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CYTOTOXIC ACTIVITY OF STIGMASTERYL ESTERS AND PRODUCTS OF THEIR THERMO-OXIDATIVE DEGRADATION AGAINST DRUG SENSITIVE AND DRUG RESISTANT HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS*

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ABSTRACT

Background. Phytosterols are mainly known as a cholesterol-lowering factor, although they form oxidation products during food storage and processing. Moreover, phytosterol oxidation products (POP) can be absorbed and found in human serum, so there is the need to investigate their impact on different kinds of cells.
Material and methods. Esters of fatty acids (oleic, linoleic and linolenic) with stigmasterol were synthetized and heated at 180°C, for 1–12 hours. The cytotoxic effect on the leukemic cells of unheated stigmasteryl esters and the mixture of compounds after heating was determined using MTT assays. POP were identified using GC-MS. The total number of POP was analysed by SPE fractionation and GC-FID separation. Dimers, trimers and oligomers in non-polar fraction were determined by gel permeation chromatography with refractive index detection.
Results. After heating, stigmasterol oxidation products were formed (up to 1.1 mg/g ester). The heating increased the potency of the compounds to reduce cell population and form POPs and oligomers in a time-dependent manner.
Conclusion. The cytotoxicity depends on the kind of ester, dose and time. The strongest cytotoxic effect was found after 72 hours of cell treatment. Among the three stigmasteryl esters tested the most cytotoxic effect was caused by stigmasteryl linoleate.

Keywords: stigmasterol, fatty acid, ester, oxidation, oligomers, cytotoxicity

Abbreviations: COP – cholesterol oxidation products, POP – phytosterol oxidation products, SOP – stigmasterol oxidation products, MTT – 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, StO – stigmasteryl oleate, StL – stigmasteryl linoleate, StLn – stigmasteryl linolenate.

INTRODUCTION

For thousands of years, people have used plants grown in their environment as food or natural medicines. It is now widely accepted that most bioactive food components are predominantly found in plant food and

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many of them have a significant beneficial impact on human health (Kris-Etherton et al., 2002). In recent years an increasing interest in phytosterols among researchers as well as consumers can be observed, especially due to their total and LDL-cholesterol lowering properties (AbuMweis et al., 2014; Jones et al., 1997). The dominant sterols in plants are β-sitosterol, campesterol, and stigmasterol and they are usually esterified with fatty acids like oleic, linoleic and linolenic, which are widely present in oils (García-Llatas and Rodríguez-Estrada, 2011). The intake of phytosterols, which are supplemented to food products reduce intestinal cholesterol absorption (Jones and AbuMweis, 2009). A molecular basis of the plant sterols' biological properties is a chemical structure, which is very similar to the cholesterol structure. Due to these characteristics, they are often recommended by dietetics as an important factor in cardiovascular disease prevention. Moreover, recent research findings on the effect of phytosterols on different cancer cells (lung, stomach, prostate, ovary, breast cancer) proved their anticancer properties (Ramprasath and Awad, 2015; Woyengo et al., 2009). Plant sterol compounds were found to act through multiple modes of action, including inhibition of cancer cell growth, apoptosis induction or invasion, metastasis and angiogenesis level reduction (Ramprasath and Awad, 2015; Woyengo et al., 2009). Because of the known beneficial biological activity of plant sterols, there is a tendency in the food industry to enrich some food products (margarine, milk, yoghurt, etc.) with them (Moreau et al., 2002). Free phytosterols are difficult to dissolve in fat (Tofiana et al., 2016), thus more lipophilic, fatty acid phytosteryl esters are usually used in the technological process of functional food production. Plant sterols and their ester absorption in the gut is limited, but certain quantities of the compounds are transported through the intestinal wall and are present in the blood. As the bioactive compounds, phytosterols may exert their activity against blood cells and blood vessels tissues or interact with biochemical plasma constituents. A previous study showed that beta-sitosterol is able to induce the apoptotic death of HAAE-2 endothelial cells (Rubiś et al., 2008), what seems to confirm the theory that an excessive intake of plant sterols and their accumulation in the body tissues may be harmful to normal cells.

There is evidence that phytosterols form oxidation products during food storage and preparation. Oxidation of phytosterols and their fatty acid esters through multiple reactions leads to primary (hydroperoxides), secondary (polar: ketones, epoxides, alcohols; nonpolar: dienes, trienes) and tertiary (dimers, oligomers, polymers) oxidation products. Most research has focused on primary and secondary polar compounds (García-Llatas and Rodríguez-Estrada, 2011; Scholz et al., 2015). However, the most abundant from oxides, formed during sterol degradation at elevated temperatures, are dimers, which are bound mostly by ether (C-O-C) or peroxy (C-O-O-C) linkages (Sosińska et al., 2014).

The oxidation of unsaturated lipids follows three stages: initiation, propagation, and termination (Porter et al., 1995). Formation of dimers, trimers, and oligomers is the final oxidation stage of lipids, which is called termination. Polymerization usually occurs at higher temperatures (150–190°C) in the presence of oxygen. The polymerization of lipids could affect the nutritional value of the product, for example, by decreasing the cholesterol-lowering efficiency of plant sterols.

Besides the effect of food enriched with phytosteryl esters, pure compounds should be studied to find the correlation between the kind of ester and their heating time and the cytotoxic activity on cell lines (Rubiś et al., 2008). Over the years the cytotoxic effect of phytosterol oxidation products (POP) was investigated and published studies show the toxic activity of POP and cholesterol oxidation products (COP). Some studies present similar effects for both, while others suggest POP have a stronger effect than COP (Adcox et al., 2001; Vejux et al., 2012). Several studies pointed to the potential chemotherapeutic effects of particular phytosterol oxides, because of selective apoptosis induction in cancer cell lines (Samali et al., 1996).

Certain phytosterol oxidation products have been demonstrated to be cytotoxic in a number of cell lines. Different mechanisms of cell death induced by POP were described, including apoptosis. It was determined that beta-sitosterol, campesterol, and stigmasterol oxides caused apoptotic cell death. The triol derivatives were found to be the most cytotoxic, but hydroxy- and keto- derivatives also induced a high level of apoptosis in the cell population tested (Awad et al., 2003). However, further studies are needed to determine the range

of cytotoxic concentrations of the compounds in food products and their absorption levels in the human body.

Thus, the aim of our study is to evaluate the cytotoxic effect of three stigmasteryl esters and the products of their thermo-oxidative degradation against the human acute lymphoblastic leukemia cell line CCRF--CEM and its multidrug resistant counterpart CCRF--VCR1000 cell line overexpressing *ABCB1* gene coding ABCB1 transmembrane protein. The biological function of the protein to transport drugs/xenobiotics outside of the cells to the extracellular environment. The result of this phenomenon is a decrease in the intracellular concentration of the drug and the absence of any therapeutic effect. To find the relation between oxidation products and the cytotoxic effect of esters after different heating times stigmasterol oxidation products and oligomers were analysed.

MATERIALS AND METHODS

Materials

Stigmasterol (≥95%), oleic acid (≥99%), linoleic acid (≥99%), linolenic acid (≥99%), 5α-cholestane (>97%), standards of cholesteryl oleate (≥98%), linoleate (≥98%), linolenate (≥97%), acetone (≥99.9%), dichloromethane (≥99.8%), n-hexane (≥97%), pyridine (≥99.8%), tetrahydrofuran (≥99.9%) with 250 ppm BHT as an inhibitor, *tetr*-butyl methyl ether (≥99.8%), toluene (≥99.8%), sodium hydroxide, silica gel (70–230 mesh, high purity), sodium methylate, the catalysts-dicyclohexylcarbodimide (DCC), 4-dimethylaminopyridine (DMAP), MTT, SDS, vincristine sulfate were purchased from Sigma-Aldrich (St. Luis, MO, USA). A silylation mixture of BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) was bought from Fluka Chemie, while the SEP-PAK amino cartridges were sourced from Waters (Milford, USA).

Cell lines

Two acute lymphoblastic leukemia cell lines were used as an experimental model: CCRF-CEM (ATCC®: CCL-119™) and its multidrug resistant counterpart CCRF-VCR1000 cell line, which was obtained using the procedure described previously (Paszel et al., 2011). Cells were grown in RPIM-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland) at 37°C in a humidified atmosphere of 5% CO_2.

Esterification

Stigmasteryl oleate, linoleate, and linolenate were obtained by chemical esterification based on the Neises and Steglich (1978) method, described in detail by Raczyk et al. (2017). The purity of the esters was evaluated by ^{1}H-NMR and GC-MS (Raczyk et al., 2017). 500 mg of the esters were heated at 180°C in 200 mL glass ampoules. The ampoules were filled with 100 mL of oxygen and closed. The samples were heated in an oven for 1, 2, 4, 8 and 12 hours. Unheated samples of the esters were also analysed in the study.

Stigmasterol oxidation products

The sum of stigmasterol oxides was quantified after transesterification, followed by SPE fractionation, GC-MS and GC-FID identification and separation, according to the procedures described by Rudzińska et al. (2014) and Raczyk et al. (2017). The oxidative derivatives were identified on a 7890A GC system (Agilent Technologies) coupled to a 5975C VL Triple-Axis mass detector (Agilent Technologies) with a DB-5MS column and quantified on a Hewlett-Packard 6890 gas chromatograph equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 mm; J&W Scientific). Samples were injected in split less mode and the column temperature was programmed as following: the initial temperature of 160°C was held for 1 min, then programmed at 40°C/min to reach 270°C, where it was held for 1 min; it was further programmed at 4°C/min to 280°C, with this final temperature being held for 25 min. Hydrogen carrier gas at a flow rate of 1 mL/min was used. The detector and injector temperatures were 300°C. The peaks of oxidized stigmasterol were identified and calculated as a sum of the oxidation products.

Polar and non-polar fraction

The separation into polar and non-polar fraction was done according to the method DGF Standard Method C-III 3e. Two fractions were collected as described by Raczyk et al. (2017).

Dimers, trimers, oligomers

Non-polar fraction of stigmasteryl esters before and after heating were dissolved in tetrahydrofuran, then

to separate and quantify monomeric, dimeric, trimeric and oligomeric fractions of the samples gel permeation chromatography in combination with refractive index detection was applied. The chromatograph was a Merck LaChrom L-7490 equipped with a L-6000A pump (Agilent Technologies, Waldbronn, Germany). The mobile phase was tetrahydrofuran with 250 ppm BHT. Separation was performed using one PL Gel 500 Å and one 100 Å column and pre-column PL Gel 5 µm at 35°C. The flow rate of the mobile phase was 0.6 mL/min, and the injection volume of the sample solution was 20 µL. Separation was performed according to Lampi et al. (2009).

MTT assay

The cytotoxic effect of the unheated stigmasteryl esters and the mixture of compounds after heating on the leukemic cells was determined using an MTT assay, which is a colorimetric test based on the reaction catalysed by the intracellular mitochondrial enzymes. Yellow tetrazolium salt MTT is reduced to the blue formazan crystals in the healthy, living cells. The intensity of the blue colour in the probe is directly proportional to the number of living cells. CCRF-CEM and CCRF-VCR1000 cells (1×10^5 cells/mL) were treated for 24, 48 and 74 hours with the compounds being tested and the mixtures of the products formed as a result of heating during for a period of 1, 2, 4, 8, 12 hours, which were dissolved in acetone and applied to the cells in the final concentrations. After the incubation, the absorbances of samples were measured using a microplate reader Multiscan FC (Thermo Scientific, Vantaa, Finland) at wavelength 570 nm with a reference wavelength 690 nm. Based on the data obtained using MTT test IC_{50} the values were calculated with the CalcuSyn software (Biosoft, Cambridge, UK). The IC_{50} factor is the concentration of the compounds which reduce the cell viability to 50%.

Statistics

All results presented in this paper represent the mean of at least two experiments ±standard deviation. All analyses were done at least in three repetitions for each sample. The differences between mean values were determined by analysis of variance (ANOVA). The post-hoc analysis was performed using Tukey's test. Differences at $P < 0.05$ were considered significant.

The statistical analyses were done using the Statistica 10.0 software (StatSoft, Inc., Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

The present study focuses on the evaluation of the three stigmasteryl esters (stigmasteryl oleate, linoleate and linolenate) activities in human acute lymphoblastic leukemia CCRF-CEM (wild type) cells and vincristine-resistant acute lymphoblastic leukemia CCRF-VCR1000 cells. The resistant subline was obtained during the long-term process of wild type CCRF-CEM cell culture in the presence of growing concentrations of the chemotherapeutic drug (vincristine). This process conducted *in vitro* under laboratory conditions mimics the development of drug resistance in the neoplastic cell population, which may occur *in vivo* as a result of cancer patients' chemotherapy. The anticancer drug insensitivity of CCRF-VCR1000 cells is related to the *ABCB1* gene overexpression (Paszel et al., 2011), which is directly related to the high level of ABCB1 protein in the cell membranes. ABCB1 is an ATP-dependent transporter protein, which is able to pump a large group of structurally unrelated drugs out of the cells. Therefore they cannot reach their molecular target and induce a therapeutic effect (Leschziner et al., 2007). Multidrug resistance phenomenon is the main cause of chemotherapy treatment failure and a serious problem for oncologists (Bontemps-Gracz et al., 2002), thus efficient ABCB1 inhibitors or cytotoxic compounds which are not the substrate of ABCB1 protein are strongly needed. Scientists instigate high numbers of molecules (natural, synthetic or semi-synthetic compounds) in their search for the most potent agents. Phytosterols interact with certain ABC proteins, as they are transported in the gut via a complex of ABCG5-ABCG8 or ABCA1 proteins (Raju, 2013). There is some evidence that plant sterols could be a promising group of compounds with potential multidrug resistance ability (El-Readi et al., 2010; Nabekura et al., 2008). This study is a continuation of studies focused on identifying stigmasteryl ester degradation products presented previously by Raczyk et al. (2017). In that study, the identification of esterified stigmasteryl esters, their degradation during heating, POP and polar compounds were analysed. In this publication, the results of the *in vitro* MTT test determining

leukemic cells' viability reduction ability of stigmasteryl esters (oleate, linoleate and linolenate) and mixtures of their thermo-oxidative degradation products were presented. The time and dose effect relationship was observed and the strongest cytotoxic effect was seen after 72 hours of cell treatment (Table 1). It should be emphasised that among the three pure, unheated esters two of them (stigmasteryl linoleate and stigmasteryl linolenate) showed significant potency against both cell lines after 72 hours of treatment, whereas the cell viability reduction activity of stigmasteryl oleate in the designed concentration range was so low that it was not possible to determine the IC_{50} value and the viability of cells treated with the substance for 72 h was around 70% (Table 1). From the two active compounds stigmasteryl linoleate was more potent, which is confirmed by lower IC_{50} values calculated for CCRF-CEM and CCRF-VCR1000 cells (79.0 ±2.1 and 41.3 ±1.2 μg mL^{-1}, respectively) compared to IC_{50} values calculated for stigmasteryl linolenate. Stigmasteryl linolenate has an approximately two times weaker effect on CCRF-VCR1000 cells and an approximately 20% weaker effect on CCRF-CEM cells than stigmasterol linoleate. Interestingly, the IC_{50} values obtained for both compounds were significantly lower in drug resistant cells than in wild type cells. In CCRF-VCR1000 treated with stigmasteryl linoleate the IC_{50} was around two times lower than the IC_{50} obtained for the wild type cells. This phenomenon should undoubtedly be interpreted as a beneficial effect. It is possible that both compounds are not a substrate of the ABCB1 protein, so they are not removed from the cells to the extracellular environment or are able to modulate the protein function or to reduce its level.

Analysis of the mixture activity of stigmasteryl esters after heating at 180°C yielded interesting data and some tendencies were observed. In general, heating increased the potency of compounds regarding cell population reduction, the formation of phytosterol oxidation products (Fig. 1) and non-polar oligomers (Table 2) in a time-dependent manner. The most pronounced effect on CCRF-CEM cells was seen for

Table 1. The half maximal inhibitory concentration (IC_{50}) of stigmasteryl esters heated at 180°C tested on lymphoblastic leukemia cell line CCRF-CEM and its multidrug resistant counterpart CCRF-VCR1000, μg mL^{-1}

Cells treated with IC_{50} for 72 h	Heating time, h					
	0	1	2	4	8	12
			Stigmasteryl oleate			
CCRF-CEM	nd	nd	nd	33.2 ±0.0	18.7 ±1.8(**)◦	14.3 ±1.9(**)◦
CCRF-VCR1000	nd	nd	nd	45.6 ±0.0(*)#	28 ±2.0(*)# (**)◦	nd
			Stigmasteryl linoleate			
CCRF-CEM	79.0 ±2.1	74.0 ±1.3(*)•	127.3 ±1.0(**)•	190.5 ±0.8(**)•	265.5 ±0.9(***)•	293 ±0.9(***)•
CCRF-VCR1000	41.3 ±1.2(**)#	38.0 ±1.6(**)# (*)•	66.8 ±1.7(**)# (**)•	83.3 ±1.4(**)# (**)•	96.3 ±1.4(**)# (**)•	106.8 ±1.1(**)# (***)•
			Stigmasteryl linolenate			
CCRF-CEM	102.3 ±0.9	81.6 ±2.0(**)•	85.8 ±1.5(**)•	48.0 ±2.2(***)•	92.8 ±1.6(**)•	144.4 ±1.7(***)•
CCRF-VCR1000	94.2 ±1.0(*)#	55.6 ±1.1(**)# (**)•	62.9 ±0.9(**)# (**)•	50.0 ±0.8(***)# (***)•	133.5 ±0.7(**)# (***)•	313 ±0.8(***)# (***)•

The table shows mean values ±SD of five independent experiments performed in triplicate. Statistical analysis was carried out by ANOVA followed by post-hoc Tukey test.
nd – not detectable in tested concentrations.
#Comparison to the CCRF-CEM cells treated with the same compound/mixture. •Comparison to the cells treated with pure compound. ◦Comparison to the cells treated with the mixture of degradation products after 4 h of pure compound heating.

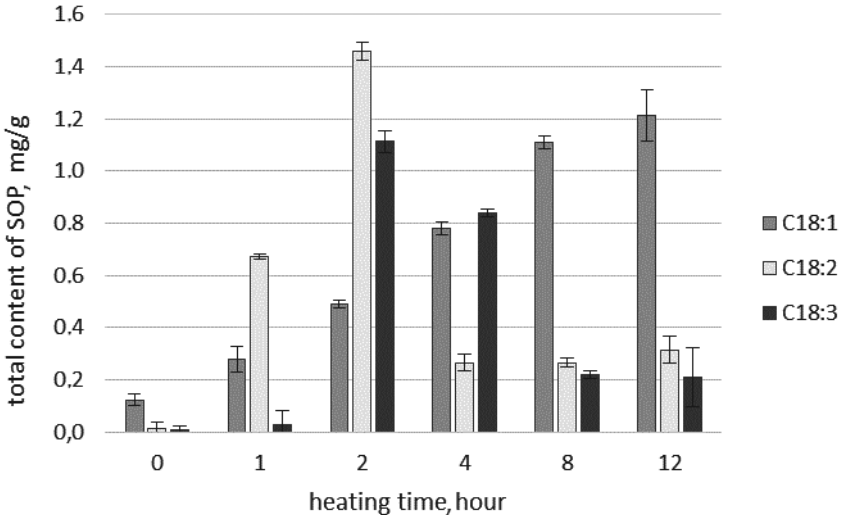

Fig. 1. The total amount of stigmasterol oxidation products (SOP) after heating at 180°C: C18:1 – stigmasteryl oleate, C18:2 – stigmasteryl linoleate, C18:3 – stigmasteryl linolenate

Table 2. Dimers, trimers and oligomers in non-polar fraction of stigmasteryl esters after heating at 180°C, %

Oligomers	Heating time, h					
	0	1	2	4	8	12
	Stigmasteryl oleate					
Dimers	0.11 ±0.01^{a}	0.24 ±0.03^{b}	0.21 ±0.01^{b}	0.32 ±0.0^{c}	0.83 ±0.03^{c}	0.45 ±0.04^{d}
Trimers	0.11 ±0.01^{a}	0.21 ±0.05^{b}	0.18 ±0.02^{ab}	1.33 ±0.02^{d}	0.96 ±0.02^{c}	0.90 ±0.02^{c}
Oligomers	0.08 ±0.02^{a}	1.41 ±0.02^{d}	1.17 ±0.01^{c}	2.98 ±0.01^{e}	3.64 ±0.04^{f}	0.75 ±0.01^{b}
	Stigmasteryl linoleate					
Dimers	0.08 ±0.06^{b}	1.69 ±0.02^{e}	1.36 ±0.02^{d}	0.77 ±0.04^{a}	0.83 ±0.03^{a}	0.63 ±0.04^{c}
Trimers	0.06 ±0.02^{b}	0.45 ±0.03^{c}	1.33 ±0.02^{a}	1.31 ±0.0^{a}	1.54 ±0.03^{e}	1.10 ±0.02^{d}
Oligomers	0.04 ±0.02^{a}	3.45 ±0.03^{d}	3.65 ±0.03^{e}	2.44 ±0.04^{b}	4.86 ±0.03^{f}	2.84 ±0.05^{c}
	Stigmasteryl linolenate					
Dimers	0.05 ±0.02^{b}	0.84 ±0.02^{c}	1.52 ±0.02^{d}	4.12 ±0.02^{a}	4.10 ±0.02^{a}	4.56 ±0.04^{e}
Trimers	0.10 ±0.05^{a}	2.36 ±0.03^{d}	1.21 ±0.02^{b}	3.21 ±0.02^{e}	3.70 ±0.05^{f}	1.73 ±0.02^{c}
Oligomers	0.04 ±0.02^{a}	0.21 ±0.03^{b}	0.72 ±0.03^{c}	2.18 ±0.02^{d}	2.60 ±0.04^{e}	0.03 ±0.02^{a}

The results present the average of duplicate analyses.
Values (means ±SD) bearing different superscripts are statistically significantly different ($P < 0.05$).

stigmasteryl oleate heated for 4 h and longer. In this case, multiple drug resistance cells were less sensitive to the oxidised compound mixture than the wild type ones. As reported by Raczyk et al. (2017), the main degradation product of stigmasteryl oleate was alpha-epoxystigmasterol. It is likely that this compound exerts a significant influence on the mixture potency, because the two other tested mixtures did not contain alpha-epoxystigmasterol and were found to be less active (Table 1). The biggest amount of stigmasteryl linoleate oxidation products was formed after 1 and 2 hours and for stigmasteryl linolenate after 2 and 4 hours of heating at 180°C (Fig. 1). Those results relate with the half maximal inhibitory concentration, which was the lowest for stigmasteryl esters heated for 1 and 2 hours. Heating the compounds for 1 hour caused their degradation and the mixture of the degradation products showed a slightly stronger cytotoxic potential to the cells of both lines compared to the pure compound. Interestingly, extending the heating time of stigmasteryl linoleate reduced the cytotoxicity of the mixture, which may be related to the significant increase in non-polar dimers and oligomers (Table 2), which are poorly-soluble in acetone. The IC_{50} values calculated for the samples treated with a mixture of oxidation products (heated for 2–12 hours) are higher than those obtained for the samples treated with a compound heated for 1 hour and increased directly proportional to the extension of heating time. Moreover, a different effect after variable heating times of stigmasteryl linolenate was noticed. The enhancement of the cytotoxic activity of the thermo-oxidative degradation products of the ester was observed for the mixtures obtained as a result of heating for 1, 2 and 4 hours, while a further extension of the heating time (8 and 12 hours) again reduced the cytotoxic potential of the degradation product mixture (Table 1). Interpreting the influence of bioactive compound mixtures on the living cells is difficult and complex. Chemical compounds applied to the cells in a combination interact and may exert synergistic or additive interaction. Furthermore, inhibition of one compound by another can be observed.

CONCLUSIONS

A time and dose effect correlation was observed. The strongest cytotoxic effect was seen after 72 hours of cell treatment. It was noted that among the three tested stigmasteryl esters the strongest cytotoxic effect was caused by stigmasteryl linoleate. Heating the ester for 1 hour caused its degradation and the mixture of degradation products showed a stronger cytotoxic potential to the cells of both lines compared to the pure compounds. Interestingly, extending the heating time of stigmasteryl linoleate reduced the cytotoxicity of the mixture, which is likely to have been related to the formation of non-polar dimers and oligomers, which are poorly soluble. The IC_{50} values calculated for the probes treated with the mixture of oxidation products (heated for 2–12 hours) are higher than those obtained for the probe treated with a compound heated for 1 hour and also increased directly proportional to the extension of the heating time.

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