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Proteins of Dialysed C-serum Supernatant Sub-fractions Elicit Anti-Proliferative Activity on Human Cancer-Origin Cells

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Dialysed latex C-serum fractions have been investigated for anti-proliferative activity against human cancer origin cell lines. The present report describes further fractionation of dialysed C-serum supernatant (DCS) and analysis of the sub-fractions that elicit anti-proliferative activity on HepG2 and MDA-MB231, the test cancer origin cell lines. DCS was fractionated through a Sephacryl S-200 column, and the eluents were pooled into four sequential sub-fractions, named DCS-F1, -F2, -F3 and -F4. Cell viability assay revealed that anti-proliferative activity was confined to DCS-F2 and -F3. Furthermore an improved anti-proliferative activity was discerned in the recombined active DCS sub-fractions. DCS-F2 and -F3 were subjected to two dimensional polyacrylamide gel electrophoresis, subsequent MALDI-TOF/TOF analysis and database searches revealed the presence of a number of proteins that have been shown to be implicated in anti-cancer activity.

Keywords: Latex C-serum; gel-filtration, cell viability assay; electrophoresis; proteins

Natural rubber latex is a complex colloid made up of rubber particles, lutoid bodies and Frey-Wyssling complexes suspended in an aqueous medium rich in proteins and other solutes. When centrifuged at high speed, fresh latex separates into three main fractions consisting of an upper layer of rubber particles, a clear aqueous phase or C (centrifuged)-serum, and a bottom fraction largely made up of lutoid bodies[1,2]. C-serum contains most of the soluble substances normally found in plant cells, including proteins, amino acids, carbohydrates, organic acids, inorganic salts and nucleotidic materials[3,4].

C-serum and its constituents have been implicated in multiple biological properties[5–9]. However, the first potential therapeutic application of C-serum was suggested when a sub-fraction of C-serum was shown to exert specific anti-proliferative properties against cell lines of malignant origin[10]. In subsequent experiments, boiling of C-serum in water bath resulted in accumulation of a

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large amount of whitish precipitate; most proteins would have precipitated along with other heat labile constituents as a result of heat denaturation. Anti-proliferative activity was found diminished in the boiled C-serum fractions, evidently, the loss of activity reflects on the heat sensitive nature of the active constituents[11]. On the other hand, dialysis against distilled water resulted in an improved anti-proliferative activity in the C-serum fractions. Increase in anti-proliferative activity in DCS and dialysed C-serum precipitate (DCP) was particularly evident against HepG2 – hepato adenocarcinoma cells; the lowest LC_{50} value was detected in dialysed C-serum supernatant (DCS)[11]. As dialysis would have eliminated most small molecular weight solutes (<3000 Da), it was then speculated that the active constituent ought to be of protein origin – the predominant macromolecules (>3000 Da) that remain in either soluble or insoluble state in the dialysis tubing. The diverse proteins of DCS and DCP were recently discerned in two dimensional polyacrylamide gel[12].

The present report describes gel filtration of DCS as a further step of fractionation, while cell viability assay serves as a guide to narrow down on the eluted sub-fractions that elicit anti-proliferative activity. The active sub-fractions were electrophoresed on a two dimensional polyacrylamide gel and the resolved protein spots were subjected to MALDI-TOF/TOF analysis, followed by searches for similar entries in protein databases.

EXPERIMENTAL

Preparation of Dialysed C-serum Fractions

Latex was collected from field grown RRIM 600 trees at the Rubber Research Institute of Malaysia Research Station, Sungai Buloh. To prepare C-serum, fresh latex collected in chilled flasks was fractionated by centrifugation at 44000 × g for 2 h at 4 ºC. The latex separates into three distinct parts on high speed centrifugation. To prepare C-serum, the upper layer (rubber cream) was carefully removed and C-serum was prepared from the remaining supernatant, based on a method previously described[1,2].

The subsequent fractions of C-serum 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 (DCP) was recovered from dialysed C-serum by centrifugation at 20000 × g for 30 minutes. Both DCP and the supernatant (DCS) were then lyophilised and kept desiccated until further use.

Chromatographic Separation of Dialysed C-serum Supernatant (DCS)

Prior to chromatography, lyophilised DCS was reconstituted (0.03 g/ml) in 0.05 M Tris-HCl pH 8.0. The UV absorbing (254 nm and 280 nm) eluent of DCS was recovered as 2 ml consecutive fractions following filtration on a Sephacryl S-200 (GE Healthcare Life Sciences, Piscataway, NJ, USA) column. The elution profiles of DCS based on optical density (254 nm and 280 nm) of the individual fractions were plotted, the consecutive UV absorbing peaks were pooled, dialysed against distilled water and then lyophilised until completion. Multiple gel filtration runs were then performed to recover sufficient quantity of pooled sequential DCS sub-fractions, named sub-fraction 1 (DCS-F1), sub-fraction 2 (DCS-F2), sub-fraction 3 (DCS-F3) and sub-fraction 4 (DCS-F4). All four DCS sub-fractions were then lyophilised and kept desiccated until further use. A portion of the lyophilised DCS sub-fractions was reconstituted with 1 × phosphate-buffered

saline (PBS; AMRESCO, Solon, OH, USA). Serial dilutions of DCS sub-fractions were performed to prepare working concentrations ranging from 2 to 2000 µg/ml.

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 C-serum or its fractions for a predetermined time points *i.e*. 24, 48 and 72 hours. The assay was developed based on the method described by Mossman[13]. 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:

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

All experimental data were derived from at least three independent experiments.

Electrophoresis, Protein Detection and Identification

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) 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. The 7 cm IPG strips, pH 3-10, containing 100 µg samples were dissolved in IPG buffer (with freshly added 18 mM DTT) and 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, 1000 V for 1 h and thereafter at 5000 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. Protein spots were excised from 2D gels, subjected to mass spectrometry (MALDI-TOF/TOF) analysis at Proteomics International Pty Ltd, Australia. The protein samples were trypsin digested and peptides extracted according to standard techniques[14]. Peptides were analysed by MALDI-TOF/TOF mass spectrometer using a 4800 Proteomics Analyzer (AB Sciex). A search was performed on the MS/MS data to identify proteins of interest against the entire taxonomy order *Viridiplantae* using Mascot sequence matching software (Matrix Science) with Ludwig NR Database.

RESULTS AND DISCUSSION

Gel Filtration of Dialysed C-serum Supernatant (DCS)

Various gel filtration matrices including Sephacryl S-200 have been successfully employed to fractionate latex sera proteins prior to further biochemical investigations[15–17]. In the present research, DCS was fractionated through a Sephacryl S-200 column, and light absorbance of the resulting individual fractions was measured at 254 and 280 nm. A graph was plotted to depict the UV absorbance values against the eluted fractions. Then, the eluted fractions of DCS were pooled into four sequential sub-fractions, which were named sub-fraction 1 (DCS-F1), sub-fraction

2 (DCS-F2), sub-fraction 3 (DCS-F3) and sub-fraction 4 (DCS-F4) (*Figure 1*). Both UV absorption value and gain of volume were taken into account to limit number of individual fractions that would make up each pooled sequential sub-fraction. The rationale was also to ensure sufficient amount of proteins (UV absorbing constituents) are present in each pooled sub-fraction to test for anti-proliferative activity using cell viability assay. In any case, each pooled DCS sub-fraction would contain a reduced range of protein population compared to the original DCS, facilitating downstream 2D-PAGE and MALDI-TOF/TOF analysis.

Anti-proliferative Activity of DCS Sub-fractions against HepG2 and MDA-MB231 Cells

A preliminary cell viability assay performed on all four DCS sub-fractions revealed that DCS-F2 and -F3 were active against HepG2 and MDA-MB231, the test human cancer origin cells, while DCS-F1 and -F4 lacked anti-proliferative activity (data not shown). Repeat assay performed with the determined concentration range (0 - 1 µg/ml) of the active DCS-F2 and -F3 sub-fractions showed that proliferation of Hs27 – non cancer origin cells, remained relatively stable, whereas that of HepG2 and MDA-MB231 cells were noticeably suppressed at 24, 48 and 72 h post-treatment. In general, DCS-F2 elicited a stronger anti-proliferative activity compared to DCS-F3; their effects on HepG2 and MDA-MB231 cells at 72 h post treatment is shown in *Figure 2*(*a* and *b*). An attempt was then made to evaluate the effects of recombined DCS-F2 and -F3 on the test cancer origin cells. Interestingly, an increased anti-proliferative activity was detected in both HepG2 and MDA-MB231 cells with the recombined active DCS sub-fractions (*Figure 2c*), which indicates a probable synergistic growth inhibitory effects of the active constituents.

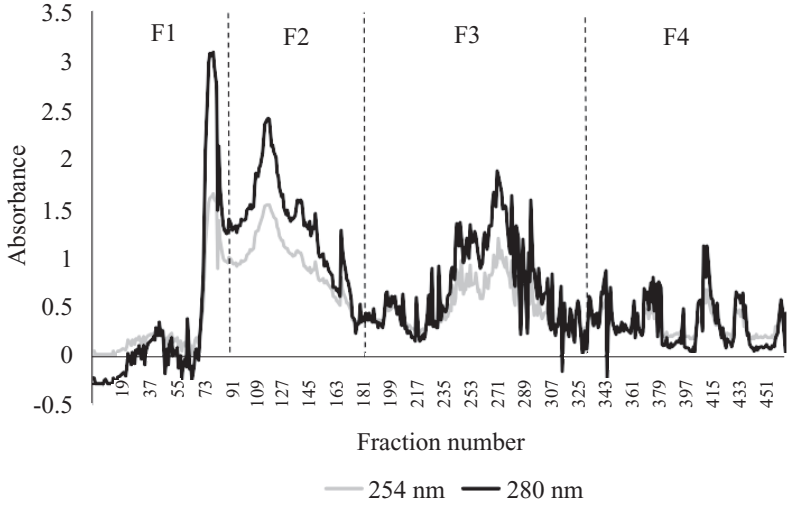

Figure 1. Elution profile of dialysed C-serum supernatant (DCS) separated through a Sephacryl S-200 column. The pooled DCS-F1 (collection tubes 1 – 90), DCS-F2 (collection tubes 91 – 177), DCS-F3 (collection tubes 178 – 331) and DCS-F4 (collection tubes 332 – 463) sub-fractions are indicated.

Proteins of the Active Dialysed C-serum Supernatant Sub-fractions

2D-PAGE and Coomassie blue staining revealed that DCS-F1 contained proteins mainly in the medium molecular weight range, DCS-F2 contained the proteins in medium to low molecular weight range, DCS-F3 contained fewer proteins and mostly in the low molecular weight region, while only one protein spot was detectable in DCS-F4 (the active sub-fractions, DCS-F2 and DCS-F3 are shown in *Figure 3*). This observation showed that sufficient molecular weight sieving of DCS proteins was achieved by the gel filtration technique. A total of 43 prominent protein spots, *i.e.* 33 of DCS-F2 origin and ten of DCS-F3 origin were selected for further analyses as among them would be candidates that elicit anti-proliferative activity. Gel plugs containing the protein spots were first digested with trypsin and the resulting tryptic peptides were subjected to MALDI-TOF/TOF analysis. Identities of 31 protein spots were determined from database searches performed on MS/MS peptide sequence data (*Table 1*). The matching proteins that were captured from the databases were then browsed for information on functionality and relevance in anticancer property.

Protease inhibitors and latex cystatin (cysteine protease inhibitor) were common constituents of both DCS-F3 and -F3. The beneficial effects of plant protease inhibitors have been attributed to their multiple regulatory activities, this include up-regulation of connexin 43 and MAPK phosphatase 1 and down regulation of EGF/TGF-α and urokinase plasminogen activator, which are invaluable markers in malignancies. With a host of other regulatory activities, protease inhibitors appear to suppress carcinogenesis at different stages including initiation, promotion and progression[18,19]. The abundance of protease inhibitors in DCS-F2 may explain the strong anti-proliferative activity elicited against HepG2 and MDA-MB231 (*Figure 2a*, *b*). Perhaps a smaller number of these proteins detected in DCS-F3 could explain its weaker anti-proliferative activity. On the other hand, the improved anti-proliferative activity in the recombined active DCS sub-fractions (*Figure 2c*) could be attributed to the increased quota of protease inhibitors, although a synergistic effect of these operating in concert with other proteins would be a more plausible explanation.

Malate dehydrogenase was among the most abundant protein detected in DSC-F2; this protein however was absent in DCS-F3. Elsewhere, malate dehydrogenase has been prominently detected in a fraction of *Gynura procumbens* that is active against breast cancer cells[20]. Malate dehydrogenase activity is generally implicated in the reversible NAD^+-dependent-dehydrogenase reaction in TCA cycle[21]. It has been reported that glucose 6-phosphate dehydrogenase activity is antagonised by malate dehydrogenase in dehydroepiandrosterone (DHEA) treated hepatocellular carcinoma rats[22]. Glucose 6-phosphate dehydrogenase is the key enzyme regulating the first and irreversible step of pentose phosphate pathway which provides ribose-5-phosphate, a precursor for DNA synthesis in cell proliferation. The demand for ribose-5-phosphate to cater for *de novo* DNA synthesis has been shown to increase during the promotion phase of carcinogenesis[22]. Therefore the abundance of malate dehydrogenase in DCS-F2 may lead to suppression of ribose-5-phasphate synthesis in HepG2 and MDA-MB231 cells, which in turn leads to suppression of cell proliferation.

Three previously characterised allergenic proteins *i.e*. Hev 2.2 (prohevein; a lectin), Hev b 7 (patatin) and profilin[23] were detected in the active DCS sub-fractions. With the exception of some plant lectins that are known to induce

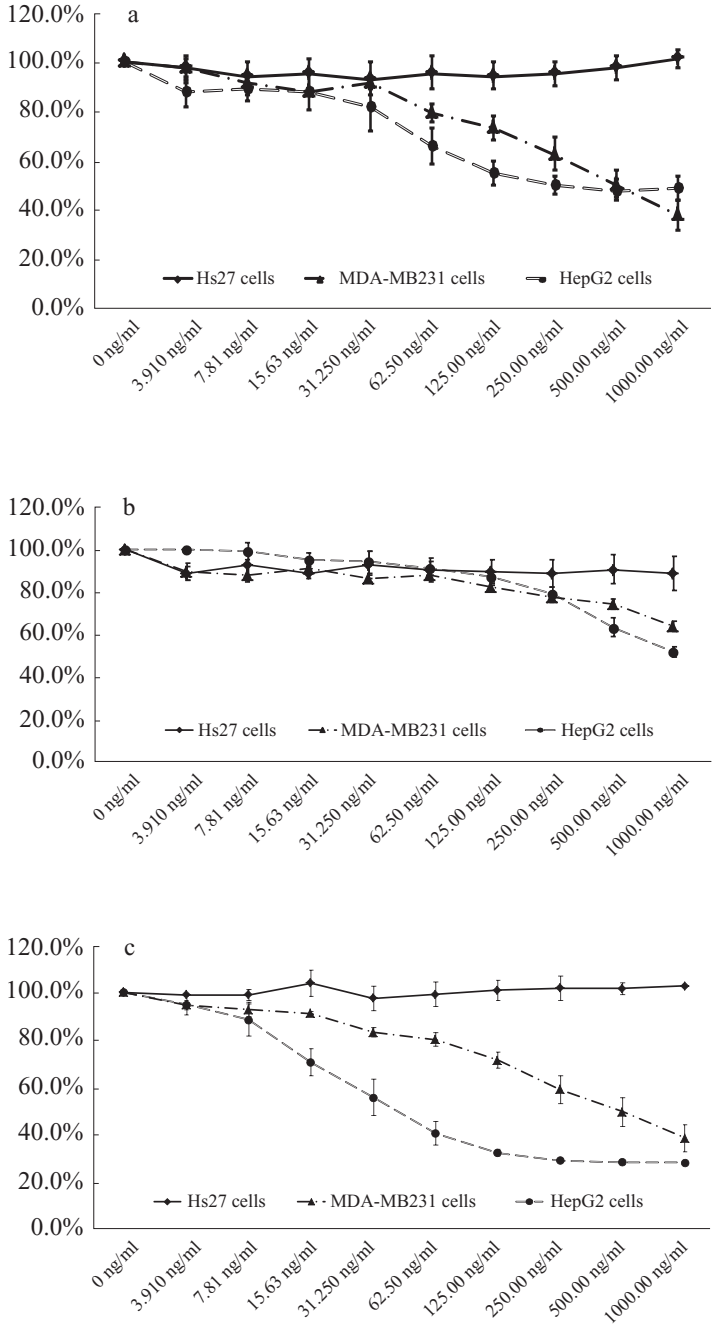

Figure 2. Viability profile of test cancer-origin (HepG2 and MDA-MB231) and non-cancer-origin (Hs27) cell lines at 72 h post treatment with determined concentrations of (a) Dialysed C-serum supernatant sub-fraction 2 (DCS-F2), (b) Dialysed C-serum supernatant sub-fraction 3 (DCS-F3), and (c) Recombined dialysed C-serum supernatant sub-fractions 2 and 3 (DCS-F2 and -F3).

TABLE 1. LIST OF IDENTIFIED PROTEINS IN DIALYSED C-SERUM SUPERNATANT SUB-FRACTION 2 (DCS-F2) AND SUB-FRACTION 3 (DCS-F3)

Gel Spot	Identified protein	Accession no.	Score	MW (kDa)	Sequence coverage (%)
DCS-F2 /1	Latex allergen Hev b 7	Q9SEM0	587	42822	30
DCS-F2 / 2	Latex allergen Hev b 7	O81984	511	42735	25
DCS-F2 / 3	Uncharacterised protein	M0RV17	113	27120	9
DCS-F2 / 4	Latex allergen Hev b 7	Q9SEM0	50	42822	10
DCS-F2 / 5	Putative uncharacterised protein	A9PF66	83	33683	4
DCS-F2 / 6	Putative uncharacterised protein	A9PF66	64	33683	4
DCS-F2 / 7	Malate dehydrogenase	F6HZK0	94	38949	5
DCS-F2 / 8	Malate dehydrogenase	F6HZK0	271	38949	19
DCS-F2 / 9	Malate dehydrogenase	B9NBW3	341	35637	28
DCS-F2 / 10	Malate dehydrogenase	F6HZK0	471	38949	29
DCS-F2 / 11	Malate dehydrogenase	FK6HZ0	207	38949	7
DCSF2 / 12	Fructose-bisphosphate aldolase	CIJ958	294	38830	9
DCS-F2 / 13	Fructose-bisphosphate aldolase	CIJ958	295	38830	9
DCS-F2 / 14	Fructose-bisphosphate aldolase	CIJ958	240	38830	9
DCS-F2 / 15	Uncharacterised protein	M0RV17	230	27120	17
DCS-F2 / 16	Uncharacterised protein	M0RV17	64	27120	9
DCS-F2 / 17	Uncharacterised protein	M0RV17	208	27120	17
DCS-F2 / 18	Profilin	I3SDB5	151	14077	24
DCS-F2 / 19	Protease inhibitor HPI	Q6XNP7	128	7542	28
DCS-F2 / 20	Protease inhibitor HP1	Q6XNP7	180	7542	31
DCS-F2 / 21	Protease inhibitor HPI	Q6XNP7	250	7542	44
DCS-F2 / 22	Protease inhibitor HPI	Q6XNP7	263	7542	44
DCS-F2 / 23	TrxH5 Thioredoxin	I1SSJ1	143	12879	33
DCS-F2 / 24	Uncharacterised protein	M1BLC8	102	14162	9
DCS-F2 / 25	TrxH5 Thioredoxin	I1SSJ1	119	12879	17
DCS-F2 / 26	Latex cystatin	E8Z9H3	296	11248	39
DCS-F2 / 27	Latex cystatin	E8Z9H3	537	11248	48
DCS-F2 / 28	Latex cystatin	E8Z9H3	297	11248	47
DCS-F2 / 29	Uncharacterised protein	M1CHX0	139	15019	17
DCS-F2 / 30	Putative uncharacterised protein	C5YHF4	186	10341	31
DCS-F2 / 31	NDK2 Nucleoside diphosphate kinase	I1SN94	259	16463	33
DCS-F2 / 32	Putative uncharacterised protein	C5YHF4	227	10341	31
DCS-F2 / 33	NDK1 Nucleoside diphosphate kinase	I1SN93	596	16338	35
DCS-F3 / 1	Protease inhibitor HP1	Q6XNP7	175	7542	41
DCS-F3 / 2	Protease inhibitor HP1	Q6XNP7	316	7542	88
DCS-F3 / 3	Latex cystatin	E8Z9H3	189	11248	29
DCS-F3 / 4	Uncharacterised protein	M0SQ12	68	10122	22
DCS-F3 / 5	Hev 2.2	Q6JYQ7	80	21855	5
DCS-F3 / 6	Hev 2.2	Q6JYQ7	73	21855	5
DCS-F3 / 7	Hev 2.2	Q6JYQ7	66	21855	5
DCS-F3 / 8	Uncharacterised protein	C5YHF4	207	10341	31
DCS-F3 / 9	Polyubiquitin	O65332	177	51316	8
DCS-F3 / 10	Peptidyl-prolyl cis-trans isomerase	F8RW92	219	17982	33

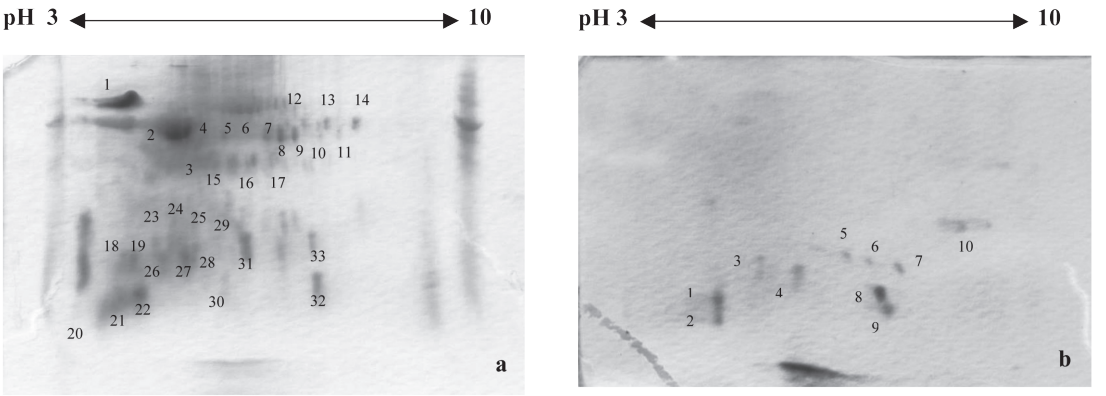

Figure 3. Coomassie blue stained protein spots of, (a) Dialysed C-serum supernatant sub-fraction 2 (DCS-F2) and (b) Dialysed C-serum supernatant sub-fraction 3 (DCS-F3), electrophoresed on a two-dimensional polyacrylamide gel (2D-PAGE). The numbers correspond to the protein spots analysed using MALDI TOF/TOF, as shown in Table 1.

apoptosis *via* activation of caspases[19], the role of the other two proteins remain elusive in cancer research. Interestingly however, Hev b 13, an allergenic esterase of latex origin[24] was recently shown to exert anti-inflammatory effects in mice with induced arthritis[25] and colitis[26], presumably by interacting with cells from the immune system, thus, raising the possibility of finding therapeutic value in other allergenic proteins.

Fructose bisphosphate aldolase detected in DCS-F2, belongs to a group of enzymes that function both in glycolysis and gluconeogenesis[27]. Members of aldolase family have been implicated in certain malignancies, for example, depletion of an aldolase isozyme was recently shown to significantly reduce lung cancer cell tumorigenicity and capability of migration[28]. To date however, there are no reports on anti-cancer activity exerted by exogenous aldolases. Similarly, thioredoxin, a protein that is involved in a spectrum of critical function in plants including programmed cell death[29] was detected in DCS-F2. This too appears to have counterparts that support cancer cell growth[30,31].

Nucleoside diphosphate kinases (NDK1 and NDK2) were detected in the active DCS sub-fractions. Transfection of NDK cDNA into highly metastatic breast, melanoma, prostate and squamous cell carcinomas and colon adenocarcinoma cells has been shown to significantly reduce the metastatic competency of the cells *in vivo*[32]. Elsewhere, intracellular NDK has been shown to interact with many proteins that are involved in cellular signal transduction in angiogenesis and tumorigenesis[33]. In addition, a number of proteins that lacked significant entries in cancer research, along with eight uncharacterised proteins and three putative uncharacterised proteins were detected in the active sub-fractions.

In conclusion, cell viability assay guided fractionation of DCS has successfully narrowed down the sub-fractions that were active against HepG2 and MDA-MB231. A number of proteins that are possible contributors to the anti-proliferative effect on human cancer origin cells have been identified from the active DCS sub-fractions. These findings call for additional preclinical investigations to

evaluate the overall beneficial effects of the active sub-fractions that would pave the way to develop an effective drug delivery system in view of eventual use in therapeutic treatment.

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