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Research Article

Alpha-Mangostin Enhances Proliferation in Sorafenib-Surviving HepG2 Liver Cancer Cells by Increasing Anti-Apoptosis and Antioxidant Markers Expressions

Melva Louisa[1,*], Meuthia Faralita Annisa[2], Pamela Basuki[2], Brigitta Cindy Lauren[2], Syarinta Adenina[3]

ABSTRACT

Background: Sorafenib is the first-line systemic option for treatment in advanced liver cancer. However, sorafenib resistance may develop rapidly, which may involve apoptosis and oxidative stress dysregulations. Several alternative treatments have been suggested to alleviate the delayed resistance of cancer cells to sorafenib, including alpha mangostin (AM). According to an earlier study, AM might be able to overcome doxorubicin resistance in hepatocellular cancer cells. **Objective**: The aim of this study was to investigate the effects of AM in sorafenib-surviving HepG2 cells, a hepatocellular carcinoma (HCC) cell line. **Methods:** Sorafenib 10 μM was used to treat HepG2 to obtain sorafenib-surviving cells. Subsequently, sorafenib surviving cells were treated with DMSO -(vehicle) or sorafenib (SF) 10 μM or AM 20 μM, or SF 10 μM + AM 20 μM. Afterward, the cells were counted, collected and extracted for RNA. The mRNA expressions of Ki-67, c-Jun, Bcl-2, Bax, Caspase-3 and -9, GPx, and MnSOD were then quantified using qRT-PCR. **Results:** Treatment of alpha-mangostin, alone or in combination with sorafenib combined enhanced the expressions of proliferation markers, Ki-67 and c-Jun. In addition, there was a marked increase in mRNA expressions of Bax and BCl2, but not Caspase-3 and -9. There were amplifications of antioxidant markers expressions, GPx, and MnSOD after AM or a combination of sorafenib and AM. **Conclusion:** Treatment of alpha mangostin in sorafenib-surviving HCC cells caused an increase in proliferation markers, which might be explained by the reduced expressions of apoptosis markers and enhancement of antioxidant markers.
Keywords: Anti-cancer drug resistance, Caspase, Hepatocellular carcinoma, Ki-67, Oxidative stress.

Melva Louisa[1,*], Meuthia Faralita Annisa[2], Pamela Basuki[2], Brigitta Cindy Lauren[2], Syarinta Adenina[3]

[1]Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

[2]Medical student, Faculty of Medicine Universitas Indonesia, Jakarta, INDONESIA.

[3]Department of Pharmacology, Faculty of Medicine, YARSI University, Jakarta, INDONESIA.

Correspondence

Melva Louisa

Department of Pharmacology and Therapeutics Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.

E-mail: melva.louisa@gmail.com

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INTRODUCTION

Liver carcinoma is the seventh most prevalent cancer globally and the second leading cause of cancer-related death worldwide.[1] By rough estimates, the number of people affected by liver cancer each year would rise to over a million by 2025.[2] Up to date, the treatment for liver cancer can either be surgical or non-surgical. The surgical treatment includes resection and liver transplantation, whereas the non-surgical approach includes locoregional therapies and systemic therapy.[2] In advanced-stage liver cancer, systemic therapy is used, including sorafenib and lenvatinib as the first line.[2,3] Sorafenib for systemic treatment has been used since its approval by the FDA in 2007, while lenvatinib was just recently favored by FDA in July 2018 as HCC first-line treatment.[3]

Sorafenib is a multiple intracellular and cell surface kinases inhibitor. The inhibited kinases have roles in angiogenesis, tumor cell signaling and apoptosis. Sorafenib has been demonstrated to be effective in wide variety of tumor cells.[4] However, the overall survival of HCC was found to only be 2-3 months longer than placebo, and drug resistance to sorafenib was shown to develop.[5] Activation of EGFR, c-Jun, autophagy, AKT activation, hypoxia, the epithelial-mesenchymal transition (EMT), cancer stem cells, dysregulated apoptosis.[6]

Studies have shown that resistance to sorafenib is marked by overexpression of anti-apoptotic markers and dysregulations of oxidative stress attenuations. Sorafenib has been reported to have Bcl-2 (B-cell lymphoma 2) family proteins and the intrinsic apoptotic pathway to be the main components for its cytotoxicity in liver cancer.[7-10] In sorafenib exposure, the depletion of Mcl-1 and Bax (Bcl-2 associated X) mitochondrial translocation has also been observed. Additionally, Bcl-xL has been associated with the growth of HCC and the development of resistance to sorafenib.[11]

Thus, in the case of resistance development, there needs to be an alternative to sorafenib. Alpha-mangostin is a natural xanthonoid extracted from mangosteen fruit's pericarp, has been shown to exhibit many pharmacological activities including anti-cancer. A study by Kritsanawong *et al.* shows that alpha-mangostin has essential roles in apoptosis as it induces inhibition to the proliferation of the cells, condensation of the nuclear, fragmentation of DNA and increased cleavage of Caspase-3 and -9, as well as decreased the expression of Bcl-2 and Mcl-1 in T47D breast cancer cell lines.[12]

A study by Cai *et al.* in HCC showed that alpha-mangostin has the effect of reversing doxorubicin resistance induced by IL-6.[13] Additionally, a study by Adenina *et al.* showed that alpha mangostin in sorafenib-surviving cells could be favorable in decreasing cell viability, yet at the same time might increase the indicators of epithelial-mesenchymal transition (EMT). Therefore, the present study was aimed further to explore the effect of alpha-mangostin in sorafenib-surviving cells, particularly on the expressions of some of the pathways affecting drug resistance, including cell proliferation, apoptosis and oxidative stress markers.

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MATERIALS AND METHODS

HepG2 cell culture

Human liver carcinoma, HepG2 were generously donated by the Eijkman Institute, Indonesia. The cultures were maintained in the medium as previously described.[14]

Sorafenib-surviving cells

Sorafenib-surviving HCC was established using sorafenib 10 µM following the methods by Adenina *et al.*[14]

Treatment of sorafenib-surviving cells

The dose for the treatment of sorafenib surviving cells with sorafenib or alpha-mangostin was based on the CC50 obtained from the previous study, which was 10 µM for sorafenib and 20 µM for alpha mangostin.[14] In brief, sorafenib (10 µM) or AM (20 µM) or combination of sorafenib 10 µM and AM µM was administered to for 24 hours. Dimethyl sulfoxide (DMSO) that was used as dissolved the drugs (vehicle) was used as control. The Luna™ automated cell counter (Logos Biosystems) was then used to count the cells. Then, the RNA was collected from the cells for qRT-PCR expression investigation.

RNA isolation and cDNA synthesis

RNA was isolated from 6 x 10^5 cells using Total RNA Mini Kit (Blood/Cultured Cell) Protocol (Geneaid) according to the manufacturers' protocol. Then, cDNA synthesis was performed using ReverTra Ace qPCR Master Mix with gDNA Remover (Toyobo) according to the provided protocol.

Analysis of mRNA expressions using qRT-PCR

The mRNA expressions of Ki-67, c-Jun, Bcl-2, Bax, Caspase-3, Caspase-9, GPx, and MnSOD expressions were performed using qRT-PCR Light Cycler 480 using Thunderbird SYBR qPCR Mix Kit (Toyobo). Beta-actin was used as the housekeeping gene. Primers used β-actin, Ki-67, c-Jun, Bax, Bcl-2, Caspase-3 and Caspase-9, GPx and MnSOD were as described previously.[15-17] The number of cDNA templates used for each sample was 100 ng. Cycle threshold (Ct) was measured automatically using the software. Ct data were later processed according to the Livak method to get the level of expressions.[18]

Data analysis

The data were presented in mean ± standard error of the mean (SEM). Analysis was done utilizing the one-way ANOVA test. The cut-off threshold for this research in determining significant differences was $p < 0.05$.

RESULTS

Marked reduction of cell viability after treatments of alpha-mangostin in sorafenib-surviving cells

In sorafenib-surviving cells, treatment with sorafenib at its CC50 still reduced cell viability but could not clear out half of the cells. However, the reduction was in a much lower efficiency when compared to alpha mangostin at its CC50 or combination of sorafenib-alpha mangostin (Figure 1).

Alpha mangostin enhanced cell proliferation markers in sorafenib-surviving cells

Cell proliferation markers expressions quantified were Ki-67 and c-Jun. Ki-67 is a marker of cell proliferation whose expression was increased in tumor tissues and associated with TGF-β1 expressions in the liver and HepG2 cells.[19] In comparison, the c-Jun marker is a proto-oncogene protein involved in cell cycle progression, tumorigenesis, and cell proliferation.[20] There was a slight increase in Ki-67 expressions after treatment of sorafenib (SF), alpha mangostin (AM), or the combination of SF and AM. However, a marked increment of c-Jun expressions was shown after treatment with alpha mangostin, alone or combined with sorafenib (Figure 2).

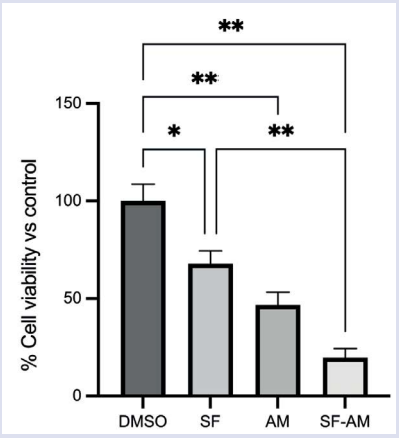

Figure 1: Percentage of cell viability vs. control in sorafenib-surviving cells after treatment with sorafenib or alpha-mangostin or combination of sorafenib and alpha-mangostin.

DMSO: dimethyl sulfoxide; SF: sorafenib 10 µM; AM: alpha-mangostin 20 µM; SF-AM: sorafenib 10 uM and alpha mangostin 20 uM ; AM: alpha mangostin 20 µM; *: $p<0.05$; **: $p<0.001$.

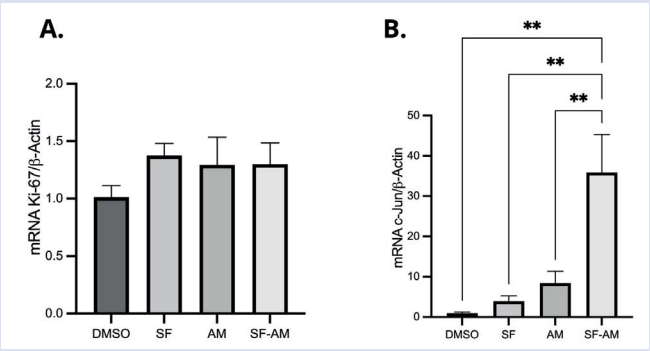

Figure 2: The mRNA expressions of cell proliferation markers in sorafenib-surviving cells after treatment after treatment with sorafenib or alpha-mangostin or a combination of sorafenib and alpha mangostin. A) mRNA Ki-67/β-actin; B) mRNA c-Jun/β-actin

DMSO: dimethyl sulfoxide; SF: sorafenib 10 µM; AM: alpha mangostin 20 µM; SF-AM: sorafenib 10 uM and alpha mangostin 20 uM; AM: alpha mangostin 20 µM; *: $p<0.05$; **: $p<0.001$.

Alteration of apoptotic expression markers after treatment with alpha-mangostin, sorafenib, and the combination of alpha-mangostin and sorafenib increased anti-apoptotic markers in sorafenib-surviving cells

We found an increase in Bax and BCl-2 mRNA expressions, with a much higher increment in BCl-2. Hence, the ratio of Bax/Bcl-2, markers that are consistent with apoptosis, were reduced after treatment with the three groups, with sorafenib showing the highest reduction. However, the change in Caspase-3 and Caspase-9 mRNA expressions was not significantly altered (Figure 3).

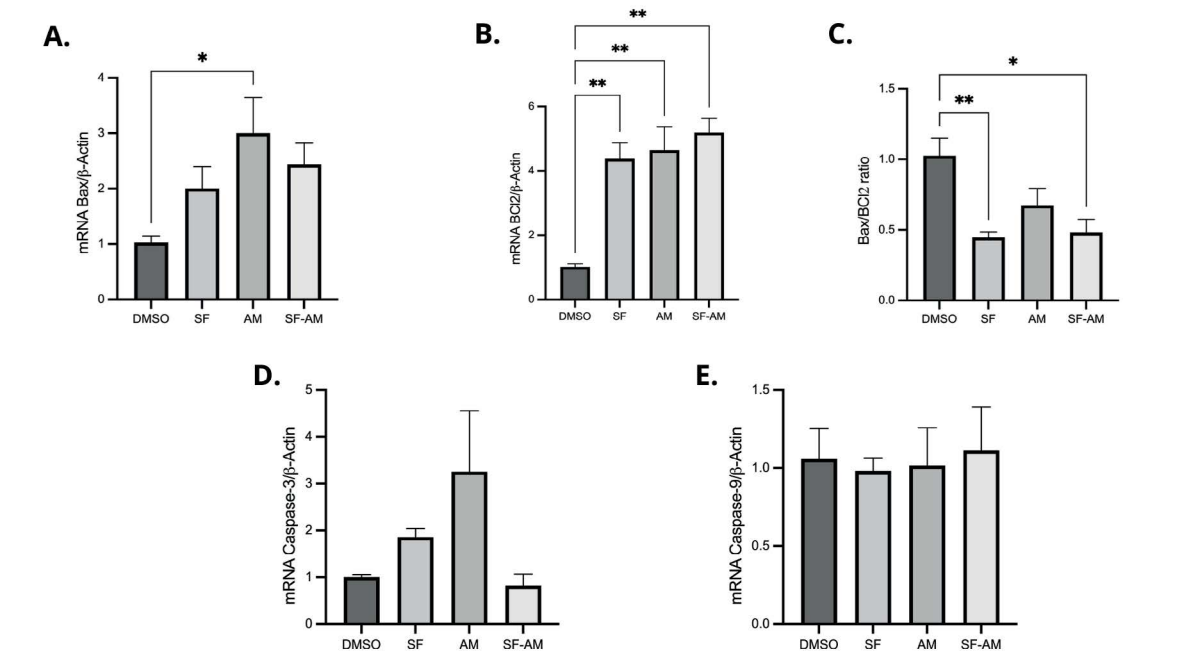

Figure 3: The mRNA expressions of apoptosis markers in sorafenib-surviving cells after treatment after treatment with sorafenib or alpha-mangostin or combination of sorafenib and alpha mangostin. A) mRNA Bax/β-actin; B) mRNA BCl-2/β-actin; C) Bax/BCl-2 ratio; D) mRNA Caspase-3/β-actin; B) mRNA Caspase-9/β-actin

DMSO: dimethyl sulfoxide; SF: sorafenib 10 μM; AM: alpha mangostin 20 μM; SF-AM: sorafenib 10 uM and alpha mangostin 20 uM; AM: alpha mangostin 20 μM; *: p<0.05; **: p<0.001.

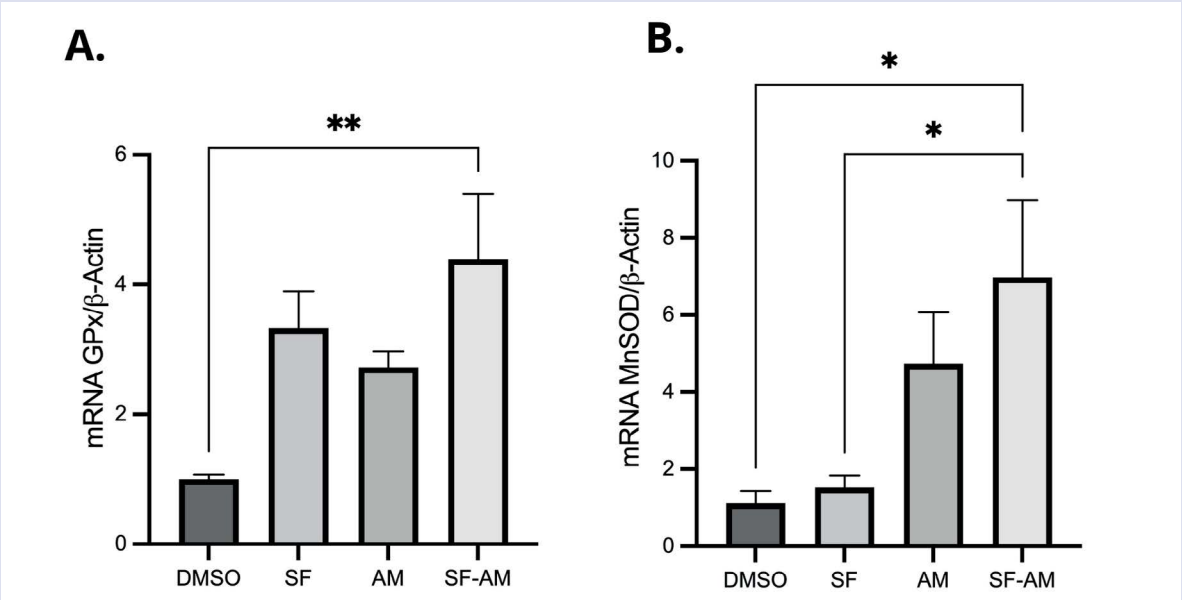

Figure 4: The mRNA expressions of oxidative-stress markers in sorafenib-surviving cells after treatment after treatment with sorafenib or alpha-mangostin or a combination of sorafenib and alpha mangostin. A) GPx/β-actin; B) mRNA MnSOD/β-actin**.**

DMSO: dimethyl sulfoxide; SF: sorafenib 10 μM; AM: alpha mangostin 20 μM; SF-AM: sorafenib 10 uM and alpha mangostin 20 uM; AM: alpha mangostin 20 μM; *: p<0.05; **: p<0.001.

A combination of sorafenib-alpha mangostin showed a distinct increase in mRNA expressions of GPx and MnSOD

The antioxidant markers quantified were GPx and MnSOD. Although both sorafenib and alpha mangostin caused an increase in mRNA expressions in both antioxidant genes, antioxidant mRNA expressions rose significantly when sorafenib-surviving cells were treated with the combination of sorafenib-alpha mangostin (Figure 4).

DISCUSSION

The previous study had shown that treatment of alpha mangostin in sorafenib-surviving cells might help decrease cell viability. However, it may also raise the signs of epithelial-mesenchymal transition.[14] The

purpose of the present study was done aiming to learn more about the alpha-mangostin's effect on drug resistance pathways such as cell proliferation, apoptosis and oxidative stress indicators in sorafenib-surviving liver cancer cells.

The present study had confirmed that in sorafenib-surviving hepatocellular carcinoma cells, sorafenib still has an effect in reducing cell viability. However, when administered in its CC50 dose, sorafenib was only reduced 20-30% of the cell viability. Nevertheless, alpha mangostin given at the CC50 dose was consistently reduced cell viability by about 50%. The combination of both drugs provides synergism in reducing cell viability.

However, the reduced cell viability after treatment with alpha mangostin or combination with alpha mangostin and sorafenib enhanced cell proliferation markers, Ki67 and c-Jun. In tumor cell proliferation, Ki67 is frequently utilized as a proliferative marker in regular pathological examinations. Ki67 has been demonstrated to have an association with tumor metastasis and clinical stage in cancer patietns. The expression of Ki67 in malignant tissues with poorly differentiated tumor cells is substantially greater than in normal tissue. Ki67-positive in advanced cancer cells are associated with patient survival.[19] The present study found a tendency to increase Ki-67 of the sorafenib-surviving cells after treatment with sorafenib, alpha-mangostin, or the combination of both drugs. This finding might explain the result of the previous study by Adenina *et al*., which showed an increase of EMT after drug treatments. Previous studies in several types of cancer had shown that increased expression of Ki-67 correlates with EMT and tumor invasiveness.[21,22]

Additionally, c-Jun, a transcription factor known as an oncogene, was remarkably increased after treatment with alpha mangostin or a combination of alpha mangostin and sorafenib. Increased expressions of c-Jun showed about three times as much when compared with alpha-mangostin alone. Overexpression of c-Jun was found to cause an increase in tumorigenesis, tumor migration in liver cancer and breast cancers.[23,24] In hepatocellular carcinoma, apoptosis is prevented by c-Jun *via* inhibition of p53 in the liver cancer cells. Data showed that apoptosis and accumulation of p53 were seen in experimentally generated c-Jun deletion in liver tumors.[25,26] The previous study demonstrates that high c-Jun plays a vital role in developing HCC resistance to sorafenib.[27] Our findings suggested that even though alpha-mangostin can be used to decrease cell viability, the consequences of the treatment were increased proliferation and the possibility of metastasis of the remaining cells.

At the same time, we found a considerable reduction of the ratio of Bax/BCl-2 ratio. However, no notable changes in Caspase-3 and Caspase-9 after treatment with alpha mangostin, alone or in combination with sorafenib. Previous research by Kritsanawong *et al*. in breast cancer T47D cells, alpha-mangostin was shown to induce apoptosis by decreasing Bcl-2 expression and increasing caspase-3 and -9 cleavage.[12]

In the case of HCC, prior research by Hsieh *et al*. shows that there is apoptosis induction by alpha-mangostin in HCC through downregulation of anti-apoptotic protein such as Bcl-2 and upregulation proapoptotic protein such as Bax as well as the activation of caspases, including caspase-9 and -3.[28] In the case of alpha-mangostin administration to sorafenib-surviving HCC, the research is still minimal. Bcl-2 protein is an anti-apoptotic protein, which in healthy cells arrest Bax action, a proapoptotic protein, therefore preventing apoptosis induction. In the presence of an apoptotic signal, BH-3 only members either induce the action of Bax or inhibit Bcl-2 action and eventually lead to apoptosis.[29] Increased expression of Bcl-2 and reduced expression of Bax might prevent apoptosis occurrence, resulting in the survival of damaged cells. The overexpression of Bcl-2 is associated with over half of various types of cancers, including in HCC.[27,30] In previous research, administration of ABT-263 (Bcl-2 and Bcl-xL inhibitor) shows sensitization of HCC to sorafenib.[11]

Caspases are crucial cysteine proteins that cleave target proteins, which lead to the success of apoptosis. In mitochondrial-dependent pathway apoptosis, the release of Bax from Bcl-2 stimulates the mitochondria to release cytochrome c. The process is continued with the activation of caspase-9 that will cleave and activate caspase-3. Specifically, caspase-3 can be activated by both extrinsic and intrinsic apoptosis pathways. Therefore, it can be considered to mediate the general characteristics of apoptosis. The under expressions of caspases have also been associated with tumors.[29,30]

Based on the Bax/Bcl-2 mRNA expression ratio analysis in our study, a marked decrease after AM treatment, therefore more resistance to apoptosis. As the inhibition of Bcl-2 and Bax act to activate mitochondria to produce cytochrome c. The decrease of the ratio may also explain the insignificant changes in caspase-9 mRNA expression and its tendency to be lowered after AM treatment. This suggests the lack of intrinsic pathway involvement—however, caspase-3 mRNA upregulation in the presence of AM indicates apoptosis activity.

Overall, this research showed the apoptosis effect induced by alpha-mangostin in sorafenib-surviving HCC, as shown by caspase-3. Nevertheless, based on insignificance change of Bax/Bcl-2 ratio and caspase-9, which are involved in the intrinsic pathway of apoptosis. This finding may suggest that the generation of apoptosis by alpha-mangostin mainly *via* the extrinsic pathway, which involves death factors such as the TNF family which the binding create death-inducing signaling complex by FADD and TRADD followed with cleavage of caspases, including caspase-8, -10 then the executioner caspase-3, -6 and -7.[29,30]

Reduced apoptosis after alpha mangostin treatment was accompanied by increased antioxidant expressions, namely GPx and MnSOD. In terms of its antitumor effects, oxidative stress and apoptotic activity are the critical mechanisms of anti-cancer effects of sorafenib.[4,6] Therefore, increased antioxidant effects in sorafenib-surviving cells can be considered as an increased risk of anti-cancer failure.

Alpha mangostin was known to have an antioxidant effect in many studies.[31,32] However, another study by Zhang *et al*. showed that alpha mangostin had a dual effect which could be either an antioxidant or a pro-oxidant, dose-dependently.[33] Studies concluded that doses closer to CC50 accentuated pro-oxidant effects.[33,34] In contrast, the study by Hafeez *et al*. showed a dose-dependent decrease in intracellular and extracellular SOR with continued induction of apoptosis in pancreatic cancer cells. The study concluded that the ability to modulate ROS by alpha mangostin depends on the type of cell studied.[35]

Based on the results obtained in the study, the potent cytotoxic effects resulting from the treatment with alpha mangostin might promote the feedback mechanism from the cells, which boosted the expressions of the antioxidant mechanism.

CONCLUSION

Alpha mangostin alone or in combination with sorafenib evidently reduced the cell viability in sorafenib-surviving hepatocellular carcinoma cells. However, the remaining cells have the characteristics of increased proliferative, anti-apoptotic and antioxidant expressions.

DATA AVAILABILITY

All contributing data in the present study are available upon request to the Corresponding Author.

CONFLICTS OF INTEREST

All authors declared no conflicts of interest.

ACKNOWLEDGEMENT

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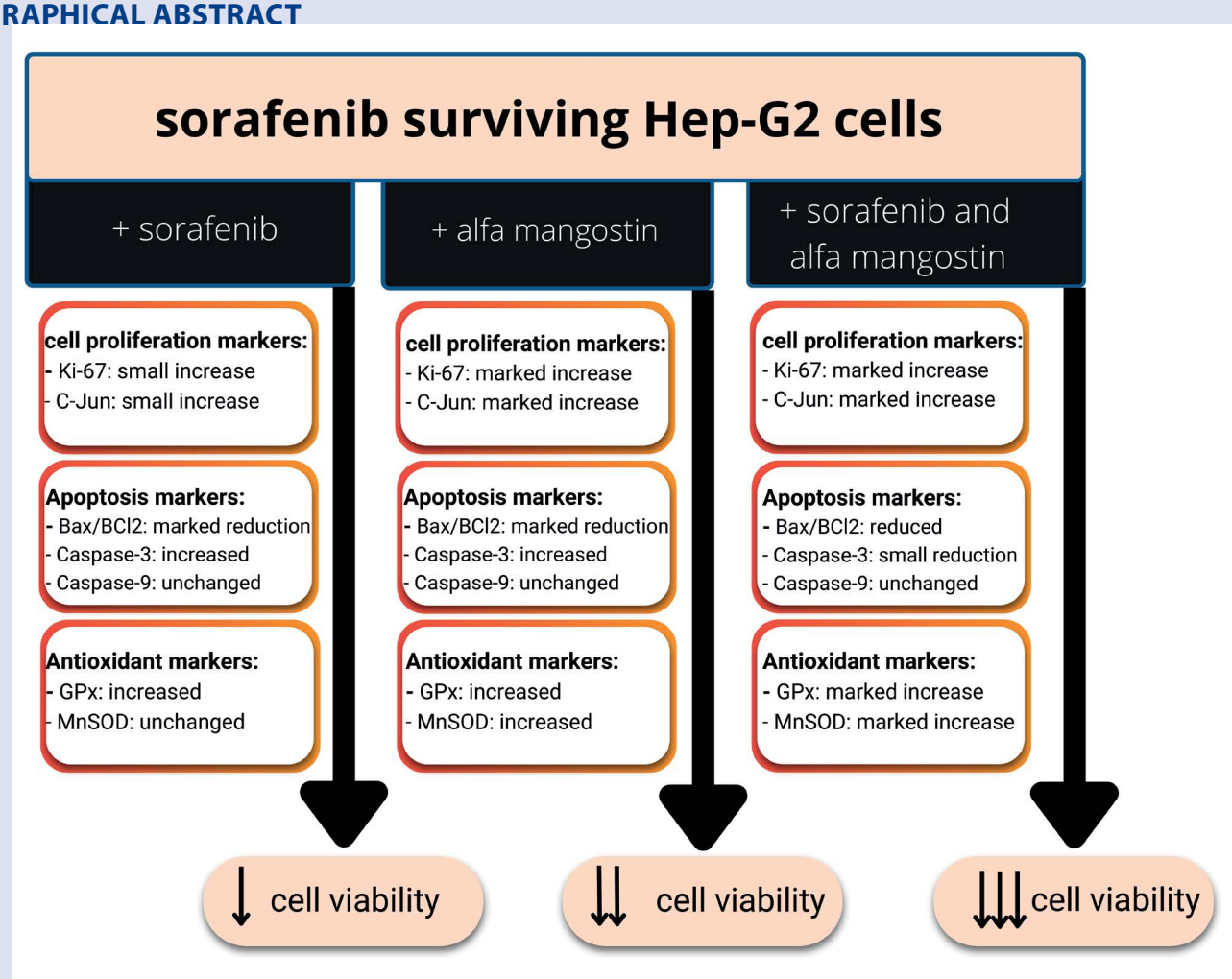
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Melva Louisa is currently an Academic staff in the Department of Pharmacology and Therapeutics Faculty of Medicine Universitas Indonesia. Apart from teaching, her current activities include being the research coordinator in her department. Her research interests include drug development studies from natural products, mainly to overcome anticancer drug resistance or toxicities.

Meuthia Faralita Annisa is currently a clinical medical student in Faculty of Medicine, Universitas Indonesia where she also completed her bachelor's degree in medicine with cum laude. Prior continuing to clinical from preclinical study in medicine, she obtained her master's degree in Cancer at Newcastle University, United Kingdom where she worked with Cancer Drug Discovery Unit Newcastle under Cancer Research United Kingdom. To date, she still has great interest and passion in Cancer research, specifically in cancer drug and discovery, and translational medicine.

Pamela Basuki is currently a clinical medical student in Faculty of Medicine Universitas Indonesia. She received her undergraduate degree in Medical Science in 2021 and was an active student who took a role as conference liaison for Journal of Asian Medical Students' Association (AMSA) among other roles. She has a lot of interest in different fields and is still studying and researching in the medical field relentlessly. She has developed an interest in pharmacology, otorhinolaryngology and more.

Brigitta Cindy Lauren is currently a fifth-year medical student in Faculty of Medicine University of Indonesia. In 2018, she participated in a student exchange program for 3 months in Leiden University Medical Center, Netherlands. She received the Bachelor of Medicine from University of Indonesia in 2021 and currently studying in Cipto Mangunkusumo National Hospital for the medical doctor degree. Her research interests are in the fields of gastroenterology, hepatology and oncology.

Syarinta Adenina hold a Master's degree in Biomedical Sciences from the Faculty of Medicine, Universitas Indonesia, and a Bachelor's degree in Medicine from the Faculty of Medicine, Universitas Sriwijaya. She is currently a lecturer in the Medical Faculty at YARSI University, working in the pharmacology department. She has experience researching hepatocellular carcinoma and publishes articles about the alpha-mangostin effect on sorafenib resistance.

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