnet post hoc test.

Results and discussion

Alpha-spinasterol has already been extracted from many plants—see Table 1. Since the authors in the literature try to determine as many lipophilic metabolites as possible in the plant material, most extractions are relatively very complex, using a variety of solvents and successive extractions (petroleum ether, acetone, alcohol, chloroform, etc.). For obtaining α-spinasterol from fractions that are analysed by GC, the following procedures were used. For

example, Szewczyk et al. (2018) extracted different parts of *Impatiens glandulifera* plant material, the extraction method included nine steps. (The plant material was air-dried.) The authors do not report the yield of α-spinasterol per gram of material, but only the mixture of α-spinasterol and chondrillasterol in mass % of total fraction. This mixture of leaf extract constitutes 69% w/w of total fraction. In the roots, they found significantly less of this mixture (9% w/w), and 44% w/w of β-sitosterol. In addition to *I. glandulifera*, the authors also extracted lipophilic material from *I. noli-tangere*. We analysed only the roots of *I. glandulifera*. Ground freeze-dried roots of *I. glandulifera* were extracted only with hexane. When repeatedly extracting the roots with hexane (Fig. 1), it is clear that we achieved 60–70% efficiency during the first extraction. The dependence of the yield on the number of extraction steps is given by an exponential function with a correlation coefficient of 0.9928—see Fig. 1. To extract the maximum amount of α-spinasterol, repeated extraction is necessary, but as shown in Fig. 1, triple extraction is completely sufficient for approx. 100% extraction of α-spinasterol. It can be seen from the chromatogram (Fig. 2) that α-spinasterol is the dominant substance in the freeze-dried roots of *I. glandulifera*. When the extracts are highly concentrated, α-spinasterol needles fall out.

The problem of broader application and use of α-spinasterol in the pharmaceutical industry or food industry lie in its low content in plants. Currently, there is available (albeit rare and expensive) oil from *Argania spinosa*, which contains, in addition to a whole range of tocopherols and fatty acids, also sterols, including α-spinasterol (El Kharrassi et al. 2018). Argan oil is mainly used in cosmetics. To obtain sufficient amount of α-spinasterol from plants, hexane:ethylacetate (4:1) or hexane-ethyl acetate-ethanol gradient are used during purification (Jeon et al. 2005; Meneses-Sagrero et al. 2017). In our case, only hexane was used for the purification. We found that the concentration of α-spinasterol in lyophilized roots of *Impatiens glandulifera*

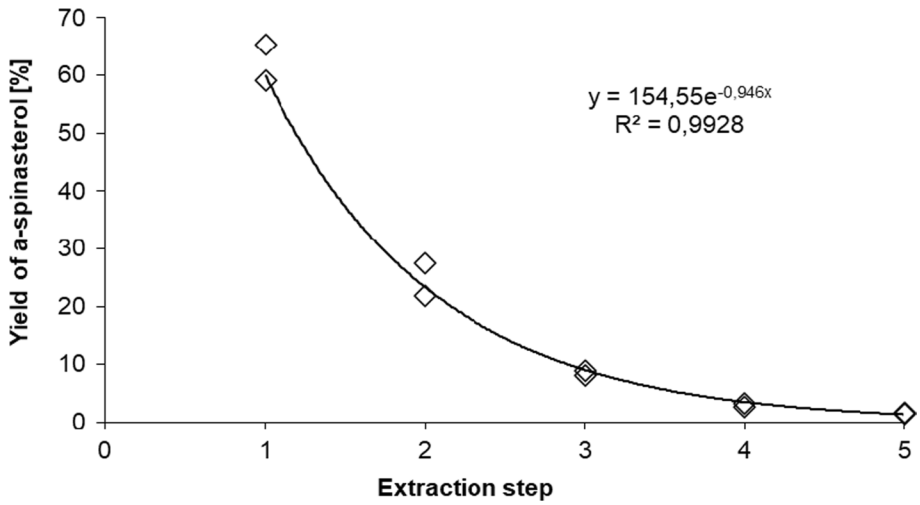

Fig. 1 Efficiency of α-spinasterol extraction using hexane

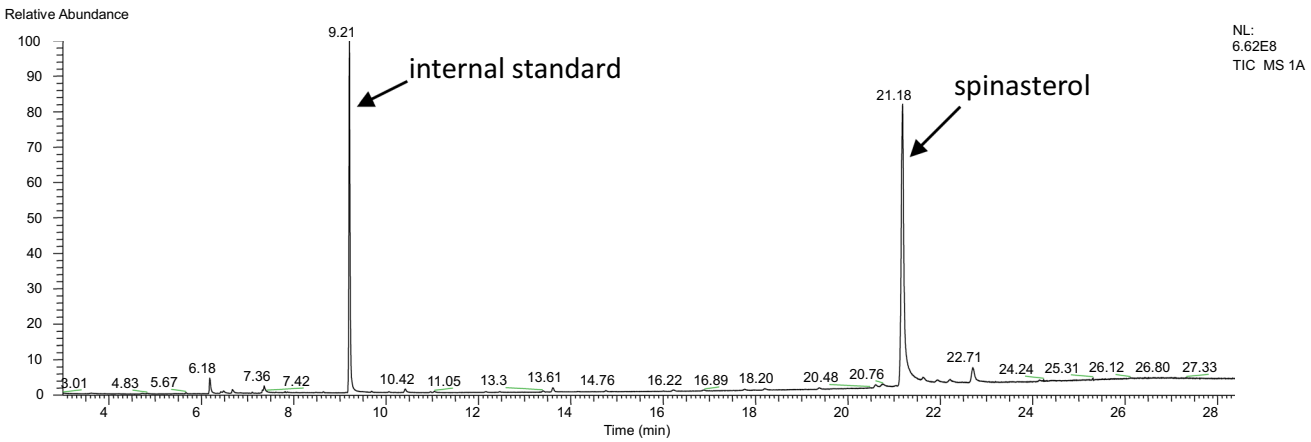

Fig. 2 TIC chromatogram of GC–MS separation of the sample A1

is quite high: in sample A 0.99%, in sample B 1.12% of lyophilized materials and the extract is very pure.

Next, we decided to prove the cytotoxic effect of hexane and α-spinasterol. Organic solvents used for extraction can influence the viability of cells treated by extracts with specific solvents. This phenomenon can differ from cells to cells; thus, the cytotoxic effect of hexane must be excluded in order to later investigate the effect of α-spinasterol (Beedessee et al. 2012). Hepatic Hep G2 and blood HL-60 cell lines were chosen as representative cells with regard to phytosterol metabolism and its effect on human body (Ling and Jones 1995). Three intervals (1, 2, and 4 days) were selected to observe whether the used concentrations of hexane could cause cytotoxic effects. Moreover, this time frame represents sufficient time for the possible biological effects of α-spinasterol. If cell viability decreases below 80% in comparison with untreated control (ISO 2009), it has cytotoxic effects. Moreover, half maximal inhibitory concentration (IC_{50}) is usually calculated for substances with biological activity; thus, we calculated the average IC_{50} as well (Somarathna et al. 2021). Figure 3 presents the cytotoxic effect of hexane on examined cells throughout the used time frame. Similar trends were observed during all intervals, in more detail, only 0.25% and 0.1% hexane concentrations have no cytotoxic effect on Hep G2 cells (Fig. 3A), while only 0.1% hexane did not influence HL-60 cells (Fig. 3B). The higher concentrations of hexane had significant cytotoxic effects on both cells. The average values of IC_{50} calculated from all intervals were determined as 0.491% (37.66 mM) for Hep G2 cells and 0.268% for HL-60 cells (19.94 mM). For hexane, an IC_{50} value of 297 mM was shown for hepatic cells, thus almost eight times higher tolerance than we observed (Zapór et al. 2015). This difference can be caused by the use of primary rat cells that means a different organism and noncancerous cells (Zapór et al. 2015). Our results proved the high cytotoxicity of hexane that should not be omitted when the effects of extracts are examined, which is not the case in some of the previously published studies (Beedessee et al. 2012; Kumar and Santhi 2012).

Taking all these previous aspects into account, we decided to perform experiments with 4.85, 2.4, 0.48, and 0.24 μg/mL of α-spinasterol with the final concentration of hexane 0.1%, 0.05%, 0.01%, and 0.005% and compare it with the respective hexane controls. This was made to detect the influence of α-spinasterol itself on cell viability. Figure 4A presents the results where α-spinasterol was not significantly

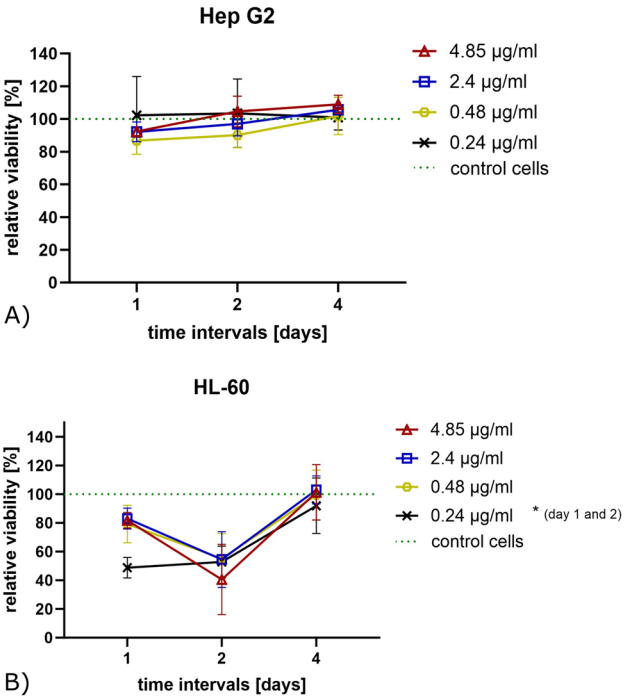

Fig. 4 Cytotoxicity of α-spinasterol. **A** Hepatic cells Hep G2, **B** blood cells HL-60. Green dotted line represents 100% viability of control cells with appropriate concentrations of hexane. Statistical significances were calculated using two-way ANOVA with Dunnet post hoc test in GraphPad Prism version 10.2.0

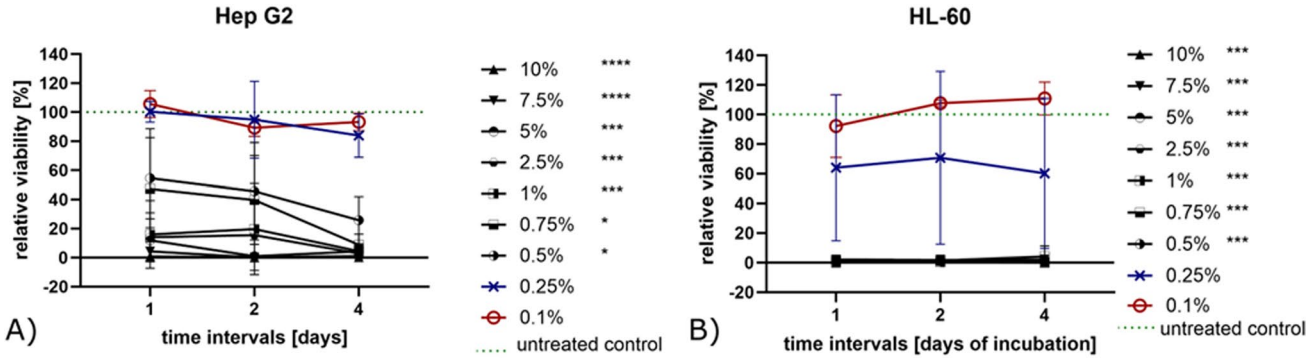

Fig. 3 Cytotoxicity of hexane at selected intervals. **A** Hepatic cells Hep G2, **B** blood cells HL-60. Green dotted line represents 100% viability of untreated control cells. Statistical significances were calculated using two-way ANOVA with Dunnet post hoc test in GraphPad Prism version 10.2.0

cytotoxic for Hep G2 cells in all examined intervals. This is a positive result in case this compound should be incorporated as a potential antimicrobial agent. It is necessary to have an agent without cytotoxic effects on cells but with inhibitory effects on microbes. Moreover, it is important to use as a low concentration of agent as possible. Previous research showed antibacterial effects of α-spinasterol at the concentration of 20–80 µg/mL to *Helicobacter pylori* (Wang et al. 2011), or 245–295 µg/mL to *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Yang et al. 2017). These data indicate that the effective concentration of α-spinasterol differs for various bacteria. Since α-spinasterol showed no cytotoxicity for hepatic cells, it could be incorporated into research focused on treatment of pathogens harmful to the liver, such as hepatitis viruses, Epstein–Barr virus, cytomegalovirus, or Leptospirosis. On the other hand, blood cells HL-60 used in our research revealed that α-spinasterol is significantly cytotoxic for them after 2 days of incubation (Fig. 4B) with a drop in metabolic activity of about 50%. However, the cytotoxic effect diminished after 4 days.

Overall, the results described above show that freeze-dried roots of *Impatiens glandulifera* are a relatively good source of α-spinasterol that can be used in further pharmacological, microbiology, or other experiments.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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