

# Toxicity Effects of Chinese Herbal ‘Five-seeds’ Formulation on Human Kidney HEK-293 and Chang Liver Cells

Y.M. Teo<sup>1</sup>, Y.H. Er<sup>1</sup>, S.M. Chye<sup>1</sup> and N. K. Wong<sup>1\*</sup>

<sup>1</sup>*School of Health Science, International Medical University, 57000 Kuala Lumpur, Federal Territory of Kuala Lumpur, Malaysia*

‘Five-seeds’ formulation (*Lycium barbarum*, *Cuscuta chinensis* Lam, *Rubus idaeus*, *Schisandra chinensis*, *Plantago asiatica*) have been used by Chinese medicine practitioner for the treatment of male infertility for a long time. The present study aims to investigate the potential toxicity effects of the individual herb used in ‘five-seeds’ formulation and as a concoction on human HEK293 and Chang liver cells, using cell viability MTT assay and SubG1 flow cytometry analysis. Percentage of cell viability from samples (1, 25, 50, 75, 100 mg/mL) decreased as the concentration increased. Half maximal inhibitory concentration (IC<sub>50</sub>) values indicated that *Cuscuta chinensis*, *Rubus idaeus* and *Schisandra chinensis* are among the most toxic herb samples on HEK293 cells, with the IC<sub>50</sub> values less than 22 mg/mL. On the other hand, *Rubus idaeus* (IC<sub>50</sub> value 20.1 mg/mL) and *Schisandra chinensis* (IC<sub>50</sub> value 17.8 mg/mL) were found to have the highest toxicity effect on Chang liver cells. *Plantago asiatica* and *Lycium barbarum* (IC<sub>50</sub> more than 67 mg/mL) are the least toxic herbs tested on both human HEK293 kidney and Chang liver cells. ‘Five-seeds’ herbal formulation had IC<sub>50</sub> values of 33 mg/mL and 38.5 mg/mL on HEK293 and Change liver cells respectively, suggesting that ‘five seeds’ formulation has modulated the toxicity effect of the mixed herbs used in this formula. Apoptotic cells in SubG1 phase were also found to be significantly low for HEK293 (13.08%) and Chang liver cells (10.17%) with the treatment of 25 mg/mL of ‘five seeds’ formulation. Toxicity effect of five-seeds’ herbal formulation has seemingly been modulated when all the five herbs mixed as a concoction, and potentially non-toxic as a concoction to HEK293 kidney and Chang liver cells. However, more investigations should be done to draw a solid conclusion.

**Keywords:** ‘five-seeds’ formulation; MTT assay; flow cytometry; HEK-293 cells; Chang liver cells; *Lycium barbarum*; *Cuscuta chinensis* Lam; *Rubus idaeus*; *Schisandra chinensis*; *Plantago asiatica*

## I. INTRODUCTION

The use of herbal medicines has been increasingly popular over the past decades. Its use continues to expand rapidly across the world. Many people now adopt herbal medicines as an additional means of treatment for many critical and chronic diseases, such as heart disease, stroke, cancer, chronic respiratory diseases and diabetes (Tilburt & Kaptchuk, 2008). This is largely because herbal medicine is less intrusive and is fundamentally a highly holistic and personalised medicine (Benzie & Wachtel-Galor, 2011).

In traditional Chinese medicine, herbal decoction or formulation is prepared by combining different herbal

components. The choice of herbal combination is based on a specific principle towards the disease. To increase male fertility, the therapeutic principle used is tonification of kidney essence. ‘Five-seeds’ formulation is prepared using *Lycium barbarum* (common name; Chinese wolfberry), *Cuscuta chinensis* Lam. (common name; Chinese Dodder), *Rubus idaeus* (common name; American red raspberry), *Schisandra chinensis* (common name; magnolia-vine) and *Plantago asiatica* (common name; Chinese plantain), which has been widely used by Chinese medicine practitioners to improve male fertility since the 13th century until today (Zhang *et al.*, 2009).

\*Corresponding author’s e-mail: wongnyetkui@imu.edu.my

*Cuscuta chinensis* Lam has been used extensively in some Asian countries to treat ageing as well as inflammation, relieve pain and increase libido while *Rubus idaeus* nourishes the kidneys and enhances the strength of Jing, improves impotence and reduce excessive urination (Donnapée *et al.*, 2014). Li (1994) reported that the water extract of *Rubus idaeus* induced Leydig cells to synthesise more testosterone and raised the levels of blood testosterone in the rats. Pharmacological studies have demonstrated that *Schisandra chinensis* has biological activities, such as hepatoprotective, antioxidant, and anti-cancer effects (Cheng *et al.*, 2013; Lee *et al.*, 2018). Lee *et al.* (2018) demonstrated that deoxyschizandrin, a compound isolated from *Schisandra chinensis*, possessed anti-cancer effects, induced cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> phase and inhibited protumoural activation of tumour-associated macrophages (TAMs). *Plantago asiatica* has been reported to remove excessive heat and increase urination, treat strangury, drain dampness, inhibit diarrhea, improve the blurred vision with nebula, and dispel phlegm from the throat (Sheng & Gong, 2017). *Lycium barbarum* had been used to enhance male fertility. A study done by Luo *et al.* (2006) showed that polysaccharides from *Lycium barbarum* could improve copulatory performance and reproductive function of mouse testicular cells due to heat damage and DNA oxidative damage.

Although herbal medicines are widely perceived as natural and harmless, many of them remain untested, especially in the aspect of toxicity and maximum consumable dosage (Ekor, 2014). This could potentially cause mortality if someone consumed herbal medicine improperly (Ekor, 2014). There have been reports on toxicity when using herbs in excessive concentration. Jiangsu New Medical College reported that LD<sub>50</sub> of the ethanol extracts of *Custata chinensis* Lam was 2.465 g/kg in mice (Jiangsu New Medical College, 1997). Feeding of 0.28 g/kg volatile oil from the *Schisandra chinensis* extracts for 1-3 hours was reported of breathing difficulty, ataxia, and death cases on the mouse model (Zhang *et al.*, 1989). Water extracts from *Plantago asiatica* on K562, U937, P3HR1, HL-60 and CCRF-CEM cells were found to have an inhibitory effect, and the IC<sub>50</sub> values are in the range from 372 to 1229 µg/mL (Chiang *et al.*, 2003).

To date, though toxicity studies on some herbs have been done, the potential toxicity when all these herbs mix together

as ‘five-seeds’ formulation is unknown. When the five herbs mix as a concoction, there could have synergistic, antagonistic or additive effects among these herbs (Hussain, 2011). Hence, it is essential to investigate the potential toxicity of the ‘five-seeds’ formulation in order to ensure the herbal medicines are harmless and also to provide insight into possible risks associated with the consumption of the Chinese medicine.

In this study, the potential toxicity of the herbal extracts from *Lycium barbarum*, *Custata chinensis* Lam, *Rubus idaeus*, *Schisandra chinensis*, *Plantago asiatica* and ‘five-seeds’ formulation were investigated on HEK-293 and Chang liver cells via the cell viability and apoptosis analysis.

## II. MATERIALS AND METHOD

### A. Materials

*Custata chinensis*, *Lycium barbarum*, *Plantago asiatica*, *Rubus idaeus*, *Schisandra chinensis* (Sheng Chang Pharmaceutical Company, Taiwan); EMSURE® ACS Dimethyl Sulfoxide (MilliporeSigma, Germany); Dulbecco’s Modified Eagle’s Medium (Gibco, Grand Island, NY, USA); Denatured Alcohol 95%; Fetal Bovine Serum (Gibco, Grand Island, NY, USA); Filter paper circles (CHMLAB, Spain); 6-well tissue culture plate (ThermoFisher, USA); 96-well tissue culture plate (TPP Techo Plastic Product AG, Switzerland); plate Human Embryonic Kidney, HEK-293 (ATCC, Virginia); Chang liver cells (ATCC, Virginia), Trypsin (Gibco, Grand Island, NY, USA); MTT; PBS.

### B. Preparation of Herbal Samples

‘Five-seeds’ herbal formulation was prepared by mixing *Custata chinensis*, *Lycium barbarum*, *Plantago asiatica*, *Rubus idaeus* in a ratio of 1:1:1:1. All herbal powders (*Custata chinensis*, *Lycium barbarum*, *Plantago asiatica*, *Rubus idaeus*) and ‘five-seeds’ herbal formulation were dissolved in 20 mL of boiling water (95°C) to prepare 250 mg/mL of stock solution. The herbal solution was then vortexed around 5 to 10 minutes and recovered by vacuum filtration. The filtrate was collected and sterilised with 0.22 µm of membrane filter, freeze-dried and stored at 4°C.

### C. Cell Line and Cell Culture

Human embryogenic kidney cells (HEK-293) and Chang liver cells were cultured in 25 mM of Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine supplemented with 10% fetal bovine serum (FBS), 110 mg/L sodium pyruvate, and pyridoxine hydrochloride. Cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator. Experiments were performed with cell cultures at approximate 70% confluency.

### D. Viability Assay Using MTT Method

Effects of *Custata chinensis*, *Lycium barbarum*, *Plantago asiatica*, *Rubus idaeus*, *Schisandra chinensis* and 'five-seeds' formulation (1:1:1:1:1) on cells viability were quantitated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A total of  $1.3 \times 10^4$  to  $2.0 \times 10^4$  cells/well with a volume of 80  $\mu$ L were seeded in a sterile flat bottom in 96-well plates (TPP, Techo Plastic Product AG, Switzerland). When cells reached 70% confluency, herbal samples were added (1- 100 mg/mL) and incubated for 24 hours. After 24 hours of treatment with the samples, 10  $\mu$ L (5 mg/mL) MTT solution was added into each well, the plates were then incubated for 4 hours under 5% CO<sub>2</sub> incubator at 37°C. The MTT reagent was removed, and the formazan crystals produced by the viable cells were dissolved in 80  $\mu$ L of dimethyl sulfoxide (DMSO) and gently shaken. The absorbance was then measured by Tecan Infinite F200 Multifunctional Microplate Plate using I-Control 1.11 software at the wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of viability was calculated as  $\frac{\text{The absorbance of treated cells}}{\text{The absorbance of untreated cells}} \times 100\%$ . The effects of the herbal extracts were expressed by IC<sub>50</sub> values (half maximal inhibitory concentration), the concentration of the herbal extracts reducing the absorbance of treated cells by 50%

against the untreated cells). Each experiment was performed in triplicate under the same conditions.

### E. SubG1 Measurement by Flow Cytometry

Cells were seeded at a density of  $5 \times 10^5$  cells/well in a sterile flat-bottom 6-well plate and upon reaching 70% confluency, cells were treated with herbal samples (1-100 mg/mL) for 24 h. The untreated cells served as controls. Floating cells and adhere cells were collected and washed with PBS and subsequently fixed with ice-cold 70% (v/v) ethanol overnight at 4°C. Upon analysis, cells were washed with ice-cold PBS, centrifuged at 1500 rpm and cells were resuspended in propidium iodide (20  $\mu$ g/mL propidium iodide and 10  $\mu$ g/mL RNase in PBS) then kept for 20 min on the ice before acquired the data on a flow cytometer. The stained cells were then analysed in a FACSCalibur flow cytometer, and the data was processed using Cell Quest Pro software. The analyses were carried out by measuring the cell percentage of different phases in the cell cycle. Cell percentage in the SubG1 phase indicates apoptotic cells.

## III. RESULT AND DISCUSSION

### A. Determination of Cell Viability

The effect of *Custata chinensis*, *Lycium barbarum*, *Plantago asiatica*, *Rubus idaeus*, *Schisandra chinensis* and five-seeds formulation (1:1:1:1:1) herbal extracts on the cell viability and proliferation rates of HEK-293 and Chang liver cells were quantitated using the MTT assay. The results of cell viability and cell toxicity were shown in Figure 1 and 2. Half maximal inhibitory concentration (IC<sub>50</sub>) values of herbal samples on HEK293 and Chang liver cells is shown in Table 1.

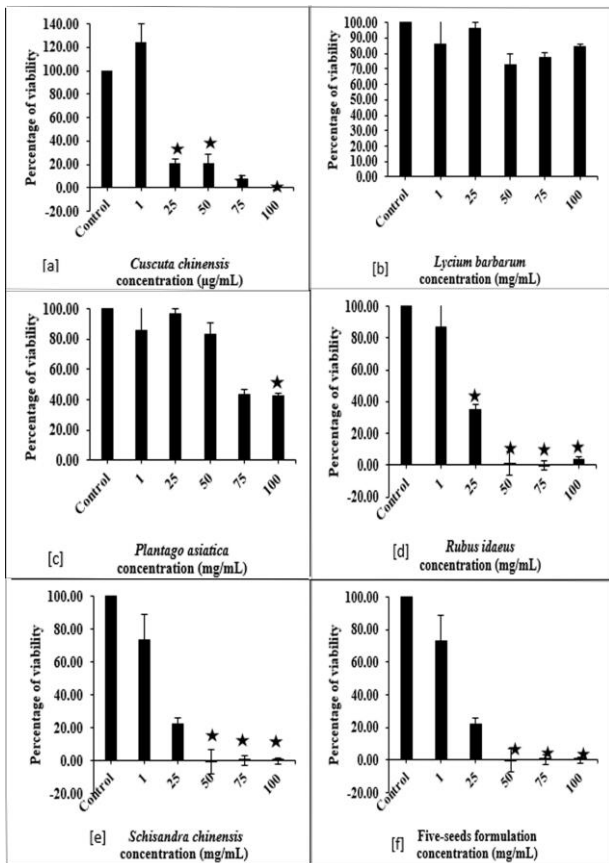


Figure 1. Percentage of cell viability of HEK293 cells after 24 h treatment with different concentration of herbal samples (1, 25, 50, 75 and 100 mg/mL): (a) *Cuscuta chinensis*, (b) *Lycium barbarum*, (c) *Plantago asiatica*, (d) *Rubus idaeus*, (e) *Schisandra chinensis*, and (f) five-seeds formulation. The results represent mean  $\pm$  SD of independent cultures in triplicate (n=3). Control represents 100 % cell viability. Asterisk indicates treatment was significantly different from the control group using Student's t-test ( $p < 0.05$ ).

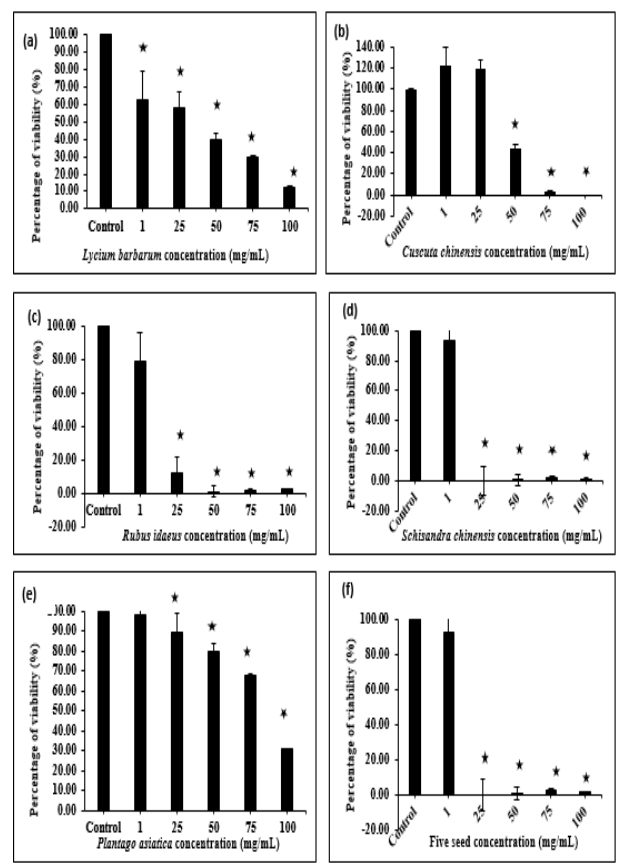


Figure 2. Percentage of cell viability of Chang liver cells after 24 h treatment with different concentration of herbal samples (1, 25, 50, 75 and 100 mg/mL): (a) *Lycium barbarum*; (b) *Cuscuta chinensis* Lam; (c) *Rubus idaeus*; (d) *Schisandra chinensis*; (e) *Plantago asiatica*; (f) five-seeds formulation. The results represent mean  $\pm$  SD of independent cultures in triplicate (n=3). Control represents 100 % cell viability. Asterisk indicates treatment was significantly different from the control group using Student's t-test ( $p < 0.05$ ).

Table 1. Doses inducing 50 % cell growth inhibition (IC<sub>50</sub>) of herbal samples on HEK-293 and Chang liver cell

Treatment	IC <sub>50</sub> (mg/mL)	
	HEK-293 cells	Chang liver cells
<i>Custata chinensis</i>	18.4	49.6
<i>Schisandra chinensis</i>	18	17.8
<i>Plantago asiatica</i>	71.5	89
<i>Lycium barbarum</i>	> 100	67.5
<i>Rubus idaeus</i>	21.2	20.1
Five-seed formulation	33	38.5

From Figure 1 and 2, it can be observed that the percentage of cell viability on HEK293 and Chang cells decreased as the concentration of herbal samples increased from 1 mg/mL to 100 mg/mL. Most of the herbal samples had significantly reduced the percentage of cell viability from 25 mg/mL onwards when compared with untreated control ( $p < 0.05$ ) on both HEK293 and Chang liver cells. From the half-maximal inhibitory concentration (IC<sub>50</sub>) values (Table 1), it is shown that *Cuscuta chinensis*, *Rubus idaeus* and *Schisandra chinensis* are among the most toxic herb samples on HEK293, with the IC<sub>50</sub> values less than 22 mg/mL. While *Plantago asiatica* and *Lycium barbarum* having high IC<sub>50</sub> values (more than 67 mg/mL) are the least toxic herbs among all herb samples tested on both HEK293 and Chang liver cells. On the other hand, *Rubus idaeus* (IC<sub>50</sub> value 20.1 mg/mL) and *Schisandra chinensis* (IC<sub>50</sub> value 17.8 mg/mL) have demonstrated to have the highest toxicity on Chang liver cells among all the herb samples tested. It is intriguing to note that 'five-seeds' herbal formulation has demonstrated to have IC<sub>50</sub> values of 33 mg/mL and 38.5 mg/mL on HEK293 and Chang liver cells respectively, indicating that the toxicity effect of the 'five-seeds' herbal formulation has somewhat been modulated when all the five herbs mixed together.

### B. SubG1 Measurement by Flow Cytometry

The toxicity effect of samples due to apoptosis can be determined by measuring the SubG1 phase of flow cytometry analysis using the propidium iodide staining method. Data from the cell cycle analysis were then presented as percentages of cells in each phase of the cell cycle, i.e., the Sub-G, G1, S and G2/M phases (Table 2 and 3). The SubG1 phase represents the apoptotic cells, while the G2/M phase represents the cells that underwent mitosis.

In the untreated control for HEK293 and Chang cells, two peaks were clearly observed at the G1 and G2/M phases (Figure 3-7), which represented 52.65 % and 18.58 % as well as 62.92 % and 23.02 % of the cells, respectively (Table 2-3). A total of 10.62 % of the HEK293 cells were in the S phase, and only 1.72 % of the HEK293 cells were in the SubG1 phase, which was apoptotic cells. While, for the untreated control for Chang liver cells, 10.50 % of Chang liver cells were in the S phase and only as low as 0.82 % (SubG1 phase) of Chang liver cells underwent apoptosis.

A similar distribution pattern was also observed when HEK293 cells were treated with herbal samples of low toxicity, i.e. *Lycium barbarum* (IC<sub>50</sub> value of >100 mg/mL). Due to the low toxicity of *Lycium barbarum*, SubG1 phase showed an only slight increase of apoptotic cells to 2.42 % and 5.28 % when treated with 25 mg/mL and 100 mg/mL respectively, of *Lycium barbarum*'s extract as compared to untreated control (1.72%). On the contrary, treatment of HEK293 cells with high toxicity herb sample; *Custata Chinese* (IC<sub>50</sub> value of 18.4 mg/mL) showed a significantly higher percentage of apoptotic cells; of which apoptotic cells were found to increase to 11.72% and 46% when treated with 25 mg/mL and 100 mg/mL of *Custata Chinese* respectively.

The high percentage of apoptotic cells in the SubG1 phase was also observed in Chang liver cells when treated with the high toxic sample, i.e. *Rubus idaeus* (IC<sub>50</sub> value of 20.1 mg/mL). SubG1 data showed that the percentage of apoptotic cells had increased significantly to 30.37% and 52.53% when Chang liver cells treated with 25 mg/mL and 100 mg/mL of *Rubus idaeus* respectively, as compared to 0.82% in the untreated control.

Worth noting that, percentage of apoptotic cells in the SubG1 phase showed that 'five seeds' formulation has modulated the toxicity effect of the mixed herbs, and the

percentage of apoptotic cells in SubG1 phase was obviously low on both HEK293 (13.08%) and Chang liver cells (10.17%) when treated with 25 mg/mL of 'five seeds' formulation; an optimal concentration close to the IC50 values of 'five seeds' formulation for both HEK293 and Chang liver cells. Astoundingly, even at a high concentration of 100 mg/mL of 'five seeds' formulation treatment on HEK293, it was found that apoptotic cells in the SubG1 phase were as low as 17.32%,

while 100mg/mL treatment on Chang liver cells was demonstrated to have 36.8% apoptotic cells.

The higher percentage of apoptotic cells observed at 100 mg/mL treatment of 'five seeds' formulation on Chang liver cells suggests 'five seeds' formulation exerts more toxic effect on the Chang liver cells than HEK293 kidney cells.

Table 2. Percentage of HEK-293 cells in the different phases of the cell cycle after treatment with *Custata chinensis*, *Lycium barbarum* and five-seeds formulation at 25 mg/mL and 100 mg/mL for 24 h

Herbs	Percentage of HEK-293 cells in each phase of cell cycle			
	SubG1	G1	S	G2/M
<b>Untreated control cells</b>	1.72	52.64	10.62	18.58
<b><i>Custata chinensis</i></b>				
25 mg/mL	11.72	33.60	10.38	17.08
100 mg/mL	46.00	16.10	2.34	0.50
<b><i>Lycium barbarum</i></b>				
25 mg/mL	2.42	37.74	12.16	28.64
100 mg/mL	5.28	31.92	9.76	14.02
<b>Five-seeds formulation</b>				
25 mg/mL	13.08	30.56	8.70	9.26
100 mg/mL	17.32	14.48	7.32	6.62

Table 3. Percentage of Chang liver cells in the different phases of the cell cycle after incubation with *Rubus idaeus* and five-seeds formulation at 25 mg/mL and 100 mg/mL for 24 h

Concentration	Percentage of Chang liver cells in each phase of cell cycle			
	SubG1	G1	S	G2/M
<b>Untreated cells</b>	0.82	62.92	10.50	23.02
<b><i>Rubus idaeus</i></b>				
25 mg/mL	30.37	25.80	13.17	1.87
100 mg/mL	52.53	1.93	1.47	0.87
<b>Five-seeds formulation</b>				
25 mg/mL	10.17	49.77	19.47	6.00
100 mg/mL	36.80	0.90	0.13	0.03

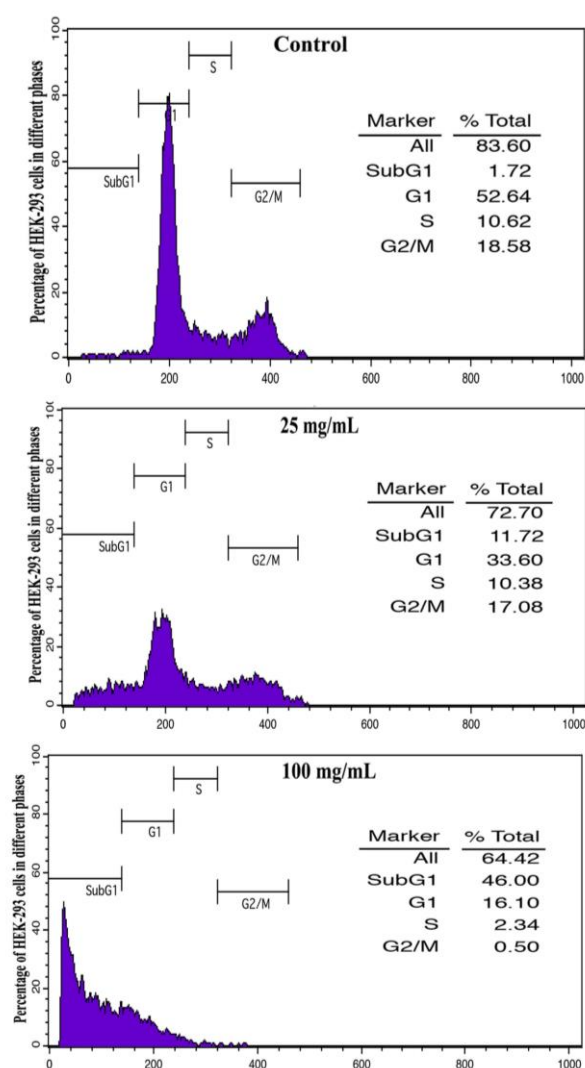


Figure 3. DNA histograms of the cell cycle for HEK293 cells treated with *Custata chinensis*, stained by propidium iodide and analysed using flow cytometry; (a) untreated control, (b) 25 mg/mL, (c) 100 mg/mL.

#### IV. DISCUSSION

Hepatic, cardiac, and nephrotoxicity are among the major concerns for organ specific drug toxicity and are the main reasons for compound termination in the drug development process (Lin & Will, 2012). Research on organ specific drug toxicity has been identified through *in vitro* and *in vivo* cytotoxicity models, and human cells are commonly used as experimental *in vitro* systems to predict human-specific drug properties (MacGregor *et al.*, 2001; Li, 2004; Li, 2001; Li *et al.*, 1999). In this study, the cytotoxicity effects of 'five-seeds' formulations were done *in vitro* mainly on the models of kidney HEK293 cells and Chang liver cells, to evaluate the toxicological effects of this herbal formulation.

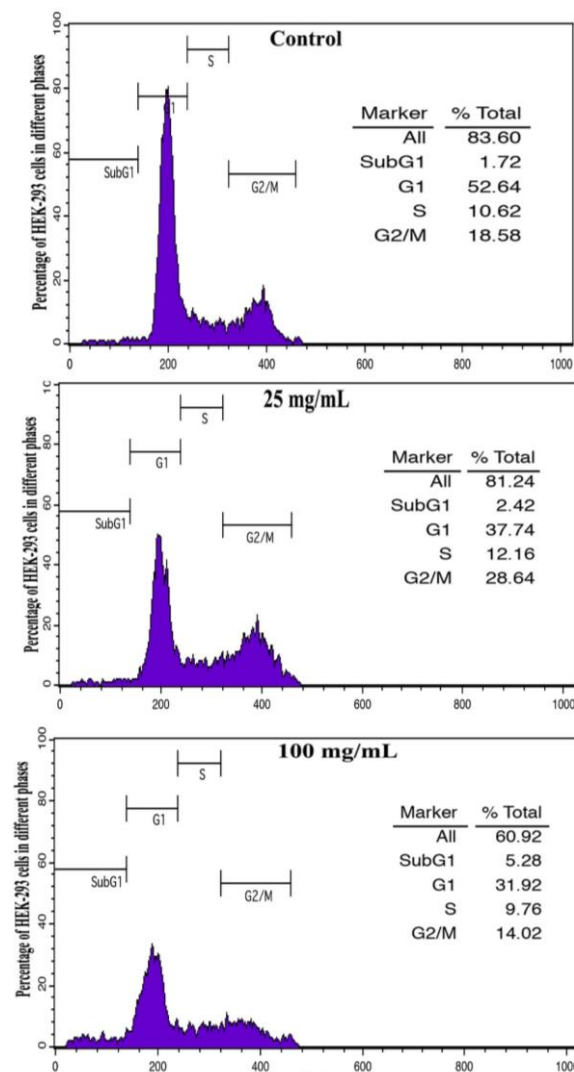


Figure 4. DNA histograms of the cell cycle for Chang liver cells treated with *Lycium barbarum*, stained by propidium iodide and analysed using flow cytometry; (a) untreated control, (b) 25 mg/mL, (c) 100 mg/mL.

Human hepatocytes are one the most useful cells to study the toxicity of drugs, as these cells conserve human specific metabolism pathways (Li *et al.*, 1999; Easterbrook *et al.*, 2001; Lee *et al.*, 1994) and have been routinely used for the evaluation of human drug properties including drug metabolism (MacGregor *et al.*, 2001; Li, 2004; Li, 2001; Li *et al.*, 1999; Easterbrook *et al.*, 2001; Lee *et al.*, 1994), drug-drug interactions (Shitara *et al.*, 2003; Li *et al.*, 1999; Li & Jurima-Romat, 1997; Lu & Li, 2001), and drug toxicity (Lloyd *et al.*, 2002; Prabhu *et al.*, 2002; Kier *et al.*, 2004). Besides, human kidney cells are also commonly used to evaluate drug toxicity because kidney cells serve as the excretory organ of metabolised drugs.

Apoptosis and inhibition of cell proliferation are the most straightforward indication to predict the toxicity of compounds. In the present study, MTT assay was done to determine the cytotoxicity of herb formulation; in which reductase enzymes in viable cells would reduce yellow tetrazolium salt to purple formazan, the absorbance of the purple formazan reflects the number of viable cells.

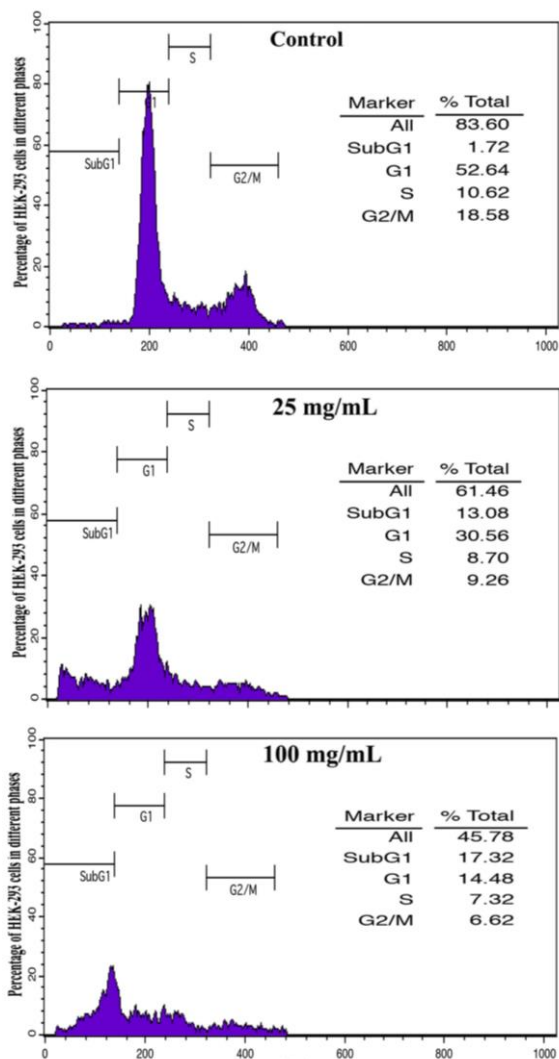


Figure 5. DNA histograms of the cell cycle for HEK293 cells treated with five-seeds' formulation, stained by propidium iodide and analysed using flow cytometry; (a) untreated control, (b) 25 mg/mL, (c) 100 mg/mL.

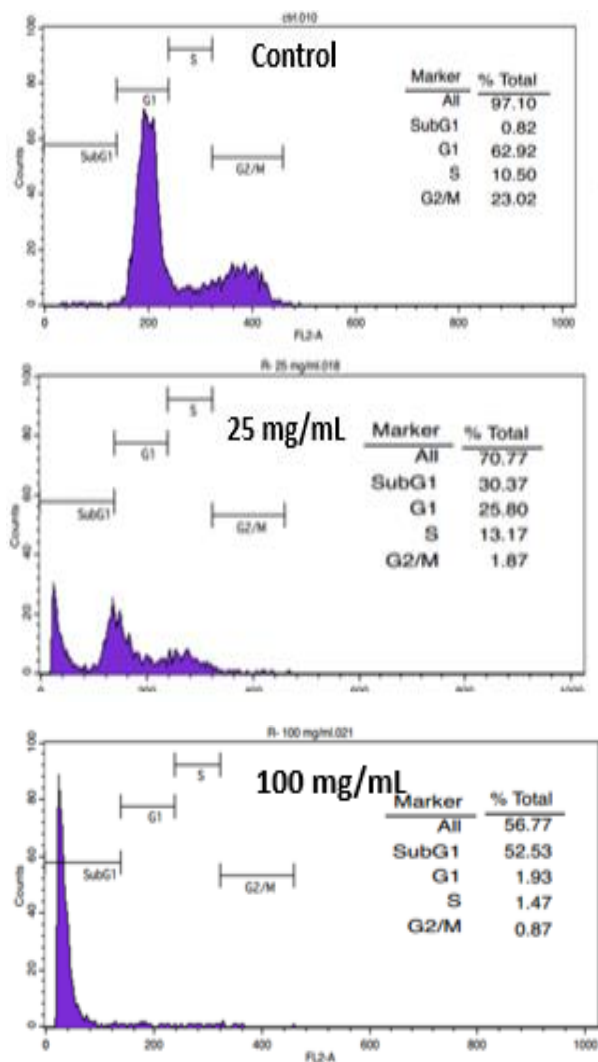


Figure 6. DNA histograms of the cell cycle for Chang liver cells treated with *Rubus idaeus*, stained by propidium iodide and analysed using flow cytometry; (a) untreated control, (b) 25 mg/mL, (c) 100 mg/mL.

The quantitative measurement of apoptosis was performed using flow cytometry. When the cells undergo apoptosis, the genomic DNA is fragmented into smaller base pairs (180 base pairs), and it would show a peak at the SubG1 phase in the flow cytometry analysis when stained with propidium iodide (PI) (Riccardi & Nicoletti, 2006; Kajstura *et al.*, 2007).

It is known that the chemical toxicity is linked with the multiple modes of cell death such as autophagy, apoptosis and necrosis (Haschek *et al.*, 2013). Autophagy is a cellular degradation process and upregulated in response to stress or nutrient deprivation, while the apoptosis and necrosis start with the increasing toxic dose and severity. If the response is excessive or prolonged intoxication to cells, it will trigger the cell death in the form of apoptosis (Haschek *et al.*, 2013).



Apoptosis is programmed cell death and is a form of cell death when healthy cells are triggered. Internucleosomal DNA fragmentation is one of the hallmarks of apoptosis which cleave the DNA into fragments of approximately 180-200 bp. It is characterised by homogeneous condensation of chromatin, cytoplasmic shrinkage, nuclear fragmentation, and blebbing of the cell membrane (Loannou & Chen, 1996). Some toxicants can induce the apoptosis and necrosis associated with higher doses and more severe toxicity (Orrenius *et al.*, 2010). Necrosis is the premature death of cells and may occur when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Necrotic cell death comprises a continuum of effects, culminating in nuclear pyknosis, karyorrhexis, and karyolysis (Loannou & Chen, 1996).

From the data of SubG1 measurement and cell viability study, it showed that percentage of apoptotic cells increased as the concentration of herb samples increased. The present data revealed that *Custata chinensis* and *Rubus idaeus* are among the most toxic samples tested on the HEK293 and Chang liver cells respectively, while, *Lycium barbarum* is the least toxic among the tested samples on both HEK293 kidney and Chang liver cells. This data is in agreement with previous research, demonstrating that water extract of *Custata chinensis* was cytotoxic on non-malignant cells (PBMC), melanoma cell line (SK-MEL-3), on and human Burkitt lymphoma (Raji) cells, with their IC<sub>50</sub> values of 3.833 mg/mL, 2.56 mg/mL and 2.06 mg/mL respectively (Ghazanfari *et al.*, 2013). Methanol fraction from *Rubus idaeus* also showed *in vitro* cytotoxic activity on two human leukaemia cell lines, J45 and HL60. *Lycium barbarum*, on the other hand, is comparatively safe to consume as the administration of *Lycium barbarum* with up to 500 mg/kg for 21 consecutive days did not result in mortalities and did not show the abnormal histopathology changes in the liver and kidney tissues in rats (van Meerloo *et al.*, 2011). The toxicological study conducted by Amagase *et al.* reported that the oral intake of the *Lycium barbarum* up to 10 mL/kg/day on rats show no toxicity, no damage and mortality on the liver, and no LD<sub>50</sub> has been found (Kim *et al.*, 2013). Based on its prolonged traditional usage without any reported toxicity, *Lycium barbarum* has been classified as a food by the Dutch authorities and the Food Safety Agent in UK (Potterat, 2010).

Notably, the IC<sub>50</sub> values of 'five-seeds' formulation on HEK293 and Chang liver cells are ranked in the middle range among all the tested herbs on both HEK293 kidney cells and chang liver cells. The data suggested that the 'five-seeds' formulation seems to modulate the inhibitory effects of cell proliferation from the other five herbal samples in this formula. As opposed to western medicine, herbal formulations are often prepared by the combination of different herbal medicines and prescribed it to the patients instead of being used singularly in large amounts. Indeed, the Chinese herbal formulations are usually used to blend the herbs to enhance their positive effects whereby able to reduce or eliminate any adverse side effects they may have (Scheid *et al.*, 2010). The Chinese practitioners always tailor the herbal formulation by employing a different combination of herbs in

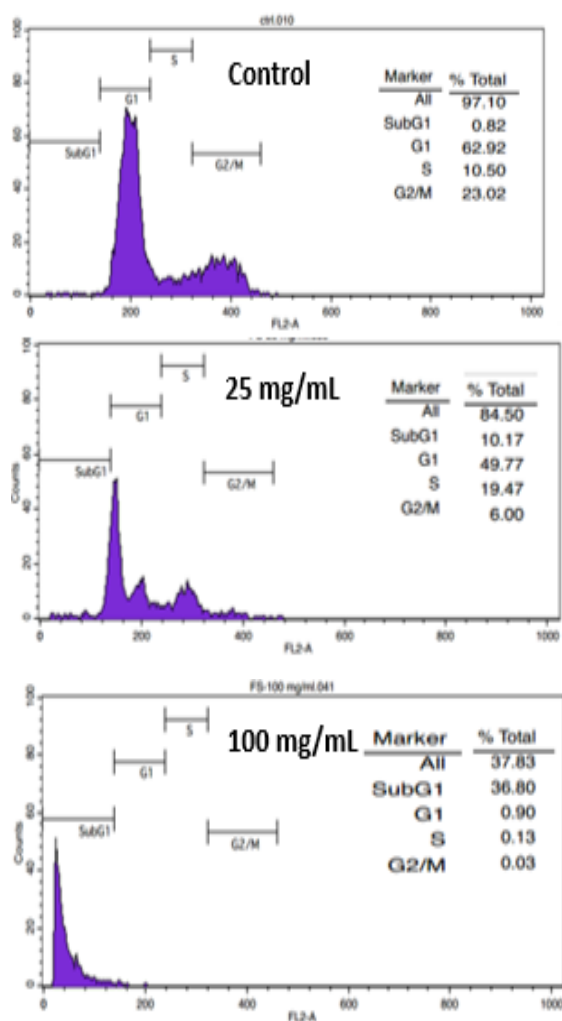


Figure 7. DNA histograms of the cell cycle for Chang liver cells treated with 'five-seeds' formulation, stained by propidium iodide and analysed using flow cytometry; (a) untreated control, (b) 25 mg/mL, (c) 100 mg/mL.

order to match precisely the signs and symptoms of each patient instead of having a standard formulation for a particular condition (Wang *et al.*, 2005). The five-seeds formulation showed a significantly low percentage of apoptotic cells on HEK293 (13.08%) and Chang liver cells (10.17%), when treated with 25 mg/mL of 'five seeds' formulation indicating five seeds' formulation, had modulated the toxicity effect when the five herbal medicine were mixed together as a concoction; therefore the percentage of apoptotic cells was lowered as compared to the treatment with *Custata chinensis* and *Rubus idaeus*.

Remarkably, 'five seeds' formulation treatment at 100mg/ml on HEK293 was found to have as low as 17.32% of apoptotic cells, and 36.8% of apoptotic cells on Chang liver cells, indicating that five seeds' formulation had less toxicity effect on HEK293 cells as compared to Chang liver cells. Combination of several herbs can definitely confer some benefits that are not available in single herbal formulation, providing better therapeutic effect and reducing the toxicity. Studies have reported that using polyherbal formulation to treat type-2 Diabetes mellitus in the rabbit model and did not show any chronic toxicity at 15 mg/kg for 90 days (Baig *et al.*, 2014). In addition, the toxicity study conducted by Rajurker *et al.* showed that no acute toxicity was observed in albino rats after consuming the polyherbal formulation up to 5000 mg/kg and no mortality was found in albino rabbits during sub-chronic toxicity studies (Rajurker *et al.*, 2009).

In parallel to our current research, we recorded that the recommended dose used by most Chinese medicine

practitioners to treat male infertility is 0.214 mg/kg per day. This recommended dosage is much lower than 25 mg/mL of 'five-seeds' formulation used in our study for the SubG1 phase flow cytometry analysis. Hence, based on the observation of this present study, 'five-seeds' formulation is potentially not toxic to the HEK293 kidney cells and Chang liver cells, though more studies to find out the minimum non-toxic dose on animal studies should be carried out to give a solid conclusion. Mechanisms of which how the 'five-seeds' formulation modulating the toxicity effect of herbs is also a crucial aspect to be investigated in the future.

## V. CONCLUSION

In conclusion, 'five-seeds' formulation has modulated the herbs toxicity effect on both HEK293 kidney and Chang liver cells, in conjunction with a significantly low percentage of apoptotic cells at 25 mg/mL treatment of 'five-seeds' formulation. Nonetheless, more toxicity studies on animal models and mechanism on how 'five-seed' formulation modulate toxicity effect among the herbal medicines in this formula should be investigated to evaluate the toxicity effect of 'five-seeds' formulation'.

## VI. ACKNOWLEDGEMENT

This study was supported by the International Medical University.

## VII. REFERENCES

- Baig, IA, Muhammed, S & Jahan, N 2014, 'Toxicity studies on herbal formulation used in diabetes mellitus', *Pakistan Journal of Pharmaceutical Sciences*, vol. 27, no. 6, pp. 2213-2220.
- Benzie, IFF & Wachtel-Galor, S (eds) 2011, 'An introduction to its history, usage, regulation, current trends, and research needs', in *Herbal Medicine: Biomolecular and Clinical Aspects*, 2nd edn, Boca Raton (FL): CRC Press/Taylor & Francis.
- Cheng, N, Ren, N, Gao, H, Lei, X, Zheng, J & Cao, W 2013, 'Antioxidant and hepatoprotective effects of *Schisandra chinensis* pollen extract on CCl<sub>4</sub>-induced acute liver damage in mice', *Food and Chemical Toxicology*, vol. 55, pp. 234-240.
- Chiang, LC, Chiang, W, Chang, MY & Lin, CC 2003, 'In vitro cytotoxic, antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*', *The American Journal of Chinese Medicine*, vol. 31, no. 2, pp. 225-234.
- Chou, WH & Integrated Chinese Medicine Holding Ltd 2007, 'Compositions and methods for prostate and kidney health and disorders, a herbal preparation', United States patent US 7223424.
- Donnapree, S, Li, J, Yang, X, Ge, AH, Donkor, PO, Gao, XM & Chang, YX 2014, '*Cuscuta chinensis Lam.*: A systematic

- review on ethnopharmacology, phytochemistry and pharmacology of an important traditional herbal medicine', *Journal of Ethnopharmacology*, vol. 157, pp. 292-308.
- Easterbrook, J, Lu, C, Sakai, Y & Li, AP 2001, 'Effects of organic solvents on the activities of cytochrome P450 isoforms, UDP-dependent glucuronyl transferase, and phenol sulfotransferase in human hepatocytes', *Drug Metabolism and Disposition*, vol. 29, no. 2, pp. 141-144.
- Ekor, M 2014, 'The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety', *Frontiers in Pharmacology*, vol. 4, pp. 177.
- Ghazanfari, T, Naseri, M, Shams, J & Rahmati, B 2013, 'Cytotoxic effects of *Cuscuta* extract on human cancer cell lines', *Food and Agricultural Immunology*, vol. 24, no. 1, pp. 87-94.
- Haschek, WM, Rousseaux, CG & Wallig, MA (eds) 2013, 'Biochemical and Molecular Basis of Toxicity', in Haschek and Rousseaux's *Handbook of Toxicologic Pathology*, 3rd edn, Academic Press, pp. 34.
- Hussain, MS 2011, 'Patient counselling about herbal-drug interactions', *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 8, no. 5S, pp. 152-163.
- Jiangsu New Medical College 1997, 'Dictionary of Chinese materia medica', Shanghai People's Hygiene Publisher, Shanghai, China, p. 2006.
- Kajstura, M, Halicka, HD, Pryjma, J & Darzynkiewicz, Z 2007, 'Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete "Sub-G1" peaks on DNA content histograms', *Cytometry Part A: The Journal of the International Society for Analytical Cytology*, vol. 71, no. 3, pp. 125-131.
- Kier, LD, Neft, R, Tang, L, Suizu, R, Cook, T, Onsurez, K, Tiegler, K, Sakai, Y, Ortiz, M, Nolan, T & Sankar, U 2004, 'Applications of microarrays with toxicologically relevant genes (tox genes) for the evaluation of chemical toxicants in Sprague Dawley rats in vivo and human hepatocytes in vitro', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 549, no. 1-2, pp. 101-113.
- Kim, EJ, Chen, Y, Huang, JQ, Li, KM, Razmovski-Naumovski, V, Poon, J, Chan, K, Roufogalis, BD, McLachlan, AJ, Mo, SL & Yang, D 2013, 'Evidence-based toxicity evaluation and scheduling of Chinese herbal medicines', *Journal of Ethnopharmacology*, vol. 146, no. 1, pp. 40-61.
- Lee, K, Vandenberghe, Y, Herin, M, Cavalier, R, Beck, D, Li, A, Verbeke, N, Lesne, M & Roba, J 1994, 'Comparative metabolism of SC-42867 and SC-51089, two PGE2 antagonists in rat and human hepatocyte cultures', *Xenobiotica*, vol. 24, no. 1, pp. 25-36.
- Lee, K, Ahn, JH, Lee, KT, Jang, D & Choi, JH 2018, 'Deoxyschizandrin, isolated from schisandra berries, induces cell cycle arrest in ovarian cancer cells and inhibits the protumoural activation of tumour-associated macrophages', *Nutrients*, vol. 10, no. 1, p.91.
- Li, Z 1994, 'Modern clinical chinese medicine', Beijing: China Medical Technological Publishers, p. 241.
- Li, AP 2004, 'In vitro approaches to evaluate ADMET drug properties', *Current topics in medicinal chemistry*, vol. 4, no. 7, pp. 701-706.
- Li, AP 2001, 'Screening for human ADME/Tox drug properties in drug discovery', *Drug Discovery Today*, vol. 6, no. 7, pp. 357-366.
- Li, AP, Gorycki, PD, Hengstler, JG, Kedderis, GL, Koebe, HG, Rahmani, R, de Sousas, G, Silva, JM & Skett, P 1999, 'Present status of the application of cryopreserved hepatocytes in the evaluation of xenobiotics: consensus of an international expert panel', *Chemico-Biological Interactions*, vol. 121, no. 1, pp. 117-123.
- Li, AP, Hartman, NR, Lu, C, Collins, JM & Strong, JM 1999, 'Effects of cytochrome P450 inducers on 17 $\alpha$ -ethinyloestradiol (EE2) conjugation by primary human hepatocytes', *British Journal of Clinical Pharmacology*, vol. 48, no. 5, pp. 733-742.
- Li, AP & Jurima-Romet, M 1997, 'Applications of primary human hepatocytes in the evaluation of pharmacokinetic drug-drug interactions: evaluation of model drugs terfenadine and rifampin', *Cell Biology and Toxicology*, vol. 13, no. 4-5, pp. 365-374.
- Lin, Z & Will, Y 2012, 'Evaluation of drugs with specific organ toxicities in organ-specific cell lines', *Toxicological Sciences*, vol. 126, no. 1, pp. 114-127.
- Lloyd, S, Hayden, MJ, Sakai, Y, Fackett, A, Silber, PM, Hewitt, NJ & Li, AP 2002, 'Differential in vitro hepatotoxicity of troglitazone and rosiglitazone among cryopreserved human hepatocytes from 37 donors', *Chemico-biological Interactions*, vol. 142, no. 1-2, pp. 57-71.
- Loannou, YA & Chen, FW 1996, 'Quantitation of DNA fragmentation in apoptosis', *Nucleic Acids Research*, vol. 24, no. 5, pp. 992-993.
- Lu, C & Li, AP 2001, 'Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague-Dawley rat, minipig, and beagle

- dog', *Chemico-biological Interactions*, vol. 134, no. 3, pp. 271-281.
- Luo, Q, Li, ZN, Huang, XL, Yan, J, Zhang, SH & Cai, YZ 2006, '*Lycium barbarum* polysaccharides: protective effects against heat-induced damage of rat testes and H<sub>2</sub>O<sub>2</sub>-induced DNA damage in mouse testicular cells and beneficial effect on sexual behaviour and reproductive function of hemicastrated rats', *Life Sciences*, vol. 79, no. 7, pp. 613-621.
- MacGregor, JT, Collins, JM, Sugiyama, Y, Tyson, CA, Dean, J, Smith, L, Andersen, M, Curren, RD, Houston, JB, Kadlubar, FF, Kedderis, GL, Krishnan, K, Li, AP, Parchment, RE, Thummel, K, Tomaszewski, JE, Ulrich, R, Vickers, AE & Wrighton, SA 2001, 'In vitro human tissue models in risk assessment: report of a consensus-building workshop', *Toxicological Sciences*, vol. 59, no. 1, pp. 17-36.
- Orrenius, S, Nicotera, P & Zhivotovsky, B 2010, 'Cell death mechanisms and their implications in toxicology', *Toxicological Sciences*, vol. 119, no. 1, pp. 3-19.
- Potterat, O 2010, 'Goji (*Lycium barbarum* and *L. chinense*): phytochemistry, pharmacology and safety in the perspective of traditional uses and recent popularity', *Planta Medica*, vol. 76, no. 1, pp. 7-19.
- Prabhu, S, Fackett, A, Lloyd, S, McClellan, HA, Terrell, CM, Silber, PM & Li, AP 2002, 'Identification of glutathione conjugates of troglitazone in human hepatocytes', *Chemico-biological Interactions*, vol. 142, no. 1-2, pp. 83-97.
- Rajurker, S, Rekhe, DS, Maini, S & Ravikanth, K 2009, 'Acute toxicity studies of polyherbal formulation (Methiorep Premix)', *Veterinary World*, vol. 2, no. 2, p. 58.
- Riccardi, C & Nicoletti, I 2006, 'Analysis of apoptosis by propidium iodide staining and flow cytometry', *Nature Protocols*, vol. 1, no. 3, pp. 1458-1461.
- Scheid, V, Bensky, D, Ellis, A, Barolet, R & Wiebrecht, A 2010, 'Chinese herbal medicine. formulas & strategies', *Deutsche Zeitschrift für Akupunktur*, vol. 53, no. 1, pp. 75-76.
- Sheng, YX & Gong, YW 2017, *Essentials of Chinese Materia Medica and Medical Formulas: New Century Traditional Chinese Medicine*, 1st edn, Academic Press.
- Shitara, Y, Itoh, T, Sato, H, Li, AP & Sugiyama, Y 2003, 'Inhibition of transporter-mediated hepatic uptake as a mechanism for drug-drug interaction between cerivastatin and cyclosporin A', *Journal of Pharmacology and Experimental Therapeutics*, vol. 304, no. 2, pp. 610-616.
- Thermo Fisher Scientific, Nuclear apoptosis assays for flow cytometry, viewed by 1 February 2018, <<http://www.thermofisher.com/my/en/home/life-science/cell-analysis/flow-cytometry/cell-health-and-viability-assays-for-flow-cytometry/apoptosis-assays-for-flow-cytometry/nuclear-apoptosis-assays-for-flow-cytometry.html>>.
- Tilburt, JC & Kaptchuk, TJ 2008, 'Herbal medicine research and global health: an ethical analysis', *Bulletin of the World Health Organization*, vol. 86, no. 6, pp. 594-599.
- Van Meerloo, J, Kaspers, GJ & Cloos, J 2011, 'Cell sensitivity assays: the MTT assay', in Cree I, (eds) *Cancer Cell Culture, Methods in Molecular Biology (Methods and Protocols)*, Humana Press, vol. 731, pp. 237-245.
- Wang, SH, Li, YM, Devinsky, O, Pacia, SV & Shachter, SC 2005, 'Traditional chinese medicine, complementary and alternative therapies for epilepsy', *Demos Medical Publishing*, pp. 177-182.
- Zhang, SC, He, B & Wang, SM 2009, 'Comparative study on the effects of Wuziyanzong Pills and Jin kui shen qi pills on Spermatogenesis function', *Zhong Guo Ji Hua Sheng Yu Xue Za Zhi*, vol. 17, no. 7, pp. 401-404.
- Zhang, TM, Wang, BE & Liu, GT 1989. 'Action of schizandrin B, an antioxidant, on lipid peroxidation in primary cultured hepatocytes', *Zhongguo yao li xue bao= Acta pharmacologica Sinica*, vol. 10, no. 4, pp. 353-356.