The effect of aqueous extracts of MRL products on viability of HepG2 liver cancer cells

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Certification of analysis

I certify that the analysis presented in this report has been conducted according standard practice for CytoGenex Laboratories.

……Dr Chris J Newton Ph.D.

(Chief Consultant, CytoGenex Ltd).

Background to analysis

A request was made to CytoGenex to determine whether the MRL nutritional support products, known as Coriolus (BN 03143), Reishi (BN 02194), Cordyceps (BN 03021) and Maitake (BN 03165) had any significant toxicity when tested with a standard toxicity screen. A system has been developed by Cytogenex laboratories based on the liver cell line, HepG2. Toxicity has a number of connotations, but Cytogenex Laboratories takes the term to mean that the test article causes '*no apparent loss of viability of cells used as an assay system'*. Viability in this context is taken to mean that cell integrity is maintained. Loss of viability for a cell population is equated with the occurrence of cell death by the mechanism of apoptosis or necrosis.

Apoptosis is the term given to the process of cell death where intracellular components are repackaged into membrane-bound apoptotic vesicles or alternatively, where the nucleus of the cell undergoes a process of contraction leading to the fragmentation of DNA. Necrosis is due a catastrophic loss of outer cell membrane function, leading to rupture and the spilling of intracellular contents into the surrounding medium or tissue. These processes are clearly apparent microscopically to the experienced eye and can also be detected by biochemical and molecular biological methods.

Methodology.

Cell culture

HepG2 cells were routinely maintained in vitro culture using the medium, minimal essential medium (MEM) containing antibiotics, glutamine and 10% heat-inactivated foetal calf serum (FCS). Prior to experiments cells were removed from stock flasks by the use of trypsin/EDTA solution and re-plated into 48 or 24 well plates at a density around 10,000/cm^2.

MRL product preparation

Of each of the biomass preparations, 10g was weighed into a 200ml, glass Duran bottle and 100ml of phosphate buffered saline (PBS) was added. The mixture was then brought to the boil on a hot plate and left for a further 15 minutes. The bottles and contents were allowed to cool to room temperature and 50ml of the contents from each were transferred to 50ml sterile plastic centrifuge tubes. These were then centrifuged at 1500g for 5 minutes. After this period the following supernatant volumes were recorded:

Coriolus	30ml	(20ml packed particulate material)
Reishi	27.5ml	(22.5ml packed particulate material)
Cordyceps	41ml	(9ml packed particulate material)
Maitake	30ml	(20ml packed particulate material)

The supernatant material in all cases was cloudy and prior to addition to cells in culture, a portion was filtered using a 0.2micron sterile filter system. In each case, only around 2ml was filtered, as the membrane soon became blocked.

Treatment of cells with aqueous MRL product extracts

(A) 'No challenge response'

To cells in 48 well culture plates 24h after 'seeding' as described above, aliquots of 50, 25 12,5, 6 and 3µl (10mg/ml- 0.6mg/ml final concentrations)

were added to duplicate wells on two parallel plates of cells. Following the addition of the filtered MRL-product extract, the plates were placed under the inverting microscope and at 400x magnification, particulate material could still be seen to be present even after 0.2 micron filtration. As it was felt that this material could interfere in a non-specific manner (adsorb growth nutrients) with cellular process, it was decided to add to parallel wells, the unfiltered material. The rational for this was that if the particulate material interfered with cell function in any way, the effect should be greater with the unfiltered extract, as it contained far more particulate material.

These plates of cells were then monitored daily (inverted microscope at 200 and 400x magnification) over a 5 day period for signs of cell death. On day 2 (48h after extract addition), apoptotic cell death was determined biochemically using the Roche ELISA^{PLUS} assay (Roche Applied Science Cat No. 1 774 425: Newton et al. 1999) and on day 5, the number of viable cells was monitored by the addition of the dye MTT. This yellow, diazo compound (see Newton et al.1995), is metabolised by live cells to give blue intracellular crystals. These can be solubilised and the absorbance can be read at 540nm in a multi-well scanner. Absorbance values are directly proportional to viable (live) cell numbers.

(B) Hydrogen peroxide (H_2O_2) 'challenge response'.

When added alone, a compound or an extract may have no effect on cell viability, however, an effect can often be 'unmasked' when a combination is made with a known toxic agent. One such toxic agent used in a multitude of studies is H_2O_2. This molecule is produced within cells as a by-product of mitochondrial metabolic processes and is therefore considered 'a physiological molecule'. At low concentrations, H_2O_2 is known to induce apoptosis and at higher concentration, it induces necrosis.

At a concentration chosen from the results obtained in (A) above, cells were pre-treated for 48 hours and then exposed to a dose range of H_2O_2. (Preliminary experiments suggested that HepG2 cells were sensitive to H_2O_2 over the range 200 to 600 μM). After 48h, estimates of apoptosis and

necrosis were made using the ELISA^{PLUS} and Lactate Dehydrogenase (LDH) assays respectively. The latter assay was performed using the Roche Cytotoxicity Detection Kit (Roche Applied Science Cat No. 1 644 793) according to the instructions provided. During necrosis the cell membrane is severely damaged, resulting in the release of intracellular contents, like LDH, into the culture medium.

Results and Discussion

(A) 'No challenge' response

Toxicity is usually observed from a period of 4hours to 48hours after first exposure to an agent. No obvious response was apparent from microscopic observations on cells over a period of 48h. This was confirmed biochemically by the lack of DNA fragmentation as detected with the ELISA plus assay. These data are provide in Appendix i Table 1. Over the subsequent 72h period, it was clear that in the presence of the higher doses of extracts (25 and 50 µl), cells were growing more slowly. After exposure to MTT on day 5 and the measurement of absorbance at 540nm, Figure 1 shows these data normalised as percentage of control (original data, given as absorbance values at 540nm, are provided in Appendix i, Table 2). For filtered extracts, from all four MRL products, a decrease in blue MTT colour formation was apparent across the whole concentration range. For the unfiltered extracts the decrease was slightly less pronounced, arguing against the idea that the particulate material might have interfered with cell growth. These observations would strongly suggest that soluble extracted components of the MRL products were responsible for the decrease in the rate of cell growth.

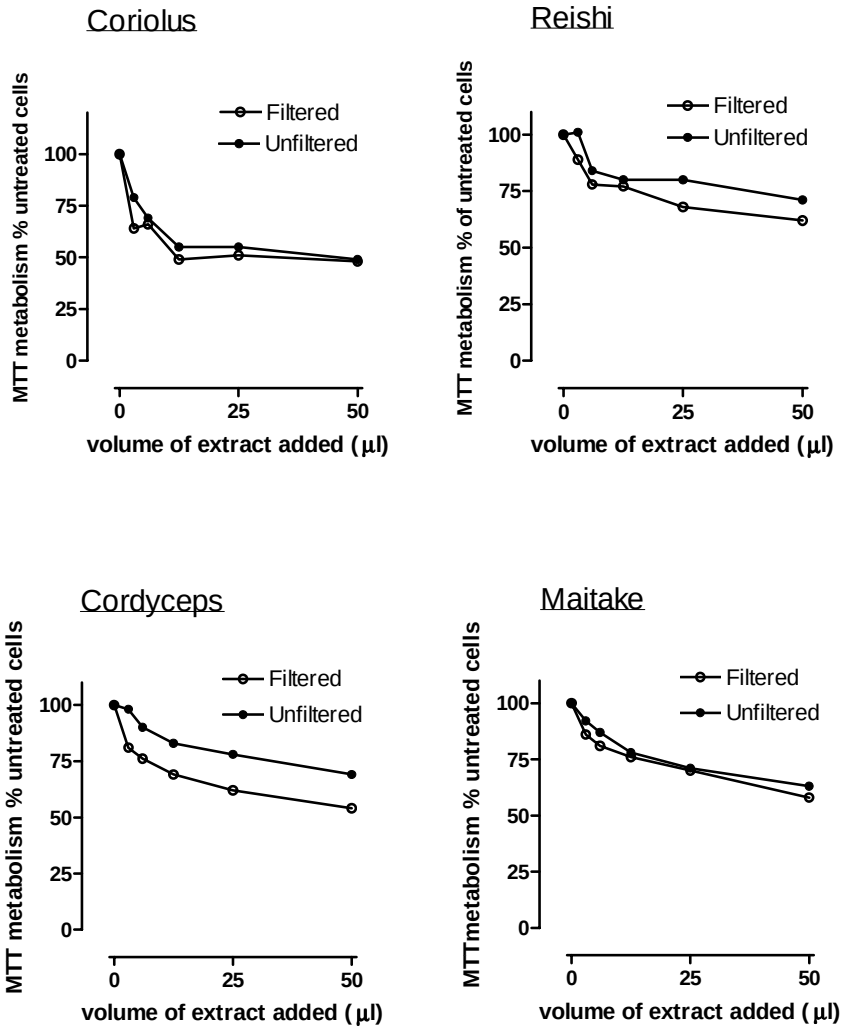

Figure 1. Effect of aqueous extracts of MRL products on the number of viable HepG2 cells maintained in culture over 5 days. Values are given as a percentage of controls for each experiment. Values along the x-axis represent the volume of extract added to 0.5ml of culture medium in which the cells were prepared. This dose range corresponds to 0.6 to 10mg/ml final extract concentrations. Original data, expressed as absorbance values at 540nm, are given in Appendix i, Table 2.

(B) H_2O_2 challenge

For these experiments, HepG2 were seeded into 24 well culture plates at a density around 10,000 per cm^2 and after 24 hours, 50µl aliquots (5mg/ml final concentration) of each of the aqueous MRL product extracts, were added to wells (apart from control wells and H_2O_2 only wells). After 48 hours, a dose range of H_2O_2 (200 to 600 µM) was added.

By microscopic observation, clear apoptosis was observed at 300 and 400 µM H_2O_2, whilst at higher doses (500 and 600 µM), cell morphology indicated that necrosis was the predominant mode of cell death. After 72 hours, MTT was added and the absorbance was measured at 540 nm, after solubilisation of the blue crystals. Figure 2 shows these results.

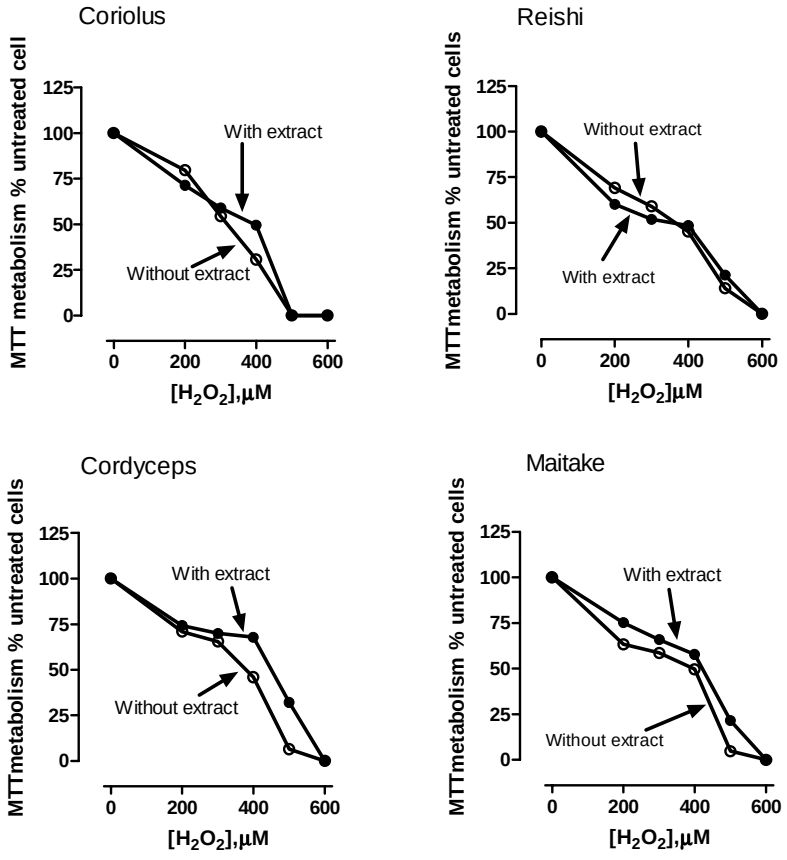

Figure 2. The response of HepG2 cells to H_2O_2 in the absence and presence of 50µl aliquots (5mg/ml final concentration) of aqueous MRL extracts.

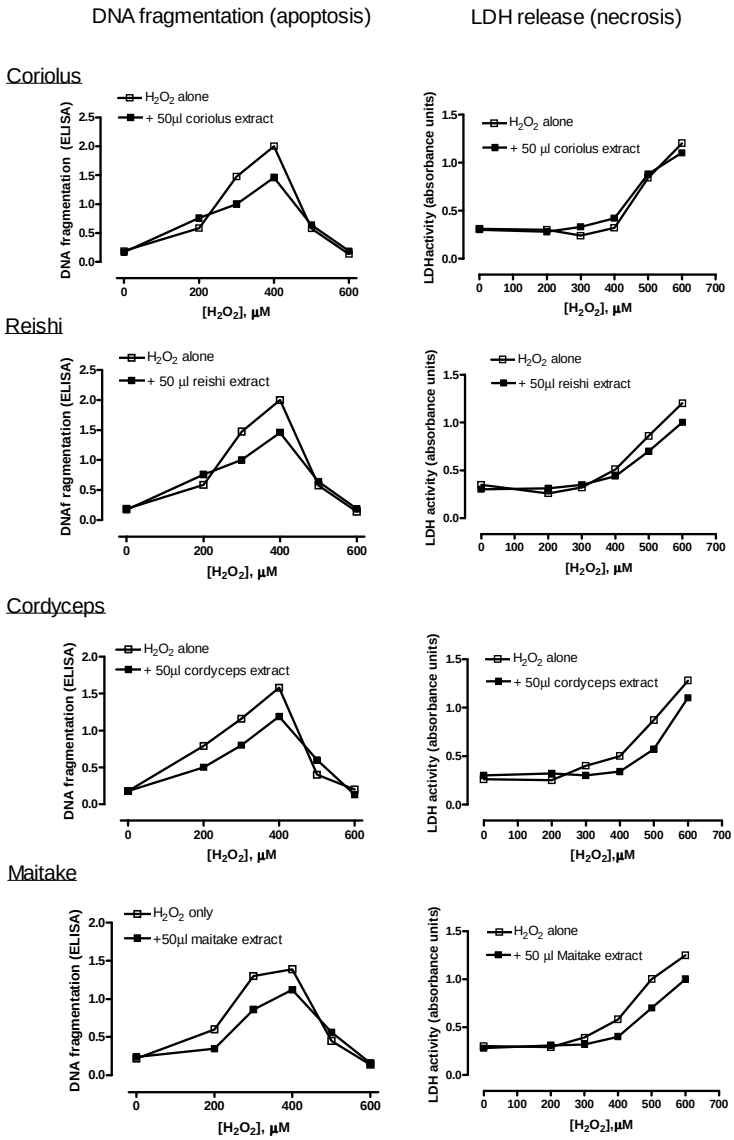

Figure 3. Apoptosis and necrosis measured in response to a H_2O_2 challenge in the absence and presence of 50µl of aqueous (5mg/ml final concentration) MRL product extracts. Values on the y-axis are given as absorbance values for untransformed data.

In a parallel experiment to that shown in Figure 2, medium was removed after 48 hours of exposure to H_2O_2 and this was used to measure LDH release, whilst the remaining monolayer of cells was processed according to the Roche ELISA^{PLUS}, apoptosis kit protocol. Figure 3 shows these data.

From data presented in Figure 2, there is the suggestion, particularly for the extract from cordyceps, that the H_2O_2-induced fall in viable cell numbers at the higher doses, is partly blocked. Although Figure 3 shows that there are less DNA fragments (indicator of apoptosis) for the extract-treated cells, it must be remembered that these cells are exposed to extract for 48 hours before H_2O_2 exposure. From the information presented in Figure 1, it is apparent that this will have the effect of reducing the initial treatment cell number, so that there will be proportionately fewer cells available to undergo apoptosis. The same reasoning can be applied to the LDH data. For all four graphs, there is a tendency for cells treated with MRL extracts to have lower values for LDH release into the culture medium. Again this is probably due to the fact that there will be lower numbers of cells present before exposure to the pro-oxidant. It is not immediately clear, therefore, from these data, why there is a smaller fall in viable cell numbers, particularly with the cordyceps extract, in the presence of 400 and 500µM H_2O_2. Usually protection at higher H_2O_2 doses is due to the prevention of necrosis. This effect is not clear from Figure 3, as it is probably masked by the difference in cell numbers at the time of treatment with oxidant. Despite these comments, the most likely explanation for the observation from Figure 1, is that the extract-induced slowing of cell growth has stabilised the cells energetically, preventing H_2O_2 from inducing such a catastrophic loss of cellular function and consequently, the onset of necrosis.

Overall conclusions

The salient experimental finding from these studies is that aqueous extracts from the MRL products, Coriolus (BN 03143), Reishi (BN 02194), Cordyceps (BN 03021) and Maitake (BN 03165) reduce the rate of growth of these HepG2 liver cancer cells. This response has nothing to do with decreasing cell viability, as the further experiments on the amount of apoptosis and necrosis in response to an H_2O_2 challenge, demonstrate.

Therefore, by the definition given in 'background', ***aqueous extracts of MRL products are devoid of toxicity in this and most probably, other cell systems***.

References

(With details and validation of methodologies used in this report)

Newton CJ. (1995) Estrogen receptor blockade by the pure antiestrogens, ZM 182780, induces death of pituitary tumour cells. J. Steroid Biochem. Mol. Biol. 55: 327-336.

Newton CJ, Drummond N, Burgoyne CH, Speirs V, Stalla GK and Atkin SL. (1999). Functional inactivation of the oestrogen receptor by the antioestrogen, ZM 182780, sensitizes tumour cells to reactive oxygen species. J. Endocrinol. 161: 199-210.

APPENDIX i

Table 1. The effects of MRL extracts on cellular DNA fragmentation measured with the ELISA^{PLUS} (apoptosis assay).

	Control	50	25	12.5	6microL
Coriolus	0.270, 0.281	0.182, 0.195	0.186, 0.192	0.262, 0.219	0.241, 0.262
Reishi	0.310, 0.292	0.210, 0.232	0.252, 0.239	0.261, 0.251	0.261, 0.282
Cordyceps	0.333, 0.327	0.261, 0.232	0.241, 0.275	0.292, 0.256	0.298, 0.310
Maitake	0.332, 0.292	0.246, 0.296	0.242, 0.221	0.259, 0.276	0.310, 0.292

Values in the table are absorbance units at 495nm. (The higher the number, the greater the amount of apoptosis).

Some cell death is always observed for untreated cells. The observation that values fall with the addition of extracts, is due to the fact that cell numbers are reduced by the presence of extracts- the fewer the number of cells, the lower the amount of basal apoptosis.

Table 2. Untransformed data from which the % control values are displayed in Figure 1. Values are absorbance units at 540nm and they are directly proportional to viable cell numbers (see Newton et al 1995).

	Coriolous (filtered)	Coriolus (unfiltered)
CTL	0.157, 0.143	0.124, 0.126
50µl	0.073, 0.071	0.066, 0.057
25	0.078, 0.074	0.080, 0.071
12.5	0.085, 0.063	0.074, 0.081
6	0.099, 0.098	0.084, 0.098
3	0.091, 0.101	0.093, 0.109
	Reishi (filtered)	Reishi (unfiltered)
CTL	0.138, 0.159	0.138, 0.128
50µl	0.085, 0.098	0.113, 0.112
25	0.099, 0.104	0.127, 0.120
12.5	0.125, 0.102	0.103, 0.119
6	0.116, 0.115	0.114, 0.128
3	0.128, 0.132	0.122, 0.139
	Cordyceps (filtered)	Cordyceps (unfiltered)
CTL	0.132, 0.155	0.154, 0.158
50µl	0.077, 0.078	0.131, 0.133
25	0.091, 0.088	0.142, 0.146
12.5	0.101, 0.104	0.134, 0.159
6	0.103, 0.115	0.144, 0.177
3	0.107, 0.125	0.166, 0.19
	Maitake (filtered)	Maitake (unfiltered)
CTL	0.185, 0.156	0.168, 0.176
50µl	0.105, 0.095	0.107, 0.123
25	0.112, 0.125	0.114, 0.132
12.5	0.121, 0.139	0.125, 0.15
6	0.132, 0.145	0.147, 0.172
3	0.137, 0.156	0.158, 0.182