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Research Article

The Effect of Subchronic Administration of Soursop (*Annona muricata*) Leaf Aqueous Extract In Bax Expression on Gastric Glandular and Non Glandular Mucosal Epithelium of Rat (*Rattus norvegicus*)

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Abstract: Soursop leaf aqueous extract (SLAE) contains acetogenin compounds which are mitochondrial complex I inhibitors. This compound can reduce cellular ATP production and induce apoptosis via bax pathways, causing side effects on cells. Gaster is a digestive organ that has direct contact with acetogenin. Gastric cells contain mitochondria and undergo physiological apoptosis.This study aims to determine the effect of subchronic administration of SLAE on bax expression on glandular/non glandular mucosal epithelium gastric of rats. This study uses posttest only control group design. The subjects were 10 female rats, Spraque-dawley strain which was divided into 2 groups; the treatment and the control group. The treatment group received extract of SLAE at a dose of 1000 mg/kgBW/day for 30 days, while the control group received aquades, both were administered using gastric tube. Observation of bax expression was performed on each Immunohistochemistry (IHC) preparation with bax antibody. The difference in bax expression between the control and treatment groups was tested by t-test. There were significant differences in the number of bax expressions in gastric glandular mucosa (p 0.038) and non-glandular gastric mucosa (p 0.027) between the treatment group and the control group. There was an effect of subchronic administration of SLWE on bax expression on mucosalepithelium for both, glandular and non-glandular of rat gaster. There were differences in the number of glandular gastric as well as non-glandular gastric mucosa epithelium which exerted bax between the control and treatment groups

Keywords: *Annona muricata* leaf, bax, gaster

Introduction

Soursop (*Annona muricata*) is a herbal plant known for its anticancer properties such as inducing apoptosis of cancer cell. The apoptosis process is primarily triggered by a decrease in energy due to inhibition of mitochondrial complex I enzymes. The main compound responsible for the effect is acetogenins, a mitochondrial complex I inhibitor that able to disrupt the oxidative phosphorylation chain [1,2]. These compounds are not only found in soursop fruit, but also resided in almost all parts of the plant, especially in the leaves [1,2].

Although this plant showed an anticancer effect, however, the inhibition of mitochondrial complex not only occurs in cancer cells, but also in healthy cells [3–7]. Therefore, various organs of the body can be affected by acetogenin compounds, including the brain and kidneys. This finding is supported by the presence of tau phosphorylation which potentially led to symptoms of neurodegenerative disorders [3]. Apparently, these caused by damage to dopaminergic neurons in the

substantia nigra and ventralis tegmenti areas of the rat brain. Moreover, soursop leaf extract may cause necrosis on the tubular cells and glomerulus of the healthy kidney through the caspase 9 mechanism [6]. Mutakin *et al*. in 2022 also mentioned an increase in apoptosis index in liver cells of rats following administration of extracts containing acetogenin [3].

In Indonesia, the habit of consuming herbal medicine as an alternative medicine leads to the frequent and prolong use of soursop leaf decoction water. This oral treatment allows direct contact with the active compounds of soursop leaves with gastric mucosa (stomach) therefore enable the prompt effects on the gastric mucosa [3]. Gaster is featured with epithelial cell renewal mechanism as well as an active apoptotic pathway. Physiologically, the stomach will lose a number of epithelial cells over time. It is estimated that 1-3% of epithelial cells in the antrum, corpus or fundus will undergo apoptosis which involves bax protein. Bax protein is expressed in the glandular gastric mucosa, especially in chief cells and glandular mucosal parietal cells [8].

In chronic uses of soursop leaves extract could damage the stomach which will lose a number of epithelial cells over time as the adverse side effects [9]. Soursop plants are suggested to induce apoptosis in healthy cells especially in highly proliferative tissue and in gastrointestinal [7]. Gavamukulya, *et al* in 2017 showed the damages of gastric wall mucus (GWM) due to the increases gastric acid production and lead in epigastric complaint [4].

Unfortunately, there are lack of studies that focus on the effect of soursop leaves on the mechanism of apoptosis involving bax proteins in the gastric epithelium. Therefore, the author interested in examining the effect of subchronic administration of soursop leaf extract (*Annona muricata* Linn) on Bax expression in the glandular mucosal epithelium and non-glandular rat gastric (*Rattus norvegicus*).

Methods

This study has obtained ethical clearance from Ethics Committee of Faculty of Medicine, Universitas Islam Indonesia with 12/Ka.Kom.Et/70/KE/XI/2019. This was a quasi-experimental study with post-test control group design. It was conducted at Laboratory of Faculty of Medicine, Universitas Islam Indonesia. Its subjects were adult female Spraque-Dawley rats (*Rattus norvegicus*). The inclusion criteria of this study were healthy and non-disabled rats, aged 3 months with a body weight of 175-300 g. Exclusion criteria were dead rats during the study.

This study consisted of two groups. The number of rats used was 10, which then divided into 2 groups and each group consisted of 5 rats. The first group was the control group and the second group was the treatment group. The control group received aquades and the treatment group received aqueous extract of soursop leaves (*Anonna muricata*) with a dose of 1000 mg/kgBW/day given for 30 days, modified from Handayani *et al*., 2015 [10]. Both were administered using gastric tube. The dependent variable is the expression of bax in the glandular mucosal epithelium and mucosa of non-glandular rat gaster. Euthanasia was performed after 30 days of treatment. The rats were anesthetized by using ketamine with a dose of 80-100 mg/kgBW administered intramuscularly on the lateral thighs of the rats. Next, a midline incision was made on the abdominal wall, followed by an incision along the median axillary line until the chest wall was opened and the heart was visible.

In this Bax antibody IHC staining, the cytoplasm of the gaster epithelial turned brownish. The obtained data were analysed by a statistical software. Data analysis using t-test by observing the difference in the number of bax expressions between groups I and II.

Result and Discussion

Result

Based on the observation of Bax expression, the brown color that appears indicates a positive reaction (+). The results of observing the number of Bax expressions are seen with an objective 1000x magnification (Table 1, Figures 1 and 2).

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Table 1. Mean of bax expression in rat gaster

	Group		P value
	Treatment	Control	
Glandular cell	6.24±2.97	2.80±0.88	0.038*
Non-Glandular cell	20.30±16.63	9.75±7.18	0.027*

*p<0.05

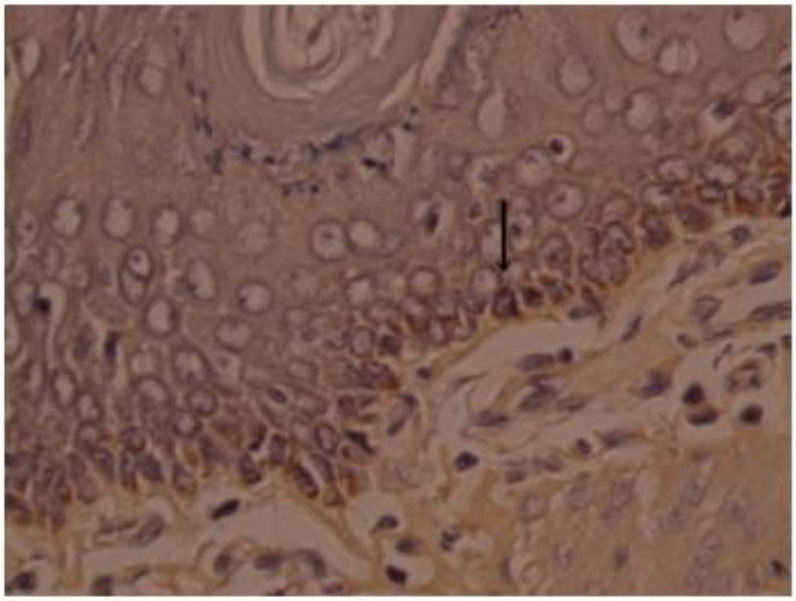

Figure 1. The histologic feature of Bax expression in non-glandular cells (Black arrow is a cell with bax (+) expression at 1000x magnification)

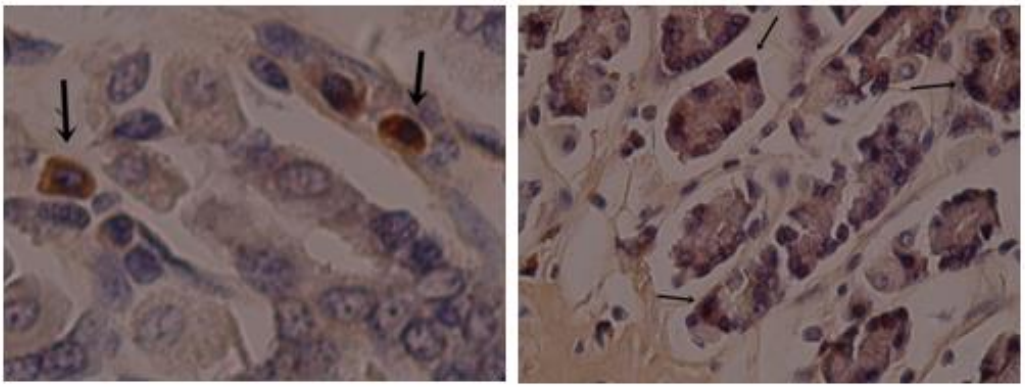

Figure 2. The histologic feature of Bax expression in non-glandular cells (Black arrow is a cell with bax (+) expression at 1000x magnification)

In the treatment group, the mean number of glandular and non-glandular cells expressing Bax was 6.24 and 20.3 respectively. Bax expression in this group was higher compared to the control group with the calculation of bax expression is presented in table 1. The results of statistical analysis with t-test showed that there were significant differences between both groups (p = 0.038 and 0.027). Administration of soursop leaf aqueous extract at a dose of 1000 mg/kgBW for 30 days can increase bax expression in gastric cells.

Discussion

Our results indicate there is an effect of giving soursop leaf aqueous extract to bax expression on the rat gaster. Treatment group rats that received extract of soursop leaf aqueous showed an increase

in bax expression in both mucosal lining, the non-glandular and glandular. In accordance with that, the control group rats also expressed bax but in a smaller amount compared to the treatment group.

This research finding supports other studies that investigate the side effects of using soursop leaves. Side effects are mainly caused by active compound in the soursop, which play role as mitochondrial acetogenin complex I inhibitor that reduce cellular energy production. This, consequently, will increase the release of calcium ions and Reactive Oxygen Species (ROS) in the tissues, therefore inducing the apoptotic process. This process is initiated by an increase in bax levels in the tissue, and ischemic conditions are a trigger for increased bax expression in gastric mucosal cells [11,12].

Acetogenins are lipophilic compounds and potent inhibitors in mitochondrial complex I which is main chain in respiratory process; consequently, energy deprivation takes place. Adenosine triphosphate (ATP) reduction will also cause cell damage in the form of increased nitric oxide (NO), ROS production, glutathione depletion (GSH), increased mitochondrial membrane permeability as well as caspase activation [3,7,12]. The excessive production of ROS leads to an increase in cell permeability, hence inducing cell apoptosis that mediated by caspase-3 and caspase-9 [3,12]. On the other hand, free radicals accumulation in the mucosal and submucosal layers of the gaster causes inhibition of cyclooxygenase activity. This is a major cause of loss of mucus and epithelial protection ability, resulting in erosion and injury of the gastric mucosa [13,14].

In healthy gastric cells, the balance between proliferation and apoptosis of gastric epithelium is strictly regulated. Apoptosis of gastric cells is a physiological condition, in which approximately 2% of gastric epithelial cells undergo this process. Following that, within 3-5 days the gastric superficial mucosal epithelial cells are normally released into the lumen of the stomach [11,15]. In this study, bax expression in the control group was less than the treatment group. Under normal conditions, bax proteins will be excreted in the glandular cells of the stomach and the gastric mucosa [8].

Consistent with the study of Li *et al.* in 2013, our result showed the involvement of pro-apotosis bax protein in inducing apoptosis after the administration of acetogenin compounds. In line with that, Arfeni's research in 2014 suggested that the administration of soursop leaves water extract for 30 days led to gastric mucosa erosion on the rats [9]. According to Wahab in 2018, the use of oral soursop leaves allows direct contact of acetogenin compounds, which can cause injury to the gaster surface. Such condition of acute injury will eventually increase the incidence of apoptosis [15]. Administration of annona muricata leaf extract at dose 1000 mg/kgBW dose significant increases the relative weight of stomach of female rat [16]. The results of the sistematic review showed that the administration of soursop water extract was safe up to a dose of 800 mg/kgBW in rat [17].

Our study suggests the existence of side effects in the use of soursop leaf water extract. This result is supported by Gayamukulnya *et al* in 2017 which stated consuming soursop in long period of time could induce side effect such epigastric pain and inhibition of healthy cell [4]. Meanwhile, Syafitri *et al* in 2017 were discovered the morphological change of rat's liver after long term consumption of soursop leaf extract, which as much as 40 mg/kgBW and 80 mg/kgBW consumption of soursop leaf extract for 60 days could decreased the central vein detachment without necrosis area and increase the amount of swollen hepatocytes [18]. In accordance to that, Wahyuningtyas in 2013 found that soursop leaf methanol extract at doses of 300 mg/kgBW/day for 30 days caused degeneration of rats hepatocytes characterized by cytoplasmic vacuolization under microscope [19]. However, Astini in 2018 contrary found that administration of soursop leaf extract at doses of 150 mg/kgBW/day for 21 days has positive impact in hepatocellular. These administration was contributes on the diameters of the islet of *Langerhans,* therefore it was recommended as adjuvant therapy on short term [20].

Besides the liver, soursop extract was shown to have side effect on other organs such as kidneys and neurons. Dayeef *et al.* in 2013 found that oral administration of soursop leaves ethanol extract for 40 days at doses of 10, 20 and 40 mg/kg/day caused an increase in caspase-9 expression in glomerular cells and renal tubular cells of the kidneys [6].

Meanwhile, recent studied by Mutakin *et al* in 2022 showed that aqueous extract of soursop had a Lethal Dose [LD)50 >5 g/kg which led to kidney damages. On the other hand, the doses of >1 g/kg soursop can cause hypoglycemic and decrease in the number of dopaminergic neurons in rat

brain. The decrease in the number of dopaminergic neurons is due to the effects of acetogenins derived from annonacin contained in the soursop root. Annonacin penetrates the brain parenchyma and inhibit the enzyme nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase (complex I) which subsequently lead to the ATP decrease. These conditions result in neuron and gliosis cell loss in the brain stem and basal ganglia. In mice, depletion of ATP causes a tau cells disruption which led to increase in tau phophorilation that could damage the neuron [3].

Another neuronal toxicity was shown by Hollerhage *et al*. (2015), a study of dietary supplements that contains of soursop extract was revealed in neurotoxic effect which 67% cell death at a 1 µg/mL of seed extract concentration. These results in line with the recent studied that active compound in soursop that is acetogens could impair the ATP production in mithocondria and inhibited the growth of promyelocyic leukemia cell on concentration 6 and 49 µg/mL. However, *in vitro* experiment showed that acetogens was able to generated the neurodegenerative taupathies on mesencephal and striatal cells through increased tau phosphorylation and reversed 3R-tau became 4R-tau [5].

Although soursop extracts have shown toxicity to various organ, Moghadamtousi *et al.* in 2015 suggest a chemopotential effect of soursop leaves ethyl acetate extract. The administration of the extract with doses of 250, 500 mg/kgBW/week for 2 consecutive weeks, has increased the expression of Bax in mice model of colon cancer induced by azoxzymethane (AOM) [21].

The previously presented studies have shown that soursop leaves have side effects or toxicity to cells both normal and cancerous. Our study supports other studies showing the presence of toxicity from the soursop extract by increasing the apoptosis process.

Further research are needed to investigate the minimum doses that leads to cell apoptosis by performing multilevel doses administration, as well as providing other apoptotic parameters such as active caspase-3 expression and applying Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) markers to calculate the number of cells undergoing apoptosis

Conclusion

We conclude that there was an effect of subchronic administration of water extract of soursop leaves (*Annona muricata L.*) on bax expression on mucosal epithelium of gastric rat (*Rattus norvegicus)*, for both glandular and non-glandular. This study shows there are differences in the number of glandular and non-glandular gastric mucosal epithelium that express bax between the control and treatment groups.

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