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Lipoxygenase Inhibitory Assay of *Averrhoa carambola* L. Leaves Extract

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Abstract : Lipoxygenase(LOX) is one of the enzymes involved in the mechanism of inflammation. Flavonoid compounds have been studied to inhibit the inflammatory pathway. Apigenin is a member of flavonoids that act as anti-inflammatory compounds. The leaves of plants *Averrhoa carambola* L. (sweet star fruit) has been known to contain apigenin. This study was conducted to test the ability of A. *carambola* L. leaves as a natural ingredient that plays a role in inhibiting inflammation. The research using in vitro LOXs enzyme inhibition method. A.*carambola* L. Leaves extracted using 70% ethanol, and fractioned with hexane, ethyl acetate, and water. Each test, sample testing LOXs enzyme inhibition with linoleate acid as the substrate. The product was measured using spectrophotometer at wavelength 234 nm and the control comparison using apigenin. The results showed that ethyl acetate fraction of A. *carambola* leaves may inhibit LOXs best with the IC_{50} value of 7.84 ± 0.03 ppm compared to other samples, 70% Ethanol Extract, water fraction, and Hexane fraction with IC_{50} values in a row 37.00 ± 0.58, 64.09 ± 1.97 and 107.71 ± 2.02 ppm. IC_{50}values of apigenin as a positive control showed IC_{50} 2.03 ± 0.831 ppm.
Keywords: Apigenin, *Averrhoa carambola* L., Lipoxygenase, inhibitor.

Introduction

There were several enzymes are known involved in a promoting inflammation pathway, such lipoxygenase. Lipoxygenases (LOXs) constitute a family of nonheme iron containing dioxygenases ubiquitously distributed in plants[1], fungi and animals, in human LOXs can be found in many cells and organs[2]. LOXs catalyze the first step in the arachidonic acid cascade that lead to the formulation of lipoxins and leukotriene involved in the variety of inflammatory responses such as asthma, rheumatoid arthritis, inflammatory bowel disease[2, 3,4]. Lipoxygenase inhibitor is any compound that can bind to the iron element or the lipoxygenase enzyme that is able to block the creation of excess iron hydroxyperoxide. By inhibiting the enzyme in inflammation pathway can prevent the inflammation. Nonsteroidal anti-inflammatory drugs (NSAIDs) was very effective to relieve pain and reduce signs of inflammation.However, reviews their side effects such as stomach bleeding, allergic reactions, kidney problems and heart problems can cause serious problems to the user's health[1, 5].The developments of safe enzyme inhibitors such as from natural materials are very important and may be useful in the treatment of various inflammatory diseases.LOX inhibitors are of interest for the modulation of these phenomena and resolution of the inflammatory processes. During LOX activity, peroxyl radical complexes are part of the reaction and may function as sources of free radicals. Thus antioxidants, such as flavonoids, capable of inhibiting lipid peroxidation and scavenging free radicals, may act as LOX inhibitors.

Flavonoids are natural polyphenol compounds in plants[6], having a variety of biological activities such as anti-inflammation, antimicrobial/antifungal action, antimutagenic, and anticancer activity *in vitro* and *in vivo*. Among these activities, anti-inflammatory activity of flavonoids has been studied to inhibit the inflammatory pathway[6, 7]Apigenin, a flavonoid abundant in various vegetables and fruits,including parsley, chamomile, and onion, has been reported to have numerous pharmacological properties and anti-inflammatory effect[6, 8]. Another plant that has anti-inflammatory activity is *Averrhoa carambola* L from (star fruit) [9]. The leaves of *Averrhoa carambola* L. has been known to contain apigenin[10]. The leaves are known, hadmany benefits for human health Such as anti-inflammatory activity[11], antioxidant activity[12], anti-ulcer[13], electrophysiological effect[14], hypoglycemic activity[15], antimicrobial activity[16], and anti-helminthic activity[17]. The acute toxicity assessment revealed that the leaves of A. *carambola* L. demonstrated low toxicity in rats and mice. Furthermore, there are no signs of toxicity present in sub-chronic evaluation[18].

Our study was conducted to test the ability of A. *carambola* L. leaves as a natural material that plays a role in inhibiting inflammation from lipoxygenase pathway, by using spectrophotometry *in vitro* method.

Experimental

Extraction:

The leaves of A. *carambola* were collected from Kelapa DuaDepok, West Java, Indonesia and identified by Botanist at the Center for Plant Conservation Botanical Gardens - Indonesian Institute of Sciences (LIPI), Bogor. A voucher specimen was deposited in the Herbarium at the Center for Plant Conservation Botanical Gardens - Indonesian Institute of Sciences (LIPI), Bogor. The air-dried leaves of A. *carambola* were extracted with 70% ethanol at room temperature for 9 days. The solvent was removed by rotary evaporation (at<55° C). The ethanolextract (41.3 g) was fractionated with n-hexane,ethyl acetate, and water. The partitions were concentrated by rotary vacuum evaporator at 40 ° C the speed of 100 RPM.

Apigenin assay:

Assay of apigenin in A. *carambola* leaves extracts and fractions using were analyzed using HPLC Waters, Sun Fire C18 column 4.6 × 150 mm, with a flow rate of 1 mL / min, 20.0 mL injection volume and detection using UV at a wavelength of 340 nm. The mobile phase used the isocratic mixture of acetonitrile: water (45:55)[19].

Lipoxygenase assay:

Lipoxygenase activity was determined using spectrophotometric methodsreported by Karl, et al (2004)[20] with slight modifications. The Buffer solution (0.2 M, pH 9.00) made of a mixture of boric acid and sodium hydroxide. The linoleic acid substrate solution made by mixing 250 μM linoleic acid 10 mL and 30 mL ethanol (concentration final substrate is 125 μM). The Enzyme solution made by dissolving the lipoxygenase in 0.2 M borate buffer, to a concentration of an enzyme that is obtained is about 10,000 U / mL, then dissolve in 400 U / mL, and to 200 U / mL (as final concentration). A stock solution of the tested sample dissolved in dimethyl sulfoxide (DMSO). The range of the best concentration of the test substance depends on the inhibitory activity. It must be tested in every case, starting with a solution that is strong enough (eg, 10 mg / mL) and then made a series of dilutions. In a final concentration for inhibition experiments that will be used is 125 μM (the same as the substrate). Measurement of the enzyme inhibitor activity should be at room temperature and measure the absorbance value was recorded at wavelength 234 nm. The performance of the assay was verified using apigenin as a positive control. The percentage of lipoxygenase inhibition was calculated as:

% Inhibition: $\frac{Abc-Abs}{Abc}$ x 100%

Ab_c was the absorbance of control and Ab_s was the absorbance of the tested sample.

Results

Apigenin Assay:

The results indicate that ethyl acetate fraction had the highest content of apigenin in the amount of 6.37%, while the ethanol extract of 0.08%, water fraction 0.02%, and hexane fraction has no contained of apigenin

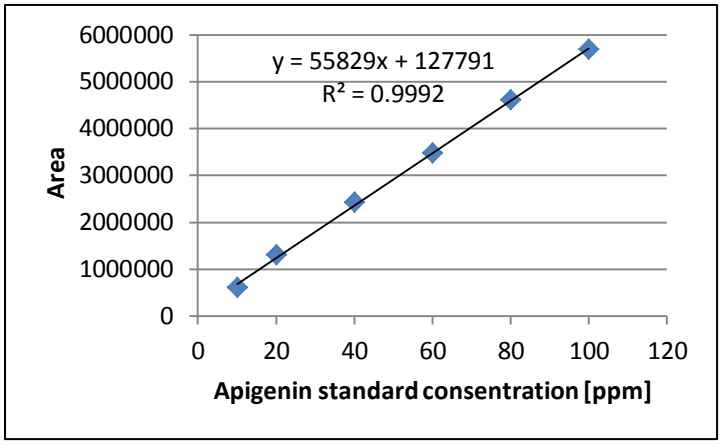

Figure 1. Apigenin standard curve concentration

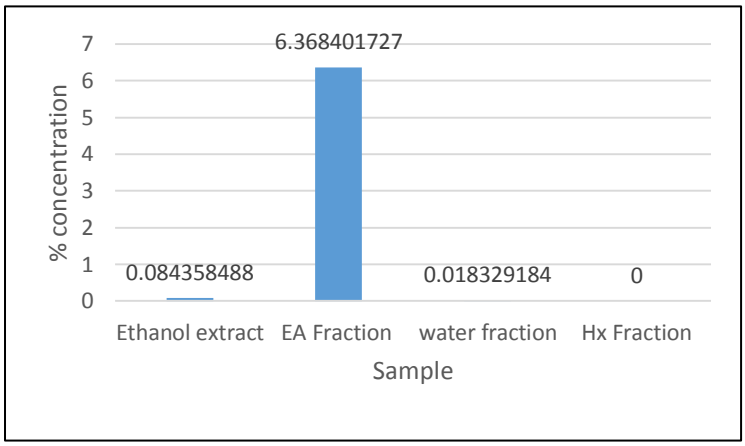

Figure 2. Apigenin concentration in sample

Lipoxygenase assay:

Table 1. IC50 Value of sample

Sample	IC50 Value
Ethanol Extract	37.00 ± 0.57
Water Fraction	64.08 ± 1.97
Hexane Fraction	107.71 ± 2.01
Ethyl Acetate Fraction	7.83± 0.03
Apigenin	2.02 ± 0.83

The results showed that the ethyl acetate fraction may inhibit LOX best with the IC_{50} value of 7.84 ± 0.03 ppm compared to other samples, Ethanol Extract, water fraction, and Hexane fraction with IC_{50} values in a row 37.00 ± 0.58, 64.09 ± 1.97 and 107.71 ± 2.02 ppm. IC_{50}values of apigenin as a positive control showed IC_{50} 2.03 ± 0.831 ppm.

Discussion

Anti-inflammatory activity of flavonoids, compounds has been studied to inhibit the inflammatory pathway. Flavonoids have been found as the most powerful inhibitor in inhibiting cyclooxygenase and lipoxygenase[7]. The mechanism of flavonoids to inhibit the inflammatory process in two ways, by inhibiting capillary permeability and inhibit arachidonic acid metabolism and the secretion of lysosomal enzymes from neutrophils cells and endothelial cells[21].

Apigenin the member of flavonoid compound (flavone) has been reported to have numerous pharmacological properties and anti-inflammatory effect. Anti-inflammatory activity ofapigenin in leaves *Averrhoa carambola* has been proven in previous study that have been done by Cabrini, et al 2011. That study showed A.*carambola* leaves contain apigenin-6-C-β-L-fucopyranoside and apigenin-6-C-(2”-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside which is called caramboflavone. The ethanol extract of A. *carambola* can inhibit inflammation by inhibiting myeloperoxidase activity[11]. This study measured that ethyl acetate fraction has the highest concentration of apigenin (6.37%). This study aimed to look at the ability to A. *carambola* leaves extract and the fractioncan relieve inflammation from other lines, which is inflammation of the lipoxygenase enzyme pathway formation. The substrate is linoleate acid. Linoleic acid has a structure similar to arachidonic acid, both of which are unsaturated fatty acids that have a methylene unit between two double bonds. Lipoxygenase is an enzyme that reacts with unsaturated fatty acids to produce hydroxy peroxide[22]. Lipoxygenase inhibition activity demonstrated by measuring the absorbance levels ofhydroxy peroxide with the spectrophotometer at a wavelength of 234 nm.

In this experiment, the positive control used apigenin, since apigenin is a compound that is contained in the leaves of starfruit[11]. Apigenin has also been proven as a compound potentially anti-inflammatory to inhibit the induction of LOX-1 that induced by TNFα[23]. Inhibition of LOX enzyme activity was measured by looking at the IC_{50} value of the inhibitory sample (A. *carambola* leaves extract and fraction). The variation concentration of apigeninthat used to test is50 – 3,125 ppm.$IC_{50}$of apigenin that result from inhibition of LOX enzymes is 7.502 μM(equal to 2.0273ppm). IC_{50} ethanol extract, ethyl acetate fraction, water fraction and hexane fraction of LOX enzyme inhibition test obtained respectively 37.00 ± 0:58; 7.83 ± 0:03; 64.09 ± 1.97; 2:08 ± 107.71 ppm (Table 1). IC_{50} values obtained in relation to the levels of apigenin. The sample that has a high level of apigenin, has small IC_{50} value, so it can be assumed that apigenin is one compound that plays a role in the inhibition of the LOX enzyme.

This research confirmed the activity of extract and fractions of *Averrhoa carambola* leaves as the inhibitor to inhibit Lipoxygenase pathway. The conclusion is *Averrhoa carambola* leave extract and the fraction can inhibit lipoxygenase pathway by in vitro spectrophotometry method. The ethyl acetate fraction may inhibit LOX best with the IC_{50} value of 7.84 ± 0.03 ppm compared to other samples. It is known the mechanisms of flavonoids to inhibit the inflammatory process in two ways, by inhibiting capillary permeability and inhibit arachidonic acid metabolism and the secretion of lysosomal enzymes from neutrophils cells and endothelial cells. Some flavonoids can inhibit the release of arachidonic acid and enzyme secretions from the lysosome membrane to block off the cyclooxygenase and lipoxygenase pathways resulting in lower levels of prostaglandin and leukotriene[21]. Further research is needed to determine the mechanism of apigenin that contain in *Averrhoacarambola* leaves extract and fraction to inhibiting inflammatory lipoxygenase enzyme pathways.

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