Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(1):187-192

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Comparative effects of chlorogenic acid and doxorubic in against expression of caspase3 in cell lines Hep-G2

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ABSTRACT

Based on reports from the International Agency for Research on Cancer (IARC) in 2008, it has been found more than 12 million new cancer cases in the world. We need an effort to discover new anticancer drugs from natural substance origin. Chlorogenic acid was isolated from Lampung Robusta coffee(Coffea Robusta). Pure experimental design in vitro using a Cell Lines Hep-G2 was conducted in the Department of Molecular Biology Cancer Hospital Dharmais. The aim of this study was to know the action mechanism of chlorogenic acid. In vitro study was using Hep-G2 cells and samples were divided into four groups; 2 control group treatment, 1 experiment group exposed to 727μM chlorogenic acid, 1 experiment group exposed to doxorubicin 1.5μM, and group treatment have been treated with 3times repetition. The expression of caspase 3 was examined at 0,8, 18 and 24. Data were analyzed with livaks method and repeated measurement. This study shows an increasing expression of caspase 3 from 0-8 hours. Caspase 3 shows the highest expression at 8 hours after doxorubicin exposure, with expression value 4.36 followed by chlorogenc acid it expression value 0.74. The caspase 3 expression was decreased after 8 hours up to 24 hours after either doxorubicin or chlorogenic acid exposure. Conclusion, IC_{50} doxorubicin (1.5μM) is moretoxic than chlorogenic acid (727μM). At 8 hours after doxorubicin and chlorogenic acid exposure, caspase 3 expression with doxorubicin (4,36) higher than chlorogenic acid (0.74).

Keywords: Chlorogenic acid, doxorubicin, expression of caspase3

INTRODUCTION

Cancer becomes a serious problem in many countries, including Indonesia. Cancer prevalence in the world tends to increase. Based on International Agency for Research on Cancer (IARC) in 2008, It has been foundmore than 12 millions new cases. The report says the number of cancer cases increases 2 times in recent 30 years. The stakeholders should overcome the causes globally with significant efforts in order to prevent the rising of prevalence case 3 times in 2030 [1].

The rising of cancer case causes the higher need for anticancer usage. The using of synthetic anticancer would threaten human health through side effect such as hair loss, significant weight loss, and hyperpigmented skin (burn skin). Doksorubicin classified as antibiotics can also be used as synthetic anticancer for hepatocellular disease.

There is a need to search for active substance that are more Eco friendly and have minimum side effect. Chlorogenic acid is an active substance that can suppress or kill hepatocellular cancer cell. Robusta coffee contains 4% chlorogenic acid [2].

Chlorogenate acid is produced from coffee by extraction, fractionation, and isolation process. It is known for its function as exogen anti-oxidant to prevent cell damage and inhibits cancer cell growth by binding with free radical

agents. The Chlorogenic acid has a role to inhibit Cell Lines Hep-G2 growth throughoxidation-reduction reactionby trapping free radical agents that eventually decreases reactive oxygen species. Chlorogenate acid induces endogenousantioxidant until its activity increases.

Apoptosis inducer categorized into 3 types; death factor, genotoxic anti cancer, and deprivation factor. Fas ligand, one of the death factor, will bind to its receptor and causes trimerization. The death domain which trimerizes in cytoplasm will bind pro-caspase 8 by FADD/MORTI to form DISC. Pro-caspase 8 which is auto activated at DISC will turn into an active enzyme. There are two caspase 3 activitation from caspase 8. First, caspase 8 directly turn procaspase 3 into caspase 3. Caspase 3 will split its cell protein include ICAD, so that CAD will be leased from ICAD. Then, it degrades DNA chromosomes. Second, caspase 8 divides Bid, pro-apoptosis molecule categorized byBcl-2 family, that will be translocated to mitochondria to extricate cytochrome c to cytosol. Cl 2 or Bcl-XL is an anti-apoptosis molecule that can inhibit the extricationof cytochrome c with unknown mechanism. Cytochrome C with Apaf-1 activates caspase 9 and caspase 9 will activate caspase 3. Caspase 3 divides some cell proteins include ICAD so that CAD will be leased from ICAD. Then, it degrades DNA chromosomes [3-4].

EXPERIMENTAL SECTION

This is a pure experimental in vitro study using cell lines Hep-G2 from Stem cell institute-Kalgen.It has been conductedsince December 2012 until March 2013 at Biology Molecular Department of Dharmais Cancer Hospital. The Hep-G2 cultured cell uses medium, that contains 10% Fetal Bovine Albumin (Sigma) and penicillin-streptomycin anti-biotic (100mg/L), the number of cells per well $0{,}5x10^4$ and plant into 96 wells in different concentration of DMSO, 0.1, 0.5, and 1%.Analyze usesElisa reader, results says DMSO 1% concentration has succeeded to grow 100% hep g2 cell.

There are 4 groups in this in vitro study; 2 control group, 1 test group which is given 727 µM chlorogenic acid and 1 test group which is given 1,5 µMdoksorubisin (D). Another group are control group and test group which is given 1,5 µM doksorubisin. All groups going through 3 repetitions. Chlorogenic acid and doksorubisin exposure will be done within 48 hours after having a cultured cell of Hep-G2 and confluent 60-80%. The dosage of IC_{50}CA is 727 µM and D 1,5 µM.

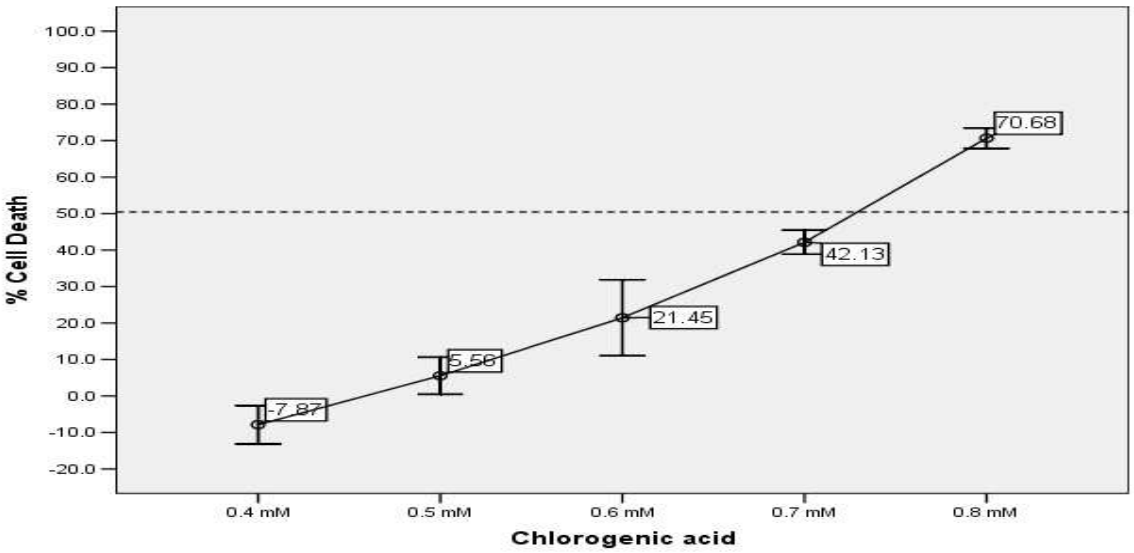
The total isolation of RNA follows Exiqon protocol and uses miRCURY™ RNA Isolation Kit–code product 300110 from Exiqon. The production of cDNA uses Universal cDNA synthesis kit product code 203300 from Exiqon. *Primer Caspase 3* (BioRad FB-6400-05 code product) is taken from Scienwerke BioRad. Its sequences are *Primer Forward Caspase 3* 5'AGA ACT GGA CTG TGG CAT TGA G 3' and Revers: 5' ATG TGC TGT GAC TGC TTG TAG ATG 3'

The cDNA synthesis needs 20µl total volume that contains :a) 5x *Reaction buffer* 4 µl, Nuclease-free water 9 µl; b) Enzyme mix 2 µl; c) Synthetic spike in with H2O 1 µl; d) *Template* total RNA (5 ng/µl) 4 µl. It will be incubated for 60 minutes at 42˚C, then reverse transcriptase did for 5 minutes at 95˚C, afterward, cooling directly at 4˚C and store it in the freezer at 4˚C as well. RT-PCR amplification is done at 10µl total volume that containsa) Sso Fast EvaGreen supermix 5 µl; b) Primer (forward and reverse) 2 µl; c) cDNA template 2 µl, and d) H_2O 1 µl. Denaturated at 94˚C for 3 minutes with certain temperature cycle. Each cycle consists of denaturation at94˚C for 30 seconds, annealing temperature for 30 seconds. Totaleach cycle consists of 40 cycles in total. The temperature can be optimized by looking at BioRad code product annex added by 3°C increased temperature and substrate3°C, then executes with software CFX-96. The Annealing temperature gradient of Caspase 3 lies between 59- 65^0C, the optimum temperature for annealingCaspase 3: 60,2 ^{0}C.

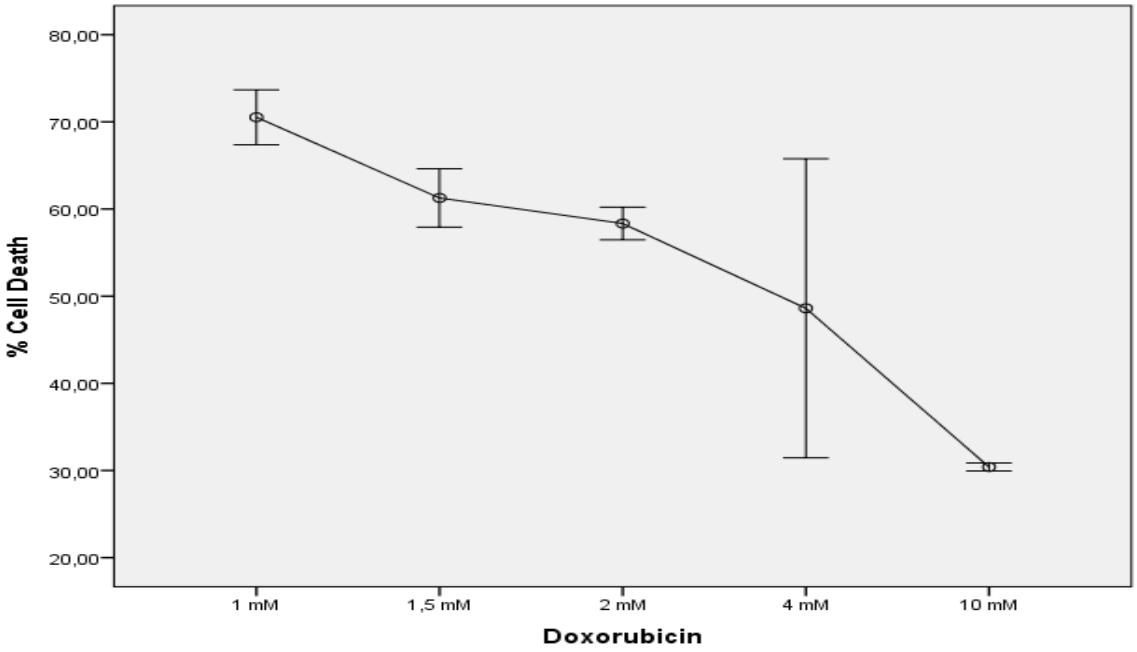
Caspase 3 expression is examined by RT-PCR CFX-96 at 0, 2th, 8th, 18th, 24th hourafter CA and D exposures, then compared to control. The data are tested by Livaks method and statistically withrepeated measurement. The caspase 3 gene expression was analyzed using a livaks method with formula: $2^{-\Delta\Delta Cq}$. The normalization can be done by subtracting each value of Cq targets before and after exposure (based on 4 different times) with each value of Cq reference to get result value ΔCq. The value of ΔΔCq is determined by subtracting ΔCq target value to ΔCq reference [5].

RESULTS

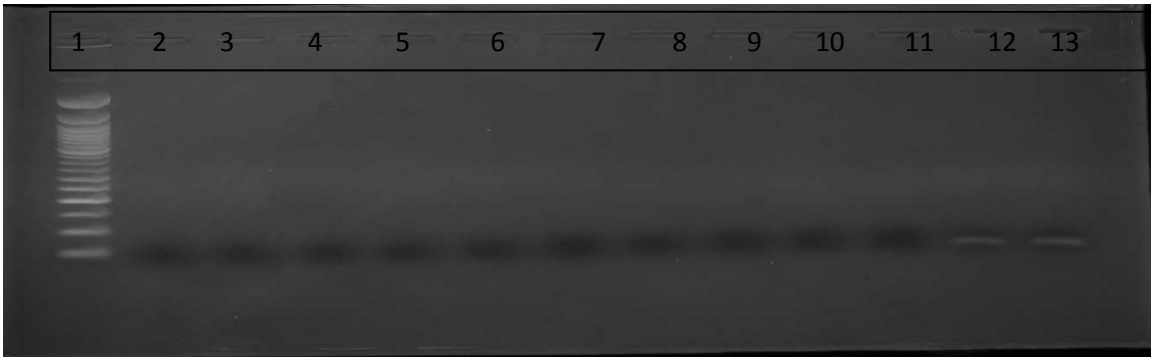
The dosage determination of IC_{50} is started from 400, 500, 600, 700 and 800 µM and chlorogenic acid IC_{50} dosage is at 727 µM By result analysis 1, 1.5, 2, 4, 10 µM and doksorubisin IC_{50} dosage is at 1,5 µM concentration



Picture 1 The growing Hep-G2 percentage and Chlorogenic acid IC_{50} Linear Regression equation



Picture 2 The growing Hep-g2 percentage and Doksorubisin IC_{50} Linear Regression equation



Picture 3 Electrophoresis of Annealing Caspase 3 temperature gradient between 63- 60,2^{0}C, with an optimum temperature of annealingCaspase3:60,2 ^{0}C

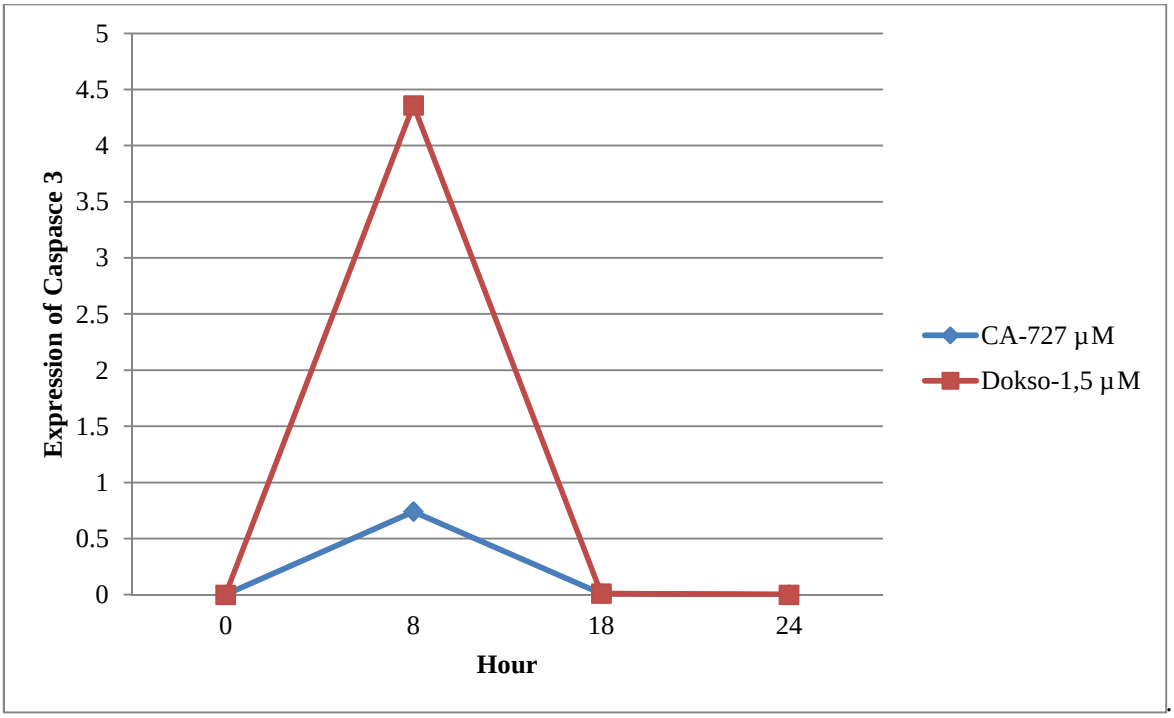
The sample at number 2-7=P0-1 and number 8-13:CA 8-1

1=Marker, suhu annealing 12-13:60,2^{0}C

To gain optimum temperature of annealing caspase 3, the study needs to be optimized through RT PCR and electrophoresis method. In purpose of comparing annealing temperature with the ribbon on electrophoresis. As clearer the ribbon looks as clearer the annealing temperature would be. The annealing temperature gradient between 63-60,2^{0}C and the optimum temperature for annealing caspase 3: 60,2 $^\circ$C for the number 12-13 picture 3. The sample for finding annealing temperature contains chlorogenic acid after 8 hours exposure and control at 0 hour.

To analyze caspase3 gene expression linearity, linear regression analysis was done with Y=3.192 - 0 .1 X and Y=0.505 + 0 .146 X.

The analysis of Caspase 3 gene expression uses Livak method with the formula: $2^{-\Delta\Delta Cq}$. Normalization was done by subtracting each value of Cq target before and after exposure(based on 4 different times) with each Cq value reference (based on 4 different times) to gain the ΔCq value. The value of ΔΔCq is determined by subtracting the ΔCq value target to ΔCq reference. The comparison of Caspase 3 expression gene which is exposed to CA with D exposure which can be seen at picture 4.



Picture 4 mRNA expression graph that exposure withCA and D based on time

DISCUSSION

Chlorogenate acid isan agent that has the capability to inhibit the Hep-G2 cancer cell growth through apoptosis induction. The role of chlorogenate acid in inhibiting Hep-G2 cell growth via oxidation-reduction reaction such as phosporilation and dephosporilation in cell level through single transmembrane segmenttyrosine kinasesreceptor by using G protein that is known as GPCR (G-Protein Couple Receptor) which has 3 subunit receptor, there are α, β, and γ [6, 7-8, 9]. β receptor is a receptor which has role in activating adenylate cyclase so that cAMP activated and then phosporilate protein serine (MAPKKK) into treonin (MAPK) then activates c-Fos-c-Jun, activates CDK4 and 6 finished byinhibiting G1 to S phase. Chlorogenate acid induces endogenous antioxidant so that endogenousantioxidant activity increases. The rising of oxidative stress increases ROS that will raise the phosporilation process [10-13].

The chlorogenate acid interventionsuppresses the oncogene gene function and induces apoptosis. Suppression gene is presented by Caspase 3 expression that causes apoptosis so that Caspase 3 activity increases, which can be seen in picture 4 [14].

There is a connection between ROS with oncogene function and suppression function. The reduction of ROS activity will decrease MAPK activity, then decrease cFos and cJun activity. C-fos and cJun activity will give impact to decrease CDK 4 and 6 activities that eventually creates inhibition of G1 phase which has big role in cell cycle

proliferation. This may cause balance instability and dominantly leads apoptosis in cell because of oncogenesuppression [14].

Gene expression can be detected quantitatively and qualitatively from RNA or protein. The examination of Caspas 3 expression in the study uses cDNA from RNA isolated by Exiqon protocol, uses miRCURY™ RNA Isolation Kit–code product 300110 from Exiqon. The gene expression of Caspase 3 is done by RT-PCR set with software CFX-96.

Gen Caspase 3 activity is measured based on Livak method with relativity basic principle and formula $2^{-\Delta\Delta Ct}$. The result seen in picture 4 which informs that chlorogenate acid and doksorubisin are able to influence caspase 3 activity by comparing time interval of 0,8,18,24 hours between before and after the procedure.

The Caspase 3 doksorubisin expression gene value is higher than chlorogenic acid. Ithappensbecause doksorubisin is an anticancer that induces caspase 3 stronger than chlorogenic acid. Doksorubisin was used as standardized anti cancer andchlorogenic acidcomparator.Although, chlorogenic acid IC50 is quite far from doxorubicyn (1,5 µM-727 µM),it can be used as chemo-preventive cancer. The action mechanism of chlorogenic acid as chemo-preventive is to inhibit free radical called anti-oxydant.

An antioxidant is a molecule that can slow or prevent oxidation reactions with other chemicals. Oxidation is a chemical reaction redox move electrons from a substance to an oxidizing agent. The oxidation reaction, can cause the onset of free radicals, may give rise to a dangerous chain reaction. Antioxidants may terminate these chain reactions by removing radical substance, and inhibit other oxidation reactions by oxidizing the substances themselves. Therefore, most of the antioxidant substances called reducing agents such as thiols or Phenols. Antioxidants can be produced in the body or obtained from the diet[14].

The parameter to show the antioxidant activities is the efficient concentration. EC is a concentration of antioxidants which can inhibit 50% sample. Any substance that has high antioxidant activity will have lowEC_{50} [15-17]. Chlorogenate acid (5,86 ppm) efficient concentration is better than vitamin C (12,2 ppm).

The apoptocyst divided into 2 types, first is the reactionbetween death receptor at legend cell surfaces and second by mitochondria pathway. Both of apoptosis waysinvolve protein cell signal throughcysteine aspartat-specific protease (Caspase) pathway that needs inisiatorcaspase protein, such as caspase 8 and caspase 9. Meanwhile, executor protein seen in apoptosis process is caspase-3 that is activated by caspase 8 and caspase 9 [18].

The conclusion, doksorubisin (IC_{50} 1,5 µM) is more toxic than chlorogenate acid(IC_{50} 727 µM).After 8 hours exposure bothactive substances, the value of doksorubisin caspase expression is higher than cholorogenic acid(0,74).

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