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Enhanced accumulation of betulinic acid in transgenic hairy roots of *Senna obtusifolia* growing in the sprinkle bioreactor and evaluation of their biological properties in various biological models

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Abstract Betulinic acid, which is found in transgenic roots of *Senna obtusifolia* (L.) H.S.Irwin & Barneby, is a pentacyclic triterpene with distinctive pharmacological activities. In this study, we report the differences in the content of betulinic acid and selected anthraquinones in transgenic *S. obtusifolia* hairy roots with overexpression of the *PgSS1* gene (SOPSS2 line) and in transformed hairy roots without this genetic construct (SOA41 line). Both hairy root lines grew in 10 L sprinkle bioreactor. Additionally, the extracts obtained from this plant material were used for biological tests. Our results demonstrated that the SOPSS2 hairy root cultures from the bioreactor showed an increase in the content of betulinic acid (38.125 mg/g DW), compared to the SOA41 hairy root line (4.213 mg/g DW). Biological studies have shown a cytotoxic and antiproliferative effect on U-87MG glioblastoma cells, and altering the level of apoptotic proteins (Bax, p53, Puma and Noxa). Antimicrobial properties were demonstrated for both tested extracts, with a stronger effect of SOPSS2 extract. Moreover, both extracts showed moderate antiviral properties on norovirus surrogates.

Keywords: transgenic hairy roots; sprinkle bioreactor; gene expression; anticancer; antimicrobial and antiviral activity

Introduction

Plant in vitro cultures are currently becoming more and more popular due to the possibility of their universal use. Single cells, organs or whole plants grown in strictly controlled and optimized sterile conditions are becoming an increasingly attractive alternative to many species of valuable plants obtained from the natural environment. [1–3] Because many medicinal plants are an irreplaceable source of various bioactive compounds, the possibility of providing uninterrupted access to the plant raw material is crucial from a pharmaceutical point of view. No interference in the natural environment, the possibility of using many strategies to increase the production of secondary metabolites by selecting cell lines, optimizing culture conditions, and usage of different elicitors, genetic transformation or cultivation in bioreactors all makes this approach increasingly used even on an industrial scale. [4] Many medically important plant species have undergone genetic transformation to induce hairy roots. Among them are representatives of the Fabaceae family, e.g. *Trigonella foenum-graecum*, *Glycyrrhiza glabra* or *Senna obtusifolia*. The latter has been used for centuries in traditional medicine thanks to a wide spectrum of health-promoting properties. It is used, among others, in the therapy of rheumatic and skin diseases, as a laxative, anti-inflammatory, analgesic, diuretic, antimicriobial, antiviral or anticancer. [5–8] It is known that this plant contain many valuable metabolites such as among others, polyphenols, alkaloids, terpenoids or antraquinones.[9–14] Our previous study demonstrated that extracts of *S. obtusifolia* induced apoptosis in leukemia cells by the cell cycle arrest, the change in expression of apoptosis-related genes (Tp53, Puma, Noxa, Bax), and decreased mitochondrial membrane potential.[15]

One of the for many years successfully used tools in biotechnology to increase the production of secondary metabolites and new valuable compounds is genetic engineering, which works by interfering with metabolic pathways. Gene encoding enzymes and some key transcription factors can be used to improve the production of the desired metabolites by overexpressing them in transgenic hairy root cultures.[16–18] It is worth emphasizing that today's tools for modifying plant genomes open up wide possibilities involving the modification of endogenous cell pathways in order to create new or improve the production of established valuable compounds.[19] Such gene transfer can be relatively easily accomplished using *Rhizobium rhizogenes*, inserting the desired gene(s) between T-DNA boundaries and transferring the resulting construct into the genome of the host plant.[20] Currently, the cloning genes of some important enzymes and global transcription factors are used quite frequently in the engineering of secondary metabolite biosynthetic pathways. Kim et al. showed that the overexpression of Panax ginseng 3-hydroxy-3 methylglutaryl-coenzyme A reductase in hairy root cultures of *Platycodon grandiflorum* enhances the accumulation of phytosterol and triterpene.[21] In turn, Sitarek et al. revealed that overexpression of AtPAP1 transcriptional factor in transgenic roots of *Leonurus sibiricus* L. showed increased production of phenolic acids with chlorogenic acid as a the major compound.[22,23] On the other hand, Jaggi et al. discovered that *Catharanthus roseus* hairy root overexpressing the peroxidase gene (*CrPrx*) increased the content of ajmalicine and serpentine.[24] One of the enzymes that play an important role in the synthesis of terpenoids is

squalene synthase (SQS), which leads to the formation of squalene from two molecules of farnesyl diphosphate (FPP). In our previous studies we presented that overexpression of *PgSS1* gene (encoding SQS) in transgenic *S. obtusifolia* hairy roots increased production of betulinic acid by interference in mevalonate pathway. [15] However, due to the currently dynamically developing pharmaceutical market, which generates a great demand for various types of compounds of medical importance, the possibility of growing hairy roots on an industrial scale is increasingly being sought.[25] For this reason, scientists and engineers cooperate intensively in the design of new solutions that allow the maximum use of the potential of plant tissues. For this purpose, special bioreactors are created to ensure optimal conditions for the growth of plant tissue, which often translates directly into its productivity.[26,27] The design of a bioreactor system is an optimizing procedure of balancing biological and engineering factors to obtain the required capacity and product quality at minimum production costs and to avoid intensive manual handling.[28] Bioreactor cultures have several advantages over agar-based cultures, with better control of the contact of the plant tissue with the culture medium and optimal nutrient and growth regulator supply, as well as aeration, filtration and circulation of the medium and culture development. It has been shown that this approach enabled an acquisition of valuable compounds such as ajmalicine, artemisinin or chicoric acid.[29–31]

In this work, we presented for the first time a strategy of cultivation of the previously selected, most productive transgenic *S. obtusifolia* hairy roots with overexpression of *PgSS1* gene in a 10-liter sprinkle bioreactor and estimating the content of betulinic acid and selected anthraquinones. The obtained plant extracts were tested for anti-cancer, anti-microbial and anti-viral properties.

Results

SOA41 and SOPSS2 hairy root lines biomass accumulation in 10 L sprinkle bioreactor

The biomass accumulation of *S. obtusifolia* roots (SOA41 and SOPSS2) growing in the 10 L bioreactor (Fig. 1) was investigated after 35 days of cultivation (Fig. 2). The biomass of hairy roots after the entire cultivation cycle was 247, ± 6 g FW and 250, ± 2g FW for the SOPSS2 and SOA41 lines, respectively, which means about 25-fold increase in the bioreactor. The dry weight (DW) of hairy roots was 25 ± 0.7 g and 25 ± 0.6 g for SOA41 and SOPSS2 lines, respectively.

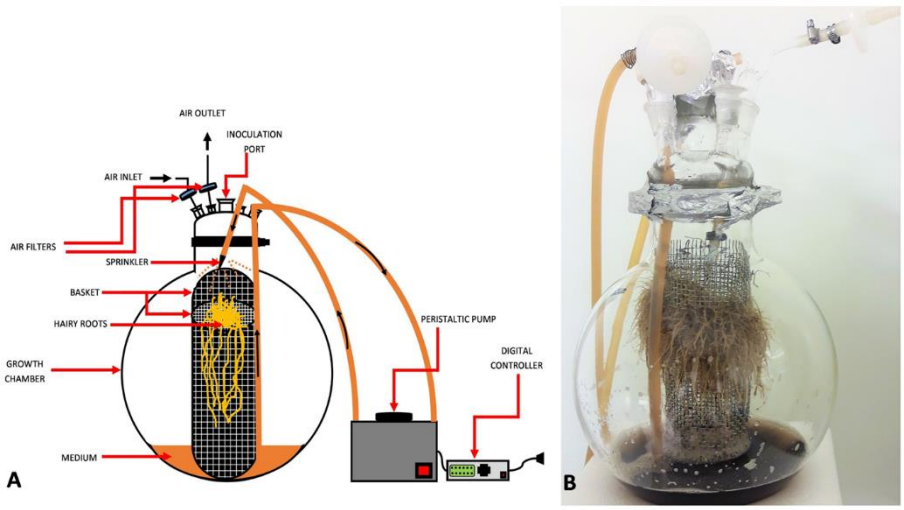

Figure 1. (A) Schematic representation of the bioreactor structure used in this experiment, (B) Bioreactor with cultured SOPSS2 transgenic hairy roots line after 35 days of cultivation.

Figure 2. SOPSS2 transgenic hairy root line cultivated in bioreactor after: (A) -7, (B) - 14, (C) - 21, (D) - 28, (E) -35 days of culture, (F) hairy roots in a basket after 35 days of cultivation, (G) - SOPSS2 transgenic hairy root biomass after the completed cultivation used for the preparation of the extract, bar=1cm.

HPLC analysis

HPLC analyses revealed that the two analyzed transformed and transgenic hairy root lines cultured in 10 L sprinkle bioreactor produced 4.21 mg/g and 38.12 mg/g of dry weight (DW) betulinic acid for SOA41 and SOPSS2, respectively. Additionally, the content of the two identified selected anthraquinones (chrysophanol and physcion) did not show any significant differences. On the other hand, changes in content have been demonstrated for emodin and aloe-emodin between the two analyzed hairy root lines (Table 1). The typical chromatograms for identified antraquinones are presented in Supplementary materials.

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Table 1. Betulinic acid and selected antraquinones content in transgenic SOPSS2 and transformed SOA41 hairy root lines of *S. obtusifolia* after 35-day cultivation in 10 L sprinkle bioreactor.

Samples from *S. obtusifolia* hairy roots	mg/g DW				
	Betulinic acid	Emodin	Aloe-emodin	Chrysophanol	Physcion
SOA41	4.21 ± 0.36^{c}	-	2.54±0.05^{b}	-	0.88±0.20^{a}
SOPSS2	38.12 ± 0.27^{d}	0.04±0.04^{a}	0.76±0.16^{c}	0.21±0.09^{b}	-

Values followed by the same letter in the same row indicate no insignificant differences $p < 0.05$. Values are presented as the means ± SD (n=3)

Molecular analysis of transformed (SOA41) and transgenic (SOPSS2) hairy roots

All analysed hairy roots showed the presence of *rol*B and *rol*C genes in a PCR reaction using isolated genomic DNA, which confirmed the presence of appropriate amplicons size (386 bp for *rol*B and 582 bp for *rolC* genes), as shown in the Fig. 3.

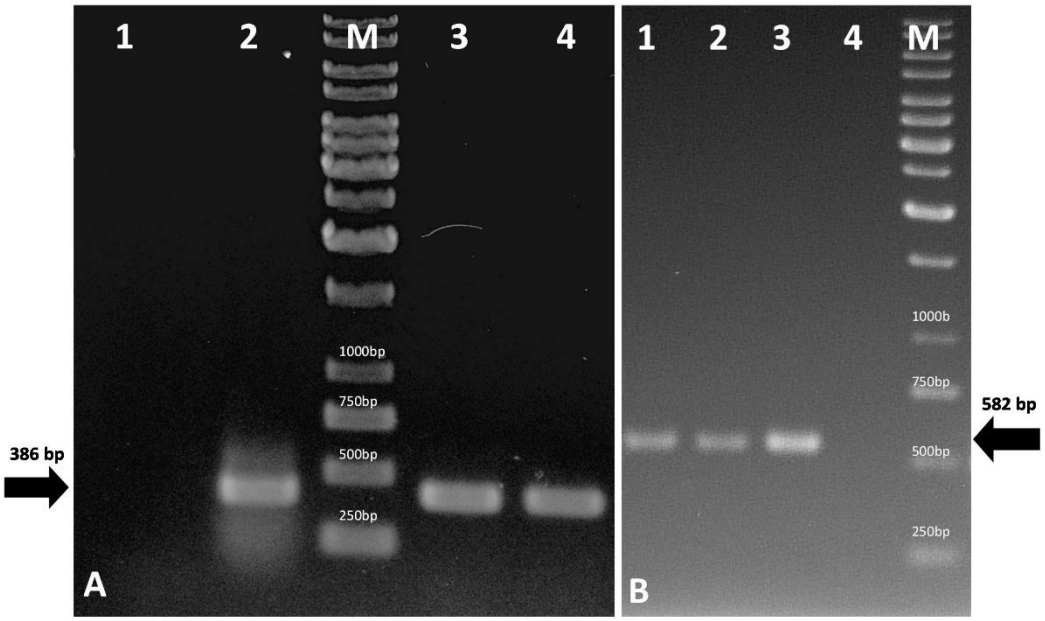

Figure 3. Polymerase chain reaction analysis of *rol*B *and rol*C genes performed on isolated genomic DNA of SOA41 and SOPSS2 hairy root lines. (A) PCR for *rol*B gene 1- negative control PCR reaction (water instead of genomic DNA), 2- positive control PCR reaction on *R. rhizogenes* A4 colony, 1 DNA marker 1kb, 3- PCR on SOA41 transformed hairy root line, 4- PCR on SOPSS2 transgenic hairy root line; (B) PCR for *rol*C gene1- PCR on SOA41 transformed hairy root line, 2- PCR on SOPSS2 transgenic hairy root line, 3- positive control PCR reaction on *R. rhizogenes* A4 colony, 4- negative control PCR reaction (water instead of genomic DNA), M-DNA marker 1 kb.

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Cytotoxic effect of SOA41 and SOPSS2 S. obtusifolia hairy root extracts on U-87MG cell line

The effect of SOA41 and SOPSS2 root extracts on cell survival in glioma (U-87 MG) cancer cell line was evaluated using MTT assay. As shown in Figure 4, both extracts exhibited cytotoxic activity on glioma cancer cell line in range tested concentrations (0.125-2 mg/mL) with IC_{50} about 0.36 mg/mL for SOPSS2 extract and with IC_{50} about 0.7 mg/mL for the SOA41 extract, respectively. A stronger cytotoxic effect for SOPSS2 extract was observed.

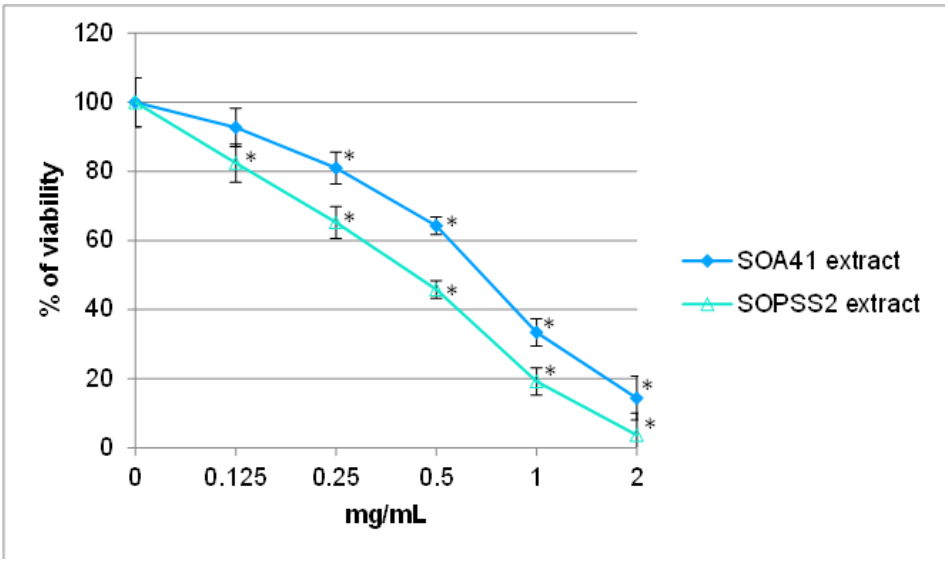

Figure 4. Cytotoxic effect of SOA41 and SOPSS2 hairy root extracts on the viability of glioma (U-87MG) cell line. Cell viability was determined on the basis of dose-response curves obtained in the MTT assay. To compare the sensitivity of cells to SOA41 and SOPSS2 root extracts, data for each cell line were presented at concentrations ranging from 0.125 to 2 mg/mL after incubation for 24 hours. All experiments were performed in triplicate and results are expressed as mean ± SD, (n=3). $p < 0.05$ SOA41, SOPSS2 vs. untreated cells

Clonogenic assay

The efficacy of the SOA41 and SOPPS2 root extract from *S. obtusifolia* on inhibition of colony formation was performed using clonogenic assay. As shown in Figure 5, both root extracts treatment gave a dramatic decrease in the colony formation potential of glioma cells in a dose-dependent manner. For IC_{30} concentration, the inhibition of glioblastoma cell proliferation was about 70% and 40% for SOA41 and SOPSS2 extracts respectively (compared to untreated control). In turn, for IC_{50} only about 20% SOA41-treated and 5% SOPSS2 maintained their proliferative potential when compared to control. Observed results suggest slightly stronger ability of SOPSS2 root extract to suppress the proliferation of glioma cancer cells.

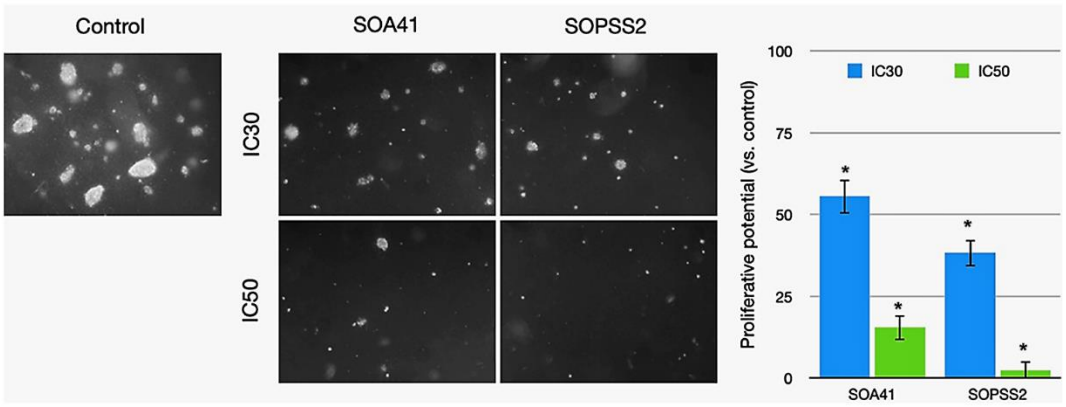

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Figure 5. Clonogenic potential of glioma cells after treatment with SOPSS2 and SOA41 root extracts in IC_{30} and IC_{50} concentrations for 24h. Photographs of a representative experiment. Cells were treated with both tested extracts by soft agar culture for 2 weeks. Clonogenic efficiency is shown as mean ± SD % of control (untreated) from 3 independent experiments,

*p < 0.05 comparison to the untreated control.

RT-PCR analysis

RT-PCR analysis was used to evaluate transgenic and transformed *S. obtusifolia* hairy root lines for the expression of *PgSS1* gene at the transcription level. The expression of PgSS1 integrated gene in transgenic SOPSS2 line was detected. Our research further indicated that PgSS1 gene was present and highly significantly expressed in the transgenic hairy roots of *S. obtusifolia* compared to roots without this construct (SOA41) (Fig. 6).

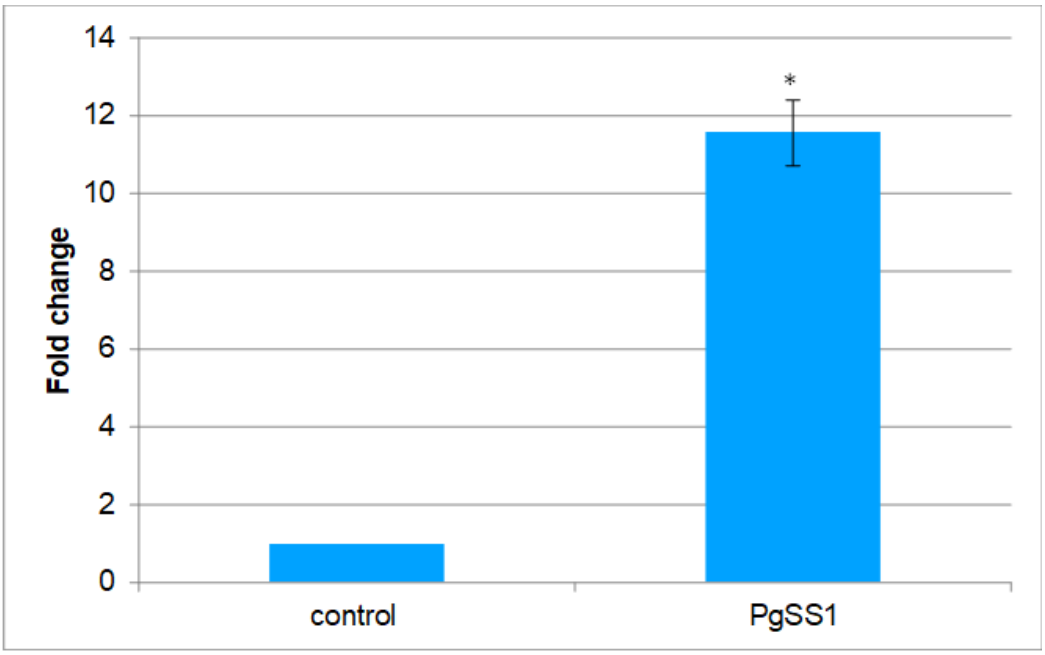

Figure 6. RT-PCR analysis expression of PgSS1 gene in transgenic hairy roots of *S. obtusifolia*. Each value represents mean ± SD (n = 3 replicates). * Statistically significant increase in gene expression compared to control, p<0.05

SOA41 and SOPSS2-mediated expression of BAX, NOXA, p53 and PUMA in U-87 MG glioma cells

To further investigate the mechanism of SOPSS2 and SOA41-induced apoptosis, the protein levels of BAX, NOXA, p53 and PUMA were examined following both extracts' exposure in vitro. After exposure of U-87 MG glioma cells to SOPSS2 and SOA41 root extracts at IC_{50} for 24 h, the protein levels of BAX, NOXA, p53 and PUMA were analyzed by Western blotting. As shown in Figure 7, expression level of apoptosis-related proteins was increased after treatment with both tested extracts. Stronger increase in apoptotic protein expression (except PUMA) was observed after treatment with SOPSS2 extract.

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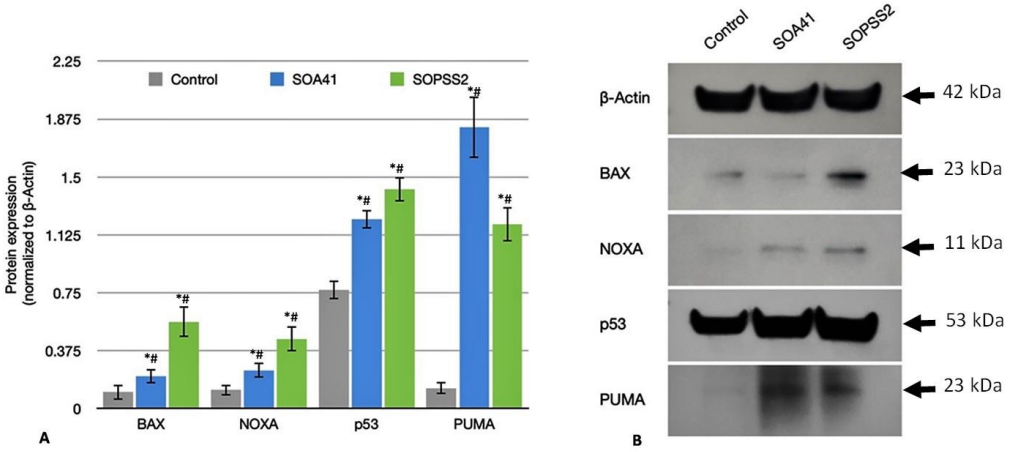

Figure 7. A) Graph presenting protein levels for BAX, NOXA, p53 and PUMA after treatment with SOA41 and SOPSS2 extracts in U-87 MG with IC_{50} after 24 h. B) Blots were reproved with an antibody for β-actin to control for protein loading and transfer. Bands shown are representative of those obtained from three independent experiments. The protein expression levels were quantified and normalized to β-actin and expressed as the fold-change to the respective control. Data are presented as the mean±SD (n=3), * indicates a significant value $p < 0.05$ as compared to control. # indicates a significant value $p<0.05$ in comparison to SOPSS2 vs SOA41 extracts.

Antimicrobial potential of SOPSS2 and SOA41 extracts from *S. obtussifolia*

The screening of antimicrobial activity of SOA41 and SOPSS2 extracts was initially assessed in vitro against two Gram-positive microorganisms (Table 2) (*Enterococcus faecalis, Staphylococcus aureus*), two Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) and two yeasts (*Candida albicans* and *Saccharomyces cerevisiae*) using the well diffusion assay. Both extracts showed moderate antimicrobial activity between 62.5 -625 µg/mL for MIC and 500-5000 µg/mL for MBC for all tested microbes, but the strongest effect for SOPSS2 and *E. faecalis* was observed.

Table 2. MIC and MBC values of SOA4 and SOPSS2 extracts in μg/mL. Data represent the median values of at least three replicates.

	S. aureus		***E. faecalis***		***E. coli***		***P. aeruginosa***		***S. cerevisiae***		***C. albicans***	
Sample	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
SOA41 extract	62.5	500	312.5	5000	625	2500	625	2500	625	1250	625	5000
SOPSS2 extract	62.5	500	156	2500	312.5	2500	312.5	2500	62.5	250	62.5	2500
POSITIVE CONTROL	1.95	500	0.49	500	0.49	500	0.98	500	15.6	500	15.6	500
	VAN		VAN		NOR		NOR		NYS		NYS	

VAN – Vancomycin; NOR – Norfloxacin; NYS – Nystatin.

Antiviral activity

The study assessed the antiviral activity of SOA41 and SOPSS2 extracts against (FCV), a human norovirus surrogate. Incubation of FCV with each extract significantly reduced its titer compared to control (Fig. 8). As shown in Figure 8, both SOA41 and SOPSS2 tested hairy root extracts showed reduction in the titer of FCV in a dose-dependent manner, where increasing concentrations of SOA41 and SOPSS2 root extracts showed increased reduction in viral titers.

Treatments at 5 mg/mL with SOA41 extract reduced infectious FCV concentration by 3.69 log while 4.17 log were reduced after 24 h treatments at 25 °C after treatments with SOPSS2 extract.

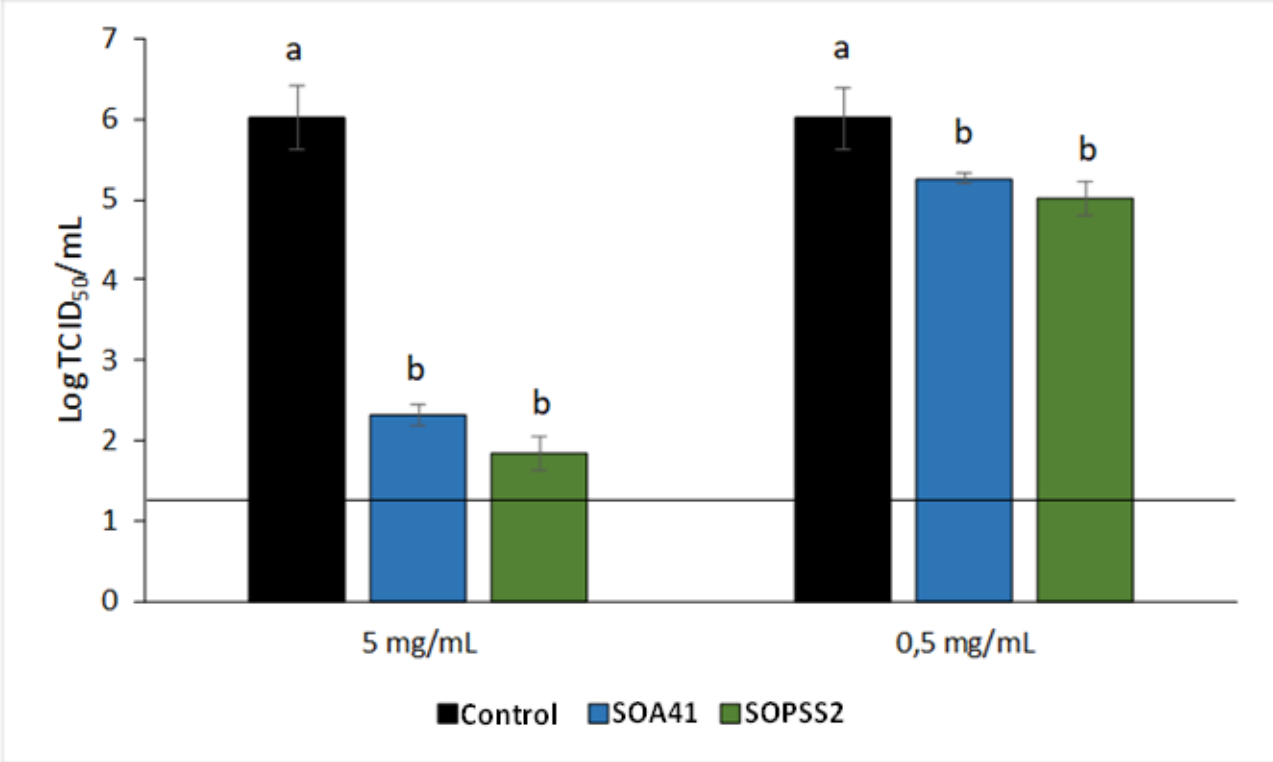

Figure 8. Reduction of feline calicivirus (FCV) titers (log TCID50/mL) exposed to extracts at 5 mg/mL or 0.5 mg/mL after overnight incubation at 25°C. Black bar: positive control. Line depicting the detection limit. Each column represents the average of triplicates. Data are presented as mean values ± SD (n=3). Within each column for each virus different letters denote significant differences between treatments ($p < 0.05$).

Discussion

The growing interest in biologically active compounds of plant origin and new possibilities of analyzing their valuable properties mean that biological sources of their synthesis are constantly being sought. Since, for many reasons, the naturally occurring plant material in the environment is not able to satisfy the demand, a lot of attention is focused on obtaining high-yield in vitro plant cultures that are an excellent source of valuable secondary metabolites. Multiple research results show that the efficient synthesis of antraquinones, phenolics and flavonoids or terpenoid indole alkaloid is possible thanks to the use of many different strategies. [32-35] Secondary metabolites, demonstrating a diverse set of properties in the plant (influencing its development, protection against the adverse effects of external factors, etc.) may also show an extremely important effect from the human point of view, such as anti-cancer, anti-bacterial or anti-viral properties. [36-41] Since these compounds are synthesized in the course of numerous and often complex biochemical transformations, the possibility of modulation of these processes is the focus of many scientists. One of the approaches widely used for this purpose is metabolic engineering. This strategy aims to interfere with the

natural metabolic pathways of the cell in order to redirect it to the synthesis of a completely new compound, strengthen the desired molecules or reduce the synthesis of undesirable products. This approach has already been successfully demonstrated in the case of isoflavonoid, camptothecin or Levodopa.[42-44] Apart from those, also terpenoids occupy a very important place among the widely used compounds of plant origin. (-) - menthol, cannabinoids, artemisinin, thapsigargin or paclitaxel, next to synthetic drugs, play an important role in modern medicine, thus creating a huge demand that is not always easy to meet.[45] Many studies also indicate betulinic acid, which is a pentacyclic triterpene, as a compound with a broad pro-health properties, including anti-cancer activity.[46] In addition to many other more or less complicated strategies in the framework of metabolic engineering leading to the overproduction of desired metabolites in plant tissues, there is an overexpression of selected genes-encoding enzymes involved in a given biosynthetic pathway. Positive research results indicating the effectiveness of such a solution are presented by Li et al., who showed that overexpression of 9-cis-epoxycarotenoid dioxygenase (NCED) being one of the key enzymes in the ABA biosynthesis pathway in higher plants results in increased accumulation of ABA and taxol compared to the untransformed cells.[47] Other studies show that overexpression of dammarenediol-II synthase gene (*PgDDS*) isolated from *Panax ginseng* in *Nicotiana tobacum* genome under the control of 35S promoter led to a high accumulation of dammarenediol-II in the roots which resulted in reduced phytosterol contents.[48] Also, Shen et al. showed that simultaneously overexpression of artemisinin biosynthetic pathway (HMGR, FPS and DBR2) genes led to production of high levels of artemisinin.[49] Experimental data showed also that overexpression of squalene synthase in plant cells can lead to increased accumulation of, among others, terpenoids.[50-52] It is worth emphasizing that, in addition to direct interference in the pathways of metabolite biosynthesis, other approaches are often used to increase the productivity of plant cultures. One of them is cultivation of plant tissue in specially designed bioreactors. So far, many examples have been described, showing the positive effect of such a culture strategy on improving hairy root productivity. The possibility of culturing plant material even on an industrial scale in bioreactors adapted to the specifics of the material to be grown (liquid phase bioreactors, gas phase or hybrid bioreactors) often contributes to the achievement of the desired yield of a valuable metabolite or other products of medical and industrial use.[53]

The aim of our studies was to compare the accumulation of betulinic acid in *Senna obtusifolia* transgenic hairy roots, exhibiting *PgSS1* gene overexpression (SOPSS2), and in transformed hairy roots without PgSS1 genetic construct (SOA41) grown in vitro in a 10L sprinkle bioreactor. In addition, we also checked the content of selected anthraquinones in both extracts. The obtained extracts were also used for examination of their biological properties. Our previous studies showed that the cultivation of *S. obtusifolia* transgenic and transformed hairy roots in 50 mL of medium in Erlenmeyer flasks was carried out with a lower biomass yield (1 g of inoculum produced about 19 g of SOA41 and 17g SOPSS2 root fresh weight after 35 days, showing a 19 and 17-fold increase, data not published) compared to the root cultured in bioreactor where the growth of transformed and transgenic hairy roots biomass after the same cultivation period was about 25 times greater than inoculum for both.[15] These results are consistent with those obtained previously for *Leonurus sibiricus* transgenic hairy roots overexpressing the transcription factor AtPAP1. In this case, 5 L bioreactor allowed for the greatest biomass acquisition compared to root culture in 300 mL, 3 L and 5 L shake flasks.[23] Also Sivakumar et al. showed that at 1L both *Artemisia annua* and *Arachis hypogaea* hairy roots had better growth in the mist reactor than shake flasks.[54] Our previous studies showed an increase of betulinic acid content in SOPSS2 hairy roots grown in liquid medium in small scale compared to SOA41 (22.71 mg/g DW and 2.78 mg/g DW respectively).[15] Cultivation of transgenic plant material under optimal conditions, including bioreactors, can be a good solution for the production of many valuable secondary metabolites for medical use and other biologically active products for industry. Dechaux and Boitel-Conti demonstrated the possibility of combining genetic engineering and culturing *Datura innoxia*

hairy roots in a bioreactor to increase tropane alkaloid accumulation.[55] Likewise, Ritala et al. showed that genetically engineered tobacco hairy roots expressing plastid-targeted geraniol synthase gene can produce geraniol. Additionally, their cultivation in a 20-liter bioreactor as demonstrated by the authors showed this compound on a larger scale.[56] In this work, apart from the differences in the content of betulinic acid, we also confirmed the content of selected anthraquinones in the studied hairy root lines. Their presence in this *S. obtusifolia* transformed hairy roots has also been demonstrated by Guo et al. and Ko et al.[57,58] In this work, we have confirmed overexpression of *PgSS1* gene in transgenic SOPSS2 hairy root line of *S. obtusifolia*. Our results are consistent with Lee et al., who showed that overexpression of squalene synthase led to enhanced triterpene and phytosterol biosynthesis in *Panax ginseng*.[50] Similar results were obtained by Seo et al., who showed that overexpression of *PgSS1* in *Eleutherococcus senticosus* causes increased phytosterol and triterpene accumulation.[59]

The next step of this work was to investigate the cytotoxic and antiproliferative effect of these two selected extracts on U-87 MG glioblastoma cell line. The results of cytotoxicity MTT and clonogenic assays confirmed that SOPSS2 extract showed a stronger effect for both extracts cytotoxicity (IC_{50} =0.36 mg/mL for SOPSS2 and 0.7 mg/mL for SOA41). In the latter test, the IC_{50} concentration inhibited proliferation of about 95% cells for SOPSS2 and 80% for SOA41 extracts in comparison to the untreated control cell line. Due to the fact that *S. obtusifolia* transgenic hairy roots with overexpression of the *PgSS1* gene were obtained by us for the first time, there is no literature data on the application of such a strategy in metabolic engineering in this species. Our previous study showed that these hairy root extracts were more cytotoxic for leukemia cells (NALM-6), exhibiting an IC_{50} value of 0.08 mg/mL for the most betulinic acid productive SOPSS2 clone. Based on these results, we suspect that betulinic acid, which is present in large amounts in combination with other identified antraquinones, transgenic *S. obtusifolia* hairy roots, may be responsible for a stronger cytotoxic effect and inhibition of cell proliferation. The cytotoxicity to neoplastic cells of extracts from non-transgenic plant material of *Cassia obtusifolia* was previously demonstrated by testing the influence of extracts on HeLa (epitheloid cervix carcinoma) and MDA MB 231 (breast cancer cells).[8] We predict that the overexpression of the squalene synthase gene in transgenic hairy roots increases the level of betulinic acid in this plant tissue. Literature data shows that this compound has anti-tumor properties against many neoplastic cells, such as equine melanoma.[60], human hepatoma (HepG2)[61], human breast cancer (MCF-7)[62] or the colorectal.[63] Additionally, our research confirmed the influence of the extracts on the changes in the expression level of apoptosis-related proteins i.e. p53, and p53 upregulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) and BCL2 Associated X, Apoptosis Regulator (BAX). This first transcription factor is crucial to many pathways, including DNA repair, cell cycle, and just apoptosis. Well known targets of p53 in the apoptotic pathway are PUMA, NOXA and BAX proteins.[64] In this work, we demonstrate a stronger influence of SOPSS2 extract on the expression level of the studied proteins, compared to the effect of SOA41 extract. It can be assumed that also in this case, this effect may be associated with biologically active compounds present in extracts, including betulinic acid, which occurred in these extracts in predominance in relation to other identified compounds. As with our previous studies, it has been shown here that the activation of apoptotic genes is stronger in cancer cells after treatment with SOPSS2 extract compared to SOA41. Our observations are also consistent with those made by Hsu et al, who showed that betulinic acid influences the expression of genes related to apoptosis.[65] Zhou et al. proved also that betulin is able to stimulate programmed cell death in colon cancer cells, demonstrating that the overexpression of NOXA enhanced betulin-induced apoptosis.[66] Additionally, Wick et al. also showed the induction of apoptosis in five human glioma cell lines. These authors, according to our results, indicate that betulinic acid enhanced the expression level of BAX protein. They demonstrated that this compound does not alter the levels of BCL-xS or BCL-xL, and leads to DNA fragmentation and cell death.[67] Based on these results, we can assume that an important component of the plant extracts tested by us,

responsible for their biological properties, is betulinic acid, which, in addition to other biologically active compounds characterized in the plant material, can demonstrate a synergistic anti-cancer effect.

The last stage of our work was to check the antibacterial, antifungal and antiviral properties of the tested extracts. Our results showed that the tested transformed (SOA41) and transgenic hairy roots (SOPSS2) extracts revealed a stronger effect on bacterial cells than yeasts and showed antiviral properties. In the case of testing the antibacterial properties, the best effect (MIC) was demonstrated for *S. aureus*. SOPSS2 extract showed stronger antibacterial properties against *E. faecalis*, *E. coli* and *P. aeruginosa* compared to SOA41 extract, which, except for the first two bacteria tested (giving similar results), showed weaker antibacterial activity for *E. coli* and *P. aeruginosa*. Additionally, the tested extracts showed significant differences in antifungal properties against the *Saccharomyces cerevisiae* and *Candida albicans* between SOPSS2 and SOA41. In the framework of this study, the antiviral activity of SOPSS2 and SOA41 was evaluated on FCV, a surrogate of human noroviruses, the leading cause of viral foodborne outbreaks. Interestingly, both extract showed strong antiviral activity with not significantly differences between extracts. Hussein et al., who tested, among others, the extract from the aerial part of *Cassia obtusifolia* for inhibitors activity on HIV-1 virus replication. The authors showed no significant effect of this extract in this study.[68] Additionally, Mohamed et al. also revealed no activity of *C. obtusifolia* leaf extracts against Newcastle Disease Virus and the Fowlpox Virus.[69] The system proposed by us, based on metabolic engineering and the use of a bioreactor to create an efficient plant synthesis model, mainly for betulinic acid, requires further research on anti-microbial properties. The search for natural systems with such properties is all the more justified, as many traditionally used antibacterial compounds are becoming less and less effective in currently used therapies, among others due to the emergence of resistance to antibiotics. Moreover, due to the lack of availability and high cost, many areas in the world lack access to traditionally used antibiotics. Many authors indicate that *S. obtusifolia* extracts exhibit broad-spectrum antimicrobial properties. Hamuel et al. conducted research on the antimicrobial properties of leaf extracts of *S. obtusifolia*, examining their effect on selected pathogenic bacteria and fungi.[70] In contrast, Farias et al. did not show the effect of the seed extract of *S. obtusifolia* on. *S. aureus*, *E. aerogenes*, *K. pneumonia*, and *P. aeruginosa* nor against the yeasts *Candida albicans*. Comparing the data obtained by these authors with our results, it can be concluded that the system of transgenic hairy roots in many cases shows a much stronger antimicrobial effect than non-transgenic plant material. *Senna obtusifolia*, as a rich source of many biologically active compounds, including those with antimicrobial activity, has been used in traditional medicine for centuries. The differences in the microbiological activity of the SOA41 and SOPSS2 extracts shown by us may be related to the differences in the content of betulinic acid, which, in combination with other compounds in the extract, exhibits different antimicrobial properties in relation to bacteria and yeasts.[71]

The currently available possibilities of modifying metabolic pathways and increasing the scale of breeding valuable plant material, in combination with the natural potential of many plant species, more and more often allow alternative plant sources of valuable biologically active compounds to be obtained. *Senna obtusifolia*, which has a wide spectrum of health-promoting properties, has been the subject of such modifications quite rarely so far. Expanding such research in the future could lead to the development of high-throughput plant in vitro cultures capable of synthesizing and accumulating a wide range of compounds used in medicine and many industries.

Conclusions

In this work, we have shown for the first time that transgenic hairy roots of *Senna obtusifolia*, overexpressing the squalene synthase gene from *Panax ginseng* (*PgSS1*) grown in a 10 L sprinkle bioreactor, may be a good source of valuable secondary metabolites. Our research revealed

the presence of selected anthraquinones as well a higher level of betulinic acid in the extracts of transgenic hairy roots (line SOPSS2) compared to roots that do not overexpress squalene synthase (SOA41) grown in bioreactor. The biological tests showed a stronger cytotoxicity, inhibition of proliferation, and also an influence on the apoptosis-related proteins level in U-87 MG glioma cells treated with SOPSS2 extract. Additionally, for SOPSS2 extract, stronger antibacterial and antifungal properties were demonstrated, while antiviral activity was confirmed for both extracts comparably. In conclusion, our studies confirmed that metabolic engineering by interfering with the pathways connected with the cultivation of transgenic plant material in larger scale in bioreactor can be an excellent tool for an increased production of valuable secondary metabolites widely used in medicine and industry.

Experimental section

Establishment and transgenic nature confirmation of the *S. obtusifolia* hairy roots

The protocol for *PgSS1*-overexpressing hairy roots induction and confirmation of their transgenic nature has been described earlier.[15] Additionally, the presence of the *rol* genes in both analyzed root lines was confirmed by PCR amplification using *rol*B (5′-GCTCTTGCAGTGCTAGATTT-3′ and 5′-GAAGGTGCAAGCTACCTCTC-3′) and *rol*C (5′-CTCCTGACATCAAACTCGTC-3′ and 5′-TGCTTCGAGTTATGGGTACA-3′) forward and reverse primers. *Rhizobium rhizogenes* A4 colony growing on the YMB (yeast, mannitol broth) agar plate were used as a positive control. The DNA from non-transformed seedlings roots was used as a negative control. Amplification conditions: Five minutes at 95 °C; 30 cycles of 45 s at 95 °C, 45 s at 49 °C (for rolB) or 52°C (for rolC), 45 s at 72 °C; finally, five minutes extension at 72 °C. Final volume of all samples was 20 µL (1 µL of each primer (10 µM), 0.5 µL of DNA (50 ng/µL), 12.5 µL of PCR Mix Plus master mix (A&A Biotechnology, Gdynia, Poland) and 11 µL of PCR Ultra-Pure Water H2O. DNA amplification was performed in a Biometra UNO II (Biometra GmbH, Göttingen, Germany) thermal cycler. The amplification products were visualized on 1.5% agarose gel stained with ethidium bromide.

RNA extraction, cDNA synthesis and real-time PCR protocol

Total RNA from the transformed (SOA41) and transgenic (SOPSS2) hairy roots was isolated with Total RNA Mini Concentrator Kit (A&A Biotechnology, Gdansk, Poland). First strand of cDNA was synthesized using a TranScriba kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's protocol. Quantitative real-time PCR analysis was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) with the use of RT-PCR Mix SYBR® (A&A Biotechnology, Gdansk, Poland). The primer sequences for the PgSS1 gene were as follows: F-5′ CCGGAGGGATCTGCTTTTC-3′ and R- 5′ATGGGAAGTTTGGGGGCAATT-3. Elongation factor 1α (EF-1α) was used as a reference gene (F5′- TGAGATGCACCACGAAGCTC-3′ and R- 5′CCAACATTGTCACCAGGAA GTG -3′). The PCR procedure was performed under the following conditions: five minutes at 95 °C, followed by 35 cycles of 95 °C by 30 s, 68 °C and 66 °C (for PgSS1 and EF-1α respectively) for each gene by 30 s and 72 °C by 45 s. Each sample was analyzed in triplicate. The levels of the genes were normalized to that of the elongation factor 1α (EF-1α) gene used to test the specificity of amplification. The expression of the genes was calculated by the comparative Ct method.[72]

Transgenic hairy root culture in a 10 L sprinkle bioreactor

Two (SOA4 and SOPSS2) root clones were selected for cultivation in the bioreactor. The first was transformed with *R. rhizogenes* A4 without recombinant vector and the second was transformed with bacteria containing pGFPGUSPlus-PgSS1 recombinant vector and exhibiting the highest betulinic acid content. Before starting the cultivation, the bioreactor was sterilized by autoclaving at 121°C for 20 minutes. Hairy roots

for bioreactor inoculation grew for 2 weeks in a shake flask culture. Hairy roots were grown separately in a 10 L bioreactor containing 2 L of Murashige & Skoog liquid media with 3% (w/v) sucrose. The roots were placed in a stainless-steel basket on a wire mesh (4mm x 4mm pore size) under a sprinkler. The spraying nozzle was located above the growing roots, which ensures optimal supply of nutrients during cultivation in this system. The medium was dispensed through the dispersion nozzle for 30 seconds with a 180 second break. Each cycle delivered approximately 60 mL of medium to the roots. The circulation of the medium was ensured using a peristaltic pump. The constant temperature of the environment was maintained by carrying out the cultivation in isolated and air-conditioned room. About 10g fresh weight (10 ± 0.15g of SOA41 and 10 ± 0.17g of SOPSS2) was used as an inoculum. Cultures in the bioreactor were carried out at 25° C ± 2° C in the dark for 35 days. Results presented are from triplicate. All results are expressed as mean ± SD.

Plant extract preparation

Two different transformed and transgenic hairy root extracts (SOA41 and SOPSS2) of *S. obtusifolia* were used in this study. The extracts were made according to the protocol described in our previous work.[15] Briefly, Extraction was performed from powdered plant material with 500 ml ethanol: water, 80:20 v/v at 35 °C with ultrasound. Then the material was extracted twice with 300 ml of the same solvent for 15 min. The extracts were filtered off and evaporated under reduced pressure and finally freeze-dried.

HPLC analysis

HPLC analysis was carried out in a 1200 Infinity Agilent Technologies Liquid Chromatograph equipped with a Column Oven and Diode Array Detector G1315D, 1260 DAD VL. HPLC grade Methanol and Acetonitrile were obtained from Fisher Chemical (Leicester, UK) and Trichloroacetic acid from Sigma (Deisenhofen, Germany). Acidified water was prepared by dissolving trifluoroacetic acid (TFA) 0.3% v/v. The extracts were dissolved in DMSO and analyzed on RP-HPLC column Eclipse XBD-C18 (4.6 x 250 mm, 5 Micron) (after injecting 20 µL), and using a gradient composed of eluent A (Methanol), eluent B (Acetonitrile) and eluent C (TFA) as follows: 0 min, 15% A, 5% B, 80% C; 2 min, 70% A, 30% B, 0% C; 10 min, 70% A, 30% B, 0% C; 15 min, 15% A, 5% B, 80% C. The flow rate was 1 mL/min and all analyses were performed at room temperature. For quantification and identification purposes, the compounds present in the samples were compared with calibration standards. Compound identification was based on retention time and ultraviolet (UV) spectra overlay. The time of analysis was 15 min, including the stabilization of the RP-18 column. The selected wavelengths were 245, 278 and 210 however, 254 was used for the analysis since it displayed high areas of detection of the compounds. The correlation coefficients for each compound were always very close to unity and the linear regression curve selected for quantification selected for each compound (for Physcion $y = 46537x + 1.0053$ $R^2 = 0.9998$, Emodin $y = 55612x + 112.93$ $R^2 = 0.9963$, Aloe emodin $y = 60308x + 339.69$ $R^2 = 0.932$, and Chrysophanol $y = 75701x + 52.348$ $R^2 = 1$). The limit of quantification (LOQ) and the limit of detection (LOD) were determined and all analyses were performed in triplicate.[73,74] Data processing was handled by Agilent OpenLAB CDS ChemStaton Workstation, revision C.01.01. Identification and quantification of betulinic acid was conducted according to our previous studies.[15]

Cell culture and MTT

U-87MG glioblastoma cell line (ATCC: HTB-14) was cultured in EMEM (Corning Inc., Corning, NY, USA) medium supplemented with 10% FBS (Lonza, Basel, Switzerland) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin) (Lonza, Basel, Switzerland). Cell line were grown in

standard culturing conditions of humidified atmosphere containing 5% CO_2 at 37°C. One day prior to the experiment cells were plated over 96-well plate ($3x10^4$ cells/well). The following day, the cells were incubated with different concentrations of SOPSS2 and SOA41 root extracts for 24h. After this time, MTT (Sigma Aldrich) was added to the final concentration of 0.5 mg/mL and incubated for 2h. Medium was then discarded and 100 µL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added. The plate was placed on the plate shaker for 10 min, and the absorbance (570 nm) was measured on GloMax microtiter plate reader (Promega, Sunnyvale, CA, USA) according to our previous study.[15] Briefly, The cytotoxicity of extracts were evaluated by the MTT assay. Briefly, U-87MG glioblastoma cells were plated in 96-well plates in culture medium containing various of plant extracts. Following this, 10 µL (final concentration 0.5 mg / mL) of MTT solution was then added to each well and the plate was incubated in 37°C. Then, the plate was centrifuged, medium was removed and 100 µL of solubilization solution was added into each well. The plate was incubated in a humidified atmosphere of an incubator. Finally, plates were read in a microplate spectrophotometer (Promega GloMax Plate Reader, Promega, Madison, WI, USA). To calculate the cytotoxic effect and IC_{50} value Graphpad software was used.

Western Blot

After a 24-hour treatment with IC_{50} concertation of SOPSS2 and SOA41, root extracts cells were collected, washed twice with cold PBS, and whole-protein lysates were isolated using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein lysates were then resolved on SDS-PAGE gel and transferred to the PVDF membrane using Trans-Blot Turbo Transfer System (Bio-RadLaboratories, Hercules, CA, USA). The membranes were then blocked with nonfat dried milk and incubated overnight with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA). The following day the membrane was washed and incubated for 1h with suitable secondary antibodies (Cell Signaling). For visualization Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used. ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/nih-image/) was used for densitometric analysis of the acquired bands.

Clonogenic assay

Plate preparation for clonogenic assay was performed as mentioned previously.[75] After treatment with IC_{50} concertation of SOPSS2 and SOA41 root extracts cells were collected, counted, and 10^3 living cells were resuspended in 0.4% w/v agar (in culture media) and applied over the plate wells prepared earlier. Medium was applied at the top of solidified agar (changed weekly). After 2 weeks colonies were stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and counted.

Antimicrobial activity

The antimicrobial properties of four extracts were assessed by the microdilution method[76] and the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values were evaluated against Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212), Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacteria and yeast (*Saccharomyces cerevisiae* ATCC 2601 and *Candida albicans* ATCC 10231) strains. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC or MFC) of the tested SOPSS2 and SOA41 hairy root extracts were determined as detailed previously.[77]

Antiviral activity

Feline calicivirus (FCV) F9 strain (ATCC VR-782) was assayed and propagated in CRFK cell line (ATCC CCL-94). Virus stock was harvested and titrated as described by Falco et al.[78] Viruses were produced in cells by centrifugation of infected cell lysates at 660 x g for 30 min. Infectious FCV were enumerated by determining the 50% tissue culture infectious dose (TCID50) with eight wells per dilution using the Spearman-Karber method.[79]

To elucidate the antiviral activity of the SOPSS2 and SOA41 root extracts, extract suspensions at 0.5 and 5 mg/mL were mixed with an equal volume of FCV suspensions (about~6 log TCID50/mL) and incubated overnight at 25 °C. Positive controls were virus suspensions added with PBS only under the same experimental conditions. Each treatment was done in triplicate. To stop the reaction Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) was added to the samples. Ten-fold dilutions of treated and untreated virus suspensions were inoculated into confluent in CRFK monolayers in 96- well plates. Then, infectious viruses were enumerated by cell culture assays as described above. The decay of FCV titres was calculated as log10 (Nx/N0), where N0 is the infectious virus titre for untreated samples and Nx is the infectious virus titre for extracts-treated samples.

Statistical Analysis

The results are expressed as mean values ±SD. The Shapiro–Wilk test was used to confirm the normality of the data. The Kruskal–Wallis with multiple comparisons of mean ranks and the one-way and two-way analysis of variance (ANOVA) with the Tukey post hoc test were used to determine differences between samples. The statistical analysis was performed using STATISTICA 13.3 software for Windows (StatSoft, Krakow, Poland). Differences of $p < 0.05$ were considered statistically significant.

Chem. Biodiversity

Supplementary Material

Author Contribution Statement

T.K.: Conceptualization, Investigation, Resources, Writing original draft; P.S.: Investigation, Methodology, review and editing; M.T.: investigation; P.R.: writing- review and editing; E.D-M.: investigation; I.F.: investigation; G.S.: writing- review and editing; T.Ś.: supervision.
All authors reviewed the final manuscript

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