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Metabolomic screening of anti-inflammatory compounds in *Acanthopanax sessiliflorus* fruit (Ogaza) extract

Gyoung-Deuck Kim[1†], Jiho Lee[1†] and Joong-Hyuck Auh[1*]

Abstract
This study investigated the anti-inflammatory compounds in Ogaza, *Acanthopanax sessiliflorus* fruit, and their extracts using metabolomic screening. Ogaza extracts were obtained in various solvents, such as 70% ethanol, 70% methanol, and water. The anti-inflammatory activity was estimated by evaluating nitric oxide production in lipopolysaccharide (LPS)-induced RAW 264.7 cells treated with the extracts. The 70% ethanol extract (EO) showed the most effective anti-inflammatory activity, inhibiting nitric oxide production by approximately 50% and downregulating iNOS expression. The 70% ethanol extract was further fractionated into three partial subfractions by preparative LC to identify the anti-inflammatory compounds. Assessment of the anti-inflammatory activity of each subfraction revealed that the third subfraction (E-F3) showed the highest inhibitory activity against nitric oxide. E-F3 effectively suppressed iNOS expression. Subsequently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and multivariate statistical analyses were performed to identify the compounds that significantly contributed to the anti-inflammatory activity of the Ogaza extract. Fourteen and 16 compounds in the negative- and positive-ion modes, respectively, were identified as significant constituents of Ogaza. Compounds like quercetin, hyperoside, acanthoside D, oleanolic acid, and scopoletin were identified as potential anti-inflammatory components in Ogaza extract. This study characterized the functional properties of *Acanthopanax sessiliflorus* fruit and indicated the possibility that other compartments of *Acanthopanax sessiliflorus* may also serve as natural sources of nutraceuticals.

Keywords Anti-inflammatory, *Acanthopanax sessiliflorus*, Hyperoside, Nutraceutical, Metabolomic

Introduction

In the food industry, bioactive compounds have gained prominence owing to their potential health benefits, and interest in their various physiological and functional activities is on the rise. As a part of this trend, many studies have assessed physiologically active substances, including phytochemicals exhibiting antioxidant, anti-inflammatory, antihypertensive, and immune-related activities [1, 2].

The inflammatory response is an essential protective mechanism. It helps defend the body by removing harmful irritants and repairing damaged tissue [3].

†Gyoung-Deuck Kim and Jiho Lee contributed equally to this work.

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Macrophages help identify the cause of inflammation, regulate the inflammatory response, and minimize cellular damage to maintain homeostasis. Nitric oxide (NO) is recognized for its ability to eliminate bacteria and tumor cells. Excessive NO production can lead to cellular damage and genetic mutations. The inflammatory response is an essential defense mechanism that can cause various diseases. Unlike acute inflammation, chronic inflammation is characterized by the overproduction of inflammatory mediators and diseases such as cardiovascular disorders, cancer, and chronic liver disease. Therefore, regulation of the inflammatory response is necessary to maintain the body's physiological balance.

Non-steroidal anti-inflammatory drugs (NSAIDs) are used instead of steroids to suppress inflammatory responses and relieve various symptoms such as pain and fever caused by inflammatory reactions. However, NSAIDs also cause adverse effects, such as gastrointestinal diseases; therefore, there is a growing interest in anti-inflammatory active substitutes from natural products, which are considered relatively less toxic and cause fewer adverse effects.

Acanthopanax species belong to the Araliaceae family and have traditionally been used as oriental medicinal herbs in Asian countries, including China, Japan, and Korea. *Acanthopanax sessiliflorus* is a native Korean Acanthopanax species. Previous studies have reported various physiological activities of the stem, bark, and roots, including anti-inflammatory [4], antioxidant [5], antitumor [6], hepatoprotective [7], and immunostimulatory effects [8]. The active compounds identified were anthocyanins, chiisanoside, eleutheroside, hyperin, sesamin, and rutin [9–11].

Recent studies have revealed that Ogaza, the fruit of *A. sessiliflorus*, which has received limited research attention compared to other plant components, harbors numerous bioactive compounds exhibiting a range of beneficial functional activities, including anti-aggregation, anti-inflammatory, antitumor, and anti-hypertension properties [10–13]. In 2019, Ogaza gained official approval as a functional food ingredient for the treatment of hypertension in Korea. In previous studies, the anti-inflammatory activity of purified triterpenoids and lignans from the 70% ethanol extract was evaluated; these compounds were identified using nuclear magnetic resonance studies, and their effectiveness was assessed by their ability to inhibit NO production [9, 14]. Additionally, the antioxidant and anti-inflammatory effects of an 80% methanol extract of Ogaza have been demonstrated [15].

Given this background, the aim of this study was to analyze the properties of Ogaza extracts obtained using 70% ethanol and water, and to compare their anti-inflammatory activities in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Furthermore, the 70% ethanol extract of Ogaza was fractionated by Prep-LC, enabling the observation of its capacity to inhibit iNOS expression, an established marker of inflammation. Through liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, anti-inflammatory compounds of Ogaza were screened, and significant compounds were identified by metabolomic screening, thereby substantiating their potential as functional materials.

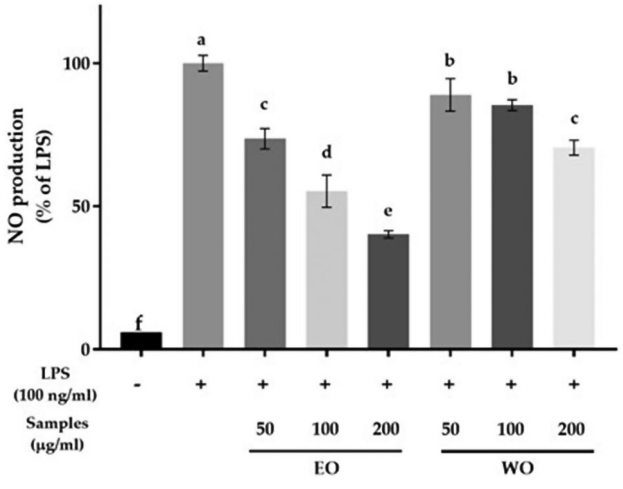

Fig. 1 Anti-inflammatory activities of different solvent extracts of Ogaza. The cells were treated with LPS and 50, 100, or 200 μg/mL of EO or WO. EO: 70% ethanol extract of Ogaza, WO: water extract of Ogaza, LPS: lipopolysaccharide. All experiments were performed in triplicate and the data were statistically assessed. Values with different superscripts are significantly different ($p < 0.05$)

Results

Anti-inflammatory activity of the extracts: inhibitory effect on NO production and iNOS expression in LPS-induced RAW 264.7 cells

The extract yield was calculated as percentage (%) of 5 g of Ogaza powder using dry weight. The extract yield was the highest with water (WO, 41.63±1.82%), followed by the 70% EtOH extract (EO, 40.70±2.68%), and then the 70% MeOH extract (MO, 34.12±2.27%). Thus, 70% EtOH and water were efficient solvents to extract compounds in Ogaza.

NO is produced by iNOS, which is expressed by immunological stimuli such as LPS, IL-1, and TNF-α. NO production is an indicator of inflammatory responses. The anti-inflammatory activity was measured based on the differences in NO production between cells treated with LPS alone and those treated with LPS and the extract samples. The highest anti-inflammatory activity was observed after 70% ethanol extract (EO) treatment, followed by water extract (WO) treatment. The decrease in

NO production in both groups was proportional to the extract concentration (Fig. 1).

iNOS expression plays a key role in the inflammatory response by generating NO from oxygen and L-arginine. To investigate the anti-inflammatory activity of the extracts, we assessed the inhibition of iNOS expression by EO and WO treatment using western blotting of LPS-induced RAW 264.7 cells. The expression of iNOS significantly increased in LPS-treated RAW 264.7 cells, and EO and WO treatment suppressed iNOS expression in a dose-dependent manner (Fig. 2). Notably, cells treated with EO showed twice the inhibition in iNOS expression compared to cells treated with LPS alone. The highest anti-inflammatory activity was observed with EO treatment, followed by WO treatment. Therefore, the metabolites from EO are considered to exhibit the highest anti-inflammatory activity in LPS-induced RAW 264.7 cells.

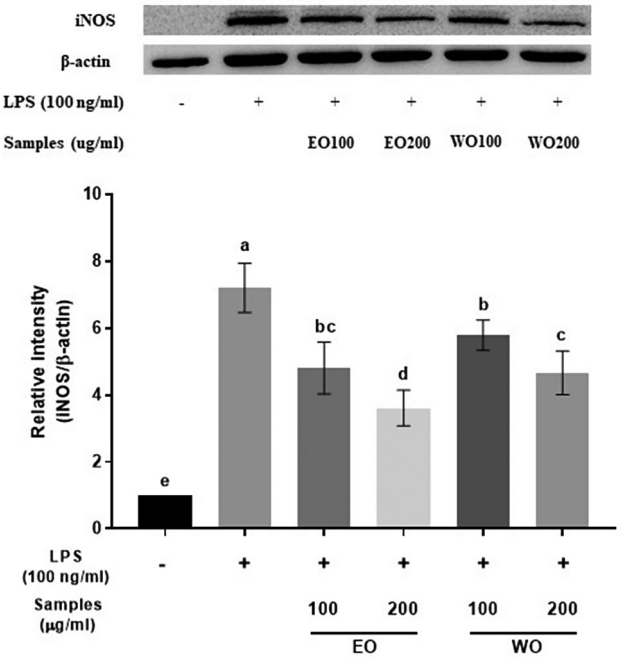

Fig. 2 Inhibition of iNOS expression by treatment with different solvent extracts of Ogaza. Cells treated with 100 and 200 μg/mL of EO and WO showed inhibition of iNOS expression. All experiments were performed in triplicate, and the data were quantified by a CS analyzer and statistically assessed. Values with different superscripts are significantly different ($p < 0.05$). EO: 70% ethanol extract of Ogaza; WO: water extract of Ogaza; LPS: lipopolysaccharide

Fractionation of the 70% ethanol extract of Ogaza (EO)

To identify the metabolites responsible for the high anti-inflammatory activity of Ogaza extract, fractionation was performed using preparative (Prep)-LC (Fig. 3). EO was separated into three subfractions based on their UV chromatograms. The first subfraction, labeled E-F1, was collected during the initial 6 min of elution. The second subfraction, labeled E-F2, was collected between 6 and 12 min of elution. The third subfraction, labeled E-F3, was collected during the 12 to 18 min of elution. The

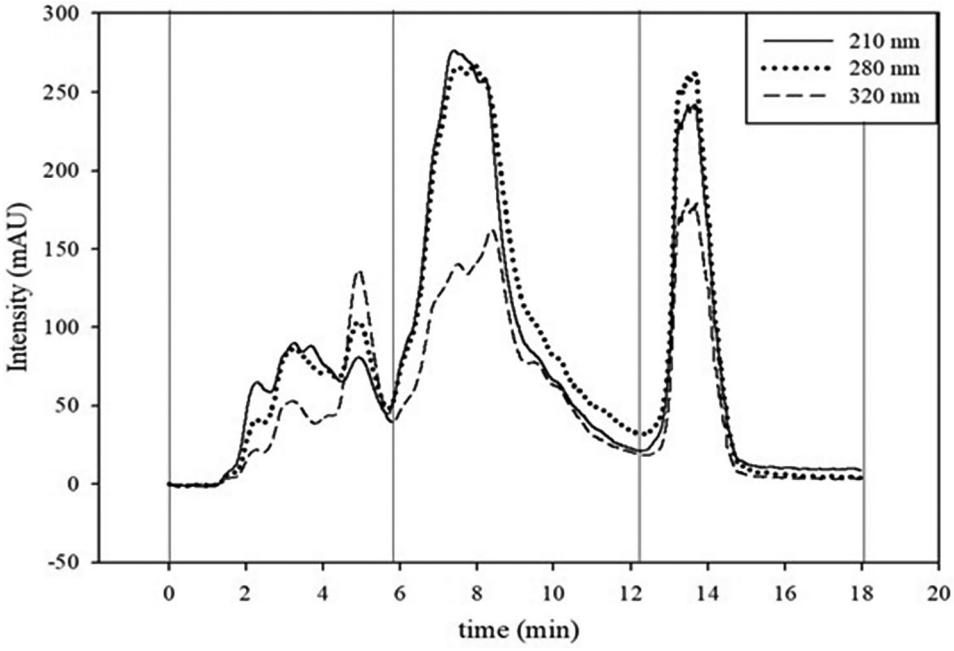

Fig. 3 Fractionation of the 70% ethanol extract. The 70% EtOH extract of Ogaza was fractionated into three parts (E-F1, E-F2, and E-F3) by Prep-LC. E-F1 is the first part of the fractionated 70% ethanol extract; E-F2 is the second part of the fractionated 70% ethanol extract; and E-F3 is the third part of the fractionated 70% ethanol extract

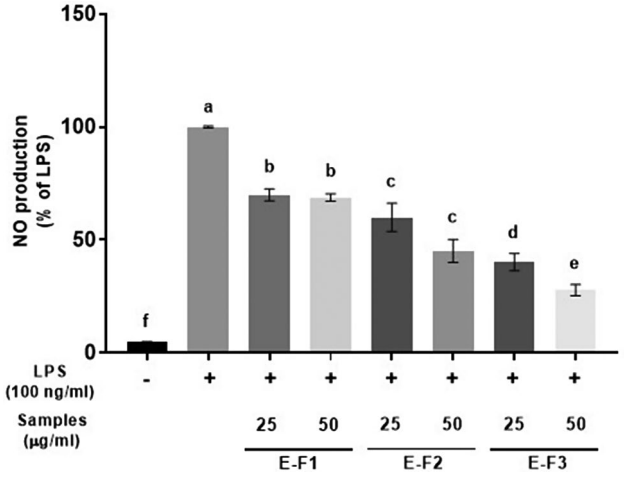

Fig. 4 Anti-inflammatory activity of fractions of Ogaza. All experiments were performed in triplicate, and the data were statistically assessed. E-F1 is the first part of the fractionated 70% ethanol extract; E-F2 is the second part of the fractionated 70% ethanol extract; and E-F3 is the third part of the fractionated 70% ethanol extract. LPS: lipopolysaccharide

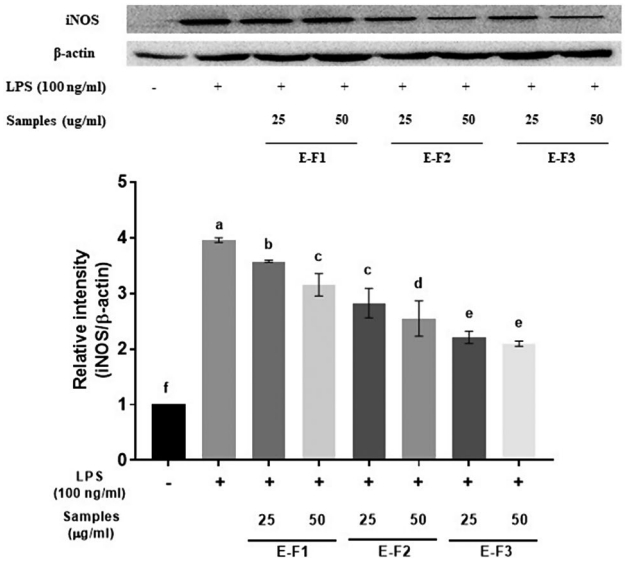

Fig. 5 Inhibition of iNOS expression by fractions of Ogaza. All experiments were performed in triplicates, and data were quantified by a CS analyzer and statistically assessed. Values with different superscripts are significantly different ($p < 0.05$). E-F1 is the first part of the fractionated 70% ethanol extract, E-F2 is the second part of the fractionated 70% ethanol extract, and E-F3 is the third part of the fractionated 70% ethanol extract. LPS: lipopolysaccharide

fractions showed different color characteristics: E-F1 was bright yellow, E-F2 was dark violet, and E-F3 was brighter and more transparent than E-F2. Hydrophilic compounds were separated in E-F1, while more hydrophobic compounds were eluted from E-F3.

Anti-inflammatory activity of the fractions: inhibitory effect on NO production and iNOS expression in LPS-induced RAW 264.7 cells

The yield of the fractions was described as the percentage (%) of the dry weight of 500 mg of 70% ethanol extract powder. The yields of E-F1, E-F2, and E-F3 were 80.17±3.96%, 2.14±0.06%, and 4.84±0.57%, respectively.

LPS-induced RAW 264.7 cells were treated with E-F1, E-F2, or E-F3 to evaluate their anti-inflammatory effects. The anti-inflammatory activities of E-F1, E-F2, and E-F3 are shown in Figs. 4 and 5. Compared with LPS-induced cells, cells treated with EF-1 and LPS showed 30% lower NO production, those treated with E-F2 and LPS showed 50% lower NO production, and those treated with E-F3 and LPS showed a considerable reduction of more than 60% (Fig. 4). Thus, the compounds present in E-F3, the hydrophobic fraction of EO, were determined to be significant anti-inflammatory metabolites in Ogaza. Therefore, the anti-inflammatory compounds extracted from 70% ethanol were more hydrophobic than hydrophilic.

Western blotting results showed a similar tendency to that observed for NO inhibition. While the expression of iNOS was inhibited by all subfractions, E-F3 showed the highest activity, and iNOS expression in cells treated with E-F3 was two-fold lower than that in LPS-treated cells. E-F3 exhibited the strongest inhibitory activity (Fig. 5).

Metabolomic analysis using multivariate statistical methods for the identification of the active metabolites in the fractions

An LC-MS/MS analysis was performed to identify the anti-inflammatory compounds in Ogaza. The results of multivariate statistical analysis indicated distinctive anti-inflammatory metabolites in Ogaza extract. The PCA plots of the subfractions showed two components that explained 98.1% and 93.6% of the variations with a total of 144 metabolites in negative ion mode and 189 metabolites in positive ion mode, respectively (Fig. 6). E-F3 was distinguished by an 80.7% principal component 1 (PC1) variance in the negative-ion mode. In the positive-ion mode, PC1 accounted for 74.6% of the variance. E-F3 was separated from E-F1 and E-F2 by PC1, whereas PC2 distinguished between E-F1 and E-F2 in both ion modes.

To determine the effective anti-inflammatory compounds in Ogaza extract, the S-plot and VIP plot derived from OPLS-DA were utilized. E-F3 was compared to E-F1 and E-F2, and several compounds (VIP values≥1.5 and $p<0.01$) indicated significant differences in E-F3.

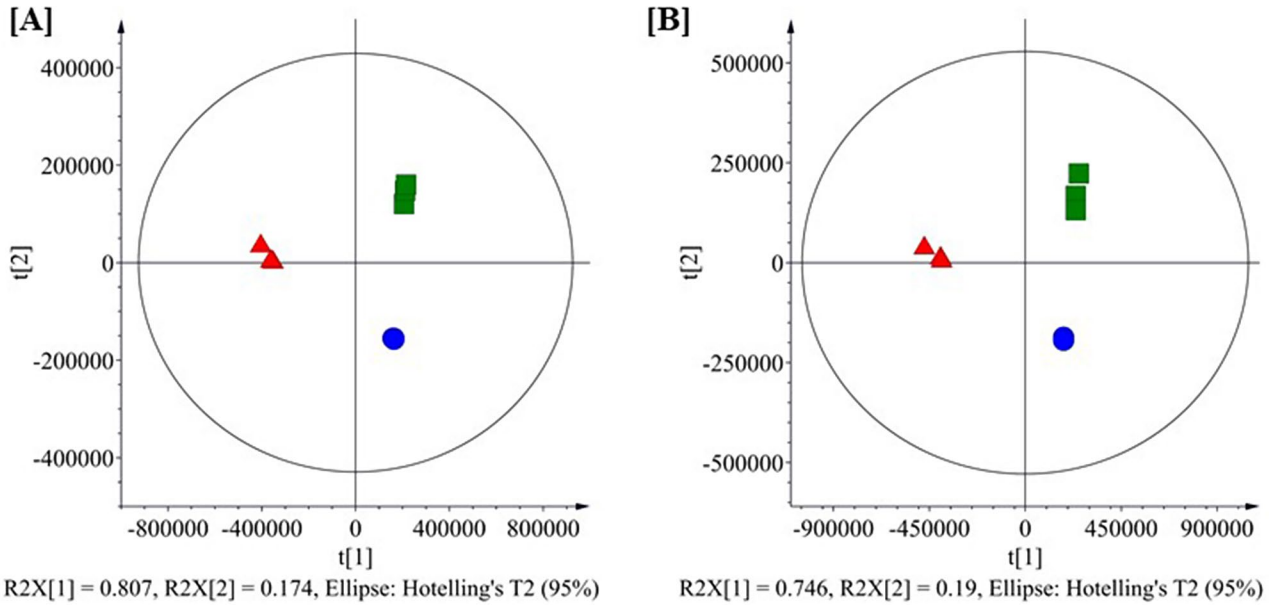

Fig. 6 The PCA score plot of the subfractions (**A**; negative-ion mode, **B**; positive-ion mode). (**A**) E-F1, E-F2, and E-F3 were divided into either side of the PCA plot by PC1 (80.7% of variance). EF-1 and EF-2 were separated by PC2 (17.4% of the variance). (**B**) E-F3, E-F1, and E-F2 were separated by PC1 (74.6% of the variance). E-F1 and E-F2 were separated by PC2 (19.0% of the variance). E-F1(●): first part of the fractionated 70% ethanol extract; E-F2(■): second part of the fractionated 70% ethanol extract; E-F3(▲): third part of the fractionated 70% ethanol extract

Overall, 14 and 16 compounds in the negative- and positive-ion modes, respectively, were selected as potential anti-inflammatory compounds in Ogaza (Fig. 7). The metabolites with m/z values of 329.233, 373.150, 461.166, 463.088, 499.306, 505.265, 517.316, 519.208, 787.266, 955.490, 999.480, and 1001.625 in the negative-ion mode and m/z values of 193.049, 303.049, 351.133, 401.159, 439.356, 453.343, 465.102, 467.315, 475.325, 483.310, 501.320, 519.331, and 1001.633 in the positive-ion mode were identified as the most characteristic markers of E-F3 in comparison with E-F1 and E-F2. Distinct compounds in E-F3 were tentatively identified to explain the anti-inflammatory activity of Ogaza. All identified compounds are indicated by their respective MS/MS fragments, VIP values, retention time (RT), and MS errors in Table 1.

Hyperoside (quercetin-3-O-galactoside, m/z 463.088), syringaresinol-diglucoside (Acanthoside D; m/z 787.266, $[M-H+FA]^-$), ganolucidic acid D (m/z 499.306), and unknown compounds including lignans and triterpenoids were identified in negative ion mode. Hyperoside (m/z 465.102), oleanolic acid (m/z 439.356, $[M+H-H_2O]^+$), quercetin (m/z 303.049), scopoletin (m/z 193.049), ganolucidic acid D (m/z 501.320), and unknown compounds were identified in the positive-ion mode. Syringaresinol-diglucoside (Acanthoside D) is a bioactive compound found in the leaves of *Acanthopanax sessiliflorus*. Syringin, also known as eleutheroside B, was detected in Ogaza [4], but it is not a potential anti-inflammatory compound. Rutin (quercetin-3-O-rutinoside) [16] was detected.

Discussion

In this study, the extracts of the fruit of *Acanthopanax sessiliflorus* (Ogaza) were obtained in different solvents, and the ethanol extract was fractionated into three subfractions. To determine the anti-inflammatory effect of Ogaza, these extracts, and subfractions were used to treat LPS-induced RAW 264.7 cells. The active compounds were identified using LC-HESI-Orbitrap-MS/MS and multivariate statistical analyses.

Five grams of pulverized Ogaza powder, equivalent to approximately 160 fruits or 2 bunches, was used for each extraction, and three biological replicates were conducted to ensure robust data and minimize the impact of biological variation. This approach is consistent with prior research, which analyzed metabolic characteristics using three biological replicates [17–19]. Thus, the small size of replicates in this study could be partially compensated by preparing pulverized biological samples, which can help mitigate the limitations of having small replicates.

The 70% ethanol extract showed anti-inflammatory activity without toxicity and was further purified into three subfractions using Prep-LC. Effective inhibition of iNOS expression was observed with the third subfraction of the 70% ethanol extract (E-F3). LC-MS/MS analysis and multivariate statistical analysis were used to identify 14 (negative-ion mode) and 16 (positive-ion mode) major

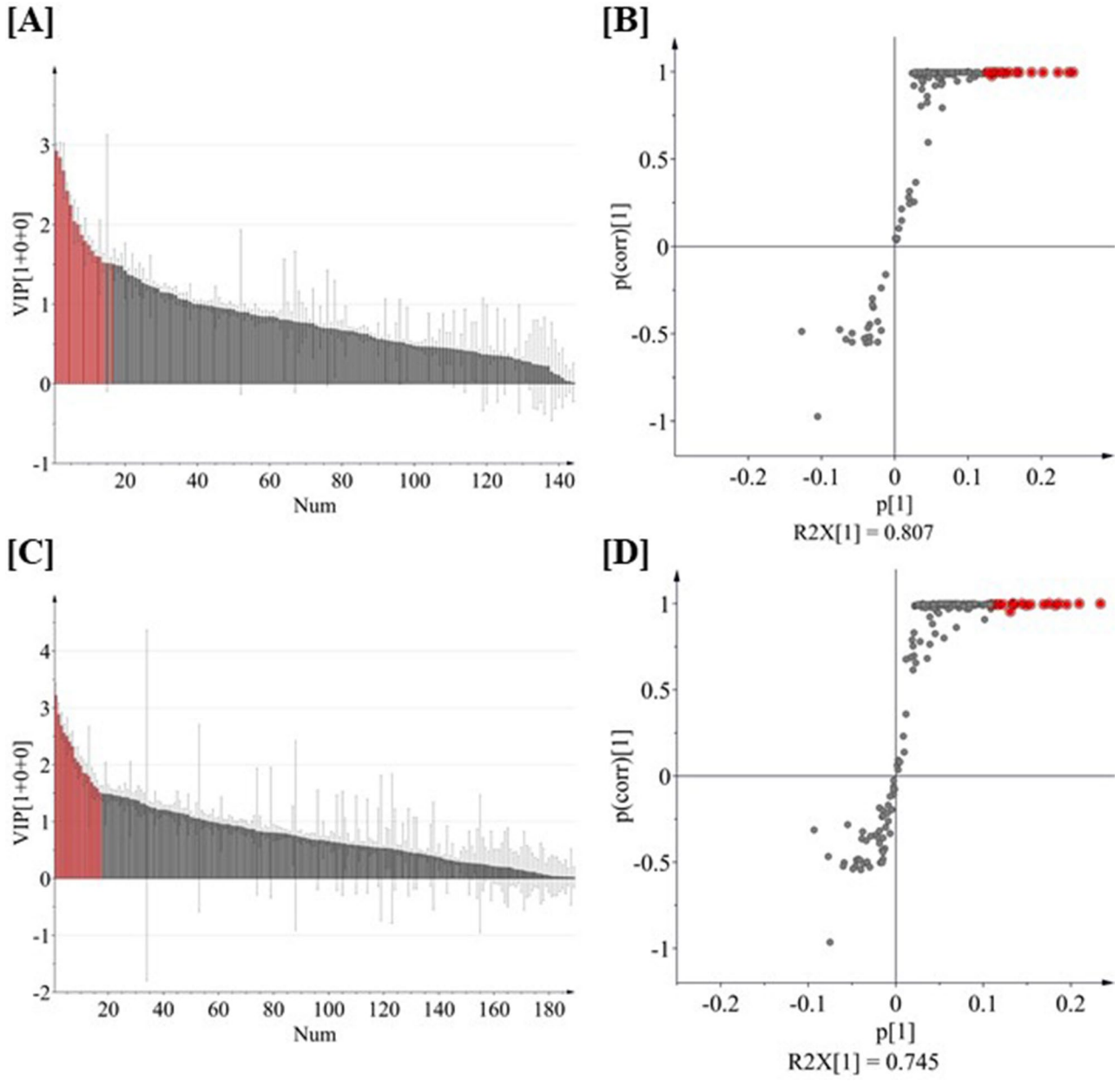

Fig. 7 The S-plot and the VIP plot generated by orthogonal partial least squares discriminant analysis (OPLS-DA) of E-F3 in comparison with E-F1 and E-F2 (**A**: the VIP plot of the negative-ion mode; **B**: the S-plot of the negative-ion mode; **C**: the VIP plot of the positive-ion mode; **D**: the S-plot of the positive-ion mode). E-F1 is the first part of the fractionated 70% ethanol extract; E-F2 is the second part of the fractionated 70% ethanol extract; and E-F3 is the third part of the fractionated 70% ethanol extract. LPS: lipopolysaccharide

compounds in E-F3, which showed the most remarkable anti-inflammatory effect, of which hyperoside, syringaresinol-diglucoside, ganolucidic acid D, oleanolic acid, quercetin, and scopoletin were identified as representative compounds in Ogaza.

Several compounds identified in this study, including flavonoids, lignans, triterpenoids, and coumarins, have anti-inflammatory properties. Quercetin is a flavonoid widely found in edible plants. Quercetin and its derivatives have various physiological activities, including antioxidant, anticancer, and anti-inflammatory activities. Various studies have reported that quercetin exhibits anti-inflammatory activities by inhibiting and blocking TNF-α-mediated proteins [20]. According to the previous study, hyperoside inhibited iNOS expression by inhibiting NF-κB translocation to the nucleus and IκB-α degradation [21]. Lignans, predominantly found in seeds and cereals, are a class of polyphenols known for their antioxidant properties. Thus, syringaresinol is a potential anti-inflammatory metabolite of Ogaza [9]. Cho et al.

Table 1 Tentative identification of potential anti-inflammatory compounds in Ogaza

Ion mode	m/z	RT (min)	Tentative identification	MS/MS fragment (acquired)	MS/MS fragment (reference)	Reference	Mass error	VIP score
+	193.049	9.93	Scopoletin	193.050, 133.029, 178.026	193.050, 133.028, 178.026	mzCloud*	-5.18	2.12
+	303.049	9.98	Quercetin	303.049, 304.053, 229.050	303.050, 304.053, 229.050	mzCloud	-3.30	2.50
-	329.233	13.44	Unknown	329.233, 171.102, 201.112	-	-	-	1.60
-	329.233	12.70	Unknown	211.133, 329.233, 171.102	-	-	-	2.42
+	351.133	11.86	Unknown	147.044, 119.049, 91.055	-	-	-	1.58
-	373.150	9.39	Unknown	59.013, 89.022, 71.013	-	-	-	1.67
-	393.212	10.28	Unknown	59.013, 101.023, 393.212	-	-	-	1.79
+	401.159	9.54	Unknown	315.086, 167.070, 330.109	-	-	-	2.04
+		10.42		315.086, 167.070, 330.109	-	-	-	1.62
+	439.356	14.41	Oleanolic acid	203.179, 191.180, 189.164	203.179, 191.179, 189.164	mzCloud	-2.28	1.98
+		13.16		203.179, 189.164, 191.179	203.179, 191.179, 189.164	mzCloud	-2.28	1.84
+	453.343	10.11	Unknown	100.112, 210.149, 182.154	-	-	-	2.69
-	461.166	8.83	Unknown	101.023, 71.013, 269.103	-	-	-	2.84
-	463.088	9.97	Hyperoside	300.027, 271.025, 301.034	300.027, 271.025, 225.030	MoNA**	0.00	2.68
+	465.102	9.97		303.050, 304.053, 85.029	303.050, 97.029, 85.029	mzCloud	-2.15	2.32
+	467.315	12.11	Unknown triterpenoid	133.101, 421.309, 119.086	-	-	-	1.69
+	475.325	10.11	Unknown	475.325, 476.328, 457.315	-	-	-	1.51
+	483.310	13.81	Unknown triterpenoid	133.101, 119.086, 121.101	-	-	-	3.22
-	499.306	13.80	Ganolucidic acid D	499.307, 87.008, 365.285	499, 481, 437	MoNA	-2.00	2.92
+	501.320		13.81	121.101, 133.101, 107.086	437.305, 483.310, 121.101	mzCloud	-1.99	2.41
+		12.81		133.101, 119.086, 107.086	437.305, 483.310, 121.101	mzCloud	-1.99	2.56
-	505.265	10.48	Caffeic acid derivative	59.013, 89.023, 71.013	-	-	-	1.52
-	517.316	12.81	Unknown	57.033, 517.347, 191.055	-	-	-	1.74
-	519.208	9.43	Unknown	59.013, 101.023, 71.013	-	-	-	2.24
+	519.331	12.81	Unknown	119.086, 185.132, 173.133	-	-	-	1.85
-	787.266	9.45	Syringaresinol-diglucoside	181.050, 417.155, 166.026	166.026, 181.050, 59.012	MoNA	-1.27	1.87
-	955.490	12.56	Unknown	485.327, 423.327, 486.332	-	-	-	2.04
-	999.480	12.11	Unknown	483.311, 484.315, 423.291	-	-	-	2.00
-	1001.625	13.81	Ganolucidic acid D dimer	499.306, 501.321, 500.310	437.305, 483.310, 121.101	mzCloud	(2.00)	1.51
+	1001.633	13.83		121.101, 133.101, 119.086	437.305, 483.310, 121.101	mzCloud	(-5.98)	2.88

* mzCloud : https://www.mzcloud.org/

** MoNA(Massbank of North America) : https://mona.fiehnlab.ucdavis.edu/

(2001) [22] reported that lignans from *Coptis japonica*, like pinoresinol, pinoresinol-glycosides, and syringaresinol-glycoside, differentially modulate inflammatory cell responses and may participate in anti-inflammatory processes by *C. japonica*. Syringaresinol-diglucoside, known as acanthoside D, has been isolated from the bark of *A. sessiliflorus* using an activity-guided approach and has anti-inflammatory activity [4]. Ganolucidic acid D and oleanolic acid are triterpenoids; only oleanolic acid exerts anti-inflammatory effects by downregulating TNF-α production and inhibiting NF-κB activation [23, 24]. Scopoletin is a coumarin that exhibits anti-inflammatory activity through the suppression of iNOS [25].

This study did not detect the previously reported representative anti-inflammatory compounds in *A. sessiliflorus*, such as acanthosessiligenin I and other compounds [7, 14, 26]. Lee et al. (2012) [15] isolated acanthosessiligenin I and other compounds by repeated fractionation using various organic solvents and columns. However, the samples used in this study were prepared relatively crudely to analyze the metabolome in the fruits of *A. sessiliflorus*, making it challenging to detect trace amounts of compounds. In this study, we screened Ogaza metabolites to identify a broader range of potential anti-inflammatory candidates compared to previous research. However, analyzing the individual compounds' anti-inflammatory activity proved challenging. Ogaza has demonstrated anti-inflammatory effects through diverse compounds, consistent with prior studies and our research findings. Future studies should focus on quantitatively comparing the anti-inflammatory activity of different Ogaza metabolites and determining the contribution of anti-inflammatory activity based on their composition.

Among the various inflammatory response pathways, iNOS induces NO production. Inflammatory mediators activate inflammatory pathways and cause cellular damage. In the present study, Ogaza extract inhibited

the inflammatory response by suppressing iNOS expression and NO production. Anti-inflammatory compounds inhibit inflammatory responses through various pathways. A previous study also reported the synergistic anti-inflammatory effects of quercetin and triterpenoid saponins [24]. Similarly, this study is expected to provide valuable data for further research on the anti-inflammatory active metabolites of Ogaza and the relevant signaling pathways. Additionally, this study characterized the functional properties of *A. sessiliflorus* fruit, suggesting the possibility that other parts of *A. sessiliflorus* may also serve as natural sources of functional materials.

Methods

Chemicals and reagents

Acanthopanax sessiliflorus fruit (Ogaza) was purchased from a local market in Gangwon, South Korea. Fresh Ogaza was freeze-dried and stored in a deep freezer (-80 ℃) until use. Dulbecco's modified Eagle medium (DMEM) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Biowest (Nuaille, France). 4-(2-Hydroxylethyl)-1-piperazine ethanesulfonic acid (HEPES), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (USA). LPS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. LC-grade solvents (methanol, ethanol, and water) were purchased from Honeywell (Burdick & Jackson, USA), while MS-grade acetonitrile, water, and formic acid (Thermo Scientific) were obtained from Thermo Fisher Scientific (USA).

Preparation of the extracts

The dried Ogaza was sourced from a local market in Gangwon Province, Korea, in 2021 and stored at -80 °C until used. The dried Ogaza were detached from their branches, and only the fruits were gathered and pulverized. Extracts of Ogaza powder (5 g, approximately 160 of dried fruits) were obtained using 100 mL of 70% ethanol, 70% methanol, and water by bath sonication (3150 R-DTH, Branson, USA) for 30 min. The extracts were centrifuged at 10,000 xg for 15 min, filtered with Whatman No. 1 filter paper (GE Healthcare, UK), concentrated with a rotary vacuum evaporator (Eyela, Japan) at 40 ℃, lyophilized to a dried powder, and stored at -50 ℃ until further experiments.

Partial purification of the extract

The 70% ethanol extract of Ogaza was fractionated by preparative LC (LC-Forte/R, YMC Co., Japan) using a YMC-DispoPack AT ODS (40 g, 25 μm, YMC Co.). The solvent system comprised distilled water (phase A) and LC-grade methanol (phase B) with a flow rate of 25 mL/min. The solvent gradient profile was as follows: 10% B for 3 min, 10–35% B for 5 min, 35% B for 10 min, and 35–100% B for 10.5 min; the run was completed in 18 min. The UV spectra were obtained at 210, 280, and 320 nm. The injection volume was 5 mL and contained 100 mg/mL of the sample.

Cell culture

Raw 264.7 cells were acquired from the Korean Cell Line Bank (KCLB, Seoul, USA). Cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES, and penicillin-streptomycin in a cell incubator at 37 ℃ in a humidified atmosphere with 5% CO_2. The cells were washed with DPBS and scraped for subculture, and 3×10^6 cells were cultured in a 10 cm dish. All cells used in this study were at passage 30.

Cell proliferation assay (MTT assay)

The MTT assay protocol described by Lee et al. [27]. was optimized in this study. RAW 264.7 cells (4×10^4 cells/well) were seeded in a 96-well plate and incubated for 12 h, and samples dissolved in dimethyl sulfoxide (DMSO) were treated for 20 h. Methylthiazoletertrazolium (1 mg/mL in DPBS, 20 μL) was added and incubated for 4 h. After removing all media, the remaining formazan crystals were dissolved in 200 μL of DMSO (Samchun Chemical, Korea). The absorbance was measured at 570 nm. Cells treated only with the medium constituted the control, and cytotoxicity was described based on the cell viability of the control.

Evaluation of anti-inflammatory activity (NO assay)

A modified NO assay protocol [28] was used. RAW 264.7 cells were seeded and incubated for 12 h. After removing the medium, the cells were treated with LPS (100 ng/mL), and the samples were dissolved in DMSO for 24 h. Subsequently, the supernatant media were collected, and 50 μL of the supernatant was mixed with a sulfanilamide solution (1% sulfanilamide [Samchun Chemical, Korea] in 5% phosphoric acid, 50 μL) for 10 min at room temperature in the dark. Then, NED solution (0.1% N-1-naphthyl ethylenediamine dihydrochloride [Kanto Chemical, Japan] in water, 50 μL) was added. The mixture was kept away from light and incubated at room temperature for 10 min. After incubation, absorbance was measured at 540 nm. Based on the sodium nitrite standard curve, the anti-inflammatory activity of the Ogaza extract samples was determined by measuring the NO production compared with that of LPS-treated cells.

Western blotting for the expression of inflammatory biomarkers

The western blotting assay was performed following a previously established method [28]. This assay was used to quantify proteins that indicate the occurrence of

inflammatory responses. For this assay, 8×10^5 cells were cultured in a 6-cm dish for 12 h and treated with the sample for 24 h. After 24 h of incubation, the cells were washed once with PBS (Bio-Rad) and harvested using 0.1% protease inhibitor added to PBS. The harvested cells were centrifuged at 13,000 × g for 5 min. Cellular proteins were extracted from cell pellets using a mammalian protein extract buffer (GE Healthcare, USA). After centrifugation at 13,000 × g for 15 min, 30 μg of protein was loaded onto an 8% polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and incubated with 5% BSA in TBST (0.075% Tween 20) for blocking.

The membrane containing the proteins was then treated with primary antibodies against iNOS (Invitrogen), COX-2 (Santa Cruz), and β-actin (Santa Cruz) and incubated overnight at 4 ℃. Treatment with secondary antibodies (donkey anti-goat IgG-HRP, Santa Cruz Biotechnology, Dallas, TX, USA; anti-mouse and anti-rabbit IgG, HRP-linked, Cell Signaling Technology, Danvers, MA, USA) was performed for 1 h at room temperature. Immunoreactive bands were visualized using an ECL solution (Bio-Rad, Hercules, CA, USA), EZ Capture MG (ATTO, Tokyo, Japan), and a CS analyzer (Ver. 3.0, ATTO).

LC-MS/MS analysis

The anti-inflammatory compounds were analyzed in 70% ethanol, 70% methanol, and water extracts using an LC-HESI-Orbitrap-MS/MS system. A Vanquish UHPLC coupled with a Q Exactive Orbitrap mass spectrometer was used for analysis using XCalibur 4.1 software (Thermo Fisher Scientific, USA). The column for sample separation was Thermo Hypersil Gold AQ (1.9 μm, 100×2.1 mm), and the column oven was set at 36 ℃ during analysis. The gradient of the binary solvent system consisting of 0.1% formic acid in MS-grade water (phase A) and 0.1% formic acid in MS-grade acetonitrile (phase B) was as follows: 5% B for 5 min, 5–100% B for over 20 min, followed by returning to the initial condition (B: 5%). The run was completed 25 min after injecting 5 μL of the sample. UV spectra were measured from 190 to 800 nm. Assessments were performed in negative-ion and positive-ion modes using a heated electrospray ionization (HESI) source. The source voltages were 3.80 kV and 2.50 kV for positive and negative ion modes, respectively. The scan range was 100–1200 m/z. The capillary temperature was 320 °C, while sheath and auxiliary gas flow rates were 40 mL/min and 10 mL/min, respectively.

Data processing and multivariate statistical analyses for metabolite identification

The LC-MS/MS data were analyzed using Compound Discoverer 3.2 software (Thermo Fisher Scientific, USA) for the metabolomic analysis of the anti-inflammatory compounds in the Ogaza extract. All raw data were processed with a spectrum properties filter ($S/N>3$) with peak alignment (intensity$\geq5\times10^6$ and feature grouping with RT≤0.2 min and MT≤5 ppm). SIMCA software (version 15.0; Umetrics, Sweden) was used for the multivariate statistical analysis, and the data were processed using Compound Discoverer. To identify differences or similarities among experimental groups using the SIMCA software, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed. Using the PCA plot, the differences in each group were distinguished, and significantly distinctive metabolites in the comparison group were detected using an S-plot derived from OPLS-DA. The variable importance for projection (VIP) value was used to compare differences between the two sample groups. All data are scaled based on the Pareto scale (bar). Non-targeted metabolite profiling of LC-MS/MS data for the identification of anti-inflammatory compounds in Ogaza was conducted using Compound Discoverer. The intensity threshold was $\geq5\times10^6$ for E-F1, E-F2, and E-F3. Group CV (%) was lower than 50 in all samples, and MS2 fragmentation patterns were used. Furthermore, mzCloud (https://www.mzcloud.org/), METLIN (https://metlin.scripps.edu), MoNA (Massbank of North America, https://mona.fiehnlab.ucdavis.edu/), PubChem (https://pubchem.ncbi.nlm.nih.gov), the MASSBANK database (www.massbank.jp), and related studies were referred for high accuracy.

Statistical analysis

All results were obtained in triplicate and expressed as the mean±standard deviation. Duncan's test ($p<0.05$) in one-way ANOVA using the SPSS software (version 12.0; Statistical Packages for Social Science, USA) was used for the statistical analysis of all experimental results.

Abbreviations

BSDMEM	Dulbecco's modified eagle's medium
DPBS	Dulbecco's phosphate buffered saline
FA	Formic acid
FBS	Fetal bovine serum
HESI	Heated electrospray ionization
iNOS	Inducible nitric oxide synthase
LC	Liquid chromatography
LPS	Lipopolysaccharide
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OPLS	DA-Orthogonal partial least squared-discriminant analysis
PC1	Principal component 1
PC2	Principal component 2

PCA	Principal component analysis
Prep	LC-Preparative liquid chromatography
RT	Retention time
VIP	Variable importance for the projection

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Author contributions
G.D.K., J.L. and J.H.A. conceived and designed the experiments; G.D.K., and J.L. performed the experiments; G.D.K., J.L. and J.H.A. analyzed the data and wrote the manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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