POTENTIAL APOPTOTIC EFFECT OF PLANTAIN EXTRACT (Plantago major L.) THROUGH INCREASING OF CASPASE-3 LEVEL ON HYPERGASTRINEMIC RAT MODEL

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ABSTRACT
This study was to evaluate the effect of Plantago major L extract. on increasing of caspase-3 level on hypergastrinemic rat (Rattus norvegicus) model. By experimental study and post test only with control group design, 20 of rats was divided in to 4 groups. Group I as a negative control was given aquadest per oral. Group II was given Plantago major L extract 50mg/200gbw rat/day per oral. Group III was given Plantago major L extract 100mg/200gbw rat/day per oral. Group IV was given Plantago major L extract 200mg/200gbw rat/day per oral. Omeprazole and Ciprofibrate have been used to induce hypergastrinemia on rats as an animal model. Blood sample was collected for measurement of gastrin and caspase 3 level after 2 month intervention. There were increasing of gastrin level of rats up to 3-4 times fold compared to normal level. Statistic analysis show that there were significantly difference of caspase-3 level between groups (p=0.041; 95%CI) Dose 200mg/200gBW rat/day per oral of Plantago major L extract are most effective to increase the caspase 3 level. This research can be concluded that administration of Plantago major L extract. can increase caspase 3 level indicate that Plantago major L extract has proapoptotic effect on hypergastrinemia rat (Rattus norvegicus) model.

KEYWORD
Apoptotic effect, Plantago major L extract, Caspase 3 level and Hypergastrinemia.
immunostimulant and anti-neoplastic\textsuperscript{1,2}. Therefore, *Plantago major* L have used as a traditional medicine for various conditions of health disorder. However, the scientific data for pharmacological effect is still poor.

The research of plantain extract for investigation anti-neoplastic effect show good efficacy as a chemoprophylactic and anti-metastatic agent for several carcinogenesis, such as breast cancer, hepatoma, ehrlich ascites tumour\textsuperscript{3-6}. Plantain extract can also inhibit to carcinogenesis of melanoma cell line tumour, renal adeno ca and adeno ca mammae\textsuperscript{7}. The ethanol extract of plantain show more effective than water extract whether in hot or cold water. Triterpenoid and flavonoid are the main active substances of the ethanol extract of plantain that have cytotoxic effect and show to inhibit carcinogenesis and to increase tumour cells apoptosis\textsuperscript{8-10}.

The triterpenoid substances of plantain extract that have chemopreventive activity consist of *ursolic acid*, *oleanic acid* and *batulinic acid* while the flavonoids are *luteoline-7-O-β-glucoside*, *apigenin*, *hispidulin* and *baicalein*. *Luteoline-7-O-β-glucoside* is the main flavonoid substance of plantain extract play the important role on carcinogenesis inhibition\textsuperscript{11}. In previously study, all of the substances shown anti cancer activity by mechanism tumour cells destruction and to induce tumour cells death (apoptosis)\textsuperscript{11-13}. These substances can induce tumour cells apoptosis through mitochondrial pathways. The mitochondrial pathway of apoptosis is begun with alteration of membrane permeability that cause proteins release include Cytochrome c, apoptosis inducing factor (AIF) and endonuclease G. Cytochrome c in conjunction with apoptosis protease activating factor (APAF-1) and pro-Caspase 9 form an apoptosome. This complex promotes the activation of Caspase 9, which in turn activates effector caspases that collectively orchestrate the execution of apoptosis. AIF\textsuperscript{14} and endonuclease G\textsuperscript{15} both contribute to DNA fragmentation and subsequent chromosomal condensation, which are hallmark features of apoptosis. Other proteins released upon mitochondrial outer membrane permeabilisation include Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-associated binding protein with low pI) and Omi/HtrA2 (high temperature requirement A2), which antagonize IAPs thereby promoting caspase activation\textsuperscript{16,17}.

*Ursolic acid*, *luteoline-7-O-β-glucoside*, *apigenin*, *hispidulin* and *baicalein* increase expression of pro apoptotic proteins and activation of *p53*, *bax* and *bak*. These substances also inhibit to anti apoptotic proteins expression, such as Bcl2, Bcl-XL, Mcl-1, *cyclin D1-D2-E* and cdk\textsuperscript{18-21}. Among the active substances of plantain extract, lutheolin is the strongest substance to induce cancer cells apoptosis. Lutheolin induce cancer cells apoptosis through several pathways are (1) to decrease level of anti apoptotic proteins, Bcl2, Bcl-XL and to increase pro apoptotic protein Bax and Bak; (2) to increase releasing of cytochrome-c continue to the activation of Caspase 9, Caspase 8, Caspase 3 and death receptor-5 (DR-5) of tumour cells\textsuperscript{22-25}; (3) to increase *TNF related apoptosis induced-ligand* (TRAIL) sensitivity to induce apaptosis of tumor cells throughout activation of Caspae 8 and Caspase 3 maturation; (4) down regulation of *X-linked inhibitor apoptosis protein* (XIAP) and inhibition of protein kinase C activity\textsuperscript{24,25}.

Hypergastrinemia has been related to gastric carcinogenesis. Hypergastrinemia is indicated by increasing serum level of gastrine up to more than 169pg/mL. Gastrin stimulates gastric acid secretion mediated by gastrin (cholecystokinin-2) receptors on the enterochromaffin like (ECL) cells\textsuperscript{26}. Gastrin also has trophic effects on the oxyntic mucosa, most notably on the ECL cells. Gastrin can stimulates the function and growth of the oxyntic mucosa of the rat as well as human. This effect has been shown to result in ECLomas in rats following long-term hypergastrinemia. Four weeks along hypergastrinemia can cause ECL cells and gastric mucosa hyperplasia. It will develop to ECLomas and gastric mucosa dysplasia if hypergastrinemia continue untill sixteen weeks. even, It can be carcinogenesis if hypergastrinemia continue untill six month and more. Hypergastrinemia induce
overexpression of genes within ECL cell, such as regenerating 1 (Reg-1)\(^2\). Reg 1 was originally identified as a regenerating growth factor for pancreatic islet beta cells\(^3\). Reg 1 is also expressed ectopically at various sites in the gastrointestinal tract, the highest expression being in the stomach especially in gastric carcinoma\(^2,3\). REG has been reported to be involved in various biologic functions including cellular proliferation, differentiation, and resistance to apoptosis during regeneration, inflammation, and tumorigenesis\(^34,36\).

Reg 1 gene encode synthesis of regenerating 1 alpha (Reg 1\(^\alpha\)) protein that induce cellular proliferation and differentiation. Reg 1\(^\alpha\) also increase activation of baclyn 2 (Bcl-2) and bad but inhibit to activation of caspases include Caspase 3. It is important to evaluate Caspase-3 as a therapeutic molecular target in use the chemotherapeutic agent of neoplasm.

Caspase-3 is a member of the interleukin-1ß-converting enzyme family, which specifically cleaves substrates at the C terminal of aspartic acid residues\(^37,38\). Caspase-3 is synthesized as an inactive proenzyme and to undergo activation process into its active form during apoptosis\(^39\). Activated caspase-3 is responsible for the cleavage of polyADP-ribose polymerase (PARP), actin and steroid regulatory element binding protein (SREBP) which relates to apoptosis\(^40,41\). Caspase-3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis\(^40\).

Hypergastrinemia also induce genes expression encode neurotrophic tyrosine kinase receptor trkB (Ntrk2) that stimulate to increase cellular proliferation but inhibit to apoptosis of gastric mucosa, result in gastric mucosal thickness. However, Six months along hypergastrinemia induce overexpression of chromogranine A (CgA) gene and histidine decarboxylase (Hdc) gene. Overexpression of these genes were related to ECL cell and gastric mucosal dysplasia and carcinogenesis\(^30,42,43\).

Hypergastrinemia can be induced by using long term omeprazole and ciprofibrate\(^42,44,45\). Omeprazole is a common proton pump inhibitor to inhibit gastric acid secretion (anti secretory drug) while Ciprofibrate is anti dyslipidemic drugs. Combination of omeprazole and ciprofibrate for four weeks can produce sinergetic effect to increase level of gastrine up to 24 time fold compared to normal level (169pg/mL)\(^42\). This study was to evaluate the potency of apoptosis effect of ethanol extract of Plantago major L on hypergastrinemic rat (Rattus norvegicus) model based on elevating of level of caspase 3. Whether the hypergastrinemia can decrease caspase-3 level and this effect can be inhibited by administration of ethanol extract of Plantago major L.

MATERIALS AND METHODS

Drugs

Ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)phenoloxyl] 2-methyl propanoic acid; Modalim, Sanofi Synthelabo Ltd, Newcastle, UK, Omeprazole; SOCID, PT SOHO, Jakarta were used for making hipergastrinemia rat model. The doses were determined through preliminary study in order to find the most appropriate dose for inducing of hypergastrinemia in rat.

Plant Extraction

Plantago major L seeds were collected from Slamet mountain, Purwokerto, Central Java, Indonesia and authenticated by Dr. Pudji Widodo MSc, Laboratory of Taxonomy Faculty of Biology, Jenderal Soedirman University. The leaves were collected and dried at room temperature, protected from dust and sunlight. Leaves and seeds were pulverized manually. Fifty grams of each plant powder was extracted in 500 ml of ethanol by maceration (48 h). The solvent was removed under vacuum at temperature below 50°C, and then the extracts were freeze-dried\(^46,47\). Dose of the plaintain extract were devided into 3 groups that are 50mg /200gBW rat/day, 100mg /200gBW rat/day, 200mg /200gBW rat/day per oral.

Animal and Experiment Protocol

The rats were housed in wire-bottom cages at 20°C and adaptation for a week at Laboratorium of Pharmacology and Therapy. 20 of rats 2-3 month age, weighing between about 190-210g was divided in to 4 groups. Group I as a negative control was given Omeprazole 8mg/200gBW rat and Ciprofibrat...
dose 12, 5 mg/200gBB rat/day per oral. Group II was given Omeprazole 8mg/200gBW rat and Ciprofibrat dose 12, 5 mg/200gBB rat/day and Plantago major L. 50mg/200gBW rat/day per oral. Group III was given Omeprazole 8mg/200gBW rat and Ciprofibrat dose 12, 5 mg/200gBB rat/day and Plantago major L. 100mg/200gBW rat/day per oral. Group IV was given Omeprazole 8mg/200gBW rat and Ciprofibrat dose 12, 5 mg/200gBB rat/day and Plantago major L. 200mg/200gBW rat/day per oral. The blood samples were collected for laboratory examination of gastrin and caspase 3 level. This protocol was approved by The Health Research Committee Faculty of Medicine University of Padjadjaran Bandung, Indonesia.

Measurement of Gastrin Level
Level of gastrin was examined to determinate whether the animals have undergone hypergastrinemia in this study. The animals were fasted overnight. Blood was collected by cardiac puncture under general anaesthesia, and serum was prepared by centrifugation after clotting at 4°C. The levels of gastrin were measured by the use of an ELISA-based kit for Mouse Gastrin (GT ELISA kit, BG-MUS11122, produced by NovaTeinBio, Inc, Cambrige, USA).

Measurement of Caspase-3 Level
Blood from cardiac puncture and serum was prepared by centrifugation after clotting at 4°C. The levels of caspase 3 were measured by the use of an ELISA-based kit (Caspase-3 assay kit, produced by Uscn Life Science Inc, Wuhan, China).

Statistics
Statistical analysis conducted with computer program of SPSS version 17. The differences of caspase 3 levels between groups of the study were tested Kruskal-Wallis test followed by Mann-Whitney test while Gasrin level was analysed by One Way ANOVA test.

RESULTS AND DISCUSSION
The evaluation of serum Gastrin level
It is important to assess serum gastrin level for determination whether the animal undergo hypergastrinemia or not. Hypergastrinemia is indicated by serum gastrin level more than 169pg/mL. In this study, the result of measurement by ELISA show that all of the animals have serum gastrin level more than 169pg/mL. It is mean than all of the animals undergo hypergastrinemia and can be involved in this study. The lowest of serum gastrin level found in Groups I as negative control(498,0 pg/mL) while the highest of serum gastrin level found in Group III (697,5 pg/mL). The average of serum gastrin level of the animals increase up to 3-4 times fold compared to normal level (Table No.1).

According to statistical analysis, there were not significantly difference of Gastrin levels (p=0,356; 95%CI) between groups. It means that there were not influences of plantain extract to gastrin levels. This result can be used as scientific explanation that the effect of plantain extract to Caspase-3 level is directly effect and it is not related to difference of gastrin levels between groups (indirectly effect). So, the analysis of effect of plantain extract to Caspase-3 levels can be continued. In the other hand, if this analysis found that there were significantly difference of Gastrin levels between groups. It mean that there were influence of plantain extract to gastrin levels and it can be assumed that the difference of Caspase-3 levels were not caused by plantain extract only but it can also be influenced by gastrin levels. Therefore, it will difficult to make true conclusion and it is unnecessary to continue to analysis of the effect of plantain extract to Caspase-3 levels.

The effect of Plantain extracts to serum Caspase-3 levels
Four month along hypergastrinemia can decrease to serum Caspase-3 levels through induction of Reg I to increase Reg 1α synthesis which can suppress to activation of caspases. The administration of plantain extract able to restrain hypergastrinemia’ effect on inhibition of Caspases activation including Caspase-3 and It can increse to serum Caspase 3 levels. Measurement of serum Caspase-3 levels by ELISA found that there were different of serum Caspase-3 levels between groups of study. The lowest of serum Caspase-3 levels was found in rat from Group II (0,020 ng/mL) while the highest of serum Caspase-3 levels was found in rat from Group II (0,020 ng/mL) while the highest of serum Caspase-3 levels was found in rat from Group II (0,020 ng/mL).
levels was found in rat from Group IV (0.860 ng/mL). Generally, the animals study in Group IV have the highest average of serum Caspase-3 levels (0.603±0.172 mg/mL) compared to the other groups. (Figure No.1 and Table No.2).

Kruskall Wallis Test used to statistical analysis and the result showed that there were significantly difference of serum Caspase-3 levels between groups (p-value=0.041;95%CI). It mean that there were significantly difference of serum Caspase-3 levels at least two groups of the study. Mann Whitney test used to determinate significance of serum Caspase-3 levels difference between two groups of this study. According to Mann Withney test, there were significantly difference of serum Caspase-3 levels between Group IV and the others. That are Group I and IV (p=0.026; 95%CI), Group II and IV (p=0.038; 95%CI) and Group III and IV (p=0.018; 95%CI). This result show that dosage 200mg/200gbw rat/day per oral of ethanol extract of Plantago major L is the most effective dosage to increase serum Caspase-3 levels on hypergastrinemia rat model.

It is possible that the increasing of serum Caspase-3 levels caused by directly effect of many active substances of plantain extract, especially triterpenoid and flavonoid. The triterpenoid compounds are ursolic acid, oleanic acid, batulinic acid while the flavonoid compounds are luteoline-7-O-β-glucoside, apigenin, hispidulin and baicalein. Both of Triterphenoid and flavonoid compounds can activate caspases as long as apoptosis process, include activation of procaspase-3 (inactive proenzyme) to be Caspase-3 as an important factor for cell apoptosis, that is as executioner caspase which is essential for the nuclear changes associated with apoptosis.

Plantain extract can activate Caspase-3 through various pathways. One of the mechanism is through activation of p53 by Ursolic acid, Oleanic acid and Apigenin which will induce cytochrom C release, apoptosis inducing factor (AIF) and mitochondrial Endonuclease G. This mechanism will continue to activation of caspases, including Caspase 3 lead to apoptosis process. Ursolic acid (3β-hydroxy-urs-12-en-28-oic-acid) is triterpenoid compounds found in the form of the free acid or as aglycones of triterpenoid saponins. Ursolic acid and its derivatives have been shown to induce apoptosis in a wide variety of cancer cells, such as hepatoma, prostate carcinoma, melanoma, leukemia and breast carcinoma. The mechanism of apoptosis effect of Ursolic acid can occur through inhibition of protein tyrosine kinases, inhibition of DNA replication, activation of caspases, induction of Ca2+ release, and by involves down-regulation of the cellular inhibitor of apoptosis gene, that is another gene known to be regulated by NFkB.

Ursolic acid has been known as a potent inhibitor of nuclear factor-κB (NFκB) activation pathway, which is activated by inflammatory agents, carcinogens, and tumor promoters. Because of the critical role of STAT3 in tumor cell survival, proliferation, and angiogenesis, it is possible that ursolic acid suppres to the STAT3 pathway for mediating those effects. Ursolic acid also down-regulated the expression of STAT3-regulated gene products, such as Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, and VEGF. It induced the inhibition of proliferation, the accumulation of cells in G1-G0 phase of cell cycle and induced cells apoptosis.

Ursolic acid has a similar molecular structure with Oleanic acid, but have different sites of the methyl group on the E loop. Oleanic acid is also a pentacyclic triterpenoid acids. Oleanic acid can induce cells apoptosis by mechanism that similar to Ursolic acid. Oleanic acid activate caspase-3 and caspase-9 which accompanied by the cleavage of poly (ADP-ribose) polymerase (PARP) in the target cells during induced apoptosis. In addition, Oleanic acid activate mitochondrial apoptotic pathway and induced G2/M cell cycle arrest through p21-mediated down-regulation of cyclin B1/cdc2. Oleanic acid also related to extracellular signal-regulated kinase-p53 signal that played a central role in OA-activated cascades responsible for apoptosis and cell cycle arrest.

Batulinic acid is other pentacyclic triterpene that shown apoptotic effect in several cancer cell lines. The mechanism of betulinic acid--induced...
proapoptotic effect through multiple pathway. Batulinic acid induce apoptosis is due to activation of selective proteasome-dependent degradation of the transcription factors specificity protein 1 (Sp1), Sp3, and Sp4, which regulate vascular endothelial growth (VEGF) and survivin expression. Betulinic acid decreases expression of VEGF and survivin which role as antiapoptotic protein. Batulinic acid also cause direct effects on the mitochondria accompanied by decreased mitochondrial membrane potential, up-regulation of death receptors, and interactions with other agents. However, direct effect on the mitochondria can induce cytochrome c release, AIF and accompanied by activation of caspases. The other compunds of plantain extract that has potent apoptotic effect is Baicalein. Baicalein is the bioactive flavonoid (5,6,7-trihydroxyflavone) has been known to cell proliferation and induces apoptosis of cancer cell lines. Baicalein induces apoptosis primarily through the mitochondria-dependent activation of the caspase-9 and caspase-3 pathways. Baicalein also promoted production of p53, BAX, cytochrome c, EndoG and AIF and reduced the levels of BCL-2 that lead to the disruption of mitochondrial membrane potential (MMP) and the release of cytochrome c from the mitochondria to the cytosol. Finally, this process will activation of caspases, including caspase-3 during inducing apoptotic process. It is well-known that mitochondrial alterations constitute a critical event of the apoptotic cascade.

These evidence are consistent to this study that administration of plantain extract can increase serum caspase-3 levels. The plantain extract have a lot of triterfenoid and flavonoid compounds, including Ursolic acid, Oleanic acid, Batulinc acid, Baicalein and the other compounds that have apoptotic effect, such as Hispidulin. We found that the effect of plantain extract for inceasing of serum caspase-3 levels is dose-dependent. Dosage 200mg/200gbw rat/day per oral of ethanol extract of Plantago major L is the most effective dosage to increase serum Caspase-3 levels on hypergastrinemia rat model. However, this effective dosage still need to develop by doing the next study with a higher dosage intervals.

Table No.1: The means of serum Gastrin levels among the groups of this study

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups of Study</th>
<th>Serum Gastrin Level (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I, Negative Control</td>
<td>570.17±54.01</td>
</tr>
<tr>
<td>2</td>
<td>Group II, 50mg of extract</td>
<td>592.08±30.79</td>
</tr>
<tr>
<td>3</td>
<td>Group III, 100mg of extract</td>
<td>622.00±67.62</td>
</tr>
<tr>
<td>4</td>
<td>Group IV, 200mg of extract</td>
<td>609.79±52.04</td>
</tr>
</tbody>
</table>

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Table No.2: Mean, Median and Deviation Standard of Serum Caspase-3 levels
Among the groups of Study

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups of Study</th>
<th>K I</th>
<th>K II</th>
<th>K III</th>
<th>K IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rerata</td>
<td>0.382</td>
<td>0.338</td>
<td>0.293</td>
<td>0.603</td>
</tr>
<tr>
<td>2</td>
<td>Median</td>
<td>0.400</td>
<td>0.451</td>
<td>0.369</td>
<td>0.594</td>
</tr>
<tr>
<td>3</td>
<td>Standar Deviasi</td>
<td>0.107</td>
<td>0.233</td>
<td>0.082</td>
<td>0.172</td>
</tr>
</tbody>
</table>

Figure No.1: The average of Serum Caspase-3 levels Among the groups of Study

CONCLUSION
The administration of Plantago major L extract can increase caspase 3 level indicate that Plantago major L extract has proapoptotic effect on hypergastrinemia rat (Rattus norvegicus) model.

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