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COMPARISON OF CYTOTOXICITY MEASUREMENTS OF *Centella Asiatica* (L.) ON HUMAN WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS *IN VITRO* VIA MTT AND PRESTOBLUE ASSAY

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ABSTRACT

Articular cartilage tissue injury can cause permanent defect due to its incapability to self-regenerate since there is limited in vascularisation. Nowadays, stem cells are used in Autologous Chondrocyte Implantation (ACI) due to their advantages in promoting recovery and regeneration of local tissue. The ability of adipose-derived stem cells (ASC) as multipotential precursor cells and its capability of self-regenerating in large numbers are very promising. Co-culturing chondrocytes and stem cells have been found to encourage chondrogenesis, suppress hypertrophic cell development and reduce osteogenic potential. The use of biomaterial such as fibrin is suitable for its natural role in recovery and regeneration of injured tissues. Therefore, in this study we co-cultured articular rabbit chondrocytes with ASC in 3-dimensional fibrin gel to study its effect on ASC chondrogenic differentiation. Human ASC and rabbit chondrocytes were isolated and cultured until passage P3 and P2 respectively. Both cells were then trypsinized and co-cultured (1:1) in fibrin gel. Fibrin gels containing cells were harvested on Day 0 and Day 7 then, digested to retrieve cells prior to Real Time-Polymerase Chain Reaction (RT-PCR) for detection of chondrogenic gene expression Collagen Type I (Col I), Collagen Type II (Col II) , Aggrecan Core Protein (ACP) and human SOX9. Haematoxylin & Eosin and immunohistochemistry staining was done to observe cells morphology and the presence of Collagen Type II proteins. The chondrogenic gene expression for both groups showed an increase as compared to Day 0 with significance level ($P<0.05$) suggesting that chondrogenesis has occurred. Collagen Type II stain coincides with its gene expression. The results suggested that by co-culturing these two types of cells, chondrocytes does have positive effect on the chondrogenic differentiation of ASC. We propose the use of ASC to replace some of the chondrocytes used in articular cartilage tissue regenerative therapy.

1.0 Introduction

Due the high cost of conventional medicines and their side effects, a wide attention has been given on the usage of herbal products. *Centella asiatica* (L.), an important herb in traditional medicine, is getting more popular worldwide recently. Largely known as pegaga in Malaysia, it has also been called as gotu kola in Sri Lanka, brahmi in Hindi, kaki kuda in Indonesia and Indian pennywort in English [1]. It is a perennial, creeper herbaceous plant, which is native to tropical countries

and belongs to the family of Apiaceae [2]. Its efficacy as herbal remedy was well known for various diseases and widely used for centuries in Ayuverdic medicine and Traditional Chinese medicine [3].

In recent years, a growing body of literature have accumulated to highlight its effectiveness to cure various ailments. Its undiscovered potentials have been widely scrutinized and it has been documented to have anti-inflammatory, anti-microbial, anti-fungal, anti-depressant, wound healing, anti-oxidant and anti-cancer effect, signifying its potential used in clinical applications [4–9]. Its mastery as nerve tonic in Ayuverdic medicine has foreseen its potential use in nerve regeneration therapy. It was found that ethanolic extract of *Centella asiatica* (L.) has the ability to increase the neurite elongation *in vitro* and to facilitate the nerve regeneration *in vivo*, which simplifies its neurotrophic activity [10]. Meanwhile, in the other study, fresh leaves extract of *Centella asiatica* was reported to increase dendritic arborization in hippocampal CA3 of rats by, which is beneficial for the improvement of learning and memory enhancement [11]. Such effects were believed to be attributed by the biological effects of its major triterpene derivatives; namely asiatic acid, madecassic acid, asiaticosside and madecassoside, though the actual mechanism involved was still uncertain [12].

Foreseeing its potential as nerve stimulant, it will be more beneficial if it can replace the role of neurotropic factors in differentiating human mesenchymal stem cells to neural lineage. However, before realizing its application, investigating its cytotoxicity effects on the stem cells is highly important, despite, the side effects of *Centella asiatica* (L.) was rarely reported [13]. This is because of their active and/or toxic constituents is poorly understood and method of their safe use is still uncertain. In addition, inconsistency in its bioactive (triterpenoid) constituents due to its geographical distribution, manufacturing and preparations have limited its applications [14,15]. Indeed, such variations would definitely reduce its quality and efficacy, which eventually leads to different outcomes. Besides, at the very high dose, *Centella asiatica* (L.) can potentially cause skin ulceration, extreme drowsiness, nausea and abortions [16,17].

Cell-based cytotoxicity analysis was designed as a screening tool to observe the direct effects of biological substances on specialized cells [18]. During the investigation, the cells will be exposed to a particular toxicant for a short period, while the changes of treated cells were monitored via its morphology, viability and proliferation [19]. Different from cell morphology, the viability of treated cells can be measured using a variety of viability assays. They include 3 - (4, 5 - dimethylthiazolyl - 2) - 2, 5 - diphenyltetrazolium bromide reduction assay (MTT assay), resazurin (7-hydroxy-10-oxidophenoxazin) reduction assay (alamarBlue® and PrestoBlue® assay) and cell death assay. These assays employed colorimetric method, which depend on the dehydrogenase activity of viable cells in reducing reductive reagents into colored product [20]. Since those assays are easy-to-perform, many studies have used it for cytotoxicity testing of *Centella asiatica* (L.) on different cells types such as kidneys cells, lymphocytes and various cancerous cells [21–23]. This study aimed to investigate the cytotoxicity effect of raw extract of *Centella asiatica* (L.), RECA, on *in vitro* human Wharton's jelly-derived mesenchymal stem cells (hWJMSCs) for the subsequent neural differentiation of the cells. The viability of treated cells was determined using MTT and PrestoBlue assay and the performances of both assays were then compared.

2.0 Materials and methods

2.1 Human Mesenchymal Stem Cells Isolation and Cultivation

This is an exploratory study that was conducted at Universiti Kebangsaan Malaysia Medical Centre, Malaysia. The use of human sample in this study was approved by the Research and Ethic Committee of Universiti Kebangsaan Malaysia (UKM 1.5.3.5/244/FF-2015-217). Human umbilical cord samples were obtained from three consented mothers undergoing full term elective caesarean delivery (n=3). The samples were processed within one hour of collection as described in the previous study [24]. The cords were rinsed several times in sterile Dulbecco's phosphate buffer saline (DPBS) (Sigma Aldrich, US) to remove any bloodstains. The Wharton's jelly part of the cord was minced and subjected for enzymatic digestion for 2 h. After complete digestion, the cell suspension was centrifuged at 5000 rpm for 5 min to obtain a cell pellet. The pellet was washed with DPBS again and subjected for another centrifugation. Then, the pellet was resuspended in low-glucose Dulbecco's modified Eagle medium (DMEM-LG) (Gibco, Life Technology, US), containing 10% fetal bovine serum (FBS) (Gibco, Life Technology, US) and cultured in T-25 cm^2 culture flask at 37˚C in humidified 5% CO_2 incubator (New Brunswick Galaxy® 170 Series, Eppendorf, USA) for expansion. Once it reached 80% confluency, it was expended into new flasks at a density of 5 x 10^3 cells/cm^2. To ensure the consistency, passage 3 cells were used throughout the experiment.

2.2 Preparation of Raw Extract of *Centella asiatica* (L.) (RECA)

Centella asiatica (L.) plant grown at Pulau Pinang, Malaysia was used in the preparation of raw extract of *Centella asiatica* (L.), RECA. In the laboratory, the herbal plant was washed, cleaned and dried in the oven at 40˚C. A total of 50 kg powdered *Centella asiatica* (L.) leaves was extracted in five batches. In each batch, 10 kg of *Centella asiatica* (L.) powder

was extracted with 95% denatured ethanol (60 L ethanol + 40 L deionized water) for 8 hours at 60˚C temperature. A total of 14.8 L of concentrated liquid extract was obtained and it subjected to freeze drying process to obtain 7.96 kg of dried extract (15.92% yield). The dried extract was stored at room temperature until further use. The bioactive compounds of RECA, which serves as biomarker for *Centella asiatica* (L.), was determined using High Performance Liquid Chromatography (HPLC) method.

2.3 Cytotoxicity Assay of RECA

For the cytotoxicity assay, hWJMSCs at passage 3 were cultured in 48 well plate in triplicates at a density of 5 x 10^3 cells/cm^2 in DMEM-LG containing 10% fetal bovine serum (FBS) for 24 h. Then, the medium was discarded and the cells were supplemented with varying concentrations of RECA (400, 800, 1200, 1600 and 2000 µg/ml) in DMEM-LG and incubated for another 24 h at 37˚C in 5% CO_2 incubator. The control group was maintained in fresh DMEM-LG medium. At the end of the assay, the morphology of the treated cells was recorded and the cell viability was measured using two types of viability assays namely Vibrant® MTT Cell Proliferation Assay Kit (Invitrogen, Life Technology, US) and PrestoBlue® Cell Viability Reagent (Invitrogen, Life Technology, US) as described below. The IC50 value (concentration of inhibitor causing 50% reduction of cell population) was calculated from concentration-dependent curve of each assays to determine the exposure limit of RECA to hWJMSCs.

Similar procedures were simultaneously applied on human Schwann cells that were purchased from ScienCell, US. The cells were treated with different concentrations of RECA ranging from 6.25-2000 µg/ml for 7 d to observe immediate and long-term effects of RECA on neural cells, in a way to determine optimal concentrations of RECA for future neural differentiation of hWJMSCs.

2.4 MTT Assay

10 µl of MTT stock solution (12mM) with 100 µl of fresh medium was pipetted into the cells and the plate was incubated for 4 h at 37˚C in the dark. Then, 100 µl SDS-HCL solution was added to the wells and incubated again for another 4 h. The absorbance of colored product formed was finally measured at 570 nm using a spectrophotometer (Power Wave XS, Bio-TEK, USA) with the value is proportional to the amount of viable cells in the test sample. Cell viability for each RECA concentrations was expressed as percentage relative to the untreated cells, which was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{average absorbance of treated cells}}{\text{average absorbance of untreated cells}} \times 100$$

2.5 PrestoBlue Assay (Colorimetric Method)

Different from MTT assay, PrestoBlue (PB) assay is much simpler and only requires a short incubation time to monitor the cell viability in culture. During this assay, 10 µl of PB solution was added to 100 µl of fresh medium, and was incubated with the cells for 2 h at 37˚C in the dark. After the incubation, the absorbance was measured at 570 nm spectrophotometrically with background subtraction at 600 nm. The absorbance value represents the metabolic activity of viable cells following the treatment. The percentage of cell viability from this assay was calculated as described above.

The mean of coefficient of variation (CV) was calculated from IC50 values obtained from each of three individual experiments in each assay. The signal-to-noise (S/N) ratio was calculated as the ratio of the absorbance of untreated cells and the signal read in the background wells (without cells).

2.6 Statistical analysis

All the experiments with hWJMSCs and human Schwann cells were set up in triplicates with the value of the results was expressed as mean ± SEM (n=3). The data for each assay was evaluated separately and analyzed using GraphPad Prism software (v.7.00). Statistical analysis of the data was carried out using Student t-test and two-way ANOVA followed by Turkey's post Hoc t-test analysis with the significance being assumed at $p<0.05$. Inter-assay variability was determined by the mean of CV for each viability assays based on the IC50 values of each individual experiment (n). The S/N ratio of each viability assays was calculated by dividing the average of absorbance signal of untreated cells (control) by the average absorbance signal of blank (wells containing medium without cells).

3. Results

Based on the HPLC analysis, four bioactive compounds were detected in RECA (Figure 1). It was found that the extract contained madecassoside (0.0060%), asiaticoside (0.0035%), madecassic acid (0.0020%) and asiatic acid (0.0017%). In spite of bioactive compounds, RECA also yielded 206.73 ± 5.53 mg/100 g GAE of total phenolic contents.

RECA treated hWJMSCs had a dose-dependent effect and change morphology (Figure 2). As the RECA concentration increase, the cytoskeleton of the cells retracted inwards, the cells rounded up and finally detached from the bottom surface of the plate at the highest concentrations (2000 µg/ml). Meanwhile, the untreated hWJMSCs (0 µg/ml) retained its large, flat to bipolar and multipolar fibroblastic-like morphology throughout the experiment.

Table 1. Summary of IC50 value, coefficients of variation (CV) and signal-to-noise (S/N) ratio in MTT and PB assays during cytotoxicity assay of RECA

	RECA		
	IC50 ± SEM (µg/ml)	CV [%]	S/N ± SEM
PB assay (2h-endpoint)	1450 ± 20.82	2.52	0.03 ± 0.007
MTT assay (4h-endpoint)	1690 ± 0.000*	0.00	0.06 ± 0.025

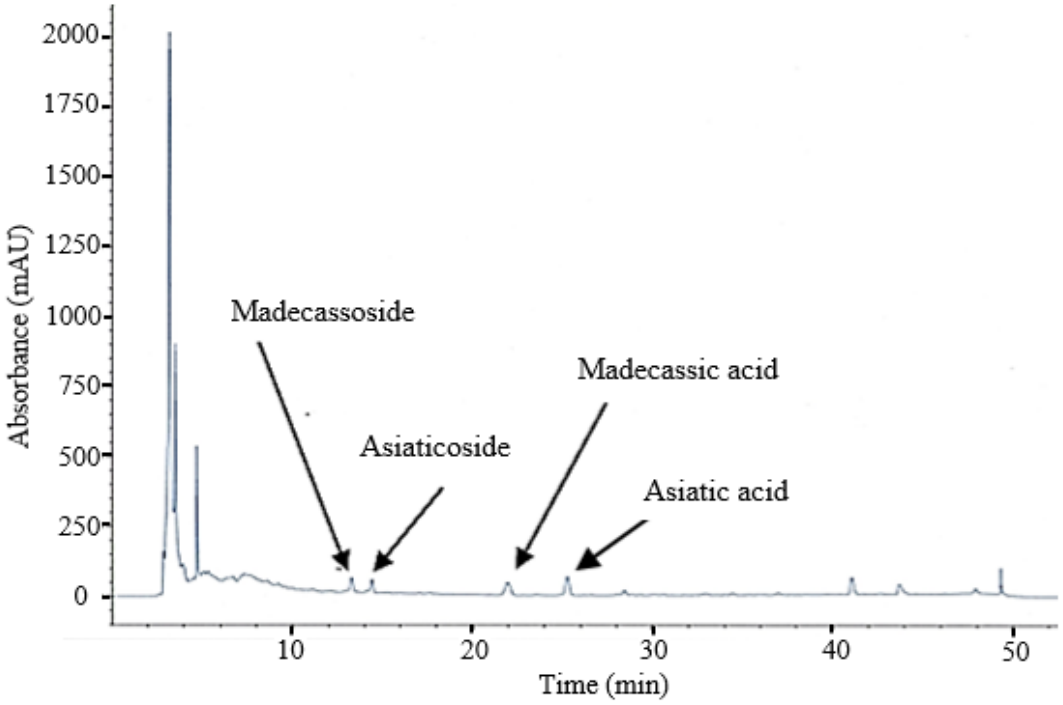

Fig.1: HPLC analysis of bioactive compounds of RECA

The cytotoxicity studies showed an inhibitory trend on hWJMSCs viability in a concentration-dependent manner after 24 h of RECA treatment. No cytotoxic effects against hWJMSCs were detected up to 1200 µg/ml. However, further increase in RECA concentration reduced the viability of hWJMSCs and at higher concentrations (1600 and 2000 µg/ml) RECA significantly inhibited the cells viability by up to 70% (Figure 3). Similar trend of RECA inhibitory effects were seen in both MTT and PB assay. Nevertheless, MTT assay showed more gradual and smooth inhibitory pattern of cell viability than PB assay as depicted in Figure 3.

The RECA concentration that causes 50% inhibition of cell viability (IC50) was calculated from both assays. Based on the concentration-response curve, the IC50 obtained from MTT assay (1690 ± 0.000 µg/ml) was significantly higher from that observed in PB assay (1450 ± 20.82 µg/ml, $p<0.05$). The inter-assay variability, expressed as coefficient of variance (CV), and the signal-to-noise ratio (S/N) were also determined to evaluate the sensitivity and reproducibility of both assays. As presented in Table 1, MTT assay has a very low CV value compared to PB assay convincingly suggesting low inter-assay variability of the assay between each run of three independent individual samples. The CV value of MTT was 2.5-fold lower than PB assay. The S/N ratio was calculated to measure the background signals on the absorbance value of the assays. The higher is the S/N value; the more sensitive is the assay. Based on the result obtained, the S/N ratio in MTT assay was (0.06 ±0.025) 2-fold higher than PB assay (0.03 ± 0.007), which suggested low background effects were detected when using MTT assay compared to PB assay.

Based on these data, MTT assay was chosen to evaluate the cytotoxicity effects of RECA on human Schwann cells, a glial

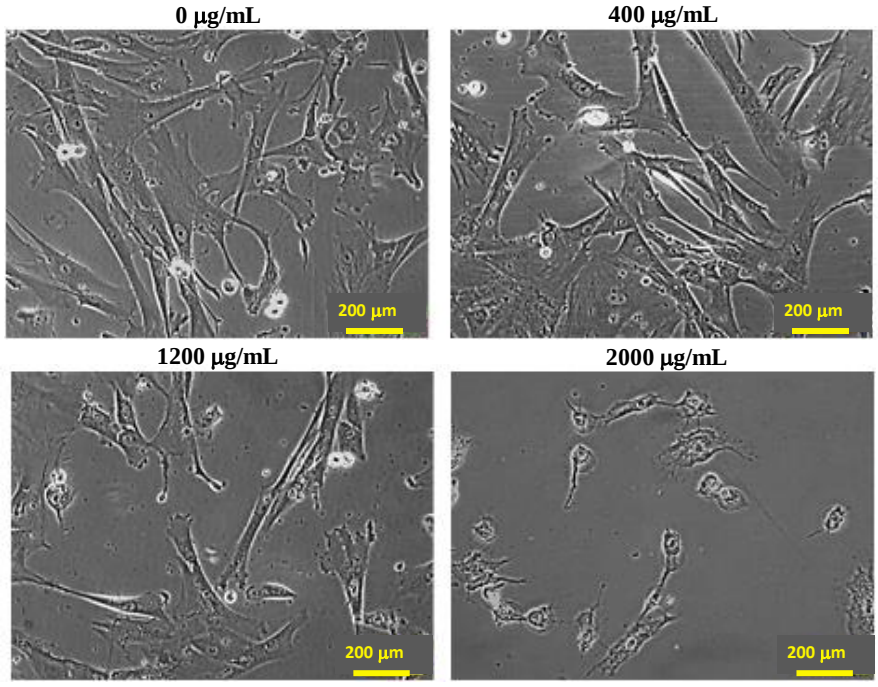

Fig. 2: Phenotypic changes of hWJMSCs following treatment with different concentrations of RECA (400, 1200 and 2000 µg/ml) (n=3). The images were captured after 24 h of RECA treatment. Scale bar, 200 µm.

cell of peripheral nervous system. As depicted in Figure 4, RECA exerted effects on the viability of human Schwann cells in a concentration-dependent manner. It showed that the viability of the cells was almost similar in all concentrations of RECA on day 1, including the untreated control. Similar trend was also seen when the cells were treated with RECA at 6.25, 25,100, and 400 µg/ml up to 7 d.

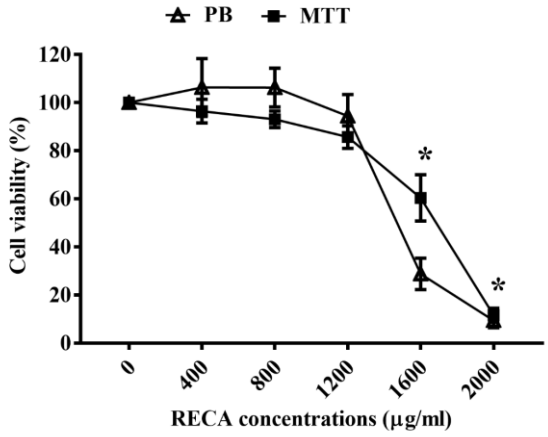

Fig. 3: Concentration-dependent effects of RECA on hWJMSCs via MTT and PB assay. The assays were performed after 24 h hWJMSCs treated with RECA at various concentrations, ranging from 400 to 2000 µg/ml. The values are represented as mean ± SEM (n=3), * indicates the significant of differences, p<0.05.

However, when treated with RECA that was beyond 800 µg/ml up to 7 d, the viability of human Schwann cells was significantly reduced. This finding demonstrated that human neural cells were intolerant to the high concentrations of RECA.

4. Discussion

The drawbacks of trophic factors has recently increased the value of natural herbs in stem cells therapy [25]. Having promising values in traditional medicines and easily accessible have eventually captured the attention of scientific community to use herbs in modern medicines. However, investigating herbal cytotoxicity at the early phase of study is very important to ensure its safety on its usage. With this regard, this study investigated the cytotoxicity effects of RECA on hWJMSCs *in vitro* in the pipeline of their applications in neural differentiation. In this study, MTT assay and PB assay were employed to observe the direct cytotoxicity effects of RECA and measure its exposure limits on hWJMSCs and human Schwann cells. hWJMSCs have been successfully isolated from human umbilical cord sample using the same materials and methods used in the previous study at our laboratory [26]. Its features as human mesenchymal stem cells have been characterized and confirmed in the aforementioned study.

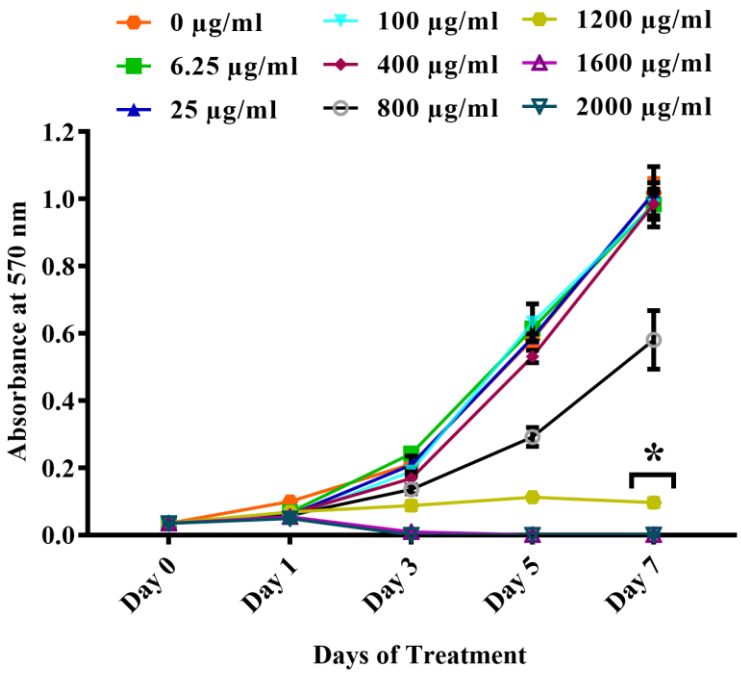

Fig. 4: Concentration-dependent effects of RECA on human Schwann cells. The viability of the treated cells was evaluated via MTT assay. * indicates the significant of differences, p<0.05.

Collectively, the present findings demonstrated that RECA exerted cytotoxic effects on hWJMSCs and human Schwann cells in a concentration-dependent manner. It was found that as the RECA concentration increased, the more inhibitory effects were seen in the viability of both cells (Figure 2 and Figure 3).

These results are in agreement with previous studies, though the analysis were detected on two different cell types [8,27]. This could be attributed by the anti-tumor and cytotoxicity properties of the triterpenes in the extract, even if the exact mechanism remains unknown [28,29]. Although those compounds exert most of the therapeutic values of *Centella asiatica* (L.), it can be harmful to cells at higher concentrations. As shown by Sampson *et al*., asiaticoside and madecassoside; major triterpenes of the *Centella asiatica* (L.), have anti-proliferative effects on keratinocyte cells [30]. Since RECA is a crude extract, other constituents such as flavonoids could also contribute to the cytotoxicity effects on both cells. In normal conditions, flavonoids, which have anti-oxidative property, act as free radicals scavengers in maintaining the homeostasis of cells [31]. However, at high concentrations it can promote cytotoxic effects [32]. The current findings revealed that both hWJMSCs and human Schwann cells are susceptible to the high concentration of RECA, where the inhibitory effects were seen particularly at 1600 µg/ml and 1200 µg/ml respectively. As the cytotoxic effects of both cells occurred at different concentrations, the data suggests RECA has a selective cytotoxicity against different cell types.

The MTT and PB assays, that were employed in this study, are cell viability assays that are commonly used in cell proliferation and cytotoxicity testing [33,34]. The cytotoxicity effects of RECA were evaluated based on the reduction in colored products formed by the treated cells. Based on the results obtained, MTT assay was found to produce more reproducible outcome in assessing the viability of the treated cells. Besides showing a gradual inhibitory pattern of hWJMSCs' viability (Figure 2), it also exhibited lower inter-assay variability (CV) and background effects (S/N ratio) in comparison to PB assay (Table 1). These findings corroborates with the previous studies [35,36], but not in agreement with Xu *et al*., who suggested that PB assay is more sensitive than MTT assay37. This discrepancy might be attributed to the different cell types used during the analysis and different modes of detection on the colored products. Since all cells have different metabolic rate, they would perform differently in an assay. In this study, PB assay was analyzed based on the absorbance approach. It has been demonstrated that colorimetric detection of PB assay is less sensitive than fluorescence version as it will generate high background effects, consequently reduces the reliability of the assay [35,38]. Pertaining to this, Gaucher and Jarraya recommended to use the fluorescence version of PB assay for the assessment of cell viability [36].

Even though colorimetric PB assay demonstrates several weaknesses, it detects the IC50 value of RECA sooner than the MTT assay in this study (Table 1). Similar finding can also be

seen in fluorometric PB assay, which was claimed to be superior to MTT in measuring the viability of human corneal epithelial cells [37]. The phenomenon can be attributed to the different mechanisms involved in each of the assays. In PB assay, the reduction of resazurin was mainly executed by mitochondrial enzymes with the involvement of cytochrome and various electron intermediate acceptors such as NADH, NADPH, FMNH and FADH [38,39]. While in MTT assay, the reduction process occurs following the reaction of mitochondrial succinate dehydrogenase [40]. Unfortunately, such reaction is not the only reaction that caused the reduction of MTT. Another evidences have signified most cellular MTT reduction was mainly mediated by the reductase activity of endoplasmic reticulum (ER) in the presence of NADH and NADPH [41]. Since metabolic rate of cells closely correlates with mitochondrial activity, hence, any physiologic interferences of cells would be detected earlier in PB assay.

Generally, the calculation of inter-assay variability (coefficient of variation; CV) and S/N ratio are not sufficient to show the superiority of MTT to PB assay or vice versa in evaluating the cytotoxicity effects of RECA. Therefore, it will be worthy if the current finding can be supported by another cell viability assay such as adenosine triphosphate (ATP) assay to validate the outcome of each assay. This is because ATP assay is known to be sensitive and less prone to the artifacts [42,43]. Thus, it is proposed that MTT or PB assay outcome should be validated with luminescence or fluorescence-based assays, which can provide more sensitive measurement to highlight the accuracy of cytotoxicity measurements of RECA on hWJMSCs.

In conclusion, RECA exerted cytotoxic effects on hWJMSCs in concentration-dependent manner. It was significantly cytotoxic to the cells at higher concentrations (>1200 μg/ml), thus, RECA concentrations of ≤1200 μg/ml would be optimal for its subsequent use in *in vitro* studies. Based on inter-assay variability and signal-to-noise ratio, MTT assay seems to be more reasonable in assessing the cytotoxicity effects of RECA on hWJMSCs. Nevertheless, other quantitative assays are needed to validate the present outcomes.

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