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The effect of *Epigallocatechin-3-Gallate* (EGCG) combined with low dose sorafenib in apoptosis and *Platelet-Derived Growth Factor Receptor* (PDGFR) expression in hepatocellular carcinoma rats

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ABSTRACT

Background: Sorafenib is a targeting therapy in Hepatocellular carcinoma (HCC) that targets Vascular Endothelial Growth Factor Receptors 1 (VEGFR-1), VEGFR-2, and VEGFR-3; Platelet-Derived Growth Factor Receptors β (PDGFR-β); c-Kit proto-oncogenes; FLT-3 genes; RET proto-oncogenes; and Raf-1 genes that have the same function in suppressing tumor cell proliferation, angiogenesis, and promotes tumor cell apoptosis; but its high cost makes necessary to reduce the dosage by combining with other agents. *Epigallocatechin-3-Gallate* (EGCG) has great potential in cancer treatment because of its anti-angiogenesis, induced *apoptosis*, and low cost. This study aimed to examine the effects of combination *EGCG* with low dose sorafenib compared to standard dose sorafenib in apoptosis and *PDGFR* expression in HCC rats.

Methods: Twenty-five male Wistar rats were randomly assigned into 4 groups: sham, control, EGCG+low dose sorafenib, and standard-dose sorafenib. Diethylnitrosamine (DEN) injection to induce HCC was carried out to all Wistar rats except for the sham group. After HCC was developed, the EGCG+low dose sorafenib group and standard-dose sorafenib group received the administration of the drugs for two weeks. PDGFR expression and apoptotic index were assessed in the last 3 groups. Data were analyzed using SPSS version 20 for Windows.

Results: There was a non-significant difference of mean PDGFR expression level between the EGCG+low dose sorafenib group and standard-dose sorafenib group ($p>0.05$); however, there was a lower significant difference of both groups compared to the control group ($p<0.05$). There was a non-significant difference of apoptotic index between low dose sorafenib+EGCG group and standard-dose sorafenib group ($p>0.05$); in addition, there were a higher not significantly different of both groups compared to the control group ($p>0.05$).

Conclusion: Combining low dose sorafenib with EGCG has a comparable effect with standard-dose sorafenib in reducing PDGFR of HCC, but not for apoptosis.

Keywords: EGCG, Sorafenib, PDGFR, Apoptotic Index, Hepatocellular Carcinoma.
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INTRODUCTION

Most patients with Hepatocellular Carcinoma (HCC) present with advanced disease, and the 5-year Overall Survival (OS) rates are only 10% for locally advanced and 3% for metastatic disease.[1-3] At the molecular level, angiogenesis in HCC results from an imbalance between drivers of vessel growth and maturation, including Platelet-Derived Growth Factor (PDGF) and those receptors.[2,4,5] In advance and metastatic HCC, targeting therapy is very important. Sorafenib as an agent for targeting therapy is an oral multikinase inhibitor that targets Vascular Endothelial Growth Factor Receptor (VEGFR) such as VEGFR-1, VEGFR-2, and VEGFR-3; PDGFR-β; c-Kit; FLT-3; RET; and Raf-1 that have the same function in suppressing tumor cell proliferation and angiogenesis and promotes tumor cell apoptosis.[2] Since 2007, sorafenib can significantly extend the median survival time of patients but only by 3–5 months.[6,7] Although single-agent sorafenib is effective for HCC, its high cost makes it necessary to combine sorafenib with other drugs to lower its dosage.

Epigallocatechin-3-Gallate (EGCG), a major polyphenol of green tea, is an anti-*angiogenesis* and induced *apoptosis*. In vitro and in vivo study with mice found that EGCG has down-regulated Bcl-2α and Bcl-xl by inactivation of NF-κB and blocks carcinogenesis, affecting a wide array of signal transduction pathways including JAK/STAT, MAPK, PI3K/AKT, Wnt, Notch, and stimulates telomere fragmentation through inhibiting telomerase activity.[8-11] EGCG induces apoptosis and promotes cell growth arrest by altering the expression of cell cycle regulatory proteins, activating killer caspases, and suppressing oncogenic transcription factors and pluripotency maintenance factors.[8-11] The previous in vivo study found that HCC mice

treated with EGCG (10 mg/kg/ BW/day) or sorafenib (10 mg/kg/BW/day) alone significantly increased the apoptosis rate.[12] Another in vitro study with human hepatic stellate cell line found that EGCG has an inhibitory effect on PDGF-induced proliferation of activated human hepatic stellate cells and the blocking of PDGF-BB binding to its receptors.[13] However, the in vivo effect of EGCG combined with low-dose sorafenib in HCC rats has never been studied.

Based on those mentioned above, this study aimed to examine the effects of *EGCG* combined with low dose sorafenib in apoptosis and PDGFR expression in HCC rats.

METHODS

Induction of HCC animals and experimental design

The experimental study was randomized control trial posttest only study design. The experimental animals were seven-week-old male Wistar rats from IPB Bogor Indonesia. We used rats for this experiment and did dose translation between laboratory animals.[14] The rats were kept in standardized animal laboratory cage at 23-25°C, 40-70% humidity with 12 hours alternating light-dark cycle. The bottom of the cage was given sawdust which was replaced every two days. The rats were fed water and standard food (AIN76) for rodents with diet formula (67.7% carbohydrate, 11.5% lipid and 20.8% protein). The rats underwent seven days of acclimatization and were observed for health and behavior. The bodyweight of the rats was measured every week. Twenty-five male Wistar rats were randomly allocated into four groups (n=25) based on the previous studies.[15,16] They were Sham group (K; n=4), control group (O; n=7), EGCG+low dose sorafenib group (X1; n=7), and standard-dose sorafenib group (X2; n=7) according to previous study.[11] All groups were induced to develop HCC by injecting 70 mg/kg/BW DEN (Sigma-Aldrich, USA) intraperitoneally, once per week for 10 weeks except sham group.[5,17] After 10 weeks of DEN injection, control group rats were euthanized by exsanguinations under general anesthesia. HCC was verified macroscopically and microscopically examination by Hematoxylin-Eosin staining. The PDGFR expression (PDGFR kit Sigma-Aldrich, USA) and apoptotic index were assessed.[18]

Sorafenib (Sigma-Aldrich, USA) was dissolved in a maximum of 1.5 ml saline (maximum 10 ml/kgBW/day) and administered orally in a drink. EGCG (Sigma-Aldrich, USA) was dissolved in a maximum of 1.5 ml saline (maximum 20 ml/KgBW/day) and administered by intraperitoneal injection.[19-21] EGCG+low dose sorafenib (X1) group received EGCG 5mg/kg/BW/day and sorafenib 5mg/kg/BW/day for 14 days. The standard sorafenib (X2) group received sorafenib 15mg/kg/BW/day for 14 days. Sham group received saline intraperitoneally. After 14 days, all experimental rats were euthanized by exsanguinations under general anesthesia using ketamine and xylazine.[11] The rats have confirmed death by diminishing heartbeats, respiratory rate, and acral reflexes. The entire liver was removed totally, observed macroscopically, and microscopically examined by Hematoxylin-Eosin staining. The PDGFR expression and apoptotic index were assessed.[18]

During DEN injection, 1 rat in the Sham group, 2 in the X1 group, and 2 in the X2 group died. All 5 rats died due to pulmonary hemorrhage. There was no death during EGCG and sorafenib administration, but on the eleventh day, the rats became weak. And we decided to sacrifice the 13 rats on the 12th day.

Enzyme-Linked Immunosorbent Assay (ELISA) of PDGFR expression

The fresh liver tissue from each rat was drawn and centrifuge and the supernatant were removed. Prepare the lysate from tissues by putting the entire liver tissue on ice as quickly as possible to prevent degradation by proteases. The tissue was placed in round bottom microcentrifuge tube or Eppendorf tubes store samples at -80°Cfor later use or keep on ice for immediate homogenization. For a 5 mg piece of tissue, add 500 µL of ice-cold lysis buffer rapidly to the tube and homogenize. The volume of lysis buffer must be determined concerning the amount of tissue present. Protein extract should not be too diluted to avoid protein loss-centrifuge for 20 min at 12000 rpm at 4°C in a microcentrifuge. The tubes were gently removed from the centrifuge and placed on ice, aspirated the supernatant and placed in a fresh tube kept on ice and the pellet was discarded.

Then the PDGFR expression was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) reader. Perform a Bradford assay, a Lowry assay, or a bicinchoninic acid (BCA) assay. Bovine serum albumin (BSA) is a frequently used protein standard. Add 200 ul of 1x Bradford reagent in each well; add 5 ul of BSA to all well. Add up to 30 µl of the sample (record volume) to each test tube. Determine the absorbance using 595 nm (VIS lamp) of wavelength. Use the sipper or using individual cuvettes. Make sure to check the setting and use the fixed wavelength.

Histologic Analysis

Liver tissue was fixed in 10% formaldehyde and embedded in paraffin. Liver tissue was sliced at 4 µm and placed on poly-L-Lysine-coated slides. After deparaffinized with xylene for 2x15 minutes, the sections were rehydrated through a decreasing ethanol series (96%,80% and 70%) for 5 minutes each step and then washed in tap water for 5 minutes. The section was stained with Hematoxylin Mayer for 20 minutes and washed in tap water for 5 minutes. The section was stained with Eosin for 30 seconds. The section was rehydrated through an increasing ethanol series (80-96%, absolute) and put into 2 places containing xylene every 15 minutes and then examined under a microscope. Apoptotic cells were identified by deeply shrunken eosinophilic cells detached from the surrounding environment with pyknotic-degenerated nuclei and were counted using a standard light microscope at a × 40 magnification. The apoptotic index was measured by a total number of apoptotic cells per 1000 cells and evaluated by 2 blinded independent pathologists based on the previous study.[18] Degree of agreement between two observers for the apoptotic index was analyzed by Cohen's kappa measurement. The Cohen Kappa values between 0.61 and 0.8 indicated a substantial and good agreement between the pathologists.

Statistical Analysis

Statistical analysis using SPSS version 20 for Windows. All data were presented as mean. The data distribution of animals' body weight was normal. The data distribution of PDGFR expression was normal. The differences in PDGFR expression between groups were analyzed using the One-Way ANOVA test and post hoc analysis using Mann Whitney. The data distribution of the apoptotic index was not normal. The differences of an apoptotic index between-group were analyzed by Kruskal-Wallis test and Post Hoc analysis using Mann Whitney. The statistically significant value was obtained using a $p<0.05$.

RESULTS

During DEN injection, 5 rats died due to lung hemorrhage. The PDGFR expression and apoptotic index were assessed after 2 weeks of EGCG and sorafenib administration. In determining and verifying the HCC in the control group after two weeks of DEN injection, we found the multiple tumor nodules macroscopically and tumor development microscopically (Figure 1). In addition, the macroscopic liver tissue among the groups was described in Figure 2. Interestingly, macroscopically observation on liver tissue found that the nodules in the control (O) group and standard-dose sorafenib group seem more prominent than the combination EGCG+low dose sorafenib group (Figure 2). However, the weight and the appearance of the nodule were not assessed in this study.

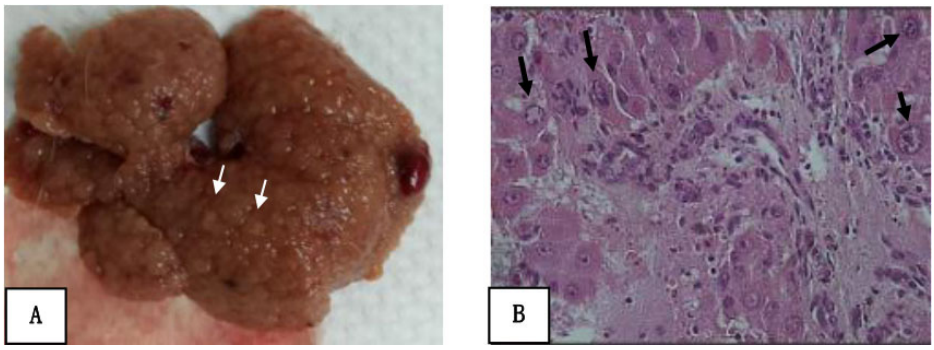

Figure 1. HCC validation in DEN-sacrifice (control) (O) group. (A) Macroscopically: the presence of multiple tumor nodules in the liver (white arrows); and (B) Microscopically: HCC was shown tumor development with oval nuclei anaplastic cell, pleomorphic nuclei, rough chromatin, and prominent nucleoli (black arrows) (40x magnification).

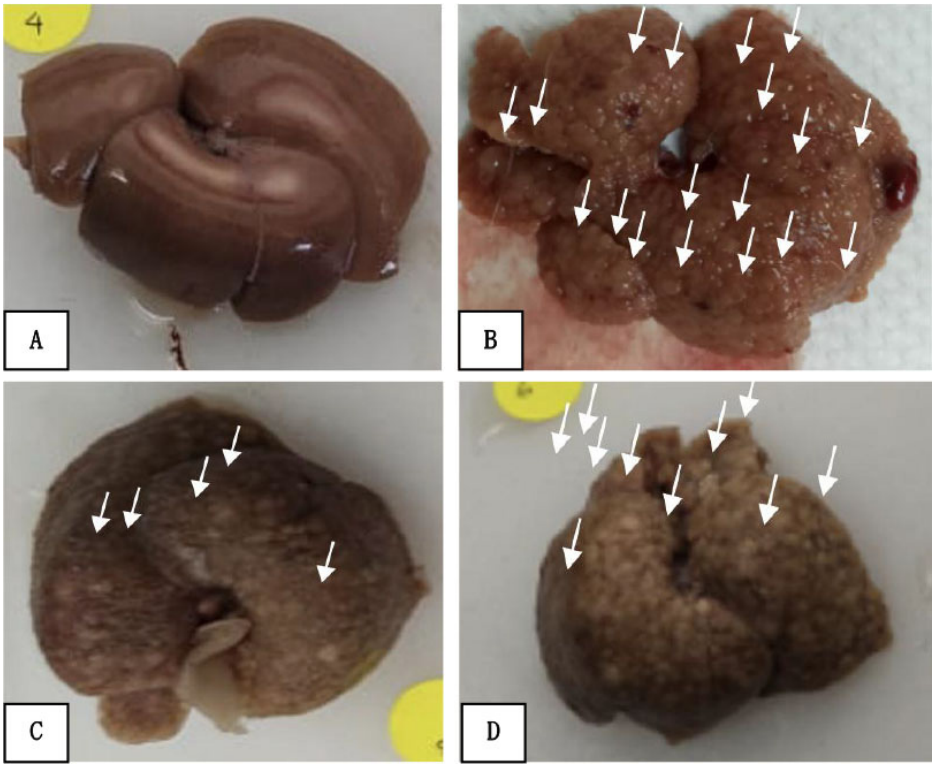

Figure 2. Macroscopic liver tissue among the groups. A) Sham (K) group; B) Control (O) group; C) EGCG+ low dose Sorafenib (X1) group; and D) Standard dose Sorafenib (X2) group. White arrows show the nodules.

The mean PDGFR expression level of the EGCG+low dose sorafenib group (X1) was not significantly different compared to the standard dose sorafenib (X2) (11.38 ug/ml and 13.46 ug/ml, respectively) ($p>0.05$). The mean PDGFR expression level of the EGCG+low dose sorafenib (X1) group and standard-dose sorafenib (X2) group were significantly lower compared to the control group (O) (16.83 ug/ml) ($p<0.05$) (Figure 3).

The mean of the apoptotic index of the EGCG+low dose sorafenib group (X1) was not significantly different compared to the standard dose sorafenib (X2) (0.22 and 0.16 respectively) ($p>0.05$). In addition, there was not a significantly higher difference between both groups compared to the control (O) group (0.22 and 0.20 respectively) ($p>0.05$) (Figure 4).

DISCUSSION

EGCG, a major green tea polyphenol, has great potential in cancer prevention because of its safety, low cost, bioavailability, and various mechanisms of action. EGCG acts on growth factors, including PDGF.[22] Previous in vivo study with mouse xenograft model used HCC-LM3 cells. Saline, sorafenib (10 mg/kg), EGCG (10 mg/kg), and sorafenib (10 mg/kg) + EGCG (10 mg/kg) were used for the *in vivo* experiments. Mice treated with EGCG (10 mg/kg/ BW/day) or sorafenib (10 mg/kg/BW/day) alone showed a significantly smaller tumor diameter than untreated mice after 30 days of treatment. Compared to the

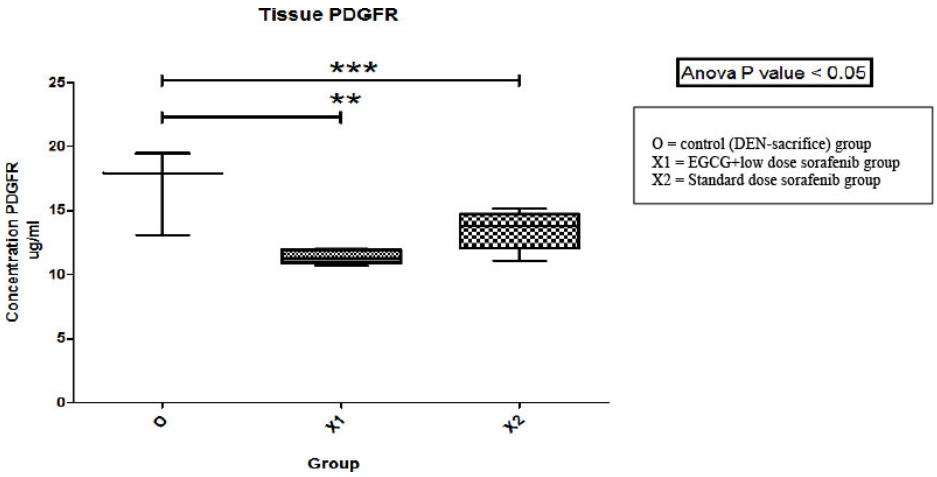

Figure 3. The PDGFR expression. There was no statistically significant difference in mean PDGFR expression level between the EGCG+low dose sorafenib (X1) and standard-dose sorafenib (X2) groups.

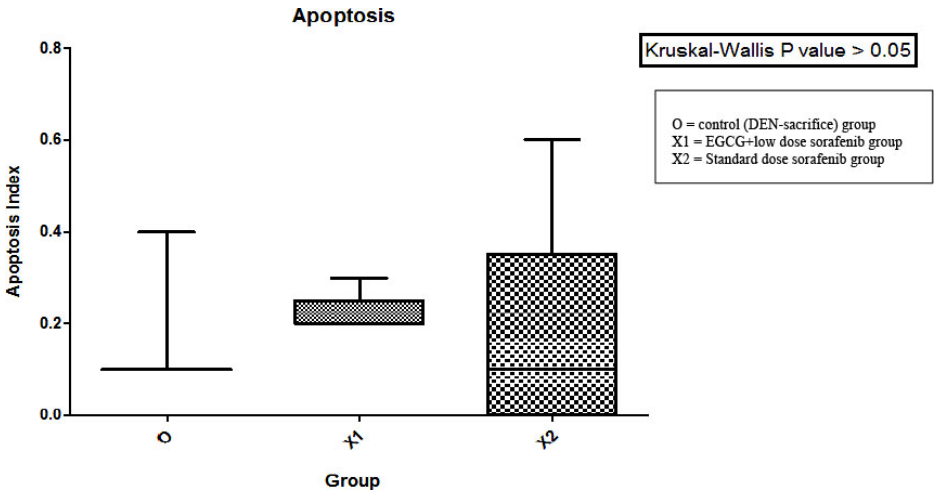

Figure 4. The apoptotic index. There was no statistically significant difference in mean apoptotic index between the low dose sorafenib+EGCG (X1) and standard-dose sorafenib (X2) groups.

sorafenib alone, combination treatment with EGCG and sorafenib significantly increased the rate of apoptosis.[12] In vitro study in fibrosis liver rats, using cultured human hepatic stellate cell line LI90 was found that EGCG has an inhibitory effect on PDGF-induced proliferation of HSC and the blocking of PDGF-BB binding to its receptor may be the mechanism behind this effect.[13] Our study analyzed the impact of combined EGCG+low dose sorafenib in PDGFR expression and apoptotic index in hepatocellular carcinoma rats. In our study, HCC was established on a 10-week intraperitoneal injection of DEN. The same effectiveness of PDGFR expression after administration of low dose sorafenib+EGCG and standard-dose sorafenib was probably allowed by blockade carcinogenesis by EGCG and sorafenib on JAK/STAT, MAPK, PI3K/AKT, that cause inhibition of NF-kB, HIF-1α, and PDGF transcription. EGCG has mechanisms, including the antiangiogenic effect, which have clarified the potential role of EGCG on hepatocarcinoma tumor growth.[11] Sorafenib also has the same target as EGCG. It works by inhibiting the activity of several tyrosine kinases involved in tumor angiogenesis and progression, including vascular endothelial growth factor receptor (VEGFR-2/3), platelet-derived growth factor receptor (PDGF-R), Flt3 and c-Kit, and also targets Raf kinases involved in the MAPK/ERK pathway and suppresses the angiogenesis, proliferation, migration, cell survival, and metastasis.[23] While PDGFR expression in the control (DEN-sacrifice) group was not decreased because there was no blockade carcinogenesis by EGCG nor Sorafenib.

Apoptotic index in our study was the same results in the EGCG+low dose sorafenib group and standard-dose sorafenib group was probably allowed by induction of apoptosis by EGCG and Sorafenib synergically. EGCG down-regulated anti-apoptotic protein such as Bcl-2α and Bcl-xl by inactivation of NF-κB and blocks carcinogenesis by affecting a wide array of signal transduction pathways, including JAK/STAT, MAPK, PI3K/AKT, Wnt, Notch and stimulates telomere fragmentation through inhibiting telomerase activity.[8-11,22] EGCG also promoted the apoptotic proteins such as caspase 9, caspase 8, caspase 7, caspase 3, PARP, Bax, Bak.[23] Sorafenib has the same target with EGCG on JAK/STAT, MAPK, PI3K/AKT, ERK. Sorafenib also reduces IF4E phosphorylation and decreases Mcl-1 protein, anti-apoptotic in MEK/ERK pathway.[23] Our study results found that apoptotic index of combined EGCG+low dose sorafenib group and standard-dose sorafenib group were not significantly different and were higher than the control group even not significantly. The similarity of an apoptotic index between EGCG+low dose sorafenib and standard-dose sorafenib group probably indicated that EGCG had a synergic mechanism with sorafenib in inducing apoptotic processes.

Nevertheless, our finding of lung hemorrhage in dead rats should be verified by histopathologic evaluation to confirm the cause of death, which was not done in this study. Further study with increasing EGCG doses or more than 14 days of drug administration will support this study. Several marker examinations should also prove the synergistic effect of EGCG and low-dose sorafenib in the HCC therapy.

CONCLUSION

A combination of low dose sorafenib with EGCG has a comparable effect with standard-dose sorafenib in reducing

PDGFR of HCC and but not for apoptosis.

CONFLICT OF INTEREST

The authors declare that the research was conducted without any financial relationships that could be a potential conflict of interest.

ETHICAL APPROVAL

The study has obtained ethical approval from the authorized Institutional Review Board of the Ethics Committee of the Mochtar Riyadi Institute of Nanotechnology (MRIN) prior to the study being conducted.

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AUTHOR CONTRIBUTION

ER designed the study, did the experimental procedure, collected and analyzed the data, and wrote the manuscript. SAP did the conceptual idea, medical aspect. IR provided advice in the methodology biomolecular aspect and reviewed the manuscript. WLA provided aspects in experimental procedure and guidance in biomolecular aspect.

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