Regulation of cell cycle transition and induction of apoptosis in HL-60 leukemia cells by the combination of *Coriolus versicolor* and *Ganoderma lucidum*

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Abstract. Medicinal mushrooms have served as the mainstay of treatment for a variety of human illnesses in Asian countries, mostly as supplements by cancer patients. Extracts prepared from *Trametes versicolor* under the trade name of I'm-Yunity exhibit anti-tumorigenic activities, as supported by inhibition of the proliferation and induction of apoptosis in malignant cells. Similar effects have also been observed for the Reishi mushroom *Ganoderma lucidum*. The two mushrooms exert their medicinal activities primarily through a family of polysaccharo-peptides. Despite the common identity in their bioactive ingredients, whether their combination might elicit an expanded efficacy and mechanism has not been investigated. In the present study, we investigated similarities and differences between extracts prepared from I'm-Yunity and from a formulation denoted I'm-Yunity-Too combining I'm-Yunity and *Ganoderma lucidum*. By assaying their anti-proliferative and anti-apoptotic effects using human promyelocytic HL-60 cells, we found that the ethanolic extract of I'm-Yunity-Too was more active in inducing cell death compared to I'm-Yunity, based on measured changes in the expression of caspase 3 and Bax. Moreover, ethanolic extracts of I'm-Yunity-Too exhibited more potent activity compared to its aqueous extracts with regard to suppression of the growth and induction of apoptosis, as assayed by the more pronounced downregulation of phosphorylation of Rb and increased cleavage of poly(ADP-ribose) polymerase (PARP) from its native 112-kDa form to the inactive 89-kDa product. These results suggested that the chemopreventive potential of I'm-Yunity may be enhanced by adding *Ganoderma lucidum* and that their bioactive ingredients potentially exhibit mechanistic synergism suggesting a more efficacious adjunct in chemotherapy.

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Introduction

Cancer remains a significant cause of morbidity and mortality in the United States and worldwide, despite advances made in recent decades on early detection and diagnosis. Increasingly, among practicing clinicians and research professionals, the one-time dominant view of seeking 'magic bullets' for cancer eradication has been supplanted by the objective of cancer prevention and management in order to improve the quality of life of cancer patients.

A reductionist approach to curtail cancer risks, thereby preventing cancer, involve the elimination or reduction, of exposure to sources of carcinogens, together with change in lifestyle and dietary habits. It is therefore notable that, although anti-tumorigenic agents abound in the diet, as revealed by epidemiological/migrant studies reporting an inverse relationship between diet and cancer incidence rate, evidence indicates that diet harbors carcinogens or their precursors. This dual benefit/risk nature of diet with regard to carcinogenesis was clearly demonstrated in a seminal report by Doll and Peto (1) who suggested that 10-70% of human cancer mortality may be associated with diet. Furthermore, whereas studies by Steinmetz and Potter (2,3) and Willett (4) portend that dietary factors contribute to one third of all cancer deaths, evidence indicates that an estimated 20-50% of all cancer types are preventable using plant-based diet strategies (5,6), in part attributed to phytochemicals and polyphenols with chemopreventive activities (7-11).

The Reishi mushroom *Ganoderma lucidum* belongs to the Basidiomycetes class of fungi whose use as an antitumor agent dated back to the Imperial Court of ancient China (12-14). The major bioactive compounds in *Ganoderma lucidum* are polysaccharides, ganoderic, ganodermic, lucidic acids and their aldehydes and alcohols, and highly oxidized lanostane-type triterpenes (15-17). I'm-Yunity™ is a proprietary method procured version of the Basidomycetes mushroom comprising a heterogeneous family of polysaccharide-protein complexes isolated from the cultivated mycelia of *Trametes* (formerly *Coriolus*) *versicolor*, which have been used as a major ingredient in traditional Chinese medicinal formulations for the prevention and treatment of chronic diseases including cancer (18-27). The two mushroom products are available as dietary supplements, principally as adjunct immune boosters and prophylactic chemopreventative agents targeting the initiation

and progression of cancers (12,20,23,28-40). Despite their similar biological activities, differences may exist, both with respect to the nature of the polysaccharides and the spectrum of synthesized and secreted secondary metabolites. Accordingly, it may be postulated that the combination of the two mushrooms elicits distinct as well as overlapping biological and molecular effects, compared to single mushroom extracts. However, this possibility has yet to be investigated. In this study, extracts prepared from I'm-Yunity and from a combined formulation denoted I'm-Yunity-Too™-containing extracts derived from I'm-Yunity and *Ganoderma lucidum* were investigated with regard to differences in: i) anti-proliferative activities; ii) cell cycle control and induction of apoptosis; iii) activities between aqueous and ethanolic extracts.

Materials and methods

Reagents. Fetal calf serum, RPMI-1640, penicillin and streptomycin were purchased from Cellgro, Inc. (Herndon, VA, USA). Any other chemicals and solvents were of analytical grade. Primary antibodies, respectively, against Rb, E2F, NF-κB p50, NF-κB p65, IκB, caspase 3, bcl 2, Bax, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against phosphorylated Rb Ser780, Ser795, Ser807, Thr821 and Thr826 were obtained from Biosource International, Inc. (Camarillo, CA, USA). Anti-PARP was purchased from Biomol International, L.P. (Plymouth Meeting, PA, USA).

Source of Coriolus versicolor and Ganoderma lucidum. I'm-Yunity and I'm-Yunity-Too capsules containing extracts of *Coriolus versicolor,* and of *Coriolus versicolor* and *Ganoderma lucidum,* respectively, (lot 3BA03020528) were provided by Integrated Chinese Medicine Holdings, Ltd. (Hong Kong, China). The mushroom products were extracted and purified from mycelia of *Coriolus versicolor* and *Ganoderma lucidum* according to Good Manufacturing Practice (GMP) standards. Quality control assays validating their authenticity and integrity were performed in government-approved testing centers in Hong Kong.

Preparation of aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too. To prepare aqueous or ethanolic extracts of I'm-Yunity and I'm-Yunity-Too, the contents of each capsule were suspended in 6 ml of water or 70% ethanol, intermittently mixed by vortexing for 60 min at room temperature, and centrifuged to remove insoluble particles. The clear supernatant was sterilized by passing through a 0.22 μm filter and stored in aliquots at 4˚C.

Cell culture and growth inhibition assay. Human promyelocytic leukemia cell line (HL-60) was supplied by American Tissue Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 and seeded at a density of $3x10^5$ cells/ml for all the experiments, as previously described (24,26,41-43). Extracts of I'm-Yunity and I'm-Yunity-Too were added to the culture media at the final doses specified. Control and mushroom extract-exposed cells were harvested at indicated times after treatment. Cell count was performed using a hemocytometer and viability was determined by trypan blue exclusion. Harvested cells were washed twice with PBS, and pellets were stored at -80˚C for subsequent analysis.

Cell cycle analysis. Cell cycle phase distribution was assayed by flow cytometry. Following a 24-, 48- and 72-h treatment of HL-60 cells with aqueous and ethanol extracts of I'm-Yunity or I'm-Yunity-Too (10 μl/ml), the cells were stained with 1.0 μg/ml DAPI containing 100 mM NaCl, 2 mM $MgCl_2$ and 0.1% Triton X-100 (Sigma-Aldrich Corp., St. Louis, MO, USA) at pH 6.8, as previously described (24,26,41-43). The DNA contents were collected and analyzed by a flow cytometer (Ortho Diagnostic, Westwood, MA, USA) and MultiCycle software from Phoenix Flow Systems (San Diego, CA, USA) was used to quantify the percentage of cells in the respective phases (G_1, S and G_2/M) of the cell cycle, as previously described (24,26,41-43). Flow cytometry was used to determine cells undergoing apoptosis, as evident by the appearance of the sub-G_1 peak (44-46).

Preparation of cell extracts and western blot analysis. For immunoblotting experiments, cells were collected and lysed in ice-cold RIPA buffer, as described in recent studies (47-50). The aliquots of lysates (20 μg of protein) were boiled with sample buffer for 5 min, and resolved by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked with TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween-20) containing 3% non-fat dried milk overnight at 4˚C. The blots were incubated overnight with various primary antibodies followed by a 1-h incubation with secondary antibodies. The blots were detected with an ECL detection system (LumiGLO Peroxidase Chemiluminescent Substrate kit, KPL Biotechnology, Inc., Gaithersburg, MD, USA) and quantified by densitometry. Actin expression was used for normalization of the sample loading, as previously described (47-50).

Results

Differential inhibition of cell growth by aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too. To determine whether aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too exert comparable anti-proliferative activities, human leukemia HL-60 cells were exposed to a single dose of either extracts for 24-72 h. Growth and cell viability were measured as described in Materials and methods. Results in Fig. 1A show that the aqueous extract of I'm-Yunity was more potent in inhibiting cell proliferation than the comparable aqueous extract of I'm-Yunity-Too. Ethanolic extracts of the latter were slightly more active than the former in suppressing cell growth and, significantly more effective in inducing cell death (Fig. 1B).

I'm-Yunity and I'm-Yunity-Too extracts affect NF-κB/IκB expression. To determine whether NF-κB plays a role on cell growth inhibition by extracts of I'm-Yunity or I'm-Yunity-Too, the expression of NF-κB/IκB was assayed by western blot analysis. Results in Fig. 2 show that the ethanolic extracts of I'm-Yunity-Too had a potent suppressive effect on the expression of NF-κB p65 throughout the duration of the experiment, and similarly inhibited IκB levels for 48- and 72 h-exposed cells. The NF-κB p65 modulatory effects were only transiently

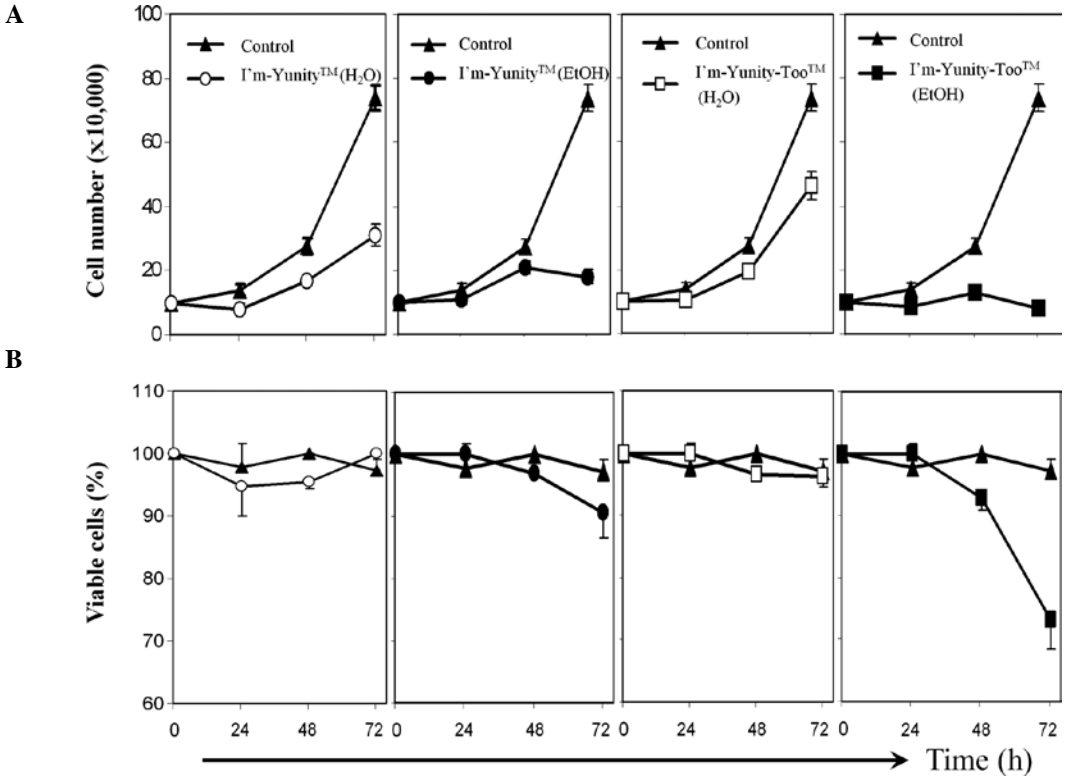


Figure 1. Effects of I'm-Yunity and I'm-Yunity-Too on HL-60 cell proliferation and viability. (A) Cells were treated with 10 μl/ml aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too. Cell numbers were assayed at 24, 48 and 72 h post-treatment using a hemocytometer. (B) Cell viability following treatment was measured as described in Materials and methods. The results are presented as mean ± SD of a minimum of three separate experiments.

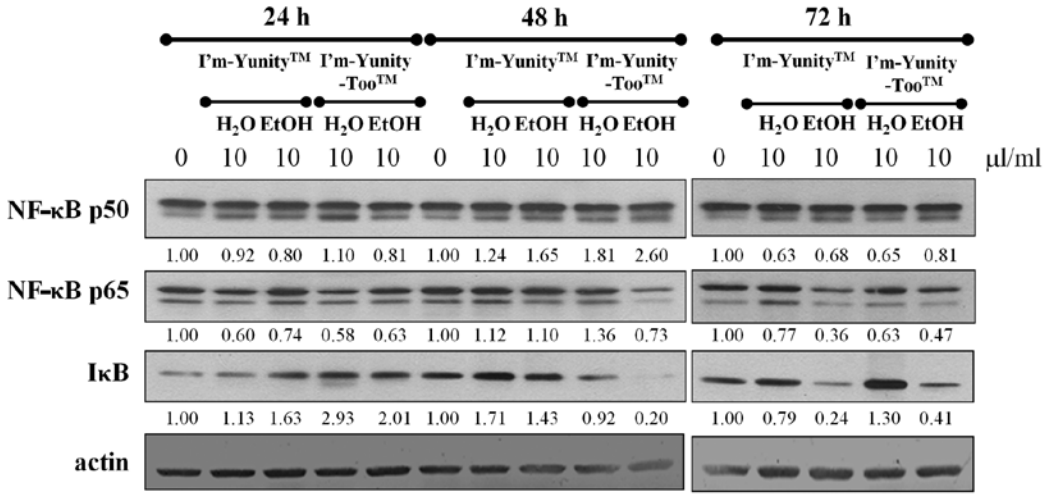
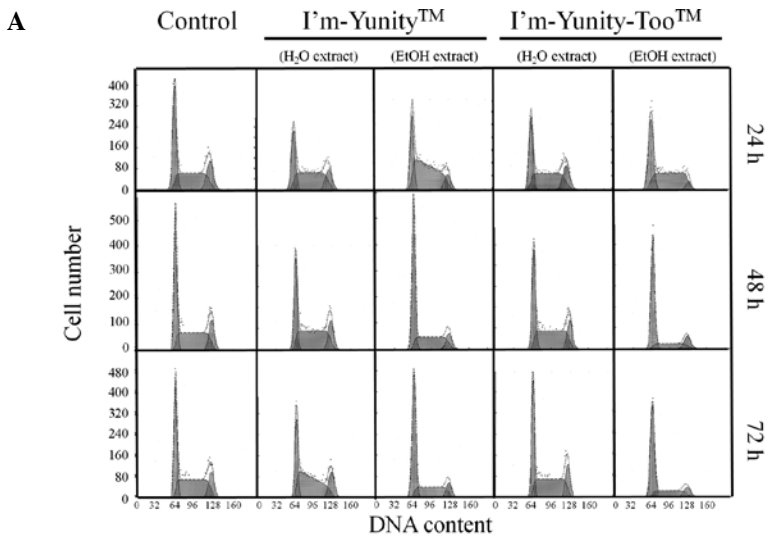


Figure 2. Effects of I'm-Yunity and I'm-Yunity-Too on NF-κB/IκB expression in HL-60 cells. Cells were treated with 10 μl/ml of aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too for 24, 48 and 72 h. The cells were collected and total protein extracts were prepared as described in Materials and methods. Aliquots of total extracts were separated by SDS-PAGE and the effects on NF-κB p50, NF-κB p65 and IκB protein expression were determined by western blot analysis, using actin as a loading control to quantify changes in the expression of the referenced genes. The intensity of the specific immunoreactive bands was quantified by densitometry and expressed as a fold difference against actin.

observed in cells treated for 24 h with aqueous extracts of I'm-Yunity or I'm-Yunity-Too, while for I'm-Yunity the ethanolic extract-exposed cell treatment for 72 h also significantly suppressed NF-κB p65 and IκB expression. These results suggest that the ethanolic extract of I'm-Yunity-Too has a significantly more pronounced effect in reducing the level of the cell survival gene, NF-κB, as compared to extracts derived from I'm-Yunity, suggesting that differential gene modulatory effects exist between the aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too.

Cell cycle control by extracts of I'm-Yunity and I'm-Yunity-Too. To examine whether I'm-Yunity- and I'm-Yunity-Too-induced cell growth inhibition involves changes in the cell cycle phase transition, flow cytometry was performed in the control and exposed cells. Results in Fig. 3A show that HL-60 cells treated



HL-60	control			I'm-Yunity™ (H_2O extract)			I'm-Yunity™ (EtOH extract)			I'm-Yunity-Too™ (H_2O extract)			I'm-Yunity-Too™ (EtOH extract)		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
24 h	37.7	44.5	17.8	29.2	55.5	15.3	26.1	64.7	9.2	31.8	50.4	17.7	39.9	55.7	4.4
48 h	38.7	46.0	15.2	30.4	53.7	15.9	54.5	35.9	9.6	34.1	51.2	14.8	66.4	21.5	12.1
72 h	35.0	50.5	14.5	26.3	57.1	16.6	56.5	33.6	9.9	34.1	50.5	15.3	61.2	28.1	10.7

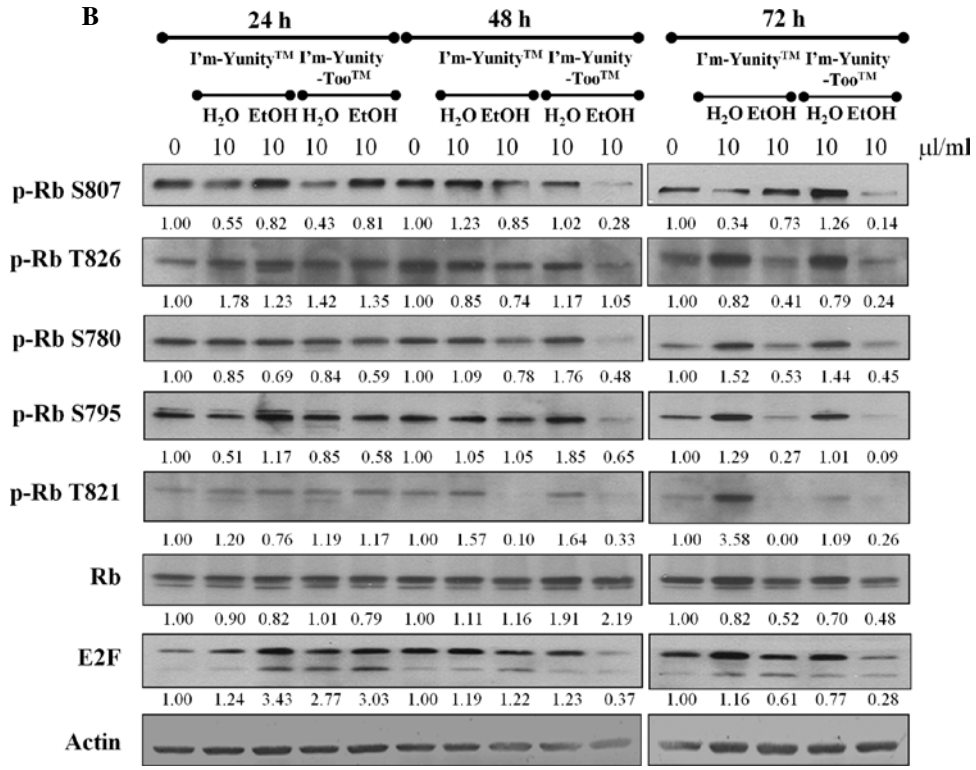


Figure 3. Effects of aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too on the changes in cell cycle phase distribution and the expression of Rb, Rb phosphorylation and E2F in HL-60 cells. (A) At 24-72 h post-treatment with 10 μl/ml I'm-Yunity and I'm-Yunity-Too extracts, the cells were harvested and cell cycle analysis was performed. (B) Expression of total Rb, E2F and phosphorylated Rb at Ser780, Ser795, Ser807, Thr821 and Thr826 was determined by immunoblot analysis. Blots were quantified; relative expression was presented as the fold difference in comparison with the level of the loading control, actin.

with ethanolic extracts of I'm-Yunity-Too were restricted in the G_1→S progression. Fig. 3A also shows a decrease in cell fraction accumulated in the G_2M phase of the cell cycle, as compared to the untreated cells. These effects were most notable in 48-72 h exposed cells. By contrast, in cells treated for 24 h by aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too, the primary cell cycle involved attenuation in the S→G_2M phase transition.

To elucidate control of G_1→S in HL-60 cells treated with aqueous/ethanolic extracts of I'm-Yunity and I'm-Yunity-Too, changes in Rb/E2F expression and the state of phosphorylation of Rb were determined. Western blot analysis demonstrated

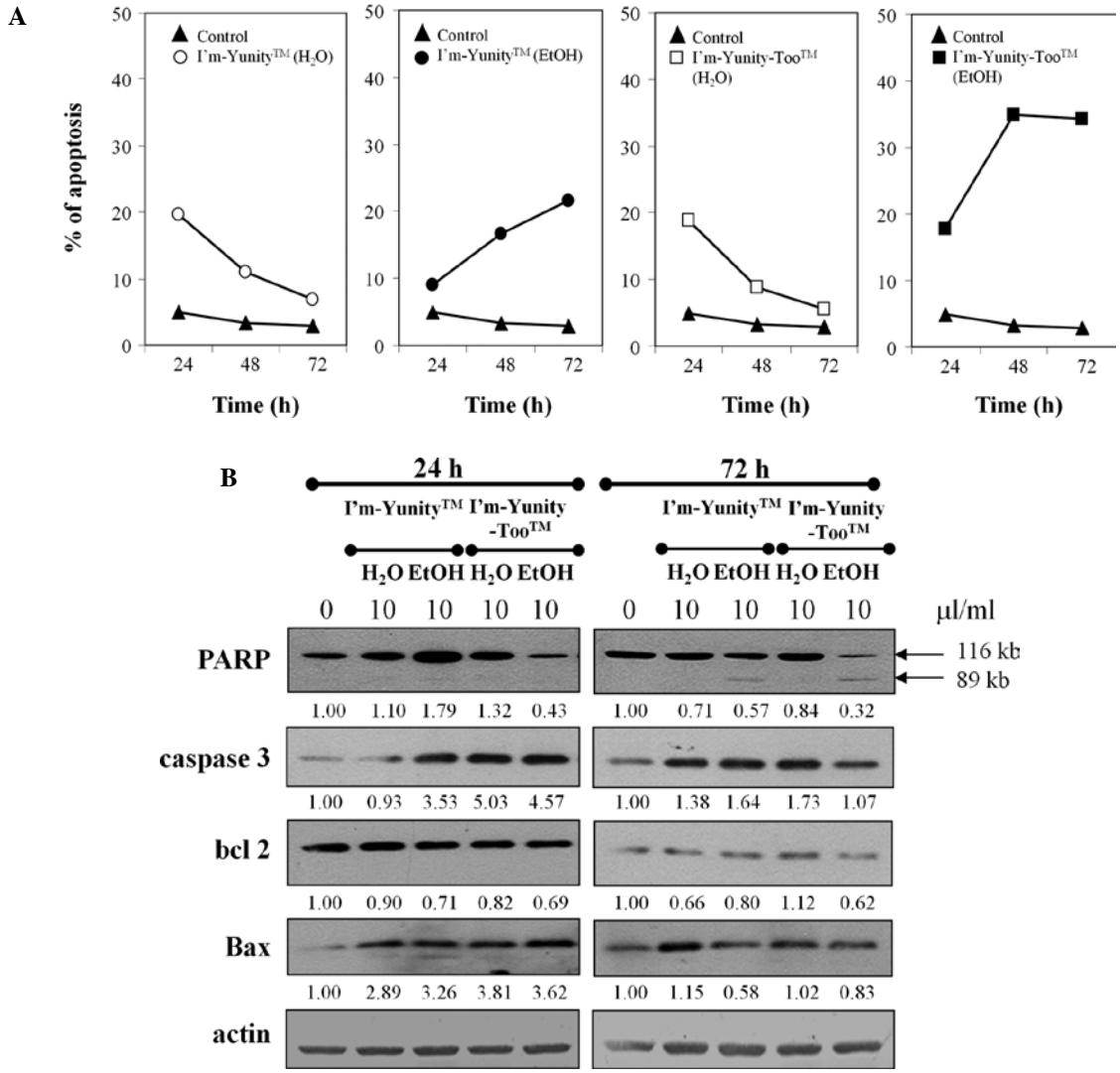


Figure 4. Effects of I'm-Yunity and I'm-Yunity-Too on the induction of apoptosis and expression of PARP, caspase 3, Bax and bcl 2. (A) HL-60 cells were treated with 10 μl/ml aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too for 24-72 h. Induction of cell death by treatment was demonstrated by the appearance of the sub-G_1 fraction in flow cytometric analysis. The extent of induction of apoptosis by treatment was calculated and presented as a percentage of the total cell population. (B) Following a 24- and 72-h treatment with 10 μl/ml I'm-Yunity and I'm-Yunity-Too extracts, cells were harvested and total protein extracts were prepared as described in Materials and methods. Aliquots of total extracts were separated by SDS-PAGE and western blot analysis was performed to reveal the cleavages of PARP from 112- to 89-kDa fragment. Expression of caspase 3, bcl 2 and bax was also determined. Blots were quantified and relative expression levels of the referenced proteins were shown as fold differences against the loading control, actin.

that while aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too did not change the expression of total Rb, they markedly reduced the levels of Rb phosphorylation at Ser807, Ser780, Thr821, as well as E2F, in 48 h-exposed cells (Fig. 3B). Furthermore, the most pronounced changes occurred in cells treated with ethanolic extracts of I'm-Yunity-Too for 48 and 72 h, reinforcing that extracts derived from the combination mushroom have biological activities that are different from the ethanolic extract prepared from the single mushroom I'm-Yunity. These findings also suggest that ethanolic extracts of I'm-Yunity and I'm-Yunity-Too exert multiple cell cycle effects in HL-60 cells manifested as specific checkpoint arrest.

Targeting the induction of apoptosis by I'm-Yunity and I'm-Yunity-Too. Results in Figs. 1B and 4A show that the ethanolic extract of I'm-Yunity-Too induced significantly more effective cell death as compared to the ethanolic extract of I'm-Yunity, leading to subsequent analysis of molecular determinants possibly contributing to such effects. Fig. 4B shows that treatment with aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too at 24 and 72 h elevated the expression of caspase 3 as well as Bax, both integral to the induction of apoptosis. Corroborative evidence for apoptosis is derived from a reduction in the expression of PARP in cells treated for 72 h with ethanolic, but not aqueous, extracts of I'm-Yunity and I'm-Yunity-Too, concomitant with the increase in the cleaved 89-kDa product from the 112-kDa precursor.

Discussion

The adjunctive potential of *Ganoderma lucidum* in the management of cancer patients has been previously reported (51). However, the mechanisms by which it exerts efficacy have not been fully elucidated. It also remains to be determined whether, when administered in combination, the mechanisms generate novel activities.

In the present study, HL-60 cells cultured *in vitro* were used to analyze the effects of I'm-Yunity (*Coriolus versicolor*) relative to I'm-Yunity-Too (*Coriolus versicolor* combined with *Ganoderma lucidum*) with regard to proliferation and induction of apoptosis. We demonstrated that I'm-Yunity and I'm-Yunity-Too significantly inhibited HL-60 proliferation, in concordance with the downregulation of NF-κB p65 expression. We also observed differences concerning changes in IκB expression between the aqueous and ethanolic extracts of I'm-Yunity and I'm-Yunity-Too. Thus, cells treated with ethanolic extracts for 72 h showed the downregulation of IκB, which was not affected in cells treated with aqueous extracts.

Studies directed at determining the mechanism controlling cell cycle progression by I'm-Yunity and I'm-Yunity-Too revealed ethanolic extracts derived from I'm-Yunity-Too-restricted cells in the G_1 compared to the untreated cells, the effects of which were most pronounced in cells treated for 48-72 h and occurred in coordination with profound loss of E2F and the downregulation of the Rb phosphorylation sites Ser780, Ser795, Thr821 and Thr826, respectively. In terms of induction of apoptosis, ethanolic extract of I'm-Yunity-Too caused more cell death, as compared to those of I'm-Yunity. We also found that ethanolic extract-treated cells induced more cell death at 72 h as compared to aqueous extract-treated cells, based on PARP cleavage which only appeared in ethanolic extract-treated cells.

Taken together, the results suggest that distinct differences exist regarding the cell and gene regulatory effects of aqueous and ethanolic extracts of I'm-Yunity-Too, as compared to comparable extracts derived from I'm-Yunity. In addition, extracts from the combination mushroom have different biological activity, compared to ethanolic extract from the single mushroom I'm-Yunity. Additional experiments and more detailed analysis are required to determine the underlying mechanisms involved.

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