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Metabolic profiling and phylogenetic analysis of medicinal *Zingiber* species: Tools for authentication of ginger (*Zingiber officinale* Rosc.)

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

Phylogenetic analysis and metabolic profiling were used to investigate the diversity of plant material within the ginger species and between ginger and closely related species in the genus *Zingiber* (Zingiberaceae). In addition, anti-inflammatory data were obtained for the investigated species. Phylogenetic analysis demonstrated that all *Zingiber officinale* samples from different geographical origins were genetically indistinguishable. In contrast, other *Zingiber* species were significantly divergent, allowing all species to be clearly distinguished using this analysis. In the metabolic profiling analysis, the *Z. officinale* samples derived from different origins showed no qualitative differences in major volatile compounds, although they did show some significant quantitative differences in non-volatile composition, particularly regarding the content of [6]-, [8]-, and [10]-gingerols, the most active anti-inflammatory components in this species. The differences in gingerol content were verified by HPLC. The metabolic profiles of other *Zingiber* species were very different, both qualitatively and quantitatively, when compared to *Z. officinale* and to each other. Comparative DNA sequence/chemotaxonomic phylogenetic trees showed that the chemical characters of the investigated species were able to generate essentially the same phylogenetic relationships as the DNA sequences. This supports the contention that chemical characters can be used effectively to identify relationships between plant species. Anti-inflammatory in vitro assays to evaluate the ability of all extracts from the *Zingiber* species examined to inhibit LPS-induced PGE_2 and TNF-α production suggested that bioactivity may not be easily predicted by either phylogenetic analysis or gross metabolic profiling. Therefore, identification and quantification of the actual bioactive compounds are required to guarantee the bioactivity of a particular *Zingiber* sample even after performing authentication by molecular and/or chemical markers.

Keywords: *Zingiber officinale*; Zingiberaceae; Ginger; Authentication; Metabolic profiling GC/MS; HPLC; Gingerols; Anti-inflammatory; TrnL-F; Rps16

1. Introduction

Ginger (*Zingiber officinale* Rosc.), a member of the tropical and sub-tropical Zingiberaceae, has been cultivated for thousands of years as a spice and for medicinal

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purposes. It is used extensively in Traditional Chinese Medicine to treat headaches, nausea and colds and in Ayurvedic and Western herbal medicinal practice for the treatment of arthritis, rheumatic disorders and muscular discomfort (Dedov et al., 2002). This species contains biologically active constituents including the main pungent principles, the gingerols and shogaols. The gingerols, a series of chemical homologs differentiated by the length of their unbranched alkyl chains, were identified as the major active components in the fresh rhizome (Govindarajan, 1982), with [6]-gingerol (5-hydroxy-l-[4′-hydroxy-3′-methoxyphenyl] decan-3-one) **1** being the most abundant. In addition, the shogaols, another homologous series and the dehydrated form of the gingerols, that result from the elimination of the OH group at C-5 and the consequent formation of a double bond between C-4 and C-5, are the predominant pungent constituents in dried ginger (Connell and Sutherland, 1969; Mustafa et al., 1993). [6]-Gingerol **1** has been found to possess various pharmacological and physiological effects including anti-inflammatory, analgesic, antipyretic, gastroprotective, cardiotonic, and antihepatotoxic activities (Bhattarai et al., 2001; Jolad et al., 2004). Due to these properties, ginger has gained considerable attention as a botanical dietary supplement in the USA and Europe in recent years, and especially for its use in the treatment of chronic inflammatory conditions.

Despite ginger's widespread medicinal and culinary uses, the authentication of ginger samples remains a difficult problem due to heterogeneity of the plant material, contamination with similar looking plants and by the purposeful adulteration of some commercial samples. Because of these problems, authentication of the raw material is very important to ensure that specific batches of dried, chipped or ground ginger are of the quality desired for the manufacture of reliable botanical dietary supplements.

To help address this problem, we investigated a large diversity of plant material within the ginger species and between ginger and several closely related species in the genus *Zingiber*, using molecular and metabolic profiling coupled with anti-inflammatory activity data. The species analyzed were selected because they have documented medicinal uses, specifically anti-inflammatory, are used as substitutes for ginger as spices/flavoring agents, or because they are sometimes mistaken in the popular market for ginger (Ando et al., 2005; Nakamura et al., 2004; Miyoshi et al., 2003; Murakami et al., 2004; Yang and Eilerman, 1999.).

2. Results and discussion

The major goals of this project were to investigate the effect of genetic diversity instead of environmental influences on the chemistry and bioactivity (anti-inflammatory activity) of ginger samples and to set a framework for the authentication of these important botanicals. To accomplish these goals, fresh frozen greenhouse-grown *Zingiber* samples were used for DNA sequence-based phylogenetic analysis, GC/MS-based metabolic profiling, HPLC quantitation of gingerols, and anti-inflammatory assays. As described in Section 4, all samples were grown at the same time under identical conditions in the same greenhouse to ensure that environmental effects were eliminated in this study.

2.1. Phylogenetic analysis based on molecular data

One of our objectives was to investigate the genetic variability of ginger obtained from different sources and of the interspecific differences between ginger (*Z. officinale*) and other medicinal *Zingiber* species. A phylogeny of 104 species in 41 genera representing all four tribes of the Zingiberaceae was reported by Kress et al. (2002). That study, which was based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid matK regions, did not include *Z. officinale* (ginger), *Zingiber mioga*, *Zingiber montanum*, *Zingiber spectabile* and *Zingiber zerumbet*. These two regions, as described in Section 4 are unsuitable for phylogenetic determination of ginger specimens. The reason is that they do not amplify well using standard primers (for the matK-trnK flanking intergenic spacer regions) or because the gene (ITS) is present in more than one copy in the genome of ginger, thus leading to undeterminable sequence data from PCR-product based sequencing runs. We, therefore, used solely the rpsl6 and trnL-F regions for our analysis of *Z. officinale*, *Z. mioga*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet* and *Alpinia galanga*. All species were chosen because of their traditional use as medicinal plants and/or because they are used as adulterants of ginger (Langner et al., 1998). *A. galanga* also served as the outgroup for our phylogenetic analyses.

In the plant specimens that we examined, the intron of rpsl6 had a total aligned length of 742 bp, and the trnL-F region contained a total aligned length of 891 bp. The combined matrix with the indel characters contained 1633 bp. Phylogenetic analysis (using maximum parsimony, see Experimental) of the joined sequences from the intron of rpsl6 and the trnL-F region resulted in a single consensus unrooted parsimonious tree (Fig. 1A). The consensus tree produced when rpsl6 and trnL-F regions were used independently to produce parsimonious trees did not differ from the tree produced when the datasets were combined (data not shown). All of the ginger (*Z. officinale*) samples contained the identical sequence over this entire region, resulting in a single, undifferentiated clade for these samples in the phylogenetic analysis, even though many of these sample lines had been obtained

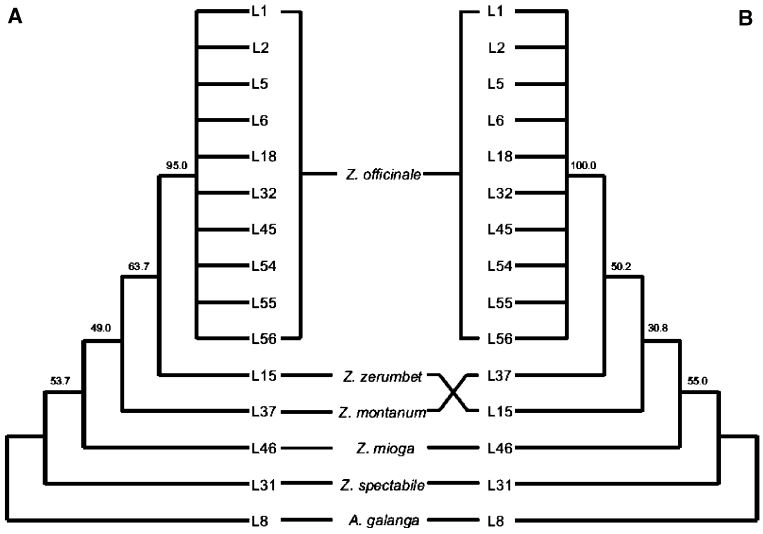

Fig. 1. (A) An unrooted phylogenetic tree of samples from genus *Zingiber* based on trnL and rps16 sequences; (B) An unrooted phylogenetic tree of samples from genus *Zingiber* based on chemical profiles identified using GC/MS. L8 from genus *Alpinia* is used as an outgroup. Numbers above the lines are bootstrap values in the phylogenetic tree.

from very different geographical origins (see Table 1). However, the sequence of the ginger samples differed from those of *Z. mioga*, *Z. montanum*, *Z. spectabile*, *Z. zerumbet*, and the outgroup *A. galanga* for both the rpsl6 intron and the trnL-F region, resulting in clear delineation of all species in our analysis. In particular, there are many single nucleotide polymorphisms in these sequences, allowing us to distinguish ginger from other species based on the sequence data. Some of these sequence differences are illustrated in Fig. 2.

2.2. GC/MS-based metabolic profiling and fingerprinting of Zingiber species

Because medicinal ginger is often sold as ground rhizome powders or as alcoholic or non-polar solvent extracts from these powders, it may be very difficult to obtain DNA evidence to demonstrate adulteration or authentication of particular samples. Thus, we evaluated the utility of chemical characters derived from metabolic profiling experiments to reconstruct the phylogeny of the medicinal *Zingiber* species and to distinguish between species. Non-polar compounds were extracted with methyl *t*-butyl ether (MTBE) and thereafter analyzed by GC/MS. Compounds detected, identified and quantified by GC/MS are listed in Table 2. Many compounds present in small quantities were not included in this analysis because they could not be readily identified due to insufficient mass spectrum quality or because their relative concentration could not be adequately evaluated.

Based on the compounds that were detected and/or identified from the different samples, we found that all

Table 1
The origin of *Zingiber* and *Alpinia* samples used in this analysis

Accession	Species	Original source	Voucher
L1	*Zingiber officinale* Rosc.	Alden Botanica, Moreno Valley, CA	S.P. McLaughlin 9501
L2	*Zingiber officinale* Rosc.	ABCO, Tucson, AZ	Flowering plant confirmed in greenhouse, not vouchered
L5	*Zingiber officinale* Rosc.	17th Street Farmers Market, Tucson, AZ	B. Lewis 217
L6	*Zingiber officinale* Rosc.	Tucson Cooperative Warehouse, Tucson, AZ	Flowering plant confirmed in greenhouse, not vouchered
L18	*Zingiber officinale* Rosc.	Stokes Tropicals, New Iberia, LA	Plants died prior to flowering
L32	*Zingiber officinale* Rosc.	Pacific: Botanicals, Grants Pass, OR	B. Lewis 218
L45	*Zingiber officinale* Rosc.	Plantation Gardens, Clermont, FL	Plants have not flowered
L54	*Zingiber officinale* Rosc.	Super K-Mart, Tucson, AZ	Plants have not flowered
L55	*Zingiber officinale* Rosc.	Trader Joe's, Tucson, AZ	Plants have not flowered
L56	*Zingiber officinale* Rosc.	Trader Joe's, Tucson, AZ	B. Lewis 216
L15	*Zingiber zerumbet* Smith	Fairchild Tropical Garden, Miami, FL.	S.P. McLaughlin 9769
L37	*Zingiber montanum* (Koenig) Theilade	Gingerwood Nursery, St. Gabriel, *LA.*	B. Lewis 208
L46	*Zingiber mioga* (Thunberg) Roscoe	Plantation Gardens, Clermont, FL	S.P. McLaughlin 9977
L31	*Zingiber spectabile* Griff.	Stokes Tropicals, New Iberia, LA	Plants have not flowered
L8	*Alpinia galanga* (L.) Sw.	Fairchild Tropical Garden, Miami, FL	B. Lewis 256

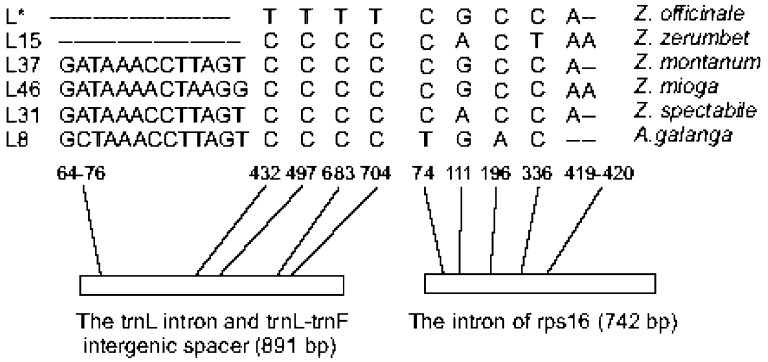

Fig. 2. Some sequence differences of the trnL intron, the trnL-trnF intergenic spacer and the intron of rps16 from *Zingiber* species and *Alpinia galanga*. L*: represents L1, L2, L5, L6, L18, L32, L45, L54, L55, and L56.

ginger (*Z. officinale*) samples showed very similar metabolic fingerprints, i.e., there was no apparent qualitative difference in their GC/MS chromatograms. These plants were grown at the same time under identical conditions in the same greenhouse. Thus, when all differential environmental factors were eliminated, the ginger samples appeared to be chemically very similar, at least at the metabolic fingerprint level (they produced the same compounds), even though these lines were originally obtained from very diverse populations around the country. This result matched the molecular data based on the joined sequences of the rpsl6 intron and the trnL-F region. At the metabolic profile level, however, clear differences between lines could be observed, where many of the compounds were found at different levels in the different ginger lines. This suggests that genetic factors control not only which specific compounds are produced, but at what levels. This is not surprising, but does suggest that ginger obtained from different sources may have significantly different levels of active compounds. This is addressed further in Section 2.3.

Interestingly, the phylogenetic trees generated using the metabolic profiling and the DNA data were almost identical in structure (see Fig. 1B). The only observed difference was that *Z. zerumbet* was found to be more closely related to *Z. officinale* based on molecular data, whereas *Z. montanum* was more closely related to *Z. officinale* based on the chemical data. However, bootstrap support for these differences was not very strong. Thus, major volatile chemical markers were very effective at distinguishing *Zingiber* species and at reconstructing essentially the same phylogeny as was obtained using the DNA sequence data, at least when the chemical characters were considered on a presence/absence basis. Other phylogenetic studies using both molecular and chemical data have been performed, such as with the genera *Peltigera* (Peltigeraceae) and *Sticta* (Stictaceae). Studies of these two genera also showed that both types of characters are useful and complementary (McDonald et al., 2003 and Miadlikowska and Lutzoni, 2000).

Many of the compounds identified in our metabolic profiling analysis could be used as marker compounds to distinguish between the different *Zingiber* species. For example, many of these compounds were only detected in *Z. officinale* samples and not in the other species that we examined. These included the gingerols and their derivatives, the shogaols and paradols, which have not been reported in any species besides *Z. officinale*, and which represent unique marker compounds for this species. In addition, citronellal **28**, (*E*)- and (*Z*)-citral (**32** and **33**), (+)-cyclosativene **37**, zingiberene **39**, α-cubebene **38**, germacrene D **43**, cedr-8-ene **46**, and α-farnesene **49**, among others (see Table 2), were also only present in *Z. officinale*. These compounds, which were not detected in the other examined *Zingiber* species, could also be used as chemical markers to distinguish *Z. officinale* from other *Zingiber* species.

Similarly, a number of other compounds were present in extracts from only one of the other *Zingiber* species. For example, 3-carene **11** and limonene **16** were detected only in *Z. zerumbet*; 1-Methyl-4-(1-methylethyl)-1,3-cyclohexadiene **13**, *trans*-4-isopropyl-1-methyl-2-cyclohexen-1-ol **25** and *cis*-4-isopropyl-1-methyl-2-cyclohexen-1-ol **26** were detected only in *Z. montanum*; and 1,4-bis(methoxy)-triquinacene **54**, was found only in *Z. mioga*. These compounds can be used as markers for the identification of ginger (*Z. officinale*) samples that have been adulterated by these other species. *Z. spectabile* did not offer any known compound that distinguished it from the other species. We observed, however, the presence of several unknown compounds that were found only in *Z. spectabile* (Table 2). These compounds, identified as DRG-GM1-N1-8.86-136-93-121 **12**, DRG-GM1-N1-9.03-134-119-105 **15** and DRG-GM1-N1-9.11-136-93-68 **17**, were named following the nomenclature rules outlined by Bino et al. (2004) for the naming of unknown compounds in metabolic profiling investigations.

We also identified a number of compounds that were not detected in *Z. officinale* but were detected in more

Table 2
Compounds detected and/or identified from *Zingiber* and *Alpinia* samples using GC/MS

RT	Compound Name	M.W.		L1-1	L2-2	L5-4	L6-1	L18-1	L32-5	L45-1	L54-2	L55-1	L56-2	L15-5	L37-3	L46-1	L31-2	L8-6
6.95	1,7,7-Trimethyl-tricyclo[2.2.1.0(2,6)]heptane	136		1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
7.06	3-Thujene	136	○	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0
7.20	1*R*-α-Pinene	136		2	2	2	2	2	2	2	2	2	2	2	2	3	2	2
7.42	*R*(−)3,7-Dimethyl-1,6-octadiene	138	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
7.51	Camphene	136		3	3	3	2	2	2	3	3	3	3	3	1	1	0	0
8.00	4(10)-Thujene	136		1	1	1	1	1	1	1	1	1	1	0	3	2	3	1
8.06	2(10)-Pinene	136		1	1	1	1	1	1	1	1	1	1	1	1	3	3	2
8.35	α-Pinene	136		2	2	2	2	2	2	2	2	2	2	2	2	1	1	0
8.62	2-Methyl-5-(1-methylethyl)-1,3-cyclohexadiene	136		1	1	1	1	1	2	1	1	1	2	1	1	1	0	0
8.74	3-Carene	136	○	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
8.86	DRG-GM1-N1-8.86-136-93-121	136	○	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
8.88	1-Methyl-4-(1-methylethyl)-1,3-cyclohexadiene	136	○	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
9.03	Cymene	134		1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
9.03	DRG-GM1-N1-9.30-134-119-105	134	○	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
9.11	Limonene	136	○	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
9.11	DRG-GM1-N1-9.11-136-93-68	136	○	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
9.12	*m*-Mentha-6,8-diene	136	○	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
9.14	3-Methylene-6-(1-methylethyl)-cyclohexene	136		3	3	3	3	3	3	3	3	3	3	0	0	3	0	0
9.16	Cineole	154		1	1	1	1	1	1	1	2	1	3	2	1	0	0	3
9.75	1-Methyl-4-(1-methylethyl)-1,4-cyclohexadiene	136	○	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0
9.91	Terpineol	154	○	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
10.34	*p*-Mentha-1,4(8)-diene	136		1	1	1	1	1	1	1	0	1	1	0	2	0	0	0
10.58	α-Linalool	154		1	2	2	1	2	1	1	2	2	1	1	1	0	0	0
11.11	*trans*-4-Isopropyl-1-methyl-2-cyclohexen-1-ol	154	○	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
11.57	*cis*-4-Isopropyl-1-methyl-2-cyclohexen-1-ol	154	○	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
11.66	(−)-Alcanfor	152		1	1	1	1	1	1	1	1	1	1	2	0	0	0	0
11.83	Citronellal	154	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
12.22	(1*S*,2*R*,4*S*)-(−)-Borneol	154	•	2	2	1	1	1	2	2	1	1	3	0	0	0	0	0
12.62	*p*-Menth-1-en-4-ol	154	○	0	0	0	0	0	0	0	0	0	0	0	3	0	1	1
12.85	*p*-Menth-1-en-8-ol	154		1	2	1	2	2	1	2	2	2	2	0	1	0	0	0
14.24	(*Z*)-α-Citral	152	•	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0
15.16	(*E*)-α-Citral	152	•	2	3	2	3	3	3	2	3	3	3	0	0	0	0	0
15.82	2-Undecanone	170	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
17.53	4-Allylphenyl acetate	176	○	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
17.64	*p*-Menth-1-en-8-ol, acetate	196	○	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
18.21	(+)-Cyclosativene	204	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
18.56	α-Cubebene or copaene	204	•	2	2	2	2	2	2	1	2	2	3	0	0	0	0	0
19.56	Zingiberene	204	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
20.10	Caryophyllene	204	○	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1
21.22	α-Caryophyllene	204	o	0	0	0-	0	0	0	0	0	0	0	3	0	0	0	1
21.34	α-Famesene (1,6,10-dodecatrine,7, 11-dimethyl-3-methylene-)	204		2	1	1	1	1	2	2	1	1	2	0	0	0	0	2
22.24	Germacrene D	204	•	2	2	1	2	1	2	1	1	2	0	0	0	0	0	0
22.32	2-Methyl-6-*p*-tolyl-2-heptene	202	•	2	3	3	2	3	2	3	3	3	3	0	0	0	0	0
22.65	Eudesma-4(14),11-diene	204		1	2	2	2	2	1	2	2	2	1	0	1	0	0	1
22.80	Cedr-8-ene	204	•	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0

(*continued on next page*)

Table 2 (*continued*)

RT	Compound Name	M.W.		L1-1	L2-2	L5-4	L6-1	L18-1	L32-5	L45-1	L54-2	L55-1	L56-2	L15-5	L37-3	L46-1	L31-2	L8-6
22.82	Cadinene	204	•	0	1	1	0	0	0	0	2	2	0	0	0	0	0	0
23.20	1-Methyl-4-(5-methyl-1-methylene-4-hexenenyl)-cyclohexene,	204	○	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
23.30	α-Farnesene (1,3,6,10-dodecatetraene,3,7,11-trimethyl-)	204	•	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0
23.36	(+)-Epi-bicyclosesquiphellandrene	204	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
23.53	Panasinsen	204	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
23.71	Cadina-1(10),4-dien	204	○	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
23.86	α-Sesquiphellandrene	204		3	3	3	3	3	3	3	3	3	3	0	2	0	0	2
25.42	1,4-Bis(methoxy)-triquinaoene	204	○	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
25.82	Caryophyllene oxide	220	○	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
28.21	Eudesm-4(14)-en-11-ol	222	○	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
31.49	2,6,9,9-Tetramethyl-2,6,10-cycloundecatrien-1-one	220	○	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
46.69	[6]-Paradol	278	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
48.37	[6]-Shogaol	276	•	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0
49.37	[7]-Paradol	292	•	2	2	2	1	2	2	2	2	2	2	0	0	0	0	0
51.11	[6]-Gingerol	294	•	3	3	2	2	2	2	2	2	3	3	0	0	0	0	0
52.85	Acetoxy-[6]-gingerol	336	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
53.95	[8]-Shogaol	304	•	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
54.48	Diacetoxy-[6]-gingerdiol	380	•	1	1	1	1	2	1	1	2	2	1	0	0	0	0	0 -
54.84	[9]-Paradol	320	•	1	1	1	1	1	2	1	1	1	1	0	0	0	0	0
56.56	[8]-Gingerol	322	•	2	2	2	1	1	2	1	1	2	2	0	0	0	0	0
59.08	[10]-Shogaol	332	•	2	2	2	2	1	2	2	2	2	2	0	0	0	0	0
60.03	[11]-Paradol	348	•	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0

Note. 0 indicates absence, 1 indicates <0.5%, 2 indicates 0.5%–5%, and 3 indicates >5% of total integrated peak area of TIC of a particular sample; • indicates compounds detected only in ginger (*Z. officinale)* samples; ○ indicates compounds not detected in ginger.

than one of the other *Zingiber* species. These included 3-thujene **3**, 1-Methyl-4-(1-methylethyl)-1,4-cyclohexadiene **21** and *p*-menth-1-en-4-ol **30**, among others (Table 2). These compounds could also be used as mar- kers to identify supposedly pure samples of *Z. officinale* that have actually been adulterated with *Z. spectabile* (or the other *Zingiber* species in which they were found).

2.3. Quantitation of gingerols using HPLC

In order to further investigate potential variation of chemical composition in samples of *Z. officinale* from different sources, three major bioactive components [6]-, [8]-, and [10]-gingerols (**1, 65**, and **68**) were quantitatively determined (metabolite target analysis). Calibration curves were derived from three independent injections of five concentrations of [6], [8]-, and [10]-gingerols versus the peak area. Linearity was found in the concentration range between 25 and 200 μg ml^{-1}, with high reproducibility and accuracy. Regression analysis of the experimental data points showed a linear relationship with excellent correlation coefficients (r^2) for [6]-, [8]-, and [10]-gingerols, being 0.999 for each, suggesting high precision in this analysis. The linear regression equations for the curves of [6]-, [8]-, and [10]-gingerols concentrations were $y = 52.85x - 95.27$, $y = 46.02x - 73.92$, and $y = 43.57x - 59.58$, respectively, where x was the concentration of standard gingerol (μg ml^{-1}) and y was the total peak area. Although the gingerols are able to undergo dehydration reactions, leading to formation of the shogaols, this only occurs at elevated temperatures or if solutions of the gingerols are dried in air. We saw no evidence for the presence of the shogaols in the standard solutions used for this ana-lysis (although we were able to detect, identify and quantify the shogaols in extracts from ginger rhizomes, see Table 2). Thus, we believe that our results are not only precise, but are accurate representations of the actual content of the gingerols in these samples.

The content of gingerols in *Z. officinale* samples, as summarized in Table 3, were determined by HPLC for 10 accessions that were obtained from different origins. The total gingerols content varied from 1.931 to 3.577 mg/g, with [6]-, [8]-, and [10]-gingerols ranging from 1.284 to 1.905, 0.220 to 0.595, and 0.310 to 1.128 mg/g, respectively (see Table 3). The observed differences were significant for most pair-wise comparisons, as determined by ANOVA analysis ($P < 0.05$). Thus, although qualitatively not different by GC/MS-based metabolic fingerprinting analysis (no difference in absence/presence of major volatile metabolites), more detailed metabolic profiles for select compounds in these *Z. officinale* samples demonstrated that the chemical compositions of these accessions were in fact quantitatively different. Because these plants were grown under identical conditions at the same time in the same

Table 3
Content (mg/g) of the major gingerols in ginger samples from different lines, as determined by HPLC

Compound	Ginger line										
	L1	L2	L5	L6	L18	L32	L45	L54	L55	L56	
[6]-Gingerol	1,669 ± 0.021	1.407 ± 0.013	1.457 ± 0.014	1.442 ± 0.013	1.454 ± 0.020	1.905 ± 0.015	1.508 ± 0.021	1.284 ± 0.022	1.388 ± 0.001	1.854 ± 0.015	
[8]-Gingerol	0.397 ± 0.004	0.220 ± 0.002	0.526 ± 0.004	0.352 ± 0.004	0.410 ± 0.010	0.580 ± 0.004	0.462 ± 0.008	0.370 ± 0.005	0.392 ± 0.001	0.595 ± 0.004	
[10]-Gingerol	0.588 ± 0.004	0.310 ± 0.003	1.021 ± 0.005	0.653 ± 0.008	0.738 ± 0.018	0.804 ± 0.006	0.805 ± 0.012	0.549 ± 0.005	0.658 ± 0.011	1.128 ± 0.008	
Total	2.654 ± 0.029	1.931 ± 0.017	3.004 ± 0.021	2.447 ± 0.024	2.602 ± 0.047	3.289 ± 0.025	2.775 ± 0.041	2.023 ± 0.033	2.438 ± 0.011	3.577 ± 0.025	

Note. All values are the average of three replicates ± standard error.

greenhouse, variation in metabolite composition could not be attributed to environmental factors. Instead, it appears that genetic variation, which we were not able to measure by sequencing two conserved genes (trnL and rps16), must be responsible for the differential accumulation of these major pungent principles. Variation in expression or activity of genes/enzymes involved in the production of these metabolites can explain these differences. Furthermore, this result supports the contention that ginger from different sources is in fact likely to possess different properties, both in flavor and pungency, but also in potential bioactivity and health benefits. Identification of genes involved in regulating the production of these compounds is a major goal of future research in this area.

2.4. Anti-inflammatory assays

To address the issue of potential variation in bioactivity within *Z. officinale*, and to determine if there were significant differences in bioactivity between different species in the genus *Zingiber*, we measured the ability of MTBE and MeOH extracts of *Zingiber* samples to inhibit LPS-induced in vitro production of PGE_2 and TNF-α in cultured human promonocytic U937 cells (Ilieva et al., 2004). All extracts from the *Zingiber* species examined were much more effective at inhibiting PGE_2 production than at inhibiting TNF-α, thus only data for PGE_2 are shown in Table 4.

No statistically significant difference was found between the ginger (*Z. officinale*) crude MeOH and MTBE extracts in their ability to inhibit PGE_2 production, as determined by one-way ANOVA. The IC_{50} for inhibition of PGE_2 production of ginger MeOH extracts ranged from 0.058 to 0.629 μg ml^{-1} and of MTBE extracts ranged from 0.062 to 0.499 μg ml^{-1}. No correlation existed ($R^2 < 0.001$) in the PGE_2 inhibitory activity between the MeOH and the MTBE extracts from individual samples. For example, the MeOH extracts from accessions L1 and L55 were among the most effective, whereas the MTBE extracts from these accessions were among the least effective (see Table 4) at inhibiting PGE_2 production. In contrast, both the MeOH and MTBE extracts from accessions L2 and L56 were among the most effective. Nevertheless, the IC_{50} for inhibition of PGE_2 production of the MeOH and MTBE extracts from most ginger samples was roughly comparable to the IC_{50} for indomethacin in our assay system. Furthermore, studies with purified gingerols, shogaols and paradols found IC_{50} values between 0.290 and 7.35 μg ml^{-1} for inhibition of inflammation response (Park et al., 1998; Young et al., 2005). These data support the observation that ginger is an effective anti-inflammatory botanical (Herderson and Panush, 1999; Srivastava and Mustafa, 1992), but raise questions regarding the nature of the compounds responsible for this activity.

Previous investigations have concluded that the gingerols are the major anti-inflammatory compounds in ginger (Park et al., 1998; Young et al., 2005). Indeed, our results demonstrate that the compounds responsible for the anti-inflammatory activity (measured in this assay system) were present in both the MTBE and MeOH extracts, which were obtained in parallel from the same ground rhizome powders. Compounds such as the gingerols, shogaols, and paradols fit this description, in that they are exactable by both MTBE and MeOH. However, detailed metabolite target analysis of ginger accessions obtained from different origins but grown under identical conditions (described above) demonstrated that gingerol concentrations varied significantly

Table 4
Anti-inflammatory activities and cytotoxicity of *Zingiber* and *Alpinia* MeOH and MTBE extracts

Accession	Species	MeOH extract			MTBE extract		
		$IC_{50}(PGE_2)$ (μg ml^{-1})	Cytotoxic dose (μg ml^{-1})	$IC_{50}(PGE_2)$/ cytotoxicity	$IC_{50}(PGE_2)$ (μg ml^{-1})	Cytotoxic dose (μg ml^{-1})	$IC_{50}(PGE_2)$/ cytotoxicity
L1	*Z. officinale*	0.058	>50	<0.001	0.335	5	0.067
L2	*Z. officinale*	0.059	>50	<0.001	0.072	0.1	0.72
L5	*Z. officinale*	0.167	>50	<0.003	0.31	0.1	3.1
L6	*Z. officinale*	0.629	50	0.013	0.483	10–50	0.01–0.048
L18	*Z. officinale*	0.074	>50	>0.002	0.077	5–10	0.008–0.015
L32	*Z. officinale*	0.146	>50	0.003	0.098	5–10	0.01–0.020
L45	*Z. officinale*	0.069	50	0.001	0.163	0.1	1.63
L54	*Z. officinale*	0.073	50	0.002	0.079	1	0.079
L55	*Z. officinale*	0.058	10	0.006	0.499	1–5	0.1–0.499
L56	*Z. officinale*	0.065	1–5	0.013–0.065	0.062	1	0.062
L15	*Z. zerumbet*	0.079	5–10	0.008–0.016	0.096	0.1	0.96
L37	*Z. montanum*	7.678	>50	0.154	33.822	50	0.676
L46	*Z. mioga*	*	>50	*	*	10–50	*
L31	*Z. spectabile*	1.171	1	1.171	0.142	1–5	0.028–0.142
L8	*A. galanga*	0.055	1–5	0.011–0.055	0.053	0.1	0.53
Ref. Compd.	Indomethacin	0.055	>5	0.011			

Note. * indicates no inhibitory activity.

between ginger samples (see Tables 3 and 4). In fact, no correlation existed between gingerol concentration (either total gingerol or for individual gingerols) and the level of PGE_2 inhibitory activity for these ginger samples (R^2 between 0.06 and 0.32 for all comparisons). Thus, total bioactivity of crude MTBE and MeOH extracts appears be the result of a combination of compounds, which appear to differ by accession. Some of these contributing compounds may well be the gingerols (or shogaols or paradols, which are derived from the gingerols), but our results clearly demonstrate that other compounds must also be involved in producing the observed anti-inflammatory activity. These could be diarylheptanoids. Curcumin, the major diarylheptanoid of turmeric, has been show to possess anti-inflammatory activity (Chainani-Wu, 2003). Ginger contains significant levels of a wide range of diarylheptanoids (Kikuzaki et al., 1991; Ma et al., 2004). Because these compounds are not amenable to GC/MS analysis, they were not included in our analysis. In addition, many species of *Alpinia* have been shown to contain a wide range of diarylheptanoids. These compounds may be responsible for the observed in vitro anti-inflammatory activity.

One major difference between the ginger MeOH and MTBE extracts was that MeOH extracts displayed much less cytotoxicity than did the MTBE extracts, as is obvious by the fact that most of these extracts were not cytotoxic at the highest concentrations measured. The compounds responsible for this cytotoxicity are likely to be rather nonpolar and non-extractable with MeOH, ruling out the gingerols, shogaols, and paradols, and most diarylheptanoids. The cytotoxic components in ginger MTBE extracts have not been identified.

Z. zerumbet MeOH and MTBE extracts also displayed good PGE_2 inhibition activity, which was comparable to most ginger samples. In contrast, other *Zingiber* species, including *Z. mioga*, *Z. montanum*, and *Z. spectabile*, showed lower anti-inflammatory activity. Interestingly, *Z. zerumbet* was the closest species to *Z. officinale* in the phylogenetic tree based on DNA sequence. In contrast, *A. galanga*, the most distant species from *Z. officinale* in the phylogenetic tree, demonstrated the highest PGE_2 inhibitory activity. On the other hand, the *A. galanga* extracts also showed strong cytotoxicity. The gingerols and related molecules are not present in *A. galanga* or *Z. zerumbet*. Thus, as discussed above, other compounds in these species must be responsible for the observed anti-inflammatory acti-vity. These compounds have not yet been identified.

3. Conclusion

Using DNA sequence data, we were able to produce a phylogeny of the medicinal *Zingiber* species. This DNA sequence-based phylogeny matched very closely the phylogeny produced from chemical markers obtained from metabolic profiling experiments using extracts from the rhizomes. Thus, chemotaxonomic investigations can be very suitable for specific groups of plants and can uncover relationships that match those determined by DNA molecular data. Furthermore, we were able to identify several compounds that may serve as important markers for specific *Zingiber* species and which may be used to identify adulterated ginger samples. However, the anti-inflammatory activity in the analyzed species did not necessarily follow the phylogeny. Although the closely related *Z. zerumbet* had similar anti-inflammatory activity when compared to *Z. officinale*, so did *A. galanga*, which was the most distant from *Z. officinale* in the phylogenetic trees. These observations, as well as the results from anti-inflammatory assays with extracts from different ginger accessions, suggest that more than one set of compounds that are not common to these plants are responsible for the anti-inflammatory activity. Lack of correlation between gingerol content and observed total anti-inflammatory activity further confirms that other compounds contribute to this activity. Some of these compounds may be diarylheptanoids, although other, as yet uncharacterized compounds may also contribute to this activity. Whether these compounds in ginger are acting alone or in synergy with the gingerols is still an open question and can only be investigated after these compounds are separated, identified and assayed. Furthermore, identification and quantitation of the actual bioactive compounds or at least measurement of the bioactivity in some assay system (such as we performed here) must be performed to ensure that a given botanical actually possesses the activity desired (Steinke et al., 1993; Gong et al., 2004), whatever that desired activity may be. These results also demonstrate the importance of having access to a living collection of closely related medicinal plants to work with when performing a comparative study of this type. The results obtained here could not have been obtained by working with commercial extracts alone.

4. Experimental

4.1. Plant material

Plant identification was performed by one of us (Steven P. McLaughlin), based on comparison to the literature of morphological characters, including rhizome structure and color, leaf size, shape, and coloring, and especially on inflorescence and flower characters. Voucher specimens were deposited in the University of Arizona Herbarium. Individual rhizomes of *Z. officinale, Z. mioga, Z. montanum, Z. spectabile* and *Z. zerumbet* were obtained from different sources (see Table 1)

and grown side by side in the same greenhouse at the University of Arizona through at least one whole year's growth seasons, from germination to dormancy. After onset of dormancy, the rhizomes were harvested and replanted to eliminate any carryover affects of prior growth conditions (from original source) on the chemical composition of the rhizomes and to ensure that uniform growth conditions were applied equally to all plant specimens that were evaluated in this investigation. The plants used for this analysis were grown in five gallon pots, in Scott's Metromix soil, and watered by drip irrigation. Fresh young leaves were collected on the same day in the middle of October (one month prior to dormancy onset) for DNA isolation and fresh rhizomes were collected on the same day in the middle of May (during the middle of the growing season). The collected plant material was immediately frozen in liquid nitrogen, and kept at −80 °C until analyzed.

4.2. Chemicals and reagents

Acetonitrile and methanol were from Burdick & Jackson (Muskegon, MI). Methyl *t*-butyl ether (MTBE, High Purity Solvent) was purchased from EMD Chemicals Inc (Gibbstown, NJ). Authentic standards of [6]-gingerol, [8]-gingerol, and [10]-gingerol were purchased from ChromaDex, Inc. (Santa Ana, CA).

4.3. Phylogenetic analysis

Genomic DNA was isolated from frozen young leaf tissue using the DNeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The isolated DNA was quantified in water using a UV spectrophotometer (Lambda 35 UV/Vis Spectrometer, Perkin–Elmer Instruments, Shelton, CT), by measuring the absorbance at 260 and 280 nm and calculating the concentration using the method from the DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit Handbook (Qiagen). The quality of the DNA samples were also examined using 1% agarose gel electrophoresis in the presence or absence of endonuclease (EcoR I) digestion. All of the DNA samples were diluted with double distilled water into working stock solutions (5 ng/μl), and stored at −80 °C before use.

Four separate DNA sequence regions were originally chosen for use in this study. These included: (1) the nuclear internal transcribed spacer (ITS) locus, (2) the flanking intergenic spacer regions of the plastid encoded genes matK-trnK, and two other noncoding chloroplast regions, (3) the *trnL-F* region and (4) the intron of *rps16*. The ITS region of ~800 bp was amplified with primer pair ITS4 and ITS5 (Kress et al., 2002). The matK region of ~2500 bp was amplified with primers trnK1F (Manos and Steele, 1997) and trnK2R (Steele and Vilgalys, 1994). The *trnL-F* region consists of the trnL intron and the trnL-trnF intergenic spacer (Taberlet et al., 1991). The entire trnL-F region of ~900 bp was amplified with primer pair tRNc and tRNf (Wallander and Albert, 2000). The intron of rps16 of 800–900 bp was amplified with primer pair rpsF and $rpsR_2$ (Wallander and Albert, 2000). PCR amplification of the ITS region produced two bands with mixed sequence in each, suggesting multiple copies of this gene region in ginger and related species. The matK regions did not amplify consistently for all the plant samples from the different species in this investigation. This made use of these two gene regions impractical for our investigation, and they were consequently dropped from further analysis, although they had been the regions used in the phylogeny reported by Kress et al. (2002), which had not included any of the species that we examined.

PCR was preformed in a 25-μl reaction volume using the Taq kit from Fisher Scientific (Fair Lawn, NJ) according to the manufacturer's directions accept that 3 mM $MgC1_2$, 0.75 U of Taq, and 5 pmol of each primer were used. These amplifying reactions were all performed in a PTC-200 DNA Engine DYAD™ Peltier Thermal Cycler (MJ Research, Waltham, MA) using the same program (an initial step of 30 s at 94 °C, followed by 30 cycles of 5 s at 94 °C, 10 s at 56 °C, 3.5 min at 72 °C, then a final 7 min extension at 72 °C).

PCR products were incubated with 3 units of exonuclease I (USB Corporation, Cleveland, Ohio) and 0.6 unit of shrimp alkaline phosphatase (USB) at 37 °C for 5 min followed by 72 °C for 15 min in order to remove unincorporated dNTPs and primers. Sequencing reactions were performed using 2 μl of enzymatically cleaned PCR product, 3 pmol/μl sequencing primer (one of the primers used in PCR amplification) and BigDye Terminator (v3.0, Applied Biosystems, Foster City, CA) in a 96 well PTC-200 thermal cycler with 35 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C. Unincorporated terminators were removed by ethanol precipitation followed by 70% ethanol wash. Purified sequencing reactions were resuspended with 10 μl of HIDI (Applied Biosystems) and loaded on a AB13730x1 automatic sequencer to separate and collect sequences.

The forward and reverse sequences were checked and edited using ChromasPro MFC Application version 1.15 (Conor McCarthy, Griffith University, Australia). Based on the forward and reverse sequences, consensus sequences from each of the two chloroplast loci were aligned separately. Alignment of the joined sequences for each sample was performed using ClustalX version 1.83 (Thompson et al., 1997). Maximum parsimony analyses of the *trnL-F* region and the intron of *rps16* sequence data were conducted using PHYLIP 3.63 (Felsenstein, 2004).

The discrete character "0" was used to represent "not detectable" and "1" was used to represent "detectable"

for compounds listed in Table 2. The Seqboot, Pars, and Consense programs in the PHYLIP 3.63 package (Felsenstein, 2004), were used to produce a consensus phylogenetic tree from these discrete characters based on parsimony. Bootstrap support for the resulting tree was also determined using these programs.

4.4. Metabolic profiling, fingerprinting and targeted metabolite analysis

Frozen fresh ginger rhizome was ground into a fine powder in the presence of liquid nitrogen with a mortar and pestle and divided into 1 g aliquots for extraction with methyl *t*-butyl ether (MTBE) or MeOH, transferred to a 4-ml glass vial, covered with 2 ml of solvent (MTBE or MeOH) and capped with PTFE lined cap. After extraction overnight at room temperature with shaking at 200 rpm, each sample was centrifuged in the same glass vial in a SORVALL RC-5 Superspeed Refrigerated Centrifuge, GSA Rotor (Du Pont instruments, Norwalk, CT, USA) at 1500 rpm for 25 min to pellet the ground plant material and then filtered through an Acrodisc® CR 13 mm syringe filter with 0.20 μm PTFE membrane. The MTBE filtrate was used for GC/MS analysis and for anti-inflammatory activity assays. The MeOH filtrate was used for anti-inflammatory activity assays. A similar extraction procedure produced MeOH extracts for gingerol quantitation by HPLC, the only difference being that the samples were extracted by sonication for 30 min instead of overnight with shaking. After that, samples were centrifuged and filtered as described above. Triplicate extracts were used for quantitation analysis.

GC/MS data were recorded with a ThermoFinnigan Trace DSQ GC/MS (ThermoElectron, San Jose, CA). The gas chromatograph was fitted with an Alltech ECONO-CAP™-EC™-5 (30 m × 0.25 mm i.d. × 0.25 μm) capillary column, with 5 m guard column. Operating conditions: column oven temperature programmed at 40 °C for 2 min, then to 100 °C at 8 °C/min, then to 280 °C at 3 °C/min, then to 300 °C at 10 °C/min and hold for 3.5 min; Injector/transfer line/ion source temperatures 220/250/200 °C, respectively; electron voltage, 70 eV. UHP helium was used as the carrier gas at a flow rate of 1.2 ml/min. Injection volume was 3 μl and split ratio was 10. Eluted compounds were identified using the NIST Mass Spectral library Version 2.0 (NIST/EPA/NIH, USA) and by referring to a publication from Jolad et al. (2004). Compound identifications indicated by the library search program as being >80% probable were viewed as being likely hits. Spectra for each eluting compound were then compared (by hand) to the standard spectrum for the best hit to determine if the molecular ion peaks and the fragmentation patterns did in fact match. If any discrepancies were observed, then the compound was designated as an unknown compound and included in Supplementary Table 1.

An Agilent HPLC system was used for gingerol quantitation. Detector: DAD; Column: Luna C18 (2), 5 μm, 25 cm × 4.6 mm (Phenomenex); Guard column: Security Guard AJO-4287 (Phenomenex); Mobil phase: nanopure water (A) and HPLC grade-CH_3CN (B); The gradient elution had the following profile: 0–8 min, 45–50% B; 8–17 min, 50–65% B; 17–32 min, 65–100% B; 32–38 min, 100% B; flow rate: 1 ml/min; temperature 48 °C; Injection volume: 20 μl; Detection: 210, 230, and 280 nm. Triplicate injections for each of three replicate extracts were performed for each accession to ensure accuracy and reproducibility in this analysis.

4.5. Anti-inflammatory activity assays

These assays were performed as previously described (Jolad et al., 2004), with a different cell type. Human promonocytic U937 cells were cultured in Iscove's Modified Dulbecco's Medium with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 20% fetal bovine serum at 37 °C, 5% CO_2. Cells (1×10^6 ml^{-1}) growing actively were distributed into 48 well plates (0.5 ml/well) and cultured with phorbol myristate acetate (PMA, 10 nM) for 24 h at 37 °C, 5% CO_2 to differentiate the cells. Cells were washed with culture media. Lipopolysaccharide (LPS, 1 μg ml^{-1}) and different concentrations of plant extract were then added to duplicate plates. Cells were cultured for another 24 h. Supernatants were taken and stored at −80 °C before assays for human TNF-α and PGE_2.

Immunoassay kits were ordered from R&D System. ODs at 450 nm for TNF-α and 405 nm for PGE_2 (570 nm reference) were measured using a Spectra max Plus plate reader (Molecular Devices, Ramsey, MT). Data were analyzed using Molecular Devices plate reader software.

For cytotoxicity assays, human promonocytic U937 cells were cultured as described above. Cells (1×10^5 cells ml^{-1}) were distributed in 96 well plates (0.1 ml/well) and cultured with PMA (10 nM) for 24 h. Cells were washed with culture media. LPS (1 μg ml^{-1}) and different concentrations of extract were added to duplicate plates. Cells were cultured for another 24 h. Then, for MTT (*C*,*N*-diphenyl-*N*′-4,5-dimethyl thiazol-2-yl tetrazolium bromide) assays, 20 μl of MTT (5 mg ml^{-1}) were added to each well. After the plates were then cultured for another 4 h, supernatants were aspirated and 100 μl of isopropanol-HCl (0.04% HCl) were added to each well. The plates were protected from the light at RT overnight. The OD was measured at 570 nm (660 nm reference). For modified tetrazolium salt XTT (sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) assay, 25 μl of XTT (1 mg ml^{-1} with phenazine methosulfate) were added to each well and the plates

were cultured for another 4 h in the dark. O.D. was measured at 450 nm (650 nm as reference wavelength).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2005.08.001.

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