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Susceptibility of Human B Lymphocyte Cell Lines to Dialysed Latex B- and C-sera Fractions

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Hevea brasiliensis *latex sera are being investigated for anticancer property. In the present cell-based assay, whole latex B- and C-sera were shown to be largely ineffective against human B lymphocytes, of both cancer origin (BDCM) and non-cancer origin (Bristol 8); albeit slight dip in cell viability were discerned at high concentrations (>100 µg/mL) of whole C-serum, and (>200 µg/mL) of whole B-serum. Interestingly, the antiproliferative activity improved in the retentate of dialysed C-serum, the increment however was more prominent in the dialysed C-serum precipitate (DCP) compared to the dialysed C-serum supernatant (DCS). DCP also showed a marginally higher specificity against BDCM (LC_{50} = 55.95 µg/mL) compared to Bristol 8 (LC_{50} = 75.62 µg/mL). However in the treatments with the retentate of dialysed B-serum, both BDCM and Bristol 8 cells remained unaffected.*

Keywords: latex B-serum; latex C-serum; dialysis; anticancer; suspension cells; MTS

Natural rubber latex, a milky white sticky emulsion (cytoplasm) that exudes upon wounding of articulated laticiferous canals in the soft bark of *Hevea brasiliensis* tree, is harvested primarily for its rubber elastomer. Rubber particles that contain the elastomer make up to about 30% w/v of fresh latex, while the vast majority of non rubber constituents are either dispersed or dissolved in the latex serum. In the processing of rubber, most non rubber constituents of the latex serum are discarded in the effluent[1,2].

Research on bioactive compounds of *H. brasiliensis* tree is actively being pursued by many[3–6]. Since the natural rubber tree can be exploited for at least 20 years, it is an ideal plant factory for recombinant protein and metabolite production. To date, a number of these compounds have been shown to be promising pharmaceuticals and nutraceuticals[7–10]. A fraction of latex B-serum has been reported positive for antiproliferative activity against cervical cancer cell line - HeLa cells[11]. More recently, fractionated latex B- and C- sera also tested positive for antiproliferative activity against a selection of cancer cell lines[12,13].

The present study is aimed to assess the anticancer properties of latex B-serum, C-serum and their dialysed retentates against

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two human B lymphocyte cell lines *i.e.* BDCM of cancer-origin and Bristol-8 of non-cancer-origin, using cell-based cytotoxicity assay. B lymphocyte is a specific subtype of white blood cells that are involved in lymphocytic leukaemia, a common type of blood cancer[14,15].

EXPERIMENTAL

Preparation of Latex B-serum, C-serum and its Subfractions

Latex was collected from RRIM 600 trees at the RRIM Research Station, Sungai Buloh. To prepare latex B-serum and C-serum, fresh latex collected in chilled flasks was fractionated by centrifugation at 44,000 × *g* at 4ºC. The latex separates into three distinct parts upon high-speed centrifugation[16]. The upper layer consisting of creamed rubber particles, was carefully removed to gain access to the clear aqueous phase below (whole C-serum), according to a method previously described[17]. The whole C-serum (WC) was then lyophilised for subsequent use. Lyophilised powder of WC was reconstituted with 1x phosphate buffered saline (PBS, Amresco, Ohio, USA). Serial dilutions of the serum were performed to prepare the working concentrations.

Latex B-serum was prepared based on a method previously described[18]. Briefly, after removal of the rubber cream and whole C-serum, the sediment (lutoids) at the bottom of the centrifuge tube was collected and resuspended 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 whole B-serum (WB) was recovered by centrifugation and lyophilised for subsequent use. Lyophilised powder of WB was reconstituted with 1x PBS. Serial dilutions of the serum were performed to prepare the working concentrations similar to WC.

Fractions of WB and WC were prepared by dialysis using SnakeSkin™ (Pierce, IL, USA) tubing with molecular weight cut-off 3000 Da, against distilled water for 48 h at about 5ºC. A whitish precipitate was recovered by centrifugation at 20,000 × *g* for 30 min; the precipitate of dialysed B- and C- sera (DBP and DCP) and the supernatant of dialysed B- and C- sera (DBS and DCS) were then lyophilised and kept desiccated until further use.

Cell Culture

Human B lymphocyte, Bristol-8 (ECACC 85011436) is an HLA-A2 positive B lymphoblastoid, non-cancer-origin cell line purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). The acute myelogenous leukemia (AML) cell line BDCM (CRL-2740™), was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco® Invitrogen) supplemented with foetal calf serum (10% v/v), penicillin (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (0.025 µg/mL) (Gibco® Invitrogen). Cultures were maintained at 37ºC in a water-saturated atmosphere containing 5% CO_2. Cell counts were performed using a Neubauer haemocytometer under light microscope and living cells were identified by Trypan blue (Gibco® Invitrogen) staining method. Approximately 5000 cells were seeded in each well of a 96-well culture plate and were starved in RPMI-1640 medium under cell culture incubation conditions for 48 h prior to cell-based assay.

Measurement of Cell Growth Inhibition

The cytotoxic effect was measured using standard CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega Corporation, Madison, USA) after the cells have been treated with respective latex sera or subfractions at various serial diluted concentrations for different time periods (24, 48 and 72 h), the assay was developed according to the method suggested by the manufacturer. The absorbance at 490 nm was read on a spectrophotometric plate reader (Multiskan spectrum, Thermo Electron Co., Waltham, Massachusetts, USA). The proportion 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[19] to obtain the LC_{50} values. All experimental data were derived from at least three independent experiments.

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.

RESULTS AND DISCUSSION

The preliminary cytotoxicity results showed that the BDCM cells (cancer-origin) were susceptible to whole C-serum (WC) and not to whole B-serum (WB) within the tested concentration range (0 – 200 μg/mL). BDCM cells were not affected by the WC treatment at 24 and 48 post treatment hours (not shown), but cell viability decreased to 70% at 100 μg/mL of WC 72 hours post treatment (*Figure 1*).

Further treatment with the dialysed B-serum supernatant (DBS) resulted in a 20% drop in viability of BDCM cells and Bristol-8 cells (non-cancer-origin cell line) at 72 h post treatment (*Figure 2*). The decrease was only apparent at the highest concentration of DBS (200 μg/mL); and the LC_{50} calculated was too high (>30 μg/mL) to be considered for further level of cytotoxicity test (data not shown). According to the American National Cancer Institute (NCI), the effective concentration of cytotoxic activity for crude extracts is LC_{50}< 30 μg/mL[20–21]. The cell viability of BDCM and Bristol-8 also remained largely unaffected when treated with the dialysed B-serum precipitate (DBP).

The retentate of dialysed latex C-serum *i.e.* dialysed C-serum precipitate (DCP) and dialysed C-serum supernatant (DCS) were generally more promising against the tested cell lines in cytotoxicity tests compared to DBS and DBP. DCP particularly showed a higher potency towards the human B lymphoblast cell lines (*Figure 3*). Cell viability dropped to about 50% when BDCM cells and Bristol-8 cells were treated with DCP at 100 μg/mL for 72 hours. Although a slight preference against BDCM was discerned, the results revealed no significant difference between the two lymphocyte cell lines. The LC_{50} value calculated using Probit analysis was 55.95 μg/mL and 75.62 μg/mL for BDCM and Bristol-8 cells respectively (*Table 1*). A similar result was observed with the tests performed with DCS but the killing effects were rather weak compared to DCP. Taken together, the results of DCP and DCS treatments suggest that the active component(s) would have been rendered inactive by inherent inhibitor(s) in WC. Devoid of inhibitor(s), the active constituent(s) in the dialysed C-serum retentate, DCP in particular exhibited antiproliferation activity against the B lymphocyte cells.

The lack of specificity in the killing of BDCM and Bristol-8 B lymphocytes suggests that DCP might affect a similar cell signalling

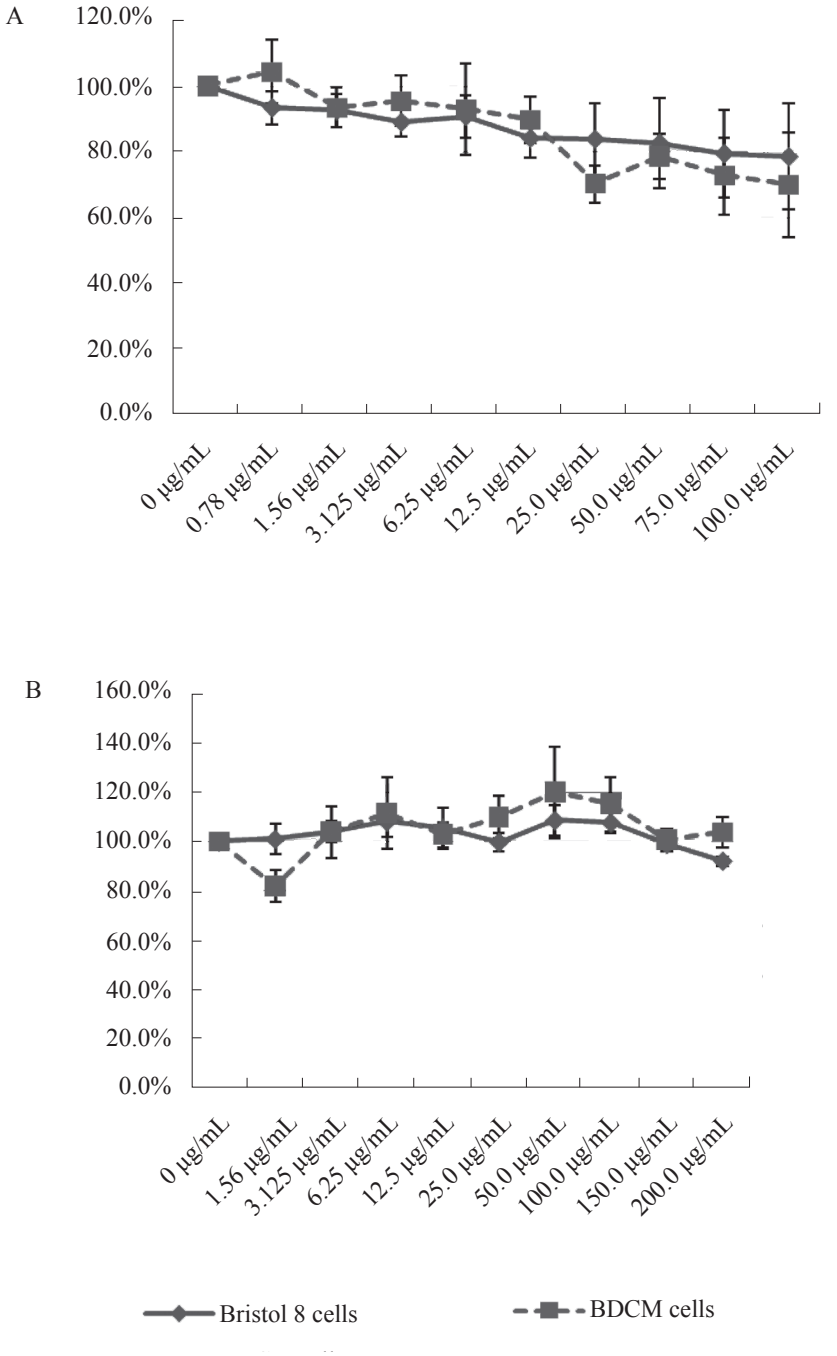

Figure 1. Measurement of cell viability of Bristol-8 and BDCM at post 72 hour treatment with whole latex sera, WC (A) and WB (B).

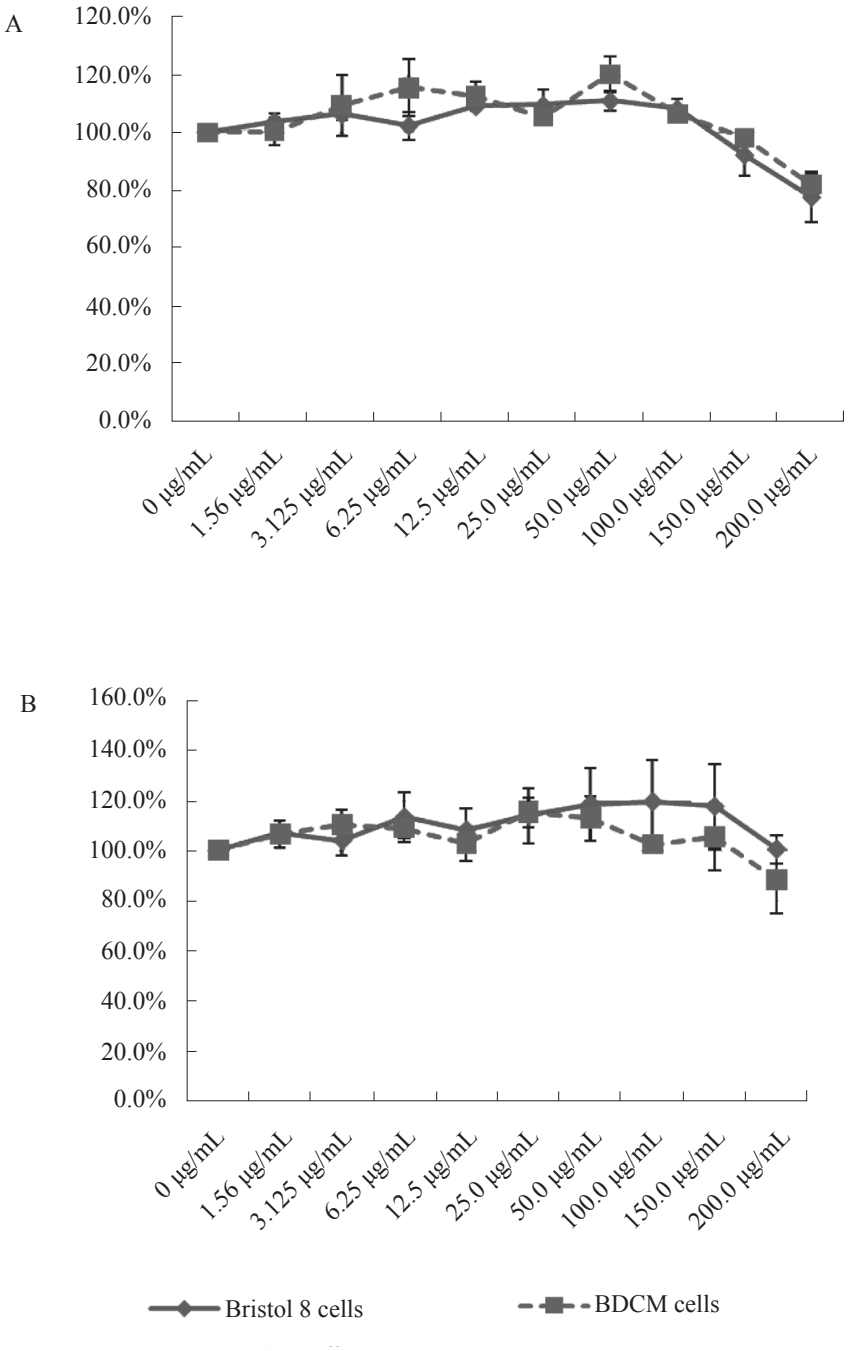

Figure 2. Measurement of cell viability of Bristol-8 and BDCM at 72 hour post-treatment with dialysed B-serum sub-fractions, DBS (A) and DBP (B) at concentrations ranging from 0-200 µg/mL.

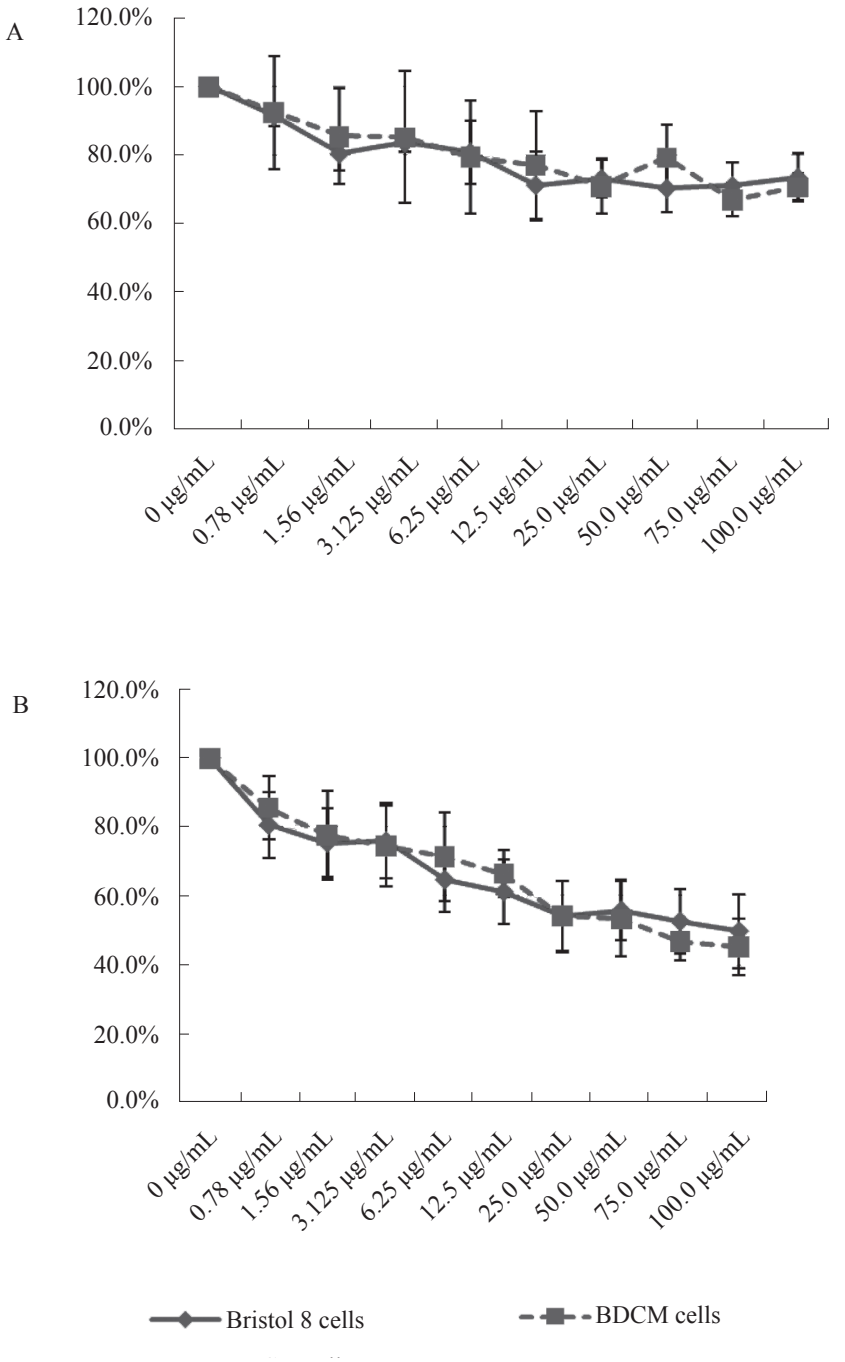

Figure 3. Measurement of cell viability of Bristol-8 and BDCM at 72 hour post-treatment with dialysed C-serum sub-fractions, DCS (A) and DCP (B) at concentrations ranging from 0-100 µg/mL.

TABLE 1. PROBIT ANALYSIS RESULTS SHOWING THE LD_{50} AT VARIOUS POST-TREATMENT TIME POINTS WITH WC, DCS AND DCP FOR BRISTOL-8 AND BDCM CELLS, WITH 95% CONFIDENCE LEVEL.

Fractions		WC		DCP		DCS	
Hours		48	72	48	72	48	72
LC_{50} (μg/mL)	Bristol 8	360 327.21	22 772.00	3211.92	75.62	N/A	4314.93
	BDCM	2 998 701.04	371.86	15 169 166.24	55.95	N/A	1854.56

N/A denotes the value was too high and therefore not included.

pathway leading to cell death in both cell lines. Previous reports had indicated that BDCM and Bristol 8 B lymphocyte cells contain human class I histocompatibility antigen, which is a prerequisite for tumour cell recognition by autologous T-lymphocytes[22,23]. This mode of recognition might lead to the introduction of the active compound(s) in DCP into the lymphocyte cells and trigger to undergo self-destruction. This explains the arguably non-specific cell death observed in both the cancer-origin (BDCM) and the non-cancer-origin (Bristol-8) cells. If proven, DCP could be employed as a secondary therapy in HLA-A2-restricted cytotoxic T-cell treatment, such as in ovarian cancer, as these cells are known to contain naturally-occurring peptides associated with HLA-A2[24].

At lower concentrations of B-serum and its subfractions, DBS and DBP, cell viability seemed to be increased (*Figure 1(B)* and *Figure 2*). This might be due to the growth promoting compounds found in these fractions and their effects were strong enough to override the effects exerted by antiproliferation compounds. This may suggest that further fractionation is needed to separate the growth promoting compounds from the antiproliferation compounds and thus these latter could be isolated and identified. The antiproliferation effects observed at higher concentrations of these fractions suggest that the potency of antiproliferation compounds in the fractions was higher compared to that of growth promoting compounds. This was translated into the observation whereby the antiproliferation effect increased with the concentration of latex fractions to overcome the growth promoting effects although the ratio of growth promoting to antiproliferation compounds remained unchanged.

In conclusion, dialysis induced precipitation of the latex whole C-serum (WC) improved the potency against human B lymphocytes, with LC_{50} = 55.95 μg/mL and LC_{50} = 75.62 μg/mL for BDCM and Bristol-8 cells respectively. Further subfractionation of DCP might result in a lower LC_{50} value. Although the current LC_{50} value for DCP is higher than that recommended by the American NCI (30 μg/mL), it has become an additional property of DCP to be potentially used as a cancer therapy adjuvant as this fraction had been proven to be active in other cancer cell lines too (unpublished data).

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