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The Inhibition of Andrographolide (*Andrographis paniculata)* on the Expression of Carbonic Anhydrase in LPS-Induced Human Leucocyte Cells

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Abstract: The aim of this work was to explore the bioactivity andrographolide, a major labdane diterpenoidal constituent of *Andrographis paniculata*, especially its influence on the expression of certain protein in LPS-induced human leucocyte cells. Human leucocyte cells were isolated from human whole blood and preincubated with andrographolide (25-200 μM) for 15 min. The preincubated cells were treated with lipopolysaccharide for 1 h afterwards. The method used in this work was SDS-PAGE, a polyacrylamide gel electrophoresis with a sodium dodecyl sulfate-containing buffer. Andrographolide inhibited the expression of certain protein which molecular weight was 36 kDa and identified as carbonic anhydrase. Since this enzyme produces and uses protons and bicarbonate ions, inhibition of this enzyme might lead to a decrease of acid production in the stomach and mild alkalosis.

Key words: Andrographolide, *Andrographis paniculata*, carbonic anhydrase, gel electrophoresis, human leucocyte cells, lipopolysaccharide

INTRODUCTION

Andrographis paniculata (Burm. F.) Nees (family: Acanthaceae) grows widely in many Asian countries such as Indonesia, China, India, Thailand and Sri Lanka. The plant is particularly known for its extremely bitter properties (often called king of bitters) and is used traditionally as a remedy against common cold, fever, inflammation etc. Andrographolide, an active component of *Andrographis paniculata*, is the major labdane diterpenoidal constituent in this plant, which has an α-alkylidene γ-butyrolactone, two olefin bonds at C8 (17) and C12(13) and three hydroxyl groups at C3, C19 and C14 (Nanduri *et al*., 2004).

Andrographolide (Fig. 1) has been reported to have anti-inflammatory activity by suppressing inducible nitric oxide synthase expression in RAW 264.7 cells (Chiou *et al*., 2000) and prevented oxygen radical production by human neutrophils (Shen *et al*., 2002). This compound inhibited NF-kappaB activation (Xia *et al*., 2004) reduced COX-2 expression induced by platelet activating factor and N-formyl-methionyl-leucyl-phenylalanine in HL60/neutrophils (Hidalgo *et al*., 2005). Andrographolide showed inhibition on COX-2 expression in LPs-induced human fibroblast cells (Levita *et al*., 2010). Direct antimicrobial activity of two ethanolic

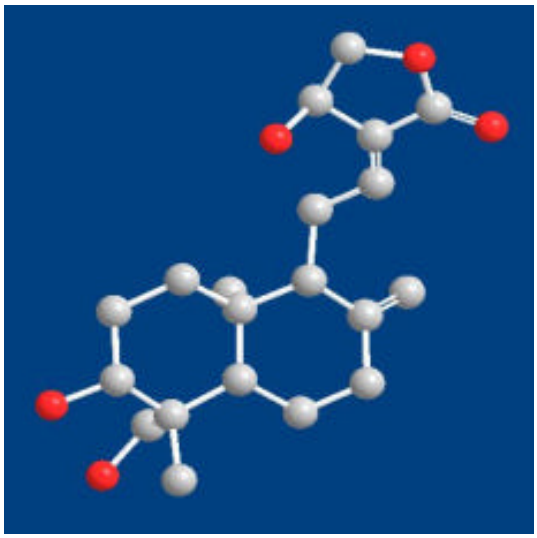

Fig. 1: Structure of andrographolide. Red balls represent oxygen atom, grey balls represent carbon atom and sticks are the bonds between the atoms

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Andrographis paniculata extracts was observed for two human pathogens, *Legionella pneumophila* and *Bordetella pertussis* (Xu *et al*., 2006).

Carbonic anhydrase is an enzyme that assists rapid inter-conversion of carbon dioxide and water into carbonic acid, protons and bicarbonate ions. This enzyme was first identified in 1933, in red blood cells of cows. Since then, it has been found to be abundant in all mammalian tissues, plants, algae and bacteria. This ancient enzyme has three distinct classes (alpha, beta and gamma carbonic anhydrase). Members of these different classes share very little sequence or structural similarity, yet they all perform the same function and require a zinc ion at the active site. Carbonic anhydrase from mammals belong to the alpha class, the plant enzymes belong to the beta class, while the enzyme from methane-producing bacteria that grow in hot springs forms the gamma class (Dutta and Goodsell, 2004). Since this enzyme produces and uses protons and bicarbonate ions, carbonic anhydrase plays a key role in the regulation of pH and fluid balance in different parts of our body. In our stomach lining it plays a role in secreting acid, while the same enzyme helps to make pancreatic juices alkaline and our saliva neutral. The transport of the protons and bicarbonate ions produced in our kidney and eyes influence the water content of the cells at these locations. Thus carbonic anhydrase isozymes perform different functions at their specific locations, and their absence or malfunction can lead to diseased states, ranging from the loss of acid production in the stomach to kidney failure (Dutta and Goodsell, 2004).

N-unsubstituted sulfonamide, such as acetazolamide, is widely used for ophthalmic disorders. Inhibition of carbonic anhydrase enzyme is believed to be the chief reason for their therapeutic effects (Chakravaty and Kannan, 1994).

Kawaai *et al*. (2009) found that acetazolamide inhibition of carbonic anhydrase activity in leukocyte showed that this drug induced changes in the intracellular calcium concentration, and extracellular calcium is thought to be a factor inducing an increase in leukocyte migration. They concluded that acetazolamide may stimulate leukocyte migration due to its participation in the regulation of intracellular pH controlled by carbonic anhydrase activity without an effect of low extracellular calcium concentration. In addition, acetazolamide was thus suggested to possibly have an anti-inflammatory effect in supporting leukocyte migration during inflammatory reactions.

In this study, we intended to explore other bioactivity andrographolide, a major labdane diterpenoidal constituent of *Andrographis paniculata*, especially its influence on the expression of certain protein in LPS-induced human leucocyte cells.

MATERIALS AND METHODS

Materials preparation: All materials used in this study were ordered on May 2010 and was available within 6-8 weeks. Andrographolide 98% 500 mg CAS 5508-58-7 for R and D use was purchased from Aldrich and was dissolved in dimethylsulfoxide (DMSO). Lipopolysaccharide (LPS) 10 mg derived from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich. Protein standard used in this work was SeeBlue^{R} Plus2 Prestained Standard (Catalog no. LC5925) from Invitrogen. Ficoll-Paque^{TM} Plus 17-1440-02 endotoxin tested was purchased from Amersham Biosciences AB Sweden.

Isolation of leucocyte cells from human whole blood: Human whole blood was obtained from healthy volunteer (Indonesia Red Cross Society, Bandung, West Java, Indonesia) which had been clinically proven to be free of Hepatitis B and C virus, *Treponema pallidum* bacteria, and HIV virus. The blood was contained in a flask with the addition of citrate phosphate dextrose adenine, as a nutrient and anticoagulant.

The isolation procedure was performed at Parasitology Laboratory, Medical Faculty, Gadjah Mada University (22-26 August 2010).

About 3 mL of Ficoll-Paque^{TM} Plus reagent was put in a 15 mL volume BD conical tube (Falcon) and was added with 7 mL human whole blood. The mixture was centrifuged for 15 min 1500 rpm at room temperature (Servall^{R} Legend RT). The buffy coat was pipetted and separated into another tube, followed by the addition of RPMI 1640 medium. The solution was centrifuged for 5 min 1500 rpm at room temperature and the pellet containing leucocyte cells was separated for further assay.

Electrophoresis gel for in vitro assay: This step was performed at Parasitology Laboratory, Medical Faculty, Gadjah Mada University on 22-26 August 2010.

Human leucocyte cells (1.5×10^{6} cells) in RPMI 1640 culture medium were placed in conical tubes. The cells were preincubated with andrographolide (25, 50, 100, 200 μM) for 15 min at room temperature, followed by stimulation with 20 μL solution of lipopolysaccharide (10 μg mL^{-1}) in DMSO for one hour to induce inflammation. After one-hour incubation, the human leucocyte cells were inserted into the wells of gel electrophoresis (SDS-PAGE), compared with the standard protein.

SDS-PAGE is the abbreviation for polyacrylamide gel electrophoresis with a sodium dodecyl sulfate-containing buffer. In SDS-PAGE method the proteins are separated in a polyacrylamide gel medium by the application of an electrical field. The buffer containing SDS, an anionic (negatively charged) amphipatic detergent, the dodecyl portion, CH_3 $(CH_2)_{11}$, is hydrophobic. The sulfate ion carries two Na^+ counter ions, making that portion of the molecule hydrophilic. When SDS binds a soluble protein, the hydrophylic portion of the SDS inserts into the hydrophobic core of the protein, causing the protein to denature by unfolding it. The Na^+ neutralizes electrostatic interactions. The negatively charged sulfate is exposed and the protein is effectively covered with negative charges. SDS binds proteins in a constant mass ratio (~1.4 g SDS/g protein) and therefore, in the presence of SDS, soluble proteins are denatured to a rod shape and posses roughly the same charge/mass ratio. Migration in an electric field depends on size, shape and charge. Since the denatured proteins have roughly the same shape and charge is reduced to a uniform negative charge, they will migrate toward the anode (positive electrode) at a rate that is dependent only on the sieving action of the polyacrylamide matrix (Spangler, 2002).

RESULTS AND DISCUSSION

Andrographolide (Fig. 1) has been reported to have anti-inflammatory activity by suppressing inducible nitric oxide synthase expression in RAW 264.7 cells (Chiou *et al*., 2000) and prevented oxygen radical production by human neutrophils (Shen *et al*., 2002). This compound inhibited NF-kappaB activation (Xia *et al*., 2004), reduced COX-2 expression induced by platelet activating factor and N-formyl-methionyl-leucyl-phenylalanine in HL60/neutrophils (Hidalgo *et al*., 2005). Andrographolide showed inhibition on COX-2 expression in LPS-induced human fibroblast cells (Levita *et al*., 2010). Direct antimicrobial activity of two ethanolic *Andrographis paniculata* extracts was observed for two human pathogens, *Legionella pneumophila* and *Bordetella pertussis* (Xu *et al*., 2006). These reviews suggested that andrographolide could be classified as a nonselective bioactive compound. It worked as inhibitor to many targets. The selectivity depends on the binding affinity of a compound to a certain target, which means the steric and surface complementarities of both molecules (the lock and key paradigm). The successful of enzyme-inhibitor complex also depends on the concentration of the inhibitor.

In this study, preincubation of andrographolide (25-200 μM) inhibited the expression of certain protein, which was showed by the disappearance of protein bands on the SDS-PAGE gel after one hour of electrophoresis (Fig. 2).

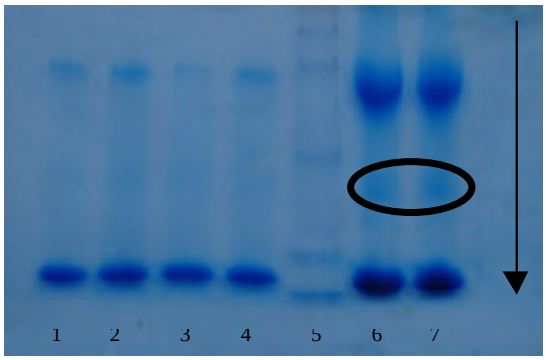

Fig. 2: Inhibiton of protein expression by andrographolide. LPS-induced human leucocyte cells preincubated with: 1: Andrographolide 25 μM, 2: Andrographolide 50 μM, 3: Andrographolide 100 μM, 4: Andrographolide 200 μM, 5:Protein marker 6: LPS-induced human leucocyte cells without andrographolide, 7: Normal human leucocyte cells without andrographolide

The result of SDS-PAGE showed that human leucocyte cells without andrographolide preincubation expressed many bands of proteins (the differences were especially showed by the black ellips in Fig. 2), while the addition of andrographolide removed the expression of that specific protein. Based on the protein marker, the missing band was identified as carbonic anhydrase's which had molecular weight 36 kDa. Andrographolide (25-200 μM) showed inhibitory on the expression of carbonic anhydrase in LPS-induced human leucocyte cells. Since this enzyme produces and uses protons and bicarbonate ions, carbonic anhydrase plays a key role in the regulation of pH and fluid balance in the body, therefore inhibition of this enzyme might lead to both advantage and disadvantage. The advantage is that inhibition of carbonic anhydrase by andrographolide can reduce acid production in the stomach, while the disadvantage is mild alkalosis. The behaviour of andrographolide on carbonic anhydrase was compared with Kawaai *et al*. (2009), who studied the effect of acetazolamide on LPs-induced leucocyte. Kawaai *et al*. (2009) concluded that acetazolamide which inhibited carbonic anhydrase activity in inflammation process could cause the change of leucocyte migration or and suggested that due to this data, acetazolamide possibly have an anti-inflammatory effect in supporting leukocyte migration during inflammatory reactions.

Maren and Swenson (1980) investigated the role of carbonic anhydrase in respiration. They studied the rate of the Bohr effect with and without carbonic anhydrase in five representative vertebrate species: spiny dogfish (*Squalus acanthias*), goosefish (*Lophius americanus*), bullfrog (*Rana catesbeiana*), white Peking duck (*Anas platyrynchos*) and man. The Bohr effect is the heterotropic interaction of hydrogen ions, carbon dioxide and oxygen upon their binding with haemoglobin. The protonation of certain oxylabile amino acids in haemoglobin causes a conformational change in the shape of the molecule, yielding a decrease in the affinity of the haem subunit for O_2. This increase in the P_{50} with acidosis (or right shift in the O_2 dissociation curve) and decrease with alkalosis enhances the exchange of O_2 and CO_2 in the tissue and gill or lung capillaries. The results clearly showed a quantitative relationship between proton generation and O_2 release when carbonic anhydrase was sufficiently inhibited by methazolamide. Total inhibition of carbonic anhydrase may be defined as the observed plateau of a dose-response study, or when the fully inhibited observed rate was equal to the calculated uncatalysed rate. When carbonic anhydrase was totally inhibited by 10^{-3}M of methazolamide, the Bohr effect rate was markedly reduced to a plateau level and the calculated uncatalysed rate of H^+ generation nearly matched O_2 release (Maren and Swenson, 1980).

CONCLUSIONS

Andrographolide inhibited the expression of certain protein which based on its molecular weight was identified as carbonic anhydrase. Since this enzyme produces and uses protons and bicarbonate ions, carbonic anhydrase plays a key role in the regulation of pH and fluid balance in the body, therefore inhibition of this enzyme might lead to a decrease of acid production in the stomach and mild alkalosis.

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The authors have declared no conflict of interest.

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