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Isolation of quinone reductase (QR) inducing agents from ginger rhizome and their *in vitro* anti-inflammatory activity

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ABSTRACT

To investigate the potential activity of ginger rhizome extracts to induce quinone reductase (QR), we performed bioactivity-guided fractionation using a murine hepatoma cell (Hepa 1c1c7) bioassay. Anti-inflammatory effects were then studied utilizing lipopolysaccharide (LPS)-stimulated mouse macrophage (RAW 264.7) cells. An ethyl acetate-partitioned fraction from ethanolic extract, rich in both QR inducing potency and anti-inflammatory activity, was subjected to repeated silica gel column and preparative thin layer chromatography to yield three compounds. The three isolated compounds were [6]-shogaol, 1-dehydro-[6]-gingerdione and hexahydrocurcumin. [6]-Dehydroshogaol, a minor component in ginger rhizome, was chemically synthesized and used for comparison in the subsequent bioassay based on its excellent QR inducing potency. Results showed that [6]-dehydroshogaol had the highest ability to induce QR activity ($CD = 9.23 \pm 0.22$ μM), followed by 1-dehydro-[6]-gingerdione ($CD = 13.24 \pm 0.45$ μM), and then hexahydrocurcumin ($CD = 68.81 \pm 3.90$ μM). Increasing QR activity in induced cells was associated with elevated expression of NQO-1 protein as confirmed by Western blot. [6]-Dehydroshogaol, [6]-shogaol and 1-dehydro-[6]-gingerdione were also potent inhibitors of nitric oxide (NO) synthesis in activated macrophages. Their IC_{50} values ranged from 5.80 ± 1.27 to 25.06 ± 4.86 μM. Hexahydrocurcumin exhibited the weakest inhibitory effect ($IC_{50} = 304.76 \pm 54.80$ μM). These findings contribute to our theoretical understanding of the potential beneficial effects of consuming ginger as food and/or dietary supplement.

1. Introduction

Cancer chemoprevention has been defined as the use of dietary and pharmacological intervention with specific natural or synthetic agents, designed to prevent, suppress, or reverse the process of carcinogenesis before the development of malignancy (Hong & Sporn 1997). Recently, increasing evidence based on *in vitro* and *in vivo* investigations have been linked to a decreased cancer risk with the consumption of certain dietary photochemicals (Bachmeier et al. 2010; Russo 2007). Among the multiple mechanisms underlying the chemopreventive potential of these constituents, xenobiotic-transforming and antioxidant defense systems are widely viewed as affording protection from cancer through the detoxification of potential carcinogens and mitigation of oxidative stress (Calabrese et al. 2008). The induction of phase II enzymes was proposed as a major mechanism of cellular protection against the toxic and reactive chemical species (Begleiter et al. 1997), and neoplastic effects of carcinogens (Wattenberg 1992). The elevation of phase II detoxification enzymes through the antioxidant response element (ARE) by dietary factors is considered more beneficial than coordinative induction of both phase I (cytochrome P450s) and phase II enzymes. Other ARE-encoded enzymes include glutathione and glucuronide conjugating enzymes, multidrug resistance protein, antioxidant defense agents such as hemeoxygenase-1 (HO-1), thioredoxin reductase, sirtuins, glutamate cysteine ligase, and several heat shock proteins (Russo 2007). Talalay (1989) and Wattenberg (1992) have reported that induction of phase II detoxification enzymes, which can modulate the threshold for chemical carcinogenesis and thereby increase cellular resistance to carcinogen exposure, was correlated with protection against chemical-induced carcinogenesis in animal models (Song et al. 1999). This was demonstrated in the stage of promotion as well as initiation (Gills et al. 2006). The regulatory regions of inducible ARE genes are activated upon binding of the nuclear factor E2-related protein 2 (Nrf2) transcription factor. Nuclear translocation of Nrf2 has been shown to be essential in the up-regulation of these protective genes as a response to oxidative stress, electrophiles, and some phytochemicals (Eggler et al. 2008).

QR (NAD(P)H: quinone reductase, EC1.6.99.2), one of the important components of phase II detoxification enzyme systems, catalyzes the two-electron reductions of a variety of quinone

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compounds. It thereby protects cells against mutagenicity and carcinogenicity that would otherwise result from free radicals and toxic oxygen metabolites generated by the one-electron reductions promoted by cytochromes P450 and other enzymes (Joseph et al. 1994). As a predominantly cytosolic FAD-containing flavoprotein, QR is inducible in the cells of many tissues such as those from the liver, lung, and colon (Wang et al. 1998). Measuring the induction of QR activity may provide an efficient approach to searching the potential chemopreventive phytochemicals from plants. This may lead to further understanding the chemopreventive mechanisms behind their actions. Many classes of plant extracts or phytochemicals have been isolated and identified as potential chemopreventive agents based on their potent QR-inducing properties. Examples include, but are not limited to sulforaphane from broccoli, curcumin from turmeric, organosulfur compounds from garlic and onion, and certain withanolides from tomatillo (Chen & Kong 2005; Surh et al. 2008).

Ginger rhizome (*Zingiber officinale* Roscoe), is used worldwide both as a spice and as a medicinal herb. Numerous studies demonstrated that ginger or its extracts present some degree of pharmacological activity including anti-inflammation, analgesic effect, anti-tumor and anti-oxidation activity (Dugasani et al. 2010; Lantz et al. 2007). Ginger contains a distinct phytochemical profile with more than 63 and 115 substances being identified from fresh and dry ginger, respectively (Jolad et al. 2004; Jolad et al. 2005). Oral consumption of ginger increases antioxidant levels and reduces oxidative damages in the liver and kidney of rats (Kota et al. 2008). Ginger extracts, specially gingerol and shogaol, exhibit antiallergenic effects by inhibiting the release of inflammatory mediators and β-hexosaminidase form RHL-2H cells (tumor analog of mast cells) without causing cytotoxicity (Chen et al. 2009). [6]-Gingerol and [6]-paradol induce apoptosis in different cancer cell lines, and aqueous ginger extract inhibits proliferation and angiogenesis of colonic adenocarcinoma cells (Brown et al. 2009). Our preliminary study also indicated that [6]-dehydroshogaol, a minor component in ginger rhizome, exhibited potent QR-inducing activity in murine hepatoma Hepa 1c1c7 cells (Imm et al. 2010). Although several pharmacological properties of ginger have been studied, there appear to be few investigations focusing on QR-inducing activity of ginger extracts or on the structure–activity relationship of isolated compounds. Thus, as a part of our ongoing screening program to evaluate the QR-inducing potentials of natural compounds, we performed a bioassay-guided fractionation using murine hepatoma cells (Hepa 1c1c7) model. The purpose of our study was to isolate potential phase II enzymes-inducing components from ginger rhizome. These isolated components were then subjected to both phase II enzymes induction and anti-inflammatory activity using a lipopolysaccharide (LPS)-stimulated macrophages (RAW 264.7 cells) bioassay.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's minimum essential medium (DMEM), α-Minimum essential medium (MEM), trypsin–EDTA (0.25% trypsin with EDTA-4Na), fetal bovine serum (FBS), and penicillin streptomycin were obtained from Gibco (Grand Island, NY). Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). NAD(P)H: quinone oxidoreductase 1 (NQO-1) and β-actin monoclonal antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO). [6]-Dehydroshogaol was chemically synthesized in our lab by the condensation of vanillin with acetone under aqueous NaOH generated dehydrozingerone, which was condensed with hexanal using $LiN(TMS)_2$ in anhydrous THF to obtain [6]-dehydroshogaol (Wu et al. 1998).

2.2. Isolation and purification of ginger extracts

Market-fresh ginger rhizome (7.756 kg) was purchased from a local grocer (Metcalfe Sentry Foods, Madison, WI) in April, 2010. Ginger rhizome was transversely sliced into ~1–2 mm pieces and first refluxed with 4 L ethyl acetate (EtOAc) for 4 h. The residue was subjected to further sequential extraction by refluxing with ethanol, and finally aqueous extraction at 60 °C for 4 h. For each extract, solvents were removed by vacuum evaporation or lyophilization to obtain dry matter. Preliminary QR inducing and anti-inflammatory (inhibition of NO evolution) bioassay revealed that only ethanolic extract had a low enough CD value (concentration required to double QR activity) of ~7 μg/mL and IC_{50} value of ~20 μg/mL to warrant further fractionation.

The ethanolic extract was suspended in H_2O and partitioned with EtOAc, and EtOAc-soluble fraction was found to be active and a portion (10.07 g) was chromatographed on a silica gel column (5.5×60 cm) using a step gradient of n-hexane-ethyl acetate (2–100% of EtOAc). Collected material was pooled according to segregation of material absorbing at 254 nm and finally to afford 14 fractions, F1–F14 (Fig. 1). Fraction F4 was resolved by flash silica gel chromatography using 40–80% of CH_2Cl_2 in hexane to obtain compound 1. Fractions F5 were loaded onto a silica gel column eluting with 0–20% of EtOAc in CH_2Cl_2, and then preparative thin layer chromatography (TLC) on silica gel using a developing solvent of 5% EtOAc in benzene to obtain compound 2. Fraction F11 was subjected to flash silica gel chromatograph using 30–60% EtOAc in petroleum ether to afford 3 subfractions (F11-1, F11-2 and F11-3). The F11-3 was repeatedly resolved on silica gel chromatography using 10–40% EtOAc in $CHCl_3$, and finally purified by reverse phase TLC to obtain compound 3. Structures of these isolated compounds were analyzed using NMR and MS by comparison with previously reported profiles. NMR spectra were obtained on Varian Unity-Inova 400 (400 MHz) spectrometer (Analytical Instrumental Center, UW-Madison, WI). High resolution ESI-MS analyses were performed on Agilent ESI-TOF Mass Spectrometer (Mass spectrometry/Proteomics Facility, Biotechnology Center, UW-Madison).

2.3. Cell culture

Murine hepatoma cells (Hepa 1c1c7) was obtained from American Type Culture Collection (ATCC, Rockville, MD), and maintained in α-Minimal Essential Medium (MEM) supplemented with 10% FBS (treated with activated charcoal to remove any traces of endogenous QR inducers), and 100 U/mL penicillin and 100 μg/mL of streptomycin

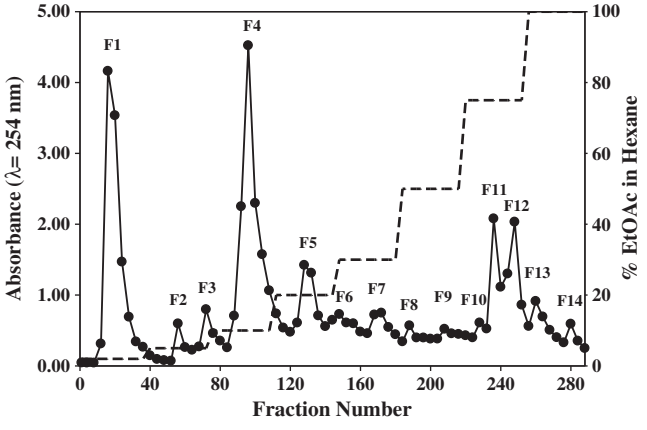

Fig. 1. Silica gel chromatographic resolution of EtOAc-soluble layer of crude ginger ethanolic extract by a gradient of hexane-ethyl acetate system (2–100% of EtOAc). Fractions were collected according to absorbance at 254 nm. Step gradient was indicated by a long dashed line.