

Anti-proliferation effect of *Hevea brasiliensis* latex B-serum on human breast epithelial cells

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Abstract: The rubber tree (*Hevea brasiliensis*) extracts are becoming increasingly visible in pharmaceutical and therapeutical research. The present study is aimed at examining the specific anti-proliferation property of *H. brasiliensis* latex B-serum sub-fractions against human breast cancer epithelial cell lines MCF-7 and MDA-MB231. The results showed that the latex whole B-serum and DBP sub-fraction exerted a specific anti-proliferation activity against cancer-origin cells MDA-MB231 but had little effect on non-cancer-origin cells. On the other hand, the anti-proliferative activity was diminished in the pre-heated B-serum fractions. With the low toxicity that the B-serum demonstrated previously in Brine Shrimp Lethality Test (BSLT), the present results suggest the potential use of the B-serum sub-fractions in cancer treatment.

Keywords: *Hevea brasiliensis*; B-serum; anti-cancer; breast cancer, epithelial cells.

INTRODUCTION

Hevea brasiliensis (natural rubber) latex contains mainly rubber particles, proteins, lipids, sugars and their derivatives such as quebrachitol, ribonucleic acids and organic salts (Chow and Draper, 1970; Bealing, 1981; Burton *et al.*, 1985; Wajant and Foster, 1996; Das *et al.*, 2002). The three main latex fractions separated by high speed centrifugation are rubber cream, centrifuged serum (C-serum) and bottom fraction that mainly consists of a vacuole-like organelle known as lutoid body. The fluid released from ruptured lutoid bodies is known as B-serum.

Latex B- and C-sera have been acknowledged as a rich source of proteins, nucleic acids, and a multitude of organic compounds (Archer *et al.*, 1969). B-serum especially contains an array hydrolases and pathogen related proteins; hevein for example have been shown to have anti-fungal activities, but the therapeutic application of the property may be limited due to the allergic effect on human being (Singh, 1980; Jan Parijs *et al.*, 1991; Kanokwiroon *et al.*, 2008). Similarly latex C-serum contains variants of hydrolases that confer anti-fungal properties (Archer *et al.*, 1969; Yeang *et al.*, 2002). In a collaborative effort, we have also detected anti-*Aspergillus niger* activity of C-serum origin, although the effective compound(s) remained unidentified (Daruliza *et al.*, 2011a). Elsewhere it has been reported that *H. brasiliensis* leaf hydroxynitrile lyase could be employed to synthesize active cyanohydrins for use as pharmaceuticals (Effenberger, 1999). Other research on

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the therapeutic potential of the plant however remained scarcely explored, and such efforts could lead to employment of its extracts as a relatively low cost resource for various anti-microbial and anti-cancer activities due to the simplicity of preparation and the abundance of the feedstocks in rubber producing regions. In the present study we examine the anti-proliferative activity on human breast cancer epithelial cells of latex B-serum, its dialyzed and pre-heated sub-fractions.

MATERIALS AND METHODS

Preparation of latex B-serum

Latex was collected from field-grown RRIM 600 trees at the Rubber Research Institute of Malaysia Research Station, Sungai Buloh. To prepare latex B-serum, fresh latex collected in chilled flasks was fractionated by centrifugation at 44,000 x g at 4°C. The latex separates into three distinct parts upon high-speed centrifugation (Moir, 1959). Latex B-serum was prepared from the bottom fraction of the centrifuged latex based on a method previously described (Hsia, 1958). Briefly, after removal of the rubber cream and C-serum, the sediment at the bottom of the centrifuge tube was collected and re-suspended in 0.4 M mannitol to aid the removal of remnant C-serum while retaining the lutoids intact. The cleansed bottom fraction was recovered after another centrifugation and subjected to alternate freezing and thawing (four times) to rupture the lutoids. The fluid from the lutoids, the B-serum was recovered by centrifugation and lyophilized for subsequent use. Lyophilized powder of latex B-serum was reconstituted with 1x phosphate buffered saline (PBS). Serial dilutions of the serum were

performed to prepare working concentrations ranging from 2-2000 µg/ml. Sub-fractions of latex B-serum were prepared by dialysis using SnakeSkin™ (Pierce, IL, USA) tubing with molecular weight cut-off 3000 Da, against distilled water for 48 hours at about 5°C. A whitish precipitate was recovered by centrifugation at 20,000 x g for 30 minutes; the precipitate (DBP) and the supernatant (DBS) were then lyophilized and kept desiccated until further use. Similar centrifugation and lyophilization procedures were employed to obtain the boiled B-serum fractions. WB were placed in a boiling water bath for 10 minutes, followed by centrifugation to yield pellet (BBP) and supernatant (BBS) sub-fractions, which were then lyophilized until completion. Reconstitution of the lyophilized BBP and BBS sub-fractions was conducted as described above for WB.

Cell culture

Hs27 (ATCC CRL-1634), MCF-7 (ATCC HTB-22) and MDA-MB231 (ATCC HTB-26) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with foetal calf serum (10 % v/v), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (0.025 µg/ml) except Hs27 was supplemented with additional 4.5 g/l of glucose. Cultures were maintained at 37 °C in a water-saturated atmosphere containing 5% CO₂. Cell counts were performed using a Neubauer haemocytometer under light microscope and living cells were identified by Trypan blue staining method. Approximately 2,500 cells were seeded in each well of a 96-well culture plate and were starved in DMEM under cell culture incubation conditions for 48 hours prior to cell-based assay.

Measurement of cell growth inhibition

The cytotoxic effect was measured using standard 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay after the cells have been treated with latex B-serum or its subfractions for various time periods (24, 48 and 72 hours); the assay was developed based on the method described by Mossman (1983). The absorbance at 570 nm was read on a spectrophotometric plate reader (Multiskan Spectrum, Thermo Electron Co., Waltham, Massachusetts, USA). The percentage of surviving cells was calculated as (OD of drug-treated sample – OD of blank)/(OD of control – OD of blank) × 100%. Dose-response curves were constructed using Probit analysis (Finney, 1962) on a Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada) to obtain the LC₅₀ and LC₈₀ values. All experimental data were derived from at least 3 independent experiments.

DNA fragmentation assay

Approximately 1 × 10⁴ cells/well in 24-well plates with concentrations 0, LC₅₀ and LC₈₀ of WB, DBP and DBS were incubated for different periods of time. Trypsinized cells were rinsed with ice-cold PBS and lysed in digestion

buffer containing: 100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, 0.5 % sodium dodecyl sulfate (SDS), and proteinase K (0.1 mg/ml) was then added and further incubated at 50°C overnight. DNA was extracted with phenol/chloroform/isoamyl alcohol (1:1:1) into the aqueous phase and precipitated with 3 M sodium acetate (pH 6.5) and 100 % ethanol. The DNA sample was dissolved in PCR grade water (Promega, WI). After mixing with 6X loading buffer (0.025% bromophenol blue, 0.025% xylene cyanole, 30% glycerol), DNA was separated on a 1.5% agarose gel electrophoresis and visualized under UV by ethidium bromide staining (10 µg/ml). The intensity of the bands was recorded with a FluorChem® FC2 Imaging System (Alpha Innotech Corporation, CA).

STATISTICAL ANALYSIS

Data are presented as mean ± S.E.M. of triplicate determinations, except when results of plots are shown, in which case a representative experiment is depicted in each figure. Comparisons between multiple groups were performed with one-way ANOVA with Tukey and Duncan corrections. Statistical significance was indicated when p < 0.05.

RESULTS

Anti-proliferation property of whole B-serum and dialyzed B-serum precipitate (DBP) sub-fraction specific to cancer-origin cells

Cell-based MTT assay with various concentrations (from 0 to 10 µg/ml) of whole B-serum (WB) or DBP sub-fraction showed that the proliferation of Hs27 (non-cancer-origin) cells was not affected, whereas that of MDA-MB231 (cancer-origin cells) was inhibited, although at different levels when treated with WB and with DBP, within the test concentration range (0 to 10 µg/ml) as shown in fig. 1. The growth of MCF-7 was not affected with WB within the tested concentration range.

Nevertheless, the anti-proliferation activity observed at 72-hours post-treatment with WB was not shown at time points 24- and 48- hours post-treatment. At these latter time points, no anti-proliferation activity was demonstrated by the treatment with WB within the concentration range examined (fig. 2).

With DBP the growth of MCF-7 was not affected at concentrations below 2.5 µg/ml at 72-hours post-treatment, but it was evident at higher concentrations (>2.5 µg/ml). This could be observed at earlier time points at 24- and 48- hours post-treatment with the sub-fraction as shown in fig. 2. The inhibition of the cell growth progressively increased with the time period of incubation at each tested concentration of the sub-fraction (figs. 1 and 2).

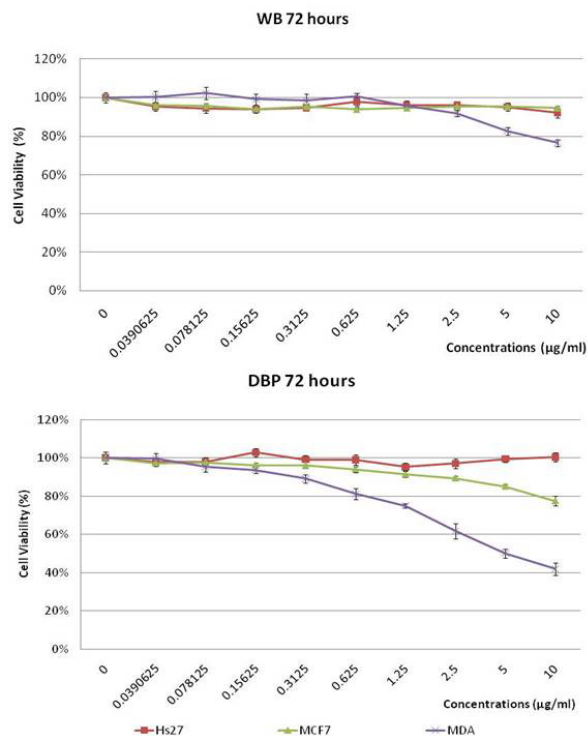


Fig. 1: Viability of Hs27, MCF-7 and MDA-MB231 at 72 hours post-treatment with WB (upper panel) and DBP (lower panel) at various concentrations.

The results obtained consistently showed that the WB and DBP, when affected the cell growth, were specific to cancer-origin cell lines (MDA-MB231 and MCF-7). Non-cancer origin cell line (Hs27) was not affected in these experiments. In both of the cases (treatments with WB and with DBP) where the growth of cells was affected, MDA-MB231 was more susceptible to the serum compared to MCF-7 (fig. 1).

MDA-MB231 was more susceptible than MCF-7 to whole B-serum (WB) and dialyzed B-serum precipitate (DBP) sub-fraction

When treated with various concentrations (from 0 to 10 µg/ml) of whole B-serum (WB) or DBP sub-fraction (fig. 1), proliferation of MDA-MB231 was affected. Results obtained from MTT cell-based assay showed that the anti-proliferation property of WB and DBP was specific to MDA-MB231 within the concentration range between 0 µg/ml to 2.5 µg/ml of serum as observed for the duration of 72 hours. Non-cancer-origin cell line Hs27 and cancer-origin breast cancer cell line MCF-7 were not affected by the sera, but nearly 60% of serum-treated MDA-MB231 cells were killed at the end of 72 hours after treatment (fig. 1).

On the other hand, only negligible anti-proliferation activity MCF-7 was discerned at DBP concentrations lower than 1.25 µg/ml, the killing effect became

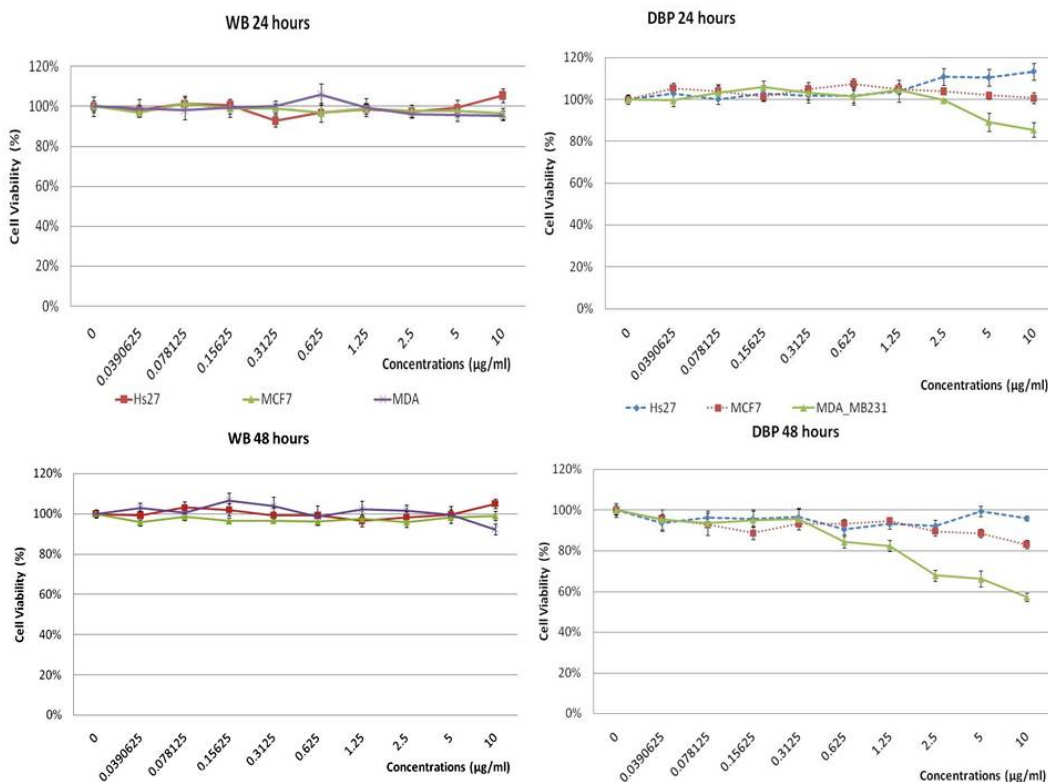


Fig. 2: Viability of Hs27, MCF-7 and MDA-MB231 at 24- and 48- hours post-treatment with WB (left panel) and DBP (right panel) at various concentrations.

increasingly evident at DBP concentrations greater than 2.5 µg/ml. Similarly, the higher killing effect in MDA-MB231 cells gained prominence with the increase of DBP sera concentrations.

The 50% lethal dose (LD₅₀) and 80 % lethal dose (LD₈₀) for MCF-7 and MDA-MB231 using whole B serum (WB) and DBP sub-fraction were calculated using Probit method on a Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, Canada). The results were shown in table 1. The results confirmed the susceptibility of MDA-MB231 towards DBP sub-fraction, for which the LD₅₀ and LD₈₀ were 5.36 µg/ml and 35.70 µg/ml respectively.

Anti-proliferation activity was not shown by DBS sub-fraction when tested on the cell lines. Cell viability at all the three time points tested remained around 100% compared to untreated cells, indicating the growth of the cells tested was not affected by the presence of the sub-fraction (fig. 3).

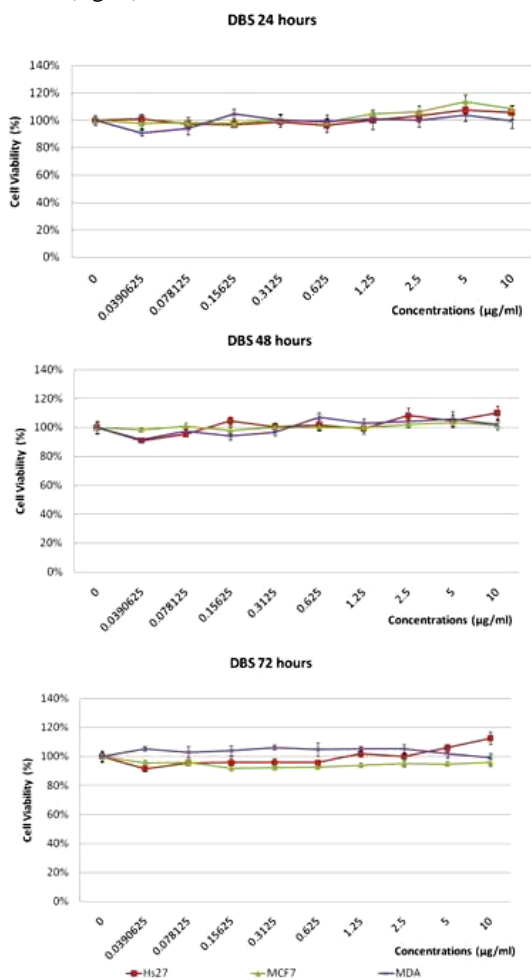


Fig. 3: Anti-proliferation activity lacking in Hs27, MCF-7 and MDA-MB231 cells at the three time points tested with DBS sub-fraction.

Anti-proliferation agents in B-serum sub-fractions were sensitive to heat treatment

No significant anti-proliferation activity was observed when HS27, MCF-7 and MDA-MB231 cells were treated with BBP and BBS, the heat treated latex B-serum sub-fractions. Fig. 4 illustrated clearly that no significant decrease of living cells across the concentrations of heat-treated latex B-serum sub-fractions on HS27, MCF-7 and MDA-MB231.

DNA laddering was not observed in extracted DNA from serum-treated cell lines

Total genomic DNA extracted from serum sub-fraction-treated cells and non-treated cells were subjected to DNA fragmentation assay. The agarose gel electrophoresis results showed the total genomic DNA smears on the gel, but no distinct DNA laddering was observed (fig. 5) as normally observed if cells had undergone apoptosis.

Table 1: Probit analysis showed the LD₅₀ and LD₈₀ for MCF-7 and MDA-MB231 treated with whole B-serum (WB) and B-serum sub-fraction DBP, with 95% confidence level.

	MCF 7 (µg/ml)		MDA-MB231 (µg/ml)	
	LD ₅₀	LD ₈₀	LD ₅₀	LD ₈₀
WB	-	-	85.86	1100.14
DBP	92.06	857.8	5.36	35.7

DISCUSSION

The results of brine shrimp lethality test performed by our group previously revealed that the toxicity level for latex B-serum was considerably low according to the classification by Meyer *et al.* (1982), with an LC₅₀ of 461.0 mg/ml (Daruliza *et al.*, 2011b) by not affecting the mortality rate of *A. salina*. This would be an additional beneficial property to the serum should it be used with its specific anti-proliferative properties against cancer-origin cells (Ong *et al.*, 2009; unpublished data).

Cytotoxicity tests performed using MTT on Hs27, MCF-7 and MDA-MB231 clearly showed the susceptibility of MDA-MB231 cells to DBP sub-fraction. At higher concentration of DBP sub-fraction, MCF-7 did show its susceptibility to the sub-fraction, but not Hs27. These results reflect on the anti-proliferation property of DBP that is specific to cancer-origin cells, MDA-MB231, MCF-7, which is in line with the findings in the assay conducted using HeLa cells (Ong *et al.*, 2009).

This is one of the important factors to consider should the active sub-fraction issued from latex B-serum be used for cancer treatment. The DBP sub-fraction fulfilled this specificity criterion at least at this very preliminary stage of drug development procedure.

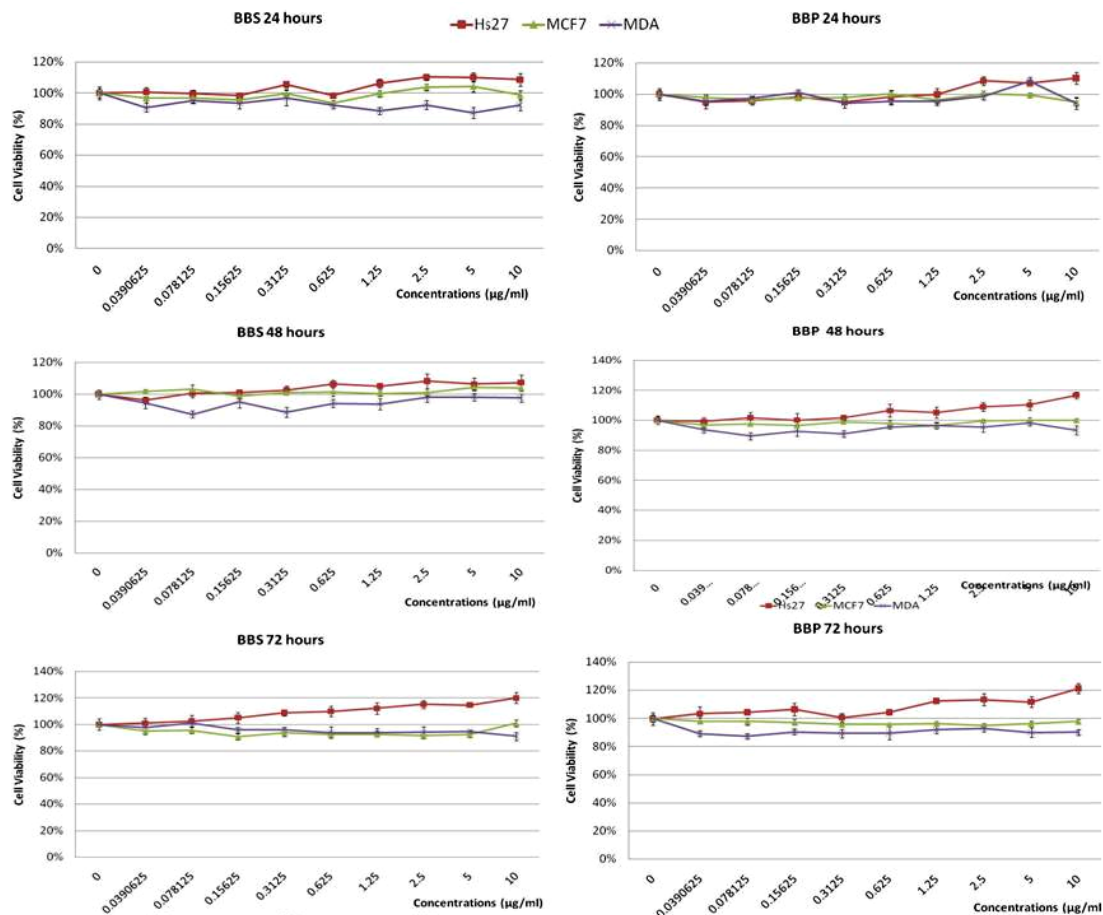


Fig. 4: Viability of Hs27, MCF-7 and MDA-MB231 at 24-, 48-, and 72- hours post-treatment with BBP (right) and BBS (left) at concentrations ranging from 0 to 10 µg/ml. The pre-heated B-serum sub-fractions, BBP and BBS showed no significant effect on the cell proliferation of the tested cell lines.

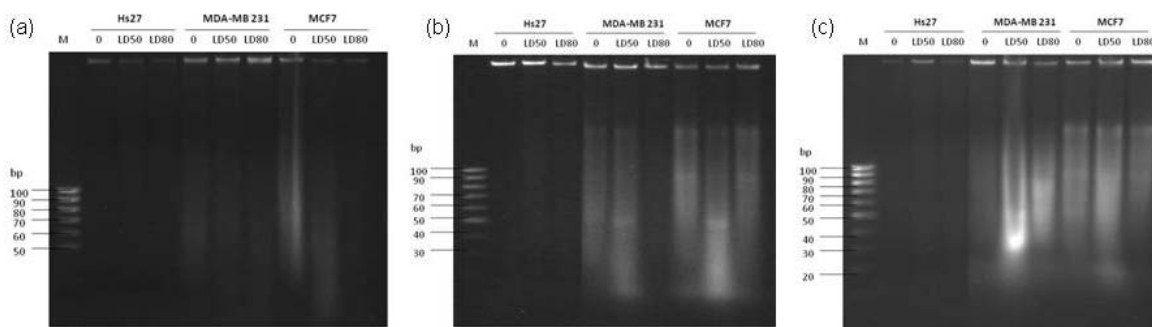


Fig. 5: Genomic DNA of DBP-treated cells separated on agarose gel electrophoresis. Figure (a), (b) and (c) show ethidium bromide staining of the DNA extracted at time points 24-, 48- and 72-hours post treatment respectively.

Unlike MCF-7 cells, MDA-MB231 cells are not defective in caspase 3 (casp3) which is the merging point of caspases regulated intrinsic and extrinsic apoptotic pathways. The lower susceptibility of MCF-7 compared to MDA-MB231 to DBP prompted us to investigate if the killing of MDA-MB231 occurred via apoptosis. However, the results from DNA fragmentation assay showed that caspase-related apoptosis was not evident in MDA-

MB231, at least within the time range of the experiment (up to 72 hours post treatment). Further studies should be conducted to elucidate the possible cell death mechanisms initiated by the latex B-serum sub-fraction.

No anti-proliferation activity was observed in the experiments using heat-treated sub-fractions, leading to the conclusion that the active substances in latex B-serum

are heat sensitive. The heat might have promoted chemical reactions to modify the initial properties of the active compound. Alternatively, the active compound might have been denatured during the heating process, such as in the case of proteins, rendering the heat-altered compounds lost the initial properties.

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