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Assessing Anti-Inflammatory Activities and Compounds in Switchgrass (*Panicum virgatum*)

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Abstract: Switchgrass is a bioenergy feedstock that potentially possesses multiple health benefits. However, the biological properties and associated bioactive compounds of switchgrass have not been adequately investigated. In the current study, we assessed the anti-inflammatory properties of switchgrass. Results from in vitro bioassays indicated that the methanolic extracts of switchgrass contained compounds exerting inhibitory effects on the expression of inflammatory mediators (TNF-α, IL-6, IL-8, and IL-10) induced in the U-937 model system. The extracts derived from four switchgrass cultivars (Alamo, Kanlow, Liberty, and Show Me) inhibited the secretion of all inflammatory mediators examined, with the only exception of the Liberty extract, which showed no significant effect on IL-10 expression. The degree of cytokine inhibition was variable, depending on the particular cultivar, the concentrations tested, and the cytokines examined. A global metabolomics approach was utilized to putatively identify possible molecules with known anti-inflammatory capacities in different switchgrass cultivars using ultra-high performance liquid chromatography with high-resolution mass spectrometry (UHPLC-HRMS). The content of multiple bioactive anti-inflammatory compounds in switchgrass was determined by liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) analyses. Our results suggest that switchgrass, particularly the Alamo and Kanlow cultivars, may represent a promising natural anti-inflammatory source for the cosmetic, nutraceutical, and pharmaceutical industries.

Keywords: switchgrass; metabolomic profiling; anti-inflammatory compounds; anti-inflammatory activity; bioactive compounds

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1. Introduction

Switchgrass (*Panicum virgatum* L.) is a terrestrial, perennial, high biomass, and warm-season C4 grass [1]. This native herbaceous plant is one of the most common species in North American tallgrass prairies, with high productivity, broad geographical adaption, low

requirements for agricultural inputs, and positive environmental impacts [1,2]. Switchgrass is valued as a multipurpose crop species that is traditionally used for forage production, soil erosion control, wildlife habitat control, and ornamental purposes [1]. Recently, this grass species has been considered as a critical cellulose source for cellulosic bioethanol production [3]. Switchgrass can also play a role in phytoremediation as a vegetative filter that can absorb pesticide residues in water systems and remove soil contaminants (e.g., herbicides, pesticides, and heavy metals) [4–6]. Other value-added applications of switchgrass include thermal conversion, pulping, and paper production [7].

Switchgrass has been utilized as a folk medicine [8] and has been proposed to possess several biological functions derived from its phytochemical properties. Switchgrass extracts contain different classes of bioactive phenolic compounds (e.g., vanillic acid, p-coumaric acid, ferulic acid, rutin, quercitrin) [9–11] that are known to exert health-promoting benefits such as antioxidant, antibacterial, anti-inflammatory, and anticancer activities. Of these potential health-promoting benefits, only the antioxidant activity of switchgrass extracts has been definitively demonstrated [10–12]. Switchgrass possesses higher antioxidant capacities than other potential bioenergy crops, such as mimosa seeds, spinach, and castor foliage, but lower antioxidant properties than mimosa foliage, sericea, velvet bean foliage, kudzu, and arunzo [12]. Ref. [11] documented two flavonoids, quercetin and rutin, as the major compounds responsible for the low-density lipoprotein oxidation inhibition activity observed in aqueous switchgrass extracts. Other potential biological activities of switchgrass (e.g., anti-inflammatory capacity) have not been adequately established. Identifying the health-promoting properties of switchgrass could result in new applications and utilizations of switchgrass that would increase the economic value of switchgrass in the biorefinery supply chain and promote the development of novel biological agents for the cosmetic and pharmaceutical industries.

Inflammation is a pathophysiological process activated by the immune system, involving a variety of cellular, molecular, and biochemical mediators in response to microbial infection or tissue injury [13]. Cytokines are inflammatory mediators (e.g., tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-8, and IL-10) that play vital roles in acute and chronic inflammation and have been widely utilized as possible indicators of systemic inflammation [14]. An initial effort to assess the anti-inflammatory properties of switchgrass was made by [15]. The authors evaluated the inhibitory effects of switchgrass extracts on the expression of monocyte chemoattractant protein (MCP)-1, a cytokine playing an important role in acute inflammation, using the mouse embryonic fibroblast cell line 3T3-L1. They observed that the switchgrass extracts reduced the expression of MCP-1, but no statistical data were provided [15]. In this study, we first evaluated the effects of switchgrass extracts on the secretion of four inflammatory mediators (TNF-α, IL-6, IL-8, and IL-10) using the human pro-monocytic cell line U-937. We then putatively identified anti-inflammatory molecules in the switchgrass extracts via a global metabolomics profiling approach and subsequently quantified bioactive compounds in the extracts using targeted analyses.

2. Materials & Methods

2.1. Switchgrass Collection

Four switchgrass cultivars, i.e., Alamo, Kanlow, Liberty, and Show Me, were obtained from the University of Missouri, Horticulture and Agroforestry Research Center (New Franklin, MO, USA). All cultivars were established from seed in 2016, and the switchgrass samples were harvested in July 2017. After harvesting, the samples were immediately stored at −20 °C until extraction.

2.2. Sample Extraction

The plant samples from each switchgrass cultivar were homogenized using a coffee grinder. Each extraction included 10 g of plant samples (dry weight basis). All experiments (cellular assays and metabolic analyses) were performed at least in triplicate through different extractions from each switchgrass cultivar. The homogenized samples were

extracted in 200 mL of methanol (HPLC grade, Fisher Scientific, Pittsburg, PA, USA) twice. The resulting extract was sonicated for 60 min at 10 °C. Subsequently, the methanolic extract was filtered through a filter (0.2 µm, Whatman Anotop, GE Healthcare, Chicago, IL, USA) to collect the supernatant. For immunoassays, the supernatant was evaporated until dryness under a flow of nitrogen. The resulting extract was resuspended in dimethyl sulfoxide (0.125 mL, DMSO, Sigma-Aldrich, St. Louis, MO, USA) and concentrated to 10,000 mg/mL.

2.3. Identification of the Anti-Inflammatory Potential of Switchgrass

Cell culture and differentiation induction. U-937 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture and differentiation induction were performed as previously described [16]. The cultures, differentiated with phorbol 12-myristate 13-acetate (PMA), were pre-treated with four switchgrass cultivars (Alamo, Kanlow, Liberty, and Show Me) at 3 concentrations (0.1, 1, and 10 mg/mL) for 2 h before stimulation with lipopolysaccharides (LPS, 1 µg/mL, *Escherichia coli* 0127:B8, Sigma-Aldrich). Two immunosuppressant agents, dexamethasone (2 µg/mL, Sigma-Aldrich) and cyclosporin A (2 µg /mL, Sigma-Aldrich), served as positive controls for the inhibition of cytokine secretion; vehicle controls were included in cultures lacking extracts or inhibitors. Twenty-two hours following LPS stimulation, the culture supernatants were collected, spun to remove cell debris, transferred to new tubes, and stored at −20 °C until analysis.

Cell viability analysis. MTT assays were conducted to determine the possible effects of switchgrass extracts on cytotoxicity and/or cell loss that can lead to reduced cytokine levels. A colorimetric cell viability assay (CGD1-1KT, Sigma-Aldrich) was used to measure mitochondrial dehydrogenase activity in the attached cells after removal of the supernatants. The MTT assays were performed as described previously [16]. Briefly, the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) substrate prepared in Dulbecco's modified Eagle medium (DMEM), high-glucose, phenol red-free (Gibco, Pittsburgh, PA, USA), containing 1% FBS was added to the cells, and incubation was performed for 3 h at 37 °C until formazan crystals were observed. The crystals were completely dissolved in acidified isopropanol by pipetting multiple times before performing the readings. A BioTek ELx808 microplate reader (BioTek, Winooski, VT, USA) was used to measure the absorbance of the formazan crystals at a wavelength of 570 nm within 30 min after the addition of the solvent. Background absorbance was measured at a wavelength of 630 nm. The MTT conversion levels were calculated by subtracting the background absorbance (630 nm) from the absorbance of the formazan crystals at 570 nm (A_{570} nm − A_{630} nm = specific MTT absorbance).

Quantification of the secretion of cytokines/chemokines by macrophages. Soluble cytokine levels were analyzed in the U-937 model system as an indicator of inflammation. Cytokines released into the supernatant were measured in PMA-differentiated, LPS-stimulated cells in the absence or presence of switchgrass extracts. Four representative cytokines, i.e., TNF-α, IL-6, IL-8, and IL-10, were chosen to reflect the anti-inflammatory as well as the proinflammatory properties of the extracts. Cytokine levels were quantitated using a Cytometric Bead Array (CBA) human inflammatory cytokine kit according to the manufacturer's procedure (BD Biosciences, San Jose, CA, USA). Samples from each treatment group were run in triplicate on a BD LSRFortessa^{TM} X-20 cell analyzer (BD Biosciences, San Jose, CA, USA) using instrument settings suggested by BD and optimized in each experiment. Cytokine levels were calculated from a standard curve for each cytokine generated from a 5-parameter logistic curve, using a curve-fitting software.

2.4. Identification and Qualification of Anti-Inflammatory Molecules in Switchgrass

UHPLC-QTOF-MS analysis. For both metabolomic profiling and quantitative analyses, the same methanolic extracts were analyzed by ultra-high performance liquid chromatography with high-resolution mass-spectrometry (UHPLC-HRMS) and tandem mass spectrometry (HPLC-MS/MS). Untargeted metabolomics analysis and the parameters used

for the UHPLC-HRMS system was conducted as described previously [17]. The methanolic extracts (2 µL per injection) obtained from the four switchgrass cultivars were injected into a UHPLC coupled with an maXis impact quadrupole-time-of-flight (QTOF) mass spectrometer (Bruker Co., Billerica, MA, USA) operated in both negative and positive electrospray ionization modes. Each treatment was analyzed in triplicate, and the solvent (methanol) was used as a control.

HPLC-MS/MS analysis. Three metabolites (quercetin, quercetin 3-glucoside, and rutin hydrate) were quantitated using an HPLC system (Water Alliance 2695, Water Co., Milford, MA, USA) coupled to a Waters Acquity TQ triple quadrupole mass spectrometer, as described in [18]. Three different extracts (replicates) from each cultivar were analyzed. The contents of the metabolites in the extracts were extrapolated using a standard curve for each associated analyte generated using authentic standards (purity > 95%, Sigma-Aldrich) at 7 concentrations ranging from 0.01 to 10 ppm, in triplicate.

2.5. Data Processing and Statistical Analysis

The results from experiments addressing cell viability and secreted cytokine levels in the treatment groups were expressed relative to the corresponding control groups. The treatment groups included PMA-differentiated, LPS-stimulated U-937 cells in the presence of switchgrass extracts or the known cytokine inhibitors dexamethasone and cyclosporin A. The respective control groups contained the extract vehicle (DMSO) or inhibitor vehicles (DMSO or ethanol), in the absence of the extracts. Data are expressed relative to the corresponding controls cultured in the presence of vehicle and in the absence of switchgrass extracts, that were set to 100%. Differences in relative cell viability and secreted cytokine levels between the treatment and the control groups were compared using a two-tailed paired t-test in SAS 9.4 (SAS Institute, Cary, NC, USA). Statistical differences between treated and control groups were determined using leas- square means at p-value < 0.01.

For untargeted metabolomics analyses, the UHPLC-MS data were analyzed as described previously [18]. Briefly, XCMS Online [19] was used for data processing to identify significant features (p-value < 0.05, fold change $\geq$ 1.5, and intensity $\geq$ 10,000) distinguishing each switchgrass cultivar from the control (methanol) across the chromatographic time domain. Parameters, statistical analysis, and compound annotation were set as described in [18]. Molecules reported to have anti-inflammatory capacities were further selected to identify the anti-inflammatory profile of switchgrass (Supplementary Table S1). Partial least-squares discriminant analysis (PLS-DA) and heat map analysis were performed to compare the anti-inflammatory profiles of switchgrass cultivars using MetaboAnalyst [20].

For targeted metabolomics analyses, differences in the concentrations of the anti-inflammatory compounds obtained from the LC-MS/MS analyses among the switchgrass cultivars were analyzed using a randomized complete block design and PROC MIXED in SAS 9.4 (SAS Institute). The switchgrass cultivar was the fixed effect, and replication was the random variable. Differences between cultivars were determined using Fisher's LSD at p-value < 0.01.

3. Results

3.1. Anti-Inflammatory Potential of Switchgrass

Cell viability analysis. MTT colorimetric assays were utilized to determine potential cytotoxic effects of the switchgrass cultivars on U-937 cell viability, which could account for reduced cytokine levels. The MTT assays were performed on the attached cells immediately after collecting the supernatants for cytokine quantitation. Two known anti-inflammatory agents, dexamethasone and cyclosporin A, were included as positive controls to demonstrate that suppression of cytokine secretion could be detected under the experimental conditions. To examine the effect of the vehicle used to prepare the switchgrass extracts, a 0.1% DMSO control group was included. This concentration corresponded to the highest amount of DMSO present in any of the extract dilutions as well as the amount used in our previous studies [16]. Likewise, ethanol and DMSO served as vehicle controls for

dexamethasone and cyclosporin A, respectively. The results in Table 1 indicate that no significant differences in cell viability were observed among any of the PMA-differentiated, LPS-stimulated U-937 groups. Specifically, cell viability was similar regardless of whether the cells were exposed to treatment (switchgrass extracts), immunosuppressant agents (dexamethasone and cyclosporine A), or vehicle (DMSO or ethanol). U-937 PMA-differentiated cells cultured in the presence of DMSO and in the absence of LPS showed similar viability to cells treated with LPS. Taken together, these findings indicate that switchgrass extracts, inhibitors, and vehicles did not show toxic effects in the U-937 model system.

Table 1. Switchgrass extracts, inhibitors, and vehicles exhibited no toxic effects in the U-937 model system. Mean ± SEM.

Treatment	Cell Viability (%)
Vehicles/controls	
LPS (1 μg/mL)	100.0 ± 4.6
LPS (1 μg/mL) + DMSO (0.1%)	101.9 ± 2.9
LPS (1 μg/mL) + Ethanol (0.2%)	99.1 ± 6.3
Anti-inflammatory agents	
Dexamethasone (2 μg/mL)	107.7 ± 3.6
Cyclosporin A (2 μg/mL)	99.1 ± 5.5
Switchgrass cultivars	
Alamo (0.1 mg/mL)	109.9 ± 6.7
Alamo (1 mg/mL)	95.5 ± 7.9
Alamo (10 mg/mL)	104.7 ± 3.0
Kanlow (0.1 mg/mL)	106.4 ± 2.7
Kanlow (1 mg/mL)	101.0 ± 4.3
Kanlow (10 mg/mL)	111.1 ± 8.9
Liberty (0.1 mg/mL)	127.2 ± 6.5
Liberty (1 mg/mL)	120.0 ± 14.8
Liberty (10 mg/mL)	107.1 ± 14.8
Show Me (0.1 mg/mL)	124.0 ± 7.3
Show Me (1 mg/mL)	111.0 ± 3.1
Show Me (10 mg/mL)	99.3 ± 2.3

Effects of switchgrass extracts on cytokine secretion. The results summarized in Table 1 demonstrated that the switchgrass extracts did not exhibit toxic effects on U-937 cells; the extracts were further analyzed for their effects on cytokine secretion as a possible indicator of inflammation. Dexamethasone and cyclosporin A, agents known to affect cytokine secretion, were included as positive controls. The switchgrass extracts were added to PMA-differentiated U-937 cells for 2 h prior to the addition of LPS, and the cultures were incubated for a total of 24 h before cell supernatant analysis. The levels of TNF-α, IL-6, IL-8, and IL-10 were determined using a quantitative flow cytometric, multiplex bead-based assay, and the results were expressed relative to samples cultured in the presence of the vehicle but without the extracts. The results, shown in Figures 1–4, illustrate that the degree of cytokine suppression depended on the switchgrass extract, the cultivar concentration, and the cytokine analyzed. Of note, the levels of all four cytokines were reduced after incubation with the extracts at the highest concentration, 10 mg/mL, for all four cultivars. The only exception was the absence of an effect of the Liberty extract on IL-10 secretion. The Alamo extracts led to a consistent, statistically significant reduction in TNF-α and IL-6 levels at all three concentrations tested. Specifically, culture with the Alamo extracts at 0.1, 1, and 10 mg/mL reduced the secretion of TNF-α to 32.3%, 47.6%, and 53.5%, respectively, and of IL-6 by 21.6%, 27.8%, 51.0%, respectively (Figures 1 and 2) compared to the control. The levels of secreted IL-8 and IL-10 were reduced to 50.8% and 32.5% of the control levels, respectively, when using the extracts at 10 mg/mL, whereas they remained unchanged at the two lower extracts concentrations. The addition of extracts of Kanlow, Liberty, and Show Me cultivars at the highest concentration reduced the secretion of TNF-α in the range of 40–60% of the control levels (50.6%, 49.8%, and 62.7%, respectively) and of IL-6 in the

range of 40–60% of the control levels (40.9%, 62.1%, and 66.4%, respectively). The effect on IL-8 was more variable and was reduced by 54.0%, 19.3%, and 48.4% of the control levels for Kanlow, Liberty, and Show Me extracts, respectively, at the highest concentration. The decrease in IL-10 response was less pronounced than that of other cytokines, corresponding to 30.9%, 19.7%, and 38.8% of the control levels (Figures 1–4). These same extracts showed minor to no effects on all four cytokines at the two lower concentrations tested. Taken together, these results show that the effect of the cultivars on cytokine suppression was dose-dependent and variable among the cultivars. While the extracts of all four cultivars showed an effect at the highest concentration tested, with only one exception, the effect at other concentrations depended on the cultivar and the individual cytokines.

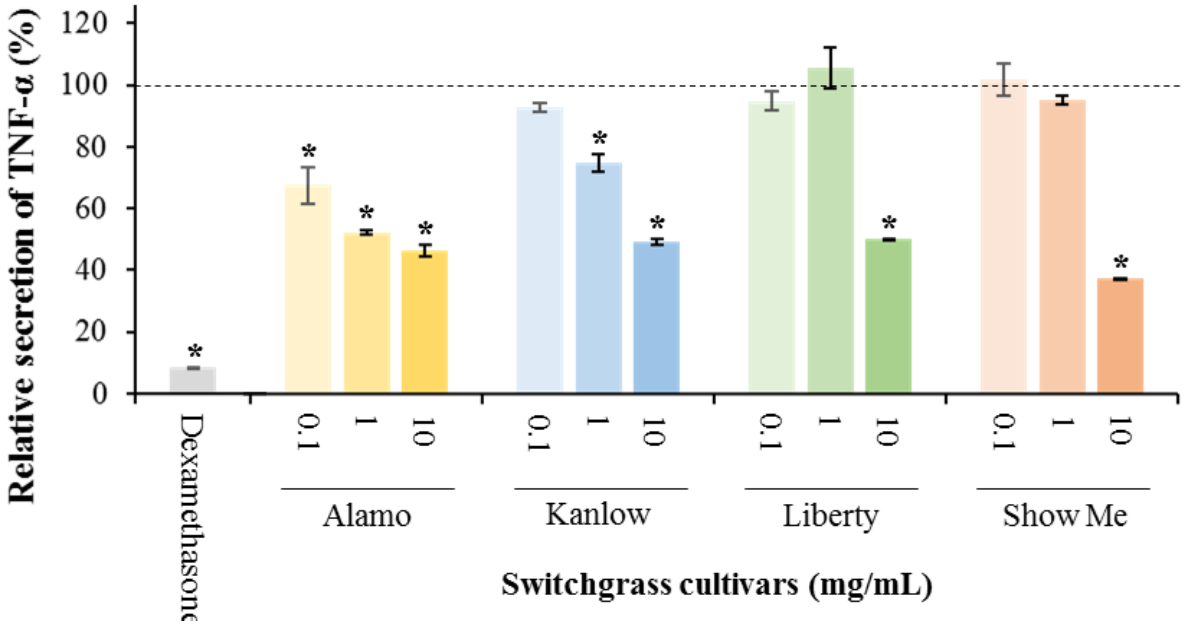

Figure 1. Effect of the switchgrass extracts on the expression of TNF-α in the U-937 model system. (*) Significant decrease ($p < 0.01$) compared to the control cells without the extract. Mean ± SEM.

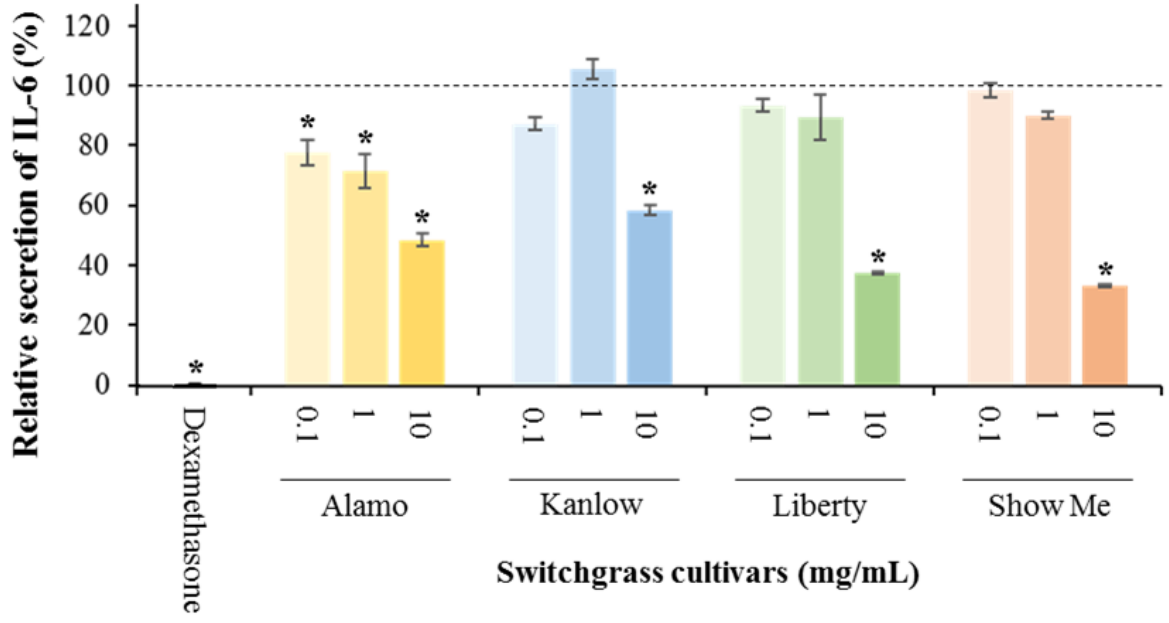

Figure 2. Effect of the switchgrass extracts on the expression of IL-6 in the U-937 model system. (*) Significant decrease ($p < 0.01$) compared to the control cells without the extract. Mean ± SEM.

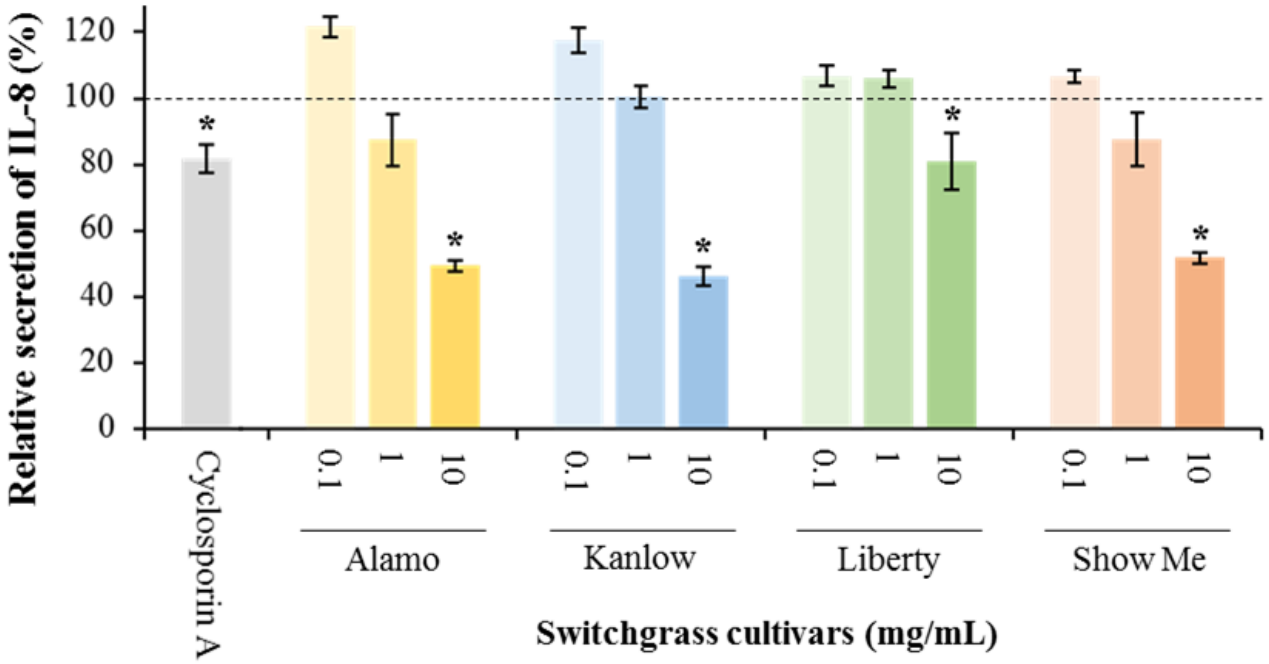

Figure 3. Effect of the switchgrass extracts on the expression of IL-8 in the U-937 model system. (*) Significant decrease ($p < 0.01$) compared to the control cells without the extract. Mean ± SEM.

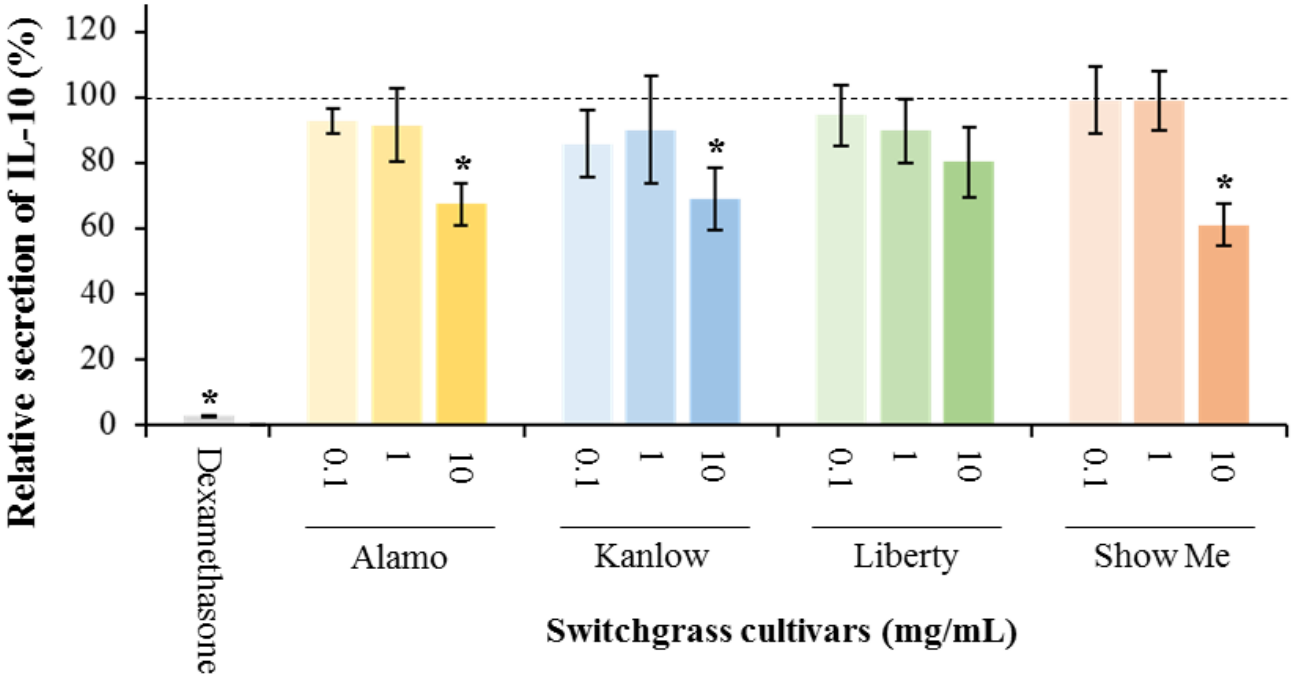

Figure 4. Effect of the switchgrass extracts on the expression of IL-10 by in the U-937 model system. (*) Significant decrease ($p < 0.01$) compared to the control cells without the extract. Mean ± SEM.

3.2. Anti-Inflammatory Compounds in Switchgrass

UHPLC-QTOF-MS analysis. The global metabolomics analysis resulted in the putative identification of 22 substances with known anti-inflammatory activity (Table 2, Supplementary Table S1). Each switchgrass cultivar appeared to contain a variety of potential anti-inflammatory molecules, whose abundance varied within specific cultivars (Figure 5). Alamo and Show Me contained the highest relative abundance of okanin 3′,4′-diglucoside, quercitrin, and aescin, while formononetin 7-O-rutinoside, tenylidone,

quercetin 3-glucoside were relatively dominant in Alamo. Kanlow contained the highest relative abundance of petunidin 3-glucoside, rutin trihydrate, and baicalin. Altholactone, bergenin, quercetin and osthenol 7-O-β-D-gentiobioside were major metabolites in Show Me, whereas dioscin, 4-hexylresorcinol, bruceine B, auranofin, and gambogic acid were most abundant in Liberty. Three metabolites, i.e., quercetin, quercetin 3-glucoside, and rutin hydrate, were further quantified using targeted analyses (Table 3).

Table 2. Putative identification of secondary metabolites with known anti-inflammatory activitiy in switchgrass through untargeted metabolomics analyses.

Compound	Retention Time (min)	Formula	Adducts	Theoretical Mass	Observed Mass	Δm (ppm)
4-Hexylresorcinol	5.75	$C_{12}H_{18}O_2$	$[M + H]^+$	194.1307	194.1302	2.68
Aescin	11.47	$C_{55}H_{86}O_{24}$	$[M - H]^-$	1130.5509	1130.543	6.96
Altholactone	8.86	$C_{13}H_{12}O_4$	$[M + H]^+$	232.0736	232.0731	2.15
Auranofin	5.22	$C_{20}H_{34}AuO_9PS$	$[M + H]^+$	678.1327	678.1335	1.2
Baicalin	6.24	$C_{21}H_{18}O_{11}$	$[M - H]^-$	446.0849	446.0858	2.08
Bergenin	5.2	$C_{14}H_{16}O_9$	$[M + NH4]^+$	328.0794	328.0745	15.03
Bruceine B	4.56	$C_{23}H_{28}O_{11}$	$[M - H]^-$	480.1632	480.1628	0.67
Coumarin	3.17	$C_9H_6O_2$	$[M + H]^+$	146.0368	146.0363	3.56
Dioscin	11.53	$C_{45}H_{72}O_{16}$	$[M + H]^+$	868.482	868.4838	1.98
Formononetin 7-O-rutinoside	8.49	$C_{30}H_{36}O_{17}$	$[M - H]^-$	668.1952	668.1946	0.91
Gambogic acid	9.94	$C_{38}H_{44}O_8$	$[M - H]^-$	628.3036	628.3045	1.47
Kaempferol-7-rhamnoside	7.34	$C_{21}H_{20}O_{10}$	$[M - H]^-$	432.1056	432.1064	1.84
Nevadensin 5-gentibioside	2.29	$C_{30}H_{36}O_{17}$	$[M - H]^-$	668.1952	668.1946	0.91
Okanin 3′,4′-diglucoside	6.96	$C_{27}H_{32}O_{16}$	$[M - H]^-$	612.169	612.1704	2.3
Osthenol-7-O-beta-D-gentiobioside	4.64	$C_{26}H_{34}O_{13}$	$[M + Na]^+$	554.1999	554.1997	0.44
Petunidin 3-glucoside	6.47	$C_{22}H_{23}O_{12}$	$[M - H]^-$	478.1111	478.112	1.91
Quercetin	6.22	$C_{15}H_{10}O_7$	$[M + H]^+$	302.0427	302.0423	1.3
Quercetin-3-glucoside	5.17	$C_{21}H_{19}O_{12}$	$[M - H]^-$	464.0955	464.096	1.22
Quercitrin	7.17	$C_{21}H_{20}O_{11}$	$[M - H]^-$	448.1006	448.1012	1.51
Rhoifolin	6.01	$C_{27}H_{30}O_{14}$	$[M + Na]^+$	578.1636	578.1643	1.29
Rutin trihydrate	4.65	$C_{27}H_{36}O_{19}$	$[M - H]^-$	610.1534	610.1543	1.57
Tenylidone	6.09	$C_{16}H_{14}OS_2$	$[M + H]^+$	286.0486	286.0477	3.31

Table 3. Amounts of anti-inflammatory compounds (mg/kg) in switchgrass determined by UPLC-MS/MS.

Polyphenols	Switchgrass Cultivars			
	Alamo *	Kanlow	Liberty	Show Me
Quercetin 3-glucoside	346.3 ± 52.1^{a}	260.6 ± 18.5^{b}	215.8 ± 38.7^{c}	155.9 ± 7.3^{d}
Quercetin	0.2 ± 0.06^{c}	0.7 ± 0.05^{b}	0.1 ± 0.01^{d}	0.8 ± 0.04^{a}
Rutin hydrate	279.4 ± 17.0^{a}	161.5 ± 11.9^{c}	243.7 ± 13.7^{b}	152.1 ± 1.1^{c}

* Mean ± SEM ($n = 3$). In each row, different letters indicate significant differences among the switchgrass cultivars ($p < 0.01$).

The partial least-squares discriminant analysis (PLS-DA) score plot showed significant differences in the anti-inflammatory metabolic profiles of the four switchgrass cultivars, which were possibly associated with a variation in the anti-inflammatory capacities of the switchgrass cultivars examined (Figures 1–4). The model quality was evaluated using a cross-validation method. The resulting R^2 and Q^2 values were 0.99 and 0.98, respectively, indicating that the model was reliable. The PLS-DA score plot with three principal components covered 94.8% of the total variability of the data (Figure 6a). The first principal components (PC1) explained 51.7% of the total variability of the data, whereas the second and third principal components (PC2 and PC3) accounted for 29.3% and 13.8% of the total variability of the data set, respectively. In the PLS-DA score plot, all four switchgrass culti-

vars were distributed separately. Alamo and Show Me shared a similar pattern according to PC1 and PC3 but were separated along PC2. Regarding PC2, Alamo and Kanlow shared relative a similar pattern and differed from Liberty and Show Me. Variable importance in projection (VIP) score plot revealed the important compounds in different cultivars (Figure 6b).

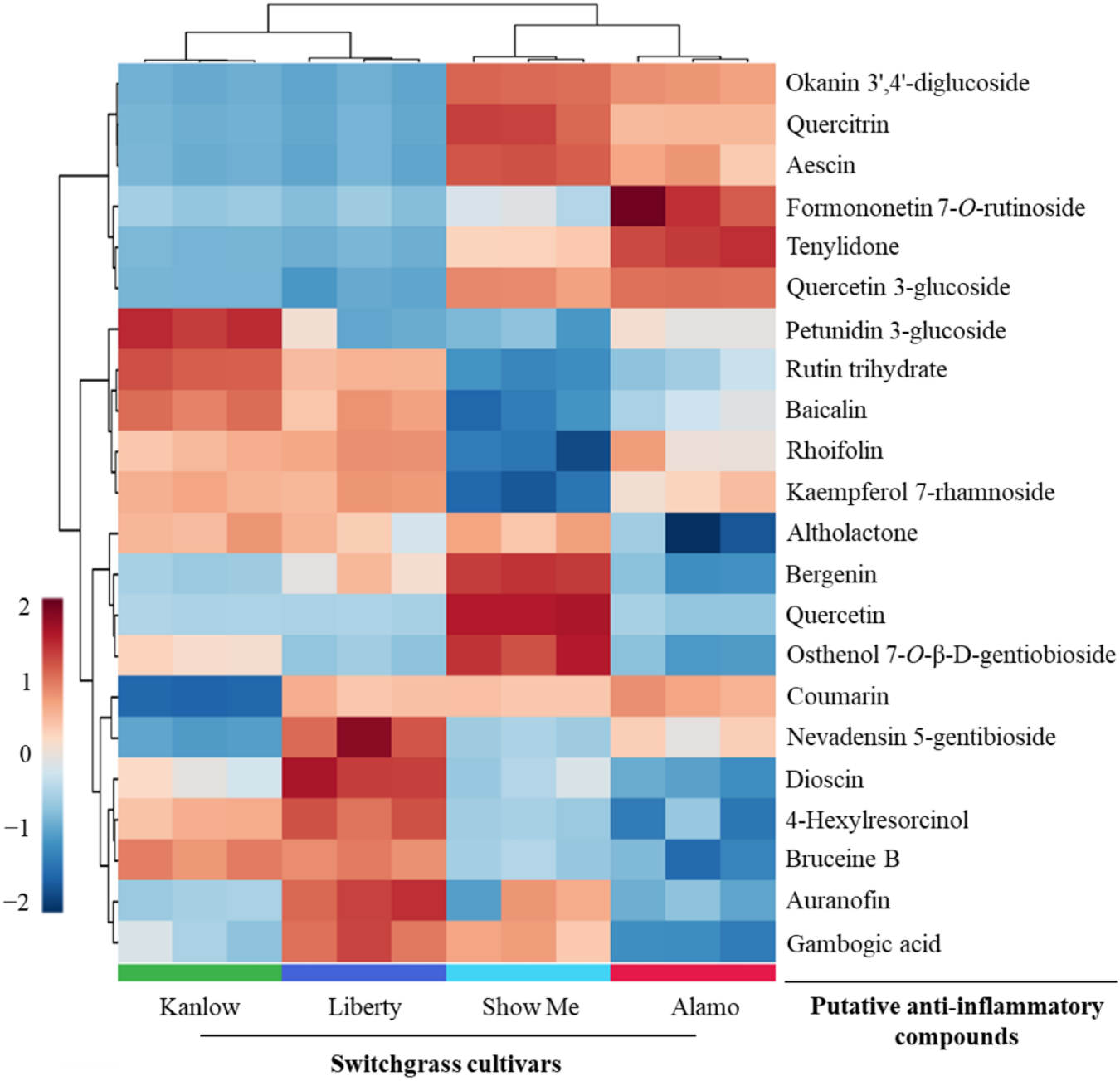

Figure 5. Relative concentrations of anti-inflammatory metabolites in switchgrass. Red indicates higher relative concentrations, whereas blue indicates lower relative concentrations.

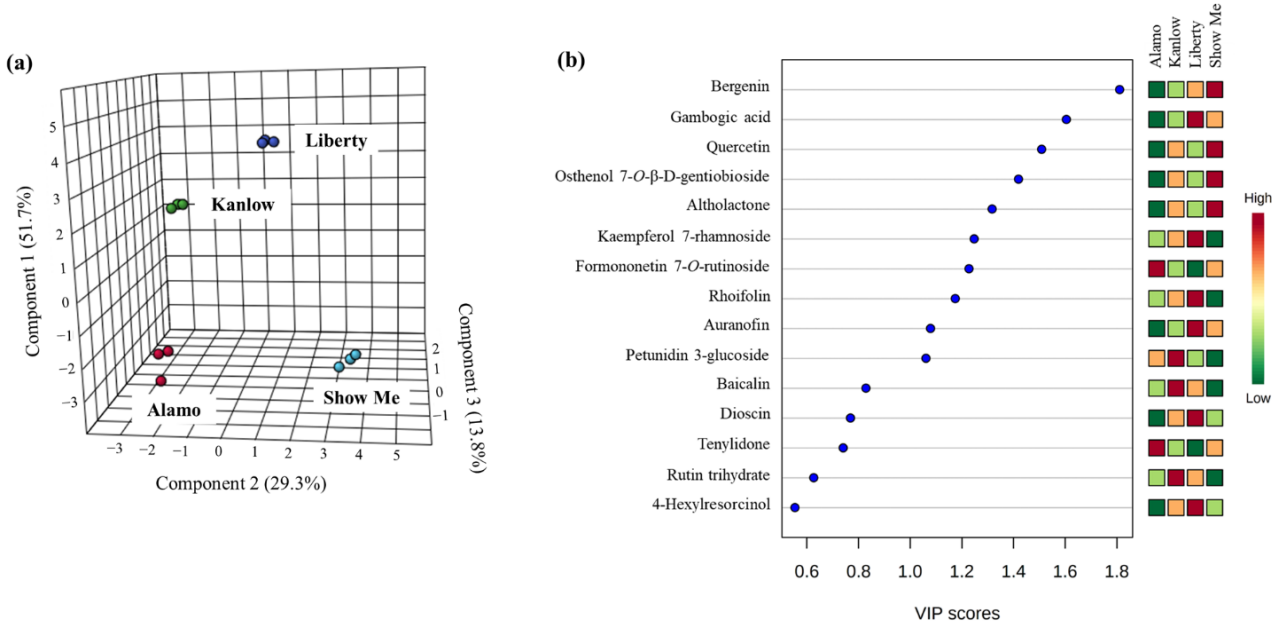

Figure 6. Partial least-squares discriminant analysis (PLS-DA) revealed differences in the metabolic profiles of the switchgrass cultivars. (**a**) PLSDA plot, (**b**) variable importance in projection (VIP) score plot to the component 1. Circles with the same color represent replicates of the metabolic profiles for each cultivar.

HPLC-MS/MS analysis. Three anti-inflammatory metabolites including quercetin, quercetin 3-glucoside, and rutin hydrate were found in the extracts of all cultivars examined (Table 3). The contents of these anti-inflammatory compounds were variable among the switchgrass cultivars. In the switchgrass extracts, quercetin 3-glucose and rutin hydrate were present at high concentrations (>150 mg/kg), whereas quercetin was found at a lower level (<1 mg/kg). Specifically, Alamo was the richest in quercetin 3-glucose (346.3 ± 52.1 mg/kg), followed by Kanlow (260.6 ± 18.5 mg/kg), Liberty (215.8 ± 38.7 mg/kg), and Show Me (155.9 ± 7.3 mg/kg). Similarly, rutin hydrate was found to be present in the highest amount in Alamo (279.4 ± 17.0 mg/kg), followed by Liberty (243.7 ± 13.7 mg/kg), Kanlow (161.5 ± 11.9 mg/kg), and Show Me (152.1 ± 1.1 mg/kg). Quercetin appeared at the highest levels in Show Me (0.8 ± 0.04 mg/kg) and Kanlow (0.7 ± 0.05 mg/kg) compared to Alamo (0.2 ± 0.06 mg/kg) and Liberty (0.1 ± 0.01 mg/kg).

Other biological activities of switchgrass. In addition to the anti-inflammatory properties, potential biological activities (i.e., antibacterial, antimycobacterial, antiproliferative, anti-tyrosinase, anti-elastase activity) of the switchgrass extracts were explored using in vitro bioassays (Supplementary Information). Our screening results revealed that the switchgrass extracts of the four cultivars (Alamo, Kanlow, Liberty, and Show Me) at the highest concentration (>0.4 mg/mL) tested exerted no significant inhibition against two bacterial strains (*Cutibacterium acnes* and *Mycobacterium smegmatis*) and two cancer cell lines (HT-29 and UCT-MEL1). Furthermore, these extracts showed no tyrosinase and elastase inhibitory effects (Supplementary Table S2).

4. Discussion

In the present study, we demonstrated that methanolic extracts of switchgrass contain compounds that inhibited the expression of inflammatory mediators (TNF-α, IL-6, IL-8, and IL-10) induced in the U-937 model system. Alamo, Kanlow, and Show Me extracts reduced the secretion of all four examined cytokines in the U-937 model system, whereas Liberty extracts decreased the secretion of TNF-α, IL-6, and IL-8 only. Cell viability was not reduced in the presence of all four switchgrass extracts compared to that of control cells without the extracts, revealing that the reduction in cytokine secretion is not a result of direct toxic effects. Furthermore, our results indicated a diverse range of anti-inflammatory

compounds potentially present in switchgrass. These compounds are likely responsible for the cytokine suppressive activities observed. Our findings suggest that switchgrass could be considered a promising source of bioactive compounds for the pharmaceutical and cosmetic industries. The identification of novel value-added byproducts and applications of switchgrass would potentially increase the sustainability of this important bioenergy crop.

We documented the variation of the cytokine suppressive capacity of switchgrass depending upon particular cultivars, concentrations tested, and cytokines examined. This is in agreement with previous reports. Tao et al. [10] reported a variation in the antioxidant capacity of switchgrass depending on the cultivar and the location where the samples were collected. Alamo was found to have higher antioxidant activities compared with other cultivars tested, including EG1101 (improved 'Alamo' cultivar) and EG1102 (improved 'Kanlow' cultivar), whereas switchgrass samples collected from different growth locations accounted for up to 20% variation in antioxidant capacity [10]. Differences in the bioactivities of switchgrass are likely associated with differences in the anti-inflammatory metabolic profiles of the switchgrass cultivars examined (Figures 5 and 6). The presence of multiple compounds might explain why the switchgrass extracts showed broad inhibitory effects that reduced the expression of all four cytokines. Additionally, the involvement of multiple compounds in cytokine secretion raises the possibility that interactions between these compounds influence the inhibitory activity and that the bioactive compounds may display synergism. Switchgrass contains carbohydrates, fatty acids, fatty alcohols, glycerol, sterols, organic acids, and other constituents (inorganic ions and alkane). Monosaccharides are the major component, representing 56–60% of total mass balance in switchgrass extracts [21,22]. Switchgrass chemical constituents may interfere with the described suppressive activities or could have inhibitory effects on IL-10 expression in the U-937 model system. Tao et al. [10] found that EG1101 and EG1102 contained similar levels of total phenolic contents but showed different antioxidant capacities and content of free sugars (sucrose, fructose, and glucose). They reported that the high levels of free sugar in EG1101 reduced the antioxidant capacities of this cultivar [10].

Our results revealed the presence of several anti-inflammatory compounds in switchgrass. Among 22 anti-inflammatory metabolites tentatively identified in switchgrass, 18 compounds, including 4-hexylresorcinol, aescin, auranofin, baicalin, bergenin, bruceine B, dioscin, formononetin 7-*O*-rutinoside, gambogic acid, kaempferol 7-rhamnoside, nevadensin 5-gentibioside, okanin 3′,4′-diglucoside, osthenol-7-*O*-β-D-gentiobioside, petunidin 3-glucoside, quercetin, quercetin-3-glucoside, rhoifolin, tenylidone, were reported for the first time possibly present in switchgrass, while other compounds have been reported previously as polyphenolic compounds in switchgrass. [11] assessed the antioxidant capacity of an aqueous extract of switchgrass and found two flavonoids, i.e., quercitrin and rutin, were the major compounds responsible for the inhibition of low-density lipoprotein oxidation. In order to further characterize the anti-inflammatory properties of switchgrass, future research will focus on the identification and purification of compounds driving the inhibition of cytokine secretion in switchgrass.

Our results also revealed that the amounts of anti-inflammatory compounds (quercetin, quercetin 3-glucoside, and rutin hydrate) in switchgrass were variable among the cultivars examined. In total, these phenolic compounds of switchgrass were found at highest abundance in Alamo, followed by Liberty, Kanlow, and Show Me. The contents of bioactive compounds in switchgrass have also been reported to be variable depending on several factors (cultivars, geographic sources, ages, and extraction procedure) [10,21,23]. Tao et al. [10] reported that Alamo contained higher levels of hydroxycinnamic acid (6.1 mg gallic acid equivalents (GAE)/g) compared with EG1101 (5.6 mg GAE/g) and EG1102 (5.3 mg GAE/g), whereas the contents of six phenolic compounds (i.e., caffeic acid, vanillic acid, p-coumaric acid, ferulic acid, rutin, and quercitrin) were found not to be significantly different among Alamo, EG1101, and EG1102. Additionally, switchgrass grown in different locations has different total phenolics contents. The total phenolics contents in Alamo, EG1101, EG1102 from four different growth locations was 6.0–10 mg GAE/g, 7.4–13.8 mg

GAE/g, 6.6–11.6 mg GAE/mg, respectively [10]. Moreover, the levels of bioactive compounds in switchgrass have been documented to be affected by extraction solvents and temperatures. Many different solvents (e.g., methanol, ethanol, water) and some methods (Soxhlet extraction, microwave) have been investigated to improve the recovery of bioactive compounds in switchgrass [11,21]. Uppugundla et al. [11] reported that 60% methanol extraction yielded higher recovery rates of rutin and quercetin compared with water extraction. Ref. [23] documented that quinic acid was found in the leaves of Alamo after extraction in benzene/ ethanol (2:1, v/v), but this compound was not detectable in the leaves after extraction in hot water. Future efforts will focus on the identification of the optimal conditions (e.g., extraction methods, solvents) to maximize the recovery of bioactive compounds in switchgrass.

5. Conclusions

We demonstrated the anti-inflammatory potential of switchgrass extracts from four cultivars (Alamo, Kanlow, Liberty, Show Me). In fact, the examined methanolic extracts of switchgrass appeared to contain bioactive compounds that suppressed the secretion of inflammatory mediators induced in the human pro-monocytic cell line U-937. Multiple anti-inflammatory metabolites in the extracts were identified and quantified. The inflammatory properties of the extracts suggest that switchgrass and its by-products could be an excellent source of raw materials for the nutraceutical and cosmetic industries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12070936/s1, Table S1: Putative identification of the secondary metabolites with known anti-inflammatory activities in switchgrass through untargeted metabolomics analyses.; Table S2: In vitro biological activities of the extracts derived from the four switchgrass cultivars. Antibacterial and antimycobacterial activities were evaluated against 2 bacterial strains Cutibacterium acnes and Mycobacterium smegmatis, respectively. Anticancer activity was investigated using human colorectal adenocarcinoma (HT-29) and human malignant melanoma (UCT-MEL-1) cell lines.

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Abbreviations

TNF-α	tumor necrosis factor alpha
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
PMA	phorbol 12-myristate 13-acetate
UHPLC	ultra-high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
QTOF	quadrupole-time-of-flight
HPLC-MS/MS	liquid chromatography–tandem mass spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

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