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Composition of Liquid Rice Hull Smoke and Anti-Inflammatory Effects in Mice

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ABSTRACT: A new liquid rice hull smoke extract with a smoky aroma and sugar-like odor prepared by pyrolysis of rice hulls followed by liquefaction of the resulting smoke contained 161 compounds characterized by GC/MS. Antioxidative, antiallergic, and anti-inflammatory activities of the extract were assessed *in vitro* and *in vivo*. At pH 5, the extract inhibited 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and suppressed nitric oxide (NO) and β-hexosaminidase releases from lipopolysaccharide (LPS)-induced RAW264.7 mouse macrophage leukemia cells and ionophore A23187-stimulated RBL-2H3 rat basophilic cells without significant cytotoxicity. 12-*O*-Tetradecanolylphorbol-13-acetate (TPA) was applied to the ears of CD-1 mice to induce inflammation (edema), which was accompanied by increases in a series of biomarkers. Topical application of 1% of the extract as well as feeding mice a standard diet with 1% extract for two weeks significantly reduced the expression of biomarkers associated with the TPA-induced inflammation. These include tumor necrosis factor-α (TNF-α), IL-1β, interleukin-1β (IL-1β), interleukin-6 (IL-6), leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), myeloperoxidase (MPO). These *in vitro* and *in vivo* findings demonstrate the potential value of rice hull smoke extract derived from a major agricultural byproduct to serve as a new biomaterial for the improvement of food quality and safety and the environment.

KEYWORDS: liquid rice hull smoke, composition, GC/MS, cytokines, edema, histochemistry, immunochemistry, inflammation, mice feeding, food quality, food safety

INTRODUCTION

Rice (*Oryza sativa* L.) is a major source of nourishment for the world's population, especially in Asia. World production of rice is estimated at around 680 million tons, equivalent to that of wheat, with The United States producing about 10 million tons and South Korea, about 7 million tons.[1] Rice hulls accounting for 20% of the rice crop are a byproduct of postharvest rice processing.[2] Rice hulls consist mainly of lignin, hemicellulose, cellulose, and hydrated silica.[3] The residual of this process is rice hull ash, consisting predominantly of silica.[2] Another byproduct of the combustion of rice hulls is the smoke that is generated. There is interest in utilizing this smoke as a new source of liquid smoke flavorings, the wood-derived versions of which are now widely used in foods including cheese,[4] meat,[5] and fish.[6]

Smoking has traditionally been used to naturally preserve food, as well as to give distinctive flavors to food. Often hardwoods such as hickory are used, but there are traditional uses of other vegetative matter, such as almond shells and dried prickly pear.[7] Liquid smoke has gained widespread acceptance in the food industry, replacing traditional smoking practices. Liquid smoke preparations have advantages over traditional smoking because they are easier to apply and provide more consistent results. In the United States, liquid smoke has been granted generally recognized-as-safe (GRAS) status as a natural flavoring.[8]

Smoke flavor is the aqueous condensate of pyrolysis products released from controlled wood burning in the absence of air. Lignin reacts to form phenolic compounds, commonly associated with the preservative (antimicrobial) qualities of smoking.[8,9] The source and composition of the wood and pyrolysis conditions can significantly affect the final product.[10,11]

Smoking can also induce the formation of potentially carcinogenic polycyclic aromatic compounds (PAHs). Because of limited solubility in aqueous solutions, they are mostly removed from the liquid smoke during the aging/purification processes. Concentrations of polycyclic compound in foods flavored with liquid smoke as opposed to traditional smoke are low.[12,13] The composition of the vegetative matter can also influence the content of these products.[14] The PAH content of rainbow trout appears to be associated with environmental pollution and not from the smoking process.[15] Antimicrobial effects of smoke have been extensively studied.[16−22]

Degradation products from rice hull lignins exhibited antioxidant activity in a DPPH radical scavenging assay.[3] Three phenolic compounds from rice hull smoke flavoring protected cells against oxidative damage and NO production.[23] No information is available on the composition and uses of smoke obtained from the pyrolysis of rice hulls. To help stimulate interest in the

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potential food uses of the liquid smoke, we investigated its composition by GC/MS, antioxidative effects in chemical and cell assays, and protective effects against inflammation in mice by topically and orally administered liquid smoke. To our knowledge, this is the first report on detailed composition and evaluation of physiological functions of rice hull liquid smoke, which will hopefully stimulate interest in potential food and other applications.

MATERIALS AND METHODS

Materials. The 1,1-diphenyl-2-picrylhydrazyl (DPPH), *p*-nitrophenyl-*N*-acetyl-β-glucosaminide, 12-*O*-tetradecanoylphorbol-13-acetate (TPA, molecular biology grade), ionophore A23187, LPS (*Escherichia coli* 0111:B4), guanidium thiocyanate, and other chemicals were purchased from Sigma (St. Louis, MO). All reagents were of analytical grade and used without further purification. The RPMI 1640 medium (solvent), Hanks balanced salt solution, fetal bovine serum (FBS), and other cell culture reagents were obtained from Hyclone Laboratories (Logan, UT). Cytokine quantification kits for the enzyme-linked immunosorbent assay (ELISA) were obtained from Biosource (Camarillo, CA). The AMV reverse transcriptase, *Taq* DNA polymerase, and dNTP mix were products of Takara Bio (Kyoto, Japan). PCR primers were custom-synthesized and purified by Bioneer (Daejeon, Korea).

Preparation of Rice Hull Smoke Extract. Dried rice hull byproduct of rice milling process was used to produce rice hull smoke extract. Smoke generation was carried out in a carbonization furnace. Pyrolysis of rice hull and smoke generation was carried out by heating the mantle at 450−500 °C for 10 days in a container described in Korean Patent Register No. 1006027320000. The process lasted from the start of the heat supply to the end of the emission of smoke. The resulting smoke was collected and conveyed to a liquefaction plant, where it passed through a cooling process at about 10 °C. The resulting liquid smoke was left standing for about one week at room temperature to remove insoluble material. The upper liquid was then recovered as the primary rice hull liquid smoke extract.

Component Analysis by GC/MS. The liquid smoke extract (50 mL) was extracted with dichloromethane (150 mL) in four steps as described for wood extracts.[24,25] The dichloromethane extract was sequentially partitioned into four fractions: (a) acidic fraction was partitioned into 5% $NaHCO_3$; (b) phenolic fraction was partitioned into 5% NaOH; (c) basic fraction was partitioned into 5% HCl; and (d) neutral fraction containing all remaining compounds soluble in dichloromethane.

The components of each fraction were then determined by GC/MS using a gas chromatograph, model 6890N (Agilent Technologies, Inc., Santa Clara, CA), equipped with a mass spectrometer detector 5975B and DB-WAX column (Agilent Technologies, stationary phase; polyethylene glycol, 30 m × 0.25 mm; i.d. 0.25 μm). The temperature was programmed at 40 °C (5 min), with an increase of 2 °C/min until 220 °C (50 min). Helium gas was used as the carrier with a flow rate of 1.3 mL/min. Both injector and detector temperature were set at 250 °C. The injection was split ratio 50:1 (acidic, neutral and phenolic fractions) and 20:1 (basic fraction), respectively. The injection volume was 1 μL. Mass spectra were recorded in electron ionization mode with ionization energy of 70 eV. Components were identified by retention times in the mass spectra and by comparing the mass spectra with those in a commercial library[26] and with others found in related studies.[24,25]

Mammalian Cell Cultures. RAW264.7 mouse macrophage cell line from the American Type Culture Collection (Manassas, VA) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS containing 100 U/mL penicillin and 100 μg/mL streptomycin. Rat basophilic leukemia cell line RBL-2H3 from Japan Hearth Science Resource Bank (Osaka, Japan) was maintained in the same medium. The cells were cultured at 37 °C in a humidified air conditioner 5% CO_2.

Mice Feeding Study. The study was similar to that described previously for black rice bran.[27] Briefly, female CD-1 mice, aged 4−5 weeks, were purchased from Orient Inc. (Seoul, Korea). After acclimation for 1 week, the mice were housed under a 12 h light/dark cycle with a temperature range of 20−22 °C and relative humidity of 50 ± 10%. Mice were fed a pelletized commercial chow diet without and with 1% smoke extract (v/w) and sterile tap water *ad libitum* during the 14 day experimental period. The mice were subjected to the same housing conditions (1 h light/dark, temperarature, humidity) as those during acclimation.

Cell Viability Assay. The effect of the extract on cell viability was determined using the described MTT colorimetric assay.[28] Briefly, the RAW264.7 cells or RBL-2H3 cells were seeded in a 96-well plate each at a density of 1×10^5 cells per well. After treatment of the extract for 48 h, MTT (0.5 mg) was added to each well. DMSO was then added onto the supernatant-drained cell layer to dissolve the intracellular chromogen. The absorbance of the supernatant was read in a microplate reader at 570 nm with a reference wavelength of 650 nm.

DPPH Free Radical Scavenging Activity Assay. DPPH scavenging activity, a measure of antioxidant activity, was determined by a described method[29] with some modifications. Briefly, ethanol solution (0.5 mL) of DPPH (0.5 mM) was mixed with the extract diluted as indicated (0.01 mL), sodium acetate buffer (1 mL, pH 5.5, 0.1 M), and ethanol (1 mL). The absorbance (A) of the mixture measured against ethanol at 517 nm was determined using a UV/vis spectrophotometer (model V-550, Jasco, Tokyo, Japan). Control solutions containing DPPH only and an ethanol blank, and solutions of the commercial antioxidant BHT as a positive control, were included in the assay. The scavenging of the DPPH radical by the liquid smoke extract was calculated according to the following formula:

$$\text{DPPH scavenging activity (\%)} = \left[1 - \frac{A \text{ of sample} - A \text{ of blank}}{A \text{ of control}}\right] \times 100$$

Nitric Oxide (NO) Generation Assay. NO formation was measured by determining the concentration of its stable oxidative metabolite nitrite using a microplate assay by a described method.[30] Briefly, RAW264.7 cells (1×10^5 cells/well) in a 96-well plate were treated simultaneously with LPS (100 ng/mL) and the extract was diluted as indicated for 48 h. After incubation, culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid) at room temperature for 15 min. The absorbance was then determined at 570 nm using a microplate reader (model 550, Bio-Rad, Hercules, CA). Sodium nitrite was used as the standard.

β-Hexosaminidase Secretion Assay. As a parameter of mast cell degranulation, β-hexosaminidase was determined by a described method.[31] Briefly, RBL-2H3 cells were seeded onto a 96-well plate at a density of 1×10^6 cells/well. After overnight culture, culture media were replaced with Tyroid buffer (200 μL; 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, pH 7.2). Appropriately diluted extract was then added and the mixture incubated for 15 min at 37 °C. To stimulate the cells, after removal of the extract by washing, ionophore A23187 was added to a final concentration of 10 μM for 20 min. The supernatant (50 μL) containing released β-hexosaminidase was recovered by centrifugation. It was then mixed with the same volume of *p*-nitrophenyl-*N*-acethyl-β-glucosaminide solution (1 mM, pH 5.2) and incubated for 1 h at room temperature. The reaction was terminated by adding sodium carbonate buffer (67 mM, pH 10.2). The absorbance of the supernatant was read at 405 nm using a microplate reader (model 550, Bio-Rad, Hercules, CA).

Inflammation Inhibition Study in TPA-Applied Mice. Inflammation in ear skin was induced by single topical application of TPA by a described method[32] with a slight modification. Rice hull smoke extract (1% (v/v), 20 μL) was applied equally to both sides of the ear followed by application 3 h later with TPA in acetone (20 μL, 160 μM).

To demonstrate that long-term daily ingestion of the smoke extract can prevent skin edema, the mice were fed a commercial chow diet supplemented with 1% rice smoke extract for 2 weeks. Changes in the ear thickness/swelling were measured at 24 h after TPA challenge using a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo, Japan). Mice were then sacrificed to obtain the ear skin using an 8 mm diameter punch. Biopsy samples taken from the ears 24 h after TPA administration were used for photochemical and immunohistochemical examination and ELISA. Mice were divided into 3 groups ($n = 10$): control group 1 (vehicle only); control group 2 (TPA only); experimental group (treatment with TPA and rice hull liquid smoke extract).

Histology and Immunohistochemistry. For histological analysis, the ear tissue of the mice was fixed with 4% paraformaldehyde in phosphate buffer (PBS, 0.5 M, pH 7.4). The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted on glass slides. The sections were then dewaxed using xylene and ethanol, and stained with hematoxylin and eosin (H&E).

For immunohistochemical analysis, the deparaffinized skin sections were treated with 3% H_2O_2 to block undesirable effects of endogenous peroxidase. Following incubation with 10% fetal calf serum in phosphate buffered saline (PBS) to reduce background staining and nonspecific antibody binding, samples were incubated with rabbit anti-mouse tryptase monoclonal antibody (Abcam, Cambridge, MA) and then labeled with horseradish peroxidase (HRP)-conjugated anti-IgG antibody (Abcam, Cambridge, MA). Tissue sections were washed, incubated in diaminobenzidine (DAB) solution, and counterstained with 0.2% modified Harris hematoxylin solution (Sigma, St. Louis, MO).

Myeloperoxidase (MPO) Assay. Ear tissue MPO activity was measured 24 h after TPA application according to the method by Bralley et al.[33] The tissues were homogenized in PBS (80 mM) containing hexadecyltrimethylammonium bromide (HTAB, 0.5%) for 45 s on ice with a tissue homogenizer (model Pro200, Pro-Scientific, Oxford, CT). The homogenate was then microcentrifuged at 14000*g* for 15 min at 4 °C. For MPO assay, an aliquot of the supernatant (30 μL) was mixed with the reaction mixture (200 μL) containing PBS-1 (100 μL, 80 mM phosphate, 140 mM NaCl, pH 5.4) PBS-2 (85 μL, 220 mM phosphate, 140 mM NaCl, pH 5.4), and H_2O_2 (15 μL, 0.017%). The reaction was started by addition of tetramethylbenzidine (20 μL, 18.4 mM) in aqueous dimethylformamide (8%), and left standing for 3 min at 37 °C. The reaction was stopped by addition of sodium acetate buffer (30 μL, 1.46 M, pH 3.0). The absorbance of the supernatant was then read in a microplate reader at 655 nm for 30 min. The reaction rate (Δ absorbance/time) was derived from an initial slope of the curve. A calibration curve was then created, showing the rate of reaction plotted against the concentration of a standard human MPO preparation (Sigma, St. Louis, MO).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Cellular RNA. Total cellular RNA was prepared from tissue samples following acid phenol guanidium thiocyanate—chloroform extraction.[34] For reverse transcription, total RNA (1 μg) was incubated with AMV reverse transcriptase (5 U) and oligo (dT_{18}) as primer (100 ng). DNA amplification was then primed in a reaction mixture containing dNTP mix (400 μM), *Taq* polymerase (2.5 U), and primer sets (20 μM each) representing target genes. PCR was conducted using a thermocycler (PTC-200, MJ Research Inc., Reno, NV) with one cycle for 5 min at 94 °C, followed by 30 cycles for 30 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C, and finally one cycle for 5 min at 72 °C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with UV illuminator. The intensity of separated bands under DNA was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

Table 1. Physical Properties of Rice Hull Liquid Smoke Extract

acidity (%)	pH	specific gravity	total solid (%)
4.03	2.3	1.007	0.026

Western Blot Analysis of Cell Proteins. The tissues were lysed and extracted in a homogenizer with RIPA buffer (50 mM Tris Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, pH 7.4). Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein kit (Hercules, CA). Bovine serum albumin (BSA) was used as standard. The tissue extracts containing proteins (30 μg) were separated on 10% polyacrylamide gels and electrophoretically transferred onto nitrocellulose membrane (Millipore, Billerica, MA).

The following primary antibodies were used for Western blot analysis: rabbit anti-mouse iNOS polyclonal antibody (Cell Signaling Tech., Danvers, MA), goat anti-mouse COX-2 polyclonal antibody (Santa Cruz, Delaware, CA), goat anti-mouse 5-LOX polyclonal antibody (Abcam, Cambridge, MA), and anti-mouse β-actin monoclonal antibody (Millipore, Billerica, MA). After blocking with 5% skim milk, membranes were incubated with each primary antibody, followed by HRP-conjugated anti-IgG antibody. Blots were developed using the ECL detection kit (Pierce, Rockford, IL). The intensity of separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan). At least three separate replicates were determined for each experiment.

ELISA of Cytokines and Eicosanoids. Extraction of cytokines and eicosanoids from ear tissue was conducted by a described method.[35] Briefly, ear tissues were homogenized in a phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM PMSF, and 10 mM EDTA. The homogenates were microcentrifuged at 14000*g* for 15 min at 4 °C to recover the supernatant. Cytokines TNF-α, IL-β, and IL-6 and eicosanoids leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) in the supernatants were measured by an ELISA (Biosource International, Camarillo, CA) as suggested by the manufacturer. The absorbance of final solution at 420 nm was measured in a microplate reader (model 550, Bio-Rad, Hercules, CA).

Statistical Analysis. The data are expressed as mean ± SD. Significant difference among the groups was obtained using SAS software with Duncan's multiple range test at $p < 0.05$

RESULTS AND DISCUSSION

Composition of Rice Hull Liquid Smoke. The procedure for the preparation of the liquid extract is similar to that used for liquid wood smoke. Table 1 summarizes selected properties of the extract. The aqueous extract was highly acidic (pH 2.3), had a specific gravity of 1.007, and contained 0.026% solids. The extract has a smoky aroma and burnt-sugar like odor.

Table 2 shows that extract contained 161 characterized compounds. However, some components remained unidentified and others were tentatively identified. Because the chemical nature of the identified compounds varied widely, we do not know which compounds are responsible for the observed bioactivity. Moreover, the safety of the liquid smoke needs to be evaluated before it can be recommended for food use.

Beneficial Effects in Cell Assays. Table 3 lists primer sets for target genes used in the described bioassays. Table 4 shows that pH 2.3-unadjusted primary extract did not adversely affect the

Table 2. Retention Times (t_R) of Identified Compounds in Acidic (AF), Phenolic (PF), Basic (BF), and Neutral (NF) Fractions of Rice Hull Liquid Smoke Extract and Relative Proportions in % Peak Area

			peak area (%)			
peak no.	t_R (min)	compd (based on mass spectral data)	AF	PF	BF	NF
1	3.965	3,3,5-trimethylheptane			0.62	
2	4.055	1-fluorododecane			0.67	
3	5.019	dodecane			1.21	
4	5.17	6-ethyl-2-methyloctane			1.21	
5	6.856	5-ethyl-2-methyloctane			1.25	
6	7.103	4,7-dimethyloctane			0.50	
7	7.566	(*E*)-9-methyl-3-undecene			1.64	
8	7.738	4-methyl-3-heptene			1.21	
9	14.67	styrene			2.07	
10	15.127	hexatriacontane			1.92	
11	18.172	tridecane			2.29	
12	19.194	1,1-dimethyl-2-propylcyclohexane			2.20	
13	19.298	2,3,8-trimethyldecane			0.69	
14	19.701	7-methyl-2-decene			2.54	
15	20.16	isotridecyl alcohol			1.78	
16	27.366	docosane			2.83	
17	30.272	hexadecane			3.09	
18	30.842	crecetane			0.70	
19	31.366	1,1′-oxybis-decane			1.25	
20	31.505	pentadecane			0.99	
21	31.965	docosane			0.91	
22	32.326	1,1′-oxybis decane			1.33	
23	37.3	pentanoic acid	1.17			
24	38.721	nonadecane			3.07	
25	41.024	pentatriacontane			0.86	
26	41.285	pentacosane			3.70	
27	41.794	eicosane			0.82	
28	42.38	dodecane			2.91	
29	42.93	dotriacontane			1.39	
30	43.329	pentacoic acid	1.25			
31	43.405	1,2-dipropylcyclopentane			0.94	
32	43.584	pentadecyl 2-propyl ester sulfurous acid			0.59	
33	44.03	geranyl ethyl ether			1.89	
34	44.318	1,1′-oxybis decane			1.17	
35	45.777	2,4-diethyl-1-heptanol			1.01	
36	47.665	2-hydroxy-1-methylcyclopenten-3-one	5.10			
37	47.672	2-hydroxy-3-methylcyclopent-2-en-1-one		2.38		
38	47.683	4,5-dihydro-1,4,5-trimethyl-1*H*-tetrazaborole				0.50
39	48.969	hexanoic acid	1.21			
40	48.981	docosane			2.92	
41	49.09	6-methyl-2-pyridinol	2.62			
42	49.091	2-methoxyphenol		3.75		
43	49.101	4-aminophenol				0.83
44	49.102	1-methyl-2(1*H*)-pyridone				1.54
45	49.506	2-pentanoic acid	0.92			
46	50.178	2-pentanoic acid	1.13			
47	51.036	2-hydroxy-3,4-dimethyl-2-cyclopenten-1-one		0.49		
48	51.132	2-methylheptadecane			0.76	
49	51.284	octacosane			3.50	
50	51.702	nonadecane			0.90	
51	52.213	tetrapentacontane			0.83	

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Table 2. Continued

peak no.	t_R (min)	compd (based on mass spectral data)	peak area (%) AF	PF	BF	NF
52	53.8	1,2,4-trimethylcyclohexane			1.15	
53	53.834	5-methyl-2-oxabicyclo[3.3.0]cot-7-en-3-one	0.83			
54	53.845	2-(2,2-diethylvinyl)thiophene		3.08		
55	53.847	2-[2,2-dimethylviny]thiophene				0.69
56	54.194	2-methylpyromeconic acid	6.05			--
57	54.215	acrolein oxime	0.33			0.33
58	54.318	*O*,*O*′-diamino-1,2-ethyleneglycol	1.99			
59	54.421	1-[2,3,4-trihydroxyphenyl]ethanone				0.52
60	56.488	phenol			1.58	
61	56.498	phenol	10.99			
62	56.506	phenol		9.40		
63	56.517	phenol				5.26
64	56.923	1*H*-pyrrole-2-carboxaldehyde				0.20
65	57.417	4-ethyl-2-methoxyphenol		1.00		
66	57.428	4-ethyl-2-methoxyphenol				2.36
67	57.429	5-isopropyl-3,3-dimethyl-2-methylene-2,3-dihydrofuran				1.25
68	58.302	docosane			2.53	
69	58.348	1,3-cyclopentadiene				0.35
70	58.664	*N*-oxide ethyl ester of nicotinic acid				1.95
71	60.114	*o*-toluidine	1.28			
72	60.117	4-methylphenol		2.51		
73	60.247	2,4-dimethylphenyl ester cyanic acid		0.53		
74	60.247	2,4-dimethylphenol				0.24
75	60.414	nonadecane			4.78	
76	60.452	[*E*,*E*]-6-[dimethylamino]-c-methyl-3,5-hexadien-2-one	2.91			
77	60.46	2-methylphenol		5.83		
78	60.469	3-methylphenol				2.94
79	60.638	1,3,5-trimethyl-1*H*-pyrazole				2.26
80	60.87	[*Z*]-2-penten-1-ol				0.30
81	61.089	2-methoxy-4-propylphenol				1.66
82	61.219	heneicosane			0.85	
83	61.237	3,6-diazahomoadamantan-9-one oxime				0.31
84	61.758	3-aminophenol				0.34
85	62.332	4,6-dimethyl-2-pyrimidianmine				0.30
86	62.876	7-methylindan-1-one				0.33
87	63.49	*trans*-bicyclo[4.3.0]non-3-en-7-one				0.53
88	63.808	3-butoxyphenol				0.61
89	63.833	tetrahydrofurfuryl alcohol	14.40			
90	64.34	4-ethylphenol		0.94		
91	64.35	3,5-dimethylphenol				0.25
92	64.49	2,3-dihydro-1,4-benzodioxin				2.91
93	64.65	2-ethylphenol		0.40		
94	65.068	1,5-dimethylcyclohexene-5-carboxaldehyde				0.24
95	65.88	2,3,5-trimethylphenol				0.45
96	66.185	2,6-di-*tert*-butyl-4-methylphenyl anti-7-bicyclo[4.1.0]heptane carboxylate				0.20
97	66.286	2-isopropylpyrazine				0.34
98	66.834	octacosane			2.06	
99	66.908	1-[2,5-dimethylphenyl]ethanone				0.97
100	68.119	2,6-dimethoxyphenol			2.55	
101	68.131	4N-ethylcytosine	3.94			
102	68.145	2,6-dimethoxyphenol				5.07
103	68.145	2,6-dimethoxyphenol		24.41		

Table 2. Continued

peak no.	t_R (min)	compd (based on mass spectral data)	peak area (%) AF	PF	BF	NF
104	68.789	4,9-dipropyldodecane			2.99	
105	68.855	2-[2′,3′-epoxy-3′-methylbuthyl]-3-methylfuran				0.89
106	69.166	4,5,6,7,8,9-hexahydro-1*H*-cyclooctrapyrazde				0.42
107	69.337	2-methyl-5-[1-methylethyl]phenol				0.23
108	69.526	5-aminoindazole				0.52
109	69.55	squalane			0.75	
110	70.082	4*H*-1-benzopyran-4-one				0.20
111	70.187	2,6-dimethoxyphenol				0.66
112	70.426	1[3*H*]-isobenzofuranone				1.09
113	70.741	3-ethyl-8-methyl-*s*-triazolo[4,3-*a*]pyrazine				0.45
114	71.422	2-methyl-methylester benzoic acid				0.52
115	71.521	6-methyl-1,2,3,5,8,8a-hexahydronaphthalene				0.60
116	71.749	2-methoxy-4-nitrobenzenamine,			1.08	
117	71.767	4-methoxy-2-nitrobenzenamine		13.88		
118	71.776	2-(methylamino)-5-nitropyridine				6.52
119	72.269	6-acetyl-beta-d-mannose				0.47
120	73.036	2-ethylbenzo[*b*]thiophene				0.80
121	73.229	1,6-dihydroxynaphthalene				1.07
122	73.494	1*H*-1,2,4-triazole	0.79			
123	73.795	*N*,*N*-dimethylpropanomide	1.04			
124	73.826	1-[2′-ethenyl-1′-cyclohexenyl]-2-propen-1-one				0.38
125	73.83	1-[2-methyl-1-cyclopenten-1-yl]-ethanone		0.46		
126	74.188	1,1′,1″-(1-ethanyl-2-ylidene)*tris*-benzene		6.54		
127	74.204	2,4,-dimethyl-3-(methoxycarbonyl)-5-ethylfuran				27.06
128	74.405	4-nitro-1-oxide benzofurazan				0.90
129	74.641	benzencarboxylic acid	17.85			
130	74.654	3-[2-pyrrolidinyl],[*S*]-pyridine				0.56
131	74.725	4,9-dipropyldodecane			1.28	
132	74.937	*N*,*N*-diaminoethane-1,2-diimine	1.00			
133	74.992	4-methyl-2*H*-pyrido[1,2-*a*]pyrimidin-2-one				0.61
134	75.224	*trans*-1-methyl-2-indanol				0.71
135	75.664	2,3,5,6-tetrafluoroaniline				0.30
136	75.95	2-hydroxy-3-nitropyridine				0.81
137	76.542	pentacosane			1.77	
138	76.871	2′,4′-dimethoxyacetophenone				1.84
139	77.044	16-keto-tetrahydrosolasodine				22.02
140	77.587	2,4-dimethyl-3[2*H*]-benzofuranone				0.26
141	77.809	4,6-dihydrothieno-[3,4-*b*]furan	2.19			
142	78.573	2-[^{13}C]methyl-[2-^{13}C]-1,3-dithian	1.10			
143	78.688	2,5-bis[1,1-dimethylethyl]thiophene				0.39
144	78.954	[R]-2-methyl-2,5-diaminopentanoic acid	1.07			
145	79.12	5-ethyl-2-methylthiazole				0.62
146	79.438	2,3-dihydro-5-phenyl-1,4-dithin				2.54
147	79.65	propenyl guaethol	2.81			
148	79.653	4-hydroxy-3-methoxybenzaldehyde		0.87		
149	79.884	benzeneacetic acid	1.06			
150	80.366	1,5-dihydro-2,4,6[3*H*]-pteridinetrione	1.20			
151	80.689	3-(2-aminoethyl)phenol				2.33
152	80.859	1-docosanol			1.68	
153	81.806	cyclohexadecane			1.77	
154	82.386	15-crown-5	1.30			
155	82.604	1-[4-(methylthio)phenyl]ethanone		2.85		

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Table 2. Continued

peak no.	t_R (min)	compd (based on mass spectral data)	peak area (%) AF	PF	BF	NF
156	85.326	1,2-benzenediol	8.05			
157	86.145	2-furancarbonitrile				3.06
158	93.398	hexadecanoic acid			2.64	
159	101.74	octadecanoic acid			3.69	
160	103.47	1,4,7,10,13,16-hexaoxacyclocotadecane			1.55	
161	104.73	3,9-diazatricyclo[7.3.0.0(3,7)dodecane-2,8-dione			2.36	

Table 3. Primer Sets Representing Seven Target Genes and the Internal Control β-Actin Gene

primer	sequence
tumor necrosis factor-α (TNF-α) sense	5′-TACTGAACTTCGGGGTGATCGGTCC-3′
TNF-α antisense	5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
interleukin-1β (1 L-1β) sense	5′-GTAGCCCACGTCGTAGCAAA-3′
IL-1β antisense	5′-CCCTTCTCCAGCTGGGAGAC-3′
interleukin-6 (IL-6) sense	5′-GAAATGATGGATGCTTCCAAACTGG-3′
IL-6 antisense	5′-GGATATATTTTCTGACCACAGTGAGG-3′
intercellular adhesion molecule-1(ICAM-1) sense	5′-TTCCTCTCTGCAAGAGACT-3′
ICAM-1 antisense	5′-TGTATCTCTCTGAAGGACT-3′
inducible nitric oxide synthase (iNOS) sense	5′-ATG TCC GAA GCA AAC ATC AC-3′
iNOS antisense	5′-TAA TGT CCA GGA AGT AGG TG-3′
cyclooxygenase-2 (COX-2) sense	5′-TCTCAACCTCTCCTACTAC-3′
COX-2 antisense	5′-GCACGTAGTCTTCGATCACT-3′
5-lipoxygenase (5-LOX) sense	5′-ATGAGCTGTTTCTAGGCATGTACC-3′
5-LOX antisense	5′-GAATAAAGTACCCCTGACCCAGCC
β-actin sense	5′-GTGGGGCGCCCCAGGCACCA-3′
β-actin antisense	5′-GTCCTTAATGTCACGCACGATTTC-3′

Table 4. Cytotoxicity of Primary (pH-Unadjusted) and pH 5-Adjusted Rice Hull Smoke Extract on RBL-2H3 Rat Basophilic Leukemia and RAW264.7 Mouse Macrophage Cells Exposed to Liquid Smoke Extract for 48 h[a]

rice hull smoke extract	extract level (%)	cell viability (%) RBL-2H3	RAW264.7
primary extract	0.1	97.21 ± 1.68 a	87.77 ± 0.49 c
	0.5	98.33 ± 0.49 a	72.61 ± 1.10 d
	1	98.89 ± 1.57 a	65.32 ± 0.33 e
pH 5 extract	0.1	98.09 ± 0.38 a	97.25 ± 2.08 a
	0.5	98.23 ± 0.33 a	97.21 ± 1.62 a
	1	98.29 ± 2.33 a	92.37 ± 2.39 b

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$.

viability of the rat basophilic leukemia cell line RBL-2H3, which ranged as follows for treatments at the three concentrations: 97.21% viability, 0.1% (v/v) treatment; 98.33% viability, 0.5% treatment; and 98.90% viability, 1% treatment. By contrast, exposure of the mouse macrophage cell line to the same treatment resulted in the following concentration-dependent reductions in cell viabilities: 87.77% viability, 0.1% treatment; 72.61% viability, 0.5% treatment; and 65.32% viability, 1% treatment. These results show a cell line-dependent resistance to acidic environmental conditions. Table 4 also shows that the pH 5-adjusted extract largely prevented loss of cell viability of the macrophage cells, which ranged from 97.25% to 92.37%, where addition of pH 5-adjusted extract was observed to prevent lowering of pH of culture media. Therefore, to avoid possible problems associated with cell viabilities, all subsequent experiments were carried out with the pH 5 extract.

Table 5. DPPH Scavenging Activity of Rice Hull Smoke Extract Compared to the Commercial Antioxidant BHT[a]

treatment	treatment level	DPPH scavenging activity (%)
BHT	10 μM	78.55 ± 0.20 d
	100 μM	86.80 ± 0.13 a
rice hull smoke extract (primary)	0.5%[b]	79.39 ± 0.33 c
	1%	82.13 ± 0.10 b

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$. [b] Percent concentration (v/v).

Table 5 compares results of scavenging of the DPPH radical by the commercial food antioxidant BHT and the rice smoke extract. At the 10 μM concentration, BHT scavenged 78.55% of the free radicals, and at 100 μM, 86.80%. At the 0.5% concentration, the liquid smoke scavenged 79.40% of the radicals, and at 1%, 82.13%. The liquid rice hull smoke exhibited

strong antioxidative properties, similar to those observed with BHT.

Table 6 shows the suppression of release by the extract of β-hexoseaminidase from ionophore A23187-stimulated rat basophilic RBL-2H3 leukemia cells. The 0.1% extract inhibited 51.12% release; the 0.5% extract, 78.53%; and the 1% extract, 83.52%. The table also shows that there was no release in the absence the ionophore (positive control) and a 100% release in its presence. The observed concentration-dependent suppression of the immune biomarker implies that the extract exhibited antiallergic properties.[36]

Table 6. Effect of pH-Adjusted Rice Hull Smoke Extract on Ionophore A23187-Stimulated β-Hexosaminidase Release from RBL-2H3 Rat Basophilic Leukemia Cells[a]

treatment	extract level (%)	inhibn of β-hexosaminidase release (%)
control − A23187[b]		100.00 ± 0.50 a
control + A23187[c]		0.00 ± 2.91 e
rice hull smoke extract (pH 5) + A23187[c]	0.1	51.12 ± 3.23 d
	0.5	78.53 ± 2.35 c
	1	83.52 ± 1.03 b

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$. [b] Acetone only was used as vehicle. [c] 10 μM solution in acetone.

Table 7. Inhibitory Effect of pH-Adjusted Rice Hull Smoke Extract on NO Production in RAW264.7 Mouse Macrophage Cells[a]

treatment	extract level (%, v/v)	μM nitrite (% inhibn)
control − LPS		4.25 ± 0.39 d (100.0)
control + LPS		46.91 ± 1.64 a (0.0)
rice hull smoke extract (pH 5) + LPS	0.1	25.86 ± 2.00 b (49.3%)
	0.5	7.92 ± 0.62 c (91.4%)
	1	3.05 ± 0.26 e (102.8%)

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$.

Table 7 shows the inhibitory effect of the rice hull smoke extract on the production of NO by the mouse macrophage cell line RAW264.7. Compared to the control value of 0% in the ionophore A23187-stimulated cells, the 0.1% extract inhibited 49.3% of NO produced by mouse macrophage cells. The corresponding value for the 0.5% extract was 91.4%, and for the 1% extract, 102.8%. The extract inhibited synthetic DPPH free radicals in vitro and NO radicals produced in cultured cells. Suppression of NO production by nitric oxide-synthase was associated with anti-inflammatory effects of methanol extracts of *Taraxacum officinale* leaves.[37]

Beneficial Effects in Mice. The inhibitory effect of the rice hull smoke extract on skin inflammation of mice was determined using the skin inflammation model induced by topical application with TPA. Table 8 shows that the rice hull smoke extract (1%) applied topically inhibited the release of three cytokines associated with the inflammation process. The 1% rice hull extract reduced cytokines TNF-α, IL-1β, and IL-6 by 78.08%, 73.86%, and 71.73%, respectively, as compared to TPA induced mice.

Table 9 shows that the 1% rice hull extract also inhibited the release of two eicosanoids from mouse ear tissues. Compared to the TPA-treatment, the inhibition of prostaglandin E_2 (PGE_2) was 38.98%, and of leukotriene B_4 (LTB_4), 45.59%. These results reinforce our conclusion that the extracts possess anti-inflammatory properties.

Table 10 shows that the 1% rice hull extract also induced a decrease in the enzyme myeloperoxidase, a representative neutrophil biomarker, in the mouse ear tissues from 270.81 U to 95.32 U, or a 65.62% reduction. The extent of decrease in this inflammation-associated enzyme[38] is similar to the above-mentioned three biomarkers. It is, therefore, likely that the inhibitory effect against myeloperoxidase is mechanistically related to the corresponding effects on the biomarkers. It may also be related to the observed reduction of TPA-induced leukocyte infiltration in

Table 8. Inhibitory Effect of pH-adjusted Rice Hull Smoke Extract on Release of Pro-Inflammatory Cytokines in Mouse Ear Tissues[a]

		pg/mL (% inhibn)		
treatment	extract level (%)	TNF-α	IL-1β	IL-6
control − TPA[b]		11.61 ± 0.00 c (100)	219.91 ± 2.63 c (100)	226.57 ± 1.44 c (100)
control + TPA[c]		85.30 ± 1.23 a (—)	1123.34 ± 24.85 a (—)	974.32 ± 26.34 a (—)
rice hull smoke extract	1	27.76 ± 1.38 b (78.08)	456.06 ± 11.31 b (73.86)	442.44 ± 20.80 b (71.13)

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$. [b] Add ethanol as vehicle. [c] 160 μM solution in acetone.

Table 9. Inhibitory Effect of pH-Adjusted Rice Hull Smoke Extract on Release of Eicosanoids in Mouse Ear Tissues[a]

		pg/mL (% inhibn)	
treatment	extract level (%)	PGE_2	LTB_4
control − TPA[b]		113.22 ± 21.75 c (100)	69.00 ± 10.00 c (100)
control + TPA[c]		988.22 ± 21.10 a (—)	295.67 ± 35.12 a (—)
rice hull smoke extract	1	647.11 ± 12.29 b (38.98)	192.33 ± 20.82 b (45.59)

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$. [b] Add ethanol as vehicle. [c] 160 μM solution in acetone.

Table 10. Inhibitory Effect of pH-Adjusted Rice Hull Smoke Extract on Myeloperoxidase Activity in Mouse Ear Tissues[a]

treatment	extract level (%)	myeloperoxidase activity (units)
control − TPA		3.39 ± 0.97 c
control + TPA		270.81 ± 21.47 a
rice hull smoke extract (pH 5)	1	95.32 ± 6.35 b

[a] Values, expressed as mean ± SD ($n = 3$), in each column with the same letters are not significantly different at $p < 0.05$.

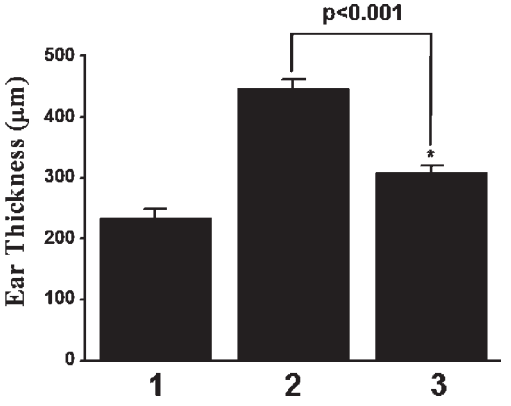

Figure 1. Changes in thickness of ear skins of mice. Rice hull smoke extract was topically applied on ear skin before application of TPA. After 24 h, increases in ear thickness were determined. Data are expressed as mean ± SD (n = 10). Lane 1, control group subjected to the topical application with PBS (20 μL) followed by topical application with acetone (20 μL) as vehicle; lane 2, control group subjected to the topical application with PBS (20 μL) followed by topical application with TPA (20 μL, 160 μM) in acetone; lane 3, experimental group subjected to the topical application with extract (20 μL, 1%) followed by topical application with TPA (20 μL, 160 μM) in acetone. *Statistically different from the group subjected to topical application of TPA alone at $p < 0.001$.

response to the treatment with the rice hull smoke extract described below.

Figure 1 shows that the TPA-induced skin swelling/thickness is dramatically reduced by application of a 1% solution of the rice hull smoke extract. The reduction is significant at the $p < 0.001$ level. This observation indicates that that extract exhibited anti-inflammatory activities *in vivo*. The protective effect is confirmed by the expressions of genes associated with the skin inflammation illustrated in Table 3 and Figures 2 and 3 and by the described reductions in levels of the pro-inflammatory mediators (cytokines, eicosanoids, NO).

Figure 2 shows results from semiquantitative RT-PCR analysis of 7 inflammatory genes treated with solvent only (lane 1), the agonist (TPA) (lane 2), and TPA and liquid smoke (lane 3) in terms of normalized relative expression (R.E.) values. Liquid smoke reduced the mRNA levels from 72% [(1.00 − 0.28) × 100] (iNOS) to 60% (5-LOX) of the pro-inflammatory genes. The mRNA of Vitamin A is reported to exhibit similar effects at the genetic level.[37]

Figures 3 shows Western blot analysis of three pro-inflammatory cytokines produced by the inflamed mouse tissue. The listed R.E. values show that liquid smoke treatment reduced protein levels of the iNOS gene by 59%. The corresponding reduction for

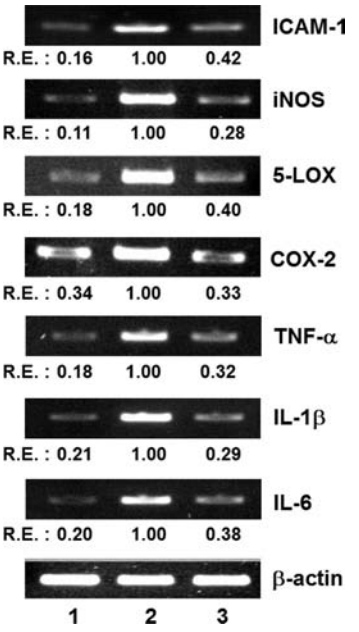

Figure 2. Semiquantitative analysis of rice hull smoke extract-modulated gene expressions for synthesis of pro-inflammatory cytokines and eicosanoids. The relative ratio of expression of each gene in ear tissues is expressed as relative expression (R.E.) value calculated from target gene/β-actin gene expression. Lane 1, control group subjected to the topical application with PBS (20 μL) followed by topical application with acetone (20 μL) as vehicle; lane 2, control group subjected to the topical application with PBS (20 μL) followed by topical application with TPA in acetone (20 μL, 160 μM); lane 3, experimental group subjected to the topical application with rice hull smoke extract (20 μL, 1%) followed by topical application with TPA (20 μL, 160 μM) in acetone. Figures represent results from at least three individual experiments.

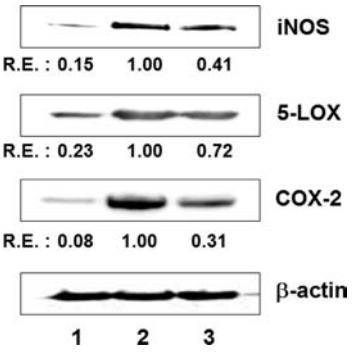

Figure 3. Western blot analysis of rice hull smoke extract-modulated NOS, COX-2 and 5-LOX protein expression in ear skin tissues. Each protein level was expressed as relative expression (R.E.) value calculated from target gene/β-actin gene expression. Lane 1, control group subjected to the topical application with PBS (20 μL) followed by topical application with acetone (20 μL) as vehicle; lane 2, control group subjected to the topical application with PBS (20 μL) followed by topical application with TPA (20 μL, 160 μM) in acetone; lane 3, experimental group subjected to the topical application with extract (20 μL, 1%) followed by topical application with TPA (10 μL, 160 μM) in acetone. Figures represent at least three individual experiments.

5-LOX was 28% and for COX-2, 69%. The β-actin gene used as an internal control was not affected by the treatments. These results confirm the mRNA data and suggest that the liquid smoke treatment reduced protein synthesis of these genes in the ear tissue.

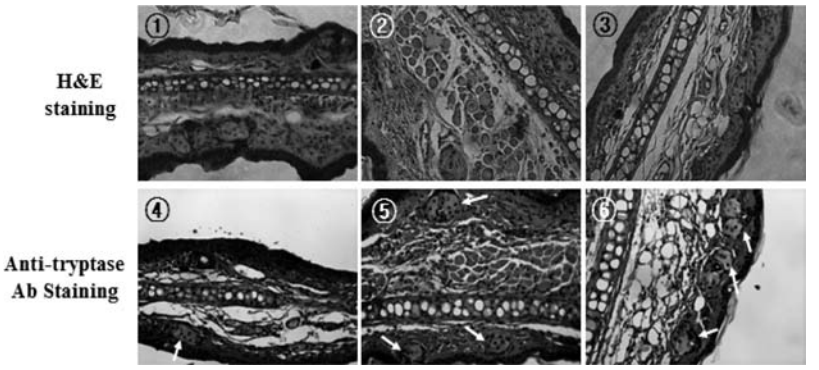

Figure 4. Hematoxylin and eosin (H&E) staining and immunostaining with anti-tryptase antibody in mouse ear skin tissues. Changes in skin thickness and infiltrated cells in dermis were observed by H&E staining (panel 1 to 3). Anti-tryptase antibody was used to immunostain the mast cells in dermis (panels 3 to 6). Histological patterns were observed under high power field microscopy (×400). Arrows in panel 4 and 6 indicate inactivated intact mast cells, and in panel 5, activated mast cells. Panels 1 and 4, control group subjected to the topical application with PBS (20 μL) followed by topical application with acetone (20 μL) as vehicle; panels 2 and 5, control group subjected to the topical application with PBS (20 μL) followed by topical application with TPA (20 μL, 160 μM) in acetone; panels 3 and 6, experimental group subjected to the topical application with extract (20 μL, 1%) followed by topical application with TPA (20 μL, 160 μM) in acetone. Figures represent results from at least three individual experiments.

Topical application of the rice hull smoke extract suppressed leukocyte infiltration in the dermis through inhibition of ICAM-1 expression as compared with that observed with the control treated with TPA alone (Figure 2). The net decrease in ear thickness/swelling in response to rice hull smoke extract equals 64.62%. In contrast to arachidonic acid, TPA is reported to elicit an inflammatory response characterized by a delayed onset and longer-lasting inflammation associated with marked leukocyte influx.[39] The extent of leukocyte infiltrations appears to be the cause of the observed change in ear thickening/swelling. This suggestion is also supported by the fact that the observed reduction rate of myeloperoxidase activity in ear tissue treated with liquid rice hull smoke was related to the observed degree of ear thickening/swelling (Figure 1, Table 10). Immunohistochemical analysis using antibody raised against tryptase identified the immunostained cells lying just beneath the dermis as mast cells. Treatment with the extract induced significant reduction in tryptase-positive mast cell levels compared to TPA challenged control group (Figure 4). The suppressive effect on activated mast cells implies that the extract can modulate the function of critical cellular components of the innate immune system.

Figure 5 shows that a standard diet supplemented with 1% smoke extract fed orally for 14 days induced significant reductions in mouse ear edema and two biomarkers associated with inflammation: prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), similar to the observed effects obtained by topical application (Table 9). There were no changes in appearance and body weight in mice fed rice hull smoke extract compared to control mice.

Mechanistic Aspects. The present study showed that the administration of the extract elicited marked changes in the expression of 7 genes, well-known to be associated with inflammation (Table 3; Figures 2 and 3). However, it is difficult to offer a detailed molecular action mechanism for attenuating the inflammatory progress triggered by TPA because of the reported complexity of the signal pathway in the inflammatory response[40,41] and of the chemical heterogeneity of the smoke extract.

In conclusion, the collective results of the present study demonstrated that administration of the extract from rice hull smoke through topical application or feeding had high inhibitory

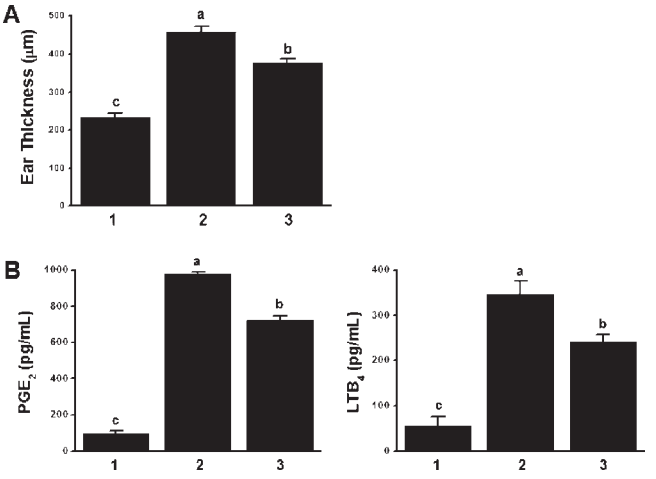

Figure 5. Effects of dietary administration of rice hull smoke extract on ear swelling due to TPA-induced skin edema in mice. (A) Measurement of changes in thickness of ear skins of mice. The extract was topically applied on ear skin before application of TPA. After 24 h, increases in ear thickness were determined. (B) Inhibitory effects of the extract on LTB_4 and PGE_2 production in TPA-induced ear tissue. Data are expressed as mean ± SD (n = 10). Lane 1, mice fed standard chow diet, then subjected to the topical application with vehicle acetone (20 μL); lane 2, mice fed standard chow diet, then subjected to the topical application with TPA (20 μL, 160 μM) in acetone; lane 3, mice fed experimental diet supplemented with 1% extract (v/w), then subjected to the topical application with TPA (20 μL, 160 μM) in acetone. Bars not sharing a common letter are significantly different between groups at $p < 0.05$.

activities in all the chemical/cellular/molecular *in vitro* and *in vivo* tests in mice designed to define antioxidative, antiallergic, and anti-inflammatory activities. These results complement our similar findings with black rice bran.[27] We do not know which or what combinations of the 161 characterized compounds of the smoke extract are bioactive in the described assays. New food uses of a major agricultural byproduct may benefit the environment, farmers, and consumers. However, it is first necessary to demonstrate that the liquid smoke is safe. The present study was designed to contribute to this assessment.

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ABBREVIATIONS USED

BHT, butyl hydroxyl toluene; COX-2, cyclooxygenase 2; DMSO, dimethyl sulfoxide; DPPH, 1-diphenyl-2-picrylhydrazyl; ELISA, enzyme-linked immunosorbent assay; GS/MS, gas chromatography/mass spectrometry; R.E., relative expression of genes and proteins; IL-1β, interleukin-1β; IL-6, interleukin-6; 5-LOX, 5-lipoxygenase; LTB_4, leukotriene B_4; NO, nitric oxide; PGE_2, prostaglandin E_2 RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanolylphorbol-13-acetate; U, units

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