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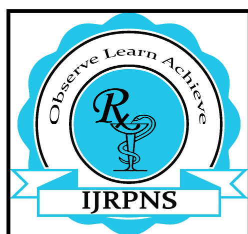

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ROLE OF CHLOROGENIC ACID FROM LAMPUNG ROBUSTA COFFEE AGAINST GENE EXPRESSION OF MIRNA (MICRO RNA) 146 A ON HEPATOCELLULAR CARCINOMA CELLS

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ABSTRACT

The use of herb to cure cancer takes a concrete breakthrough. It means that it is very essential to have a development on herb referring to a standar quality, efficacy and safety. Therefore, we need an analysis against the mechanism of herb on molecular level. Chlorogenic Acid (CA) is an active compound isolated from traditional plants from robusta coffee that is used as a chemopreventive therapy Hepatocellular Carcinoma (HCC), allegedly works as an anticancer and prevents the cell from destruction, also inhibits the growth of the cancer cells through the inhibition of free radicals. The research was done by in vitro, by using the Cell Lines Hep-G2 series 1886, obtained from Riken Cell Bank-Tohoku University. The aim of the research is how to understand the mechanism of CA in inhibiting HCC growth by using the model of Cell Lines Hep-G2 series 1886 through a series of gene expression. The type of research is experimental on 72 sample groups. 1 group consists of 250 thousands cancer cells. The research uses 3 doses of CA: 727, 500 and 250 µM, with 3 times repeated measurement, and then compared after and before the exposure. The role of those doses CA against Hep-G2 is analysed by comparing the expression of miRNA 146 A, before and after the exposure on 0, 8, 18 and 24 hours. The data is tested statistically with different test, t, repeated measurement, pearson and multiple linear regression.The lowest expression decrease of miRNA 146 A happened in the group 24 hours after the exposure at doses of 727 µM CA (0.85), followed by 500 µM (1.28) and the highest expression increase happened at a dose of 250 µM (1.61), continued with a statistical test at the 8th and the 18th hour Cq values miRNA 146 A significant difference $p<0.05$ at all doses CA (727,500 and 250 µM).The conclusion of the research is CA plays the important role on the reduction of miRNA 146 A expression.

KEYWORDS

Chlorogenic Acid, Hepatocellular Cancer and miRNA 146 A.

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INTRODUCTION

It is needed a concrete and continuing effort to invent some new anticancer compound with effective and selective activity and safe for the body. There are about 40,000 plants in the world and 30,000 are reported founded in Indonesia. But only 940 kinds can be utilized as a traditional treatment.[1]

The use of herb to cure cancer takes a concrete breakthrough. It means that it is very essential to have

a development on herb referring to a standard quality, efficacy and safety. Therefore, we need an analysis against the mechanism of herb on molecular level.

The research is a molecular biology research that is beneficial for the health and it has just been invented because the CA active has got high capacity in reducing the growth of HCC cell (Hepatocellular Carcinoma) with the model of Cell Lines Hep-G2.[2, 3, 4]

MicroRNA (miRNA) is a new ribonucleotide acid with non-protein-coding characteristic, 19-25 base pairs size, acts in inhibiting gene expression. MiRNA was developed around the year of 2000 and invented by Victor Ambros.[5]

On mammals, miRNA was founded as a long RNA transcript (among hundreds of nucleotides dozens of kilobase), called as a mikroRNA primer (pri-miRNA), like the structure of hair ribbon. Pri-miRNA, consisting of miRNA with multiple coding order, is cut shorter becomes precursor miRNA (pre-Mirna). The reaction is done by complex protein which is called microprocessor, involving Drosha with Rnase III enzyme. Pre-miRNA is a short loope with ~70 nucleotides size, on 2 nucleotides that are connected on the position of 3′UTR.This is the pre-RNA exported from nucleus by Exportin-5, and was cut in sitoplasma by the support of Rnase III enzyme, producing 19-25 multiple miRNA nucleotides. MiRNA with some unstable pairs are divided into RNA induced silence complex (RISC), consisting of TAR RNA Binding Protein (TRBP) and Ago2 protein (Argonaute).[6]

The role of CA in inhibiting the growth of Cell Line Hep-G2 using the gene path of miRNA 146A as a symbol and showed by the decrease of gene activity of miRNA based on the research done by Gang Chen, Dianzhong Luo, Yiwu Dang and Zhenbo Feng in 2010.[7]

The research is a molecular biology research that beneficial for the health related with miRNA 146 A, can be used as a diagnostic, prognosic and connected to HCC therapy. The research used Cell Line Hep-G2 (1886 series) obtained from Riken Cell Bank-Tohoku University-Japan.

The target of nuclear kappa beta (NF-KB) gene is miRNA 146 A and miRNA 146 A activated by NF-KB, if the activity of NF-KB is increased, miRNA. A will be increased too. MiRNA inhibits the regulation (down regulation) against Interferon Regulatory Factor-5 (IRF5), Signal Transducer and Activator of Transcription-1 (STAT-1), and inhibits the regulation (up regulation) Interleukin-1 Receptor-Associated Kinase 1 (IRAK-1), Interleukin-1 Receptor Associated Kinase 2 (IRAK-2) and TNF Receptor Associated Factor (TRAF-6). The target of TRAF 6 gene is (TGF-beta activated kinase 1/MAP3K7 binding protein 2) TAB 1/2 and (Triticum aestivum kinase) TAK 1. The target of TAB ½ and TAK 1 genes are to inhibit the gene (inhibitor of kappa light polypeptide gene enhancer in B-cells/kinase epsilon) IKKe. The target of IKKe gene is to enhibit (inhibitor kappa beta) Ikb.[8]

The primary liver cancer is the fifth cancer mostly happens in Indonesia. The cause is still unidentified but it mostly happens on high-risk population with liver sirrhosis, hepatitis virus B active, hepatitis virus B carrier and hepatitis virus C. The model of HCC used in the research is by in-vitro by using Cell Lines Hep-G2 obtained from Riken Cell Bank- Tohoku University Japan.

The problems faced by the patients are: a long-term therapy, unsatisfying treatment, expensive medicines with high-risk of side effect.

It is hoped that the research can be tested on animals and continued with a clinical test CA, resulting in a phytopharmaca product, produced massively by the industries.

MATERIAL AND METHOD

Cell Lines

Cell lines Hep-G2 (RBC 1886) was a kind gift from Dr.Arief B Witarto from Riken Cell Bank-Tohoku University Japan.

Cell Lines Hep G-2 growing on culture media to reach confluent 60-80%, coming from the 3rd passage. The sample is divided into 4 main groups (0, 8, 18 and 24 hours), each group consists of 18 wells and each of it consists of 250 thousand Hep-G2 cells. The sample is given CA 727, 500 and 250 µMdoses, compared before and after exposure, with 3 times repeated measurement. The total sample is 72 groups of Cell Lines Hep-G2, intervened by CA and checked by RT-PCR.[9]

MiRNA Isolation and Quantification

Total RNA was isolated from Hep-G2 with RNA extraction kit (miRCURYTM RNA Isolation Kit-Cell and plant Exiqon, Code Number 300110, Denmark) and reverse-transcribed to cDNA with Primescript RT Reagent Kit (miRCURY LNATM Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit Exiqon, Code Number 203300, Denmark). The primers for miRNA 146A were Code Number 204688 from Exiqon (forward and reverse), and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed using SYBR Master Mix with Code Number 203450 (Exicon).

The optimation for reference gene miRNA 146 A candidate, is done by the support from RT-PCR CFX96 machine, using 4 reference gene candidates, they are mir (micro RNA)-16, mir-21, mir-103 and mir-423-3p. Each mir is represented by 8 samples (4 samples before intervention, and the others after intervention) and the sample total after 2 times checkings (running) reference gene is 104 samples, including 4 NTC (non templete controle) and 4 spikes. The RT-PCR checking to find reference gene candidate, is analysed by using software Bio-Rad CFX Manager 2.1 (Hercules, California) and Normfinder.[10]

After the reference gene candidate and optimum temperature are achieved, continued by adding 3 doses of Chlorogenic Acid on each well, based on the time decided. (0, 8, 18 and 24 hours) continued by RNA isolation, cDNA process and checked by RT-PCR CFX-96 machine.

Statistical Analyses

The data is analysed with the repeated measurement, after the normalisation data with kolmogorov-smirnov and the data distribute normally. If it doesn't distribute normally, transformation data is needed. If in measurement repeated there are some differences in groups, the pairwise comparisons analysis must be done. It is done on 0, 8th, 18th and 24th hours to know the time different among the gene expression.

To analyse the role of CA against the growth of Cell Lines Hep-G2, the test of multivariate regression model is done against each variable until we get the dose value with the highest resitor and symbolized by correlation coeficient R2.

RESULT

The MTT test is a cytotoxic test to assess the effect of CA against Hep-G2. The optimization on the test was done with variety of time and dose to obtain the optimal concentrate and time. The MTT test is started with 6, 24, 48, 72 and 96 hours after the exposure as shown in Figure No.1. Based on the previous research, it is concluded that CA could inhibit the growth of Hep-G2 as much as 50% after 24 hours exposure.

Furthermore, the researcher determined the value of IC_{50} CA which is obtained on the concentration of 727 µM. The Researcher addedd some variety doses of CA on 500 and 250µM to analyze them against the gene expression of miRNA 146 A, as shown in Figure No. 2.

The analysis of gene expression of miRNA 146 A used Livak method with the formula $2^{--\Delta\Delta Cq}$. The normalization is done by subtracting each value of Cq target before and after the exposure (based on 4 timing differentiates) with each value of Cq reference (based on 4 timing differentiates), to get the value of ΔCq. The value of ΔΔCq is determined by subtracting ΔCq value target with ΔCq reference.

The influence of CA doses: 727, 500 and 250 µM against the expression of miRNA 146 A on 0, 8, 18, and 24 is shown in Figure No.3. The expression of miRNA 146 A decreased from 0 to 24 hours.

Based on the comparison on each exposure (0, 8, 18 and 24 hours), the lowest expression of miRNA is on the group after 24 hours exposure on the dose of 727 µM with the expression value is 0.85, and then it is increased on the dose of 500 µM with the value of 1.28 and the highest expression on the dose of 250 µM with the value is 1.61.

Furthermore, continued by the analysis on before and after the CA exposure, which is done by a repeated anova test, continued by analyzing correlation of each variables.

The Pearson correlation test is done, between the dose of CA against the Cq value of miRNA 146 A against exposure timing, as shown in Table No.1.

The result of pairwise comparison test, as include on Table No.2, obtained that the Cq value of miRNA 146A (250 μM) on 0 hour is different from the Cq value on the 8th hour with p=0,032 and p<0, 05.
The Cq value of miRNA 146A (250 μM) on the 8th hour is different from the Cq value on the 24th hour with p= 0,026 and p<0,05. The same thing happens on Cq value of miRNA 146A (250 μM) on the 18th hour is different from Cq value on the 24th hour with p=0,006 dan p<0, 05.
The result of multiple comparison-tamhane test, as shown in Table No.3. The test was done to analyze the exposure timing against CA doses and the expression of miRNA 146A is different on the 8th hour and 18th hour on the doses 727 and 250 μM with p<0, 05.
The result of multiple linear regressions is shown in Table No.4. It explains that CA strongly acts against the decrease of gene expression of miRNA 146A with the determinant coefficient R^2 0,924. Based on the value of adjusted R square 70.7%, the decrease of gene expression of miRNA is influenced by CA.

DISCUSSION

The normality testing measured by excel, it is known that the data distributes normally with the value COV, less than 30%. The continuation testing is done by testing the role of CA against the time differentiate and testing some doses against the time value Cq miRNA 146 A.
Based on the viability test Hep-G2 against the time differentiate: 6, 24, 48, 72 and 96 hours, is concluded that 50% of Hep-G2 is destroyed after 24 hours CA exposure. The viability test of Hep-G2 against CA dose is needed to determine the value of IC_{50} that shows the dose is destroying 50% Hep-G2 and the value is IC_{50} 727 μM.
The optimation of reference gene candidate, is done by the support from RT-PCR CFX96 machine, using 4 reference gene candidates, they are mir (micro RNA)-16, mir-21, mir-103 and mir-423-3p. Each of it is represented by 8 samples (4 ones before the intervension and the others are after) and the number of total sample in 2 checkings (running) of reference gene is 104 samples, including 4 non templete controle (NTC) and 4 spikes. The checking result of RT-PCR to find reference gene candidate analysed by software Bio-Rad CFX Manager 2.1 (Hercules, California) and Normfinder.
Based on the Normfinder analysis, the smallest stability (the most stable) is 0.049 for mir-103, meanwhile the best and the most stable reference gene candidates are mir-103 and mir-423-3p. Then, the researcher analysed the result of Normfinder software and t test. It is concluded that the choosen reference gene candidate for miRNA 146 A exposured with CA is mir-423-3p.[11-12]
CA decreases the gene expression of miRNA 146 A on Hep-G2. The lowest expression on group 24 after CA exposure, are on 727 μM dose (0.85), followed by 500 μM (1.28) and the highest expression on 250 μM dose (1.61). On the 8th and the 18th hour the value of Cq miRNA 146 A is different p<0.05 on all CA doses (727, 500 and 250 μM). The gene expression of miRNA 146 A is decreased from 0 to 24 hours because of CA exposure. If we compare the exposure time (0, 8, 18 and 24 hours), the lowest expression of miRNA 146 A on group 24 hours after CA exposure are on 727 μM dose (0.85), followed by 500 μM (1.28) and the highest one is on 250 μM dose (1.61).
Based on the testing of F repeated anova on table 1, CA influences the expression of miRNA 146 A is different on 8, 18 and 24. It happens because the CA exposure that inhibits the growth of Hep-G2 cells. It means, The less SBY green read by RT-PCR, the bigger the Cq value, if we compare with control (without CA exposure). CA strongly acts against the enhancement of gene expression of miRNA 146 A with the determinant coefficient R2 0.924 and adjusted R square value 70.7%. The role of CA against decrease gene expression of miRNA 146 A on Hep-G2 cell, is trigerred by oxidation-reduction, phosphorylation and dephosphorylation of cellular level by transmembrane segment tyrosine kinases receptor by protein G, called G-Protein Couple Receptor (GPCR) with 3 receptor subunits α, β and γ.[13] Receptor β is the receptor that activates the adenilat siklase, causing cAMP activated and phosphorylation serine protein MAPKKK into treonin (MAPK) which activate c-Fos-c-Jun, activates CDK 4 and 6 and ended by the inhibition of G1 phase to S.[14] CA induces antioxidant endogen, that makes

antioxidant endogen increased. The increasement of oxidation stress will increase ROS that improves phosphorylation process.ROS destroys the cell balance. It makes the phosphorylation activity increased; the NF-KB activity is increased caused by P50 and P65 genes that makes miRNA activity increased. It informs that the target of NF-KB gene is miRNA 146 A. On the other hand, miRNA 146 A has got feed back negative activity against IRAK 1, 4 and TRAF 6 and the final target is NF-KB. There is another pathway saying that the target of miRNA 146 A is NF-KB through c-Myc and ERK5.[14-15] ROS is very identical with oncogenic process. If ROS activity is increased, the NF-KB expression and miRNA 146 A will be increased too. MiRNA inhibits the regulation of IRF5, STAT-1, IRAK 1, IRAK 2 and TRAF-6. If miRNA activity is increased, the activities of IRF5, STAT-1, IRAK 1, IRAK 2 and TRAF-6 will be decreased.[8, 16-18]

Table No.1: The F Repeated Anova miRNA 146 A test

S. No	Variation Source	Statistic	F	p
1	miRNA 146 A Factor	Greenhouse-Geiser	4,519	0,29
2	The role of CA to the time value of miRNA 146 A	Sphrecity Assumed	4,519	0,012

According to The test of F Repeated Anova the value $p<0.05$ on miRNA 146 A, The Chlorogenic Acid is different/changeable against the time differential Cq miRNA 146A, before and after the exposure.

Table No.2: The significance of miRNA 146 A against time differentiate

S.No	Comparison	miRNA 146 A-727	miRNA 146 A-500	miRNA 146 A-250
		p	p	p
1	0 hour vs 8 hours	NS	NS	0.032
2	0 hour vs 18 hours	NS	NS	NS
3	0 hour vs 24 hours	NS	NS	NS
4	8 hours vs 18 hours	NS	NS	NS
5	8 hours vs 24 hours	NS	NS	0.026
6	18 hours vs 24 hours	NS	NS	0.006

Note : The value p is calculated based on pairwise comparisons test. It works if $p< 0.05$. NS: Not Significant

Table 2 displays the value of Cq miRNA 146 A (250 μM) on the 0 hour is different from the value on the 8th hour with $p= 0.032$ and $p<0.05$. The value of Cq miRNA (250 μM) on the 8th hour is different from the value on the 24th hour with $p= 0.026$ and $p<0.05$. The value of Cq miRNA (250 μM) on the 18th hour is different form the value on the 24th hour with $p=0.006$ and $p<0.05$.

Table No.3: The significance between miRNA 146 A against dose differentiate of CA and timing

S. No	Comparison	miRNA 146 A
		p
1	0 hour vs 727 µM	NS
2	0 hour vs 500 µM	NS
3	0 hour vs 250 µM	NS
4	8 hours vs 500 µM	0,051
5	8 hours vs 250 µM-727 µM	0,035
6	18 hours vs 727 µM-250 µM	0,035
7	18 hours vs 500 µM-727 µM	NS
8	24 hours vs 727 µM	NS
9	24 hours vs 500 µM-250 µM	NS

Note: The value of p is calculated based on multiple comparisons-Tamhane test. It works if $p<0.05$. NS: Not Significant

Table 3 displays the timing of CA exposure against miRNA 146 A, it works on the 8th and 18th hour on the dose of 727, 500 and 250 µM with $p< 0.05$.

Table No.4: The role of Chlorogenic Acid against miRNA 146 A.

	R	R Square	Adjusted R Square	Durbin - Watson
The role of CA vs miRNA 146 A	0,924	0,853	0,707	1,566

Table 4 displays the data, Chlorogenic Acid is strongly decrease the miRNA 146 A gene expression with the determinant coeficient R2 0.924. Based on the value of adjusted R square 70.7%

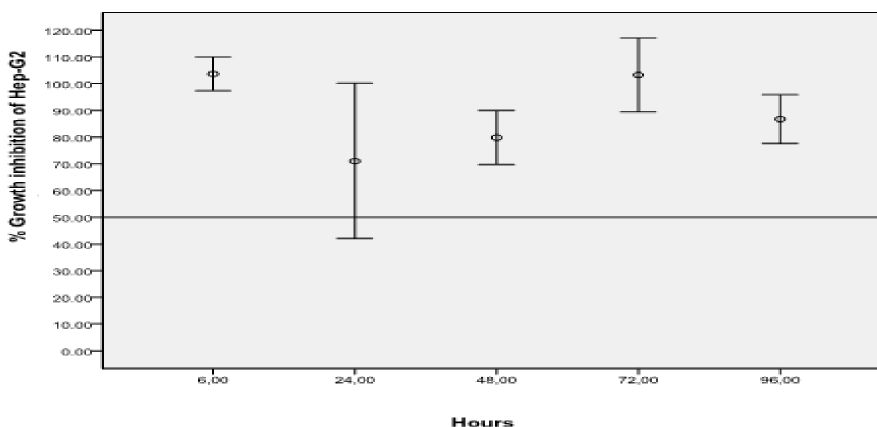

Figure No.1: The percentage of Hep-G2 reduction growth against Chlorogenic Acid time-based

It illustrates the viability test Hep-G2 against Cholorogenic Acid on 6 hours, 24 hours, 48, 72 and 96 hours. Based on the data above, The Chlorogenic Acid IC50 is achieved after 24 hours of AC exposure.

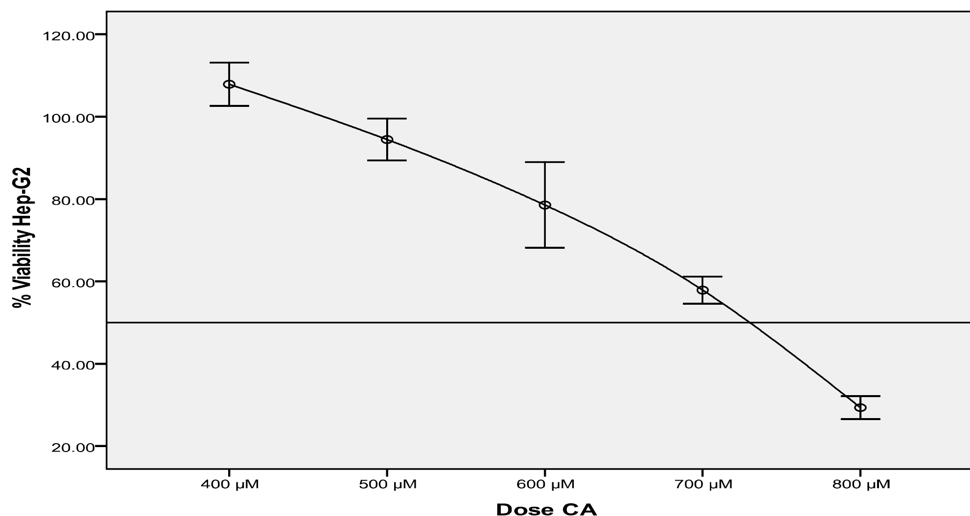

Figure No.2: The percentage of Hep-G2 alive and Linear Regresion Equation of IC50 Chlorogenic Acid

Based on the data obtained statistically, the researcher gets the value IC50 Chlorogenic Acid on the concentration of 727 µM. After that, the researcher added the dose variation 500 and 250 µM to analyse the dose variation against gene expression.

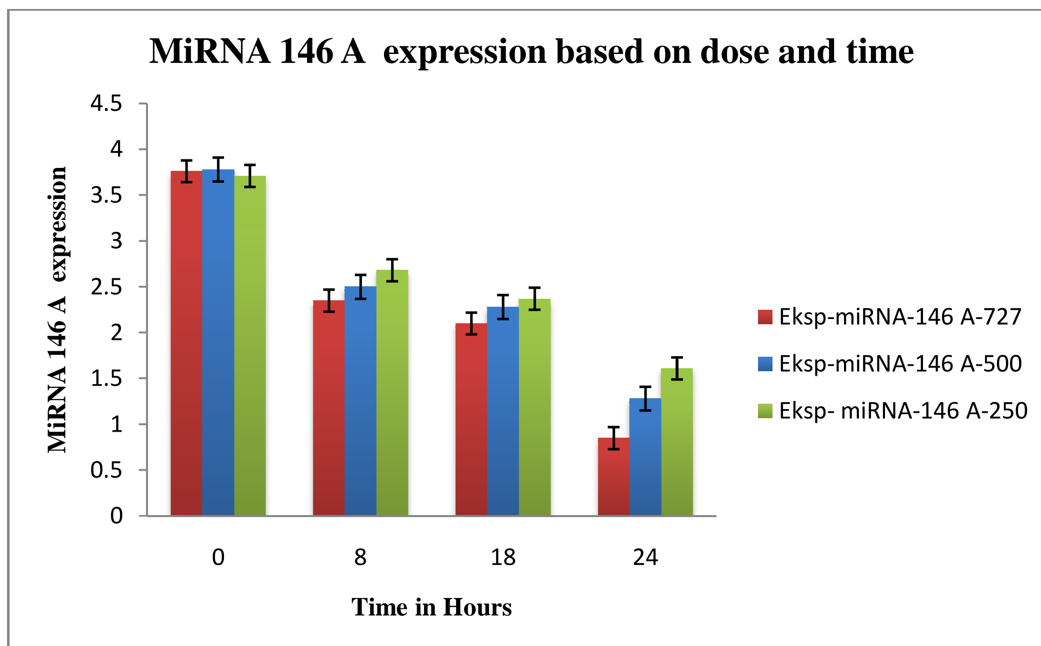

Figure No.3: miRNA 146 A expression based on dose 727 Chlorogenic Acid, 500, 250 µM on 0, 8, 18, 24 hours

It illustrates the expression of miRNA 146 A decreases from 0 to 24 hours. The lowest miRNA 146 A expression on group 24 after CA exposure is on 727 µM dose (0.85), then to be increased to 500 µM dose (1.28) and the highest expression on 250 µM dose (1.61).

CONCLUSION

1. Chlorogenic Acid is toxic against Cell Lines Hep-G2 with the value IC_{50}= 727 μM.
2. There is a decreasement of miRNA 146 A gene expression on Cell Lines Hep-G2 exposured by Chlorogenic Acid
3. Mir-423-3p be used as reference gene for miRNA 146 A on CA

Suggestion

1. It is used as basic data for molecular research of Hep-G2 series 1886 cell
2. It is used as a scientific information to support Oncogenic theory
3. The CA through in-vitro is anti-cancer and can be used to support the Hepatocellular carcinoma treatment

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Firman F.Wirakusumah.

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FOOTNOTES

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