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ORIGINAL ARTICLE

Permeabilization of *Calendula officinalis* L. hairy root cultures for the release of accumulated triterpenoid saponins

Jakub Kamiński[1] · Piotr Bujak[1] · Marek Długosz[1]

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Abstract

Triterpenoid saponins, which are glycosidic derivatives of oleanolic acid, demonstrate numerous pharmacological properties. The hairy root cultures of marigold accumulate these phytoanticipins mainly in vacuoles, which may contain up to 40% of the triterpene glycosides synthesized in the cytoplasm. Dimethyl sulfoxide, Tween 20 (T_{20}), Tween 80 (T_{80}), and Triton X-100 (Tx100) were used as potential surfactants, allowing for an increase in the release of saponin into the culture medium. T_{20} at concentrations of 0.3–1.0% (v/v) caused a higher saponin content in the liquid medium, from 52 to 61 times for CH9, 15 to 22 times higher for CC16, and seven to twelve times higher for CH2, compared to the respective control cultures. DMSO was efficient toward the CC16 line, providing a 3–7 times higher saponin content for 0.5% and 1.0% (v/v) surfactant concentrations, respectively. The suitability of Triton X-100 for triterpenoid saponin could not be determined with the method used in this experiment, and there was a serious contamination of the analyzed samples. The ultrasound method accelerated surfactant action, and only for the CH9 line did it result in an increase in the secretion of glycosides to almost three-fold in the case of T_{80} and over two-fold in the case of T_{20}. The conditions that were least harmful for the roots were: ultrasound, Tween 80, and T_{80} interacting with US. The permeabilized cultures, after 30 days of growth in the new medium, obtained fresh biomasses similar to the control or a reduction by the maximum of one-fifth for CH9. For the second line (CC16), the growth parameters were reduced twice. Polyoxoethylene sorbitan monolaurate was found to be the most powerful surfactant, and a proposed concentration and time of action allowed for culture viability only for the CC16 line. The young parts of the root tips generated a new culture, with the growth being reduced by 77% (FW) and 82% (DW).

Key message

Polysorbates and ultrasounds allow to increase by 22-61 times triterpenic saponins content in medium of marigold hairy root cultures. Tween20 was powerful, while Tween80 did not weaken cultures regrowth.

Keywords Permeabilization · Detergents · Ultrasound · Hairy roots · Triterpenoid saponins · Marigold

Abbreviations

CC	line derived from Cotyledon
CH	line derived from Hypocotyl
DMSO	Dimethyl Sulfoxide
DW	Dry Weight
FW	Fresh Weight
GLC	Gas-Liquid Chromatography
HR	Hairy Root Culture
MeOA	Methyl Ester of Oleanolic Acid
½ MS	Murashige and Skoog (1962) medium
MS	Mass Spectrometry
OA	Oleanolic Acid
PGR	Plant Growth Regulator
T_{20}	Tween 20
T_{80}	Tween 80
TLC	Thin-Layer Chromatography
Tx-100	Triton X-100
US	Ultrasound

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✉ Marek Długosz
mm.dlugosz@uw.edu.pl

[1] Department of Plant Biochemistry, Faculty of Biology, University of Warsaw, Miecznikowa 1, Warsaw 02-096, Poland

Introduction

The plant kingdom offers a rich source of valuable metabolites with broad biological effects. Due to the diverse chemical structures and properties related to plants, they are widely used as pharmaceuticals, fragrances, dyes, biopesticides, and food additives (Chandran et al. 2020; Lengai and Muthomi 2018; Derbassi et al. 2022). Unfortunately, the vast majority of these compounds are stored in intracellular compounds, mainly in the vacuoles. The necessary stage to obtain these compounds is, therefore, the destruction of subcellular structures, followed by a multi-stage extraction and purification procedure. Thus, the use of this type of source seems to be profitable only for expensive cytostatics (vinblastine, vincristine, or paclitaxel) operating at low concentrations.

Marigold (*Calendula officinalis* L.) is a well-known herbaceous species that has been cultivated for centuries. The richness and diversity of biologically active substances produced in the tissues is representative of the *Asteraceae* family, making this plant an attractive research model with applications in the pharmaceutical and food industries (Patil et al. 2022). Triterpenoid saponins are specially favored here due to their numerous properties and their participation both in plant chemical defense and in their interaction with the environment. This active ingredient belongs to phytoanticipins and is constantly present in/on plant tissues, with the possibility for rearrangement in response to stress (Chowdhary and Tank 2022). The main shortcomings that complicate the procurement of triterpenoid saponins with industrial production are variable and dependent on accumulation according to environmental conditions, complex structures and difficulties in chemical synthesis, and hard purification from the total extract. However, protocols that have been developed over the last few years aim to overcome the barriers mentioned above. The following seem to be particularly useful: the transfer of triterpenoid biosynthesis to yeast cells (Zhuang et al. 2017), and the optimization of bioreactor cultures using airlift systems (Thanh et al. 2014) or a transient system based on *A. thaliana* protoplasts for the modification of the triterpenoid pathway (Johnson et al. 2014).

Permeabilization seems to be a different and less complicated solution that enables access to compounds locked in the compartments of the plant cell. In order to release compounds from vacuoles in plant cells, two membrane barriers must be penetrated: the plasma membrane and the tonoplast. Breaking up the continuity of cell membranes takes place mainly through the use of chemical agents–compounds such as surfactants or detergents (Komolpis et al. 1998; Patade et al. 2017). Unlike organic chemicals such as methanol (Chugh and Eudes 2008) or chloroform (Brodelius 1998), they do not cause a loss of cell viability. The most commonly used detergents for this purpose are the Triton X family, polysorbates (Tween), and DMSO.

Dimethyl sulfoxide (DMSO) is a polar aprotic solvent that is quite miscible with water-soluble organic compounds, and it is also classified as a detergent. Its most common application is the cryoprotection of biological material. Due to its molecular structure, it easily penetrates into the cells and protects cell structures from mechanical damage by ice crystals during freezing (Notman et al. 2006). Its amphiphilic properties and the ability to loosen the structure of protein–lipid membranes mean that DMSO is readily used as an excellent cell permeabilization agent (Sanz et al. 2000). Recently, it has been combined with ultrasound as a method for the effective extraction of polyphenols from fruit waste, with potential industrial uses (Selvakumar et al. 2021). The use of DMSO as a factor causing plant cell permeabilization was successful for *Coleus blumei* cells, and rosmarinic acid was obtained (Park and Martinez 1992). This important compound, known for its antioxidant properties, was released outside of the cells in amounts that accounted for 66% of the total content synthesized in cells. An important step for its high viability after permeabilization was the preconditioning of cells using 0.1% DMSO.

The second group of nonionic surfactants is the Tween series, comprising polysorbates like Tween 20, 40, 60, and 80, with the difference being the length of the fatty acid chain. Tween 20 is polyoxyethylene sorbitan monolaurate ($C_{58}H_{114}O_{26}$), and it contains 20 oxyethylene units. Its molecule consists of a polar ethylene tail, a polar polyethylene head, and an ester of lauric acid with a non-polar carbon chain. Tween 80 represents a similar structure, but it contains oleic acid instead of lauric acid, which makes T_{80} more sensitive to oxidation. Both compounds break protein–lipid and lipid–lipid connections, and they do not significantly affect protein activity (Liang et al. 2015; Banfalvi 2016). Their properties have frequently been used in a variety of industries: food, pharmacy, cosmetics, and agriculture. The hairy roots of *Panax ginseng* after media supplementation with T_{80} significantly increased the ginsenoside content outside of the tissues (Liang et al. 2015). In addition, the authors demonstrated the features of a typical elicitor for this detergent: it caused an increase in the expression of dammarenediol synthase – a key enzyme for triterpenoids synthesis. This acceleration was simultaneously concerned with gene expression and enzymatic activity. Another representative of the polysorbates, T_{20}, was used as a permeabilizing agent for important tropane alkaloid isolation from *Datura innoxia* Mill. hairy root culture (Boitel-Conti et al. 1995, 1996). At various detergent concentrations, the increased presence of hyoscyamine and scopolamine was

detected in the medium; however, tissue viability was possible after 24 h treatment with 2% T_{20} (Boitel-Conti et al. 1995).

Triton X is the group of detergents to which the widely used Triton X-100 belongs, together with many other lesser-known homologous members. They all contain a p-tert-octylphenyl hydrophobic moiety, while the hydrophilic part consists of a mixture of polyethylene oxide chains. Except for protein solubilization, Tx-100 was efficient in obtaining proteins from cells and their organelles, and to permeabilize cells before the transfection process (Gennuso et al. 2004; Rajagopal et al. 2002). Polar groups break hydrogen bonds in cell membranes, which leads to disturbances in their integrity, and, ultimately, to destruction. It has been shown that membranes containing a lot of cholesterol and its derivatives are more resistant to the effects of Triton (Fontaine et al. 2007; London and Brown 2000). Tx-100 was successfully utilized for releasing betacyanines from a suspension culture of *Beta vulgaris* without damaging the cells. This beet culture were able to regrow and retain red pigment production utility; however, this was reduced by a quarter (Trejo-Tapia et al. 2007).

The permeabilization of plant tissues and cells for the purpose of obtaining valuable metabolites seems to be made more difficult by the presence of a cell wall, which does not occur in the structures of animal cells. For this reason, the effect of detergent was supported by the action of an additional physical factor, for example, ultrasound (US). The mechanism of sound waves at 20 kHz to 1 GHz relies on the generation of cavitation bubbles near the surfaces of cell walls, which leads to higher local temperatures and pressure conditions. These mechanical, thermal, and chemical factors improve solvent penetration into the cell, and they increase mass transfer and diffusion through the cell envelope (Chemat et al. 2011). Various effects of US, in terms of obtaining plant special metabolites, were observed. For the oldest and most famous industrially developed plant-derived product – shikonin – the use of US improved both effects: synthesis and secretion to the culture medium (Lin and Wu 2002). The mentioned suspension culture of *Lithospermum erythrorhizon* demonstrated a two-dimensional operation: membrane permeability and the enhanced activity of phenylalanine ammonia lyase and p-hydroxybenzoic acid geranyl transferase (enzymes involved in shikonin synthesis).

As mentioned above, different permeabilization agents have been shown to increase the release of special compounds from plant cultures. However, no such application has been described for *Calendula officinalis* L. In the current research, the effect of three different detergents on the viability of marigold hairy root cultures and release of saponin was analyzed for the first time. At a further step, ultrasound were used in combination with two types of polysorbates for a more effective release of triterpene saponin accumulated in tissues. The total triterpene saponins were determined as the aglycone form: oleanolic acid. The regenerative capacity of the culture after the action of detergents and/or ultrasound was also assessed.

Materials and methods

Plant material

Hairy root (HR) cultures of *Calendula officinalis* L. were obtained during the procedure described earlier (Długosz et al. 2013). The lines selected for permeabilization were: CH2, CH9, and CC16, after 90 and 130 subcultures, derived from hypocotyl (CH) or cotyledon (CC).

Established lines of HRs were subcultured every 3–4 weeks via the transfer of one piece cut off as long as possible (usually 30–50 mm) a piece of the light part of the root with all its branches to a 50 mL Erlenmeyer flask with 25 mL of fresh ½ MS (Murashige and Skoog 1962) medium. The liquid medium was half-strength of macroelements, free of plant growth regulators (PGR) and lactalbumin hydrolysate, standard set of vitamins, and contained 2% (w/v) of sucrose. The pH of the medium was adjusted to 5.7 with 0.1 M NaOH before autoclaving for 17 min at 121 ˚C. Marigold cultures were maintained in darkness at 24 ± 2 ˚C on a rotatory shaker operating at 120 rpm.

Hairy roots permeabilization

Cultures were prepared in 300 mL Erlenmeyer flasks containing 100 mL ½ MS liquid medium and an inoculum was three pieces of the light yellow part of the root with all its branches (30–50 mm). The permeabilization effect was performed on 30-day-growth cultures (the stationary phase). The first experiment included the use of the three most commonly exploited surfactants: Tween 20 (Sigma) T_{20}, DMSO (Sigma), and Triton X-100 (Sigma), at a concentration from 0.1 to 1% (v/v). Permeabilizing agents were applied directly using sterile pipette tips without sterilization. After surfactant addition, HR cultures (after 90 subcultures) were agitated for the next 24 h. The control culture was HR, which on day 30 was given 1% (v/v) fresh 1/2 MS medium instead of detergent. The second experiment was performed on cultures after 120 subcultures, using Tween 20 and 80 (Loba Chemie), intensified through the action of ultrasound. Hairy root cultures at the stationary phase (30 days) were treated with 1% (v/v) of the proposed surfactants (T_{20} or T_{80}) and exposed on ultrasound (US) over 10 min. The source of the ultrasound was a device produced by EMAG technologies® (model Emmi 40), generating US at 250 W power

and a frequency of 45 kHz. The control consisted of HR not exposed to detergents, which were treated with 1% fresh medium (1/2MS) on the 30th day. Each sample was treated with maximal power obtained using a generator equipped with a water bath. Before each treatment of the cultures with ultrasound, the water temperature was measured before the device was turned on and after 10 min of operation. After each cycle, the heated water was replaced with new water at room temperature. After detergents and US application hairy root cultures were placed on rotatory shaker operating at initial parameters for 24 h.

Biomass measurement and saponin extraction

After 24 h, surfactant action cultures were separated on tissue and medium via filtration using a Büchner funnel under vacuum. The tissue was weighed (fresh mass) and left to dry at room temperature. The dry weight (DW) measurement was made after at least 7 days of drying at room temperature when no changes in weight readings were no longer observed. The volume and the pH of the medium were determined, and then 30 mL of 1-butanol was added to the medium to guard against microbial contamination, and to saponin for the first step of extraction. In the next two successive extractions, 1-butanol/water yielded a total saponin content that was released into the medium. The dry tissues of HRs were ground with methanol and boiled under reflux over 1 h. Both extracts, butanolic and methanolic, were evaporated to dryness under reduced pressure and subsequently hydrolyzed with 10% HCl in methanol. The hydrolysate, after methanol distillation and three rounds of dilution with water, were extracted with 40 mL of diethyl ether and purified using thin-layer chromatography (TLC) on silica-gel plates with chloroform: methanol (95:5, v/v) as the mobile phase. Oleanolic acid (OA) was methylated with an ether solution of diazomethane (Janiszowska and Kasprzyk 1977) and detected as a methyl ester (MeOA) via gas–liquid chromatography (GLC).

Quantification of oleanolic acid

The quantitative measurement of OA was performed using gas–liquid chromatography (GLC) at 270 °C on a Shimadzu GC-2014 instrument equipped with a flame ionization detector (FID). Samples were applied via split injection (1:5) on a ZB-1 30 m × 0.25 mm × 0.25 µm column (Phenomenex). The temperature of the injector and detector was 290 °C. Nitrogen was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. Peak identification and the quantification of oleanolic acid were carried out by referring to a calibration curve prepared with an authenticated sample of MeOA as the standard (Długosz et al. 2013). In addition, the methyl ester of OA was identified using GLC–MS on an Agilent Technologies 7890 A gas chromatograph coupled with a 5975 C mass spectrometric detector (for details, see Długosz et al. 2018).

Cultures after permeabilization reconditioning

Hairy root lines after 130 subcultures, which had been treated with detergent boost via ultrasound, were checked for their viability. Three young tips of roots (as long as possible a piece of the light part of the root with all its branches) were collected after permeabilization and resuspended in 100 mL of fresh ½ MS medium to begin a new culture using a 300 mL flask. Fresh, dry weight, and saponin content in the tissue/medium were determined after 30 days of culture growth.

Statistical analysis

All experiments were performed in triplicate. A one-way analysis of variance (ANOVA) was used to verify the statistical significance between the treatment groups. The differences between means were evaluated using Fisher multiple-range test, with statistical significance set at $P \leq 0.05$. Results are shown as means ± standard error (± S.E.). All statistical analyses were performed using Microsoft Office Excel 2010 and Statgraphics® 4.1.

Results

The permeabilization effect on HR

DMSO

Two lines that had been treated by increasing the concentration of dimethyl sulfoxide exhibited different effects from tissue permeabilization. Line CH2 showed an outflow of the OA glycosides into the culture medium only at the lowest (0.1%) concentration of DMSO (Fig. 1a). The glycosides content in the other DMSO-treated cases oscillated around the control level in the medium (Fig. 1a), however 0.3% DMSO reduced the content inside the tissue by one third (Fig. 1b) with no obvious increase in the medium. Line CC16 released the most saponins at the two highest concentrations of DMSO: 0.5% and 1%, which were 3-fold and almost 7 times more than in the control culture, respectively (Fig. 1c). All concentrations used caused a slight increase in the glycoside content inside the tissue, but the effect was significant only for 0.3% compared to the culture that was not treated with DMSO (Fig. 1d). Its concentrations of 0.1% and 0.3% generated twice and 3.5 times higher saponin concentrations in the medium than the control culture. In the

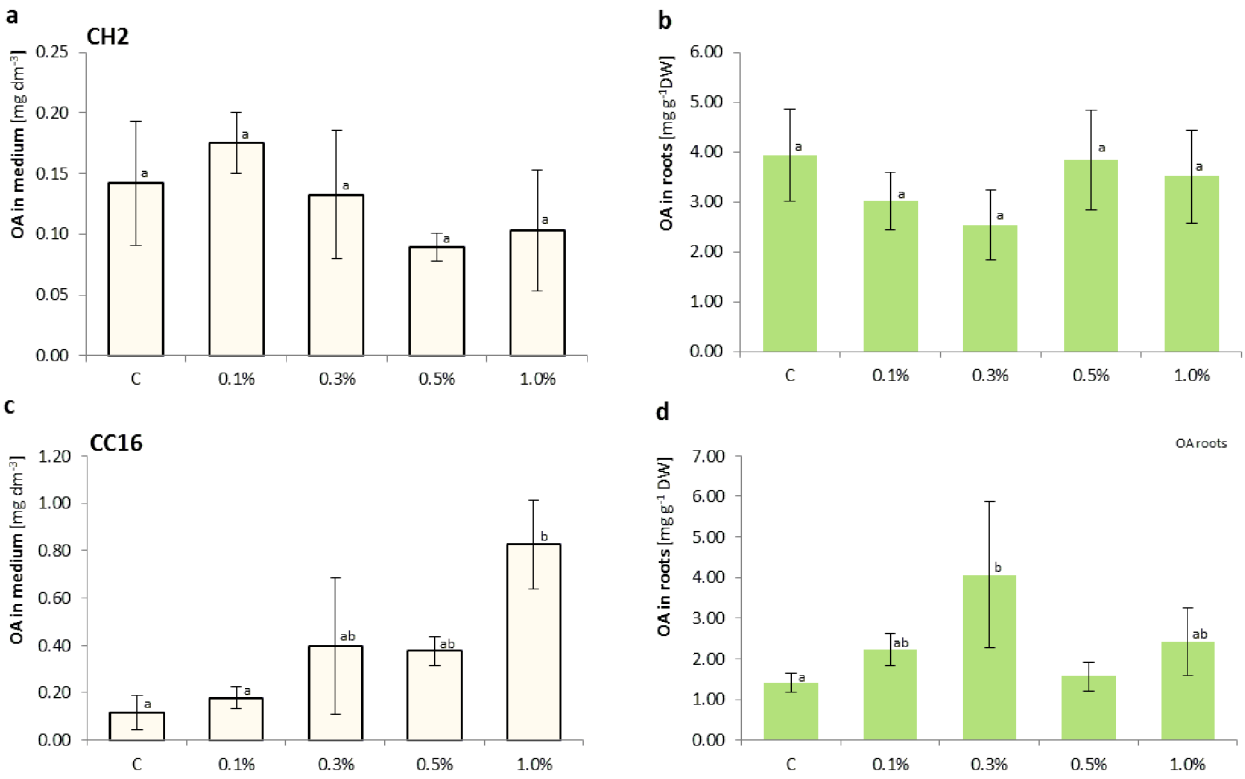


Fig. 1 Effects of dimethyl sulfoxide (DMSO 0.1-1.0% v/v) on total saponin (OA) production in hairy root cultures of *C. officinalis***a)** CH2, released into the medium; **b)** CH2, accumulated in roots; **c)** CC16, released into the medium; **d)** CC16, accumulated in roots. Mean ± standard error of three replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test

preliminary experiment, the lowest (0.1%) concentration of DMSO was tested during one subculture (29 days) for CH2, CH9, and CC16 (data not presented). Line CH2 showed a two-fold higher saponin content inside the tissue at the end of the culture, while in the medium, it was unchanged from the control culture. The other two lines released more saponin to the medium: CH9 released 2.7 times more, but this was unchanged in the tissue, and CC16 released 9.5 times more, with a more than two-fold reduction in tissue content.

Tween 20

Tween 20, the most popular surfactant, showed a permeabilizing effect that accelerated with a rise in concentration for all of the tested hairy root lines. The release of significant amounts of triterpene saponins to the medium took place in cases where the addition of the above-mentioned detergent from 0.3 to 1% was applied; however, minor differences among the lines were noticeable (Fig. 2a, c, e). The average contents of saponin secreted into the medium, taking into account the achieved increases in dry matter for the control cultures, were 4.05%, 0.17% and 0.65% of the total content of OA glycosides, respectively, for CH2, CH9 and CC16 (calculated from the data in Fig. 2; Table 1). However, such strong differentiation for the CH2 and CH9 lines was not reflected in the levels of saponins remaining in the root tissue. In both cases, the determined amounts of OA do not differ significantly from the control culture (Fig. 2b, d). In the case of CH2, surfactant addition enhanced saponin concentration in the medium by from over six- to over eleven-fold at 0.3–1.0% of T_{20} (Fig. 2a), and it was 54 and 61 times higher, respectively, for CH9 at the analogous T_{20} concentrations (Fig. 2c). Line CC16 showed a growth in glycosides content in the medium, from 11.91% (15 times) for 0.3% T_{20} to 34.55% (22 times) for 1% T_{20} (Fig. 2e). This latter case was closely coupled with a reduced saponin concentration in the hairy roots tissue (Fig. 2f).

Triton X-100

This nonionic surfactant containing a polyethylene oxide chain proved to be unsuitable for saponin determination when using the method employed here. Triton constantly accompanied the butanolic and ethereal extracts obtained from the post-culture liquids. The mutual interaction between MeOA and Tx100 derivatives continued, as seen in

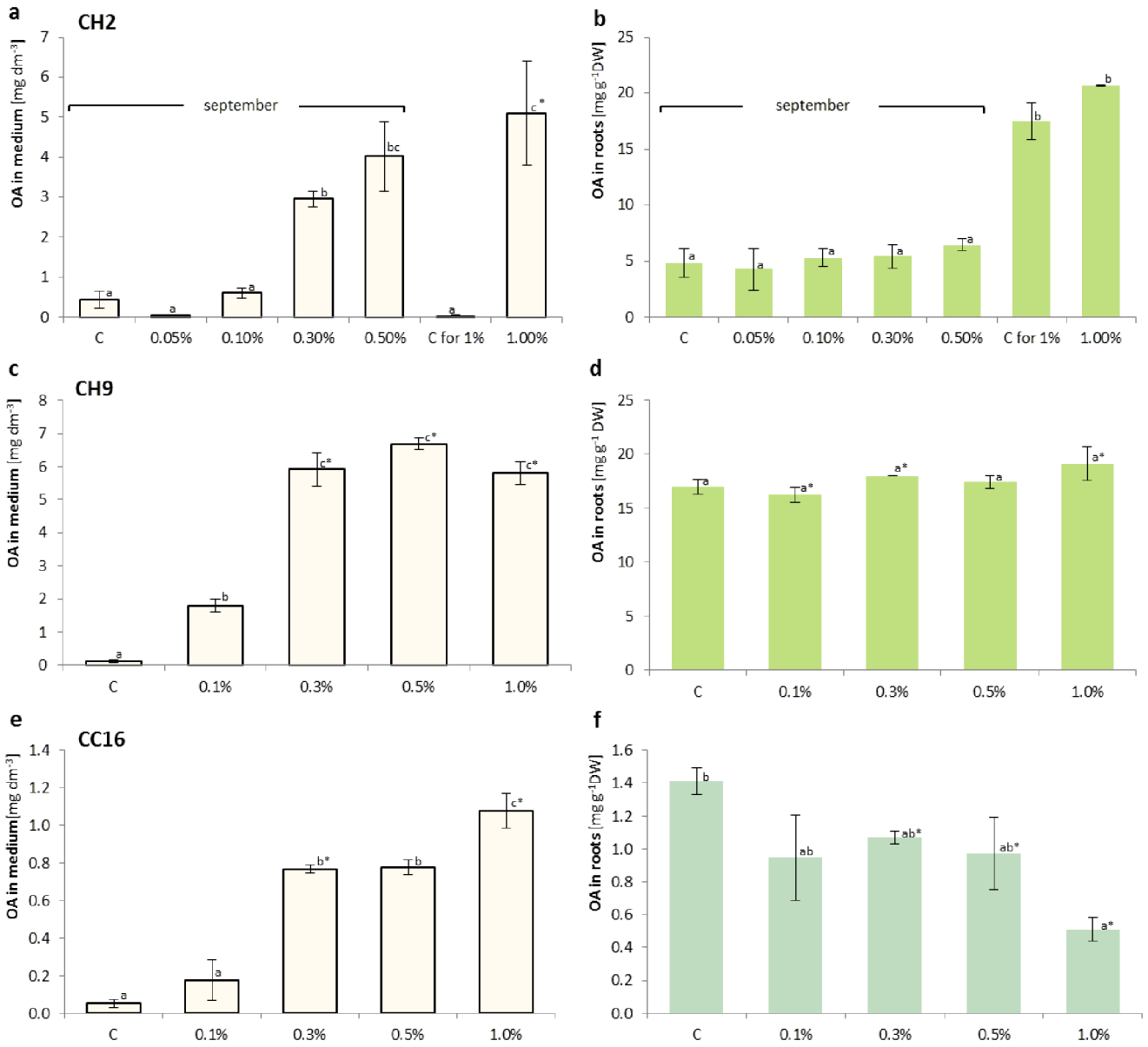


Fig. 2 Effects of Tween 20 (T_{20} 0.05-1.0% v/v) on total saponin (OA) production in hairy root cultures of *C. officinalis***a)** CH2, released into the medium; **b)** CH2, accumulated in roots; **c)** CH9, released into the medium; **d)** CH9, accumulated in roots; **e)** CC16, released into the medium; **f)** CC16, accumulated in roots. Mean ± standard error of three (* two) replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test. [September] - experiments were conducted in September (first month of culture slower growth period), remaining experiments were conducted in the optimal growth of the HR culture

gas–liquid chromatography (GLC), where it manifested as an enlarged surface of the peak at around the retention time of MeOA (data not presented). Inferring the effectiveness of permeabilization solely on the basis of the depletion of saponin in the tissue seems insufficient (Fig. 3).

Surfactants and ultrasound combined action

Two lines, CH9 and CC16, were examined for the cooperation between chemical and physical methods of permeabilization. In most cases of CH9 hairy root cultures subjected to various variants of permeabilization, they showed an increase in the release of oleanolic acid glycosides into the medium (Fig. 4a). The use of the detergent Tween 80 increased the secretion of OA glycosides to the medium by over 1.3-fold compared to the control culture, and the addition of Tween 20 caused an almost 3-fold increase in the concentration of this compound in the medium. Compared to the use of both types of Tween alone, supporting their action with ultrasound resulted in an increase in the secretion of glycosides

Table 1 Effects of detergents on the growth of hairy root lines of *C.officinalis*

Agent	Line	Agent conc.[%]	Fresh weight [g]	Dry weight [g]	DW [%]
DMSO	CH2	0.0	10.70 ± 0.62^{a}	0.84 ± 0.08^{a}	7.83 ± 0.47^{a}
		0.1	11.16 ± 0.30^{a}	0.96 ± 0.04^{a}	8.57 ± 0.09^{a}
		0.3	11.46 ± 0.17^{a}	1.02 ± 0.03^{a}	8.87 ± 0.31^{a}
		0.5	10.37 ± 0.37^{a}	0.84 ± 0.10^{a}	8.09 ± 0.64^{a}
		1.0	11.74 ± 0.69^{a}	0.95 ± 0.11^{a}	8.06 ± 0.59^{a}
	CC16	0.0	13.99 ± 0.25^{ab}	1.09 ± 0.02^{a}	7.80 ± 0.19^{a}
		0.1	15.30 ± 0.47^{b}	1.12 ± 0.01^{a}	7.33 ± 0.15^{a}
		0.3	15.34 ± 0.70^{b}	1.12 ± 0.02^{a}	7.33 ± 0.29^{ab}
		0.5	13.16 ± 0.48^{aa}	1.06 ± 0.05^{a}	8.08 ± 0.13^{b}
		1.0	14.09 ± 0.79^{ab}	1.14 ± 0.04^{a}	8.11 ± 0.19^{b}
T_{20}	CH2	^{s}0.0	$3.71 \pm 0.82^{ab*}$	$0.21 \pm 0.07^{ab*}$	5.72 ± 0.04^{a}
		^{s}0.05	$2.28 \pm 0.05^{ab*}$	$0.15 \pm 0.01^{ab*}$	6.22 ± 0.50^{ab}
		^{s}0.1	$2.74 \pm 0.62^{ab*}$	$0.18 \pm 0.05^{ab*}$	6.18 ± 0.24^{ab}
		^{s}0.3	$3.00 \pm 0.09^{ab*}$	$0.20 \pm 0.00^{ab*}$	6.27 ± 0.37^{ab}
		^{s}0.5	$4.04 \pm 0.68^{ab*}$	$0.24 \pm 0.04^{b*a}$	5.93 ± 0.14^{a}
		1.0	$1.90 \pm 0.00^{a*}$	$0.14 \pm 0.00^{a*}$	5.93 ± 0.08^{b}
		0.0	$5.52 \pm 0.16^{b*}$	$0.33 \pm 0.01^{c*}$	6.95 ± 0.21^{a}
	CH9	0.0	5.18 ± 0.25^{abc}	0.39 ± 0.04^{abc}	7.46 ± 0.61^{a}
		0.1	5.99 ± 0.48^{c}	0.43 ± 0.04^{bc}	7.16 ± 0.11^{a}
		0.3	5.42 ± 0.39^{bc}	0.44 ± 0.04^{c}	8.08 ± 0.45^{a}
		0.5	4.14 ± 0.30^{a}	0.32 ± 0.02^{a}	7.61 ± 0.23^{a}
		1.0	4.40 ± 0.49^{ab}	0.32 ± 0.03^{ab}	7.37 ± 0.12^{a}
	CC16	0.0	7.46 ± 0.53^{a}	0.56 ± 0.03^{a}	7.49 ± 0.18^{ab}
		0.1	6.65 ± 0.81^{ab}	0.48 ± 0.06^{ab}	7.27 ± 0.14^{a}
		0.3	6.74 ± 0.27^{ab}	0.53 ± 0.01^{ab}	7.92 ± 0.26^{a}
		0.5	5.73 ± 0.30^{a}	0.42 ± 0.03^{a}	7.38 ± 0.23^{ab}
		1.0	5.52 ± 0.63^{a}	0.41 ± 0.05^{a}	7.47 ± 0.10^{ab}
Tx-100	CH2	0.0	7.83 ± 1.21^{a}	nd	-
		0.1	10.61 ± 1.01^{ab}	nd	-
		0.3	10.34 ± 0.77^{ab}	nd	-
		0.5	10.58 ± 2.02^{ab}	nd	-
		1.0	12.54 ± 0.63^{b}	nd	-
	CH9	0.0	5.76 ± 0.98^{a}	nd	-
		0.1	5.77 ± 0.68^{a}	nd	-
		0.3	4.98 ± 0.99^{a}	nd	-
		0.5	6.82 ± 0.42^{a}	nd	-
		1.0	6.89 ± 0.48^{a}	nd	-
	CC16	0.0%	4.92 ± 0.57^{a}	0.35 ± 0.05^{a}	7.13 ± 0.26^{a}
		0.1%	4.28 ± 0.23^{a}	0.35 ± 0.02^{a}	8.14 ± 0.19^{b}
		0.3%	4.23 ± 0.27^{a}	0.33 ± 0.03^{a}	7.87 ± 0.42^{ab}
		0.5%	4.02 ± 0.27^{a}	0.33 ± 0.02^{a}	8.30 ± 0.35^{b}
		1.0%	3.99 ± 0.17^{a}	0.34 ± 0.02^{a}	8.48 ± 0.34^{b}

Mean ± standard error of three (* two) replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test. [S] – experiments were conducted in September (first month of culture slower growth period), remaining experiments were conducted in the period of the optimal growth of the HR culture, T_{20} - Tween 20, Tx-100 - Triton X-100, [nd] - not determined

by almost three times in the case of T_{80}, and over two times in the case of T_{20}. The combined permeabilization methods compared to the control culture resulted in a total increase in the saponins secretion into the medium by over 3.5-fold (2.05 to 7.54) with T_{80}, and up to 6-fold (2.05 to 13.15) with T_{20}. The exception for the first line was permeabilization with ultrasound alone. This physical method generated the release of over 37% less glycosides into the medium than was found for the control culture (Fig. 4a). However, this fact was interesting from the point of saponins residue in the tissue culture: their content was the most greatly lowered of all the tested variants of permeabilization (Fig. 4b). The average decrease in the content of saponins in the remaining cases ranged from 22.7% (T_{20}) to 27.8% (T_{80} US) compared to the control culture.

The hairy root culture marked as a CC16 produced and accumulated over eight times less saponins (taking into account the achieved increase in biomass and secretion into

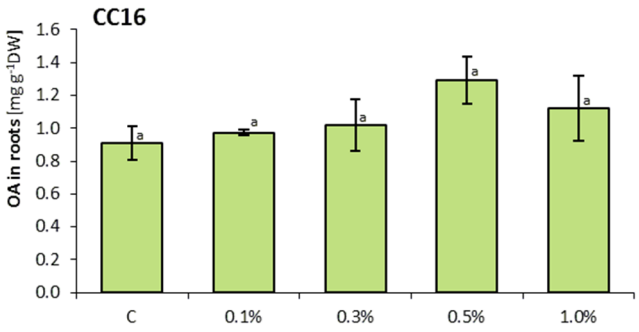


Fig. 3 Effects of Triton X-100 (Tx-100 0.1-1.0% v/v) on total saponin (OA) accumulation in hairy root cultures of *C. officinalis*, line CC16. Mean ± standard error of three replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test

the medium) than line CH9. In all of the cases, the cultures of the CC16 line subjected to various variants of permeabilization showed an increase in the release of oleanolic acid glycosides into the medium, compared to the control culture (Fig. 4c). Contrary to CH9, the use of ultrasound resulted in a four-fold increase in OA secretion to the medium. The use of the detergents T_{80} and T_{20} respectively resulted in an over three-fold increase, and the largest: an over eight-fold increase in the value of glycoside released outside the tissue. However, the use of ultrasound alongside detergents caused a 12.4% reduction in OA secretion to the medium for T_{80} and reduction of 1.5% for T_{20}, but the difference was not statistically significant. When analyzing the above effect from the level of tissue saponin residues exposed to surfactants and ultrasound, different conclusions can be drawn. The tissues of cultures treated with both types of surfactants assisting with the temporal action of ultrasound contained fewer saponins than those treated with surfactants alone: 31.5% for T_{80} and over 45% for T_{20} (Fig. 4d). Generally, almost all cases of permeabilization showed higher (10.0–61.4%) saponin contents in the roots tissue than in the control (untreated) culture. Tween 20 accelerated via ultrasound lowered the saponin content in tissue by over 23% compared to the control.

The older HR cultures (130 sc.) were treated with two polysorbates that were assisted using ultrasound and then restarted (reconditioned) in control media. These were also analyzed for saponin content. Line CH9 maintained a saponin synthesis rate that was completely unchanged compared to the control; however, earlier contact with T_{80} stimulated an enhancement in the tissue concentration (Fig. 5b). Only

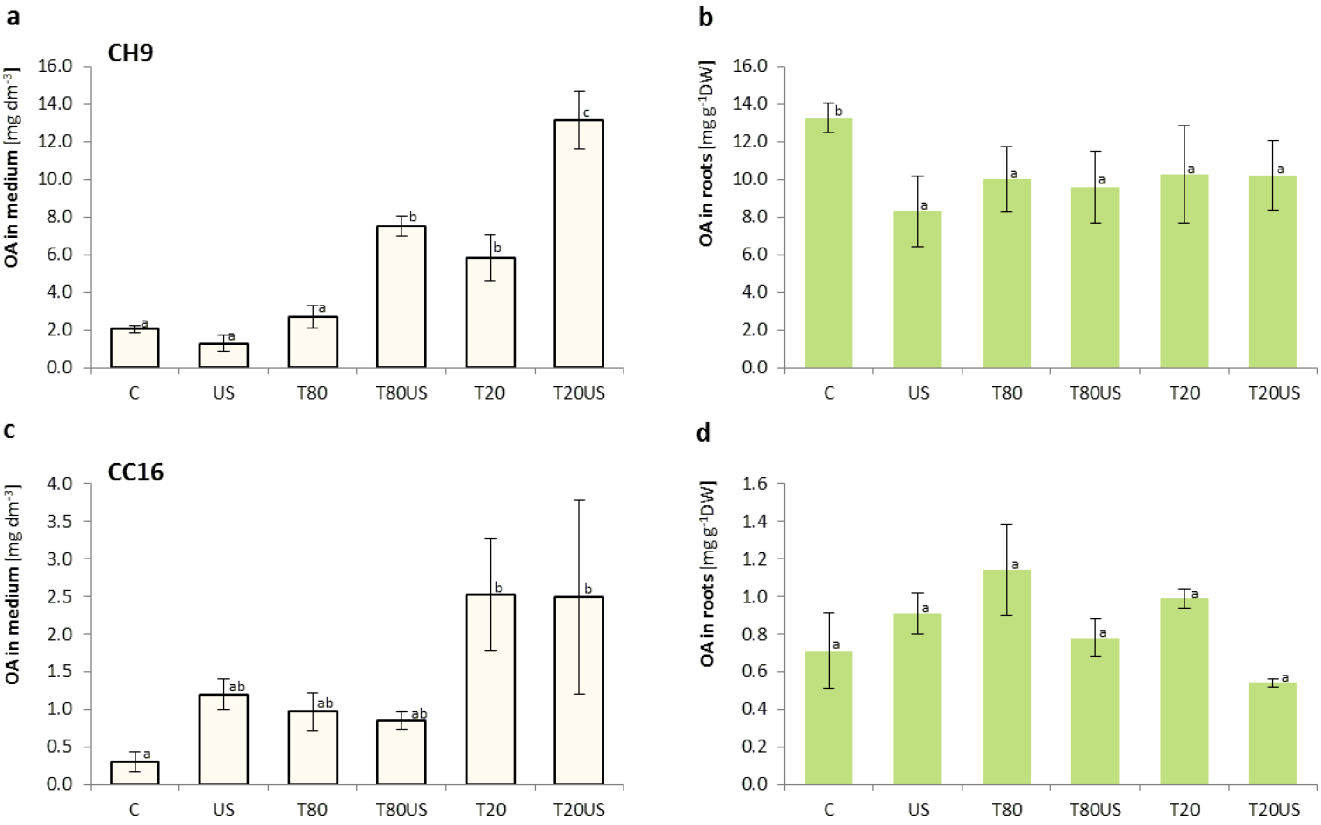


Fig. 4 Effects of ultrasounds and polysorbates interaction on total saponin (OA) production in hairy root cultures of *C. officinalis***a)** CH9, released into the medium; **b)** CH9, accumulated in roots; **c)** CC16, released into the medium; **d)** CC16, accumulated in roots. Mean ± standard error of three replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test

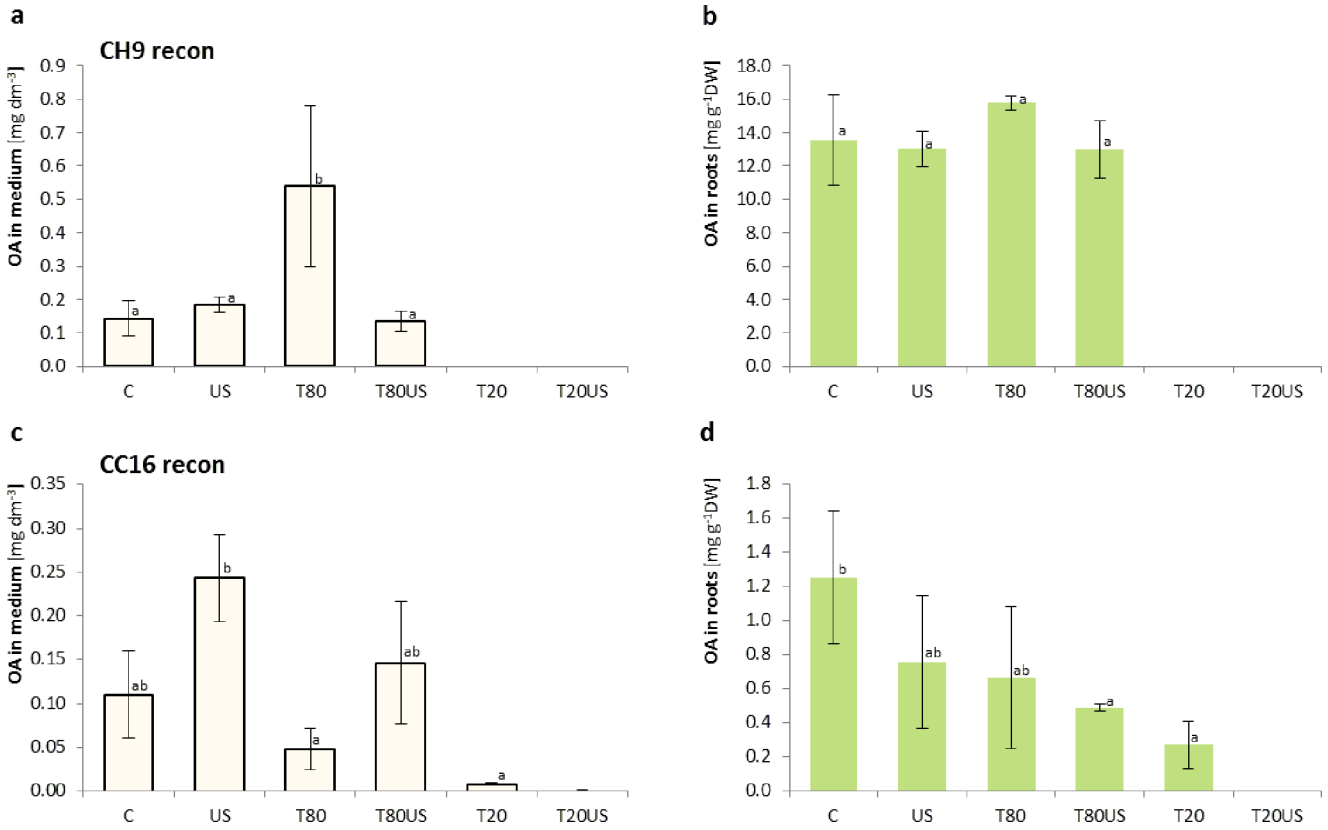


Fig. 5 Total saponin (OA) production in *C. officinalis* hairy root cultures reconditioned after permeabilization **(a)** CH9, released into the medium; **(b)** CH9, accumulated in roots; **(c)** CC16, released into the medium; **(d)** CC16, accumulated in roots. Mean ± standard error of three replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test

the inoculum derived from the roots treated with T_{20} did not initiate new biomass. The regenerated line showed diminished glycosides release into the medium, where the culture that regenerated after the T_{80} treatment secreted at least three times more glycosides than the other cultures (Fig. 5a). The second line (CC16) generated roots with a decreasing glycoside content in order: from US, T_{80}, and T_{80} + US to T_{20}; however, this was not statistically significant (Fig. 5d). The saponin secretion for this line after its restitution was noticeably larger than in the control culture for cases after US and T_{80} US treatment (Fig. 5c).

Quality of the hairy roots culture

Most of the surfactants used caused a significant reduction in fresh and dry matter content (HRs after 90 subcultures) in relation to the values obtained using the corresponding control cultures. At 0.1–0.3%, the ionic surfactant DMSO improved tissue growth (FW and DW) regardless of the line (Table 1) in most cases, however the change was not statistically significant. The tested parameters were diminished for 0.5%, and surprisingly, they increased after 1.0% DMSO addition. However, the CH2 line treated with T_{20} showed an upward trend for both mass parameters, with an increased concentration of surfactant for cultures collected in September (Table 1). The other two lines presented slight fluctuations of fresh and dry weights at a low concentration of T_{20}, and this decreased by 15–26% for the two last concentrations (Fig. 2c-f). Lines treated with Triton X-100 achieved similar values of DW to the control; however, FW decreased with an increasing surfactant concentration, from 13 to 19% for CC16, or it showed higher values (32–60%) for CH2, rising with the highest Tx100 concentration for the CH9 line (Table 1). The use of DMSO did not significantly affect the appearance and condition of the hairy root cultures of *C. officinalis*. The only observable change was a darker color in the air-dried tissues of the CC16 line after treating them with 0.5% and 1% (v/v) concentrations (Fig. 6a, b). The level of tissue hydration in both lines also did not change (data not presented). The appearance of the CH2 line after T_{20} treatment of the culture shows the degeneration of morphology, progressing with an increase in the detergent concentration. The roots achieved a larger cross-sectional diameter and a glassy structure, indicating an increase in their hydration (Fig. 7). Among the three Tx100-treated lines, only CC16

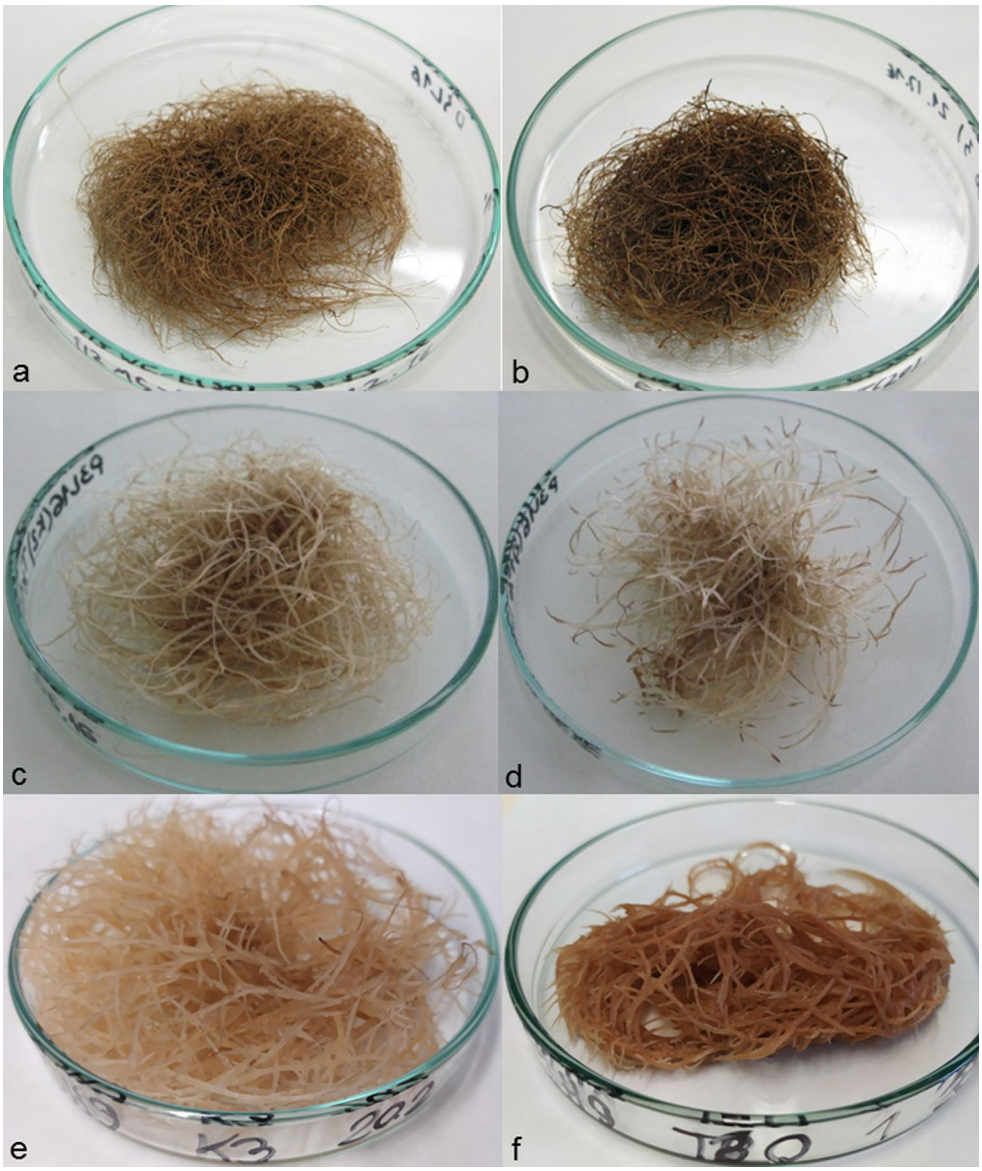


Fig. 6 Response to detergents alone or assisted with ultrasound (US), **(a)** control culture line CC16 after 31-day growth; **(b)** 30-day culture line CC16 after 24 h treatment with 1% DMSO; **(c)** control culture line CC16 after 31-day growth; **(d)** 30-day culture line CC16 after 24 h treatment with 1% Tx-100; **(e)** control culture line CH9 after 31-day growth; **(f)** 30-day culture line CH9 after 24 h treatment with 1% T_{20} assisted with US during first 10 min

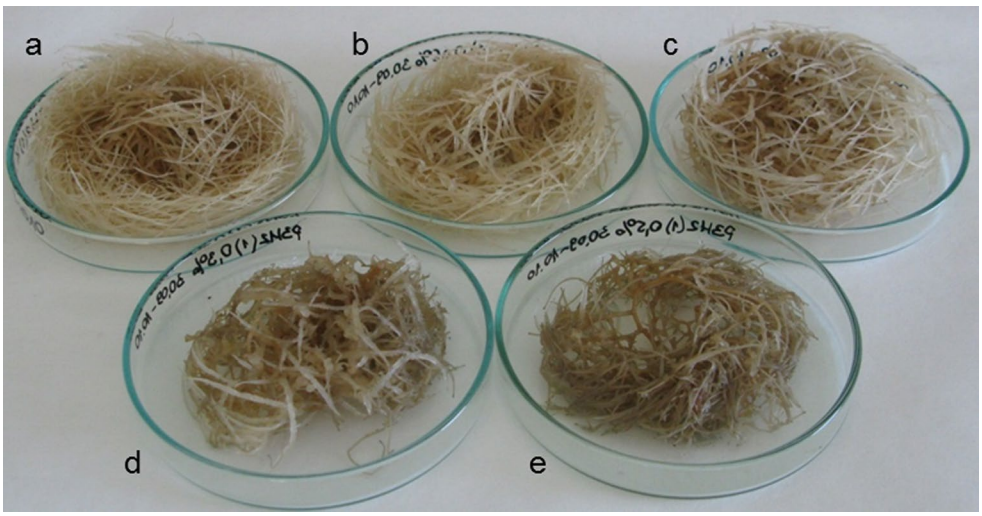


Fig. 7 Response to Tween 20 (T_{20}) of hairy root culture, line CH2, **(a)** control culture after 31-day growth; culture 30-day after 24 h treatment with **(b)** 0.05% T_{20}; **(c)** 0.1% T_{20}; **(d)** 0.3% T_{20}; **(e)** 0.5% T_{20}

showed morphological changes, manifested by a darkening of the root tips and a reduced biomass (Fig. 6c, d).

Subsequent trials with hairy root cultures (130 subcultures) treated with both types of Tween detergents and ultrasound obtained line-dependent growth parameters. Although the two lines achieved maximal FW and DW (8.7 and 9.1% for CH9, and 3.2% and 7.7% for CC16 over the control, respectively) after the US, only line CC16 responded positively to T_{80} supplementation (Table 2). With the second surfactant, in accordance with the US, the permeabilized cultures for both lines decreased in growth by a maximum of 30.7% DW for CC16. The morphology and properties of hairy root tissues differed depending on the method of permeabilization. Line CH9 did not change in tissue color after treatment with US; however, it was characterized by a thinner root diameter, less resilience, and less volume being taken up compared to the control culture. The tissue treated by T_{80} was slightly darker and medium springy. The strongest effect on culture morphology was found for the permeabilization variants based on T_{20}. The roots were characterized by a much darker color, and their volume and elasticity were clearly reduced (Fig. 6e, f). Although line CC16 generated roots with a finer structure, its reaction was very close to CH9.

An important step, restarting the new cultures in fresh media allowed for the determination of the real impacts of chemical and/or physical factors generating the perforation of the cell layer. The conditions that were least harmful for the roots were: ultrasound, Tween 80, and T_{80} interacting with US. The hairy root cultures after 30 days of growth in new ½ MS medium yielded a fresh biomass that was similar to the control, or that was reduced by a maximum of one-fifth for CH9 (Table 2). The quality of the reconditioned cultures after permeabilization did not differ from the control culture; however, the effect of T_{20} prevented the initiation of the new one. For the second line (CC16), the growth parameters were reduced twice (Table 2). Polyoxoethylene sorbitan monolaurate (T_{20}) turned out to be the most powerful surfactant, and a proposed concentration and time of action only for the CC16 line allowed for hairy roots culture viability. The young part of root tips generated a new culture, with the growth being reduced by 77% (FW) and 82% (DW) (Table 2). Their pigmentation was less intense than the untreated control culture, with a clearly reduced biomass for the culture being reconditioned after T_{20} treatment, and no growth for T_{20} that was accelerated with US (Fig. 8).

Table 2 Effect of ultrasound and polysorbates interaction on the growth of the hairy root lines of *C.officinalis*

Line	Agent	Fresh weight [g]	Dry weight [g]	DW [%]
CH9	Control	7.85 ± 0.17^{bc}	0.55 ± 0.01^{bc}	6.97 ± 0.12^{bc}
	US	8.24 ± 0.42^{c}	0.60 ± 0.03^{c}	7.33 ± 0.22^{c}
	T_{80}	6.43 ± 0.42^{a}	0.43 ± 0.03^{a}	6.67 ± 0.00^{ab}
	T_{80} +US	6.89 ± 0.58^{ab}	0.47 ± 0.05^{ab}	6.86 ± 0.14^{b}
	T_{20}	6.74 ± 0.14^{ab}	0.42 ± 0.01^{a}	6.24 ± 0.01^{a}
	T_{20} +US	6.68 ± 0.42^{ab}	0.42 ± 0.02^{a}	6.24 ± 0.21^{a}
CC16	Control	6.74 ± 0.49^{ab}	0.52 ± 0.04^{c}	7.70 ± 0.17^{b}
	US	6.96 ± 0.10^{b}	0.56 ± 0.02^{c}	7.99 ± 0.19^{b}
	T_{80}	6.82 ± 0.18^{ab}	0.54 ± 0.05^{c}	7.88 ± 0.44^{b}
	T_{80} +US	6.44 ± 0.53^{ab}	0.48 ± 0.05^{bc}	7.44 ± 0.28^{b}
	T_{20}	5.60 ± 0.57^{a}	0.36 ± 0.05^{a}	6.37 ± 0.24^{a}
	T_{20} +US	5.69 ± 0.34^{a}	0.36 ± 0.01^{ab}	6.36 ± 0.21^{a}
After recondition				
Line	**Agent**	**Fresh weight [g]**	**Dry weight [g]**	**DW [%]**
CH9	Control	7.84 ± 0.18^{b}	0.63 ± 0.03^{c}	7.98 ± 0.23^{b}
	US	7.61 ± 0.27^{ab}	0.58 ± 0.04^{bc}	7.56 ± 0.36^{ab}
	T_{80}	6.88 ± 0.24^{a}	0.47 ± 0.01^{a}	6.88 ± 0.36^{a}
	T_{80} +US	7.46 ± 0.42^{ab}	0.53 ± 0.03^{ab}	7.14 ± 0.14^{ab}
	T_{20}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	T_{20} +US	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CC16	Control	7.04 ± 0.09^{b}	0.62 ± 0.02^{c}	8.88 ± 0.35^{b}
	US	7.40 ± 1.07^{b}	0.52 ± 0.05^{bc}	7.11 ± 0.37^{a}
	T_{80}	5.56 ± 0.64^{ab}	0.37 ± 0.06^{abc}	6.65 ± 0.44^{a}
	T_{80} +US	5.37 ± 2.01^{ab}	0.35 ± 0.15^{ab}	6.36 ± 0.33^{a}
	T_{20}	1.64 ± 1.26^{a}	0.11 ± 0.08^{a}	6.86 ± 0.45^{a}
	T_{20} +US	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Polysorbates (T_{20}, T_{80}) concentration was 1%, US duration was 10 min. Mean ± standard error of three (* two) replicates. Means with common letters within the line are not significantly different at $P \leq 0.05$ according to Fisher multiple range test, [nd] - not determined

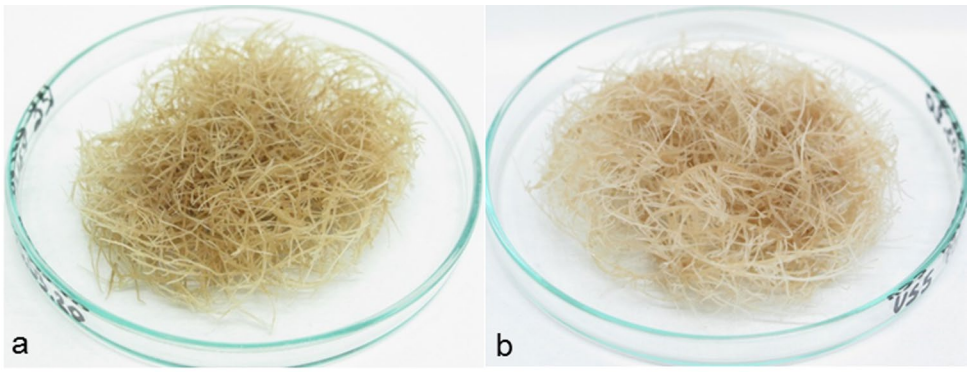


Fig. 8 Morphology of reconditioned hairy root culture after combined effect of polysorbates and/or ultrasounds (US), line CC16; **(a)** control culture after 30-day growth; **(b)** culture (30 d) after US

Discussion

Tween 20, Triton X-100, and DMSO

Permeabilization to obtain triterpene saponin was performed on hairy root cultures on the 30th day of subculture. Previously, we found that the highest accumulation of triterpenic saponin occurred at the stationary phase of the culture growth (Długosz et al. 2013). This method seems appropriate as the contents inside the tissue of the metabolite have numerous pharmacological properties; however, the older tissues have poorer reconstruction abilities, and due to the secondary growth of the root, access by substances causing its permeabilization may be more difficult. During the primary experiment, the most commonly used mild detergents of various structures were tested. As the smallest amphiphilic particle, dimethyl sulfoxide was recognized as an effective carrier for many molecules applied inside the cell through complex biological membrane structures (Notman et al. 2006). This potential was therefore checked as a factor for increasing cell permeability to cell-synthesized compounds such as phytopharmaceuticals. Parr et al. (1984) reported a wide concentration range of DMSO (1–30%) in *Cinchona ledgeriana* suspension cells producing quinoline alkaloids. The effects were already visible when using a concentration of 2% (v/v) DMSO; however, a concentration of above 20% was necessary for the complete permeabilization of all cells. It was also shown that the tonoplast, in this case, was more resistant to permeabilization than the cell membrane. After a 2 h incubation at a concentration of 10% (v/v) DMSO, around 10% of intracellular alkaloids were obtained, and at a concentration of 30% (v/v), it reached up to 25%. However, the concentrations that effectively release alkaloids cause permanent cell damage. A suspension culture of *Panax notoginseng* showed an increased content of extracellular saponin after DMSO (1–10%) treatment (Zhong et al. 1997). The most important step that improved both cell viability and saponin concentration in the medium was culture preconditioning with the lowest DMSO concentration over five subcultures. As was shown from our preliminary results (data not presented), the presence of a low concentration of DMSO during the growth of the subculture in some lines can act as an elicitor, and in others, it improves only the saponin secretion. In our present study, marigold hairy roots showed line-dependent differences for saponins released from tissues in response to the concentration of applied DMSO. The highest concentrations (0.5–1.0%) boosted saponin movement outside of the tissue for one of the treated lines. In contrast, the other was more efficient at the lowest concentration of permeabilizing factor. There was no significant deterioration in the growth of cultures, regardless of the line. A negative impact of 1% DMSO was also not found for *Catharanthus roseus* seedlings subjected to 35-day co-cultivation (Bonzom et al. 1998).

Polysorbates, with their most commonly used representative – T_{20} – have been proven to be efficient permeabilization agents for hairy root cultures of marigold. Regardless of the lines and their productivities, the highest concentrations of T_{20} are the most effective for increasing the content of marigold saponin in the medium. Gontier et al. (2002) demonstrated the same trend for the alkaloid content in *Datura inoxia* Mill. cultivated under hydroponic conditions. Hyoscyamine and scopolamine were released about 7-fold more intensively after Tween application. There was no significant difference in the permeabilization duration: 12, 24, and 48 h generated comparable concentrations of alkaloid in the medium. In the current report, we decided to determine the contents of compounds after 24 h of permeabilization. A hairy root culture (CH2) treated for 29 days with 0.1% T_{20} doubled saponin accumulation inside the tissue without significantly increasing its level in the medium (data not presented). At a low concentration, the action of the detergent may, therefore, resemble an elicitor, as was mentioned above for DMSO. Tanshinones (diterpene quinone pigments) and phenolic compounds were 67% and 5-fold higher, respectively, in *Salvia miltiorrhiza* hairy root culture media after permeabilization with 1% (w/v) T_{20} (Siu and Wu 2014). For the *Salvia* culture, apart from an increase in the secretion of both types of compounds, a significant stimulation of total tanshinones synthesis was also found.

Triton X is not as popular among nonionic detergents as polysorbate T_{20} for phytopharmaceutical release from plant cells or tissues. Mongrand et al. (2004) suggested that the greater qualitative and quantitative diversity of sterols in the membranes of plant cells makes it difficult to determine the precise parameters of their permeabilization. This contrasts with animal cells and fungal structures, where cholesterol and ergosterol are mainly present. The current results for saponin obtained from marigold hairy roots showed that it was not the permeabilization conditions that seemed to be crucial. The extraction and determination of saponins in a liquid medium did not remove the Triton, which increased the peak area of the determined oleanolic acid methyl ester. Other compounds, like alkaloids, were efficiently acquired from *C. roseus* through permeabilization with Tx100 at the lowest concentration. Ajmalicine that accumulated in hairy roots had a 16-fold increase for their efflux outside of the tissue at the lowest concentration of Tx100 (Thakore et al. 2013). The short-time action of Tx100 induced a release of 30% betacyanines from *B. vulgaris* cell culture, slightly reducing cell viability while increasing the growth rate of the culture for the next subculture (Trejo-Tapia et al. 2007).

Interaction between ultrasound and polysorbates

According to our knowledge, this is a first report on the combined use of ultrasound with detergents to obtain an enhancing effect for permeabilization in plant tissue culture. We decided to choose Tween 20 and Tween 80 as the most frequently used polysorbates in plant cultivation and biotechnology, which are here combined with ultrasound. Ultrasound, next to pulsed electric fields and high hydrostatic pressure, belong to the main physical permeabilization methods that have been successfully applied to plant cells and tissues (Cai et al. 2012). The significant advantages of this method include a low application cost, the simplicity of use, the relatively high resistance of tissues and even plant cells, and promising efficacy in many plant culture systems (Gaba et al. 2008). Our previous results confirmed the effectiveness of this physical technique; however, the saponins increment in the medium and tissue conditions depends on the time exposure to US and the cell line (Alsoufi et al. 2019a). Suspension cultures were more sensitive to prolonged exposure to US, so they needed several seconds of exposure for anthraquinone release, as was the case with *Morinda citrifolia* (Komaraih et al. 2005). Tissue and organ cultures can withstand much longer exposure times, up to several minutes (Liu et al. 2012; Khezri et al. 2022). Low-energy US enhanced both the synthesis and secretion of shikonin in *L. erythrorhizon* suspension cultures (Lin and Wu 2002). This natural dye, classified as naphthoquinones, increased its extracellular concentration, not only as a result of permeabilization, but also due to the stimulation of enzymes that are key to its synthesis: phenylalanine ammonia lyase and p-hydroxybenzoic acid geranyltransferase. In several cases, it has also been proven that ultrasound does not affect the release of plant metabolites. They can then often be assigned the role of elicitors: the induced synthesis pathways of diterpenes in *Corylus avellana* (Safari et al. 2012) and phenolic acids in *Echinacea purpurea* (Liu et al. 2012); however, the final product was not detected outside of the tissue.

Studies conducted on the release of hyoscyamine from the hairy roots of *Datura stramonium* confirm some of the effects of Tween 20 that were observed in this report (Khelifi et al. 2011). The thornapple tissues were exposed to the same 24 h incubation period with the permeabilizing agent. Tween 20 resulted in the release of at least 25% more hyoscyamine into the medium, and it also caused a 25% decrease in tissue dry weight compared to the control culture. A similar loss of dry weight after the use of T_{20} in both *Calendula* lines was approximately 24% and 30%, respectively, compared to the control. In the *D. stramonium* culture, a tissue browning effect was also reported; it was assessed that this phenomenon was the result of the onset of necrosis. However, *Datura* hairy roots remained viable after permeabilization with T_{20}, unlike those of *C. officinalis* examined in the current research. Previous reports have shown that the *Datura* genus remains viable even after one day of incubation with a medium containing 2% T_{20}, and that this detergent stimulated the secretion of alkaloids into the liquid medium (Boitel-Conti et al. 1995).

As was confirmed by our results, a more gentle and definitely more effective degree of permeabilization were achieved with second type of Tween – T_{80}. This detergent did not cause tissue necrosis; moreover, in the case of the CH9 line, it resulted in a higher percentage of dry weight content in the reconditioned tissue. Although T_{80} was secondary in terms of effectiveness compared to T_{20}, it maintained the plant tissue in excellent condition. The support of detergents with ultrasound in this line not only increased the amount of saponin released into the medium, but it also increased the mass of reconditioned tissue after the permeabilization culture. The second line (CC16) responded completely differently under identical conditions. Most of the published reports have confirmed the mild and effective action of this surfactant. In *Glycyrrhiza uralensis* hairy roots, 2% of T_{80} increased the contents of licochalcone A and flavonoids by 9- and 11-fold, respectively. The mentioned compounds were released outside of the tissue at 98% and 94% efficiency, respectively, for licochalcone A and flavonoids. The authors have suggested that T_{80} functions as an elicitor: elevated mRNA levels and the activities of three enzymes that are crucial for the phenylalanine metabolism pathway.

Similarly, in marigold, this surfactant does not affect tissue growth (Zhang et al. 2011). The complex effect of T80 was also confirmed for the hairy roots of *P. ginseng*. The long-term presence of detergent in the medium promotes the release of ginsenosides accumulated in the tissues. After four weeks, the concentration of this main bioactive constituent of *P. ginseng* in the medium reached 76% of the total amount of compounds produced. In the case of the control, the amount of ginsenosides released outside was negligible; moreover, compared to the control, the total level of ginsenosides was increased approximately 3-fold under the T_{80} treatment. This detergent had no significant effect on root viability and morphology (Liang et al. 2015).

Conclusion

Permeabilization adds to the theme of determining the biotechnological uses of marigold. In addition to the elicitation (Alsoufi et al. 2019a, b; Rogowska et al. 2022), influence of macronutrients (Długosz et al. 2018), plant growth regulators (Markowski et al. 2022) current reports complement the subsequent tools that have been adapted to the hairy roots culture of *C. officinalis* for the synthesis of triterpenic saponins. These multidirectional, biologically active compounds accumulated inside the cells in the vast majority of cases. Therefore, it becomes necessary to use methods that release them from the inside, which is often associated with the destruction of plant tissues. Tween 20 turned out to be the most effective agent for perforating cell membranes for the two lines tested. However, its effect was associated with a negative impact on the viability of cultures, which is contrary to the idea of maintaining the continuity of the process and obtaining secondary metabolites without cultures termination. An excellent alternative may be permeabilization based on T_{80}, which in the CH9 resulted in the release of a significant amount of OA glycosides into the medium, while maintaining tissue viability. Supporting the action of detergents with short-term use of ultrasound, more effective permeabilization effects were achieved, but only for the CH9 line. However, for the CC16 line, physical permeabilization generated using ultrasound turned out to be attractive; although it did not ensure the maximum content of saponins in the medium, it did not inhibit the culture growth.

The temporary permeabilization of plant tissues by using popular and safe detergents and/or ultrasound seems to be an attractive way of obtaining bioactive compounds. From an ecological and economic point of view, that solution can be included with the so-called "green extraction" method (Bitwell et al. 2023). This allows us to exclude a number of imperfections that accompany classic extraction methods: a high-energy input, low selectivity, a large volume of toxic organic waste, and the risk of environmental pollution. These issues appear to be absolutely crucial when it comes to using these valuable compounds in human nutrition and in the food industry.

Author contributions Conceived and designed the experiments: MD. Performed the experiments: JK, PB, MD. Analyzed the data: JK, PB, MD. Contributed reagents/materials/ analysis tools: JK, PB, and MD. Wrote the paper: MD. All authors read and approved the final manuscript.

Data availability The datasets generated during the current study will be made available by the corresponding author on reasonable request.

Declarations

Conflict of interests The authors declare no conflict of interest.

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