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Cell Viability Assay Guided Fractionation of Natural Rubber Latex Sera

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Natural rubber latex sera have been investigated for anti-proliferative property on cancer cell lines. The present report describes fractionation of latex B- and C-serum using cell viability assay (anti-proliferative activity) as a guide to narrow down on the active constituents. Dialysis of B- and C-serum against distilled water caused some of its constituents to precipitate, then a brief centrifugation separated their fractions – dialysed B-serum precipitate (DBP), dialysed B-serum supernatant (DBS), dialysed C-serum precipitate (DCP) and dialysed C-serum supernatant (DCS). Boiling of B- and C-serum resulted in extensive precipitation; a brief centrifugation separated the boiled B- and C-serum precipitate from their supernatant. Cell viability assay performed on two human cancer-origin cell lines revealed a marked increase in anti-proliferative activity in the dialysed C-serum fractions and in DBP but diminished in DBS as well as in the boiled B- and C-serum fractions. Chromatographic separation of DBP, DCP and DCS followed by cell viability assay will shed light if sub-fractions with further improved anti-proliferative activity are attainable.

Keywords: Latex B-serum; latex C-serum; dialysis; cell viability assay; anti-proliferative activity

Natural rubber latex is a milky white sticky emulsion (cytoplasm) that exudes upon wounding of articulated laticiferous canals in the soft bark of *Hevea brasiliensis* tree. Fresh latex, after tapping from the tree, may be divided into three main fractions by high-speed centrifugation; these are a white upper layer, an aqueous phase (C-serum) and the so-called 'bottom fraction'[1,2]. The top layer comprises rubber micro-particles stabilised by an adsorbed layer of protein and phospholipids. The C-serum contains most of the soluble substances normally found in plant cells, including amino acids, proteins, carbohydrates, organic acids, inorganic salts and nucleotidic materials. The 'bottom fraction' consists largely of lutoid particles but also contains varying amounts of other organelles or particulate components of normal plant cells having a density greater than that of C-serum[3]. The fluid content of the 'bottom fraction' is called B-serum[4].

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So far, investigations on natural rubber latex sera (B- and C- serum) and its constituents have revealed multiple biological activities such as anti-fungal, anti-inflammatory, anti-oxidant and lipolytic properties[5–13]. Potential anti-cancer therapeutic application of natural rubber latex was first suggested when B- and C-serum were shown to exert a specific anti-proliferative property against a malignant cell line, in cell viability assay[14]. This is encouraging as latex sera is a relatively low-cost resource particularly in rubber producing regions *vis-à-vis* its potential use as an anti-cancer therapeutic.

This report describes the sequential fractionation of latex B- and C-serum; cell viability assay serves as a guide to direct fractionation towards isolation of the active constituents.

EXPERIMENTAL

Materials

Preparation of latex B- and C-serum. Latex was collected from field grown RRIM 600 trees at the Rubber Research Institute of Malaysia Experiment Station, Sungai Buloh. To prepare latex B- and C-serum, fresh latex collected in chilled flasks was fractionated by centrifugation at 44,000 × g for 2 h, at 4ºC. The latex separates into three distinct parts on high-speed centrifugation, as shown in *Figure 1*. To prepare whole C-serum (WC), the upper layer (rubber cream), was carefully removed and WC was prepared from the remaining supernatant, based on a method previously described[1,2]. Latex B-serum was prepared from the bottom fraction of the centrifuged latex based on a method previously described[15]. 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 lutoid particles intact. The cleansed bottom fraction was recovered after another centrifugation and subjected to alternate freezing and thawing (four times) to rupture the lutoid particles. The clear brownish fluid from the lutoid particles, the whole B-serum (WB) was recovered by centrifugation. Both WB and WC were lyophilised until completion and kept in a desiccator for subsequent use.

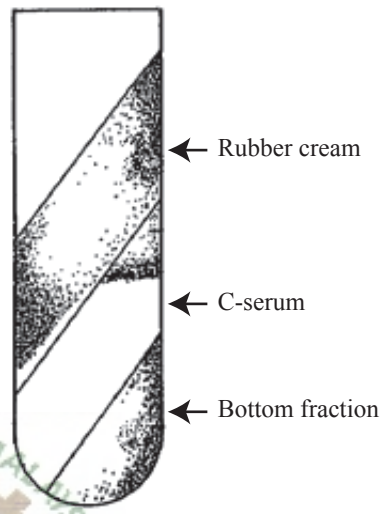

Figure 1. Fractionation of fresh latex by high-speed centrifugation. The positions of rubber cream, C-serum and bottom fraction are marked.

Preparation of dialysed B- and C-serum fractions. A portion of the lyophilised WB and WC were reconstituted with 1 × phosphate-buffered saline (PBS; AMRESCO, Solon, OH, USA). Serial dilutions of WB and WC were performed to prepare working concentrations ranging from 2 to 2,000 µg/mL. The subsequent fractions of WB and WC were prepared by dialysis using SnakeSkin™ (Pierce, IL, USA) tubing with molecular weight cut-off 3,000 Da, against distilled water for 48 h at about

5ºC. A whitish precipitate was recovered from dialysed B and C-serum by centrifugation at 20,000 × g for 30 minutes. The precipitates (DBP and DCP) and the supernatants (DBS and DCS) were then lyophilised and kept desiccated until further use. Working solutions for dialysed fractions were prepared as described above.

Preparation of boiled B- and C-serum fractions. WB and WC were placed in a boiling water bath for 10 minutes. A centrifugation procedure at 20,000 × g for 30 min was employed to separate the boiled B- and C-serum fractions. The recovered boiled B-serum precipitate (BBP), boiled B-serum supernatant (BBS), boiled C-serum precipitate (BCP) and boiled C-serum supernatant (BCS) fractions, were then lyophilised until completion. Reconstitution and working solutions of the lyophilised BBP, BBS, BCP and BCS were as described above for WB and WC.

Measurement of Cell Growth Inhibition in Cell Viability Assay

Cytotoxic effects were measured using standard 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma Chemical Company, St. Louis, MO, USA) after cell treatment with WB, WC or its fractions, for pre-determined time points *i.e*. 24, 48 and 72 hours. The assay was developed based on the method described by Mossman[16]. Absorbance (OD) at 570 nm was read using a spectrophotometric plate reader (Multiskan spectrum; Thermo Electron Co., Waltham, MA, USA) and proportions of surviving cells were calculated as shown in *Equation 1*.

$$\frac{\text{OD of drug-treated sample - OD of blank}}{\text{OD of control - OD of blank}} \times 100\% \quad \ldots 1$$

Dose–response curves were constructed using Probit analysis[17] to obtain LC_{50} values. All experimental data were derived from at least three independent experiments.

Electrophoresis and Protein Detection

Two-dimensional electrophoresis was carried out with isoelectric focusing for the first dimension on IPGphor (GE Healthcare Life Sciences, Piscataway, NJ, USA), and SDS-PAGE for the second dimension with Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturers' instructions. Briefly, the 7 cm IPG strips, pH 3-10 L, containing 100 µg samples were dissolved in IPG buffer (with freshly added 18 mM DTT) and were placed in porcelain strip-holders. The strips were overlaid with ~1 mL mineral oil and rehydration was performed at 20ºC for 16 hours. This was followed by isoelectric focusing at constant voltage of 500 V for 30 min, 1,000 V for 1 h and thereafter at 5,000 V for 1 hour. After isoelectric focusing, the strips were soaked in equilibration buffer for 10 min before loading on to 12.5% SDS-polyacrylamide slab gels and electrophoresed at constant current of 30 mA, for 45 minutes. Upon completion, the gels were stained with Coomassie brilliant blue R250 to detect the separated proteins.

RESULTS AND DISCUSSION

The results of brine shrimp lethality test performed by our group previously indicated that the toxicity level for latex B-serum (WB)[11] and C-serum (WC)[12] were considerably low with an LC_{50} value of 461.0 mg/mL and 98.4 mg/mL, respectively; LC_{50} values >1000 µg/mL were considered non-toxic[18]. This prompted us to perform cell viability assay, which revealed that WB and WC could elicit anti-proliferative

activity on a number of adherent cancer-origin cell lines but inactive against a non-cancer origin cell line that served as negative control (unpublished results). Perceived as highly susceptible among the cancer-origin cells, MDA MB231 – breast cancer cells that recorded an LC_{50} value of 85.9 µg/mL at 48 h post-treatment with WB[19] and HepG2 – hepato adenocarcinoma cells that recorded an LC_{50} value of 889 ng/mL at 48 h post-treatment with WC[20] were chosen as marker cells to evaluate subsequent B- and C-serum fractions. At this point, it was evident that the LC_{50} value of WC is well within the 30 µg/mL threshold set by the American National Cancer Institute for potential crude plant extracts[21]. Then, WC was dialysed against distilled water to eliminate small molecules (<3,000 Da), as it is known to contain a multitude of small organic molecules including sugar derivatives[22] that may interfere with the cell viability assay. The process also resulted in a proteinaceous precipitate within the dialysis tubing.

A brief centrifugation separated the dialysed C-serum precipitate (DCP) from the dialysed C-serum supernatant (DCS). Anti-proliferative activity against HepG2 improved after dialysis, in both DCP and DCS. Probit analysis revealed that LC_{50} values at 48 h were significantly reduced to 2 ng/mL for DCS and 280 ng/mL for DCP, from the original value of 889 ng/mL recorded for WC[20] (*Figure 2* and *Table 1*). The very low LC_{50} value especially in DCS reflects on the enrichment of active constituents achieved at this stage of fractionation. With the high specificity to HepG2, there seems to be a potential for application of dialysed C-serum fractions in the treatment of hepatocellular carcinomas. These findings also justified further fractionation of DCP and DCS in view of targeting HepG2 and narrowing down on the active constituents. As both DCP and DCS had been shown to elicit slightly different anti-proliferative patterns[20], it is also plausible that these fractions are harbouring different active constituents.

As observed earlier in WC, dialysis of WB also induced precipitation and a brief centrifugation was employed to recover the dialysed B-serum precipitate (DBP) and dialysed B-serum supernatant (DBS). Cell viability assay showed that anti-proliferative activity against MDA MB231 improved nearly sixteen fold in DBP with LC_{50} value at 48 h lowered to 5.4 µg/mL, whereas the activity largely diminished in DBS[19] (*Figure 2* and *Table 1*). Thus far, DBP had fulfilled the specificity criterion against MDA MB231 at least at this very preliminary stage of drug development procedure. Further fractionation of DBP coupled with cell viability assay with MDA MB231 will confirm if sub-fractions with lower LC_{50} values are attainable.

The LC_{50} values obtained for MDA MB231 treated WB and its dialysed fractions, and for HepG2 treated WC and its dialysed fractions are summarised in *Table 1*. Another noteworthy point is that anti-proliferative activity had diminished in the boiled B- and C-serum fractions[19,20], which reflects on the heat sensitive nature of active-constituents. Evidently boiling results in denaturation and precipitation of most B- and C-serum proteins, which were then recovered in the BBP and BCP. Thus it is tempting to speculate that active constituent(s) in DBP and in the dialysed C-serum fractions are of protein-origin – the predominant macromolecules (>3,000 Da) that are retained within the dialysis tubing. The diversity of proteins in the dialysed B- and C-serum fractions is shown in *Figure 3*. Chromatographic separation followed by cell viability assay will prove if further enriched DBP, DCP and DCS sub-fractions are attainable, which would be invaluable for *in vivo* comparative analysis with that of known anti-cancer agents.

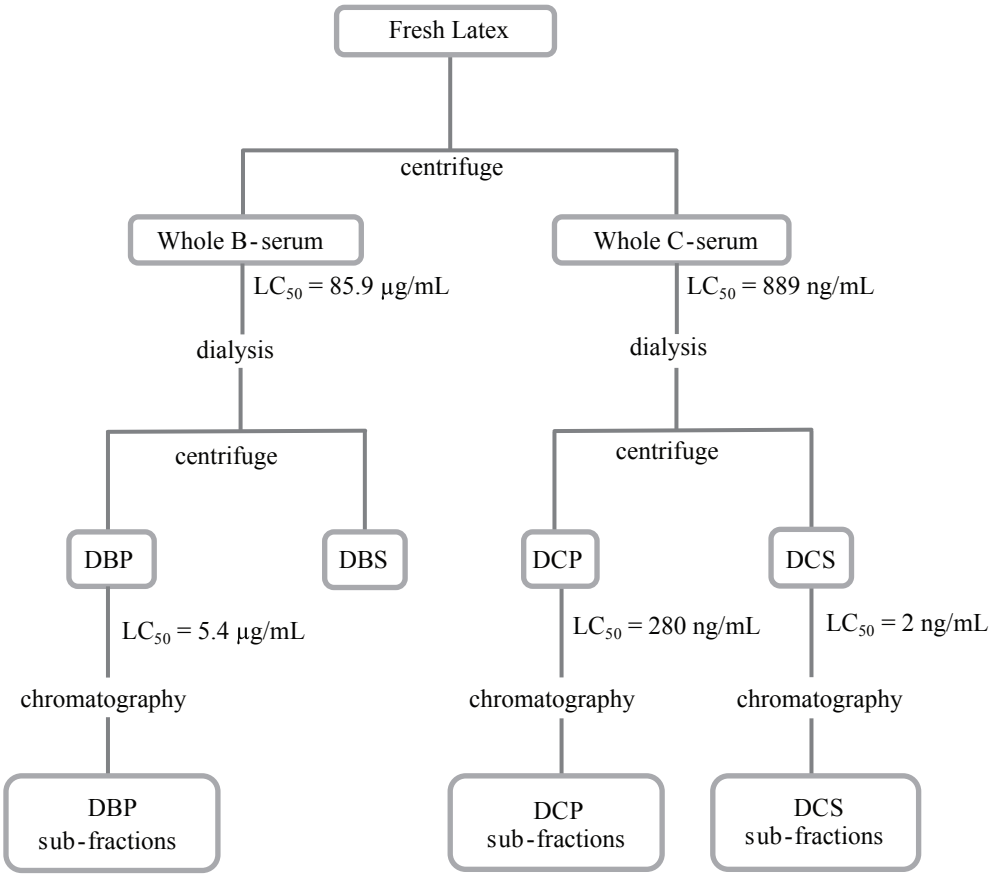

Figure 2. Cell viability assay guided fractionation of latex sera. Fractionation of B-serum is guided by potency against MDA MB231, while fractionation of C-serum is guided by potency against HepG2. DBP: dialysed B-serum precipitate; DBS: dialysed B-serum supernatant; DCP: dialysed C-serum precipitate; DCS: dialysed C-serum supernatant.

TABLE 1. LC_{50} VALUES OF WHOLE B-SERUM (WB), DIALYSED B-SERUM PRECIPITATE (DBP) AND DIALYSED B-SERUM SUPERNATANT (DBS) TESTED WITH MDA MB231, AND OF WHOLE C-SERUM (WC), DIALYSED C-SERUM PRECIPITATE (DCP) AND DIALYSED C-SERUM SUPERNATANT (DCS) TESTED WITH HepG2

		WB	DBP	DBS	WC	DCP	DCS
MDA-MB231	LC_{50}	85.9 µg/mL	5.4 µg/mL	N/A	-	-	-
HepG2	LC_{50}	-	-	-	889 ng/mL	280 ng/mL	2 ng/mL

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

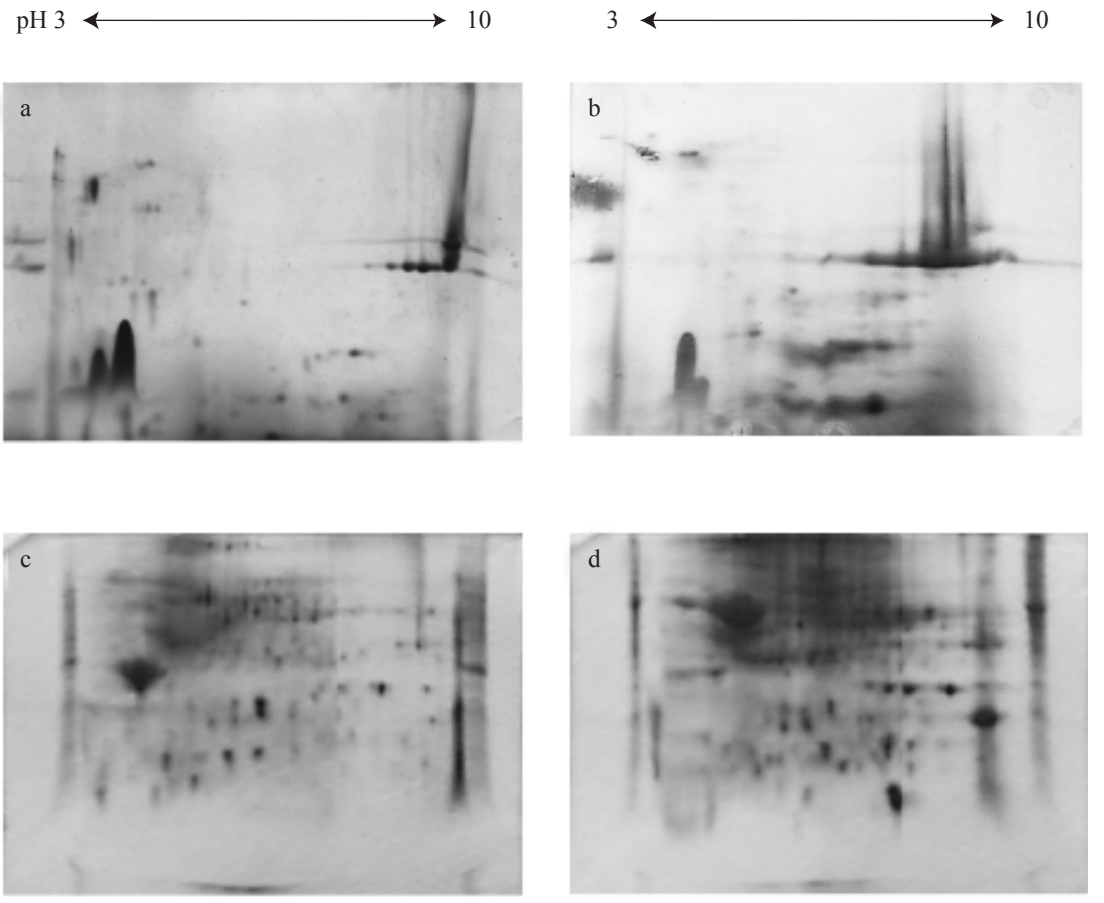

Figure 3. Coomassie blue staining of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of (a) dialysed B-serum precipitate, (b) dialysed B-serum supernatant, (c) dialysed C-serum precipitate and (d) dialysed C-serum supernatant.

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