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Polyozellin Isolated from *Polyozellus multiplex* Induces Phase 2 Enzymes in Mouse Hepatoma Cells and Differentiation in Human Myeloid Leukaemic Cell Lines

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Induction of cellular phase 2 detoxifying enzymes is associated with cancer preventive potential. Quinone reductase (QR) has been used as a prototype for anticarcinogenic phase 2 enzymes because of its widespread distribution in mammalian systems, large amplitude of inducer response, and ease of measurement in murine hepatoma cells. Methanol extract of *Polyozellus multiplex*, which shows a strong QR induction potential, was subjected to bioassay-guided fractionation, and polyozellin (PM1) appeared to be a major active component. In in vitro cultured cells (hepa1c1c7 and BPRc1 cells), polyozellin enhanced QR, glutathione S-transferase (GST) activities, and glutathione (GSH) content in a dose-dependent manner. The compound also significantly promoted differentiation of HL-60 human promyelocytic emia cells. In conclusion, polyozellin deserves further in vivo study to evaluate its potential as a cancer preventive agent.

KEYWORDS: Glutathione-S-transferase; quinone reductase; polyozellin; cancer prevention

INTRODUCTION

Induction of phase 2 enzymes such as quinone reductase (QR; NAD(P)H/quinone-acceptor oxidoreductase (EC1.6.99.2)) and glutathione *S*-transferase (GST) is closely associated with the prevention of chemical carcinogenesis (*1*). Phase 2 enzymes are involved in detoxification of carcinogens, bioactivation of some anticancer drugs, stabilization of tumor suppressor(s), and prevention of oxidative damage caused by various chemicals. Accordingly, there has been considerable interest in screening natural products for their capacity to induce quinone reductase activity, and many diverse chemical agents such as oxidizable diphenols, isothiocyanates, and even hydrogen peroxide have been found to do so (*2*). Thus, it is anticipated that the plant kingdom may be a rich source of QR inducers awaiting discovery.

Biochemical studies have already demonstrated that the NQO1 gene, encoding QR, contains two distinct regulatory elements in its 5′-flanking region, one of which is an antioxidant responsive element (ARE), also called the EpRE (electrophile response element), and the other is a xenobiotic response element (XRE), called the AhRE. QR induction through the XRE involves binding a ligand to the aromatic hydrocarbon receptor, AHR. The aryl hydrocarbon (Ah) receptor (AhR) is a member of the basic helix−loop−helix PER−ARNT−SIM (PAS) transcription factor family and translocates to the nucleus upon binding to Arnt (*3*). The AHR/Arnt dimer interacts with DNA sequences known as XREs to regulate QR expression. Both NQO1 and CYP1A1 genes can be induced by TCDD and polycyclic aromatic hydrocarbons, while induction of NQO1 is largely dependent upon the ability of bifunctional inducers such as dye, Sudan I, and β-naphthoflavone to first undergo conversion to oxidative labile metabolites through a functional P450-dependent mechanism. However, it seems that some compounds, known as monofunctional inducers, induce only QR without affecting the expression of phase 1 enzymes. It is known that some pro-carcinogens, such as benzo[*a*]pyrene, are ultimately converted into carcinogenic agents by the action of phase 1 enzymes. Accordingly, BPRc1 cells, a mutant defective in the Ah receptor function, may be a good model system for screening for substances that induce phase 2 enzymes alone. For instance, the 1,2-dithiol-3-thiones, a class of five-membered cyclic sulfur compounds, which have chemotherapeutic and chemoprotective properties, have been reported to elevate quinone reductase and glutathione levels in BPRc1 cells as well as hepa1c1c7 cells (*2*). Our preliminary study to screen for QR inducers was performed over 200 kinds of herbal extracts in hepa1c1c7 and BPRc1 cells. The methanol extract of *Polyozellus multiplex*, an edible mushroom, showed a significant QR

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induction in both cell lines. The bioassay-guided fractionation identified polyozellin (PM1) as a potent QR inducer. The modulation of cancer preventive biomarkers including phase 2 enzymes and cell differentiation by polyozellin is described herein.

MATERIALS AND METHODS

Materials. All cell culture reagents and fetal bovine serum were obtained from Gibco BRL (Gaitherburg, MD). Hepa1c1c7 and BPRc1 cells were from American Type Culture Collection (Rockville, MD). All other chemicals were of reagent grade.

The mushroom, which was identified as *Polyozellus multiflex* (*4*, *5*) by macro- and microscopic method, was collected in mountain Odae, Korea and deposited in Division of Applied Biology and Chemistry, College of Agriculture and Life Sciences, Kyungpook National University, Daegu, Korea.

Cell Culture. Hepa1c1c7 and its mutant (BPRc1) cells were plated at density of 3×10^5 and 5×10^5 cells per 100-mm plate in 10 mL of α-MEM supplemented with 10% FBS, respectively. The plates were normally incubated for 3−4 days in a humidified incubator in 5% CO_2 at 37 °C. Cells were cultured for 48 h and exposed to various concentrations of herbal extract for another 24 h, followed by biochemical assays.

Biochemical Assays. QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (*6*). Glutathinone-*S*-transferase activity was assayed by the method described by Habig (*7*), with 1-chloro-2,4-dinitrobenzene as a substrate. Total cellular GSH was determined by rate measurements in a recycling assay (*8*). In brief, cells were grown for 24 h in 96-well plates (10 000 per well for hepa1c1c7 and BPRc1 cells), exposed to serial dilutions of polyozellin for 24 h, and finally lysed in 50 μL of 0.08% digitonin. One-half of the wells were used for protein determination. The other half received 50 μL of ice-cold metaphosphoric acid (50 g/liter) in 2 mM EDTA to precipitate cellular protein. After 10 min at 4 °C, plates were centrifuged at 1500*g* for 15 min and 50 μL of the resulting supernatant fractions was transferred to the corresponding wells of a parallel plate. To each of these wells, 50 μL of 200 mM sodium phosphate buffer, pH 7.5, containing 10 mM EDTA, was added, and GSH content was measured by rate measurements in a recycling assay. *tert*-butylhydroquinone (TBHQ, 20uM or 3.3 ug/mL), a known QR inducer, was used as a positive control.

Western Blot. This was performed on cytosolic fractions prepared from cultured cells to estimate the level of QR according to a protocol described previously (*9*). Electrophoresis was carried out in a Bio-Rad Mini-Protein II Cell apparatus (Bio-Rad Co., Hercules, CA), using a discontinuous buffer system. Electroblotting was performed in a semi-dry blotting unit (Trans-BlotR SD Blotting Kit, Bio-Rad). Aliquots (about 3 μg of protein) of cytotolic fraction of cells was loaded onto 10% SDS-polyacrylamide gel, and transferred onto polyvinylidene difluoride (PVDF) membranes in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol). The membrane was blocked with blocking buffer containing 5% skim milk in 0.1% tween 20 in PBS (PBS/T). The antibody against QR (200 μg/mL), a kind gift from Dr. Anil Jaiswal at Baylor College of Medicine, was diluted by 1:1000 with dilution buffer (5% skim milk in PBS/T), added to the membrane, and incubated for 4 h at room temperature. After washing six times with PBS/T, blots were incubated in washing buffer (PBS/T) with a secondary antibody for 1 h at room temperature, followed by washing eight times with PBS/T. Washed blots were incubated with horseradish peroxidase-conjugated secondary antibody anti-rabbit IgG, which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and exposed onto a film. Antibody against rat β-tubulin was used as a loading control.

Assay of Differentiation. The potential of an agent to promote cell differentiation was assayed by the nitro blue tetrazolium (NBT)-reducing activity, as reported by Suh and co-workers (*10*). In brief, HL-60 cells (1.2×10^5 cells/well) were plated onto a 24-well plate with 1 mL of EMEM containing 10% FBS. After 18 h of incubation, an agent dissolved in DMSO was added into each well, followed by 98 h of incubation at 37 °C, 5% CO_2. After incubation, the cells were mixed with an equal volume of freshly prepared solution, containing 1 μg/mL phorbol-12-myristate-13-acetate (Sigma) and 2 mg/mL NBT (Sigma), and incubated for 60 min at 37 °C. A minimum of 100 cells was counted, and the percentage of NBT-positive cells was assessed under the inverted microscope.

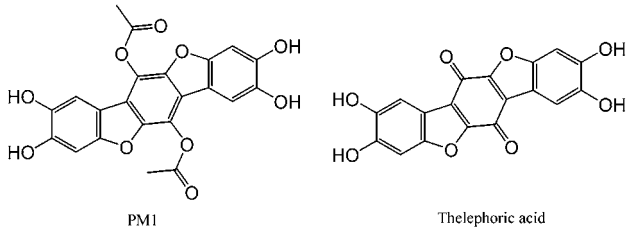

Figure 1. Structure of polyozellin (PM1) and thelephoric acid.

Extraction and Purification of QR Inducer from *Polyozellus multiplex.* *Polyozellus multiplex* was collected in Mt. Odae, Korea and deposited in Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, Korea. The dried fruiting body of the *P. multiplex* (1 Kg) was refluxed in 3 L of methanol (MeOH) for 3 h at 70 °C. The extract was evaporated to dryness and the residue (204 g) was suspended in water to partition with benzene, chloroform, and ethyl acetate (EtOAc), consecutively. The EtOAc soluble layer was evaporated, and the resultant residue (2.1 g) was washed with 100 mL of EtOAc. The residue (1.2 g) was suspended in 500 mL of MeOH, and then the suspension was centrifuged at 3000*g* for 5 min. The precipitate was discarded and the supernatant was concentrated in vacuo. The residual material was further purified on a Senshu Pak ODS HPLC. Elution with 65% MeOH containing 1% acetic acid produced 512 mg of polyozellin as a grayish powder. The purity of polyozellin was checked by HPLC (uBondapak C18, 3.9- × 150-mm, linear gradient elution with 1% HOAc in water and 1% HOAc in MeOH for 50 min, 0.8 mL min^{-1}) equipped with PDA and ELSD (evaporative light scattering detector) (tR, 17.28 min). Under UV 254 nm, the purity was more than 98% as was calculated by peak areas. Thelephoric acid was isolated from *P. multiflex* and identified in the previous work (*11*). The purity was more than 97% under the above HPLC conditions (tR, 15.09 min).

Identification of Polyozellin. The molecular formula of polyozellin was determined as $C_{22}H_{14}O_{10}$ on the basis of FAB-MS and ^{13}C NMR. The ^1H NMR spectrum showed two broad singlet protons at 9.20 and 8.70 ppm with two aromatic methine singlets at 7.20 and 7.12 ppm and an acetyl singlet at 2.53 ppm. On the basis of these spectral data, polyozellin was postulated as polyozellin (6,12-diacetoxy-2,3,8,9-tetrahydroxybenzo[1,2-b;4,5-b′]bisbenzofuran), as presented in **Figure 1**. The structure was confirmed by the comparison of its spectral data with those in the reported reference (*12*). The analytical properties of the compound were as follows:

$FeCl_3$ positive. ^{13}C NMR (100 MHz, DMSO-d_6+chloroform-*d*, δ ppm from TMS): 168.0 (carbonyl), 151.0 (4a, 10a), 146.4 (3, 9), 142.8 (2, 8), 137.6 (5a, 11a), 130.8 (6, 12), 116.9 (6a, 12a), 113.5 (6b, 12b), 106.1 (1, 7), 98.8 (4, 10). ^1H NMR (400 MHz, DMSO-d_6+chloroform-*d*, δ ppm from TMS): 9.20 (2H, brs, phenolic OH), 8.70 (2H, brs, phenolic OH), 7.20 (2H, s, H-1 and H-7), 7.20 (2H, s, H-4 and H-10), 2.53 (6H, s, methyl). FAB-MS (*m*/*z*): $[M^+ + 1]$ 439. In the literature, ^{13}C NMR (100 MHz, DMSO-d_6+chloroform-*d*, δ ppm from TMS): 168.02 (carbonyl), 150.74 (4a, 10a), 146.58 (3, 9), 142.85 (2, 8), 137.59 (5a, 11a), 130.80 (6, 12), 116.94 (6a, 12a), 113.51 (6b, 12b), 106.17 (1, 7), 98.76 (4, 10). ^1H NMR (400 MHz, DMSO-d_6+chloroform-*d*, δ ppm from TMS): 9.23 (2H, brs, phenolic OH), 8.78 (2H, brs, phenolic OH), 7.21 (2H, s, H-1 and H-7), 7.14 (2H, s, H-4 and H-10), 2.54 (6H, s, methyl).

Statistical Analysis. Statistical significance of enzyme activity, GSH content, and degree of cell differentiation data was tested by analysis of variance, followed by Duncan's multiple range test, using SPSS software (SPSS Inc, Chicago, IL). $P \leq 0.05$ means statistical difference among treatment groups.

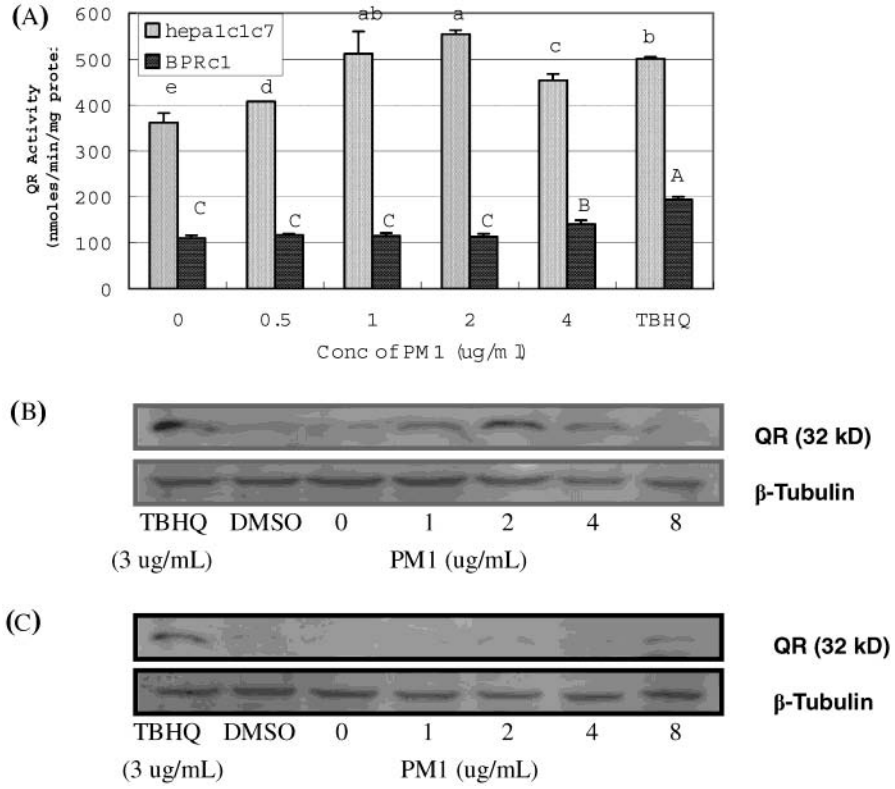

Figure 2. Dose-dependent QR induction by PM1 in hepa1c1c7 and BPRc1 cells. Hepa1c1c 7 (3×10^5/plate) and BPRc1 cells (5×10^5/plate) were plated into a 100-mm culture dish, incubated 48 h, and exposed to various concentrations of polyozellin and TBHQ (3.3 ug/mL), a positive control, for 24 h, following QR assay and western blot. A, QR activity; B, QR expression in hepa1c1c7 cells; C, QR expression in BPRc1 cells. Values are means ± sd, $n = 3$. Means with different letters differ, $P < 0.05$. Small letters (a–e) denote differences in hepa1c1c7 cells; capital letters (A–C) denote differences in BPRc1 cell.

RESULTS AND DISCUSSION

Our preliminary study showed that the extract of *Polyozellus multiplex* had a good QR induction potential. Furthermore, Kim and co-workers (*13*) demonstrated that the feeding of *Polyozellus multiplex* extract with 0.5 and 1.0% doses in a diet induced GST activity in the livers and stomachs of Wistar rats. An activity-guiding separation using solvent fractionation and chromatography generated 512 mg of a final grayish powder from 1 kg of dried raw material. The spectroscopic data, including ^{1}H- and ^{13}C NMR, and EI-MS of this compound were completely identical to those of PM1 (**Figure 1**). As shown in **Figure 2**, polyozellin induced QR activity in a dose-dependent manner in both hepa1c1c7 and BPRc1 cells, although in hepa1c1c7 cells, the enzyme activity appeared to be reduced at the concentration above 4 μg/mL, perhaps due to cytotoxicity. To confirm that the increased QR activity by polyozellin treatment was caused by the increased QR expression, western blotting by using QR-specific antibody was performed. As shown in **Figure 1**, parts **B** and **C**, immunoblot analysis demonstrated a significant increase in the levels of QR by treatment with 1, 2 μg/mL in hepa1c1c7 cells and 8 ug/mL in BPRc1 cells, consistent with the dose-dependent induction of QR activity (**Figure 1A**). polyozellin appeared to be more effective QR inducer than TBHQ, a known monofunctional QR inducer, at the similar concentration (**Figure 2**).

GST activity was also induced by polyozellin in a dose-dependent manner in both cell lines (**Figure 3**), although its induction ratio was less prominent than that of QR. polyozellin also caused a maximum 1.7 and 2.5-fold induction in GSH content in hepa1c1c7 and BPRc1 cells (**Figure 4**).

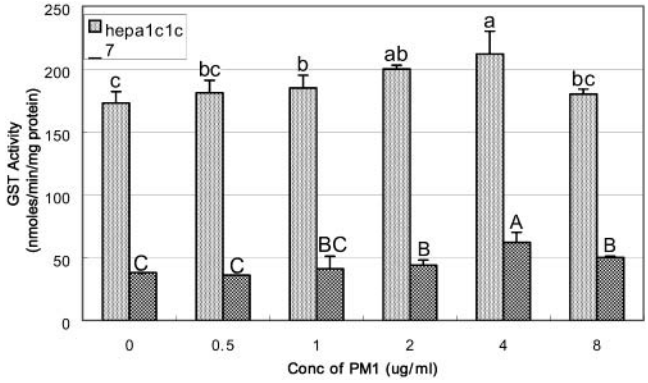

Figure 3. Dose-dependent GST induction by polyozellin (PM1) in hepa1c1c7 and BPRc1 cells. Hepa1c1c 7 (3×10^5/plate) and BPRc1 cells (5×10^5/plate) were plated into a 100-mm culture dish, incubated 48 h, and exposed to various concentrations of polyozellin for 24 h, followed by GST assay. Values are means ± sd, $n = 3$. Means with different letters differ, $P < 0.05$. Small letters (a–c) denote differences in hepa1c1c7 cells; capital letters (A–C) denote differences in BPrc1 cells.

The activity promoting the differentiation of HL-60 human promyelocytic leukaemia cells, one of the chemopreventive biomarkers, was assessed by NBT-reducing assay, in which the percentage of NBT-positive cells was calculated (**Figure 5).** Polyozellin at the concentration of 25 ug/mL, significantly enhanced the percentage of NBT-positive cells, suggesting a strong activity inducing tumorigenic cell differentiation, although its activity was lowered at the concentration of 50 ug/mL maybe due to cytotoxicity.

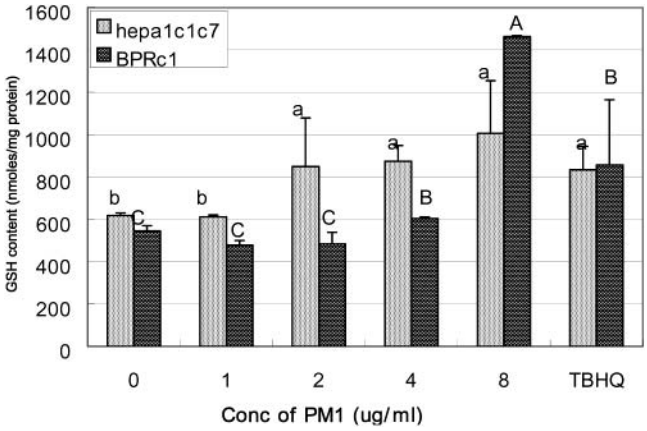

Figure 4. Effect of PM1 treatment on cytosolic GSH content in hepa1c1c7 and BPRc1 cells. Hepa1c1c 7 (3×10^5/plate) and BPRc1 cells (5×10^5/plate) were plated into a 100-mm culture dish, incubated 48 h, and exposed to various concentrations of polyozellin and TBHQ (3.3 ug/mL), a known QR inducer, for 24 h, followed by the determination of GSH content in the cells. Means with different letters differ, $P < 0.05$. Small letters (a, b) denote differences in hepa1c1c7 cells; capital letters (A–C) denote differences in BPRc1 cells.

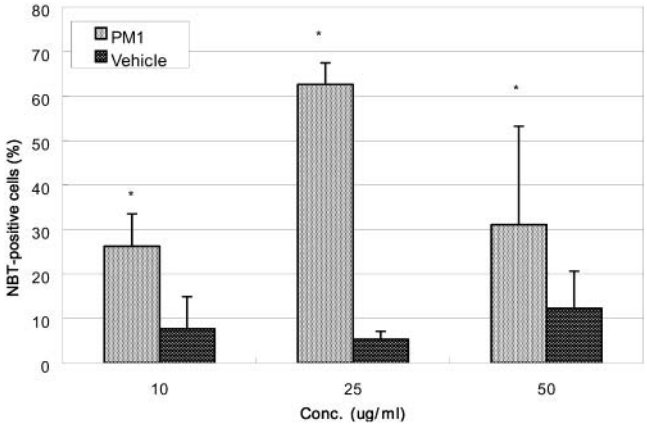

Figure 5. Induction of HL60 human promyelocytic leukaemia cells by PM1. 1.2×10^5 HL-60 cells/well were plated into a 24-well plate with 1 mL of EMEM containing 10% FBS per well, incubated for 18 h at 37 °C, and treated with polyozellin for another 4 days, followed by NBT assay. Values represent means ± SD. *, $p < 0.05$ versus vehicle.

Although *Polyozellus multiplex* extract has been shown to have a cancer preventive potential including induction of glutathione *S*-transferase, increasing glutathione level, and a significant reduction in proliferating cell nuclear antigen-labeling index of the glandular stomach epithelium from rats insulted with MNNG, the active agents had not been identified (*13*). The current study demonstrated that one of the major components responsible for induction of phase 2 enzymes appeared to be polyozellin. Phase 2 detoxification enzymes, including QR and GST, which are involved in the removal of various carcinogens and surrogate biomarkers for chemopreventive potential, were significantly induced by polyozellin. However, the potential of polyozellin to induce QR should be underestimated by the assay method used in this study, in which 2,6-dichlorophenolindophenol was used as a substrate. In fact, a large discrepancy in QR induction by TBHQ in hepa1c1c7 cells was observed according to assay methods (*14*, *15*).

NAD(P)H/quinone-acceptor oxidoreductase (QR) is an electrophile processing metabolic enzyme (*14*). It is widely distributed, is primarily cytosolic, and catalyzes the reduction of a wide variety of quinones. QR is induced coordinately with phase 2 metabolizing enzymes by a variety of compounds that protect rodents from the toxic, mutagenic, and neoplastic effects of carcinogens (*16*−*19*). In the current study, polyozellin was more effective in inducing QR than GST. There is a large body of evidence that monitoring this enzyme is a convenient screening method for anti-carcinogenic activity.

GST, a phase 2 enzyme, is involved in catalyzing the conjugation of GSH to a variety of electrophilic compounds, including carcinogens and other xenobiotics. This enzyme might play a critical role in preventing animals and humans from developing tumors, which may be caused by chemical carcinogens present in the environment (*14*).

Meanwhile, polyozellin significantly induced HL-60 cell differentiation, indicative of cancer preventive potential. For instance, retinoic acid and its analogues, effective HL-60 differentiation inducers, are clinically effective against acute promyelocytic leukemia (*20*).

For screening potential detoxifying enzyme inducers, animal models have been traditionally regarded as the ideal models. However, the cost, the time consumption, poor absorption, and/or first pass metabolism may limit their applicability in the early-stage of drug discovery process (*16*). Cell-based models, which are relatively less expensive, more time efficient, and without the complications of poor oral absorption and/or metabolism, may offer some advantages for high-throughput screening of large number of samples or compounds. Although the activity of polyozellin in animal model was not assessed due to limited availability of the compound, it needs to be done prior to taking consideration as a chemopreventive measure.

Polyozellin was previously reported to have inhibitory activity against prolyl endopeptidase (*12*). Recently, Song and Raskin (*21*) reported the isolation of a new benzofuran dimer, 5,6,5′,6′-tetrahydroxy[3,3′]bibenzofuranyl-2,2′-dicarboxylic acid dimethyl ester (kynapcin-24) from *Polyozellus multiplex*, which showed noncompetitive inhibitory activity against prolyl endopeptidase, with an IC_{50} value of 1.14 uM.

Thelephoric acid (**Figure 1**), which was isolated as a PEP inhibitor from the same mushroom (*11*) and similar to polyozellin in its chemical structure except the lack of two acetyl groups, was not effective in QR induction, suggesting a crucial role of acetyl groups attached to benzofuran dimer in the activation of phase 2 enzyme expression.

Taken together, our data strongly suggest that polyozellin has great anti-carcinogenic potential because it induced the activities of detoxifying phase 2 enzymes and stimulated the differentiation of cancerous cells.

ABBREVIATIONS USED

Arnt, Arylhydrocarbon hydroxylase (Ah) receptor nuclear translocator; GSH, glutathione; GST, glutathione S-transferase; α-MEM, alpha-modified minimal essential media; EMEM, Eagle's minimal essential media; NBT, nitroblue tetrazolium; NQO (or OR), NAD (P) H/quinone-acceptor oxidoreductase; PBS, phosphate buffered saline; PM1, polyozellin; QR, quinone reductase; TBHQ, *tert*-butylhydroquinone.

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