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Co-Extraction of Policosanols and Phytosterols from *Sorghum bicolor* subsp. *bicolor*: A Mild Approach Unveiling New Bioactive Molecules

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Abstract

Phytochemicals have recently gained considerable attention for their therapeutic and nutraceutical potential. Particularly, policosanols and phytosterols have shown promising lipid-lowering effects through distinct mechanisms. Therefore, the combination of these two compound classes should offer synergistic benefits, enhancing cholesterol reduction. Despite various protocols having been developed for extracting these compounds from plant matrices, challenges remain regarding yields, high purity, non-toxicity and general biocompatibility of extracts. Tackling these aspects, this study provides an efficient co-extraction and purification method for policosanols and phytosterols from *Sorghum bicolor* subsp. *bicolor*, a plant rich in both such compounds. The newly developed protocol involved crude lipid extraction, saponification, column chromatographic purification and compound identification using gas chromatography coupled with mass spectrometry (GC/MS). High yields for both policosanols and phytosterols were obtained with fractions pure and rich in a wide variety of compounds of both classes, some of which have never been described before for the species. Moreover, analyses revealed, for the first time, the presence of a variety of terpenes. The biocompatibility of the extracts has been evaluated as well, through MTT-based in vitro assays. The novel, promising approach would allow us to obtain compound-rich and safe extracts, suitable for nutraceutical applications.

Keywords: *Sorghum bicolor*; extraction; policosanols; phytosterols; terpenes; toxicology assays

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1. Introduction

The use of phytochemicals in medicine and nutraceutical sciences has received significant attention, becoming increasingly popular for treating various diseases and pathologies [1]. Phytochemicals are currently defined as biologically active organic substances found in plants, used for their recognized health-promoting properties and potential therapeutic applications [2,3]. Phytochemicals encompass a broad spectrum of structurally diverse compounds, primarily polyphenols, carotenoids, alkaloids, glycosylates, policosanols, and phytosterols [4]. Specifically, both policosanols (**PCs**) and phytosterols (**PSs**)

are currently considered as plant-based bioactive compounds exhibiting significant potential health benefits, obtanied by modulating lipid metabolism and supporting cardiovascular health through different and complementary mechanisms of action [5].

PSs are essential components of the lipid bilayer of cell membranes [6], consisting of 28 or 29 carbon atoms in the main structure. Despite some differences in the composition of the side chain, they are similar to cholesterol both structurally (exhibiting a four-ring steroid nucleus, a 3β-hydroxyl group and often a 5,6-double bond) and functionally (phospholipid bilayers stabilization in cell membranes, according to Uddin [7].

PCs, instead, are a group of long-chain monohydric primary alcohols varying from 20 to 36 carbon atoms, extracted mainly from plant waxes [8]. Some recent studies have described an enhanced lipid-lowering efficacy when combining **PCs** and **PSs**. This effect is supposedly related to their different but synergistic mechanisms of action [9]. Indeed, **PSs** act on dietary cholesterol competitively, inhibiting its absorption in the intestine and leading to reduced LDL cholesterol levels in the bloodstream [10]. **PCs**, instead, inhibit hepatic cholesterol synthesis, enhancing LDL receptor activity and reducing platelet aggregation [11,12]. Moreover, the latter are also supposed to decrease the absorption of dietary cholesterol [13]. The combination of **PCs** and **PSs** thus seem to lower both endogenous and exogenous cholesterol levels in the bloodstream. Therefore, obtaining combined extracts of **PCs** and **PSs** represents a particularly promising approach to increase and facilitate their use as nutraceuticals.

The co-extraction of **PCs** and **PSs** applying a single ad hoc procedure and stating from a biomatrix particularly rich in both represents the most efficient and least time-consuming way to obtain extracts highly concentrated in **PCs** and **PSs** [14].

Several extraction techniques have been extensively documented for each of the two classes of compounds. The focus has been on the yield of the obtained extracts, considering the low bioavailability of a great part of them [15], as well as on their purity and composition. Only limited information is available, on the contrary, on the biocompatibility of **PC** and **PS** extracts, which could potentially hinder the possibility of testing and using them for nutraceutical applications [16].

For both **PCs** and **PSs**, solvent extraction resulted to be the most effective method, despite the conflicting opinions regarding the number of extracted compounds and the obtained yields [7,16–21]. The solvents most often used for the extraction of PSs are n-hexane (HX), petroleum ether (PE), ethanol (EtOH) and dichloromethane (DCM). For **PCs**, instead, the most commonly used solvents include methanol (MeOH) and ethanol (EtOH) [20,22]. Soxhlet extraction is generally the method of choice [23–26]. After extraction, different saponification and chromatographic techniques are usually utilized for purification and/or fractioning, with specific protocols for each class of compounds [27–29] choosing solvents with respect to regulatory aspects [30].

Consdiering the biomatrices used for **PCs** and **PSs** extractions, plant sources rich in saccharides such as sugarcane, wheat germ, rice bran and other cereals are mainly used for isolating **PCs**. **PSs**, instead, are particularly abundant in vegetable oils, nuts and whole grains [31,32]. Among the species containing the two above mentioned classes of compounds, *Sorghum bicolor* (L.) Moench subsp. *bicolor* and, specifically, the agricultural sorghum grain type (hereafter *S. bicolor*) resulted to be particularly rich in both [33–35]. It is a drought-tolerant cereal initially grown primarily in the United States, where it was used for livestock feed as well as for ethanol and flour production for human consumption [36,37]. Currently, sorghum grains are a dietary staple in the Americas, Asia, Australia and Africa, and sorghum ranks as the fifth most cultivated cereal globally [38]. It is one of the major gluten-free cereal grains [39] and stands out from other major cereal grains for the high content of different bioactive compounds [40–42] alongside its essential components,

such as starch, fat, proteins and non-starch polysaccharides. *S. bicolor* contains, among others, vitamin E, carotenoids and phenolic compounds [43,44], as well as policosanols and phytosterols [33–35]. Yet, no efficient co-extraction protocols are currently available in the literature to obtain enriched extracts of policosanols and phytosterols from *S. bicolor*. A summary and comparison of the already described extraction procedures for sorghum and other relevant biomasses containing policosanols and phytosterols are reported in Table S1 (Supplementary Materials).

In this work, an ad hoc co-extraction and purification protocol is proposed to obtain high-yield extracts enriched in both phytosterols and policosanols from *S. bicolor*. Following the devised work-flow reported in Figure 1, crude lipid extracts were obtained. The unsaponifiable fraction was then purified, and the obtained extracts were characterized using gas chromatography coupled with mass spectrometry (GC/MS), to evaluate both qualitatively and quantitatively the content of compounds of interest. An MTT-based in vitro cell viability assay was carried out to verify the biocompatibility of the extracts.

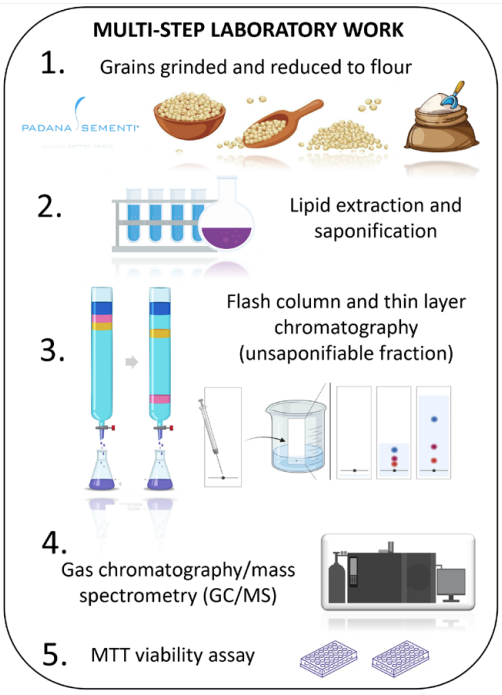

Figure 1. Schematic representation of the multi-step procedure tested during the study.

2. Results and Discussion

2.1. Extraction Yields

S. bicolor grains were extracted according to the multi-step procedure described in detail in the Materials and Methods section (for details please see Figure 1 and Section 3). Specifically, total lipids were intially obtained by crude lipid extraction. Then, saponification and purification by flash column chromatography were performed, starting from crude lipid extracts, to obtain policosanol (**PC**) and phytosterol (**PS**) fractions.

The lipid extraction yielded an amount of crude lipids in the range of 50 mg/g with respect to dry biomass, corresponding to 5.5% to 6.2% (*w*/*w*). The amount of policosanol (**PCs**) and phytosterols (**PSs**) obtained after saponification and purification from the crude lipid extracts ranged from approx. 11 mg/g to 13 mg/g and 12 mg/g and 18 mg/g of initial dried material, respectively. These yields obtained for both **PCs** and **PSs** are among the highest ever reported for *S. bicolor* [18,31,45–48] (Table S2, Supplementary Materials). Interestingly, de Morais Cardoso [49] reported for *S. bicolor* a maximum value of about 0.74 mg/g of initial dry mass material for policosanols and of 0.03 mg/g of initial dry mass material for phytosterols.

Moreover, Mohamed [50] in a more recent review, reported lower amounts obtained by Soxhlet extraction. Higher amounts are generally obtained for the kernels, as indicated by Leguizamón [18] and Hwang [31], reporting mean yields of 0.25 mg/g of aliphatic long chain alcohols and 0.75 mg/g of sterols (Table S1, Supplementary Materials).

The difference in yields can partially be attributed to obvious natural and cultivation-related factors, including plant variety, the cultivation modes, the places of growth and the development and harvest time [29,49,51]. Moreover, differences in extraction protocols are also known to affect the relative yields [51–53]. In this light, the choice of solvent is crucial for the selective extraction of compounds [45]. Lipid solvent extraction with $CHCl_3$ and MeOH has been described as an efficient procedure for extracting **PCs** and **PSs** from other cereals [29,54] (Table S1). Harrabi [29] obtained for **PCs** extracted from maize/corn a yield of 0.11 mg/g, which is very high when compared to what has been obtained using other solvents and protocols (Table S2, Supplementary Materials). The yields obtained in this study are thus not surprising as such, but are actually in line with a recent report that claims a rather high amount of **PCs** and **PSs** in *S. bicolor* [55]. The higher yields obtained in this work can be at least partially explained considering the longer extraction times applied in this procedure. Indeed, it has already been extensively stated that longer extraction times could increase the efficiency of the extraction process [54]. Another important contrast to Harrabi [29], who crushed kernels directly in a mortar with the extraction mixture and then immediately centrifugated them, lies in the fact that in this study the kernels were reduced to flour and then extracted for 3 h under constant agitation. A third factor that could have influenced the amounts of extractable **PCs** and **PSs** is the storage and pre-treatment procedures applied on the biomass. In this study, the whole sorghum grain kernels were dried at 40 °C for 72 h to reach a constant weight. Considering that wet milling was shown to cause a significant reduction in the extraction yields of several bioactive compounds in cereals [40,56], the drying pre-treatment procedure of kernels could have played an important role in the increase in final yields. Moreover, also achieving the denaturation of phospholipases, by simply keeping the samples in a container in a water bath, rather than dissolving them in subsequently boiled water, could have contributed in achieving high final yields [57].

As a note of caution, however, the saponification following the ad hoc extraction protocol by Harrabi [29] needs to be considered. It acts on wax residuals still present in the whole lipid extracts, and hence also on the waxes (co-)extracted from of *S. bicolor*, which are quite abundant on the kernel surface [58]. As such, this protocol could lead to an increase in the amounts of **PCs** and **PSs**, but also to the presence of fatty acids (**FAs**) in the extracts. However, according to the extract characterization carried out by GC/MS, as reported in Section 2.2, fatty acids (**FAs**) were present only in rather low amounts, i.e., 5.1% and 5.3%, respectively, in the **PC** and **PS** extracts, thus suggesting that the above-mentioned transformation processes related to the saponification step did not significantly influence the final yield of policosanols and phytosterols.

Therefore, the overall procedure applied herein, i.e., the storage, pre-treatment and extraction, resulted to be very effective for the co-extraction of both **PCs** and **PSs** in *S. bicolor* using a $CHCl_3$-MeOH mixture (Table S1, Supplementary Materials) [50]. This is particularly interesting given that the developed procedure is based on solvent extraction, currently considered a low yielding method in comparison with supercritical fluid extraction (SFE) with CO_2 [14,59–61].

2.2. GC/MS Extract Characterization

Particularly interesting results were obtained for the generated **PCs** and **PSs** extracts also when analyzing the composition of policosanol and phytosterol contents in the ex-

tracts by GC/MS. Amongst the studies reported in Table S2 (Supplementary Materials), only Tuhanioglu [48] used GC/MS for the qualitative description of *S. bicolor* extractives, evidencing the presence of a quite low number of compounds for both policosanols (**PCs**, **4**) and phytosterols (**PSs**, **3**).

The total ion chromatogram (TIC) of policosanols enriched fraction (**PCEF**) and sterols enriched fraction (**PSEF**) are shown in Figure 2a,b. Both chromatograms displayed numerous and rather intense peaks; smaller and not assigned ones can be mainly ascribed to alkanes, and alkenes also detected in the blank fraction (Figure S1, Supplementary Materials). The blank fraction was isolated as a fraction of extractives that neither contained policosanols nor phytosterols.

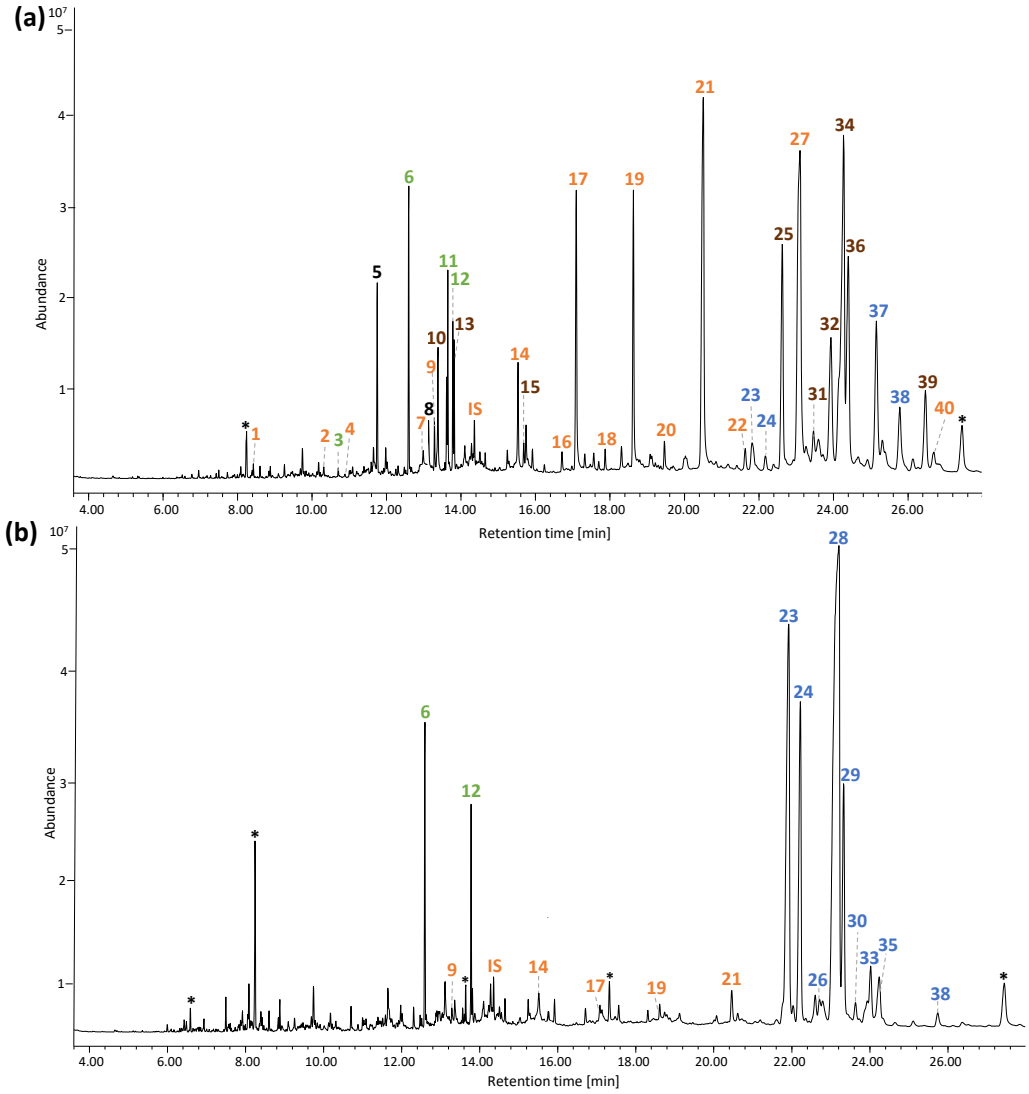

Figure 2. TIC-GC/MS chromatogram of *S. bicolor* extracts relative to the two isolated and purified fractions: (**a**) **PCEF** and (**b**) **PSEF**. Peaks of identified compounds are numbered according to Table 1. Orange = policosanols; green = fatty acids; brown = terpenes; blue = phytosterols; black = other compounds. * = contaminants; other no-labeled peaks are mainly ascribable to alkenes present in blank fraction.

Table 1 reports the list of all compounds identified in the chromatograms, the richness of which, in terms of variety of compounds in the extracts, clearly appears.

Table 1. Identified compounds (listed based on retention order) in the GC/MS chromatograms of *S. bicolor* extracts relative to the two isolated and purified fractions, i.e., **PCEF** and **PSEF**. The category of the identified molecules is reported. A semi-quantitative analysis was performed and the percentages relative to the sum of the area of all the compounds belonging to each category are reported.

Entry	t_{ret} (min)	Compound	PCEF	PSEF
1	8.7	1-Dodecanol (TMS)	X	
2	10.3	1-Tetradecanol (TMS)	X	
4	11.2	1-Pentadecanol (TMS)	X	
7	12.7	1-Heptadecanol (TMS)	X	
9	13.3	1-Octadecanol (TMS)	X	X
IS	14.3	1-Eicosanol (TMS)	X	X
14	15.5	1-Docosanol (TMS) §	X	X
16	16.2	1-Tricosanol (TMS)	X	
17	17.1	1-Tetracosanol (TMS)	X	X
18	17.8	1-Pentacosanol (TMS) §	X	
19	18.6	1-Hexacosanol (TMS)	X	X
20	19.5	1-Heptacosaol (TMS) §	X	
21	20.5	1-Octacosanol (TMS)	X	X
22	21.6	1-Nonacosanol (TMS) §	X	
27	23.0	1-Triacontanol (TMS)	X	
40	26.7	1-Dotriacotanol (TMS) §	X	
% Policosanols			**42.6**	**2.6**
23	22.1	Campesterol (TMS)	X	X
24	22.4	Stigmasterol (TMS)	X	X
25	22.6	Glutinol *		
26	22.7	Ergost-7-en-3β-ol (TMS)		X
28	23.2	β-Sitosterol (TMS)		X
29	23.3	Fucosterol (TMS) §		X
30	23.5	Isofucosterol (TMS)		X
33	24.0	5α-Stigmast-7-en-3β-ol (TMS)		X
35	24.2	[(3β)-stigmasta-7,24(28)-dien-3-yl]oxy] (TMS)		X
37	25.1	9,19-Cyclo-3β-lanostan-3-ol, 24-methylene (TMS)	X	
38	25.8	Citrostadienol (TMS)	X	X
% Phytosterols			**15.4**	**92.1**
10	13.4	Phytol (TMS)	X	
13	13.9	Farnesol (TMS)	X	
15	15.7	Nerol (TMS)	X	
31	23.6	β-Amyrin (TMS)	X	
32	23.9	Epilupeol (TMS)	X	
34	24.1	Lupeol (TMS)	X	
36	24.4	Lupeol *	X	
39	26.4	Friedooleanan-3α-ol *	X	
% Terpenes			**34.7**	**-**
3	11.1	Myristic acid (TMS)	X	
6	12.6	Palmitic acid (TMS)	X	X
11	13.7	Oleic acid (TMS)	X	
12	13.8	Stearic acid (TMS)	X	X
% Fatty acids			**5.1**	**5.3**
5	11.7	Methyl palmitate	X	
8	13.1	Methyl stearate	X	
% Esters			**2.4**	**-**

* These compounds were not found in the respective derivatized form possibly due to their lower stability with respect to the underivatized. § Indicate the newly described compounds for the species.

The semi-quantitative analysis was performed to hightlight the main trends among the identified classes of compounds, whereas the determination of their absolute cocentration was beyond the scope of this study. In this approach, peak areas were used for relative comparison without applying compound-specific calibration. Since MS detection is characterized by compound-dependent response factors, arising from differences in ionization efficiency, fragmentation patterns, and detector response, peak areas cannot be easily correlated with actual concentrations. Accordingly, the percentages relative to the sum of the area of all the compounds belonging to each category are reported in Table 1. Thus,

comparing the obtained results with those of other studies using different techniques to analyze the composition of **PCs** and **PSs** extracts [18,46], e.g., mainly GC-FID, some relevant differnces can be observed, especially for phytosterols such as campesterol, stigmastyerol and β-sitosterol, that were commonly detected only at trace levels.

From a qualitative point of view, the chromatogram of **PCEF** (Figure 2a) was characterized by four main classes of molecules: fatty acids (**FAs**) (11–14 min) and related esters due to transesterification, various aliphatic alcohols (**AAs**), distributed over the entire time interval, terpenes (**Ts**) (10–25 min), and phytosterols (**PSs**) (22–26 min). Aliphatic alcohols, especially in the form of policosanols (**PCs**) (C24-C34) dominated the profile, showing the entire series from C20 to C32 and accounting for 42.6% of the total detected compounds. In particular, 1-tetracosanol (**17**), 1-hexacosanol (**19**), 1-octacosanol (**21**) and 1-triacontanol (**27**) resulted in being the most abundant. Nevertheless, the presence of other **PCs** was also detected (compounds **14**, **16**, **18**, **20**, **22**, and **40**). At higher retention times, rather intense peaks ascribed to a great variety of **Ts** have been identified, ranging from monoterpenes (nerol (**15**)), diterpenes (phytol (**10**)), sesquiterpenes (farnesol (**13**)) and the widest group of triterpenoids (glutinol (**25**), β-amyrin (**31**), epilupeol (**32**), lupeol (**34**, **36**), friedooleanan-3α-ol (**39**)) for a total of 34.7%. Sorghum lipid content detected in this fraction was principally constituted by fatty acids, such as palmitic (**6**), oleic (**11**) and stearic (**12**) acid. Phytosterols, instead, were present only in low quantities, i.e., 15.4% (Table 1).

With respect to the GC/MS profile of **PSEF** (Figure 2b), the first part, visible between 13 min and 21 min, still displayed small quantities of some policosanols already detected in the **PCEF**, while at higher retention times, between 22 min and 26 min, **PSs** were featured in rather intense concentrations, accounting for 92.1%. Amongst them, campesterol (**23**), stigmasterol (**24**), β-sitosterol (**28**), and fucosterol (**29**) were the most abundant.

Both the main extract fractions **PCEF** and **PSEF** obtained upon fractioning and purifying the unsaponifiable part of the crude lipid extract produced from *S. bicolor* seeds appeared to be particularly rich in terms of bioactive compounds, with 40 different molecules detected in them, out of which 27, i.e., more than 67%, were **PCs** and **PSs**. Also these results indicate the good performance of the extraction protocol developed in this study.

Notably, **PCEF** is effectively dominated by **PCs**. The entire series from C20 to C32 is present, with 15 different compounds detected overall, some of which are not yet specifically described for *S. bicolor*. Indeed, the most abundant compounds reported in **PCEF**, i.e., 1-tetracosanol (**17**), 1-hexacosanol (**19**), 1-octacosanol (**21**) and 1-triacontanol (**27**) had already been reported for this cereal [18], while for 1-docosanol (**14**), 1-pentacosanol (**4**), 1-heptacosanol (**7**), 1-nonocosanol (**22**), and 1-dotriacotanol (**40**), no documented presence in a sorghum grain matrix exists.

Also, the composition of the **PSEF** revealed some interesting results. As already stated, the matrix appeared to be richer, in terms of different compounds, than those described in the literature for *S. bicolor* (e.g., [18,49,62,63]). Besides some quite common **PSs**, such as campesterol (**23**), stigmasterol (**24**), and β-sitosterol (**28**), the effects of which for cholesterol control have already been described (e.g., [64,65]), some less common sterols were also detected. Among them, fucosterol (**29**) appears to be of particular interest. It is a phytosterol found primarily in various marine algae, particularly in brown seaweeds [66,67]. Even though it has already been described for different cereals of the sorghum genus (e.g., [68–71]), it has never been detected in larger amounts in *S. bicolor* grains.

Interestingly, in both **PCEF** and **PSEF**, terpenes (**Ts**) also appeared to be quite abundant, forming a surprisingly rich matrix. While Agustina [72] had already included **Ts** in the main groups of secondary metabolites present in *S. bicolor* seeds, a detailed characterization, such as that presented in Table 1, has not been reported so far. Several **Ts** are already known for their aromatic properties and potential health benefits [73–75]. In this regard, recent

studies have suggested that some **Ts**, such as nerol, may play a role in reducing cholesterol levels and improving lipid profiles [76–78]. Therefore, focusing on cholesterol lowering, the presence of nerol (**15**) in the obtained extracts appeared as particularly interesting and promising [79,80].

2.3. Effect of *PCEF* and *PSEF* Administration on Cell Viability

The toxicity of plant extracts enriched in bioactive compounds is a crucial point to consider with respect to their use in the nutraceutical field. Another aspect to be taken into account is solvent-based extraction [81–83]. As noted by Sulaiman [84], the testability and bioactivity of a potential nutraceutical are mainly determined by the solvents used during the extraction process [85,86]. The ad hoc procedure defined in this study employs various solvents that are classified as toxic, such as methanol, chloroform, and n-hexane. Yet, the protocol applied for solvent removal, i.e., automated distillation using a rotary evaporator, was efficient enough to remove solvents essentially quantitatively. A cell viability assay was performed administrating increasing concentrations between 25 and 250 µg/mL of **PCEF** and **PSEF** d for 48 h to Caco2/HT29 co-cultured cells, used as an in vitro model usually applied to resemble the gut barrier. In parallel, a group of cells was treated with ezetimibe (**EZT**), which influenced the cholesterol uptake through the inhibition of Niemann-Pick C1-Like 1 (NPC1L1) transporter. Ezetimibe is the only safe and well-tolerated currently approved NPC1L1 inhibitor for the treatment of hypercholesterolemia and it is usually used as a positive control for other potential inhibitors screening. Compared to **EZT**, which reduces the cell viability by 20%, none of the treatments with sorghum grain lipid extracts influenced the Caco2/HT29 viability, indicating their safety (Figure 3). Even if merely preliminary, these results prove that the obtained final extracts enriched in policosanols and phytosterols, i.e., **PCEF** and **PSEF**, seem to be non-toxic, suggesting the importance of the extraction procedure for the toxicity of the final extract, as already stated by Tsirigka [87].

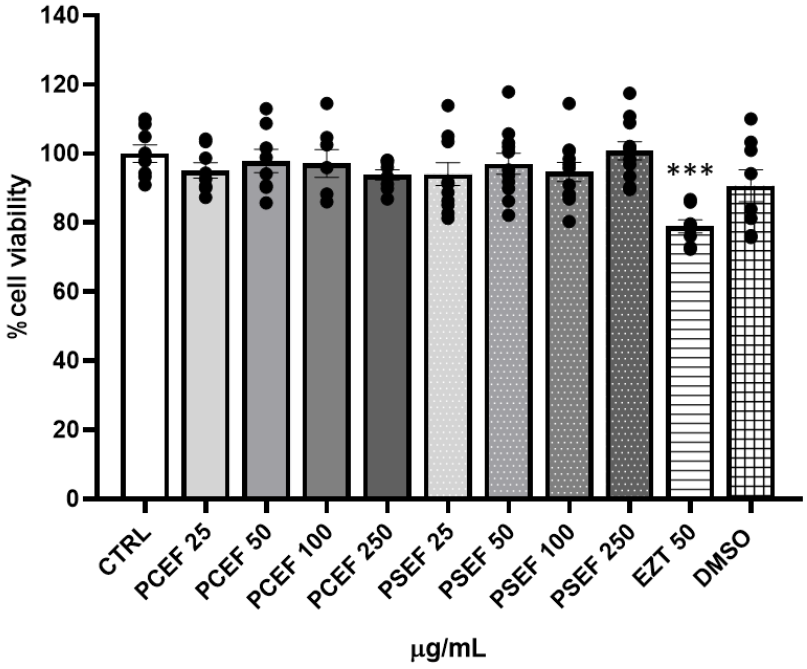

Figure 3. Evaluation of cell viability. Caco2/HT29 cells were co-cultured for 7 days before 48 h of treatment with ezetimibe (**EZT**) (50 µg/mL) and increasing concentrations (25–250 µg/mL) of **PCEF** and **PSEF**. MTT assay was performed to evaluate cell viability. Dimethyl sulfoxide (DMSO) was also assessed as control. All data are expressed as mean ± SEM (standard error of the means) *** $p < 0.001$ versus CTRL (untreated cells).

3. Materials and Methods

3.1. Raw Material and Chemicals

S. bicolor seeds (2 batches of the var. Diamond) were provided by Padana Sementi Elette s.r.l. (Tombolo, Italy). Chloroform ($CHCl_3$) (≥99% purity, stabilized), methanol (MeOH) (HPLC grade) and ethanol (EtOH) (95% purity) were purchased from VWR International s.r.l. (Milan, Italy) and used without further purification. Low boiling petroleum ether (lbPE) (analytical grade), diethyl ether (DE) (ACS reagent, anhydrous, ≥99.0%) pyridine (py) (anhydrous, ≥99% purity), dimethyl sulfoxide (DSMO) (biological grade), anhydrous sodium sulphate (ACS reagent), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (≥99% purity), silica gel (230–400 mesh), and 3-(4,5-dimethylthiazol-2-yl)diphenyltetrazolium bromide (≥99% purity) were purchased from Merck (Darmstadt, Germany) and used without further purification. Other reagents for cell cultures and cell viability tests were provided from Euroclone (Pero, Italy).

3.2. Crude Lipid Extraction

Total lipids were extracted according to a protocol based on a modification of the extraction method reported by Folch [88]. Five replicated extracts were considered for the study. Each of them was obtained merging, prior to saponification, the total lipidic material that resulted from 5 different extractions. Each extraction was performed starting from a mixture of 3 g of seeds from 2 different batches (N = 5). Therefore, each lipidic extract used for saponification was referred to 15 g of initial dry biomass. Operationally, seeds were desiccated for 72 h at 45 °C and then ball-milled, reducing them to a fine powder (60-mesh sieve). The obtained powder was transferred in 50 mL chemical-resistant plastic tubes and immersed for 30 min in a thermostatic bath at 60 °C to allow the complete denaturation of phospholipases [57]. Then, 20 mL of a mixture of chloroform and methanol at a ratio of 2:1 (volume/volume—hereafter v/v) was added to the powder, and the obtained solution was maintained in constant agitation for 1 h at room temperature. The homogenate was filtered over a 25 µm pore filter paper and diluted with microfiltered MilliQ water to reach a 1:5 (v/v) ratio. Then, the mixture was centrifuged at 1000 RCF for 15 min. The organic phase, containing total lipids, was kept and the remaining solution was extracted twice adding 5 mL of the $CHCl_3$–MeOH mixture followed by 5 mL of microfiltered water, centrifuging the obtained solution at 1000 RCF for 5 min and keeping the organic phase after each extraction. The solvents were removed from the obtained extract using a rotary evaporator with the water bath set to 40 °C. Lipids were finally stored at −20 °C.

3.3. Saponification

Phytosterols and policosanols were obtained by saponifying the crude lipid extracts, according to the procedure described by Harrabi [29]. Lipids were initially resuspended in a 12% potassium hydroxide ethanolic solution (KOH in EtOH, 1:10 weight/volume–hereafter w/v). The solution was then heated at 60 °C for 90 min in a thermostatic bath. After 1 h of cooling, MilliQ water was added at a 1:10 (w/v) ratio, with respect to the weight of the dried extract, and the unsaponifiable matter was extracted four times with the same volume of low boiling petroleum ether. Finally, the combined ether extracts were washed with an equivalent volume of aqueous ethanol solution (EtOH-H_2O 1:10 v/v), considering the weight of the initial lipid extract, and the isolated organic phase was dried using anhydrous sodium sulfate. The extract was then concentrated in a rotary evaporator, and then the dry residues of the unsaponifiable fraction were stored at −20 °C for further analyses and fractioning.

3.4. Flash Column and Thin Layer Chromatography

Phytosterols and policosanols were separated by flash column chromatography, according to Blunt [89]. Separation was performed using a 30 mm diameter column with silica gel 230–400 mesh (for n grams of sample, ~60 n grams of silica gel were used) as stationary phase and n-hexane–diethyl ether (65:35 v/v) as eluent (~300 mL for packing and elution), according to Roge [90]. After an initial column packing, the sample (~100 mg) was loaded using the dry loading method, with previous dissolution of the sample with chloroform (~5 mL) in silica (~100 mg) and subsequent solvent evaporation to obtain a powder [90]. Overall, 24 fractions having a volume of 14 mL were collected.

After fractionating, one-dimensional analytical thin layer chromatography (TLC) was performed to individuate the fractions effectively containing the compounds of interest, using silica gel 60 F254 plates with n-hexane–diethyl ether (65:35 v/v) as mobile phase (about 10 mL was used for each sample). UV light was used for the detection of the fractions containing the compounds of interest (fractions from 5 to 9 for policosanols and from 10 to 18 for phytosterols), in addition to charring with Hanessian's Stain [91]. To correctly identify policosanols and phytosterols, 1-eicosanol and lanosterol were used as references, respectively.

3.5. Extraction Yield Calculation

The recovery yields of total lipids as well as those of phytosterols and policosanols (mg/g) were calculated starting from the mass of recovered dried extracts. Specifically, the mass values of both total lipids and those of the phytosterol and policosanol fractions (mg) were divided by the initial dry mass of the grain sample (g), as reported in Leguizamón [18].

3.6. GC/MS Analysis

The GC/MS method, used for determining the composition of the policosanol and phytosterols extracts, was chosen according to Irmak [92].

The dried extracts of phytosterols and policosanols obtained after flash column chromatography were re-dissolved in 500 µL of *n*-hexane and 5 µL of 1-eicosanol (IS, 50 ppm solution in n-hexane). Then, the silanization was performed using 15 µL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and 15 µL of anhydrous pyridine at room temperature for 30 min. The derivatized extract solution was injected into the GC/MS system composed of a 8860 N Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with a 5977 B Mass Selective single quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). A DB-5 fused silica capillary column (stationary phase (5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 µm, Agilent J&W Columns (Agilent Technologies, Santa Clara, CA, USA) was used for chromatographic separation. The injection volume was 1 µL, and the injection port was held at 280 °C and operated in spitless mode. The flow was kept constant at 1 mL/min (carrier gas He, purity 99.995%), using the following temperature protocol: initial temperature 80 °C for 2 min, 20 °C/min to 200 °C for 2 min, 20 °C/min to 280 °C for 3 min, 20 °C/min to 300 °C for 10 min. The interface temperature was kept at 280 °C while the ion source and quadrupole temperature were kept at 230 and 150 °C, respectively. The MS operated in electron ionization mode (70 eV), in positive mode scanning in the range from 35 to 700 m/z. The NIST MS Search 2.4 (2020) (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral database was utilized for tentative identification of compounds (Table 1). Only components that exhibited a match value >80% were included.

3.7. Cell Culture

Cellular tests were performed on a co-culture settled in a mixture that was 70:30 of Caco-2 (ATCC® HTB-37™ o BS TCL 87) and HT29 (BS TCL 132) human epithelial colorectal adenocarcinoma cell lines, as previously described in the literature [93,94]. Cells were separately grown in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and maintained at 37 °C in a humidified 5% CO_2 incubator. A density of 1×10^4 cells/well was used for the seeding procedure in 96-well plates. Reaching confluence, Caco-2 cells express the characteristics of enterocytes while HT29 cells present features of absorptive and mucus-secreting cells, resembling the morphology of a gut barrier.

3.8. Cell Viability Assay

Cells were seeded and maintained in culture for seven days before treatment with increasing concentrations (25–250 µg/mL) of the two different lipid extracts of sorghum grains: **PCEF** (policosanols enriched fraction) and **PSEF** (sterols enriched fraction). In comparison, a group of cells were treated with ezetimibe 50 µg/mL. Ezetimibe concentration was chosen according to previous studies [95,96]. Finally, dimethyl sulfoxide (DMSO), which was used to dissolve the phytoextracts, was also evaluated at the higher concentration tested. After 48 h treatment, cell viability was evaluated by MTT assay [97], measuring the cellular capacity to reduce 3-(4,5-dimethylthiazol-2-yl)diphenyltetrazolium bromide into blue formazan products by various mitochondrial dehydrogenase enzymes. MTT stock solution (5 mg/mL) was added to each plate to a final concentration of 1.2 mM and cells were incubated for 90 min at 37 °C. After removing the MTT solution, the reaction was interrupted by adding EtOH. The optical density was measured using a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) multi-detection microplate reader (app. software: BMG LABTECH MARS—Microplate reader software—OMEGA SERIES) at a wavelength of 570 nm and a reference light of 600 nm. Cell viability was expressed as a percentage against untreated cell lines used as controls. All data are expressed as mean ± SEM (standard error of the means). The values were compared to the negative control (untreated cells) using Dunnett's multiple comparisons test following one-way ANOVA calculation. A p-value < 0.05 was statistically significant.

4. Conclusions

The protocol developed in this study for the co-extraction of policosanols (**PCs**) and phytosterols (**PSs**) from *S. bicolor* allowed us to obtain two extracts rich in policosanols (**PCEF**) and phytosterols (**PSEF**) respectively. The fraction of **PCs**, 42.6% in **PCEF**, and **PSs**, 92.1% in **PSEF**, appeared to be rather well-separated, and contain the respective bioactive compounds in truly predominant quantities. Moreover, the yields of the **PCEF** and **PSEF** were significantly higher than any other reported so far in the literature. On this matter, it can be speculated that the extraction process carefully optimized in each single step, probably in combination with the use of particularly rich batches of *S. bicolor*, lead to the observed 10-fold-higher isolated amounts of **PCs** and **PSs**. Moreover, the extracts showed a remarkable purity in terms of comprised compound classes. The chemical profiling of *S. bicolor* achieved via GC/MS analyses, revealed the presence of some policosanols (1-docosanol, 1-pentacosanol, 1-heptacosanol, 1-nonacosanol and 1-dotriacontanol) and phytosterols (fucosterol) not yet detailed discussed for this species. Moreover, several compounds, whose positive contribution in regulating cholesterol levels has been already assessed in the past, were detected, most noteworthy also in the form of terpenes (**Ts**), thus increasing the potential health benefits of the fractions. Finally, according to the first results

of the biological tests, the obtained extracts were shown to be biocompatible in standard cell-viability tests in a concentration range of 25–250 μg/mL for **PCEF** and **PSEF**, respectively.

Supplementary Materials: The supporting information can be downloaded at https://www.mdpi.com/article/10.3390/molecules31040727/s1, Table S1: Comparison of selected extraction procedures of other relevant cereals biomasses containing policosanols and phytosterols. Table S2: Comparison of selected extraction procedures of sorghum. Figure S1: TIC-GC/MS chromatogram of blank fraction.

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