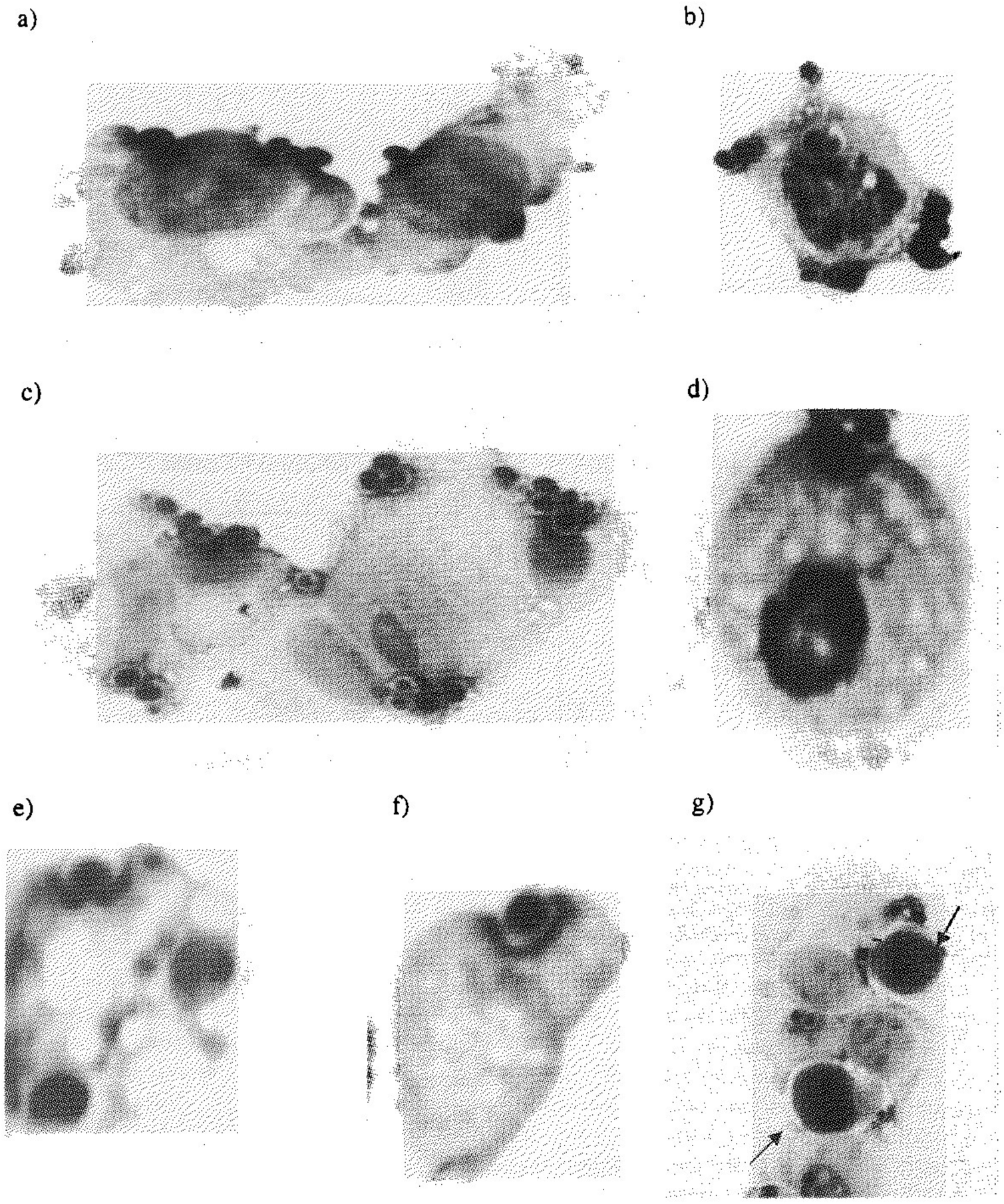

Fig. 5(a - g) Morphological examination of apoptotic MGN-3-treated MCF-7 cells using Giemsa-stained cytospin preparations.

Notice tumor cells exhibiting membrane blebbing and early nuclear condensation (a & b).
Preparation also showed cells with severe nuclear condensation which was located in an eccentric position (c & d).
Finally, nuclear fragmentation occurs (stained red) (e).
Nucleus then disappears (f).
Notice dead tumor cells stained with trypan blue (g).
Also, notice yeast (stained blue) has been ingested inside the tumor cells.
Figs. 5a, c&g ×400. b, d, e&f ×1000

ground of MCF-7 alone (3.4%). The effect of BioBran/MGN-3 on phagocytosis-induced cell death was found to be dose-dependent (**Figure 7**). BioBran/MGN-3 at 100 μg/m*l* showed an increase of MCF-7 apoptic cells (35.4%), as compared to MCF-7 cells and yeast. At 500 μg/m*l*, it further increased (40.1%), however, at 1000 μg/m*l*, it began to decline (33.04%).

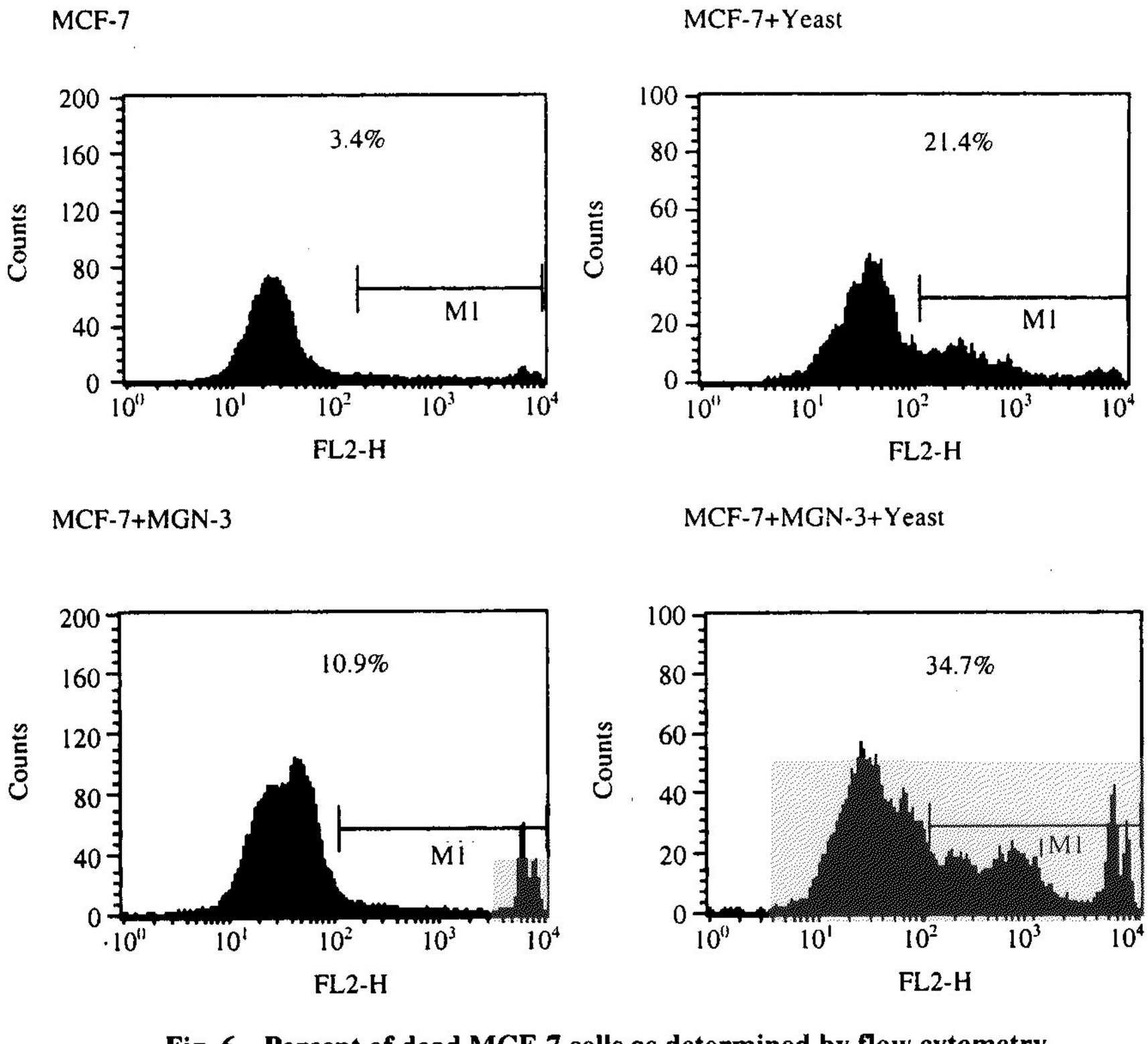

Fig. 6 Percent of dead MCF-7 cells as determined by flow cytometry.

MCF-7 cells were cultured with yeast at the ratio of 1:10 in the presence or absence of MGN-3 (500 μg/m*l*) for 2 hr, and cell survival was determined by flow cytometry using propidium iodide (PI) technique.
In this technique dead cells pick up PI and florescence.
The number in the histgrams represents the percent of dead cells.

4. Effect of BioBran/MGN-3 on apoptosis of other BCC lines

We examined the accelerative effect of BioBran/MGN-3 on apoptosis of other BCCs post phagocytosis of yeast. Survival in ZR-75 and HCC70 cell lines, was examined at 0.5 hr post culture with yeast. Results in **Figure 8** demonstrated that phagocytosis of yeast resulted in increased percentages of dead cancer cells: ZR-75 (21%) and HCC70 cells (11%). BioBran/MGN-3 treatment caused a significant increase in the expression of apoptic ZR-75 cells (48%), while the effect was less noticeable in HCC70 cells (20%). BioBran/MGN-3 alone demonstrated an appreciable increase in percent apoptotic ZR-75 cells (11%) and HCC70 (10%), as compared to background of cancer cells alone (3.5-5%).

5. Activation of caspases

A. Effect of yeast phagocytosis: To determine whether the decreased survival of tumor cells is due to yeast-induced necrosis or apoptosis, activation of caspases was determined. MCF-7 and HCC70 cells were incubated with yeast for 2 hr and activation of caspases 8, 9 and 3 was determined by flow cytometry. **Figure 9** is a representative of histograms that illustrate the yeast induced activation of both caspase 8

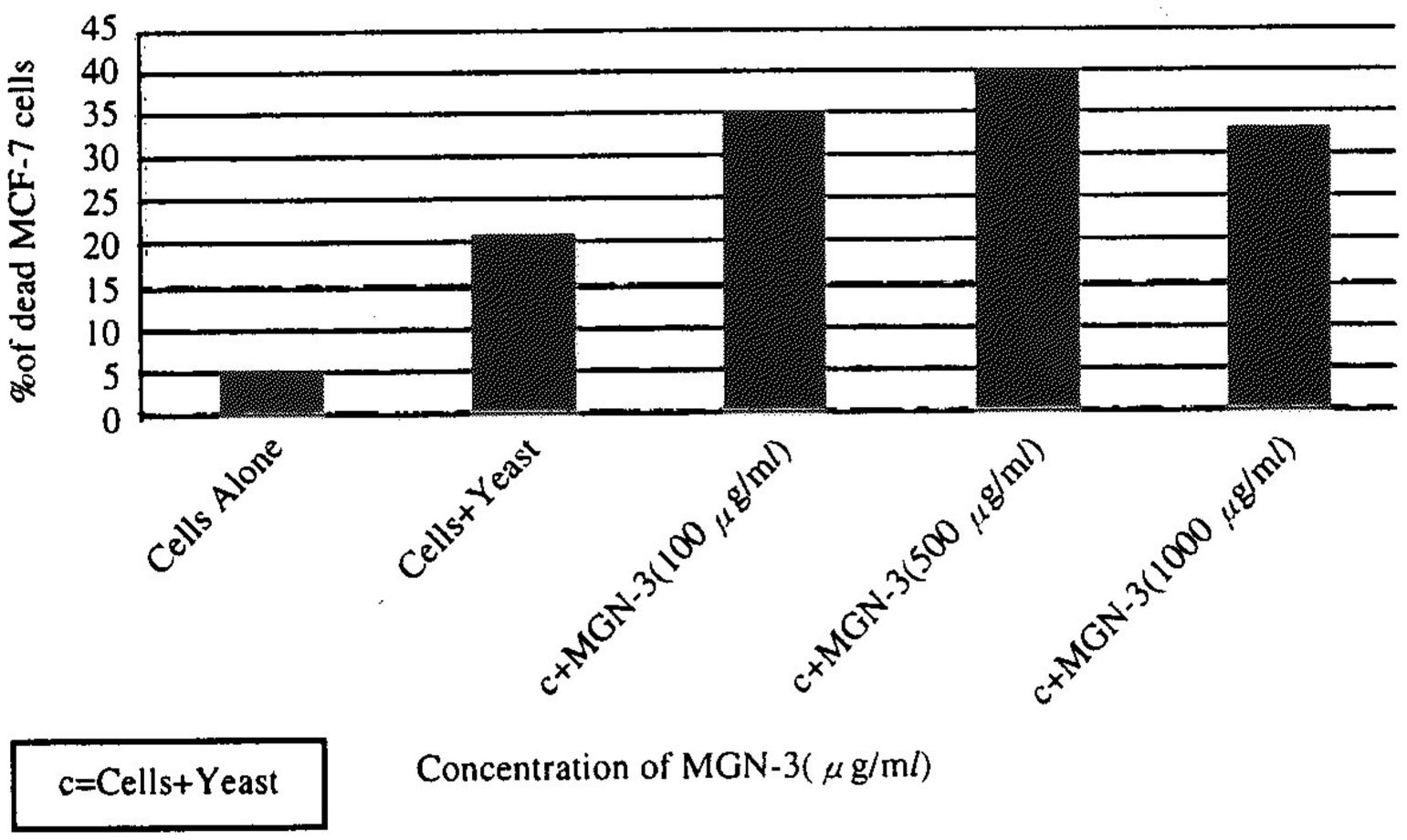

Fig. 7 Action of MGN-3 at different concentrations on apoptosis of MCF-7 cells.

MCF-7 cells were cultured with yeast at the ratio of 1:10 in the presence of MGN-3 (100-1000 μg/ml). Cancer cell survival was determined by flow cytometry using propidium iodide (PI) technique. In this technique dead cells pick up PI and florescence.

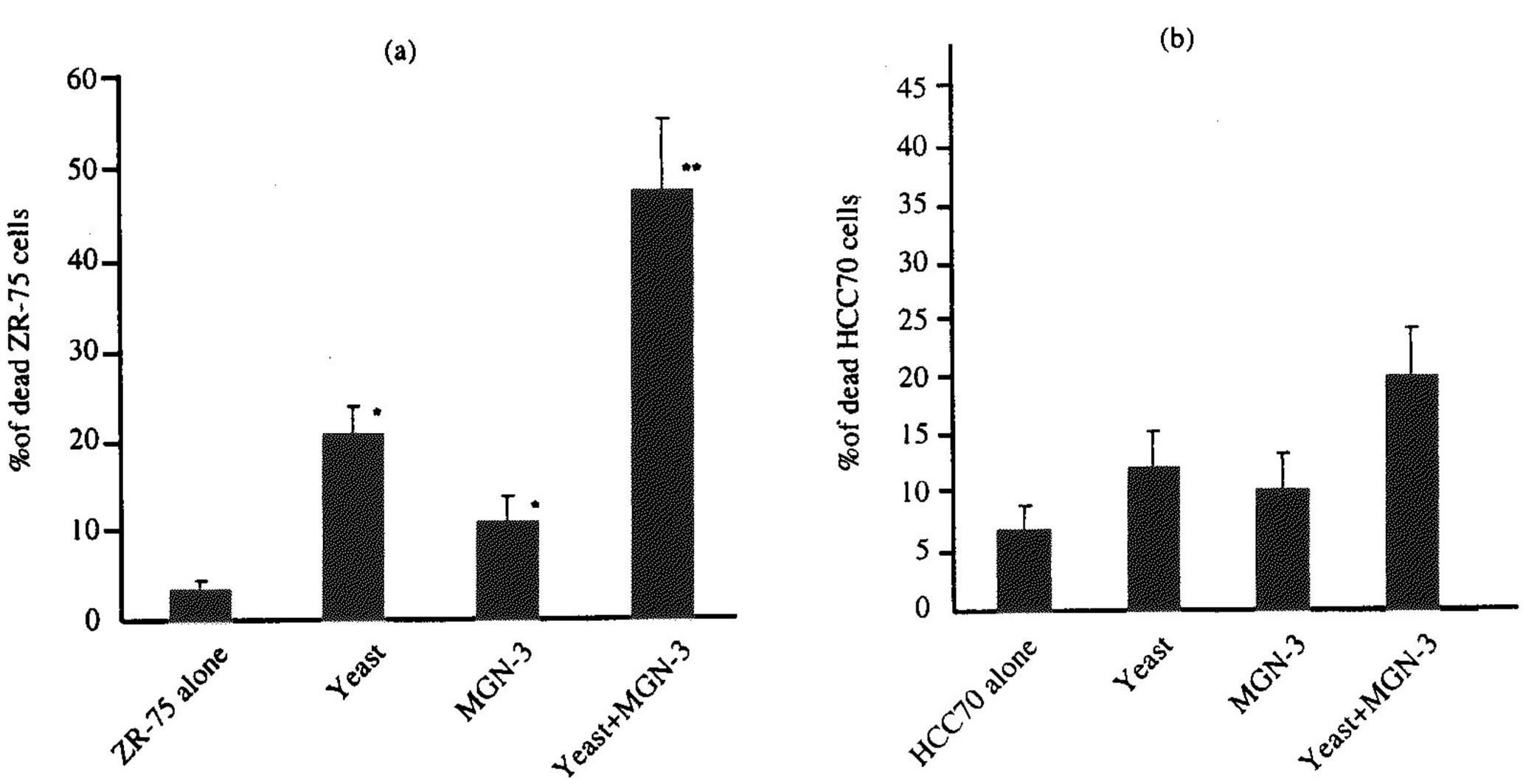

Fig. 8 Effect of MGN-3 on phagocytosis-induced apoptosis in ZR-75 and HCC70 cells as determined by flow cytometry.

Cancer cells were cultured with yeast at the ratio of 1:10 for 0.5 hr in the presence or absence of MGN-3 (500 μg/ml), and survival of ZR-75 cells (a) and HCC70 cells (b) was determined by flow cytometry using the propidium iodide (PI) technique. In this technique dead cells pick up PI and floresence.

*P < 0.05 as compared to control untreated cells. **P < 0.01 as compared to cells + yeast or cells + MGN-3.

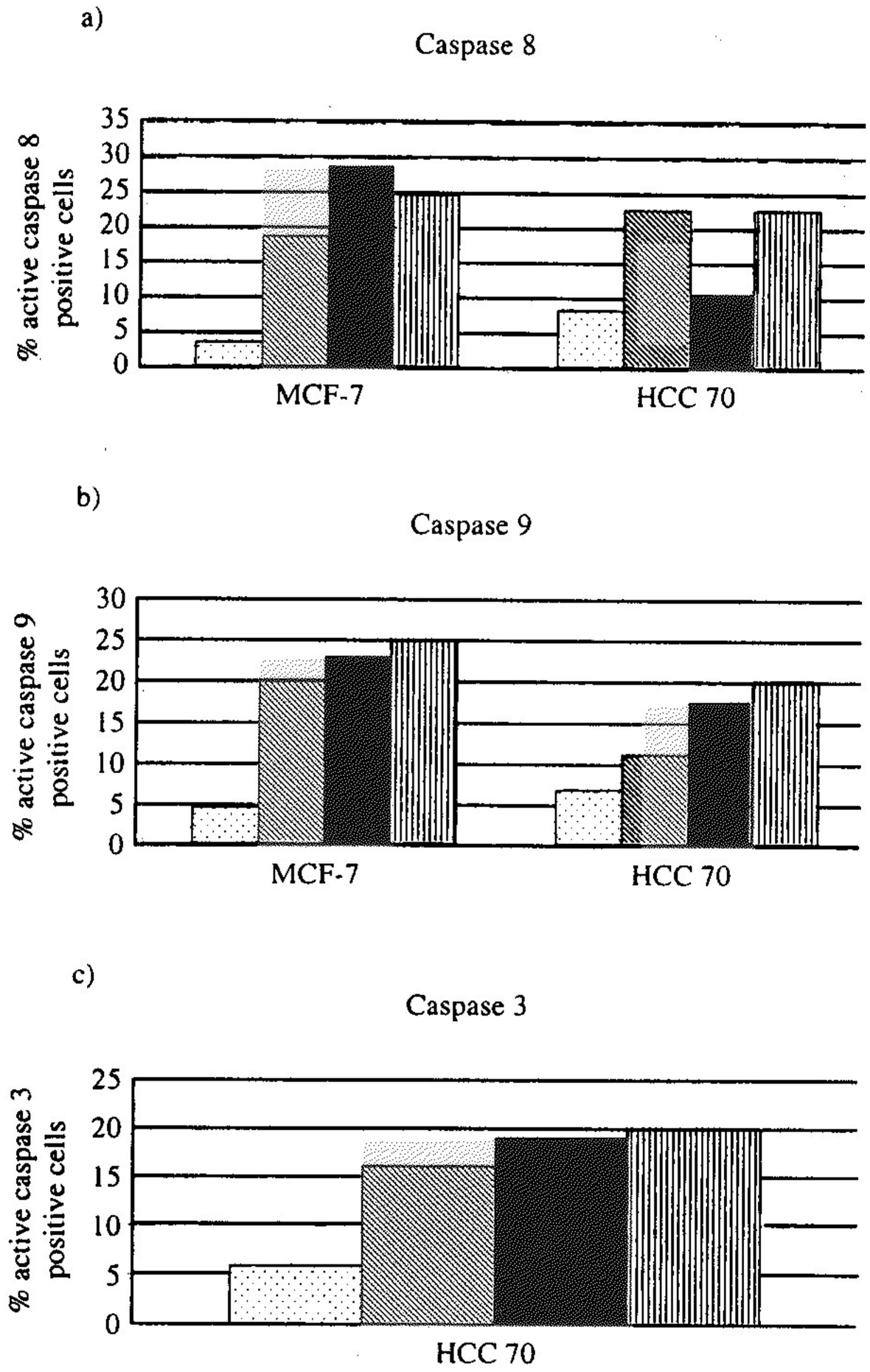

Fig. 9 Activation of caspases in MCF-7 and HCC70 cells post treatment with MGN-3.

Cancer cells were incubated with yeast in the ratio of 1:10 for 2 hr in the presence and absence of MGN-3 (500 μg/m*l*). Intracellular active caspase 8 (Fig. 9a), caspase 9 (Fig. 9b), and caspase 3 (Fig. 9c) were determined with casp glow caspases determination kit using FACScan.

Activation of caspases was examined in the following groups: cells alone (▨), cells + yeast (▩), cells + MGN-3 (■), and cells + yeast + MGN-3 (▥).

and caspase 9 in MCF-7 cells, suggesting that death of MCF-7 cells is due to apoptosis. A similar observation was made in HCC70 cells where an increase in caspase 8, 9, and 3 post culture of the cells with yeast was observed.

B. Effect of BioBran/MGN-3: In order to determine the steps in yeast-mediated apoptosis that are affected by BioBran/MGN-3, we examined the activation of caspase 8 and caspase 9. MCF-7 cells were co-cultured with yeast in the presence or absence of BioBran/MGN-3 for 2 hr. The proportion of cells with active caspases 8 and 9 was determined with a Caspatag caspase detection kit, using FACScan. The proportions of MCF-7 cells with active caspase 8 and caspase 9 were 3.5% and 4.9%, respectively. Phagocytosis of yeast

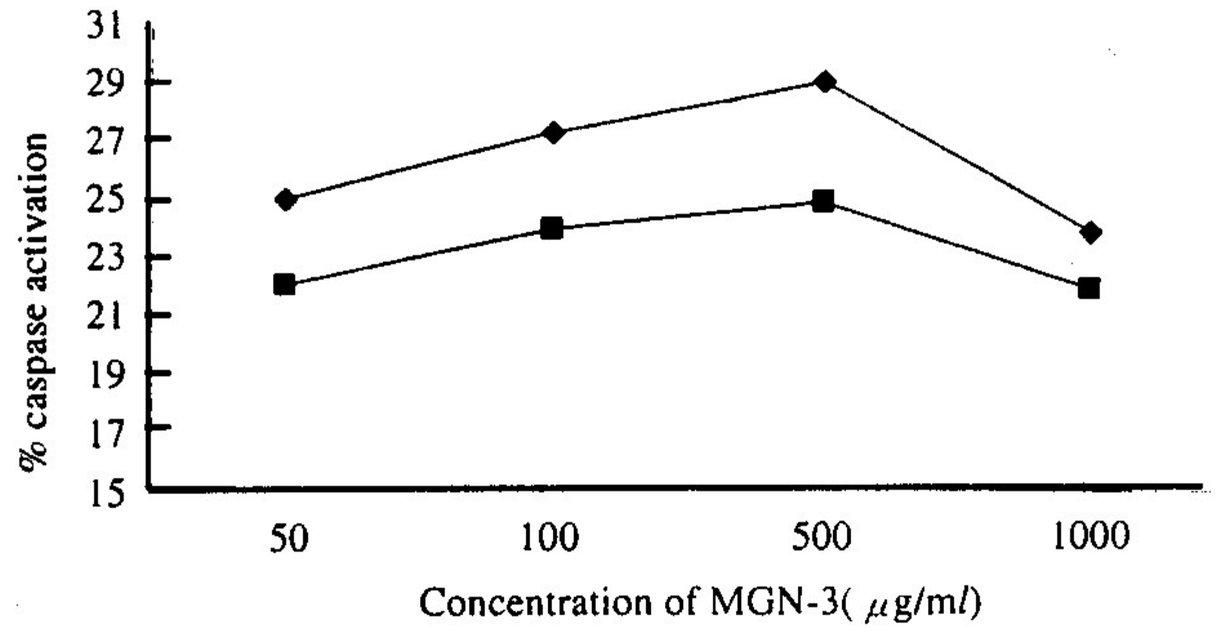

Fig. 10 Activation of caspases 8 and 9 of MCF-7 cells post treatment with MGN-3 at different concentrations.
MCF-7 cells were incubated with yeast in the ratio of 1:10 and treated with MGN-3 at different concentrations (50-1000 μg/m*l*). Intracellular active caspase 8 (◆) and caspase 9 (■) were determined with casp glow caspases 8&9 determination kit using FACScan.

by MCF-7 cells resulted in an increased proportion of cells exhibiting active caspase 8 (18.9%), and caspase 9 (20%). MGN-3 enhanced phagocytosis-induced activation of caspase 8&9. The action of BioBran /MGN-3 on induction and enhancement of apoptosis in HCC70 cells was also associated with activation of caspases (**Figure 9**). BioBran/MGN-3 has a negligible effect on the activation of caspase 8 in HCC70 cells, but it increased the proportion of cells with active caspase 9 and caspase 3.

C. Dose- dependant effect of BioBran/MGN-3: The effect of BioBran/MGN-3 on the level of caspases of MCF-7 cells was shown to be dose-dependent. The data demonstrates that the proportion of cells with increased active caspase 8 was higher in BioBran/MGN-3 (500 μg/m*l*) treated cells (29.2%), as compared to untreated control (18.9%). A similar trend of activation with caspase 9 was noted (**Figure10**).

Discussion

Results of this study reveal that modified arabinoxylan rice bran (BioBran/MGN-3) accelerates the level of phagocytosis of yeast by MCF-7 cells and significantly enhances apoptosis by MCF-7 cells. Several investigators reported phagocytosis by different types of cancer cells of nonphagocytic origin [10-19] including breast cancer cells (BCCs). These BCCs demonstrated their ability to phagocytize latex beads and fluorescent Matrigel [21,22]. We extended these studies and showed that MCF-7 cells are also able to phagocytize yeast [23], and that phagocytosis subsequently resulted in apoptosis in BCCs [24]. Several studies demonstrated that the expression of apoptosis in the human professional phagocytic cells can be modified in response to phagocytosis of microorganisms [6-9]. There is a great need to further study the factors that accelerate the expression on apoptosis in BCCs, post ingestion of microorganisms.

Recent studies demonstrate that several natural biological response modifiers (BRMs) induce apoptosis against a variety of cancer cell lines. These include: HL-60 cells by [6]-gingerol and [6]-paradol (ginger derivatives) [32], erythroleukemia, prostate, and BCC lines by *dl*-alpha-tocopherol (vitamin E) [33], histiocytic lymphoma U937, and prostate adenocarcinoma PC 3 lines by PC SPES (a Chinese herbal preparation) [34], cervical carcinoma cells by Coprinus disseminatus [35], and human endometrial adenocarcinoma cells by a lipoprotein fraction of rice bran [36]. Moreover, the growth of human BCCs (MCF-7 and MDA-231) has

been inhibited by ergosterol (extract of baker's yeast) [37].

BioBran/MGN-3 is a polysaccharide and its main chemical structure has been identified as an arabinoxylan with a xylose in its main chain and arabinose polymer in its side chain [25]. Earlier studies revealed that BioBran/MGN-3 enhances human NK cell activity *in vivo* [26], increases T and B cell proliferation [25], and increases TNF-α and IFN-γ production [27]. In addition, BioBran/MGN-3 is able to induce cancer cell apoptosis [28]. In this study, BioBran/MGN-3 demonstrated an additional anticancer characteristic that accelerates yeast phagocytosis-induced apoptosis in cancer cells. BioBran/MGN-3 increases the levels of attachment and phagocytosis as early as 0.5 hr post culture MCF-7 cells with yeast. The mechanism(s) by which BioBran/MGN-3 induces this effect is not fully understood, but it could be attributed to changes in cancer cell receptors that are involved in attachment/phagocytosis. Whether the receptors found in professional phagocytic cells such as mannose, Fc and C3 receptors [38] are the same ones that govern phagocytosis of yeast by cancer cells is under investigation.

BioBran/MGN-3 also accelerates the expression of apoptosis in BCCs post phagocytizing yeast. Giemsa-stained cytospin preparations show apoptotic BioBran/MGN-3-treated MCF-7 cells, post phagocytizing yeast. Cancer cells illustrate membrane blebbing and nuclear condensation. This is followed by nuclear fragmentation that subsequently disappears, and apoptic cells acquire the trypan blue stain. It was noted, in the three BCC lines used, that the percentage of apoptosis in cancer cells co-cultured with yeast in the presence of BioBran/MGN-3 was higher than that of either yeast or BioBran/MGN-3 treatment alone. This could be attributed to a synergistic apoptic effect of yeast alone in addition to the direct apoptic effect of BioBran/MGN-3. The mechanism by which BioBran/MGN-3 enhances apoptosis in BCCs after phagocytizing yeast may involve the FAS/FAS Ligand system. The apoptic effect of FasL post phagocytosis of microorganisms has been investigated recently. Baran et al [9] reported release of FasL from monocytes post phagocytosis of *Staphylococcus aureus*, and these FasL induce apoptosis of phagocytic monocytes and, to some extent, the bystander cells. We have recently investigated the effect of BioBran/MGN-3 on death receptor-induced apoptosis in the human leukemic HUT-78 cell line [28]. HUT-78 cells that were pre-treated with BioBran/MGN-3 and then with the agonistic antibody against death receptor (Fas, CD95) revealed enhanced anti-CD95 induced apoptosis.

Alternatively, BioBran/MGN-3 may exert its effect through activation of caspases. Data from the present study showed that BioBran/MGN-3 treatment of MCF-7 cells up regulates the activation of proximal caspases 8 and 9; this occurred in a dose-dependent manner that was maximized at 500 μg/m*l*. The mechanism by which BioBran/MGN-3 activates caspases may be due to its increased production of tumor necrosis factor a (TNF-α), and interferon-γ (IFN-γ) [27]. Both cytokines are known to induce tumor cell death, or modulate cell death via activation of caspases [39-41]. MCF-7 cells do not express caspase 3 [42]; this suggests that a caspase 3 independent pathway that causes the DNA fragmentation in MCF-7 cells may exist. In addition, studying the mitochondria membrane potential and Bcl level may be helpful in investigating the mechanism by which BioBran/MGN-3 causes apoptosis of MCF-7 cells.

Data in the present study reveal a differential response among BCC lines towards the augmentory effect of BioBran/MGN-3 on enhancing apoptosis in cancer cells, post culture with yeast. Metastatic BCCs, such as MCF-7 and ZR-75, are more responsive than HCC70. The reason for this phenomenon is not known, but it could be attributed to the difference in the mechanisms of apoptosis in these cell lines. BioBran /MGN-3 increased activation of caspases 8 and 9 in MCF-7 cells, a cell line that lacks caspase 3. On the other hand, HCC70 cells treated with BioBran/MGN-3 demonstrated an increase in caspases 3 and 9 but

not caspase 8. This suggests that treatment with BioBran/MGN-3 caused apoptosis in MCF-7 cells through both intrinsic and extrinsic pathways. On the other hand, apoptosis in HCC70 cells may occur only through mitochondrial activation. A similar apoptic pathway was noted in mouse macrophages upon ingestion and digestion of *E. coli*, activating caspases 3 and 9, but not caspase 8 [43].

The National Cancer Institute has frequently highlighted the critical need for cancer treatments with greater specificity for cancer cells and less toxicity for normal tissue; it strongly encourages the exploration of such therapies. In the present study, we introduced a novel approach to BCC therapy using *S. cerevisiae*, a heat-killed non-pathogenic yeast, and BioBran/MGN-3, a safe product made from arabinoxylan derived from rice bran. We believe that the results of the present study may have implications for the treatment of breast cancer.

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