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## Plant Extracts of the Family Lauraceae: A Potential Resource for Chemopreventive Agents that Activate the Nuclear Factor-Erythroid 2-Related Factor 2/Antioxidant Response Element Pathway

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### Abstract

Cells and tissues counteract insults from exogenous or endogenous carcinogens through the expression of genes encoding antioxidants and phase II detoxifying enzymes regulated by antioxidant response element promoter regions. Nuclear factor-erythroid 2-related factor 2 plays a key role in regulating the antioxidant response elements-target gene expression. Hence, the Nrf2/ARE pathway represents a vital cellular defense mechanism against damage caused by oxidative stress and xenobiotics, and is recognized as a potential molecular target for discovering chemo-preventive agents. Using a stable antioxidant response element luciferase reporter cell line derived from human breast cancer MDA-MB-231 cells combined with a 96-well high-throughput screening system, we have identified a series of plant extracts from the family Lauraceae that harbor Nrf2-inducing effects. These extracts, including *Litsea garrettii* (ZK-08), *Cinnamomum chartophyllum* (ZK-02), *C. mollifolium* (ZK-04), *C. camphora* var. *linaloolifera* (ZK-05), and *C. burmannii* (ZK-10), promoted nuclear translocation of Nrf2, enhanced protein expression of Nrf2 and its target genes, and augmented intracellular glutathione levels. Cytoprotective activity of these extracts against two electrophilic toxicants, sodium arsenite and H<sub>2</sub>O<sub>2</sub>, was investigated. Treatment of human bronchial epithelial cells with extracts of ZK-02, ZK-05, and ZK-10

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### Conflict of Interest

There are no conflicts of interest to declare.

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significantly improved cell survival in response to sodium arsenite and H<sub>2</sub>O<sub>2</sub>, while ZK-08 showed a protective effect against only H<sub>2</sub>O<sub>2</sub>. Importantly, their protective effects against insults from both sodium arsenite and H<sub>2</sub>O<sub>2</sub> were Nrf2-dependent. Therefore, our data provide evidence that the selected plants from the family Lauraceae are potential sources for chemopreventive agents targeting the Nrf2/ARE pathway.

## Keywords

Lauraceae; *Cinnamomum*; *Litsea*; plant extracts; Nrf2/ARE pathway; chemoprevention

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## Introduction

Despite the progress in early diagnosis and treatment, cancer is still a major public health problem around the world. Carcinogenesis is recognized as a multiple-step process that is composed of three typical stages, which includes initiation, promotion, and progression [1]. Intervention of the carcinogenic processes with natural or synthetic chemicals is an effective approach to inhibit the incidence of cancer. Hence, the term “chemoprevention”, coined in the 1970s, is defined as the strategy of blocking or slowing the onset of premalignant tumors with relatively nontoxic chemicals (so-called chemopreventive agents). Chemopreventive agents are able to protect cells and tissues from exogenous or endogenous carcinogens. This might be achieved via the induction of phase II detoxifying and antioxidant enzymes, such as NQO1,  $\gamma$ GCS, GST, and HO-1, a process mediated by the ARE located in the promoter region of these genes [2]. Nrf2 is a member of the cap “n” collar subfamily of transcription factors, and contains a basic leucine zipper DNA-binding domain [3]. Under basal conditions, Nrf2 is sequestered by Keap1 in the cytosol, and maintained in a low constitutive level through constant ubiquitination and degradation by the 26S proteasome. Upon the exposure of cells to chemopreventive agents, Nrf2 translocates into the nucleus, binds to the ARE sequence, and activates the transcription of many cytoprotective genes [4,5]. These ARE-containing target genes encode intracellular redox-balancing proteins and phase II detoxifying enzymes, including NQO1,  $\gamma$ GCS, and HO-1, that are involved in GSH synthesis, the elimination of ROS, and xenobiotic metabolism [6]. Hence, the Nrf2/ARE pathway represents a vital cellular defense mechanism against damage caused by oxidative stress and xenobiotics, and facilitates cell survival under stressful conditions triggered by toxic insults. Studies with Nrf2-deficient mice highlight the protective role of the Nrf2/ARE pathway against oxidative stress and xenobiotics. Nrf2 null mice are more susceptible to xenobiotics and toxicants compared with wild-type littermate mice [7–10]. Based on the cytoprotective functions of the Nrf2/ARE pathway, it is concluded that Nrf2 activators have the potential to act as chemopreventive agents.

Characterization and isolation of Nrf2-inducing compounds from natural resources represents a rational strategy for chemoprevention. Presently, many natural products have been identified as Nrf2 activators, including SF (cruciferous vegetables) [7,11], curcumin (turmeric roots) [12,13], cinnamaldehyde (cinnamon) [14,15], resveratrol (giant knotweed plant, grapes, and others) [16], (-)-epigallocatechin-3-gallate (green tea) [17], zerumbone (ginger) [18], lycopene (tomato) [19], carnosol (rosemary) [20], and quercetin [21]. Besides

these well-studied molecules, discovery of novel plant-derived ingredients with Nrf2-inducing effects is still a research hotspot, and some natural products, covering lignans [22], flavonoids [23,24], terpenoids [25,26], and quinones [27], have recently been identified to be activators of the Nrf2/ARE pathway. Hence, identifying Nrf2 activators from natural resources with a high potency, yet low toxicity profile, is the first critical step in our journey of achieving disease prevention. Cinnamonyl-based secondary metabolites, represented by cinnamaldehyde, are mainly distributed in the genus *Cinnamomum* (family Lauraceae), and have been verified as an important group of chemopreventive agents for the induction of the Nrf2/ARE pathway [14,15,28]. Considering the phytotaxonomy and biosynthesis, plants belonging to the same family or genus produce secondary metabolites containing similar substructures because of their similarity in the biosynthetic pathways [29]. Therefore, it can be expected that many molecules with similar structures to cinnamaldehyde are to be found within the family Lauraceae derived from homologous biosynthetic reactions, and that these molecules might be capable of activating the Nrf2/ARE pathway. Accordingly, the plants of the family Lauraceae have been collected, and their effects on the Nrf2/ARE pathway have been evaluated. In this study, the cytoprotective properties of the plant extracts from the family Lauraceae have been confirmed using cell-based models, demonstrating that these plants are potential sources for the purification of novel chemopreventive agents targeting the Nrf2/ARE pathway.

## Results

Twelve plant species of the family Lauraceae, covering the genera *Persea*, *Litsea*, and *Cinnamomum*, were extracted with EtOH and subjected to an ARE-luciferase reporter gene assay. Plant species, voucher ID, evaluated plant part, and extract yields are summarized in Table 1. To examine whether these plant extracts activate the Nrf2/ARE pathway, we carried out a high-throughput cell-based screening for plant extracts that are capable of inducing the ARE-dependent response using our established human breast cancer cell line MDA-MB-231-ARE-Luc [30]. The EtOH extracts of *L. garrettii* (ZK-08), *C. chartophyllum* (ZK-02), *C. mollifolium* (ZK-04), *C. camphora* var. *linaloolifera* (ZK-05), *C. burmannii* (ZK-10), and *C. porrectum* (ZK-12) potently increased the ARE-luciferase activity more than 3-fold over the control under the treatment of nontoxic doses (Fig. 1D,E,G,H,J, and L). Moderate inductions of ARE-luciferase activity (2- to 3-fold) were observed in cells treated with the extracts of *L. glutinosa* (ZK-06), *L. monopetala* (ZK-07), *C. tenuipilum* (ZK-03), and *C. bejolghota* (ZK-09) (Fig. 1B,C,F, and I). However, the extracts of *P. americana* (ZK-01) and *C. burmannii* f. *heyneanum* (ZK-11) did not induce the ARE-dependent luciferase activity (< 1.5-fold) (Fig. 1A and K). A noticeable decrease of the ARE-luciferase expression was observed with some extracts at higher doses (Fig. 1D,E, and G–J). This is likely due to the cytotoxicity detected when cells were treated with high doses of these extracts (Fig. 2S, Supporting Information)

Based on their potent induction of the transcriptional activity of Nrf2 in the high-throughput ARE-dependent luciferase assay, the plant extracts of ZK-08, ZK-02, ZK-04, ZK-05, and ZK-10 were further evaluated for their effects on the Nrf2/ARE pathway. First, we investigated the ability of these plant extracts (ZK-08, ZK-02, ZK-04, ZK-05, and ZK-10) to induce the expression of Nrf2 and its downstream genes NQO1 and  $\gamma$ GCS in MDA-MB-231

cells. Immunoblot analyses were carried out to measure protein levels of Nrf2, NQO1, and  $\gamma$ GCS after the cells were treated with several doses of extracts for 16 hours. The plant extracts of ZK-08, ZK-02, ZK-04, and ZK-05 upregulated the protein levels of Nrf2, NQO1, and  $\gamma$ GCS in a dose-dependent manner (Fig. 2A–D). The plant extract of ZK-10 enhanced protein expression of Nrf2 and NQO1, but did not influence the  $\gamma$ GCS protein level when cells were treated for 16 hours (Fig. 2E).

Next, an indirect immunofluorescence analysis was performed to detect Nrf2 localization in response to extract treatment. Not surprisingly, Nrf2 proteins were localized mainly in the cytoplasm in the untreated group, and were accumulated in the nucleus upon exposure to the plant extracts ZK-08, ZK-02, ZK-04, ZK-05, and ZK-10, as well as SF (Fig. 3).

Nrf2-mediated cell protection against oxidative stress is partially achieved by regulating the intracellular redox status [31]. To confirm the induction of the Nrf2/ARE pathway by the plant extracts, we compared intracellular reduced GSH levels in cells left untreated or treated with each plant extract. In normal HBE cells, treatment with each plant extract resulted in a 1.2- to 1.5-fold increase in the reduced GSH level (Fig. 4), implying that these plant extracts augmented the cellular redox capacity.

Next, we investigated the cytoprotective effect of these plant extracts against the environmental toxicant and human carcinogen arsenic in the form of As(III). Since the lung is one of the target organs for arsenic-induced cancer, an immortalized primary lung epithelial cell line HBE was used for this study. The HBE cells were either left untreated or were pretreated with several doses of each plant extract for 8 hours, and further co-treated with 20 or 40  $\mu$ M As(III) for an additional 24 hours before measuring the cell viability using an MTT assay. Pretreatment followed by co-treatment with the extracts of ZK-02, ZK-05, and ZK-10 evidently improved cell survival in response to 20 or 40  $\mu$ M As(III) treatments (Fig. 5B and D–E). Nevertheless, no protective effects against As(III)-induced cytotoxicity were observed after exposure of HBE cells to the extract of ZK-08 or ZK-04 (Fig. 5A and C). To further evaluate whether protection conferred by the active extracts (ZK-02, ZK-05, and ZK-10) against As(III)-induced toxicity was Nrf2-dependent, we knocked down Nrf2 in HBE cells utilizing siRNA and subsequently measured the cell viability. Immunoblot analysis verified the ability of Nrf2-siRNA to downregulate Nrf2 expression (Fig. 5G). Plant extracts can no longer confer cyto-protection in the cells whose Nrf2 expression was knocked down by Nrf2-siRNA (Fig. 5H–J). Taken together, these results indicated that protection from the plant extracts against As(III)-induced toxicity required the activation of the Nrf2/ARE pathway. We further analyzed the effects of these plant extracts on H<sub>2</sub>O<sub>2</sub>-induced toxicity. Consistent with the results of the active extracts ZK-02, ZK-05, and ZK-10 in As(III)-induced toxicity, these four extracts also exerted protective effects against HBE cell damage induced by 100 and 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Fig. 6B and D–E). Notably, the extract of ZK-08 defended cells against H<sub>2</sub>O<sub>2</sub>- but not As(III)-induced toxicity (Fig. 6A). The extract of ZK-04 failed to attenuate H<sub>2</sub>O<sub>2</sub>-induced toxicity (Fig. 6C), which is consistent with its ineffectiveness against As(III)-induced toxicity. To validate that the protection conferred against H<sub>2</sub>O<sub>2</sub>-induced cell death was attributed to the activation of the Nrf2/ARE pathway, a similar cell viability analysis was performed in HBE cells transfected with Nrf2-siRNA. As

shown in Fig. 6G–J, the protective effect of the active extract ZK-08, ZK-02, ZK-05, or ZK-10 was lost, verifying that Nrf2 is responsible for the protective effect of each extract.

## Discussion

Carcinogenesis is a complex process in which normal cells are transformed into malignant cancer cells, caused by the exposure of carcinogens. Chemicals that activate signaling pathways involved in the removal of carcinogen metabolism, neutralization of ROS, and repair of DNA damage could be used for chemoprevention to eliminate insult to normal cells, and prevent or reverse carcinogenesis [32]. Induction of phase II detoxifying and antioxidant enzymes, such as NQO1, GST,  $\gamma$ GCS, and HO-1, are essential for the elimination of exogenous or endogenous carcinogens [33]. It has been established that the ARE is the key regulatory element for the expression of these aforementioned genes, and the transcription of these genes is activated by the binding of Nrf2 to their respective ARE sequence contained within the promoter region. Indeed, activation of the Nrf2/ARE pathway has been regarded as an effective strategy for preventing or reversing carcinogenesis [2], which has been verified in Nrf2 null mice [7,10,34].

Cinnamon, which originates from the bark of eight *Cinnamomum* species, has been used in traditional Chinese medicine for improving immunity, promoting blood circulation, and relieving pain. Cinnamon powder is sold as a dietary supplement in many countries to promote sugar metabolism, and maintain heart and circulatory health. Other plants from the family Lauraceae were adopted as antidiabetic and anti-inflammatory drugs in Chinese folk medicine. The benefits reported from traditional uses of these plants might be derived from the activation of the Nrf2/ARE pathway, because of the presence of cinnamonyl-based ingredients [14,15,28,35,36]. Here, we identified several extracts of the family Lauraceae as Nrf2 activators. These plant extracts enhanced the expression of Nrf2 and its downstream antioxidant and phase II enzymes, such as NQO1 and  $\gamma$ GCS, and augmented the cellular redox capacity by upregulating GSH levels. More importantly, they were capable of protecting HBE cells against As(III) and H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in an Nrf2-dependent manner.

These plant extracts, with the exception of ZK-01 and ZK-11, induced ARE-dependent luciferase activity (Fig. 1). We further evaluated the plant extracts with Nrf2-inducing effects of greater than 3-fold using immunoblot analysis and immunofluorescence techniques (Figs. 2 and 3). These results convincingly indicated that the plant extracts ZK-08, ZK-02, ZK-04, ZK-05, and ZK-10 were capable of activating the Nrf2/ARE pathway. Importantly, the Nrf2-inducing effects of these plants were not attributed to cinnamaldehyde, as verified by the TLC profile assuring the absence of cinnamaldehyde in these plant extracts (Figs. 3S and 4S, Supporting Information). These results imply that novel Nrf2 activators, other than cinnamaldehyde, exist in these plants. Previous phytochemical investigations of the genera *Litsea* and *Cinnamomum* led to the isolation of flavonoids, alkaloids, terpenoids, phenols, and lactone-type ingredients [37]. Taken together, results from the current study combined with analysis of reported structures from these two genera support the notion that these plants contain Nrf2 activators.

GSH, as the most abundant thiol-containing molecule in most cells, plays important roles in the cellular antioxidant defense system [38]. Similar to SF, the plant extracts enhanced the cellular reducing capacity by elevating GSH levels (Fig. 4). Increased GSH levels maintain a reducing environment within cells, and prevent DNA damage and apoptosis caused by insults from oxidants and xenobiotics [39].

In this study, we have demonstrated the effectiveness of plant extracts (ZK-02, ZK-05, and ZK-10) in conferring cytoprotection against As(III) acute toxicity via induction of the Nrf2/ARE pathway (Fig. 5). Arsenic is a human carcinogen, and chronic exposure to arsenic results in the formation of skin, lung, and bladder cancer [40,41]. It has been shown that arsenic exerts its carcinogenic effect through promotion of ROS and RNS generation, DNA damage, and mutagenesis [42–45]. Pretreatment with plant extracts ZK-02, ZK-05, or ZK-10 enhanced cell survival in response to As(III) treatment in a cytotoxicity protection assay. Furthermore, the protective role of these plant extracts is Nrf2-dependent, and their function in suppressing the As(III)-induced toxicity was abolished when the expression of Nrf2 was blocked by Nrf2-siRNA. This result demonstrates the important role of the Nrf2/ARE pathway for eukaryotic cells counteracting oxidative stress, inflammation, and apoptosis caused by arsenic insults [30,34,46,47]. Therefore, the coordinated induction of Nrf2 downstream events including upregulation of phase II detoxifying enzymes and enhancement of cellular reducing capacity, confers protection against As(III)-induced toxicity in HBE cells.

Consistent with the results in the As(III)-toxicity model, the plant extracts ZK-08, ZK-02, ZK-05, and ZK-10 suppressed cytotoxicity in an Nrf2-dependent manner when HBE cells were treated with H<sub>2</sub>O<sub>2</sub> (Fig. 6). ROS, such as H<sub>2</sub>O<sub>2</sub>, hydroxyl radicals, and the superoxide ion, are highly reactive, causing damage to cells by oxidizing DNA, lipids, and proteins [48]. They are thereby regarded as potentially mutagenic and carcinogenic substances because of their detrimental behavior on macromolecules [49,50]. Since the elucidation of the pivotal role of the Nrf2/ARE pathway in cellular redox homeostasis, there has been mounting evidence supporting its action in eliminating the harmful effects of ROS [51–54]. Consistently, our study demonstrated that upregulation of Nrf2 and its downstream genes by plant extracts promoted cell viability in response to H<sub>2</sub>O<sub>2</sub> treatment.

In summary, these findings strongly suggest that plants from the family Lauraceae contain ingredients that activate the Nrf2/ARE pathway, and are potential resources for discovering novel chemopreventive agents. Future directions call for an activity-guided purification and structure elucidation of these specific Nrf2-inducing compounds within these extracts.

## Materials and Methods

### Plant material and extraction

The plants of the family Lauraceae were collected from Xishuangbanna, Yunnan Province, China, in September 2011, and identified by Prof. Lan Xiang, School of Pharmaceutical Sciences, Shandong University. These plants were identified by comparison of their characteristics in plant morphology and taxonomy with those described in Flora of China.



Voucher specimens (voucher ID, see Table 1) of the plants have been deposited at the Laboratory of Pharmacognosy, School of Pharmaceutical Sciences, Shandong University.

Crushed aerial parts or leaves of plant materials (50 g) were extracted under reflux for 2 hours with EtOH (Tianjin Fuyu Chemical) ( $2 \times 500$  mL), and then EtOH was removed under reduced pressure. The yield of each extract is presented as a percentage of weight of dried plant material, and is summarized in Table 1.

### Cell cultures

Human breast carcinoma MDA-MB-231 cells (ATCC) were maintained in Eagle's MEM (Cellgro) supplemented with 10% FBS (Atlanta Biologicals), 2 mM HEPES (Gibco), and 6 ng/mL bovine insulin (Sigma). HBE cells (ATCC) were cultured in MEM supplemented with 10% FBS. All mammalian cells were incubated at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

### Cell viability

As(III) (Sigma) and H<sub>2</sub>O<sub>2</sub> (Mallinckrodt Chemicals)-induced toxicity was determined using an MTT (Sigma) assay. HBE cells ( $4.0 \times 10^4$  cells/well) were seeded in a 96-well plate and treated with several doses of extracts for 8 hours, and then co-treated with extracts and As(III) or H<sub>2</sub>O<sub>2</sub> for an additional 24 hours. After the addition of 20  $\mu$ L 2.0 mg/mL MTT solution, the cells were incubated at 37°C for 3 hours, and absorbance was measured at 570 nm on the Synergy 2 plate reader (BioTeK). All samples were carried out in triplicate for each experiment and the data represent the mean  $\pm$  SD.

### Luciferase reporter gene assay

The MDA-MB-231-ARE-Luc, a stable ARE luciferase reporter cell line previously established in our lab, was used for identification of the plant extracts with Nrf2-inducing effects [30]. The cells were seeded in a 96-well plate and treated with several doses of plant extracts, as well as the positive control SF (2.5  $\mu$ M, Fig. 1S, Supporting Information) (Sigma, catalog # S6317, -% purity) for 16 hours. Then, cells were lysed in passive lysis buffer (Promega), and the luciferase activity was measured using an assay buffer [25 mM glycylglycine (Sigma), 15 mM MgSO<sub>4</sub> (Sigma), 500  $\mu$ M ATP (Sigma), 250  $\mu$ M luciferin (Thermo Scientific), and 250  $\mu$ M CoA (Sigma)] in the Synergy 2 plate reader (BioTeK). The reporter gene assay was carried out in triplicate, and the data are presented as the mean  $\pm$  SD.

### Immunoblot analysis

Antibodies for Nrf2, NQO1, GCS, and  $\alpha$ -tubulin were purchased from Santa Cruz Biotechnology. MDA-MB-231 cells were seeded in D35 dishes and treated with plant extracts for 16 hours. To detect the protein expression in total cell lysates, cells were washed with PBS buffer and lysed in sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS (J.T. Baker), 10% glycerol (Sigma), 100 mM dithiothreitol (Sigma), 0.1% bromophenol blue (Fisher Biotech)]. After sonication, samples were electrophoresed through SDS-polyacrylamide gels and transferred onto nitrocellulose membranes for immunoblot analysis.

### Indirect fluorescence staining

Cells were grown on glass coverslips in D35 dishes. After treatment with extracts for 8 hours, cells were fixed with methanol and washed with PBS. Cells were then incubated with an anti-Nrf2 antibody and Alexa Fluor 488 anti-rabbit IgG antibody (Invitrogen-Molecular Probes), as well as Hoechst 33342 dye. Fluorescent images were visualized with the Zeiss Observer.Z1 microscope with the Slidebook computer program (Intelligent Imaging Innovations).

### Glutathione assay

Intracellular reduced GSH concentration was measured using the QuantiChrom GSH assay kit from BioAssay Systems. All the procedures were carried out according to the manufacturer's instructions. All samples were carried out in triplicate for each experiment and the value from the untreated group was set as 1. The data represent the mean  $\pm$  SD.

### Statistical analysis

ANOVA and post hoc multiple comparison Bonferroni test were used to determine the significant difference between two groups. Results are presented as the mean  $\pm$  SD.  $P < 0.05$  was considered to be significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Abbreviations

<b>ARE</b>	antioxidant response element
<b>As(III)</b>	sodium arsenite
<b>ATCC</b>	American Type Culture Collection
<b>FBS</b>	fetal bovine serum
<b>GCLM</b>	glutamate-cysteine ligase, modifier subunit
<b><math>\gamma</math>GCS</b>	$\gamma$ -glutamylcysteine synthetase
<b>GSH</b>	glutathione
<b>GST</b>	glutathione S-transferase
<b>HBE</b>	human bronchial epithelial
<b>HO-1</b>	heme oxygenase-1



<b>Keap1</b>	kelch-like ECH-associated protein 1
<b>MEM</b>	minimal essential medium
<b>MTT</b>	3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide
<b>NQO1</b>	NAD(P)H quinone oxidoreductase 1
<b>Nrf2</b>	nuclear factor-erythroid 2-related factor 2
<b>PBS</b>	phosphate buffered salt
<b>ROS</b>	reactive oxygen species
<b>SF</b>	sulforaphane
<b>siRNA</b>	small interfering RNA

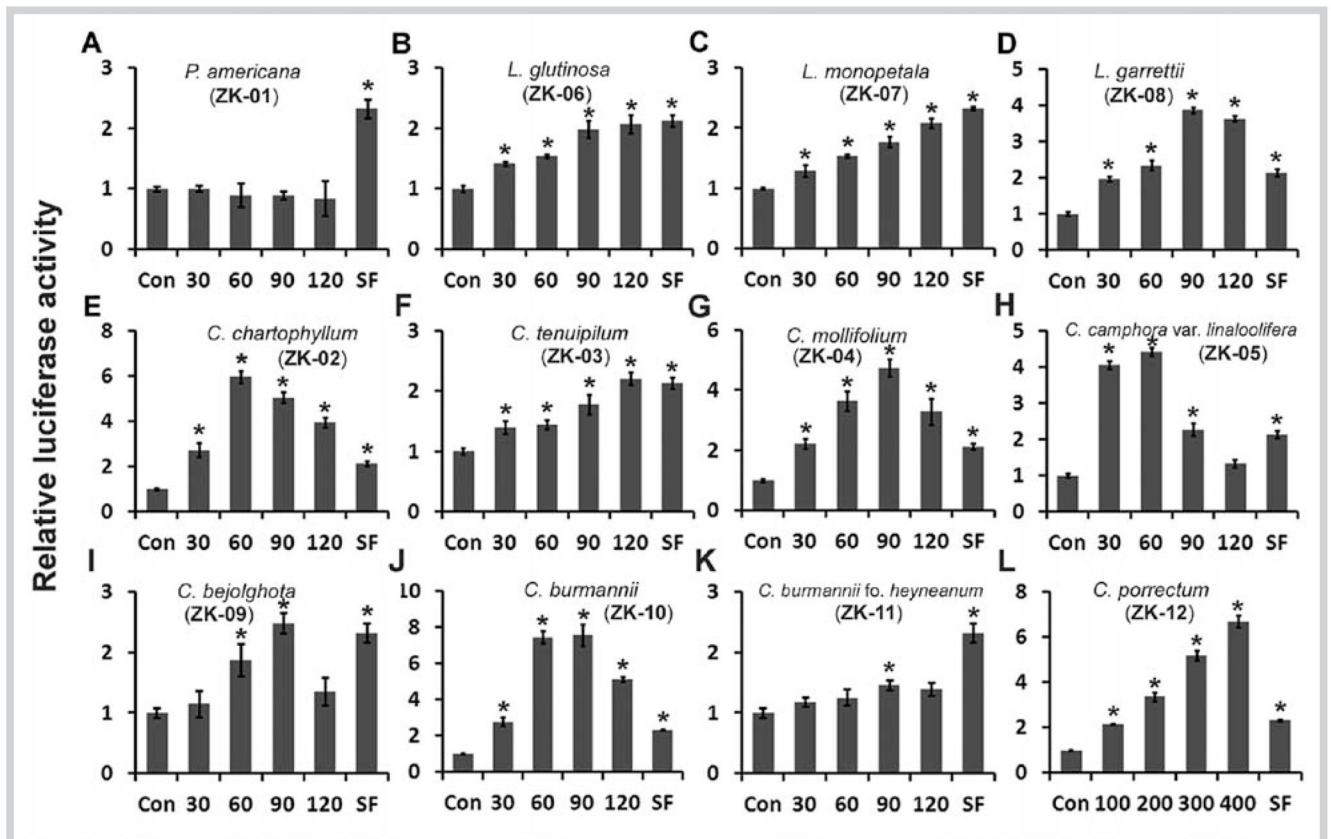
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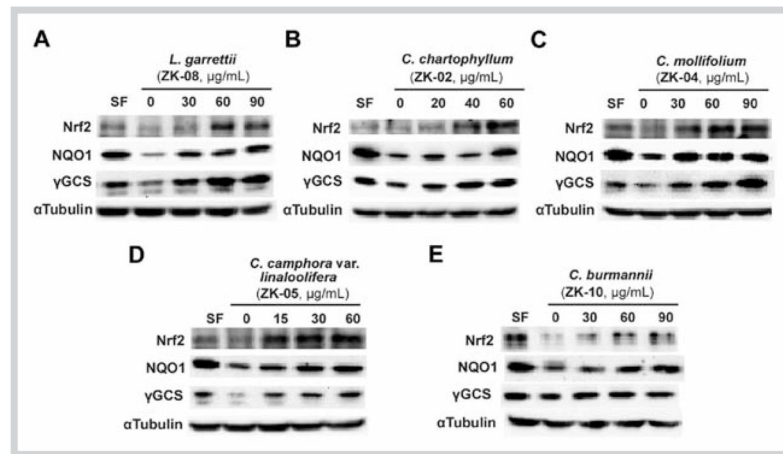
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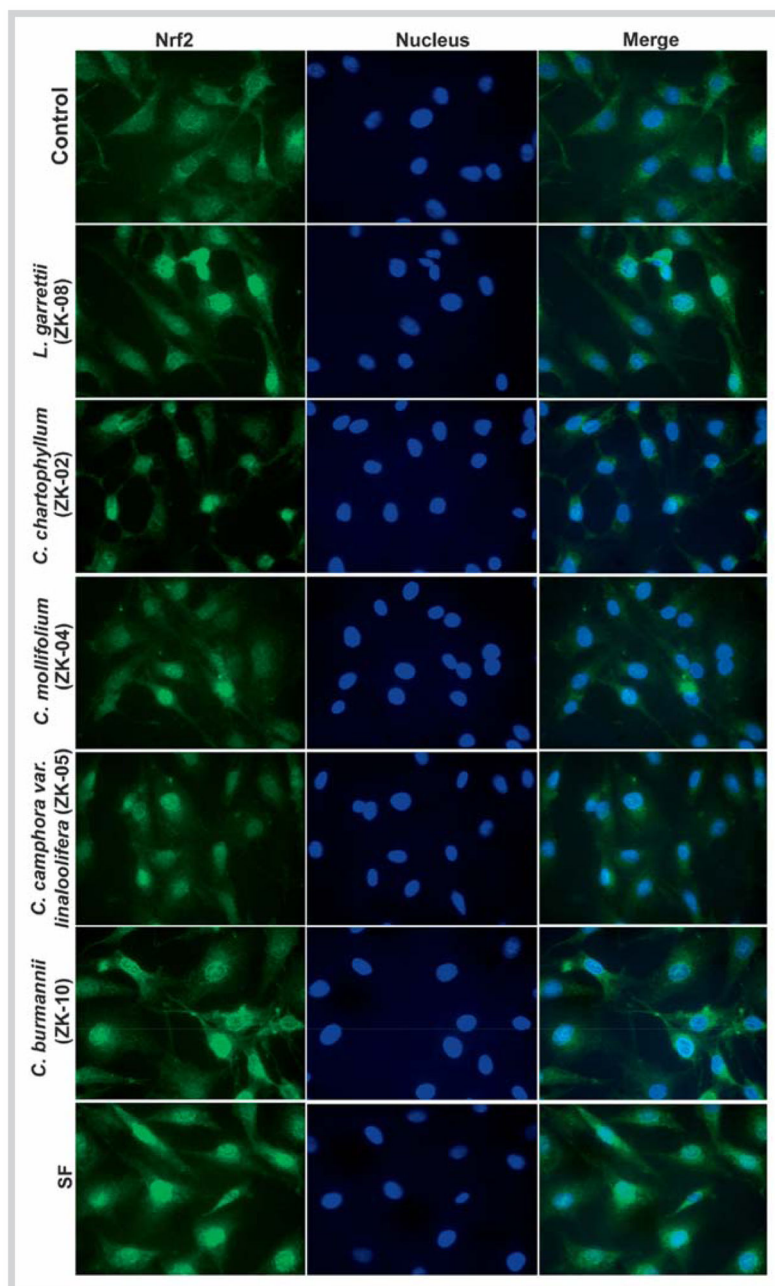
**Fig. 1.**

Luciferase reporter gene assay of the plant extracts from the family Lauraceae. The stable MDA-MB-231 cells expressing antioxidant response element-luciferase were seeded in 96-well plates and treated with several doses of each plant extract ( $\mu\text{g}/\text{mL}$ ) or sulforaphane ( $2.5 \mu\text{M}$ ) for 16 hours before analysis of luciferase activity. Results are presented as the mean  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$  vs. "Con" group.

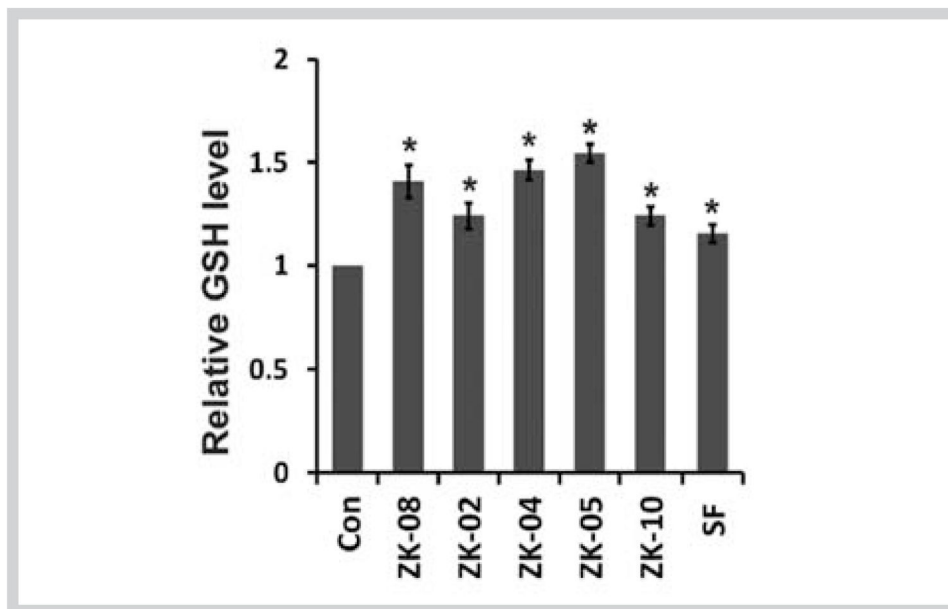


**Fig. 2.** Effects of active plant extracts on protein levels of nuclear factor-erythroid 2-related factor 2 and its downstream genes in MDA-MB-231 cells. Total cell lysates from MDA-MB-231 cells treated with each plant extract or sulforaphane (2.5  $\mu$ M) for 16 hours were subjected to immunoblot analysis with antinuclear factor-erythroid 2-related factor 2, anti-NAD(P)H quinone oxidoreductase 1, anti- $\gamma$ -glutamylcysteine synthetase, and anti-tubulin antibodies. Data shown are representative of two independent experiments.

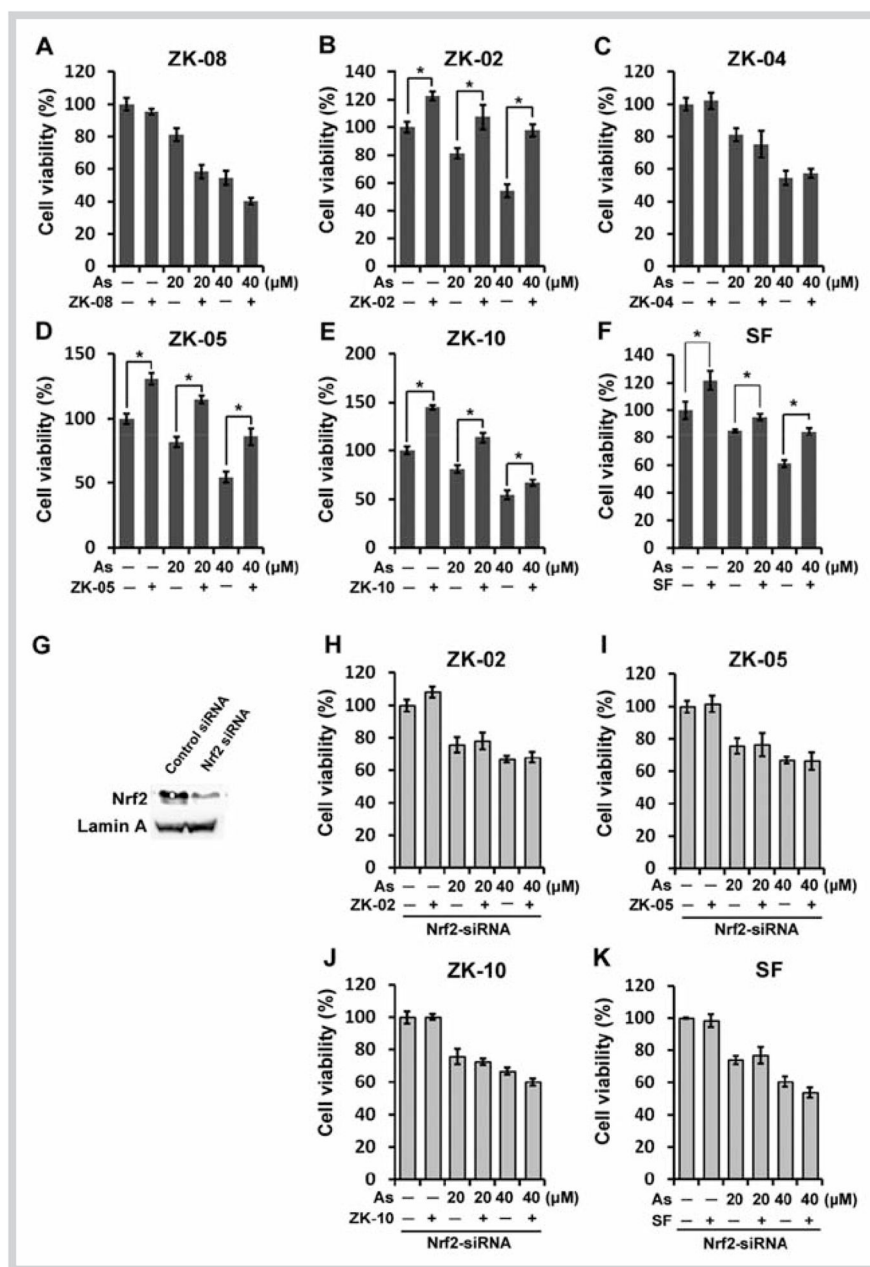




**Fig. 3.** Active Lauraceae plant extracts activated the nuclear translocation of nuclear factor-erythroid 2-related factor 2 in MDA-MB-231 cells. The concentrations of extracts were 60  $\mu\text{g}/\text{mL}$  for ZK-02 and ZK-05, and 90  $\mu\text{g}/\text{mL}$  for ZK-04, ZK-08, and ZK-10. The cells were treated with each active extract or sulforaphane (2.5  $\mu\text{M}$ ) for 8 hours, and then subjected to indirect fluorescence staining. Data shown are representative of two independent experiments. (Color figure available online only.)



**Fig. 4.** Active Lauraceae plant extracts upregulated intracellular reduced glutathione levels in human bronchial epithelial cells. The concentrations of extracts were 60  $\mu\text{g}/\text{mL}$  for ZK-02 and ZK-05, and 90  $\mu\text{g}/\text{mL}$  for ZK-04, ZK-08, and ZK-10. The cells were treated with each active plant extract or sulforaphane (2.5  $\mu\text{M}$ ) for 24 hours. The reduced glutathione concentration was measured by the QuantiChrom glutathione assay kit. Data presented are the mean  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$  vs. “Con” group.



**Fig. 5.** Active Lauraceae plant extracts protected human bronchial epithelial cells against sodium arsenite-induced toxicity. **A–F** Cell survival in human bronchial epithelial cells untreated or pretreated with ZK-08 (60 μg/mL), ZK-02 (40 μg/mL), ZK-04 (60 μg/mL), ZK-05 (30 μg/mL), ZK-10 (60 μg/mL), and sulforaphane (3.0 μM) for 8 hours, and then treated with 20 or 40 μM sodium arsenite in the absence or presence of half of the pretreated doses of Lauraceae extracts and sulforaphane (2.0 μM) for 24 hours. **G** Immunoblot analysis of nuclear factor-erythroid 2-related factor 2 protein level in human bronchial epithelial cells 36 hours after transfection with nuclear factor-erythroid 2-related factor 2-small interfering RNA. **H–K** Cell survival was performed as described in **A–F** in human bronchial epithelial

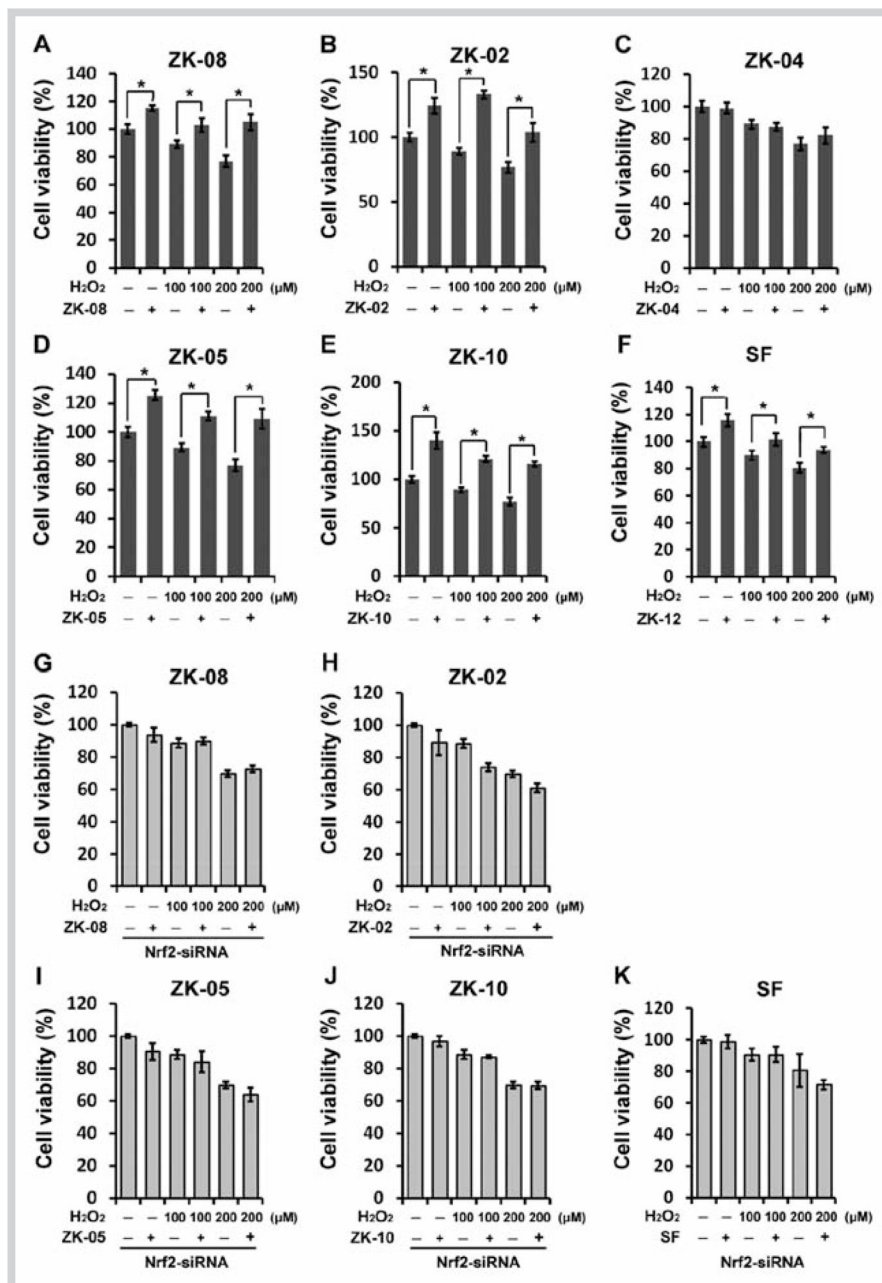
cells transfected with nuclear factor-erythroid 2-related factor 2-small interfering RNA. Values shown are the mean  $\pm$  SD of experiments run in triplicate. \*  $P < 0.05$  vs. plant extract-treated group.

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**Fig. 6.** Active Lauraceae plant extracts protected human bronchial epithelial cells against H<sub>2</sub>O<sub>2</sub>-induced toxicity. **A–F** Cell survival in human bronchial epithelial cells untreated or pretreated with ZK-08 (60 μg/mL), ZK-02 (40 μg/mL), ZK-04 (60 μg/mL), ZK-05 (30 μg/mL), ZK-10 (60 μg/mL), and sulforaphane (3.0 μM) for 8 hours, and then treated with 100 or 200 μM H<sub>2</sub>O<sub>2</sub> in the absence or presence of half of the pretreated doses of Lauraceae extracts and sulforaphane (2.0 μM) for 24 hours. **G–K** Cell survival was performed as described in **A–F** in human bronchial epithelial cells transfected with nuclear factor-

erythroid 2-related factor 2-small interfering RNA. Values shown are the mean  $\pm$  SD of experiments run in triplicate. \*P < 0.05 vs. plant extract-treated group.

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Table 1

Plants of the family Lauraceae tested in the present study.

Genus	Plant species	Abbreviated ID	Voucher ID	Plant part	Extract yields (%)
<i>Persea</i>	<i>P. americana</i> Mill	ZK-01	XSBN2011-ZK-01	Aerial part	8.50
<i>Litsea</i>	<i>L. glutinosa</i> (Lour.) C.B. Rob.	ZK-06	XSBN2011-ZK-06	Aerial part	4.27
	<i>L. monopetala</i> (Roxb.) Pers.	ZK-07	XSBN2011-ZK-07	Aerial part	5.99
	<i>L. garrettii</i> Gamble	ZK-08	XSBN2011-ZK-08	Aerial part	6.46
<i>Cinnamomum</i>	<i>C. chartophyllum</i> H. W. Li	ZK-02	XSBN2011-ZK-02	Aerial part	6.71
	<i>C. tenuipilum</i> Kosterm	ZK-03	XSBN2011-ZK-03	Aerial part	8.00
	<i>C. mollifolium</i> H. W. Li	ZK-04	XSBN2011-ZK-04	Aerial part	6.27
	<i>C. camphora</i> var. <i>linaloolifera</i> Y. Fujita	ZK-05	XSBN2011-ZK-05	Aerial part	5.91
	<i>C. bejolghota</i> (Buch.-Ham.) Sweet	ZK-09	XSBN2011-ZK-09	Aerial part	8.10
	<i>C. burmannii</i> (C.G. et Th. Nees) Bl.	ZK-10	XSBN2011-ZK-10	Aerial part	7.22
	<i>C. burmannii</i> fo. <i>heyneanum</i> (Nees) H.W. Li	ZK-11	XSBN2011-ZK-11	Leaf	8.37
	<i>C. porrectum</i> (Roxb.) Kosterm	ZK-12	XSBN2011-ZK-12	Aerial part	6.72