INTRODUCTION

Micro RNA (miRNA) is a new ribonucleotide acid of non protein coding, containing 19-25 pairs of bases and plays a role in inhibiting gene expression. MiRNA was discovered by Victor Ambros and had been developed since year 2000 [1, 2].

We interested in studying miRNA because it can be used as cancer diagnosis and therapy. To get the best results, the study should determined miRNA 146 A as the target gene, looking for reference genes and optimization of protocols. Use of unreliable reference genes for normalization may lead to inaccurate quantification of miRNAs [3, 4] and the use of a single reference genes for normalization leads to large errors and inappropriate result [5, 6].

RT-PCR at this time has become a necessity to support molecular biology research and before doing experiment we perform optimization. To optimize reference gene of microRNA there are several approaches or optimization that recommended by previous researchers. Some miRNAs that have been recommended are hsa-mir-103, hsa-mir-423-3p, hsa-mir-191, hsa-mir-16, hsa-mir-423-5p, and hsa-let-7a [2, 4, 7-8]. Based on the data that has been collected, we selected four candidates for reference genes those are mir-16, mir-21, mir-103, and mir-423-3p.

Chlorogenic acid can be isolated from various traditional medicines; strawberry, betel leaves and coffee. Chloropreventive chlorogenic acid isomerizations have not been reported. Isomerization of chlorogenic acid have been reported with 3 isomerizations formed between certain cinnamic acid (trans) and (L)-quinic acid at positions 1 (OH), tetrahydroxycyclohexanecarboxylic 3.4/5 acid. Isomerization of chlorogenic acid have been reported with 3 quinic acid isomerizations at position 3, (3-CQA), 4 (4-CQA) and 5 (5-CQA). Isomerization at positions 1 and 6 have not been reported.

Chlorogenic acid molecular weight is 354, it is important to know for the calculation of the therapeutic dose to be used for cell lines Hep-G2. Chlorogenic acid as a potent anti cancer, antioxidant in vitro and therefore contribute to the prevention of Type 2 diabetes mellitus, cardiovascular disease, antiviral, antibacterial, antifungal, and chemopreventive.

MATERIALS AND METHODS

Chlorogenic acid

structurally, chlorogenic acid/CGA [chlorogenic acid] are esters formed between certain cinnamic acid (trans) and acid (L)-quinic (1L-1 (OH), tetrahydroxycyclohexanecarboxylic 3.4/5 acid. Isomerization of chlorogenic acid have been reported with 3 quinic acid isomerizations at position 3, (3-CQA), 4 (4-CQA) and 5 (5-CQA). Isomerization at positions 1 and 6 have not been reported.

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Cultured hep-G2

Hep-G2 cell obtained from SCI-Kaligen Jakarta, culture performed with medium (DMEM/F12, Gibco containing 10% Fetal Bovine Albumin (Sigma) and antibiotics penicillin-streptomycin (100 mg / L). Cells were incubated at 5% CO2 and 37°C. Passage was performed on day 3.

Hep-G2 cell viability

Performed by culturing Hep-G2 cells with medium (DMEM/F12, Gibco) containing 10% Fetal Bovine Albumin (Sigma) and antibiotics penicillin-streptomycin (100 mg / L), the number of cells per well 0.5 x 10^4 and planted in 96 well with DMSO different concentrations of 1%, 0.5% and 1%. Were analyzed using Elisa Reader and in DMSO concentration of 0.1% Hep-G2 cells can live 100%.

MTT assays

We used MTT product from sigma, Principle of MTT method was the separation of tetrazolium MTT salt (3-(4,5 dimethyl-thiazole-2-yl) -2,5-diphenyltetrazolium bromid) by reductase succinate tetrazolium enzyme system which was in mitochondria living cells so that purple formazan crystal was formed. The color intensity could be read with ELISA Reader. A living Cell still have active metabolism activity so that MTT will be disparted by reductase succinate tetrazolium enzyme in cell's mitochondria and formed purple formazan crystal. The result from toxicity test of chlorogenic acid was analyzed using excel. The results was in the form of living cells percentage (cell viability) with the equation Y=189.81-193.67X using excel. The results was in the form of living cells percentage

Chlorogenic acid intervention

Chlorogenic acid interventions was performed at 48 hours after Hep-G2 cell cultured and 60-80% confluent. Assessment was done at the 0, 2nd, 8th, 18th, and 24th hours.

Total RNA isolation

Isolation of total RNA was performed by Exiqon protocol, using miRCURY™ RNA Isolation Kit with code of product 2030110. Isolates obtained from 1 ml of Hep-G2 cell culture which had been resuspension, then made pellet by centrifugation at 1500 rpm for 5 min. Pellets stored at -70°C. Concentration and purity of RNA was assessed using the Gene Quant RNA/ DNA with code 80-2105-20.

Preparation of cDNA

Total Isolation of RNA is performed by Exiqon protocol using RNA Isolation Kit™ miRCURY with code of products 203300 from Exiqon (stage 1 to stage 5).

After having obtained total isolate RNA, conversion into cDNA was performed using a cDNA synthesis kit Universal protocol 203300.

Determination of mir-16

Mir-16 (product code number 204409) obtained from Exiqon through PT.Scienceswerk.

Determination of mir-21

Mir-21 (product code number 204230) obtained from Exiqon through PT.Scienceswerk.

Determination of mir-103

Mir-103 (product code number 204663) obtained from Exiqon through PT.Scienceswerk.

Determination of mir-423p

Mir-423-3p (product code number 204488) obtained from Exiqon through PT. Scienceswerk.

Validation RT-PCR

RT-PCR machine used Bio Rad CFX96, with protocol number 10203300. Data were analyzed with Bio Rad CFX Manager (Bio Rad, Jakarta). RT-PCR performed with a final total volume of 10 μl consisting of: a) 5 μl SYBR Green master mix, b) 1 μl primer mix, c) 4 μl cDNA template. Followed by initial denaturation at 95°C for 10 minutes. The miRCURY LNA™ Universal RT-PCR microRNA protocol is a two part protocol consisting of strand cDNA synthesis and Real time PCR amplification.

RT-PCR amplification of miRNA 146 A and mir-423-3p from Hep-G2 was performed using the CFX-96 system with the SYBR Green kit (Exiqon, Denmark, USA). Data were analyzed using the Bio-Rad CFX manager 2.1 software (Applied Bio Rad, Denmark, USA). RT-PCR was carried out in a 20 μl final volume containing: a) 5x Reaction buffer 4 μl, Nuclease free water; b) 9 μl Enzyme mix 2 μl; c) Synthetic spike in, optional replace with H2O if omitted 1 μl; d) Template total RNA (5 ng/μl) 4 μl. Following Incubate for 60 minutes at 42°C, heat inactivate the reverse transcriptase for 5 minutes at 95°C, immediately cool to 4°C and store at 4°C or freeze.

RT-PCR amplification was carried out in a 10 μl final volume containing: a) SYBR Green master mix 5 μl; b) PCR primer mix 1 μl; c) Diluted cDNA template 4 μl. Following initial denaturation at 95°C for 10 minutes, temperature cycling was initiated. Each cycle consisted of denaturation at 95° for 10 second, annealing at 60°C for 1 minute. A total of 40 cycles was carried out.

Data analysis

Data was analyzed using Bio-Rad CFX Manager 2.1 software (Hercules, California). The relative expression of reference genes (mir-16, 21, 103 and 423-3p) was normalized using Norm Finder software [10] and universally applicable quantification model based on efficiency correction, error propagation and multiple reference gene normalization [11-12].

Average values of triplicate Cq were converted to relative quantities for Norm Finder [10].

RESULTS

To examine the effect of chlorogenic acid on cell morphology, HepG2 cells were treated with 727 μM chlorogenic acid for 24 hours and morphological changes were observed by microscopy. The images showed that the chlorogenic acid induced severe morphological changes of cell death including rounding and shrinkage of cells (Figure 3). The morphological difference with Hep-G2 control that attach to the plate (figure 2).

The MTT test is a cytotoxic test to assess effect of chlorogenic acid against Hep-G2. The optimization of the test was done with variety of time and dose to obtain the optimal concentration of IC50 against Hep-G2. The opt imization of the test was done with variety of time and dose to obtain the optimal concentration of IC50 against Hep-G2. The optimiztion of the test was done with variety of time and dose to obtain the optimal concentration of IC50 against Hep-G2. The optimiztion of the test was done with variety of time and dose to obtain the optimal concentration of IC50 against Hep-G2. The optimiztion of the test was done with variety of time and dose to obtain the optimal concentration of IC50 against Hep-G2.

To determine the control gene from target genes of miRNA, we do a series of preliminary test based on the literature that has been published. The results are shown in figure 5, 6, 7, 8 and analyzed using NormFinder software.

Figure 5 shows mir-423-3p Cq values, the red line (control) and the blue line (chlorogenic acid) for 24 hours. Based on the results, it is concluded that chlorogenic acid could inhibit growth of Hep-G2 as shown in figure 4 [13]. Furthermore, we determined the value of IC50 chlorogenic acid which is obtained on the concentration of 727 μM. After IC50 was determined, we proceed to assess the viability of Hep-G2 with 3 concentrations of DMSO 0, 1, 5 and 1%. The best concentration for viability cell was DMSO 0,1%.

To determine the control gene from target genes of miRNA, we do a series of preliminary test based on the literature that has been published. The results are shown in figure 5, 6, 7, 8 and analyzed using NormFinder software.

Figure 6 shows mir-16 Cq values, the red line (control) and the blue line (chlorogenic acid) for 24 hours. Based on the results, it is concluded that chlorogenic acid could inhibit growth of Hep-G2 as shown in figure 4 [13]. Furthermore, we determined the value of IC50 chlorogenic acid which is obtained on the concentration of 727 μM. After IC50 was determined, we proceed to assess the viability of Hep-G2 with 3 concentrations of DMSO 0, 1, 5 and 1%. The best concentration for viability cell was DMSO 0,1%.
Fig. 2: Cells hep-G2 at 0 hour (without chlorogenic acid)

Fig. 3: Cells hep-G2 at 24 hours after treated chlorogenic acid

Fig. 4: Percentage of hep-G2 viability and linear regression equation to determine IC₅₀ doses of chlorogenic acid

Fig. 5: The expression mir-423-3p after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

Fig. 6: The expression mir-16 after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

Fig. 7: The expression mir-21 after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

Figure 7 shows mir-21 Cq values, the red line (control) and the blue line (chlorogenic acid) with dose 727 µM. Based on the results of the t test with P=0.246 higher than 0.05, there is no difference between the value of Cq mir-21 control and mir-21 treated with 727 µM dose chlorogenic acid.

Fig. 8: The expression mir-103 after treated with 727 µM chlorogenic acid, red line for chlorogenic acid and blue line for control

Figure 8 shows mir-103 Cq values, the red line (control) and the blue line (chlorogenic acid) with dose 727 µM. Based on the results of the t test with P=0.440 higher than 0.05, there is no difference between the value of Cq mir-103 control and mir-103 treated with 727 µM dose chlorogenic acid.
**CONCLUSION**

These results are very useful for researchers who will conduct the study using Hep-G2 cells with an active compound chlorogenic acid. Based on the data that has been discussed, we conclude the reference genes candidate for miRNA 146 A treated chlorogenic acid is mir-423-3p.

**CONFLICT OF INTERESTS**

Declared None

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**Author’s contribution**

Conception and design, obtaining of funding, provision of study materials, collection, assembly, analysis, and interpretation of the data, statistical expertise, drafting of the article: AS, HH, HT. Critical revision and final approval of the article: ST, HS, FW.

**REFERENCES**