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Food Grade Fungal Stress on Germinating Peanut Seeds Induced Phytoalexins and Enhanced Polyphenolic Antioxidants

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ABSTRACT: The effects of food grade fungus *Rhizopus oligosporus* stress on phytochemicals and phytoalexins of germinating peanut seeds were investigated by comparing the metabolic profiles of ungerminated (UG), germinated (G), and germinated seeds under fungal stress (GS). Three types of peanut seeds with different skin color (red, reddish brown, and black) were compared in the process. The polyphenolic contents were analyzed and correlated with antioxidant capacity for specific free radicals including peroxyl radical $ROO^{\bullet}$ (ORAC), hydroxyl radical $HO^{\bullet}$ (HORAC), superoxide radical $O_2^{\bullet-}$ (SORAC), and DPPH radical. The polyphenolic fingerprints analyzed by HPLC and LC-MS^n showed that phenolic acids (coumaric, sinapinic, and ferulic acids derivatives) were the major group of phenolic compounds in ungerminated seeds. G or GS increased the level of phenolic acids, phytoalexins, and antioxidant capacity values in reddish and red peanuts but not in black peanuts. From the LC-MS^n spectral data, 45 compounds were identified tentatively in the germinated peanuts, including 14 coumaric acids, 3 ferulic acids, 4 sinapinic acids, 2 hydroxybenzoic acids, 1 caffeic acid, 2 flavonoids, and 19 stilbenoids derivatives. Reddish brown germinated peanuts produced the highest amount of phytoalexins after GS with 55 compounds detected. Forty-five of these compounds were suggested as stilbenoid phytoalexins derivatives. The high content of phytoalexins may enhance the bioactivity of peanut seeds as functional food ingredients.

KEYWORDS: Peanut, groundnut, fungal stress, phytoalexins, phytochemicals, antioxidant capacity

INTRODUCTION

Phytoalexins are secondary metabolites that plants synthesize for self-defense against microbial infections; they have shown great promise in chronic disease prevention.[1,2] The most well-known example is resveratrol, an induced phytoalexin found in yeast-infected grape skin. Resveratrol may have interesting potential as a calorie restriction mimic. Furthermore, it has been shown to be a good antioxidant and an anti-inflammatory agent.[3,4] However, foods containing significant levels of resveratrol are limited to red grape wine/juice.[5] Glyceollin is another induced phytoalexin in soybean, which has many health benefits. We have demonstrated that germinating soybeans stressed with tempeh starter led to great enrichment of glyceollins among other isoflavones biosynthesized by the beans.[6] We extended this food grade bioprocessing technology to peanut seeds, and reported herein are our findings.

Peanuts are a widely consumed food. They have recently attracted greater attention because of their health-promoting properties attributed to the numerous bioactive components such as unsaturated fatty acids, vitamin E, folate, phytosterols, phenolic acids, procyanidins, and selenium.[7] Peanut seeds, when induced by pathogenic infections, fungal elicitors, and UV light, could produce inducible phytoalexins such as stilbenoid derivatives (resveratrol, arachidins, 3′-isopentadienyl-3,5,4′-trihydroxystilbene, SB-1, chiricanine A, and arahypins), and pterocarpanoid derivatives (e.g., aracarpene-1 and aracarpene-2).[8−12] Stress-induced stilbenoid phytoalexins from peanuts are of considerable interest because of their biological activities and possible therapeutic value for chronic diseases. Resveratrol oligomers are less studied but have demonstrated some potential as disease-preventing ingredients.[13,14] There is no report on stressing germinating peanuts by using *Rhizopus oligoporus*, a popular food grade microbe for food fermentation in the Southeast Asia region.

MATERIALS AND METHODS

Reagents. Food grade fungus, *R. oligosporus*, was bought from PT Aneka Fermentasi Industri (Bandung, Indonesia). Caffeic acid (98%), catechin (98%), *p*-coumaric acid (98%), ferulic acid (99%), genistein (98%), hydroxybenzoic acid (99%), *trans*-resveratrol (98%), sinapic acid (98%), Trolox (97%), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and fluorescein were purchased from Aldrich (Milwaukee, WI), Folin−Ciocalteau (FC) reagent and gallic acid were purchased from Sigma (Louis, MO). The 96-well polystyrene microplates with flat bottom were purchased from Fisher Scientific (Nunc, Rochester, NY). *n*-Hexane, methanol, and other solvents were of spectroscopic-grade or high-performance liquid chromatography (HPLC) grade from commercial sources.

Instruments. A Synergy HT microplate reader (Biotek, Winooski, VT) was used in six antioxidant capacity-related assays. Sample dilution and reaction solution mixing were performed manually or by the Precision 2000 automatic liquid handling systems (Biotek). HPLC analysis was carried out on a Waters HPLC system (Milford, MA) with an Alliance 2659 separation module, a 2996 photodiode array (PDA) detector, and a Waters C18 column (5 μm, 4.6 mm × 250 mm, Atlantis, Ireland). LC-MS spectra were acquired using a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA) equipped with a TSP 4000 HPLC

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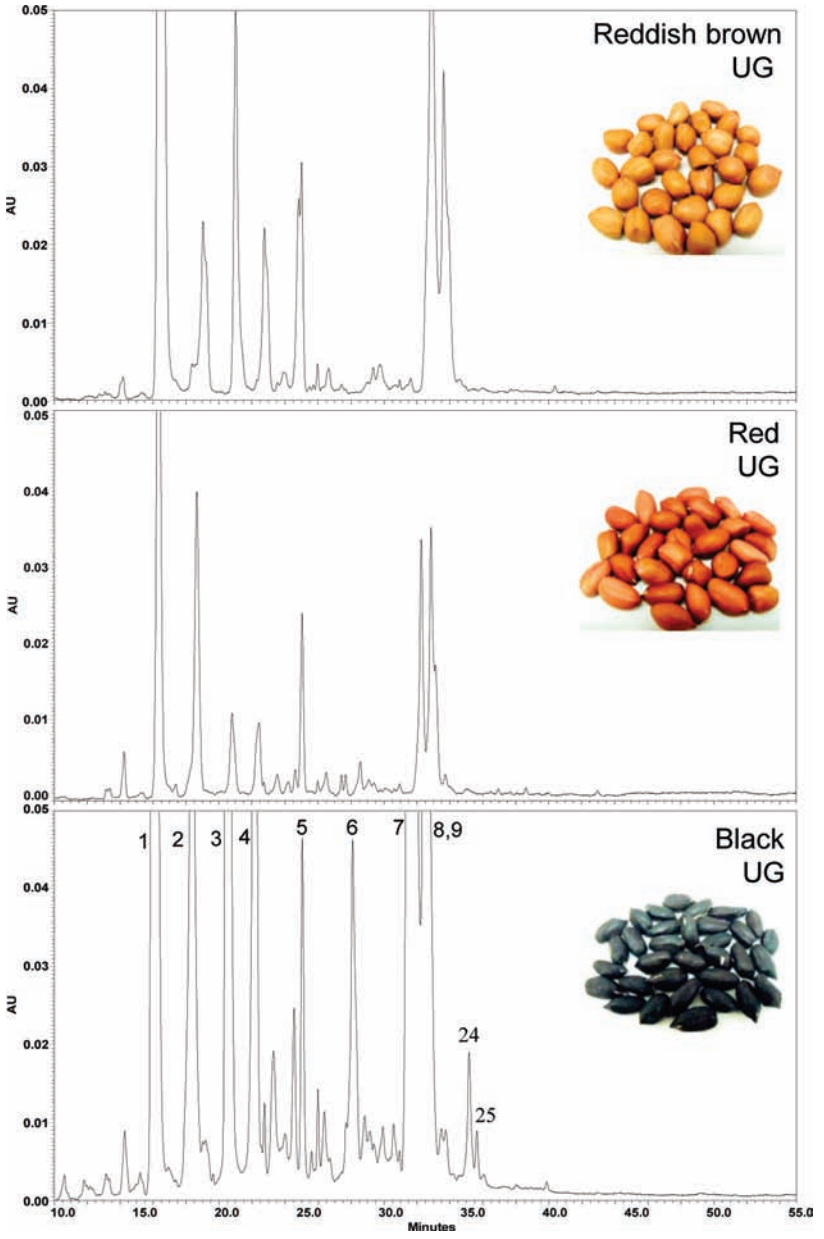

Figure 1. HPLC chromatograms (310 nm) of three types of peanut seeds in acetone/ethanol/water (2:2:1) extracts. Tentative identification of compounds is listed in Table 1.

system and an electrospray ionization (ESI) source, which consisted of a P4000 quaternary pump, a UV6000LP PDA detector, and an AS3000 autosampler.

Peanut Germination and Fungal Inoculations. Peanut (*Arachis hypogaea* L.) seeds with three classes of skin colors (reddish brown, red, and black) were collected from the supermarket in China (Beyond Organic, Bejing, China). The seed germinations and fungal inoculations were carried out according to the method described previously.[6] In brief, *R. oligosporus* culture powder (0.2 g) was suspended at 25 °C in the dark in a test tube with 25 mL of sterilized water for 24 h to obtain a spore suspension. Seeds were surface sterilized with 70% ethanol for 3 min and then rinsed with water before they were imbibed for 24 h at room temperature (25 °C). Each soaked seed was carefully peeled without injuring the radicle of seed and surface of the endosperm. The inoculated seeds were placed on a sterile plastic Petri dishes (90 mm × 13 mm) lined with two autoclaved filter papers moistened with 5 mL of prepared fungal culture suspension solution (germination and fungal stress, GS) or 5 mL of sterile water (germination, G). The deactivated seeds were prepared by heating in an oven at 150 °C for 20 min. The deactivated seeds were placed in the Petri dishes with (deactivated seed with fungal stress, DS) or without (deactivated seed without fungal stress, D) fungal culture. The Petri dishes were sealed with parafilm and incubated for 3 days at 25 °C in the dark. Two milliliters of fungal culture solution or sterile water was added into the two group Petri dishes of GS/DS and G/D, respectively, each day. Ungerminated (UG) seeds were also prepared with identical procedures. Four replicates were conducted for each sample.

Sample Preparation Procedures. Two to four seeds of each peanut samples were collected from day one to day three, weighed in a 15 mL screw-cap tube, and then extracted with 5 mL of acetone/ethanol/water (2:2:1; v/v) containing 0.1% acetic acid on a shaking incubator at 200 rpm and room temperature for 12 h. The mixtures were

Table 1. Peak Assignments of Compounds in GS Peanuts Presented According to Retention Time, Maximum UV Absorption, and Molecular Ions

peak	LC-RT (min)	tentative identification	UV bands (nm)	ESI^+ $[M + H]^+$	ESI^- $[M - H]^-$
1	9.1	coumaric acid derivative 1	314	147, 165	163, 295
2	10.6	coumaric acid derivative 2	314	147, 297	163, 277
3	12.6	coumaric acid derivative 3	314	147	163, 295
4	13.0	coumaric acid derivative 4	314	251	163, 295
5	16.2	coumaric acid derivative 5	314	309	163
6	18.9	coumaric acid derivative 6	314	291	163, 289
7	21.8	coumaric acid derivative 7	314	147, 309	163, 277, 441
8	23.2	sinapinic acid derivative 1	318	207	223
9	23.7	feruloyl malic acid derivative 1	318	177, 291	193, 307
10	12.0	hydroxybenzoic acid derivative 1	260, 302		137
11	14.1	coumaric acid derivative 8	313		132, 163, 307
12	14.7	coumaric acid derivative 9	311	134,	132, 163, 307
13	16.0	sinapinic acid derivative 2	305	207	205
14	10.1	coumaric acid derivative 10	314	147, 297	163, 277
15	19.4	coumaric acid derivative 11	313	133	163
16	19.5	catechin	282	291, 245	203, 245, 289
17	19.8	feruloyl malic acid derivative 2	318	291	193
18	20.0	sinapinic acid derivative 3	305	207	223
19	20.4	caffeic acid derivative 1	240, 324	163	179, 135
20	21.0	hydroxybenzoic acid derivative 2	260, 302	152	137
21	26.3	sinapinic acid derivative 4	318	165,207	223
22	26.7	feruloyl malic acid derivative 3	327	177, 291	193
23	27.4	piceid derivative 1	307	391	389, 227
24	29.2	coumaric acid derivative 12	314		277, 441
25	29.4	coumaric acid derivative 13	315	147	163, 295
26	29.5	*cis*-resveratrol	309	229	227
27	36.4	3′-isopentadienyl-3,5,4′-trihydroxystilbene derivative 1	297	295	293
28	36.5	3′-isopentadienyl-3,5,4′-trihydroxystilbene derivative 2	298	295	293
29	37.8	arachidin-3 derivative 1	336	297, 923, 241	921, 239, 295
30	27.9	*trans*-resveratrol	306, 318	229	227, 453
31	36.5	arachidin-1 derivative 1	323	313	311
32	37.1	arachidin-2 derivative 1	325	297	
33	30.8	arahypin-3 derivative 2	337	229	227, 329
34	31.3	coumaric acid derivative 14	314	147	145, 163
35	32.5	genistein	260	271	269
36	34.2	SB-1 derivative 1	325	345	343
37	34.7	SB-1 derivative 2	322	343	341
38	35.1	arahypin-4 derivative 1	310	229, 313	311, 227
39	35.6	3′-isopentadienyl-3,5,4′-trihydroxystilbene derivative 3	295	295	
40	39.3	arahypin-5 derivative 1	341	295	293
41	39.5	arahypin-1 derivative 2	326	281	279
42	40.6	chiricanine A derivative 1	317		279
43	41.6	chiricanine A derivative 2	316	281	279
44	42.8	KODE glyceryl esters	282	351, 355, 369	353, 367
45	47.5	KODEs	280	295	293

centrifuged at 5000 rpm for 10 min. The supernatant was collected and stored at −20 °C for analysis of antioxidant capacity, total phenolics, and total flavonoids. The supernatant was filtered through a Sartorius Minisart polytetrafluoroethylene (PTFE) membrane (0.45 μm) before using phytochemical HPLC and LC-ESI-MS analysis.

Quantification of Antioxidant Capacity and Total Phenolics. Six antioxidant capacity related values including total phenolics content (TPC), total flavonoid content (TFC), oxygen radical ($ROO^•$) absorbance capacity (ORAC), hydroxyl radical ($HO^•$) absorbance capacity (HORAC), superoxide radical ($O_2^{•-}$) absorbance capacity (SORAC), and DPPH radical scavenging capacity values were measured. TPC was measured based on the Folin−Ciocalteau method according to Wu et al.[15] Gallic acid (50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/L; correlation coefficient, r = 0.999) was used to establish the standard

curve, and the results were expressed as gallic acid equivalents (mg GAE/100 g FW). TFC was determined using a colorimetric method described previously.[16] Twenty microliters of sample or (+)-catechin standard solution (50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/L) was mixed with 80 μL of distilled water in each well of a 96-well polypropylene plate, followed by adding 6 μL of 5% (w/v) $NaNO_2$ solution. After 5 min, 6 μL of 10% (w/v) $AlCl_3$ solution was added and allowed to stand for another 1 min before adding 40 μL of 1.0 M NaOH. The mixture was toped up to 200 μL with distilled water. The absorbance was measured immediately against the blank (the same mixture without the sample) at 510 nm. The results were calculated and expressed as catechin equivalents (mg CAE/100 g FW). Hydrophilic ORAC procedure was based on previous report.[17] The results were expressed as Trolox equivalents (μmol TE/100 g FW). The HORAC assay was based on a previous report.[18] SORAC protocol was based on a Zhang et al. report,[19] and results were expressed as units SOD equivalent/100 g FW. The DPPH scavenging capacity was determined as previously described.[20] The analysis of variance was carried out with the SAS statistical program (version 9.00, SAS Institute Inc., Cary, NC), and differences between the means of treatments were determined by Duncan's multiple range test at $P < 0.05$.

Detection of Phytochemical by PDA and MS. HPLC analysis was performed on a Waters apparatus equipped with PDA detector. The detection wavelength was set from 210 to 800 nm. The separation was accomplished on a Waters C_{18} column (5 μm, 4.6 mm × 250 mm) with water (A), acetonitrile (B), and 2% acetic acid in water (C) as the mobile phase. The column temperature was 30 °C. The injection volume was 20 μL. Solvent C composition was maintained constant at 5%. The other 95% of the eluents were contributed from mobile phase A and B with gradients as follows: 0−1 min, isocratic with A 95% (of total A and B); 1−8 min, A decreased from 95 to 85%; 8−24 min, A decreased from 85 to 70%; 24−34 min, A decreased from 70 to 40%; 34−50 min, A decreased from 40 to 20%; 50−55 min, A decreased from 20 to 5%; 55−58 min, A increased from 5 to 95%; and 58−60 min, A was kept constant at 95%. The flow rate was 1.0 mL/min. The LC conditions for LC-MS analysis used solvent A (water with 0.05% acetic acid) and B (acetonitrile with 0.05% acetic acid) as the mobile phase. The gradient was identical to those used for HPLC analysis above. The injection volume of each sample was 20 μL. For ESI-MS, both the positive and the negative ion modes were used for further characterization of the phytochemicals. The capillary temperature and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was supplied at 80 psi as the sheath gas and at 20 psi as the auxiliary gas. Full scan mass spectra from m/z 50 to 2000 were recorded with a scan speed of one scan per second.

Phytoalexin Extraction and Detection by HPLC and LC-MS. The reddish brown peanut seeds (500 g) were germinated with *R. oligosporus* stress at 25 °C in the dark for 3 days. The peanut samples were homogenized in acetone/ethanol/water (2:2:1; v/v) and then extracted with the same solvent mixture for three times on a shaking incubator at 200 rpm and room temperature for 12 h at each time. The extraction solutions were concentrated in a rotary evaporator at 50 °C. The concentrated residue was transferred to a silica gel column (35 cm × 6 cm, silica gel 60) for fractionation. The column was pre-equilibrated with hexane and then successively eluted with a hexane and hexane/ethyl acetate (7:3) mixture at a flow rate of 5 mL/min. Each fraction was collected (100 mL). After HPLC detection, the fractions containing the phytoalexins were combined and further analyzed by LC-ESI-MS. The LC-ESI-MS conditions were similar with those used for phytochemical detection above, except the LC mobile phase gradient. The gradient was as follows: 0−1 min, isocatic with 95%; 1−8 min, A decreased from 95 to 65%; 8−15 min, A further decreased from 65 to 55%; 15−50 min, A decreased from 55 to 40%; 50−55 min, A decreased further from 40 to 10%; 55−58 min, A increased from 10 to 95%; and 58−60 min, A remained at 95%.

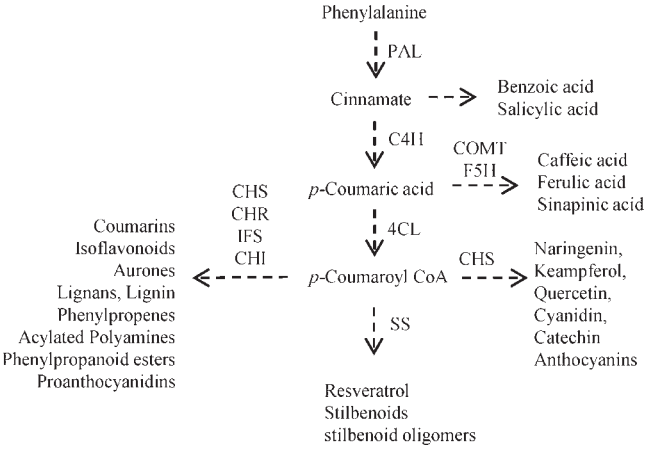

Figure 2. Proposed phenylpropanoid biosynthetic pathway in germinated peanuts under food grade fungus *R. oligosporus* stress. PAL, phenylalanine ammonialyase; C4H, cinnamate 4-hydroxylase; F5H, ferulic acid 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; COA, coenzyme A; 4CL, 4-coumarate:CoA ligase; SS, stilbene synthase; CHS, chalcone synthase; CHR, chalcone reductase; IFS, isoflavone synthase; and CHI, chalcone isomerase.

RESULTS AND DISCUSSION

Peanut skin color varies usually from light brown to deep red. However, peanuts with different skin colors have been cultivated and consumed widely during the past decade in China, such as black, purple black, white, red, and multicolored, although most of them are rarely seen in the marketplace worldwide. To investigate the difference between phytochemicals in peanut seeds with different skin colors, three of the types of peanut were studied; for future comparison purposes, samples of each peanut seeds were kept at −80 °C in a freezer in our lab.

Stressed peanuts can produce a wide range of phytoalexins. Using *R. oligosporus* as a food grade elicitor, the germinating peanuts of three different skin colors were processed, and their phytoalexin profiles were extracted, fractionated, and characterized by HPLC and LC-MS. We applied an analytical method for the comprehensive profiling of semipolar metabolites in the acetone/ethanol/water (2:2:1; v/v) extract of the three cultivars of peanuts. HPLC-PDA and ESI-MS detectors were used to tentatively identify the secondary metabolites. The masses or MS fragments information were matched with compounds reported in literatures and supplemented by UV, HPLC retention time, and reference compounds, including caffeic acid, catechin, *p*-coumaric acid, ferulic acid, genistein, hydroxybenzoic acid, *trans*-resveratrol, and sinapic acid, as well as existing metabolite databases including PubChem, Kegg Ligand database, Massbank, and Scifinder Scholar.

Profiles of Phenolic Acids in Peanut Seeds. For ungerminated peanut seeds, HPLC chromatograms (310 nm) of acetone/ethanol/water extracts of three different types of peanut seeds are given in Figure 1. The HPLC-PDA detector allowed us to record the UV/vis spectrum in the range of 210−800 nm, but only the chromatograms at 310 nm are presented in this study since the most number of peaks are observed at this wavelength in all peanut samples. LC-MS analysis reveals that phenolic acids

Table 2. TPC, TFC, ORAC, HORAC, SORAC, and DPPH Values of Ungerminated Seed (UG), Germinated Seed (G), and Germinated Seed with Fungal Stress (GS)[a]

		TPC		TFC		ORAC		HORAC		SORAC		DPPH	
peanut	treatment	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
black	UG0d	144.8 K	3.6	18.54 j	0.40	2335.5 hi	16.3	651.3 b	19.5	54.9 h	2.0	3.54 n	0.10
	G1d	184.7 F	5.6	24.23 i	0.89	2279.1 i	99.0	594.3 c	14.1	30.0 k	2.1	6.96 hi	0.06
	G2d	165.6 Hi	6.6	24.58 i	1.29	2892.3 f	83.4	663.6 b	33.7	20.4 l	1.1	6.16 ijk	0.21
	G3d	174.2 Gh	7.1	18.93 j	0.91	2984.2 f	123.6	690.5 ab	61.1	12.5 l	1.8	5.09 kl	0.26
	mean	174.9		22.64		2718.5		649.5		21.0		6.11	
	GS1d	194.1 E	8.6	14.80 kl	0.55	2439.6 gh	20.3	663.2 b	25.1	33.6 jk	2.8	6.28 ij	0.37
	GS2d	165.8 Hi	6.2	15.22 kl	0.75	3313.3 e	122.0	568.8 c	31.5	13.9 l	0.4	7.69 gh	0.62
	GS3d	158.5 Ij	5.0	15.69 kl	0.80	3288.7 e	125.9	687.4 ab	20.3	14.1 l	0.1	5.55 jkl	0.20
	mean	172.8		15.21		3013.9		639.8		20.5		6.52	
red	UG0d	126.9 L	3.4	13.59 l	0.51	690.4 n	0.9	126.8 i	2.3	36.4 ijk	1.3	4.87 lm	0.21
	G1d	155.8 J	2.7	27.25 h	0.23	1339.4 m	28.9	216.7 h	10.8	55.6 h	1.1	6.46 ij	0.30
	G2d	174.3 Gh	4.4	36.89 f	0.39	1685.2 l	54.9	337.0 g	23.3	66.3 g	3.5	10.36 ef	0.10
	G3d	194.1 E	8.6	24.77 i	0.91	2084.4 j	84.9	364.2 fg	29.1	37.6 ijk	1.3	10.53 ef	0.71
	mean	174.8		29.61		1703.0		306.0		53.1		9.15	
	GS1d	160.1 Ij	4.8	33.80 g	1.61	1393.3 m	47.6	235.5 h	12.1	42.1 ij	2.2	8.71 g	0.13
	GS2d	181.1 Gf	5.7	57.34 d	1.16	3580.2 d	86.2	381.1 f	16.4	250.1 b	6.7	18.47 c	0.99
	GS3d	228.0 B	10.9	75.73 c	2.11	4670.6 b	155.9	520.6 d	41.8	142.0 d	12.8	11.44 e	0.33
	mean	189.7		55.67		3214.7		379.1		144.7		12.92	
reddish brown	UG0d	130.1 L	3.0	17.06 jk	0.37	1383.6 m	60.9	229.5 h	5.5	94.3 f	4.7	3.90 mn	0.08
	G1d	160.6 Ij	4.0	34.45 g	0.89	1914.5 k	32.6	357.2 fg	29.3	104.6 e	5.9	9.78 f	0.40
	G2d	194.7 E	8.2	44.42 e	0.64	2502.4 g	18.2	451.9 e	16.6	45.5 i	2.9	18.79 bc	2.00
	G3d	208.4 D	5.8	45.98 e	0.67	3384.0 e	97.9	586.1 c	20.2	40.7 ij	4.1	12.50 d	1.23
	mean	187.9		41.65		2600.3		465.0		63.6		13.77	
	GS1d	218.5 C	10.1	35.96 fg	0.61	3360.8 e	103.0	369.5 fg	25.5	144.1 d	6.0	10.86 ef	1.03
	GS2d	220.0 Bc	12.2	95.31 b	4.18	3859.2 c	134.5	530.9 d	17.8	318.5 a	18.2	19.70 b	1.33
	GS3d	253.2 A	5.1	113.35 a	4.73	5031.6 a	155.9	713.2 a	38.8	196.6 c	16.1	23.67 a	1.26
	mean	230.5		81.52		4083.9		537.9		219.7		18.13	

[a] The data are expressed as means ± standard deviations ($n = 4$), and G and GS are measured during the 3 days. The means of treatments are compared by Duncan's multiple range test at $P < 0.05$, and different letters show significant differences. TPC values are expressed as gallic acid equivalents (mg GAE/100 g fresh weight sample), TFC values are expressed as catechin equivalents (mg CAE/100 g FW), ORAC values are expressed as Trolox equivalents (μmol TE/100 g FW), HORAC are expressed as mg GAE/100g FW, SORAC values are expressed as units SOD equivalent/100 g FW, and DPPH scavenging capacity values are expressed as mg GAE/100 g FW.

are the major group of phenolic compounds in ungerminated seeds. LC retention time, maximum UV absorption, fragment ion masses in positive-ion ($[M + H]^+$) and negative-ion ($[M - H]^-$) modes of compounds identified tentatively are listed in Table 1.

A majority of the phenolic acids are found to be coumaric acid derivatives (peaks 1−7), sinapinic acid (peak 8), and ferulic acid (peak 9). This result is consistent with previous reports that *p*-coumaric acid accounted for 40−68% of the total phenolic acids in all peanut protein products, and roasted peanuts contain *p*-coumaric acid greater than 100 mg/kg.[21] Coumaric acid derivatives are characterized by common maximal UV absorption at 309−314 nm[22] and by MS peak at 147 ($[M + H - H_2O]^+$ from dehydration of the coumaric acid ion ($[M - H]^-$, 163). Meanwhile, sinapinic acid derivatives are identified by main product ions at ($[M + H - H_2O]^+$, 207), ($[M - H - H_2O]^-$, 205), and ($[M - H]^-$, 223) and ferulic acid at ($[M + H - H_2O]^+$, 177) and ($[M - H]^-$, 193).

For higher plants, these phenolic acids are covalently bound to polysaccharides in cell walls, acting as cross-linkers between the lignins polymers and the hemicellulose and cellulose. Coumaric acid plays a central role in the phenylpropanoid biosynthetic pathway (Figure 2), which starts from the conversion of phenylalanine to *trans*-cinnamic acid, which subsequently hydroxylates to form *p*-coumaric acid. *p*-Coumaric acid is a precursor of 4-coumaroyl-CoA, which serves as a substrate to form the basic skeleton of all flavonoid derivatives.[23] Coumaric acid could also be used as a precursor in the production of resveratrol in microorganisms (e.g., food-grade yeast *Saccharomyces cerevisiae*) genetically modified with genes of the phenylpropanoid pathway.[24]

As can be seen in Figure 1 and Table 2, black peanuts have higher contents of phenolic acids, total phenolic (TPC), and total flavonoids (TFC) than reddish brown and red peanuts. These translate to its higher ORAC and HORAC. However, the SORAC and DPPH radical scavenging capacity do not show proportional increase (Table 2). For ORAC and HORAC values, black peanut (2335 μmol TE/100 g, 651 mg GAE/100 g) has a much higher value than that of reddish (1384 μmol TE/100 g, 229 mg GAE/100 g) and red peanuts (690 μmol TE/100 g, 126 mg GAE/100 g). In addition, black peanuts are reported to be

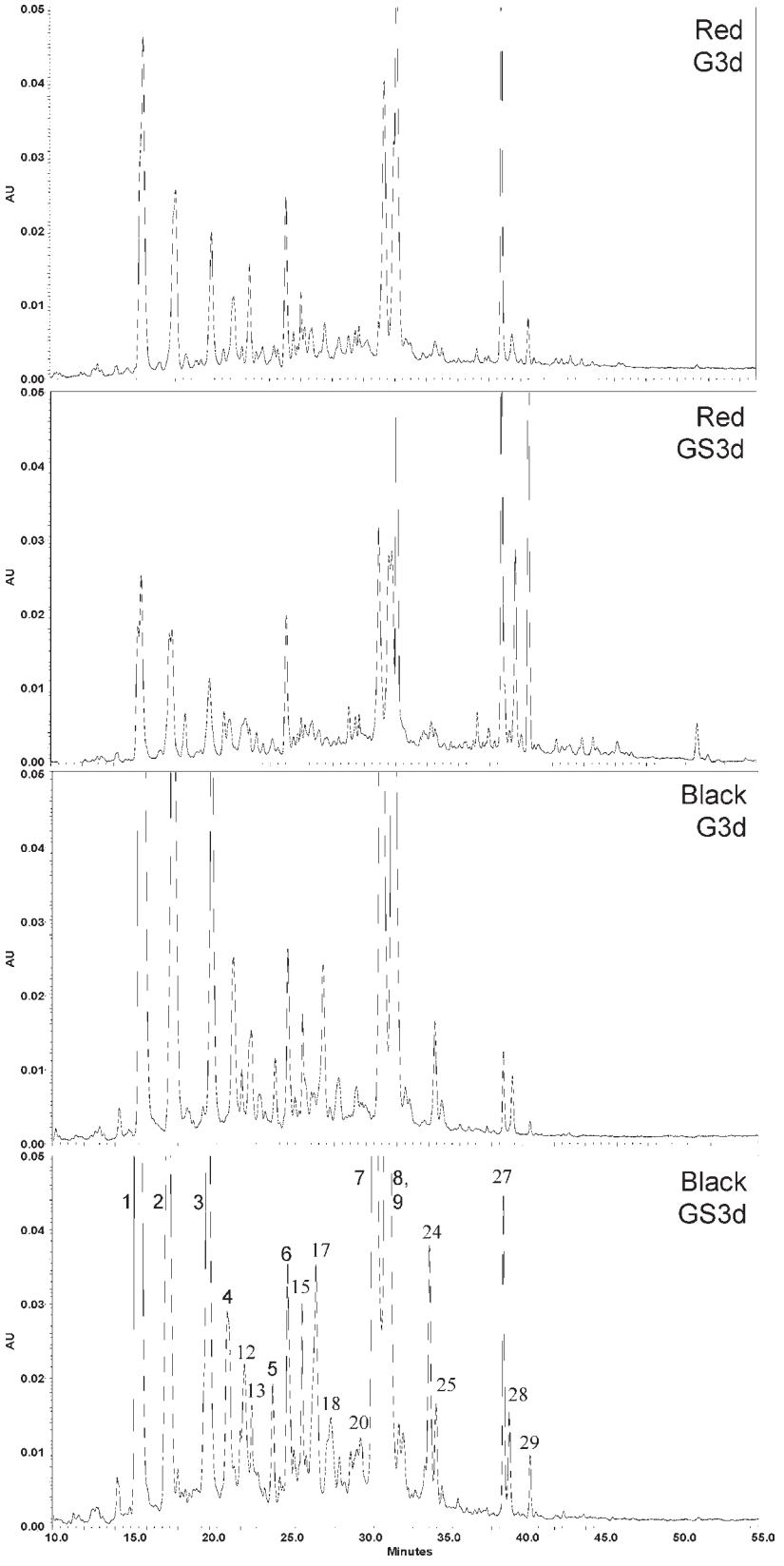

Figure 3. Comparative HPLC chromatograms (310 nm) between red and black peanuts after 3 days of germination with or without fungal inoculation (G or GS). The peak assignments of corresponding compounds are presented in Table 1.

rich in mineral elements, such as potassium (7136 μg/g), iron (58.16 μg/g), selenium (14.24 μg/g), and zinc (38.92 μg/g), which are higher than the levels in red peanut.[26] Therefore, black peanuts may have a potential as a new functional food due to its

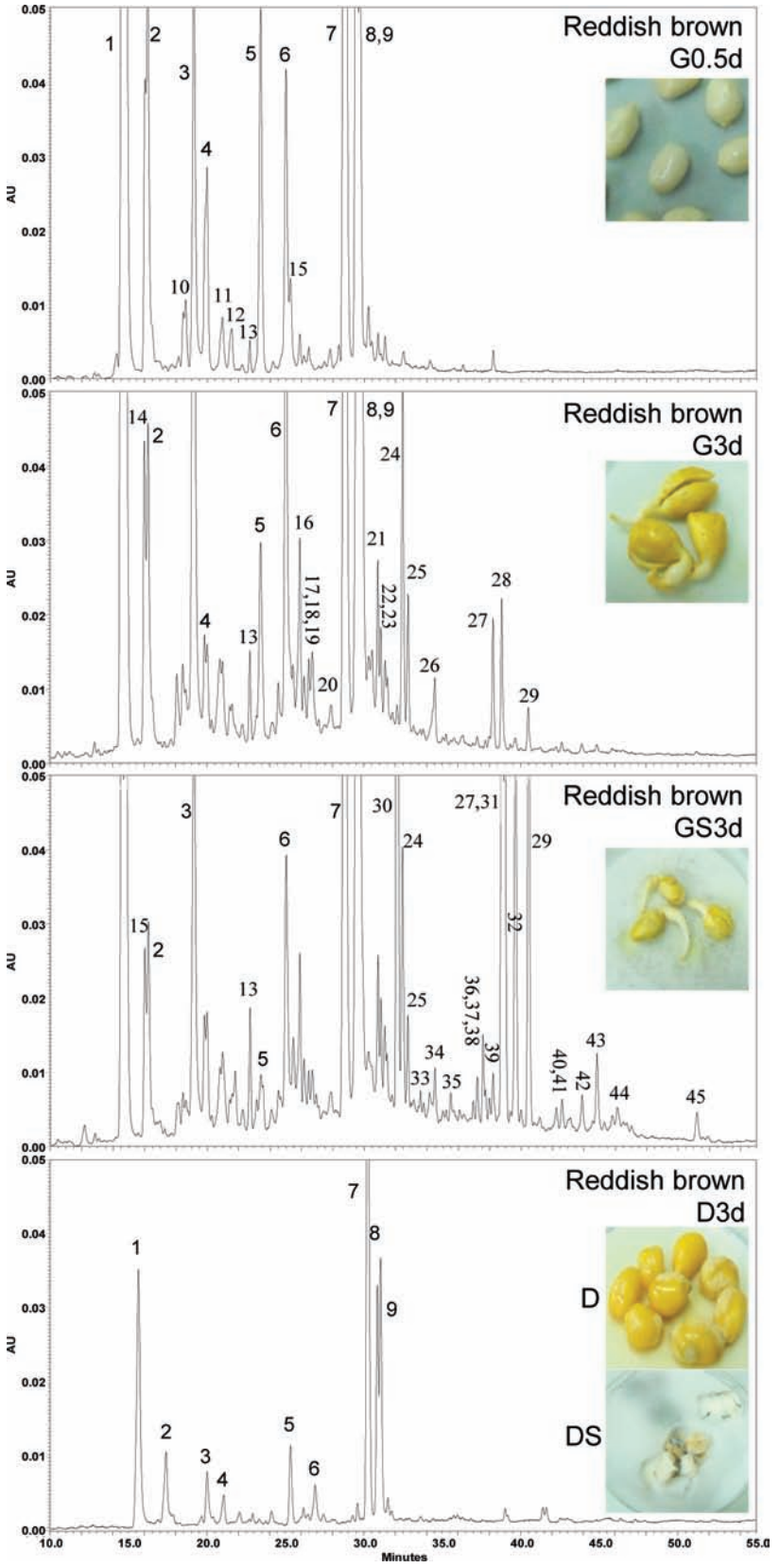

Figure 4. HPLC chromatogram (310 nm) of germinated reddish peanut after 12 h (G0.5d) and 3 days (G3d) without *R. oligosporus* stress, 3 days with the fungal stress (GS3d), and thermal-deactivated seeds inoculated without (D) or with (DS) fungal stress for 3 days (D3d). The peak assignment is shown in Table 1.

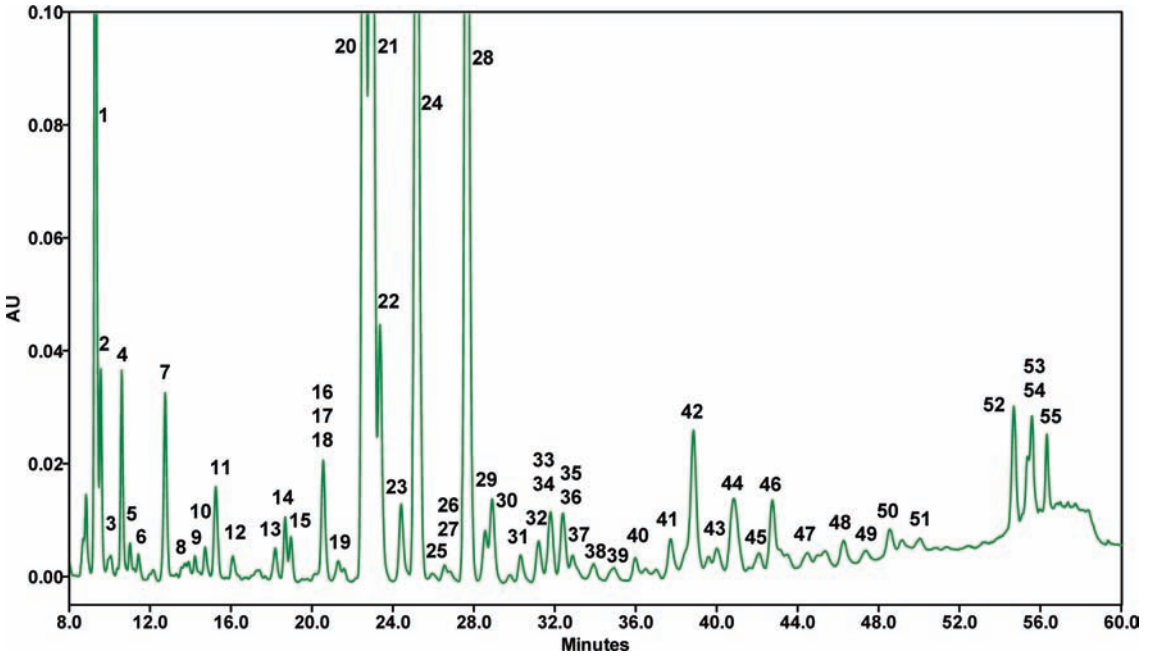

Figure 5. HPLC chromatogram (310 nm) of peanut phytoalexins in germinated peanuts under food grade fungus *R. oligosporus* stress. The tentative identification of corresponding compounds is listed in Table 3.

rich mineral contents. Our measured values are found to be smaller than U.S. Department of Agriculture database for TPC and ORAC of peanut raw.[25] However, our results may be different if the sample is the whole seed.

Polyphenolic Profiles in Germinated Peanuts. In this study, a total of 45 compounds (Table 1) are identified tentatively in the GS (germinating seeds with fungal stress) samples; these include 14 coumaric acid derivatives, 3 ferulic acids, 4 sinapinic acids, 2 hydroxybenzoic acids, 1 caffeic acid, 2 flavonoids, and 19 stilbenoids derivatives. Among the metabolites detected, the most abundant compounds are phenolic acid compounds (10−32 min, Figures 3 and 4) and stilbenoid phytoalexins (32−55 min). Both classes of secondary metabolites are receiving considerable attention from producers and consumers due to their antioxidant activity and anti-inflammation activity.[2]

Figure 3 presents a comparative HPLC chromatogram between red and black peanuts after 3 days of germination with or without fungal inoculation (G or GS). There are several compounds proposed as phytoalexins that are generated under GS and G such as peaks 27 and 28 (isopentadienyl-3,5,4′-trihydroxystilbene), peak 29 (*trans*-arachidin-3), and peak 30 (*trans*-resveratrol). In addition, some new phenolic acid derivatives are also produced during G and GS, such as caffeic acid (peak 19), hydroxybenzoic acids (peaks 10 and 20), and catechin (peak 16) and genistein (peak 35).

Figure 4 shows HPLC chromatogram of germinated reddish peanut after 12 h (G0.5d) and 3 days (G3d) of germination without *R. oligosporus* stress, 3 days with the fungal stress (GS3d), and thermal-deactivated seeds inoculated without (D) or with (DS) fungal stress for 3 days (D3d). To confirm whether these newly released compounds are produced by fungi themselves or by peanuts in response to the fungal action, the D3d peanuts are analyzed, and it is found that these compounds are not present. Thus, we conclude that the 19 stilbenoids are phytoalexins produced by the peanuts during the germination under fungal stress. In addition, we found that there is a significant decrease in phenolic acids in deactivated peanut after 3 days in comparison to the ungerminated seeds (Figure 4). However, not much difference is found in the phytochemicals between the deactivated peanut with or without fungal inoculation (data not shown). This suggests that thermal processing could reduce phenolic acid compounds.

Overall, among the three peanuts, the maximum changes in the production of phytochemicals are observed in reddish brown peanuts during G and GS, followed by red peanuts, while black peanuts seemed to have little change from the HPLC analysis. For red peanuts, G and GS could increase polyphenolic compounds and concentrations, which may be responsible for the significant increase in TPC, TFC, ORAC, HORAC, SORAC, and DPPH values in the corresponding seeds (Table 2). For TPC in GS reddish peanut, the maximum value is 230 mg GAE/100 g FW, which is 1.8 times higher than that of ungerminated seed. Similarly, the TFC maximum value is 81 mg CAE/100 g FW in GS reddish peanut, an increase of 4.8 times; ORAC is 4084 μmol TE/100 g FW or 2.9 times increase; SORAC is 220 units SOD equivalent/100 g FW or 2.3 times; and DPPH is 18 mg GAE/100 g FW or 4.6 times. However, for HORAC, the maximum value belongs to ungerminated black peanut (651 mg GAE/100 g FW), which is higher than that of GS reddish peanut (538 mg GAE/100 g FW).

The sprouts from mung bean and yellow soybean are the most popular traditional food. The ORACs of mung bean and yellow soybean sprout obtained from a supermarket in Singapore were 1606 and 3126 μmol TE/100 g FW, respectively,[27] and soybean sprout is 962 μmol TE/100 g FW.[25] In this study, the ORAC of GS germinated peanuts was 4084 μmol TE/100 g FW. Germinated peanuts are not a typical food item in this part of the world, although they may have some health benefits due to the unique phytoalexin contents.

Table 3. Peak Assignments of Phytoalexins in GS Peanuts Presented According to Retention Time, Maximum UV Absorption, and Molecular Ions

peak	LC-RT (min)	tentative identification	UV bands (nm)	ESI^+ $[M + H]^+$	ESI^- $[M - H]^-$
1	8.92	*trans*-resveratrol	307, 320		227
2	9.12	arachidin 2 derivative 1	326	297	295, 457
3	9.5	arahypin 2 derivative 1		331	329
4	10.1	arachidin 3 derivative 1	338	297	295, 457
5	10.4	arahypin 4 derivative 1		311, 313	295, 315, 325
6	10.8	cyanidin	280	287	285
7	12.1	naringenin	277		271
8	12.8	arahypin 2/3 derivatives 1		331	329
9	13.5	hesperetin	289		301
10	14.0	chiricanine A derivative 1	308	225, 281	223, 227, 279, 453
11	14.6	unknown	260		299
12	15.3	arahypin 1 derivative 1		281	225, 279
13	17.4	SB 1 derivative 1	260, 357	345	299, 329, 343, 389
14	17.8	SB 1 derivative 2	262, 360	465	299, 343, 361
15	18.2	arahypin 3 derivative 1	300		329
16	19.8	arachidin 2 derivatives 2	322	297	295
17	19.8	aracarpene	322	301	299
18	19.8	arachidin 1 derivatives 3	322	313	311
19	20.6	acacetin	332		283
20	21.8	arachidin 1 derivative 4	343	313	311
21	22.2	3′-isopentadienyl-3,5,4′-trihydroxystilbene derivative 1	296	295	293
22	22.6	arachidin 2 derivatives 3	324	297	295
23	23.6	arachidin 1 derivatives 5	346	309, 311, 313	309
24	24.4	arachidin 2 derivative 4	326	297	295
25	25.1	arahypin 7 derivatives 1		623	621
26	25.8	arachidin 2 derivatives 5		297	295
27	25.8	arachidin 1 derivatives 6		313	311
28	26.9	arachidin 3 derivative 2	337	297, 591	295, 589
29	27.7	unknown	345		325
30	28.1	3′-isopentadienyl-3,5,4′-trihydroxystilbene derivative 2	296		295, 355
31	29.5	arahypin 7 derivative 2	331	311	311, 621
32	30.4	arahypin 6 derivative 1	276, 320	607	605
33	31.1	arahypin 5 derivative 1	341	238	293
34	31.1	arahypin 4 derivative 2	341	238	313
35	31.6	arachidin 1 derivatives 7	328	313	311
36	31.6	SB 1 derivatives	328		343
37	32.3	arahypin derivatives 1	340	394, 667	313, 619
38	33.3	arahypin 7 derivatives 3	335	623	621
39	34.4	dimeric stilbenoids derivatives 1	330	605	311, 603, 621
40	35.5	dimeric stilbenoids derivatives 2	333	603, 621, 623	311, 619, 621
41	37.4	dimeric stilbenoids derivatives 3	335	311, 621, 623	309, 311, 603, 619, 621
42	38.5	arahypin 7 derivative 4	262, 347	623	621
43	39.6	arahypin 6 derivatives 2		607	605
44	40.4	arahypin derivatives	324	621, 623	295, 327, 619, 621
45	42.0	arahypin 6 derivatives 3		607	605
46	42.6	arahypin 6 derivative 4	269, 345	607	605
47	44.0	arahypin 6 derivatives 5		311	605
48	46.2	arachidin 2 derivatives 6	324	589, 591	295, 587, 592
49	47.0	dimeric stilbenoids derivatives 4		299	297, 621, 653
50	48.6	dimeric stilbenoids derivatives 5	341	623	311, 621, 657
51	50.1	arachidin 2 derivatives 7		399, 631	295, 587
52	54.4	13-*Z*,*E*-KODE	279	195, 249, 295	293
53	55.2	13-*E*,*E*-KODE	279	195, 249, 296	293

Table 3. Continued

peak	LC-RT (min)	tentative identification	UV bands (nm)	ESI^+ $[M + H]^+$	ESI^- $[M - H]^-$
54	55.2	9-*E*,*Z*-KODE	279	199, 249, 295	293
55	56	9-*E*,*E*-KODE	276	199, 249, 295	293

Phytoalexins in Germinated Peanuts. To identify the possible phytoalexins generated, the phenolic acids and fats are removed by silica column chromatography with hexane and ethyl acetate. The HPLC chromatogram of phytoalexins is shown in Figure 5, and their LC retention time, maximum UV absorption, fragment ion masses in positive-ion ($[M + H]^+$) and negative-ion ($[M - H]^-$) modes are listed in Table 3.

In total, there are 55 compounds detected. Forty-five of these compounds are suggested to be stilbenoid phytoalexin derivatives, 3 flavonoids (cyanidin, peak 6; naringenin, peak 7; and hesperetin, peak 9), 2 unknown compounds (peaks 11 and 29), 4 oxooctadecadienic acids (KODEs, peaks 52−55),[6] and 1 peanut pterocarpanoid phytoalexin−aracarpene (peak 17).[8] Furthermore, resveratrol (peak 1), arachidin 1 (peak 20), isopentadienyl-3,5,4′-trihydroxystilbene (peak 21), arachidin 2 (peak 24), and arachidin 3 (peak 28) are at concentrations that are among the most abundant in GS peanuts.

It has been reported that Georgia Green peanut kernel, when challenged by *Aspergillus flavus*, produced the highest level of resveratrol (2.91 mg/g) at 48 h, while SB-1 concentration (4.51 mg/g) peaked at 72 h,[12] higher phytoalexin concentrations are accumulated with longer incubation times.[11] Prior reports showed that germination increased amino acids, sucrose, and glucose contents of peanut kernels. This might improve sprout taste and flavor preference.[28] Peanuts are prone to be colonized by *A. flavus* and *Aspergillus parasiticus* since the germinating condition is generally under high temperature and moisture. The fungi can generate aflatoxins, thus rendering the resulting peanut toxic, a major concern to consumers. Peanut sprout itself is safe to consume as shown in an in vivo toxicological and nutraceutical assessment of germinated peanuts and revealed no obvious growth hazard or health toxicity in female Sprague−Dawley rats, which were fed with basal diets supplemented with different amounts of peanut sprouts for 18 weeks.[29]

Stilbenoids are a common structural motif of phytoalexins induced from peanuts. The previously reported ones include arachidins 1−3, 3′-isopentadienyl-3,5,4′-trihydroxystilbene, SB-1, chiricanine A, and arahypins 1−7.[9−12] In the plant kingdom, there are more than 1000 stilbenoids ranging from monomeric stilbenes, bibenzyls, bisbibenzyls, phenanthrenoids, and other stilbene oligomers.[14] These stilbenoid phytoalexin resveratrols and their derivatives have received much attention over the past decade because of their potential health-promoting benefits. In vitro and in vivo studies using various human disease models have demonstrated diverse bioactivities, including antioxidant, antimicrobial, antimalarial, and anti-inflammatory properties, cardio- and neuroprotection, immune regulation, cancer chemoprevention, and lifespan extension.[4,5,13,30] Food grade fungal elicitation of these stilbenoids in peanuts, a popular and healthy food, may be a good way to enrich these bioactive compounds for human consumption, provided that the safety issue is addressed properly.

In conclusion, the HPLC-PDA and LC-ESI-MS based approach is important in the metabolic profiling of peanuts due to the highly diverse phytochemicals present. Our metabolic profiling analysis uncovered many metabolites in the acetone/ethanol/water extracts of the three peanuts. A total of 45 compounds in raw sprouts (mainly phenolic acids) and approximately 50 phytoalexins in GS reddish brown peanut are detected on the basis of their chromatographic retention, UV absorption, positive and negative MS fragments, and data from the literature. In addition, G or GS germinated peanuts produce much higher concentrations of phenolic acids, their derivatives and phytoalexins, along with higher TPC, TFC, ORAC, HORAC, SORAC, and DPPH values than peanut seeds. Our results form a basis for further study to examine the potential of stress germinated peanuts as a source of complex phytoalexins that are otherwise hard to obtain in large quantity. The exact chemical structures of the phytoalexins require further characterization with preparative scale compound isolation. In addition, the nutritional and functional quality is also in need of further investigation, together with the potential food safety evaluation of the products derived from the bioprocessed seeds.

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