

# Effect of Various Natural Products on Growth of Bladder Cancer Cells: Two Promising Mushroom Extracts

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## Abstract

Despite the availability of several therapeutic options, a safer and more effective modality is urgently needed for treatment of bladder cancer. Specific immunotherapy is effective, but severe side effects limit its clinical use and underscore the need for unconventional therapies using less toxic substances. Many natural substances are touted for their medicinal aspects and side effect profiles, and some of these have been well characterized for their biological and medicinal properties. Accordingly, the effects on bladder cancer cells *in vitro* were investigated. Eight commercially available natural products were tested for possible effects on the growth of human bladder cancer T24 cells. This study demonstrated that two mushroom extracts, GD- and PL-fractions, induced a significant (>90%) growth reduction in 72 hours, whereas the remaining six products had no effect. Interestingly, non-toxic concentrations of the GD- or PL-fractions, when combined with a non-toxic concentration of vitamin C, became highly cytotoxic, resulting in >90-percent cell death. Thus, vitamin C appears to act synergistically with these fractions to potentiate their bioactivity (cytotoxicity). No other products tested demonstrated such a synergistic potentiation with vitamin C. The present study indicates that GD- and PL-fractions appear to have the most potent cytotoxic effect on human bladder cancer T24 cells. It is thus plausible that these substances could be used, solely or combined with conventional modalities, for the treatment of superficial bladder cancer. (*Altern Med Rev* 2007;12(1):63-68)

## Introduction

Bladder cancer is the second-most common urological malignancy in the United States, next to prostate cancer.<sup>1</sup> Currently, transitional cell carcinoma (TCC) is the most prevalent type (~90%) of bladder cancer. Annually, 50,000 new cases are diagnosed and more than 10,000 people die of this disease.<sup>2</sup> Approximately 80 percent of TCC presents as superficial bladder tumors, while 15 percent are invasive and five percent present as metastatic disease.<sup>1</sup> Although endoscopic transurethral resection (TUR) is often performed as the primary therapy, 50-75 percent of patients have a recurrence within five years and approximately 10 percent progress to invasive disease.<sup>3</sup> TCC is characterized by a multifocal origin and an acquired resistance to drugs, factors that likely contribute to the high incidence of disease relapse.<sup>4,5</sup> Thus, the primary therapeutic objectives are to prevent multiple recurrences, as well as progression to a more advanced, invasive disease.

Several cytotoxic and immune-modifying agents are used intravesically to treat bladder cancer. Among currently available therapeutic options, intravesical administration of Bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, is the most effective immunotherapy for superficial bladder cancer and carcinoma *in situ*.<sup>6,7</sup> BCG therapy has been shown to alter disease progression, reduce recurrence, and increase survival.<sup>8</sup> Adjuvant intravesical BCG therapy following TUR is now the established therapy for superficial bladder cancer, resulting in a 40-percent

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reduction in cancer recurrence, compared to other viable options (<20-percent reduction).<sup>8,9</sup> However, this benefit should be measured against potentially severe side effects, including cystitis, fever, allergic reactions, sepsis, and even death.<sup>10,11</sup> These drawbacks do limit BCG therapy in clinical practice and demonstrate the need for a non-toxic, safe, effective treatment modality with minimal side effects.

Various medicinal aspects of natural agents have been claimed or passed down through folklore. Such natural substances include herbs, mushrooms, flowers, fruits, plant seeds, seaweeds, algae, teas, bark, shark cartilage, etc. In some cases, sufficient scientific studies have not been performed to demonstrate the biological properties of these natural substances, although several studies have characterized the medicinal properties of some natural agents. For example, the maitake (*Grifola frondosa*) mushroom has been extensively studied.<sup>12</sup> In particular, its antitumor activity was well-documented in a study using tumor-bearing mice,<sup>13</sup> which demonstrated activation of various immune effectors including macrophages, cytotoxic T-lymphocytes, and natural killer cells.<sup>14</sup> Accordingly, this preliminary study investigates whether commercially available natural products have potential anticancer effects on human bladder cancer T24 cells *in vitro*.

## Materials and Methods

Human bladder cancer T24 cells, derived from a patient with TCC, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in McCoy's 5a medium containing 10-percent fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). Culture medium was routinely changed every 3-4 days and the passage of cells was performed weekly. For experiments, cells were seeded in six-well culture plates at an initial cell density of  $1 \times 10^5$  cells/mL. T24 cells were cultured for 72 hours with varying concentrations (0-1,000 µg/mL) of eight natural, commercially available products (Table 1). Cell morphology/appearance was monitored daily and cell number and viability were assessed at 72 hours using the trypan blue exclusion test and Alamar Blue cell viability test (Biosource International, Camarillo, CA), respectively.

Table 1. Natural Agents Tested

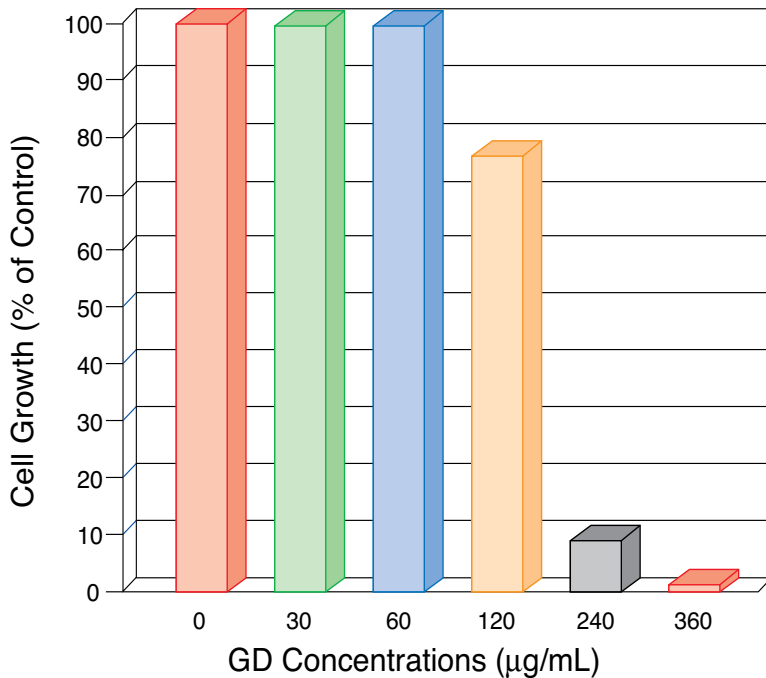
Name of Agent	Description of Agent*
GD-fraction	Active fraction of maitake ( <i>Grifola frondosa</i> ) mushroom
PL-fraction	Extract of meshimakobu ( <i>Phellinus linteus</i> ) mushroom
YBG	Extract of yeast cell wall
ARBX	Arabinoxylan from rice bran
ABE	Extract of <i>Agaricus blazei</i> Murill mushroom
ASC	Mixed powder of <i>Agaricus blazei</i> Murill mushroom and shark cartilage
MSK	Mixed powder of three kinds of mushrooms and three herbs
AHC	Mycelial extract of several mushrooms

\*Only GD-fraction was originally in a liquid form while the remaining seven products were in a powder form, which was dissolved in water (20 mg/mL). All eight products were sterilized through a 0.2 µm syringe filter and diluted appropriately with culture medium (if necessary) before use.

## Results

This dose-dependent study reveals a 25-percent reduction in cell growth with 120 µg/mL of GD-fraction and >90-percent growth reduction (due to cell death) with  $\geq 240$  µg/mL of GD (Figure 1). No effects were observed with GD concentrations up to 60 µg/mL. Similarly, PL-fraction induced 30-, 80-, and >90-percent growth reduction at 50, 100, and  $\geq 200$  µg/mL, respectively (Figure 2). PL at 20 µg/mL had no effect.

**Figure 1. Dose-Dependent Effects of GD-Fractions on T24 Cell Growth**



T24 cells were cultured with GD-fractions at the given concentrations, and cell growth was assessed at 72 hours. The data are mean of three separate experiments.

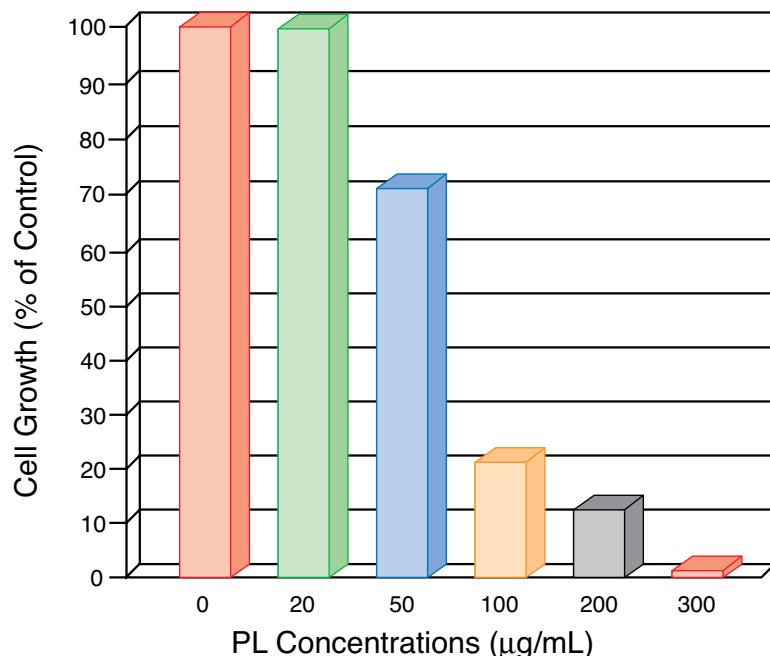
Besides potential cytotoxic activity of vitamin C, it has also been proposed that vitamin C might be capable of modulating the bioactivity of  $\beta$ -glucan.<sup>15</sup> Of note, a possible stimulatory effect of vitamin C on BCG-mediated immune response in patients with bladder cancer has been postulated.<sup>16</sup> The current study examined whether vitamin C might actually potentiate the cytotoxic effects of the GD- and PL-fractions (containing  $\beta$ -glucan and other proteoglycans), as well as the other six agents. It was revealed that as low as 30  $\mu\text{g/mL}$  of GD-fraction combined with 200  $\mu\text{M}$  (non-toxic concentration) vitamin C was nearly as effective as 240  $\mu\text{g/mL}$  of GD alone (Figure 1), resulting in >90-percent cell death (Table 3). Similarly, the combination of 20  $\mu\text{g/mL}$  of PL-fraction and vitamin C induced >90-percent cell death (Table 3). Because the given concentration (200  $\mu\text{M}$ ) of vitamin C alone had no effect on T24 cells (Table 2), it might have primarily served to augment the bioactivity of both GD- and PL-fractions,

In contrast, five other products (see Table 1 for product description) – ARBX, ABE, ASC, MSK, and AHC – were found to have little effect on T24 cell growth, even at the highest concentration of 1,000  $\mu\text{g/mL}$  (Figure 3). Interestingly, YBG at 20  $\mu\text{g/mL}$  showed a severe cytotoxic effect (>90% cell death). However, since YBG was originally prepared with vitamin C, this result should be interpreted with caution. Our pilot study confirmed that vitamin C by itself had a significant cytotoxic effect on T24 cells, especially when its concentration went beyond 500  $\mu\text{M}$  (Table 2). In fact, calculations revealed the above-lethal dosage of 2,400  $\mu\text{M}$  (2.4 mM) of vitamin C was included in 20  $\mu\text{g/mL}$  of YBG. Thus, a cytotoxic effect of YBG is more likely attributable to an extremely high concentration of vitamin C, not to its bioactive component ( $\beta$ -glucan).

**Table 2. Effects of Vitamin C alone on T24 Cell Viability**

Vitamin C ( $\mu\text{M}$ )	Cell Viability (% of Control) at 72 hours
0	100
100	100
200	100
300	~75
500	~50
700	~10
1,000	0

**Figure 2. Dose-Dependent Effects of PL-Fractions on T24 Cell Growth**



T24 cells were cultured with PL-fractions at the given concentrations, and cell growth was assessed at 72 hours. The data are mean of three separate experiments.

even at their non-toxic concentrations. Yet no other agents tested demonstrated enhanced cytotoxic effects in combination with vitamin C (data not shown).

## Discussion

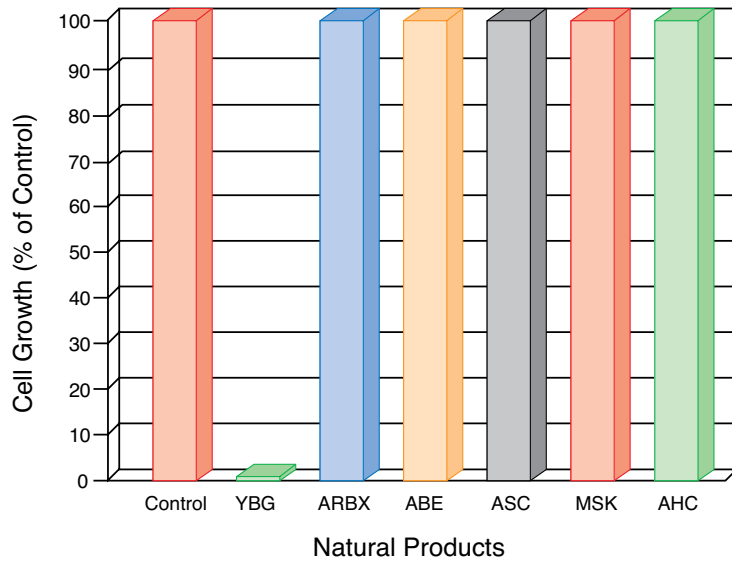
In search of a safer, more effective treatment for resilient bladder cancer with a high recurrence rate, this study investigated the potential anticancer effects of various natural agents on human bladder cancer T24 cells. Among eight products tested, two agents – GD- and PL-fractions – demonstrated potent cytotoxic activity on these cancer cells, inducing almost complete cell death in 72 hours (Figures 1 and 2). The other natural products tested failed to provide anticancer activity alone or in conjunction with a non-toxic concentration (200 µM) of vitamin C.

The cytotoxic activity of GD- and PL-fractions was enhanced when combined with vitamin C (Table 3). GD-fraction at 30 µg/mL (non-cytotoxic alone) induced >90-percent cell death in combination with 200 µM vitamin C (non-cytotoxic alone). PL-fraction at 20 µg/mL also led to >90-percent cell death when combined with vitamin C. Thus, vitamin C appears to act synergistically with these fractions to potentiate their bioactivity. In other words, the bioactivity of GD- and PL-fractions containing β-glucan (proteoglycan) can be synergistically augmented with vitamin C, becoming highly cytotoxic to bladder cancer cells.

The exact mechanism of this vitamin C-induced synergistic cytotoxic effect has not yet been elucidated, although exploratory studies by these authors are currently underway. One recent report found oxidative stress and the resultant generation of free radicals to be one of the primary mechanisms of high-dose, vitamin C-mediated cytotoxicity. The researchers concluded: “Vitamin C selectively killed cancer but not normal cells, using concentrations that are achievable by I.V. administration, via generation of free radical (H<sub>2</sub>O<sub>2</sub>) from vitamin C as the electron donor (as a prooxidant).”<sup>17</sup>

In the current study, YBG exhibited a significant cytotoxic effect, seemingly attributable to a cytotoxic dose (2,400 µM) of vitamin C in the product, rather than to the β-glucan content. YBG containing 20 µg/mL β-glucan and 2,400 µM vitamin C induced cell death (>90%) equivalent to GD (30 µg/mL β-glucan)- and PL (20 µg/mL β-glucan)-fractions with 200 µM vitamin C (Table 3). This exemplifies the need for extra caution when interpreting experimental data. It is important to determine whether cytotoxic effects exerted by certain samples resulted exclusively from the ingredient (e.g., β-glucan) being tested or from the addition of other ingredients (e.g., vitamin C).

**Figure 3. Effects of Other Natural Products on T24 Cell Growth**



T24 cells were cultured with six different products at the given concentrations, and cell growth was assessed at 72 hours. The data are mean of three separate experiments. YBG (20 µg/mL) was supplemented with a lethal dose of 2,400 µM of vitamin C, while other five products (1,000 µg/mL) contained no such additive.

YBG= Yeast cell wall extract  
 ARBX= Arabinoxylan from rice bran  
 ABE= Extract of Agaricus blazei Murill mushroom  
 ASC= Mixed powder of Agaricus blazei Murill mushroom and shark cartilage  
 MSK= Mixed powder of three kinds of mushrooms and three herbs  
 AHC= Mycelial extract of several mushrooms

Although the remaining five products appeared to be ineffective on T24 cells, the possibility that these products, due to potential specificity for different cancer cell lines, could be effective on other human malignancies cannot be ruled out. In order to ascertain the clinical efficacy for bladder cancer, *in vivo* studies are warranted.

In the meantime, this *in vitro* study suggests both GD- and PL-fractions could be useful in the intravesical therapy most often used for bladder cancer patients. Since this therapy directly introduces drugs/agents into the bladder through a catheter inserted into the urethra, they may directly affect cancer cells in the bladder. Because certain chemotherapeutic drugs or BCG are usually given intravesically for this type of cancer, it is plausible that GD/PL-fractions may demonstrate cytotoxic activity when introduced intravesically. However, actual effective clinical dosage of these fractions needs to be

established and potential side effects carefully examined and addressed.

Regarding the safety of GD- and PL-fractions in their potential clinical utility, they should be quite safe, originating from the non-poisonous maitake and meshimakobu mushroom, respectively. These mushrooms have been consumed dietarily or medicinally for many years in Asian countries, including Japan, China, and Korea. In fact, maitake still contributes to an important part of Japanese daily life, while meshimakobu has been recently approved as a prescription drug in Korea. In addition, although their medicinal properties were initially claimed in anecdotes and folklore, a number of scientific studies conducted over the past 20 years have confirmed and elucidated some proposed medicinal, biological, and clinical effects.

In conclusion, this preliminary study demonstrates the extracts of two types of mushrooms (GD- and PL-fractions), compared to other natural products tested, appear to have potent cytotoxic effects *in vitro*

**Table 3. Synergistic Effects of Vitamin C on GD- and PL-Fractions**

β-Glucan (µg/mL) / Vitamin C (µM)		Cell Viability (% of Control) at 72 hours
Control	0/0	100
Vitamin C	0/200	100
GD-fraction	30/0	100
GD + Vitamin C	30/200	<10
PL-fraction	20/0	100
PL + Vitamin C	20/200	<10
YBG*	20/2,400	<10

\* YBG containing a lethal dose of vitamin C (2,400 µM) is shown for a comparison.

on T24 cells. It is conceivable that GD- and PL-fractions could be used in the future, solely or combined with conventional modalities, for treatment of superficial bladder cancer. Further exploration of such natural agents is warranted.

### Acknowledgement

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