Int J Pharm Bio Sci 2013 July; 4(3): (B) 1325 - 1332

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ANTI TUMOUR EFFECT OF AQUEOUS EXTRACT FROM *HELIX ASPERSA*

IBTISSEM EL OUAR[1*], CORNELIA BRAICU[2], DALILA NAIMI[1], ALEXENDRU IRIMIE[3,4] AND IOANA BERINDAN-NEAGOE[2,5]

[(1)]Laboratory of genie microbiology and application, University Constantine 1- road Ain el bey –Constantine-25000- Algeria-
[(2)]Department of Functional Genomics and Experimental Pathology, Cancer Institute "Ion Chiricuta", Cluj-Napoca, Romania
[(3)]Department of Surgery, University of Medicine and Pharmacy, "I. Hatieganu", Cluj-Napoca, Romania,
[(4)]Department of Surgical Oncology, University of Medicine and Pharmacy, "I. Hatieganu", Cluj-Napoca, Romania
[(5)]Department of Immunology, University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania

ABSTRACT

The garden snail *Helix aspersa* is a big land snail found widely in the Mediterranean countries; it has a high economic and nutritional value. The aim of this study is to investigate the anticancer activity of *Helix aspersa* extract against breast cancer cells Hs578T. Our assays showed that the extract had a high cytotoxicity against tumour cells; it acts by inducing necrosis and downregulating BcL2 expression. These results are novel and demonstrate that *H. aspersa* extract may serve as a source of a new anticancer drug or as an associated treatment in chemotherapy that inhibits BcL2 expression.

KEY WORDS: ***Helix aspersa*, bioactive molecule, apoptosis, BcL2, tumour cells, Hs578T cell line.**

IBTISSEM EL OUAR
Laboratory of genie microbiology and application, University Constantine 1- road Ain el bey –*Constantine-25000- Algeria-*

Corresponding author

INTRODUCTION

Breast cancer is one of the most and common malignancy and leading causes of related women's death worldwide; it is recognized to be a molecularly heterogeneous disease. Epidemiological studies have reported that only 10 to 15% of all breast cancer cases are caused by genetic predisposition whereas the remaining 80-90% cases are attributed to environmental, reproduction, lifestyle, stress and diet [1] - [2]- [3]- [4] The treatment usually consists of various combinations of surgery, radiation therapy and chemotherapy but despite these therapeutic options, cancer remains associated with high mortality. The synthetic compounds can prevent, suppress or reverse the progression of cancer [5] but these drugs are very expensive. For these reasons, it is necessary to explore new natural drugs that are inexpensive. Therefore, different research data have shown that natural foods may have the ability to serve as modulators of tumorigenecity [6]- [7]. Snails are traditional and luxury food with a high economic and nutritional value. They are mainly consumed by western European countries; France, Italy, Germany, Spain and the UK[8]. One of the most consumed species is *Helix aspersa,* a big Mediterranean land snail with a shell diameter of approximately 45mm.The nutritional value of *H. aspersa* has been largely reported but few studies have focused on the therapeutic aspects of these nutrients. Snails have been used in medicine since the antiquity and prepared by several methods, they are recommended for stomach pain, vertigo, nephritis, respiratory and cardiovascular diseases[9]. Nowadays, lectins from snails are used as a marker for metastasis tissues in breast and colon cancers [10]- [11]. In this context, the aim of the present study is to evaluate the anti tumour effect of the extract from *H. aspersa* on Hs578T cell line and to determine its mode of action by studying the apoptotic process, which is the first mechanism against tumour cells.

MATERIALS AND METHODS

Cell culture

Human breast cancer Hs578T cell line was obtained from the laboratory of functional genomic and experimental pathology of oncologic institute "Ion Chirucita" (Cluj Napoca-Roumania). The cell line derived from a human breast carcinoma (a 74 years Caucasian woman). The cells were cultured at 37°C and humidified atmosphere of 5% CO_2 , in Dulbecco's Modified Eagle's Medium high glucose supplemented with 1% non essential amino acids, 1% L- glutamine (200mM), 1% gentamicin (10 mg/ml), 1% insulin and 10% fetal bovine serum. All reagents were purchased from Sigma Aldrich, Germany.

Aqueous extract preparation

H. aspersa were dissected and homogenized. 3 volumes of water per volume of wet tissues were then added to the homogenate. After 24 hours the crude extract was filtered and centrifuged for 10 mn at 5000g.

The supernatants were collected and used to treat cells. (Supernatant pH was evaluate to 6.8).

Cytotoxicity assay

Viability of breast cancer cells was measured using the colorimetric MTT assay; briefly cells were seeded into 96 well plate for 24 hours in the presence of varying dilution of snail extract 10%, 5%, 2,5%, 1%, 0,5% , 0,1% and 0,01%. Cell viability was then evaluated on the basis of ability of their mitochondria to reduce terazolium salt 3-(4,5-dimethylthiazoly-2)-2,5-diphenyltetrazolium bromide (MTT) into Formosan crystal. Formosan formation is proportional to the number of functional cell mitochondria [12] and cell viability was determined by comparison with untreated cells.

Apoptosis assay

Calcein AM/PI staining

After 24 hours of treatment, cells were stained for 20 min with Calcein AM (Invitrogen, code C1430) and Propidium iodide (PI) (Calbiochem, JA1654) and analysed under objective X10 of the fluorescence inverted microscope *Leica*.

Annexin staining

Following treatment of 24 hours with *H. aspersa* extract, cells were trypsinized (with trypsin 0,25% for 5 min) and the suspension was transferred from 6 wells plates to eppendorf tubes, centrifuged at 3000g for 5 min, then washed with 200 µl phosphate buffer saline and centrifuged for another 5 min. After that, cells were resuspended in 200 µl of Annexin binding buffer with 2 µl of Annexin V-Cy5. The samples were incubated for 10 min away from light and the fluorescence was detected by bio analyzer Agilent 2100. Cell apoptosis kit was obtained from Agilent Cell Reagents, code 5067-1519.

RT- PCR for BcL2 gene

In order to study Bcl2 expression cells were treated with the extract at different times 4, 8 and 24 hours. Total cellular RNA was isolated using TRiagen (Sigma, Germany) and converted to cDNA with The Random Hexamer Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnosis, Germany) following manufacturer's instruction.

Quantitative RT-PCR was performed using TaqMan Master Kit and light cycler. Amplification was performed with 40 cycles with the following conditions: The cycles were initiated by denaturing the DNA at 95 °C for 10s, followed by annealing reaction at 55 °C for 20s and extending at 72 °C for 1s. After the last cycle a final extending at 72°C for 45s. All samples were run in triplicates and the β actin was amplified as an internal control. Primers used are: BcL2: Right 5'ATCTTTATTTCATGAGGCACGTT (TibMolBiol, code 934115), Left: 5'TTGACAGAGGATCATGCTGTACTT (TibMolBiol code 934116) B-actin: sense 5'AGGAATGGAAGCTTGCGGTA (TibMolBiol, code 881493), anti sense: 5'AATTTTCATGGTGGATGGTGC (TibMolBiol, code 881492)

Quantification of relative gene expression was done using the competitive threshold cycle C_T method. C_T values were averaged for triplicate wells and subtracted from corresponding C_T of internal reference RNA to obtain ΔC_T values. The averaged control ΔCT was subtracted from the experimental ΔC_T to yield $\Delta\Delta C_T$. The fold change was calculated as $2^{-\Delta\Delta CT}$ for experimental Vs control [13].

Statistics

Data were expressed as mean ±SD from at least three separate experiments performed on triplicate samples. The differences between experimental conditions and controls were analysed using test t ($p<0.05$ is considered statistically significant). Statistical analyses were carried out using Graph Pad Prism software (free trial).

RESULTS

Effect of H. aspersa extract on Hs578T growth

Incubation of cells with *H. aspersa* extract for 24 hours induced an interesting reaction in Hs578T breast cancer cells. The extract showed a particularly high toxicity at 10 and 5%. The IC_{50} value in Hs578T cells was evaluated at around 0. 9118% (Fig.1) For the next experiments and to study the mode of action of the extract we have chosen the dilution (1v:100w) 1% (≈IC_{50}).

Evaluation of mode of action of H. aspersa extract

To show whether the growth inhibition induced by *H. aspersa* extract was caused by induction of apoptosis, cells were stained first with PI and Annexin, then we have studied the expression of the principle regulator of mitochondrial pathway in apoptosis, BCL2. PI has been accepted and widely used as the appropriate method to measure apoptotic cells.

The PI penetrates into the damaged cell, intercalates with DNA double helix to emit a red fluorescence (excitation 535 nm and emission 617 nm). At the same time Calcein AM stains only the viable cells and labels the cell body by green fluorescence. When viewed using a fluorescence microscope with 645 nm filter, cells that have lost membrane integrity showed contrasting red PI staining from their nuclei (Fig.2). Moreover, the number of cells in treated wells was greatly decreased. Thus, cells with PI were damaged by necrosis or underwent the apoptotic program. In order to determine if *H. aspersa* extract induces necrosis or apoptosis in breast cancer cell line Hs578T, cells were first stained by annexin V, then the expression of the RNAm (Massenger RNA) for BcL2 was evaluated by RT-PCR. The result of Annexin staining shows that the fraction of apoptotic cells in non treated wells is similar to those in treated wells. Consequently the extract does not kill cells by an apoptotic mechanism.

Effect on BcL 2 expression

To determine the effect of *H. aspersa* extract on BcL2 expression, cells were treated with the IC_{50} dose. As shown in Fig.4 treatment with the extract markedly suppressed BcL2 expression. After 8 hours of treatment the BcL2 expression was significantly decreased ($p = 0.001$); the suppression being about 67%. Similarly, after 24 hours BcL2 expression was significantly inhibited ($p<0.01$) with about 40% of control.

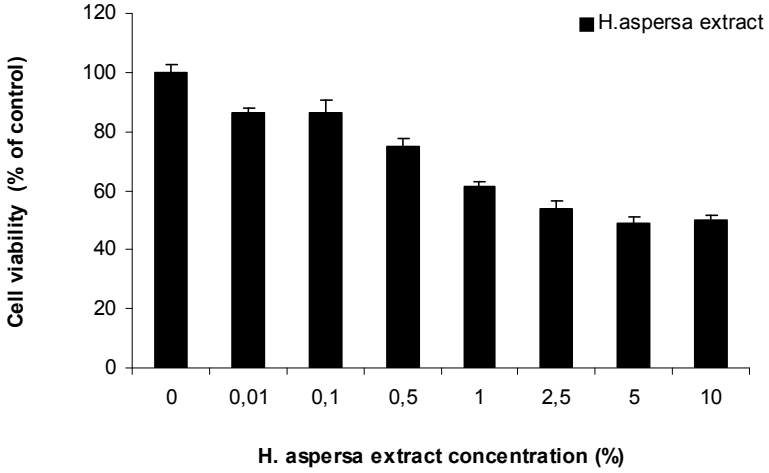

Figure 1
Effect of H. aspersa extract at 1% on cell viability of Hs578T cells after 24 hours of treatment

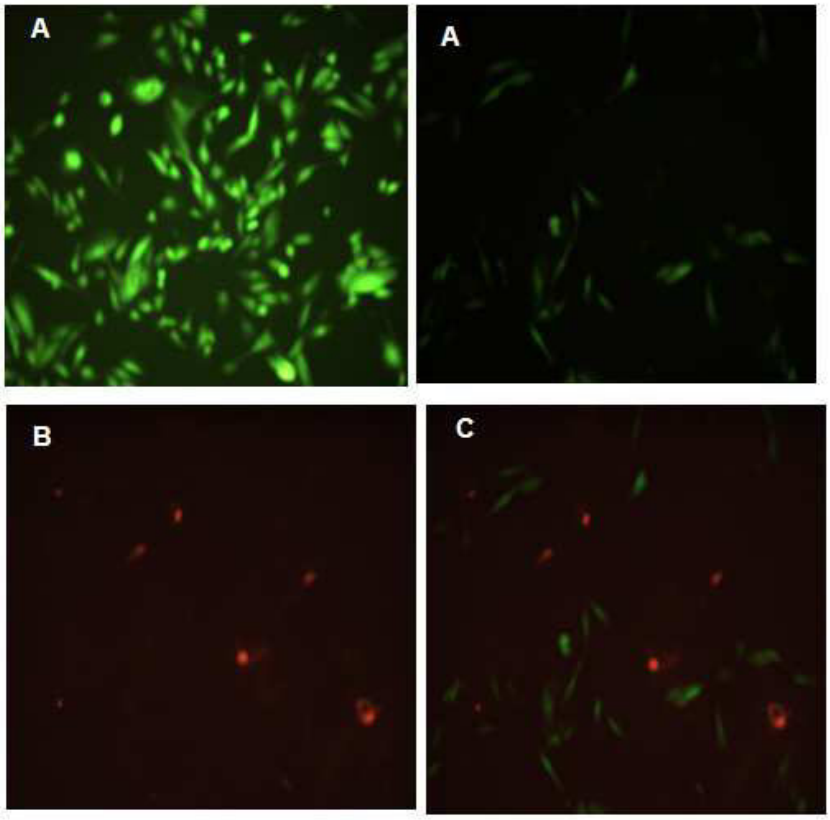

Figure 2

Microscope fluorescence image of Hs578T cells treated with H. aspersa extract (at 1%) and stained by Calcein AM/ PI: (A) Calcein labeled live cells in control cells (left) and treated cells (right) ; (B) PI stained red nuclei with emission at 645 nm in Hs578Tcells treated by H. aspersa; (C) composite overlay fluorescence for A right and B.

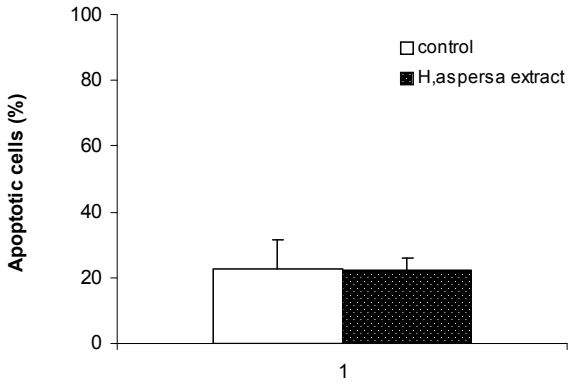

Figure 3

Analysis of apoptosis by Annexin V, the results are expressed as percentage of apoptotic cells.

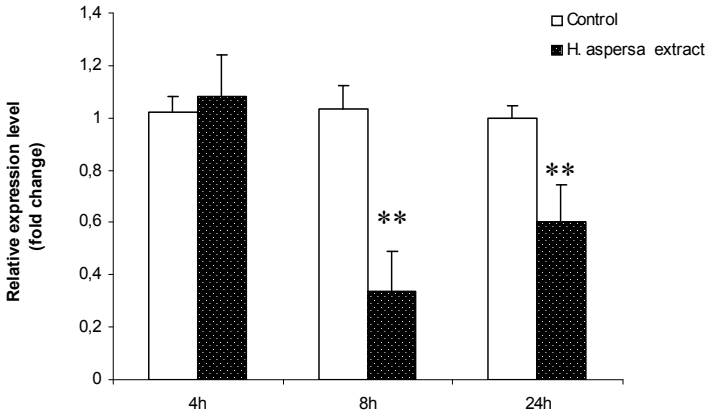

Figure 4

Effect of H. aspersa extract on expression of BcL2 RNA$_m$: Treatment of breast cancer cells by the aqueous extract at 1% decreases significantly the expression of BcL2 RNA$_m$ after 8 (p = 0.001) and 24 (p<0.01) hours of treatment compared to controls. (** significant p<0.01)

DISCUSSION

The initial screening for determination of anticancer activity was to employ the MTT assay; the results showed that *H. aspersa* extract has a great cytotoxic effect against breast cancer cells. The extract both induces necrosis and significantly decreases BcL2 expression and at very low dose. BcL2 is one of the main cell cycle regulators that promote proliferation by preventing programmed cell death. The overexpression of BcL2 was associated with diminution of apoptosis in more aggressive malignant phenotype and tumour resistance to cytotoxic drugs. This overexpression was observed in several cancers including hematologic malignancies as well as a range of solid tumours; nasopharyngeal, colorectal, prostate and breast cancer where the overexpression of BcL2 occurs in 40-80% of breast cancer tissues [14]- [15];[16]. Thereby, BcL2 is an attractive new anti cancer target. Actually the bioactive compounds mostly compromise the phenols, peptides and polysaccharides. *H. aspersa* contains a high level of protein (60-70%). It is very rich in essential amino acids lysine, leucine, valine and non essential amino acids, glutamic acid, alanine and aspartic acid [17]- [8], the extract also contains polyphenols (data not shown).

Thus, our results showed that the extract induces necrosis which is associated with BcL2 downregulation. In this case it seems that the apoptotic process has been prevented and the anti tumour effect of *H. aspersa* extract may be resulted from the action of the polyphenols that have anti inflammatory, antioxidant and anti carcinogenic activities, or from the action of peptides, lectins or polysaccharides that bind to some surface molecules. In this context, the tannins which are a family of polyphenols soluble in water have been shown to induce apoptosis in breast cancer cells MCF-7 and Hs578T[18]-[19] and myeloid leukemia cells HLC 60 and K562 [20]. They also modulate the expression of apoptosis regulatory proteins; they downregulate BcL2 and BcLxL expression and enhance the expression of pro apoptotic factors Bax and Bak [21]- [22]. In addition, a lectin isolated from *Helix pomatia* immune system has been shown to bind to complex glycans on the surface of cancer cells, like ganglioside GD2, a well known tumour associated carbohydrate antigen [23]- [11]. A lectin

from *Musca domestica* has been reported to induce apoptosis in MCF-7 cells [24]. Moreover different lectins isolated from plants have been shown an anti-tumour activity against HeLa cells, HT29 and MCF-7 cells [25]- [26]. Otherwise, *H. aspersa* extract may act by modulating reactive oxygen species (ROS). Therefore a high level of ROS can lead to lipid peroxydation, damage to cellular membranes, inactivation of caspase enzymes and necrotic cell death [27]. In contrast, a low level of ROS leads to apoptotic cell death. Also, it has been known that BcL2 overexpression can protect cells from ROS mediated apoptosis. However the mechanism by which BcL2 prevented ROS induced apoptosis is unknown[28]. One of the recent hypotheses is that ROS act by down-regulating BcL2 levels because BcL2 is critical for the anti apoptotic activity. Decreasing BcL2 could be a mechanism of sensitizing the cells to apoptosis [28]- [29] Taken together, these results are novel and promising. They call for further investigations especially with the triple negative cell line Hs578T. This phenotype is the most aggressive and malignant subtype of breast cancer and its treatment is limited.

CONCLUSION

Our results demonstrate that *H. aspersa* extract has a high cytotoxicity against breast cancer cells. The extract can be used as an associated treatment, in chemotherapy that enhances tumour sensitivity by downregulating BcL2; since that tumour resistance to agents like doxorubucine and Texans was correlated with an overexpression of BcL2. Although, extended *in vivo* studies are needed to be performed, the present study opens new perspectives in the search for a new natural anticancer drug.

REFERENCES

1. Colditz G.A. & Frazier AL: Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus. Cancer Epidemiology, Biomarkers &Prevention: 4 (5): 567-571. ISSN 1055-996,(1995).
2. Madigan M., Ziegler R., Benichou J., Byrne C. & Hoover R: Proportion of breast cancer cases in the United States explained by well established risk factors. J. Natl. Cancer Inst, 87: 1681-1685, (1995)
3. Antonova L., Aronson K. & Muller C R: Stress and breast cancer from molecular biology to molecular biology. Breast cancer research, 13: 208, (2011)
4. Place AE., Huh SJ & Polyak K.: The microenvironment in breast cancer progression biology and implication for treatment. Breast cancer research, (13): 227, (2011)
5. Amin A & Moussa M: Merits of anti cancer plants from the Arabian Gulf region. Cancer therapy. (5): 55-66, (2007)
6. Hemaiswarya S & Doble M: Potential synergism of natural products in treatement of cancer. Phytother. Res, 20 (4): 239-49, (2006)
7. Kannan A., Hettiarachchy N. & Narayan S: Colon and breast anti cancer effects of peptide hydrolysates derived from Rice Bran. The open bioactive compound Journal, (2): 17-20, (2009)
8. Cagiltay F., Erkan N., Tosun D. & Selcuk A: Amino acid, Fatty acid, vitamin and mineral contents of Edible garden snail (Helix aspersa). Journal of Fisheries Sciences, 5(4), 354-363, (2011)
9. Bonnemain B: Helix and drugs: Snails for western health care from antiquity to the present, eCAM, 2(1) 25-28, (2005)
10. Walker RA: Helix pomatia and prognosis of breast cancer. Br. J. Cancer, (68): 453-454, (1993)
11. Lescar J., Sanchez JF., Audfay A., Coll JL., Breton C., Mitchell EP. & Imberty A.: Structual basis for recognition of breast and colon cancer epitopes Tn antigen and forssman disaccharide by helix pomatia lectin. Glycobiology, 17(10): 1077-1083, (2007)

12. Mosman T: Rapid colorimetric assay for cellular growth and survival:Application for proliferation and cytotoxicity assays. J. Immunol. Methods, (65):55-63, (1983)
13. Livak K J & Schmittgen TD: Analysis of relative gene expression data using real time PCR and the $2^{-\Delta\Delta}C_T$ Method. Methods (25): 402-408, (2001)
14. Krajewski S., Krajwska M., Shabaik A., Wang HG., Irie S., Fong L. & Reed JC: Immunohistochemical analysis of in vivo patterns of BCLX expression. Cancer Res, (54): 5501-5507, (1994)
15. Olopade OI., Adeyanju MO., Safa AR., Hogos F., Mivk R., Thompson CB. & Recant WM: Overexpression of BCLX protein in primary breast cancer is associated with high tumor grade and nodal metastases. Cancer J. Sc. Am. (3): 230-237, (1997)
16. Schlotter C., Vogt U., Allgayer H. & Brandt B: Molecular targeted therapies for breast cancer treatment. Breast cancer research, 10:211, (2008)
17. Ozogul y., Ozygul F. & Olgunoglu A: Fatty acid profile and mineral content of wild snail Helix pomatia from the region of south of the turkey. Eur. Food. Res.Technol. (221): 547-549, (2004).
18. Bawadi HA., Bonsode RR., Trappey A., Truax RE & Losso JN: Inhibition of CaCo-2 colon, MCF-7 and Hs578T breast and Du145 prostatic cancer cell proliferation by water-soluble black bean condensed tannins. Cancer Lett, (218): 153-162, (2005)
19. Soyocak A., Cosan D.T., Basaran A., Veysi Gunes H. & Degirmenci I: Evaluation of Bax protein in breast cancer cells treated with tannic acid. Dicle Medical Journal, 38(1): 1-6, (2011)
20. Liu M.J., Wang Z., Lie HX., Cong Wu R., Liu Y Z & Wu Q Y: Mitochondrial dysfunction as an early event in the process of apoptosis induced by woodfordin I in human leukemia K569 cells. Toxicology and Applied pharmacology, (194): 141-155, (2004)
21. Selvendiran K., Koga H., Ueno T. et al.: Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hematoma cells: an implication for the anti tumor potential of flavonoids. Cancer Res, 66: 4826-4834, (2006)
22. Lee HJ., Wang CJ., Kuo HC., Chou FP., Jean LF, Tseng TH: Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/ Bak and activation of JNK. Toxicol. Appl. Pharmacol, 203:124-131, (2005)
23. Sanchez JF., Lescar J., Chazalet V., Audfray A., Gagnon J., Alvarez R, Breton C., Imberty A. & Mitchell EP: Biochemical and structural analysis of helix pomatia agglutinin a hexameric lectin with a novel fold. The journal of Biological Chemistry, 281(29): 20171-20180, (2006)
24. Cao X., Huob Z., , Luc M., Maod D., Zhaoe Q., Xuf ., Wangg C & Zengh B: Purification of lectin from larvae of the fly, Musca domestica, and in vitro anti-tumor activity in MCF-7 cells . Journal of Insect Science: Vol. 10 | Article 164, (2009)
25. Zhao C, Sun H, Tong X., & Qi Y: An antitumour lectin from the edible mushroom Agrocybe aegerita. Biochem. J, (374): 321–327, (2003).
26. Yu, L. G., Fernig, D. J., Smith, J. A., Milton, J. D. & Rhodes, J. M: Reversible inhibition of proliferation of epithelial cell lines by Agaricus bisporus (edible mushroom) lectin. Cancer Res. (53): 4627–4632, (1993)
27. Kannan, K. & Jain, SK: Oxidative stress and apoptosis. Pathophysiology 7, 153–163, (2000).
28. Hockenbery, DM., Oltvai, ZN., Yin, XM., Milliman, CL. & Korsmeyer, SJ: BCL2 functions in an antioxidant pathway to prevent apoptosis. Cell, (75): 241–251, (1993)
29. Hildeman DA., Mitchell T., Aronow B., Wojciechowski S., Kappler J: Control of BCL2 expression by reactive oxygen species. PNAS. 100 (25): 15035-1504, (2003).