

## OPTIMIZATION OF SODIUM DODECYL SULPHATE AS A FORMAZAN SOLVENT AND COMPARISON OF 3-(4,-5-DIMETHYLTHIAZO-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY WITH WST-1 ASSAY IN MCF-7 CELLS

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**Submitted:** 04-05-2014  
**Revised:** 07-07-2014  
**Accepted:** 25-09-2014

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### ABSTRACT

A 3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a method that used to measure cell viability. It is based on the conversion of MTT by succinic dehydrogenase enzyme into insoluble formazan. Dissolution of formazan by using proper solvent is the most important step of MTT assay to obtain valid and reliable data. In this study, we observed several solvents [isopropanol, dimethyl sulfoxide (DMSO), and sodium dodecyl sulphate (SDS)] to validate MTT assay by using MCF-7 cells. The observation was performed by MTT addition at concentration of 0.5µg/µL, 3-4h cells incubation at 37°C, dissolution of formed formazan crystal and absorbance measurement at 570nm. The result showed that formazan completely dissolved in DMSO and 10% SDS. The most advantage of using SDS was it avoided the removal of partially dissolved formazan. In this observation, we also found that pH was a very important factor in SDS solution that affected the reaction. The use of optimal condition on MTT assay by SDS-0.01M HCl and SDS-0.025M HCl as formazan solvents showed that IC<sub>50</sub> of curcumin were 32.3±0.78µM and 24.08±1.72µM respectively, while WST-1 assay resulted IC<sub>50</sub> of curcumin 80.69±5.35µM. Altogether, this study strongly indicated that SDS-0.01M HCl was the best formazan solvent for MTT assay.

**Key words:** DMSO, MCF-7 cells, MTT assay, sodium dodecyl sulphate, WST-1 assay.

### INTRODUCTION

Cell viabilities are very important on cell kinetic study (Bernard *et al.*, 2003), cell treatment guideline (Haustermans *et al.*, 1997) as well as cell proliferation or cytotoxicity assessment (Graham-evans *et al.*, 2003). Mammalian cells can be counted by direct or indirect counting method. Direct method can be performed by using high technology equipment such as flowcytometry (Weaver, 1998) or handy automatic counter (Ongena *et al.*, 2010). However, these kinds of equipments are expensive and have not been supported for high throughput screening so that indirect methods has been widely used.

As indirect method, MTT assay, had been developed based on conversion of yellow MTT substrate into dark blue formazan (Mosmann, 1983; Berridge and Tan, 1993). The blue formazan that formed is proportional to

cell viability. The disadvantage of this assay is incompletely soluble formazan crystal in culture media so that solvent addition is needed to dissolve it. Several formazan solvents such as isopropanol (Mosmann, 1983), sodium dodecyl sulfate (SDS) (Tada *et al.*, 1984) and DMSO (Twentyman and Luscombe, 1987) have been introduced in MTT assay. Each of solvent has its characteristic so that the application to dissolve formazan is different.

Soluble forming formazan crystal reagent, such as XTT (Scudiero *et al.*, 1988), MST (Berg *et al.*, 1994), and WST, have been developed (Berridge *et al.*, 2005). However MTT assay nevertheless still has been used recently, especially because it has been widely developed to enhance data accuracy and reproducibility (Jiao *et al.*, 1992; Itagaki *et al.*, 1998). In this study we performed the optimization of MTT-formazan solvents that

commonly used, i.e. SDS, to be compared with isopropanol and DMSO. The best solvent was used to study the comparison of MTT assay and WST-1 assay on IC<sub>50</sub> curcumin determination.

## **MATERIAL AND METHODS**

### **Cell culture and reagent**

MCF-7 breast cancer cells were grown in DMEM medium (Biowest/Sigma) with supplementation of 10% of FBS (Biowest/Sigma), 100,000 U penicillin and 100µg/µL streptomycin (Invitrogen). Cells were maintained in a humid CO<sub>2</sub> incubator with addition of 5% CO<sub>2</sub> at 37°C and passaged every 3-4 days. MTT was obtained from Invitrogen, WST-1 reagent was from Roche, DMSO and SDS were from Applichem, Isopropanol and HCl were from Merck.

### **Study of MTT-formazan solvents**

To observe the needs of MTT substrate for MTT assay, MCF-7 cells were seeded at density of 1x10<sup>3</sup> to 15x10<sup>3</sup> cells/well. For further assay, MCF-7 cells were seeded at density of 5 x10<sup>3</sup> cells/well. Each of 5x10<sup>3</sup> cells/well were seeded onto 96-well plate and incubated for 48h in CO<sub>2</sub> incubator. Following incubation, culture media was discarded and MTT was added at concentration 0.5µg/µL. MTT reactions were proceeded for 3-4h before addition of formazan solvents (DMSO, isopropanol, and SDS). DMSO and isopropanol were applied to solve formazan after discarding of culture medium with or without washing of formazan with 1xPBS. After adding 100µL DMSO or isopropanol, plates were shaken and the absorbance was read at 570nm. On the other hands, 10% SDS in 0.01, 0.05, and 0.1M HCl were applied directly as formazan solvents at the end of MTT reaction, respectively. Following overnight incubation in the dark, plates were shaken and absorbance was read at 570nm.

### **Time dependent-formazan solution stability**

Overnight incubated cells were washed with PBS and added with 100µL of working media containing MTT (0.5µg/µL). Cells were incubated for 4h. For DMSO solvent observation, the media was removed and cells

were washed with PBS. The formazan crystals that formed at the bottom of the well were solved with 100µL of DMSO. The absorbance was measured at various time points (0, 5, 10, 20, 30, 60 and 120min). For SDS – HCl solvent observation (10 % SDS in 0.01 MHCl), 100µL of HCl SDS was added. The absorbance was measured at 0, 1, 2 and 18h. Each measurement performed at 570nm. The observations were applied in three replications at three different times.

### **Optimization of SDS solution as a formazan solvent**

To determine the effect of HCl concentration, SDS in 0.01, 0.025 as well as 0.05M HCl were used to dissolved formazan crystal. Measurement of absorbances were done at 570nm. To investigate the maximum wavelength that gives highest value, wavelength scan of formazan solution in SDS-HCl was carried out. A density of 50,000 cells/well was seeded onto 6 well plate and incubated for 48h. Following MTT reaction, formazan dissolution was performed by using 10% SDS in 0.01 and 0.025M HCl. Wavelength scan of formazan solution was done at 300 to 700nm. Standard formazan (Sigma) solution in SDS 0.01M HCl was also used as a positive control.

### **MTT antiproliferation assay on curcumin by using SDS-HCl as formazan-solvents compared to WST-1 assay**

MCF-7 cells were seeded at density of 5,000 cells/well and incubated overnight. Then, various concentration of curcumin (Sigma) solution 5, 10, 20, 40, 50 and 70µM were added to the cells. The cells were continued to incubate for the next 24h. The serial DMSO concentrations were also applied to the cells as control for curcumin solution. For MTT assay, at the end of incubation, medium containing curcumin was discarded by inverting the plate and cells were washed with 1xPBS. As much as 50µg/100µL MTT reagent was added and incubated again for 3-4h before addition of 10% SDS-0.01M HCl as well as 10% SDS-0.025M HCl. Absorbance of each well was measured at 570nm the next day. In addition, for preliminary WST-1 assay, 5-10% of WST-1 reagent in 100µL culture medium was added into each well after discarding of

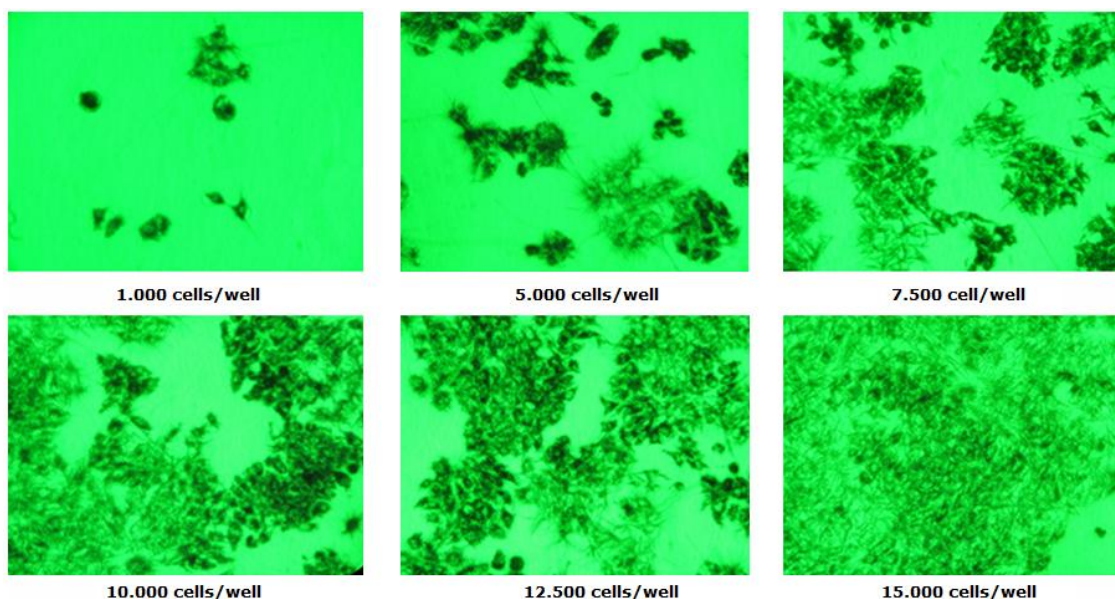


Figure 1. Formation of formazan in various cell numbers. The picture was taken after the cells incubated for 48 hour at 5% CO<sub>2</sub> and 37°C which followed by 0.5µg/µL of MTT addition and 4 hours incubation.

culture medium containing curcumin. The absorbance of soluble formazan crystal was measured at 450nm directly after 0.5, 1, 2 and 4h incubation respectively. After optimization, incubation of 10% of WST-1 was done for 0.5-1h. Absorbance values were converted to percentage of cell viabilities according to the formula=  $(OD_{curcumin} - OD_{blank}) / (OD_{DMSO} - D_{blank}) \times 100\%$

### Statistical analysis

The data was expressed as mean ± SD each performed in triplicate. Student t tests were carried out to analyze the difference significances between IC<sub>50</sub> values of curcumin obtained by MTT assay by using 10% SDS-0.01 and SDS-0.025M HCl and WST assay from three independent experiments.

## RESULTS AND DISCUSSION

### Study of MTT-formazan solvents

The determination of MCF-7 cell number required in the test, various number of cells (1,000; 5,000; 7,500; 10,000; 12,500; and 15,000 per well) were seeded and 0.5µg/µL of MTT substrate was added. The result showed that the formazan produced in every living cell for all cell numbers. Altogether, the amount of

formazan formed is proportional to the number of living cells (Figure 1).

Best formazan solvent was determined by comparing isopropanol, DMSO, and SDS. The results informed that different solvent resulted in various color of dissolved formazan ranging from purple, dark purple, and yellow (Figure 2). Isopropanol and DMSO were applied to the well after culture medium discarding and PBS washing. This step based on previous study which stated that culture medium may retard the solubilization of formazan and generated protein precipitate after interaction with solvents (Twentyman and Luscombe, 1987). Various colors were caused by solvent rapidity in dissolving formazan. Our result was in line with a study that reported by Twentyman and Luscombe (1987) which stated that DMSO is best solvent to dissolved formazan. DMSO quickly dissolved formazan and produced bright purple color which showed high optical density at 570nm. We found that isopropanol dissolved formazan slowly, but not completely even by shaking and resuspending steps. Isopropanol produced pale purple formazan solution and showed lower optical density. SDS was applied directly to

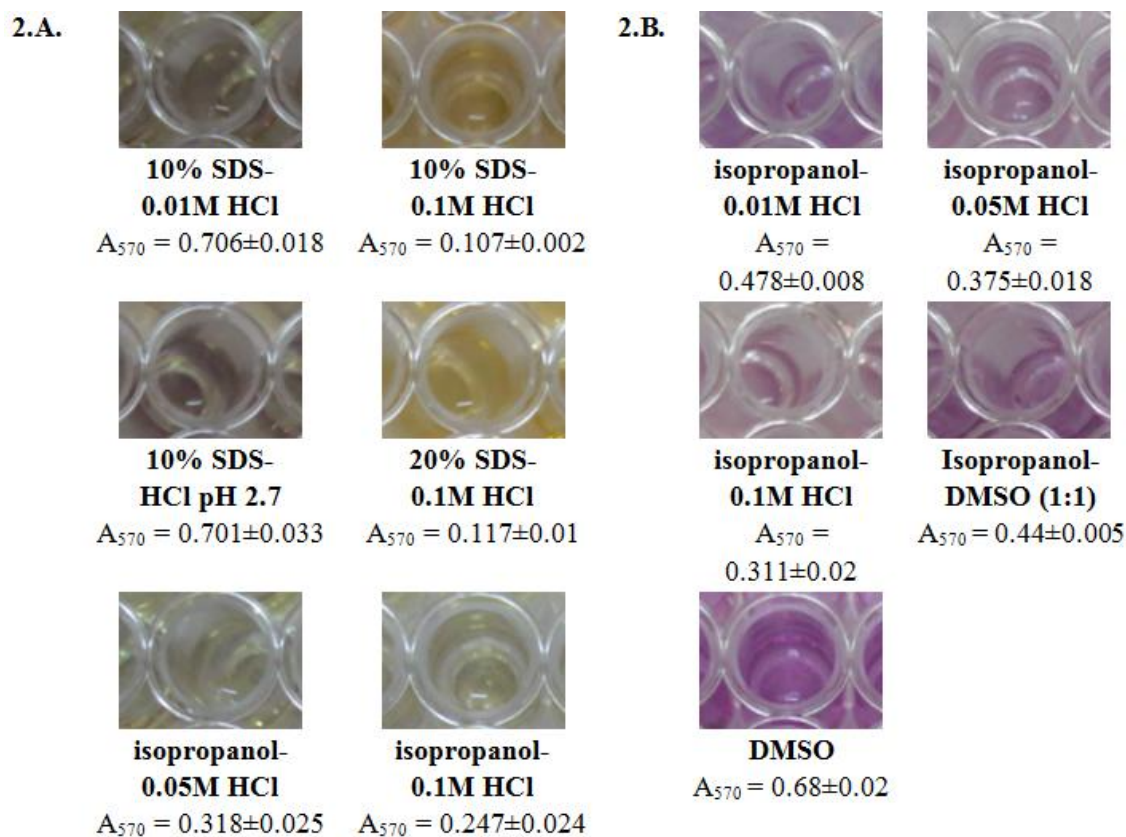


Figure 2. Various colour of culture media and dissolved formazan after addition of MTT-formazan solvents. A) without culture media discarding and B) by media discarding and PBS washing. Cells were seeded at densities  $5 \times 10^3$  cells/well. MTT solution was added at concentration  $0.5 \mu\text{g}/\mu\text{L}$ .

formazan in culture medium and incubated overnight to generate homogenous formazan solution. It produced different color of formazan solution, depend on HCl concentration in SDS solution. With more HCl concentration, the formazan solution become more yellowish (Figure 2). However, even though DMSO is the best formazan solvent, its application in MTT assay has disadvantage. It led to formazan crystals lost due to culture medium discarding and PBS washing. The use of SDS solution gave more advantage because it can be applied directly to formazan crystal in culture medium.

In our preliminary study, formazan was dissolved in solvents as follows: 1) isopropanol-0.01M HCl, 2) isopropanol- 0.05M HCl, 3) isopropanol- 0.1M HCl, 4) isopropanol-DMSO (1:1), 5) DMSO, 6) SDS 20%- 0.1M HCl, 7) SDS 10%- 0.01M HCl (pH2.53), 8) SDS 10%-

0.1M HCl pH 2.7. Solvents number 1-5 were added to formazan crystal after discarding the medium and washing with PBS, while solvents number 6-8 were added directly to formazan in the culture medium. The different color of formazan solutions that were resulted was in line with its optical density (OD) at 570nm. The highest OD was generated by the use of SDS 10%- 0.01M HCl, SDS 10%-HCl pH 2.7, and DMSO with the optimal cell number approximately 5,000 cells/wells (Figure 3).

#### Optimization of SDS as formazan solvent

SDS is one of the best choices for the formazan solvent based on its ability on avoiding formazan crystal lost. Various HCl concentrations need to observe in order to optimize solubility. We checked 10% of SDS in 0.01; 0.025; 0.05; and 0.1M of HCl. Each concentration was added to MTT formazan

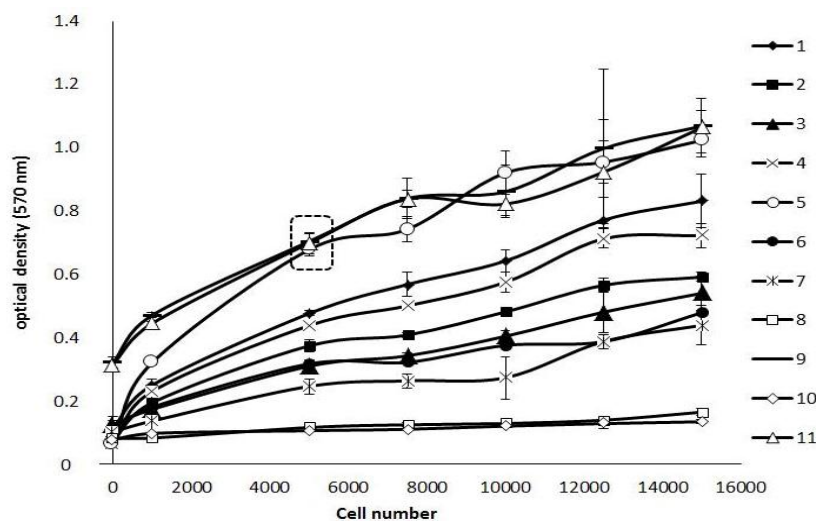


Figure 3. Optical densities of dissolved formazan from different cells numbers after solvent addition. Formazan solvent 1-5 in sequence: isopropanol-HCl 0.01M; isopropanol-HCl 0.05M; isopropanol-HCl 0.1M; isopropanol:DMSO (1:1) and DMSO were added after discarding of culture media. Formazan solvent 6-11 in sequence: isopropanol-HCl 0.05M; isopropanol-HCl 0.1M; SDS 20%-HCl 0.1M; SDS 10%-HCl 0.01M (pH 2.53); SDS 10%-HCl 0.1M; SDS 10%-HCl pH 2.7 were added to each well without discarding culture media.

and the mixture was incubated overnight in the dark. The OD measurement informed that higher concentration of HCl in SDS solution resulted in lower OD. The use of 0.05 and 0.1M of HCl gave no effect on curcumin treated MCF-7 (Figure 4A) and the best concentration of HCl was 0.01. This best concentration was used in optimization of wavelength for formazan measurement. Wavelength scanning result showed a peak at 571nm (Figure 4B). However, we found that the value of maximum wavelength was also influenced by the concentration of formazan in solution. The wavelength scanning of formazan from 0.01M HCl and 0.025M showed less difference of wave crest value. But the use of 10 % SDS - 0.01M HCl gave higher optical density and zero value at 650nm (Figure 4C).

#### Cytotoxicity Assay of Curcumin by MTT and WST-1 Assay

Validation of our optimized MTT assay was performed by curcumin cytotoxicity test. Serial concentrations of DMSO series were used as curcumin solvent control. Because the

use of HCl to eliminate the effect of phenol red in the media, to determine the effect of its concentration, two different concentration (10% SDS in 0.01M and 0.025M HCl) were tested. the absorbance value generated by the formazan solution in 10% SDS - 0.01M HCl is higher than the formazan solution in 10% SDS - 0.025M HCl. Curcumin  $IC_{50}$  calculations were  $32.3 \pm 0.78$  for 10% SDS - 0.01M HCl and  $24.08 \pm 1.72 \mu M$  for 10% SDS - 0.025M HCl. To compare the results with WST-1 assay, we developed preliminary tests such as WST-1 reagent estimation and optimum incubation time.  $IC_{50}$  value of curcumin obtained from WST-1 assay was  $80.69 \pm 5.35 \mu M$  (Figure 6).

SDS solution is widely use to stop MTT reaction. However, the detail procedures are sometimes different. Young *et al.* (2005) reported the use 20% SDS- 0.2M HCl and Ramachandran *et al.* (2005) reported the use of 10% SDS-0.01M HCl. Our study informed that HCl concentration in SDS solution influenced the absorbance values. HCl concentration that higher than 0.01M resulted in more yellowish,



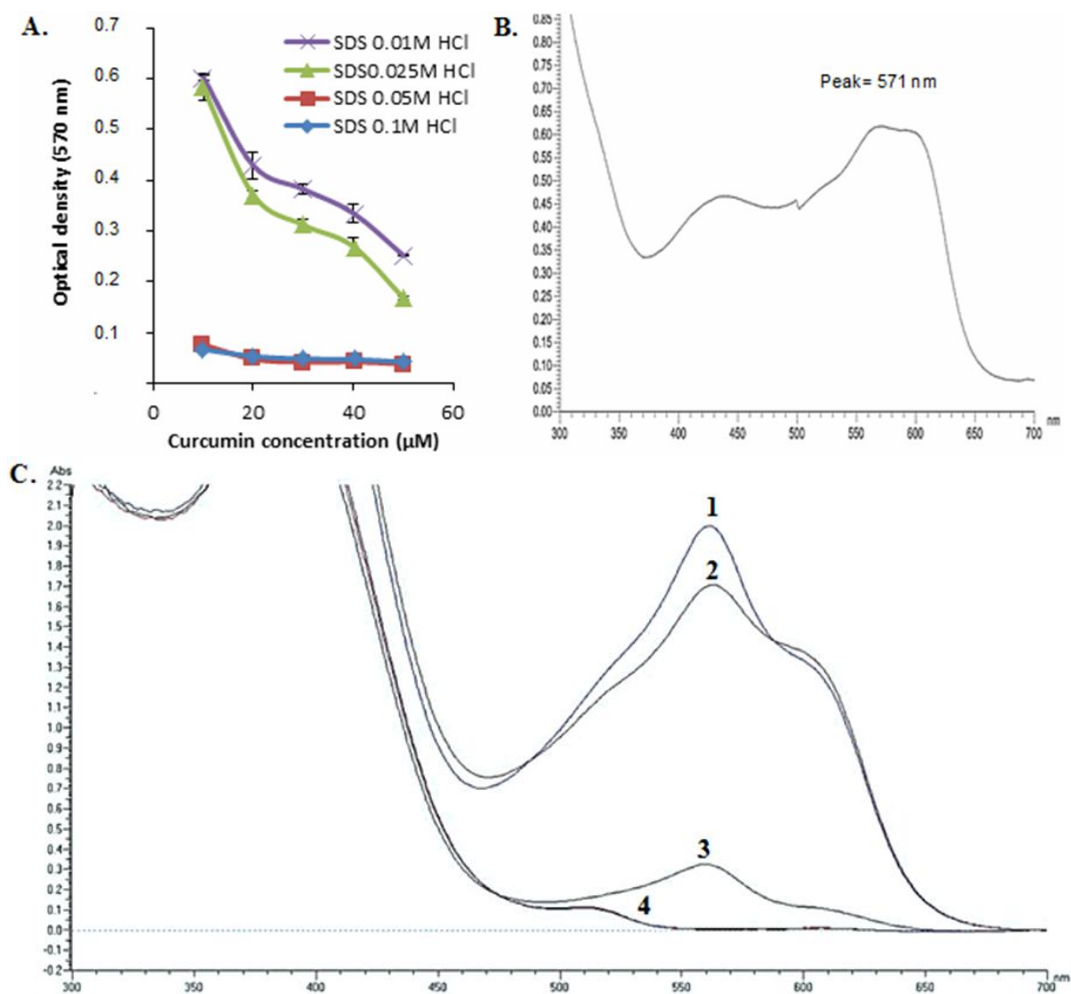


Figure 4. Optimization of SDS solution as a formazan solvent. A: determination of HCl concentration effect in SDS. 0.01, 0.025 as well as 0.05M HCl were used. B: Wavelength scan of standard formazan solution in SDS 0.01M HCl as a control. C: maximum wavelength determination for dissolved formazan. Formazan of each well was solved by using 1) 10% SDS in 0.01M and 2) 0.025M HCl, 3-4) MTT in media solved by 10% SDS in 0.01M and 0.025M HCl as blanks.

and 0.05 and 0.1M of HCl produced yellow color and different cells viability was no longer observed. Our study also informed that  $\text{IC}_{50}$  of curcumin from 10% SDS- 0.01M HCl or 10% SDS-0.025M HCl in MTT assays and WST-1 assay were significantly different.

WST-1 is a soluble tetrazolium that produces soluble formazan. WST-1 is cell impermeable thus minimize the toxicity to the cells. Moreover, WST-1 is not absorbed by the cells and reduction of WST-1 into formazan performs extracellular (Berridge *et al.*, 2005).

Gold standard methods for proliferation assay in mammalian cells are by the use of radioactive compounds such as [ $^3\text{H}$ ]thymidine or [ $^{125}\text{I}$ ]Iododeoxyuridine that can incorporate into DNA during cell proliferation (Mosmann, 1983; Russel and Vindelov, 1998). However, these methods cannot be used in general laboratories. In addition, it is difficult to carry out thousand assays in one day. Three methods as alternatives to radioactive assay are MTT, XTT, and sulforhodamine B (SRB) assays (Boyd, 1997).

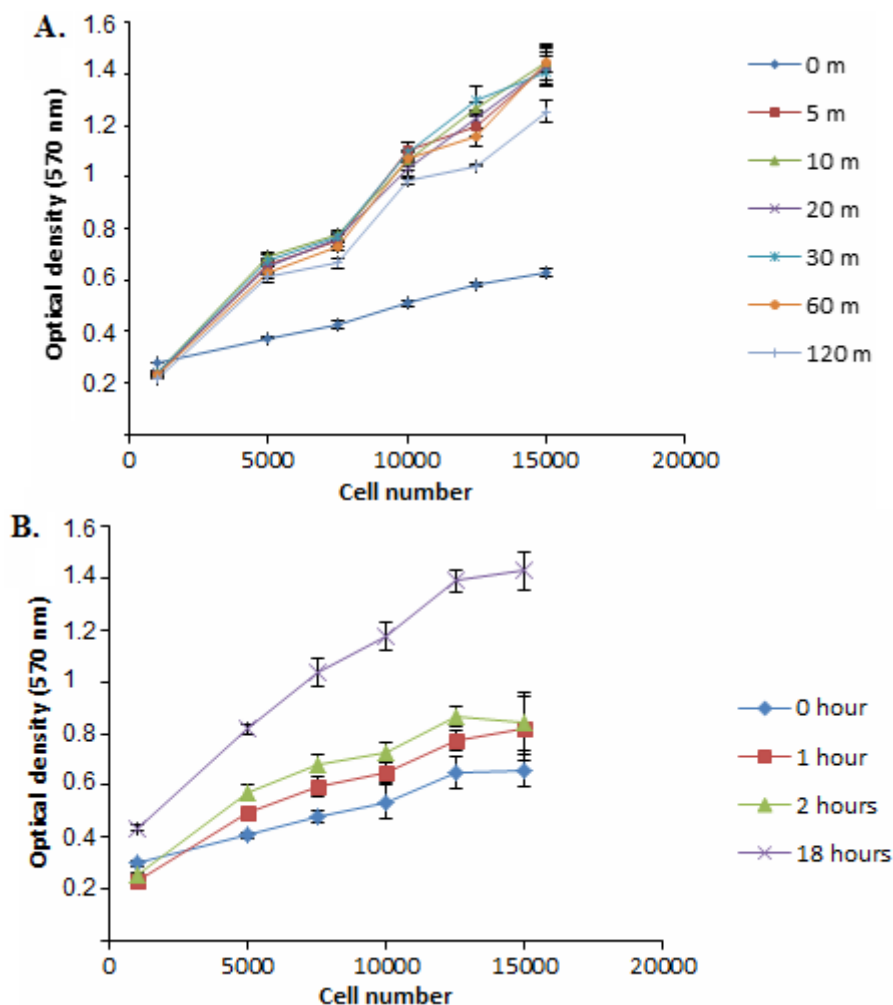


Figure 5. The Solubility and stability of dissolved formazan in A) DMSO and B) SDS-0.01M HCl.

Similar to WST-1 assay, XTT produces soluble formazan that diminish solubilization step of formazan. Unfortunately that It has higher background compare to MTT assay. The result is also affected by end point incubation of XTT in the cells. Another method, SRB assay, has performed enhanced screening capacity and reproducibility compare to XTT and MTT assay although its application is more laborius (Rubinstein *et al.*, 1990). Some of MTT assay are using DMSO as formazan solvent (Zhong *et al.*, 2012). In our study, the use of SDS as formazan solvent showed more stabile formazan solution compare to DMSO. The use of SDS as formazan solvent also showed more reproducible data than DMSO (data not shown).

Zou *et al.* (2011) reported that IC<sub>50</sub> of curcumin (24 hour incubation in MCF-7 cells obtained by MTT assay) by using isopropanol as formazan solvent is approximately 40µM. Zhong *et al.* (2012) reported that IC<sub>50</sub> of curcumin for 48 hours incubation in the same cells was about 50 µM by using DMSO. Moreover, Ramachandran *et al.* (2005) stated that curcumin shows IC<sub>50</sub> about 29µg/mL or 78.72µM by MTT assay with SDS 10%-HCl 0.01M. In addition, Zaidi *et al.* (2011) tested curcumin cytotoxicity in the same cells for 48 hours by SRB assay and reported IC<sub>50</sub> curcumin about 35 µM. Various IC<sub>50</sub> result may due to different application of MTT assay, such as use formazan solvent, incubation time, compound solvent, and also PBS washing.

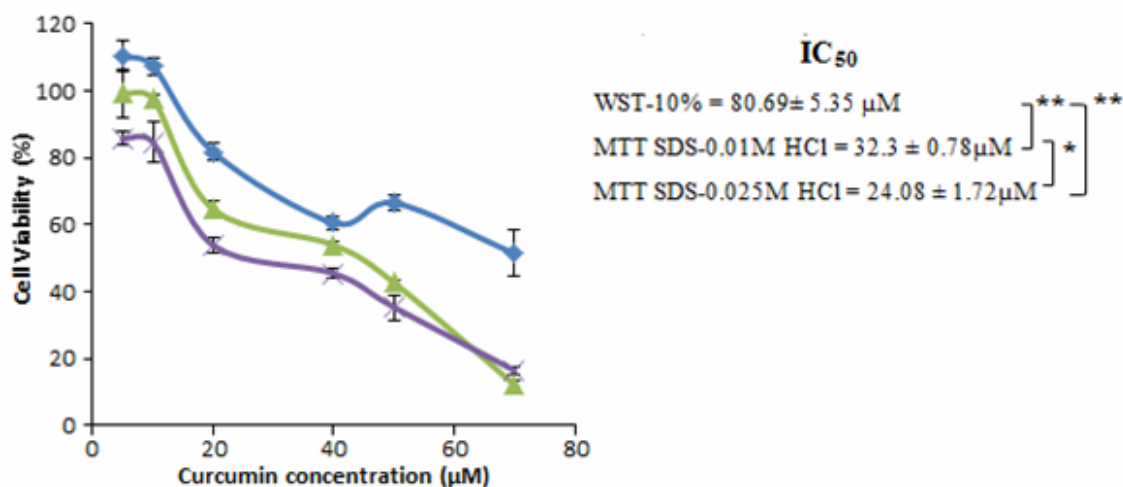


Figure 6. MTT antiproliferation assay on curcumin compared to WST-1 assay. ◆ = 10% WST in medium ■ = 5% WST in medium ▲ = MTT assay by SDS-0.01M HCl × = MTT assay by SDS-0.025M HCl. \*The difference was considered to be very statistically significant. \*\*The difference was considered to be extremely statistically significant. P<0.05.

It has been known that several solvents show low cytotoxicity, and DMSO shows more cytotoxicity compare to ethanol (Klawitter *et al.*, 2012).

MTT assay with the use of 10% SDS solution in 0.01 M HCl as a formazan solvent is a convenient method to obtain reliable data. However, for best result of MTT assay on different cell lines, optimization of cell number, needs of MTT substrate, and HCl concentration in SDS solution should be performed (Boyd, 1995). The use of phenol red free medium will allow SDS without HCl addition. On the other hands, serial concentration of compound solvent should be performed as control. In this case, DMSO showed low cytotoxicity to MCF-7 cells. Trivedi *et al.* (1990) reported that DMSO showed cytotoxicity to HeLa cells.

## CONCLUSION

Our study of DMSO, isopropanol and SDS as formazan solvent for MTT assay recommended SDS as the best solvent. HCl concentration in SDS solution should be further studied to obtain valid and reliable data. The use of HCl concentrations that higher than 0.025 M lowered the absorbances significantly and undifferentiated accumulating formazan that formed by viable cells. IC<sub>50</sub> values of

curcumin obtained from MTT assay by using 10% SDS - 0.01M HCl, MTT assay by using 10% SDS - 0.025M HCl, and WST-1 assay were significantly different.

## ACKNOWLEDGEMENT

This research was supported by DIPA 2013 grant of Indonesian Institute of Sciences (LIPI). Authors are very grateful to Dr. Andria Agusta from Research Center for Biology LIPI as well as Dr. Apon Z. Mustofa, Ms. Larasati and Mr. Khairul Anam from Research Center for Biotechnology LIPI for precious technical assistances.

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