Note

In vitro Anti-inflammatory Effects of Edible Igusa Soft Rush (Juncus effusus L.) on Lipoxygenase, Hyaluronidase, and Cellular Nitric Oxide Generation Assays: Comparison with Matcha Green Tea (Camellia sinensis L.)

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Soft rush (Juncus effusus L. var decipiens), known as igusa, is locally cultivated as an edible organic crop, and the dried powder is applied to the processing of unique foods in Japan. The current study investigates the anti-inflammatory effects of edible soft rush using lipoxygenase and hyaluronidase assays, and an activated macrophage cell model in vitro. Matcha green tea powder was tested for comparison. Hot-water and ethanol extracts of soft rush as well as matcha showed comparable lipoxygenase inhibition, with IC50 values of 123 to 145 μg/mL. For the hyaluronidase assay, IC50 values of the samples were 1.16 mg/mL or more. Macrophages cultured in the presence of hot-water and ethanol extracts of soft rush showed strongly suppressed nitric oxide production (IC50 of 120 μg/mL and 35.2 μg/mL, respectively) compared to matcha in a lipopolysaccharide-activated cell model. These results support the potential usefulness of edible soft rush powder for anti-inflammatory purposes.

Keywords: soft rush, Juncus effusus L., igusa, anti-inflammation, lipoxygenase, macrophage

Introduction

Juncus effusus L. var decipiens (Juncaceae), namely soft rush, is a perennial herb widely grown in the marshes of mostly temperate and sub-tropical areas. The aerial part of soft rush, called igusa in Japan, has been used as a folk medicine for antiphlogistic, diuretic and other medicinal purposes (Keys 1976; Hotta et al., 1989; Okada et al., 2002). Further studies have disclosed the multifaceted functions of this plant in antifungal (Hanawa et al., 2002), gastrointestinal spasmyloytic (Di et al., 2014), anticancer (Ishiuchi et al., 2015) and anxiolytic activities (Wang et al., 2014). These effects have been attributable to the rich content of indigestible dietary fiber (Morita et al., 2002), and major/minor biologically active constituents such as flavonoids, coumarins, terpenes, stilbenes, sterols, phenolic acids, carotenes, phenanthrenes and their derivatives (El-Shamy et al., 2015). Among them, luteolin, a type of flavonoid, and its derivatives are the characteristic chemicals present in Juncaceae plants (Williams and Harborne 1978; Ishiuichi et al., 2015). In recent decades, this herbal soft rush has been cultivated as an edible organic crop, processed into dry powder, and marketed as unique health foods such as herbal powdered tea, candy, ice cream, and noodles in the local Kyushu area. However, the beneficial health properties of this edible herbal plant and its processed products have not been fully established.

Green tea (Camellia sinensis L.), native to China and India, has been popularly consumed for centuries. A number of human studies suggest that green tea contributes to a reduction of lifestyle-related disease risks, cardiovascular disease and some forms of cancer (Butt et al., 2015; Maheswari et al., 2015). Previous reports demonstrated the anti-inflammatory effects of green tea leaves and...
polyphenols on arachidonate cascade-based (Choi et al., 2002; Roy et al., 2008) and macrophage-mediated immune responses (Lin et al., 2006; Byun et al., 2010). Special powdered green tea, namely *matcha* (*maccha*), is used in the traditional Japanese tea ceremony, and is also consumed as various processed foods, for example, candy, ice cream, cookies, cakes, chocolates, and other snacks and drinks in Japan. *Matcha* is rich in epigallocatechin gallate (EGCG), and differs in catechin composition from other regular green teas because it is traditionally grown under 90% sunlight shade (Weiss et al., 2003).

Lipoxygenases constitute a family of key enzymes that produce inflammatory leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids in a number of pathophysiological processes such as asthma, psoriasis, cancer metastasis and atherosclerosis (Samuelsson 1983; Lewis et al., 1990; Henderson 1994). Due to the involvement of this enzyme in various inflammation-related processes, there is considerable interest in examining whether natural products can inhibit the lipoxygenase pathway of the arachidonic cascade (Gleason et al., 1986; Komoda et al., 2004). Hyaluronidases are a family of key enzymes involved in human tissue remodeling during allergy and other inflammatory responses (Kakegawa et al., 1988; Mio and Stern, 2002). Previous reports show that the action of activated hyaluronidase can be involved in allergic inflammation in line with the histamine release from mast cells (Sawabe et al., 1992; Asada et al., 1997). Macrophages derived from blood monocytes play an important role in defense. Cytokines and/or microbial compounds activate macrophages, in which excess nitric oxide (NO) can be generated by inducible NO synthase (iNOS); thus, the NO level reflects the inflammation state of macrophages *in vitro* and *in vivo* (Nathan, 1992; Bogdan, 2001).

In this study, we investigated the anti-inflammatory effects of hot-water and ethanol extracts of soft rush powder in lipoxygenase and hyaluronidase assays, and an activated macrophage cell model *in vitro*. To delineate the functionality of edible soft rush powder, inhibitory effects of two other extracts from *matcha* green tea powder were examined for comparison.

**Materials and Methods**

The soft rush cultivar (*Juncus effusus* L. var *decipiens*) ‘Okayama 3’ has been registered and categorized in Japan (Okamoto et al., 2006). Dried leaf powder of this plant (700 mesh, 20 μm size, specified), cultivated, processed, and marketed in Kyushu Yatsushiro area (Kumamoto, Japan), was obtained from Inada Co., Ltd. (Kumamoto, Japan) in March 2011. A *matcha* product (Fukuoka, Japan), one of Kyushu Yame-branded green tea powders, was purchased at a retail store (Kumamoto, Japan). Hyaluronic acid sodium salt from *Streptococcus equi*, hyaluronidase from bovine testis type IV-S, and lipopolysaccharide (LPS) from *Escherichia coli* were products of Sigma-Aldrich Co. (St. Louis, MO, USA). Lipoxygenase (lipoxidase) from soybean was a product of Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). RAW 264.7 mouse macrophage-like cells (ECACC91062702) and fetal bovine serum (FBS) were from DS Pharma Biomedical Co. Ltd. (Osaka, Japan) and Gibco-Invitrogen (Carlsbad, CA, USA), respectively. Cell-counting kit-8 was from Dojindo Labs (Kumamoto, Japan). All other chemicals were of the highest grade commercially available.

Extracts of soft rush powder were prepared by hot-water and ethanol extractions as described with a slight modification (Oliveira et al., 2009). Briefly, 5.00 g of soft rush powder was extracted with 50 mL of MilliQ water at 95 – 100°C for 20 min. After centrifugation at 2,500 x g for 10 min, the precipitate was extracted twice more. The hot-water extract was obtained from the filtrate of these supernatants after lyophilization. For the ethanol extract, 5.00 g of another soft rush powder was extracted with 50 mL of ethanol at room temperature for 10 min. After centrifugation at 2,500 x g for 10 min, the residual soft rush was successively extracted twice more. The ethanol extract was obtained from the filtrate of these supernatants after vacuum evaporation. Two more extracts of *matcha* green tea powder were similarly prepared.

Amount of total polyphenol was determined by an established procedure (Singleton and Rossi, 1965). The assay mixture was subjected to colorimetric measurement at 600 nm using a grating microplate reader (SH-1000Lab; Corona Electric, Ibaraki, Japan). Chlorogenic acid was used as the standard sample for a calibration curve. Background absorption for each determination was carried out to control for the interference of photometric absorption of samples/reagents.

Amount of total flavonoid was determined by an established procedure (Chang et al., 2006). The assay mixture was subjected to colorimetric measurement at 510 nm. (+)-Catechin was used as the standard sample.

Amount of tannin was determined by the ferrous-tartrate method (Sriwilaijaroen et al., 2012). The assay mixture was subjected to colorimetric measurement at 540 nm. Ethyl gallate was used as the standard sample.

Lipoxygenase assay was performed based on the decolorization of methylene blue during the enzymatic reaction (Nishida et al., 2014). The reaction was started by enzyme addition, allowed to proceed for 0 min and 30 min at room temperature in a 96-well multiplate. The assay mixture was used for colorimetric measurement at 660 nm.

Hyaluronidase assay was performed using hyaluronic acid as the substrate, based on the detection of liberated N-acetyl glucosamine end group (Nishida et al., 2014). The reaction was started by the addition of hyaluronic acid in the standard assay mixture containing hyaluronidase for 40 min at 37°C. Colorimetric measurement was carried out at 585 nm with *p*-dimethylaminobenzaldehyde reagent.

For the cellular NO generation assay, RAW 264.7 mouse macrophage-like cells, seeded in individual wells of a 96-well culture plate at a density of 0.5 x 10^4 cells in 100 μL per well. Cells were pre-incubated for 3 h, and thereafter incubated with another
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Table 1. Yield of hot-water and ethanol extracts from edible *igusa* soft rush powder or *matcha* green tea powder, and amounts of total polyphenol, total flavonoid, and tannin.

<table>
<thead>
<tr>
<th>Yield of extract (%)</th>
<th>Polyphenol (CAE)</th>
<th>Flavonoid (CE)</th>
<th>Tannin (EGE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft rush, hot-water extract</td>
<td>6.44</td>
<td>84.6 ± 7.0<em>a</em></td>
<td>27.2 ± 1.0<em>b</em></td>
</tr>
<tr>
<td>Soft rush, ethanol extract</td>
<td>3.32</td>
<td>82.9 ± 4.8<em>a</em></td>
<td>21.5 ± 0.4<em>b</em></td>
</tr>
<tr>
<td>Matcha, hot-water extract</td>
<td>32.9</td>
<td>298 ± 15<em>b</em></td>
<td>47.3 ± 2.1*</td>
</tr>
<tr>
<td>Matcha, ethanol extract</td>
<td>7.84</td>
<td>205 ± 9*</td>
<td>31.5 ± 1.2<em>c</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amount (µg per mg extract)</th>
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<tbody>
<tr>
<td>Soft rush, hot-water extract</td>
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<tr>
<td>Soft rush, ethanol extract</td>
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<tr>
<td>Matcha, hot-water extract</td>
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<tr>
<td>Matcha, ethanol extract</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Amount (g per 100 g material powder)</th>
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<tbody>
<tr>
<td>Soft rush, hot-water extract</td>
</tr>
<tr>
<td>Soft rush, ethanol extract</td>
</tr>
<tr>
<td>Matcha, hot-water extract</td>
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<tr>
<td>Matcha, ethanol extract</td>
</tr>
</tbody>
</table>

Data shown represent mean ± S.D. of four experiments. Values not sharing a common superscript letter are considered significantly different at *P* < 0.05. CAE, chlorogenic acid equivalent; CE, (+)-catechin equivalent; EGE, ethyl gallate equivalent.

culture medium (25 µL) containing individual test samples plus 100 ng/mL LPS. After incubation for 24 h, the culture medium was collected by centrifugation and the level of NO therein was measured by the Griess method (Wang et al., 2008). Briefly, the collected culture medium (100 µL) was mixed with the same amount of Griess reagent in a new 96-well microplate. The level of NO generated was determined colorimetrically at 550 nm. Cytotoxicity was determined using the commercial Cell-counting kit-8.

Measurement of iNOS activity was conducted as previously reported (Hu et al., 2008). Briefly, RAW264.7 cells were stimulated with 100 ng/mL LPS for 24 h. Thereafter, cells were washed with fresh medium to deplete the stimuli, and incubated in the presence of test samples plus 1 µg/mL cycloheximide for an additional 24 h. The supernatants were collected and the level of NO, the end product of cellular iNOS, was measured by the Griess method.

For statistical analysis, the values are expressed as mean ± standard deviation of four different experiments. Data in part were analyzed using the statistical add-on software program (Statcel; OMS Co., Saitama, Japan) for Excel 2004 (Microsoft Corp., Redmond, WA, USA). Statistical differences were considered significant at *P* < 0.001 using a Student’s *t*-test.

For multiple comparisons, the presence of interaction/influence between two categories of test samples by two-way analysis of variance (ANOVA) was first confirmed. A post-hoc Bonferroni-Dunn test with one-factor ANOVA was next conducted and considered significant at *P* < 0.05.

Results and Discussion

Hot-water and ethanol extractions yielded 0.322 g (6.44%) and 0.166 g (3.32%), respectively, from 5.00 g of soft rush powder (Table 1). For *matcha* powder, hot-water and ethanol extractions yielded 1.01 g (32.9%) from 3.07 g and 0.240 g (7.84%) from 3.06 g of the powder, respectively. Lower yields in soft rush may be due to insoluble ingredients such as dietary fiber (Morita et al., 2002). The extraction time from 20 to 40, or even 80 min with hot water results in highly efficient solubilization of many catechins and caffeine from green tea leaves (Ziaedini et al., 2010). Because the extraction method for soft rush is not widely established, hot-water extraction for 20 min was carried out in this study. Notably, ethanol, water, methanol and aqueous ethanol, in this order, have been frequently used as solvents for extracting polar compounds such as phenolic compounds (Alam et al., 2013). Among them, ethanol is an organic and nontoxic solvent. Thus, it might have the highest frequency of use for extraction purposes. To obtain these food concentrates and compare their functionality, we chose hot-water and ethanol extractions in this study.

As compiled in Table 1, the phenolic parameters of the extracts were determined. In soft rush, the amounts of total polyphenol in 1 mg of hot-water and ethanol extracts were 84.6 µg and 82.9 µg as chlorogenic acid equivalent, respectively. Amounts of total flavonoid in 1 mg of hot-water and ethanol extracts were 27.2 µg and 21.5 µg as (+)-catechin equivalent, while the levels of tannin were 10.6 µg and 2.53 µg as ethyl gallate equivalent, respectively. Amounts of these phenolic parameters were significantly lower than those for *matcha*. When extraction yield is taken into consideration (Table 1), 0.545 g and 0.275 g of polyphenol can be extracted from 100 g of soft rush by hot-water and ethanol extractions, respectively. Thus, hot-water and ethanol extractions from 100 g of soft rush yield 0.175 g and 0.0714 g of flavonoid, and 0.0686 g and 0.00838 g of tannin, respectively. Notably, luteolin, a type of flavonoid, and its derivatives are the characteristic chemicals present in Juncaceae plants (Williams and Harborne 1978; Ishiuchi et al., 2015). Meanwhile, *matcha* possesses green tea-specific catechin flavonoids such as EGCG (Weiss et al., 2003).
We first investigated whether soft rush can inhibit lipoxygenase activity. The hot-water and ethanol extracts demonstrated concentration-dependent inhibitions and reached a maximum of 89.6% and 98.1% in the enzymatic assay, respectively (Fig. 1A). The calculated half-maximal inhibitory concentrations (IC$_{50}$) were 123 μg/mL by hot-water extract and 143 μg/mL by ethanol extract. The standard sample, nordihydroguaiaretic acid (NDGA), showed an IC$_{50}$ of 4.65 μg/mL. Hot-water and ethanol extracts from matcha also demonstrated concentration-dependent inhibitions, with IC$_{50}$ of 138 μg/mL and 145 μg/mL, respectively (Fig. 1B). Among IC$_{50}$ values obtained from these four extracts, no significant difference was observed by multiple comparison test. It is necessary to account for the extraction yield (see Table 1) when presenting IC$_{50}$ values for powdered extracts. When extraction yield is taken into account, hot-water and ethanol extractions of soft rush, respectively, provided IC$_{50}$ of 1.91 mg/mL and 4.31 mg/mL at the powder level. The corresponding IC$_{50}$ of matcha are lower at 0.419 mg/mL and 1.85 mg/mL, respectively. Previous reports have indicated that inhibitors of lipoxygenase play a suppressive role in acute inflammation (Yamazaki et al., 1998) and histamine-induced extravasation in vivo (Giannaras et al., 2005). Intake of green tea catechins suppresses acute inflammation in vivo through lipoxygenase inhibition (Choi et al., 2002). However, the bioactive components of soft rush extracts responsible for the inhibitory effect in this assay require clarification.

We next investigated whether soft rush can inhibit hyaluronidase activity. The hot-water extract showed only 15.3% maximum inhibition at the concentration range from 500 to 2,500 μg/mL (Fig. 2A). In contrast, the ethanol extract demonstrated 88.7% of the maximum inhibition, with IC$_{50}$ of 1.33 mg/mL. The standard sample, tannic acid, showed an IC$_{50}$ value of 94.5 μg/mL. In addition, hot-water and ethanol extracts of matcha demonstrated concentration-dependent inhibitions and their calculated IC$_{50}$ values were 1.49 μg/mL and 1.16 mg/mL, respectively (Fig. 2B). The IC$_{50}$ value obtained from the ethanol extract of soft rush showed no significant difference vs. the IC$_{50}$ values obtained from two matcha extracts after a multiple comparison test. When extraction yield is taken into account, hot-water and ethanol extractions of soft rush, respectively, provide IC$_{50}$ of 38.8 mg/mL and 40.1 mg/mL, while those IC$_{50}$ of matcha are lower at 4.53 mg/mL and 14.8 mg/mL at the powder level. Previous studies demonstrate that patients with burns, septicemia, or shock have rapid increases in circulating hyaluronic acid levels because of hyaluronidase activation (Engstrom-Laurent and Hellstrom, 1990; Ferrara et al., 1991; Onarheim et al., 1991). Tea polyphenol catechin acts as an anti-inflammatory by inhibiting
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Macrophages derived from blood monocytes play an important role in defense. When macrophages are activated by cytokines and/or microbial compounds, excess NO can be generated; thus, NO level reflects the inflammation state of macrophages (Nathan 1992; Bogdan 2001). To gain insight into the physiological relevance of soft rush and its potent anti-inflammatory effects, RAW264.7 mouse macrophage-like cells were incubated for 24 h in the presence of these extracts plus LPS. We performed a cytotoxic assay using a commercial kit. With concentrations ranging from 25 to 500 μg/mL, hot-water and ethanol extracts from soft rush demonstrated no significant cytotoxicity until 250 μg/mL and 100 μg/mL, respectively (data not shown). Similarly, hot-water and ethanol extracts from matcha showed no significant cytotoxicity until 250 μg/mL and 100 μg/mL, respectively (data not shown). Under these non-toxic concentrations, the extracts were subjected to a cell-based NO production assay.

Incubating the cells in the presence of two soft-rush extracts demonstrated concentration-dependent and significant inhibitions (Figs. 3A and 3B). The calculated IC$_{50}$ values of hot-water and ethanol extracts were 120 μg/mL and 35.2 μg/mL, respectively. This result revealed a 3.4-times stronger inhibitory effect of the ethanol extract than the hot-water extract. The inhibitory effects of matcha extracts were much weaker than for soft rush (Figs. 3C and 3D). The IC$_{50}$ values of these extracts were estimated to be 250 μg/mL or more. These results suggest the greater utility of soft rush than matcha for suppressing macrophage-oriented inflammation. When extraction yield is taken into account, hot-water and ethanol extractions of soft rush, respectively, provide IC$_{50}$ values of 1.86 mg/mL and 1.06 mg/mL, representing a 1.8-times stronger inhibitory effect of the ethanol extraction than the hot-water extraction. Our preliminary study demonstrated NO production in activated macrophages could be strongly suppressed by lipoxygenase inhibitor NDGA (data not shown), which was supported by another report (Imai et al., 1993). Therefore, soft rush and its possible constituent(s) may, in part, contribute to the concomitant attenuation of NO and lipoxygenase pathways in macrophage cells.

In activated macrophages, excess NO is generated by iNOS (Nathan, 1992; Bogdan, 2001). It is of interest to investigate...
whether soft rush can suppress the iNOS catalytic activity. To gain insight into the NO suppressing mechanisms, our next challenge was to examine the effect of these extracts on iNOS enzymatic activity. Under this experimental setting, iNOS protein in RAW264.7 cells was pre-stimulated for 24 h, and further induction was blocked by the addition of the translation inhibitor cycloheximide (Hu et al., 2008). All four extracts from soft rush and matcha showed no inhibitory effect against iNOS enzymatic activity in RAW264.7 cells (data not shown). Statistical analyses showed no significant differences in these test samples vs. control. Nevertheless, further investigation is needed to determine iNOS mRNA and protein expression as well as other inflammation-related cytokines for delineating the anti-inflammatory mechanisms in detail. As a possible interpretation, soft rush might play a role as an NO scavenger. Further study is needed to clarify the bioactive compounds present in soft rush responsible for the anti-inflammatory effects in these assays.

The bioactive compounds present in the hot-water and ethanol extracts of soft rush powder responsible for the anti-inflammatory effects remain to be fully clarified. Comparison between determined phenolic parameters (see Table 1) and biological activities (see Fig. 1) in these two soft rush extracts leads to an interpretation that polyphenols and flavonoids may be the target compounds possessing lipoxygenase inhibitory activity due to their comparable values at the extract level. Based on our results, low amounts of tannin might interfere with hyaluronidase activity and cellular NO generation (see Table 1 and Figs. 2, 3). Interestingly, similar trends were observed between the two extracts from matcha.

As described in previous reports, luteolin was isolated and identified as one of the major phenolic flavonoids of soft rush (Williams and Harborne 1978; Ishiuchi et al., 2015), while catechin and EGCG are the major phenolic flavonoids/tannin from green tea leaves (Weiss et al., 2003). To gain insight into the active compounds, these three compounds were examined by the lipoxygenase and cellular NO generation assays. For the lipoxygenase assay, different concentrations of luteolin (ranging from 0, 1 to 100 μM) demonstrated a dose-dependent inhibition with IC50 of 7.91 μM, while catechin and EGCG (ranging from 0, 5 to 500 μM) showed enzyme inhibitions with IC50 of 277 μM and 31.2 μM, respectively (data not shown). For the cellular NO production assay, luteolin (ranging from 0, 2.5 to 50 μM) showed 6.34% NO productivity at 50 μM, with IC50 of 2.63 μM. Because catechin and EGCG respectively, demonstrated 61.6% and 69.6% NO productivity at 50 μM, their IC50 values were estimated to be greater than 50 μM (data not shown). Our results support a previous paper in which luteolin strongly inhibited lipoxygenase and cellular NO generation compared to catechin among different classes of flavonoids (Lee and Kim, 2010). Therefore, luteolin may be a good candidate and may play an important role in the anti-inflammatory mechanism(s) of soft rush in these assays. Notably, catechin, but not luteolin, inhibited hyaluronidase activity (Lee and Kim, 2010). As summarized in a review paper, a wide range of major/minor constituents have been identified in soft rush plants, i.e., flavonoids, coumarins, terpenes, stilbenes, sterols, phenolic acids, carotenoids, phenanthrenes and their derivatives (El-Shamy et al., 2015). Nevertheless, qualitative and quantitative analyses of luteolin as well as other possible biologically active candidates such as flavonoid/tannin are needed to prove the functional differences between hot-water and ethanol extracts of soft rush.

It is notable that undesired cytotoxic effects of soft rush and matcha were found at the higher concentrations tested; 67% and 79% cell viability with 500 μg/mL hot-water extracts of soft rush and matcha, respectively. In the case of ethanol extracts, both samples showed greater toxicity of 45% and 42% cell viability at 250 μg/mL, respectively. Matcha has a long history of safety and continues to be popularly consumed at present in Japan. However, a systematic review was undertaken of possible risks and adverse side effects resulting from the intake of highly concentrated green tea supplements (Sarma et al., 2008). Thus, the effectiveness and safety of soft rush powder or green tea should be given proper consideration when determining the optimal dosage.

In this report, we demonstrated the in vitro anti-inflammatory effects of extracts of edible soft rush powder using a lipoxygenase assay and an activated macrophage cell model. Hot-water and ethanol extracts from soft rush and matcha showed comparable lipoxygenase inhibition. Incubating LPS-activated macrophages in the presence of two extracts from the soft rush strongly suppressed NO production compared to the matcha extracts. However, it should be noted that this study has examined efficacy at the extract level. Extensions of this work are warranted to fully elucidate the multi-faceted contributions of this unique agricultural plant for beneficial health use.

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References
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